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LA THÈSE A ÉTÉ
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THE POLYADENYLATION OF RAT mRNA DURING
MAMMARY GLAND DEVELOPMENT

Susan A. Nadin-Davis

Thesis submitted to the School of Graduate Studies of the
University of Ottawa in partial fulfillment of the require-
ments for the degree of Doctor of Philosophy





UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

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ABSTRACT

Two characteristics of the polyadenylation of mRNA from rat mammary gland at several developmental stages were studied: 1. The length of poly(A)⁺ tracts in poly(A)⁺ RNA; 2. The distribution of two types of milk protein mRNA sequences - 9S whey protein and 15S casein - between poly(A)⁺ and poly(A)⁻ RNA fractions. This was determined by hybridization of these RNA fractions to cDNA probes prepared from mammary 9S and 15S mRNAs. Poly(A) tracts from all glands studied had sizes ranging from 25-150 nucleotides. Tracts from 8 day pregnant gland showed a unimodal distribution with a peak at 80 residues, but by 16 days of gestation the proportion of smaller poly(A) tracts was increased. At 7-8 days and 11-15 days of lactation, a bimodal poly(A) tract distribution was found with size peaks at 40-45 and 80 residues. On involution the smaller size class was being lost. It was concluded that a specific poly(A) size class appeared in the gland during peak activity. The distribution of the 9S and 15S mRNA sequences between mammary poly(A)⁺ and poly(A)⁻ RNAs was determined for five developmental stages; both sequences exhibited a similar trend. At 7-8 days of lactation, levels of the poly(A)⁻ mRNA were low (about 6-10% of total sequence) but in early pregnancy and involution this level appeared to be larger (up to 60-65%). It might be concluded that there are significant changes in the proportions of poly(A)⁺ and poly(A)⁻ 9S and 15S mRNAs during gland development; however, this statement must be made cautiously since these measurements exhibited high variation and production of high levels of poly(A)⁻ RNA due to the artefact of RNA degradation is difficult to disprove. This study has shown that the proportion of poly(A)⁻ 15S casein mRNA present in the rat lactating mammary gland is lower than reported previously, and that 9S whey protein mRNA shows a similar distribution between poly(A)⁺ and poly(A)⁻ RNAs.

ACKNOWLEDGEMENTS

Many people have supported my work in preparation for this thesis. I would like to thank Charles Seguin for his technical assistance, especially with the preparation of purified mRNA sequences. My thanks are also due to Ian Parke for performing the DNA analysis of mammary gland extracts and to Andres Lozano who performed the experiments to investigate the presence of 5' caps in mammary RNA samples.

I would also like to express my appreciation to the supervisor of this project, Dr. V.A. Mezl, who spent many hours with me in fruitful discussions on the progress and direction of this research and who was always ready to provide moral support during the research blues. Finally, I would like to acknowledge the support of the Muscular Dystrophy Association of Canada through the provision of a pre-doctoral fellowship during a period of four years.

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LIST OF ABBREVIATIONS

10A	Oligoadenylate consisting of 10 residues
60A	Polyadenylate consisting of 60 residues
90A	Polyadenylate consisting of 90 residues
113A	Polyadenylate consisting of 113 residues
207A	Polyadenylate consisting of 207 residues
9S cDNA	cDNA prepared from 9S mammary mRNA
12S cDNA	cDNA prepared from 12S mammary mRNA
15S cDNA	cDNA prepared from 15S mammary mRNA
2x poly(A) - RNA	unbound RNA from two affinity columns
4x poly(A) - RNA	unbound RNA from four affinity columns
AMV	Avian Myeloblastosis virus
bis	N,N'-methylene-bis-acrylamide
cDNA	complementary DNA
Cot	product of concentration of nucleotides (M) x time (s)
DEAE-cellulose	Diethylaminoethyl-cellulose
DTT	dithiothreitol
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EMC virus	Encephalomyocarditis virus
Hepes	4-(2-hydroxy ethyl)-1-piperazine-ethane sulphonic acid
hnRNA	heterogeneous nuclear RNA
m ⁷ G ⁵ 'p	7-methylguanosine-5'-phosphate
mRNA	messenger RNA
Pipes	1,4-piperazine-diethanesulphonic acid
Poly(A) ⁺	polyadenylated
Poly(A) ⁻	nonpolyadenylated
S.D.	standard deviation
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylenediamine
Tris	tris(hydroxymethyl)amino methane
VSV	Vesicular Stomatitis Virus
X. laevis	Xenopus laevis
α-LA	alpha-lactalbumin
incorporation of RNA	incorporation of label into translation product directed by RNA

INTRODUCTION

The central dogma of biochemistry states that mRNA molecules direct the production of protein molecules as specified by the precise base sequences contained within the genomic DNA. Thus mRNA provides an essential link between the hereditary information contained within a cell and its expression. A good understanding of the process of gene expression and its control must involve detailed knowledge of the structure and properties of mRNA molecules and the means by which synthesis (transcription) and use (translation) of mRNA is controlled.

All RNA molecules consist of ribonucleoside monophosphates linked by 5'-3' phosphodiester linkages, but mRNA molecules bear particular characteristics apparently not shared by other RNA species (1). Mature mRNA molecules contain both coding and non-coding regions; for each molecule the former directly specifies the primary sequence of a specific polypeptide according to the genetic code while non-coding regions function as regulatory sequences and determine the reading frame of the coding sequence. Specific mRNA characteristics include: a 'cap' structure consisting of a 7 methyl guanosine residue (m^7G) attached in a 5'-5' triphosphate linkage to the 5' terminal nucleotide and a homopolynucleotide sequence composed entirely of adenosine monophosphate (AMP) residues and attached to the 3' terminal of the mRNA molecule. This sequence

of AMPs is usually referred to as the poly(A) segment or poly(A) tail - due to its position in the molecule. It is this property of mRNA molecules which is the subject of this study. This introductory review will describe current knowledge of the prevalence, properties and possible function of poly(A) chains and should provide a framework for the interpretation of the experimental data presented in this thesis.

The existence of poly(A) rich RNA was first proposed by Edmonds and Abrams (2). They reported the ability of a partially purified enzyme preparation from calf thymus nuclei to incorporate ATP into polynucleotide; the enzyme preferred ATP over other nucleoside triphosphates and produced polynucleotides linked by 3'→5' phosphodiester bonds. This work was later recognized as the first report of the enzyme responsible for poly(A) synthesis, poly(A) polymerase. Nine years later, Edmonds and Caramela (3) reported the isolation and characterization of AMP-rich polynucleotides from Ehrlich ascites cells. This report is a milestone in poly(A) research since it introduced several methods which have become standard tools in this area. For example, AMP-rich polynucleotides were separated from total cellular RNA using an affinity resin of polythymidylate oligomers attached covalently to cellulose. The basis of this separation is described later (see "oligo(dT)-cellulose affinity chromatography"). The AMP-rich polynucleotides purified from Ehrlich Ascites cell RNA were heterogenous in size, having sedimentation coefficients ranging from 4s to 18s. RNase T₁ treatment (which cleaves RNA molecules at guanine

nucleotides) removed much of the higher molecular weight material but the smaller polynucleotides remained; this observation suggested that at least some of the AMP-rich segments were covalently attached to heteroribopolynucleotides. The use of RNases A and T_1 , which cleave RNA molecules specifically at pyrimidine and guanine nucleotides respectively, has become a standard procedure for isolating poly(A) sequences from RNA molecules. RNase T_2 treatment or non-specific alkaline hydrolysis cleaves poly(A) segments. Thus the presence of poly(A) chains within ribopolynucleotides is indicated by the presence of sequences resistant to RNase A and RNase T_1 treatment, but susceptible to RNase T_2 (4-6).

In 1970, Lim and Canellakis (7) found a polypurine sequence highly enriched in adenine, in rabbit reticulocyte mRNA but this polypurine sequence was not associated with 18S and 28S rRNA, nor with 5S RNA. An adenine-rich sequence was also associated with duck globin mRNA (8). Lee et al. discovered AMP-rich polynucleotides in rapidly-labelled polyribosomal RNA (mRNA) obtained from mouse Ehrlich ascites cells (9). At that time, poly(A)-rich sequences were also found to be associated with several viral mRNAs : eg. Vaccinia, Herpes Simplex, SV40 and VSV mRNAs (10).

Since these initial studies, most mRNAs have been found to contain poly(A) chains; organisms which exhibit this mRNA characteristic include yeast, the simple multicellular organism *Volvox* (11), the cellular slime mold (12), several plants including pea and cotton, sea urchin embryos (13) and tissues of higher organisms, e.g. HeLa cells and rabbit brain. Even

mammalian and insect mitochondrial mRNA molecules apparently bear poly(A) sequences. For a comprehensive listing of the occurrence of poly(A) in living organisms, see Karpetsky et al.(10).

There has been some controversy over the occurrence of poly(A) in prokaryote RNA. Perry et al.(14) were unable to detect any poly(A) associated with E. coli polysomal RNA, but three years later researchers did detect poly(A) in certain prokaryote RNAs under specified conditions. E. coli poly(A)⁺ RNA has been isolated (15), and poly(A) sequences were found associated with Caulobacter crescentus RNA (16) and with sporulating Bacillus subtilis mRNA (17). The poly(A) associated with prokaryote mRNA did appear to be generally shorter in length than that present in eukaryote mRNA (compare 10-45 residues with up to 200). Recently, Gopalakrishna et al.(18) isolated RNA from Bacillus brevis and E. coli by a single-step procedure and detected poly(A)⁺ RNA bearing poly(A) sequences of much greater length than found previously in prokaryotes. They suggest that the inability to detect poly(A) and the shortness of the poly(A) sequences isolated from prokaryotes in earlier work could be an artefact of the isolation procedures employed.

In summary, poly(A) sequences are associated with RNA from a wide variety of organisms, and they appear to be ubiquitous components. All the available evidence suggests that poly(A) sequences are associated with mRNA and its precursors, but not tRNA or rRNA (11, 19, 20). Advances in

RNA purification procedures, based to a large extent on the poly(A) content of RNA, have enabled researchers to produce highly purified mRNA preparations. Several high abundance mRNAs from specialized tissues are known to be polyadenylated; these include mRNAs directing synthesis of hen oviduct ovalbumin (21), trout testis protamines (22), rat α -lactalbumin (23), rat caseins (24), α -, β - and γ -actins (25), rat albumin (26), embryonic chick myosin heavy chain (27), soybean leghaemoglobin (28) and rabbit globin (7). A notable exception are HeLa cell histone mRNAs which do not contain poly(A) chains (29).

The finding that poly(A) sequences are an integral part of mRNA molecules suggested that their origin was closely associated with mRNA synthesis. A high percentage of Ehrlich ascites cell poly(A) was found to be in the nucleus (3) and an enzyme capable of poly(A) synthesis had been demonstrated in calf thymus nuclei (2). A nuclear origin of eukaryote poly(A) is strongly supported by studies on HeLa cell mRNA synthesis. HeLa cell, hn RNA (heterogeneous nuclear) was shown to contain poly(A) chains of similar sizes (150-200 residues) to those associated with cytoplasmic mRNA, but poly(A) was not present in nucleolar RNA which is thought to consist essentially of rRNA (30). On the basis of pulse-labelling experiments using [^{3} H] adenosine, Darnell et al. suggested a precursor/product relationship between hnRNA poly(A) and mRNA poly(A) (31, 32).

A. concentration of the drug, actinomycin D, which inhibited over 90% of hnRNA and mRNA synthesis, inhibited poly(A) synthesis only 60-75%. Actinomycin D apparently had an immediate effect on hnRNA synthesis but a delayed effect on poly(A) synthesis; it was concluded that poly(A) synthesis is partially independent of transcription (31). The drug cordycepin (3'-deoxyadenosine) suppressed [³H]-uridine labelling of mRNA but not that of hnRNA (33); [³H]-adenosine labelling of both hnRNA and mRNA in the presence of cordycepin caused a preferential reduction in poly(A) labelling compared to that observed in the absence of the drug (31). To explain these observations it was proposed that cordycepin interfered with poly(A) synthesis specifically by acting as an adenosine analogue. Jelinek et al. have shown that cordycepin inhibits nuclear synthesis of HeLa cell poly(A) (34).

On the basis of studies like those described above, it has been proposed that mRNA is synthesised as large precursors (hnRNA) by transcription of nuclear DNA, and nuclear mRNA maturation, (now known to include polyadenylation, capping and shortening of the transcripts) occurs before transport of mature mRNA to the cytoplasm (34). Nucleocytoplasmic transport may require, or be regulated by, the poly(A) chain (see "Possible Functions of Poly(A)"). More recent studies on specific mRNA sequences support this scheme; a 155 poly(A)⁺ globin mRNA precursor has been detected in chick red blood cells (36). Molloy and Darnell (37) have compared the poly(A)

chains of HeLa cell hnRNA and mRNA after isolation using just RNase T₁. In both cases, the poly(A) chain contained about 195 adenyate residues, two uridylate residues and one or two cytidylate residues. This suggests nucleotides adjacent to the poly(A) chain are similar for mRNA and hnRNA, and this is again consistent with the proposed product/precursor relationship.

There has been some disagreement on the exact timing of nuclear polyadenylation and whether all nuclear poly(A) is exported to the cytoplasm. Evidence pertaining to these issues, as they relate to possible poly(A) function is discussed later. However, it is now accepted that hnRNAs are precursors of mRNAs and that poly(A) tracts are a component of both types of molecules. With the exception of the histone mRNAs, poly(A)⁺ RNA became synonymous with mRNA (38) and methods for mRNA purification have taken advantage of the ease with which one can select for the homopolynucleotide, poly(A). Relatively non-specific resins, e.g. cellulose nitrate and millipore filters, which were used previously for mRNA purification (39, 40) have been replaced with more specific affinity resins: oligo(dT)-cellulose and poly(U)-sepharose.

Poly(A) Affinity Chromatography

(i) Oligo(dT)-cellulose

This resin consists of cellulose bound covalently to oligodeoxythymidylate sequences 10-15 residues in length. The use of this resin to purify globin poly(A)⁺ mRNA was first fully described in 1972 (41). Briefly, a total reticulocyte RNA extract was applied to an oligo(dT)cellulose column in binding buffer containing 0.5M KCl. At 0.5M KCl and room temperature poly(A):oligo(dT) hybridization occurred and poly(A)⁺ RNA was selectively retained by the column; other RNA components were washed through in the binding buffer. After an intermediate column wash with 0.1M KCl buffer, poly(A)⁺ RNA was eluted with a "no salt" buffer.

Although the procedure for poly(A)⁺ mRNA purification using oligo(dT)-cellulose always follows this basic protocol, many modifications have been reported. In some cases, the intermediate salt wash is omitted (42), and sometimes SDS(0.1-0.5%) is included in the buffers, either to limit RNase activity or possibly to reduce non-specific RNA binding to the resin (43). Hames and Perry used an application buffer which contained 0.1M KCl and 0.1% sarkosyl, a detergent similar to SDS (44).

The optimal use of oligo(dT)-cellulose resins has been investigated. Mercer and Naora (40) showed that the cellulose itself could bind significant amounts of rRNA and synthetic poly(A); this binding capacity increased with rising salt

concentration; oligo(dT)-cellulose had a 150-fold higher poly(A) binding capacity than cellulose and again poly(A) binding increased with rising salt concentration. The authors showed however that lower ionic strength binding buffers provide optimal conditions for poly(A)⁺ purification using oligo(dT)-cellulose since the higher the salt concentration, the greater is the proportion of non-specific rRNA binding to the resin. Poly(A) can also be eluted from oligo(dT)-cellulose columns by increased temperature rather than by reduced ionic strength. Temperatures of 50-60° C., at a salt concentration of 0.4M NaCl were required (40). A combination of increased column temperature and reduced ionic strength could also be used.

Bantle et al. demonstrated nonspecific binding of poly(A)⁻ RNA to oligo(dT)-cellulose - this RNA was eluted with poly(A)⁺ RNA at low ionic strength(45). Furthermore, strong non-specific binding of poly(A)⁺ RNA, which is not disrupted by normal elution procedures, was detected. Both types of non-specific binding were abolished by prewashing the resin with small amounts of RNA. Singer and Penman (46) reduced non-specific binding of RNA to oligo(dT)-cellulose resin by including yeast tRNA with their HeLa cell RNA preparations. A more indirect form of non-specific RNA binding to this resin can occur if poly(A)⁺ RNA and rRNA are allowed to aggregate before application of a total RNA sample to the resin (45). This results in indirect association of the rRNA with the resin, and contamination of the poly(A)⁺ RNA eluate with

rRNA. This problem can be eliminated by treatment of RNA preparations with dimethyl-sulfoxide and heating which disrupts RNA aggregates prior to loading on oligo(dT) cellulose columns. Heating of RNA preparations at 65-70°C. for a few minutes before application to affinity resins is often used to reduce rRNA contamination of poly(A)⁺ RNA preparations (47).

Recently, Ochoa et al. have studied the effects of ionic strength, salt type, pH and temperature on poly(A) binding to affinity resins, such as oligo(dT)-cellulose, in order to better understand the physical forces involved (48).

Despite the minor technical difficulties described above, efficient poly(A)⁺ and poly(A)⁻ RNA separation is obtained with oligo(dT)-cellulose. Passage of HeLa cell polysomal RNA through a single column efficiently separated poly(A)-containing RNA from poly(A)⁻ RNA, as measured by the presence of RNase-resistant polynucleotide in bound and non-bound fractions (49). Mercer and Naora obtained highly purified poly(A)⁺ RNA after a single passage of total RNA through oligo(dT)-cellulose; 95-98% of the poly(A)⁺ RNA rebound during a second passage through the column (40).

It is not surprising that the strength of poly(A) binding to oligo(dT)-cellulose appears to depend on the length of the poly(A) sequence. Elution of a rabbit globin poly(A)⁺ mRNA from oligo(dT)-cellulose with 0.1M to zero KCl gradient yielded a discontinuous elution of globin mRNA (50). Analysis of eluate fractions with a poly(A) assay (using [³H]-poly(U) -

see p.24) indicated a non-uniform poly(A) content; mRNA containing the larger poly(A) chains was eluted at lower ionic strength. Rhoads (47) partially fractionated ovalbumin poly(A)⁺ mRNA into molecules containing shorter and longer poly(A) sequences by stepwise thermal elution of the sample from an oligo(dT)-cellulose column. The poly(U) sepharose resin has been more widely used to separate mRNAs of different poly(A) chain lengths (see next section).

Oligo (dT)-cellulose can also be used to isolate poly(A)⁺ mRNP; the bound protein does not generally prevent poly(A): oligo(dT) interactions but it may alter optimal operating conditions (51). Occasionally, oligo(dT)-cellulose is used in batch-wise procedures rather than in columns (46). The ability of poly(U)-cellulose to separate poly(A)⁺ and poly(A)⁻ RNAs has been examined; it is less selective than oligo(dT)-cellulose and is less attractive for routine use since poly(U) is readily lost from the resin (40).

(ii) Poly(U) Sepharose

This resin has been used extensively in mRNA purification; hen oviduct ovalbumin and rat liver albumin mRNAs have both been purified by protocols including poly(U) sepharose chromatography (21, 26). More drastic conditions are required to elute poly(A)⁺ RNA from poly(U)-sepharose compared to oligo(dT)-cellulose. Mercer and Naora (40) found that "no salt" buffer

was unable to elute more than a small fraction of bound poly(A) from poly(U)-sepharose even at high temperatures (75°C.). A combination of high temperature and high salt (0.5M NaCl) did elute most of the bound poly(A). More effective recovery of poly(A) was obtained by washing the resin with solutions containing 90% formamide at room temperature. It is suggested that poly(A) and rRNA bind non-specifically to sites on the Sepharose other than poly(U), and this is responsible for the difficulty encountered in eluting bound RNA from this resin. This association is abolished only by high ionic strength and high temperature (consistent with the idea that poly(A) associates strongly with charged groups other than poly(U) in this resin) (40).

RNA samples are generally applied to poly(U)-sepharose columns in buffers containing 0.3-0.7M NaCl, and up to 1% SDS (52, 21, 26). Formamide concentrations of up to 25% have been included in the application buffer (52). Once the rRNA has been removed by washing, poly(A)⁺ RNA is eluted with 70-90% formamide-containing solutions (21, 52). Firtel et al. reported that poly(A)⁺ mRNA from Dictyostelium discoideum was eluted from poly(U) sepharose equally well by 70% formamide solutions at room temperature or by water at 50°C (53). Urea-containing solutions are also used occasionally to elute material from this resin (54). Phillips et al. have recently studied the specificity and sensitivity of poly(A) binding to poly(U) agarose; poly(U)-sepharose had essentially similar properties (55). Other polynucleotides (e.g. poly(C) and poly(G)) bound to the resin at 1% of the level of poly(A) under their

conditions. Studies using poly(A)/oligo(A) sequences of defined length indicated that sequences smaller than 10 nucleotides long will not selectively bind to the resin; very small oligo(A) sequences therefore cannot be retained and isolated by this method. Sequences of up to 20-30 nucleotides in length may not be retained by oligo(dT)-cellulose (56, 57) and in this respect, poly(U) sepharose would appear to be the resin of choice. ♡

Poly(U)-sepharose has been used on a number of occasions to fractionate poly(A)⁺ RNA into RNAs containing different lengths of poly(A). Bynum and Volkin fractionated HeLa cell RNA into six preparations containing from 0.8 to 19.5% poly(A) by stepwise elution using a discontinuous KCL gradient, 1% SDS and 90% formamide solutions (58). Thermal elution of rat liver poly(A)⁺ RNA from poly(U) sepharose in low salt yielded three RNA populations varying in poly(A) chain size (59).

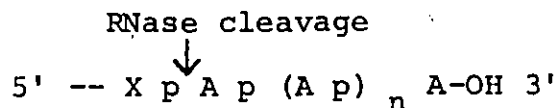
Other methods for isolating poly(A)⁺ RNA from total RNA preparations have been reported. Nitrocellulose filters (9) and poly(U)-impregnated fiberglass filters have been used (60). Greenberg and Perry (61) used a novel procedure, in which poly(U) was annealed with the poly(A) of total RNA and hybrid-containing RNA was isolated on hydroxylapatite columns which separate single- and double-stranded RNA. However, the high selectivity and ease of use of oligo(dT)-cellulose

and poly(U)-sepharose affinity resins makes them the best available method for isolation of poly(A)⁺ RNA from total RNA preparations. These resins are now routinely used in mRNA purification and RNA fractionation into poly(A)⁺ and poly(A)⁻ RNA.

CHEMICAL CHARACTERISTICS OF POLY(A)

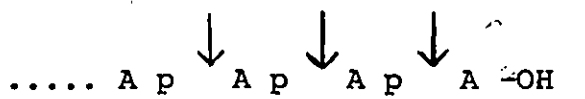
It is now clear that the poly(A) sequence is covalently attached to mRNA molecules; denaturing conditions alone are not sufficient to separate poly(A) from the mRNA molecule (62). Isolated poly(A) chains have been detected in mRNA preparations only after enzymatic treatment with RNase A and T_1 (5., 62). Solas et al (63) claimed the existence of "free poly(A) chains" in preparations of African Swine Fever virus RNA, but these chains had a very short average length (14 molecules) and I believe it possible that they were artefacts of degradation. Several approaches suggest that the poly(A) tract constitutes the 3' terminal section of the mRNA molecule.

Early evidence for a 3' terminal location of poly(A) came from alkaline hydrolysis of [^3H]-labelled poly(A) isolated from Xenopus laevis embryo mRNA by RNase digestion. Digestion of mRNA by RNases A and T_1 produces an intact poly(A) chain as indicated below:



The nucleotide adjacent to the 5' end of the poly(A) sequence is removed by RNase T_1 if it contains guanine or by RNase A if it contains uracil or cytosine. Alkaline hydrolysis, which attacks ribonucleotide sequences of the 3' phosphate residue to yield a mixture of 2' and 3' ribonucleo-

tide monophosphates, attacks poly(A) thus:



It is clear that since neither enzyme treatment nor alkaline hydrolysis are capable of producing ribonucleosides, any adenosine residues present in alkaline hydrolysates of RNase-isolated poly(A) sequences must have originated from the 3' terminal of the mRNA molecule. Dina et al. showed that alkaline hydrolysis of Xenopus laevis poly(A) yielded AMP and small levels of adenosine; they concluded that in this system poly(A) is 3'-terminal (61).

Exoribonucleases which act specifically at the 3' terminus have been used in studying poly(A) chain location. Molloy et al. (64) treated both L cell and HeLa cell mRNA preparations with 3'exoribonuclease and found that the rate of poly(A) hydrolysis was greater than the rate of hydrolysis of the rest of the molecule. This demonstrated that at least the bulk of the poly(A) was located nearer the 3' than the 5'-terminal. Polynucleotide phosphorylase, which can hydrolyse RNA in the 3'→5' direction, destroyed the poly(A) sequence of vaccinia and HeLa cell mRNAs quicker than the heteropolynucleotide portion of these molecules (65). Furthermore, the enzyme was active on RNase-isolated poly(A), indicating that this sequence contained a 3'-OH terminal (the enzyme activity requires a free 3' end).

[³H]- Labelling of the 3'-OH termini of RNA molecules can be achieved very specifically by periodate oxidation of the terminal followed by [³H]-labelled borohydride reduction (66 - 68). When HeLa cell mRNA and hnRNA were [³H]-labelled at their 3' termini by this method, a high proportion of the label was recovered in the poly(A) sequences obtained from these RNAs (69). These experiments suggested that while the majority of these poly(A) sequences were 3' terminal, a group of short poly(A) sequences, (about 20 residues in length, and present only in hnRNA) might not be located at the 3' end. These findings of short internal poly(A) sequences in hnRNA were later confirmed (70). This method of introducing [³H]-label at the 3'-OH termini of RNA molecules has also indicated a 3' end location for poly(A) chains of polio virus RNA (4) and mouse globin mRNA (71). Other researchers have introduced [32p]-label specifically at the 3' end of viral RNA and shown this to be incorporated into a 3' terminal poly(A) sequence (72). In this instance the [³²P]-label was introduced by ligation of [5'-³²P]-labelled CTP to RNA using T₄ RNA ligase.

Although poly(A) sequences are generally regarded as 3' terminal, occasional exceptions to this rule have been reported. The existence of short internal poly(A) in HeLa cell hnRNA has already been mentioned. Studies of poly(A) from Sendai virus mRNA have suggested that although this poly(A) tract

is located at the 3' terminal, the mRNA molecule is "masked" or "blocked" at the 3' end and is thus insensitive to polynucleotide phosphorylase degradation (73). It is possible that mRNA secondary structure may have caused this resistance to enzymatic attack.

The 3'- terminal location of poly(A) chains has enabled development of "in vitro" methods to specifically remove poly(A) from mRNA molecules. Purified polynucleotide phosphorylase, at appropriate temperature and pH, selectively removes poly(A) from poly(A)⁺ RNA and leaves the heterogeneous portion of the RNA molecule intact (74, 75). In an alternate method, the poly(A) chain is annealed to oligo(dT); the resulting hybrid can be selectively removed by an RNase specific for RNA-DNA hybrids, e.g. RNase H (76).

It is also possible to synthesise poly(A) chains onto RNA 3' termini in vitro using ATP and the enzyme ATP: polynucleotidylexotransferase (77, 78). Such in vitro methods for poly(A) removal from mRNA and poly(A) addition to RNA primers have proved to be valuable tools in the study of poly(A) function (see "Possible Functions of Poly(A)").

RNase T₂ digests of poly(A) chains usually consist only of adenosine monophosphate and small amounts of adenosine. The presence of a modified nucleotide has been detected in one instance, i.e. in maize poly(A)⁺ RNA (79). This modified nucleotide was present as a dinucleotide which resisted

RNAse T₂ digestion, and it was tentatively identified as an adenylic acid derivative containing 2'-O-methyl ribose. This modified nucleotide was in an internal position in the poly(A) chain.

This last observation emphasises one potential criticism of the RNAse digestion method used for poly(A) sequence isolation. If poly(A) sequences contained single nucleotides or oligonucleotides of guanine, cytosine or uracil within their structure, these nucleotides would be removed during digestion and long poly(A) sequences interspersed with other nucleotides would be detected as smaller homogeneous sequences. At present there appears to be no satisfactory means of overcoming such a difficulty. In a later section the presence of oligo(U) tracts in certain mRNAs will be discussed.

POLY (A) TRACT SIZE

One of the earliest methods used to determine the size of poly(A) tracts was to determine the fraction of the mRNA molecule consisting of poly(A). Poly(A) can be detected and quantitatively assayed by its resistance to RNase degradation or by hybridization with [^3H]-poly(U) (described below). The size of a mRNA molecule may be determined readily by gel electrophoresis or sedimentation. From a knowledge of the proportion of poly(A) in the mRNA molecule and mRNA size, the poly(A) tract length can be estimated. By this method, the poly(A) tract of purified Encephalomyocarditis viral RNA was estimated to be 38 nucleotides long (80) and several preparations of human reticulocyte globin mRNA were shown to contain poly(A) tracts up to 120 nucleotides long (81). The drawback to this method is that only an average poly(A) size can be determined for each mRNA preparation and no indication of poly(A) size range is provided.

Other methods of sizing rely on isolation of poly(A) tracts from mRNAs using RNases A and T_1 ; these two enzymes cleave phosphodiester bonds of polynucleotides at pyrimidines and guanines, and therefore treatment of poly(A)⁺ RNA with a mixture of these enzymes digests the entire molecule except for the poly(A) tracts. After such digestion of poly(A)⁺ mRNA preparations, the remaining poly(A) tract may be isolated and

sized by standard procedures. RNase digestion of poly(A)⁺ mRNA is performed at 37° C at high ionic strength (0.1-0.3M NaCl); a relatively high salt concentration is needed to maintain enzyme specificity and prevent poly(A) degradation (82, 83). Poly(A) tracts have been recovered from the digestion mix by ethanol precipitation using E. coli tRNA as carrier (82) (84) or by phenol/chloroform extraction in 1% SDS followed by gel filtration (85). In some instances, poly(A) chains have been isolated by passing the digestion mix through oligo(dT)-cellulose or poly(U)-sepharose affinity columns (83, 86). One criticism of this method of recovery is that it might result in loss of any short poly(A) tracts (less than 15-20 nucleotides) released by the digestion; they would not be retained by these resins and would be lost from the poly(A) tract preparation.

Alkaline hydrolysis of isolated poly(A) tracts causes release of adenosine monophosphate and small amounts of adenosine representing the 3' terminal residue (see p. 15). Since each poly(A) chain can yield only one adenosine residue on hydrolysis, the molar ratio of adenosine monophosphate: adenosine is a measure of average chain length. Using this method, yeast mitochondrial RNA was shown to bear poly(A) chains of average length 20-28 nucleotides (86) and Xenopus laevis embryonic mRNA contained poly(A) sequences of 75-100 nucleotides (6). The drawback to this approach is that it yields only average values.

Darnell et al. assessed the actual distribution of HeLa cell poly(A) tract length by sucrose gradient centrifugation (32). The most practical method for sizing of poly(A) tracts, and the one now usually employed, is polyacrylamide disc gel electrophoresis. This procedure has been fully described elsewhere (85, 84). A potential hazard in the assignment of size to poly(A) tracts on the basis of their mobility on polyacrylamide gels is the finding that poly(A) exhibits anomolous behaviour on such gels compared to heteropolynucleotides such as 4S and 5S RNAs, i.e. poly(A) migrates much more slowly than RNA of a similar size (87, 88). Accurate estimates of the length of poly(A) tracts therefore require comparison with synthetic poly(A) sequences of known length, and if 4S or 5S RNAs are used as size markers a considerable adjustment is necessary, otherwise the length of the poly(A) tracts will be overestimated (88). Early measurements of poly(A) tract size did use heteropolynucleotides as size markers (7, 8, 30), and the accuracy of these results is now in question.

In these sizing experiments, poly(A) detection is facilitated by the incorporation of radioisotope into poly(A) prior to electrophoresis. Several methods by which radioisotope may be introduced into poly(A) are described in the following section.



METHODS FOR DETECTION OF POLY(A)

In general, the amounts of poly(A) sequence available from mRNA preparations are too small to be detectable by ultra violet absorbance at 260nm. This problem is usually overcome by introducing radiolabel into the poly(A) either in vivo or in vitro.

Vournakis et al. (89) isolated ^{32}P -labelled poly(A) from silkworm chorion protein mRNA after in vivo ^{32}P -labelling of RNA; poly(A) sequences of 10-60 nucleotides were detected.

In vivo ^3H -labelling of globin mRNA poly(A) using [^3H]-adenosine has also proved successful (83). When the poly(A) sequence is isolated from unlabelled mRNA, there are several ways to introduce a radioisotope label into the poly(A) chain in vitro.

The periodate oxidation - borohydride reduction method has already been mentioned as a useful method for introducing [^3H]-label at the 3' end of a poly(A) tail. Mondal et al (90) used this procedure to label poly(A) of chick embryo myosin heavy chain mRNA; this sequence was found to be 170 nucleotides long. Poly(A) sequences may also be labelled at their 5' terminus; Negruk et al. introduced ^{32}P -label into poly(A) from Barley Stripe Mosaic Virus RNA using [γ - ^{32}P]-ATP and polynucleotide kinase. This poly(A) ranged in size from 8 to 30 residues (91). [^{32}P]-label can be introduced at the 3' terminal of RNA using T_4 RNA ligase and [$5'$ - ^{32}P]pCp (72);

this label is resistant to removal by RNase digestion. Arendes et al. introduced [^{3} H]-label into isolated poly(A) tails by methylation with [^{3} H]-dimethyl sulfate; it was claimed that methylated poly(A) migrated in an identical manner to non-methylated poly(A) and by this method poly(A) of rat liver mRNA and mouse L5178Y cell mRNA was 150 nucleotides long (92).

A widely used method for detection of poly(A) sequences, and one which can be used in conjunction with poly(A) sizing, is the [^{3} H]-poly(U) assay, first described by Gillespie et al. (93) and later developed by Bishop et al. (94). In this assay, poly(A) is allowed to anneal with a molar excess of [^{3} H]-poly(U); unhybridized [^{3} H]-poly(U) is then removed by digestion with RNase A. Residual hybrids are precipitated, filtered and measured by liquid scintillation counting. Since [^{3} H]-poly(U) is present in excess, all the poly(A) in the assay is hybridized, and the amount of [^{3} H]-label protected from RNase digestion is a direct quantitative measurement of the levels of poly(A) present. Under the annealing conditions of this assay both poly(rA) and poly(dA) chains are detected. For example, Bishop et al. (94) noted low levels of binding of poly(U) to duck DNA, presumably due to the presence of poly(dA) sequences, and Gillespie et al. (93) have suggested that RNA preparations containing significant amounts of DNA should be treated with DNase as well as RNase during the poly(U) assay so as to eliminate any contribution of the DNA to poly(rA) measurements.

Since the development of the [^3H]-poly(U) assay, it has been used extensively in poly(A) research. It has found application in determining the in vivo subcellular location of poly(A) sequences (in situ hybridization (95)) and in quantitative measurements of poly (A) in RNA preparations (24) (76). Its ease of use makes it particularly useful for detection of poly(A) in sizing experiments (after gel electrophoresis and elution of gel slices), it has the further advantage that it does not require the introduction of label until after electrophoresis and thus the size of the poly(A) molecules is unaffected by the introduction of radioisotope. In lieu of in vitro labelling of poly(A), the [^3H]-poly(U) assay is the method of choice for detection of poly(A) sequences.

Poly(A) Shortening In Vivo

The studies on poly(A) sizing described above show that this mRNA characteristic can vary in length widely from one species to another. A further complication which has been discovered from such studies is that poly(A) size can change during the lifetime of a mRNA molecule. Pulse-labelling and pulse-chase labelling of HeLa cell mRNA using [^3H]-adenosine suggested that once a mRNA molecule reaches the cell cytoplasm, its associated poly(A) sequence is continually shortened (96). Most of the evidence supporting poly(A) shortening

has come from studies on globin mRNA.

Gorski et al. found that globin mRNAs exhibited different affinities for oligo(dT)-cellulose and millipore filters and that this was due to poly(A) heterogeneity (56).

Three poly(A) size classes of lengths 35-45, 55-65 and 75-120 nucleotides were detected. 95% of their mRNA preparations contained poly(A) chains with lengths of 65 nucleotides or less (56, 97). The kinetics of in vivo [³²P]-labelling of mouse globin mRNA indicated that the smaller poly(A) chains originated from the larger ones by a shortening process (98). Merkel et al. found that while poly(A) chains from newly synthesised globin mRNAs of nucleated erythroid mouse spleen cells were 150 molecules in size, those of globin mRNAs from circulating reticulocytes were only 40-60 nucleotides long (83). Similar observations were made for rabbit globin mRNA, although the actual size of the poly(A) sequences were different (85). These results suggest that poly(A) shortened with age. Other studies on the poly(A) of "old" and "new" mRNAs of different organisms also support this hypothesis, e.g. Physarum (99), Chironomus (100) and yeast (101, 102). Egyhazi et al. (100) proposed that poly(A) shortening was not age-dependent but was a stochastic process.

There is evidence to suggest that poly(A) shortening

is a discrete process involving endonucleolytic removal of poly(A) (103), possibly in 10-20 nucleotide increments (104). It is also possible that some poly(A) shortening occurs by an exonucleolytic process (see p.38 for description of a poly(A)-specific exonuclease).

Although Sheiness and Darnell (96) initially suggested that HeLa cell mRNA poly(A) shortening was not prevented by mRNA translation inhibition, they later reported that the translation inhibitor emetine inhibits both mRNA turnover and poly(A) shortening (103). It was therefore inferred that poly(A) shortening could be a consequence of mRNA translation. Amino acid deprivation of a strain of Neurospora crassa to the extent that protein synthesis within the organism was severely restricted, resulted in a population of poly(A)⁺ mRNAs with larger and more heterogeneous poly(A) sequences compared to those of growing control cell mRNAs. It was again concluded that poly(A) chain shortening might be associated with mRNA translation (105).

Poly(A) shortening appears to be a common occurrence and probably occurs in most living systems. The significance of Poly(A) size and shortening and their relation to poly(A) function will be discussed later. (see "Possible functions of poly(A)").

THE EXISTENCE OF NON-POLYADENYLATED mRNA

Research by Darnell and associates in the early 1970s on HeLa cell mRNA suggested that all mRNAs of these cells, with the sole exception of histone mRNAs, bore a poly(A) tail (38). The subsequent finding that poly(A) sequences were associated with a large number of mRNAs of other tissues and species strengthened the hypothesis that poly(A)⁺ RNA and mRNA were synonymous; poly(A)⁻ RNA was thought to consist entirely of non-messenger RNA (e.g. rRNA, tRNA) and the histone mRNA sequences.

It has since been recognized that poly(A)⁻ mRNA does exist. It is defined as mRNA which does not bind to oligo(dT)-cellulose or poly(U)-sepharose affinity resins and which therefore lacks poly(A) sequences longer than 10-20 nucleotides in length. This absence of poly(A) has, in some cases, been verified using the [³H]-poly(U) assay. The possible existence of poly(A)⁻ mRNA in HeLa cells was first suggested by the pattern of [³H]-adenosine incorporation into poly(A)⁺ and poly(A)⁻ RNA under conditions which inhibited rRNA synthesis. Milcarek et al. (106) found that poly(A)⁻ RNA and poly(A)⁺ RNA were labelled with similar kinetics, and had similar sedimentation profiles; furthermore poly(A)⁻ RNA was associated with polyribosomes but behaved like mRNA in being released from polyribosomes by EDTA or puromycin

treatment. It was concluded that up to 30% of HeLa cell mRNA was not polyadenylated, and this fraction was too large to consist solely of histone mRNAs. Since cDNA prepared from poly(A)⁺ HeLa cell mRNA did not hybridize significantly with poly(A)⁻ RNA, it appeared that these two mRNA populations contained essentially different sequences.

Although there were valid objections to the work of Milcarek et al which stemmed from the use of 5-fluorouridine to specifically inhibit rRNA synthesis, poly(A)⁻ mRNA was also detected in mouse L cells (107) and in sea urchin embryos (108) by different approaches.

The sea urchin embryo has been a useful system for study of poly(A)⁻ mRNA, since after fertilization mRNA synthesis is initiated but rRNA synthesis is delayed. Pulse chase labelling experiments therefore result in labelling of poly(A)⁺ and poly(A)⁻ mRNA, but not rRNA. These studies showed that sea urchin embryos contain two forms of non-histone mRNA, poly(A)⁺ and poly(A)⁻, and these mRNAs have similar turnover rates (108). Studies on whether or not similar sequences are contained in poly(A)⁺ and poly(A)⁻ mRNAs of sea urchin embryos have been performed by two methods; hybridization of both mRNA preparations to cDNA prepared against poly(A)⁺ mRNA and electrophoretic analysis of the in vitro translation products of both mRNA preparations.

Two studies, by the first and second methods respectively, indicated that poly(A)⁺ and poly(A)⁻ mRNAs consisted of essentially different sequences (108, 109), while two other

investigations suggested that there was complete sequence overlap between these mRNA populations (110, 111). Contradictory results have been obtained in other systems too, e.g. HeLa cells. Kaufmann et al. (112) have suggested that the interpretation of hybridization measurements between cDNA (prepared from poly(A)⁺ RNA) and both poly(A)⁺ and poly(A)⁻ mRNAs is unclear; qualitative comparisons of these populations are therefore best performed by analysis of their translation products. By this method, Kaufmann et al. showed that a small number of proteins are produced only by poly(A)⁻ mRNA, a larger number are made by both mRNA populations and most proteins are produced by poly(A)⁺ mRNA only (112). This result has since been supported by Milcarek (113).

Poly(A)⁻ mRNA has now been identified in many systems, particularly in mammalian tissues, but mRNA population studies have yielded no clear trend with respect to the distribution of coding sequences between poly(A)⁺ and poly(A)⁻ mRNAs. Extensive and possibly complete overlap of coding sequences between poly(A)⁺ and poly(A)⁻ mRNAs was reported for mouse kidney (114) and AKR mouse embryo cells (115). Specific sequences did not necessarily occur with similar frequency in the two populations. Moffet and Doyle (116) divided mouse liver mRNA sequences into three

classes: those specific to poly(A)⁺ mRNA, a small number specific to poly(A)⁻ mRNA and those which are found in both mRNA populations. Translation studies suggested a similar pattern in rat ascites hepatoma cells (117) and in rat brain (118). Up to 60% of pea seedling leaf polysomal mRNA was poly(A)⁻ (119) and poly(A)⁺ and poly(A)⁻ mRNAs of pea seedling translated into similar major polypeptides. Cellular slime mold mRNA existed in poly(A)⁺ and poly(A)⁻ forms; there was much sequence overlap between these mRNA populations with actin as a major translation product of both (12).

Several highly abundant mRNAs coding for specified proteins exist in poly(A)⁺ and poly(A)⁻ forms. Up to 60% of lactating rat mammary gland casein mRNA was reported to be poly(A)⁻ (42) and α -lactalbumin mRNA existed in poly(A)⁺ and poly(A)⁻ forms in the lactating human mammary gland (120). Only 70% of hen oviduct ovalbumin mRNA was retained by poly(U)-sepharose, i.e. 30% of this mRNA was poly(A)⁻ (21). Similarly 20-25% of rat liver albumin mRNA was poly(A)⁻ (26). The parsley plant mRNA coding for phenylalanine ammonia lyase has also been reported to exist as poly(A)⁺ and poly(A)⁻ mRNA (121).

Studies on the polyadenylation of trout testis protamine mRNA are of special interest since protamines replace histones in the chromatin of trout sperm cells during development. Three different protamine polypeptides have been isolated

and all three were encoded in poly(A)⁺ and poly(A)⁻ mRNAs (22, 122). A cDNA probe, prepared against highly purified poly(A)⁺ protamine mRNA, was used to measure the distribution of poly(A)⁺ and poly(A)⁻ forms of the mRNA in three subcellular compartments of trout testis cells: nuclei, polysomes and postribosomal supernatant (123). Poly(A)⁻ protamine mRNA constituted less than 10% of total cellular protamine mRNA. In the nucleus, almost half of the protamine mRNA sequences were poly(A)⁻, possibly due to the presence of incompletely processed sequences. In the cytoplasm, levels of poly(A)⁻ protamine mRNA were much lower than those of the poly(A)⁺ form (<10%) but while the latter was distributed evenly between polysomes and postribosomal supernatant, the poly(A)⁻ form was almost entirely in the polysomes. Purification of poly(A)⁻ protamine mRNA has been achieved (76). Protamine mRNA is one of the few mRNAs which has been purified in a poly(A)⁻ form.

It is worthy of note that although histone mRNAs are generally nonpolyadenylated, reports of the existence of poly(A)⁺ histone mRNAs in amphibian oocytes (123a, 123b) and chick erythrocytes (123c) have been made.

The significance of the existence of poly(A)⁻ mRNA is quite unclear. Since poly(A) chains associated with several mRNAs are shortened with time, it has been suggested that poly(A)⁻ mRNA arises from poly(A)⁺ mRNA by complete loss of the poly(A) sequence. In agreement with this,

Zimmerman et al. (124) have reported that Drosophila poly(A)⁻ mRNA is present in a polyadenylated form in the nucleus. Iatrou and Dixon (123) have proposed that all protamine mRNA is initially polyadenylated but during translation, the poly(A) sequence is gradually shortened until it is eventually lost and then the resulting poly(A)⁻ mRNA leaves the polysomes and is probably rapidly degraded. This would explain some of their findings with respect to the sub-cellular location of poly(A)⁺ and poly(A)⁻ protamine mRNA.

This theory would not explain the existence of mRNA sequences which predominantly exist in a poly(A)⁻ form, (e.g. moderately abundant sequences in lactating guinea-pig mammary gland (125)), and here it is necessary to postulate that mRNAs other than histone mRNAs can remain nonadenylated in the nucleus and enter the cytoplasm in this form. What mechanisms operate to differentiate the mRNAs to be polyadenylated must remain speculative at this time. However, several of the findings described above indicate that poly(A)⁻ mRNA is likely to be an important component of total mRNA and suggestions that poly(A)⁻ mRNA is a non-functional intermediate in poly(A)⁺ mRNA degradation cannot be fully justified. Possible relationships between poly(A)⁺ and poly(A)⁻ mRNAs are discussed further in a recent review (126).

POLY(A) POLYMERASE AND SYNTHESIS OF POLY(A) SEQUENCES

The existence of an enzyme capable of synthesising long poly(A) sequences was first demonstrated in calf thymus nuclei (2). This report detailed partial purification of an enzyme (now named poly(A) polymerase) which catalysed the synthesis of adenine polynucleotides linked by 5'-3' phosphodiester bonds from ATP. Purified calf thymus nuclear poly(A) polymerase had a molecular weight of 150,000 daltons and exhibited a marked preference for ATP as substrate over other nucleotide triphosphates. It was activated by Mg^{2+} and Mn^{2+} (127). In vitro the enzyme added poly(A) sequences of 100 to 200 residues to several polynucleotide primers including poly(A), tRNA and any RNA fragment bearing a free 3' terminal. DNA was not used as a primer for poly(A) synthesis (128). A more detailed description of the poly(A) polymerase reaction, its primer requirements and properties of the purified enzyme are provided in a review article (129).

The poly(A) polymerases of several other species, including E. coli, yeast, Krebs ascites cells and rat liver, have also been examined (129), and there are clear differences in size and activity between these enzymes. Nuclear poly(A) polymerase of rat liver and hepatoma has been extensively studied, and interestingly the enzyme of the normal and diseased tissue differed in size and reaction kinetics; it

appeared that in the diseased state, there was an alteration in the activity of poly(A) synthesis (130). Studies have suggested that rat liver poly(A) polymerase activity could be regulated by polyamines (131), phosphorylation of the enzyme (132) and by binding or non-binding of the enzyme to chromatin (133). Rabbit poly(A) polymerase activity has been shown to be stimulated by in vivo treatment of rabbits with the steroid hormones estrogen and progesterone (134); this increase was believed to be due to an increase in enzyme synthesis as well as stimulation of pre-existing enzyme (135). This small number of reports already indicates that complex control mechanisms may operate to regulate poly(A) polymerase activity. In view of the interest in the biological significance of poly(A) sequences, further research on the control mechanisms operating on poly(A) polymerase and poly(A) synthesis should be most productive.

Although it was presumed that poly(A) polymerase should be found only in nuclei of eukaryotic cells, since poly(A) synthesis was thought to occur exclusively in the nucleus, reports of cytoplasmic poly(A) polymerase have become numerous (129). Much of the support for cytoplasmic polyadenylation came from studies of polyadenylation in fertilised eggs. Immediately after fertilization of a sea urchin egg, the poly(A) content of the egg increased twofold but little RNA synthesis occurred at this time,

and it appeared that poly(A) was added onto pre-existing mRNA molecules which had been synthesised and stored in the cytoplasm prior to fertilization (136 - 138). Enucleation of sham-fertilized sea urchin eggs did not prevent this poly(A) synthesis, therefore the rise in poly(A) content of the egg was independent of transcription (139, 136). Cytoplasmic fractions of both unfertilised sea urchin eggs and four cell embryos were capable of incorporating ATP into poly(A) sequences covalently attached to RNA primers; this observation is the most direct evidence yet obtained in support of cytoplasmic poly(A) polymerase in sea urchin eggs (140).

There is evidence to support a poly(A) elongation process distinct from de novo synthesis in the cytoplasm and nuclei of mouse sarcoma cells (141). Two distinct cytoplasmic poly(A) polymerases, one present free in the cytosol and the other associated with ribosomes, have been isolated from Ehrlich ascites tumour cells (142). A poly(A) synthesising activity was identified in wheat germ cytoplasmic lysates (143).

The mRNAs of several viruses whose life-cycle occurs entirely in host cell cytoplasm, bear poly(A) sequences, e.g. reovirus (144). Vesicular stomatitis Virus (VSV) mRNA bears poly(A) chains of up to 200 nucleotides in length (145) and synthesis of this poly(A) is apparently dependent on a cytoplasmic host cell poly(A) polymerase. The sig-

nificance of cytoplasmic forms of poly(A) polymerase with respect to poly(A) function is discussed later (see "Possible Functions of Poly(A)").

The precise signal(s) indicating the site of polyadenylation of a nuclear mRNA precursor has not been elucidated, but specific nucleotide sequences occurring in the polyadenylation region may play a role. The mRNAs coding for rabbit and human α - and β -globulin, mouse immunoglobulin light chain, chicken ovalbumin, rabbit and human α - and β -globin all contained the hexanucleotide sequence 5'-AAUAAA-3' (146). In each case this sequence was 20 residues upstream of the poly(A) attachment site, and its conservation and common position in all these mRNAs would indicate a possible function in processing events such as polyadenylation (147, 148). Other workers have reported the presence of the AAUAAA sequence in adenovirus mRNAs (149, 150), SV40 mRNA (151) and bovine growth hormone mRNA (152). Strong evidence for the involvement of this hexanucleotide sequence has come from studies on the polyadenylation of mutated SV40 mRNAs (153). Deletion mutations in the 3' non-coding region between the -AAUAAA - sequence and the normal poly(A) site resulted in movement of the polyadenylation site so that polyadenylation always occurred 11-19 nucleotides away from the hexanucleotide AAUAAA-. Deletion of a 16 residue sequence, including part of this hexanucleotide, prevented polyadenylation. More recent studies on the possibility

that the -AAUAAA- sequence is a polyadenylation signal are contradictory. Cole and Santangelo (154) support this hypothesis but polyadenylated mRNAs lacking this sequence have been detected for chicken histone H5 (155) and mouse dihydrofolate reductase (156). Other poly(A) addition signal sequences have been proposed; all are rich in uridylic acid (157, 158).

Although the enzyme responsible for poly(A) synthesis is now well characterized in many systems, it is clear that the precise signals controlling the cellular location, timing and RNA location of polyadenylation are not understood. It is of interest in this respect that the existence of several enzymes, which preferentially degrade poly(A) chains, has been reported. Two ribonucleases which specifically degrade poly(A) have been purified from guinea pig adrenal cells (159). Three poly(A) degrading enzymes were found in calf thymus nuclei (160); one was an exoribonuclease which degraded single- or double- stranded poly(A) in preference to heteropolynucleotides (161) and the other two were endoribonucleases (160, 162). Phosphorylation of a calf thymus poly(A) endoribonuclease has been shown to stimulate this enzyme activity (163). Poly(A)-specific endoribonuclease IV and poly(A) polymerase of calf thymus have been reported to exist as a complex together with G-actin and tubulin; these proteins both inhibited the enzyme activities (160).

The existence of complexes of poly(A) synthesising and degrading activities clearly provides potential for fine control of poly(A) metabolism; this is likely to be especially important during cell growth and development (see "Poly(A), development and aging"). Further studies on the inter-relationships between the enzymes involved in poly(A) metabolism and the control mechanisms operating on these enzymes are needed since these may provide a better understanding of poly(A) function.

POSSIBLE FUNCTIONS OF POLY(A)

A great deal of research and effort has been directed towards the elucidation of the function of poly(A). Processes likely to be influenced by poly(A) are nucleocytoplasmic transport of RNA, mRNA translation and mRNA stabilization. Evidence for and against these possible roles of poly(A) will be discussed in this section.

(i) Nucleocytoplasmic transport and/or nuclear processing of RNA

Most of the evidence supporting a transport function has been provided by studies on HeLa cell mRNA metabolism. As early as 1971, HeLa cell labelling studies indicated a nuclear site of poly(A) synthesis followed by transfer of this poly(A) attached to mature mRNA into the cytoplasm (31). This sequence of events prompted the suggestion that poly(A) chains might be actively involved in either selection of the RNA sequences destined to be transported into the cytoplasm and/or the transport process itself.

Salditt-Georgieff et al. reported that most HeLa cell hnRNA molecules are polyadenylated promptly after synthesis (164). It had already been shown that polyadenylation of adenovirus 2 RNA in host cell nuclei preceded the splicing events which convert this large molecule into smaller mature mRNAs (165). It was suggested that there was a causal

relationship between polyadenylation and processing (e.g. splicing) of RNA. In 1980, Bina et al published a theoretical paper which proposed that poly(A) might facilitate processing and splicing of RNA by promoting formation of triple-stranded structures within the mRNA precursor and thereby aligning splicing sites in preparation for cleavage and ligation of the molecule (166). There is now mounting evidence that poly(A) is not required for RNA processing. Zeevi et al. reported that although polyadenylation usually precedes splicing nuclear adenovirus 2 RNA splicing can occur in the absence of poly(A) synthesis (167). Spohr et al. detected nonadenylated but shortened nuclear RNA precursors of globin mRNA in immature duck erythrocytes; this suggests that in this system polyadenylation is preceded by certain processing events (168).

One of the earliest suggestions regarding poly(A) function was made by Adesnik and Darnell (29). On the basis that histone mRNAs were the only HeLa cell mRNAs known to lack poly(A) and these mRNAs were able to exit from the cell nucleus more quickly than other poly(A)⁺ mRNAs, they suggested that the poly(A) chain played a role in nucleocytoplasmic transport. This idea was supported by the observed labelling kinetics of HeLa cell RNA, which were reported to indicate that all cytoplasmic poly(A) originated from the nucleus and that all poly(A) was transported to the cytoplasm (169). Perry et al. claimed that there was substantial nuclear turnover of HeLa cell poly(A)⁺ hnRNA

and not all nuclear poly(A)⁺ RNA entered the cytoplasm (170).

Some of the strongest evidence supporting a transport function for poly(A) resulted from studies on the effect of the drug cordycepin (3'-deoxyadenosine). This drug was found to inhibit synthesis of HeLa cell mRNA but not hnRNA synthesis in these cells (33). This action was later suggested to be due to specific inhibition of poly(A) synthesis (the drug is an adenosine analogue) and a correlation between inhibition of poly(A) synthesis and reduced appearance of mRNA in the cytoplasm during cordycepin treatment supported the idea of a nucleocytoplasmic role for poly(A) (31, 34). Similar conclusions were drawn from in vitro studies of the effect of cordycepin on rat liver nuclei (171).

Studies on nucleocytoplasmic transport of a 75S RNA synthesised from Balbiani rings of Chironomus tentans have indicated that the polyadenylated form of this RNA exited into the cytoplasm rapidly, but the nonadenylated form remained in the nucleus for long periods and never entered the cytoplasm (172). The author concluded that polyadenylation of the 75S RNA transcript was essential for its transport into the cytoplasm.

In spite of these observations, several studies suggest that polyadenylation itself is not sufficient to ensure nucleocytoplasmic RNA transport. Comparisons of the complexity of cytoplasmic and nuclear poly(A)⁺ RNAs in Xenopus

liver and mouse Friend cells indicated that not all poly(A)⁺ RNA sequences exit from the nucleus (173, 174). Interpretation of the results obtained with cordycepin is dependent on the specificity of this drug and this specificity has been questioned by Maale et al. (175). These authors claimed that the primary effect of cordycepin was inhibition of hnRNA synthesis and not inhibition of poly(A) synthesis as reported by Darnell et al. (31). Finally, Zeevi et al. have shown that nonadenylated forms of nuclear adenovirus type 2 RNA leave the nucleus with a similar exit time to adenylated forms, and they concluded that poly(A) does not have a role in this transport process (176). Other factors which make this hypothesis unattractive is the discovery of poly(A) tracts in association with mRNAs that never pass through nuclear membranes (ie. prokaryote mRNA and the mRNAs of viruses which restrict their life cycle to the host cell cytoplasm). The existence of cytoplasmic forms of poly(A) polymerase (see p.35) also argue against an exclusively nuclear role of poly(A) sequences.

Although there is now little evidence in support of nucleocytoplasmic transport of RNA as a general function of poly(A), it is possible that poly(A) does modulate this process in some situations, e.g. balbiani ring RNA. The only recent evidence for a transport role of poly(A) came from a report that the nuclear-membrane enzyme, nucleoside

triphosphatase, which may supply the energy requirement for nucleocytoplasmic transport, was stimulated by poly(A) and poly(A)⁺ mRNA but not by poly(A)⁻ RNA (177). The significance of this report remains to be clarified and recent experimentation on poly(A) function has concentrated on possible effects of poly(A) on mRNA translational efficiency and stabilization.

(ii) Efficiency of Translation

Early research on the translation of poly(A)⁺ and poly(A)⁻ RNA samples (see "Nonadenylated mRNA") showed that the presence of a poly(A) tract was not essential for mRNA translation in vitro. This result has been supported by experiments on specific mRNAs in which the translation of poly(A)⁺ native and synthetic poly(A)⁻ mRNAs was compared. Poly(A)⁻ mRNA was prepared by RNase H digestion of the corresponding poly(A)⁺ mRNA after hybridization to poly(dT) (see p.18). Using this method, poly(A)⁺ and poly(A)⁻ forms of rabbit globin mRNA (178) and poliovirus mRNA (179) exhibited similar in vitro translational efficiencies. Deadenylated mouse L cell mRNA, prepared by limited 3'-exoribonuclease digestion of L cell poly(A)⁺ mRNA, had a similar template activity in vitro compared to its native undigested form (180). Nevins and Joklik isolated naturally occurring vaccinia virus poly(A)⁻ mRNA and showed that in vitro this RNA was utilised as efficiently as the poly(A)⁺ form (181).

Poly(A)-rich and poly(A)-poor rat liver mRNAs, prepared by differential oligo(dT)-cellulose chromatography and bearing poly(A) tracts of average lengths 173 and 40 nucleotides respectively translated equally well in both the wheat germ cell-free system and in Xenopus laevis oocytes (182). This result suggested that over the size range examined poly(A) tract length has little effect on mRNA translation.

Munoz and Darnell reported an experiment in which in vitro translation of a native poly(A)⁺ mRNA was compared with that of poly(A)⁺ mRNA which had been hybridized to poly(U) to form a poly(A): 2 poly(U) structure at the 3' terminal (183). No differences in translational activity between the two mRNA preparations were observed, and it was concluded that poly(A) played no role in the translation process.

Two other reports gave opposite results. Deadenylated forms of mouse globin mRNA (184) and hen oviduct ovalbumin mRNA (185) did not translate as efficiently as their polyadenylated counterparts; translational efficiency was reduced by up to 50% on loss of poly(A). In both studies, deadenylated mRNA was prepared from poly(A)⁺ mRNA by limited digestion with polynucleotide phosphorylase and evidence was presented suggesting that while poly(A) tract removal was complete, the coding portion of both types of mRNA was left intact.

