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Characterisation of the 1B4 Antigen

Thesis submitted to the
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CONTENTS

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Abstract

Processes occurring within the nucleus are thought to be organised by a matrix of protein and RNA. The individual protein components of this structure are not well defined. This work describes the characterisation of an antigen identified by the 1B4 antibody, which was created as part of an effort to identify novel nuclear proteins. This antibody was raised against a nuclear matrix extract of HL-60 cells to specifically target proteins which are components of, or associated with, the nonchromatin structure of the nucleus. It detects a 100 kDa protein in mitotic HeLa cells. I have performed experiments to verify that this antigen is a nuclear matrix protein in which I extracted cells *in situ* and detected the 1B4 antigen by immunofluorescence. These results were consistent whether the salt buffers used to prepare the nuclear matrices contained sodium chloride or ammonium sulphate. Further experiments involved immunofluorescence studies in which I compared the distribution of the 1B4 antigen to distributions of proteins with known functions related to pre-mRNA splicing. Concentration of certain nuclear proteins in specific domains within the nucleus suggests that functions associated with them are compartmentalised, and there are several examples of proteins with related functions sharing the same nuclear domain. Consistent localisation of the 1B4 antigen to a nuclear domain associated with a certain nuclear function would imply that the function of the 1B4 antigen is related to that of proteins within that domain.

The 1B4 antigen is distributed in 20-50 irregularly-shaped foci connected by thin fibrils throughout the nucleoplasm excluding the nucleolus. This distribution is similar to that described for several factors involved in pre-mRNA splicing, which have also been

identified as associated with the nuclear matrix. Pre-mRNA splicing is the removal of intron sequences from primary RNA polymerase II transcripts, and is known to be performed by small nuclear ribonucleoprotein particles (snRNPs) and several non-snRNP splicing factors. I found that the 1B4 antigen colocalised exactly with the non-snRNP splicing factor SC35. I verified this observation by confocal microscopy. The distribution of the 1B4 antigen partially colocalised with that of the snRNPs identified by the Y12 antibody. The 1B4 antigen also colocalised with SC35 through the cell cycle.

One model of the spatial distribution of factors involved in pre-mRNA splicing holds that the speckle domains are the sites of splicing factor storage, while splicing occurs along the fibrils and in the spaces connecting the speckle domains. Inhibition of transcription in HeLa cells results in the re-arrangement of the speckled domains into fewer, rounder structures lacking connecting fibrils. It is thought that the lack of nascent transcripts to be spliced results in less need of active splicing factors, and thus an accumulation of them in the speckles. I treated HeLa cells with actinomycin-D, α-amanitin, or 5,6-dicloro-1-ß-D-ribofuranosylbenzimidazole to inhibit transcription. I observed that the 1B4 antigen and SC35 maintained their colocalisation when the distributions of these antigens were rearranged. This colocalisation was also observed in cells recovering from transcriptional inhibition. The level of transcription in these cells was assayed by ^{3}H-uridine incorporation.

I also studied the distributions of the 1B4 antigen, SC35, the snRNPs identified by the Y12 antibody, and the hyperphosphorylated form of the large subunit of RNA polymerase II (Pol IIo) in other cell types. L6E9 rat myoblasts can be induced to leave the

cell cycle and fuse into myotubes without a concomitant decrease in their overall level of transcription. The distributions of the speckled domains of the 1B4 antigen, snRNPs, or Pol IIo were not altered in these cells upon differentiation. Unexpectedly, SC35 was almost undetectable by immunofluorescence labelling in these cells.

I also compared the distribution of the 1B4 antigen to that of SC35, the snRNPs, and Pol IIo in splenocyte cultures. These cultures are largely composed of B-lymphocytes which respond to concanavalin A via T-cell help. Stimulation by concanavalin A is thought to simulate the response of B-lymphocytes to antigen *in vivo*, resulting in an increased level of transcription in these cells. I determined that cells were stimulated by monitoring their level of transcription and observing their morphology. The 1B4 antigen did not appear to colocalise with either the snRNPs or SC35 in these cells. Its distribution appears similar to that of Pol IIo by conventional light microscopy. The lack of SC35 staining in these cells was unexpected and cannot be easily explained, though there are several reports in the literature of cell lines in which the distribution of splicing factors differs from that reported for HeLa cells.

The observed relationship between the 1B4 antigen and SC35 in HeLa cells suggests that the 1B4 antigen has a function related to pre-mRNA splicing and/or the spatial compartmentalisation of splicing factors in the nucleus. For more direct functional studies, it would be very useful to know the DNA sequence corresponding to the 1B4 antigen. I have described a cDNA with two zinc finger motifs identifed by the 1B4 antibody in Appendix II.

List of Abbreviations

CIAP	calf intestinal alkaline phosphatase
CSK	cytoskeletal buffer
DAB	diaminobenzidine
DNase	deoxyribonuclease
DRB	5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol bis-(ß-aminoethyl ether)N,N,N',N'-tetraacetic acid
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HCl	hydrochloride
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
hnRNA	heterogeneous nuclear ribonucleic acid
hnRNP	heterogeneous nuclear ribonucleoproteins
IPTG	isopropylthio-ß-D-galactoside
MEM	minimum essential medium
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIPES	piperazine-N,N'-bis(2-ethane-sulfonic acid)
PMSF	phenylmethylsulfonylfluoride
Pol IIo	hyperphosphorylated RNA polymerase II large subunit
poly(A)$^+$	polyadenylated messenger ribonucleic acid
mRNA	messenger ribonucleic acid
RNA	ribonucleic acid
RNase	ribonuclease
RNP	ribonucleoprotein
SDS	sodium dodecyl sulfate
snRNA	small nuclear ribonucleic acid
snRNP	small nuclear ribonucleoprotein particle
TBS	Tris buffered saline
TCA	trichloroacetic acid
TPCK	N-tosyl-L-phenyl chloromethyl ketone

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1. INTRODUCTION

1.1 Definition of the nuclear matrix

Berezney and Coffey (1974, 1977) were the first to identify a nuclear structure which they proposed to provide a framework for maintaining the shape of the nucleus and a foundation for nuclear organization. Initial skepticism towards the nuclear protein matrix proposed by Berezney and Coffey (1974) arose from the difficulties that researchers had faced when attempting to study isolated nuclei. Berezney and Coffey's matrix was studied in isolated nuclei that had been treated with DNaseI to loosen chromatin, after which low and high (2M sodium chloride) salt buffers were used to elute the partially digested chromatin and associated proteins. This extraction procedure was used to show that both newly synthesised DNA and transcribed sequences were associated with a nuclear matrix, which would provide a structural basis of nuclear organisation (Berezney and Coffey, 1977; Jackson *et al.*, 1981). Critics argued that the extraction procedure had caused artefactual binding of DNA and RNA to nuclear proteins. Nuclear matrix extraction protocols and knowledge of the role of the matrix in nuclear processes have evolved and overcome the criticism that met Berezney and Coffey's original proposal.

Several factors need to be considered when working with nuclei. The temperature and ionic environment to which isolated nuclei are exposed can generate artefacts. The estimated concentrations of DNA and RNA in the nucleus are in the order of 100 μg/ml, and the concentration of protein is thought to be considerably higher (Cook, 1988; Jack and Eggert, 1992). Such high concentrations of macromolecular solutes were thought to cause the formation of artefactual associations under non-physiological conditions. A subset of

proteins within isolated nuclei exposed to temperatures at or slightly below the physiological temperature become insoluble to extraction by detergents or high salt buffers (Jack and Eggert, 1992). Chromatin is soluble in low salt (one tenth the physiological salt concentration), insoluble at physiological salt concentrations, and again soluble at high salt concentrations (Hughes *et al.*, 1995). The addition of "stabilising" cations such as Mg^{2+} can reduce aggregation of chromatin, but this activates degradative nucleases (Cook, 1988), and produces artefactual attachments of chromatin to nuclear proteins (Hughes *et al.*, 1995).

Direct evidence supporting the existence of the nuclear matrix came from observations made using embedment-free electron microscopy (Penman, 1995). In conventional electron micrographs, chromatin masks other nuclear components, though Puvion and Bernhard (1975) had used the EDTA regressive stain technique to observe structures they believed to be ribonucleoprotein (RNP) domains in the interchromatin regions of the nucleus. Capco and colleagues (1982) found that they could observe small portions of the nuclear matrix in whole mount preparations of cells with flat, thin nuclei that had been extracted with Triton-X 100 to remove soluble proteins. However, few interior regions could be seen. This technique was improved upon four years later by Fey and colleagues (1986), who used resinless section electron microscopy in conjunction with a modified extraction procedure to identify a dense three-dimensional lattice of thick filaments bounded by the nuclear lamina. Their protocol included a mild initial Triton X-100 treatment, followed by a relatively gentle extraction with 0.25 M ammonium sulphate. The final steps of this protocol were sequential digests with DNase and RNase. This allowed

them to extract chromatin from the nucleus while retaining components such as the heterogeneous nuclear ribonucleoproteins (hnRNP), that could be lost using harsher extraction procedures. These researchers observed that the lattice of filaments was coated with 20-30 nm electron-dense particles that they suggested to be hnRNP in cells extracted using their technique. Further treatment of this interior matrix with RNase depleted the 20-30 nm particles and resulted in a distorted nuclear matrix morphology showing aggregation of the interior fibres. They noted that the morphology of the matrix observed using the original 2M NaCl extraction procedure of Berezney and Coffey (1974, 1977) was much different from theirs, particularly because the original extraction method removed the proteins associated with hnRNA.

Researchers designing nuclear matrix extraction protocols based upon that of Berezney and Coffey (1974, 1977) had noted that extracting the nuclear matrices of cells attached to a substratum (grown on coverslips rather than floating free in suspension) prevented the altered morphology caused by the collapse of intermediate filaments onto nuclear structures in preparations observed by light microscopy (Chaly *et al.*, 1985; Staufenbiel and Deppert, 1984). He and colleagues (1990) also extracted the whole cell rather than isolated nuclei, and were able to further refine the morphological definition of the nuclear matrix. Their protocol used the Triton-X 100 permeabilisation, DNase I digestion, and 0.25 M ammonium sulphate extraction of Fey and colleagues (1986) to obtain the RNP-containing nuclear matrix. They further extracted this structure by gradually increasing the ionic strength to 2M NaCl before the final nuclease digests. This uncovered highly branched structures with diameters of 9 and 13 nm which were termed

core filaments. Seventy percent of nuclear RNA, consisting of both ribosomal RNA precursors and high molecular weight (20 kb) heterogeneous nuclear RNA (hnRNA), were retained in the preparation of the core filaments. These core filaments are believed to be the underlying structure of the matrix. The interior nuclear matrix is bordered by the nuclear lamina and its attached intermediate filaments at the nuclear periphery.

An approach which averts the problem of chromatin aggregation under isotonic conditions is the use of agarose microbeads to encapsulate cells permeabilised in buffers possessing near physiological salt concentrations (Jackson *et al.*, 1988). More than 90% of the chromatin can be removed by electroelution. This technique has been used to visualise sites of *in vivo* DNA replication by electron microscopy of thick resinless sections of HeLa cells (Hozák *et al.*, 1993). This approach overcomes the problem of polymerase aggregation during chromatin extraction (Martelli *et al.*, 1990), and allows recovery of nearly all polymerase activity (Hozák *et al.*, 1993). As originally suggested by Berezney and Coffey in 1975, it is now accepted that active sites of replication are associated with the nuclear matrix. In this context, nuclear structure and function are closely related.

Since the original, widely-criticised report of a nuclear protein matrix by Berezney and Coffey in 1974, this structure is now accepted as a genuine and integral nuclear component. Berezney's current research concerns the identification and characterisation of nuclear matrix proteins, and the visualisation of functional domains within the *in situ* nuclear architecture (Berezney *et al.*, 1996). Nuclear function is now studied within a structural context, and colocalisation of related nuclear components is regarded as significant.

1.2 The nuclear matrix and mRNA metabolism

Functions associated with the nuclear matrix include DNA organisation (reviewed by Nelson *et al.*, 1986), DNA replication (Hozák *et al.*, 1993; reviewed by Hughes *et al.*, 1995), transcription (Jackson and Cook, 1985; Fakan *et al.*, 1986; Jackson *et al.*, 1993; reviewed by Hughes *et al.*, 1995), and pre-mRNA processing (Zeitlin *et al.*, 1987; Smith *et al.*, 1989; reviewed by Spector 1993; Huang and Spector, 1996). The nucleus is compartmentalised into numerous domains having specific biochemical functions which, lacking membrane boundaries, are identified by their composition using antibodies and oligonucleotide probes. Antibodies raised against nuclear cell fractions have been used to identify nuclear proteins that are components of the nuclear matrix as defined by their retention in nuclear matrix preparations. Characterisation of these protein functions and distributions within the nucleus is essential for understanding nuclear organisation and metabolism.

Two proteins important to mRNA metabolism are RNA polymerase II and the splicing factor SC35. The hyperphosphorylated form of the largest subunit of RNA polymerase II (Pol IIo) has been identified as a nuclear matrix protein (Bisotto *et al.*, 1995; Bregman *et al.*, 1995; Mortillaro *et al.*, 1996). RNA polymerase II transcripts are co-transcriptionally capped at their 5' ends, and post-transcriptionally polyadenylated at the 3' end (Lewis *et al.*, 1995). Removal of introns from pre-mRNAs (splicing) is performed by small nuclear ribonucleoprotein particles (snRNPs), aided by numerous non-snRNP splicing factors (reviewed by Green, 1991; Sharp, 1994). SC35 is an essential, non-snRNP splicing

factor (Fu and Maniatis, 1990), which has also been shown to resist detergent and nuclease extraction (Bisotto *et al.*, 1995). SC35 is a member of a family of arginine/serine-rich splicing factors termed SR proteins, which are important in spliceosome assembly and in splicing regulation (reviewed by Fu, 1995). A subpopulation of the largest subunit of Pol IIo colocalizes with SC35, being distributed in 20-50 nuclear foci or speckles connected by thin fibrils that are excluded from the nucleolus (Bisotto *et al.*, 1995). RNA polymerase II is also found diffusely distributed throughout the nucleus, excluding the nucleolus. However, only the foci or speckles are retained in nuclear matrix preparations.

The speckled distribution is characteristic of several proteins involved in pre-mRNA processing (reviewed by Spector, 1993; Huang and Spector, 1996). This distribution has been observed in several cultured cell lines. Moreover, a similar organisation of snRNPs has been observed in the nuclei of higher plants (Beven *et al.*, 1995), suggesting that this organisation of splicing-related factors is a key aspect of pre-mRNA metabolism in eukaryotes.

The snRNPs as identified with the Y12 antibody (Lerner *et al.*, 1981) are diffusely distributed in the nucleoplasm in addition to being concentrated in speckles. They are also found in 0-6 subnuclear domains termed coiled bodies (Andrade *et al.*, 1991; Carmo-Fonseca *et al.*, 1992). Y12 is a monoclonal antibody which, like anti-Sm autoantibodies produced by patients with autoimmune diseases, recognises 17, 26 and 27 kDa polypeptide components conserved among the U1, U2, U4/U6 and U5 (spliceosomal) snRNPs (Wooley, Zukerberg, and Chung, 1983; Petterson *et al.*, 1984). All SC35 domains are coincident with the speckles identified with the Y12 antibody (Carmo-Fonseca *et al.*,

1991a; Spector *et al.*, 1991); SC35 is excluded from coiled bodies (Carmo-Fonseca *et al.*, 1992). The distributions of these structures in a mammalian nucleus is represented in Figure 1-1.

Beyond the apparent colocalisation of a subset of speckles identified by the Y12 antibody, SC35, and Pol IIo, the capability of Y12 and other antibodies against splicing factors to immunoprecipitate Pol IIo indicates that splicing factors associate with Pol IIo *in vivo* (Kim *et al.*, 1997). Since complexes containing Pol IIo and snRNPs can also be prepared from mitotic cell extracts, it is unlikely that the association is dependent upon transcriptional activity (Kim *et al.*, 1997).

The speckled domains containing SC35 observed by light microscopy correspond to structures identified by electron microscopists as interchromatin granules (20-25 nm in diameter), while the connections between speckles correspond to perichromatin fibrils (reviewed by Fakan *et al.*, 1996; Puvion and Puvion-Dutilleul, 1996). Immunogold labelling studies related early studies of these three structures to the localisations of specific antigens (Spector *et al.*, 1983; Fakan *et al.*, 1984). Like the coiled bodies (0.5-1 μm diameter), interchromatin granules and perichromatin fibrils were first identified by electron microscopy. The function of coiled bodies is unknown. The lack of poly(A)$^+$ mRNA and splicing factors within these structures makes it unlikely that they are a site of pre-mRNA splicing (Bohmann *et al.*, 1995). The function of nuclear speckles (interchromatin granules) has been debated. Pre-mRNA splicing factors are thought to be stored in interchromatin granules, while splicing takes place along the perichromatin fibrils which are thought to represent emerging nascent transcripts (Fakan, 1994; reviewed by Huang and

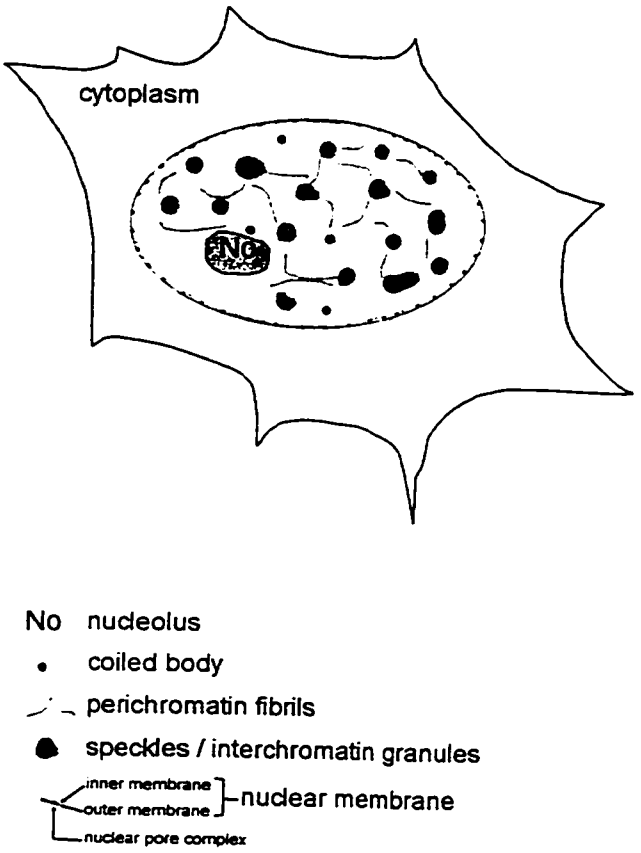

Figure 1-1. Cartoon showing the spatial organisation of subnuclear domains in the mammalian nucleus. There are typically 30-40 interchromatin granules (IG) or speckles in the region outside of the nucleolus. These are connected by perichromatin fibrils (PF). Zero to six coiled bodies (CB) may be present.

Spector, 1996). Recent evidence supporting this view includes the finding that the colocalisation of pre-mRNA splicing factors and RNA transcripts in the nucleus is dependent upon the presence of introns (Huang and Spector, 1996). Furthermore, the speckles do not contain nascent RNA (Pombo and Cook, 1996).

Another class of proteins important to RNA metabolism are the heterogeneous nuclear ribonucleoproteins (hnRNP) (reviewed by Dreyfuss *et al.*, 1993). Pre-mRNAs are a subset of the heterogeneous nuclear RNAs (hnRNAs) synthesised by RNA polymerase II. HnRNPs are defined as proteins that bind hnRNA *in vivo* but are not stable components of other ribonucleoprotein complexes such as the snRNPs (Weighardt *et al.*, 1996). Recent studies characterising individual hnRNP proteins indicate that they have roles in pre-mRNA splicing (Gattoni *et al.*, 1996; Mahé *et al.*, 1997) and are components of the nuclear matrix (Holzmann *et al.*, 1997; Mattern *et al.*, 1996). Most of the approximately 20 major hnRNPs known in human cells are diffusely distributed throughout the nucleoplasm excluding the nucleolus. None have been identified with the speckled distribution characteristic of SC35.

The distributions of several proteins important to mRNA metabolism are altered during mitosis. SC35, the spliceosomal snRNPs, and Pol IIo are all diffusely distributed in mitotic cells. In addition, they are concentrated in a small number of bright round foci termed mitotic clusters (Leser *et al.*, 1989). The function of these foci, which are most prominent in metaphase/anaphase, is not known. They do not correspond to coiled bodies: the 80 kDa protein p80 coilin which is generally considered to be a specific marker for coiled bodies is diffusely distributed in mitotic cells (Andrade *et al.*, 1993; Carmo-Fonseca

et al., 1993). As transcription is suspended during mitosis, the Pol IIo and splicing factors are not active in pre-mRNA metabolism, since they are diffusely distributed and concentrated in mitotic granule clusters.

The distributions of SC35, the spliceosomal snRNPs and Pol IIo are also rearranged in cells exposed to drugs that inhibit transcription. Exposure to drugs that inhibit transcription by all RNA polymerases (actinomycin-D) or specifically inhibit RNA polymerase II (α-amanitin) has been observed to result in an altered distribution of splicing factors, with the SC35-defined speckled domains becoming larger and rounder, concomitant to the loss of connecting fibrils (reviewed by Spector, 1996). This effect has been shown to be reversible by using the drug 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole (DRB), which inhibits RNA polymerase II and can be "washed out" as it easily passes through cell membranes (Tamm *et al.*, 1976). These observations support the model of splicing factors being shuttled from the sites of storage (speckles) to sites of pre-mRNA processing (fibrils) (Spector, 1993). The rationale for this is that inactive splicing factors would be concentrated in storage sites in the absence of nascent transcripts.

Since Pol IIo and splicing factors have been determined to be retained in nuclear matrix preparations, it is highly suggestive that the nuclear matrix is involved in mRNA metabolism. The nuclear matrix is a dynamic structure that is rearranged during the cell cycle and in response to such stimuli as the inhibition of transcription. The involvement of the nuclear matrix with pre-mRNA metabolism may be indirect, as the speckled domains of matrix-associated proteins are likely to be significant sites of Pol IIo and splicing factor storage, rather than sites of active synthesis and processing of mRNA.

1.3 Rationale for characterization of the 1B4 antigen

The 1B4 antibody is an IgE secreted by a hybridoma cell line established by M. Paulin-Levasseur. This cell line is one of a library prepared by Paulin-Levasseur and colleagues, using a nuclear matrix extract from HL-60 cells to immunize balb/c mice. At the time that this study was begun, immunofluorescence studies had shown that the 1B4 antigen is conserved in several cell types (Figure 1 in Appendix I), including those of plant origin. The 1B4 antigen is distributed in irregularly shaped speckles connected by thin fibrils in the nucleus excluding the nucleolus in all cell types tested. This distribution has also been observed by electron microscopy (Figure 2 in Appendix I).

Interest in nuclear antigens having speckled distributions initially arose from the finding that sera from patients with certain autoimmune diseases identified antigens localized in speckles. Researchers believed that characterising autoantigens would help to elucidate the etiology of autoimmune disease (Tan, 1991). Before the Y12 hybridoma was created, the Sm antigen was defined as the target of sera from a patient with systemic lupus erythematosus (Lerner and Steitz, 1979). Autoimmune diseases have characteristic spectra of autoantibodies specific for certain intracellular antigens. From the serum of a single patient, autoantibodies directed against nuclear matrix components are often found in conjunction with other autoantibodies, including anti-U1 snRNP antibodies, in patients with mixed connective tissue disease, systemic lupus erythematosus, and rheumatoid arthritis (Deng *et al.*, 1996). While the etiology of these autoimmune diseases is still unknown, researchers hope characterising the proteins identified by autoantibodies will eventually contribute to a comprehensive understanding of autoimmune disease (Deng *et al.*, 1996).

Though it was once thought that the nucleolus is the only sub-nuclear domain with a specialised function, the discovery of antibodies which label specific nuclear constituents, and the development of *in situ* molecular probe technology have led to the current view that the nucleus contains several distinct extranucleolar compartments. Several researchers have approached the problem of assigning functions to nuclear domains by inferring that nuclear components with coincident distributions have related functions (Berezney *et al.*, 1996; Clemson and Lawrence, 1996). The non-snRNP splicing factor SC35 colocalises with the large subunit of RNA polymerase II, and with a subset of domains containing snRNPs (Bisotto *et al.*, 1995). Biochemical assays have been used to verify that RNA polymerase II, spliceosomal snRNPs, and SC35 have interrelated functions. Transcription by RNA polymerase II is coordinated with pre-mRNA splicing by the spliceosomal snRNPs (Mortillaro *et al.*, 1996; Vincent *et al.*, 1996). SC35 is required for the first step of the splicing reaction and can influence splice site selection (reviewed by Fu, 1995). The similarity of the speckled distribution identified with the 1B4 antibody to the distributions of factors involved in pre-mRNA splicing suggested that it would be worthwhile to determine whether the 1B4 antigen might have a function related to pre-mRNA metabolism.

The observation that the 1B4 antigen is conserved in several cell types of diverse origin indicates that its function would relate to a process that is not specific to a single cell type. Highly conserved proteins are often involved in vital cell functions. Pre-mRNA processing is an important nuclear function, and factors associated with it are highly conserved. Homologues to several splicing factors have been identified in yeast (*e.g.* Bennett and Reed, 1993; Chiara *et al.*, 1994; Fabrizio *et al.*, 1994; Lauber *et al.*, 1996),

Drosophila (*e.g.* Kim *et al.*, 1992; Heinrichs and Baker, 1997), and plants (*e.g.* Beven *et al.*, 1995; Concha *et al.*, 1995). Antibodies to the snRNPs and SC35 have been used to stain *Chironomus tentans* salivary gland chromosomes (Baurén *et al.*, 1996; Wieslander *et al.*, 1996), and to identify structures containing splicing factors in amphibian germinal vesicles (*e.g.* Wu *et al.*, 1991; Wu *et al.*, 1996). Since antibodies to known splicing factors are such useful tools in cell biology, it follows that the protein identified by the 1B4 antibody should be investigated for its potential to add to the growing knowledge of nuclear function and structure.

While the SC35 antibody was raised against spliceosomes and later found to recognise a component of the nuclear matrix (Bisotto *et al.*, 1995), the 1B4 antibody was raised against a nuclear matrix extract of HL-60 cells. Verification that the 1B4 antigen is a component of the nuclear matrix was an essential part of the characterisation of this antigen. It was also important to establish whether the distribution of 1B4 is coincident with, or only similar to, that of SC35. To this end, I performed double-labelling immunofluorescence studies using the 1B4 antibody in addition to either the anti-SC35 antibody or the Y12 antibody to the snRNPs, and further assessed my observations by confocal microscopy.

The speckled distributions of several factors involved in pre-mRNA splicing are altered during mitosis or when transcription is inhibited with drugs (reviewed by Spector, 1996). Though the exact function of the speckled domains is still debated (see Huang and Spector, 1996; Clemson and Lawrence, 1996), the factors maintain colocalisation when their distributions are altered, and this has been interpreted as evidence of related functions (*e.g.* Antoniou *et al.*, 1993; Carmo-Fonseca *et al.*, 1992; Lallena and Correas, 1997). I

compared the distribution of 1B4 to that of SC35 and the spliceosomal snRNPs in HeLa cells through the cell cycle and in HeLa cells treated with drugs that inhibit transcription. Furthermore, I attempted to quantify the "fewer, larger" speckles seen in drug-treated cells.

To ensure that the redistribution of antigens observed in HeLa cells could be attributed to the inhibition of transcription with drugs, I used three different drugs having different modes of action. I also followed the fate of the 1B4 antigens treated with reversible transcriptional inhibitors during a recovery period. Throughout these experiments, I measured the level of transcription present in these cells by assaying their capacity to incorporate tritiated uridine. Similar studies studying the rearrangement of the speckled domains did not assay the level of transcription in drug-treated cells (*e.g.* Carmo-Fonseca *et al.*, 1991a, 1991b; Spector *et al.*, 1993).

Though the link between transcription and splicing is widely accepted (Mortillaro *et al.*, 1996; Spector *et al.*, 1996), I thought it would be interesting to study the distribution of splicing factors in cells undergoing complex physiological processes. Splenic lymphocytes in a quiescent state have a very low level of transcription. They can be stimulated to enter the cell cycle by the plant lectin concanavalin A, upon which their level of transcription is greatly increased in conjunction with an increase in cell size and volume of the cytoplasm relative to the nuclear volume (Setterfield *et al.*, 1983). The staining intensity of snRNPs observed using the Y12 antibody has been shown to increase in stimulated cells (Chaly, *et al.*, 1988; Daev *et al.*, 1994), though the distribution of SC35 in murine lymphocytes has not previously been reported. I compared the distribution of the 1B4 antigen to those of SC35 and snRNPs in murine lymphocytes using confocal microscopy. The increase in

transcription that accompanied lymphocyte stimulation was monitored by ^{3}H-uridine incorporation.

I was also interested in examining the distributions of the 1B4 antigen and splicing factors in a cell line that undergoes a complex physiological process without a concomitant change in the level of transcription. The rat myoblast cell line L6E9 (Yaffe, 1968) is capable of irreversibly withdrawing from the cell cycle upon differentiation into myotubes. However, differentiated myotubes in culture continue to synthesise mRNA and proteins at pre-fusion levels (Krauter *et al.*, 1979; Benoff and Nadal-Ginard, 1980). I observed the distribution of the 1B4 antigen and splicing factors in both cycling and differentiated L6E9 cells, which has not been previously reported in the literature.

Immunofluorescence colocalisation data indicating that a nuclear protein is restricted to a certain domain of the nucleus can be interpreted as suggesting that the protein is therefore likely to have a function related to those of other proteins localised in that domain. If the 1B4 antigen were consistently found to colocalise with splicing factors, then this would indicate that its function is related to pre-mRNA splicing. I also wanted to use the 1B4 antibody to obtain direct evidence of its function. The molecular weight and DNA sequence coding for a protein are very useful characteristics which can be the basis of experiments to elucidate the function of a protein. Unfortunately, the 1B4 antibody does not bind denatured protein preparations of interphase cells, though a 100 kDa signal is obtained in Western blots of mitotic HeLa cell extracts. I have performed several experiments attempting to obtain a signal with the 1B4 antibody in interphase cells. I have also contributed towards the cloning of this protein. Since the clone I worked with has not

yet been validated as encoding the 1B4 antigen, the methods used and results obtained thus far are included in Appendix II. My supervisor, M. Paulin-Levasseur, is collaborating with the group of B. Chabot at the Université de Sherbrooke to test whether the 1B4 antigen participates directly in the splicing reaction.

2. MATERIALS AND METHODS

2.1 Cell culture

2.1.1 HeLa cells

HeLa (CCL2) or HeLa S3 (CCL2.2) cells obtained from the American Type Culture Collection (Rockville, MD) were grown in monolayer culture in modified Eagle's minimum essential medium (αMEM; Gibco, Burlington, ON) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and 1% antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin, Gibco) at 37°C, in 5% CO_2.

To inhibit transcription, HeLa (CCL-2) cells were treated with actinomycin-D (50 μg/ml stock in 95% ethanol) at concentrations of 0.5 and 0.05 μg/ml for 1, 2, 4, and 8 hour exposures. DRB (3.5 mg/ml stock in 95% ethanol) was used at a final concentration of 25 μg/ml for a 2 hour exposure. α-Amanitin (1 mg/ml stock in 95% ethanol) was used at a final concentration of 5 μg/ml for a 5 hour exposure. All drugs were supplied by Sigma (St. Louis, MO). Control cells were treated with equivalent concentrations of ethanol. To allow recovery from 2 hour treatments of 0.5 μg/ml actinomycin-D or 25 μg/ml DRB, drug-containing medium was replaced with pre-warmed αMEM and the cells incubated at 37°C for a further 2-18 hours.

Cells were synchronised for the preparation of a mitotic cell extract for Western blotting. Nocodazole (750 μg/ml stock in 95% ethanol; Sigma) was diluted to a final concentration of 75 ng/ml in the medium of subconfluent cell cultures. This drug inhibits microtubule polymerisation (Zieve *et al.*, 1980). After 20 hours exposure, mitotic cells were harvested by mechanical agitation. Interphase cells, which adhere more strongly to

the bottom of 90 mm petri dishes, remained attached to the dish while the mitotic ones were collected with the overlying medium. The preparation of proteins from cell homogenates for Western blotting is described in Section 2.7.

2.1.2 L6E9 rat myoblasts

Cycling L6E9 cells obtained from Dr. M. McBurney of the University of Ottawa were grown at a sub-confluent density in monolayer culture at 37°C in 5% CO_2. To induce differentiation into myotubes, the concentration of fetal bovine serum present in the medium (αMEM supplemented with 10% heat-inactivated fetal calf serum and 1% antibiotics as for HeLa cells) was decreased from 10% to 2% of the total volume and the cell density allowed to increase for 3 days so that fused cells were seen as described by Yaffe (1968).

2.1.3 Murine splenocytes

Murine splenocytes were isolated from female balb/c mice aged 8-12 weeks. The mice were sacrificed by cervical dislocation, and the spleens aseptically transferred to a fine mesh screen in a 60 mm petri dish with 4 ml complete medium (RPMI (Flow Laboratories, Mississauga, ON) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine (Sigma), and 1% antibiotics. Cells for each experiment were obtained from a single mouse. The spleens were squashed with a blunt object until they appeared colourless, indicating that the splenocytes present had been released into the medium. The splenocyte-containing medium was passed through a 20G1½ needle three times to break up clumps of cells, and the cell solution in RPMI laid over 4 ml cold FBS as a distinct layer

for 5 minutes on ice. The top layer was then drawn off and centrifuged for 5 minutes at 2500 rpm. After discarding the supernatant, the cell pellet was resuspended in 4 ml cold (4°C) 0.15 M ammonium chloride (NH_4Cl) and set on ice 10 minutes to lyse erythrocytes. This solution was underlaid with 4 ml cold FBS before centrifuging 5 minutes at 2500 rpm. The supernatant containing lysed erythrocyte debris was discarded and the beige cell pellet resuspended in 10 ml complete medium. Cells were seeded at 2 x 10^6 cells/ml/cm^2. Counts of viable cells were determined by their ability to exclude trypan blue (0.8% trypan blue in 4.5% NaCl). Cells were incubated at 37°C, 5% CO_2 in upright 50 ml canted neck flasks with the lids loosely capped, and fed each day with 10% of the initial seeding volume of complete medium.

Splenocytes were stimulated the day after isolation with 5 μg/ml concanavalin A (Sigma). Unstimulated controls were maintained in parallel with each stimulated flask of cells.

2.2 Immunofluorescence

Monolayer cultures were seeded on sterile glass coverslips and fixed at a sub-confluent density. Round cells (splenocytes) were attached to coverslips that had previously been coated with 0.1% poly-L-lysine (Sigma) for 5 minutes. This involved setting a concentrated, small volume of cells on dry prepared coverslips for 10 minutes at room temperature in a humidity chamber, then rinsing briefly prior to fixation.

Cells were routinely rinsed twice for 30 seconds in phosphate-buffered saline (PBS; 1.3 mM NaCl, 50 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 7.0) before being fixed for 3

minutes in 3% phosphate-buffered (pH 7.0) paraformaldehyde (JBS, EM grade, Dorval, PQ), as described by Chaly and colleagues (1984). The 1B4 antigen is also well preserved in cells fixed and permeabilised in cold (-20°C) methanol for 10 minutes. After two 30 second rinses in PBS, cells fixed in paraformaldehyde were rinsed three times for 4 minutes in 1 mg/ml sodium borohydride (BDH, Toronto, ON) in PBS to reduce free aldehyde groups. They were then permeabilised in PBS containing 0.2% Triton X-100 (BDH) for 20 minutes. Following another three 4 minute rinses in PBS, the primary antibody diluted in PBS was applied for 1 hour at room temperature. This was rinsed three times for 4 minutes with PBS before the secondary antibody was applied. Cells were also incubated with the secondary antibody for 1 hour at room temperature. In double-labelling immunofluorescence experiments, the second set of primary and secondary antibodies were applied following the first set, such that the staining procedure for single-labelling was repeated with different antibodies. As in single-labelling experiments, cells were rinsed three times for 4 minutes with PBS before applying antibodies, and incubations were conducted for 1 hour at room temperature. All steps of the staining procedure were carried out at room temperature.

Cells were counterstained with 1 μl/ml Hoescht 33258 in PBS prior to mounting in 0.1% *p*-phenylene diamine in 50% glycerol/PBS (v/v), pH 7.0.

2.2.1 Antibodies

As mentioned in Section 1.3, the 1B4 antibody is a murine monoclonal antibody secreted by the 1B4 hybridoma cell line established by M. Paulin-Levasseur. This antibody

was analysed with an isotyping kit which recognises IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, and IgE (Innogenics, Antwerp, Belgium). The 1B4 antibody is an IgE with κ light chains. Hybridoma supernatant was used undiluted for immunofluorescence staining, and used directly or at a 1:10 dilution for immunoblotting.

The anti-SC35 antibody described by Fu and Maniatis (1990) is an IgG1 that recognises the 35 kDa non-snRNP splicing factor SC35. A sample of anti-SC35 hybridoma supernatant used for preliminary experiments was generously supplied by Dr. T. Maniatis of Harvard University. Hybridoma supernatant for further experiments was obtained from cells ordered from the American Type Culture Collection (ATCC CRL-2031). For immunofluorescence experiments, hybridoma supernatant was typically diluted 1:10 in PBS. It was used direct for Western blotting.

The Y12 antibody described by Lerner and colleagues (1981) is an IgG2a that recognises the 17, 26, and 27 kDa polypeptide components conserved among the U1, U2, U4/U6 and U5 snRNPs (Wooley, *et al.*, 1983; Petterson *et al.*, 1984). The hybridoma cell line secreting this antibody was obtained from Dr. D.L. Brown of the University of Ottawa. It is described in the American Type Culture Collection catalogue, but is not distributed through that organisation. For immunofluorescence experiments, hybridoma supernatant was typically diluted 1:10 in PBS. It was used direct for Western blotting.

A monoclonal (IgG) antibody to the trimethylguanosine (m3G) cap of U1, U2, U4, U5 and U6 snRNAs was obtained from Oncogene Science (distributed by Cedarlane Laboratories, Hornby, ON). It was used at a dilution of 1:500 to stain nuclear matrix preparations.

The monoclonal (IgG2a) antibody CC-3, which recognises Pol IIo, was originally described by Thibodeau and Vincent (1991). An aliquot was generously provided by Dr. M. Vincent of the Université Laval. I diluted the hybridoma serum concentrate 1:75 for immunofluorescent staining. I diluted the same batch of antibody 1:2000 - 1:10000 for immunoblotting.

The 2H12 antibody is an IgG1 which recognises a phosphoepitope of a human isoelectric variant of the nucleolar protein B23 (Paulin-Levasseur *et al.*, 1995). This antibody was used as a positive control at a dilution of 1:20 (hybridoma supernatant dated 01.11.94) in the immunofluorescence assay to determine if the reactivity of the 1B4 antibody is dependent upon phosphorylation of its epitope (Section 2.3). I also used it as a positive control for immunoprecipitation experiments, as it does precipitate itself.

In double-label immunofluorescence studies involving the 1B4 antibody and antibodies against the snRNPs, SC35, m3G-capped snRNAs, or Pol IIo, the 1B4 antibody and its secondary were applied following incubations with the other primary antibody and its secondary. A rat anti-mouse antibody specific for the IgE heavy chains conjugated to fluorescein isothiocyanate (FITC; Serotec, Toronto, ON) was used at a dilution of 1:100 to identify the 1B4 antigen in double-labelling studies. This antibody did not cross-react with the sheep anti-mouse CY3-conjugate (Jackson Immunoresearch, West Grove, PA) used at a dilution of 1:400 to recognise IgG primary antibodies to the snRNPs, SC35, m3G-capped snRNAs, and Pol IIo. For single-label immunofluorescence using the 1B4 antibody, a FITC-conjugated goat anti-mouse Ig was used (Cappel, Durham, NC) at a dilution of 1:100. A donkey anti-mouse IgG conjugated to CY3 (Jackson Immunoresearch) was

diluted 1:400 for single-label staining of snRNPs, SC35, Pol IIo, 2H12, or the m3G cap of snRNAs. All secondary antibodies were diluted in PBS.

2.2.2 Conventional light microscopy and confocal laser scanning light microscopy

Stained cells were viewed using a Zeiss Axiophot microscope equipped for epifluorescence illumination. Fields of greater than 200 cells were viewed and immunofluorescent staining distributions recorded for representative cells. Photographs were taken using Ilford XP2 400 film (Mobberley, England), or Kodak Elite II Ektachrome 400 film (Toronto, ON). Colour slides reproduced as figures in this thesis were scanned at a resolution of 72 dots per inch (dpi). Confocal images were obtained using an upright Leica CLSM system (Leica, West Germany) equipped with an Ar/Kr mixed gas laser. Cells were scanned briefly to find the plane with the most speckles in focus. This single plane was scanned using a filter which allowed both FITC and CY3 luminescence to be detected simultaneously.

2.3 Assay to test phosphodependence of the reactivity of the 1B4 antibody to its epitope

The assay of Thibodeau and Vincent (1991) was used to determine whether the reactivity of the 1B4 epitope is sensitive to dephosphorylation. Cells grown to sub-confluent density on glass coverslips were rinsed with Tris buffered saline (TBS; 50 mM Tris base, 150 mM NaCl) pH 7.5, fixed for 15 minutes in cold (-20°C) methanol, and rinsed again in TBS pH 7.5. Treatment with 100 units/ml calf intestinal alkaline

phosphatase (CIAP; 713 023, Boehringer Mannheim, West Germany) was carried out in Tris-$MgCl_2$ buffer (100 mM Tris base, 1 mM $MgCl_2$, pH 8.0) containing protease inhibitors (5 μg/ml antipain, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonlyfluoride (PMSF); all from Sigma) for 2 hours at 37°C followed by 1 hour at room temperature. Control cells were exposed to 50 mM tetra-sodium pyrophosphate (BDH) in addition to CIAP or left in Tris-$MgCl_2$ buffer with protease inhibitors for the same incubations at the same temperatures and durations as the CIAP-treated cells. Following these treatments, cells were stained with either the 1B4 or 2H12 antibodies according to the procedures for regular immunofluorescence (Section 2.2), except that TBS pH 7.2 was used in place of PBS pH 7.0.

2.4 Peroxidase staining and quantification of speckled distributions

Cells on coverslips were fixed in paraformaldehyde and permeabilised as described in Section 2.2 (Chaly *et al.*, 1984). Following a 1 hour incubation with primary antibody (1B4 undiluted or anti-SC35 diluted 1:10 in PBS), cells were rinsed 3 times for 4 minutes in PBS pH 7.0. A biotinylated sheep anti-mouse Ig (Cappel) antibody was then applied at a dilution of 1:200 in PBS pH 7.0 for 30 minutes. Following 3 rinses in PBS pH 7.0, streptavidin conjugated to horseradish peroxidase (Amersham, Oakville, ON) was applied at a dilution of 1:200 in PBS pH 7.4 with 0.15% gelatin for 15 minutes. After this step the cells on coverslips were rinsed 3 times in PBS pH 7.4 with 0.15% gelatin. The colour was developed in the dark with a solution containing 0.5 mg/ml diaminobenzidine (DAB; Sigma) and 0.02% hydrogen peroxide in PBS pH 7.4. Following three rinses in PBS pH

7.4, the coverslips were passed through 70% and 95% alcohol rinses, and allowed to completely air-dry. They were then mounted in DPX medium (BDH).

The speckles, which appeared brown, were viewed by light microscopy using a Zeiss Axiophot microscope in conjunction with a VIDAS image analysis system. The number of distinct spots in 200 nuclei were counted for each treatment group.

2.5 Nuclear matrix preparation

Two different protocols for preparing nuclear matrices were followed. The first was described by Chaly and colleagues (1985), while the second was that of Fey and colleagues (1986). Using either protocol, matrices were prepared from HeLa cells grown on sterile glass coverslips by either submerging the coverslips in approximately 5 ml of the appropriate solution in a weighboat, or by applying a small amount of solution to the coverslip in a humidity chamber (nuclease digestions). A 4 minute incubation time was consistently used for all rinses or washes. Matrix preparations were fixed in 3% paraformaldehyde (pH 7.0) for 5 minutes, and stained with antibodies to the 1B4 antigen, SC35 (positive control), snRNPs, or the m3G caps of small nuclear RNAs (negative control).

As described by Chaly and colleagues (1985), protease inhibitors (1 mM PMSF, 1 mg/ml soybean trypsin inhibitor, 2 mM sodium tetrathionate dihydrate; all ordered from Sigma) were included in all solutions, which were adjusted to pH 7.4 unless otherwise noted. Medium was rinsed from cells with sucrose-Tris-magnesium buffer (STM; 0.05M sucrose, 2 mM Tris base, 0.4 mM $MgCl_2$, 5 mM KCl), pH 6.2, at 25°C. Soluble proteins

were extracted with 0.5% Triton X-100 in STM for 25 minutes. After washing in STM, cells were treated with 10 μg/ml deoxyribonuclease I type IV from bovine pancreas (DNase I; E.C. 3.1.4.5, Sigma) in STM at 4°C for 30 minutes. Cells were rinsed in 4 ml low magnesium buffer (LMB; 0.04 mM $MgCl_2$, 2 mM Tris base) for 15 minutes on ice. Then 4 ml volumes of 2M high-salt buffer (HSB; 2M NaCl in LMB) and 4M HSB (4M NaCl in LMB) were sequentially added very slowly for 15 minute incubations. Digestion with either 50 μg/ml DNase I or 50 μg/ml DNase I in combination with 50 μg/ml ribonuclease A type IIA from bovine pancreas (RNase A; E.C. 3.1.4.22, Sigma) in LMB was carried out at 4°C for 20 minutes. Nuclear matrix preparations were rinsed in STM briefly before fixation.

To prepare nuclear matrices according to the protocol of Fey and colleagues (1986), I prepared a stock of cytoskeletal buffer (CSK; 300 mM sucrose, 10 mM piperazine-N,N'-bis(2-ethane-sulfonic acid) (PIPES; pH 6.8), 3 mM $MgCl_2$, 1 mM ethylene glycol bis-(ß-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), 1.2 mM PMSF). All incubations were performed at 4°C unless otherwise specified. After an initial rinse in PBS pH 7.0, cells were extracted with CSK containing 0.5% Triton X-100, 2 mM vanadyl adenosine, and 100 mM NaCl for 3 minutes. They were then rinsed for 4 minutes in CSK containing 100 mM NaCl prior to a 5 minute extraction using CSK containing 250 mM ammonium sulfate. A DNase I (100 μg/ml) digestion was performed at 20°C for 20 minutes in CSK containing 2 mM vanadyl adenosine, and 50 mM NaCl. Cells were then extracted for 5 minutes in CSK containing 2 mM vanadyl adenosine, 50 mM NaCl, and 250 mM ammonium sulfate. Following a rinse in CSK containing 2 mM vanadyl adenosine and 100

mM NaCl, cells were incubated at 20°C for 10 minutes in a solution of CSK containing 25 μg/ml RNase A and 100 mM NaCl. They were then rinsed in CSK containing 100 mM NaCl before fixation.

2.6 Assay of level of transcription

Transcription was assayed by ^{3}H-uridine incorporation. HeLa cells in a 10 ml volume seeded at 2 x 10^{5} cells/dish 48 hours prior to the assay were incubated with a 50 μCi pulse of ^{3}H-uridine for 2 hours. After the 2 hour period the medium was discarded and the cells scraped from the dish with a rubber policeman in 3 ml of EGTA buffer (10 mM Tris acetate pH 7.5, 150 mM NaCl, 1 mM EGTA). Triplicate 1 ml samples were obtained from each dish as the suspended cells were distributed into the wells of a cell harvester. Splenocytes seeded at a density of 2 x 10^{6} cells/ml were divided into triplicate 1 ml aliquots and pulsed with 5 μCi of ^{3}H-uridine for 2 hours, after which each 1 ml of medium with the cells in suspension was loaded into a well on the cell harvester. Cells (HeLa or splenocytes) deposited on 2.4 cm Whatman glass microfibre filters were rinsed 3 times with cold saline solution (70 mM NaCl), twice with cold 5% trichloroacetic acid, and once with cold 90% ethanol. The filters were submerged in CytoScint scintillation fluid (ICN Biomedicals Inc., Irvine, CA) and analyzed with a Tri-Carb 2500 TR Liquid Scintillation Analyzer (Canberra Packard Canada).

2.7 Immunoprecipitation and Western blotting

Homogenates of whole HeLa cell proteins were prepared by scraping cells grown in monolayer culture from a dish with a rubber policeman in EGTA buffer at 4°C. Cell pellets were rinsed with EGTA buffer and transferred to 1.5 ml Eppendorf tubes. After a 5 minute spin at 3000 rpm, the supernatant was removed and the cell pellet was then suspended in 10% TCA and incubated at 40°C for 15 minutes. Precipitated proteins were again spun down (5 minutes, 3000 rpm), rinsed with 5% TCA, spun down, resuspended in cold (-20°C) 99% acetone, and spun again. After removing the acetone supernatant, the pellet was allowed to dry briefly before being resuspended in Laemmli buffer (2% sodium dodecyl sulfate (SDS; Bio-Rad), 10% glycerol, 10 mM Tris pH 6.9, 25 mM DTT, 0.005% bromophenol blue; Laemmli, 1970). Protein samples were stored at -80°C and sonicated briefly before use. I also prepared homogenates omitting the TCA precipitation step. As these did not produce an improved signal with the 1B4 antibody, I routinely included the precipitation step when preparing proteins for Western blotting.

To prepare isolated nuclei, cells harvested and washed in EGTA buffer were resuspended in sucrose solution (250 mM sucrose, 50 mM triethanolamine pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF). After rupturing plasma membranes by 10-15 strokes in a homogeniser, cells were centrifuged at 6000 rpm for 20 minutes at 4°C. Proteins were precipitated from these nuclei with TCA as described for whole cells.

For immunoprecipitation experiments, I prepared HeLa whole-cell homogenates from 5 dishes of confluent cells as described except that I suspended the sample in 1 ml immunoprecipitation buffer (0.15 M NaCl, 10 mM Tris pH 7.5, 2 mM sodium fluoride,

2 mM sodium pyrophosphate, 1 mM PMSF, 1 mM N-tosyl-L-phenyl chloromethyl ketone (TPCK), 5 mM ammonium vanadate) instead of Laemmli buffer. Half of this was used for each immunoprecipitation trial. All steps were carried out with gentle agitation at 4°C. The sample was first cleared for nonspecific binding to protein-A sepharose (add 7.5 μl protein-A sepharose for each 100 μl of protein solution) for 1 hour. The protein-A was pelleted by a very brief spin, and the supernatant taken into a new tube. Primary antibody was added at a 1:10 dilution to the total sample volume and incubated for 1 hour. I used the 1B4 antibody for these experiments, as well as the 2H12 antibody, which served as a positive control. The secondary antibody (Cappel rabbit αmouse IgG) was added at a 1:200 dilution to the total sample volume and incubated for 1 hour. After allowing the secondary antibody to bind to the primary antibody, protein-A sepharose was added (7.5 μl per 100 μl sample volume) and incubated for 1 hour. The sample was spun just long enough to obtain a pellet below a clear supernatant. The supernatant was removed and a portion of it mixed with 5X concentrated Laemmli buffer so that the final concentration of Laemmli buffer in the sample was 1X for use as a negative control. The immunoprecipitate pellet was rinsed 3 times with 1 ml of immunoprecipitation buffer by drawing it through a needle. After removing the supernatant immunoprecipitation buffer used in the third rinse, the pellet was suspended in 40 μl of Laemmli buffer. The sample was heated to 80°C for 10 minutes to release the antibody bound to protein A before PAGE and Western blotting.

Polyacrylamide gel electrophoresis (PAGE) was performed according to standard protocols (Harlow and Lane, 1988). Mini-gels were cast using Bio-Rad equipment (Richmond, CA). Samples were run at 100V through the stacking gel (5% acrylamide),

after which the voltage was raised to 200V while the samples passed through the resolving gel (12% acrylamide). A duplicate of each gel was stained with Coomassie blue to estimate the amount of protein loaded. Biotinylated or Coomassie blue-stained standards (Bio-Rad), were used to estimate apparent molecular weights (Weber and Osborn, 1969).

For Western blots, proteins separated by PAGE were transferred onto nitrocellulose membrane (Schleicher and Schuell, Keene, NH; distributed in Canada by Xymotech Biosystems, Toronto, ON) overnight at 4°C using a 40V potential difference. Protein transfer was checked using Ponceau red to detect proteins on the nitrocellulose membrane. Carnation milk in PBS-Tween (5% w/v milk in PBS with 0.05% Tween-20) was used as a blocking agent, after which the blot was incubated in primary antibody diluted in PBS-Tween. Incubations with the 1B4 or 2H12 antibodies were typically performed for 1 hour with gentle agitation at room temperature. The immunoreactivity of the Y12, anti-SC35, and CC-3 antibodies were also tested by Western blotting, using them in 1 hour incubations with gentle agitation at room temperature. Alternately, the primary antibody was sometimes left overnight with gentle agitation at 4°C. Between incubations, blots were rinsed three times (10 min) with PBS-Tween, using gentle agitation at room temperature. The secondary antibody used was an biotinylated anti-mouse Ig (Amersham) at a dilution of 1:5000 in PBS-Tween. Following a 45 minute incubation with streptavidin peroxidase (Amersham) diluted 1:2000 in PBS-Tween, the blots were rinsed in PBS-Tween, then twice in PBS. Signals were detected with the use of an Amersham ECL kit and Hyperfilm.

As mentioned in the Introduction (Section 1.3), the 1B4 antibody does not produce a signal in Western blots of interphase HeLa cell homogenates. A dot blot was performed

under non-denaturing conditions by Mrs. Guoxiang Chen. This involved dotting 1-2 μl of urea extract (5 mg/ml in PBS) from HeLa S3 cells onto nitrocellulose. The nitrocellulose was immunoblotted with the 1B4 antibody at a dilution of 1:20, followed by the same detection system used for Western blots. The urea extract was prepared by Dr. M. Paulin-Levasseur using the protocol of Paulin-Levasseur and colleagues (1989).

Blencowe and colleagues (1994) used the denaturation/renaturation protocol of Vinson and colleagues (1988) to successfully renature the epitope of a nuclear matrix protein. This procedure involves denaturing proteins transferred to nitrocellulose with guanidine hydrochloride (HCl), then renaturing them by gradually rinsing away this reagent before blotting as usual. All steps are carried out with gentle agitation at 4°C. The blot was first rinsed for 10 minutes with binding buffer (25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), pH 7.9). This was decanted, and the blot treated with binding buffer containing 6 M guanidine HCl for 10 minutes. The guanidine HCl was gradually removed by diluting its concentration 1:1 with binding buffer and allowing a 5 minute rinse. The buffer covering the blot was then diluted again for a total of 5 rinses with binding buffer containing an exponentially decreasing quantity of guanidine HCl. Two final 10 minute rinses with fresh binding buffer were carried out before blocking. The blot was incubated with primary antibody overnight at 4°C.

A variation of this protocol described by Celenza and Carlson (1986) was also used. Proteins transferred to nitrocellulose were first blocked with 5% Carnation milk in 30 mM HEPES pH 7.5 for 1 hour at 25°C. Proteins were denatured (7 M guanidine HCl, 50 mM

Tris HCl, 50 mM DTT, 2 mM ethylenediamine tetra-acetic acid (EDTA), 0.25% Carnation milk, pH 8.3) for 1 hour at 25°C. The renaturation step involved a rinse in a guanidine HCl-free buffer (50 mM Tris HCl, 100 mM NaCl, 2 mM DTT, 2 mM EDTA, 0.1% Nonidet P-40, 0.25% Carnation milk, pH 7.5) for 16 hours at 4°C. After rinsing the blot for 30 minutes with 5% Carnation milk in 30 mM HEPES pH 7.5, the blot was incubated with antibodies and streptavidin as per regular Western blotting.