Attempts to assess the relative translational efficiency of poly(A)⁺ and poly(A)⁻ mRNAs in vivo have involved measurements of the subcellular distribution of the two forms of the mRNA. In neuroblastoma cells both polysomal and non-polysomal mRNAs consisted of 70-75% poly(A)⁺ RNA and the remainder was poly(A)⁻ (186). There was therefore no preferential enrichment of poly(A)⁻ mRNA in either subcellular fraction. Iatrou and Dixon (123) observed preferential enrichment of poly(A)⁻ protamine mRNA in trout testis polysomal RNA compared to the postribosomal component. Both studies indicated that on the basis of subcellular location poly(A)⁻ mRNA was translated with equal or even greater efficiency than poly(A)⁺ mRNA.

In summarizing these translation studies, there is only very limited evidence to suggest that poly(A)⁺ and poly(A)⁻ mRNAs are translated with different efficiencies, and most reports indicate there is no difference at all. However, two in vitro studies suggested there may be long-term differences between the translation of poly(A)⁺ and poly(A)⁻ mRNAs. A comparison of poly(A)-rich and poly(A)-poor EMC viral mRNA translation in Ehrlich ascites cell-free extracts indicated that differences in translation of the two mRNAs became apparent only later in the reaction (187). It was proposed that poly(A) might function to stabilize mRNA molecules and this idea was supported by a study involving preincubation of poly(A)⁺ and deadenylated globin mRNAs in

a Krebs II ascites translation system (178). It appears likely that an effect of poly(A) on mRNA translation is due to functional stabilisation of the molecule rather than any direct effect on the rates of initiation, elongation and termination,

(iii) mRNA Stabilization

Some of the most convincing experiments supporting a poly(A) role in mRNA stabilization are based on comparisons of the translational capacities of poly(A)⁺ and poly(A)⁻ mRNAs in X. laevis oocytes. This work has been performed by two collaborating groups from Belgium (Huez et al.) and Israel (Soreq et al.).

Most of this work has utilised rabbit globin mRNA in its native poly(A)⁺ form and in a deadenylated (poly(A)⁻) form produced by limited digestion of poly(A)⁺ mRNA by polynucleotide phosphorylase (188). These poly(A)⁺ and poly(A)⁻ mRNAs were initially translated in a Krebs II ascites tumour cell-free system. Initially the two mRNAs were functionally comparable but later in the reaction the poly(A)⁻ mRNA was not as active as the poly(A)⁺ mRNA (189, 190). A major limitation of cell-free systems such as that from Krebs ascites cells is that they will support translation for only 1-2 hours (189). Since it was felt necessary to examine the translation of these mRNAs for longer time periods than 2 hours, these studies were continued using the in vivo translation system provided by intact Xenopus laevis oocytes. This system will support translation of mRNAs injected into the oocyte for

several days (189); e.g. poly(A)⁺ globin mRNA was translated, as measured by incorporation of [³H]-histidine into globin polypeptide, at a high level for over two days. Two days after introduction of poly(A)⁻ globin mRNA into oocytes, this mRNA was translated at a rate over 10 fold lower than that of poly(A)⁺ globin mRNA, even though initially the two mRNAs were translated equally (190,189). It was concluded that the poly(A) sequence of mRNA is necessary to ensure long-term functional stability. The possibility that the increased rate of loss of poly(A)⁻ mRNA function was due to a higher rate of mRNA sequence degradation was investigated. The amounts of poly(A)⁺ and poly(A)⁻ globin mRNA sequences remaining in X.laevis oocytes at various times after their introduction were measured using a cDNA probe prepared against poly(A)⁺ globin mRNA. Fifty-six hours after injection into oocytes, about 85% of the poly(A)⁻ globin mRNA was no longer able to hybridize to globin cDNA and had presumably been degraded, while the poly(A)⁺ globin mRNA sequences remained almost entirely preserved (191). Loss of translational activity of the poly(A)⁻ mRNA was therefore shown to correlate with loss of mRNA sequence, and these results supported a role for poly(A) in stabilization of mRNA sequences.

One potential objection to the validity of these studies was the possibility that the enzymatic process used for

the production of poly(A)⁻ mRNA from its poly(A)⁺ form was causing some degradation or changes in the mRNA coding sequence itself as well as removing the poly(A) tracts. It was therefore necessary to demonstrate that the apparent differences observed between poly(A)⁺ and poly(A)⁻ mRNAs were in fact due to poly(A) tail-removal and were not simply an artefact due to the enzyme treatment. This was achieved by readenylation of the poly(A)⁻ globin mRNA in vitro using the E. coli enzyme ATP:RNA adenylyl transferase; this enzyme synthesised a 30-nucleotide long poly(A) sequence onto the 3' terminal of the poly(A)⁻ mRNA (192). This reconstituted globin mRNA behaved similarly to native globin poly(A)⁺ mRNA in X. laevis oocytes; in other words readenylation restored functional stability to poly(A)⁻ mRNA (192). This observation strongly suggested that the observed differences between these poly(A)⁺ and poly(A)⁻ mRNAs were due solely to the presence and absence of poly(A) sequences.

The effect of varying poly(A) chain length on both functional and structural stability of mRNA has also been investigated in the X. laevis oocyte system. Nudel et al. (193) prepared rabbit globin mRNA bearing poly(A) segments of various lengths by treatment of native globin mRNA with polynucleotide phosphorylase under a variety of conditions. Oligo(dT)-cellulose chromatography of the digests resulted in seven different globin mRNA preparations bearing poly(A)

tracts of approximately 16, 21, 32, 39, 68, 95 and 122 nucleotides. The functional stability of each of these mRNAs was compared to that of native poly(A)⁺ mRNA containing poly(A) of length 149 nucleotides and poly(A)⁻ globin mRNAs in X. laevis oocytes. All the mRNAs bearing a poly(A) segment of 32 residues or more were of comparable stability to poly(A)⁺ mRNA while the mRNA with a 16 residue oligo(A) tail had similar stability to poly(A)⁻ mRNA. The mRNA with a 21 adenylate residue tail exhibited a stability in between that of native poly(A)⁺ and poly(A)⁻ mRNAs. It appeared that mRNA stabilization by poly(A) was independent of poly(A) length provided it was greater than 30 residues. Below this size, the poly(A) tail did not effectively protect its associated mRNA sequence (193). The poly(A) tail was also found to stabilise globin mRNA in intact human HeLa cells (194). Native globin mRNA was translated in these cells for over 26 hours, but globin synthesis was undetectable after injection of deadenylated globin mRNA into HeLa cells.

The generality of poly(A) tail stabilization of mRNA sequences has been investigated using mRNAs other than globin mRNA, e.g. HeLa cell histone mRNAs. Native poly(A)⁻ and poly(A)⁺ histone mRNAs (prepared by polyadenylation of poly(A)⁻ mRNA using ATP:RNA adenylyltransferase) were compared in X. laevis oocytes (195). When native poly(A)⁻ histone mRNA was introduced into oocytes, it was trans-

lated at a high rate for the first seven hours, but the rate declined over the next 13 hours and by 43 hours histone synthesis was hardly detectable. Synthetic poly(A)⁺ histone mRNA, which contained a poly(A) sequence of 40-50 residues, was translated at a high rate for over 19 hours and translation was still high at 43 hours. These observations are clearly consistent with a poly(A) role in mRNA stabilisation.

However, native poly(A)⁺ and deadenylated interferon mRNAs were translated equally well in X. laevis oocytes (196); in both cases interferon synthesis was reduced to undetectable levels 24-48 hours after introduction of mRNA into the oocytes, and it appeared that this interferon mRNA was especially labile whether or not it was polyadenylated.

It has been proposed that degradation of deadenylated mRNA is linked to the translation process. The degradation of deadenylated α -globin mRNA in X. laevis oocytes, as measured by an inability to hybridize to a globin cDNA probe, was significantly higher in the presence of haemin (197). Since haemin stimulates α -globin mRNA translation and itself has no degradative effect on globin mRNA, this higher rate of degradation could be related to the translation process. Other reports that poly(A)⁻ mRNA must be translated in order to allow its degradation have been made (189).

Although the work of Huez, Soreq and associates has provided most of the evidence suggesting a role in

mRNA stabilization for poly(A) tracts, supporting research from other groups is available. A correlation between mRNA half-life and the rate of shortening of mRNA-associated poly(A) has been reported for adenovirus mRNA (198), and mammalian mitochondrial mRNA (199).

Differences in the turnover kinetics between poly(A)⁺ and poly(A)⁻ mRNAs of mouse L cells were reported in an early study by Perry and Kelley (200); poly(A)⁻ mRNA had a lower mean lifetime than poly(A)⁺ mRNA and it was suggested that poly(A) may have a stabilising role during the period in the cell cycle when histone poly(A)⁻ mRNAs are destroyed.

A few reports have suggested that poly(A) does not stabilize mRNA. Naturally-occurring non-polyadenylated mRNAs of reovirus were all found to be as stable in X. laevis oocytes as other native poly(A)⁺ mRNAs (201). On the basis that poly(A)⁻ reovirus mRNA was functionally more stable than poly(A)⁻ globin and histone mRNAs, these authors argued that mRNA characteristics other than the poly(A) segment (e.g. the cap structure) might be involved in mRNA stabilization. Comparison of the translation products of poly(A)-rich and poly(A)-poor mRNAs of Dictyostelium discoideum before and after treatment in vivo with concentrations of actinomycin D that inhibited mRNA synthesis indicated that short-lived mRNA sequences occurred

predominantly in the poly(A) rich fraction. It was therefore concluded that mRNAs bearing short or even no poly(A) tracts were more stable than those with long poly(A) sequences (202)!

In spite of the few reports to the contrary, the evidence that poly(A) sequences play a role in stabilization of mRNA molecules is strong and this proposed function is the most popular of all the suggested roles for poly(A). Of course, it must be remembered that there is no a priori reason why poly(A) role should be limited to one process; it is conceivable that poly(A) can have several roles and the predominant role could vary from one system to another. A review by Littauer and Soreq summarises current ideas and thinking on poly(A) functions (203).

Possible mechanisms by which poly(A) could mediate RNA stabilization have been investigated recently, and a short summary of these studies is presented here.

(iv) Possible Mechanisms of mRNA Stabilization by Poly(A)

It is generally assumed that in order to stabilize mRNA, poly(A) sequences inhibit RNase activity. In a relatively early study, Allende et al. (204) examined the rates of degradation of a variety of RNAs after injection into X. laevis oocytes. Poly(A) sequences, either attached

or unattached to mRNA coding sequences, were especially resistant to degradation but they did not appear to appreciably protect heteropolynucleotide sequences covalently attached to them. Huez et al. concluded that in order to protect histone poly(A)⁺ mRNA against loss of functional stability, the poly(A) sequence had to be covalently attached to the mRNA (195). Injection of free poly(A) (100-200 residues in length) together with poly(A)⁻ histone mRNA into X. laevis oocytes, resulted in no protection of the mRNA compared to poly(A)⁻ histone mRNA injected alone. Thus, in this system, poly(A) did not protect the mRNA molecule by simple competitive inhibition of ribonuclease (195). Hieter et al found that human spleen endoribonuclease activity was reduced in vitro by the inclusion of synthetic poly(A) or several poly(A)⁺ mRNAs including rabbit globin mRNA, but not by poly(A)-free globin mRNA (205). Furthermore, inclusion of polyadenylated 5S rRNA, but not 5S rRNA, reduced endonuclease activity, the extent of RNase inhibition depending on the length of the poly(A) segment added to 5SRNA.

These studies were generally supported by Karpetsky and co-workers who have examined the effects of 5S rRNA and polyadenylated 5S rRNA on several ribonuclease activities. Compared to 5S rRNA, poly(A)⁺ 5S rRNA inhibited the endonuclease activity of RNase^(C) A and RNases from human plasma, human spleen, Citrobacter and Emerobacter, but in some cases inhibition was very dependent on ionic strength (206).

The evidence on whether or not poly(A) must be covalently linked to 5S rRNA in order to effect inhibition of RNase activity is confused. One report stated that free poly(A) was unable to protect 5S rRNA from degradation (206) but another report did not support this statement (205). Karpetsky et al. have reported that short chains as small as 18 residues protected 5S rRNA when covalently bound to this molecule and lengthening the poly(A) tail increased the inhibition (207). Furthermore the presence of poly(A) covalently bound to a small percentage of molecules in a 5S rRNA preparation was apparently sufficient to protect all rRNA molecules. Poly(A) mediated inhibition of RNase activity was reported to be reversed by polyamine (208, 205) and it has been suggested that variations in poly(A) length and polyamine concentration could determine the overall role of RNase activity in vivo and hence the rate of RNA degradation (208).

The physiological significance of these in vitro findings using poly(A)⁺ 5S rRNA as a model substrate for poly(A)⁺ remains to be demonstrated, particularly since in vivo studies in X. laevis oocytes (195) yielded quite different conclusions. One potentially important finding is that poly(A) addition dramatically changed 5S rRNA secondary structure (206). There are indications that poly(A) also influences secondary structure of mRNAs. Thermal

denaturation studies of poly(A)⁺ and poly(A)⁻ forms of rabbit globin mRNA showed that poly(A)⁺ mRNA had a highly ordered secondary structure compared to poly(A)⁻ RNA (209). Jeffery and Brawerman suggested that the poly(A) sequence associates with other regions of mouse sarcoma mRNA since limited digestion of polysomal RNA by RNase A gave rise to specific structures consisting of poly(A) and heteroribonucleotides (210).

When considering possible mechanisms by which poly(A) tracts might effect their function it is of interest to discover the existence of several types of RNA which contain tracts rich in UMP residues. The presence of uridylate-rich oligonucleotides was first shown in HeLa cell hnRNA (211), and these structures were later shown to be present in cytoplasmic mRNA of HeLa cells (212). Molloy has divided HeLa cell mRNA into four classes: poly(A)⁺-oligo(U)⁺, poly(A)⁺-oligo(U)⁻, poly(A)⁻-oligo(U)⁺ and poly(A)⁻-oligo(U)⁻ (213). The two oligo(U)⁻ mRNAs constituted about 85% of the total mRNA population, and thus only 15% of HeLa cell mRNAs contained an oligo(U) tract. Oligo(U) tracts isolated from HeLa cell RNA by RNase T₁ treatment consisted of 80-90 mol % UMP and had lengths of 20-50 nucleotides (212, 213). UMP-rich tracts have been found in RNAs of other systems: nuclear and cytoplasmic RNAs of cultivated hamster fibroblasts (214) and sea urchin embryos

(215). Tomaszewski (216) has compared the translation products of a wheat embryo poly(A)⁻-oligo(U)⁺ RNA fraction with those of poly(A)⁺ RNA from the same source and found that there were distinct differences. Nichols and Pryhitka have reported the presence of UMP-rich tracts up to 70 residues in length in rat liver RNA (217).

Oligo(U) and poly(U) tracts have also been demonstrated in RNAs which do not appear to function as mRNA. Dobrzanska et al reported the presence of UMP-rich tracts 10-150 nucleotides long in wheat embryo RNA (218); this RNA originated from the nucleus but only a fraction of the nuclear form resisted degradation to reach the cytoplasm. Bester et al have isolated two types of translational control RNA from embryonic chick nuclei; one of these translation inhibiting RNAs consisted of up to 50% UMP and was capable of forming stable hybrids with poly(A)⁺ RNA (219). Finally a series of small RNAs (4.5-5.5S) differing essentially in the number of uridyate residues attached to their 3' terminals has been reported (220). These RNAs associated with the genome of spleen focus forming virus (SFFV) and the poly(A)⁺ RNA of SFFV-infected and non-infected rodent cells (220, 221). It has been proposed that these UMP-rich non-messenger RNAs function as poly(A) binding sequences and either effect or modulate poly(A) functions such as mRNA stabilization or RNA nucleocytoplasmic transport (221, 228). Regulatory functions have also been

proposed for oligo(U) sequences contained within hnRNAs and mRNAs. There is evidence that oligo(U) and poly(A) of HeLa cell RNA are involved in secondary structures since these oligo(U)⁺ RNAs would bind to poly(A)sepharose only after disruption of hydrogen bonding by formaldehyde denaturation (222).

Investigation of possible mechanisms of poly(A) action have been significantly stimulated by the discovery of proteins which have a high affinity for poly(A) tracts. The following section summarises present knowledge of the characteristics of poly(A) binding proteins and discusses their biological significance in relation to poly(A) function.



POLY(A) BINDING PROTEINS

The first report of specific poly(A) tract binding proteins came in 1972 when Kwan and Brawerman isolated a complex composed of poly(A) and protein after limited RNase digestion of polysomes from mouse sarcoma ascites cells (223). This complex had a sedimentation coefficient of 12-15S, compared to 4S for free poly(A) from these polysomes. It was disrupted by pronase treatment, thereby indicating some protein content and it contained exposed poly(A) since the intact complex was capable of binding poly(U). Blobel reported that the mRNAs of mouse L cells and rat hepatocytes were tightly associated with two different proteins (224). The larger protein, 78,000 daltons, was bound specifically to the poly(A) tracts of these mRNAs. Since these two early reports, there has been extensive analysis of the proteins associated with specific mRNAs. Most mRNAs associate with several different polypeptides and some of these are specifically bound to the poly(A) sequence. Only the proteins which appear to associate specifically with poly(A) are discussed here.

Tomcsanyi et al. discovered a total of 6 polypeptides ranging in nuclear weight from 50,000 to 90,000 daltons which associated with poly(A) of rat liver mRNA (225). Duck globin mRNA poly(A) has been found to associate with a 73,000 dalton protein (226) and a protein of 76,000 daltons

which bound strongly to poly(A)-sepharose was present in Ehrlich ascites cell cytoplasm (227). Poly(A) of wheat mRNA was shown to associate with two proteins of molecular weights 36,000 and 52,000 daltons (228). Smaller poly(A) binding proteins were found in Artemia salina; the major component was 38,000 daltons and the minor component was only 23,500 daltons (229). The association of these proteins with poly(A) is frequently dependent on the subcellular location of the mRNA. Poly(A) of duck globin mRNA bound to the 73,000 dalton protein only when in polysomes and not when in free mRNP particles (226). In general, the proteins associated with translationally active and inactive globin mRNA (polysomal vs nonpolysomal) are quite different. However, the poly(A) binding protein of Artemia was present in nonpolysomal poly(A)⁺ mRNP (229) and poly(A) binding proteins of Physarum polycephalum were entirely restricted to free mRNP (230). Polysomal mRNA of Physarum contained short poly(A) sequences of about 15 residues, while non-polysomal poly(A) tracts were 65 residues long. There is evidence that entry of Physarum mRNA into polysomes might result in a concomitant loss of most of the poly(A) tract and hence loss of the poly(A) binding proteins. In this system it appeared that the process of polysome production was responsible for both the disruption of poly(A)/protein complexes and poly(A) degradation (231), and these observations are in agreement with those suggesting that poly(A)

shortening is a product of mRNA translation (see p.27).

Attempts have been made to characterize and purify poly(A)-binding proteins. The 38,000 dalton protein of *Artemia* has been purified to near homogeneity (232); it has approximately 357 amino acids, is high in glycine and contains dimethylarginine. Rat liver poly(A)-binding proteins have been found to be high in acidic and hydrophobic amino acids (225). Other studies have examined the association of poly(A) and specific poly(A)-binding proteins. Tomcsanyi et al. showed that poly(A) of poly(A)-protein complexes from rat liver mRNA was only 50% degraded by RNase T₂, thus indicating that the poly(A) was only partially protein covered (225). It was suggested that several molecules of the 73,000 dalton protein, which bound poly(A) of polysomal duck globin mRNA, were lined up along each poly(A) tract since limited nuclease digestion of the poly(A)-protein complex fragmented the poly(A) sequence periodically (233). A similar model was suggested for *Artemia* poly(A) protein complexes where each protein molecule was calculated to bind to 9-11 adenylate residues (132). Goldenberg et al. also showed that globin mRNA sequences both adjacent and non-adjacent to the poly(A) sequence were protected by poly(A) binding proteins (233). Baer and Kornberg favour a model in which the poly(A)-protein complex is composed of repeating units of poly(A)-protein structure, but they proposed that each structural unit contains about 27 adenylate residues (234).

Recently, they purified a 75,000 dalton protein from rat liver (235). This protein was similar to the poly(A)-binding protein described previously (224) and when it was combined with purified poly(A) in vitro poly(A)-protein complexes exhibiting a 27 residue periodicity were formed. This reconstitution was specific for poly(A). Adams et al. have suggested a different model in which a single molecule of the Physarum protein covers the 5' terminal of the poly(A) sequence and the adjacent 45 residues (236). They detected no periodicity in the poly(A)/protein complex of Physarum.

By studies involving UV-light-induced crosslinking of proteins to RNA, poly(A) of mouse L cell RNA was shown to combine with a 78,000 dalton protein when in cytoplasmic mRNA but with a 60,000 dalton protein when in hnRNA (237, 238). It appeared that in these cells a different set of proteins was associated with cytoplasmic mRNA and its nuclear precursors. In the cytoplasm continuous exchange of mRNA bound proteins with free cytoplasmic proteins has been proposed, and the poly(A)-protein complex might constantly be in a dynamic state.

The functional importance of poly(A) binding proteins is suggested by the finding that a high degree of conservation is found between the proteins of duck, mouse and rabbit (239). Standart et al. suggested that poly(A)-binding proteins have a purely structural role analogous to the histones of chrom-

atin. Rose et al. found several similarities between rat poly(A) polymerase and HeLa cell poly(A)-binding protein, e.g. in amino acid composition, molecular weight and antigenic properties (240). They proposed that poly(A)-binding proteins are responsible for poly(A) synthesis and/or elongation. Other poly(A)-binding protein preparations have failed to exhibit poly(A) polymerase activity, e.g. the 38,000 dalton protein of Artemia had no RNase or poly(A) polymerase activity (232). Other observations have suggested a transport role for poly(A)-binding proteins; eg. cordycepin treatment of HeLa cells not only prevented labelling of new poly(A) but also inhibited the appearance of ³⁵S-methionine label in the 75,000 dalton poly(A) binding protein of newly-formed poly(A)/protein complexes (241). Chisick et al. studied mRNA transport out of isolated nuclei and found that cordycepin triphosphate-induced transport inhibition was restricted to poly(A)⁺ RNP (242). It was suggested that the poly(A)/protein complex is involved in transport of poly(A)⁺ mRNA from nucleus to cytoplasm in vivo. Three proteins of molecular weight 78,000, 100,000 and 120,000 daltons were associated with the poly(A) of nuclear RNA in this system. DeHerdt et al. recently demonstrated nuclear accumulation of the cytoplasmic poly(A)-binding protein of Artemia after injection into X. laevis oocytes (243). On this basis it was suggested that this protein is functional in

both cytoplasmic and nuclear events, and this could include nucleocytoplasmic transport.

Alternatively, poly(A)/protein complexes may function to protect mRNA sequences from RNase-mediated degradation. The poly(A) sequence of mRNA in mouse sarcoma polysomes was not degraded by snake venom exonuclease, but after deproteinization this mRNA was hydrolysed extensively (244). Similar results were obtained using free cytoplasmic nucleoprotein particles, therefore protein/mRNA associations are likely to be responsible for this protection. Polysomal poly(A) was also degraded by cytoplasmic S-180 cell extracts and this degradation was increased by snake venom exonuclease. It appears that this S-180 cell cytoplasm contained a factor which interfered with poly(A)/protein associations thereby increasing the susceptibility of poly(A) to exonuclease attack. It was suggested that poly(A)/protein interactions could prevent exonucleolytic attack on poly(A)⁺ mRNA, but cytoplasmic factors modulated such protection by destabilizing mRNA/protein associations. Müller et al. examined the effect of poly(A)-binding proteins on the susceptibility of poly(A) sequences to two enzymes which preferentially degrade poly(A): alkaline exoribonuclease and endoribonuclease IV (see p. 38) (245). The degradation of the poly(A) sequence of mouse cell mRNA was reduced for exoribonuclease and totally abolished for endoribonuclease when the poly(A)

was contained in polysomes or associated with poly(A)-binding proteins. In the absence of protein both enzymes degraded the poly(A) rapidly. The two proteins associated with poly(A) of mouse cell mRNA (79,000 and 51,000 daltons) were clearly able to protect poly(A) against RNase degradation.

This short summary illustrates that many systems contain a 73,000-78,000 dalton poly(A)-binding protein; in some cases (e.g. Artemia) smaller proteins are found in their place. No consensus can be drawn regarding the subcellular distribution of these proteins; it is likely that different protein populations associate with the poly(A) sequence depending on whether the RNA molecule is nuclear or cytoplasmic and polysomal or free, and in some situations no protein associates with this sequence. Likely structural models have been proposed for the poly(A)-protein complex and possible functions for these proteins have been discussed. It is of popular opinion that these proteins play an important regulatory role in the expression of mRNA molecules but the mechanism by which this is achieved requires much further study. They may, for example, protect the 3' terminal region of mRNA molecules and thereby protect this terminal from RNase-mediated hydrolysis. Poly(A)-protein interactions themselves could be regulated by a large range of factors and could thus act as a sensitive post-transcriptional control of gene expression (e.g. protein phosphorylation (246)). Further studies on poly(A)-protein interactions and their biological effects are clearly of importance.

CHANGES IN POLY(A) DURING GROWTH AND DEVELOPMENT

In view of the suggestion that poly(A) tracts might regulate mRNA function (e.g. via mRNA stabilization) it is a possibility that changes in mRNA polyadenylation occur during cell growth and development. Polyadenylation of mRNAs has been studied in a small number of systems in order to evaluate the effects of growth, development and aging.

Sogin and Saunders found a reduced poly(A) content in stationary-phase cells of Saccharomyces cerevisiae compared to exponentially growing cells (247). The reduction in poly(A) content of non-growing cells was due to both an overall reduction in the number of sequences containing poly(A) and also a relative reduction in the amount of the larger poly(A) tracts. Sajdel-Sulkowska and Kumar also found higher amounts of poly(A) in growing mouse 3T3 cells compared to resting cells (248). Furthermore, there was a different subcellular distribution of poly(A) in the two states; growing cells contained more poly(A) in the nuclear and polysomal fractions but less in the soluble cytoplasmic fraction compared to resting cells. However, in contrast to the results of Sogin and Saunders, these researchers noted that growing 3T3 cells contained shorter poly(A) sequences than resting cells, and they suggested that an increase in

poly(A) processing accompanied growth.

One developmental process which has been investigated with respect to poly (A) size and content is oocyte production. Cabada et al. showed that mature X. laevis oocytes contained poly(A)⁺ mRNA bearing two size classes of poly (A) with a mean size of 20 and 60 residues (84). Only the larger poly(A) size class was found in previtellogenic oocytes. The smaller poly(A) size class accumulated from vitellogenesis through to oocyte maturation; and this accounted for a 10-fold increase in the number of poly(A)⁺ RNA molecules per oocyte during oogenesis. Other researchers reported that during progesterone induced X. laevis oocyte maturation there was a rise in poly(A) content (249, 82). This was due in part to a slight increase in the number of poly(A) sequences but the rise was predominantly caused by cytoplasmic extension of pre-existing poly(A) sequences by 10-30 residues. After germinal vesicle breakdown, there was extensive degradation of poly(A) sequences. Sagata et al. extended these studies to post-fertilization events. During early development, there were many rises and falls in poly(A) content with an upward trend overall (250); by the 'heartbeat' stage three poly(A) size classes of mean size 100, 80 and 20 residues could be detected, and as development progressed the proportion of the largest poly(A) size class increased. The smallest

poly(A) size class appeared to be associated with translationally inactive mRNA. Fromson and Duchastel found that a high proportion of sea urchin embryo polyribosomal RNA was not polyadenylated, but that during development the proportion of poly(A)⁺ polyribosomal RNA increased (251).

The poly(A) content of mouse oocytes and embryos has been found to change in a manner resembling that of sea urchin oocytes and embryos with an overall accumulation of poly(A) (252). This was achieved with very little change in poly(A) size and was therefore due to changes in the number of poly(A) sequences (253).

In at least one situation, reduced poly(A) production has been found to correlate with reduced poly(A) polymerase activity (254). Liver and brain of 25 day old rats contained significantly more poly (A) polymerase activity than those tissues from 300 day old rats; this reduction in poly(A) polymerase activity during ageing was consistent with the reduction of poly(A)⁺ RNA synthesis and hence the reduced protein synthesis of the tissues of older rats.

Another process which has been investigated with respect to polyadenylation is the series of events accompanying "heat-shock". Exposure of Drosophila to a temperature of 37°C. (heat-shock) causes major transcriptional changes in Drosophila cells. The size of the poly(A) tail attached to mRNAs made after heat shock was reported to be similar to

that attached to control mRNAs (255). However, heat-shock did cause a rapid and dramatic reduction in the cytoplasmic poly(A) content of these cells, and this appeared to correlate with degradation of up to 80% of the mRNA. More detailed study of this cytoplasmic poly(A) decay suggested two components (256): the first comprised 46% of the total cytoplasmic poly(A) and it decayed with a half life of only 10 min., while the rest of the poly(A) was more stable and its half-life could not be determined. At this time there was a rapid increase in nuclear poly(A) to 2-3 times the normal level; the nuclear poly(A) was believed to be added to preexisting nonadenylated primers.

The differentiation process has occasionally yielded interesting patterns of cellular poly(A) size and content. During terminal differentiation of rat L₆E₉ myoblast cells into myotubes, a rapid rise in the level of mRNA coding for MHC (myosin heavy chain) protein was reported (257). Once high levels of this protein have been produced, MHC and MHC mRNA synthesis were rapidly reduced and the induction of MHC mRNA was therefore transient. This short-lived mRNA was associated with a very short poly(A) tract of less than 20 residues (257). If poly(A) size does indeed control mRNA lifetime, it would not be surprising that such a short-lived mRNA bore a very short poly(A) chain, since the mRNA did not need long term protection from degradation.

7 Lectin-induced differentiation of lymphocytes was accompanied by an initial doubling of RNA polyadenylation followed by increased rates of RNA synthesis. The average poly(A) tract length was slightly shortened on stimulation and the synthesis of several proteins (e.g. actin, tubulin and calmodulin) was stimulated. Up to 50% of actin synthesis was due to poly(A)⁻ mRNA whereas the other two proteins were encoded in mRNAs that were predominantly poly(A)⁺ (258). mRNA sequences coding for different proteins therefore responded differently with respect to polyadenylation upon stimulation of cell differentiation.

However, not all cells have exhibited changes in poly(A) in response to growth and differentiation. Mouse neuroblastoma cells exhibited similar patterns of poly(A) size and poly(A) processing whether the cells were growing (controls) or were treated with agents to inhibit growth by raising intracellular cAMP levels (259).

The studies described in this section suggest that variations in poly(A) content and size can occur during growth and development, but no general conclusions can be reached. One of the major drawbacks of many of these studies is that polyadenylation of mRNAs coding for specific products could not be measured in these systems and overall measurements of poly(A) content are difficult to interpret during growth due to concomitant increases in mRNA content. Changes in poly(A) size do appear to correlate with changes in

cellular growth or development but not always in a consistent manner.

In this work a developmental process has been used to study mRNA polyadenylation of specific sequences. The process, milk protein synthesis by the mammary gland, will be described in detail before the aims of this research are discussed.

MILK PROTEINS AND THEIR SYNTHESIS BY THE MAMMARY GLAND

The mammary gland functions essentially to provide newborns with a well-balanced supply of nutrients in the form of milk and this function (lactation) is required only in the period immediately following parturition. In virgin and nonpregnant animals the gland is not well developed but during pregnancy the gland proliferates as measured by increases in the tissue's DNA content (260), and there is a significant increase in the relative numbers of milk secretory (alveolar) cells. By the end of gestation or the beginning of lactation the gland has fully differentiated into a milk secreting organ. The lactating gland consists of large numbers of alveoli, each composed of a central lumen surrounded by a single layer of alveolar cells. Milk is secreted into the alveolar lumina which connect by a system of ducts to the teat through which the young obtain milk. During gestation the alveolar cells accumulate mitochondria, polysomes (261) and rough endoplasmic reticulum (262) and they assume the typical characteristics of secretory cells. This growth and differentiation of the mammary gland is closely correlated with pregnancy in order that milk secretion may be initiated immediately after parturition. Gland development and milk production are both under complex hormonal control involving both protein and steroid hormones.

Oka and Topper reported that in vitro mouse mammary cells required insulin, corticosteroid and prolactin for normal development (262). The precise hormonal requirements appear to differ from one species to another, but in all cases studied prolactin is found to be a very important regulator of lactation. For a more detailed review of the physiology of the mammary gland and the endocrine controls operating on this system, see Mepham (263).

The biosynthesis and composition of milk has been reviewed in detail (263,264) and only a brief description is given here. Although there are large quantitative differences in certain milk components between species, qualitatively milk is quite similar from one species to another. The major milk carbohydrate is a disaccharide, lactose, which is readily synthesised from glucose in alveolar cells. The final and rate-limiting step in lactose biosynthesis is catalysed by the enzyme lactose synthetase in the reaction:



This enzyme has been resolved into two polypeptide components (265). Catalytic activity is found in only one component; this isolated polypeptide catalyses the addition of galactose to N-acetylglucosamine in place of glucose. The second component has a regulatory role; it modifies the catalytic activity of the first component by increasing the enzyme's affinity for glucose so that the production of

lactose by the above reaction is favoured. This regulatory polypeptide is α -lactalbumin (α LA) and it is one of the major milk proteins (267). α LA is a whey protein, which means that it is not precipitated from milk by acidification to a pH of 4.5, unlike the caseins. Besides regulating lactose synthesis, α LA provides a source of amino acids for the new-born.

The caseins are a series of acidic phosphoproteins which are characterised by a high content of essential amino acids, and they constitute a major fraction of the proteins of milk. In experimental terms, the caseins are defined as proteins that precipitate from milk at a pH of 4.5; most of the caseins contain carbohydrate residues. They are principally nutritive in function but it is believed that through the formation of casein micelles in the presence of calcium and phosphate ions these proteins may play a role in concentrating these ions in milk (263). Other proteins which are found in milk in small amounts include blood proteins like iron-binding protein, serum albumin and immunoglobulins.

Milk lipids consist predominantly of triglycerides with smaller amounts of phospholipids, sterols and cerebroside. Milk contains several ions including Na^+ , K^+ and Cl^- and the secretion is isotonic with plasma; water makes up 80% or more of milk weight (264).

The following detailed description of the major milk proteins will be restricted to the rat forms, since this is the system used in the present study. The period of gestation lasts for 20 days in the rat, while the following period of lactation is completed by 18-20 days.

Rat α -Lactalbumin.

One of the first reports on the isolation and characterization of α -LA from rat milk came in 1977 (268). The protein was reported to contain carbohydrate which appeared to result in anomolous behaviour of this protein on SDS polyacrylamide gel electrophoresis. An average of 11 carbohydrate residues per mole protein was reported, although this might vary from one protein molecule to another since multiply charged forms of the protein were observed. McKenzie and Larson found up to six different forms of α -LA and these were believed to represent two genetic forms with variation in carbohydrate content resulting in three forms of each gene product (269). Prasad et al. resolved rat α -LA into three charge forms by ion exchange chromatography, but the differences resided in the carbohydrate portion of these molecules, not in the amino acid composition (270). All forms were active in the lactose synthetase reaction. The carbohydrate portion of rat α -LA was reported to occur at Asn-45 and subsequent work has provided a possible structure for this polysaccharide (271).

Qasba and Chakrabartty (272) claim to have purified two α -LAs differing in molecular weight. α -LA₁ had a molecular weight of 21,500 daltons irrespective of the method of determination, while α -LA₂ was 16,000 daltons when measured by sedimentation analysis and 22,500 daltons by SDS polyacrylamide gel electrophoresis. Both forms were

glycoproteins and they exhibited identical immunological activities. It was proposed that αLA_1 was a precursor of αLA_2 , since mild proteolysis apparently converted αLA_1 to αLA_2 . Recently, Prasad et al. reported the entire amino acid sequence of a rat αLA (273); the protein consisted of 140 amino acid residues compared to 123 for the αLAs of most other species. Compared to other αLAs , the rat protein has a 17 amino acid extension at the carboxyl terminus; it is proposed that the extension could have arisen through a termination codon mutation in the rat αLA gene. These direct amino acid sequence data are in good agreement with that predicted from the nucleotide sequence of a cloned cDNA prepared against rat αLA mRNA (274). However the question of whether or not multiple genetic forms of this protein exist has not yet been resolved.

The mRNA coding for rat αLA has been isolated from 3-5 day lactating mammary gland (23). A wheat germ cell-free translation system was used to assay mRNA activity and the translation product of αLA mRNA was identified with the use of specific αLA anti-serum. Of the total mammary poly(A)⁺ mRNA translation products, 40-50% were immunoprecipitated with anti-serum. The mRNA coding for αLA has been prepared from total mammary RNA by poly(A) affinity chromatography and subsequent size fractionation of poly(A)⁺ RNA by sucrose gradient centrifugation. αLA mRNA was shown

to be present in a distinct size class of mRNA (8-10.5S) (23); in vitro translation of this mRNA gave a translation product identical to purified α LA in a fingerprint analysis (275). Until recently, 8-10S mammary RNA was believed to consist exclusively of α LA mRNA; it now appears that this RNA contains mRNA for a second whey protein (see Chapter 5).

Rat Caseins

Caseins are defined as acid precipitable milk proteins and separation of the rat forms has been achieved by DEAE-cellulose chromatography (42,276). This resolved three separate proteins of molecular weights 42,000 (casein 1), 30,000 (casein 2) and 25,000 daltons (casein 3) (42). The last casein was partially resolved into two forms - these probably differ only in their carbohydrate or phosphate content. However, caseins 1, 2 and 3 appeared to be different proteins based on size and amino acid content (276,278), but their amino acid sequences have not been determined. A purified casein mix was used to generate specific anti-serum which could be used to assay for casein products in cell-free translation systems directed by rat mammary RNA (42).

Casein mRNA was found to reside in the 12S and 15-16S poly(A)⁺ RNA molecules of mammary RNA, and purified fractions of these mRNAs showed that the 15S mRNA coded for the two largest caseins (molecular weights 42,000 and 30,000 daltons)

while the 12S mRNA coded for the smallest casein (molecular weight 25,000) (24). These mRNAs had molecular weights of 450 and 320 kilodaltons respectively. The purity of these mRNAs was monitored by electrophoresis on 3% agarose-urea gels and examination of the cell-free translation products of these mRNAs. The 15S casein mRNA contained at least 90% casein mRNA activity, but the 12S casein mRNA did not appear to be as pure. Further resolution of the 15S mRNA doublet into two components coding for caseins 1 and 2 respectively has not been achieved and all 15S mRNA preparations therefore consist of two mRNAs.

Rosen and Barker have reported the preparation and characterization of cDNAs prepared against 12S and 15S casein mRNAs (278); appropriate reaction conditions yielded almost full copy 15S cDNA (~1300 nucleotides). This 15S cDNA hybridized with 15S casein mRNA in a pseudo first order reaction with a $\text{Rot } \frac{1}{2}$ of $0.0023 \text{ mol. s.}^{-1}$. Total lactating mammary RNA hybridized 166 times more slowly than purified 15S casein mRNA and this indicated the degree of purification obtained for this casein mRNA. Hybridization of 15S cDNA with rat genome DNA indicated that casein mRNA was transcribed from non-repetitive DNA sequences (i.e. a single copy gene), both in the lactating gland and in tumour tissue (278).

Variation of Milk Proteins and their mRNAs throughout Mammary Gland Development

Specific radioimmunoassay measurements in the 4 day lac-

tating rat gland by Qasba and Nakhasi showed that α LA made up 0.6% of total protein while caseins 1, 2 and 3 made up 6.6%, 1.0% and 1.8% respectively of total protein (275). These proteins were present in similar proportions in rat milk. There was up to a ten fold difference in the amounts of these proteins present in the fully lactating gland, but the milk protein mRNAs were present in the gland in approximately equal amounts. Quantitation of 9S α LA mRNA and 15S casein mRNA in total mammary RNA was performed both by analysis of wheat germ cell-free translation products and by hybridization to cDNA 9S and cDNA 15S probes. Both analyses indicated that total 15S casein mRNA was present in approximately double the amounts of 9S α LA mRNA, but it must be remembered that the 15S mRNA consisted of two separate types of mRNA; α LA mRNA made up 0.9% of total RNA and 15S casein mRNA made up 1.8%. The difference in proportions of the milk proteins and their corresponding mRNAs has prompted the suggestion that postranscriptional controls operate during milk protein production (275).

Rosen and Barker reported the quantitation of the 15S mRNA in total mammary RNA from glands at various stages of gestation, lactation and involution (278). Total RNA of 5 day pregnant gland contained 0.027% casein mRNA and this rose slowly during pregnancy and lactation until it reached 0.52% at 8 days of lactation, a rise of 19 fold. The absolute amount of casein mRNA reported here is a little lower than

that found by Qasba and Nakhasi (275). After 7 days of involution, the casein mRNA content had dropped to 0.014% (half of the 5 day pregnant value). Nardacci et al. also measured levels of rat α LA and total casein mRNAs throughout gestation and lactation (276). Total mammary RNA was translated in a cell-free system; the milk protein translation products were quantitated after isolation with milk protein anti-serum and separation by SDS/polyacrylamide gel electrophoresis. This method, which measured only biologically active mRNA, indicated that whereas casein mRNA levels increased throughout pregnancy and up to 15 days of lactation, α LA mRNA exhibited only a small rise over pregnancy but increased substantially from 2-15 days lactation. On weaning for 6 days both mRNA levels dropped from lactating levels and approached the amounts observed in early pregnancy.

The most comprehensive study of quantitative changes in milk proteins and their respective mRNAs throughout gestation and lactation of rat mammary gland was undertaken by Nakhasi and Qasba (279). Protein levels were expressed in μ g/ μ g DNA. The levels of α LA and casein proteins increased dramatically (5-25 fold) especially during the latter half of gestation but remained reasonably constant for the first 14 days of lactation. Casein 1 was the most abundant protein with levels about 5 times higher than those of either α LA or casein 2. Casein 3 levels were slightly higher than

those of casein 2. All four milk proteins rose 3-4 fold in amounts between days 12-16 of lactation but this rise was short-lived and levels quickly declined thereafter.

Levels of the mRNAs coding for α LA and the two largest caseins were measured by cDNA hybridization of total RNA from glands at various stages in development

(279). This provided a measure of total sequence present but not all of this mRNA is necessarily functional in vivo. There was an increase in α LA mRNA in the first week of gestation followed by a decline and then a steady rise until the lactating level (0.8-1.2% total RNA) was reached. In the first day of gestation, α LA mRNA was less than 0.05% of total RNA, thus a 20 fold increase in this mRNA was observed. Casein mRNA levels rose throughout gestation with lactating levels being reached by day 20 of gestation. This increase, from 0.1% total RNA at day 1 of gestation to 2-3% during lactation, represented a 20-30 fold accumulation. Both milk protein mRNAs exhibited peak levels at 12 days of lactation and thereafter levels declined.

Since mammary gland milk proteins are secreted from the alveolar cells, it is likely that they are synthesised and packaged like other secretory proteins, i.e. via a mechanism like the signal hypothesis (280). This hypothesis suggests that secretory proteins are synthesised on ribosomes associated with the endoplasmic reticulum (hence rough endoplasmic reticulum - RER) and that during peptide production, a signal sequence of high hydrophobicity is made

at the N-terminal. This sequence allows the peptide to traverse the ER membrane but in the process it is cleaved from the rest of the polypeptide molecule. The protein then progresses along the endomembrane system (through the golgi apparatus) and into secretory vacuoles which release the enclosed proteins from the cell by reverse pinocytosis.

There is evidence supporting such a mechanism for the milk proteins. Translation of mRNA from 13-15 day lactating rat mammary gland in a wheat germ cell-free system resulted in synthesis of immunoprecipitable α LA; this peptide was not glycosylated and it contained a 19 amino acid signal peptide sequence at the amino terminal (281). When dog pancreas microsomes were included in the translation, the resulting α LA lacked the signal peptide sequence and was apparently core glycosylated. Processing and glycosylation of the protein was linked temporally to translation and resulted in a protein which was protected from proteolysis, presumably because the protein was sequestered within microsomes (281). Similar observations have been made for rat caseins (282). Casein phosphorylation, which occurs at specific serine/threonine residues, is now known to occur through the action of a Mg^{2+}/Ca^{2+} dependent kinase localised in the Golgi apparatus (283).

Milk Protein cDNA clones

Recently, cDNA clones for rat α LA and the three individual caseins have been prepared (234, 284). The α LA cDNA clone has been sequenced and this yielded an amino acid sequence for part of the α LA protein (274). The three casein cDNA clones studied represent 77%, 95% and 99% of the mRNA sequence for caseins 1, 2 and 3 respectively (284). Restriction site maps for all three casein clones have been reported (285) but only the casein 2 clone has been completely sequenced (286). This nucleotide sequence enabled prediction of the entire amino acid sequence of the protein and has indicated the existence of a 15 amino acid signal peptide which exhibits a very high degree of conservation compared with the signal peptide of ovine caseins. Much more limited sequence homology was evident for the rest of the casein sequence. Restriction enzyme mapping and heteroduplex analysis of the three casein cDNA clones both suggested that there is wide sequence divergence amongst the casein genes (285).

Now that cDNAs for the individual caseins are available, more thorough analysis of the accumulation of these mRNAs during mammary gland development in vivo and hormonal treatment of organ explants in vitro will be possible. Until now, studies of the 12S casein mRNA have been hampered by a lack of sufficiently

specific 12S cDNA, and it was impossible to study the two 15S casein mRNAs individually. Preliminary work on the effects of prolactin and hydrocortisone on the individual casein mRNAs has been reported (287). In early pregnancy, it appeared that the relative proportions of these mRNAs changed dramatically, but thereafter their levels increased coordinately.

Hormonal Control of Milk Protein Synthesis

The changing levels of mammary gland ~~milk~~ proteins and their corresponding mRNAs during pregnancy, lactation and involution are due to the complex effects on this tissue of several hormones. Studies of this tissue especially using in vitro systems have attempted to dissect the actions of individual hormones on lactogenesis and specifically on milk protein synthesis. Early studies on pregnant mouse mammary gland in vitro suggested that insulin, hydrocortisone and prolactin acted synergistically to stimulate milk protein synthesis (288). The action of insulin in this system might be simply to maintain cell viability, and this hormone might not have any specific effects on milk protein synthesis. Both hydrocortisone and prolactin had a stimulatory effect on rat mammary gland lactogenesis but, in contrast, progesterone inhibited both casein synthesis and casein mRNA accumulation (289), and it has been suggested that the sudden drop in circulating progesterone at birth is the

trigger for the commencement of lactation.

The mechanism of prolactin action has been investigated using explants of rat mammary gland taken from pregnant animals (290). Prolactin addition to this tissue caused casein mRNA accumulation, measured with the cDNA 15S probe, within one hour of hormone treatment and mRNA accumulated for up to 48 hours thereafter. In this system, hydrocortisone was not essential for prolactin-induced casein mRNA accumulation, but this hormone did potentiate the prolactin effect. Progesterone inhibited casein mRNA accumulation when given with prolactin. There is evidence that prolactin increased casein mRNA levels in the mammary gland in two ways. The hormone increased casein mRNA transcription rates by 2-4 fold but it also increased casein mRNA half-life by 17-25 fold (291). It was therefore suggested that casein mRNA accumulation in response to prolactin occurred by a combination of transcriptional and post-transcriptional events.

Possible modes of action of prolactin have been reviewed (292). There is little evidence to suggest that prolactin acts on its target tissue by the classical peptide hormone pathway, via activation of adenylate cyclase. There is evidence that at least part of the prolactin molecule is internalized by mammary alveolar target cells. An internalized hormone-receptor complex could act directly or through a chemical mediator. Polyamines, cyclic nucleotides and

prostaglandins have all been suggested as possible mediators of prolactin action but the evidence in each case is not strong (293).

In agreement with studies on casein synthesis, Ray et al (294) found that long-term rat mammary cell cultures would produce α LA only in the presence of insulin, prolactin and corticosterone. The importance of these hormones in stimulation of milk protein synthesis by the rat is thus clear, but the precise mechanisms by which these hormones elicit such specific effects remains to be determined.

The Polyadenylation of Milk Protein mRNAs

Measurements of rat casein mRNA translation during purification from total mammary RNA have indicated that up to 60% of this mRNA was not retained by oligo(dT)-cellulose chromatography. It therefore appeared that a large fraction of casein mRNA contained either no poly(A) tails or poly(A) tails of less than 20 nucleotides in length (42). Up to 50% of lactating ewe and pseudopregnant rabbit casein mRNAs have also been reported to exist in poly(A) deficient forms (295, 296). Rat α LA mRNA has been partially purified using poly(U)-sepharose columns, and therefore was in part polyadenylated, but no indication of the recovery of α LA mRNA from these columns was reported (23). Human α LA mRNA has been reported to exist in polyadenylated and nonpolyadenylated forms at various stages of pregnancy, lactation and involution. The poly(A)⁻ form appeared to be present

at a concentration of approximately one hundredth that of the poly(A)⁺ form at all stages, but values of the absolute amounts present were not given (120).

The size of the poly(A) segment attached to 15S casein mRNA has been determined (24). [³H]-label was introduced into 15S casein mRNA using periodate oxidation/[³H]-borohydride reduction and the resulting [³H]-labelled poly(A) segments ranged from 15 to 150 residues, with a number average size of 42 residues. No report of the length of the poly(A) associated with rat α LA mRNA has been presented.

AIMS OF THIS RESEARCH

Developmental processes often provide the researcher with good model systems with which to investigate cellular control mechanisms. Since during development at least one cellular function is being either stimulated or depressed. In view of the roles postulated for poly(A) sequences, it would seem reasonable to suppose that mRNA polyadenylation could be modified during cell development. Indeed, studies discussed previously have indicated that there is a variation in mRNA polyadenylation during cell growth, development and differentiation but many of these studies were unable to study polyadenylation of specific mRNAs and therefore only very general observations were possible.

In this study, polyadenylation of specific mRNAs has been investigated during tissue development in attempts to further understand the functioning and significance of the poly(A) sequence in development. The system chosen for this analysis was the rat mammary gland and there are several reasons why this tissue is a good choice for such a study. First of all, the development of the mammary gland has been extensively studied already and the major gene products of the developed tissue, the milk proteins and their corresponding mRNAs have been characterized. The milk proteins represent a series of genomic products, the

production of which is highly stimulated during gland development. Two milk protein mRNAs, 9S whey protein and 15S casein mRNAs, are both readily purified from the lactating gland and this permits synthesis of cDNA probes directed specifically against these sequences. Once such probes are available, they can be used to measure the amounts of these mRNA sequences in a RNA population. Another reason for choosing the rat mammary gland system is that this is one of the few systems in which there is good evidence for post-transcriptional control of protein synthesis. Prolactin is believed to effect casein mRNA accumulation by both an increased rate of transcription and a reduced rate of degradation; in other words prolactin stabilises casein mRNA and this effect is believed to be specific for the milk proteins. One possible function of poly(A) is to stabilise mRNA molecules; so it is conceivable that the stabilisation of milk protein mRNAs by prolactin might be mediated by differences in the polyadenylation of these molecules compared to non-stabilised mRNAs, e.g. they might receive longer poly(A) sequences. A final advantage in the use of the mammary gland system is that the development process is reversible; once the production of milk is no longer required, the gland involutes and it returns to the pre-pregnant state both structurally and biochemically. It is therefore possible to study both the onset (during pregnancy) and offset (during involution) of milk protein production,

as well as peak synthesis during lactation.

Preliminary studies of 15S casein mRNA had indicated that up to 60% of the sequence existed as poly(A)⁻ mRNA in the lactating rat gland; this is an unusually high level of poly(A)⁻ mRNA for an abundant sequence (see Introduction, p.31).

In this study three main questions have been asked:

(i) Does a high percentage of the whey protein 9S mRNA also exist as poly(A)⁻ mRNA in the lactating gland ?

(ii) Do the relative amounts of poly(A)⁺ and poly(A)⁻ mRNAs for each milk protein change during development and, if so, are the two types of mRNA affected co-ordinately in this respect ?

(iii) Are there variations in the size of the poly(A) tracts associated with total mammary RNA during development, and is any such variation reversible during gland regression ?

The results of a series of studies of this nature should show whether or not mammary gland development is accompanied by any changes in the degree of polyadenylation of its mRNAs. The nature of any changes detected may suggest possible mechanisms which are responsible for the changes.

CHAPTER 1

FRACTIONATION OF TOTAL MAMMARY RNA BY OLIGO(dT)-CELLULOSE

AFFINITY CHROMATOGRAPHY

As indicated in the preceding page, one of the major goals of this work involved determination of the distribution of milk protein mRNAs between poly(A)⁺ and poly(A)⁻ RNA populations. In order to perform this analysis, a method which efficiently separates total RNA into poly(A)⁺ and poly(A)⁻ RNA fractions was needed. It has already been stated in the introduction that the best procedure currently available for this separation is affinity column chromatography. Two types of resin are routinely employed in this procedure: oligo(dT)-cellulose and poly(U)-sepharose.

In this chapter optimal conditions for the use of oligo(dT)-cellulose for RNA fractionation are described; this was necessary since, as described in the introduction several modifications of the original procedure (41) have been reported. In all recent studies on the distribution of poly(A)⁺ and poly(A)⁻ mRNA sequences, the effectiveness of RNA fractionation by oligo(dT)-cellulose has been monitored by an independent poly(A) assay (111,116). In the analysis presented here RNA fractionation has been monitored by the [³H]-poly(U) assay for poly(A).

The initial experiments performed to evaluate oligo(dT)-cellulose affinity column chromatography utilized mammary

RNA extracted from 11-15 day lactating rat glands. There were several reasons for using this developmental stage first. The production of the milk protein mRNAs was reported to peak around 12 days after parturition (279); maximal amounts of the milk protein mRNAs are therefore present at this time. High levels of mRNA were advantageous for evaluation of affinity column RNA fractionation and for later work which required purification of specific milk protein mRNAs in order to produce specific cDNA probes (see chapters 5 and 6).

Two methods for extraction of total RNA from rat mammary gland have been reported. The method of Nakhasi and Qasba (279) has the advantage that it removes DNA from the extract, but this procedure does involve considerable sample processing. It might be speculated that this processing could lead to selective loss of certain RNA species and/or increased sample degradation. Indeed these authors did find high variations in the extent of hybridization of their RNA samples with cDNA probes, which led to the normalization of the hybridization curves for presentation. This observation might be explained by sample degradation. Rosen and coworkers have provided the best data with respect to the characterization of their mammary RNA samples. Furthermore, these researchers made the original observation of the high levels of poly(A)⁻ casein mRNA in

their mammary RNA preparations. It was therefore decided to use the method of Rosen et al (297) to prepare all mammary RNA extracts.

MATERIALS

All animals used were Sprague-Dawley female rats obtained from Charles River, Montreal, Que. The day of parturition was assigned as day 1 of lactation. Pups remained with the mother until the time of sacrifice and only lactating females with at least 10 pups were used. The entire mammary glands of the female rats were removed after anaesthesia and decapitation. The mammary tissue was frozen rapidly on dry ice, weighed and stored at -70°C.

Phenol (U.S.P.) was from Baker Chemical Co., Phillipsburg, N.J., U.S.A. It was distilled (only the material distilling at 179-182°C was collected) and stored at -20°C until use. SDS was from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Materials for polyacrylamide gel electrophoresis (acrylamide, bis, ammonium persulfate and TEMED) were from Bio-rad Laboratories Ltd., Mississauga, Ont.

Oligo(dT)-cellulose (type 2) was from Collaborative Research Inc., Waltham, Mass., U.S.A. When not in use this resin was stored in 0.02% sodium azide at 4°C. (5')Poly(A), potassium salt type 1, and Bovine pancreas RNase A (type 1-A, 60 Kunitz units/mg), were from Sigma.

[³H]-Poly(U) [specific activity 39μCi/ mole P] was from Miles Laboratories Ltd., Rexdale, Ont. It was stored at -20°C; between 70-75% of the total [³H]-label in these preparations was acid precipitable.

METHODS

(i) Extraction of RNA from rat mammary gland

RNA extraction was performed as described previously (297). Frozen tissue was homogenized with a Waring blender (3 x 20 sec. runs) in 6-7 volumes of extraction buffer (0.025 EDTA, pH 8.0, 0.075M NaCl, 0.5% SDS). The homogenate received 5 volumes of phenol saturated with extraction buffer; this mixture was blended thoroughly, transferred to centrifugation bottles and cooled for 15 minutes in methanol/ice. Centrifugation of the homogenate at 8,000g (HS-4 rotor, Sorval) for 35-40 minutes at 4°C separated the phenolic

and aqueous phases. The aqueous phase was removed and reextracted once with 1 volume of phenol saturated with buffer and twice with 1 volume of a mix of chloroform and phenol saturated with buffer (1:1). The first interphase was sometimes re-extracted in three volumes of extraction buffer and 1 volume of chloroform and phenol saturated with buffer (1:1). The final aqueous phase was brought to 0.2M NaCl and received 2 volumes of cold 95% ethanol. Any long strands of DNA remaining were spooled out and the RNA preparation was precipitated overnight at -20° C.

RNA precipitates were collected by centrifugation (HS-4 rotor, Sorval) at 3300g for 30-40 minutes at 4°C. Ethanol was removed, and the pellet was washed twice with 95% ethanol before being dried in vacuo. RNA was dissolved in low salt buffer (10mM Tris-HCl, pH 7.6, 1mM EDTA) and its UV absorbance measured in 0.2M phosphate buffer, pH 6.8, RNA solutions had 230/260 and 280/260 ratios of 0.5 or less. RNA amounts were calculated on the basis that a 1 mg/ml solution has an absorbance of 25 at 260 nm (298). For similar measurements of poly(A), an absorbance of 30 for a 1 mg/ml solution was used (299).

(ii) Oligo(dT)-cellulose affinity column chromatography

The oligo(dT)-cellulose column (12 mm x 25 mm, 1 gm oligo(dT)-cellulose) was washed thoroughly with sterile water and equilibrated with high salt binding buffer (0.4M NaCl, 10mM Tris-HCl, pH 7.6, 1mM EDTA). The total mammary

RNA solution was brought to 0.4M NaCl and applied to the column at an RNA concentration not exceeding 2mg/ml and at a flow rate of 0.2-0.5 ml/min except where otherwise indicated. The flow rate could be adjusted readily by raising or lowering the effluent tube. When the solution was applied to one column only, the unbound RNA solution was collected in an Ehrlenmeyer on ice. When the solution was applied to two columns, the unbound RNA fraction from the first column was applied directly to the second column and the final effluent was collected in an Ehrlenmeyer. Columns were washed separately with binding buffer until all the unbound RNA was removed as determined by the UV absorbance of the column wash. Bound poly(A)⁺ RNA was eluted separately from each column in low salt buffer. The poly(A)⁺ RNA and unbound RNA fractions were collected by ethanol precipitation and their amounts determined by UV absorbance as described above. As indicated in the text, the two column fractionation was sometimes repeated with the unbound RNA sample.

(iii) [³H]-Poly(U) assay for Poly(A)

This poly(A) assay was modelled on previously reported assays (94, 76). [³H]-poly(U) (0.057 Ci/assay) was hybridized with either 20-70µg total RNA, 1-5µg poly(A)⁺ RNA, 150-300 µg poly(A)⁻ RNA or 10-200ng poly(A) standard. The hybridization was carried out in 0.3M NaCl, 0.03M Na

citrate, pH 7.2 and 5mM MgCl_2 in a total volume of either 0.3 ml or 0.45 ml. After incubation at 30°C. for 30 minutes, 8 μl RNase A (200 $\mu\text{g}/\text{ml}$) were added and the incubation was continued at 30°C. for 45 minutes. The assay received 300 μg carrier yeast RNA and was brought to 10% TCA with 0.15-0.2ml 30% TCA; remaining hybrids were allowed to precipitate at 0°C for at least 90 minutes. The precipitates were collected on Whatman GF/C filters and they were washed with cold 5% TCA (2 x 10ml) and cold 95% ethanol (2 x 10 ml). Washed filters were dried and the precipitated hybrids were hydrolysed with 200 μl 0.5N NaOH for one hour at 80°C; after neutralization with 200 μl 0.5N HCl, the filters received 5ml Ready-solv (Beckman, Toronto, ONT). Filter-containing vials were shaken for 15 minutes at room temperature and counted in an Isocap 300 liquid scintillation system (Nuclear-Chicago) at an efficiency of 30-35%. A counting period of 10 minutes was generally used.