3. RESULTS

3.1 The 1B4 antigen is a nuclear matrix component of interphase cells

In interphase HeLa cells, the 1B4 antigen was distributed in 30-50 irregularly-shaped foci or speckles throughout the nucleoplasm excluding the nucleolus (Figure 3-1 a,d). These speckles appeared to be connected by thin fibrils. Similar distributions have been observed in other cell lines stained with the 1B4 antibody. These include differentiated and undifferentiated HL60 cells, wheat spheroblasts, 3T3 mouse fibroblasts, PTK_2 kangaroo rat kidney cells, and undifferentiated P19 murine embryonal carcinoma cells (Appendix I, Figure 1). This observation corresponds to the foci seen in electron micrographs of immunoperoxidase-labelled HeLa cells (Appendix I, Figure 2).

Like the 1B4 antigen, SC35 was also distributed in speckles connected by thin fibrils throughout the nucleoplasm excluding the nucleolus of HeLa cells. The distributions of the 1B4 antigen and SC35 in HeLa cells were found to be exactly coincident by confocal scanning laser microscopy (Figure 3-1 a-c). Most speckles in the nucleus came into focus in a distinct plane, as documented precisely by Carter and colleagues (1993).

The distribution of the 1B4 antigen in interphase cells showed partial colocalisation with that of the snRNPs identified by the Y12 antibody (Figure 3-1 e-f). The Y12 antibody labels the U1 snRNPs, which are diffusely distributed throughout the nucleoplasm excluding the nucleolus (Carmo-Fonseca *et al.*, 1991a, 1991b, 1992; Matera and Ward, 1993). The other spliceosomal snRNPs (U2, U4/U6, U5) labelled with the Y12 antibody are concentrated in 30-50 irregularly shaped foci. Electron microscopy studies have shown that the spliceosomal snRNPs are present in the perichromatin fibrils connecting the

interchromatin granules (speckles) (reviewed by Fakan, 1994). However, the diffuse distribution of the U1 snRNP in the nucleoplasm made the fibrils indistinguishable from this background in cells stained with the Y12 antibody and viewed by light microscopy.

As previously reported with the anti-SC35 antibody (Carmo-Fonseca *et al.*, 1991a; Spector *et al.*, 1991), the 1B4 antibody labelled a subset of the foci identified by the Y12 antibody (Figure 3-1 d-f). The 1B4 antigen and SC35 colocalise exactly, and the Y12 foci from which SC35 is excluded have been identified as coiled bodies (Carmo-Fonseca *et al.*, 1992). Thus it can be deduced that the Y12-labelled foci which 1B4 is excluded from are coiled bodies.

The speckled immunofluorescent staining pattern of the 1B4 antigen in interphase cells was maintained in all nuclear matrix preparations with little decrease in staining intensity. This result was reproducible regardless of whether sodium chloride (Figures 3-2, 3-3, 3-4, 3-5, and 3-6) or ammonium sulphate (Figures 3-7, 3-8, and 3-9) were used to remove non-matrix components. Identical results were obtained using the anti-SC35 antibody, which was expected given that SC35 has been previously reported to be a nuclear matrix protein by researchers using a similar assay (Bisotto *et al.*, 1995). Nuclear matrix preparations labelled with both the 1B4 and Y12 antibodies showed that the colocalisation of speckles present in interphase cells was maintained (Figure 3-6).

Using the protocol of Chaly and colleagues (1985), diffuse nucleoplasmic staining seen in interphase nuclei stained with the Y12 antibody was removed gradually through the nuclear matrix extraction, while snRNPs concentrated in foci were retained in the nuclear matrix preparation. In nuclear matrix preparations labelled with both the 1B4 and Y12

antibodies, the colocalisation of the majority of the speckles was maintained. Bright foci which were not labelled with the 1B4 antibody were also evident (Figure 3-6). These foci were not observed in the nuclear matrix preparations double-labelled with 1B4 and anti-SC35. It is likely that these foci were coiled bodies. Coiled bodies and interchromatin granules are preserved in nuclear matrix preparations (Puvion-Dutilleul *et al.*, 1995). In nuclear matrix preparations made using the protocol of Fey and colleagues (1986), the results were similar, with slightly more of the diffuse nucleoplasmic staining retained after the final step (compare p and s in Figure 3-4 to n in Figure 3-7).

The stages of the nuclear matrix extraction protocol of Chaly and colleagues were described in detail in their 1985 publication. The results I obtained using this protocol were as described by these authors with respect to the morphology of nuclear structures in phase-contrast micrographs (far right columns in Figures 3-2, 3-3, 3-4, and 3-5). Intact nuclei had the same overall size, shape, and distinct periphery as nuclei of cells taken during the various stages of the extraction. After the first extraction with DNase I, the nuclei had a grainy texture, with residual nucleoli appearing as dark, irregularly shaped structures (*e.g.* Figure 3-2 (f)). Thread-like interchromatinic reticulum structures were also visible during and after the high-salt extraction stages.

I monitored chromatin extraction during nuclear matrix preparation by staining with Hoescht 33258. In agreement with the results of Chaly and colleagues (1985), I observed that Hoescht staining in the cells fixed after the DNase I and low magnesium buffer steps showed diffuse nuclear staining with bright, irregular masses of condensed chromatin (*e.g.* Figure 3-2 e-h). Chaly and colleagues reported that after the 1M NaCl extraction, Hoescht

staining was absent or barely detectable, and did not show their results. I also found this, as shown by the black panels in Figures 3-2 to 3-5 k,n,q and t.

Staining for the m3G-cap structure decreased throughout the various stages of the protocol of Chaly and colleagues. From the Triton X-100 extraction through to the first DNase I extraction, the staining pattern faded slightly, but retained its speckled distribution (Figure 3-5 a,d, and g). In the high salt buffer extraction steps, staining was concentrated around the residual nucleoli and reticular fibres, with a lesser intensity of staining present after the second high salt buffer extraction (Figure 3-5 j and m). In assays in which DNase I was the final treatment, a very pale, indistinct distribution was seen (Figure 3-5 p). Nuclear matrix preparations performed using both DNase I and RNase A in the final step did not appear to contain any m3G-capped RNA as detected by this antibody (Figure 3-5 s).

Using the protocol of Fey and colleagues (1986), a very faint level of Hoescht staining was visible through all stages of the protocol (Figures 3-7, 3-8, and 3-9). This extraction protocol was less harsh than that of Chaly and colleagues, since it did not remove all detectable DNA. However, the level of staining was quite faint. To obtain an image on the negative, photographs of the Hoescht staining in these cells were overexposed. There was still so little contrast between the image and the background that in some photographs the exposure adjustment required in printing results in a pale grey background with the DNA barely visible. Fey and colleagues presented electron micrographs of nuclear matrix preparations, so it was difficult to directly compare my results at every step of the procedure to theirs. As with the protocol of Chaly and colleagues, the cells extracted with Triton X-100 were morphologically not very different from non-extracted cells in phase-

contrast micrographs (*e.g.* Figure 3-7 a-d). Fey and colleagues showed a transmission electron micrograph of a Triton X-100 extracted nucleus which appeared less dense than that of a control cell. They claimed that 70% of phospholipids and soluble cell proteins had been removed after this step. Further extraction with 0.25 M ammonium sulphate was shown by Fey and colleagues to remove protein structures exterior to the nucleus except for the intermediate filaments. I observed nuclei after this step to have a grainy texture in phase-contrast micrographs, with similar dark staining masses that were probably residual nucleoli (*e.g.* Figure 3-7 e-h). Hoescht staining after this step was of similar intensity as the Triton X-100 extracted cell nuclei. These cells did not show the foci of brighter staining of chromatin masses seen in the preparations of Chaly and colleagues following the DNase I and low magnesium buffer steps. Digestion with DNase I was followed by extraction with 0.25 M ammonium sulphate, removing 94% of the DNA, greater than 95% of the histones, and 70% of the nonhistone nuclear proteins (Fey *et al.*, 1986). Eighty percent of the hnRNA remained tightly associated with this structure. In my results, the DNA staining is very faint after this step, and thin reticular fibers were visible in the nucleus (*e.g.* Figure 3-7 i-l). The final step of Fey and colleagues' protocol is a brief digestion with RNase A, which removes 97% of the hnRNA from the RNP-containing matrix, and causes a marked distortion of inner nuclear morphology (*e.g.* Figure 3-7 m-p).

Staining for the m3G-capped RNA species in the protocol of Fey and colleagues decreased through the successive stages of the extraction (Figure 3-9). Faint staining was visible in the DNase I-extracted cells, although it did appear to be concentrated within residual nucleolar aggregates. Following the final RNase A treatment, no staining was

visible with this antibody (Figure 3-9 n). The same preparation double-labelled with the 1B4 antibody showed bright staining of foci undiminished by the extraction procedure (Figure 3-9 m).

Thus it has been shown that the 1B4 antigen is retained in the nuclear matrix regardless of the matrix extraction protocol used. By the same criterion, the snRNPs identified by the Y12 antibody, SC35, but not the m3G cap structures, are also nuclear matrix components.

Figure 3-1. Confocal micrographs of HeLa cells double-labelled with the 1B4 antibody and either the anti-SC35 or Y12 antibody: a) 1B4 immunofluorescence alone, b) anti-SC35 immunofluorescence alone, c) overlapping signal obtained by combining signals a) and b), d) 1B4 immunofluorescence alone, e) Y12 immunofluorescence alone, f) overlapping signal obtained by combining signals d) and e). Yellow areas indicate colocalisation of green and red labelling. Bar = 3.4 μm.

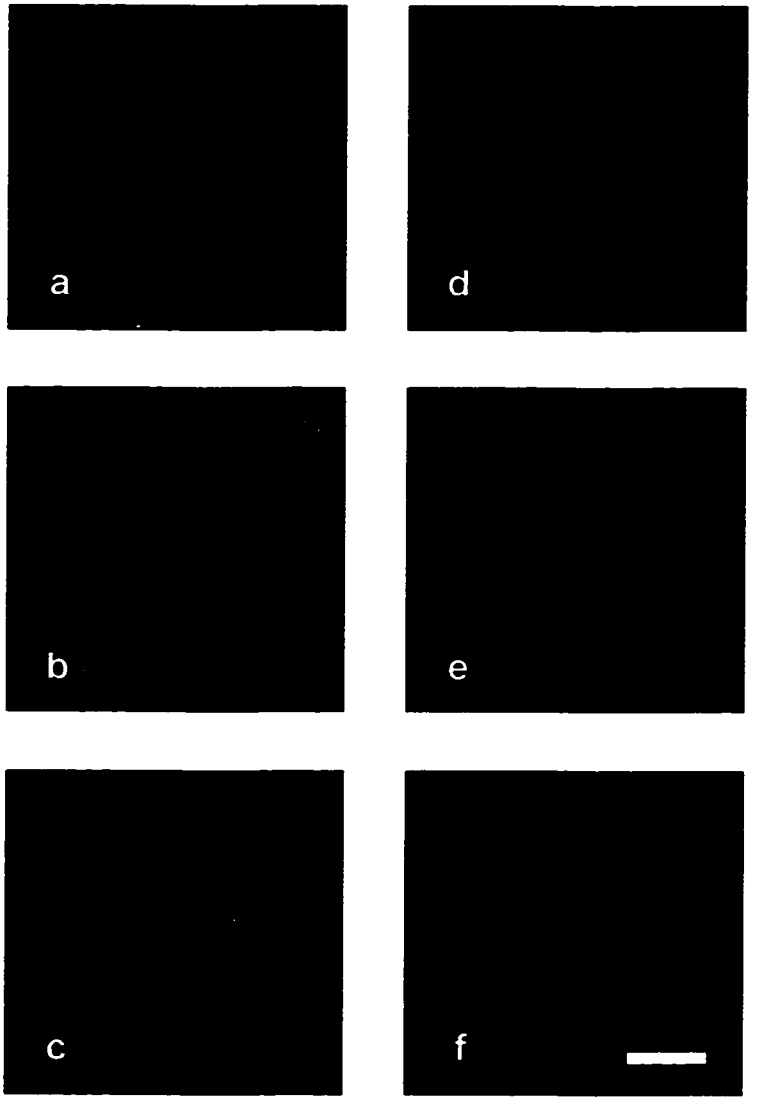
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Figure 3-2. Nuclear matrix extraction preparations by procedure of Chaly and colleagues (1985). Immunofluorescence labelling with the 1B4 antibody (a,d,g,j,m,p,s), Hoescht 33258 counterstain (b,e,h,k,n,q,t), and phase-contrast (c,f,i,l,o,r,u) are shown for HeLa cells fixed at successive stages of the extraction procedure: extraction with Triton X-100 (a-c), brief treatment with DNase (d-f), rinse with low magnesium buffer (g-i), 1M NaCl extraction (j-l), 2M NaCl extraction (m-o). The final step was either treatment with DNase (p-r) or treatment with DNase and RNase (s-u). Bar = 5.4 μm.

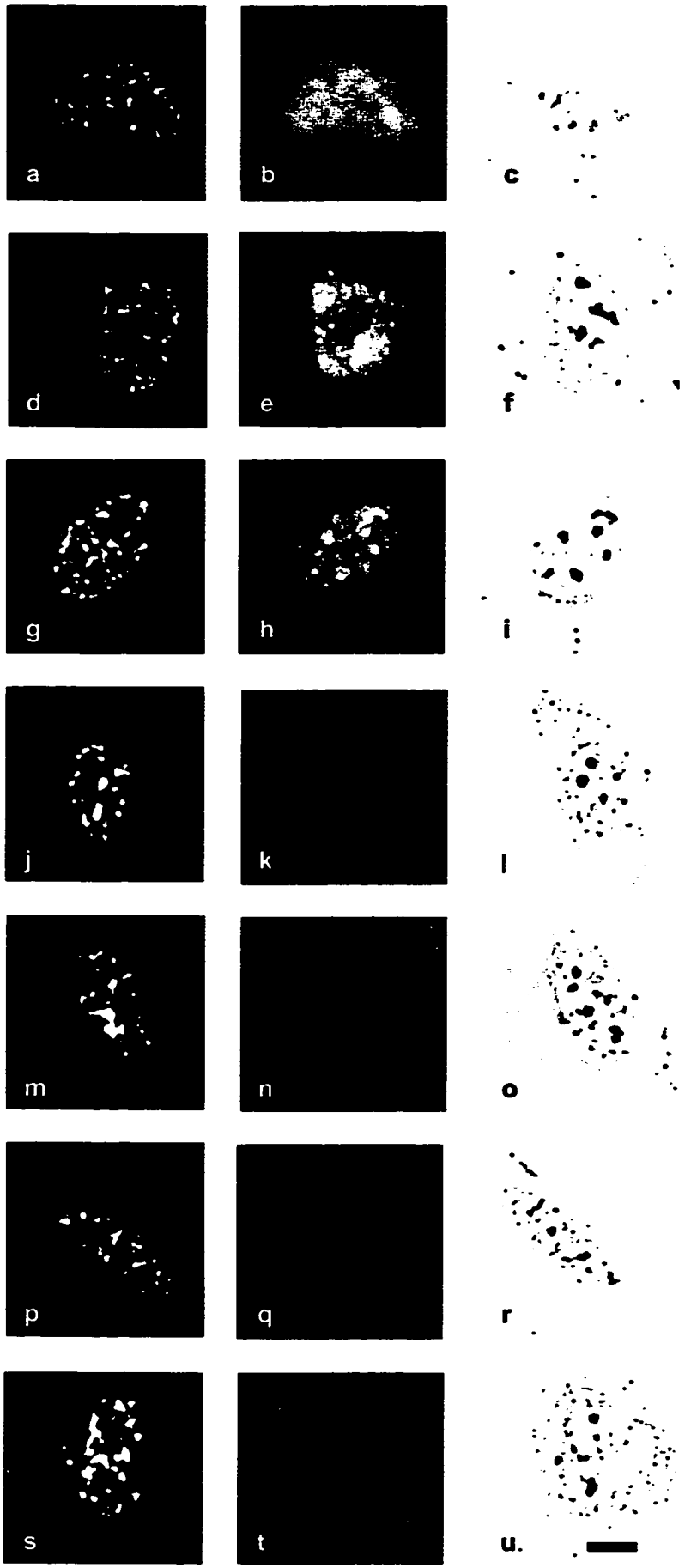
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Figure 3-3. Nuclear matrix extraction preparations by procedure of Chaly and colleagues (1985). Immunofluorescence labelling with anti-SC35 (a,d,g,j,l,n,p), Hoescht 33258 counterstain (b,e,h,k,n,q,t), and phase-contrast (c,f,i,k,m,o,q) are shown for HeLa cells fixed at successive stages of the extraction procedure: extraction with Triton X-100 (a-c), brief treatment with DNase (d-f), rinse with low magnesium buffer (g-i), 1M NaCl extraction (j-l), 2M NaCl extraction (m-o). The final step was either treatment with DNase (p-r) or treatment with DNase and RNase (s-u). Bar = 5.4 μm.

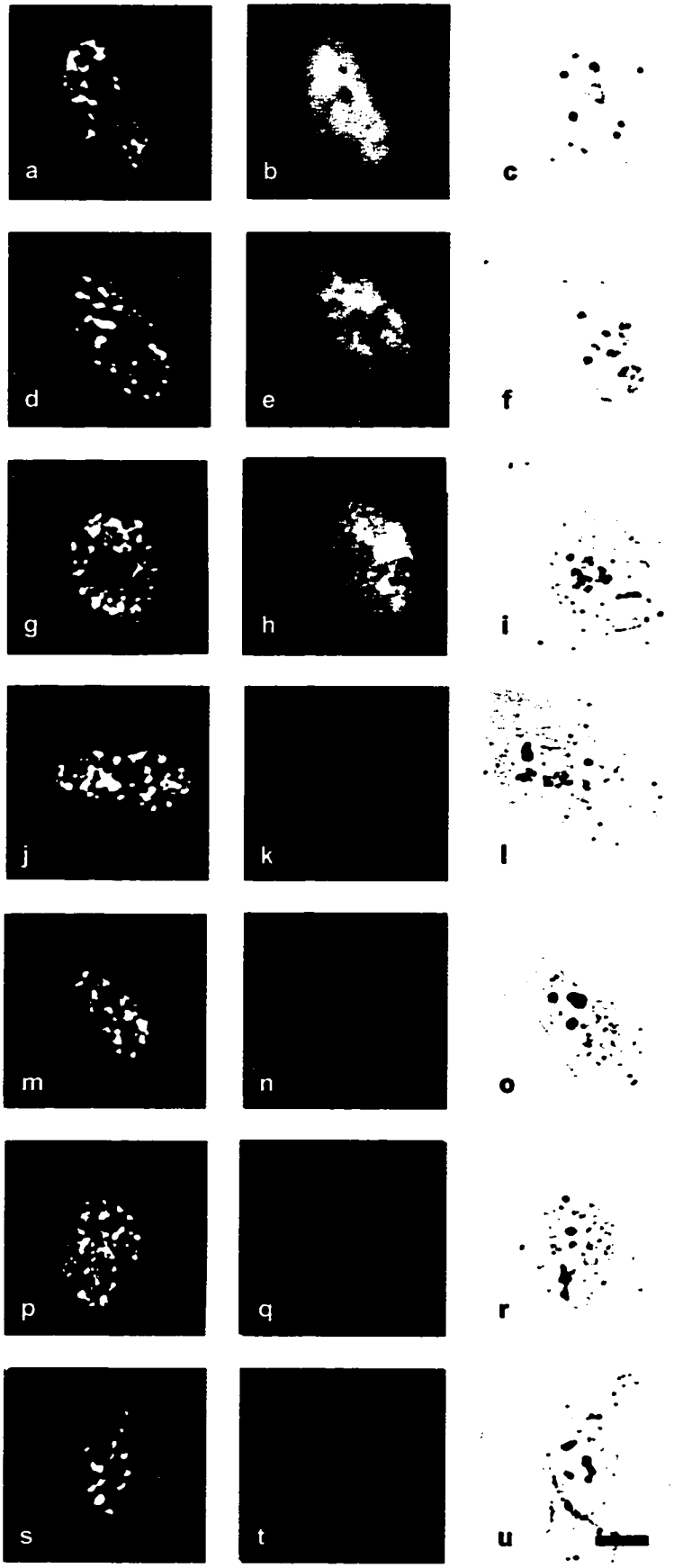
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Figure 3-4. Nuclear matrix extraction preparations by procedure of Chaly and colleagues (1985). Immunofluorescence labelling with the Y12 antibody (a,d,g,j,m,p,s), Hoescht 33258 counterstain (b,e,h,k,n,q,t), and phase-contrast (c,f,i,l,o,r,u) are shown for HeLa cells fixed at successive stages of the extraction procedure: extraction with Triton X-100 (a-c), brief treatment with DNase (d-f), rinse with low magnesium buffer (g-i), 1M NaCl extraction (j-l), 2M NaCl extraction (m-o). The final step was either treatment with DNase (p-r) or treatment with DNase and RNase (s-u). Bar = 5.4 μm.

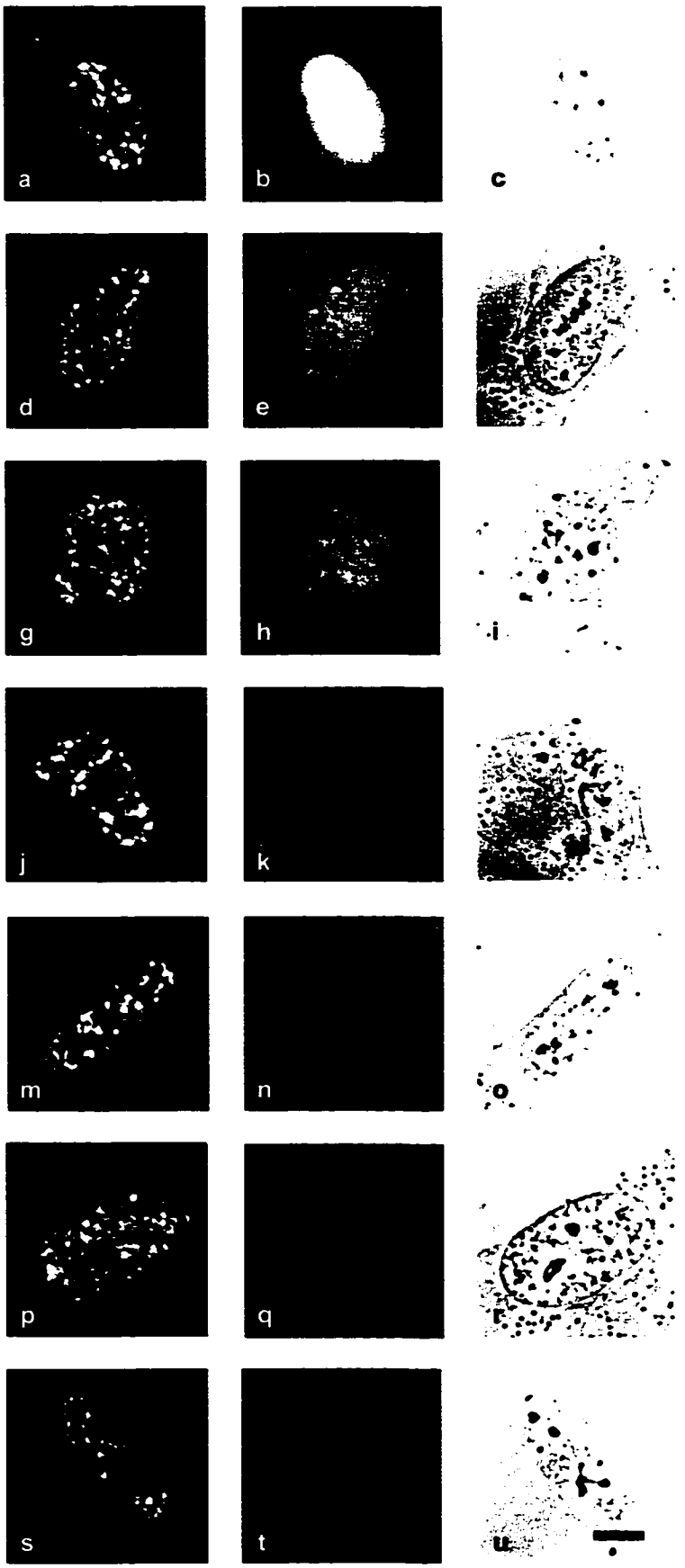
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Figure 3-5. Nuclear matrix extraction preparations by procedure of Chaly and colleagues (1985). Immunofluorescence labelling with the anti-m3G cap antibody (a,d,g,j,m,p,s), Hoescht 33258 counterstain (b,e,h,k,n,q,t), and phase-contrast (c,f,i,l,o,r,u) are shown for HeLa cells fixed at successive stages of the extraction procedure: extraction with Triton X-100 (a-c), brief treatment with DNase (d-f), rinse with low magnesium buffer (g-i), 1M NaCl extraction (j-l), 2M NaCl extraction (m-o). The final step was either treatment with DNase (p-r) or treatment with DNase and RNase (s-u). Bar = 5.4 μm.

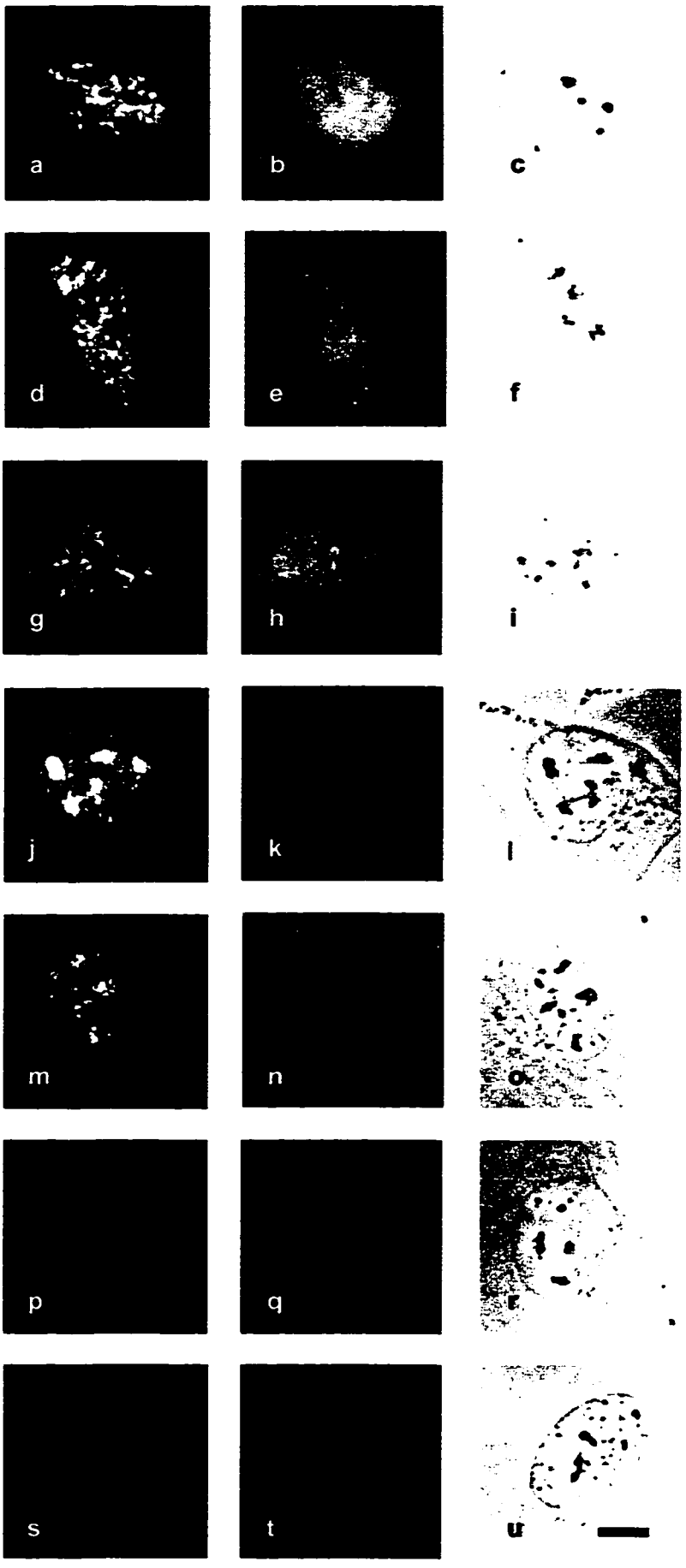
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Figure 3-6. Nuclear matrix extraction preparation by procedure of Chaly and colleagues (1985). This HeLa cell was double labelled with both the Y12 and 1B4 antibodies after the final (DNase and RNase treatment) step of the extraction procedure. The secondary antibody which bound to Y12 was conjugated to CY3 (red), while the secondary antibody which bound to 1B4 was an FITC conjugate (green). The regions of Y12 and 1B4 colocalisation appear yellow. Bar = 7.2 μm.

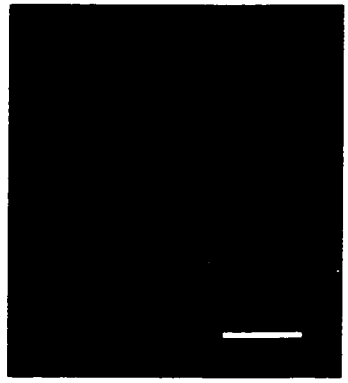

Figure 3-7. Nuclear matrix extraction preparations by procedure of Fey and colleagues (1986). Immunofluorescence labelling with the 1B4 antibody (a,e,i,m), Y12 antibody (b,f,j,n), Hoescht 33258 counterstain (c,g,k,o), and phase-contrast (d,h,l,p) are shown for HeLa cells fixed at successive stages of the extraction procedure: extraction with Triton X-100 (a-d), ammonium sulphate extraction (e-h), DNAse I treatment terminated by addition of ammonium sulphate (i-l), RNase digestion (m-p). Bar = 5.4 μm.

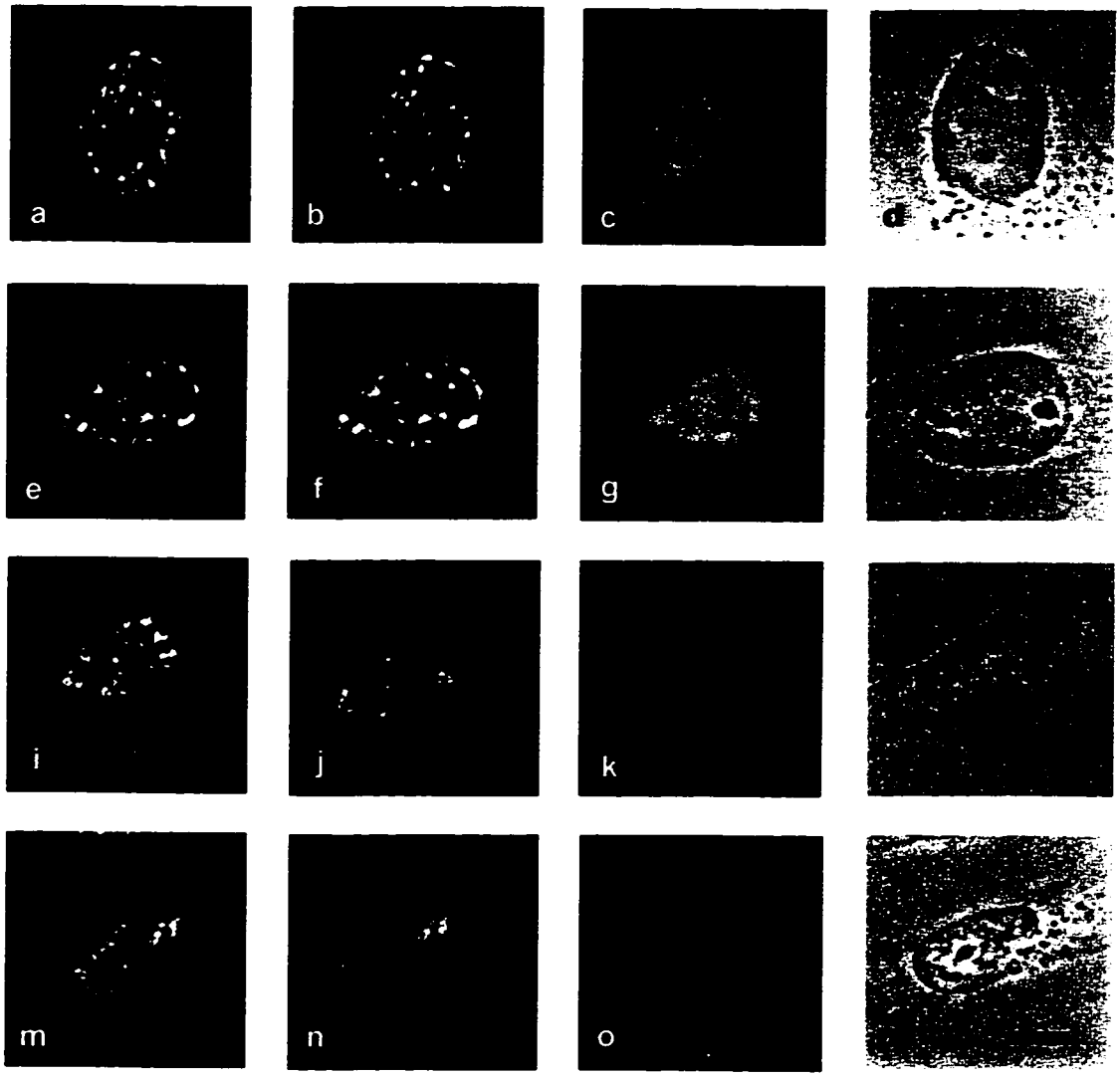
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Figure 3-8. Nuclear matrix extraction preparations by procedure of Fey and colleagues (1986). Immunofluorescence labelling with anti-SC35 (a,d,g,j), Hoescht 33258 counterstain (b,e,h,k), and phase-contrast (c,f,i,j) are shown for HeLa cells fixed at successive stages of the extraction procedure: extraction with Triton X-100 (a-c), ammonium sulphate extraction (d-f), DNAse I treatment terminated by addition of ammonium sulphate (g-i), RNase digestion (j-l). Bar = 5.4 μm.

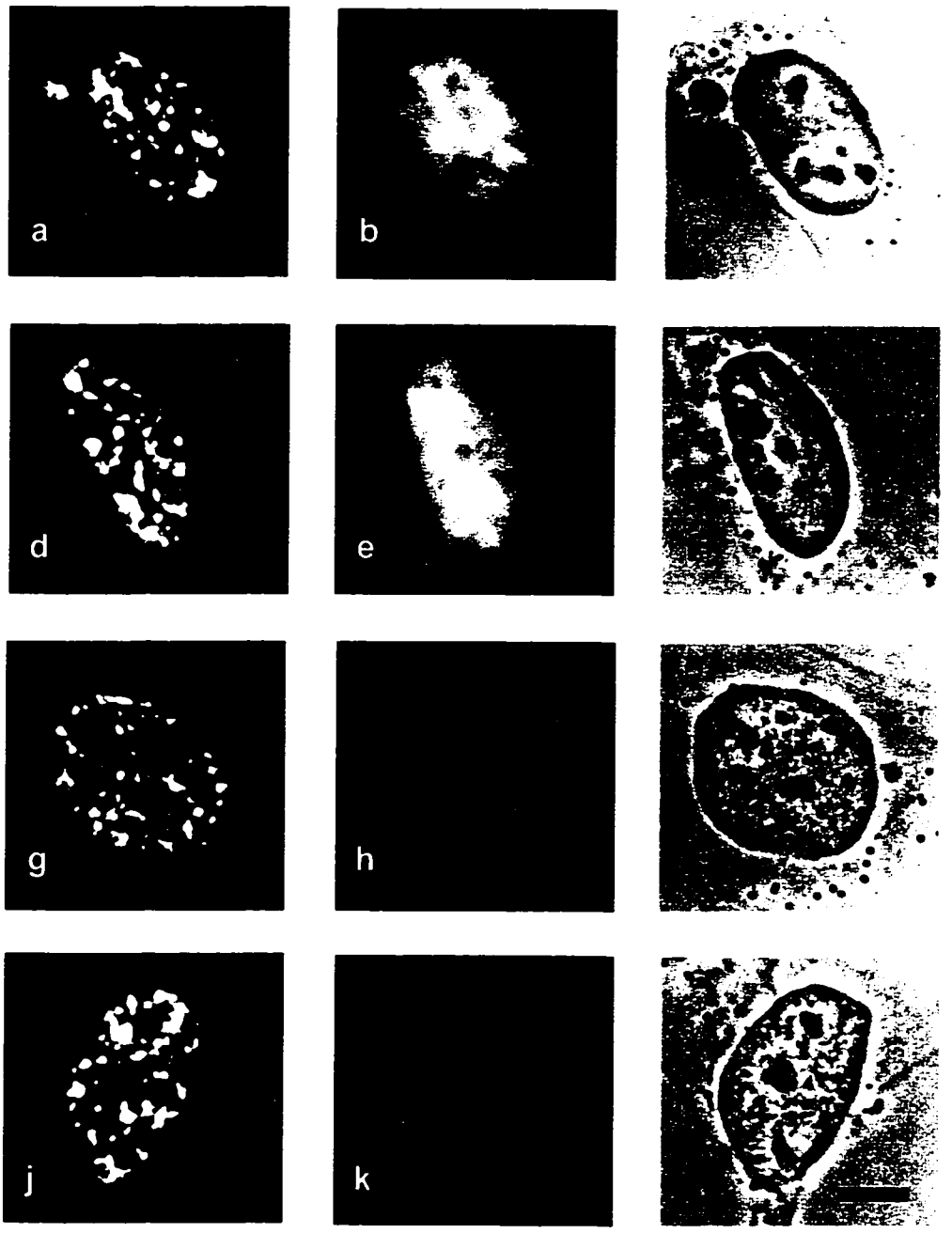
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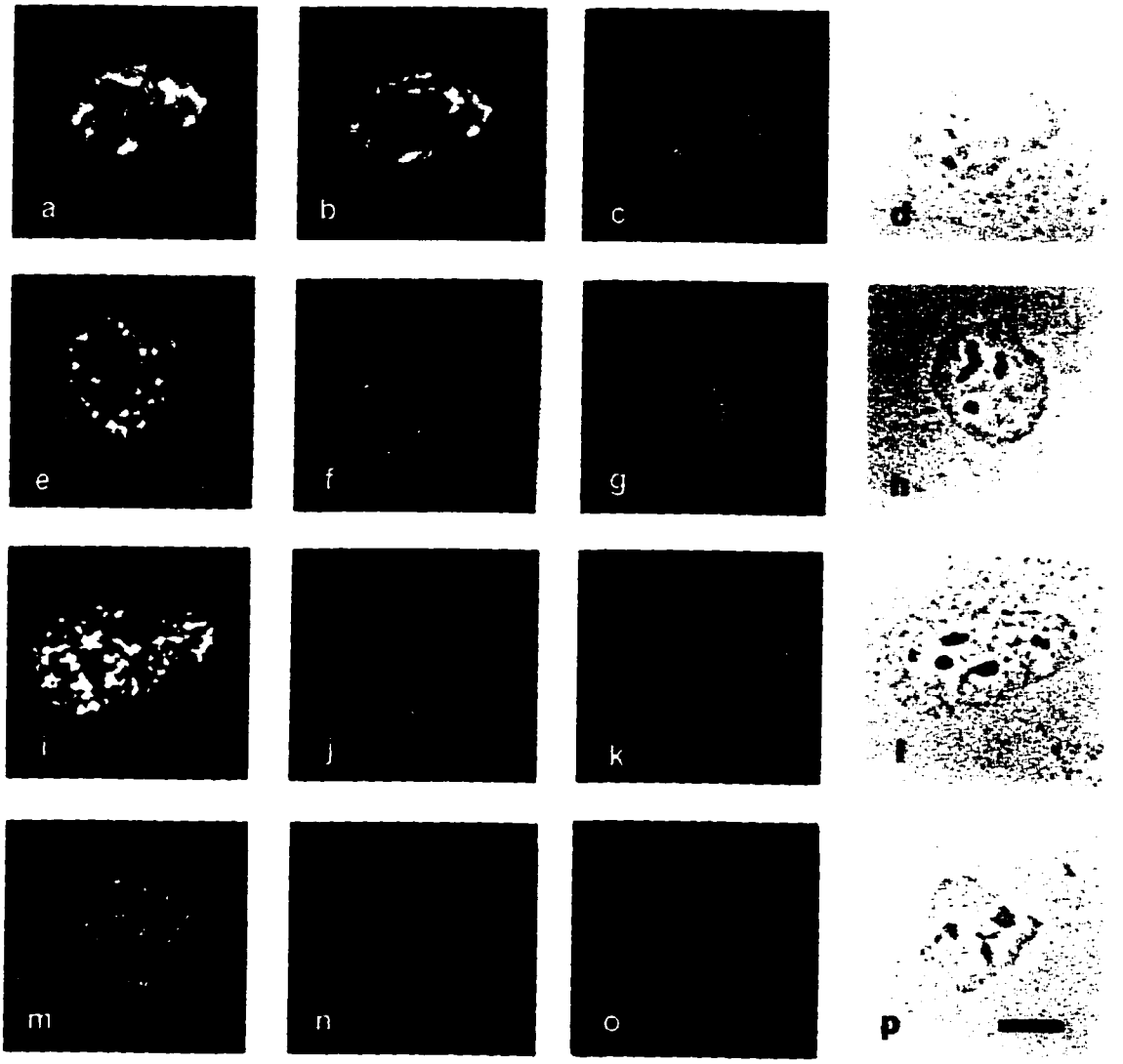
Figure 3-9. Nuclear matrix extraction preparations by procedure of Fey and colleagues (1986). Immunofluorescence labelling with the 1B4 antibody (a,e,i,m), m3G cap antibody (b,f,j,n), Hoescht 33258 counterstain (c,g,k,o), and phase-contrast (d,h,l,p) are shown for HeLa cells fixed at successive stages of the extraction procedure: extraction with Triton X-100 (a-d), ammonium sulphate extraction (e-h), DNAse I treatment terminated by addition of ammonium sulphate (i-l), RNase digestion (m-p). Bar = 5.4 μm.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3.2 Fate of the 1B4 antigen through the cell cycle

3.2.1 Distribution of the 1B4 antigen through the cell cycle in relation to snRNPs and SC35

The distribution of the 1B4 antigen through mitosis is shown in Figures 3-10, 3-11, and 3-12. In early prophase, chromosomes begin to condense. By mid- to late prophase, chromosome condensation is completed. In prophase cells, the 1B4 antigen appeared diffusely distributed throughout the nucleoplasm, as did SC35 and the snRNPs. These distributions were maintained through prometaphase, when the nuclear envelope disassembled.

In metaphase cells in which the chromosomes were aligned at the equatorial plane, it was apparent that the 1B4 antigen did not associate with the condensed chromosomes or mitotic spindle. A few (0-6) small, round foci were sometimes visible in metaphase cells, usually near the cell periphery (Figure 3-10). They were not all in the same plane of focus. Antibodies to SC35 and the snRNPs also labelled these foci, which have been termed mitotic granule clusters by Leser and colleagues (1989). The 1B4 antigen, SC35, and the snRNPs were all diffusely distributed in these cells. The relative intensity of the diffuse 1B4 or Y12 labelling was greater than that of anti-SC35: in photographs of fields containing both mitotic and interphase cells, mitotic cells labelled with anti-SC35 did not fluoresce brightly enough to be photographed at an exposure sufficient to photograph an adjacent interphase cell. If the exposure was based upon the fluorescence intensity of an interphase cell, a mitotic cell in the field would barely be visible. In photographs of fields containing both mitotic and interphase cells labelled with either the Y12 or 1B4 antibodies,

the mitotic cells are clearly visible and sometimes even overexposed at exposure times optimised for interphase cells. For these observations, having both an interphase and mitotic cell in the same field served as an internal control for fluorescence intensity.

In anaphase A as the sister chromatids were separating to opposite poles of the spindle, the diffuse distributions of 1B4, SC35, and snRNPs were maintained. The mitotic granule clusters present in metaphase and some anaphase A cells were not present during anaphase B, when the cells became elongated. Nuclear membranes reformed around the decondensing chromosomes of daughter nuclei in telophase (Figure 3-11). The 1B4 antigen, SC35, and the snRNPs observed within the daughter nuclei rearranged into the speckled distribution in early G1. In cells still connected by a midbody visible by phase contrast microscopy (completing cytokinesis), the spaces between the speckles were diffusely labelled rather than clearly defined as fibrils.

Figure 3-10. The distributions of splicing-related antigens through prophase and prometaphase. A cell in early prophase (a-d) is shown stained with the 1B4 antibody (a), the CC-3 antibody (b), and counterstained with Hoescht (c). The phase contrast image (d) is also shown; bar = 5.4 μm. A cell in mid-prophase (e-h) is shown stained with the 1B4 antibody (e), the Y12 antibody (f), and counterstained with Hoescht (g). The phase contrast image (h) is also shown; bar = 5.4 μm. A cell in late prophase (i-j) is shown double-labelled with the Y12 and 1B4 antibodies. The secondary antibody used to detect Y12 was a CY3 conjugate (red), while the antibody used to detect 1B4 was a FITC conjugate (green). Regions where the Y12 and 1B4 staining distributions overlap appear yellow. Hoescht counterstain of this cell is shown (j); bar = 7.2 μm. A cell in prometaphase (k-m) is shown stained with the 1B4 antibody (k), counterstained with Hoescht (l), and as a phase contrast image (m); bar = 5.4 μm. A cell in prometaphase (n-o) is shown double labelled with anti-SC35 and the 1B4 antibody (n). The secondary antibody used to detect anti-SC35 was a CY3 conjugate (red), while the antibody used to detect 1B4 was a FITC conjugate (green). Regions where the anti-SC35 and 1B4 staining distributions overlap appear yellow. Hoescht counterstain of this cell is shown (o); bar = 7.2 μm.

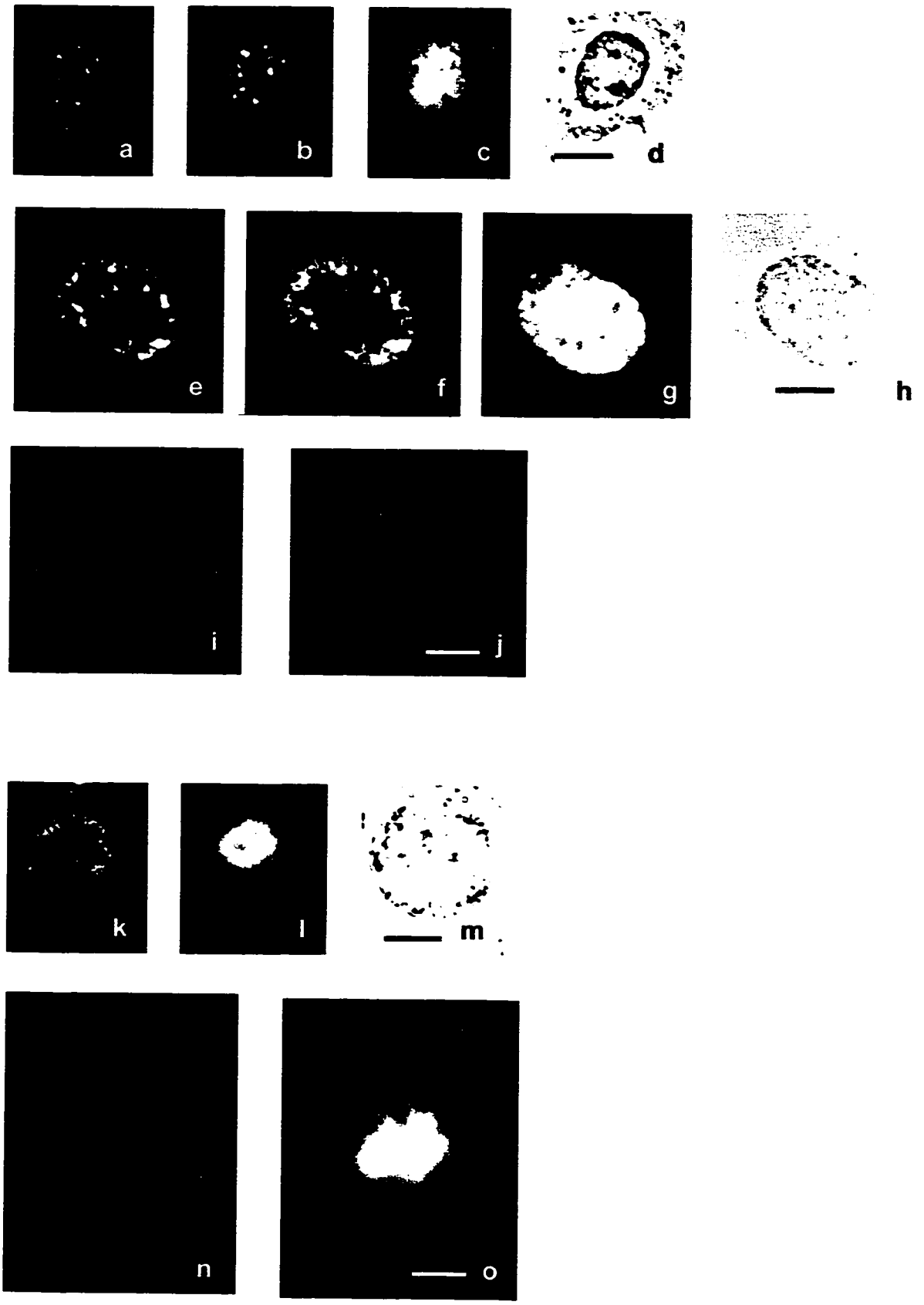
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Figure 3-11. The distributions of the 1B4 antigen, snRNPs, and SC35 in metaphase cells. Interphase cells are shown in each frame so that the relative intensities of staining of metaphase cells can be estimated. In (a-d), cells are shown labelled with the 1B4 antibody (a), and anti-SC35 (b). The cells in (e-g) are shown labelled with the Y12 antibody (a).Each pair of cells is also shown counterstained with Hoescht (c, f), and as a phase contrast images (d,g). Bar = 5.4 μm.

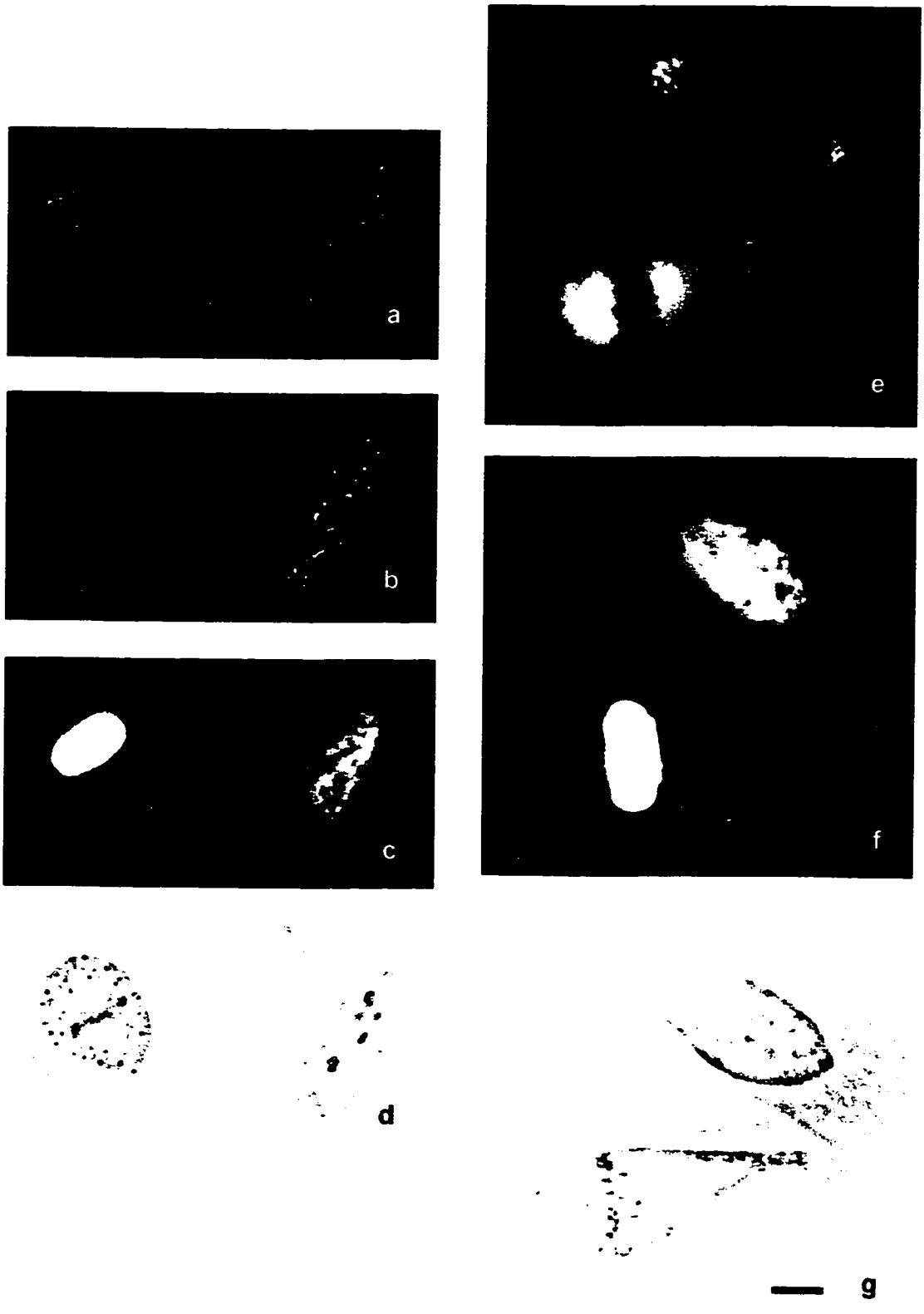
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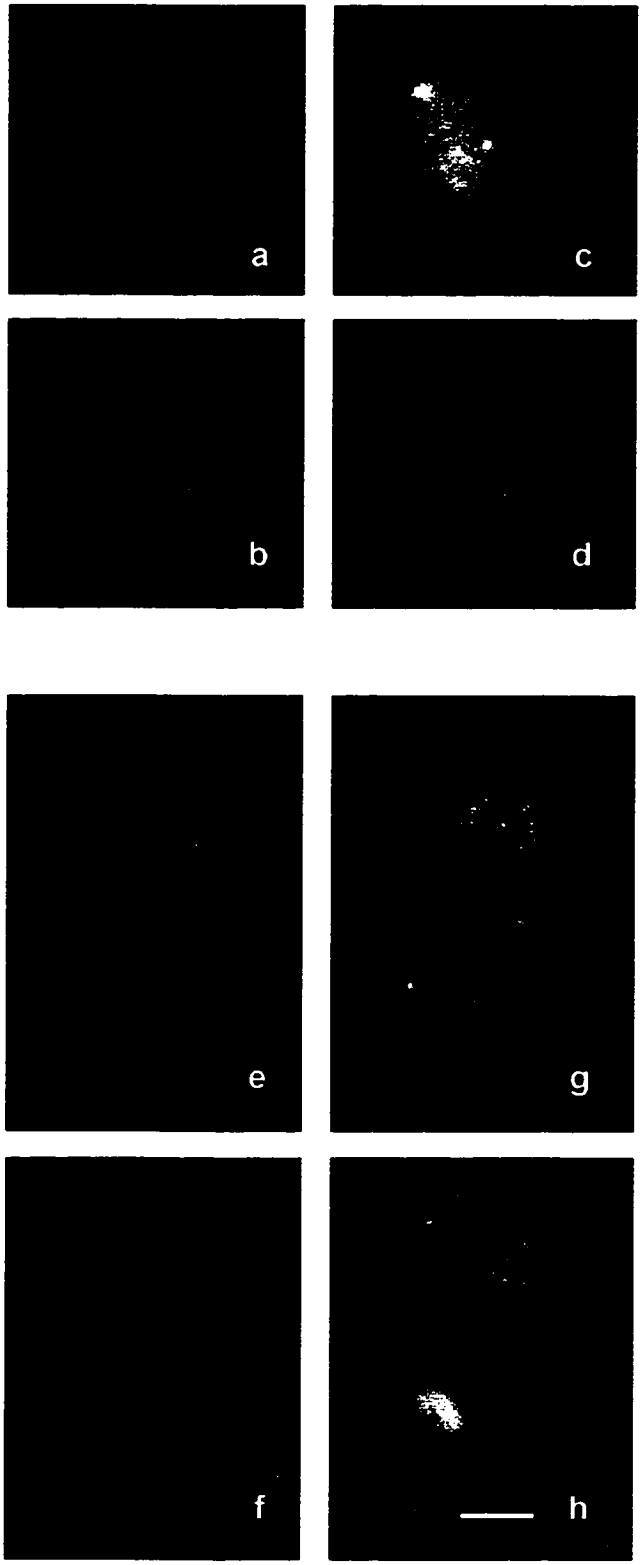
Figure 3-12. The distribution of the 1B4 antigen and SC35 in anaphase (a-d) cells and in cells undergoing cytokinesis (e-h). Cells are shown labelled with either the 1B4 antibody (a,e), or anti-SC35 (b,f). The superimposed 1B4 and anti-SC35 images (c,g) were obtained by double-exposing the film. The secondary antibody used to detect anti-1B4 was a FITC conjugate (green), while the secondary antibody used to detect SC35 was a CY3 conjugate (red). In the double-exposed frames, regions where the 1B4 and anti-SC35 staining distributions overlap appear yellow. Hoescht counterstain of this cell is shown (d,h); bar = 7.2 μm.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3.2.2 Cell-cycle specific detection of the 1B4 antigen by Western blotting

The 1B4 antibody did not detect a signal in Western blots of either whole interphase cell homogenates or isolated nuclei. Denaturation/renaturation protocols (Celenza and Carlson, 1986; Vinson *et al.*, 1988) using guanidine hydrochloride were not effective for recovering this antigen. The 1B4 antibody did not immunoprecipitate the 1B4 antigen; this problem may be due to the lack of commercially available anti-IgE antibodies suitable for immunoprecipitation. It is very rare to produce a hybridoma that secretes an IgE antibody, as this type of antibody is uncommon. The concentration of IgG antibodies in serum is 6-16 mg/ml, while the concentration of IgE antibodies is only 10-400 ng/ml (Harlow and Lane, 1988). A 100 kDa signal was seen in whole cell homogenates of mitotic cells synchronised with nocodazole (Figure 3-13 x). There were additional signals present, but these were also observed in control experiments in which the secondary antibody and streptavidin were used, and thus are considered to be background. The 1B4 antigen signal was not seen in Western blots of whole cell homogenates (Figure 3-13 x), or in cytoplasmic or nuclear fractions of interphase cells (Figure 3-13 x-x2).