(iv) Disc gel electrophoresis of mammary RNA preparations

Mammary RNA preparations were electrophoresed on SDS polyacrylamide disc gels (85 mm x 6 mm) containing 2.2% acrylamide, 0.11% bis, 0.2% SDS, 0.07% ammonium persulfate and 0.07% TEMED. The buffer system of Falvey et al. (300) (36mM Tris, 30mM NaH_2PO_4 , 1mM Na_2EDTA , 0.2% SDS, pH7.8) was

used. Gels were preelectrophoresed at 3.5mA/gel for 1 hour. RNA samples (15-20µg in 5-50µl) were applied in electrophoresis buffer containing 5% sucrose and 0.003% bromophenol blue. The gels were run for 3-4 hours (3.5mA/gel $\approx$ 50 Volts) until the blue dye almost reached the bottoms of the gels. Gels containing electrophoresed RNA samples were scanned at 254nm using an ISCO UA-5 gel scanner.

(v) DNA assay

DNA was determined by the method of Burton (301). Calf thymus DNA (Sigma) was used as a standard.

RESULTS

(i) Extraction of total RNA from 11-15 day lactating rat mammary gland

In four separate phenol extractions of RNA from lactating mammary gland (see methods) yields were 6.9, 7.3, 6.8 and 9.6 mg RNA/gm wet tissue; average yield was 7.5 mg RNA/gm tissue $\pm$ 1.32 (standard deviation). When total RNA was analysed on 2.2% polyacrylamide SDS gels as in Figures 1b and 2a two major UV absorbance peaks corresponding to 18S and 28S rRNAs were present. From the reported molecular weights of eukaryote rRNAs (18S rRNA = 0.71×10^6 daltons, 28S rRNA = 1.90×10^6 daltons) and the obser-

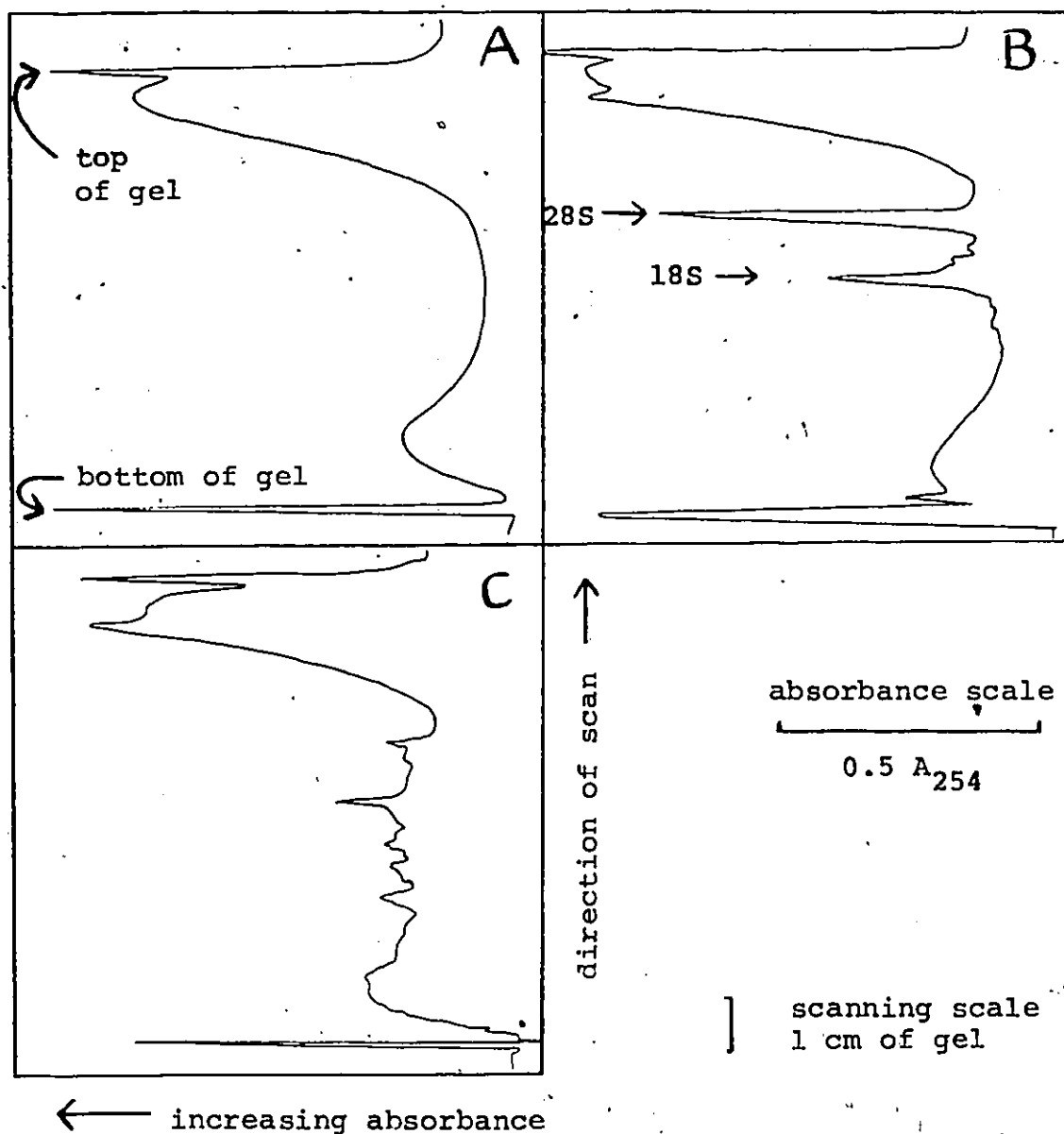


Figure 1. Analysis of RNA preparations by 2.2% polyacrylamide SDS disc gel electrophoresis.

RNA (15µg/gel) was electrophoresed on 2.2% polyacrylamide SDS disc gels (see Methods). Gels were scanned (at 254 nm) at a rate of 150 cm/hr. Tracings of three gel scans are shown: (A) gel run without sample - blank gel ; (B) total mammary RNA preparation (C) preparation of unbound RNA run through an oligo(dT)-cellulose column in high salt buffer - the initial sample was heat-treated before application to the column.

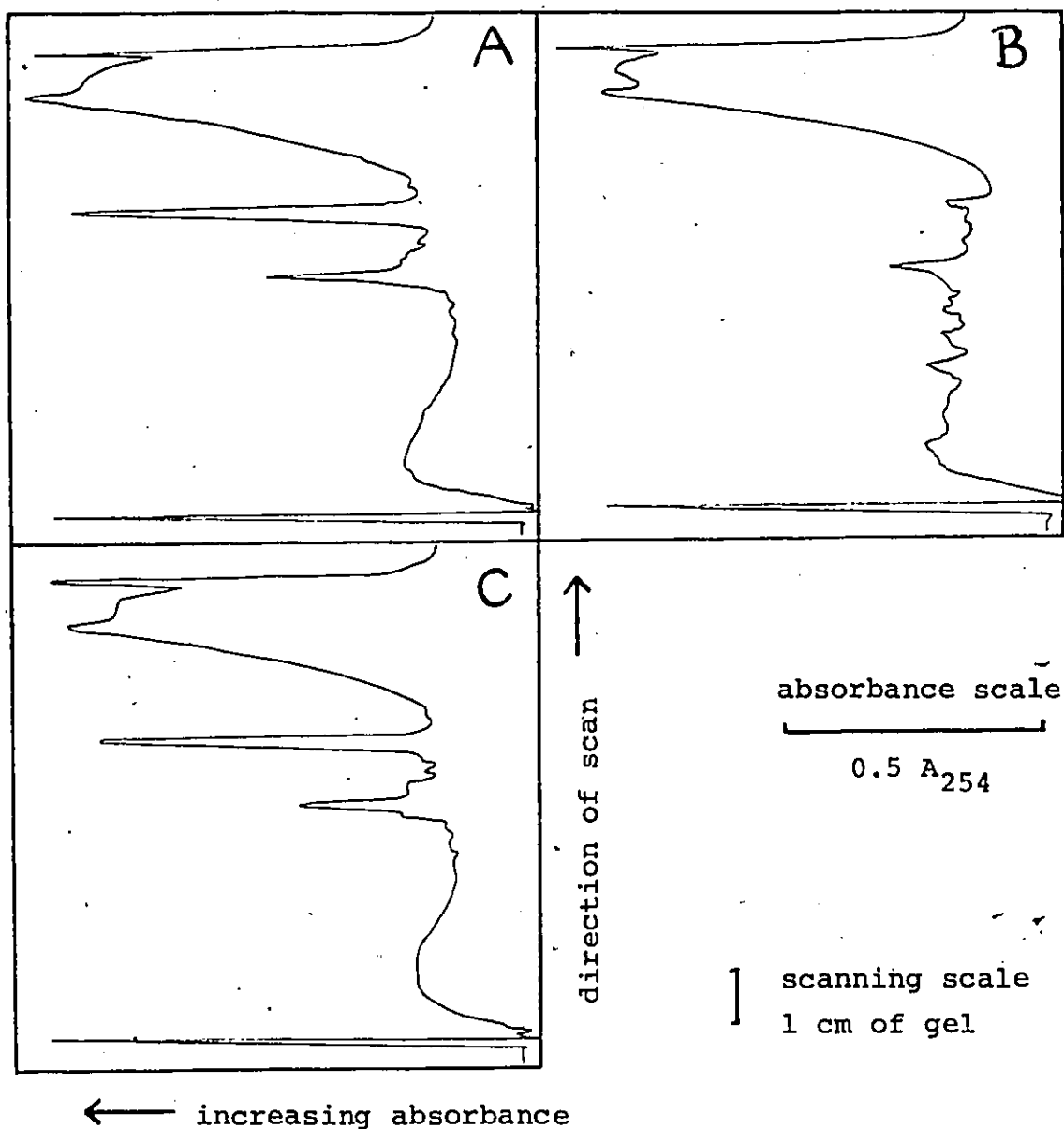


Figure 2. The Effect of various treatments on structure of Total Mammary RNA

Total RNA preparations were run on 2.2% polyacrylamide SDS disc gels (15 μ g RNA/gel) as described in Methods. After electrophoresis gels were scanned at 254 nm and tracings of the resulting profiles are shown. RNA treatment before electrophoresis was as follows: (A) passage of RNA through oligo(dT)-cellulose column in low salt buffer; (B) heated to 70°C (5 min.), then cooled at 0°C; (C) incubated at 37°C (1 hr) .

vation that they are in a 1.1 molar ratio (302) it may be calculated that the ratio of the 18S/28S absorbance peaks should be about 0.4:1. The observed values of 0.45 and 0.38 are in close agreement to this. These profiles in Figs. 1b and 2a show that intact total RNA can be produced by this extraction protocol, as judged by analysis of the rRNA peaks. When blank gels were electrophoresed by this method significant UV absorbance was observed at the bottom and top of the gel (Fig. 1a). As a result, it is difficult to observe molecules smaller than 18S and larger than 28S with this system. Figures 1 and 2 will be discussed further.

Analysis of these total RNA preparations by the Burton procedure (see methods) indicated that significant amounts of DNA were present in these preparations. The amounts of DNA present varied from 20-40% of total nucleic acid as shown in Table 1. The presence of DNA in these samples is consistent with the findings of Matusik and Rosen (290); their mammary RNA preparations contained 25-35% DNA. The presence of DNA in the present mammary RNA samples had to be taken into consideration during subsequent analysis of these preparations (see chapters 4 and 8).

(ii) Initial observations on RNA fractionation using oligo(dT)-cellulose columns

A survey of the literature on the use of oligo(dT)-cellulose columns indicates that several factors can effect

TABLE 1: DNA CONTENT OF TOTAL MAMMARY "RNA" PREPARATIONS

RNA preparation	concentration (mg/ml)		%DNA
	total nucleic acid	DNA only	
1 total	3.72	1.21	33
2 total	5.92	2.37	40
3 total	3.5	0.71	20
4 total	5.5	1.04	19

Total nucleic acid concentration of RNA preparations was measured by absorbance at 260nm. The DNA content of the samples was determined by the method of Burton as described in methods. The final column expressed the DNA content of these samples as a percentage of total nucleic acid.

poly(A)⁺ RNA yield, e.g. sample size, or more precisely the ratio of RNA sample/resin weight, flow rate and the concentration of salt in the binding buffer. Overloading of the resin with sample, or a high rate of sample flow through the resin may result in an unbound RNA fraction which is not completely depleted of poly(A)⁺ RNA. It follows that a RNA preparation may require more than one passage through oligo(dT)-cellulose in order to remove all poly(A)⁺ RNA species. The exact conditions required for complete separation of poly(A)⁺ RNA from total RNA must be determined by each investigator for the RNA preparations under study.

Initial investigations on the use of oligo(dT)-cellulose therefore examined the effects of sample flow rate and of the passage of a single RNA sample through more than one column. It became obvious after some experience with these columns that the flow rate of a solution through the resin was reduced when the solution contained RNA. Initially flow rates were measured during the binding buffer wash, but in later studies the column flow rate was measured during sample application. The flow rates presented in Table 2 were obtained with binding buffer only and are thus 2-3 times faster than the actual rate during sample application.

Table 2 summarizes the results of passing four total

TABLE 2: THE EFFECT OF FLOW RATE DURING OLIGO(dT)-CELLULOSE
CHROMATOGRAPHY ON POLY(A)⁺ RNA RETENTION BY THE COLUMN

Initial flow rate (ml/min)	Poly(A) ⁺ RNA retained (mg)	Poly(A) ⁺ RNA yield (%)
3.3	0.097	0.48
2.0	0.178	0.88
1.25	0.236	1.17
1.8	0.223	1.11

RNA samples (20.1mg) were heat-treated (70°C , 5-10 min., followed by cooling at 0°C), brought to 0.4M NaCl and applied to single oligo(dT)-cellulose columns as described in methods. After removal of unbound RNA with high salt buffer, retained poly(A)⁺ RNA was eluted with low salt buffer and measured by UV absorbance at 260nm. Poly(A)⁺ RNA yield is expressed as a percentage of total RNA applied to each column. The initial flow rate was determined during the high salt buffer wash and before sample application. Actual flow rates during passage of sample through the column will be lower than these initial values (see text).

mammary RNA preparations (20.1 mg each) through one oligo (dT)-cellulose column. The principal observation from this table is that the flow rate of a sample through a column can significantly affect the final poly(A)⁺ RNA yield. At a binding buffer flow rate of 3.3ml/min.

(corresponding to a rate of over 1 ml/min. during RNA application) poly(A)⁺ RNA yield (0.48%) was about half that obtained with flow rates of 2 ml/min. or less (0.88-1.11%).

It was concluded that flow rates of 1.8 ml/min. (corresponding to a rate of 0.75 ml/min. during RNA application) or less, permits optimal column retention of poly(A)⁺ RNA.

Higher yields of poly(A)⁺ RNA (1.85) were achieved with a slightly smaller RNA sample, and this suggested that these RNA preparations might require passage through more than one column to remove all poly(A)⁺ RNA. A fraction of

larger total RNA samples (63 mg) did bind to the resin on a second column run. It was therefore necessary to determine the number of rounds of oligo(dT)-cellulose column chromatography needed to remove all poly(A)⁺ RNA from a total mammary RNA preparation of specified size. Before such an analysis was performed, it was examined whether or not affinity column chromatography yielded RNA preparations which retained their primary structure.

(iii) Conditions of affinity chromatography which yield intact unbound RNA fractions

Since the distribution of poly(A)⁺ and poly(A)⁻ mRNA sequences can be significantly changed by pronounced RNA degradation (see chapter 6), it was necessary to confirm that passage of RNA samples through oligo(dT)-cellulose did not result in pronounced degradation of these samples. Unfortunately routine size determinations of poly(A)⁺ RNA are not practical since relatively small amounts of material are available (1-2% of total RNA) and mRNAs exist in a range of sizes. However, the size of certain poly(A)⁺ RNA preparations was determined by sucrose density centrifugation and shown to be consistent with the reported size of the milk protein mRNAs (see chapter 5). Size analysis of the translation products of poly(A)⁺ mRNAs also indicated that this RNA was of the correct size (see chapters 3 and 8). For routine determination of the intactness of RNA run through affinity columns, the unbound RNA fraction was used; these samples contain rRNAs which are sensitive indicators of degradation due to their large size. A polyacrylamide SDS disc gel electrophoresis, followed by UV absorbance scanning, were used to monitor RNA size (see methods). Figure 1b shows a profile of total mammary RNA before application to a column; discrete 18S and 28S rRNAs are present. The sharpness of these peaks

and their ratio (approx. 0.4:1) demonstrate that no significant RNA degradation has occurred during extraction. Figure 1a is a profile of a blank gel run without RNA sample, and this shows that the absorbance background of these gels contributes significantly to the overall profiles shown in Figure 1. Peaks of high absorbance are apparent at the bottom and particularly at the top of these gels and the presence of these peaks makes it impossible to comment on the presence of nucleic acid species much smaller than 18S rRNA (e.g. tRNAs) and larger than 28S rRNA (e.g. DNA) in these samples. It was not possible to eliminate the irregular absorbance background seen on scanning these gels thus it was always necessary to compare sample profiles with the profile of a blank gel. Attempts to eliminate this absorbance included gel preelectrophoresis and variation of the relative amounts of acrylamide and the catalysts TEMED and ammonium persulfate. A recent company's statement suggests that a potential source of UV-absorbing components in this procedure is the black rubber grommet used in the disc gel electrophoresis apparatus. Grommets lose UV absorbing material into the buffer during electrophoresis and this can contaminate the gel (303). Figure 1c is a profile of unbound RNA produced by passage of heat-treated total RNA through oligo(dT)-cellulose, followed by ethanol precipitation. This RNA preparation exhibits substantial loss of structure; the discrete rRNA peaks have been lost and a series of ribonucleotides of various lengths were

generated. Attempts to prevent this degradation by passage of an unbound RNA effluent from an oligo(dT)-cellulose column directly into a mixture of phenol saturated with extraction buffer and chloroform (1:1) followed by immediate extraction of the RNA were not successful in preventing significant RNA degradation.

A series of experiments was designed to determine the step in affinity column chromatography responsible for the observed loss of RNA structure. The RNA profiles of several aliquots of a total RNA preparation, treated in different ways and run on 2.2% polyacrylamide SDS gels, are presented in Figure 2. When a total RNA preparation (5.9mg) was passed directly through an oligo(dT)-cellulose column in low salt buffer, without prior heat treatment, the unbound RNA fraction maintained its discrete structure (Figure 2a). It was concluded that RNA degradation was not occurring during passage through the columns. Heating a total RNA preparation for just 5 minutes at 70°C., followed by cooling at 0°C., caused extensive RNA degradation (Figure 2b). The reason for this high sensitivity of these mammary rRNA species to heating is not understood; heat treatment of RNA samples is often performed before affinity column chromatography (see introduction) and there are no reports indicating that this treatment causes the severe disruption of primary structure seen here. The possibility that the high susceptibility of these total RNA

preparations to heating was due to contamination with small amounts of nucleases which had not been removed during phenol extraction was studied. Protease treatment of the samples prior to heat treatment did not prevent this effect (data not shown); moreover, incubation of total RNA preparations at 37° C. for 1 hour (conditions most favourable for RNase activity) did not result in significant RNA degradation (Figure 2c).

In summary, the total RNA preparations from lactating rat mammary gland examined here appeared to be degraded by even short exposure to high temperatures (70°C.) and this unusual property of rat mammary RNA is discussed further in the discussion to this chapter. Since intact poly(A)⁻ RNA preparations were required in order to carry out meaningful analyses of these RNA fractions, affinity column chromatography was routinely performed without prior heat treatment of samples in all experiments. By omitting the heating step, intact total RNA preparations routinely yielded intact unbound RNA.

(iv) The [³H]-poly(U) assay for poly(A)

To assess the separation of mammary RNA preparations into poly(A)⁺ and poly(A)⁻ fractions by affinity chromatography an accurate poly(A) assay was required. The [³H]-poly(U) assay adopted for this purpose is described fully in "methods" and the theoretical basis of this assay

was discussed in the introduction (see p.24). The protocol routinely used for this assay was based on data from initial experiments in which optimal conditions for poly(A): poly(U) hybridization and digestion of non-hybrid poly(U) were determined. Figure 3 indicates results of one experiment performed under the conditions described in the legend; a linear standard curve with a slope of 158 cpm/ng poly(A) was obtained using 0-275 ng poly(A). The assay background, obtained with 0ng poly(A), was 1750 cpm and the assay was linear up to approximately 46,000 cpm, a value corresponding to about 67% of the TCA precipitable cpm in the assay. Subsequent experiments indicated that the linearity of the standard curve was lost when over 70% of the TCA-precipitable cpm in the assay were hybridized with poly(A) and precipitated.

Studies on the effect of varying hybridization time and digestion conditions on the final cpm value obtained with the assay are summarised in Table 3. Similar results were obtained with hybridization times of 15 and 30 mins. (rows 1 and 2); the small difference observed between these two values is not significant with an assay background of 2,000 cpm. The use of several digestion times - 15, 30 and 45 minutes at 30°C. (lines 3,4 and 5) - produced similar results too. It is noteworthy that the enzyme was effective at both 0°C and 30°C (lines 2 and 4 respectively) and performing the digestion with double the usual enzyme

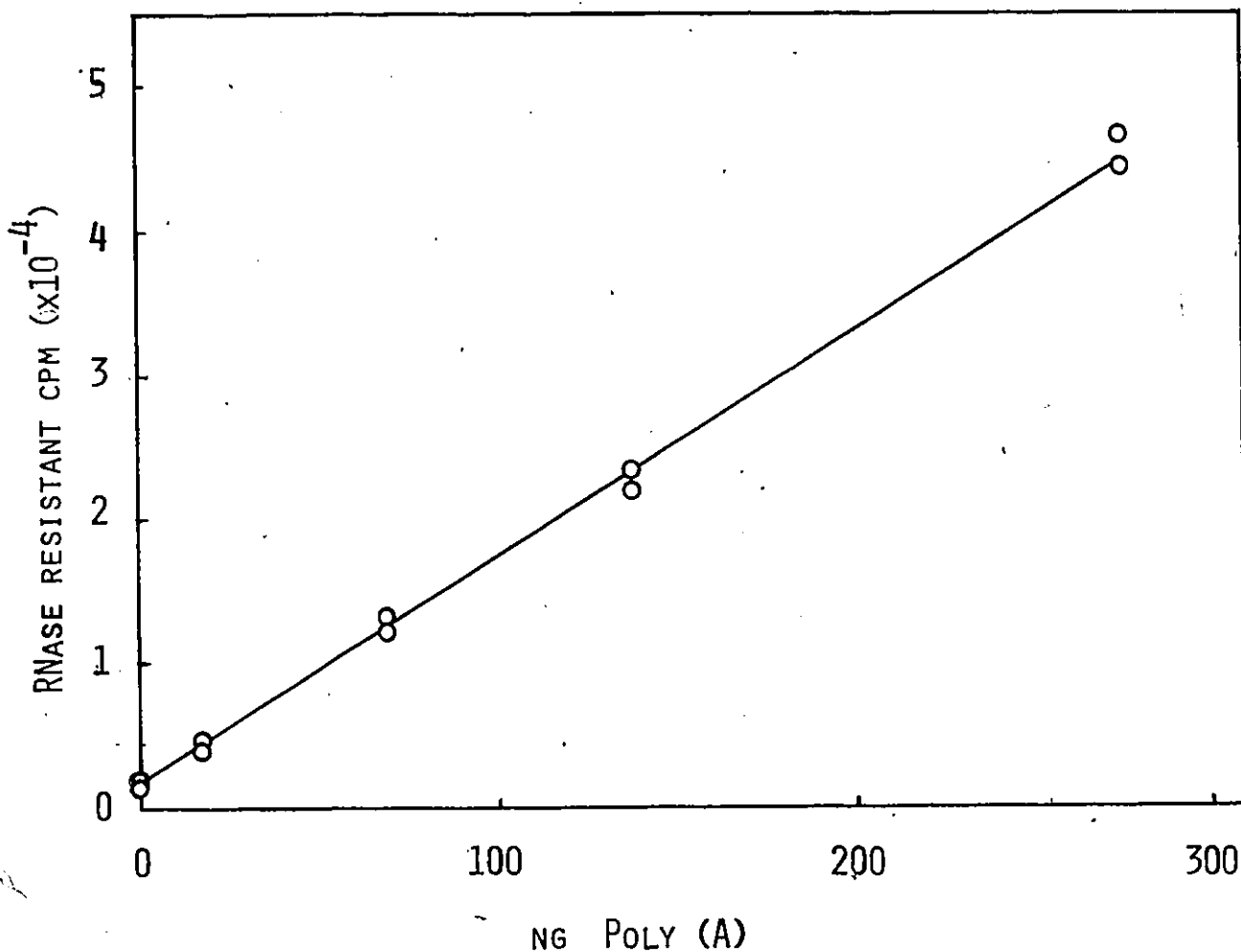


Figure 3. Standard Curve for $[^3\text{H}]$ -Poly(U) Assay of Poly(A).

A $[^3\text{H}]$ -poly(U) assay was performed as described in the text. The indicated amounts of synthetic poly(A) were hybridized with 0.114 μCi $[^3\text{H}]$ -poly(U) at 30°C for 30 min. After a 30 minute RNase A digestion (0.3 μg enzyme/assay) at 0°C, the remaining hybrid was acid precipitated. The precipitate was collected on GF/C filters and counted (see Methods).

TABLE 3: THE EFFECT OF VARIOUS CONDITIONS OF HYBRIDIZATION AND RNase DIGESTION ON THE [³H]-POLY(U) ASSAY FOR POLY(A).

Expt. no.	Duration of hybridization (mins)	Duration of RNase digestion (mins)	Temperature at which digestion performed (°C)	Amount of enzyme used (mg/assay)	[³ H]-label in hybrid precipitate (cpm)
(i)	15	30	0	0.3	20240 ± 140
	30	30	0	0.3	22250 ± 590
(ii)	30	15	30	0.3	22400 ± 700
	30	30	30	0.3	22990 ± 1000
	30	45	30	0.3	22590 ± 280
(iii)	30	30	0	0.6	21690 ± 660

A [³H]-poly(U) assay was performed in a volume of 0.2ml as described with the indicated modifications in hybridization time, RNase digestion time and digestion temperature. The assay contained 138ng synthetic poly(A), and 0.114 μ Ci [³H]-poly(U) equivalent to 68,300 cpm of TCA precipitable [³H]-label. The amount of [³H]-label appearing in the TCA precipitate is expressed as the mean of duplicate determinations ± the range. The background of this assay, obtained by omission of the poly(A) from the assay, was 2,000 cpm.

concentration at 0°C. again gave a similar final result (lines 2 and 6). The reason for investigating these two digestion temperatures is that both conditions have been used previously; Bishop et al. (94) digested their poly(A) assays at 0°C while Gedamu et al. (76) digested at 30°C. The data of Figure 3 and Table 3 both indicate that under the conditions being employed, the [^3H]-poly(U) assay can measure poly(A) accurately.

Subsequent experiments investigated whether or not this assay could be used to measure the poly(A) content of RNA fractions of oligo(dT)-cellulose columns. It appeared unlikely that assay conditions which are satisfactory for the measurement of standard poly(A) are necessarily adequate for the measurement of poly(A) in RNA samples, especially in RNA samples containing low amounts of poly(A). In this case, large quantities of RNA must be present in the assay and modification of the RNase digestion conditions could be required. In order to provide appropriate samples to test the [^3H]-poly(U) assay, the following fractionation was performed. A total RNA preparation (74.4mg) was run through four oligo(dT)-cellulose columns as described in methods. The final unbound RNA was designated "4x poly(A)⁻". The poly(A)⁺ RNA bound by each column was eluted and saved for separate analysis. An aliquot of the unbound RNA effluent from the second column ("2x poly(A)⁻ RNA") was also saved for analysis. The total unfractionated RNA, the four poly(A)⁺ RNA fractions and the two unbound poly(A)⁻ fractions were

all analyzed with the [^3H]-poly(U) assay under the conditions determined above. Using synthetic poly(A) as a standard, the hybrid cpm produced by each sample was converted into poly(A) content. These data are presented in Table 4.

[^3H]-poly(U) analysis of these RNA fractions suggests, from the data of Table 4, that a total RNA preparation containing 146 μg poly(A) has been fractionated into four poly(A)⁺ samples containing a total of 118 μg poly(A) and a final unbound RNA fraction with 156 μg poly(A) remaining! These results are clearly inconsistent; the most likely cause of this inconsistency was an overestimate of the poly(A) content of the unbound RNA fractions. Up to 210 μg of unbound RNA were included in the [^3H]-poly(U) assay and these are amounts which are in vast excess to the amounts of poly(U) used. These large quantities of RNA would be competing with the [^3H]-poly(U) for digestion by RNase and could cause the digestion to be limiting.

In a separate experiment, doubling the level of RNase A in the digestion (from 0.3 μg /assay to 0.6 μg /assay) did significantly reduce the observed percentage of poly(A) in the final unbound RNA fraction from 0.09% to 0.035%. This observation confirmed that digestion was limiting in these assays; furthermore the inconsistency between assays (compare 0.09% with the value of 0.277% shown in Table 4) indicated a problem.

TABLE 4: MEASUREMENT OF THE POLY(A) CONTENT OF TOTAL AND FRACTIONATED RNA PREPARATIONS BY THE [³H]-POLY(U) ASSAY

RNA type	Sample size (mg)	Poly(A) in sample (%)	Poly(A) in sample (μg)	Total poly(A) retained by columns (μg)	Expected amount of poly(A) in final unbound RNA (μg)
initial RNA	74.4	0.196	146.0		
Poly(A) ⁺ RNA eluted from columns:					
1	0.603	8.86	53.4	118.4	
2	0.810	4.74	38.4		
3	0.381	3.60	13.7		
4	0.371	3.48	12.9		
unbound RNA washed from columns:					
2	63.18	0.364	230.0		
4	56.37	0.277	156.0		4 27

A total RNA preparation was run through 4 oligo(dT)-cellulose columns to yield four separate poly(A)⁺ RNA fractions and a final unbound (4x poly(A)⁻) RNA. These RNA preparations and an aliquot of the unbound RNA effluent from the second column (2x poly(A)⁻ RNA) were assayed for poly(A) under the conditions used for the standard poly(A) curve (Fig. 3). Comparisons with the poly(A) standard were used to calculate the amount of poly(A) in each sample. The expected amount of poly(A) in the final unbound RNA preparation was determined by subtracting the amount of poly(A) retained by the four columns from that in the initial RNA preparation.

Table 5 summarises data from a series of [^3H]-poly(U) assays performed in the "4x poly(A)" unbound RNA fraction; these studies were performed to determine optimal assay conditions for analysis of this sample. Assays were performed using three amounts of RNA sample (i) 47 μg , (ii) 94 μg , (iii) 188 μg . Hybridization was performed as usual at 30°C for 30 min. Digestion was done with increased levels of enzyme (0.94 μg /assay) and at a temperature of 30°C. For each RNA amount, digestion was allowed to proceed for 30, 45 and 60 minutes. All the RNase-resistant cpm obtained in experiments (i)-(iii) are low compared to previous results. The thirty minute value is in all cases higher than the 45 and 60 minute cpm, but it is not clear how significant this is due to the background value of 417 cpm. Certainly, complete digestion has probably been achieved in these samples by 45 minutes, since only very small and insignificant differences are seen between the 45 and 60 minute values. Only the highest amount of RNA used (188 μg RNA) gave cpm values above background in this assay. Using a cpm of 659 (average of 45 and 60 minute digestion values) for this sample, 188 μg of unbound RNA contains approximately 2.5ng poly(A); this represents a poly(A) content of 0.0013%. This value is much lower than those previously obtained (see Table 4). The limitation of enzyme digestion in this assay is most clearly demonstrated if the results of experiments (i)-(iii) in Table 5 are compared to those of (iv). In section (iv)

TABLE 5: THE EFFECT OF SEVERAL RNase DIGESTION CONDITIONS
ON THE RESULTS OF [³H]-POLY(U) ASSAY OF AN UNBOUND RNA
FRACTION FROM OLIGO(dT)-CELLULOSE CHROMATOGRAPHY

Expt. no. #	Sample in assay (µg)	RNase digestion time (mins.)	RNase digestion temperature (°C)	[³ H]-label in hybrid precip- itate (cpm)
(i)	47.0	30	30	345
		45	30	279
		60	30	251
(ii)	94.0	30	30	480
		45	30	439
		60	30	394
(iii)	188.0	30	30	733
		45	30	634
		60	30	684
(iv)	47	30	0	11974
	94	30	0	19948
	188	30	0	22923

An unbound RNA preparation which had been run through four oligo(dT)-cellulose columns (see Table 4) was analysed by the [³H]-poly(U) assay. Hybridization was performed at 30°C. for 30 min. The RNase A digestion was performed using 0.94 g enzyme/assay (i) - (iii) and 0.6µg enzyme/assay (iv) at the indicated temperatures and times. The background of the assay (using 0ng poly(A)) was 417 cpm; each assay contained 0.057 Ci [³H]-poly(U) equivalent to approx. 42,300 cpm. The assay volume was 0.3 ml.

the three RNA sample amounts were assayed under conditions similar to those in (i)-(iii) except that only 0.6 μ g enzyme was included per assay and the digestion was performed over 30 minutes at 0°C, the temperature used by Bishop et al (94). All samples yield very high RNase-resistant cpm. This discrepancy in results must be due to a large difference in the rate of enzyme activity at the two incubation temperatures. It should be recalled that for the purpose of producing a standard curve using synthetic poly(A), a 30 minute incubation using 0.3 μ g enzyme at 0°C was sufficient. However, when analyzing large amounts of RNA with this assay, much higher rates of digestion are needed. This has been achieved by increasing the levels of enzyme used, and increasing both incubation time and temperature.

To verify the results of the [³H]-poly(U) assay at high RNA concentration an internal standard of synthetic poly(A) was included with an aliquot of the RNA sample. When the two components are present together in the assay, the [³H]-label in the hybrid should equal the sum of the [³H]-hybrid label for each of the two components. The results of these mixing experiments are shown in Table 6. The internal standard used was 34.5ng poly(A), which when tested separately in the assay yielded a hybrid precipitate of 4,073 cpm. Three types of RNA sample were used: total mammary RNA, a poly(A)⁺ RNA fraction and the unbound 4xpoly(A)⁻ RNA.

TABLE 6: THE USE OF AN INTERNAL STANDARD TO VERIFY THE USE OF THE [³H]-POLY(U) ASSAY ON FRACTIONATED RNA PREPARATIONS

Sample RNA		Amount of [³ H]-label in hybrid:		Expected amount of [³ H]-label in hybrid:
type	µg in assay	(a) without internal standard (cpm)	(b) with internal standard (cpm)	(c) with internal standard (sample value +4070 cpm) (cpm)
total	37.2	7620	12320	11690
poly(A) ⁺	3.25	5790	9726	9860
unbound	235	820	5360	4890
*unbound	235	820	5198	4890

A [³H]-poly(U) assay was performed on initial and fractionated RNA preparations in the absence (a) and presence (b) of an internal standard. Hybridization was at 30°C for 30 minutes and the RNase digestion (0.94µg enzyme/assay) was at 30°C for 45 minutes. The internal standard consisted of 35ng poly(A) which itself gave a value of 4070 cpm. in the assay. The expected amount of [³H]-label in the precipitate when both the sample RNA and the internal standard were included in the assay (c) was determined by adding 4070 cpm to the values given in (a). The assay background (with 0ng poly(A)) was 420 cpm. * - indicates 1.41µg enzyme/assay were used.

preparation. The hybrid [^3H]-label given by each sample when tested separately by the assay is shown in column (a). Column (b) indicates the hybrid label cpm obtained when both the sample and the external standard are included in the assay. Column (c) presents the expected hybrid cpm, obtained by adding together the value for the internal standard (4073 cpm) and the sample value given in column (a). There is good agreement between the values presented in columns (b) and (c). This observation indicates that the digestion conditions employed (0.94 μg enzyme/assay at 30°C for 45 minutes) are not limiting, and that the [^3H]-poly (U) assay may be used under these conditions to determine the poly(A) content of RNA preparations up to RNA amounts of 235 μg . This conclusion is substantiated by the fact that increasing the RNase amount to 1.41 μg per assay gave a result similar to that obtained with 0.94 μg per assay (Table 6). In subsequent studies, the [^3H]-poly(U) assay is used to measure poly(A) content of RNA preparations as low as 5-20 parts per million (per mole nucleotide).

(v) The effect of binding buffer salt concentration and sample size on the efficiency of oligo(dT)-cellulose column chromatography

This chapter has already indicated some of the conditions needed for effective oligo(dT)-cellulose affinity

chromatography of rat mammary RNA: appropriate flow rate and sample size, and omission of heat-treatment to maintain RNA integrity. Previous fractionations described in this work were performed with a binding buffer containing 0.4M NaCl. It was now possible to perform experiments to investigate the effect of the ionic strength of the binding buffer on the effectiveness of RNA fractionation using this resin, since a sensitive method for poly(A) detection had been optimized. As indicated in the introduction, lowering the binding buffer salt concentration is reported to reduce the proportion of non-specific RNA binding to oligo(dT)-cellulose. A series of fractionations was therefore performed in order to compare the effects of binding buffers containing 0.4M NaCl and 0.15M NaCl.

Two mammary RNA preparations were divided into aliquots. Each aliquot was fractionated by passage through four oligo(dT)cellulose columns using binding buffer containing either 0.4M or 0.15M NaCl. Both the final unbound RNA and the four poly(A)⁺ RNA fractions retained by the columns were collected and their amounts determined by UV absorbance. Table 7 indicates the total poly(A)⁺ RNA yield from all four columns for each fractionation. In total, six fractionations were performed - three using binding buffer containing 0.4M NaCl and three using buffer containing 0.15M NaCl. Fractionation of preparation #1 under the two conditions gave reasonably similar yields - use of the

TABLE 7: THE EFFECT OF BINDING BUFFER SALT CONCENTRATION
ON RNA RETENTION DURING OLIGO(dT)-CELLULOSE CHROMATOGRAPHY

Fractionation no.	Total RNA applied. (mg)	Sum of the RNA retained by four columns using:	
		0.4M NaCl (mg)	0.15M NaCl (mg)
1a	74	2.17	
1b	74		1.96
2a	44	1.47	
2b	44	1.56	
2c	44		1.03
2d	44		0.98

Two total RNA preparations were divided into aliquots. Each aliquot was passed through four oligo(dT)-cellulose columns, and bound RNA was eluted from each column separately with low salt buffer.

For each fractionation, the sum of the RNA retained by the four columns is given; RNA was measured by UV absorbance (see Methods). Oligo(dT)-cellulose chromatography was performed as detailed in Methods except that two different salt concentrations were used in the high salt buffer: 0.4M NaCl and 0.15M NaCl.

lower ionic strength buffer reduced the yield of retained RNA by about 200 μ g (10% of the total yeild). Smaller amounts of initial RNA were used in the fractionation of preparation #2 and much greater differences were observed here. Use of the high ionic strength binding buffer yielded about 1.5 mg retained RNA while use of lower ionic strength buffer consistently gave only 1.0mg retained RNA.

The reduced amounts of poly(A)⁺ RNA obtained when oligo(dT)-cellulose fractionation is performed with binding buffer containing 0.15M NaCl could be due at least in part to a reduced contamination of this fraction by rRNA. This was supported by electrophoretic analysis of poly(A)⁺ RNAs prepared under the two conditions (data not shown). An attempt to further reduce rRNA contamination of bound poly(A)⁺ RNA fractions was made. This involved washing the oligo(dT)-cellulose column with buffer containing 50mM KCl at 4 °C. (304) subsequent to removal of nonbound RNA in 0.15M NaCl and before elution of poly(A)⁺ RNA with low salt buffer. This wash removed only very small amounts of RNA from the column and it was considered ineffective in purifying the poly(A)⁺ RNA retained by the column.

It is clear that the effectiveness of oligo(dT)-cellulose chromatography cannot be judged solely on the basis of high yields of column bound material in view of the possibility of variable levels of contamination of the samples with rRNA. The six RNA fractionations described

above were therefore all analysed using the [^3H]-poly(U) assay. In each case, the poly(A) content of the initial RNA, the four poly(A)⁺ RNA fractions and the final unbound poly(A)⁻ RNA was determined. Table 8 summarizes the data thus obtained; it also includes data for two other fractionations of total RNA preparations. The poly(A) content of each sample is expressed as a percentage of the total poly(A) found in the starting material. The data of Table 8 indicate that there are two factors influencing the efficiency with which poly(A)⁺ RNA is removed from total RNA: sample size and the salt concentration in the column binding buffer.

Sample size appears to be the most important factor. When 74mg total RNA (equivalent to 102 μg poly(A)) was applied to the first oligo(dT)-cellulose column only 40-50% of the poly(A) in the sample was retained and smaller amounts were still retained by subsequent columns. When the sample was reduced to 44mg RNA (51 μg poly(A)), 59-88% of the poly(A) in the sample was retained on the first column. This percentage rose to 90-100% when only 21-22mg total RNA were applied. This effect might be explained by an overloading of the oligo(dT)-cellulose resin with sample in the case of the larger samples (see discussion); however, a high proportion of the poly(A) content of all total RNA preparations is retained provided the sample is

TABLE 8: DETERMINATION OF THE POLY(A) CONTENT OF BOUND AND UNBOUND RNA FRACTIONS PREPARED BY OLIGO (dT)-CELLULOSE CHROMATOGRAPHY - PREPARATION OF POLY(A)⁺ AND POLY(A)⁻ RNAS.

Expt: RNA no. and prep no.	Amount of RNA used (mg)	Poly(A) retained:				Final poly(A) ⁻ fraction	
		Col 1	Col 2	Col 3	Col 4	poly(A) content	(ng poly(A) / mg RNA)
		(% of poly(A) in initial unfractionated RNA)				(% of initial)	
1a	74	40.	27	8.6	4.8	1.17	21.0
1b*	74	51	22	8.9	0.5	0.46	7.0
2a	44	59	27	8.2	4.3	2.1	29.0
2b	44	63	24.5	9.3	4.0	0.8	12.0
2c*	44	68	16	8.9	ND	2.0	28.0
2d*	44	88	11	5.5	ND	1.9	27.0
3	21	90	ND	-	-	0.36	3.4
4	22	100	ND	-	-	0.23	2.4

Total RNA was passed through successive oligo(dT)-cellulose columns as described in "methods". The poly(A) content of the final unbound RNA and of each RNA sample retained by a column is expressed as the percentage of the poly(A) in the initial RNA preparation. Poly(A) content was determined by the [³H]-poly(U) assay (see methods). ND - none detected (the amounts of poly(A)⁺ RNA retained by this column were too small to be analyzed by the [³H]-poly(U) assay). (-) = column chromatography not carried out. * = applied with binding buffer containing 0.15M NaCl.

run through a sufficient number of columns. Four oligo(dT)-cellulose columns were adequate to remove 80% or more of the poly(A) from up to 74mg of the initial RNA preparation.

In most of the fractionations detailed in Table 8, overall poly(A) recovery was over 90%, and thus poly(A) recovery is essentially quantitative.

The use of binding buffer containing 0.15M NaCl instead of 0.4M NaCl did routinely result in a slightly higher recovery of poly(A) on the first column and a lower poly(A) recovery on later columns, particularly on column four. These findings are again consistent with the idea that a lowered salt concentration reduces contamination of poly(A)⁺ RNA fractions with rRNA. The total yield of the poly(A) in all the column bound RNA fractions did not appear to be greatly affected by the ionic conditions used in the chromatography. Both salt conditions would appear to be sufficient to allow complete retention of poly(A)⁺ RNA by this resin.

The [³H]-poly(U) assay also provided important information regarding the nature of the final unbound RNAs produced by these fractionations. In all cases, the unbound RNA fraction was substantially depleted of poly(A) and there appeared to be little difference between the poly(A)⁻ RNAs prepared under the two ionic strength conditions used for

running the affinity columns. The highest level of poly(A) found in these preparations constituted of only 2.1% of the poly(A) present in the starting material and several unbound RNA fractions contained much lower levels; the lowest recorded was 0.23%. In terms of absolute amounts, these percentages represented 29-2.4ng poly(A) per mg RNA. These values are so low that even using large quantities of unbound RNA in the poly(A) assay, the resulting hybrid [³H]-label is close to background values. The large variability in the poly(A) content of these samples may be due in part to the fact that measurements of their poly(A) content are made at the limits of sensitivity of the [³H]-poly(U) assay. For all practical purposes these final unbound RNA preparations may be regarded as poly(A)⁻.

DISCUSSION

In earlier reports on purification of rat α LA and casein mRNAs (275, 42), complete recovery of poly(A)⁺ RNA was deemed to have occurred after passage of total RNA through one oligo(dT)-cellulose column since passage of the resulting unbound RNA through a second column did not result in further RNA retention. In the present studies, it was necessary to evaluate RNA fractionation by affinity column chromato-

graphy, using an independent measure of poly(A) content, since it was essential for subsequent analyses to show that the unbound RNAs produced were free of poly(A) and that most or all of the poly(A) in the total RNA preparations was recovered in the poly(A)⁺ fractions. RNA preparations which failed to bind to oligo(dT)-cellulose could still contain significant amounts of oligo(A), if a proportion of the poly(A) in these samples was present as sequences shorter than 20 nucleotides.

Table 8 summarises the fractionation of several rat mammary RNA preparations by oligo(dT)-cellulose in terms of poly(A) recovery. Two major conclusions may be drawn from this data. First, with the 1 g. oligo(dT)-cellulose columns used here, the number of rounds of chromatography needed to remove a high proportion of the poly(A) from the RNA fractions depended on initial sample size. Large samples (44mg RNA and more) required passage through 3-4 columns, depending on the precise salt conditions, while smaller samples (21-22mg RNA) needed just 1-2 column runs for complete fractionation. The poly(A) assay indicates that these columns appear to be capable of binding no more than 50µg poly(A) on each passage of a total RNA preparation. This value is much lower than that quoted by the producers of this resin, Collaborative Research (305). They claim that type 2 oligo(dT)-cellulose binds 1,200µg or more poly(A) per g. resin. Collaborative Research measured

poly(A) retention by this resin using synthetic poly(A) sequences of length 290 nucleotides (mol.wt. 100,000 daltons). It will be shown (Chapter 4) that the steady-state length of the poly(A) sequences present in the rat mammary preparations is much shorter than 290 nucleotides. If each poly(A) sequence is retained by one oligo(dT) chain, then the total amount of poly(A) retained by oligo(dT)-cellulose will be very dependent on poly(A) length; use of long poly(A) tracts to determine poly(A) retention will result in large overestimates of the capacity of the resin to bind much shorter poly(A) sequences. This factor may be contributing to the discrepancy observed between the observed and quoted capacities of this resin.

The second major observation to be made from Table 8 is the small amount of poly(A) retained in the final unbound RNA fractions. The amount of poly(A) remaining in these preparations is no greater than 2.1% of the initial poly(A) content in all cases, and in many instances the level is much lower - 0.23% is the smallest value observed. This degree of depletion of poly(A) from these preparations compares favourably with the levels of depletion reported by other researchers investigating the presence of poly(A) mRNA. Ragg et al. (121) fractionated a plant RNA preparation by oligo(dT)-cellulose chromatography, and showed that the unbound RNA fraction contained less than 5% of the

poly(A) of the starting material: poly(A) was measured by the relatively insensitive method of determining resistance to RNases A and T₁. Brandhorst et al (111) showed that a poly(A)⁻ RNA preparation from sea urchin embryo RNA contained only 4% of total poly(A), using the [³H]-poly(U) assay. Moffett and Doyle (116) were able to prepare poly(A)⁻ RNA from mouse liver which contained no more than 1% of the poly(A) in the original RNA extract; poly(A) was again determined by [³H]-poly(U) hybridization. It is clear that the poly(A)⁻ RNA preparations produced here from mammary RNA have been depleted of poly(A) to an extent comparable to that reported by other researchers, and that these preparations may be used for the detection of poly(A)⁻ mRNA. As discussed previously, measurements of the poly(A) content of the unbound RNA fractions were carried out at the limits of sensitivity of the [³H]-poly(U) hybridization assay, and this may partly explain the degree of variability in the reported levels.

Actual values of the poly(A) content of these samples will be lower than those reported in Table 8 since poly(A) sizing experiments performed on these poly(A)⁻ RNA samples did indicate that some of the [³H]-poly(U) hybridization could be due to binding of [³H]-poly(U) to DNA sequences (see Chapter 4).

One unexpected observation in this work was the apparent



heat-sensitivity of the rRNA species. Exposure of rRNA to temperatures of 60-70°C. for short time periods (2-10 minutes) has not been reported to adversely affect the structural integrity of RNA. No satisfactory explanation for this effect was obtained, and it appeared that rat mammary mRNA was not heat-sensitive since heat-treatment was routinely performed on RNA preparations prior to sucrose density centrifugation. (Chapter 5), and mRNA/cDNA hybridizations were performed over several hours, or even days, at 68°C. (See Chapter 6). The problem was not pursued since simple omission of the heat treatment of total RNA before oligo(dT)-cellulose chromatography permitted preparation of structurally intact poly(A)⁻ RNA.

CHAPTER 2

THE USE OF POLY(U) SEPHAROSE AFFINITY COLUMN
CHROMATOGRAPHY TO FRACTIONATE TOTAL MAMMARY RNA

In Chapter 1 it has been demonstrated that passage of rat mammary RNA preparations through successive rounds of oligo(dT)-cellulose affinity chromatography is effective in separating these preparations into poly(A)⁺ RNA fractions and poly(A)⁻ RNA fractions depleted of 98% or more of the poly(A) present in the starting material. The major drawback to this procedure was the fact that if large quantities of RNA (40mg or more) were to be processed in this way, several rounds of chromatography had to be performed. Since this work required fractionation of a large number of RNA samples into poly(A)⁺ and poly(A)⁻ preparations (see Chapter 7, developmental study), it was obviously desirable from a practical standpoint to determine if a more efficient process was available. Since there are some reported advantages of the resin poly(U) sepharose, over oligo(dT) cellulose (discussed below), the use of poly(U) sepharose for fractionation of rat mammary RNA was evaluated.

One reported advantage of poly(U)-sepharose is that it is capable of binding, and thus retaining, shorter poly(A) sequences than the oligo(dT)-cellulose resin. It is claimed that poly(U) sepharose can bind oligo(A) sequences as small as 10-15 nucleotides (55); oligo(dT)-cellulose

can only bind poly(A) sequences longer than 20 nucleotides (56, 101). RNA preparations containing short oligo(A) sequences (10-20 residues) should thus be fractionated by poly(U)-sepharose, since oligo(dT)-cellulose will be unable to retain the short oligo(A) chains, and separation of poly(A)⁺ and poly(A)⁻ RNA species will be incomplete with the latter resin. A few reports of the existence of RNAs bearing poly(A) tracts of 10-25 residues which were retained by poly(U) sepharose but not oligo(dT)-cellulose have appeared in the literature (25, 84). It has also been claimed that the use of poly(U)-sepharose produces poly(A)⁺ RNAs which exhibit reduced contamination by rRNAs compared to RNAs retained by oligo(dT)-cellulose (21).

These reported advantages of poly(U)-sepharose were the two main reasons for studying the effectiveness of this resin for fractionation of rat mammary RNA. The experiments described in this chapter were again carried out using total mammary RNA from the glands of 11-15 day lactating rats.

MATERIALS

Poly(U)-sepharose 4B was obtained from Pharmacia Fine Chemicals, Dorval, Que. It was stored as a dry powder at -20°C until column preparation. The first columns made with this resin were tall and narrow (11mm x 120 mm) but

the flow rate of solutions through columns of this dimension was too slow to be practical, particularly during passage of concentrated RNA solutions through the resin.

The columns which were finally used had dimensions of 25 mm x 20 mm and flow rates of up to 1 ml/min could be obtained with them. When not in use, columns were stored in buffer containing 0.02% sodium azide and 0.2% SDS at 4°C.

Formamide was obtained from several sources, including Matheson, Coleman and Bell (Norwood, Ohio, U.S.A.), Bethesda Research Laboratories (Gaithersburg, Md, USA) and Eastman Kodak Company (Rochester, N.Y., U.S.A.). Formamides were stored at 4°C. All these preparations of formamide had high absorbance values in the UV range (OD_{260} greater than 1.0). Attempts were made to improve the formamide by the method reported by Tibbetts et al. (306). Norite A charcoal (Fisher, Fairlawn, N.J., U.S.A.) was added to formamide (2 gm charcoal/100 ml formamide) and the suspension was stirred for two hours. Charcoal was removed by filtration through Whatman number 1 filter paper. Formamide was also treated with Chelex 100 (Biorad) and a mixed bed resin AD501-X* (Biorad) using 5 gm of each resin per 100 ml formamide. After stirring for two hours, the resins were removed by filtration. Formamide treated by this procedure was stored frozen at -20°C. Although this procedure

did reduce the UV absorbance of these formamide preparations slightly, the absorbance, measured at 270nm, remained high (1.0 O.D.).

SDS was obtained from two sources: Sigma and BDH Chemicals (Ville St.Laurent, Que). SDS from the latter company was 98% pure, while that from Sigma was only 60-70% pure. The ethanol precipitation experiments were performed with SDS from Sigma; use of the purer grade SDS in place of Sigma SDS gave comparable results in RNA precipitations from formamide.

METHODS

(i) Poly(U) -Sephadex chromatography

Poly(U) -sephadex columns were run using the buffer system of Sehgal et al. (196). Total RNA preparations, at a concentration not exceeding 2.5mg/ml, were applied to the column in 0.4M NaCl, 10mM Tris-HCl, pH7.4, 10mM EDTA and 0.2% SDS. After thorough washing with this buffer to remove unbound RNA, poly(A)⁺ RNA was eluted. This was initially accomplished by a three step elution using (a) salt free buffer (10mM Tris-HCl pH 7.4, 10mM EDTA and 0.2% SDS); (b) salt-free buffer containing 50% (v/v) formamide; (c) salt-free buffer containing 90% (v/v) formamide. In later experiments, for reasons discussed in the text, elutions (a) and (b) were omitted. Column bound and unbound RNA fractions were collected by ethanol precipitation, dissolved

in low salt buffer and their concentration was measured by UV absorbance at 260nm.

(ii) Ethanol precipitation of RNA from formamide-containing solutions

For the purpose of investigating the effects of formamide on RNA precipitation in ethanol, precipitations were performed as follows. RNA samples were brought to a final volume of 4 mls (8 mls for the study of RNA concentration effects) containing 10mM Tris-HCl, pH7.4, 10mM EDTA, 0.2% SDS in the appropriate volumes of water and formamide to obtain the required formamide concentration. Samples were then brought to 0.2M NaCl using stock 5M NaCl and two volumes of cold 95% ethanol were added. After overnight precipitation at -20°C, the precipitates were sedimented in a swinging bucket Sorval HS-4 rotor at 6500 RPM (8130g max) for 30 minutes at 4°C. The ethanol layer was removed and the precipitate washed once using 2ml cold ethanol and the same centrifugation conditions. A second wash was performed by centrifugation in a fixed angle Sorval SA-600 rotor at 14,000 RPM (28,000 g max) for 25 minutes at 4°C. The final ethanol wash was removed and the RNA precipitates were dried. Each pellet was dissolved in 0.4ml low salt buffer and RNA concentration was determined by UV absorbance.

The methods for extraction of total mammary RNA and

the [^3H]-poly(U) assay for poly(A) were as described previously (Chapter 1, Methods).

RESULTS

(i) Initial studies on the use of poly(U)-sepharose: poly(A)⁺ RNA elution required 90% formamide solutions

In the literature, there is a wide variation in the reported conditions required to elute bound RNA from poly(U)-sepharose. Conditions range from the use of water (53) to 70% (21) or 90% (52) formamide solutions. It was thus necessary to determine in the initial experiments the type of elution buffer required to remove bound RNA from this resin.

The studies shown in Table 9 compare the yield of poly(A)⁺ RNA when heat-treated total mammary RNA was passed through oligo(dT)-cellulose or poly(U)-sepharose columns. In agreement with previous studies, oligo(dT)-cellulose retained about 2% of the RNA applied under binding conditions; the retained poly(A)⁺ RNA was readily eluted with low salt buffer. Elution of bound RNA from poly(U)-sepharose was initially performed under three different conditions: the salt-free buffer eluted only 79 μg RNA (0.42% of RNA applied to the column) and further elution with 50% and 90% formamide solutions liberated only 96 μg and 105 μg respectively. It is evident that a high recovery of bound RNA from poly(U)-sepharose columns requires highly denaturing

TABLE 9 COMPARISON OF POLY(A)⁺ RNA YIELD AFTER FRACTIONATION
OF TOTAL MAMMARY RNA USING OLIGO(dT)-CELLULOSE AND POLY(U)-
SEPHAROSE

Resin	No. of columns used	Eluate from column #	Poly(A) ⁺ RNA eluted			final yield (%)
			salt-free buffer (μg)	50% form (μg)	90% form (μg)	
T	2	1	342	-	-	1.84
		2	28	-	-	0.15
U	1	1	79	96	105	1.51
U	2	1	-	-	481	2.59
		2	-	-	71	0.38

Total mammary RNA preparations (18.6mg) were applied to affinity columns as described. RNA was applied to oligo(dT)-cellulose buffer (T) in 0.4M NaCl and poly(A)⁺ RNA was eluted in low salt buffer (Chapter 1, methods). Poly(U)-sepharose (U) columns were run as described (see methods), poly(A)⁺ RNA was eluted by up to three conditions as indicated. Samples were heat-treated before application to the resin and one or two columns were used sequentially as shown. (-) indicates that this elution was not performed. Final poly(A)⁺ RNA yields are expressed as a percentage of the initial RNA sample.

conditions of the type provided by a 90% formamide solution. Subsequent poly(U)-sepharose elutions were therefore performed with 90% formamide only, as indicated in the third fractionation shown in Table 9. A high yield of poly(A)⁺ RNA was obtained using 90% formamide in this case; 18.6mg total RNA gave 481µg poly(A)⁺ RNA (2.59%) on the 1st column but only 71µg (0.38%) on the second column. It appears that 18.6 mg total RNA has almost all its poly(A)⁺ RNA removed after two passages through poly(U) sepharose.

During these studies, which indicated that formamide solutions are required in order to elute column bound RNA, certain problems associated with the use of formamide became apparent. It has already been stated that commercially available formamide has a significant absorbance in the UV range (230-280 nm). The 90% formamide solutions used to elute bound RNA from the column had an optical density of 0.8 or higher; since RNA elution is usually monitored by optical density measurements this was a serious problem and it was necessary to collect relatively large volumes of column eluate and precipitate the RNA before measurement of the RNA in the eluate could be performed. As will be shown later, the inability to measure the yield of poly(A)⁺ RNA directly after elution from the column is a serious handicap, since recovery of poly(A)⁺ RNA after passage through poly(U)-sepharose and after ethanol precipitation cannot be determined at each step. Furthermore, a single ethanol precip-

itation was often not sufficient to remove all traces of formamide and reprecipitation of RNA samples was sometimes necessary.

(ii) Fractionation of RNA by Poly(U)-sepharose can give low poly(A) yields

A total RNA preparation (51mg) was applied to two poly(U)-sepharose columns in sequence using Sehgal's buffer system (see methods). No heat treatment was used. After washing through the rRNA, bound RNA was eluted separately from each column with the buffered 90% formamide solution. Both unbound and bound RNA fractions were collected by precipitation and assayed for poly(A) content using the [^3H]-poly(U) assay. In this experiment, the second column yielded no significant amount of poly(A)⁺ RNA - it appeared that the first column removed all the poly(A)⁺ RNA in the sample. When the initial RNA, the poly(A)⁺ RNA fraction from the first column and the unbound RNA were all assayed for their poly(A) content, overall poly(A) recovery after fractionation was poor. The initial RNA contained 64 μg poly(A); 30-35 μg were recovered in the poly(A)⁺ RNA fraction and no poly(A) could be detected in the unbound (2x poly(A)⁻) RNA fraction. This represents a poly(A) recovery of only 50%.

Possible reasons for this result were investigated.

Initial studies looked at the possibility that the poly(A) assay was not providing true values due to the presence of small amounts of poly(U) in the RNA samples. Loss of poly(U) from poly(U)-sepharose during washing of these columns has not been reported although poly(U) is reported lost from poly(U)-cellulose (40). If small amounts of unlabelled poly(U) were introduced into the poly(A): [^3H]-poly(U) hybridization, unlabelled poly(U) would compete with the [^3H]-poly(U) for hybridization to poly(A). The assay would yield low values for the poly(A) present under these conditions.

In Table 10, it is shown that salt-free buffer which had been passed through poly(U)-sepharose in the absence of RNA had no effect on [^3H]-poly(U) detection of synthetic poly(A). Assay of RNA samples in the presence and absence of an internal standard were also consistent. These results suggest that there are not sufficient amounts of poly(U) in these samples to interfere with the [^3H]-poly(U) assay.