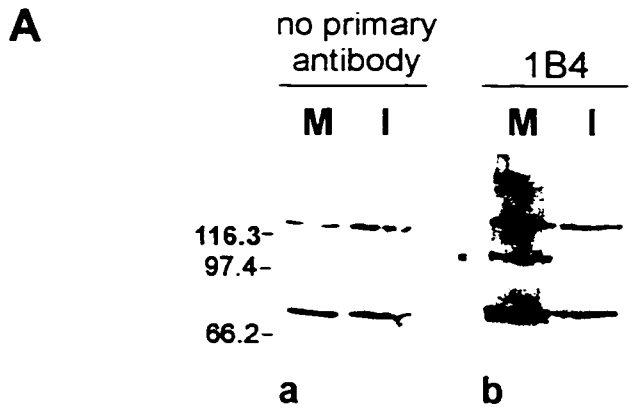
The anti-SC35, Y12, and CC-3 antibodies were used in Western blots of the cell homogenates tested with the 1B4 antibody (Figure 3-13). The anti-SC35 antibody did not detect a signal in any of the fractions tested. Spector and colleagues (1991) detected a 35 kDa doublet in Western blots of SC35 protein that had been purified to homogeneity by conventional and immunoaffinity chromatography methods. The Y12 antibody identified 27, 28, and 78 kDa proteins in extracts prepared from cells in either mitosis or interphase. The 27 and 28 kDa proteins were present in both the cytoplasmic and nuclear fractions.

The CC-3 antibody strongly labelled high molecular weight (estimated 255 kDa) protein in both interphase and mitotic cell extracts. The signal on the Western blot prepared from mitotic cells was stronger and included additional lower molecular-weight bands not present on the interphase blot. The approximately 255 kDa protein present in interphase cells was found in the nuclear fraction and absent from the cytoplasmic fraction. These results are in agreement with the results of Thibodeau and Vincent (1991), giving assurance that the lack of 1B4 signal in nuclei is not a result of a poor protein preparation.

3.2.3 The reactivity of the 1B4 antibody to its epitope has not been determined to be phosphodependent

Cells treated *in situ* with calf intestinal alkaline phosphatase were stained with either the 1B4 or 2H12 antibodies (Figure 3-14). The 2H12 antibody detects human B23 in a phosphorylation-dependent manner. Treatment with alkaline phosphatase prevented this antibody from labelling its epitope. Staining with the 1B4 antibody was unaffected by alkaline phosphatase treatment. Control cells exposed to 50 mM tetra-sodium pyrophosphate in addition to CIAP or left in Tris-$MgCl_2$ buffer with protease inhibitors for the same incubations at the same temperatures and durations as the CIAP-treated cells appeared to have the same 2H12 staining intensity as cells routinely fixed and permeabilised for microscopy.

Figure 3-13. Western blots of HeLa cell extracts using the (A) 1B4, or (B) 1B4, anti-SC35, Y12, and CC-3 antibodies. The small black square marks the position of the signal obtained with the 1B4 antibody in mitotic cells (100 kDa). HeLa cell extracts prepared from mitotic (M) or interphase cells (I) are shown in Figure 3-13 A . Identical blots were probed with either the secondary antibody followed by streptavidin (a), or the 1B4 antibody followed by the secondary antibody and streptavidin (b). This demonstrates that the signal obtained with the 1B4 antibody is specific. In Figure 3-13 B, a sample standard lane is shown to the left, with the molecular weights marked in kilodaltons. Whole-cell homogenates were prepared from (a) mitotic and (b) interphase cells. Protein extracts were also prepared from interphase cells divided into (c) cytoplasmic and (d) nuclear fractions.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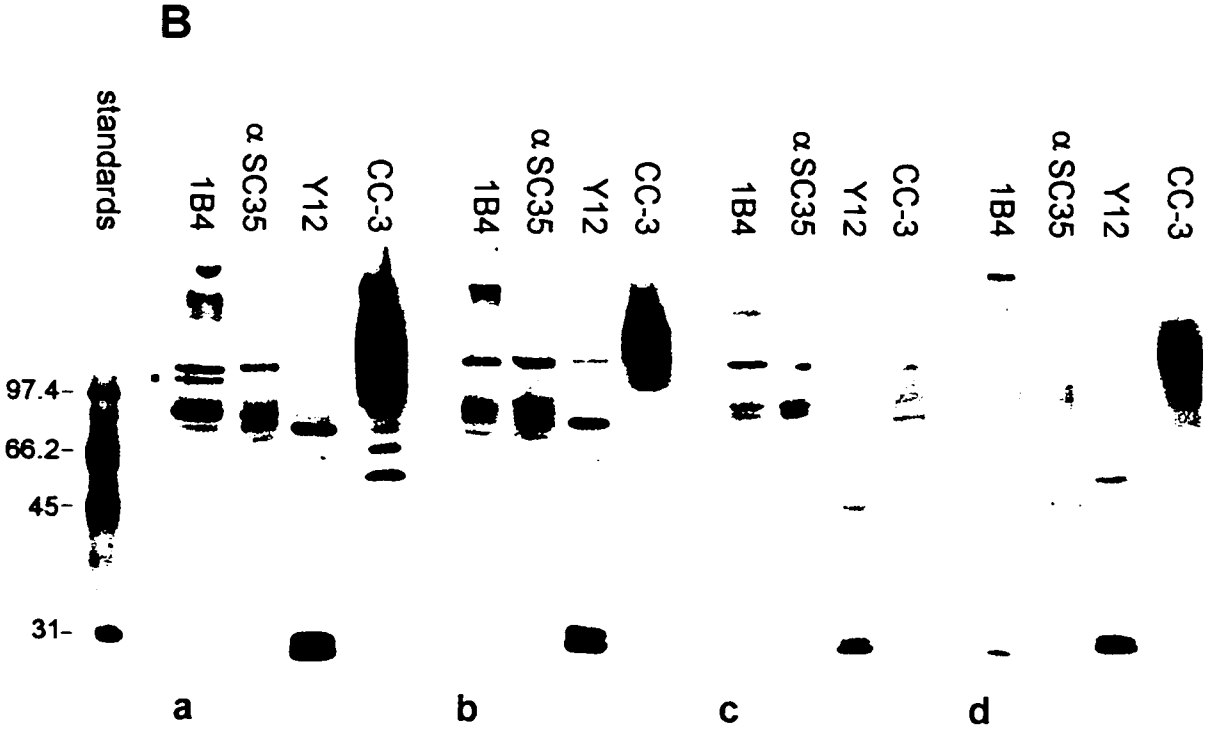
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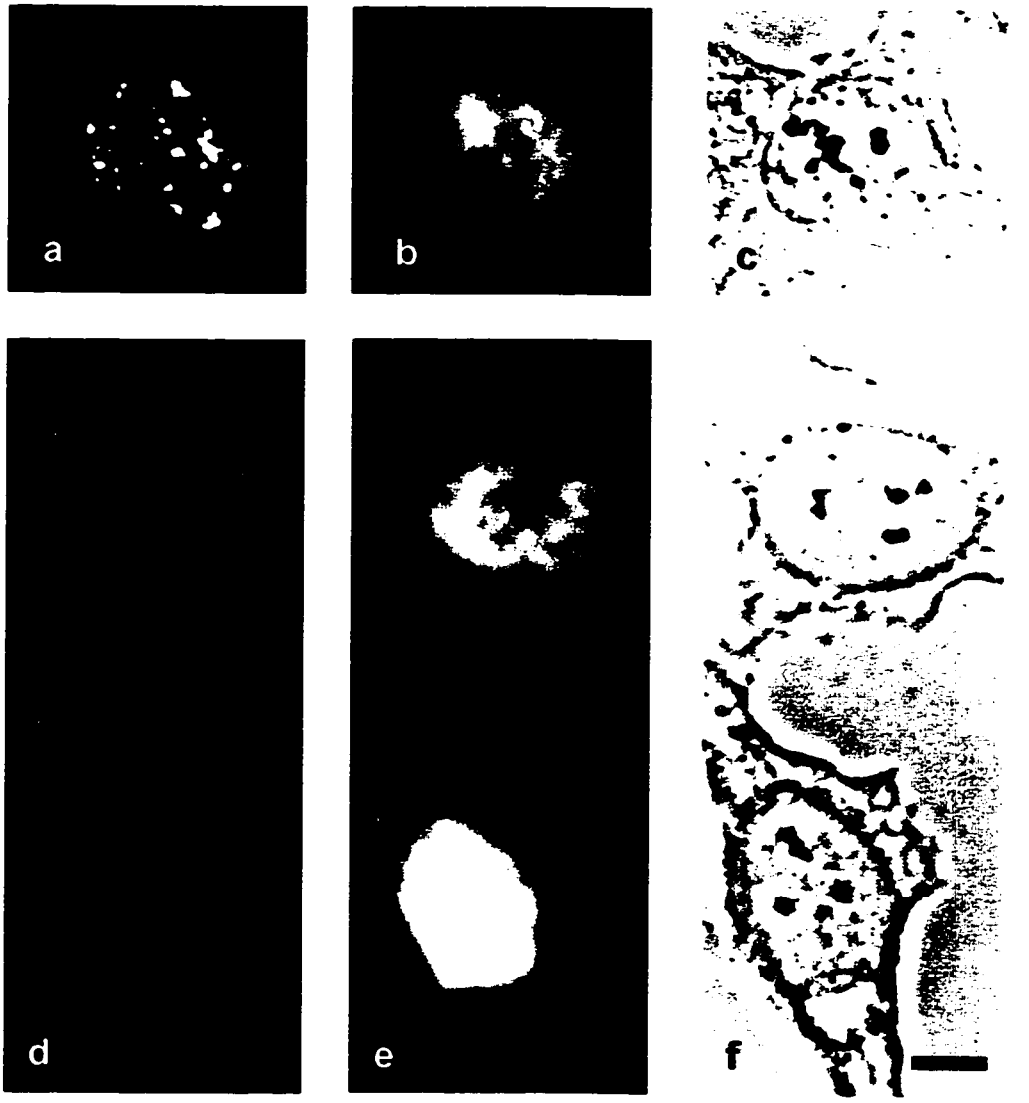
Figure 3-14. Immunofluorescence labelling of HeLa cells exposed to calf intestinal alkaline phosphatase before fixation. Cells were stained with either (a) 1B4, or (d) 2H12 antibodies. Hoescht counterstain (b,e) and phase-contrast (c,f) images are shown. Bar = 5.4 μm.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3.3 The effect of decreased transcription upon the 1B4 antigen

3.3.1 The distribution of the 1B4 antigen, snRNPs, and SC35 in HeLa cells exposed to drugs that inhibit transcription

Three drugs with different modes of action were used to inhibit transcription in HeLa cells. All three have been documented as causing splicing-related antigens such as SC35 to be rearranged into fewer, larger speckles (*e.g.* Carmo-Fonseca *et al.*, 1991a; Bregman *et al.*, 1995; Spector, 1996). Actinomycin-D intercalates into double-stranded DNA resulting in inhibition of transcription by RNA polymerases (Sobell, 1985). DRB inhibits transcription by inhibiting protein kinases that phosphorylate the large subunit of RNA polymerase II (Stevens and Maupin, 1989). Since DRB rapidly penetrates cell membranes, it can be rapidly washed out of cells to reverse the transcriptional block (Tamm *et al.*, 1976). α-Amanitin irreversibly and selectively inhibits RNA pol II as a result of binding to and its largest subunit (Lindell *et al.*, 1970, Nguyen *et al.*, 1996; Rudd and Luse, 1996). The effects of these drugs upon the distribution of the 1B4 antigen as compared to SC35 or the snRNPs was studied using double-labelling immunofluorescence.

The exact colocalisation of the 1B4 antigen and SC35 was maintained in all actinomycin-D treatment groups. These observations have been confirmed by confocal microscopy (Figure 3-15). The partial colocalisation of 1B4 and the snRNPs was also maintained. In cells treated with either 0.5 or 0.05 μg/ml actinomycin-D for only 1 hour, it was difficult to discern any difference in the arrangement of the speckles. However, the observation of fewer, larger speckles lacking connecting fibrils was more pronounced in the 2 hour treatment groups. The effect of the lower (0.05 μg/ml) dose of actinomycin-D upon

the distributions of the 1B4 antigen, SC35, or the snRNPs was less obvious upon visual inspection (Figures 3-16 and 3-17 i-l and m-p) in comparison to the higher (0.5 μg/ml) dose of this drug (Figures 3-16 and 3-17 a-d and e-h). The appearance of large, rounded speckles lacking interconnecting fibrils was very obvious in cells treated for 4 or 8 hours with 0.5 μg/ml actinomycin-D (Figures 3-18 and 3-19). For these exposures, a less noticeable effect was also seen for the 0.05 μg/ml treatment groups. As the actinomycin-D was dissolved in ethanol, controls for the effect of ethanol alone are shown in Figure 3-20.

The effect of drug treatment and recovery was studied using DRB. Two hour treatments with 25 μg/ml DRB produced an effect similar to that observed in cells treated with 0.5 μg/ml actinomycin-D for 2 hours. The speckles appeared to be larger and lack interconnecting fibrils (Figure 3-21). The exact colocalisation of the 1B4 antigen and SC35 was maintained, as was the partial colocalisation of the 1B4 antigen and a subset of the snRNPs. An ethanol control is not shown, as the volume of ethanol delivered to these cells with the DRB was less than that administered in the actinomycin-D treatments. Cells recovering from DRB treatment regained the speckled distribution characteristic of untreated cells within two hours. The exact colocalisation of 1B4 and SC35 was maintained throughout the recovery period. In contrast to the recovery seen with DRB, cells which had been treated for 2 hours with 0.5 μg/ml actinomycin-D did not regain their interconnecting fibrils. After an extended (18 hours) period of time, their chunky speckles appeared to fragment into a greater number of smaller speckles than was initially observed (Figures 3-22 and 3-23).

The rearrangement of speckles in cell treated with 5 μg/ml α-amanitin for 5 hours

was subtle (Figure 3-24). The observed exact colocalisation of the 1B4 antigen with SC35 was maintained, as was the partial colocalisation with the snRNPs. As this drug is irreversible and specific for RNA polymerase II, this experiment verified that the effects seen with either actinomycin-D or DRB may be attributed to RNA polymerase II inhibition.

Figure 3-15. Confocal micrograph of HeLa cells double-labelled with the 1B4 antibody and either the anti-SC35 or Y12 antibody: a) 1B4 immunofluorescence alone, b) anti-SC35 immunofluorescence alone, c) overlapping signal obtained by combining signals a) and b), d) 1B4 immunofluorescence alone, e) Y12 immunofluorescence alone, f) overlapping signal obtained by combining signals d) and e). Yellow areas indicate colocalisation of green and red labelling. Bar = 3.4 μm.

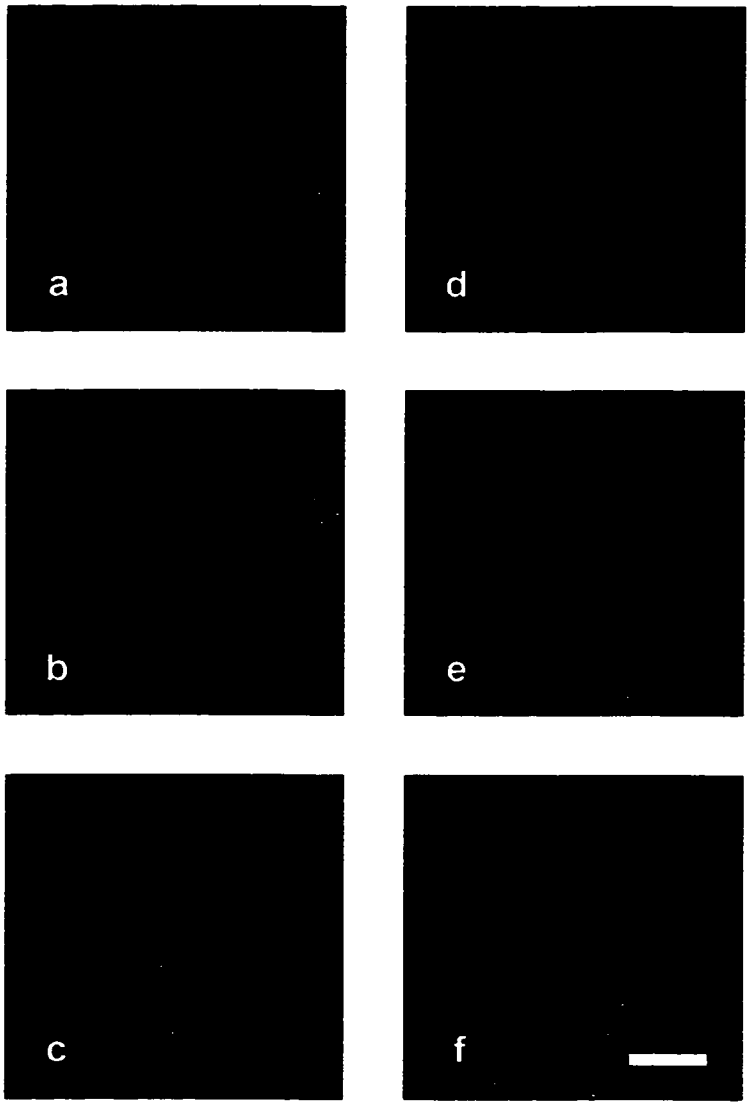
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Figure 3-16. HeLa cells treated with actinomycin-D show altered patterns of speckles identified with the 1B4 and anti-SC35 antibodies. Cells were double-labelled with the 1B4 antibody (a,e,i,m,q) and anti-SC35 (b,f,j,n,r), and counterstained with Hoescht 33258 to label DNA (c,g,k,o,s). Phase contrast micrographs are (d,h,l,p,t). The 0.5 μg/ml dose of actinomycin-D was given for 2 hours (a-d) or 1 hour (e-h). The 0.05 μg/ml dose was given for 2 hours (i-l), or 1 hour (m-p). A cell not exposed to actinomycin-D is shown for comparison (q-t). Bar = 5.4 μm.

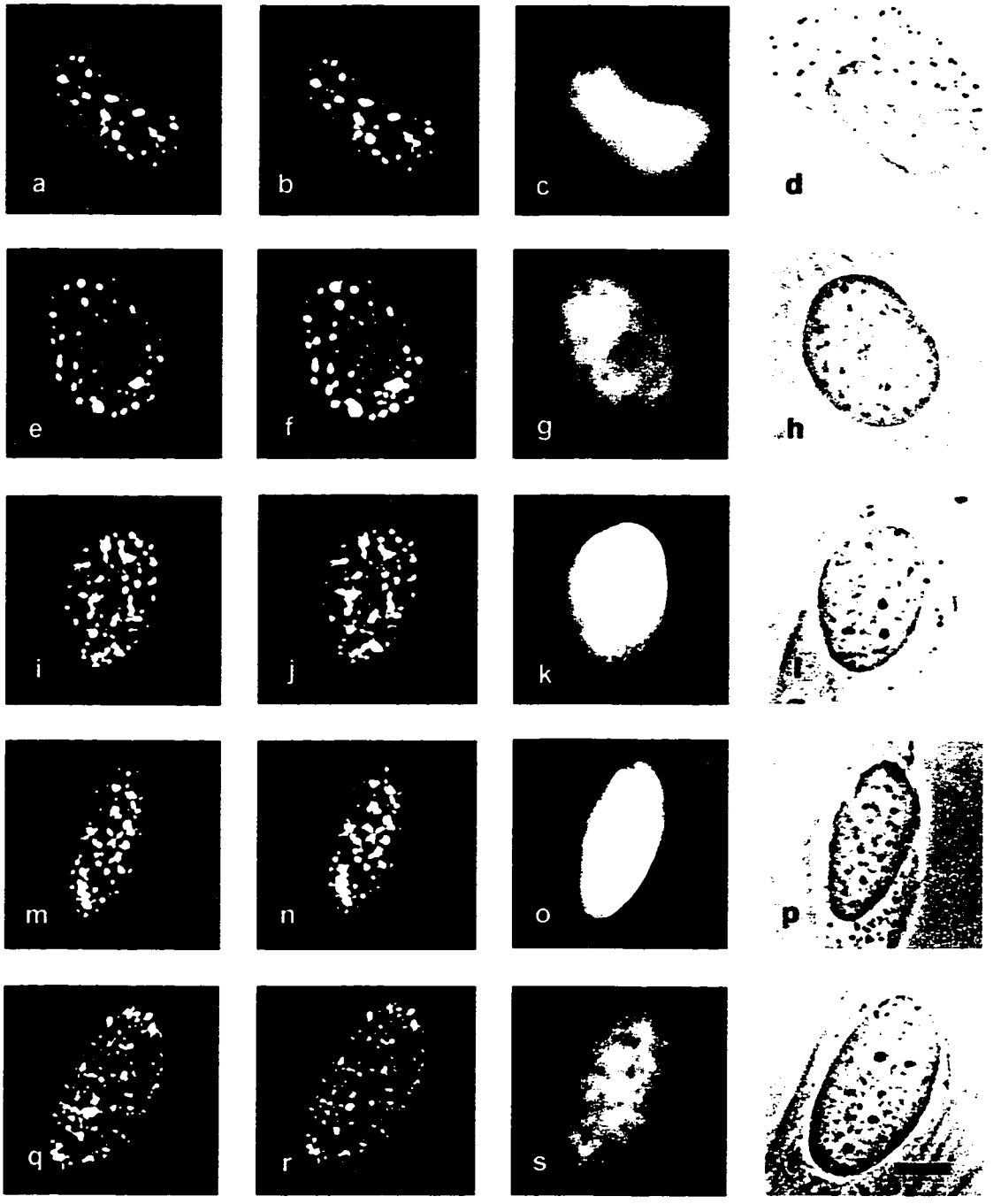
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Figure 3-17. HeLa cells treated with actinomycin-D show altered patterns of speckles identified with the 1B4 and Y12 antibodies. Cells were double-labelled with the 1B4 antibody (a,e,i,m,q) and Y12 (b,f,j,n,r), and counterstained with Hoescht 33258 to label DNA (c,g,k,o,s). Phase contrast micrographs are (d,h,l,p,t). The 0.5 μg/ml dose of actinomycin-D was given for 2 hours (a-d) or 1 hour (e-h). The 0.05 μg/ml dose was given for 2 hours (i-l), or 1 hour (m-p). A cell not exposed to actinomycin-D is shown for comparison (q-t). Bar = 5.4 μm.

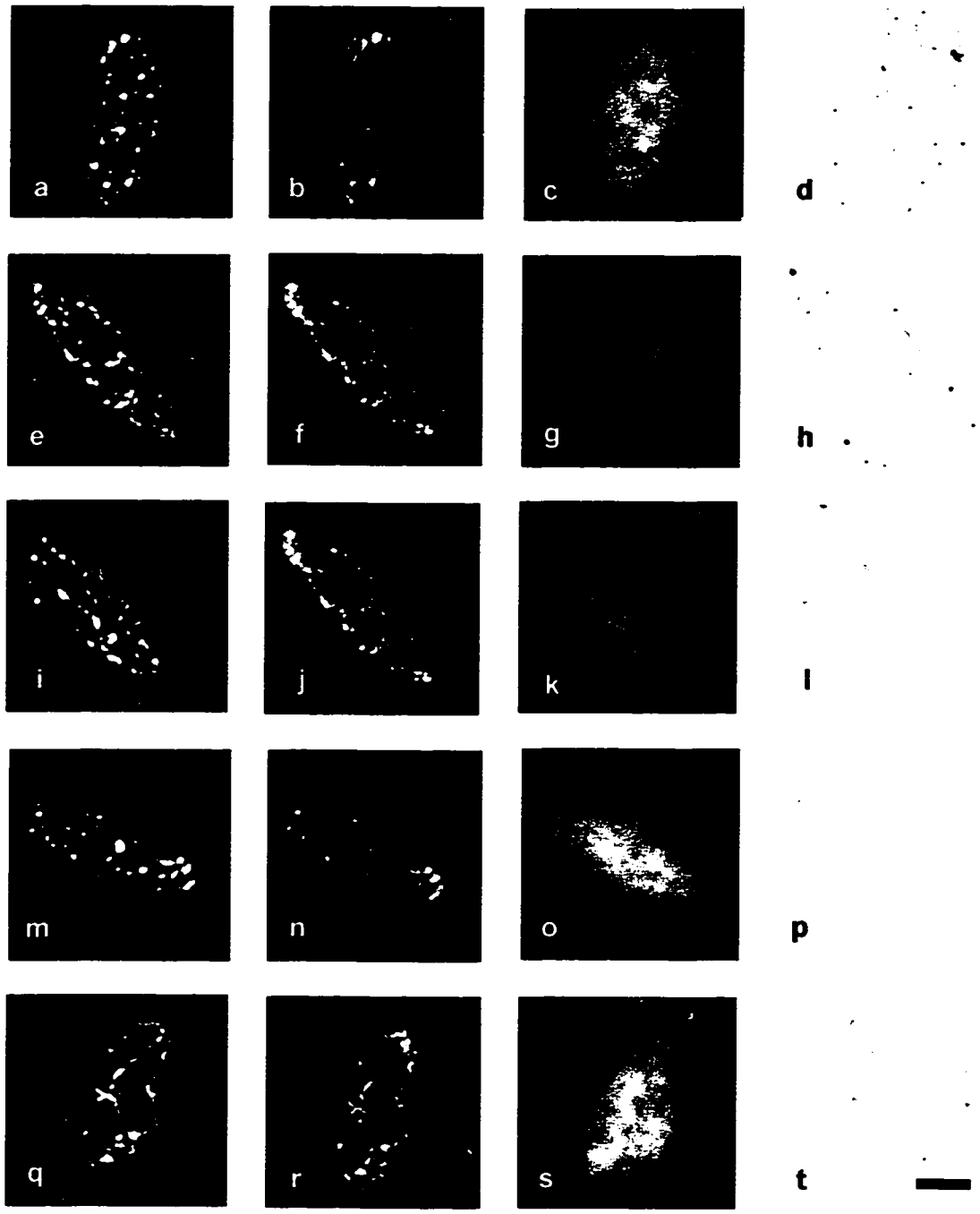
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Figure 3-18. HeLa cells treated with actinomycin-D show altered patterns of speckles identified with the 1B4 and anti-SC35 antibodies. Cells were double-labelled with the 1B4 antibody (a,e,i,m,q) and anti-SC35 (b,f,j,n,r), and counterstained with Hoescht 33258 to label DNA (c,g,k,o,s). Phase contrast micrographs are (d,h,l,p,t). The 0.5 μg/ml dose of actinomycin-D was given for 8 hours (a-d) or 4 hours (e-h). The 0.05 μg/ml dose was given for 8 hours (i-l), or 4 hours (m-p). A cell not exposed to actinomycin-D is shown for comparison (q-t). Bar = 5.4 μm.

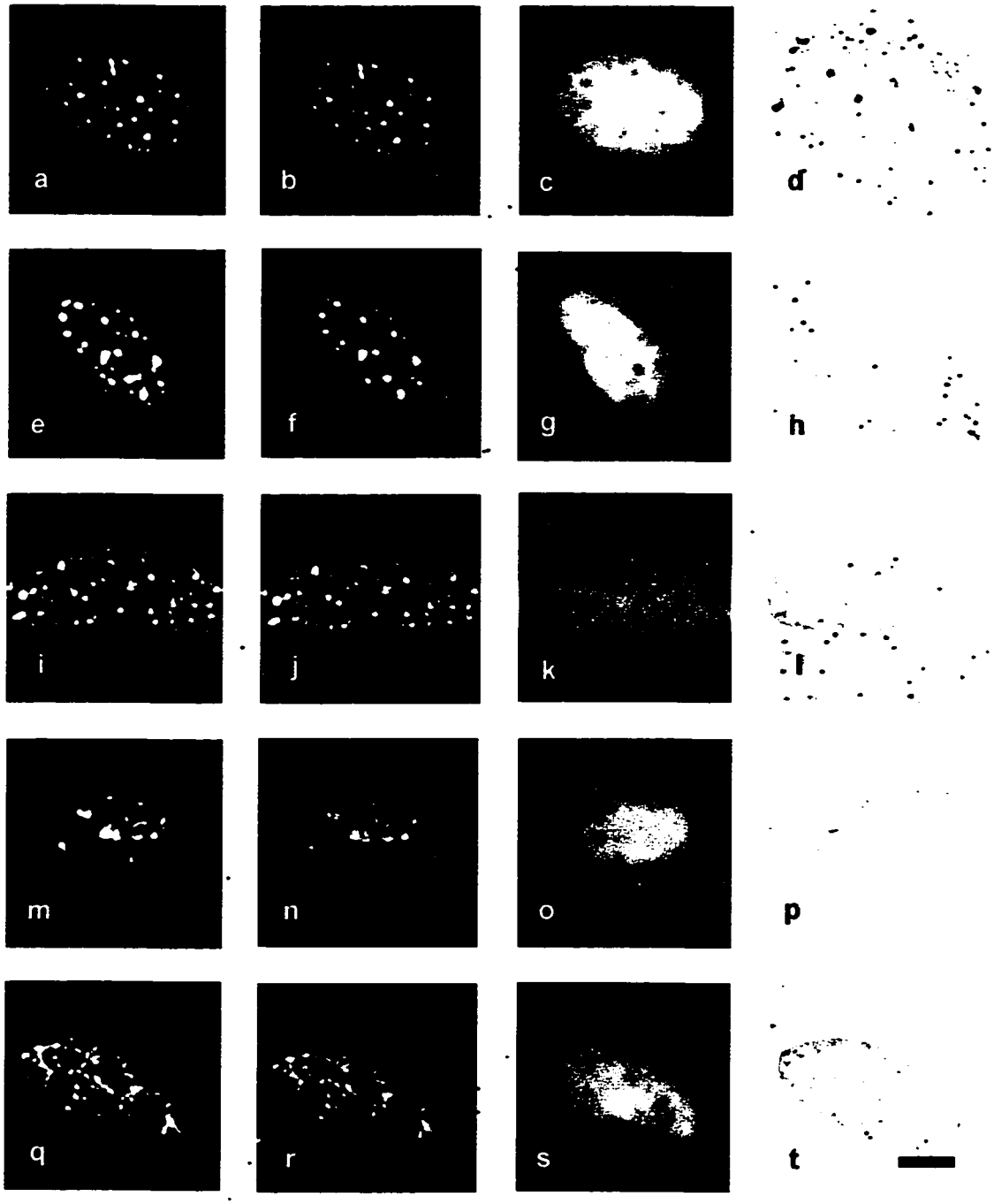
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Figure 3-19. HeLa cells treated with actinomycin-D show altered patterns of speckles identified with the 1B4 and Y12 antibodies. Cells were double-labelled with the 1B4 antibody (a,e,i,m,q) and Y12 (b,f,j,n,r), and counterstained with Hoescht 33258 to label DNA (c,g,k,o,s). Phase contrast micrographs are (d,h,l,p,t). The 0.5 μg/ml dose of actinomycin-D was given for 8 hours (a-d) or 4 hours (e-h). The 0.05 μg/ml dose was given for 8 hours (i-l), or 4 hours (m-p). A cell not exposed to actinomycin-D is shown for comparison (q-t). Bar = 5.4 μm.

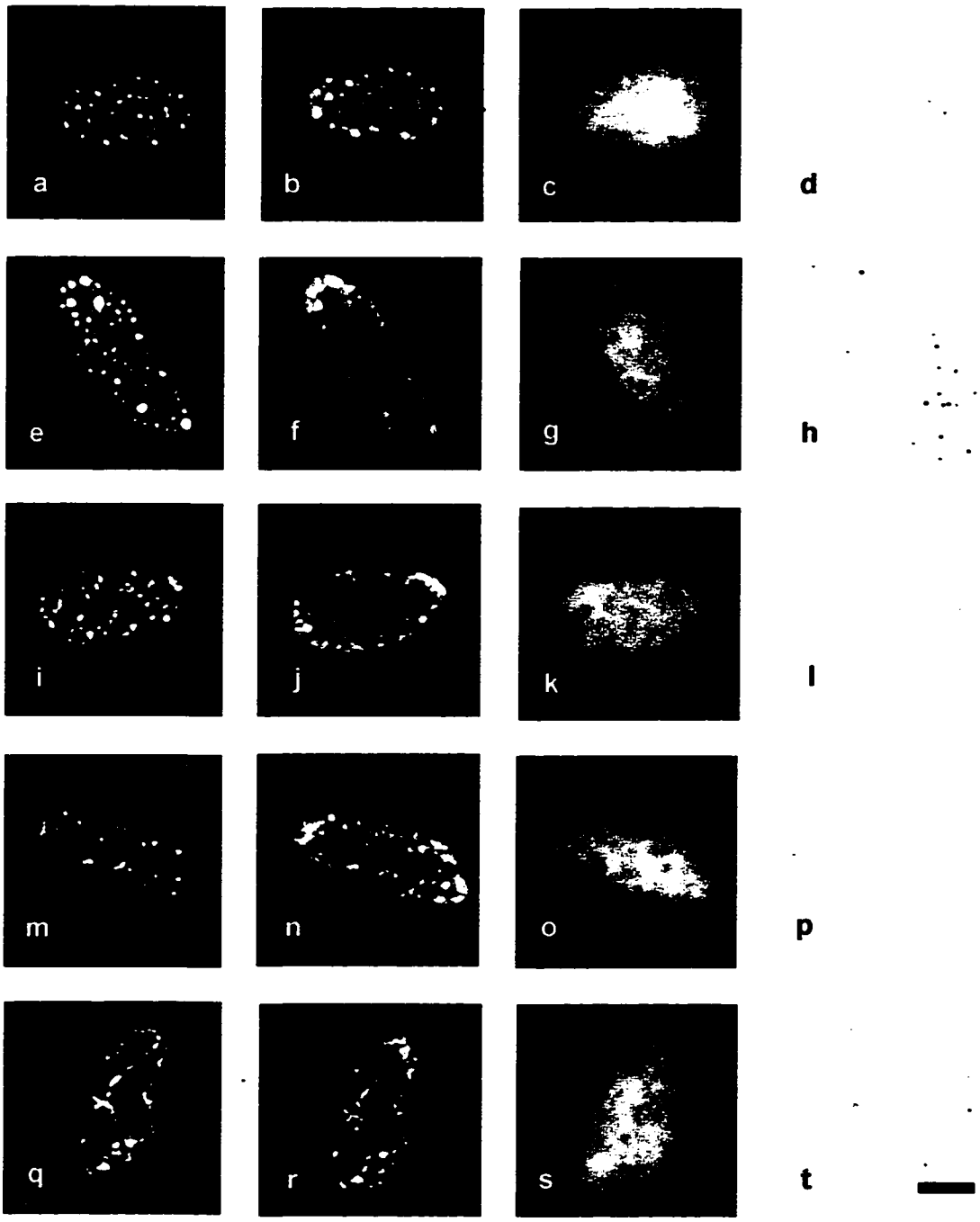
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Figure 3-20. HeLa cells exposed with 1% ethanol to control for ethanol exposure accompanying actinomycin-D treatment. Cells double-labelled with the 1B4 antibody (a,e,i,m) and anti-SC35 (b,f,j,n) are shown for exposures of 8 hours (a-d), 4 hours (e-h), 2 hours (i-l), and 1 hour (m-p). Bar = 5.4 μm.

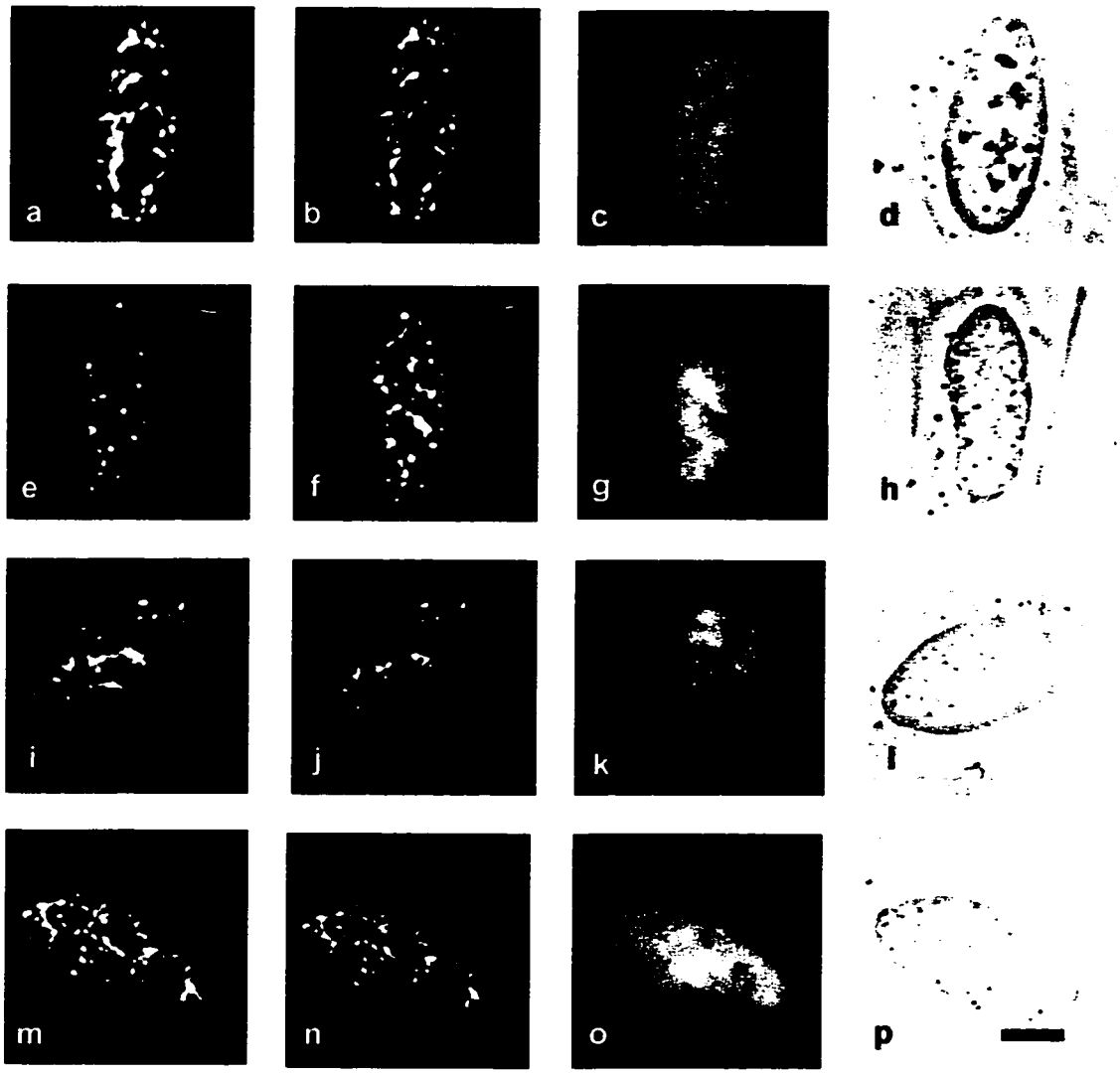
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Figure 3-21. HeLa cells treated with 25 μg/ml DRB for 2 hours and allowed to recover regain the appearance of untreated cells. These cells were double-labelled with the 1B4 antibody (a,e,i,m) and either anti-SC35 (b,j,n) or Y12 (f). The DNA is counterstained with Hoescht 33258 (c,g,k,o), and phase contrast micrographs are also shown (d,h,l,p). The 2 hour treatment with DRB is shown in (a-d) and (e-h). The cell shown in the next row (i-l) was treated with DRB for 2 hours and allowed to recover for 1 hour. Recovery for 2 hours after a 2 hour exposure is shown in (m-p). Bar = 5.4 μm.

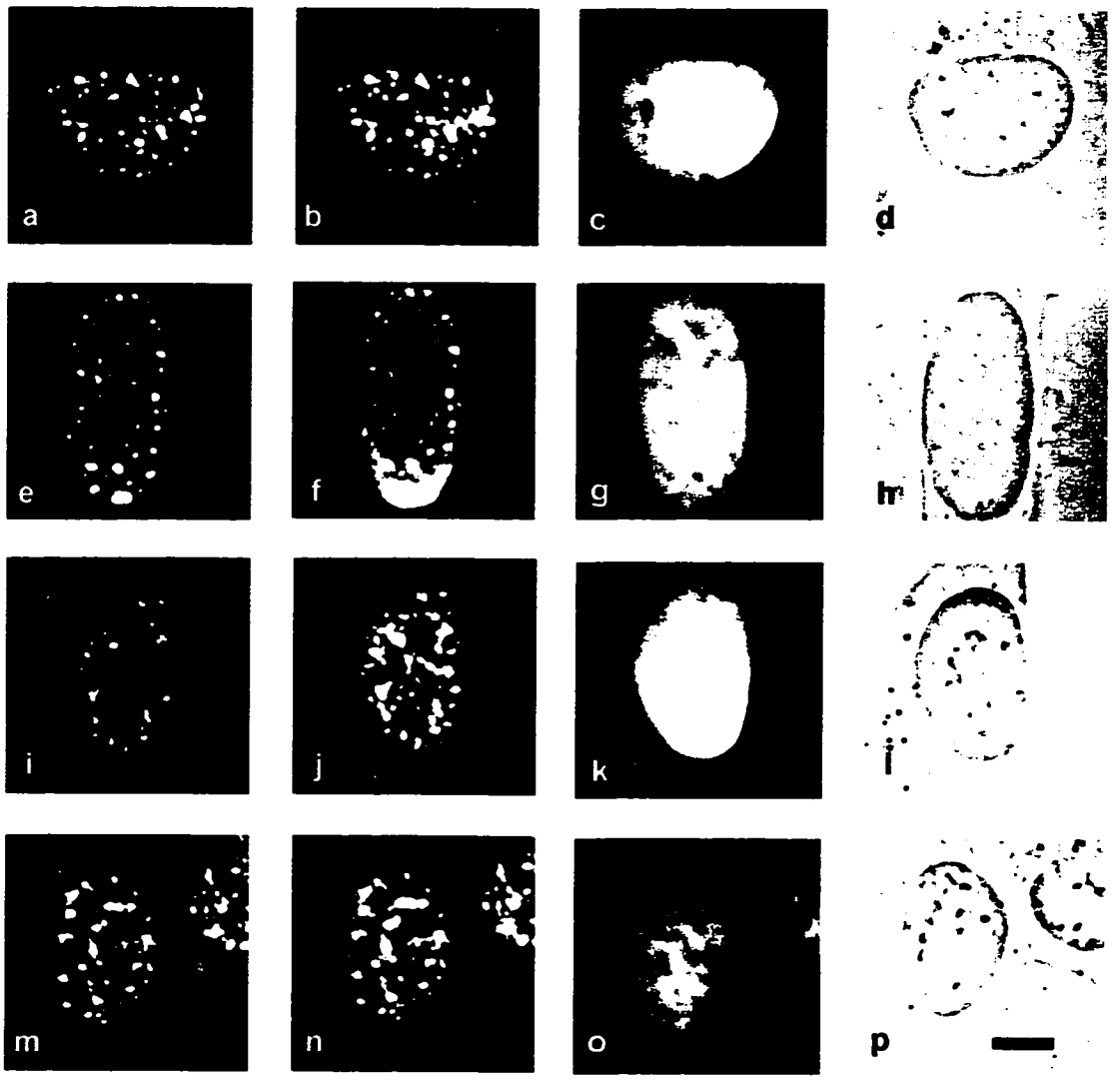
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Figure 3-22. HeLa cells treated with 0.5 μg/ml actinomycin-D for 2 hours and allowed an extended recovery period do not regain the appearance of untreated cells. These cells were double labelled with the 1B4 antibody (a,e,i,m,q) and anti-SC35 (b,f,j,n,r). Hoescht counterstaining for DNA (c,g,k,o,s) and phase contrast (d,h,l,p,t) are also shown. A representative cell treated for 2 hours with 0.5 μg/ml actinomycin-D (a-d) is shown with cells recovering from the 2 hour treatment for 2 hours (e-h), 4 hours (i-l), 10 hours (m-p) or 18 hours (q-t). Bar = 5.4 μm.

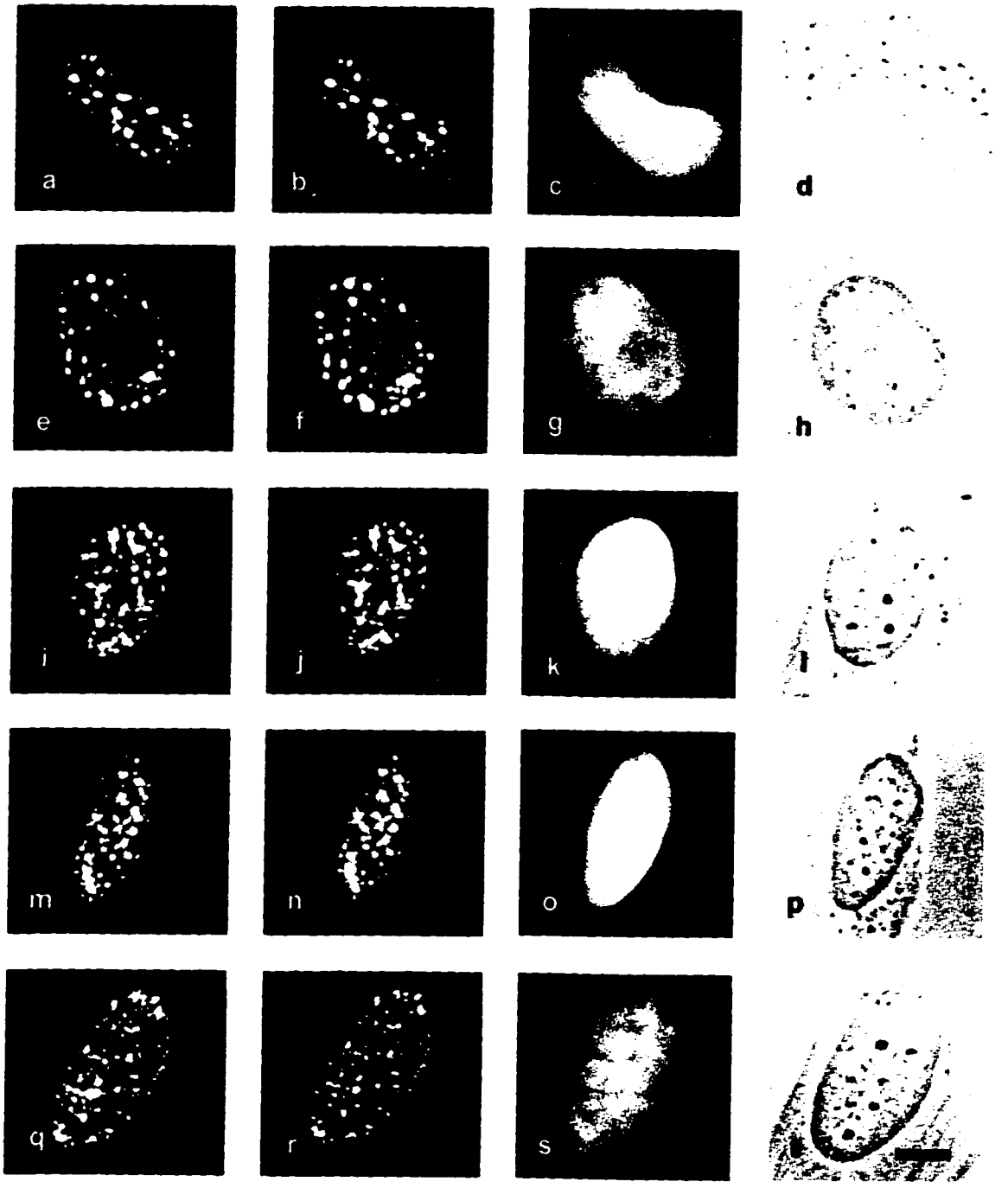
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Figure 3-23. HeLa cells treated with 0.5 μg/ml actinomycin-D for 2 hours and allowed an extended recovery period do not regain the appearance of untreated cells. These cells were double labelled with the 1B4 antibody (a,e,i,m,q) and Y12 (b,f,j,n,r). Hoescht counterstaining for DNA (c,g,k,o,s,w) and phase contrast (d,h,l,p,t) are also shown. A representative cell treated for 2 hours with 0.5 μg/ml actinomycin-D (a-d) is shown with cells recovering from the 2 hour treatment for 2 hours (e-h), 4 hours (i-l), 10 hours (m-p) or 18 hours (q-t). Bar = 5.4 μm.

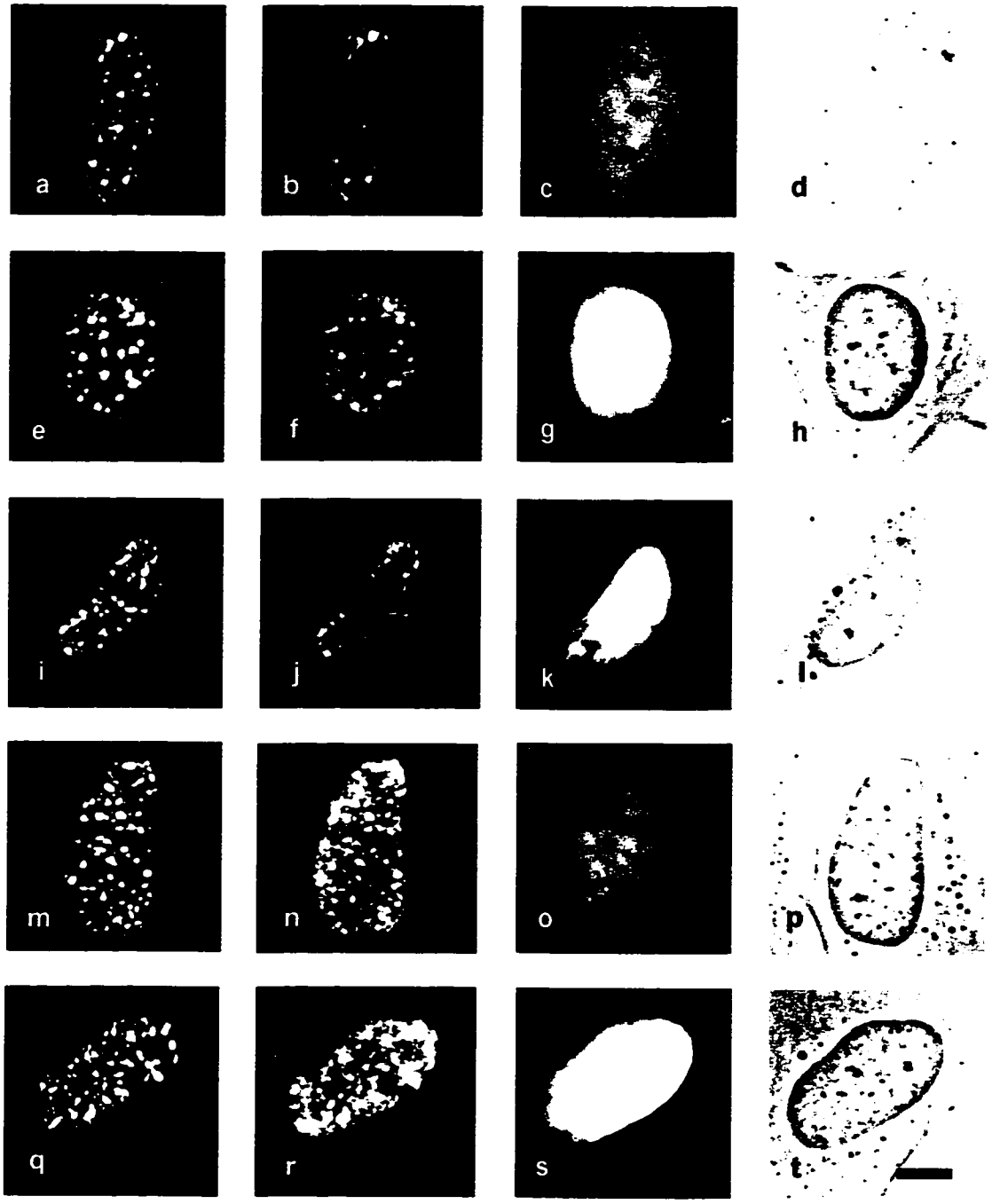
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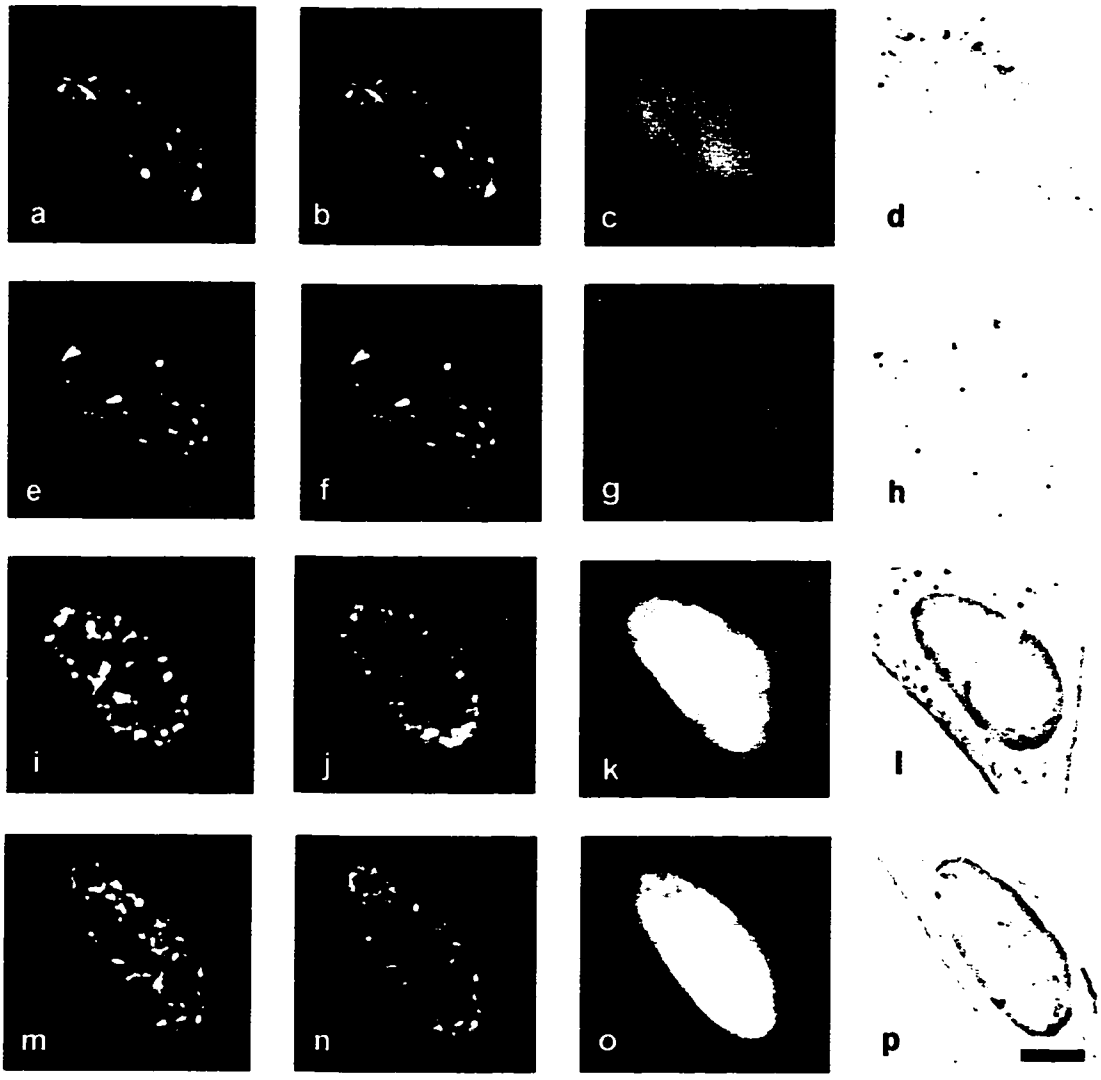
Figure 3-24. HeLa cells treated with 5 μg/ml α-amanitin for 5 hours are difficult to distinguish from untreated cells. These cells are double-labelled with the 1B4 antibody (a,e,i,m) and either anti-SC35 (b,f) or Y12 (j,n), counterstained with Hoescht 33258 (c,g,k,o) and also shown by phase contrast microscopy (d,h,l,p). The cells shown represent those treated with α-amanitin (a-d),(i-l), and ethanol controls (e-h), (m-p). Bar = 5.4 μm.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3.3.2 Assay of level of transcription in HeLa cells exposed to drugs that inhibit transcription

The overall level of transcription was assayed to correlate changes in the rearrangement of the speckled distribution described in section 3.2.1 to the effectiveness of the drugs. Table 3-1 illustrates the relative levels of transcription in HeLa cells exposed to actinomycin-D, DRB, and α-amanitin. The 2 hour treatment with 0.5 μg/ml actinomycin-D and the 2 hour treatment with 25 μg/ml DRB reduced the level of transcription to 17% and 12% of the control level respectively. Exposing cells to 0.5 μg/ml actinomycin-D for longer periods of time continued to lower the level of transcription, with a levels of 24% observed at 4 hours and 2% at 8 hours. These levels reflect the observed rearrangements of speckles in cells exposed for prolonged exposures with 0.5 μg/ml actinomycin-D. However, due to the small number of repetitions, these observed trends are unlikely to be statistically significant. The level of transcription in cells treated with α-amanitin decreased to only 41% of the control level, which corresponds to the lesser rearrangement of the speckled distribution observed by microscopy.