A different explanation for the low poly(A) recovery from poly(U) sepharose column chromatography was provided by studies on the recovery of RNA by ethanol precipitation from formamide-containing solutions. This work is summarised in the following section.

(Legends to Table 10)

- (i) [^3H]-poly(U) assays were performed in the presence of 110 ng synthetic poly(A) and 0.15-0.30 ml of binding and elution buffers which had been passed through poly(U) - sepharose. The volume of the assay was increased to 1.2 ml to permit relatively large volumes of solution to be included. A high concentration of MgCl_2 (16.7mM) was included in the assay to counteract the EDTA in the column buffers. The assay was performed as described in methods (Chapter 1). The values of precipitated cpm shown are the mean $\pm$ the range for duplicate determinations.
- (ii) A [^3H] poly(U) assay was performed on initial and fractionated RNA in the absence (a) and presence (b) of an internal standard. The assay was performed as described in methods, Chapter 1, s.(iii). The internal standard, 31ng poly(A), gave 4245 cpm in the assay. The expected amount of [^3H]-label in the precipitated hybrid when both the sample RNA and the internal standard were included in the assay was determined by adding 4243 cpm to the values in (a). The assay background (with 0ng poly(A)) was 530 cpm.

TABLE 10. EVIDENCE AGAINST INTERFERENCE IN THE [³H]-POLY (U) ASSAY BY POLY(U) IN SAMPLES PREPARED BY POLY(U) SEPHAROSE COLUMN CHROMATOGRAPHY

(i) Effect of including column eluate in the poly(A) assay

Addition to assay	[³ H]-label in hybrid precipitate (cpm)	proportion of precipitated [³ H]-label (%)
none	20,000 [±] 1100	100
0.3ml binding buffer which had been passed through column #1	18,500 [±] 105	91
0.3ml binding buffer which had been passed through column #2	19,900 [±] 160	98
0.15 ml buffered 90% formamide solution which had been passed through column #1	18,400 [±] 480	91

(ii) Analysis of RNA fractions produced with poly(U)-sepharose by the [³H] poly(U) assay with or without an internal standard

Sample RNA		Amount of [³ H]-label in the hybrid when the assay contained:		Expected amount of [³ H]-label in (b)
type	µg in assay	(a) sample RNA (cpm)	(b) sample RNA and internal standard (cpm)	(cpm)
total	28	4790	8290	9035
unbound	151	160	4930	4405
poly(A) ⁺	0.55	3220	7680	7465

(iii) Recovery of RNA from formamide-containing solutions:
optimization of ethanol precipitation

The high degree of variability observed in the recovery of RNA from formamide solutions made it necessary to carry out a large number of precipitations before any conclusions could be drawn. All the precipitations performed can be grouped under three sets of conditions:

- (a) precipitation from the type of solution that would be obtained from a poly(U) sepharose column eluate (90% formamide, 10mM Tris-HCl pH7.4, 10mM EDTA, 0.2% SDS) after dilution with water to the indicated formamide concentration. Under these conditions SDS concentration varied with the formamide concentration.
- (b) Precipitation from solutions of varying formamide concentration but constant SDS concentration (0.2%).
- (c) Precipitation from solutions of varying formamide concentration in the absence of SDS.

The reason for investigating these three conditions was to determine whether or not the SDS present in the precipitations was contributing to, or causing, the low RNA recoveries, since initial observations suggested that this could be a factor. However, RNA precipitations carried out in the absence of formamide showed that increasing amounts of SDS (Sigma) up to a concentration of 0.5%, did not affect recovery (see Table 11). These data show that when 149 µg RNA were precipitated under the conditions described, recovery was about 82% in the absence of SDS,

TABLE 11: EFFECT OF SDS ON RECOVERY OF RNA AFTER PRECIPITATION
FROM AQUEOUS SOLUTION

SDS CONCENTRATION (%)	RNA YIELD (%)
0	81.6 ± 5.1
0.05	84.3 ± 2.1
0.10	74.1 ± 1.8
0.15	82.6 ± 0.0
0.20	73.4 ± 7.1
0.30	82.9 ± 0.7
0.40	78.2 ± 3.9
0.50	83.1 ± 2.6

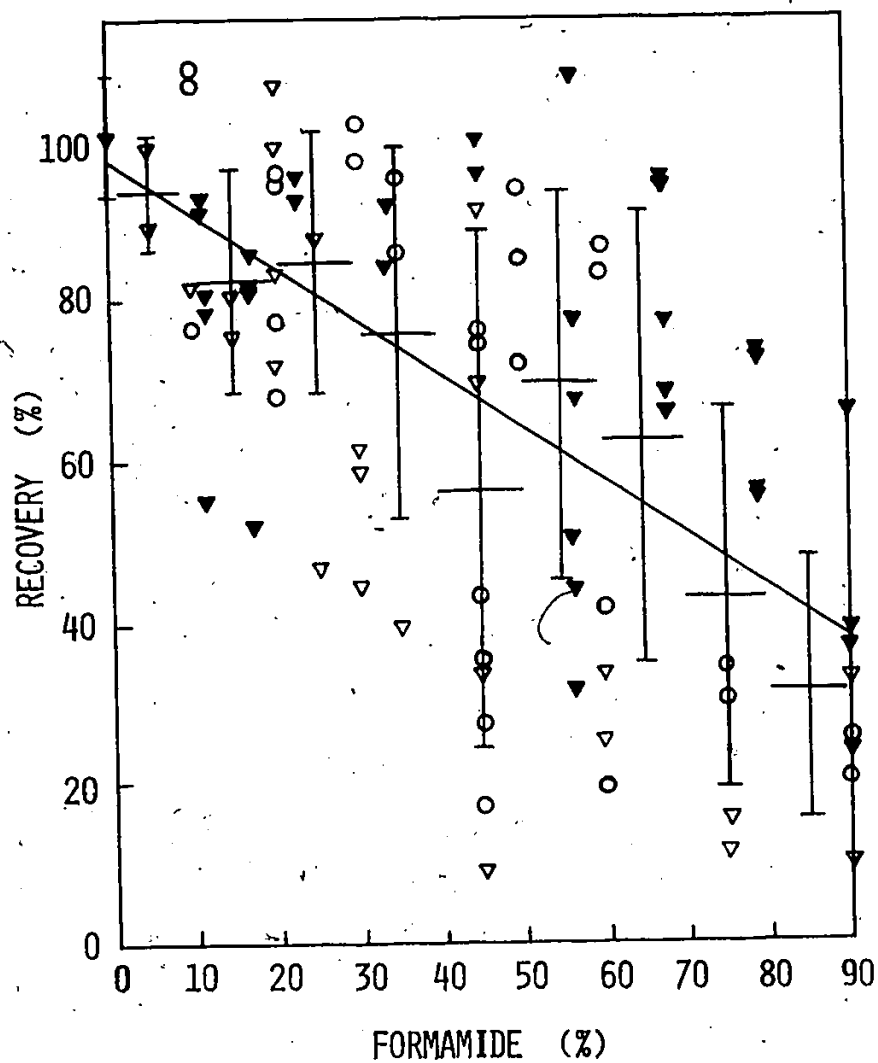
TOTAL MAMMARY RNA (149µg) was precipitated from 4ml low salt buffer containing the indicated SDS concentration. Precipitation was performed as described in Methods. Recoveries were measured by absorbance (260nm) measurements and expressed as a percentage of the amount of starting material (149µg). The value given for each SDS concentration is the mean ± the range for duplicate determinations. The SDS used was from Sigma (see Materials).

and this remained fairly constant over the range of SDS concentrations tested. It remained a possibility that the low RNA recoveries observed might be due to the combined effects of SDS and formamide, and this is why the three sets of conditions were investigated.

Figure 4 summarizes the results of studies on recovery of RNA after precipitation from formamide-containing solutions. Several control precipitations performed in the absence of formamide yielded an average recovery of $85.6\% \pm 6.8\%$ (standard deviation); this recovery has been adjusted to 100% and all other recoveries have been expressed as a percentage of this control. Figure 4 shows that with increasing formamide concentration, RNA yield progressively dropped and values as low as 10% of the control were obtained using 70-90% formamide solutions. No difference between the three sets of conditions was observed and therefore the data for all three conditions were combined to produce the regression line. This line indicates that overall there is a 7% decrease in RNA recovery for each 10% increment in the formamide concentration. However, the high level of variability observed in Figure 4 severely restricts the usefulness of this regression value. Even though the mean recovery might be considered acceptable up to formamide concentrations of 70%, the standard deviations show that when the formamide concentration exceeded 30%

Figure 4. Effect of Formamide on RNA Precipitation.

Precipitation of 148 μ g of mammary RNA from a final volume of 4 ml was carried out as described in Methods, from either 90% formamide containing 10 mM Tris-HCl (pH 7.6), 10 mM EDTA, 0.2% SDS diluted with water to the indicated concentration of formamide (∇) or at a final concentration of 10 mM Tris-HCl, 10 mM EDTA either in the presence of 0.2% SDS (∇) or in the absence of SDS (O). The recovery is expressed as the % of the RNA that precipitated at 0% formamide in each experiment. In the absence of formamide the RNA used gave a recovery of $85.6\% \pm 6.8\%$ S.D. ($n=12$). All the values were used to calculate the regression line² (recovery % = -0.66 formamide % + 96% ; $r = -0.67$, $p < 0.001$) and the mean recovery and its standard deviation for each 10% interval of the formamide concentration.



the variability was already so high that the precipitation was unreliable.

The formamide used in these studies was from Matheson, Coleman and Bell; it was charcoal and resin treated before use since it was found that treated formamide gave 10-20% better recoveries than non-treated formamide. The two other types of formamide available (from Eastman and BRL) showed essentially similar properties to the MCB material when used in RNA precipitations; this effect is thus not limited to one type of formamide. It was of interest to determine whether low RNA yields after precipitation from formamide solutions were associated with a selective loss of smaller molecules. Electrophoresis on 2.2% polyacrylamide SDS gels of RNA samples before and after precipitation showed that even at very low recoveries a constant 18S/28S rRNA ratio was maintained (data not shown). While this indicates no preferential loss, it remains possible that smaller molecules (eg tRNAs) were selectively lost during this procedure.

To test the effect of RNA concentration on recovery from formamide solutions, the recovery of 18-370µg total RNA from 8 ml 45% formamide solution was assessed. The solutions (at a final RNA concentration of 2.3µg/ml to 46µg/ml) contained 0.1% SDS and were equivalent to a two-fold dilution of poly(U)-sepharose column eluates. Precipitation was carried out as described (see methods) and the RNA yield was determined by UV absorbance measurements. The yields for the low RNA

concentrations (which had low optical densities) were confirmed in a separate experiment in which each sample was supplemented with 8,700 cpm of [^3H]-poly(U). In this case yield was based on ^3H -label recovery. This latter method overcame the difficulty of measuring very dilute RNA concentrations by UV absorbance. A control precipitation performed with 298 μg RNA in the absence of formamide gave a recovery of 88.5%. In the presence of 45% formamide, preparations containing 74-370 μg RNA gave similar recoveries and even the lowest amount of RNA tested (18 μg) showed only a 20% reduction in yield.

It was concluded that the principal consideration when RNA is to be precipitated from poly(U) sepharose column eluates is not the amount and/or concentration of the RNA sample, but the concentration of the formamide in the solution. The formamide concentration study (Fig. 4) shows that RNA recovery from such solutions can be optimized by appropriate dilution of the formamide solution before precipitation. Although dilution to 45% formamide was often sufficient to permit high RNA recovery, reliable recoveries were not ensured at formamide concentrations greater than 30%.

(iv) Fractionation of total mammary RNA using poly(U) sepharose

Two preparations containing 44 mg total mammary RNA were each run through two poly(U)-sepharose columns as

described in methods. Poly(A)⁺ RNA was eluted from the columns in buffered 90% formamide solutions but was diluted threefold with water before ethanol precipitation. Reduction of the formamide levels to 30% in these solutions was done to ensure high RNA recovery on precipitation. Table 12 summarises the recovery of poly(A)⁺ and poly(A)⁻ RNA fractions obtained under these conditions. These fractions and the initial RNA preparation were assayed for poly(A) content by the [³H]-poly(U) assay, and this information is also provided in Table 12.

The results of the two experiments are very similar. Passage of 44 mg total RNA through two poly(U)-sepharose columns yielded a total of 1.73 mg and 2.04 mg of poly(A)⁺ RNA using the modified precipitation technique (see previous page). In both experiments, over 90% of the RNA applied to the columns was recovered. Poly(A) recovery was also high; two passages through poly(U)-sepharose was sufficient to remove 98% or more of the poly(A) from total RNA and over 90% of this poly(A) was recovered as poly(A)⁺ RNA. The poly(A)⁻ RNA preparations produced contained respectively 0.95ng and 2.1ng poly(A)/mg RNA; these values are comparable to those obtained after four passages of 44 mg total RNA through oligo(dT)-cellulose (see Table 8, Chapter 1).

TABLE 12: FRACTIONATION OF TOTAL MAMMARY RNA BY POLY(U)
SEPHAROSE COLUMN CHROMATOGRAPHY

Fraction- ation	Total RNA		Poly(A) ⁺ RNA eluted		Poly(A) ⁻ RNA	RNA recovery in column fractions (%)
	applied		Col. 1	from Col. 2	(unbound)	
2e	mg RNA	44	1.05	0.68	40.9	97
	μg poly(A)	(50.6)	(28.2)	(21.6)	(0.49)	(99)
2f	mg RNA	44	1.12	0.92	38.5	92
	μg poly(A)	(50.6)	(34.1)	(10.7)	(1.1)	(91)

Two preparations containing 44mg total mammary RNA were each run through two poly(U)-sepharose columns as described in methods. Poly(A)⁺ RNA was eluted with 90% formamide and diluted to 30% formamide before ethanol precipitation. Poly(A)⁺ and unbound (2x poly(A)⁻) RNAs were collected by ethanol precipitation and assayed for poly(A) using the [³H]-poly(U) assay (see methods). The total poly(A) content of each sample is given in brackets (μg) underneath the yield (mg) of the RNA sample. The final column indicates the recovery in the bound and unbound fractions (expressed as a percentage of the total applied).

DISCUSSION

An unexpected problem encountered during the use of poly(U)-sepharose was the effect of high concentrations of formamide on ethanol precipitation of RNA. This effect can greatly reduce the recovery of poly(A)⁺ RNA obtained after elution from a column using solutions containing up to 90% formamide. A detailed study of this effect showed that reproducible recovery of RNA from solutions containing over 30% formamide was not obtained and at high formamide concentrations (70-90%) very low RNA recoveries could be obtained (10-30%).

This problem has not been previously reported; however Shapiro and Schimke do mention that during ovalbumin mRNA purification, poly(A)⁺ RNA was eluted from poly(U)-sepharose with solutions containing 70% formamide and these solutions were diluted to a formamide concentration of 30% before ethanol precipitation (21). No reason for this dilution was given, but it is possible that these workers had observed formamide effects similar to those described here.

One reason that this effect might have escaped attention in other work is that poly(A) recovery is not usually monitored during poly(A)⁺ mRNA preparation. The recovery of mRNA



is measured by cell-free translation before and after total RNA fractionation and as will be shown in Chapter 3, such measurements may not be quantitative. A few researchers have avoided the use of 70-90% formamide solutions by employing high concentrations of urea (54) or high temperature (59) to elute poly(A)⁺ bound RNA from poly(U) sepharose. These elution conditions were not investigated in this study.

Between 2.4mg and 3.6mg total RNA can be processed by 1 ml of swollen poly(U) sepharose gel (307). The columns used in this work should therefore be capable of processing 24-36mg total RNA; this appears to be the case, since two columns were required to bind all the mRNA contained in a preparation of 44 mg total RNA. There is therefore close agreement between the predicted and actual amounts of mRNA which can be retained by this resin.

A comparison of the products of oligo(dT)-cellulose and poly(U) -sepharose chromatography is possible using Table 8 (Chapter 1) and Table 12. In both cases, poly(A) recovery in the poly(A)⁺ RNA fractions is generally 90% or higher, and contamination with rRNA is comparable using the two types of resin (data not shown). Furthermore, the unbound RNA fractions are depleted of poly(A) to the same degree; only 1-2% of the poly(A) in the initial sample is retained in these unbound samples. The two types of resin thus yield comparable products; a slight advantage in the use of poly(U)-

sepharose was that fewer column runs were required to process mg. amounts of RNA compared to oligo(dT)-cellulose.

Other factors made oligo(dT)-cellulose the resin of choice. Poly(U)-sepharose contains ribonucleotide sequences (poly(U)) and is therefore less stable than oligo(dT)-cellulose which contains deoxyribonucleotides. It follows that poly(U)-sepharose cannot be stored in swollen form as long as oligo(dT)-cellulose. The technical problems associated with the use of formamide in the elution buffer have already been mentioned. For these reasons, and for the fact that the two resins yielded similar RNA fractions (their mRNA content is discussed in chapter 6), all subsequent experiments involving fractionation of total RNA into poly(A)⁺ and poly(A)⁻ RNA preparations were performed with oligo(dT)-cellulose (see chapter 7). In spite of the claims made regarding the efficacy of poly(U)-sepharose use (26), many recent studies have utilized oligo(dT)-cellulose to prepare poly(A)⁺ RNA (27,308). It would appear that oligo(dT)-cellulose is the more popular resin for preparation of poly(A)⁺ RNA.

CHAPTER 3

TRANSLATION OF RAT MAMMARY RNA PREPARATIONS IN A WHEAT GERM CELL-FREE SYSTEM

To demonstrate the presence of mRNA in an RNA preparation, it is necessary to show that this sample is functional and can be translated into protein. There are several in vitro systems available for translating RNA; the two most frequently used are the rabbit reticulocyte lysate and the wheat germ cell-free system. The latter method was used here to assay translation of total, poly(A)⁺ and poly(A)⁻ RNAs. The sizes of the radioisotope-labelled translation products of these reactions were studied both by disc and slab gel electrophoresis to verify that they were comparable to the sizes obtained by others working with rat mammary gland mRNAs, and to assess the major products made by poly(A)⁻ RNA since this has not been reported previously. Attempts were also made to estimate the relative proportions of poly(A)⁺ and poly(A)⁻ mRNAs in total mammary RNA based on the relative incorporation of [³H]-label into acid insoluble material on translation of poly(A)⁺ and poly(A)⁻ RNA fractions. The major reason for this analysis was to determine whether overall translational activity was distributed between poly(A)⁺ and poly(A)⁻ RNA fractions in a manner similar to that reported for casein mRNA (42).

Finally, evidence is presented that both poly(A)⁺ and poly(A)⁻ rat mammary mRNAs bear an intact 5' region by showing that both include a 5' cap structure. The presence of a 5' cap on both species helps to confirm that these mRNAs are intact species, and it further indicates the probable importance of the poly(A)⁻ mRNA as a functional species.

MATERIALS

Wheat germ was from a local health food store. Dithiothreitol (DTT) was from Boehringer Mannheim (Dorval, Que). Sephadex G-25 (course) was from Pharmacia (Dorval, Que). Most of the other reagents used in the translation, for example Hepes, ATP, GTP, phosphocreatine, creatine phosphokinase (132units/mg protein) and several unlabelled amino acids, were from Sigma (St. Louis, Missouri). The [³H]-amino acids - L-[³H(N)]-leucine (specific activity 50.4Ci/mmol) and L-[³H(N)]-phenylalanine (specific activity 46.7Ci/mmol) - were from New England Nuclear (Lachine, Que). L-[³⁵S]-methionine (specific activity 1210Ci/mmol) was from Amersham (Oakville, Ont). Q β RNA was from Miles (Rexdale, Ont); poly(U) (sodium salt) and the cap analogue 7-methyl guanosine-5'-phosphate (m⁷G^{5'}p) were from Sigma (St. Louis, Missouri). Materials for polyacrylamide gel electrophoresis including Coomassie blue R.250 were from Biorad (Mississauga, ONT) as was the standard protein

marker mix. This mix contained lysozyme (14,300), soybean trypsin inhibitor (21,000), carbonic anhydrase (30,000), ovalbumin (45,000), bovine serum albumin (68,000), phosphorylase (94,000). The figure in brackets following each protein is the molecular weight in daltons. Urea, TCA, mercaptoethanol and 30% hydrogen peroxide were all from Baker (Phillipsburg, N.J.). X-ray film was type X-Omat AR-5 from Eastman Kodak Company (Rochester, N.Y. USA).

METHODS

(i) Wheat germ cell-free translation

Wheat germ was prepared essentially as described (309). Wheat germ (2gm) was ground with mortar and pestle and ground glass for 1 minute and suspended in 6 ml grinding buffer (20mM-Hepes, pH 6.35, 15mM Magnesium acetate, 1mM DTT, 100mM KCl). The paste was spun for 8 min. at 22,000g (Sorval SS-34 rotor) and 1.5 ml of resulting supernate was applied to a Sephadex G-25 column (260mm x 12 mm) pre-equilibrated with column buffer (200mM Hepes, 50mM Magnesium acetate, 1mM DTT, 100mM KCl - pH brought to 7.7 with 1N KOH and K^+ content thus added was taken into account). The void volume was collected as 1 ml fractions and the fraction with the highest UV absorbance was used as the wheat germ extract.

The complete translation system had a final volume of 50 μ l and contained 20mM Hepes, pH 7.7, 100mM KCl, 3mM magnesium acetate, 2mM DTT, 1mM ATP, 0.1mM GTP, 8mM phosphocreatine, 100 μ g of creatine kinase/ml, each of the 19 unlabelled amino acids at 30 μ M, the labelled amino acid at 0.5-1.0 μ M (L-[4,5-³H(N)]-leucine, 50.4Ci/mmol, or L-[ring-2,6-³H(N)]-phenylalanine, 46.7Ci/mmol), 25 μ l wheat germ extract and the appropriate amount of RNA sample. After 1 hour at 25°C, 5-10 μ l samples were analysed by precipitation on 3MM Whatman paper as previously described (298) and the remainder of the translation mix was stored frozen at -70°C. The filter paper precipitates were boiled in 5% TCA to hydrolyse aminoacyl tRNA and washed. The filter papers were counted in a toluene-base scintillator (0.5% 2,5-diphenyl-oxazole, 0.05% 1,4-bis-(5-phenyloxazol-2-yl) benzene in toluene).

The amounts of RNA included in the translation assay varied; the usual range was 5-20 μ g of total or poly(A)⁺ RNA and 0.5-3.0 μ g poly(A)⁺ RNA.

The translations performed with [³⁵S]-methionine as the labelled amino acid were done under similar conditions to those described above, except that the total assay volume was reduced to 20 μ l. L-[³⁵S]-methionine (1210Ci/mmol) was at a concentration of 0.3 μ M in the translation; this is equivalent to about 6 μ Ci/assay.

(ii) Analysis of translation products by polyacrylamide
SDS disc gel electrophoresis

Gels (90mm x 0.6 mm) contained 10% acrylamide 0.27% bis, 1 mM DTT, 0.2M sodium phosphate, pH 7.0 and 0.2% SDS. Translation mixtures and standard protein markers were applied to gels in buffer (0.2M sodium phosphate, pH 7.0, 0.2% SDS)also containing 5% sucrose and bromophenol blue. Gels were electrophoresed overnight at 2.5mA/gel, until the blue dye had run through 4/5ths of the length of the gels. Gels were stained for 1 hour in 0.25% Coomassie blue, 45% methanol, 10% acetic acid and destained overnight in 25% methanol, 8.25% acetic acid. Gels bearing [³H]-labelled translation products were sliced using an electric gel slicer (Hoefer Scientific Instruments, Cal., U.S.A.) into 1 mm slices. Two gel slices were combined per vial and dissolved in 300µl 30% hydrogen peroxide over several hours at 60-70°C. Counting was performed in 3 ml Readysovlv (Beckman, Toronto,ONT.).

(iii) Analysis of translation products by polyacrylamide
SDS slab gel electrophoresis

The slab running gel was composed of 16.5% acrylamide, 0.2% bis, 1 mM DTT, 4M urea, 0.1% SDS, 0.375M Tris-HCl, pH 8.8. A stacking gel consisting of 3.75% acrylamide, 0.625% bis, 4M urea, 0.1% SDS, 0.125M Tris-HCl, pH 6.8, was employed. The appropriate volume of each [³⁵S]-methionine translation

assay was applied to the gel in 4M urea, 0.5% SDS, 0.5% mercaptoethanol, 0.6M Tris, pH 8.8, and bromophenol blue, after heating at 60°C for 20 minutes. Standard protein markers were treated in a similar manner. The gel was run overnight (18 hours) at 50V; the blue dye migrated about 13 cm. from the top of the running gel in this time. The gel was stained for 4 hours in 0.2% Coomassie blue, 10% TCA, 40% methanol and destained overnight in 10% acetic acid, 40% methanol. After rinsing in water, the gel was placed in Enhance (New England Nuclear) for 1 hour and in water for a further hour. Finally, the gel was dried (about 2 hours) on a Savant Instruments SGD-200 slab gel dryer. X-ray film was placed over the gel for 4-5 days and developed to allow visualization of the [³⁵S]-labelled protein bands.

(iv) Removal of the cap from the 5' terminal of mRNA

Decapping of mRNAs was performed by A. Lozano; the method employed was that of Rose and Lodish (310), in which periodate oxidation was followed by β -elimination with aniline.

RESULTS

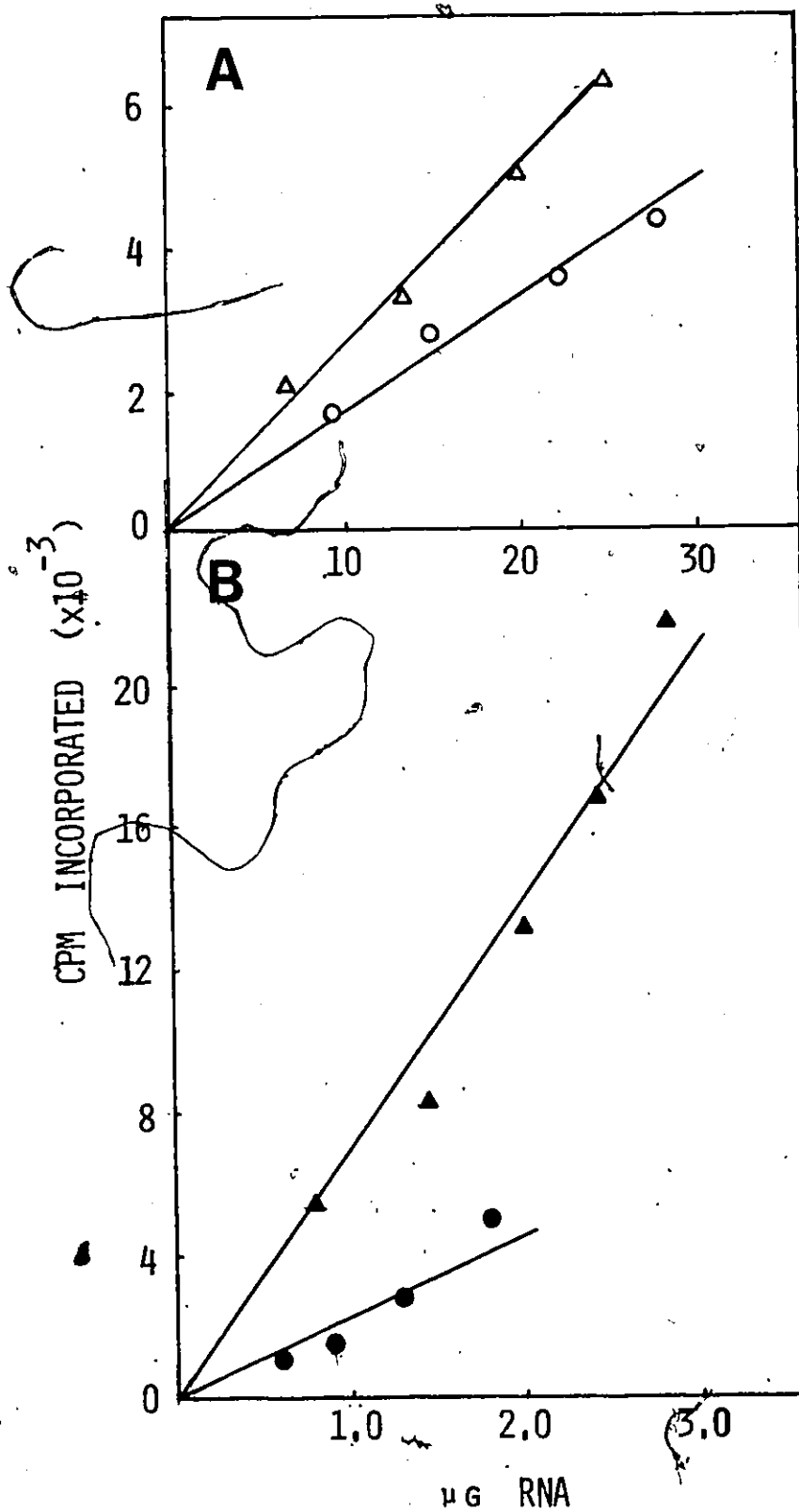
(i) Wheat germ cell-free translation of poly(A)⁺ and poly(A)⁻ RNA preparations

The RNA preparations prepared as described in Chapters

1 and 2 using oligo(dT)-cellulose and poly(U) sepharose affinity chromatography were translated in the heat germ cell-free system. Translational activity was shown to reside in both poly(A)⁺ and poly(A)⁻ RNA fractions. Figure 5 shows RNA concentration curves for cell-free translation of two poly(A)⁺ RNA preparations and their corresponding poly(A)⁻ RNAs. Samples from fractionations 1a and 3 (see table 8, chapter 1) have been translated using a range of RNA amounts (0.6-3.0 µg for poly(A)⁺ RNA and 6-28 µg for poly(A)⁻ RNA). Over the range of RNA concentrations tested both poly(A)⁺ and poly(A)⁻ RNAs yielded a linear plot of [³H]-label incorporated (cpm) against µg RNA in the assay. These concentrations of RNA are thus appropriate for quantitative measurement of translational activities of these samples, and allow comparison between different RNA preparations. The poly(A)⁺ and poly(A)⁻ RNA samples of fractionation 1a (triangles) exhibited higher translational activities than the corresponding preparations from fractionation 3 (circles). This may represent a real difference in translational activities of these two sets of samples but it is pertinent to note that these data were obtained from two different translation assays. Since different wheat germ supernatants vary significantly in translational activity, meaningful comparisons of the translational activities of RNA preparations

Figure 5. Translation of several concentrations of Poly(A)⁺ and Poly(A)⁻ RNAs produces linear incorporation of labelled amino acid.

The incorporation of [³H]-phenylalanine into acid-precipitable cpm is plotted against μ g RNA for (A) Poly(A)⁻ RNA, (B) Poly(A)⁺ RNA, when translated in a Wheat germ cell-free system as described (see Methods). The incorporated cpm were determined from 10 μ l spottings. Samples tested were: (●) poly(A)⁺ RNA, fractionation 3; (o) poly(A)⁻ RNA, fractionation 3; (▲) poly(A)⁺ RNA, fractionation 1a; (Δ) poly(A)⁻ RNA, fractionation 1a. Background values, obtained by omitting RNA from the translation, have been subtracted from the incorporations. Background values were 300 cpm (●, o) and 700 cpm (▲, Δ).



must be performed with a single wheatgerm supernatant. Alternatively, mRNA standards can permit direct comparisons of the extent of [^3H]-label incorporation between different translations.

Table 13 presents the results of cell-free translation of several of the RNA preparations, produced as described in chapters 1 and 2. For each RNA fractionation studied, the combined poly(A) $^+$ RNA samples and the corresponding poly(A) $^-$ RNA preparation were translated as described using various amounts of each RNA but not exceeding those used to provide Figure 5. Relative mRNA activity is expressed as the cpm incorporated/ μg RNA/ $10\mu\text{l}$ spotting for 6 sets of RNA fractionations. Taking account of the total RNA in each fraction, the relative mRNA activity may be converted to total mRNA activity; a similar analysis has been performed for mouse liver poly(A) $^+$ and poly(A) $^-$ RNA fractions (116). The final column of the table gives the percentage of total mRNA activity in each fraction. In all cases, the poly(A) $^+$ RNA sample has a much higher relative mRNA activity (10-50 fold) than the corresponding poly(A) $^-$ RNA; however when the large amounts of poly(A) $^-$ RNA are taken into account, over half (56-86%) of the total mRNA activity of each fractionation resides in the poly(A) $^-$ RNA preparation; this represents a significant amount of poly(A) $^-$ mRNA.

Attempts to confirm the calculations of poly(A) $^-$

TABLE 13: ANALYSIS OF POLY(A)⁺ AND POLY(A)⁻ RNA FRACTIONS BY TRANSLATION IN THE

WHEAT GERM CELL-FREE SYSTEM

Sample and Fraction	Relative mRNA activity (cpm/μg)	RNA in fraction (mg)	Total mRNA activity in fraction (cpm x 10 ⁻³)	Distribution of mRNA activity between fractions (%)
1a poly(A) ⁺ poly(A) ⁻	1800 170	2.17 56.40	3900 9570	29 71
2a poly(A) ⁺ poly(A) ⁻	3250 163	1.47 36.80	4780 6210	43 57
3 poly(A) ⁺ poly(A) ⁻	2120 118	0.41 16.80	860 1980	30 70
2b poly(A) ⁺ poly(A) ⁻	4630 156	0.98 36.40	4510 5680	44 56
2e poly(A) ⁺ poly(A) ⁻	1930 124	1.78 40.90	3440 5060	40 60
4 poly(A) ⁺ poly(A) ⁻	1380 138	0.33 19.50	450 2690	14 86

The translational activity of various RNA fractions prepared as indicated in Tables 8 and 12 was determined with the wheat germ cell-free system using [³H]-phenylalanine (46.7 Ci/mMol) as described in "Methods". Each assay contained 0.5-2.0μg of the pooled poly(A)⁺ RNAs or 8-30μg of poly(A)⁻ RNA. Activities were determined from the linear portion of the translation curve.

mRNA amounts presented in Table 13 involved translation of combined poly(A)⁺ and poly(A)⁻ RNA samples. Specified amounts of poly(A)⁺ and poly(A)⁻ RNAs were translated together and separately in the wheat germ cell-free system. Mixing experiment data for two sets of RNA fractionations (2e and 3) are given in Table 14. In each case the poly(A)⁺ RNA fractions were translated using various amounts of RNA (0.8-2.5μg) in the presence and absence of a constant amount of the corresponding poly(A)⁻ RNA. From Table 14 it is clear that when the incorporation obtained with two combined samples (column 5) is compared to the sum of the incorporation of the individual samples (columns 2 and 3) a discrepancy is found. The combined samples gave higher incorporations than was obtained by summing individual incorporations, as seen on comparison of columns 4 and 5. If this increase in total translational activity of the mixed samples is attributed to a more efficient translation of the poly(A)⁺ mRNA in the presence of poly(A)⁻ RNA (which includes rRNAs and tRNAs), poly(A)⁺ mRNA incorporation can be determined under the same conditions used to translate poly(A)⁻ mRNA (see column 6, Table 14). The incorporation directed by poly(A)⁺ mRNA in the presence of poly(A)⁻ RNA (column 6) compared to that observed in the absence of poly(A)⁻ RNA (column 2) is expressed as a ratio (column 7). Up to a two-fold increase in translational activity for poly(A)⁺ mRNA is observed in the presence of poly(A)⁻ RNA. Using the higher translational activities calculated for these two poly(A)⁺ RNA fractions, the percentage

TABLE 14: TRANSLATION OF COMBINED POLY(A)⁺ AND POLY(A)⁻ SAMPLES IN THE WHEAT GERM CELL-FREE SYSTEM

FREE SYSTEM							
Poly(A) ⁺ RNA in assay (μg)	Incorporation of poly(A) ⁺ RNA (cpm x 10 ⁻³)	Incorporation of poly(A) ⁻ RNA (cpm x 10 ⁻³)	Expected incorporation of combined sample ^b (cpm x 10 ⁻³)	Observed incorporation of combined sample ^a (cpm x 10 ⁻³)	Calculated incorporation of poly(A) ⁺ RNA ^c (cpm x 10 ⁻³)	Increased activity of poly(A) ⁺ RNA in combined sample ^d	Distribution of mRNA activity between RNA fractions on the basis of increased activity of the poly(A) ⁺ RNA ^f (%)
Sample 2 ^e							
1.3	5.0	2.0	7.0	11.7	9.7	1.9	57 43
1.9	8.7	2.0	10.7	14.2	12.2	1.4	53 57
2.2	11.5	2.0	13.5	17.7	17.7	1.4	59 41
2.6	14.1	2.0	16.1	22.8	20.8	1.5	59 41
Sample 3 ^e							
0.8	5.5	2.1	7.6	11.4	9.3	1.7	48 52
1.5	8.4	2.1	10.5	20.5	18.4	2.2	49 51
2.0	13.2	2.1	15.3	23.5	21.4	1.6	45 55

^a A constant amount of the corresponding poly(A)⁻ RNA was used with each poly(A)⁺ RNA (8.2 μg for sample 2e and 6.7 μg for sample 3).

^b Expected incorporation = (incorporation of poly(A)⁺ RNA) + (incorporation of poly(A)⁻ RNA).

^c Calculated incorporation = (observed incorporation of combined sample) - (incorporation of poly(A)⁻ RNA).

^d Increased activity of poly(A)⁺ RNA = (calculated incorporation of poly(A)⁺ RNA) / (incorporation of poly(A)⁺ RNA).

^e From Tables 8 and 12.

^f Poly(A)⁺ and poly(A)⁻ mRNA activities were each calculated using the formula:

activity = (incorporation of RNA/μg RNA in assay) x μg RNA in total sample. For poly(A)⁺ RNA, calculated incorporation (c) was used; for poly(A)⁻ RNA observed incorporation (a) was used. Values of total μg RNA in each sample are given in Table 13. Each mRNA activity has been expressed as a percentage of the sum of both activities.

Two preparations of poly(A)⁺ RNA were translated in the wheat germ cell-free system at several RNA concentrations, both in the presence and absence of a constant amount of the corresponding poly(A)⁻ RNA fraction. The translations were performed using [³H]-phenylalanine at 2 μCi/assay (see methods).

distribution of poly(A)⁺ and poly(A)⁻ mRNA activity for these samples was revised (see column 8, Table 14). These values probably represent a more accurate measurement of mRNA activity between these fractions than do the values in Table 13.

The data of Table 14 indicate that measurements of translational activity of different types of RNA preparations by the wheat germ cell-free system must be critically evaluated before they can be used to estimate relative amounts of mRNA activity in these RNA preparations.

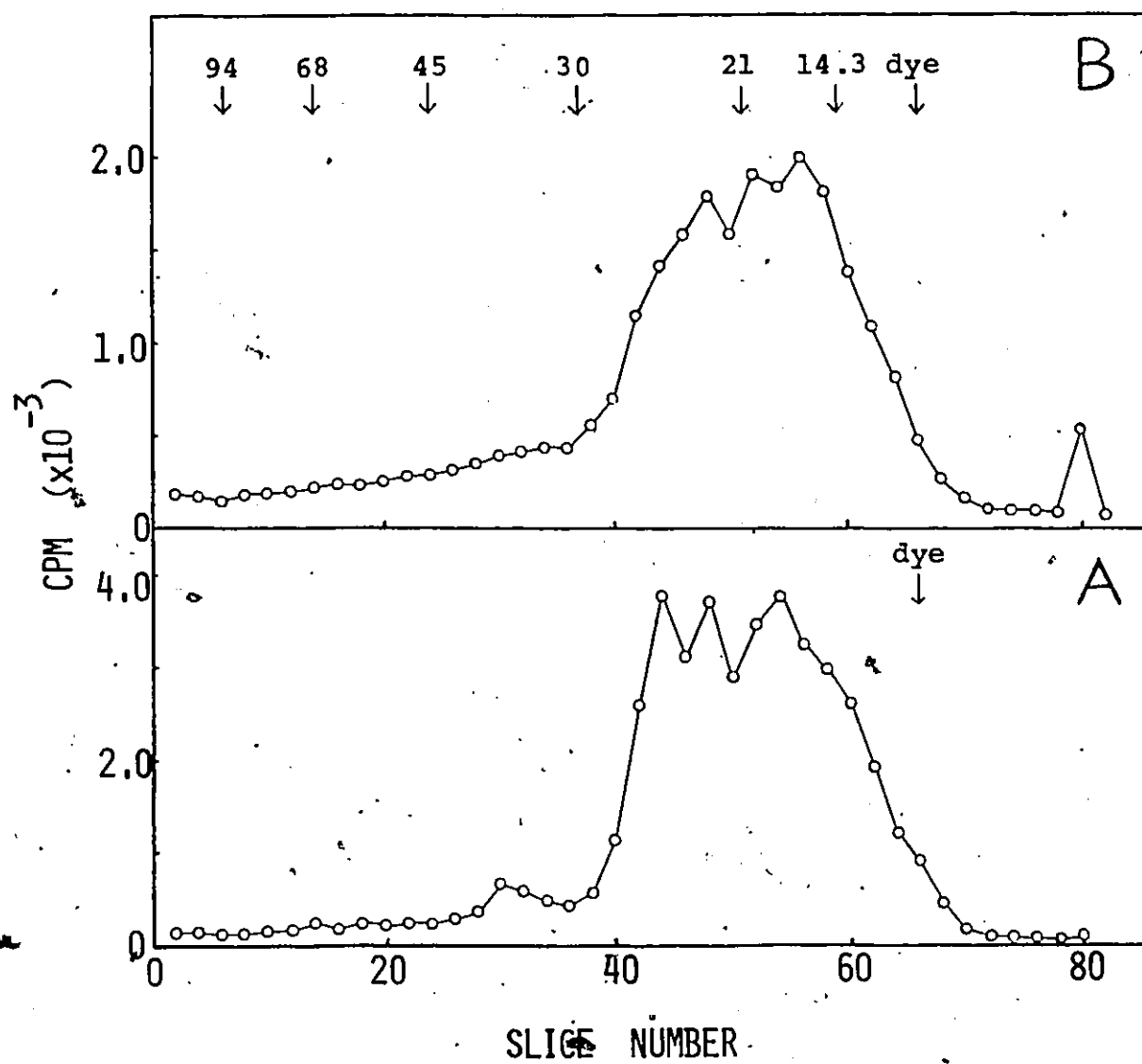
(ii) Analysis of translation products by SDS/polyacrylamide gel electrophoresis

Initial analysis of the cell-free translation products of mammary RNA fractions was performed by disc gel electrophoresis as described in Methods. A small number of aliquots from the translations described above were used. Figure 6 shows two gel profiles of the translation products of poly(A)⁺ RNA (Fig. 6a) and poly(A)⁻ RNA (Fig. 6b); both samples were from fractionation #3. The profiles obtained with poly(A)⁺ and poly(A)⁻ RNA translations were quite similar; in both cases the translation products were virtually all smaller than the 30,000 dalton protein standard. While this size range included α LA and the two smaller caseins, the larger casein (42,000 daltons) was not observed in significant quantity. Unfortunately these gels did not resolve specific protein products and all the labelled product appeared as one large band. Although the profiles in Figure 6 did suggest overall similarity in the protein products

Figure 6. Analysis of the products of Mammary RNA Translation by disc gel electrophoresis.

Two mammary RNA preparations which had been translated in the Wheat germ cell-free system using [^3H]-phenylalanine as described in Figure 5 were analysed further. The translation products were run on 10% polyacrylamide SDS gels, and the electrophoresed gels were sliced and counted as described (see Methods). The resulting profiles are shown. (A) translation products of poly(A) $^+$ RNA, fractionation 3. A 30 μl aliquot of the translation mix was applied to the gel - this was equivalent to 50,740 acid insoluble cpm and recovery of these cpm from the gel was 88%.

(B) translation products of the poly(A) $^-$ RNA from fractionation 3. A 40 μl aliquot of the translation (containing 25,375 acid insoluble cpm) was applied to the gel and recovery of the label was complete.



of the two RNA fractions, minor differences could not be detected and the individual milk proteins were not well defined. It was thus deemed necessary to use a polyacrylamide gel system with much higher resolving power to examine the translation products of these samples.

(iii) Analysis of translation products by SDS/polyacrylamide slab gel electrophoresis.

To carry out fluorography, [^{35}S]-labelled translation products were prepared in the wheat germ cell-free system using [^{35}S]-methionine. (see Methods). The [^{35}S]-labelled translation products directed by several mammary RNA preparations were run on a slab gel as described, and a photograph of the resulting fluorogram is shown in Figure 7. (This experiment contains translation data for RNA fractions from several stages in gland development -- see Chapter 7 for further details.) The lanes of interest here are 8, 9 and 10; these show respectively the translation products of the total, poly(A) $^{+}$ and poly(A) $^{-}$ RNAs of the 7-8 day lactating gland. Several discrete [^{35}S]-labelled protein bands were observed in these lanes; these proteins have been assigned molecular weights on the basis of the mobility of the standard protein markers. All three lanes exhibited a very similar pattern; the fainter pattern of the poly(A) $^{-}$ RNA products was due to the application of much smaller amounts of incorporated [^{35}S]-label onto this lane.

(NEXT PAGE IS 175)

Figure 7. Analysis of the products of Mammary RNA
Translation by slab gel electrophoresis

Translations were performed as described in methods using [^{35} S]-methionine, and the translation products were run on a slab gel. The data shown here is more fully described in Chapter 8, and this gel is reproduced there with a longer exposure period. The lanes of interest here are lanes 8 - 10 which contain the translation products of total, poly(A)⁺ and poly(A) RNA from 7-8 day lactating gland. The positions of the standard protein molecular weight markers are indicated by arrows and their molecular weights are given in kilodaltons. About 100,000 acid insoluble cpm were applied to each lane:

94→
68→
45→
30→
21→
14.3→

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

It can be seen from Figure 7 that each translation contains four major products. When measured from the top of the running gel, these bands occur at 3.1 cm (43,000 daltons) 4.4cm (30,000 daltons), 5.0 and 5.2 cm (26-25,000 daltons) and 6.3 cm (18,500 daltons). The molecular weights of each protein band were assigned on the basis of the protein marker standard curve.

The two largest translation products appear to represent the two largest caseins encoded by the 15S rat mammary mRNA, and the 26,000/25,000 dalton doublet corresponds to the smallest casein encoded by the 12S rat mammary mRNA. The smallest band of 18,500 daltons is the right size to be α -lactalbumin. The assignment of these translation products to particular protein species is discussed in detail in chapter 5, where these data are compared to the products of rat mammary RNA translations reported previously.

For the purpose of this chapter, it is shown that the total, poly(A)⁺ and poly(A)⁻ RNA fractions yield similar translation products and therefore, in agreement with Rosen, it may be concluded that a significant fraction of the milk protein mRNAs is in a non-polyadenylated form, and that most of mammary mRNA codes for milk protein.

(iv) Evidence that poly(A)⁺ and poly(A)⁻ mRNAs are capped

There are several methods for demonstrating the presence of a 5'-cap in mRNA preparations. In this study two procedures were employed. It has been shown that cap analogues, e.g. 7-methylguanosine-5'phosphate ($m^7G^{5'}p$), inhibit the in vitro translation of capped mRNAs but not uncapped RNAs (311). Thus, a demonstration that the presence of a cap analogue inhibits in vitro translation of mRNA preparations is indirect evidence that these mRNAs do bear a cap. A method specific for removal of the cap structure from mRNAs has been reported (310); this involves a β -elimination of the oxidised cap structure. It is known that capped mRNAs incorporate amino acids better than the corresponding non-capped species in vitro. If treatment of a mRNA preparation by the uncapping procedure causes a significant drop in its translational activity, this is evidence for the initial presence of a cap.

Several RNA preparations, including mammary poly(A)⁺ and poly(A)⁻ RNAs, purified 9S and 15S mRNAs and partially purified 12S mRNA (see chapter 5 for mRNA purification protocol) were analysed for the presence of a 5' cap. Poly(A)⁺ rabbit globin mRNA (prepared as reported by Mezl and Hunt (298)) was used as a standard, since this mRNA is known to be capped (304). All these RNA preparations were translated in the in vitro wheat germ translation system:

(1) with or without the inclusion of $m^7G^{5'}p$ in the assay, (2) before and after decapping. The results of these studies are presented in Table 16 and Figure 8.

Figure 8 shows the effect of increasing concentrations of $m^7G^{5'}p$ on the in vitro translation of several RNAs. A concentration as low as $0.1mM m^7G^{5'}p$ was sufficient to inhibit translation of rabbit globin mRNA and purified mammary mRNA fractions by about 90%. Translation of an uncapped control, QB RNA was partially inhibited (60% inhibition) at $0.1mM m^7G^{5'}p$ but increasing concentrations of cap analogue did not cause any further increase in inhibition, and a clear difference in the response of QB RNA and the other mRNA preparations is apparent. Poly(U), which also lacks a cap, was affected by the cap analogue to an even smaller degree; there was a small and gradual increase in the inhibition of translation of this polynucleotide in response to increasing concentrations of cap analogue. The data of Figure 8 show that in the case of capped mRNAs, e.g. globin, a concentration of $0.5mM m^7G^{5'}p$ in the wheat germ cell-free translation system is sufficient to inhibit translation by 90% or more as expected from a previous study (311).

Table 16 summarises available data on the effect of (1) $0.5mM m^7G^{5'}p$ and (2) chemical decapping on the translation of various mammary mRNAs and control templates using the wheat germ cell-free system. All the mammary

Figure 8. Effect of Increasing Concentrations of 7-methylguanosine-5'-phosphate on the Translation of several mRNAs and control templates.

Translations were carried out using the wheat germ cell-free system as described (see Methods), and acid insoluble cpm was determined using 10 μ l spottings. m⁷G^{5'}p was included in the translations at the indicated concentrations. The RNA samples studied were: (+) rabbit globin mRNA, (o) rat mammary 9S mRNA, (Δ) rat mammary 12S mRNA, ($\square$) rat mammary 15S mRNA, ($\bullet$) phage Q β RNA and ($\blacktriangle$) poly(U). Each translation contained 1-2 μ g mRNA, 11 μ g Q β RNA or 18 μ g poly(U). All translations were carried out using [³H]-leucine at 2.5 μ Ci/assay except for the poly(U) translation which was performed with [³H]-phenylalanine (2 μ Ci/assay). The figure shows the % inhibition of the amino acid incorporation at increasing concentrations of m⁷G^{5'}p.

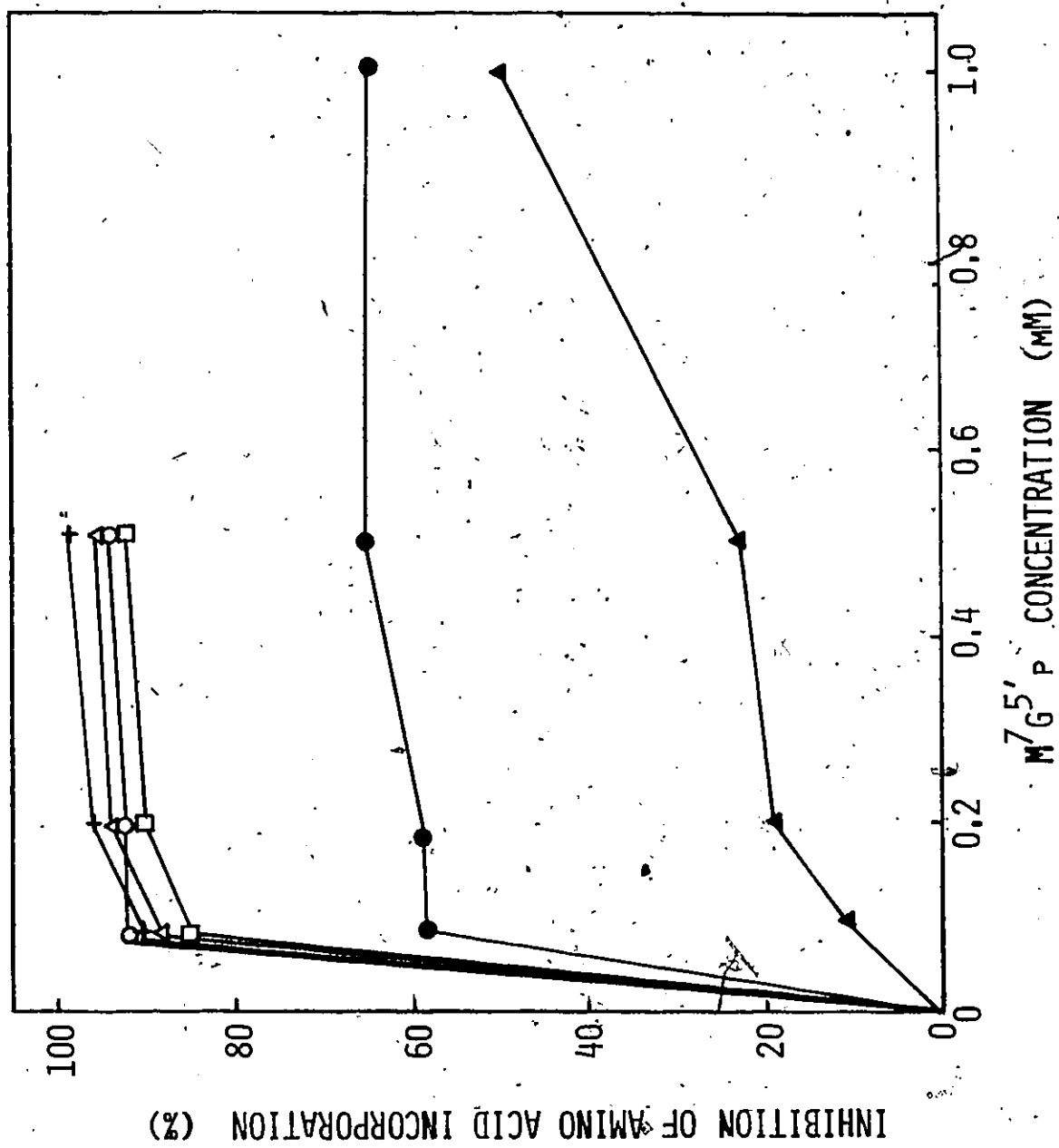


TABLE 16: EVIDENCE FOR THE PRESENCE OF 5' CAPS IN VARIOUS
MAMMARY RNA PREPARATIONS

Type of RNA in trans- lation assay	Normal Incorporation (cpm x 10 ⁻³)	Reduction in incorporation by 0.5mM m ⁷ G ^{5'} p (% inhibition)	Reduction in incorporation by β -elimination (% inhibition)
Rabbit globin mRNA	66.1	98	90
Mammary poly(A) ⁺ RNA	14.4	98	90
Mammary poly(A) ⁻ RNA	14.4	96	72
9S mRNA	26.5	91	-
9S mRNA*	7.0	94	91
12S mRNA	15.1	95	-
12S mRNA*	2.0	83	94
15S mRNA	25.4	97	-
15S mRNA*	7.2	93	92
Q β RNA	1.7	66	30

Translations were performed in the wheat germ cell-free system as described (see Methods) using [³H]-leucine (2.5 μ Ci per assay) in the presence or absence of m⁷G^{5'}p. Poly(A)⁺ and poly(A)⁻ mammary RNAs were from fractionation 1b (see Table 8). Purified 9S and 15S mRNAs were prepared from poly(A)⁺ mammary RNA by two successive rounds of sucrose gradient centrifugation (see Chapter 5). 9S* and 15S* RNA were produced by a single gradient and are partially purified. 12S and 12S* RNAs were prepared similarly, but the final 12S mRNA was still not highly purified. Sufficient amounts of the purified mRNAs were not available for the β -elimination procedure. Each translation contained 1-2 μ g RNA except the Q β RNA translation which contained 11 μ g RNA per assay.

mRNAs examined, including poly(A)⁺ and poly(A)⁻ RNA fractions and the 9S, 12S and 15S mRNAs behave in a similar manner to rabbit globin mRNA. The presence of 0.5mM m⁷G^{5'}p inhibits translation by over 90% and decapping causes 90% or more inhibition of incorporation (except for poly(A)⁻ RNA which was inhibited by 72%). This is in contrast to the noncapped control RNA, QB mRNA, the translation of which was inhibited by only 66% in the presence of cap analogue and by just 30% subsequent to treatment with the decapping procedure. Clearly, these mammary RNAs behave as if they are capped rather than uncapped species. This conclusion is of particular importance in the case of mammary poly(A)⁻ RNA, because it is the first reported evidence for the presence of 5' caps in this RNA fraction. The finding of 5' caps in this RNA, together with the analysis of its translation products by gel electrophoresis, indicates that the only difference between poly(A)⁺ and poly(A)⁻ mammary RNAs is the presence of a poly(A) tract.

DISCUSSION

These translation data show that both poly(A)⁺ and poly(A)⁻ RNA fractions of total RNA from 7-8 day and 11-15 day lactating rat mammary gland contain significant translational activity when tested in the wheat germ cell-free system. As expected, the poly(A)⁺ RNA fractions

are highly enriched in translational activity compared to total and poly(A)⁻ RNAs, when expressed per μ g RNA. Relative mRNA activity was between 10 and 50 fold higher in the poly(A)⁺ RNA fraction compared to the corresponding poly(A)⁻ RNA fraction.

Attempts were made to measure quantitatively the total amount of mRNA activity in all of the poly(A)⁺ and poly(A)⁻ RNA fractions which had been prepared previously (Chapters 1 and 2). For each RNA fraction, translational activity was measured at RNA concentrations which gave linear incorporation. Specific translational activities were thus calculated. An estimate of the absolute amounts of mRNA activity were then determined by adjusting for the total RNA in each fraction. The results of this analysis, presented in Table 13, suggested that between 56% and 86% of the total mRNA activity of these rat mammary RNA preparations was present in the poly(A)⁻ RNA fractions, i.e. as poly(A)⁻ mRNA. If sample 4 is ignored for the time being (reasons for this will be given later - see chapter 6) this percentage remained high at 56-71%.

This observation was consistent with a previous report by Rosen that, when assayed in an in vitro translation system, only 40% of rat casein mRNA activity was retained by oligo(dT)-cellulose. He concluded that a high proportion of this mRNA was nonpolyadenylated. Since the casein mRNAs constituted a large fraction (about 50% (42)) of

the total mRNA present in these preparations, and the fluorogram shown in Figure 7 indicated that all major translation products exhibit a similar distribution between poly(A)⁺ and poly(A)⁻ RNA fractions these two observations appeared to be in close agreement.

Further translational analysis of certain poly(A)⁺ and poly(A)⁻ RNA fractions in mixing experiments complicated the interpretation of the analysis described above. The data of Table 14 suggested that mRNA in the poly(A)⁺ and poly(A)⁻ RNA fractions may be translated with different efficiencies. Such an observation was not entirely unexpected. Previously, it was reported that the presence of large quantities of rRNA in cell-free translation assays caused an inhibition of translation (112). In these experiments, an opposite effect was observed; the presence of poly(A)⁻ RNA, which includes rRNAs and tRNAs, appeared to stimulate poly(A)⁺ mRNA translation. The reason for this effect is unknown; it is possible that the tRNA species present in the poly(A)⁻ RNA preparations were increasing the efficiency of translation of the wheat germ cell-free system, and overcoming any inhibitory effect of the rRNAs. Despite attempts to correct for this problem (see Table 14), the results of Table 14 would indicate that comparison of the translational activity of different types of RNA preparations in the wheat germ cell-free system is not strictly quantitative. Determination of the relative distribution

of mRNA sequences between poly(A)⁺ and poly(A)⁻ RNA preparations is therefore best done by other methods, eg. by hybridization to specific cDNA probes (see Chapter 6).

Slab gel electrophoresis of the translation products of mammary gland RNA fractions (Figure 7) showed that they did include proteins of the sizes expected for complete rat milk proteins. Although several smaller peptide products were present in these translations, the predominant products were full length milk proteins, thus indicating that at least most of the mRNA molecules in these preparations were essentially intact. Indeed, the translation profiles for the purified mRNAs indicated that premature termination of translation did occur in this system and contributed significantly to the amount of the smaller peptides produced. The poly(A)⁺ and poly(A)⁻ RNAs exhibited essentially similar profiles and this would argue against the suggestion that the poly(A)⁻ RNA fractions consisted entirely of degraded poly(A)⁺ RNA. More detailed discussion of these translation product profiles is presented in Chapters 5 and 8.

Other evidence to suggest that poly(A)⁺ and poly(A)⁻ mammary mRNAs were similar except with respect to polyadenylation consisted of a study of the nature of their 5' termini. Both RNA preparations translated in the wheat germ cell-free system as though their mRNAs consisted predominantly of capped species. While neither this analysis, nor the translation product profiles could provide evidence to entirely preclude small

levels of mRNA degradation in these samples, they did indicate that there were no very large differences in the intactness of the poly(A)⁺ and poly(A)⁻ RNA species.

CHAPTER 4

THE SIZE OF THE POLY(A) SEQUENCES ASSOCIATED

WITH RAT MAMMARY RNA

One of the goals of this research was to determine the size of the poly(A) sequences associated with rat mammary RNA at various stages in development of the gland. In the introduction, the methods available for performing such an analysis were discussed. In this work, sizing was performed by enzymatic removal of the poly(A) sequence from the RNA molecule and disc gel electrophoresis of the isolated poly(A) tracts followed by their detection with the [^3H]-poly(U) assay. In this chapter, the technical details of this procedure are examined and the poly(A)⁺ RNA fractions prepared from 11-15 day lactating rat mammary gland (see chapters 1 and 2) are analysed by this method. In these initial studies, the poly(A)⁻ RNA fractions were also analyzed by the poly(A) sizing method; this was done to determine whether or not the very small levels of poly(A) detected in those preparations were due to the presence of very short oligo(A) tracts or small numbers of unretained poly(A) tracts.

Since this method does not indicate whether the poly(A) sequences analysed are at the 3' terminal, attempts were made to show this by [^3H]-labelling of the 3' terminal of mammary RNA before poly(A) removal. [^3H]-label was

introduced by periodate oxidation of these molecules followed by reduction using sodium borohydride containing [^3H]-label.

MATERIALS

RNase A (Bovine pancreas , type 1A 60 Kunitz units/mg protein), RNase T₁ (Aspergillus oryzae grade IV 566000 units/mg protein) and RNase T₂ (Aspergillus oryzae grade VII, 1375 units/mg protein) were all obtained from Sigma (St. Louis, Missouri), as was E. coli tRNA. Materials for polyacrylamide gel electrophoresis were from Biorad (Mississauga, Ont); the SDS used for preparing the gels was from BDH (Ville St Laurent, Que) and was 98% pure while that used for other purposes was from Sigma. Use of the less pure SDS (Sigma) in gel preparation resulted in a peak of UV absorbance in the middle of the gels which interfered both with the detection of the poly(A) size standards and the [^3H]-poly(U) analysis.

Poly(A) preparations of defined length characteristics were obtained from Miles Biochemicals (Rexdale, Ont). The poly(A) standards had modal lengths of 207, 113, 90 and 50 nucleotides. A 10 nucleotide oligo(A) sequence was from Collaborative Research (Waltham, Mass). [^3H]-poly(U) (specific activity 39 $\mu\text{Ci}/\mu\text{mole P}$) was from Miles.

Sodium meta-periodate was from Sigma. [^3H]-borohydride (sodium salt, specific activity 50-500mCi/mmol) was from Amersham (Oakville, Ont.).

METHODS

(i) Isolation of poly(A) sequences by RNase digestion of mammary RNA

Digestion of RNA to liberate poly(A) tracts was performed using RNases A and T₁ by a modification of the procedure of Merkel et al (312). Samples consisted of 3-10 µg poly(A)⁺ RNA or 1.8-3.0 mg poly(A)⁻ RNA. They were digested at 37°C for 30 minutes in 0.3M NaCl, 10mM Tris-HCl, pH 7.4, 5mM MgCl₂ using 25 µg RNase A/mg RNA. For some poly(A)⁻ RNA samples, DNase I was also included at 15 µg/mg RNA for reasons discussed in Results. Digests were brought to 10mM EDTA using 0.2M EDTA (pH 7.0) and digested with RNase T₁ (1.25 units/µg RNA) at 37°C. for 30 minutes. The digestion was stopped by bringing the solution to 0.2% SDS, and remaining poly(A) tracts were extracted with 0.45ml of a phenol and chloroform (1:1) mix. The resulting aqueous phase was brought to 0.2M NaCl and received 50 µg E. coli tRNA carrier; poly(A) was ethanol precipitated from the aqueous phase and taken up in 0.12 ml water for disc gel electrophoresis.

(ii) Poly(A) sizing.

This was performed using SDS polyacrylamide disc gels (85mm x 6 mm) containing 10% acrylamide and 0.25% bis in the buffer solution of Falvey et al (300) supplemented with 0.2% SDS. Samples were applied to the gels in the

same buffer containing 5% sucrose and Bromophenol blue in a final volume of 0.16 ml. Electrophoresis was at 4.5mA/gel; the duration of the run was 4.5 hours for samples from poly(A)⁺ RNA and only 2.5 hours for samples from poly(A)⁻ RNA. The bromophenol blue marker dye ran 7.0cm and 4.0cm respectively from the top of the gel under these conditions. Gels were cut into 1 mm slices with a gel slicer (Hoefer Scientific Instruments) and 4 slices (poly(A)⁺ RNA samples) or 5 slices (poly(A)⁻ RNA samples) were combined per fraction. Each fraction was eluted for 18 hours at room temperature in 0.8 ml of 0.3M NaCl, 0.03M Na citrate (pH 7.2). Aliquots of 0.5 ml from each eluted fraction were combined with 100μl containing 0.3M NaCl, 0.03M Na citrate, pH 7.2, 30mM MgCl₂ and 0.023μCi [³H] poly(U). A poly(A) assay was then performed as described in Chapter 1 (methods), with the indicated modifications of assay volume (0.6 ml) and of the amount of [³H]-poly(U) included in the assay. Slightly higher levels of enzyme were included (12μl RNase A (200μg/ml) was used per assay).

Poly(A) standards were run on parallel gels. The position of the peak of each poly(A) size class was determined by scanning each gel with an Isco UA-5 gel scanner at 280nm. The high UV absorbance background observed for these gels at 254nm made it necessary to scan these gels at 280nm. As indicated in the results section, several poly(A) standards were also assayed by the [³H]-poly(U) assay to verify that the absorbance peak did in fact

correspond to maximal poly(U) binding.

(iii) RNase T₂ digestion of poly(A) tracts

Poly(A) tracts which had been isolated from mammary RNA by digestion with RNases A and T₁ were treated with RNase T₂ (0.5 units/ μ g RNA, including carrier) for 2 hours at 37°C. in 0.1M sodium acetate, pH 4.5, 2mM EDTA. The enzyme was removed by phenol extraction. The resulting aqueous phase was ethanol precipitated using 50 μ g E. coli tRNA carrier as described above.

(iv) [³H]-labelling of mammary RNA

This was performed by a modified method of De Wachter and Fiers (66). Poly(A)⁺ RNA samples (20-94 μ g), E coli. tRNA (25 μ g) or the 90A standard (3-30 μ g) were diluted with 0.5ml 0.01M sodium phosphate, pH 6.0. The solution was brought to 6mM sodium periodate (with 15 μ l of 0.02M NaIO₄) and left at room temperature in the dark for 2 hours, to permit 3' terminal oxidation of the sample. Each sample received 1 ml 0.5M sodium phosphate buffer, pH 7.5 and 45-65 μ l 1N NaOH containing 170-230 μ Ci [³H] NaBH₄. This ensured that the pH of the reduction reaction was maintained between 7.7 and 8.0. Contrary to the protocol of De Wachter and Fiers, sodium periodate destruction by ethylene glycol was not performed. This step was also omitted by Randerath et al. (393). Occasionally, propylene

glycol was included before addition of borohydride, but this did not result in any increased [^3H]-label incorporation into the sample. The reduction was allowed to proceed for four hours and stopped by addition of 150 μl glacial acetic acid. The reaction mixture was dialysed against low salt buffer for 3 hours to remove most of the free [^3H]-label; it was then ethanol precipitated.

Digestion of [^3H]-labelled samples to obtain isolated poly(A) tracts was as described above, using RNases A and T_1 . RNase T_2 digestion was also performed, as described above, in certain cases. [^3H]-labelled digests were run on 10% acrylamide/SDS gels; gels were sliced and 4 slices were combined per vial. [^3H]-label was eluted from the gel slices in 0.5ml 0.5N NaOH at room temperature overnight. After neutralization with 0.5ml 0.5N HCl, [^3H]-label was counted in 5 ml Ready-solv (Beckman).

RESULTS

(i) Mobility of Poly(A) standard markers on SDS/polyacrylamide gels

To assign nucleotide length to the poly(A) tracts isolated from mammary RNA, a standard curve of poly(A) length vs. mobility had to be prepared. Since samples from poly(A) $^+$ and poly(A) $^-$ RNA had to be analysed using different

running conditions (see methods) standard poly(A) marker mobilities were determined for both running times. Figure 9 shows the observed relationship between mobility and log. nucleotide length of the five poly(A)/oligo(A) size classes used. It can be seen that there is the expected linear relationship between these two characteristics at both running times. The open and closed squares indicate the position of E. coli tRNA when run on these gels for 2.5 and 4.5 hours respectively. Although this molecule consists of about 75 nucleotides, it migrates as if it were made up of only 26-27 nucleotides relative to the poly(A) size markers. This anomalous behaviour of poly(A) sequences compared to heterogeneous RNA strands on acrylamide gels has been reported previously (see discussion). Figure 9 shows that these conditions are suitable for size analysis of the poly(A) tracts from mammary RNA; the standard curve in Figure 9 covers a size range of 10-200 nucleotides, which is sufficient for this analysis.

(ii) Poly(A) sizing after [³H]-labelling of the 3' termini of RNA samples

Periodate oxidation of poly(A)⁺ RNA followed by reduction with [³H]-borohydride has been used to introduce [³H]-label into poly(A) since this sequence is located

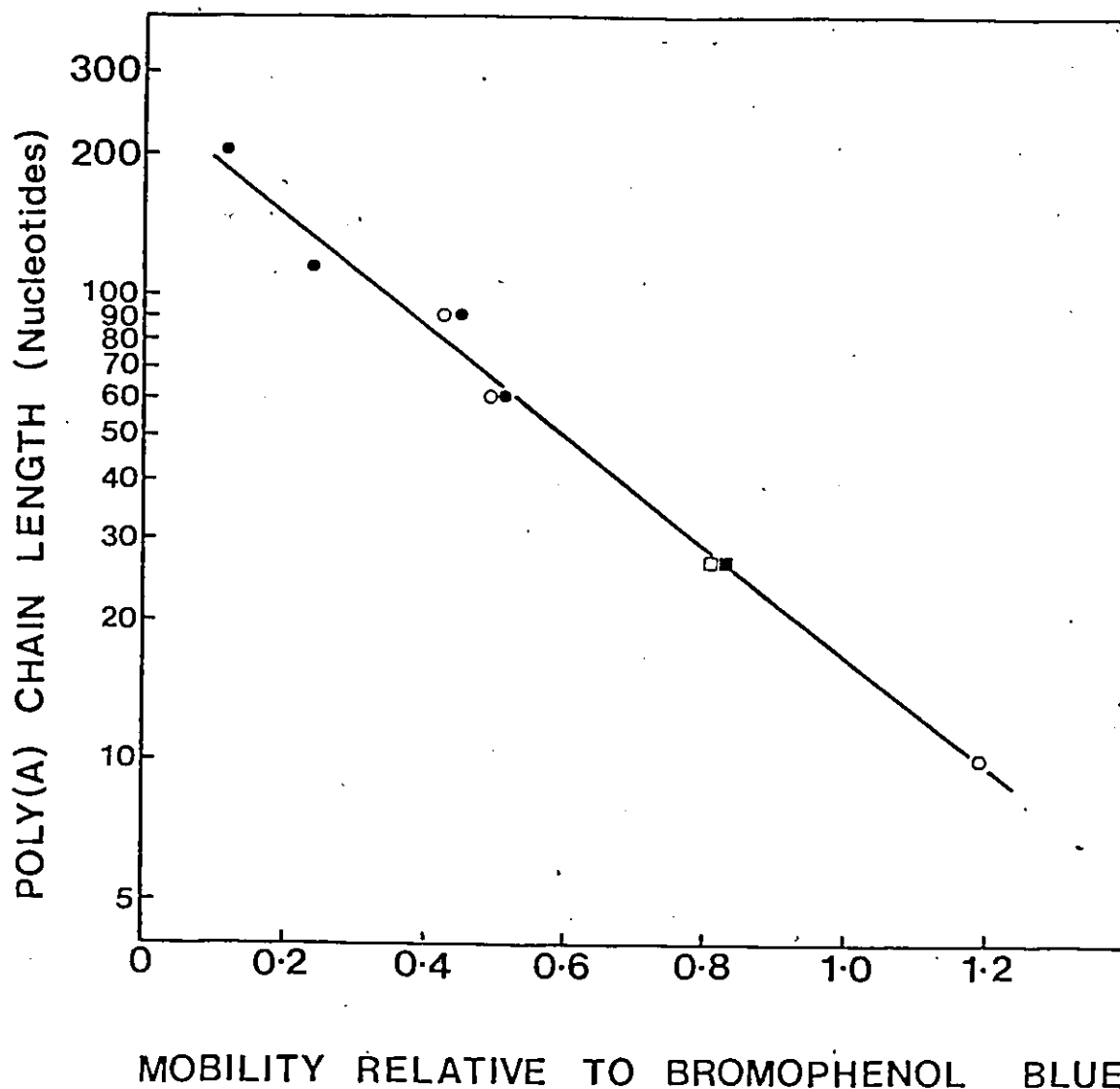


Figure 9. Electrophoretic Analysis of Poly(A) size standards

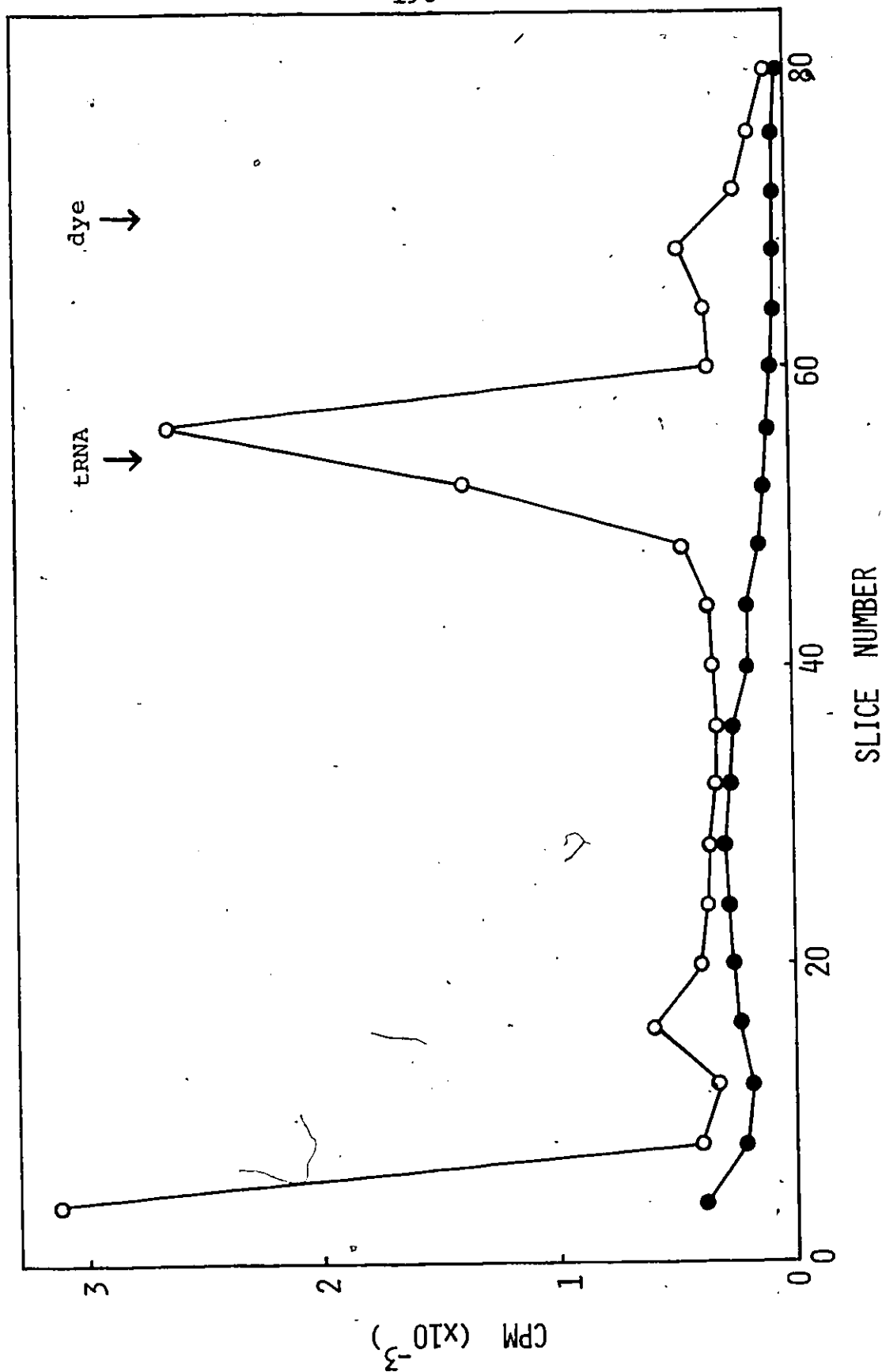
Five poly(A) size classes were electrophoresed on 10% polyacrylamide SDS gels (see Methods). Electrophoresis was performed for 2.5 hours (○) or for 4.5 hours (●). The position of each size class was determined by gel scanning at 280 nm and the position of the modal size length is expressed as its mobility relative to bromophenol blue dye. The position of *E. coli* tRNA after 2.5 hours (□) and after 4.5 hours (■) of electrophoresis is indicated.

at the 3' terminus of the RNA molecule. Conversely, [^3H]-label incorporation into poly(A) by this method demonstrates that the poly(A) is 3'-terminal. This method therefore may yield extra information about the position of the poly(A) chain in the RNA molecule compared to techniques which detect poly(A) only after its removal from RNA. Once labelled, poly(A) can be isolated by RNase digestion and sized on gels; detection of the sequences is readily performed by virtue of the incorporated label.

Initial experiments on [^3H]-labelling of RNA were performed on E. coli tRNA. An aliquot (25 μg) of this RNA was labelled as described in Methods. After ethanol-precipitation, the sample was taken up in 0.25 ml water and divided into two equal fractions: the first was reprecipitated while the second was digested with RNases A and T_1 and reprecipitated. This digestion treatment should result in loss of all [^3H]-label from the acid-precipitable fraction and provides a control when running these samples on acrylamide gels. The two samples were finally each taken up in 0.135 ml water and 5 μl aliquots were used to determine TCA-precipitable cpm. The undigested sample contained a total of 103,350 cpm which were acid precipitable, this value was reduced to 18,000 cpm in the digested sample. Figure 10 shows the profiles of these two samples after they had been electrophoresed on 10% acrylamide SDS

Figure 10. Electrophoresis of [³H]-labelled E. coli tRNA

E. coli tRNA was [³H]-labelled using the periodate oxidation borohydride reduction procedure described in Methods. This labelled preparation was divided into two aliquots: one aliquot received no further treatment but the second was digested with RNases A and T₁. The electrophoretic profile of 2.3µg [³H]-labelled tRNA before (o) and after (●) RNase digestion is shown. Recovery of [³H]-label from the gel which contained the undigested tRNA was only 40% of the acid insoluble cpm applied. The position of unlabelled tRNA run on a parallel gel and detected by its UV absorbance is indicated (see arrow).



gels. The [^3H]-labelled undigested tRNA sample exhibited a peak of [^3H]-label at a position comigrating with unlabelled tRNA run on a parallel gel. It therefore appears that [^3H]-label was successfully incorporated into tRNA; this peak was absent from the profile of the digested control sample. The only unsatisfactory aspect of these profiles is the low recovery of [^3H]-label from these gels. About 19,000 cpm was applied to the gel in Figure 10 but the tRNA peak contained only 4,500 cpm. There was a significant amount of label at the top of the gel (about 3,000 cpm) and recovery from the very top of the gel is likely to be poor if material barely enters the gel. The nature of this material is unknown, but a high background in this region of the gel was often observed.

It has thus been shown that [^3H]-labelling of *E. coli* tRNA is obtained under the conditions of labelling described. Calculation of the expected incorporation of [^3H]-label cpm showed that on the basis of the TCA-precipitable cpm values obtained, the observed [^3H]-label incorporation was essentially quantitative, however, a large fraction of this label was not recovered from the gel.

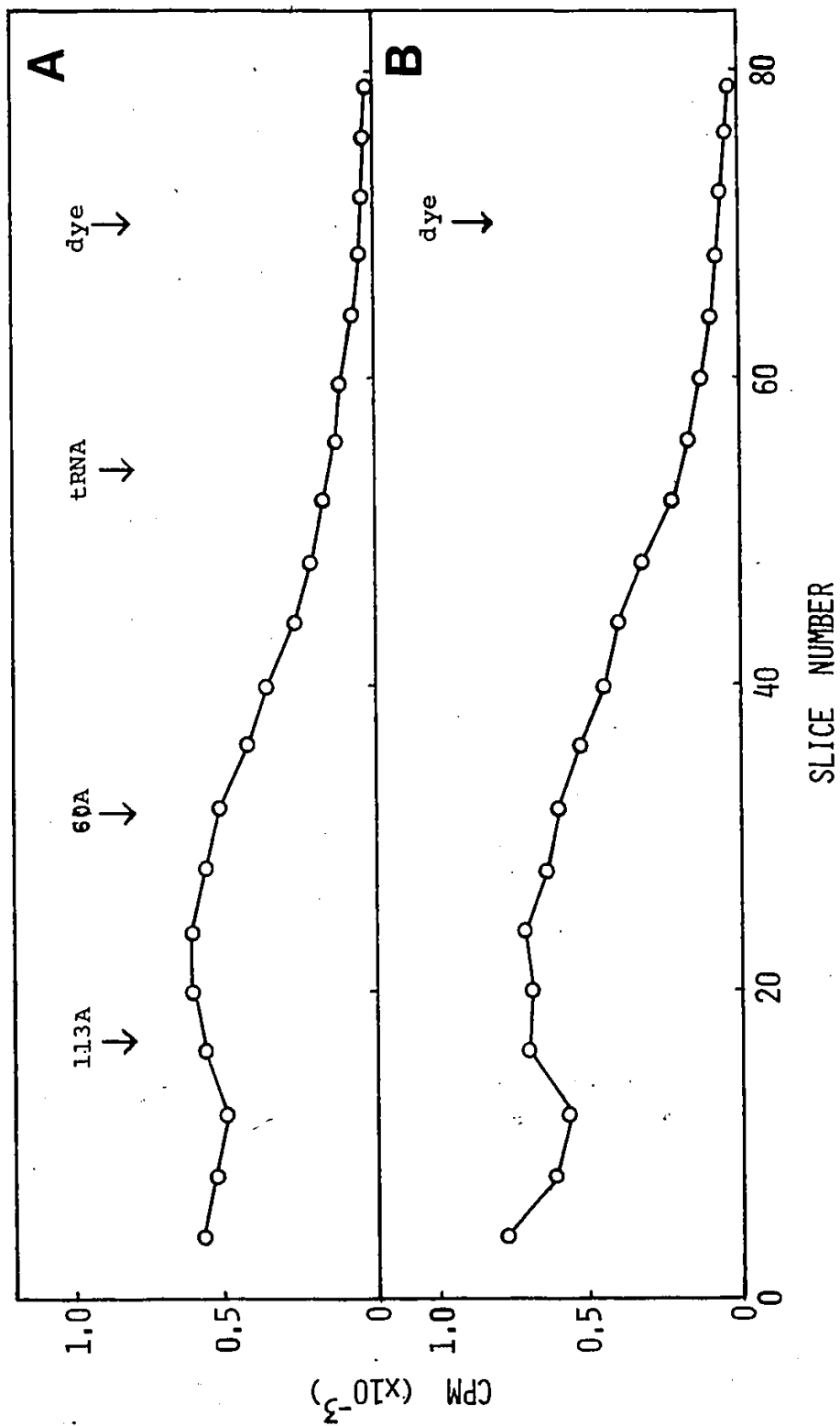
Several attempts were made to introduce [^3H]-label into poly(A)⁺ RNA. In one experiment, a poly(A)⁺ RNA sample (94 μg) from fractionation 2c was treated by the same

method used to label tRNA (see methods). After recovery from ethanol precipitation[§], the sample was digested with RNases A and T₁ (see methods) and reprecipitated. The digested RNA was divided into two aliquots: the first was precipitated and the second was RNase T₂ digested before precipitation. Measurements of the amount of acid precipitable label remaining in the two preparations were not encouraging. The first sample, which should represent intact [³H]-labelled poly(A) tracts contained only 14,700 cpm, while the second control sample containing no poly(A) sequences gave 20,400 acid precipitable cpm. This suggested there was no specific incorporation of [³H]-label into poly(A) tracts, and indeed the profiles of these two samples after gel electrophoresis were identical (see Figures 11a and 11b). The broad peak of [³H]-label observed in the upper half of these profiles is presumably a background, since it is present in both samples, including the RNase T₂ control. Since many other attempts to label mammary RNA samples were unsuccessful, the applicability of this technique to poly(A) sequences was therefore confirmed using the 90A standard.

About 30 µg of 90A standard was [³H]-labelled as described and collected by precipitation and divided into two aliquots. One aliquot was RNase T₂ treated and reprecipitated as a control, the other was just reprecipitated. These samples retained respectively 13,150 and 35,750 cpm of [³H]-label which were acid precipitable

Figure 11. Electrophoretic Profile of [^3H]-labelled Mammary Poly(A) Tracts

Mammary poly(A)⁺ RNA was [^3H]-labelled (see Methods) and digested by RNases A and T₁ to release poly(A) sequences. This sample was divided into two equal aliquots: one aliquot was further digested with RNase T₂. Both samples were electrophoresed on 10% polyacrylamide SDS disc gels and [^3H]-label was eluted from gel slices and counted. The resulting profiles for poly(A) tracts (A) with RNase T₂ treatment, (B) without RNase T₂ treatment are shown. The positions of several poly(A) size markers are indicated by arrows at the top of Figure 11A.

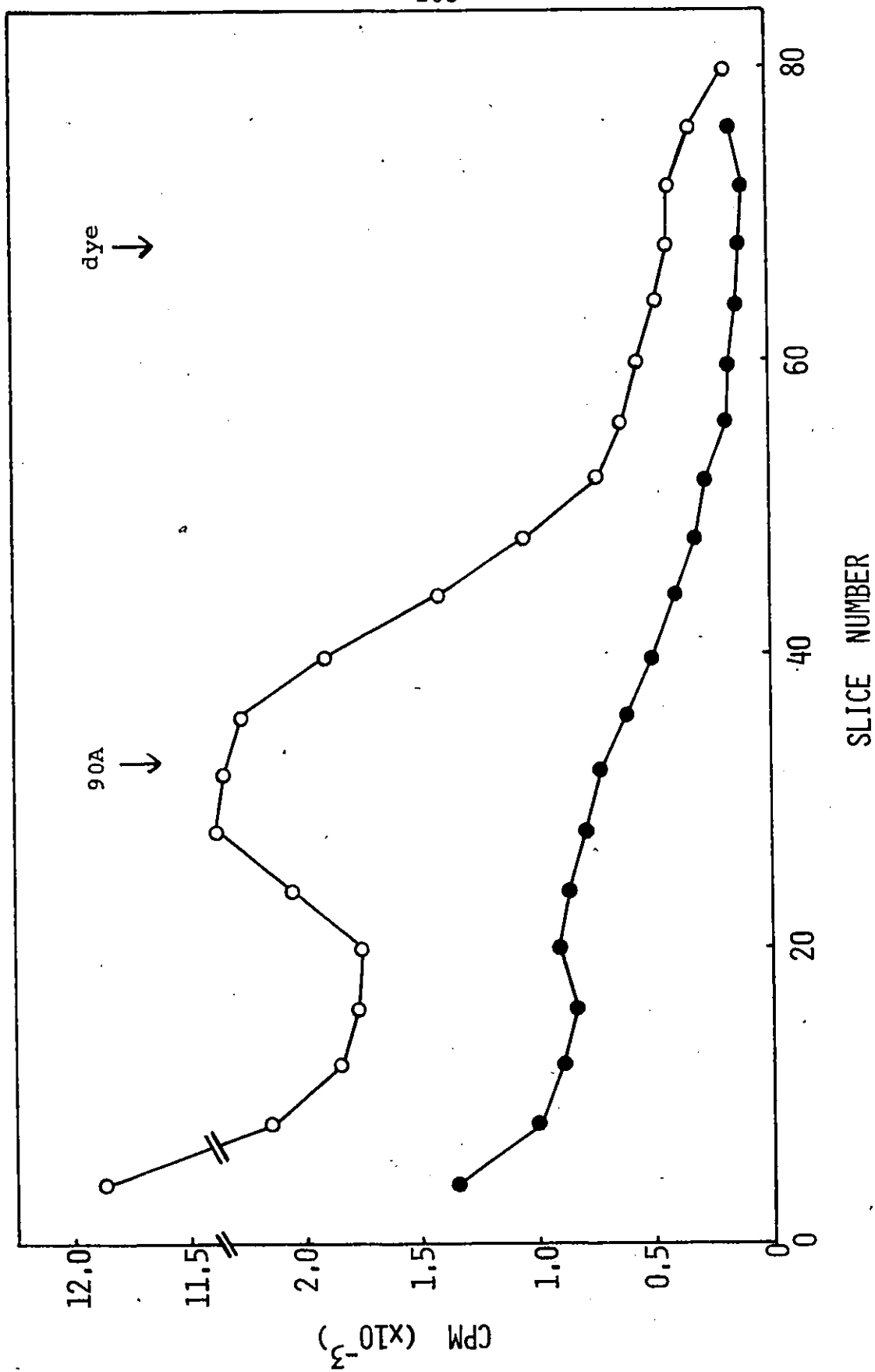


and the gel profiles of these samples are shown in Figure 12. The lower profile shows the RNase T₂ treated control which should contain only non-specific background label; this profile is very similar to those shown in Figure 11. The upper profile is that of the labelled poly(A) sequence, which contains a [³H]-label peak at the position corresponding to that of unlabelled 90A standard. A very high level of label at the top of this gel interferes with the upper part of the 90A peak (see Figure 10). This labelled material which does not migrate into the gel, appears to be lost after RNase T₂ treatment.

The results of Figure 12 indicate that the periodate oxidation and borohydride reduction method can be successfully used to label poly(A) tracts; however, in order to incorporate amounts of label which can be readily detected above the background profile of these gels, about 15μg of 90A standard were needed. Another experiment using 3μg poly(A) gave a typical "control" gel profile presumably because the amounts of incorporated label were so low. The 15μg 90A standard sample corresponds to amounts of poly(A)⁺ RNA in the order of 0.3mg since these samples seldom consist of more than 5% poly(A) as determined by the [³H]-poly(U) assay (see Chapters 1 and 2). It therefore was clear that for this [³H]-labelling procedure to be useful in poly(A) sizing experiments, large quantities of poly(A)⁺ RNA were required, unless the reaction

Figure 12. Electrophoresis of [³H]-labelled 90A standard

The 90A size standard (30µg) was [³H]-labelled (see Methods) and this sample was divided into two equal aliquots; one of these aliquots was RNase T₂ treated. Both samples were run on 10% polyacrylamide SDS disc gels which were sliced and the radioactivity eluted. The resulting profiles of [³H]-label are shown for the undigested (o) and digested (●) 90A standard. The position of unlabelled 90A, run on a parallel gel and detected by UV absorbance, is indicated.



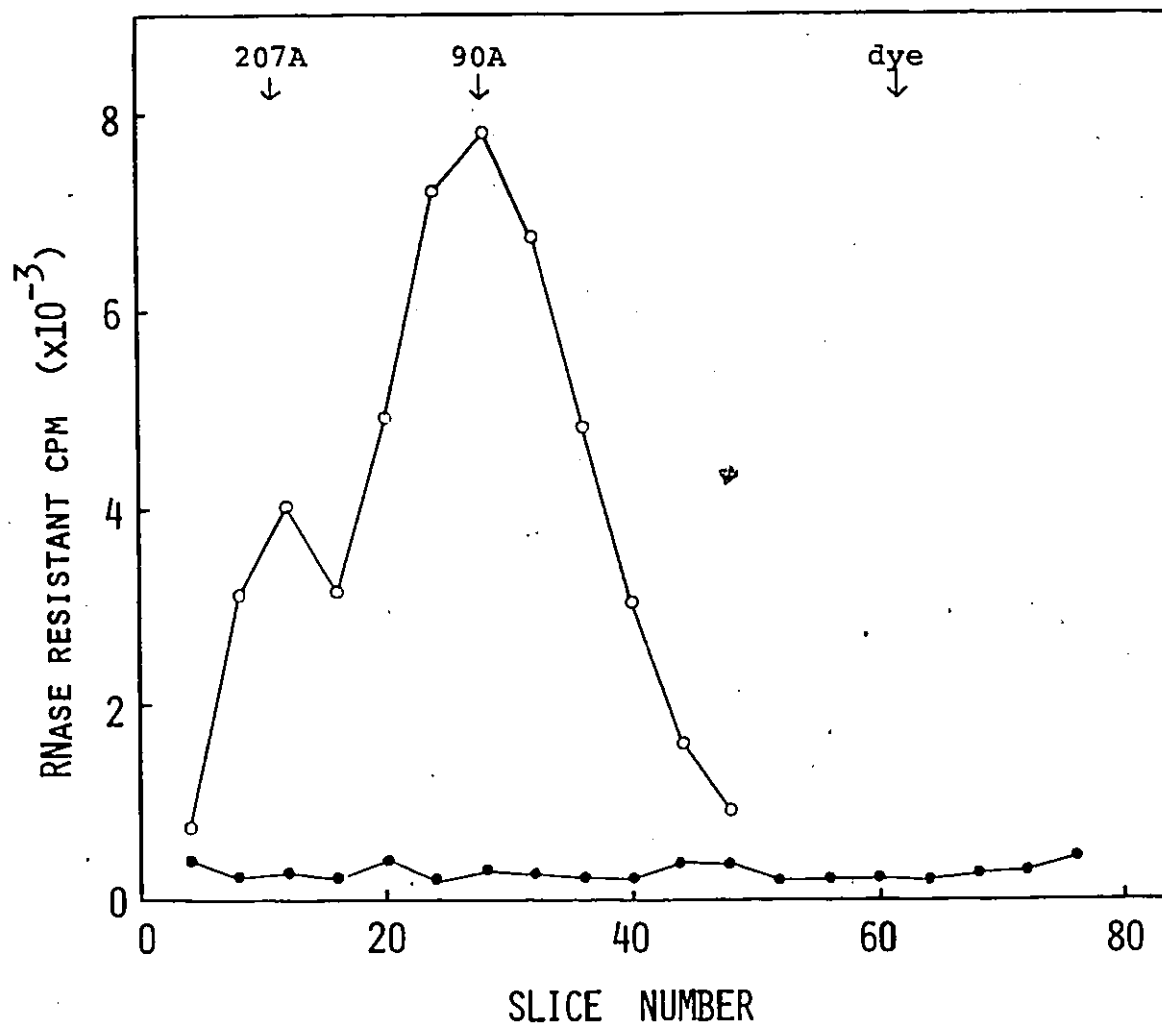
could be made much more efficient. It is pertinent to note here that whereas [^3H]-label incorporation into tRNA was 100%, that into the 90A standard was only 30-40%. In view of these problems, this method of poly(A) tract detection was not pursued further and efforts were concentrated on sizing experiments which utilised the [^3H]-poly(U) assay for poly(A) detection.

(iii) Poly(A) sizing with the [^3H]-poly(U) assay

The effectiveness of this procedure was determined by analysing two poly(A) standard size markers. A 10A standard (about 1.0 μg) and the 90A standard (0.6 μg) were digested with RNases A and T_1 as described (see methods), and collected by ethanol precipitation with E. coli tRNA carrier. The digests were electrophoresed as indicated using the short running time for the 10A standard and the longer electrophoresis for the 90A standard. After gel slicing of these two gels, poly(A) tracts were eluted from the slices and assayed with [^3H]-poly(U). The results of this experiment are shown in Figures 13 and 14. Figure 13 indicates that the 90A peak is contained within the top 48 fractions of the gel; maximum [^3H]-poly(U) binding occurs in slices 25-28 and under these conditions of electrophoresis, this corresponds to the 90A standard peak position when determined by UV absorbance. Further-

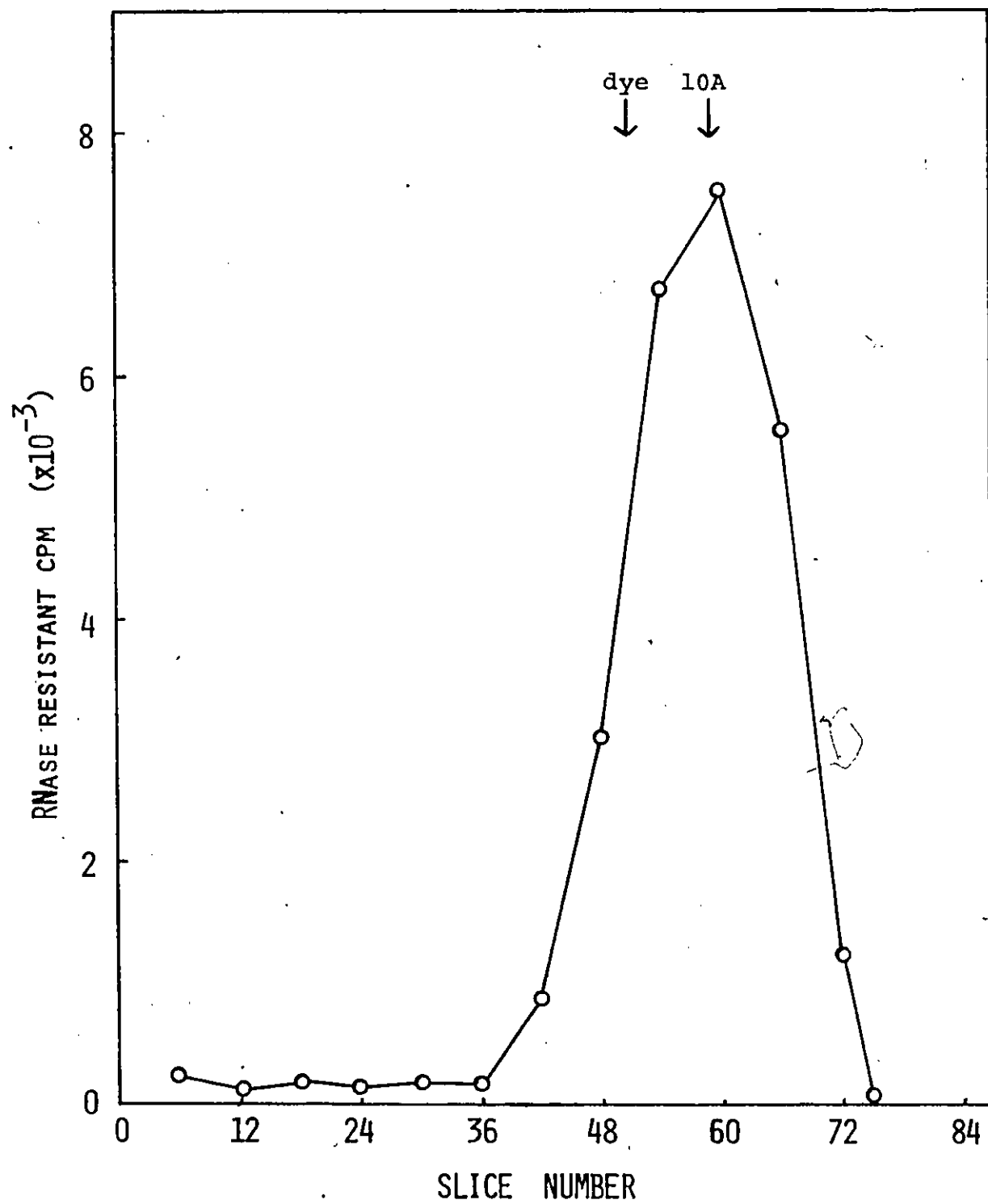
Figure 13. The Profile of the 90A size standard as detected by the [³H]-poly(U) assay after treatment of the standard with the poly(A) sizing procedure

The 90A standard (0.6μg) was digested with RNases A and T₁ (see Methods) and collected with E. coli tRNA carrier. The sample was run by polyacrylamide SDS disc gel electrophoresis and the gel was sliced. A [³H] poly(U) assay was performed on gel slice eluates (see methods) and the resulting profile is shown (○). The position of undigested 90A standard, run on a parallel gel and detected by UV absorbance, is indicated. Estimated poly(A) recovery was 85%. A [³H] poly(U) assay was also performed on a gel which received only E. coli tRNA carrier (●).



- Figure 14. The profile of the 10A size standard as detected by the [³H] poly(U) assay after treatment of the standard with the poly(A) sizing procedure

The 10A standard (1.0μg) was digested with RNases A and T₁ (see Methods) and electrophoresed as described. A [³H] poly(U) assay was performed on the gel (see Methods) and the resulting profile is shown. The position of undigested 10A standard, run on a parallel gel and detected by UV absorbance is indicated. Poly(A) recovery was estimated to be 42%. The electrophoresis was for 2.5 hours only.



more, a very similar profile was observed on electrophoresis of undigested 90A standard followed by detection using the [³H]-poly(U) assay. These observations not only confirmed that UV absorbance and [³H]-poly(U) binding were equivalent for detection of poly(A) standards, but that poly(A) standards maintain their initial size when treated by the entire assay procedure, including the RNase digestion.

This is important, since it demonstrates that shortening of poly(A) tracts does not occur during the procedure.

Recovery of the 90A standard from the gel slices was good (about 85% of applied), thus the elution conditions appear to be adequate. This was also tested using the 207A standard; this size class was chosen since the largest poly(A) tracts will be slowly eluted from the gel.

Elution of this 207A standard from an electrophoresed gel for 18 hours and 41 hours, followed by [³H]-poly(U) assay of 50μl aliquots of the eluate, results in two very similar profiles; the longer elution period resulted in no more than a 10% increase in the poly(A) levels detected, and this difference was close to experimental variation (data not shown).

This result, together with the high recoveries obtained with poly(A) tracts from poly(A)⁺ RNA samples and the 90A standard, shows that poly(A) elution from 1mm gel slices was essentially complete after 18 hours. A control sample

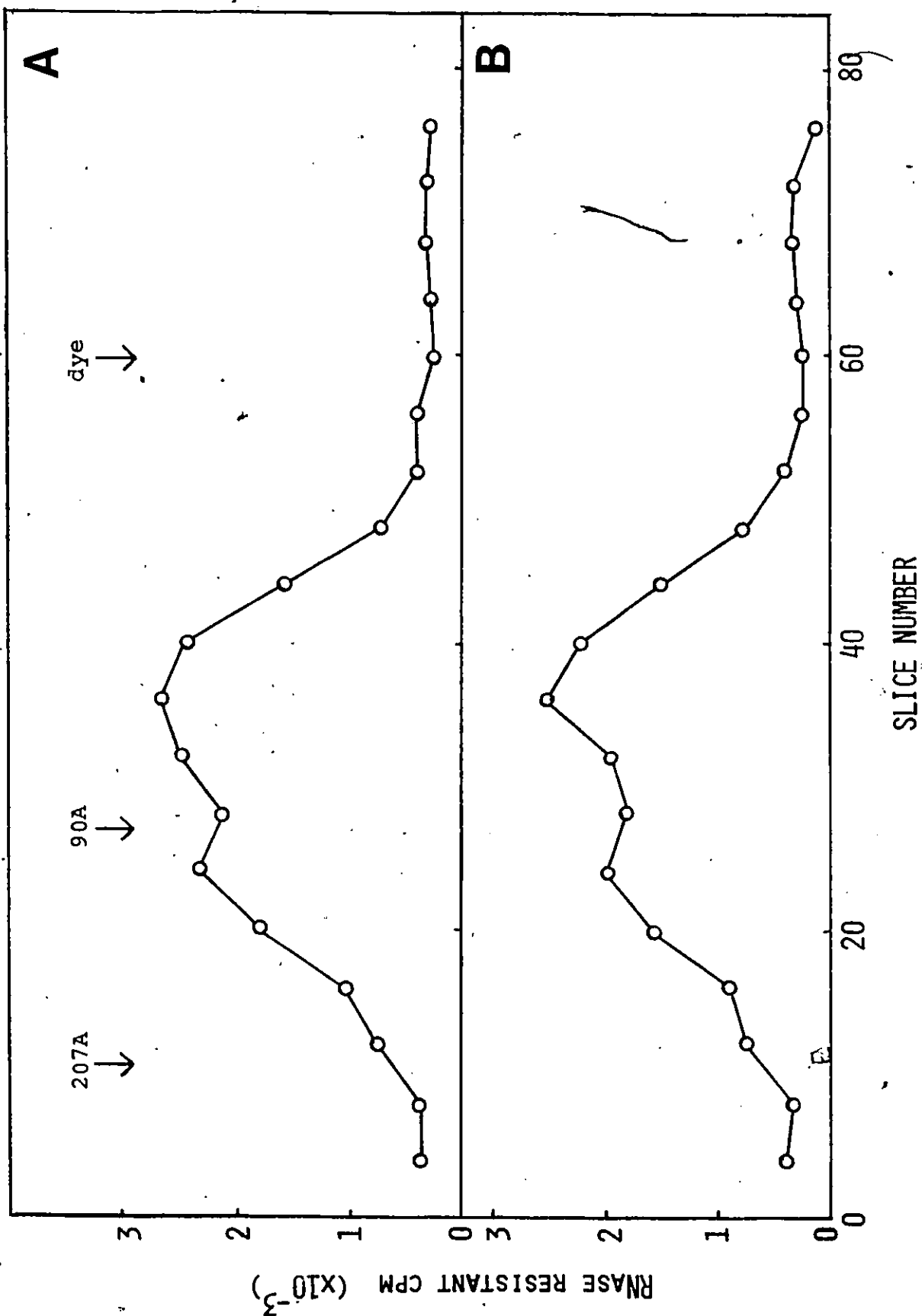
containing only 50µg E. coli tRNA was run on one gel and assayed with [³H]-poly(U). The purpose of this was to study whether or not the carrier RNA interfered with the assay in any way; the closed circle profile in figure 13 indicates that the tRNA peak does not interfere with the assay and this control gel yields a low and consistent background.

Figure 14 is a [³H]-poly(U) hybridization profile of a 10A standard which had been processed by the entire poly(A) sizing protocol, including RNase digestion. As for the 90A standard, the peak of [³H]-poly(U) RNase-resistant cpm coincided with the peak of UV absorbance of an aliquot of the 10A standard run on a parallel gel. This experiment again shows that [³H]-poly(U) binding and UV absorbance are equivalent. It also demonstrates that oligo(A) sequences as small as 10 nucleotides in length can successfully be detected by this system; however, it should be noted that recovery of the 10A standard, as measured by [³H]-poly(U) binding to gel slice eluates, was low (about 43%) compared to recoveries of larger poly(A) tracts. It is possible that this assay does not reproducibly detect tracts much smaller than 10A, since very small chains will not readily hybridise to poly(U) or be precipitated. It is possible that such factors could already be causing the rather large losses observed for the 10A standard.

Two typical size profiles of the poly(A) sequences from mammary poly(A)⁺ RNA of 11-15 day lactating rat gland are given in Figure 15. The first profile (Fig. 15a) was prepared from a poly(A)⁺ RNA sample obtained using oligo(dT)-cellulose chromatography (fractionation 2a) and the second profile (Figure 15b) was prepared from the same initial RNA but using poly(U)-sepharose chromatography (fractionation 2e). The two profiles are clearly similar and are representative of all the poly(A) size profiles obtained with poly(A)⁺ RNA from lactating gland. A comparison of the poly(A) size profile from poly(A)⁺ RNA contained in first and second column eluates suggested that the profile was similar irrespective of the number of columns through which the sample had been passed before being retained. All significant amounts of the poly(A) sequences, as measured by RNase-resistant [³H]-poly(U), occur in a size range no larger than 200 nucleotides and no smaller than about 25 nucleotides. The poly(A) size distribution is clearly bimodal with peaks occurring at sizes of about 80 and 40-45 nucleotides. The smaller poly(A) size class peak is generally slightly higher than the larger size class peak, indicating that the 40-45 nucleotide class must be present in amounts twice those of the larger size class. It therefore appears that a range of poly(A) sizes is present in association with mammary RNA but certain size classes do predominate.

Figure 15. Sizing of Poly(A) Tracts from Poly(A)⁺ RNA of Rat Mammary Gland.

Two poly(A)⁺ RNA samples were digested with RNases A and T₁ (see Methods) and electrophoresed for 4.5 hours as described. Gel slice eluates were assayed by the [³H] poly(U) assay. The profiles shown were obtained using: (A) 3.9μg poly(A)⁺ RNA, fractionation 2a (see Table 8); (B) 7.5μg poly(A)⁺ RNA from fractionation 2e (see Table 12). Poly(A) recoveries from the gels were 105% and 94% respectively. The positions of two poly(A) size markers, run on parallel gels and detected by UV absorbance, are indicated.

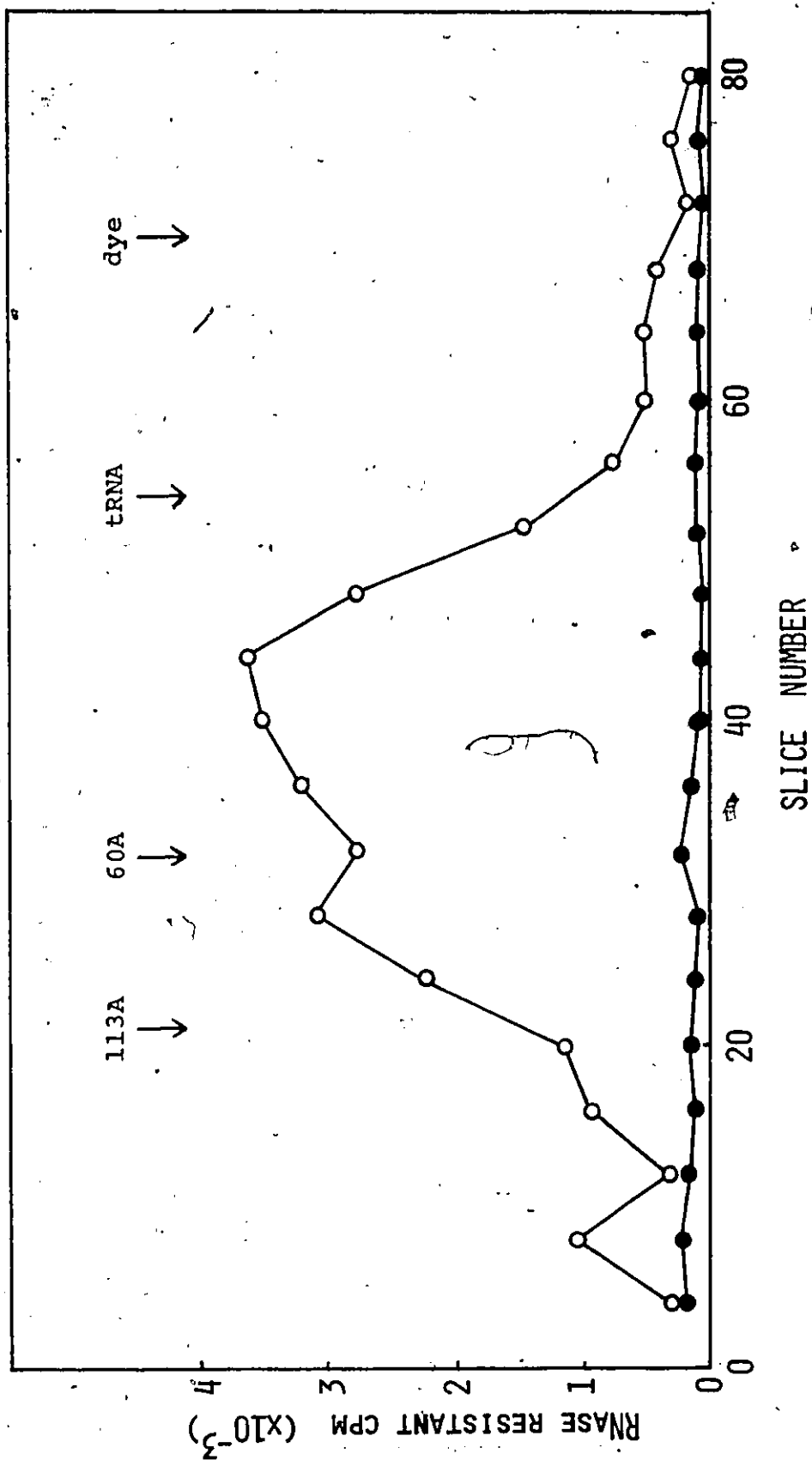


In figure 16, the [^3H]-poly(U) gel profile of a poly(A) sample from poly(A)⁺ RNA (fractionation 1a) is compared before, and after, RNase T₂ treatment (this enzyme cleaves at adenosine residues). This control experiment confirms that RNase resistance of [^3H]-poly(U) is a measurement of poly(A) and not an artefact of the procedure.

As mentioned earlier in this chapter, poly(A)⁺ RNA samples were also analysed by the poly(A) sizing method. In figure 17, two gel profiles of [^3H]-poly(U) binding of RNase digested poly(A)⁺ RNA samples are presented. The two profiles were obtained using the same sample (fractionation 2d); one was digested with RNases A and T₁ only while the other was also digested with DNase I (included in the digest buffer during RNase A treatment). The difference between these two profiles is striking. The profile obtained without DNase treatment is essentially close to background values, and no poly(A)/oligo(A) size classes are apparent. This means that recovery of [^3H]-poly(U) binding for this gel profile was poor; according to previous measurements 49ng of poly(A) were applied to the gel. Not surprisingly, RNase T₂ treatment of this sample subsequent to treatment with RNases A and T₁ resulted in a similar profile. When DNase was included in the digest, a large peak of [^3H]-poly(U) binding was detected close to the

Figure 16. Effect of RNase T₂ treatment on the poly(A) tracts of Mammary poly(A)⁺ RNA

Poly (A)⁺ RNA (9.6μg) from fractionation 1a (see Table 8) was digested with RNases A and T₁. The resulting sample was divided into two equal aliquots, and only one was treated with RNase T₂ (see Methods). Both samples were electrophoresed as described for 4.5 hours, and each gel was analysed by the [³H] poly(U) assay (see Methods). The two profiles obtained show the poly(A) tract profile of this mammary RNA sample before (o) and after (●) RNase T₂ treatment. Poly(A) recovery was 114% and 0% respectively. The positions of several poly(A) size markers are indicated.



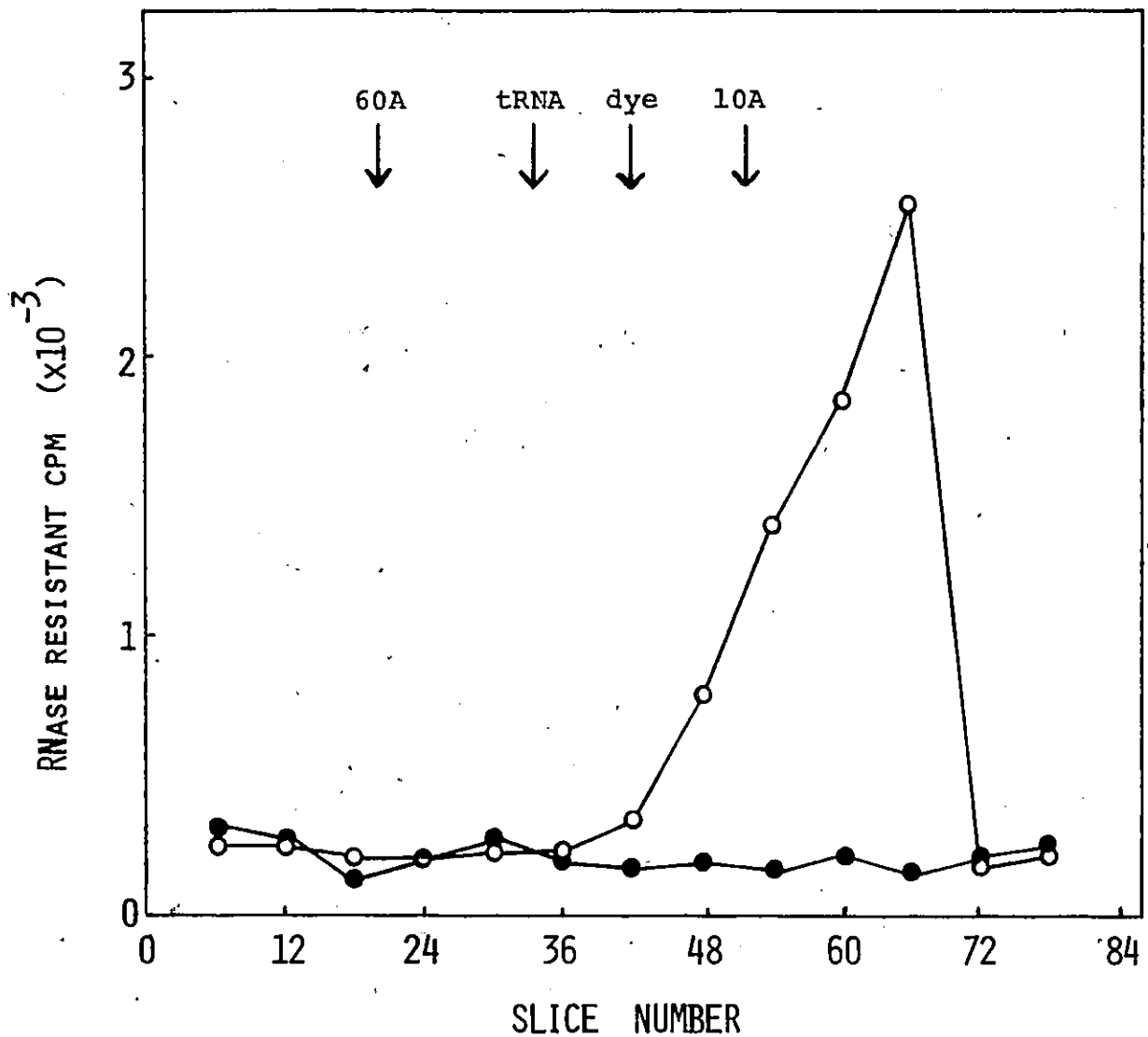


Figure 17. Sizing of "Poly(A)" from Mammary Poly(A)⁻ RNA

Poly(A)⁻ RNA (1.8mg, fractionation 2d - see Table 8) was digested with RNases A and T₁ in the presence (o) or absence (●) of DNase 1 (see methods). The digests were each electrophoresed for 2.5 hours as described and the gels assayed for poly(A) using [³H] poly(U) (see Methods). The positions of several size markers which were run on parallel gels are indicated.

bottom of the gel, in an area corresponding to oligonucleotides smaller than 10 residues. Recovery of [^3H]-poly(U) binding for this profile was obviously much higher; in fact the estimated recovery of poly(A) was 71ng, giving a yield of over 100%.

The effect of DNase in these assays suggested that a significant portion of the [^3H]-poly(U) binding detected in this sample was due to DNA binding of poly(U), and was not due exclusively to adenylate sequences in RNA. Before concluding that the presence of DNA in these samples was contributing to [^3H]-poly(U) binding activity, it was necessary to show that DNase I treatment of poly(A)⁻ RNA was not producing artefactually small poly(A) tracts due to contamination of the DNase I preparation with a poly(A)-degrading nuclease. Evidence against the presence of such a ribonuclease in these DNase I preparations was provided by inclusion of DNase I in the poly(A) sizing protocol for both a poly(A)⁺ RNA sample and the 60A standard. The presence of DNase did not change the profiles obtained with these two samples (data not shown). It was therefore concluded that the DNase I used did not contain any RNase activity which affected this assay.