Table 3-2 shows the relative level of transcription in cells allowed to recover from a 2 hour 25 μg/ml DRB treatment. The levels of transcription of cells treated with 0.5 μg/ml actinomycin-D and given the same opportunity to recover are included for comparison. Actinomycin-D does not pass easily through cell membranes but may disengage from its intercalation with DNA. The level of transciption of cells exposed to DRB rapidly increased to 70% of the control value in a 2 hour recovery period. The level of

transcription in cells exposed to actinomycin-D remained at only 13% of the control value after a 2 hour recovery period, and increased to only 37% of the control value after 18 hours. As described in Section 3.2.1, it is interesting that the DRB-treated cells regained the appearance of untreated cells within 2 hours, while those exposed to actinomycin-D never did regain the interconnecting fibrils between speckles.

Table 3-1. Measurements of the effect of actinomycin-D, DRB, and α-amanitin upon HeLa cells. Tritiated uridine incorporation is expressed as a percentage relative to a control value of 100%. The results of repeated trials are averaged in the final row.

	0.5 μg/ml actinomycin-D			0.05 μg/ml actinomycin-D			25 μg/ml DRB	5 μg/ml α-amanitin
hours of exposure	2h	4h	8h	2h	4h	8h	2h	5h
per cent relative rate of trans-cription	18 15 17	24 24	2 2	33 47	9 58	40 45	12 12	76 54
average	17	24	2	40	34	43	12	65

Table 3-2. Measurement of the capability of HeLa cells to recover from exposure to drugs that inhibit transcription. Tritiated uridine incorporation is expressed as a percentage relative to a control value of 100%. The results of repeated trials are averaged in the final row.

	25 μg/ml DRB		0.5 μg/ml actinomycin-D			
hours of exposure	0h	2h	0h	2h	10h	18h
per cent relative rate of trans-cription	12 12	76 65	19 15 17	14 12	33 34	43 32 36
average	12	70	17	13	34	37

3.3.3 Quantification of speckles in HeLa cells exposed to drugs that inhibit transcription

The alterations in the speckled distribution of cells exposed to drugs that inhibit transcription was quantified in an attempt to verify the apparent decrease in the number of speckles seen by immunofluorescence microscopy. HeLa cells exposed to some of the drug treatments were stained using DAB as a chromogen (Section 2.4). The same staining pattern seen with the 1B4 and anti-SC35 antibodies by fluorescence microscopy was observed, except that some speckles appeared to have pale centres, and that the chromosomes appeared stained in mitotic cells. Control cells to which no primary antibody were applied were not stained with DAB (Figure 3-25). The advantages of this staining technique over fluorescence microscopy were that the intensity of the stain did not fade with time, and that the cells could be permanently mounted. As the cells were dehydrated before being mounted in DPX medium, there was a distinct plane in which the speckles were in focus. This flattening of the cells did not cause them to appear very different than cells viewed by light microscopy: most of the speckles in cells viewed by light microscopy appear to be arranged in a plane parallel to the substratum. This observation has been documented by Carter and colleagues (1993). Upon detailed examination, the actual number of speckles did not decrease as much as expected, with a notable trend seen only in the extreme treatment groups.

Counts of the number of speckles in 200 1B4/peroxidase-stained cells in each actinomycin-D treatment group showed large decreases in the number of speckles only in cells treated with drugs for 4 or more hours (Table 3-3). The rearrangement of the speckled

distribution observed by visual inspection is more on account of the loss of connecting fibrils than because of a change in the actual number of speckles. As the fibrils connecting the speckles clear away, they appeared to form distinct fine spots which were indistinguishable from speckles, since dark foci were counted without using a set criterion such as a minimum size. The tendency for speckles to be aggregated near each other also contributed to the impression that there are much fewer larger ones in cells treated with transcriptional inhibitors. The number of speckles alone cannot be used to quantify the rearrangement of the speckled distribution.

Table 3-3. Mean number of speckles identified with the 1B4 antibody in the nuclei of cells stained using diaminobenzidine as a chromogen. Averages of the number of speckles in the nuclei of 200 cells are shown; the data was not normally distributed.

actinomycin-D dose	mean number of speckles after various hours of exposure			
	0h	1h	2h	8h
0.5 μg/ml	35	31	31	24
0.05 μg/ml	35	32	31	23

Figure 3-25. Immunoperoxidase labelling of HeLa cells with the 1B4 antibody (a), and a control cell from a slide to which no primary antibody was applied (b). Since diaminobenzidine was used as a chromogen, the speckled distribution of the 1B4 antigen is visible as dark spots by phase contrast microscopy. Bar = 5.4 μm.

a b

3.4 The distributions of the 1B4 antigen, snRNPs, and SC35 in L6E9 cells and murine splenocytes

3.4.1 Differentiation of L6E9 cells

Cycling L6E9 cells and those induced to differentiate by serum starvation were observed by both conventional and confocal microscopy. Differentiated cells characteristically were multinucleated, as differentiation into myotubes involves the fusion of adjacent cycling cells. The distributions of the 1B4 antigen or snRNPs in either cycling or differentiated cells were similar to those seen in HeLa cells, with prominent speckles visible against an unstained (1B4) or diffuse (Y12) background (Figures 3-26 and 3-27). Large, chunky speckles were seen in some differentiated cells. However, this observation was also noted in cells in sparse patches on the coverslips. As these were isolated from other cells and therefore incapable of differentiating, it cannot be concluded that the slight alteration in the appearance of the speckles is a result of fusion.

Staining with anti-SC35 in either cycling or differentiated cells was very faint, and difficult to photograph. SC35 appeared to be distributed in grainy speckles. Some larger grains appeared to colocalise with the 1B4 antigen by visual inspection of cells on the light microscope. This is very difficult to see in images obtained by confocal microscopy, or by light microscopy using a regular (50 second) exposure time. The anti-SC35 antibody was used undiluted rather than at the 1:10 dilution used to stain other cells, and the same detection system and fixation was used. HeLa cells fixed and stained in parallel to L6E9 cells showed the bright labelling of nucleoplasmic speckles typically detected with this antibody. By extending the exposure time to 3 minutes, a speckled distribution largely

coincident with that of 1B4 was obtained (Figures 3-26 and 3-27). It is difficult to judge whether the small foci that do not colocalise with 1B4 are significant, as a 3 minute exposure was not used for any observations with HeLa cells and may introduce errors.

Figure 3-26. Cycling L6E9 cells show the speckled distribution of the 1B4 antigen, pol IIo, and snRNPs seen in HeLa cells. However, staining for SC35 was extremely faint and grainy. The photograph of a cell stained with anti-SC35 shown here were taken with a 3 minute exposure time. These cells were double-labelled with the 1B4 antibody (a,e,i), and either Y12 (b), CC-3 (f), or anti-SC35 (j). Counterstaining with Hoescht 33258 for DNA (c,g,k), and phase contrast (d,h,l), are also shown. Bar = 5.4 μm.

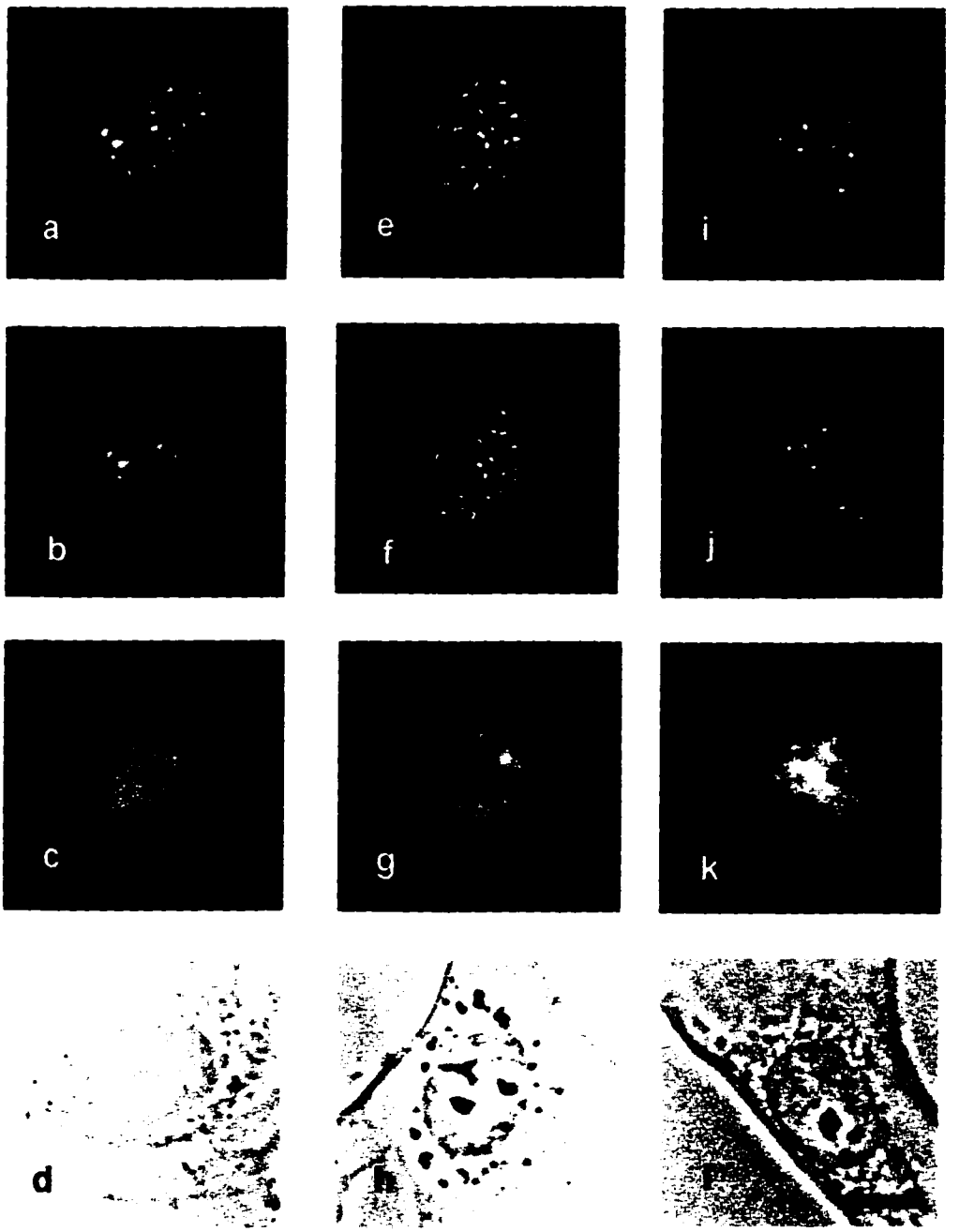
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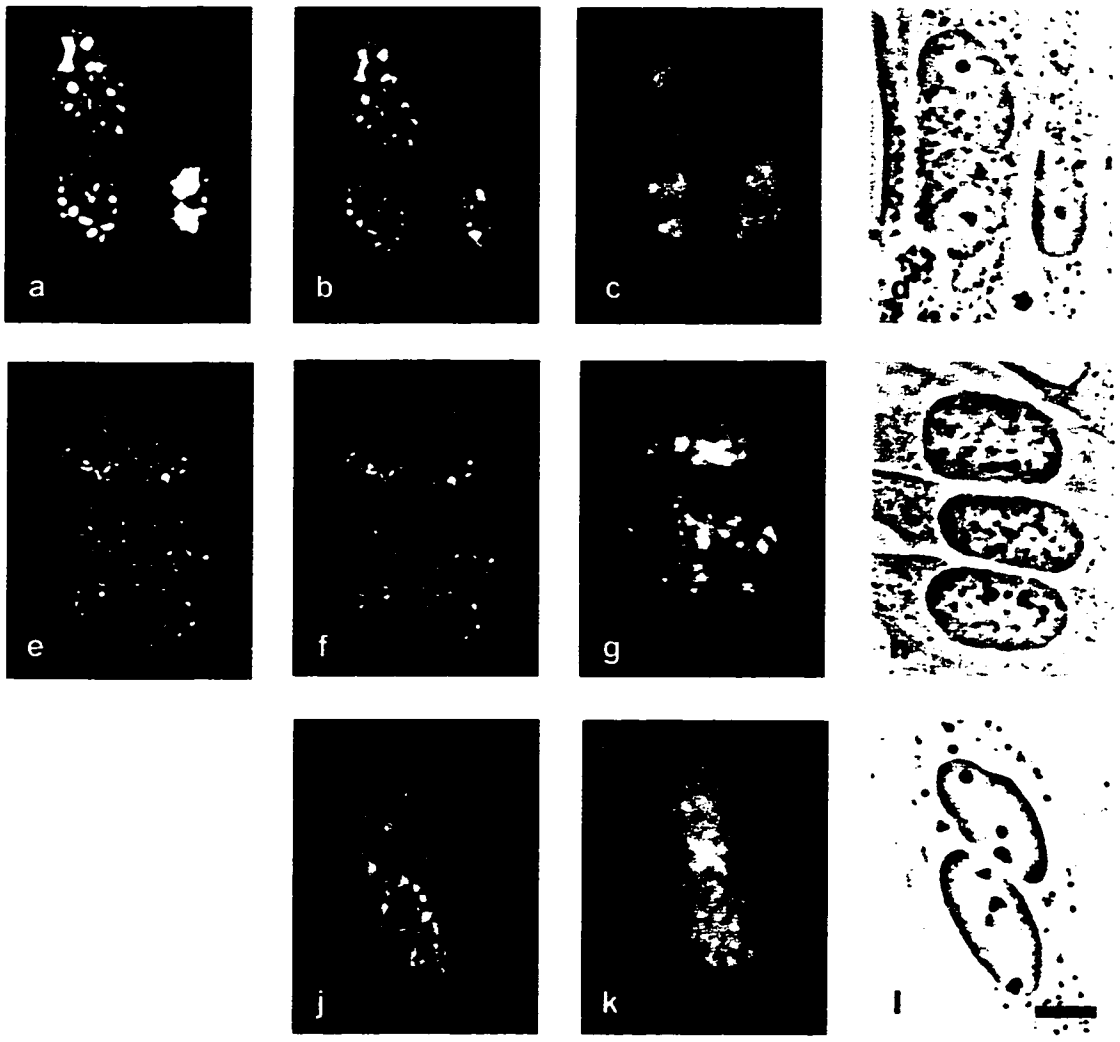
Figure 3-27. Differentiated L6E9 cells show the speckled distribution of the 1B4 antigen, pol IIo, and snRNPs seen in HeLa cells. However, staining for SC35 was extremely faint and grainy. The photograph of a cell stained with anti-SC35 shown here were taken with a 3 minute exposure time. These cells were double-labelled with the 1B4 antibody (a,e), and either anti-SC35 (b), CC-3 (f), or Y12 (j). Counterstaining with Hoescht 33258 for DNA (c,g,k), and phase contrast (d,h,l), are also shown. Bar = 5.4 μm.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3.4.2 Stimulation of murine splenocytes

Primary splenocyte cultures stimulated with concanavalin A increased in both the total cell volume and in the relative ratio cytoplasmic to nuclear volume (Figure 3-28). The level of transcription 24 hours post-stimulation was 5.6-fold the level of transcription in non-stimulated cells, which is similar to the 6-fold increase in stimulation reported by Daev and colleagues (1994) at 24 hours post-stimulation. However, the almost 20-fold higher level of uridine incorporation reported by Daev and colleagues was not reached at 48 hours post-stimulation. In the cultures I studied, the levels of transcription at 48 and 72 hours post-stimulation were 9.1 and 10.7-fold respectively. My values are averages of three trials in which the variability between trials was high, though the trend in each trial was of increasing transcription with the greatest increase occurring between the 24 and 48 hour times post-stimulation. Thus I have not used statistics to determine the significance of the increase.

Chaly and colleagues (1988), and Daev and colleagues (1994) reported that the intensity of staining with the Y12 antibody increased with time up to a maximum 72 h after stimulation, while control cells (unstimulated) remained relatively dim. I observed Y12 staining that was faintly diffuse through the nucleus with some focal regions of increased intensity in stimulated cells. These observations were also noted with the CC-3 antibody. However, only small numbers of cells were observed by confocal microscopy, so it is difficult to reach conclusions regarding the extent of the increase in intensity. The distribution of the 1B4 antigen appeared similar to that of pol IIo identified with the CC-3 antibody in both its increased intensity with stimulation and distribution in the nucleus

(Figure 3-29). Confocal microscopy was used in attempts to determine whether the distributions of the snRNPs and 1B4 antigen overlap. However, the intensity of staining was very faint with the 1B4 antibody. Confocal microscopy requires that distributions of antigens to be compared are labelled with equal intensity.

Staining for SC35 in splenocytes was extremely faint as compared to the intensity seen in HeLa cells stained by the same procedures. I used the anti-SC35 hybridoma supernatant directly (I diluted it 1:10 or more to stain HeLa cells) and observed only a faint, diffuse staining. There did not appear to be foci of concentrated staining within the cell as visualised by confocal scanning of serial sections. This staining was so faint as to be visible to the naked eye but difficult to capture as either a confocal image or photograph. Brighter labelling was visible to the naked eye in stimulated cells, though this level was still too dim to be photographed.

Figure 3-28. Splenocytes stimulated with concanavalin-A show increased total cell volume and an increase in the relative ratio of cytoplasmic to nuclear volume in phase contrast micrographs. Stimulated cells at 24 hours (a), 48 hours (b), 72 hours (c) post-stimulation with concanavalin-A are shown compared to an unstimulated control (d) kept in parallel with the 48 hour post-stimulation group. Bar = 3.4 μm.

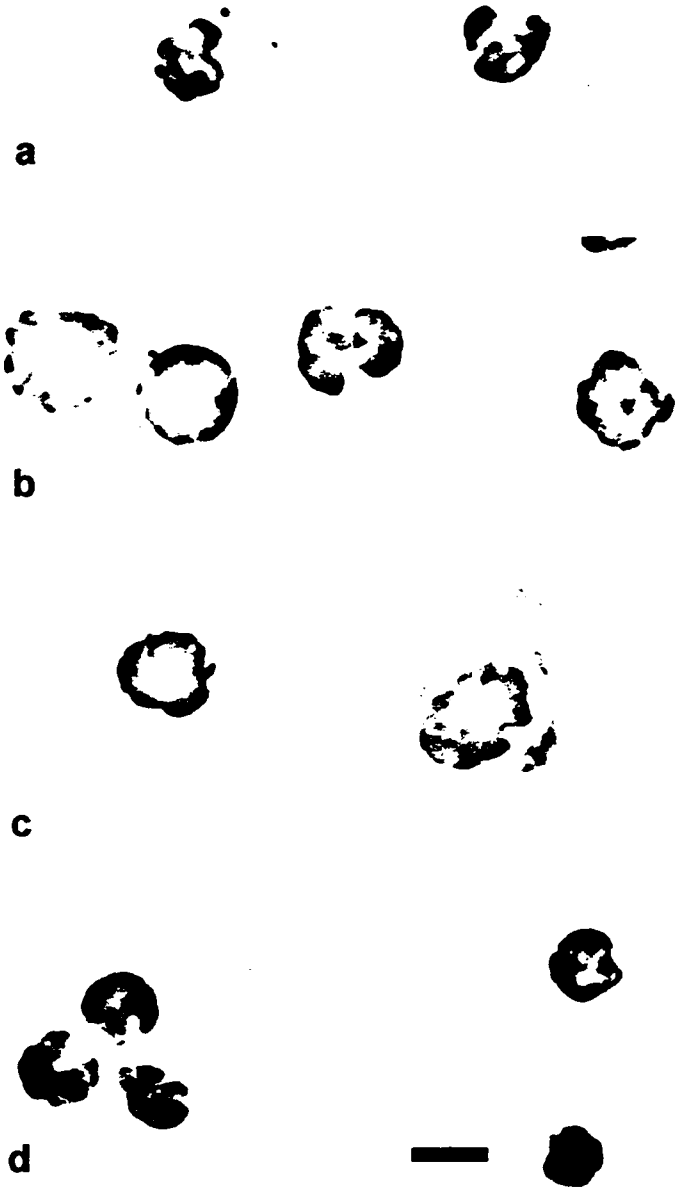
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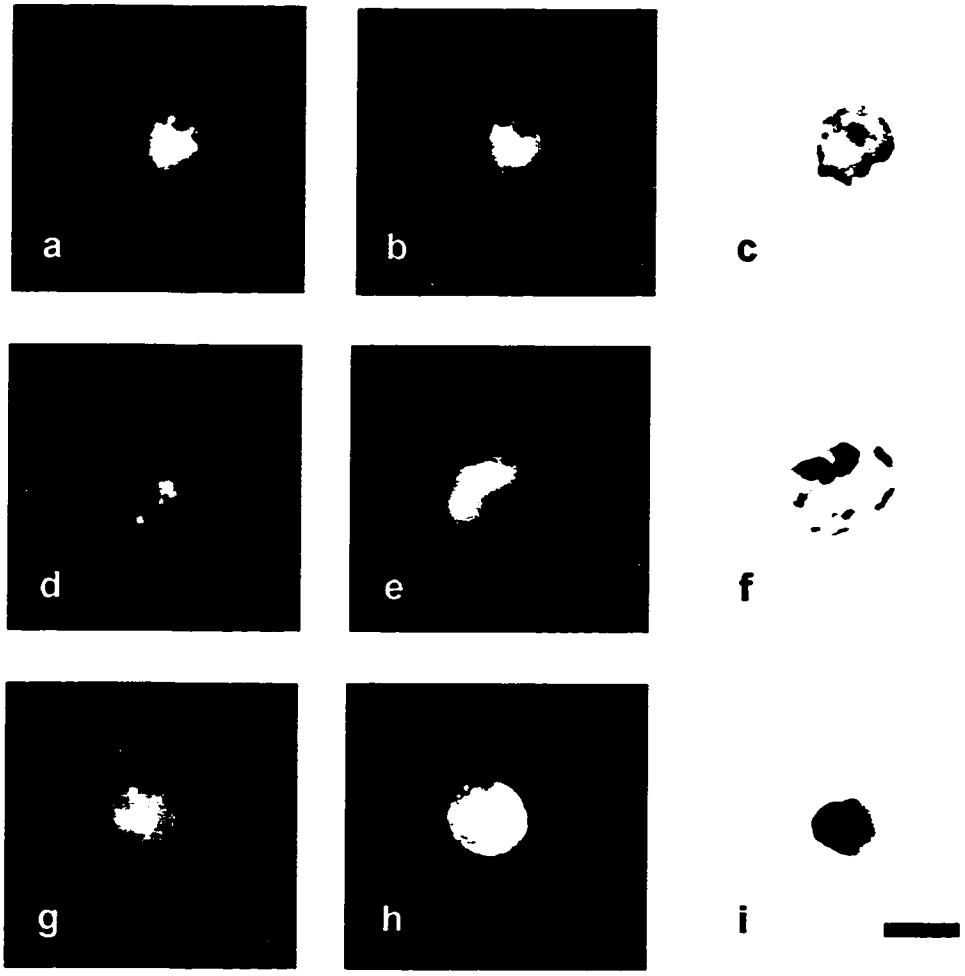
Figure 3-29. Splenocytes double-labelled with the 1B4 antibody (a,d,g) and CC-3 (b,e,h) are shown 48 hours (a-c) and 72 hours (d-f) post-stimulation. An unstimulated control (g-i) kept in parallel with the 48 hour post-stimualation group. Phase contrast micrographs are panels (c,f,i). Bar = 3.4 μm.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4. DISCUSSION

4.1 The 1B4 antigen is a nuclear matrix component of interphase cells

The speckled distributions of the 1B4 antigen and SC35 seen in interphase cells were maintained in nuclear matrix preparations (Figures 3-2, 3-3, 3-7, and 3-8). SC35 has been characterised as a nuclear matrix protein (Bisotto *et al.*, 1995; Huang and Spector, 1996). Speckles and foci labelled with the Y12 antibody were also observed in nuclear matrix preparations. The foci labelled with the Y12 antibody which probably represent coiled bodies were not labelled by the 1B4 antibody in nuclear matrix preparations (Figure 3-6). Interestingly, observations of the distributions of these antigens were consistent regardless of whether the protocol used to prepare nuclear matrices was that of Chaly and colleagues (1985) or Fey and colleagues (1986).

Chaly (1988) reported that her nuclear matrix preparations appeared to be composed of extensive fibrogranular networks in conventional electron microscopic preparations. This contradicted the findings of Fey and colleagues (1986). This group reported that the nuclear matrix prepared with sodium chloride did not retain the network of fibrils and granules seen in nuclear matrix preparations made using ammonium sulphate. They also stated that resinless sections were superior to conventional electron microscopy preparations for viewing nuclear matrix preparations. In the discussion of their results, Fey and colleagues proposed that nuclear matrices prepared with sodium chloride would be useful for observing chromatin loop attachment sites. Nuclear matrices prepared with ammonium sulphate would be suitable for studying tissue-specific nuclear composition, structure, and function because they retained proteins associated with hnRNA. In the protocol of Chaly,

sodium tetrathionate is included as a protease inhibitor, serving to stabilise the matrix architecture by promoting the formation of disulfide bridges (Chaly, 1988).