The results of RNase T₂ treatment of poly(A)⁻ RNA samples, which had previously been digested with DNase I and RNases A and T₁, complicated this analysis further. Two [^3H]-poly(U) binding profiles are shown in Figure 18; that with the closed circles was obtained with a sample which was treated with DNase I, RNases A and T₁ and finally RNase T₂. The open circle profile was prepared by a similar treatment except that the RNase

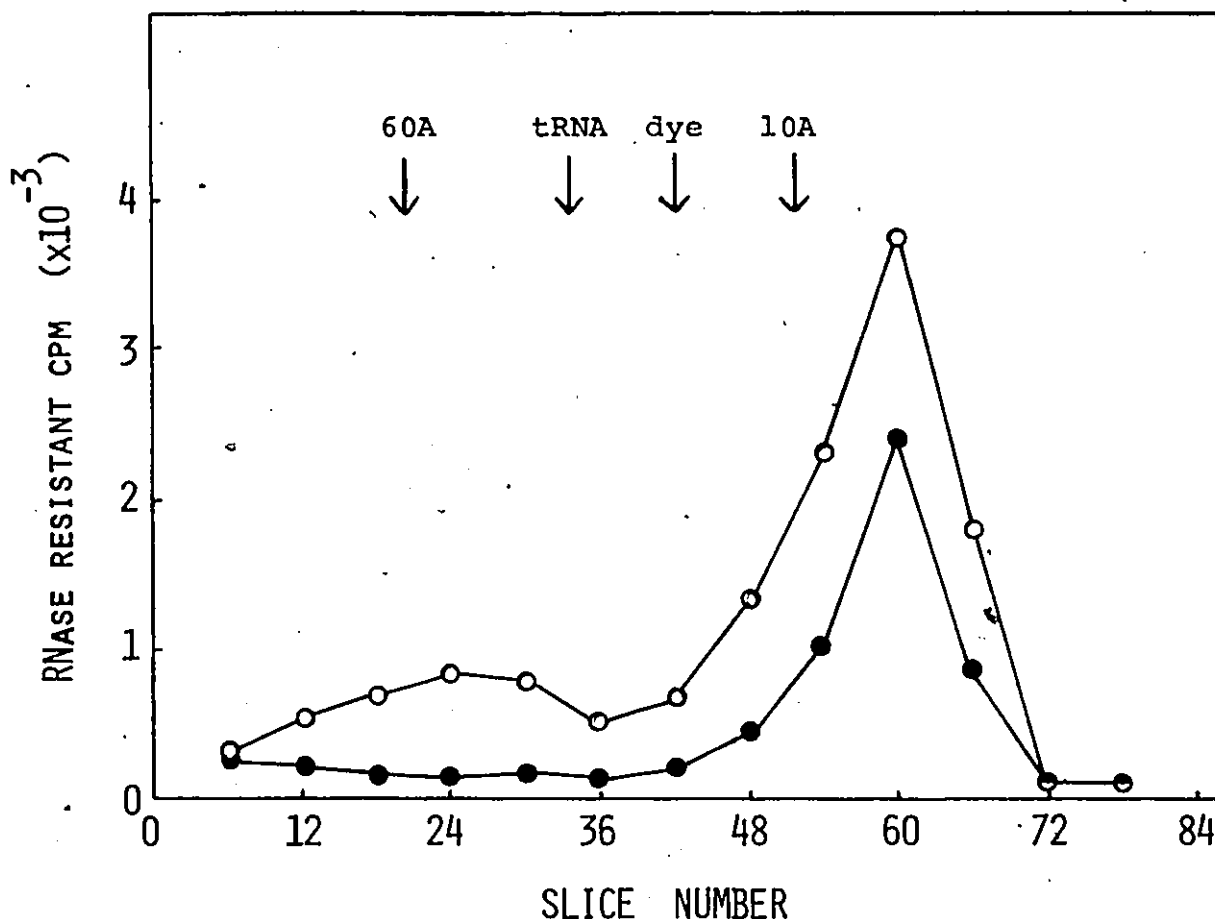


Figure 18. Analysis of Mammary Poly(A)⁻ RNA by the Poly(A) sizing procedure - Effect of RNase T₂ treatment

Poly(A)⁻ RNA (4.9mg, fractionation 2a - see Table 8) was digested with DNase I and RNases A and T₁ (see Methods). The sample was divided into two equal aliquots and one was treated with RNase T₂. The digests were electrophoresed for 2.5 hours as described and each gel was analysed by the [³H] poly(U) assay. The profiles shown are the results with (●) and without (o) RNase T₂ treatment. The positions of several markers run on parallel gels are indicated.

T_2 treatment was omitted. RNase T_2 treatment appeared to reduce the size of the peak near the bottom of the gel, but did not remove it entirely. This result could suggest that this peak of small oligonucleotides consisted of a mix of oligo(α A) and oligo(δ A).

The results of poly(A) sizing of mammary poly(A)⁻ RNA presented here were representative of all samples studied. It may be concluded that these poly(A)⁻ RNAs do not contain any significant amounts of poly(A) larger than 10-15 nucleotides, but they may contain very short adenylate tracts (<10 nucleotides).

DISCUSSION

It has been shown that when several poly(A) size classes were analyzed by electrophoresis under the conditions described, a plot of log. (nucleotide size) vs. mobility was linear; this standard curve can be used to assign a nucleotide size to poly(A) tracts of unknown length. The use of poly(A) tracts of defined size for this purpose has already been reported and this earlier work also made important observations regarding the relative mobilities of poly(A) tracts and heterogeneous RNA molecules. Burness et al. found that E. coli tRNA migrated as a 25 nucleotide molecule relative to poly(A) of defined size, although tRNA molecules actually consist of about 75 nucleotides (88). This anomalous behaviour of poly(A) tracts on acrylamide gels compared to hetero-

geneous RNA molecules has been confirmed elsewhere (85) and in this work. By comparison with the poly(A) size standards, tRNA was found to have a gel mobility corresponding to 26-27 nucleotides (see Figure 8), in close agreement with Burness et al. In view of the large discrepancy between actual and observed sizes of heterogeneous RNA, when compared with poly(A) on SDS/polyacrylamide gels, it is necessary to use poly(A) tracts of defined length as standards for poly(A) length determination. Small heterogeneous RNAs (e.g. tRNA, 5S RNA) can only be used as size markers provided the differences in relative mobility on polyacrylamide gels between RNA and poly(A) tracts are taken into account (88) .

The poly(A) standard curve which was obtained has been used to determine the size of poly(A) chains associated with RNA samples. Poly(A) sizing was successfully performed by isolation of poly(A) tracts after digestion with specific RNases, electrophoresis of these tracts and detection with the [^3H]-poly(U) assay. The poly(A)⁺ RNA samples analysed here contained a range of poly(A) sizes between 25 and 150 nucleotides. It is noteworthy that no sequences smaller than about 25 nucleotides were detected in the poly(A)⁺ RNA samples irrespective of their method of preparation. The absence of tracts in the size range of 15-25 nucleotides (none were detected in the poly(A)⁻

RNA fractions either) explains why the use of poly(U)-sepharose for affinity column chromatography had no advantage over oligo(dT)-cellulose in the fractionations of total mammary RNA described in chapters 1 and 2. Both resins are very effective in retaining poly(A) sequences longer than 20 nucleotides, only in the 10-20 nucleotide length range, is the use of poly(U)-sepharose believed to be advantageous. No affinity resin is capable of retaining oligo-nucleotides smaller in length than 10 residues, and thus any oligo nucleotides of 3-10A residues present in total mammary RNA will remain in the poly(A)⁻ RNA fractions. By extrapolation of the poly(A) standard curve, the peak of [³H]-poly(U) binding activity present in DNase and RNase-treated poly(A)⁻ RNA samples is estimated to correspond to a size of 4-5 residues. This assumes that the standard curve remains linear in this range. This is probably the smallest oligonucleotide which can reasonably be detected by the poly(A) sizing method. The source of the poly(U) binding material for the poly(A)⁻ RNAs is not absolutely clear; experiments which included RNase T₂ and DNase treatments of poly(A)⁻ RNA samples would suggest that the poly(U) binding is provided both by oligo(dA) and oligo(rA) sequences. In the classic paper on the [³H]-poly(U) assay by Bishop et al. (94), they reported that high amounts of DNA did give a positive reaction in

the assay. Very large quantities of poly(A)⁻ RNA (up to 2.5 mg per experiment) had to be used in the sizing procedure because the poly(A) content of these samples is so low. Since these samples contain significant amounts of DNA (see Chapter 1, table 1) interference in the [³H]-poly(U) assay by DNA or its digestion products remains a possibility. The chances of a short oligo(A) sequence or even a sequence highly enriched in A residues being present in rat mammary-DNA are real. If such sequences were present they could be detected in this procedure after DNase 1 treatment. DNase 1 tends to digest DNA into mononucleotides and a series of oligo nucleotides; limit digests have a mean chain length of a tetranucleotide, although chains up to octa-nucleotides may be present (314). The enzyme is particularly inactive with respect to di- and tri-nucleotides particularly if they do not contain a 5' Purine-Pyrimidine linkage and sequences of purines are not digested well (315). Oligo(dA) species might therefore survive DNase 1 treatment and be detectable by the sizing procedure.

The important observation to be drawn from these results is that poly(A)⁻ RNA contains no poly(A) sequences of the sizes (25-150 nucleotides) found in poly(A)⁺ RNA. The very small oligo(A) sequences which are present may not all be of RNA origin and whatever their source; they are probably not related to the long poly(A) sequences present

in poly(A)⁺ RNA. In view of the small size of the oligo(A) sequences detected, it is likely that some of them originate from internal positions in their nucleic acid sources, and thus are not 3' terminal.

It has been reported that when poly(A) tracts of HeLa cells are newly synthesised, they are 200 nucleotides long (37). Poly(A) shortening does occur, however, and measurements of the steady state size of poly(A) tracts have indicated the presence of much shorter sequences; for example, globin mRNA of circulating reticulocytes bears poly(A) tracts ranging from 35-120 residues in length, with the smaller size class apparently resulting from processing of the longer tracts (98). The steady state size of poly(A) sequences from rat mammary RNA has also been shown to be much shorter than 200 nucleotides. Two size classes around 40-45 and 80 residues predominate in this population. These sequences may have arisen by processing of larger poly(A) tracts, but this can only be shown by in vitro pulse chase labelling experiments and these were not performed. The 3'-terminal position of these poly(A) tracts could not be verified in these experiments because of the problems associated with [³H]-labelling of RNA samples by the periodate oxidation/borohydride reduction method. Rosen was successful in using this method to incorporate [³H]-label into poly(A) sequences associated with purified 15S rat casein mRNA (24). The size of these [³H]-labelled tracts was

similar in range to that observed for total mammary poly(A)⁺ RNA, although the purified mRNA did not show a pronounced bimodal size distribution of poly(A) sequences as was found in this study. On the basis of this result, and all the wealth of evidence in favour of the 3' terminal location of poly(A) in virtually all other systems, it is very likely that the poly(A) sequences analysed here are 3' terminal. The inability of the [³H]-labelling method to label these sequences appears to be due to a low efficiency of the reaction rather than the result of a peculiar characteristic of mammary RNA poly(A).

In theory, this [³H]-labelling method could determine whether or not the very small oligo(A) sequences present in poly(A)⁻ RNA were 3' terminal. It is likely that a substantial proportion of them were from internal positions and therefore would not have been labelled. In practice this point would be difficult to resolve due to the problems discussed above.

CHAPTER 5

PURIFICATION OF 9S WHEY PROTEIN AND 15S CASEIN mRNAs OF RAT MAMMARY GLAND

In order to prepare cDNA probes complementary to specific milk protein mRNA sequences for the analysis of the distribution of these two types of milk protein sequences between polyadenylated and non-polyadenylated RNA fractions purified milk protein mRNAs were required. In this chapter the purification of two mRNA fractions- 9S whey protein mRNA and 15S casein mRNA- from poly(A)⁺ RNA of lactating rat mammary gland is achieved by velocity sucrose gradient centrifugation. The purity of the mRNA fractions thus obtained was verified qualitatively by electrophoresis of their translation products synthesised in a wheatgerm cell-free system (this chapter) and by cross-hybridization of the corresponding cDNAs (next chapter).

MATERIALS

Sucrose (grade 1) was obtained from Sigma (St. Louis, Missouri). It was essentially RNase free.

METHODS

(i) Preparation of rat mammary poly(A)⁺ RNA

11-15 day lactating rat mammary glands were phenol-extracted as described in Chapter 1 to obtain total mammary RNA. These RNA preparations were fractionated by 1 or 2

rounds of oligo(dT)-cellulose column chromatography (see Chapter 1) to yield poly(A)⁺ RNA preparations.

(ii) Sucrose gradient centrifugation

Sucrose gradient centrifugation was performed as previously described (316) (298). Linear sucrose gradients (4-20% w/v) containing 1mM EDTA, 10mM Tris-HCl, pH 7.2, 0.1M NaCl were prepared at 4°C using an Isco model 570 gradient maker. First fractionation gradients had a volume of about 38ml; second fractionation gradients had a volume of 18.5 ml.

Samples (up to 0.6mg RNA) were heat treated at 68°C. for 5-10 minutes, followed by cooling at 0°C. They were subsequently applied to the top of the gradients in a volume not exceeding 0.6 ml. Omission of the heat treatment step resulted in gradient profiles which apparently contained a much higher proportion of large RNA molecules. This could be due to aggregation of RNA molecules, a phenomenon which is reduced by heating (47); all samples were therefore heat-treated before application to gradients. Preparative gradients were run at 27,000 RPM (131,000g) in a Beckman SW27 rotor and analytical gradients were run at 27,000 RPM (135,000g) in a Beckman SW27.1 rotor. See the legends to Figures 19 and 20 for the precise running temperatures and times.



Gradients were fractionated by upward displacement with 50% sucrose through an Isco UA-5 absorbance monitor (which produced an UV absorbance (254nm) profile of each gradient); the gradients were finally collected in autoclaved glass tubes in a fraction collector. The 38ml gradients were divided into 30-31 x 1.23ml fractions; 18.5ml gradients were divided into 35 x 0.47ml fractions. Corresponding fractions from separate gradients were pooled, brought to 0.2M NaCl and precipitated with 2 volumes of ethanol at -20°C. Precipitates were collected (see methods, chapter 1), dried and dissolved in Low Salt buffer.

The S values assigned to the peaks of UV absorbance obtained from these gradients were calculated by linear regression using mammary 4S tRNA and 18S rRNA as markers. It has been shown for these gradients that there is a linear relationship between the S value of an RNA species and the distance it migrates down the gradient (298).

Wheat germ cell-free translations were performed as described in Methods, Chapter 3, using [³⁵S]-methionine as the radioisotope source. Urea/polyacrylamide/SDS slab gel electrophoresis was also as described previously (Methods, Chapter 3).

RESULTS

- (i) Fractionation of mammary poly(A)⁺ RNA on sucrose density gradients.

A typical profile of lactating rat mammary poly(A)⁺ RNA after a single sucrose gradient ultracentrifugation is shown in Figure 19. This profile contains four major absorbance peaks: the most rapidly sedimenting peak (fractions 23-25) corresponded to 18S rRNA by comparison to total mammary RNA run on a separate gradient (see arrow). The position of 4S tRNA can be assigned (see arrow) by a similar comparison. The three other peaks, which occur in fractions 11-14, 15-17 and 18-20, are shown to have sedimentation values of 9S, 12S and 14S respectively and may tentatively be assigned as 9S αLA, 12S casein and 15S casein mRNAs respectively (23) (24). Although the largest mRNA fraction was found to have a sedimentation value of 14S, it will be referred to as 15S in keeping with reported values. This variation in observed S values is within experimental error for the gradients employed.

Although a single gradient run is sufficient to demonstrate the presence of three major size classes of mRNA in these poly(A)⁺ RNA samples, further sucrose gradient centrifugation was clearly required to resolve these preparations into discrete size classes. For each of the three size class peaks, appropriate gradient fractions prepared from several (6-12) individual preparative gradients were combined and rerun on analytical sucrose gradients. Figure 20 shows the

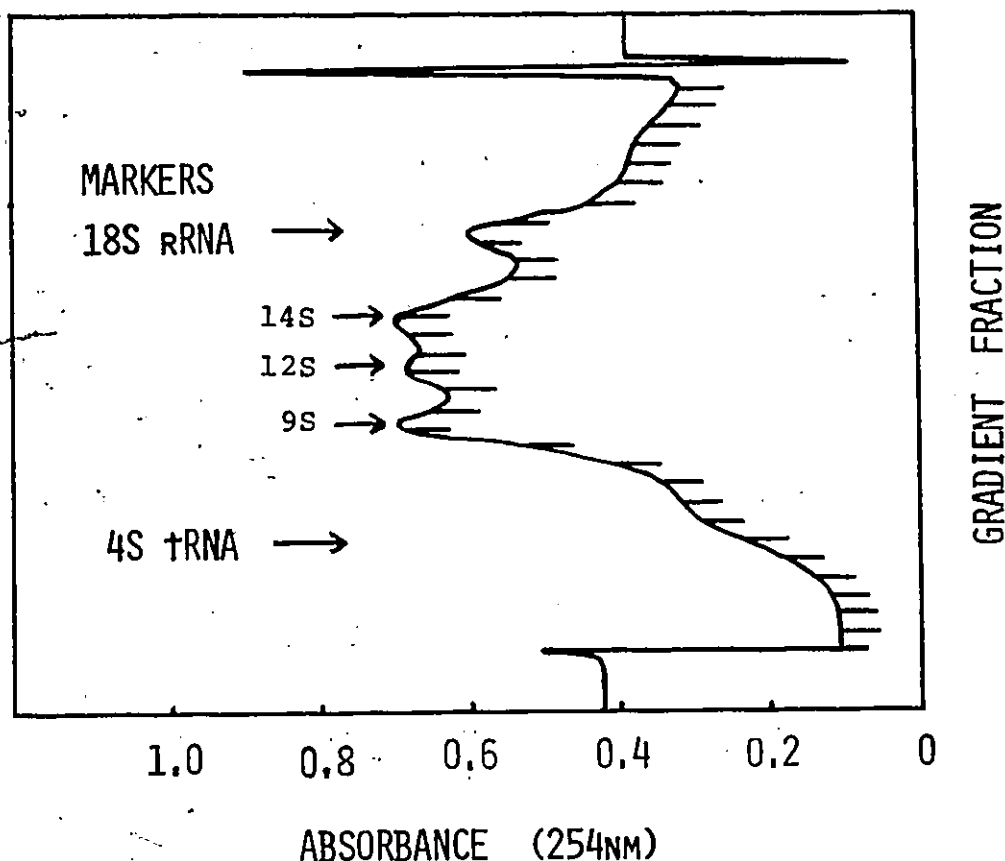


Figure 19. The Absorbance profile obtained on Sucrose Gradient Centrifugation of Mammary Poly(A)⁺ RNA.

Poly(A)⁺ RNA (0.62 mg) was run on a 38ml preparative sucrose gradient in a SW 27 rotor (Beckman) at 27,000 RPM (131,000g) for 20 hours at 8°C (see Methods). The gradient was fractionated as described and the resulting absorbance (at 254 nm) profile is shown. The positions of 4S tRNA and 18S rRNA were determined by centrifugation of total Mammary RNA on a parallel gradient. The sedimentation values of the three major peaks were determined by linear regression using the 4S and 18S RNA markers.

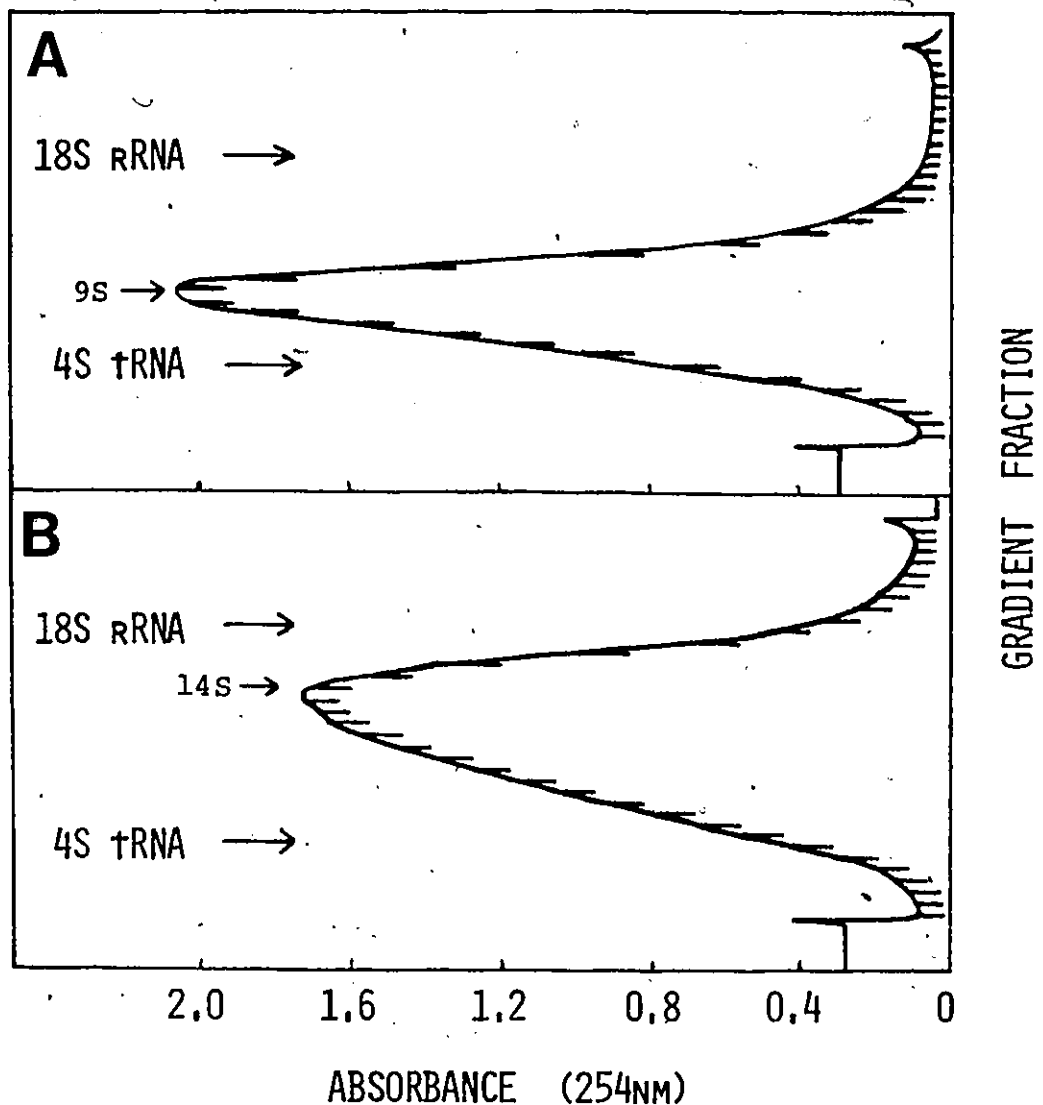


Figure 20. The profile of partially purified mRNA preparations after centrifugation on SW 27.1 sucrose gradients

Two mammary RNA samples were run on analytical sucrose gradients at 27,000 RPM (135,000g) at 11°C for 23 hours; the profiles obtained on fractionation are shown. The samples were: (A) 457µg partially purified 9S mRNA (B) 534µg partially purified 15S mRNA. Both samples had been recovered from preparative gradients similar to that shown in Figure 19. The 4S and 18S RNA markers were from total mammary RNA run on a parallel gradient.

absorbance profiles obtained after running (a) 9 S peak fractions, (b) 15S peak fractions on a set of analytical gradients. The 9S mRNA fraction has a UV absorbance peak at fractions 13-15; this corresponds to a sedimentation value of 9S. The 15S mRNA fraction has an absorbance peak at fractions 19-21, corresponding to a value of 14S. The profiles presented in Figure 19 further confirm the sedimentation values assigned to these fractions and they show that a significant purification of these mRNA size classes is already achieved after fractionation of the first round preparative gradients.

To reduce cross-contamination of these mRNA preparations, fractionation of the two profiles in Figure 19 was performed as follows. The major 9S preparation was taken from the peak fractions and those adjacent fractions on the small-size side of the peak (fractions 10-15). The major 15S preparation was prepared from fractions 19-23 (the peak fractions and those on the large-size edge of the peak). By this method, it was hoped to significantly reduce 9S and 15S mRNA cross-contamination as well as 12S mRNA contamination of both preparations. A 12S RNA fraction from preparative gradients peaked at fractions 15-17 under these gradient conditions.

Using this fractionation procedure the yield of 9S and 15S mRNA fractions from poly(A)⁺ RNA were as follows.

After a single preparative type gradient, 9S mRNA yield was generally around 10-15% and the yield of 15S mRNA was comparable at 10-20%. On rerunning these mRNA sequence enriched fractions on analytical size gradients, 9S mRNA recovery ranged from 26-40%, but 15S mRNA recovery was rather lower (20-25%). From these numbers it is possible to calculate that 1 mg poly(A)⁺ RNA yields approximately 26-60 µg 9S mRNA and 20-50 µg 15S mRNA after two successive rounds of sucrose gradient centrifugation.

(ii) Analysis of the translation products of sucrose gradient RNA fractions by slab gel electrophoresis

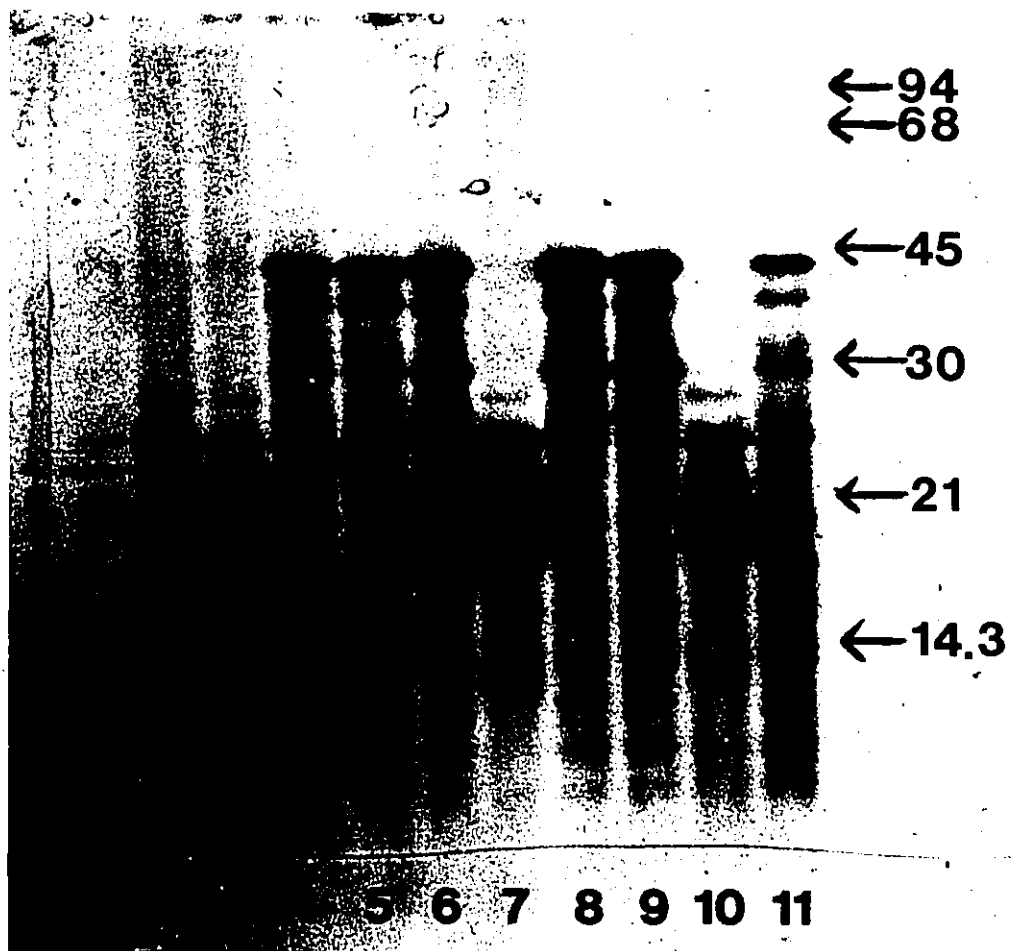
Although the 9S and 15S mRNA fractions from analytical sucrose gradients (see Figure 20) appear to consist of different mRNA species, the gradient profiles gave little indication of the extent of any cross-contamination of the two mRNA preparations. The nature of final sequence-enriched preparations of 9S and 15S mRNAs was determined by translation in a wheat germ cell-free system using [³⁵S]-methionine as the isotope. The [³⁵S]-methionine labelled protein products were run on an urea-SDS-polyacrylamide slab gel and detected by fluorography. For comparison, an aliquot of unfractionated poly(A)⁺ RNA and several intermediate preparations (from a single gradient run) were also translated; their products were electrophoresed, on the same gel.

All these poly(A)⁺ RNA preparations were very active in the wheat germ cell-free system; they incorporated 400,000-800,000 cpm/ μ g RNA and thus provided adequate incorporation to permit fluorography.

Figure 21 shows the results of fluorography of a slab gel on which have been run the translation products of the poly(A)⁺ RNA fractions described above. The pattern of translation products yielded by total poly(A)⁺ RNA is shown in lane 6. From the standard molecular weight curve provided by the protein standards, the principal proteins in lane 6 correspond to bands of molecular weights 41,500, 30,000, 24,500-26,000, and 19,500 daltons. The largest two proteins (30,000 and 41,500 daltons) were assigned to the two larger caseins, the proteins migrating around 25,000 represented the smallest casein and the 19,500 molecular weight band was initially attributed to α LA. These assignments were based on previous reports of the size of milk proteins when prepared in a cell-free translation system and they are described in greater detail in the discussion. It may be concluded here that the translation products of unfractionated poly(A)⁺ mammary RNA show the size pattern predicted from previous studies. The translation products of 9S mRNA are shown in lane 7, and those of a corresponding 15S mRNA fraction were run in lane 8. It is clear from these two translation patterns that (a) the 9S mRNA preparation does not contain significant amounts of 15S mRNA; (b) the 15S

Figure 21. Analysis of the Wheat germ cell-free translation products of Mammary mRNA by Slab Gel Electrophoresis

Total mammary poly(A)⁺ mRNA and several mRNA size fractions, prepared by sucrose gradient centrifugation, were translated in the wheat germ cell-free system using [³⁵S]-methionine. The translation products were electrophoresed on an urea **SDS** polyacrylamide slab gel and they were visualised by fluorography (see Methods, Chapter 3). The translation products shown were prepared from the following samples: lane 1, no mRNA; lanes 2 and 3, 9S mRNA fractions; lanes 4 and 5, 15S mRNA fractions; lane 6, total poly(A)⁺ mRNA; lane 7, highly purified 9S mRNA; lane 8, highly purified 15S mRNA; lane 9, a 15S mRNA prepared by taking wide gradient cuts; lane 10, intermediate 9S mRNA (after a single centrifugation of total poly(A)⁺ mRNA); lane 11, intermediate 15S mRNA fraction. Between 100,000 and 200,000 cpm of acid insoluble [³⁵S]-label was applied to each lane. The positions of the standard marker proteins, which were detected by Coomassie blue staining of the gel prior to fluorography, are indicated with their molecular weights (in Kilodaltons).



mRNA preparation contains little 9S mRNA. Small amounts of 12S mRNA have remained in the 9S mRNA fraction, but the 15S mRNA preparation contains little 12S mRNA.

Lane 9 shows the ~~translation~~ profile of another 15S mRNA preparation which was prepared by taking wider gradient fraction cuts than with the sample represented in lane 8 and this is evident from the translation profiles.

Lanes 2-5 contain translation products of RNA preparations from a different set of gradients. Lanes 2 and 3 are 9S mRNA translation products and lanes 4 and 5 are of 15S mRNA products. Although these preparations have been fractionated by the two gradient protocol described, they are not purified to the extent of the preparations analysed in lanes 7 and 8, even though their gradient profiles were comparable. This indicates the importance of monitoring the purity of sucrose gradient fractions of RNA by factors independent of RNA size. Finally lanes 10 and 11 contain translations of intermediate RNAs, prepared by a single gradient run. Lane 10 is of a 9S mRNA fraction and lane 11 is of a 15S preparation, and these preparations are clearly of intermediate purity between initial mRNA (lane 6) and final mRNA fractions (lanes 7 and 8). Lane 1 represents a control translation run in the absence of exogenous mRNA.

The most highly purified mRNA fractions produced are represented in lanes 7 and 8 of Figure 21; these fractions

were used for cDNA probe preparation as described in the next chapter.

DISCUSSION

Previous reports have shown that the principal translation products of poly(A)⁺ mRNA from lactating rat mammary gland are three caseins of molecular weights 42,000, 29,000 and 24,500 - 25,000 daltons (24) (42) (279) and α LA of molecular weight 21,500 - 22,500 (23). The sizes of these translation products are in close agreement with the sizes of the milk proteins as isolated from rat milk. In vivo the secreted proteins are glycosylated and this might be expected to result in slightly anomalous behaviour of these processed proteins on polyacrylamide gels. Indeed, Rosen (24) has reported a slight difference in mobility on such gels between the second largest casein (29,500 daltons) isolated from milk and the second largest translation product (28,500 daltons) directed by rat mammary mRNA. This difference is attributed to glycosylation of the protein synthesised in vivo (wheat germ cell-free systems would not be expected to process its translation products by carbohydrate addition); the two proteins are believed to be otherwise identical.

Rosen (24) has shown that the two largest caseins of

molecular weights 42,000 and 29,500 daltons, are synthesised by mammary mRNA sedimenting at 15S. Separation of this 15S mRNA into its two composite species each coding for one of the two largest caseins is not possible by sucrose gradient centrifugation. Recently, the preparation of three cDNA clones specifying the nucleic acid sequence of the three rat caseins 1, 2 and 3 (now generally referred to as α -, β - and γ -caseins) was reported (284), and it would appear that cloning techniques are the only available means for preparing pure preparations of each casein gene sequence. Attempts to purify the 12S mRNA, coding for casein 3 (24-25,000 daltons) by sucrose gradient centrifugation was not highly successful either in this work or previously (24, 278).

The results of this chapter have shown that in close agreement with previous findings the three largest proteins encoded by lactating rat mammary poly(A)⁺RNA have molecular weights 41,500 daltons, 30,000 daltons and 24,500-26,000; the two largest proteins (which correspond to caseins 1 and 2) are produced by 15S mRNA. At the position of the smallest casein (casein 3), a doublet was consistently observed. This is explained either by the existence of multiple forms of this protein or by production of incomplete peptide chains by the in vitro translation system.

Rat mammary α LA is reported to have a molecular weight of 21,500-22,500 daltons and is encoded in mRNA sedimenting

at 8.3 - 10.5S (23). The smallest predominant polypeptide produced on in vitro translation of poly(A)⁺ rat mammary RNA had a molecular weight of approximately 19,000 daltons and this was initially assigned as α LA since this peptide was produced by the in vitro translation system containing a 9S mRNA fraction. However, this band was unusually diffuse compared to standard protein markers in this size range on the gel, and close examination of this band (see lane 6) suggested that it might contain two bands. Several reports have indicated that α LA exists in many forms, but the available evidence suggests that this heterogeneity resides in the glycosylation of the molecule, rather than in the amino acid sequence (268, 270, 272). Since in vitro translation systems are unable to process translation products by carbohydrate addition, heterogeneity in the protein after synthesis in vitro would not be expected. Most recently a different explanation for the apparent heterogeneity of this polypeptide in figure 21 came from studies on the clones of rat milk protein cDNAs. In 1981, Richards et al. prepared and characterised a cDNA clone (pLA32) which they believed represented the α -lactalbumin sequence (284, 285). In 1982, these authors acknowledged that this clone had been incorrectly designated as coding for α LA mRNA and the clone was renamed pX32 (287). It has now been found that 9-10S poly(A)⁺ rat mammary RNA may be resolved

into two components and only one of these codes for α LA; the second protein encoded by this 9-10S mRNA appears to be a whey protein (287); thus, it now appears likely that all rat mammary 9-10S mRNA is made up of two components, and this would provide an explanation for the heterogeneous and very diffuse polypeptide product made by in vitro translation of the 9S mRNA prepared in these studies. Thus the 9S mRNA fractions prepared in this study are described as 9S whey protein mRNAs to indicate the heterogeneity of these preparations.

For the assessment of the purity of the 9S and 15S mRNA preparations in a more quantitative manner, cDNA probes were prepared using each preparation and these were hybridised back against both mRNA preparations. In this way, the degree of cross-contamination between these two preparations could be determined. This analysis is presented in chapter 6.

CHAPTER 6

PREPARATION OF SPECIFIC cDNA SEQUENCES AND THEIR USE FOR MEASUREMENT OF mRNA SEQUENCES IN MAMMARY RNA SAMPLES

To determine the relative amounts of specific milk protein mRNA sequences in poly(A)⁺ and poly(A)⁻ mammary RNA fractions, cDNA probes prepared against purified mRNA sequences are required. This chapter describes the synthesis and characterization of such cDNA probes and their use for measuring the levels of 9S and 15S mRNAs in mammary RNA preparations. The importance of this analysis has already been indicated in "Aims of this Research"; the developmental stage studied by this method in this chapter is the 11-15 day lactating gland and in later studies (see Chapter 8) these values are compared to those obtained using glands at other developmental stages.

MATERIALS

Materials for the reverse transcriptase reaction were from the following sources: AMV reverse transcriptase was from Life Science Inc (St. Petersburg, Fla); unlabelled deoxy-nucleotides dATP, TTP, dGTP and dCTP, actinomycin D, E. coli tRNA, type XXI and bovine serum albumin were all from Sigma (St. Louis, Missouri); oligo(dT)12-18 was from Collaborative Research (Waltham, Mass.). DTT was from

Boehringer Mannheim (Dorval, Que). [5-³H]-dCTP, (tetra-sodium salt (specific activity 30Ci/mmol or 16.8Ci/mmol) was from ICN (Irvine, Cal.). Where the higher specific activity material was used, it was diluted with unlabeled dCTP to bring the specific activity down to 19.5Ci/mmol.

Phenol (U.S.P.) was from Baker (Phillipsburg, N.J.) and it was redistilled before use. Sephadex G-100 (Coarse) was from Pharmacia (Dorval, Que). Sucrose (for gradients) was grade 1 from Sigma.

The materials for hybridization and nuclease S₁ digestion included calf thymus DNA (type I), Pipes and S₁-deoxynuclease (3600I Units/ml) from Sigma (St. Louis, Missouri); S₁ nuclease was diluted to 20 IU/ml using S₁ buffer (see methods) and glycerol (1:1) before use. Yeast RNA carrier was obtained by phenol extraction of Yeast RNA (grade VI, Sigma); RNA yields were about 40% of the initial material.

METHODS

(i) Synthesis of cDNA by reverse transcription of purified mRNA fractions

Reverse transcriptase reactions, performed in a volume of 25 or 50µl, contained 50mM Tris-HCl, pH 8.3, 6mM Magnesium acetate, 60mM NaCl, 10mM DTT, 0.4mM of each of the deoxynucleotides dATP, dTTP and dGTP, 50-100µg/ml BSA, 20mg/ml oligo(dT)₁₂₋₁₈, 50µg/ml actinomycin D, 2-8µg poly(A)⁺.

mRNA, and 134 μ Ci (reaction 1) or 50 μ Ci (reaction 2) [5-³H]-dCTP, of specific activity 16.8Ci/mmol (reaction 1) and 19.5Ci/mmol (reaction 2). The synthesis was initiated by addition of 1 μ l reverse transcriptase (20.9U/ μ l).

Reactions were incubated at 30°C for 45 minutes and terminated by addition of 20 μ l 0.2M EDTA (pH 7.0). RNA was hydrolysed by incubation at 68°C for 10 min. after bringing the reaction to 0.12N NaOH with 250 μ l 0.14N NaOH. Reactions were cooled and neutralised with 22 μ l 1N HCl; 75 μ g *E. coli* tRNA were added as a carrier. Measurements of [³H]-dCTP incorporation were then performed by spotting a 2 μ l aliquot of each reaction onto 3MM filter paper (Whatman), which was placed in cold 10% TCA, 0.9% sodium pyrophosphate for 15-20 minutes. Filter papers were washed with 10% TCA, 0.9% sodium pyrophosphate (5 x 40ml) and 95% ethanol (2 x 40 ml). After rinsing with ether, filter papers were air dried and counted in 4 ml scintillant (0.5% 2,5-diphenyloxazole and 0.05% 1,4-bis-(5-phenyloxazol-2-yl) benzene in toluene) at a counting efficiency of approximately 30%.

The reverse transcriptase reactions were phenol extracted twice using 200-300 μ l of phenol saturated with water. The pooled aqueous phases were passed through a column of Sephadex G-100 (prewashed with sterile water) in order to remove unreacted [³H]-dCTP. The [³H]-label running through the column in the void volume was collec-

ted and stored at -70°C . cDNAs produced had specific activities of $1.2-1.3 \times 10^7$ cpm/ μg .

(ii) Alkaline sucrose gradient analysis of cDNA preparations

These gradients were prepared according to Mezl et al. (317). They were run at 40,000 RPM in a Beckman SW60 Ti rotor for 15 hour at 4°C . Gradients were fractionated into 16 x 0.2ml fractions and [^3H]-label determined in each fraction using a 2 μl aliquot. A 16S marker ([^3H]-SV40 component III DNA) from Bethesda Research Laboratories (Gaithersburg, Md U.S.A.) was run on a parallel gradient.

(iii) Solution hybridization of cDNA probes with mammary RNA preparations

Hybridizations were performed in silicone-treated capillary tubes containing 50 μl of 0.18M NaCl, 10mM Pipes (pH 6.8), 0.1% SDS (from BDH, 98% pure), 2mM EDTA (pH 7.0), about 0.5 μg *E. coli* tRNA, 425-800 cpm of [^3H]-cDNA (about 0.05ng) and various amounts of RNA. The reaction mixtures were denatured at 100°C . for 10 minutes and then hybridized at 68°C . for 5 hours. Each reaction was washed into 1 ml of S_1 buffer (30mM sodium acetate (pH 4.5), 0.1M NaCl, 1mM Zinc sulphate) containing 10 μg heat-denatured calf thymus DNA. Reactions were digested with 0.12 I.U. S_1 nuclease at 45°C . for 45 mins. Hybrids

were precipitated with 150µg of carrier yeast RNA for at least 2 hours by bringing the reaction to 10% TCA, the precipitate was collected on Whatman GF/C filters and washed with 5% TCA (2 x 10 ml) and with 95% ethanol (2 x 10 ml). Dried filters were incubated at 70-80°C. for 1 hour in 200µl 0.5N HCl to hydrolyze the precipitated cDNA. After neutralization with 200µl 0.5N NaOH, filters received 5ml Ready-solv (Beckman) and were mechanically shaken for 15-20 minutes at room temperature before counting in an Isocap 300 liquid scintillation counter with an efficiency of about 35%. Total input cDNA was determined for several samples by omission of S₁ nuclease.

Two types of RNA excess hybridization analyses were performed. The initial cDNA characterizations (see Figure 22) were performed by Cot curve analysis. As described previously (298), the data were fit to the equation

$$D_H = B + \sum F_i (1 - e^{-K_i \text{Cot}})$$

where D_H is the fraction of cDNA in the hybrid, B is the fraction of cDNA hybrid that is present as a background, F_i is the fraction of cDNA hybridised in the i th transition and K_i is the apparent rate constant for this transition. $\text{Cot} \frac{1}{2}$ values were calculated from the first order rate constant; these provide a measure of the concentration of the mRNA sequences in the sample being hybridised.

Further cDNA characterization and use of these probes in sample investigation were performed by the hybridization analysis described by Mezl et al. (317). In this method, in which the data are plotted as %hybrid vs. ng RNA in the hybridization, only the linear portion of the hybridization curve is used for quantitation. The advantages of this method are (a) it is very sensitive, (b) it does not require subjective curve drawing and (c) an estimate of the analytical variation is possible. The hybridization data were fit to a straight line by linear regression; the slope of the sample hybridization was compared to that of the standard mRNA hybridization and the ratio of these two values indicated the amount of mRNA sequence in the RNA sample.

RESULTS

(i) Reverse transcription of purified 9S and 15S mRNA preparations

A typical cDNA synthesis, (carried out as described in methods) using the two highly purified mRNA fractions prepared as indicated in Chapter 5, is summarised in Table 17. The reaction resulted in the incorporation of 5.7×10^6 cpm of [^3H]-dCTP into 9S cDNA and an incorporation of 3.39×10^6 cpm into 15S cDNA. This is equivalent to cDNA amounts of 0.425 μg and 0.254 μg respectively with a specific activity of 1.3×10^7 cpm/ μg . When these cDNA

**TABLE 17: SYNTHESIS OF cDNA BY REVERSE TRANSCRIPTION
OF PURIFIED 9S and 15S RAT MILK PROTEIN mRNAs**

	1	2	3	4	5
Sample in reaction type (µg)	[³ H]-dCTP in reaction (cpm)	*[³ H]-dCTP incorporated into cDNA (cpm)	[³ H]-label incorporated into cDNA (%)	Amt. of µgDNA/µg mRNA synthesised (µg)	µgDNA/µg mRNA (%)
9S 2.2	38 x 10 ⁶	5.7 x 10 ⁶	15.0	0.43	20
15S 2.0	46 x 10 ⁶	3.39 x 10 ⁶	9.9	0.25	13

Reverse transcription reactions were carried out in a volume of 25 µl as described (see methods), using [³H]-dCTP of specific activity 19.5Ci/mmol.

Incorporation of [³H]-dCTP was measured by counting the acid precipitable [³H]-label (counting efficiency of 30%). These cpm values were converted to soluble cpm values (counting efficiency of 40%) (values presented here*) in order to permit calculation of the proportion of total [³H]-label incorporated (column 3). In column 5, the cDNA yield is expressed as a percentage of the amount of mRNA included in the synthesis. The calculated specific activity of these two cDNAs is 1.3×10^7 cpm/µg.

yields are expressed as a percentage of the amount of mRNA present in the assay, the transcription level is estimated at 20 % and 13 % respectively; such levels are representative of a good efficient synthesis (284).

The free [³H]-dCTP present in these cDNA preparations was removed by passage of the preparations through Sephadex G-100 columns (20 x 1cm) prewashed with sterile water. Recovery of cDNA (collected in the void volumes) from these columns was 80% or higher; about 75-80% of the [³H]-label cpm present in these void volume fractions was acid precipitable.

The size of the cDNAs prepared in these transcription reactions was determined in one instance by analysis on alkaline sucrose gradients. The resulting gradient profiles indicated that the length of the cDNA prepared from 9S mRNA peaked at 6.7S (equivalent to 530 nucleotides) while the cDNA prepared from 15S mRNA gave a rather broad size peak around 9.3S (equivalent to 1204 nucleotides)- (data not shown). The alkaline sucrose gradient profiles show that the cDNAs synthesized here do not contain any significant amount of small sequence (less than 100 nucleotides) and that they consist predominantly of almost full length copies of the mRNA templates. Full length copies of these mRNA templates should be 600 nucleotides long (for 9S mRNA) and 1300 nucleotides (for 15S cDNA) (275, 278). Unfractionated cDNAs were used for hybridization, since the shorter transcripts do not affect these analyses (278).

(ii) CDNA Hybridization.

Figure 22 shows a Cot curve analysis of both cDNA probes when hybridized to each mRNA template. The data have been fitted to a first order curve as described in methods and the $Cot\frac{1}{2}$ for each curve was calculated. The $Cot\frac{1}{2}$ values obtained were $2.5 \times 10^{-3} \text{ Mol. s. l}^{-1}$ for the 9S cDNA hybridized to its template and $4.5 \times 10^{-3} \text{ Mol. s. l}^{-1}$ for hybridization of 5S cDNA to the 15S mRNA template. These values are in close agreement with the values of $Cot\frac{1}{2}$ previously reported for the hybridization of 9S and 15S cDNA probes to their purified mammary mRNA templates (275) (273). The degree of cross contamination of the 9S and 15S mRNA preparations is indicated by the heterologous hybridizations shown in Figure 22, but more accurate characterization of these samples is provided in Figure 23.

The hybridizations shown in Figure 23 use only the linear portion of the curves (% hybrid vs. ng RNA) in their analysis. By this method the relative amount of mRNA sequence in an RNA sample is determined from the slope of the regression line (see methods). These data show that the 9S mRNA contains about 10% 15S mRNA (Figure 22b) and that the 15S mRNA contains about 1.5% 9S mRNA (Figure 22a). These values of the cross-contamination of these two mRNA species are in good agreement with the findings of the Cot curve analysis and they indicate that these preparations are of comparable purity to those used in other studies (275) (278). For further details of this analysis see Appendix.

Figure 22. Hybridization kinetics of 9S and 15S cDNAs

RNA excess hybridizations were carried out under the conditions described and the data were fitted to a first order curve (see methods). The arrows mark the $Cot_{1/2}$ values obtained by curve fitting. The root mean square of each curve fit is between 2% and 3%. The figure shows both the hybridization of the 9S cDNA to 9S mRNA template ((●) $Cot_{1/2} = 2.5 \cdot 10^{-3}$) and with 15S mRNA ((○) $Cot_{1/2} = 7.9 \cdot 10^{-4}$) and the hybridization of 15S cDNA with the 15S mRNA template ((■) $Cot_{1/2} = 4.5 \cdot 10^{-3}$) and with 9S mRNA ((□) $Cot_{1/2} = 2.6 \cdot 10^{-4}$).

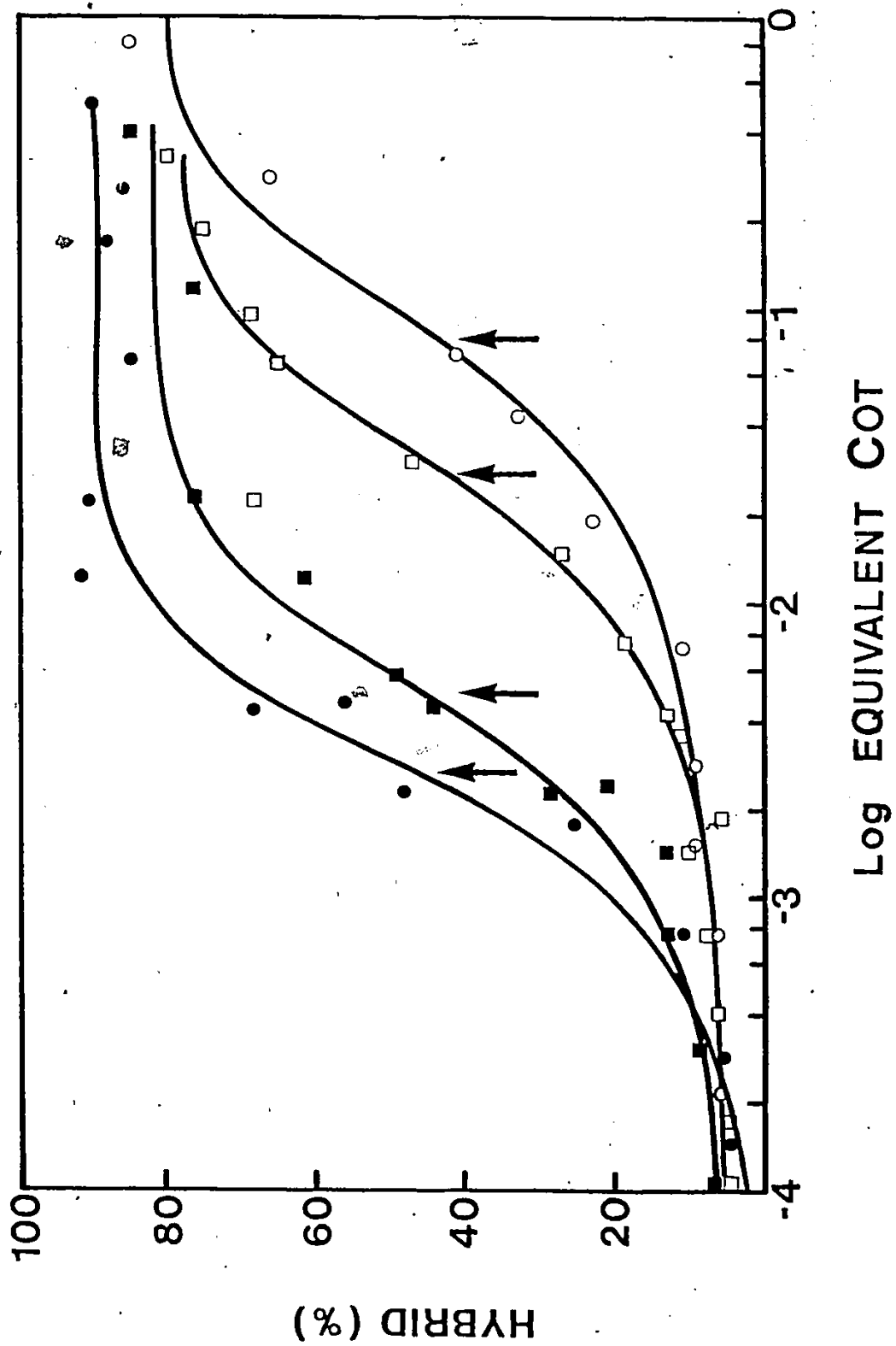
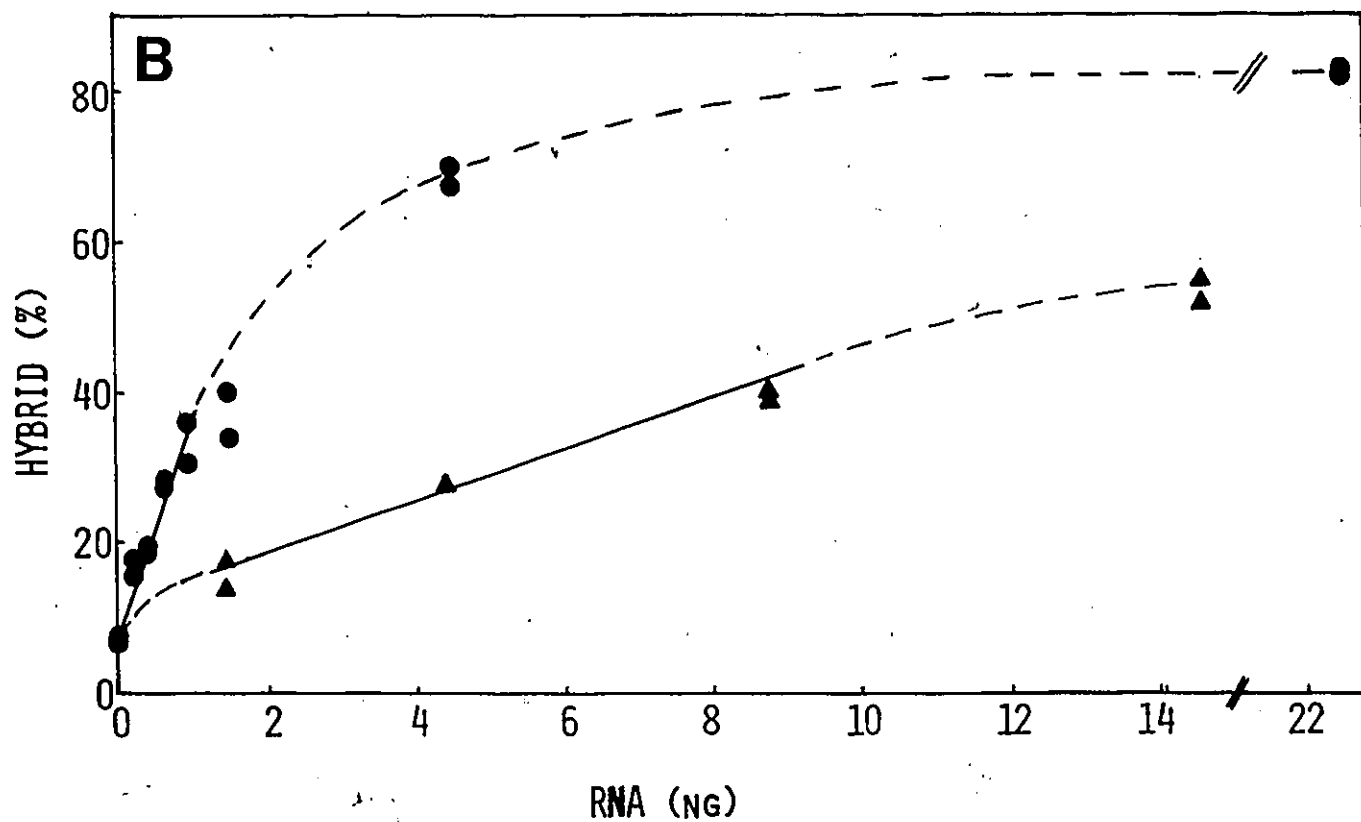
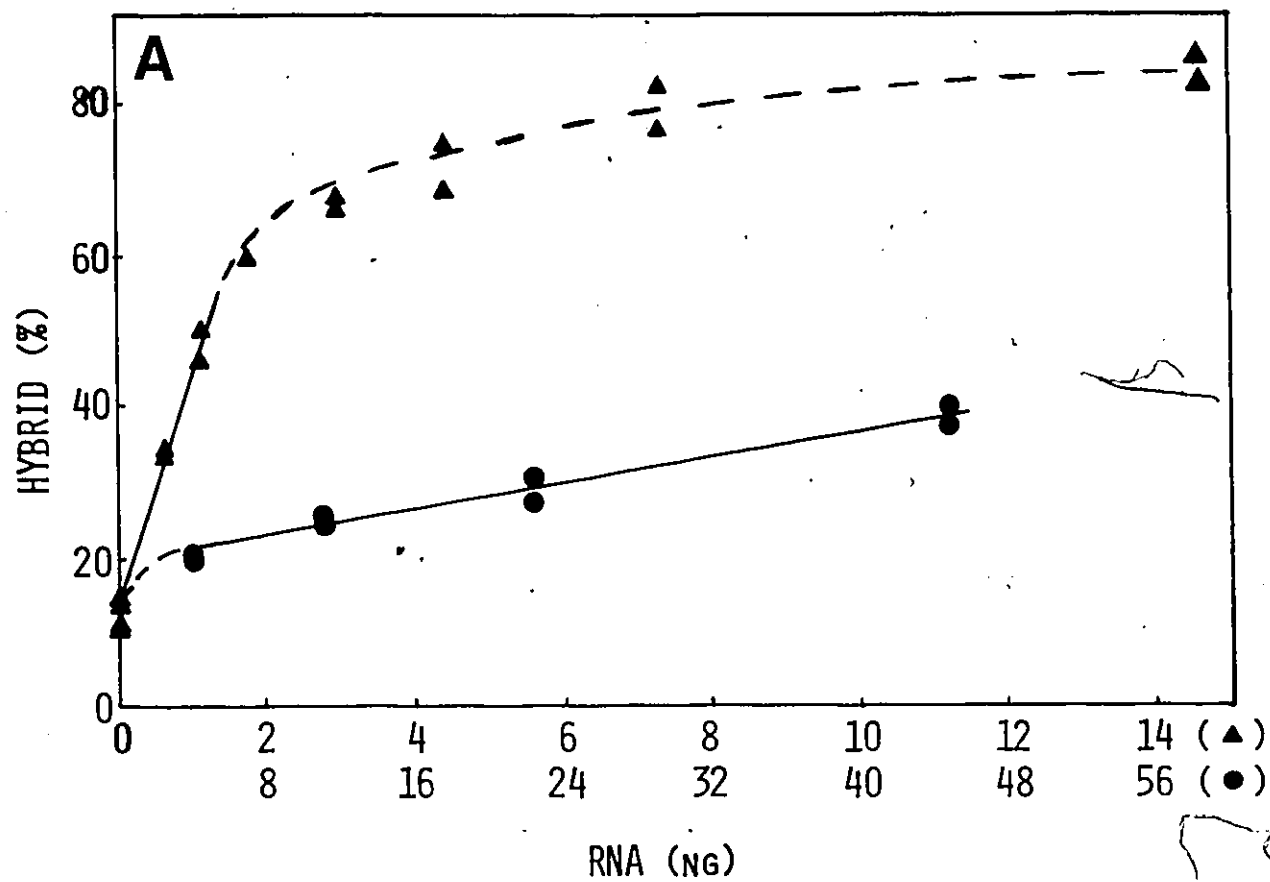


Figure 23. Characterization of 9S and 15S cDNAs by hybridization

RNA / cDNA hybridization was performed by the second procedure described in Methods.

(A) The 9S cDNA (425 cpm) was hybridized with the original 9S mRNA template (▲) or with 15S mRNA (●). The slopes of the linear portion of these curves indicates that there is 1.5% 9S mRNA in 15S mRNA; extrapolation of the 15S mRNA curve to the ordinate indicates that there is 7.5% 15S cDNA in the 9S cDNA. (B) The 15S cDNA (800 cpm) was hybridized with the 15S mRNA template (●) or with the 9S mRNA (▲). The slopes of these curves indicate that there is 10.4% 15S mRNA in 9S mRNA; extrapolation of the linear part of the 9S mRNA curve to the ordinate suggests that there is 7.2% 9S cDNA in the 15S cDNA.





As described previously (317), the hybridization analysis of the type presented in Figure 23 may be used to estimate the purity of the cDNAs. In Figure 23a, the point of intersection of the linear portion of the 15S mRNA curve (solid line) with the ordinate indicates that there is about 7.5% 15S cDNA in the 9S cDNA; similarly from Figure 23b, it is estimated that the 15S cDNA probe contains 7.2% 9S cDNA. Although it is usually assumed that the purity of a cDNA is identical to the purity of the mRNA from which it was made, the results shown here suggest that when small differences are being measured, the purity of a cDNA may not necessarily correspond to the purity of the mRNA. For further details of this analysis see Appendix.

The hybridizations shown in Figures 22 and 23 indicate that the purified 9S and 15S mRNAs and their corresponding cDNAs do not exhibit very significant levels of cross-reactivity; thus these cDNA probes may be used to determine the distribution of specific mRNA sequences between poly(A)⁺ and poly(A)⁻ mammary RNA preparations.

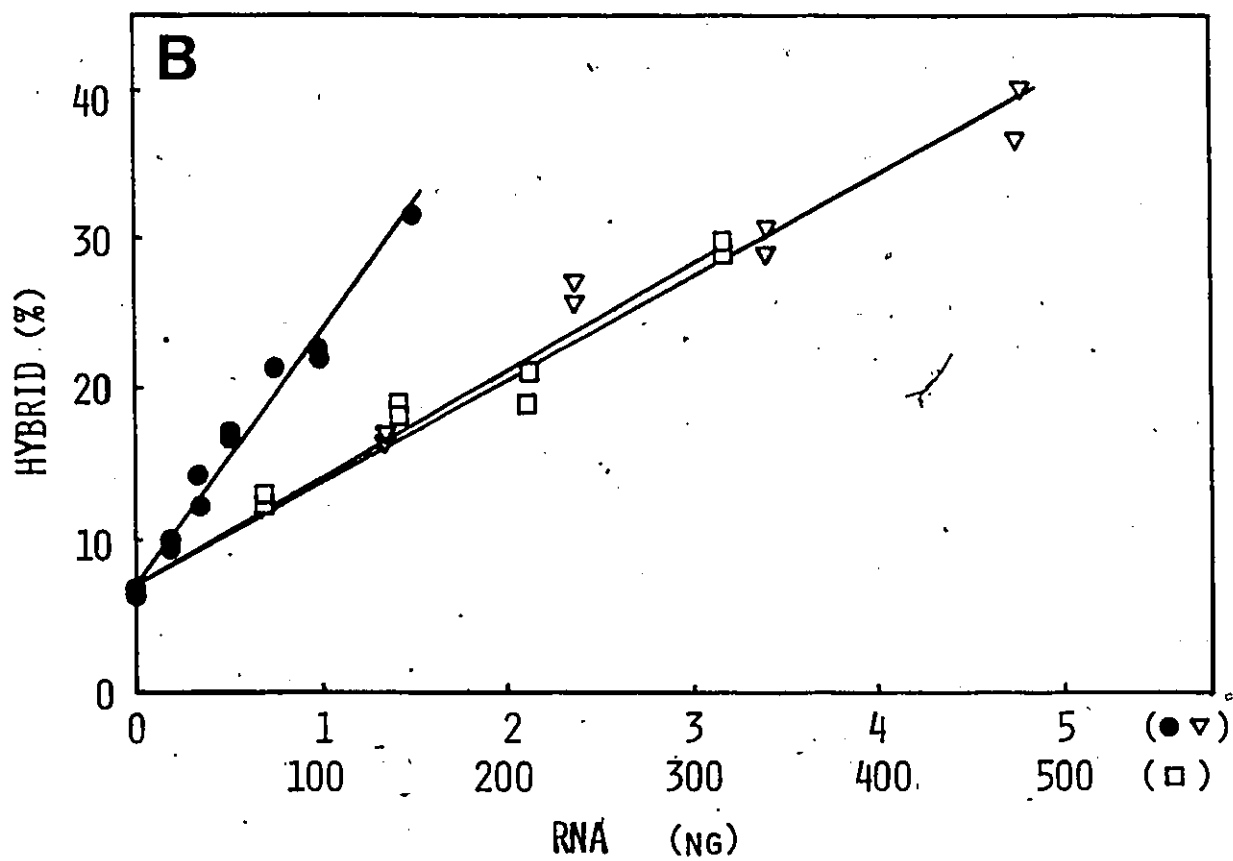
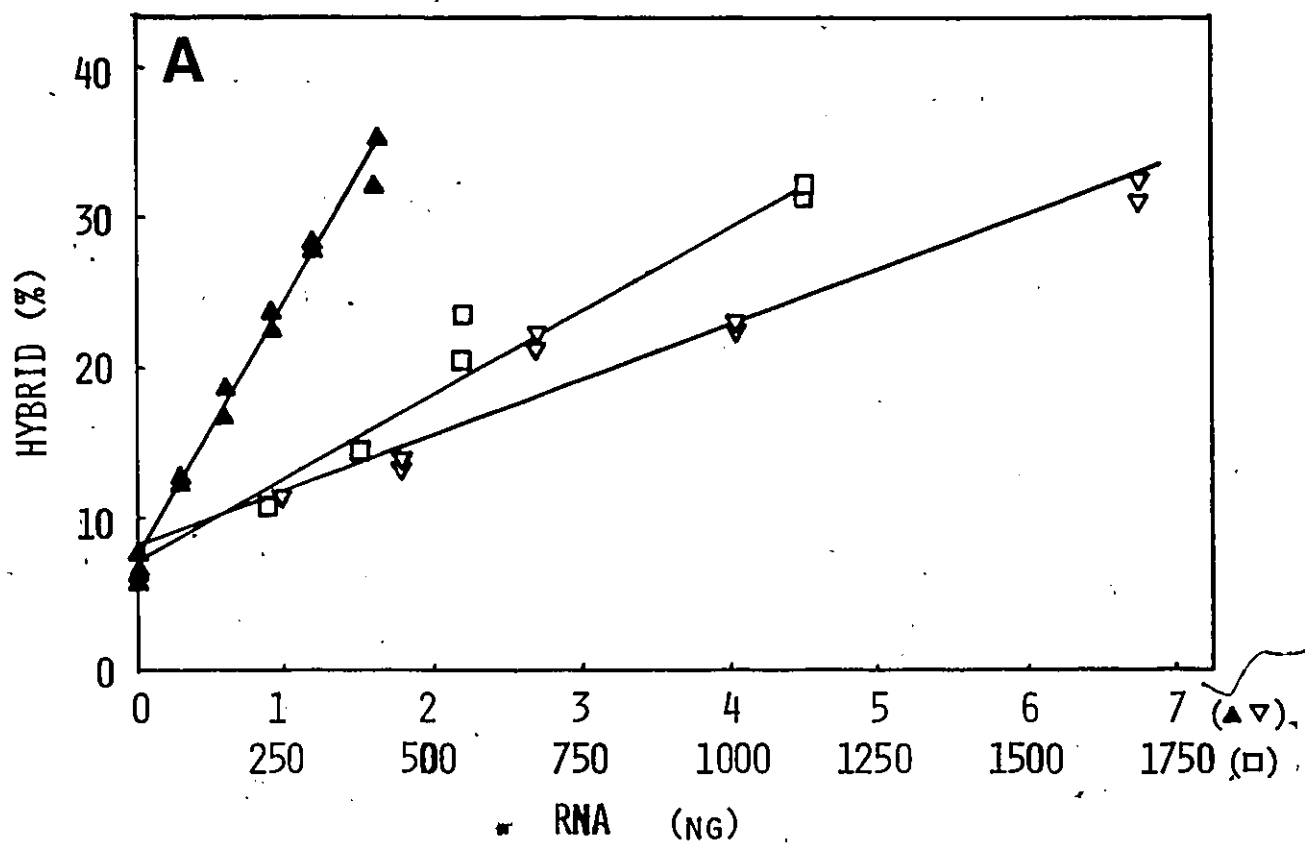
Examples of the use of these probes in these analyses are presented in figure 24. Aliquots of pooled poly(A)⁺ RNAs and the final poly(A)⁻ RNA from fractionation 3 were hybridized with (a) 9S cDNA and (b) 15S cDNA. Standard mRNA hybridizations are included in the figures for comparison. For each sample, the concentration of the specific mRNA

Figure 24. Hybridization of 9S and 15S cDNAs to Mammary poly(A)⁺ and poly(A)⁻ RNA fractions.

Hybridizations were performed as described in methods using the analysis of the type shown in Figure 23.

(A) The 9S cDNA was hybridized with 9S mRNA template (▲) and with poly(A)⁺ RNA (▼) and poly(A)⁻ RNA (□) from 11-15 day lactating mammary gland (fractionation 3, see Table 8). The amount of 9S mRNA in each sample was determined from the regression line for the 9S mRNA standard (%hybrid = 17.45%/ng 9S mRNA + 7.2%).

(B) The 15S cDNA probe was hybridized with standard 15S mRNA (●) and with the poly(A)⁺ RNA (▼) and the poly(A)⁻ RNA (□) of fractionation 3. The 15S mRNA standard gave a regression line (%hybrid = 16.9%/ng 15SmRNA + 7.3%), which in comparison with the curves for the sample RNAs was used to calculate the amount of mRNA in these samples.



sequence being studied is determined by comparison of the slope of the standard mRNA hybridization with that for the sample hybridization. Thus, in figure 23a the poly(A)⁺ RNA fraction contains 238ng 9S mRNA/μg RNA sample and the poly(A)⁻ RNA fraction contains 1.24ng 9S mRNA/μg RNA due to reductions in the slope of the hybridization curve of 4.4 fold and 800 fold respectively for the sample RNAs. Similarly the 15S mRNA content of these fractions is 429ng/μg poly(A)⁺ RNA and 4.61ng/μg poly(A)⁻ RNA.

Several poly(A)⁺ and poly(A)⁻ RNA fractions prepared as described in Chapters 1 and 2 were analysed by cDNA hybridization in this manner. Using the values obtained for the concentrations of these mRNA sequences in these mammary RNA fractions and adjusting them for the amounts of RNA present in each sample, a measure of the absolute amount of mRNA sequence present in each RNA fraction was obtained. In Figure 25 the amounts of mRNA sequence present in the poly(A)⁺ and poly(A)⁻ RNA fractions of six different fractionations are expressed as μg of mRNA sequence per mg of initial unfractionated RNA. This unit of measure was chosen so as to allow direct comparison between poly(A)⁺ and poly(A)⁻ samples of the same and different fractionations; furthermore, it allows easy calculation of absolute amounts of the mRNA sequence present in total unfractionated RNA, a measure that would not be available if the results were expressed in the form of a ratio.

Figure 25. Distribution of 9S and 15S mRNA between Poly(A)⁺ and Poly(A)⁻ RNA fractions of 11-15 day lactating Mammary gland.

Several of the poly(A)⁺ and poly(A)⁻ RNA fractions of lactating mammary RNA prepared as described in Chapters 1 and 2 (see Tables 8 and 12) were hybridized to the 9S and 15S cDNAs. From this hybridization data (of the type shown in Figure 23) the 9S and 15S mRNA levels were calculated for each RNA fraction. The results are expressed as the amount of poly(A)⁺ sequence (open bar) and the amount of poly(A)⁻ sequence (shaded bar) per mg of unfractionated RNA. The error bars represent the standard deviation for the 4 - 8 determinations carried out for each sample. The percentage of the total sequence present in the poly(A)⁻ RNA is given above the bar showing the poly(A)⁻ mRNA content.

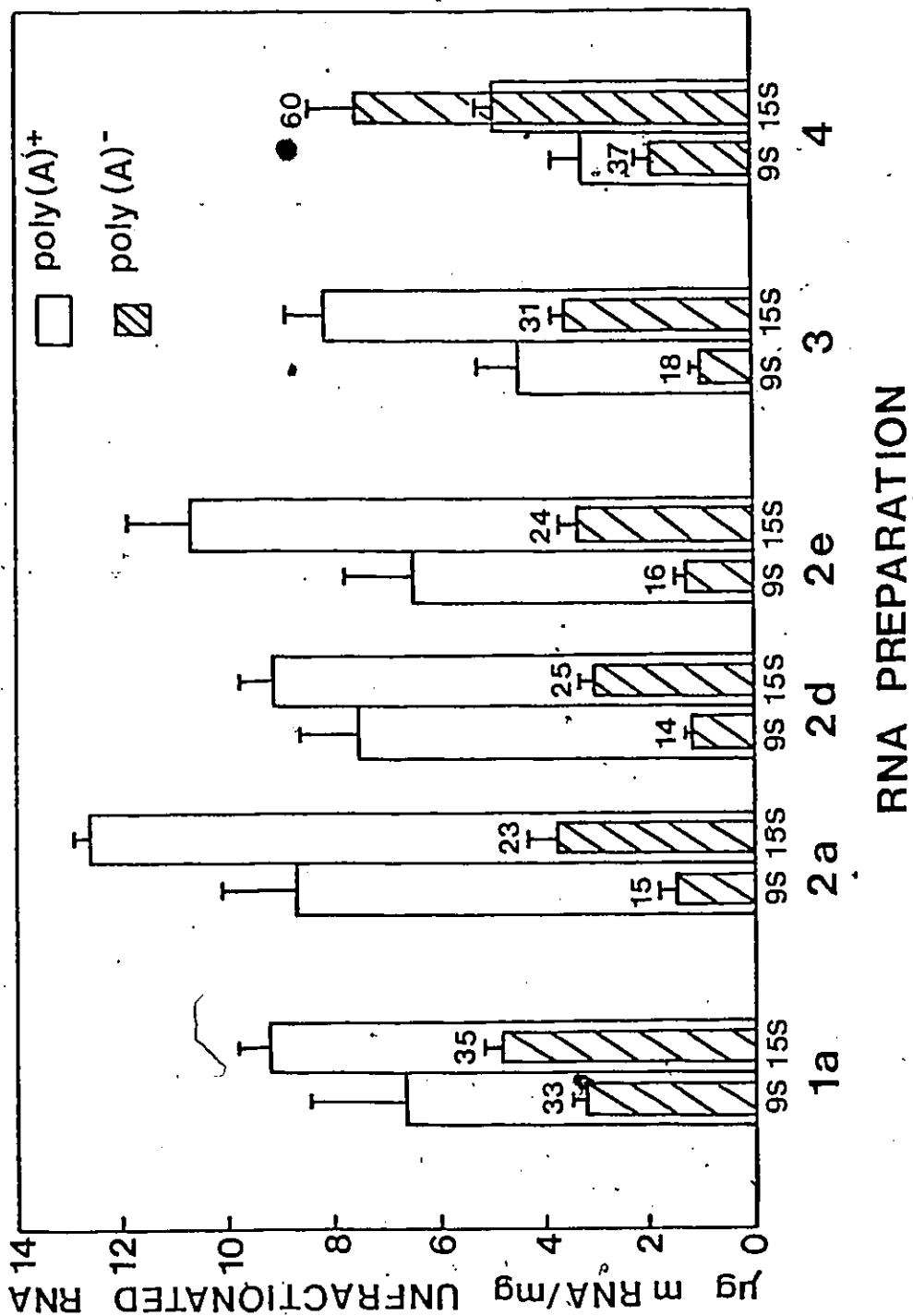


Figure 25 shows that the 9S and 15S milk protein mRNA sequences are not distributed equally between the poly(A)⁺ and poly(A)⁻ RNAs and the poly(A)⁺ forms of these mRNAs predominate. If the results of five of these fractionations are averaged (1a, 2a, 2d, 2e and 3) only $19 \pm 8\%$ of the total 9S and $28 \pm 5\%$ of the 15S mRNAs are found in the poly(A)⁻ fraction. Thus on average, about 75% of each sequence is present as polyadenylated mRNA at this stage in lactation (11-15 days). This observed distribution of specific mRNAs was not dependent on the fractionation procedure employed as indicated by a comparison of the three different fractionations (2a, 2d and 2e) performed on aliquots of one total RNA extract. (Fractionations 2a and 2d (Chapter 1, Table 8) had been performed by four rounds of oligo(dT)-cellulose chromatography using a binding buffer salt concentration of 0.4M and 0.15M respectively and fractionation 2e (Chapter 2, Table 12) was performed by two rounds of poly(U) sepharose chromatography. The similarity in the mRNA contents of these three sets of fractionations, particularly when expressed as a percentage of the total sequence in each RNA fraction, shows that these results are not dependent on the method of sample preparation. Fractionation 4 gave results which were quite different from those of the other samples analyzed (Figure 25); in this case 40% of the α LA mRNA and 60% of the 15S

casein mRNA were present in the poly(A)⁻ RNA sample. This sample had also exhibited a particularly high level of poly(A)⁻ mRNA activity on translation in the wheat germ system (Chapter 3, Table 13). An explanation for this anomolous behaviour of sample 4 became apparent when the unfractionated RNA of sample 4 was compared with other total RNA preparations by UV scanning of the RNAs after electrophoresis on 2.2% polyacrylamide SDS gels. Unlike the other samples, Sample 4 contained significantly degraded rRNA peaks and its UV absorbance profile was similar to that of other degraded mammary RNAs. (see Chapter 1, Figures 1 and 2). It appeared that during its preparation sample 4 had been exposed to a limited nuclease treatment. This exposure had not affected the sample's total mRNA activity to any extent, but it had significantly reduced the polyadenylation of these mRNAs.

The data of Figure 25 also yields information on the total amount of mRNA sequence present in unfractionated mammary RNA. This is readily calculated from the amount of the sequence in corresponding poly(A)⁺ and poly(A)⁻ RNA preparations. Thus in these RNA extracts 9S mRNA constituted 0.55-1.0% of total mammary RNA and the 15S mRNA constituted 1.2-1.6%. These values are in good agreement with previous measurements of the amounts of these sequences in total rat mammary RNA (275).

DISCUSSION

It has been shown that the cDNAs prepared from purified 9S and 15S mRNAs do not exhibit a significant degree of cross reactivity, and indeed the small levels of cross-hybridization observed in the heterologous reactions are likely due predominantly to the presence of some 12S mRNA in both purified mRNAs. These DNA preparations may therefore be used to measure amounts of specific mRNA sequences present in total and fractionated RNA samples. Preparations of 12S mRNA, which had been prepared by sucrose gradient centrifugation as described in chapter 5, were also used to prepare 12S cDNA. Unfortunately hybridization analysis of this probe indicated that it contained significant amounts of other sequences (9S and 15S) and it could not be used in the hybridization analysis presented here. Other researchers have also had difficulty trying to obtain reasonably pure mammary 12S mRNA (278). An effective separation of three mRNA species which differ in size by relatively small levels, as is the case with rat mammary mRNA, is not readily accomplished by methods such as sucrose gradient centrifugation and even the preparative gel electrophoresis used by Rosen was not successful (24). Thus, in this work only the smallest and largest mRNAs were used, but this was sufficient to give a cDNA probe for each type of milk protein (whey protein cDNA and casein cDNA).

The hybridization analysis of several mammary poly(A)⁺ and poly(A)⁻ mRNAs has indicated that on average 20-30% of the two milk protein mRNAs is nonpolyadenylated and the remainder exists in a poly(A)⁺ form. These values are quite different from the proportions of translational activity detected in the poly(A)⁺ and poly(A)⁻ fractions (see Chapter 3, Table 13). Since the milk protein mRNAs constitute a high proportion of total mRNA content of lactating rat mammary gland, this difference in distribution of specific sequences and overall translational activity between the poly(A)⁺ and poly(A)⁻ RNAs, was surprising. However, as was indicated in Chapter 3, quantitative comparison of the translational activities of poly(A)⁺ and poly(A)⁻ mRNAs by the in vitro system described was likely to be inaccurate judging from the mixing experiments data of Table 14.

The degree of nonpolyadenylation of rat 9S and 15S mRNAs is therefore smaller than first reported (42). It is unclear whether the small differences in mRNA sequence distribution between poly(A)⁺ and poly(A)⁻ RNAs observed for 9S and 15S mRNAs are real or whether they are entirely due to experimental variation. With the small number of fractionations analyzed, the observed difference is statistically significant when analysed by the Student's

T test ($t' = 4.52$, D.F. = 4) ($0.02 < p < 0.01$).

The results obtained with fractionation 4 demonstrate the importance of using intact RNA samples for analysis of mRNA polyadenylation. Analysis of sample 4 indicated that whereas the mRNA translational activity of these fractions was normal, the integrity of the rRNAs and mRNA polyadenylation was not, and it seems that the poly(A) tract of poly(A)⁺ mRNA is especially susceptible to nuclease in these preparations. In this respect it is of interest to note that the distribution of the smaller mRNA sequences is not as unusual as that of the larger sequence; this result is consistent with the observation of sample degradation which would be expected to effect larger molecules to a greater extent. Bergman and Brawerman have described a treatment of polysomes with nuclease that causes little fragmentation of mRNA but considerable loss of poly(A) (318); they suggest that accidental exposure to nuclease activity could interfere with measurements of mRNA polyadenylation. Since sample 4 also exhibited a loss in rRNA structure, only preparations which contained intact rRNAs were analysed by cDNA hybridization, so as to minimise the possibility of measuring artefactually increased levels of poly(A)⁻ mRNA.

CHAPTER 7

POLYADENYLATION OF RAT MAMMARY RNA DURING DEVELOPMENT

Chapters 1-6 have described the development of techniques necessary to perform the goals of this research.

Two major questions may now be asked:

- (1) Is there any variation in Poly(A) tract length during mammary gland development?
- (2) Are there changes in the distribution of specific milk protein mRNA sequences between the poly(A)⁺ and poly(A)⁻ RNA populations during gland development?

As discussed in "Aims of This Research" the rationale behind this research is to investigate the possibility that variation in mRNA polyadenylation might be a mechanism for post-transcriptional control of gene expression. This chapter will be concerned with examining the size of poly(A) during gland development.

Four stages in mammary gland development were chosen for the purpose of this study: 8 days and 15 days of gestation, 7-8 days of lactation and 2 days of involution. At 8 days of gestation, milk protein mRNA levels have begun to rise but they are still very low compared to later levels, and this stage in gestation represents a very early point in gland stimulation (279). By 16 days of gestation the gland has differentiated very significantly

and milk protein mRNA levels are increasing rapidly in readiness for parturition (279). The 7-8 day lactating gland represents very active mammary tissue. There has been disagreement as to the precise point of maximal mRNA levels in the rat gland; while Nakhasi and Qasba reported a rapid decline in milk protein mRNA sequences (expressed as a percentage of total RNA) after 12 days of lactation (279), Rosen has found that milk protein mRNA sequences (expressed as $\mu\text{g mRNA/gm tissue}$) increase continually up to 18 days of lactation (287). It was therefore of interest to compare the 7-8 day lactating stage to the 11-15 day lactating stage (analysed in chapters 1-6). Finally, the shutdown of milk production and loss of milk protein mRNA sequences is represented by the 2 day involuting gland. It is known that after weaning the return of the milk protein mRNA levels to the non-pregnant amount is very rapid and almost complete by 6-7 days (42, 276). In order to ensure that the involuting glands studied still contained significant milk protein mRNAs but were clearly in involution, the glands were taken two days after weaning. These four stages in pregnancy, lactation and involution represent the major stages in mammary gland physiology and should permit the observation of any changes in mRNA polyadenylation in relation to mRNA function which might occur during gland development.

MATERIALS

Female Sprague Dawley rats were obtained from the usual source (see Materials, Chapter 1). Day one of pregnancy was defined as the day after mating and the stage of pregnancy was confirmed on the day of sacrifice by measurement of the average length of the foetus after removal from the uterus (319). The day of parturition was the first day of lactation. Pups were removed from the mother at 18-20 days of lactation and the involuted gland was taken 48 hours later.

Gland removal and storage was as described previously (See Methods, Chapter 1). Yields of mammary tissue ranged from 3-5g (wet weight) per animal at the 8 day pregnant stage, 6-9g at 16 days of gestation, 14-18g at 7-8 days of lactation and 20-25g at involution.

METHODS

All methods used were as described previously. For details of the RNA extraction, DNA determinations, oligo(dT) cellulose chromatography, and the [^3H]-poly(U) assay, see Methods, chapter 1. Poly(A) sizing was performed as detailed in Methods, Chapter 4.

RESULTS

- (i) Preparation of RNA samples from rat mammary gland at various stages of gland development

During gestation and early lactation the nucleic acid content of the rat mammary gland increases significantly (279), and this is reflected in the RNA yields obtained in these studies.

A summary of the results of RNA extraction using the mammary glands of rats at four stages of development is presented in Table 18. Very significant differences in nucleic acid yield are observed when the extractions of gland from different development stages are compared. The yield is only 0.09-0.18% of tissue wet weight in early gestation (8 days), but this rises 2-3 fold by late gestation (16 days). At 7-8 days of lactation, the recovery is increased 6-9 fold above early pregnant levels and at this highly active stage in the gland's development RNA constitutes up to 1% of wet tissue weight. Although the involuted glands studied had the highest wet tissue weight of all stages investigated (possibly due to a high milk content), just 48 hours after induction of involution it appears that nucleic acid content has dropped back to late pregnant levels.

Analysis of these RNA extracts by the Burton method (see Chapter 1, methods) indicated that these preparations contained significant amounts of DNA, particularly the extracts from early pregnant gland where DNA constituted over two thirds of the nucleic acid content of these preparations (see Table 18). The lactating gland extracts

**TABIE 18: THE RESULTS OF RNA EXTRACTION OF RAT MAMMARY GLANDS
AT FOUR STAGES IN DEVELOPMENT**

Stage of gland	Sample #	Nucleic acid yield (% wet tissue weight)		DNA levels (% total nucleic acid)		Poly. A levels (% total nucleic acid)	
8 day pregnant	1	0.094		65		0.062	
	2	0.12	0.134 ±	96	78.5 ±	0.077	0.071 ±
	3	0.18	0.036	69	14.2	0.078	0.008
	4	0.14		84		0.066	
16 day pregnant	1	0.32		57		0.091	
	2	0.27	0.295 ±	56	56.3 ±	0.093	0.095 ±
	3	0.29	0.021	59	2.5	0.098	0.004
	4	0.30		53		0.098	
7-8 day lactating	1	0.93		27		0.119	
	2	0.69	0.8 ±	30	29.3 ±	0.137	0.132 ±
	3	0.78	0.121	31	2.0	0.130	0.011
involuting	1	0.25		44		0.080	
	2	0.30	0.29 ±	44	44 ±	0.093	0.087 ±
	3	0.30	0.027	44	0	0.090	0.006
	4	0.31		44		0.084	

Nucleic acid extraction of mammary glands at the indicated developmental stages was performed as described (methods, Chapter 1). Total nucleic acid content was measured by UV absorbance (260 nm) and DNA content was measured by the Burton method (see Chapter 1). It is assumed that the amount of RNA in these extracts is the difference between the amounts of total nucleic acid and DNA. The amount of poly(A) present in these preparations was determined using the [³H]-poly(U) assay. Values after each group are the mean ± S.D.

exhibited the lowest DNA content, and preparations from 16 day pregnant and involuted glands contained intermediate amounts of DNA. This variation in DNA content of these extracts is probably a consequence of changes in the relative amounts of DNA and RNA present in the gland at various stages in development (see discussion). In spite of the DNA content of these preparations no steps were taken to remove the DNA, since this would involve significantly more sample handling. They will be referred to as RNA extracts, as has been done previously by Rosen (42). The presence of significant amounts of DNA in these samples must be taken into account especially in the hybridization analysis (see chapter 8).

Table 18 also indicates the levels of poly(A) in these samples as a percentage of total nucleic acid. These data suggest that there is a two-fold increase in levels of poly(A) of mammary gland RNA extracts between the 8 day pregnant gland and the 7-8 day lactating gland. However, when the DNA content of the extracts is taken into account and poly(A) content is expressed as a percentage of total RNA, then very similar values (0.15-0.25%) are obtained for all extracts. It is likely that there are no significant differences between the different stages in terms of poly(A) levels per mg RNA.

Aliquots of the total RNA extracts described in Table 18 were fractionated into poly(A)⁺ and poly(A)⁻ RNA fractions by oligo(dT)-cellulose chromatography and these fractionations are summarised in Table 19. To ensure that only two columns were needed to effect complete fractionation (see Chapter 1, Table 8), the amount of RNA applied to the columns in each experiment was kept below 30 mg. In each fractionation described in Table 19 the first column retained at least 70% of the poly(A) in the total sample (measured by the [³H]-poly(U) assay) and much reduced amounts of poly(A) were retained by the second column. In the case of the early and late pregnant RNA extracts, much lower amounts of RNA were applied (8-15mg) since only limited amounts of material were available. The amounts of material retained by the second columns during fractionation of 8 day pregnant rat gland extracts were so small that they were not retained for analysis. In general, poly(A) recovery in column retained fractions is around 90% of applied poly(A) and these recoveries are comparable to those observed in earlier studies (see Tables 8 & 12). Recoveries from the 8 day pregnant gland extracts were not quite so high with a range of 73-90%; a possible reason for this observation is that since smaller amounts of material were used at this stage, recovery of RNA during ethanol precipitation was not as efficient as usual.

TABLE 19: FRACTIONATION OF RNA EXTRACTS FROM MAMMARY GLANDS
AT FOUR STAGES IN DEVELOPMENT BY OLIGO(dT)-CELLULOSE CHROM-
ATOGRAPHY)

Stage	Sample	Total poly(A) retained on two oligo(dT)- cellulose columns (% of poly(A) in initial unfractionated RNA)	Final Poly(A) RNA fraction: poly(A) remaining (% of initial)	ng poly(A)/ mg RNA
8 day pregnant	1	74	0.92	6.6
	2	73	1.26	9.6
	3	90	1.25	11.3
	4	73	0.89	7.1
		77.5 ± 8.4	1.08 ± 0.20	
16-day pregnant	1	92.4	2.14	20
	2	90.9	2.02	21
	3	92.5	1.18	13
	4	89.8	0.56	5.8
		91.4 ± 1.3	1.48 ± 0.74	
7-8 day lactating	1	86.1	1.22	16
	2	98.2	2.03	29
	3	89.3	1.43	20
		91.2 ± 6.3	1.56 ± 0.42	
involut- ing	1	103.8	0.90	8.4
	2	91.3	1.20	12.3
	3	93.6	1.13	11.0
	4	100.0	0.92	9.0
		97.2 ± 5.8	1.04 ± 0.15	

The RNA extracts prepared as described in Table 17 were fractionated by oligo(dT)-cellulose chromatography (see Methods, Chapter 1). The amounts of nucleic acid applied to the columns in these fractionations are as follows: 8 day pregnant stage 8.7-13.4 mg; 16 day pregnant stage, 15 mg; 7-8 day lactating stage, about 25 mg; involuting stage, about 24 mg. These fractionations were performed using binding buffer containing 0.4M NaCl. Column bound and unbound samples were collected and assayed for poly(A) using the [³H]-poly(U) assay. The value shown after each grouping bracket is the mean $\pm$ S.D.

The unretained RNA preparations consistently contained 2% or less of the initial poly(A) content, and this corresponds to 21 ng poly(A) per mg RNA or less. The depletion of poly(A) from these preparations is therefore comparable with earlier observations using 11-15 day lactating rat gland RNA extracts (see Tables 8 and 12). It has been shown in earlier poly(A) sizing studies, that the poly(A)⁻ RNA preparations produced by oligo(dT)-cellulose fractionation in this work contain no poly(A) tracts and few oligo(A) sequences of any significant size. This analysis was not repeated with the poly(A)⁻ RNA samples from mammary glands of different stages.

(ii) Length of poly(A) tracts found in mammary gland RNA of various stages in development

Aliquots of the poly(A)⁺ RNA preparations of each developmental stage were treated by the poly(A) sizing procedure as described in chapter 4. Figure 26 shows four gel profiles of poly(A) tracts, as detected by the [³H]-poly(U) analysis of gel eluates, using mammary RNA from glands at the following stages in development: a) 8 day pregnant, b) 16 day pregnant, c) 7-8 day lactating, d) involuting.

The poly(A) tracts from 8 day pregnant gland RNA

Figure 26. Sizing of the Poly(A) Tracts obtained from Poly(A)⁺ RNAs of Mammary Gland at four stages in development

Poly(A) tracts were isolated by RNase digestion of poly(A)⁺ RNA and sized by electrophoresis on polyacrylamide gels as described previously (see Methods, Chapter 4). After electrophoresis, gels were sliced and gel eluates were assayed for poly(A) using [³H] poly(U).

The samples analysed were as follows:

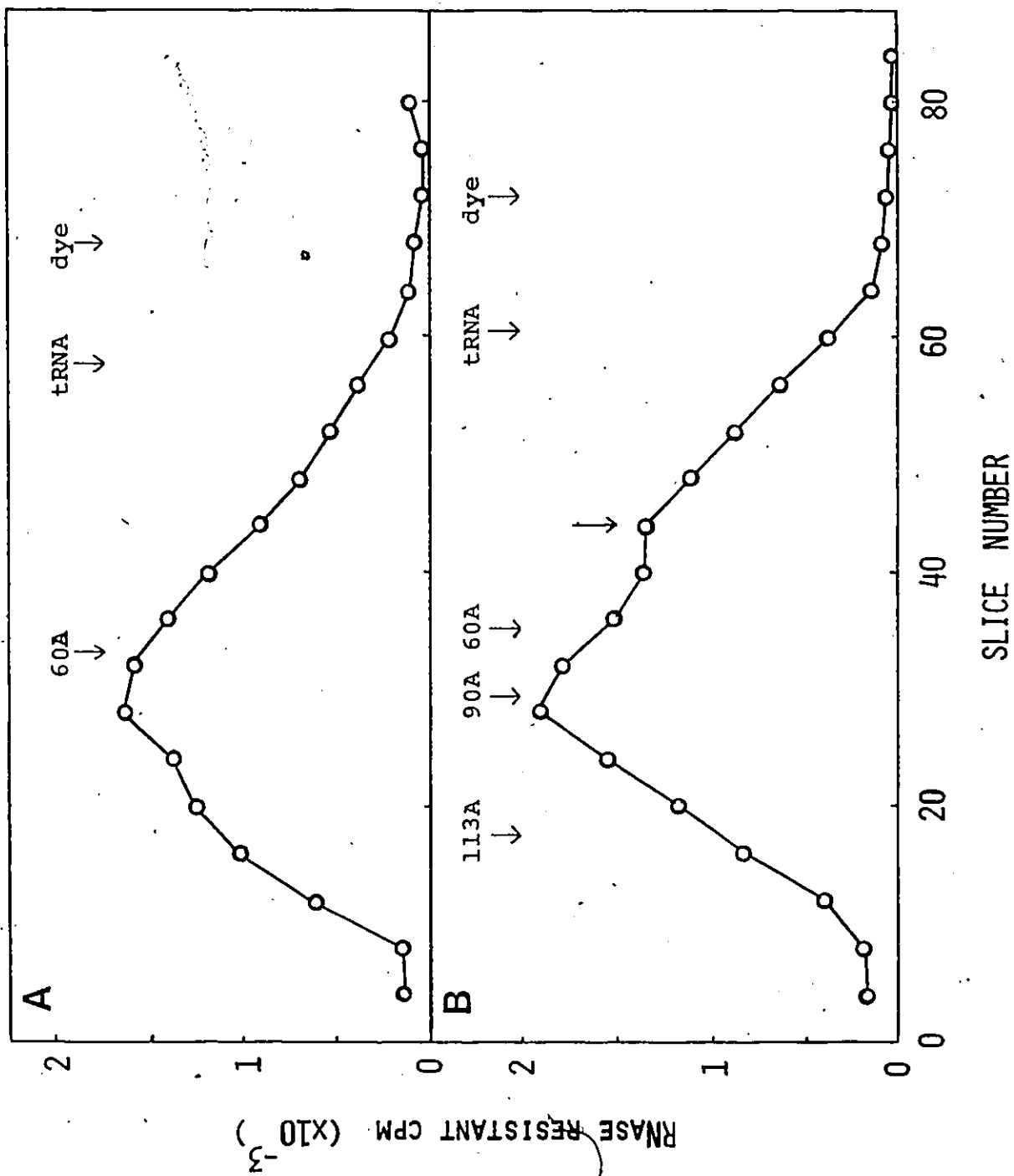
(A) 199ng poly(A) from 3.95µg poly(A)⁺ RNA, sample 2 of 8 day pregnant gland

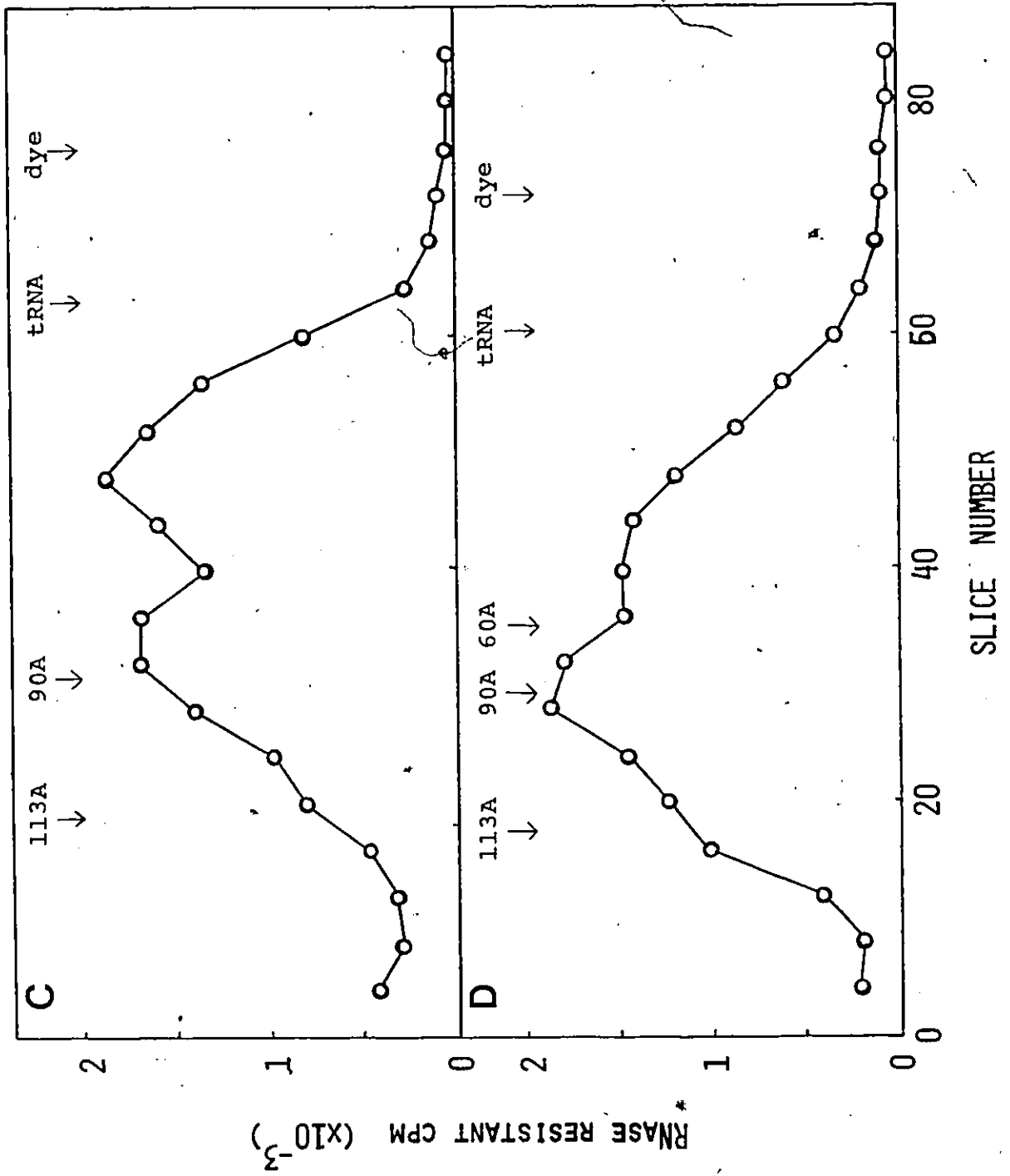
(B) 248ng poly(A) from 8.1µg poly(A)⁺ RNA, sample 4 of 16 day pregnant gland

(C) 317ng poly(A) from 9.3µg poly(A)⁺ RNA, sample 3 of 7-8 day lactating gland

(D) 244ng poly(A) from 8.6µg poly(A)⁺ RNA, sample 2 of involuting gland.

Poly(A) recovery from each gel was 82%, 77%, 69% and 78% respectively. The positions of several poly(A) size markers are indicated.





(Figure 26a) have a size range similar to that of the tracts from RNA of 11-15 day lactating gland; these tracts are no smaller than 25 nucleotides long and are as large as about 160 nucleotides. The modal tract length occurs at about 80 nucleotides and the distribution of these tract sizes is clearly unimodal. The poly(A) size distribution of tracts from 16 day pregnant gland RNA (Figure 26b) is rather similar to that for the tracts of 8 day pregnant gland RNA in terms of range (25-160 nucleotides) and modal length (80 nucleotides). However in this second profile there is a suggestion of the appearance of a second size class of poly(A) tracts peaking at a length of approximately 45 nucleotides (see arrow).

Figure 26c is a poly(A) profile of RNA from 7-8 day lactating gland; this profile is similar to those observed using 11-15 day lactating gland RNA but it is included here to permit easy comparison with the profiles using mammary RNA of glands at the other developmental stages. This profile is bimodal with size class peaks around 80 and 45 nucleotides. Furthermore there appears to be proportionally less of the very large tracts; a rise in the profiles in Figure 26c does not occur until slice number 16 (corresponding to a tract length of 145 residues) while in the other profiles of Figure 26 tracts of up to 160-165 residues are detected. Another interesting aspect of the profile in Figure 26c is that although it

contains an extra peak of poly(A) tracts of smaller size, there is no change in the length of the smallest poly(A) tract observed (still around 25 nucleotides). However there is a sharper drop in the poly(A) tract profile in the 25-35 residue size range in Figure 26c compared to the other profiles in this figure. This would suggest that there might be an important reason for the absence of poly(A) tracts shorter than 25 nucleotides (see discussion). Figure 26d shows that the size profile of poly(A) tracts obtained from the involuting gland exhibits an intermediate form between the pregnant gland and lactating gland poly(A). There is a reduction in the relative amount of the smaller poly(A) tracts and it appears as if the poly(A) tract size is in the process of returning to the early pregnant gland form with just a single size peak at about 80 nucleotides.

Poly(A) tract size was examined using at least three different RNA extracts from mammary gland at each of the four developmental stages studied. The gel profiles shown in Figure 26 were representative of each stage. In order to summarise all the available data on poly(A) tract size, a large:small tract ratio was calculated for each profile. Large poly(A) tracts were defined as those sequences with a mobility relative to blue dye of < 0.55 (larger than about 56 nucleotides) and small tracts were defined as the poly(A) sequences smaller than 56 nucleotides.. Each gel profile

was divided into two portions (greater and smaller than 56 nucleotides) and the total RNase-resistant cpm present in each portion was calculated. These data are presented in Table 20, together with the calculated large:small tract ratio for each poly(A) sizing experiment performed. The final column of Table 20 presents the mean $\pm$ S.D. for each developmental stage; these figures illustrate the large differences in the relative amounts of poly(A) tracts larger and smaller than 56 nucleotides in mammary gland of different stages in development. The standard error values calculated for these data indicate that there are very significant differences in poly(A) size in these developmental groups, particularly between early pregnancy and lactation.

Figure 26 demonstrates that the functional development of the rat mammary gland is associated with the appearance of a distinct, relatively short population of poly(A) sequences, which apparently peaks at a size of about 45 residues. This finding prompted further experimentation to determine whether the poly(A) tracts associated with milk protein mRNAs consisted entirely of the smaller size poly(A) population. To answer this question the two purified abundant mRNAs, 9S whey protein mRNA and the 15S casein mRNA - were analysed by the poly(A) tract sizing procedure.

Figure 27 shows that the profiles of the poly(A) tracts of both mRNAs are similar. The profile again

TABLE 20: VARIATION IN THE RATIO OF LARGE AND SMALL POLY(A)
TRACTS IN RAT MAMMARY GLAND AT SEVERAL STAGES OF DEVELOPMENT

Stage	Sample	RNase resistant cpm present in poly(A) tracts:			mean $\pm$ S.D.
		larger than 56 nucleo- tides	smaller than 56 nucleo- tides	Large:Small tract ratio	
8-day pregnant	1	9663	5183	1.86	2.05 $\pm$ 0.15
	2	9137	4268	2.14	
	3	9391	4269	2.20	
	4	9982	5011	1.99	
16-day pregnant	1	9102	5042	1.81	1.56 $\pm$ 0.25
	3	8917	6733	1.32	
	4	9525	6172	1.54	
7-8 day lactating	1	8370	7842	1.07	1.11 $\pm$ 0.05
	2	8595	7378	1.17	
	3	8208	7456	1.10	
11-15 day lactating	1a	8494	9904	0.86	1.02 $\pm$ 0.13
	2a	11249	9696	1.16	
	2e	9735	9009	1.06	
	3	11885	12959	0.99	
invol- uting	1	9230	5620	1.64	1.52 $\pm$ 0.11
	2	9652	6441	1.50	
	4	8893	6278	1.42	

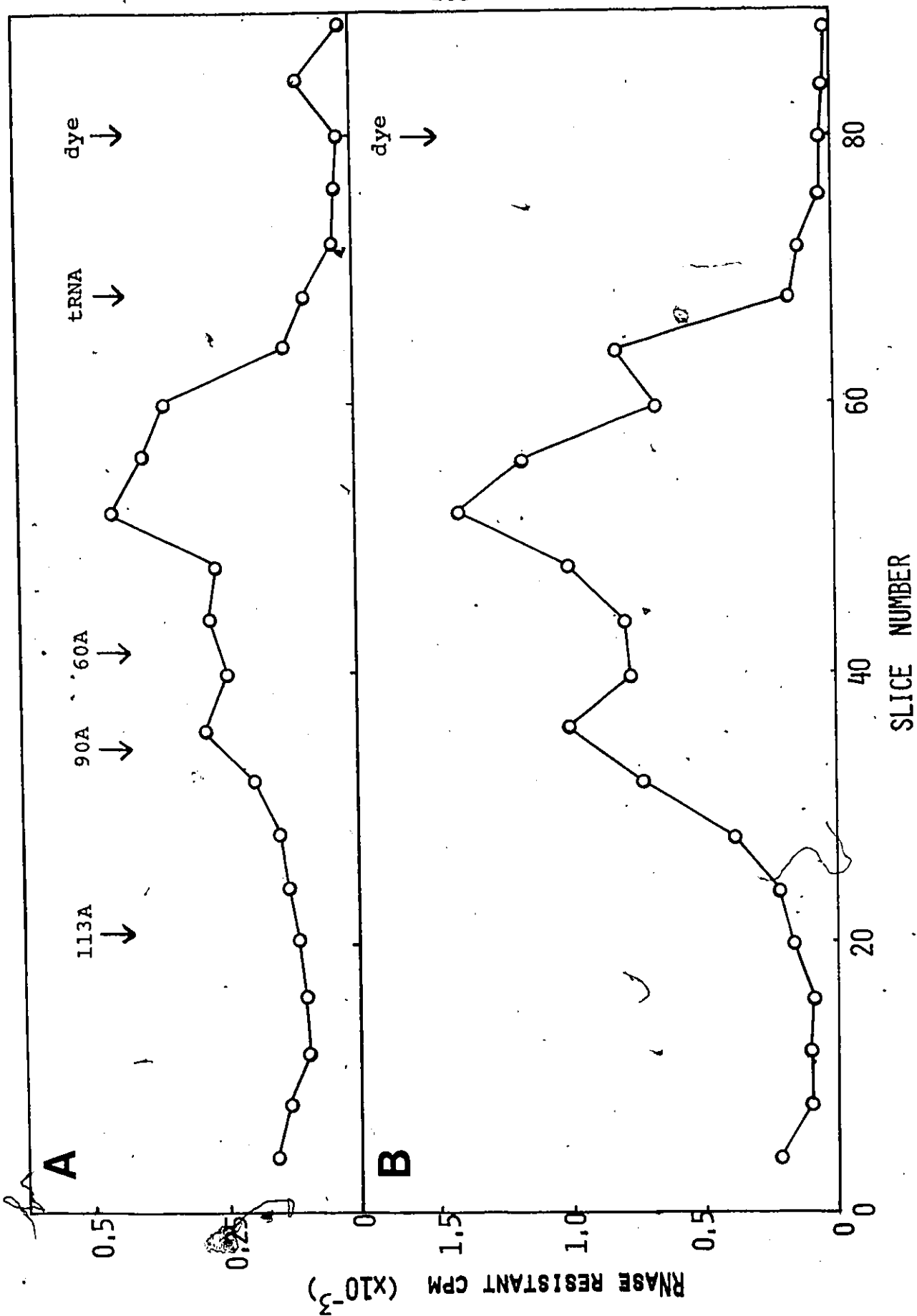
Poly(A)⁺ RNAs of the indicated samples were subjected to poly(A) sizing as described (see Methods, Chapter 4). Each profile thus obtained was divided into two portions: tracts larger than 56 nucleotides (corresponding to a relative mobility of 0.55) and tracts smaller than 56 nucleotides. The sum of the RNase resistant cpm in each portion of the profile is presented, and these values were used to calculate the large:small tract ratio for each sizing experiment.

Figure 27. Sizing of Poly(A) Tracts obtained from
purified milk protein mRNAs

Poly(A) tract sizing was performed as described previously (see Methods, Chapter 4), using the tracts isolated from 9S whey protein mRNA and 15S casein mRNA. These two preparations were the highly purified mRNAs described in Chapter 5. The two profiles shown were obtained with:

- (A) 50ng poly(A) from 2.2 μ g 15S casein mRNA
- (B) 123ng poly(A) from 3.5 μ g 9S whey protein mRNA.

Poly(A) recovery from the gels was at least 89%. The positions of several poly(A) size markers are indicated.



exhibits a bimodal form with peaks at 75-80 and 42 residues. This is in good agreement with the sizes of the two peaks in the poly(A) profile from lactating gland RNA. The major difference between the profiles in Figure 27 and the profile of Figure 26c is that in the former there is a relative enrichment of the shorter poly(A) tract size class. Clearly the milk protein mRNAs contain the same range of poly(A) tract sizes as total mammary poly(A)⁺ mRNA, but the milk protein mRNAs contain proportionately higher amounts of the shorter poly(A) size class. This suggests that the shorter poly(A) size class is associated with the milk protein mRNAs and perhaps other mRNAs directly related to the lactation process, while the other constitutive mRNAs carry only the longer poly(A) size class throughout the gland's entire developmental process. This hypothesis could explain the difference in the profiles of Figure 26c and Figure 27.

In summary, it has been shown that mammary gland development does involve changes in the polyadenylation of its mRNA. During induction of lactation and milk protein synthesis, a distinct poly(A) size class appears in the gland and it is speculated that this size class is restricted to milk protein mRNAs. Loss of this poly(A) size class parallels the loss of milk protein mRNA sequences during involution.

DISCUSSION

An explanation for the variable content of RNA and DNA in nucleic acid extracts of mammary gland of various developmental stages is provided by Nakhasi and Qasba (279). They show that between 8 and 16 days of gestation, the RNA/DNA ratio of rat mammary gland increases 3-fold and during lactation it increases another 4-fold. This variation in the RNA/DNA ratio is reflected in the relative RNA and DNA contents of the nucleic acid extracts of glands at the four stages analysed; this changes 10-12 fold between the 9 day pregnant and 7-8 day lactating stage. The increase in RNA content of the gland throughout pregnancy and lactation would also explain the higher yield of material in the latter stages of development.

As discussed early in this chapter, one goal of this work was to determine whether there is any change in the length of poly(A) tracts associated with mammary RNA during the gland's development. It has been shown that native poly(A)⁺ globin mRNA is more stable in X. laevis oocytes than poly(A)⁻ globin mRNA (190). The poly(A) tracts associated with globin mRNA, and other mRNAs, are gradually reduced in length during mRNA aging (98) and this is likely the process by which poly(A)⁻ mRNA is produced (123). This hypothesis would suggest that one

means of prolonging mRNA half-life is to extend the length of its associated poly(A) tract and therefore prolong the period required to completely remove the poly(A) and convert the mRNA into its poly(A)⁻ form. One might therefore expect to see an overall increase in the steady-state length of mammary RNA poly(A) during maximal stimulation of this tissue (during lactation) if increased polyadenylation of milk protein mRNAs is the mechanism by which their half-lives are increased.

The poly(A) tract profiles shown in Figures 26 and 27 clearly show that this is not the case; the poly(A) sequences associated with the abundant mRNAs (predominantly milk protein mRNAs) during lactation are enriched in smaller-sized tracts compared to those present in the pregnant gland.

The size of the poly(A) tract found associated with 15S casein mRNA in this study is in quite close agreement with the size profile determined by Rosen (24). In Rosen's study, the poly(A) tracts were oxidised by periodate and labelled at their 3' termini using [³H]-borohydride, before removal from the mRNA by RNase digestion. The gel profile of this [³H]-poly(A) indicated a size range of about 15-150 residues, with a peak around 35-45 residues.

In the present study, it has been found that the size distribution of poly(A) tracts associated with a 9s mRNA fraction is similar to that of the casein mRNA; the length

of the poly(A) tract attached to mammary 9S mRNA had not been reported previously. This similarity in size of the two poly(A) populations suggests that the mRNAs for both types of milk proteins are handled by the alveolar cell in a similar manner with respect to polyadenylation.

It will be recalled from the general introduction that studies in X. laevis oocytes indicated that a poly(A)⁺ mRNA was stabilised maximally by poly(A) tracts of over 30 residues in length; when the poly(A) sequence was reduced to 21 residues the resulting mRNA was destabilised and further reduction of the sequence to 16 residues produced a mRNA which was as unstable as poly(A)⁻ mRNA (193). It appears that once poly(A) tract length is reduced below 25-30 residues, its effectiveness in stabilising mRNA is reduced. In this respect it is of interest to note that in all the mammary RNA samples analysed in this work, the smallest poly(A) tract observed was 25-30 residues long; furthermore, Rosen did not find any poly(A) tracts smaller than 20 residues. These observations are explained by the findings with the X. laevis system; the absence of mRNAs bearing short poly(A) tracts is due to their relative instability. This would also suggest that the conclusions obtained with Xenopus are applicable to mammalian systems.

Recent measurements of the size of poly(A) tracts from rat liver and hepatoma tissues show that normal liver RNA contains poly(A) tracts from 25 to 140 nucleotides long with a single peak around 80 nucleotides (320). The normal liver size distribution is very similar to that observed for poly(A) of early pregnant gland RNA while in hepatoma RNA there is an increased proportion of tracts longer than 110 residues. In these rat liver studies, it appears that the faster growing tissue contains longer poly(A) sequences. Mattei et al. speculate that since hepatoma tissue contains a higher proportion of non-translated mRNA sequences compared to normal liver, the increase in steady-state poly(A) tail length in hepatoma might be a consequence of reduced mRNA utilization (320). It had already been proposed that poly(A) shortening might be a function of mRNA utilization and if this were so, this would complicate attempts to correlate mRNA half-life with poly(A) tract length. The final conclusions regarding mammary poly(A) tract length are discussed in Chapter 9.

CHAPTER 8: DISTRIBUTION OF 9S AND 15S mRNA SEQUENCES
BETWEEN POLY(A)⁺ AND POLY(A)⁻ MAMMARY RNA DURING GLAND
DEVELOPMENT

This chapter is concerned with answering the second question posed at the beginning of chapter 7: it investigates the distribution of mRNA sequences for the two types of abundant milk proteins between poly(A)⁺ and poly(A)⁻ RNA forms at the four stages of development studied. It was hoped that this analysis would provide further evidence to suggest that changes in mRNA polyadenylation (or more precisely the lack of polyadenylation) would accompany mammary gland development.

The analysis has been performed using the specific cDNA probes that were prepared and used in previous experiments (see Chapter 6). To confirm the presence of mRNA in these samples, both poly(A)⁺ and poly(A)⁻ RNA preparations were translated in a wheat germ cell-free system and the identity of the translation products was confirmed by their analysis on urea SDS polyacrylamide slab gels.

METHODS

(i) Preparation of rat mammary DNA

A 2.25ml aliquot of the poly(A)⁻ RNA fraction of sample 3 from 8-day pregnant rat mammary gland (see Table 19) was

used; this contained about 3.7 mg DNA and 1.6 mg RNA. The RNA portion of this sample was destroyed by alkaline hydrolysis; the sample was brought to a pH of 12.8 by addition of 1.0 ml 0.5N NaOH, and it was incubated at 68° C. for 20 mins. The pH of the solution remained unchanged at the end of the incubation. The solution was neutralised with 0.5N HCl and extra buffering capacity was provided by addition of 10µl 0.2M EDTA (pH 7.0). This treatment is sufficient to completely hydrolyse RNA molecules but it leaves DNA molecules intact (321). The DNA was collected by ethanol precipitation; about 4.0 mg of DNA were recovered.

(ii) Nucleic acid hybridization to the cDNA probes

RNA excess hybridizations were performed as described previously (see Chapter 6) except that a final salt concentration of 0.3M NaCl was used. Hybridization of mammary DNA to the 9S cDNA was also performed at 0.3M NaCl. A number of technical difficulties were associated with the hybridization of mammary DNA because it was necessary to attain particularly high Cot values in this analysis.

Long incubation times (up to 5 days at 68°C) were necessary to achieve Cot values of 10^3 - 10^4 and during this time significant degradation of the sample could occur. Furthermore, very concentrated DNA solutions (6-7mg/ml) are required to maintain DNA sequence excess, and these solutions

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are viscous and difficult to pipet. A DNA/cDNA ratio of 3,000,000 was maintained in this hybridization; Rosen and Barker achieved a ratio of 9,000,000 in their DNA excess hybridization using 15S casein cDNA (278).

The data obtained in the mammary DNA:9ScDNA hybridization have been fit to a first order curve using the available computer program as described (Chapter 6). Although the DNA-cDNA hybridization is second order, the curve obtained using the first order equation is not very different from that obtained with second order kinetics, particularly in the middle of the curve which is the region used to determine $Cot\frac{1}{2}$ (322).

(iii) Translation of RNA samples in the wheat germ cell-free system

Wheat germ cell-free translations were performed as indicated previously (see Chapter 3). The results of chapter 3 had already shown that at the 11-15 day lactating stage both poly(A)⁺ and poly(A)⁻ RNA fractions contained significant translational activity. This result was reconfirmed for the RNA fractions prepared from several stages of mammary gland development (see Chapter 7, Tables 18 and 19). Translations were performed using [³H]-leucine (2μCi/assay) and total, poly(A)⁺ and poly(A)⁻ RNAs from each developmental stage. All RNA preparations tested

exhibited translational activity, although the specific activities of total and poly(A)⁻ RNA from 8 day pregnant gland were very low. There were differences in the specific incorporation obtained with total RNA preparations from glands at different stages as expected from a previous study (279), with a range of 320 cpm/μg RNA in early gestation to 4900cpm/μg RNA during lactation. Poly(A)⁻ RNA showed a similar trend but with a smaller range (300-400 cpm/μg RNA). Poly(A)⁺ RNA from glands at all stages of development exhibited much higher specific activities; typical values were 6,300 cpm/μg RNA at 8 days of gestation and 42,000 cpm/μg RNA at 7-8 days of gestation. This range in poly(A)⁺ RNA specific activity (7 fold) is unexpectedly large, since an in vitro translation system should translate poly(A)⁺ RNAs of different stages equally. This variation in specific translational activity could be explained in part by variations in rRNA content of the samples, and/or variation in the proportion of leucine incorporated into the proteins encoded by poly(A)⁺ mRNAs from different developmental stages. The translational activities of RNA fractions from 16-day pregnant and involuting glands gave corresponding intermediate values in all cases.

Although cell-free translations indicated the presence of mRNA in all these RNA preparations, both in the poly(A)⁺ and poly(A)⁻ fractions, the nature of the translation products was of interest. For this purpose, wheat germ

cell-free translations were also performed with [^{35}S]-methionine (6 μCi /assay) and the products were analysed by slab gel electrophoresis and fluorography as described previously (see Chapter 3).

RESULTS

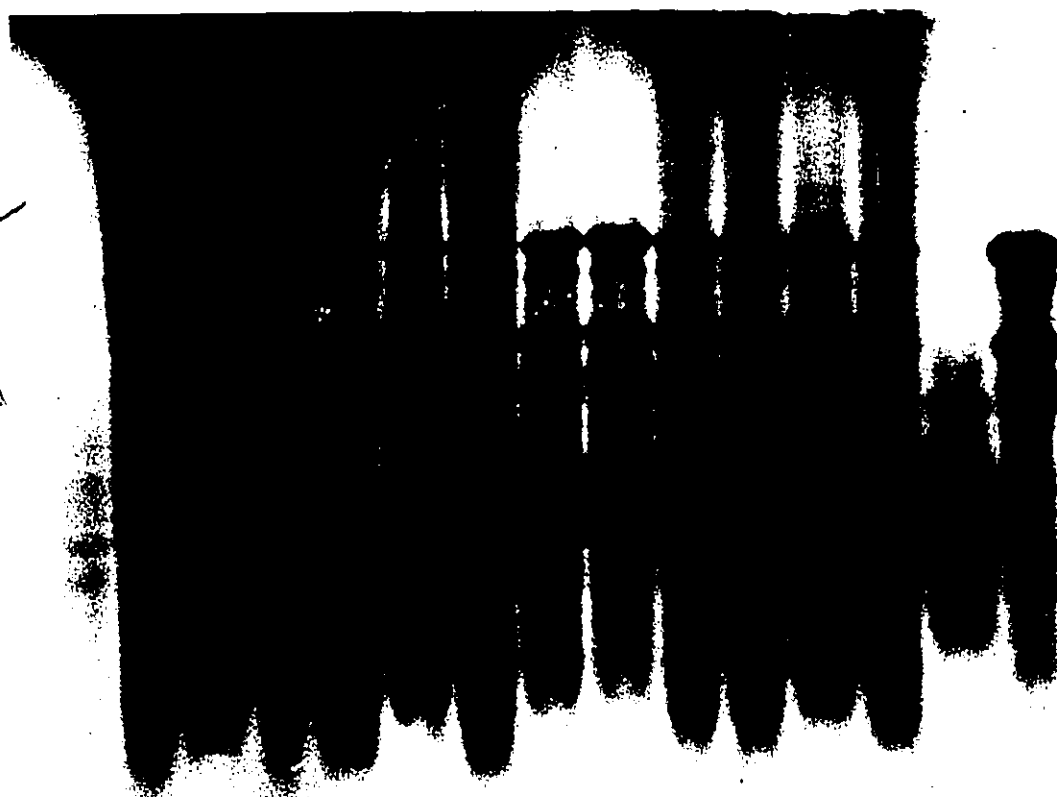
(i) Analysis of the translation products of mammary RNA fractions obtained from glands at different developmental stages

A total, poly(A) $^{+}$ and poly(A) $^{-}$ RNA sample from each development stage was translated in the wheat germ system using [^{35}S]-methionine and the resulting translation products are shown in Figure 28. A fluorogram of this same gel, prepared with a shorter exposure period, was shown in Chapter 3 (Figure 7) to demonstrate the similarity of the translation products of poly(A) $^{+}$ and poly(A) $^{-}$ RNA from 7-8 day lactating gland. In Figure 28 a longer exposure is shown to visualize more clearly some of the less prominent translation products. The translation products of the two purified 9S and 15S mRNAs (see Chapter 5) are presented for comparison.

Figure 28 shows that for almost all the samples analysed, the predominant translation products were proteins of molecular weights 42-43,000 daltons, 30,000 daltons and

Figure 28. Electrophoretic analysis of the Wheat germ cell-free translation products of Mammary mRNA from gland at several stages in development.

Total, poly(A)⁺ and poly(A)⁻ RNA fractions of one gland extract from each of the four developmental stages studied were translated in a wheat germ cell-free system containing [³⁵S]-methionine (see methods). The translations were run on an urea SDS polyacrylamide slab gel together with translations of purified 9S and 15S mRNAs. Translation products were visualised by fluorography. The RNAs used to provide these products are: lane 1, no mRNA; lanes 2 - 4, total, poly(A)⁺ and poly(A)⁻ RNAs from 8 day pregnant gland; lanes 5 - 7, total, poly(A)⁺ and poly(A)⁻ RNAs of 16 day pregnant gland; lanes 8 - 10, total, poly(A)⁺ and poly(A)⁻ RNAs from 7 - 8 day lactating gland; lanes 11 - 13, total, poly(A)⁺ and poly(A)⁻ RNAs from involuting gland; lane 14, 9S mRNA; lane 15, 15S mRNA. Between 20,000-50,000 cpm of acid insoluble [³⁵S]-methionine label were applied to each lane. The positions of the standard protein markers are indicated by arrows and the molecular weight of each protein is given in kilodaltons.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

25-26,000 daltons. These proteins have already been assigned as caseins 1, 2 and 3 respectively (see Chapters 3 and 5). One can also see the 18,500 dalton product of 9S mRNA in the lactating and involuting gland RNAs, however there appears to be very little of this product in the translations of pregnant gland RNA. These results indicate that during pregnancy there is a relatively high proportion of casein/ α IA mRNA sequences compared to the proportions seen later in development. Total and poly(A)⁻ RNAs of 8 day pregnant gland gave very low levels of incorporation, and it is difficult to detect specific bands of translation product for these two samples (lanes 2 and 4); on close inspection of these lanes very faint bands at the position of the two larger caseins can be seen. The early (8 day) and late (16 day) pregnant gland RNAs gave translation products other than the caseins (see lanes 3 and 6). This is not surprising since the milk protein mRNAs should constitute a lower proportion of total mRNA sequence in pregnancy than during lactation.

The most important observation to be made from Figure 28 is that the poly(A)⁺ and poly(A)⁻ RNAs code for proteins of quite similar sizes. This suggests that any poly(A)⁻ milk protein mRNA sequences measured by cDNA hybridization (see below) do not consist entirely of degradation products of poly(A)⁺ RNA; indeed, many poly(A)⁻ mRNA sequences must be full length since complete milk proteins are made by this RNA. Furthermore the

finding that the poly(A)⁺ RNAs are translated predominantly into full-length milk proteins reduces the possibility that variations in the translational activity of these samples are due to variable levels of RNA degradation.

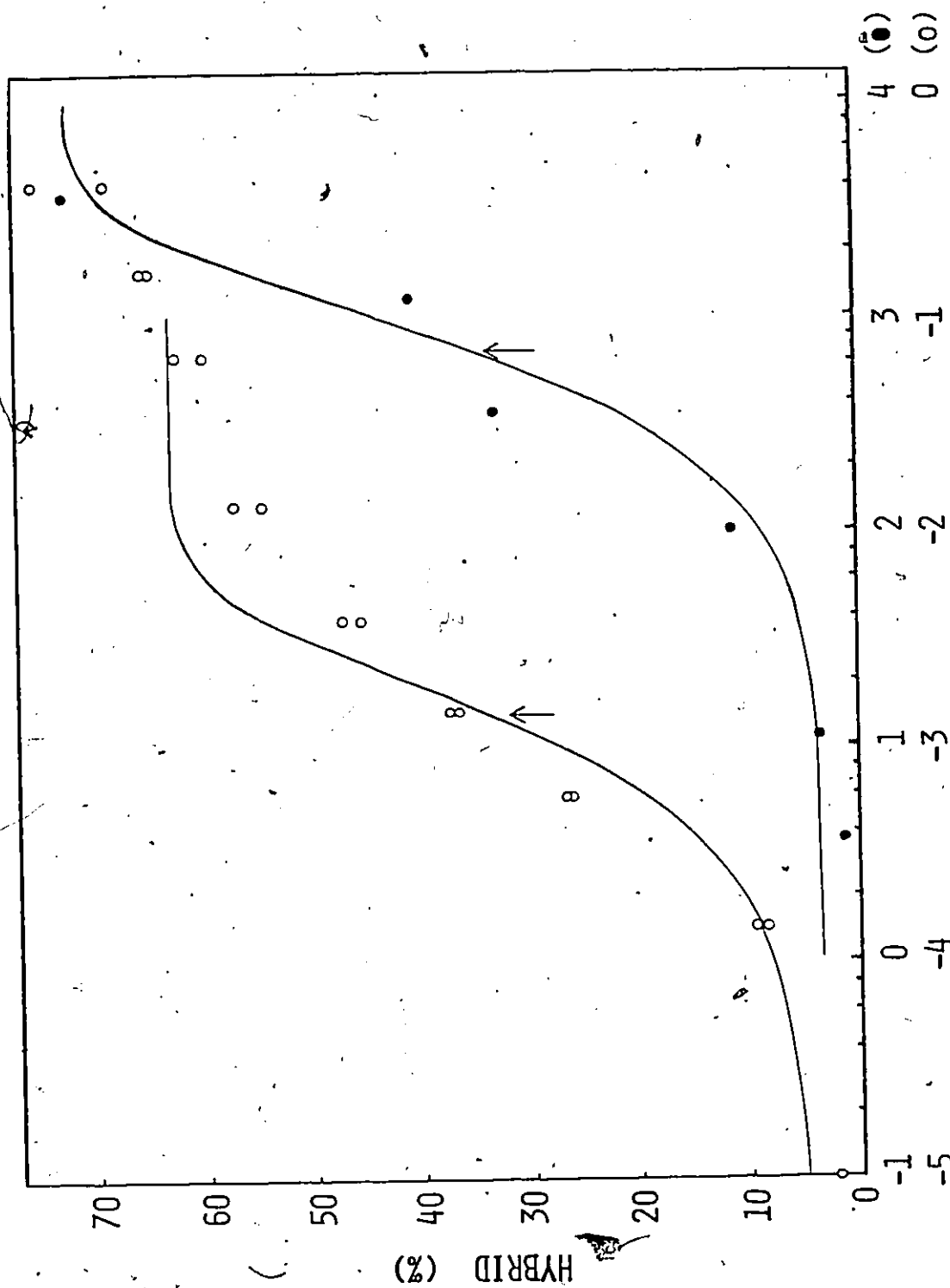
(ii) Hybridization of 9S cDNA to rat mammary DNA

Several of the mammary RNA preparations contain large amounts of DNA (see Table 18) particularly the samples from the early pregnant gland in which mRNA levels are still very low. Although there is evidence that the 15S casein mRNA sequences are transcribed from unique DNA (278), similar information is not available for the 9S mRNA. It was conceivable that some correction to the hybridization data might be necessary if the poly(A)⁻ RNA samples contained very low concentrations of milk protein mRNAs and were enriched in milk protein DNA sequences. This possibility was evaluated by hybridization of rat mammary DNA (prepared as indicated in Methods) to the 9S cDNA probe.

The Cot curve representing this hybridization is given in Figure 29. Due to the practical difficulties encountered in achieving Cot values greater than 10^3 , the upper part of the curve is poorly defined. From the best curve fit to this data, a $\text{Cot}_{\frac{1}{2}}$ of 780 mol.s.l^{-1} was obtained compared to a $\text{Cot}_{\frac{1}{2}}$ value of $1.4 \times 10^{-3} \text{ mol.s.l}^{-1}$ for the 9S mRNA standard. Since the DNA:cDNA hybridization was performed at 0.3M

Figure 29. Hybridization of 9S cDNA to Mammary DNA

Mammary DNA was prepared as described (see Methods) and hybridized to 9S cDNA at a DNA/cDNA ratio of 3×10^6 (see Methods). The hybridization data points were fitted to a first order equation and a $Cot_{1/2}$ value was determined from the first order rate constant. The curves shown are for hybridization of mammary DNA (●) and of 9S mRNA template (o); $Cot_{1/2}$ values are indicated by the arrows. To permit the drawing of these two curves in the same figure, two scales were used for Log. Cot: the scale of -5 to 0 refers to the 9S mRNA hybridization (o) and the scale of -1 to 4 refers to the mammary DNA hybridization (●).



NaCl, this corresponds to an equivalent $Cot\frac{1}{2}$ value of 1800 mol.s.l⁻¹ (323). This $Cot\frac{1}{2}$ value is comparable to values obtained for other mRNAs that are transcribed from unique sequence DNA (278, 324) and on this basis it is concluded that the 9S mRNA sequence is contained in the rat genome as unique DNA.

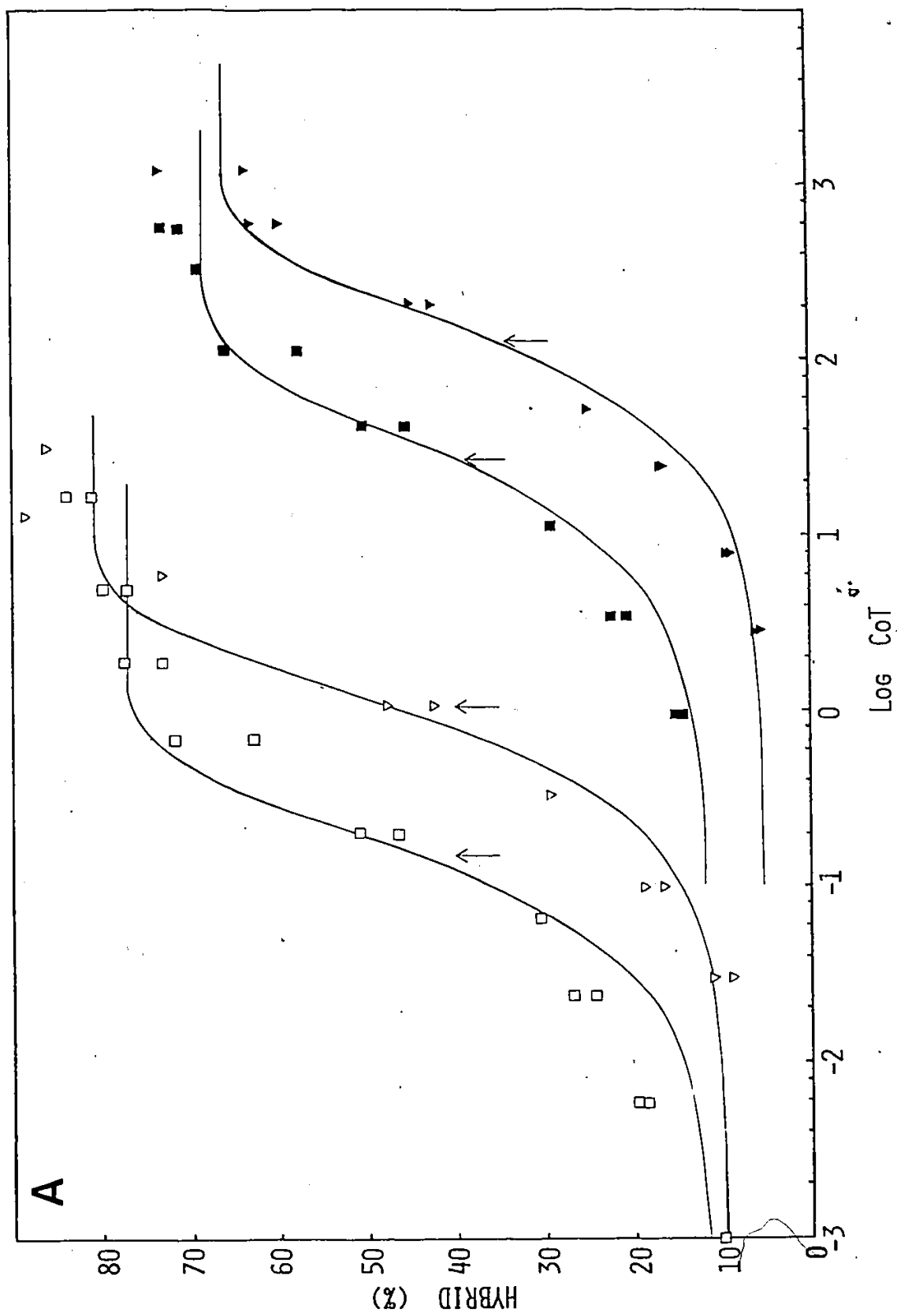
In the following section it will be shown that no poly(A)⁻ RNA samples give $Cot\frac{1}{2}$ values approaching a value of 780 and therefore contribution of the DNA sequences to these hybridizations is negligible.

(iii) Measurement of the 9S and 15S mRNA concentrations of mammary RNA fractions by hybridization to cDNA probes

The poly(A)⁺ and poly(A)⁻ RNA samples prepared from total mammary RNA of glands at the four developmental stages studied (Tables 18 and 19) were analysed by hybridization to the 9S and 15S cDNA probes. As indicated previously, where fractionation of a total mammary RNA sample produced more than one poly(A)⁺ RNA sample, all poly(A)⁺ RNAs were pooled for hybridization analysis; this ensures that the poly(A)⁺ RNA sample analysed is truly representative. Examples of the hybridizations performed using 9S and 15S cDNAs are shown in Figures 30 and 31 respectively. For each type of hybridization the analysis of only one sample for

Figure 30. Hybridization of 9S cDNA to Mammary Poly(A)⁺ and Poly(A)⁻ RNA fractions from glands at several stages of development

Mammary RNA samples (prepared as indicated in Chapter 7) were hybridized to 9S cDNA. Hybridizations were performed as described (see Methods) using 1000cpm of cDNA per assay. The data were fitted to first order curves and $Cot_{1/2}$ values were determined. The data shown were obtained using the following samples: (▽) Poly(A)⁺ and (▼) Poly(A)⁻ RNA of sample 4, 8 day pregnant gland; (□) Poly(A)⁺ and (■) Poly(A)⁻ RNA of sample 4, 16 day pregnant gland; (○) Poly(A)⁺ and (●) Poly(A)⁻ RNA of sample 2, 7-8 day lactating gland; (Δ) Poly(A)⁺ and (▲) Poly(A)⁻ RNA of sample 4, involuting gland. Pregnant gland data are shown in Figure 30A and The lactating and involuting gland data are shown in Figure 30B. The hybridization of 9S mRNA template is also shown (x). $Cot_{1/2}$ values are indicated by arrows. The root mean square of each curve fit is between 1.5% and 6%.



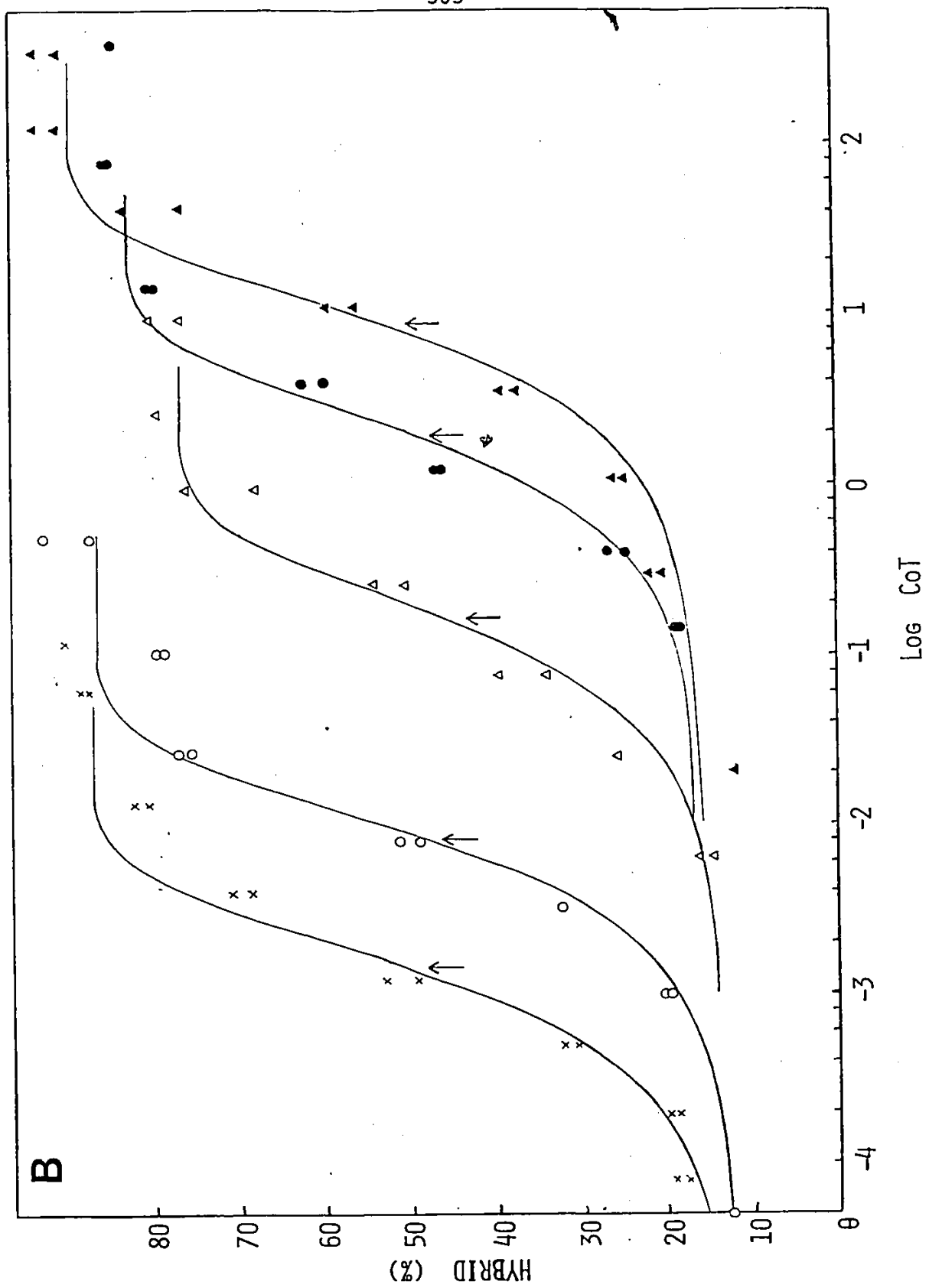
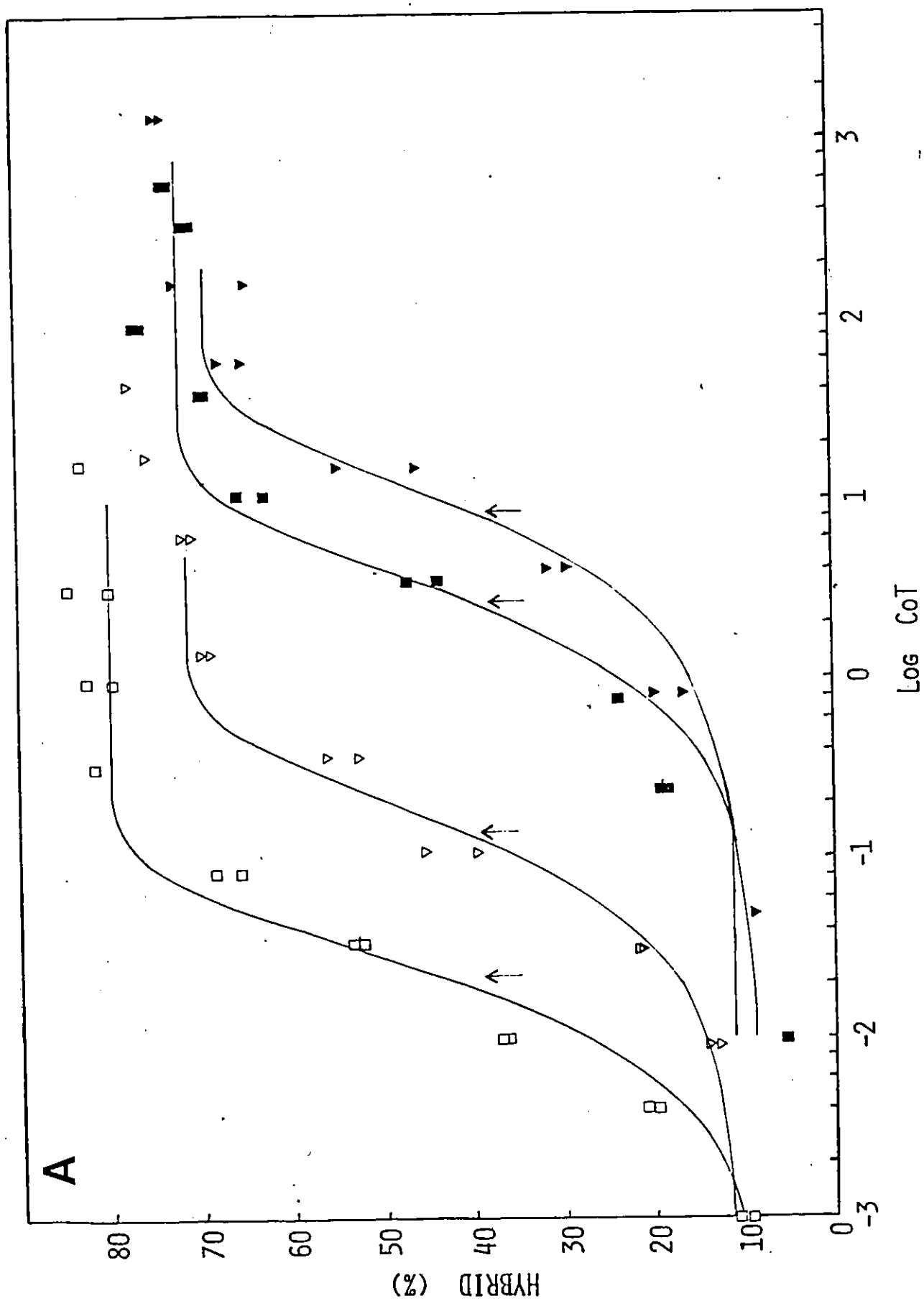
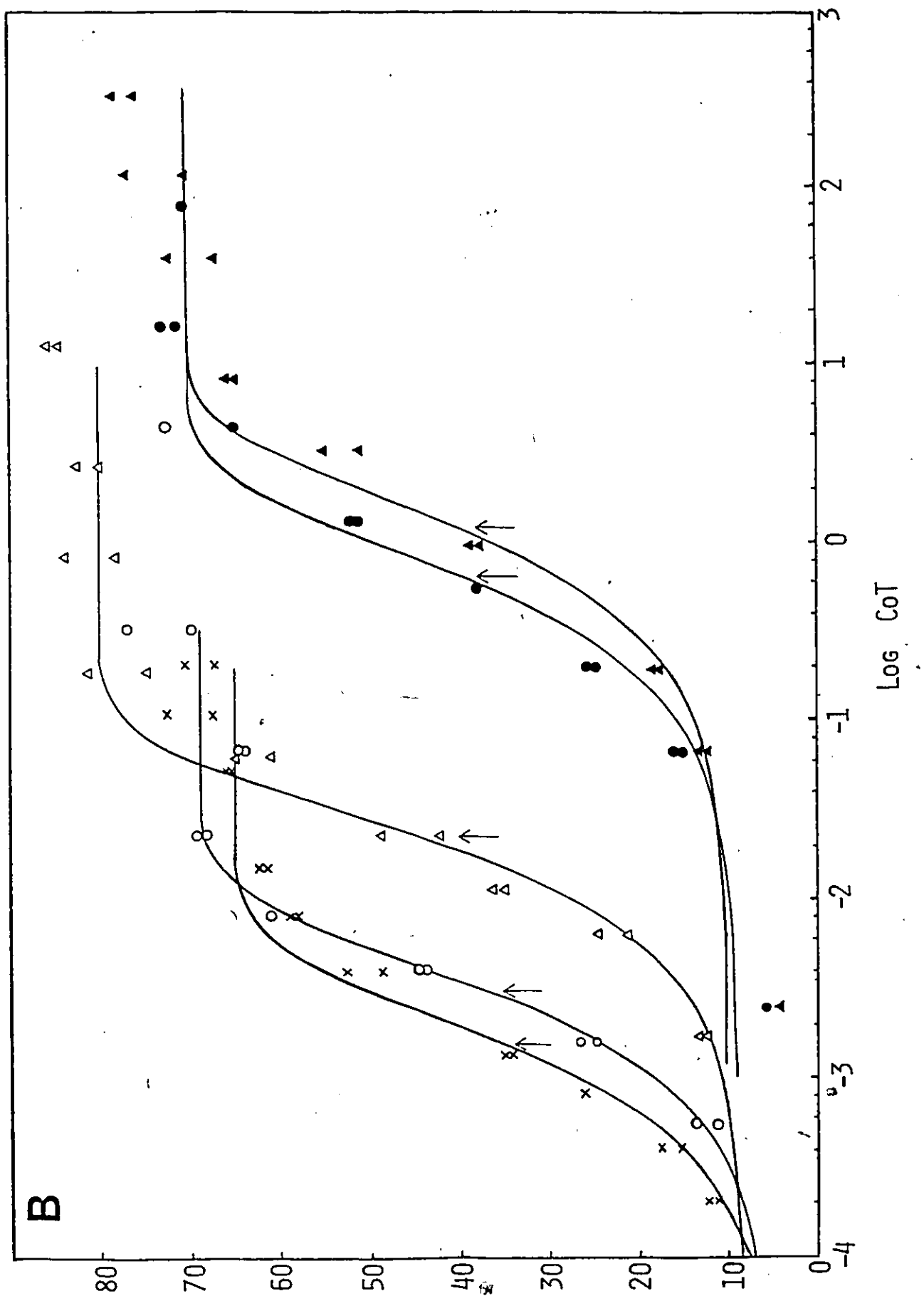


Figure 31. Hybridization of 15S cDNA to Mammary Poly(A)⁺ and Poly(A)⁻ RNA fractions from glands at several stages of development

Mammary RNA samples (prepared as indicated in Chapter 7) were hybridized to 15S cDNA. Hybridizations were performed as described (see Methods) using 1000cpm of cDNA per assay. The data were fitted to first order curves and $Cot_{1/2}$ values were determined. The data shown were obtained using the following samples: (▼) Poly(A)⁺ and (▼) Poly(A)⁻ RNA of sample 2, 8 day pregnant gland; (□) Poly(A)⁺ and (■) Poly(A)⁻ RNA of sample 4, 16 day pregnant gland; (○) Poly(A)⁺ and (●) Poly(A)⁻ RNA of sample 1, 7-8 day lactating gland; (Δ) Poly(A)⁺ and (▲) Poly(A)⁻ RNA of sample 4, involuting gland. Pregnant gland data are shown in Figure 31A and the lactating and involuting gland data are shown in Figure 31B. The hybridization of 15S mRNA template is also shown (x). $Cot_{1/2}$ values are indicated by arrows. The root mean square of each curve fit is between 1.5% and 6%.





each developmental stage is shown. In this way, examples representative of all the hybridizations are illustrated.

The data of Figures 30 and 31 have been fitted to first order curves and $Cot\frac{1}{2}$ values (see arrows) have been calculated from the first order rate constant. One can see that the rates of these reactions are such that the DNA present in these samples does not contribute significantly to these hybridizations. The $Cot\frac{1}{2}$ value for each sample was therefore compared directly to the $Cot\frac{1}{2}$ of the standard mRNA template in order to calculate the concentration of mRNA sequence in that sample. As described previously, from a knowledge of the concentration of mRNA sequence in a sample and the total RNA present, absolute amounts of mRNA sequence can be determined for each sample.

The results of the hybridization analyses performed on these samples in the manner indicated in Figures 30 and 31 are given in Table 21 (for 9S cDNA) and Table 22 (for 15S cDNA). These results are expressed as μg mRNA sequence per mg. unfractionated RNA for both poly(A)⁺ and poly(A)⁻ RNA fractions as described in detail previously (Chapter 6).

The following observations may be made from Tables 21 and 22:

a) As expected from previous studies there are very substantial increases in the amounts of the milk protein mRNA sequences between early gestation and lactation; this is

LEGEND TO TABLE 21:

Poly(A)⁺ and Poly(A)⁻ RNA fractions from mammary gland at the stage indicated were analysed by hybridization to 9S cDNA as indicated in Figure 30. From the $Cot\frac{1}{2}$ values obtained, the absolute amounts of 9S mRNA in each sample were determined. The values shown after each group are the mean $\pm$ S.D. Total 9S mRNA has been calculated by summing the amounts of mRNA in the poly(A)⁺ and poly(A)⁻ RNA fractions. The 11-15 day lactating gland data were taken from the results shown in Chapter 6.

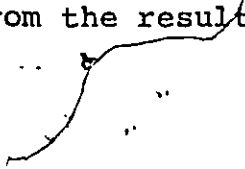


TABLE 21: RELATIVE AMOUNTS OF 9S mRNA SEQUENCE IN POLY(A)⁺ AND POLY(A)⁻ RNA FRACTIONS OF MAMMARY GLAND AT SEVERAL STAGES IN DEVELOPMENT

Stage	Sample	Amount of 9S mRNA in:			9S mRNA in poly(A) ⁻ RNA	
		Poly(A) ⁺ RNA	Poly(A) ⁻ RNA	total	(% of total mRNA sequence in poly(A) ⁺ and poly(A) ⁻ RNA)	
		(μg mRNA/mg unfractionated RNA)				
8 day pregnant	1	0.004	0.009	0.013	0.013	67
	2	0.009	0.013	0.022	0.024	59
	3	0.018 ± 0.008	0.012 ± 0.002	0.030 ± 0.008		40
	4	0.022	0.009	0.031		28
16 day pregnant	1	0.088	0.047	0.135		35
	2	0.093	0.034	0.127	0.301	27
	3	0.450 ± 0.186	0.061 ± 0.014	0.511 ± 0.199		12
	4	0.365	0.065	0.430		15
7-8 day lactating	1	10.79	0.85	11.64	13.83	7.3
	2	11.01	1.01	12.02	± 3.46	8.4
	3	17.03	0.79	17.82		4.4
11-15 day lactating	1a	6.68	3.21	9.89	8.06	33
	2d	7.57	1.18	8.75	± 2.26	14
	3	4.56	0.98	5.54		18
involuting	1	0.31	0.10	0.41		25
	2	1.29	0.21	1.50	0.64	14
	3	0.12 ± 0.54	0.13 ± 0.05	0.25 ± 0.58		51
	4	0.26	0.14	0.40		35

LEGEND TO TABLE 22

Poly(A)⁺ and Poly(A)⁻ RNA fractions from mammary gland at the stage indicated were analysed by ~~hybridization~~ to 15S cDNA as indicated in Figure 31. From the $Cot\frac{1}{2}$ values obtained, the absolute amounts of 15S mRNA in each sample were determined. The values shown after each group are the mean $\pm$ S.D. Total 15S mRNA has been calculated by summing the amounts of mRNA in the poly(A)⁺ and poly(A)⁻ RNA fractions. The 11-15 day lactating gland data were taken from the results shown in Chapter 6.

TABLE 22: RELATIVE AMOUNTS OF 15S mRNA SEQUENCE IN POLY(A)⁺ AND POLY(A)⁻ RNA FRACTIONS OF MAMMARY

GLAND AT SEVERAL STAGES IN DEVELOPMENT									
Stage	Sample	Amount of 15S mRNA in:		Poly(A) ⁺ RNA	Poly(A) ⁻ RNA	Total	15S mRNA in poly(A) ⁻ RNA		
		Poly(A) ⁺ RNA	Poly(A) ⁻ RNA				(% of total mRNA sequence in poly(A) ⁺ and poly(A) ⁻ RNA)		
8 day pregnant	1	0.088	0.15	0.23	0.23	0.65	65		
	2	0.170	0.47	0.26	0.18	0.43	60	40 ± 26	
	3	1.05	±0.44	0.14	±0.05	1.19	12		
	4	0.56	0.18	0.74		0.42	24		
16 day pregnant	1	2.21	0.81	3.02		2.70	27		
	2	1.93	2.05	0.42	0.66	2.35	28	24 ± 4.9	
	3	2.16	±0.16	0.88	±0.22	3.04	29		
	4	1.88	0.52	2.40		±0.38	22		
7-8 day lactating	1	14.84	1.69	16.53		15.46	10		
	2	13.97	1.79	15.76	1.92	±1.25	11	12.3 ± 3.2	
	3	11.80	2.28	14.08	±0.32		16		
11-15 day lactating	1a	9.25	4.86	14.11	3.85	12.73	35		
	2d	9.17	3.05	12.22	±0.92	±1.21	25	30.3 ± 5.0	
	3	8.21	3.65	11.86			31		
involuting	1	2.64	0.86	3.50		2.71	22		
	2	1.71	0.71	2.42	0.89	±0.60	29	33.5 ± 10.7	
	3	1.12	0.98	2.10	±0.14		47		
	4	1.79	1.02	2.81			36		

followed by a drop in the levels of these sequences on involution. Examination of the standard deviations of these values does however show that there can be large variations in the amounts of specific mRNAs present in mammary RNA even when prepared from glands of rats at similar stages in gestation and lactation. This variation is particularly noticeable in the 9S hybridization data for the 16 day gestation samples (Table 21).

b) The levels of 15S casein mRNA are consistently higher than levels of 9S mRNA. The difference between these two sequences is minimal during maximal gland activation, i.e. at lactation, where the 15S casein mRNA sequence is present at a level only 1.5 fold higher than that of the 9S mRNA. At other stages of gland development, 9S mRNA levels are 10-30 fold lower than those of casein mRNA. It therefore appears that the 9S mRNA sequence accumulates at a later stage during gestation but is lost at an earlier stage during involution compared to the 15S mRNA sequence; in other words the levels of these two sequences are not co-ordinately controlled throughout gland development. This observation is in agreement with the analysis of the translation products of mammary RNA prepared from glands at different stages (see Figure 28).

c). The most important observation to be made from these data is the relative amounts of poly(A)⁺ and poly(A)⁻ milk protein sequences present at different stages of gland development. The proportion of poly(A)⁻ milk protein mRNA is not constant throughout mammary gland development; it appears to be at a minimum (about 5-10% of total milk protein mRNA) during lactation while during gestation and involution this proportion rises (up to 65%). Study of these data would suggest that there is an inverse relation between total levels of milk protein mRNA and the proportion of that mRNA which is in the poly(A)⁻ RNA fraction.

To determine the significance of the changes in relative poly(A)⁻ milk protein mRNA levels during gland development, statistical analysis of the data of Tables 21 and 22 was performed. An analysis of variance on the data of Table 21 did indicate a significant variation between groups ($p = 0.015$). A similar analysis of the data of Table 22 indicated no significant difference between groups ($0.2 < p < 0.15$). The reason for a lack of significance in the latter case is most likely the high variability in the percentage of poly(A)⁻ 15S mRNA in early pregnant gland preparations. This group yields total 15S mRNA sequence levels of 0.23 - 1.10 $\mu\text{g}/\text{mg}$ total RNA and percent poly(A)⁻ 15S mRNA sequences of 12-65%. A student's T test indicates there is no significant difference between the

8 day gestation group and the 7-8 day lactating group in Table 22, but by this test there is a significant difference between the 7-8 day lactating group and all other groups ($0.025 < p$ in all cases). Close examination of the early gestation group of Table 22 indicates that within this group there is a trend in which high mRNA levels are associated with a low proportion of poly(A)⁺ mRNA levels. It is possible that the trend observed within this group is obscuring the overall trend because of the high variability of the early pregnant group.

It is of interest here to compare the 7-8 day lactating values with those of 11-15 day lactating samples which have been included in Tables 21 and 22 to permit ready comparison. The percentage of poly(A)⁺ mRNA in the latter samples was 14-33% for 9S mRNA and 23-35% for 15S mRNA. These values are rather higher than the percentages obtained for the 7-8 day lactating samples (4.4-8.4% and 10-16% respectively). The total amount of each milk protein mRNA is higher at the 7-8 day stage than the 11-15 day stage and one could therefore conclude that milk protein levels have begun to decline at the later stage. The 7-8 day lactating stage is thus the stage containing the highest mRNA levels studied, and this result is in agreement with Nakhasi and Qasba who showed a rapid decline in milk protein mRNA sequences after day 12 of lactation (279).

(iv) Recovery of mRNA sequences after affinity chromatography

[³H]-poly(U) analysis of the RNA fractions used in these studies had indicated that poly(A) recovery in the poly(A)⁺ RNA samples was high after mammary RNA fractionation using oligo(dT)-cellulose (Table 19). This does not show that mRNA recovery in column bound and unbound RNA fractions is quantitative. It was therefore important to show that in these developmental studies mRNA sequence recovery from total mammary RNAs was close to complete and that the observed variations in the relative proportions of poly(A)⁺ and poly(A)⁻ mRNAs during gland development were not due to poor sample fractionation.

To determine mRNA recovery from several fractionations, it was necessary to analyse unfractionated mammary RNAs to determine the levels of total mRNA sequence in these samples. These analyses were performed by hybridization with the 9S cDNA probe. The major reason for choosing this sequence is that the 9S mRNA is smaller than 15S casein mRNA and therefore more readily lost during sample handling. It would be expected that 15S casein mRNA recovery will be at least as high as 9S mRNA, if not higher.

Four unfractionated RNA samples, one from each of the four developmental stages studied, were analysed by

TABLE 23 DETERMINATION OF 9S mRNA RECOVERY AFTER OLIGO(dT)-
CELLULOSE FRACTIONATION OF MAMMARY RNA BY HYBRIDIZATION OF
TOTAL, POLY(A)⁺ AND POLY(A)⁻ RNAs TO 9S cDNA

Stage of development	Sample	mRNA in total RNA sample applied to column (μ g)	mRNA recovered in poly(A) ⁺ and poly(A) ⁻ RNA fractions (μ g)	recovery (%)
8 day pregnant	2	0.221	0.193	87
16 day pregnant	3	10.84	8.12	75
7-8 day lactating	3	470.0	431.0	92
involuting	2	36.52	35.7	98

Total unfractionated, poly(A)⁺ and poly(A)⁻ RNA samples from the indicated mammary gland extracts (see Tables 18 and 19) were hybridized with 9S cDNA as indicated in Figure 30. Using the $Cot_{1/2}$ values determined from this analysis the absolute amount of mRNA contained in each sample was calculated. The amount of 9S mRNA contained in total unfractionated RNA is compared to the combined amount of 9S mRNA in the poly(A)⁺ and poly(A)⁻ RNA fractions recovered from the oligo(dT)-cellulose columns. The overall mRNA recovery is expressed as a percentage of that applied to the column.

hybridization to 9S cDNA and the $Cot\frac{1}{2}$ values thus obtained for these samples were used to calculate the amount of 9S mRNA sequence present in the initial samples. These values are compared directly to the amount of sequence recovered in both the poly(A)⁺ and poly(A)⁻ RNA fractions as shown in Table 23. All the recoveries determined here were equal to or greater than 75%; therefore high and satisfactory recoveries were observed for RNA obtained from all stages.

Thus the data of Table 23 show that the changes in proportion of poly(A)⁻ milk protein mRNAs during mammary gland development cannot be attributed to loss of sequence from a particular fraction during the fractionation procedure.

DISCUSSION

Translation analysis of RNA preparations from mammary gland of several developmental stages clearly demonstrated the progressive increase in translational activity of mammary RNA throughout gestation and lactation. Nakhasi and Qasba have already shown that total mammary RNA preparations exhibit a ten fold increase in translational activity between virgin and 12 day lactating rat gland preparations (279).

The major translation products of the poly(A)⁺ and poly(A)⁻ RNAs were essentially similar in all cases (with the exception of the low 9S mRNA product during gestation). This observation suggested that the poly(A)⁻ RNA preparations were not significantly degraded compared to the poly(A)⁺ RNAs; both RNAs translated into full-length milk proteins as the major products. These translations also indicated that the poly(A)⁻ milk protein sequences detected by hybridization did not contain extra sequences (eg. introns); this suggested that these poly(A)⁻ sequences were not newly synthesised, unprocessed nuclear RNA. Although the precise relationship between poly(A)⁺ and poly(A)⁻ forms of the same mRNA has not been demonstrated in any one system, several investigators have proposed that poly(A)⁻ mRNA is produced as a consequence of poly(A) tract removal from poly(A)⁺ mRNA. The analysis of translation products shown in Figure 28 is consistent with this hypothesis, but the possibility that the poly(A)⁻ mRNA is a completely separate species, unrelated to poly(A)⁺ mRNA, can not be eliminated.

The major goal of this chapter was to analyse the distribution of 9S whey protein and 15S casein mRNAs between poly(A)⁺ and poly(A)⁻ mammary RNAs as a function of gland development. Before the results of such an analysis could be discussed it was necessary to eliminate the possibility that the relatively high proportion of poly(A)⁻ mRNA in certain RNA preparations (as found for example in early

gestation) was not a consequence of cDNA binding to milk protein sequence in mammary DNA. The $\text{Cot}\frac{1}{2}$ of 9S cDNA hybridization to mammary DNA was found to be 780 mol.s.l^{-1} at 0.3M NaCl (equivalent $\text{Cot}\frac{1}{2}$ of $1800 \text{ mol.s.l}^{-1}$). This very high value of $\text{Cot}\frac{1}{2}$ indicated that:

- 1) The rate of cDNA:DNA hybridization was too slow to be a factor in the hybridization analyses of poly(A)⁻ RNA since even in early gestation mRNA sequences are present at many fold the DNA level; (Tables 21 and 22).

- 2) This $\text{Cot}\frac{1}{2}$ value is in close agreement with the $\text{Cot}\frac{1}{2}$ value of $1372 \text{ mol.s.l}^{-1}$ obtained by Rosen and Barker for hybridization of 15S casein cDNA to rat mammary DNA (278). Packman et al. calculated the $\text{Cot}\frac{1}{2}$ for an unique duck globin gene sequence to be $1500 \text{ mol.s.l}^{-1}$ (324). It may therefore be concluded that like the 15S casein gene sequence, the 9S gene sequence is contained in the genome as unique DNA.

The analysis of variance and student's T test studies performed on the group data of Tables 21 and 22 suggest that while there is a significant variation between groups in Table 21 (for 9S mRNA), the situation was less clear-cut with the data in Table 22 (for 15S casein). It was believed that a possible trend for these data was being obscured by the high variation in the early gestation group. It was observed that the total mRNA sequence and the relative proportion of poly(A)⁻mRNA in this group both show large variations but these two measurements do change

inversely. A better analysis of the data of Tables 21 and 22 might therefore be achieved by investigating whether there is an inverse relation between relative poly(A)⁻ mRNA levels and the amount of total mRNA sequence. Graphical plotting of the data in this manner clearly showed there was no linear correlation; however, there was definitely a trend towards a higher proportion of poly(A)⁻ mRNA sequences, especially at low mRNA sequence levels. The data for the 9S mRNA sequence were again more convincing than those for the 15S mRNA. It is postulated that especially high poly(A)⁻ mRNA levels appear at low levels of mRNA sequence, but since the levels of 9S mRNA detected in these studies are lower than those for the 15S mRNA, this trend is more readily apparent for the 9S sequence. This hypothesis would suggest that a high proportion of these sequences exists as non-polyadenylated mRNAs at low mRNA levels but as the total level of each mRNA rises, the proportion of the poly(A)⁻ forms of this mRNA is reduced.

CHAPTER 9

CONCLUDING DISCUSSION

The major aim of this work was to investigate whether rat mammary gland development is associated with changes in mRNA polyadenylation. It has been shown that two specific changes in this mRNA characteristic appeared to parallel stimulation of the gland and its synthesis of milk components.

The first change was the appearance of a specific poly(A) size class (40-45 residues in length) in total poly(A)⁺ mammary mRNA; levels of this poly(A) size class were highest during peak lactation and much reduced or absent on involution or in early pregnancy. It has been speculated that this particular size class of poly(A) might be restricted to milk protein mRNAs, since these sequences were found to be enriched in the 40-45 residue poly(A) tracts compared to total lactating mammary poly(A)⁺ mRNA. Proof of this suggestion would require further investigation of the translation products directed by mammary mRNAs bearing long and short poly(A) tracts respectively.

The finding of shorter poly(A) tracts in mammary gland during the period of high protein synthesis was consistent with earlier reports that poly(A) shortening might be associated with mRNA translation. Indeed poly(A) shortening might be a necessary consequence of translation. Could such observations be reconciled with the hypothesis that the poly(A) tracts

associated with mammary RNA act to stabilise these mRNA sequences? Investigations of poly(A) function performed in X. laevis oocytes have indicated that in order to effectively stabilise mRNA sequences, the associated poly(A) tracts need only be about 30 residues long (193). Thus 40 residue poly(A) tracts could stabilise mRNAs as well as 80 residue tracts and all the poly(A) tracts observed in mammary gland at the various developmental stages studied would be equally effective in this respect.

The relatively high proportion of smaller poly(A) tracts observed during lactation might be a consequence of a specific mechanism in the mammary cell which restricted the appearance of tracts smaller than 25 residues, thereby increasing the levels of tracts in the 40-45 residue size range. There are several means by which poly(A) shortening might be strictly controlled in the mammary cell during lactation: eg. by reduction of the activity of specific poly(A) nucleases (see p. 38); by stimulation of poly(A) elongation processes such as that described by Brawerman and Diez (141) or by the action of poly(A) binding proteins which are known to modulate poly(A) metabolism (see p. 64). Mechanisms such as these might lengthen the lifetime of a poly(A) tract and thereby effect mRNA stabilisation but at the same time cause an overall reduction in poly(A) length. The observed changes in poly(A) tract length during gland development are therefore not inconsistent with a mechanism of increased mRNA stabilisation by changes in mRNA

polyadenylation.

The second observed change in mammary mRNA polyadenylation was with regard to the proportion of 9S and 15S milk protein mRNA sequences in poly(A)⁺ and poly(A)⁻ forms. Both sequences exhibited a trend in which the percentage of the poly(A)⁻ form was relatively high at early gestation and involution (up to 65%) but lower during lactation, especially at 7-8 days lactation (about 5-10%). This series of observations must be evaluated cautiously in view of the technical problems associated with this analysis as discussed previously (see Abstract). If poly(A) tracts do function in mRNA stabilisation however, a particularly low proportion of poly(A)⁻ milk protein mRNA sequences during high levels of gland stimulation would be consistent with this hypothesis.

It has already been stated (see "Aims of this Research") that the hormone prolactin appears to have post-transcriptional effects on the milk protein mRNAs. Prolactin has been reported to increase milk protein mRNA half-life in rat mammary gland in vitro (291) and it is therefore possible that an in vivo function of prolactin is to stabilise these mRNAs. Little is known about the mechanism by which prolactin mediates such an effect. In view of the observations made on the function of poly(A) tracts in mRNA stabilisation (see Introduction) and the changes in mammary mRNA polyadenylation during development as reported in this study, it is possible that the

post-transcriptional actions of prolactin are mediated by the poly(A) tracts of milk protein mRNAs. It appears that mRNA stabilisation in response to prolactin is specific to the milk protein sequences and there are means by which changes in polyadenylation could be specific eg. by variation of the nature of 3' non-translated sequences in mRNAs (203).

A direct link between the action of prolactin on milk protein mRNA stability and the observed changes in mRNA polyadenylation have not been shown in this study, but the interrelationship of these processes and their potential involvement in the control of gene expression are clearly of great interest. It is believed that this research has demonstrated specific changes in mRNA polyadenylation in the developing mammary gland and this should permit future research to examine more thoroughly the mechanisms by which these changes occur and their relative importance in the control of gene expression.

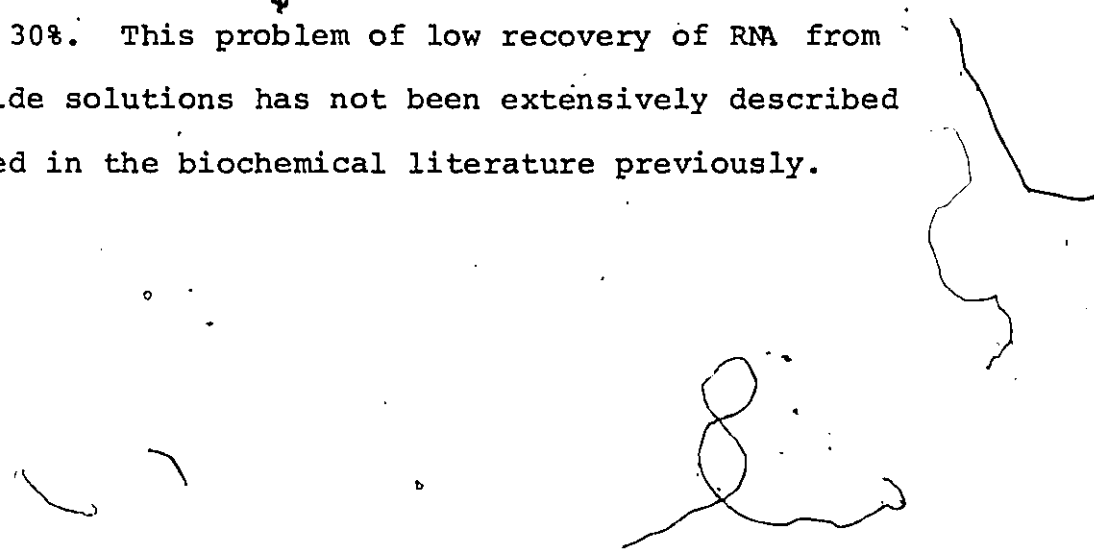


Contributions to Knowledge

1. The proportion of poly(A)⁻ 15S casein mRNA has been shown to vary from 10-30% of total 15S mRNA sequence in lactating rat mammary gland. This proportion is significantly smaller than the 60% reported in a previous study. The earlier analysis was performed by mRNA translation studies in a wheat germ cell-free system; the present report has shown that measurements based on such an analysis may be inaccurate. The present study has utilised the more accurate technique of RNA hybridization to a specific 15S cDNA probe in order to measure levels of 15S mRNA sequence in poly(A)⁺ and poly(A)⁻ fractions of total lactating mammary RNA. The relative amounts of poly(A)⁺ and poly(A)⁻ 9S whey protein mRNA have also been reported. This is the first time that the distribution of both 9S and 15S mRNAs between poly(A)⁺ and poly(A)⁻ RNA fractions has been reported in the same study. This has allowed strict comparisons between the two sequences with respect to their polyadenylation.
2. Specific changes in the characteristics of mRNA polyadenylation during mammary gland development have been demonstrated. The poly(A) tract size profiles from glands at five developmental stages indicated that very active lactating tissue was enriched with a poly(A) size class peaking at 40-45 residues; relatively inactive gland contained predominantly larger tracts with a modal length of about 80 nucleotides.

During these studies, the size of the poly(A) tracts associated with purified rat 9S whey protein mRNA was reported for the first time. The distribution of both 9S and 15S mRNA sequences between poly(A)⁺ and poly(A)⁻ mammary RNAs during gland development was investigated. There was evidence that there might be an inverse relation between the percentage of sequence in the poly(A)⁻ RNA and total levels of the sequence in the gland.

3. During the use of poly(U) sepharose columns to fractionate total mammary RNA into poly(A)⁺ and poly(A)⁻ fractions, it was found that recovery of RNA from solutions containing high concentrations of formamide was irreproducible and often unacceptably low. In order to ensure efficient recovery of RNA on ethanol precipitation from formamide containing solutions, the solution should be diluted to a formamide concentration not exceeding 30%. This problem of low recovery of RNA from 70-90% formamide solutions has not been extensively described or investigated in the biochemical literature previously.



APPENDIX

Determination of the purity of mRNA and cDNA preparations by measurement of their cross-reactivities in RNA:cDNA hybridization reactions.

This section summarises the molecular basis of the use of the hybridization data presented in Figure 23 to measure the degree of cross contamination of purified mRNAs and their corresponding cDNAs. This type of analysis of these hybridization curves has been performed in previous studies (317).

For ease of illustration, the hybridization of 9S cDNA with its 9S mRNA template (▲) and the 15S mRNA preparation (●) has been reproduced here (see Figure 23a), and this is used as an example of these calculations. The following text makes several references to the curves shown on p.330.

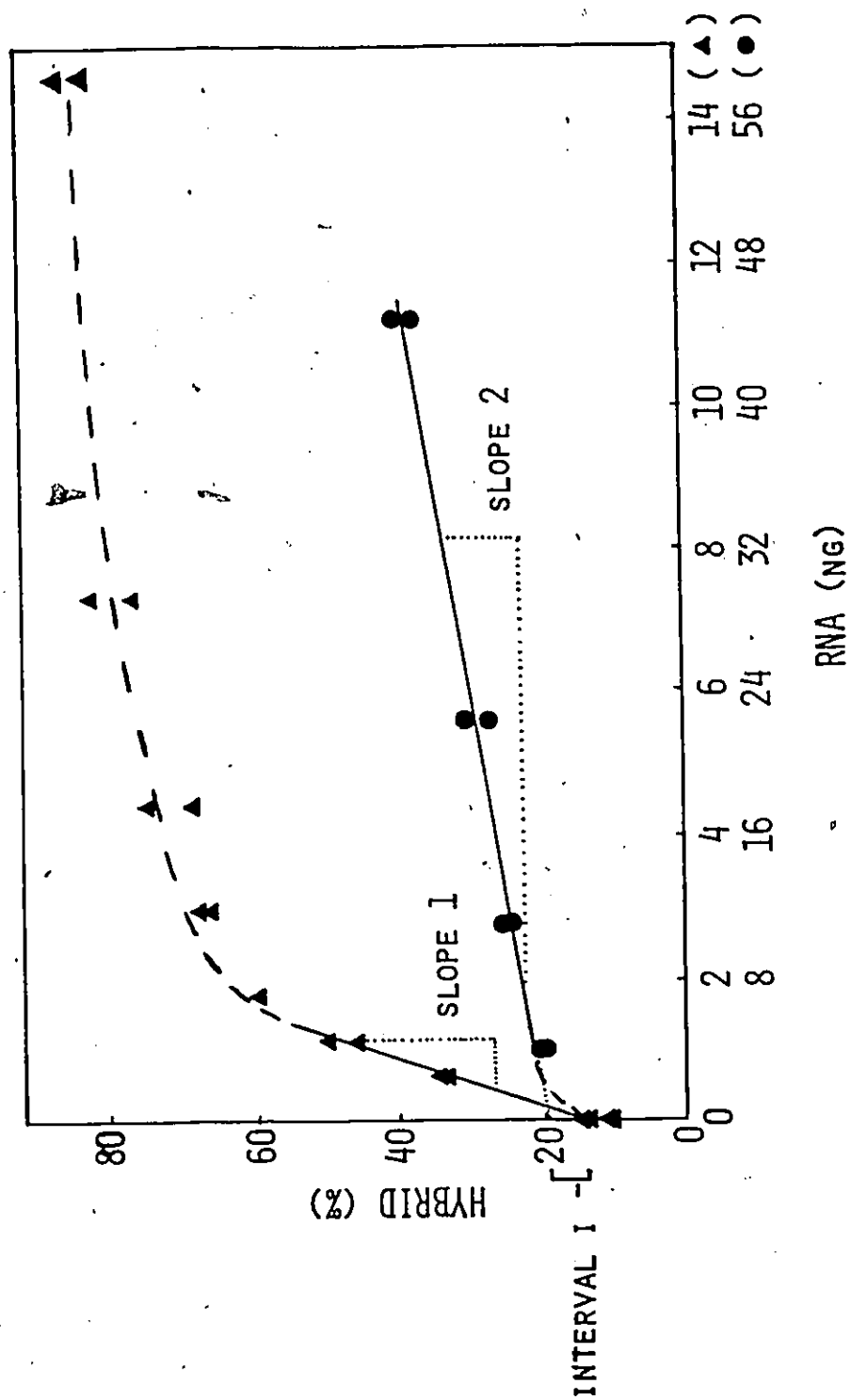


Figure 23a reproduced from page 256.

9S mRNA hybridization

This curve represents only the hybridization of 9S cDNA with 9S mRNA. The linear part of this curve (slope 1) provides a standard measure for the rate of hybridization of 9S mRNA with this cDNA. All other RNAs containing 9S mRNA will hybridise to this cDNA but with a lower rate ie. a smaller slope, depending on the proportion of 9S mRNA in the sample.

15S mRNA hybridization

The hybridization curve for the heterologous reaction is composed of two components: the hybridization of 9S cDNA: 9S mRNA (section a) and the hybridization of 15S cDNA: 15S mRNA (section b). These two components are resolved as follows:-

(a) The linear portion of the curve corresponds to the hybridization of the 9S cDNA with small amounts of contaminating 9S mRNA sequence present in the 15S mRNA preparation. By comparison of the slope of this curve (slope 2) with slope 1, the relative concentration of the 9S mRNA sequence in the 15S mRNA is determined. For example, in this case slope 1 has a value of 30% hybrid/ng RNA and slope 2 gives a value of 0.43% hybrid/ng RNA. Thus the heterologous reaction occurs at a rate of approximately 1.5% of that of the homologous reaction and it is concluded that the 15S mRNA preparation is 1.5% 9S mRNA.

(b) The portion of the curve at very low RNA amounts does not appear to be linear - extrapolation of the linear portion to the ordinate provides a "0ng RNA" value of 19% compared to the true "0ng RNA" value of 13%. This 6% difference is indicated in the illustration as "Interval 1". This interval represents hybridization of the 15S cDNA present in the 9S cDNA preparation with the large amounts of 15S mRNA present in the heterologous reaction. Even at the lowest 15S mRNA concentration studied, hybridization of the 15S cDNA contaminant in the 9S cDNA is essentially complete due to the large excess of the 15S mRNA sequence. Fortunately, this component is a small and constant level of hybridization and it is readily separated from the 9S mRNA hybridization by extrapolation of the linear section of the curve to the ordinate. The level of 15S cDNA measured here is 6% of the 9S cDNA, but since the hybridization curves proceed to only 85% completion, this value is adjusted to 7.5% on correction to 100% cDNA.

Using the data provided by Figure 23b in similar calculations, corresponding values may be calculated for the levels of 15S mRNA sequence in 9S mRNA and the levels of 9S cDNA in the 15S cDNA preparation.

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