Neither Chaly nor Fey stained their nuclear matrix preparations with the Y12 antibody. Bisotto and colleagues (1995) stained nuclear matrices prepared with ammonium sulphate using the Y12 antibody. They reported that Y12 labelling was not observed by light microscopy at the end of the extraction procedure. However, earlier studies using sodium chloride-based nuclear matrix extraction protocols and the Y12 antibody claimed that the snRNPs localised in speckles were components of the nuclear matrix (Vogelstein and Hunt, 1982; Spector *et al.*, 1983). I observed that the Y12 labelling of the speckles and foci was retained using either the ammonium sulphate or sodium chloride extraction procedures. If Y12 labelling were maintained only in nuclear matrices prepared using the protocol of Chaly, it might be argued that the sodium tetrathionate had promoted artefactual disulphide bridge formation rather than stabilising existing protein architecture. Though I did not examine my nuclear matrix preparations by electron microscopy, the retention of faint Hoescht staining for DNA in the nuclear matrices prepared using the protocol of Fey and colleagues (1986) suggests that this protocol is not as harsh as sodium chloride extractions.

My observations of the markedly decreased intensity of staining for the m3G cap structure during nuclear matrix extraction procedures are in agreement with those of Bisotto and colleagues (1995). They did not show their results with this antibody or report whether the staining appeared to localise at the residual nucleoli at intermediate steps of the extraction. Re-arrangement of the m3G cap staining distribution in unfixed cells in my

experiments was an artefact which should not be confused with the redistribution of splicing factors upon inhibition of transcription. HeLa cells treated with drugs to inhibit transcription were maintained as regular cultures aside from the presence of drugs in the medium. Nuclear matrix extractions were performed in non-physiological buffers, on ice rather than at 37°C, and outside of the 5% CO_2 atmosphere that HeLa cells require. Following the extraction procedure, the cells were fixed prior to staining. It is likely that the m3G-capped RNAs were mechanically trapped at the aggregated nucleolar structures. The key observation in evaluating nuclear matrix preparations is the maintenance of labelling distribution and intensity. Provided that the signal is not redistributed diffusely throughout the cell, maintained labelling intensity is evidence that the antigen being detected has not been removed from the cell by the extraction procedure. The maintained Y12 labelling in speckles of nuclear matrix preparations would probably represent snRNP polypeptides rather than assembled snRNPs. Sites to which several snRNP polypeptides bind have been identified for several snRNA species. However, some proteins are believed to bind to the snRNPs via protein-protein interactions (reviewed by Lührmann *et al.*, 1990). The nature of the associations of the snRNPs with the nuclear matrix is not well defined. Carmo-Fonseca and colleagues (1991) concluded that the foci labelled by both antibodies to the snRNP polypeptides (Y12) and m3G caps of snRNAs represented assembled snRNP particles. Thus the loss of the m3G cap structure in nuclear matrix preparations while Y12 labelling is maintained indicates that some snRNP polypeptides maintain their association with the nuclear matrix even with the loss of at least a portion of the snRNA. It is not possible to determine whether the stem/loop structural elements to which several snRNP

polypeptides bind are protected from ribonuclease digestion by these proteins. The retention of SC35 and the snRNPs concentrated in foci in nuclear matrix preparations implicates the nuclear matrix as an anchor for the speckled domains. With respect to a current model of pre-mRNA metabolism in the nucleus, these results imply that active splicing factors along the perichromatin fibrils and in the nucleoplasm are not as securely associated with the nuclear matrix as those concentrated in interchromatin granules.

4.2 Fate of the 1B4 antigen, snRNPs and SC35 during the cell cycle

The colocalisation of the 1B4 antigen and SC35 in interphase cells was maintained as cells progressed through the cell cycle (Figures 3-10, 3-11, and 3-12). This finding is consistent with the partial colocalisation of the 1B4 antigen and the snRNPs. The behaviour of SC35 and the snRNPs through the cell cycle has previously been documented (Spector and Smith, 1986; Leser *et al.*, 1989; Spector *et al.*, 1991; Spector, 1994; Ferriera *et al.*, 1994; reviewed by Huang and Spector, 1996). Cells entering mitosis show a diffuse snRNP or SC35 distribution that is excluded from condensed chromatin. Transport of snRNPs into daughter nuclei correlates with the onset of transcription occurring in late telophase (Ferriera *et al.*, 1994). SC35 and the 1B4 antigen remain in the cytoplasm of interphase cells in early G1 after snRNPs have re-entered the nucleus (Spector *et al.*, 1991). Ferriera and colleagues (1994) stated that the snRNPs leave mitotic granule clusters while SC35 remains in them. Very faint staining for SC35 in the nucleus may be indicative of the presence of this antigen, though other groups have considered that this protein may be present but not detected by immunofluorescence labelling. I observed the 1B4 antigen in

foci that colocalised with SC35 in telophase cells. The characteristic speckled distribution with thin fibrils apparent between the speckles was seen for these antigens in late telophase cells showing decondensed chromatin. This is consistent with the proposed function of speckles as storage sites for splicing factors if late telophase cells contain excess SC35. Unfortunately, quantitative measures of levels of transcription in early and late telophase cells are not feasible. Ferriera and colleagues visualised *in situ* transcription sites in non-synchronised cells using the method of Jackson and colleagues (1993). They did not observe the nascent transcription sites visualised by brominated uridine in prometaphase through to early telophase.

It has been established that the mitotic granule clusters are not coiled bodies. Studies using the Y12 antibody and sera specific for p80 coilin found that coiled bodies disassemble during mitosis, and reform in daughter nuclei in late G1 (Andrade *et al.*, 1993; Carmo-Fonseca *et al.*, 1993; Ferriera *et al.*, 1994). This lag accounts for some observations of interphase cells without coiled bodies. I observed coiled bodies stained with the Y12 antibody as distinct foci that were most clearly visible in confocal micrographs or colour photographs. They were also clearly visible in colour photographs of nuclear matrix preparations from which diffuse staining of snRNPs in the nucleoplasm was removed (Figure 3-6). Exclusion of the 1B4 antigen from coiled bodies suggests that its function is not directly related to the function of these structures.

However, the function of the coiled bodies is not well defined. Gall and colleagues (1995) argued that the coiled bodies seen in mammalian cells are homologous to structures seen in *Xenopus* egg extract, and in both amphibian and insect germinal vesicles. All of

these structures contain spliceosomal snRNPs and a unique 80 kDa protein termed p80 coilin, as well as several nucleolar proteins. Several lines of evidence suggest that the function of coiled bodies is related to nucleolar function (Raska *et al.*, 1990; Jiménez-García *et al.*, 1994; Malatesta *et al.*, 1994; reviewed by Bohmann *et al.*, 1995). Recent evidence suggests that coiled bodies are structurally heterogeneous. Not all coiled bodies contain all of the proteins identified within these structures (Bauer and Gall, 1997).

Since the 1B4 antibody clearly detected the 1B4 antigen in interphase cells by immunofluorescence, the lack of a signal detected with the 1B4 antibody in Western blots of interphase cells does not indicate cell cycle-specific expression. Antibodies that work well in immunofluorescence labelling studies while not producing a signal on Western blots are not uncommon. Studies have been published describing the distributions of nuclear matrix proteins which are not identified by immunoblotting (*e.g.* Chaly *et al.*, 1984). Of the antibodies used in this study for immunofluorescence labelling, only the Y12 antibody against the snRNPs produced a clean signal much stronger than the background due to the detection system. The CC-3 antibody produced a very strong signal and identified multiple bands in mitotic cell homogenates, in agreement with Thibodeau and Vincent (1991). The αSC35 antibody did not produce a signal in whole cell homogenates or isolated nuclei. This finding has been confirmed by other researchers (Dr. M. Vincent, personal communication). Spector and colleagues (1991) specified that preparations of immunoaffinity-purified proteins were used for Western blotting. Carmo-Fonseca and colleagues (1991a) stated that they obtained an approximately 35 kDa signal with αSC35, and that this antibody also weakly labels multiple other bands. They did not publish these

results.

Western blots using the 1B4 antibody identified a 100 kDa protein which must contain the 1B4 epitope in extracts prepared from mitotic cells (Figure 3-13). No signal was obtained in blots of proteins prepared from unsynchronised (predominantly interphase) cells. Isolating nuclei to select for nuclear proteins did not produce results. However, the 1B4 antibody does detect a signal in dot-blots of interphase cell extracts prepared under non-denaturing conditions. This indicates that the 1B4 antibody only binds efficiently to its epitope in interphase cells under non-denaturing conditions. Unfortunately, denaturation/renaturation protocols (Celenza and Carlson, 1986; Vinson *et al.*, 1988) that were reported to be successful with other nuclear matrix antigens (Blencowe *et al.*, 1994) did not allow the 1B4 antibody to bind to its epitope.

Though it was not possible to test whether the reactivity of the 1B4 antibody was dependent upon phosphorylation by Western blotting, I did attempt to assay this by immunofluorescence. Cells treated with alkaline phosphatase *in vivo* and stained using the 1B4 antibody did not show a decrease in fluorescence intensity. The intensity of immunofluorescence staining for B23, a known phosphoprotein (Paulin-Levasseur *et al.*, 1995), was greatly decreased by this procedure (Figure 3-14). However, this is not conclusive evidence that binding of the 1B4 antibody is independent of phosphorylation of the 1B4 antigen. If binding of the 1B4 antigen is dependent upon phosphorylation, fluorescence intensity of cells stained with the 1B4 antibody may be decreased by other phosphatases having different specificities.

It would be interesting to know whether the 1B4 antigen is a phosphoprotein

because of the importance of phosphorylation in regulating known splicing factors. The αSC35 antibody has been shown to detect a phosphoepitope by Western blotting. SR proteins including SC35 are hyperphosphorylated 3-5 fold in metaphase as compared to interphase cells (Gui *et al.*, 1994). Gui and colleagues (1994) identified and cloned a serine kinase, SRPK1, which specifically induced the disassembly of nuclear speckles. This implies that the change in distribution of splicing factors through the cell cycle is regulated by phosphorylation. Furthermore, high levels of SRPK1 inhibited *in vitro* splicing reactions. Gui and colleagues (1994) proposed that excess SRPK1 might either cause hyperphosphorylation and/or prevent dephosphorylation of SR proteins, and that phosphorylation/dephosphorylation of SR proteins is required for splicing. It was previously known that pre-mRNA splicing could be positively and negatively regulated *in vitro* by reversible protein phosphorylation (Mermoud *et al.*, 1994). Lyon and colleagues (1997) inhibited protein dephosphorylation in HeLa cells by exposing them to low levels (10 nM) of okadaic acid for 24 hours. Okadaic acid specifically inhibits Ser/Thr protein phosphatases. They observed that the distribution of SC35 was not altered in okadaic acid-treated cells, while spliceosomal snRNP components accumulated within the nucleolus.

Protein kinases other than SRPK1 that are active upon SR proteins include DNA topoisomerase I (Rossi *et al.*, 1996), and the Clk/Sty protein kinase (Colwill *et al.*, 1996). Though DNA topoisomerase I does not have an obvious kinase domain, it does phosphorylate specific serine residues in the SR domain of SC35 (Rossi *et al.*, 1996). The distribution of SC35 was not studied in cells treated with DNA topoisomerase I inhibitors, though its level of phosphorylation was decreased. The Clk/Sty protein kinase associates

with SR splicing factors, and regulates their distribution *in vivo*. Overexpression of active Clk/Sty caused the speckled domains detected with either αSC35 or Y12 to be rearranged to a diffuse distribution. Furthermore, transfection experiments showed that a kinase-inactive mutant of Clk/Sty was localised to the discrete speckled domains of the nucleus in which SC35 is concentrated. This could indicate that this kinase is stored in the speckled domains when not active, as has been proposed for splicing factors.

4.3 Re-organisation of the domains containing the 1B4 antigen and SC35 in HeLa cells exposed to drugs that inhibit transcription

Researchers studying the effect of actinomycin-D on nuclear and nucleolar structure by electron microscopy reported that it causes chromatin to aggregate, interchomatin granules to clump together, and nucleolar components to segregate (Carmo-Fonseca *et al.*, 1991b). The clumping of interchromatin granules is visible by light microscopy as the appearance of larger speckles lacking interconnecting fibrils (reviewed by Huang and Spector, 1996). As it had been reported that there appeared to be fewer speckles in cells upon inhibition of splicing and transcription (Huang *et al.*, 1994; O'Keefe *et al.*, 1994), I counted the number of speckles per nucleus of HeLa cells treated with transcriptional inhibitors. However, I found a notable decrease in the number of speckles only in the most extreme treatment groups. One and 2 hour treatments with 0.5 μg/ml actinomycin-D were not distinguishable on the basis of the number of speckles in the nuclei of 200 cells (Table 3-3). By visual inspection, I observed that larger cells tended to have a larger number of speckles within them, which would account for the non-normal distribution of my data. It might have been more informative to calculate the relative surface areas of the speckles as

compared to the surface area of the entire nucleus to better quantify the observation of "fewer" speckles. However, the time required to define the surface area of each individual nucleus made this approach unfeasible. It was evident that the drug treatments were effective mainly by the alteration in the shape and size of the speckles and by the loss of fibrils connecting them.

I observed that the 1B4 antigen maintained its colocalisation with SC35 in all drug treatment groups. Though the function of nuclear speckles is controversial, they are thought to represent a dynamic structure where RNA splicing factors are stored and/or reassembled (Wansink *et al.*, 1993; Huang and Spector, 1996a; Gama-Carvalho *et al.*, 1997). Though the speckled domains are not transcriptionally active, they are often surrounded by a few transcription sites (Pombo and Cook, 1996). It is interesting that Pol IIo colocalises with SC35, and that these antigens redistribute simultaneously into enlarged speckles lacking interconnections upon inhibition of transcription (Bregman *et al.*, 1995). Other studies have provided strong evidence that Pol IIo associates with snRNPs identified by the Y12 antibody (Kim *et al.*, 1997), and several of the SR family of proteins, of which SC35 is a member (Mortillaro *et al.*, 1996; Kim *et al.*, 1997). Moreover, there is evidence for a functional relationship between the carboxy-terminal domain of RNA polymerase II and pre-mRNA splicing (Du and Warren, 1997). A recent model for RNA synthesis suggests that RNA polymerase II is stationary, while the DNA template slides past a fixed polymerisation site (Cook, 1994). This is in agreement with observations that Pol IIo is a nuclear matrix protein. Since the nuclear matrix is a structural component of the nucleus, it would be expected to organise the chromosomes in addition to interacting with them

(Nelson *et al.*, 1986).

Other nuclear matrix proteins distributed in nuclear speckles have been proposed to have functions related to pre-mRNA metabolism. For example, De Cárcer and colleagues (1995) identified protein 4.1 as a nuclear matrix component of mammalian cells which colocalises with SC35 (1995). This group went on to study the distributions of protein 4.1 and SC35 during and after release from transcriptional inhibition, and found that the colocalisation was maintained (Lallena and Correas, 1997). Their finding that protein 4.1 and SC35 coprecipitated from RNase-digested HeLa cell nuclei led them to hypothesise that the association between these proteins could be related to a structural role for protein 4.1, in which protein 4.1 would modulate the distributions of related proteins (Lallena and Correas, 1997). Antibodies commonly used in immunoprecipitation studies belong to the IgG subtype. However, the 1B4 antibody belongs to the IgE subtype. It is unfortunate that secondary antibodies suitable for immunoprecipitating the IgE subtype are not commercially available.

It was known that actinomycin-D causes clumping of interchromatin granules before these structures were known to correspond to speckles seen by light microscopy (Carmo-Fonseca *et al.*, 1991b). The finding of Carmo-Fonseca and colleagues (1991b) that HeLa cells exposed to 5 μg/ml actinomycin-D showed U1 snRNP capped around the nucleolus were not confirmed in this study. Though the Y12 antibody recognises the other spliceosomal snRNPs in addition to U1, the petal-like formation around the nucleolus should still have been visible. This formation does not contain SC35 (Carmo-Fonseca *et al.*, 1991a). Perhaps the ten-fold lower dose of actinomycin-D used in my study was

insufficient to cause an accumulation of U1 snRNP around the nucleolus.

The two hour treatment with 0.5 μg/ml actinomycin-D produced an effect similar to the 2 hour treatment with 25 μg/ml DRB. This dose of DRB had been shown previously to rearrange the speckled distribution (Spector *et al.*, 1983; Puvion *et al.*, 1984; Huang *et al.*, 1994). The rearrangement of speckles identified by the 1B4, Y12, and αSC35 antibodies is apparent in the observations made by light microscopy (Figures 3-13, 3-14, 3-15, 3-16, 3-17 and 3-19) and corresponds to the decreased level of transcription (Table 3-1). After two hours, the level of transcription in the actinomycin-D-treated cells was 17% of the control (100%) level, while the level in the DRB-treated cells was 12%. This similarity was useful for comparing the recovery of HeLa cells to each of these transcriptional inhibitors.

Cells recovered from 2 hour exposures to either 0.5 μg/ml actinomycin-D or 25 μg/ml DRB at different rates, as would be expected since DRB passes through cell membranes much more easily than actinomycin-D (Table 3-2). After a 2 hour recovery period, the level of transcription in cells exposed to DRB had risen to 70% of the control value, while the level of transcription in cells exposed to actinomycin-D was only 13%, a decrease from the 17% observed before "recovery" began. This is lower than the 24% level of transcription observed in cells exposed to 0.5 μg/ml actinomycin-D for 4 hours. However, since there were few repetitions averaged, these differences of 13% to 24% may not be statistically significant.

The cells recovering from DRB exposure showed a speckled distribution much like untreated controls, with speckles connected by thin fibrils (Figure 3-19). The cells allowed to recover from actinomycin-D exposure never did regain the appearance of untreated cells

(Figures 3-20, and 3-21). After an 18 hour recovery, the level of transcription had increased to only 37% (Table 3-2), and the speckles appeared small, fragmented, and higher in number than in the control cells. The fate of actinomycin-D-treated cells over an extended time course such as this have not been reported in the literature.

Actinomycin-D inhibits all three RNA polymerases at the doses used (Perry and Kelley, 1970), while α-amanitin inhibits only RNA polymerase II at the low dose (5 μg/ml for 5 hours) used. This dose of α-amanitin was reported to cause speckles labelled with anti-SC35 (Spector *et al.*, 1993) to be rounder and lack connecting fibrils. When used at a dose of 50 μg/ml for 5 hours (Carmo-Fonseca *et al.*, 1992; Spector, 1996), it caused much more dramatic rearrangements of speckles. My results do not show a rearrangement of the speckled distribution comparable to the results obtained with 0.5 μg/ml actinomycin-D (Figure 3-22), in agreement with the decrease in [^{3}H]-uridine incorporation to 65% of the control (Table 3-1). As other researchers have reported the amount and duration of drugs used but not the measured levels of transcription, it is difficult to compare my data with theirs.

4.4 The distributions of the 1B4 antigen, snRNPs, and SC35 in L6E9 cells and murine splenocytes

Studying the distribution of splicing factors in cells treated with drugs that inhibit transcription was an early approach taken by several groups interested in RNA processing and nuclear architecture (*e.g.* Carmo-Fonseca *et al.*, 1992; Spector *et al.*, 1983, 1993). Researchers identifying nuclear matrix proteins (*e.g.* Chaly *et al.*, 1988; De Cárcer *et al.*, 1995; Lallena and Correas, 1997) have used this approach to suggest that these proteins

may be involved in pre-mRNA splicing. The distributions and rearrangements of splicing factors in differentiating cells or those having a unique physiology is an emerging field of study.

For example, the PC12 cell line can be induced to differentiate into neurons by exposing them to nerve growth factor (Sahlas *et al.*, 1993). The majority (99%) of undifferentiated PC12 cells showed the typical snRNP distribution observed in HeLa cells. The distribution of snRNPs in differentiated cultures resembled a spherical shell at the nuclear periphery. This spherical shell distribution was also seen in dorsal root ganglia isolated from newborn mice (Sahlas *et al.*, 1993). The speckled SC35 distribution was not altered upon differentiation in these cells. In another publication, Ferriera and Carmo-Fonseca (1996) studied the distributions of snRNPs, SC35, and coiled bodies in hamster embryos. They found that large clusters of interchromatin granules that were labelled with the anti-SC35 antibody were not stained with antibodies to snRNPs in pronuclei. SnRNPs imported into the pronuclei following fertilisation were concentrated in coiled bodies. The assembly of coiled bodies and clusters of interchromatin granules was independent of the onset of embryonic transcriptional activity in that study. These studies are interesting in that they describe cell systems in which the snRNPs and SC35 behave differently than in HeLa cells.

There is also evidence that HeLa cells treated with drugs that inhibit transcription may be representative of some types of differentiation. Antoniou and colleagues (1993) studied the distribution of snRNPs and SC35 in murine erythroleukemia cells. These cells are erythroid progenitors which can be terminally differentiated in culture by the addition of

dimethylsulfoxide. Total levels of transcription decreases to the point of nearly stopping by 6 days after differentiation is induced. In uninduced cells, the snRNPs were diffusely distributed throughout the nucleus, excluding the nucleolus. Six days after induction of terminal erythroid differentiation, the snRNPs appeared in a clumped pattern similar to the distribution I observed 72 h post-stimulation in murine splenocytes. This group also used the polyclonal antibody to p80 coilin to identify coiled bodies. They found that the coiled bodies colocalised with the snRNPs identified with the Y12 antibody in undifferentiated cells, and at 2 and 4 days post-induction. However, at 6 days post-induction, the coiled bodies appeared distinct from the snRNP clumps as observed by confocal microscopy. They observed SC35 to be distributed in its typical speckled pattern in undifferentiated cells, and noted only minor alteration (fewer, larger speckles) 6 days after induction of differentiation. These researchers noted that the altered distribution of snRNPs in differentiated cells, including their exclusion from coiled bodies, was similar to what had previously been observed in HeLa cells treated with transcriptional inhibitors. This led them to treat uninduced murine erythroleukemia cells with α-amanitin (50 μg/ml, 5 hour exposure) to determine whether the rearrangement of snRNPs they observed could be attributed to the decreased level of transcription. They found that inhibition of transcription in these cells resulted in a redistribution of snRNPs into large clumps that were not coincident with coiled bodies. The clumped distribution of snRNPs in differentiated murine erythroleukemia cells was confirmed to correspond to interchromatin granule clusters by electron microscopy. Thus, the distributions of splicing factors in these cells upon inhibition of transcription was similar regardless of whether transcription was reduced by

differentiation or drug treatment.

I observed that the distribution of the 1B4 antigen in L6E9 cells did not change upon differentiation of these cells into myotubes. L6E9 myotubes are produced by fusion of adjacent L6E9 cells which have irreversibly withdrawn from the cell cycle (Yaffe, 1968; Nadal-Ginard, 1978). The rates of mRNA, protein, and ribosomal protein synthesis in myotubes are quantitatively similar to the rates in undifferentiated myoblasts, though the predominant gene products are muscle-specific proteins (Krauter *et al.*, 1979; Benoff and Nadal-Ginard, 1980). As observed in HeLa cells, most speckles labelled with the Y12 antibody were coincident with the foci labelled by 1B4. The faint immunofluorescence labelling of the splicing factor SC35 was an unexpected result. SC35 has been reported to be present in several cell lines. Its function in pre-mRNA splicing is presently understood to involve mediating interactions between splicing components bound to the 5' and 3' splice sites in the first step of spliceosome assembly (Fu and Maniatis, 1992). It is unfortunate that the αSC35 antibody does not perform well in Western blots, as it would be interesting to determine whether the lack of staining is due to decreased expression or masking of the epitope.

Chaly and colleagues are the only group to have published reports of splicing factor distributions in murine splenocyte cultures exposed to concanavalin A (Chaly *et al.*, 1988; Daev *et al.*, 1994). They have described labelling with the Y12 antibody as increasing in intensity upon stimulation. The labelling was distributed in patches in the nucleoplasm in unstimulated cells. Stimulated cells showed bright patches and a diffuse nucleoplasmic labelling. My observations of cells labelled with the 1B4, CC-3, and Y12 antibodies were

in agreement with these observations. Unfortunately, the intensity of staining obtained with the 1B4 antibody was much lower than that of the Y12 or CC-3 antibodies due to the titre of the antisera (Figure 3-29). Splenocytes are round, relatively small cells in which staining distributions are difficult to visualise with the clarity easily obtained in HeLa cells. I observed the staining distributions of these antigens by confocal microscopy. While viewing serial sections taken at 1 μm intervals through the cells, there appeared to be distinct patches labelled with the 1B4 antibody which did not colocalise with Y12 labelling in stimulated cells. However, the difference in intensity of labelling obtained with these antibodies made superpositioned images an unreliable indicator of colocalisation. Composite images of the average signal in the sections through the cell were not intense enough to provide convincing evidence of these observations and were not shown.

The increased intensity of the snRNPs as identified with the Y12 antibody in stimulated splenocytes agrees with the rationale that increasing the rate of transcription would increase the demand for splicing factors. Huang and Spector (1996) have argued that a high level of transcription can result in an accumulation of splicing factors along perichromatin fibrils, giving the appearance of interchromatin granule clusters (speckles). Thus the lack of colocalisation of the 1B4 antigen and snRNPs might be attributed to the fact that they are present in speckles with different functions for purposes that are unique to splenocyte biology. It is unfortunate that I was unable to test whether either the 1B4 antigen or the snRNPs colocalised with coiled bodies in these cells.

Unexpectedly, anti-SC35 labelling in splenocytes was very faint. There are no published reports of the distribution of SC35 in splenocytes or similar culture systems. As

with the lack of staining intensity in L6E9 cells, this observation cannot be simply attributed to a lack of requirement of the SC35 antigen function in these cells.

Setterfield and colleagues (1983) categorised human lymphocytes as morphotype I (unstimulated), II (partially stimulated), or III (fully stimulated). Chaly and colleagues (1988) also used this classification system to describle splenocyte cultures exposed to concanavalin A. In agreement with the group of Chaly, typical unstimulated cells or those after 24 hours exposure to concanavalin A appeared to belong to morphotype I. Types II and III corresponded to my observations of typical cells 48 or 72 hours post-stimulation with concanavalin A (Figure 3-28). I used the ^{3}H-uridine incorporation assay to verify that my cultures were responding to concanavalin A stimulation. As would be expected for primary cultures, the variability in repeated assays (one mouse spleen was used for each assay) was higher than I observed in repeated assays with HeLa cells. Daev and colleagues (1988) reported results from a single representative experiment. It is likely that my data differs from the much higher value they obtained after 48 hours post stimulation because I did not divide the rates of transcription by the number of cells to correct for cell viability. If concanavalin A decreased cell viability with time in their cultures, this factor could account for the discrepancy between my results and theirs. They did not comment on such an observation in their papers. I did observe that my stimulated cultures typically contained more clumps of cells clustered together. These were visible in the culture dish but not in immunofluorescence preparations because the larger clumps of cells did not adhere well to the coverslips coated with poly-L-lysine. I believe that the uncertainty of counts of clumps of cells would distort the data of the level of transcription in these cells more than not

accounting for dying cells. I suspended cells to be counted in trypan blue and did not see large numbers of nonviable cells. The fact that the rate of transcription in these cultures increased clearly indicates that the splenocytes were responding to concanavalin A. My observations of stimulated splenocyte morphology agree with those previously published. Daev and colleagues' use of a single representative experiment rather than an average suggests to me that it is not uncommon for the increase in transcription which accompanies stimulation to vary.

My results from experiments with splenocyte cultures and L6E9 cells indicate that the distribution of splicing-related antigens in the nucleus is complex and not always directly affected by the apparent level of transcription, as might be suggested from the HeLa cell results alone. There are studies in the literature which describe cell systems in which splicing factors have unusual distributions (Sahlas *et al.*, 1993), or in which the rearrangement of their distributions is not coincident with altered levels of transcription (Ferriera and Carmo-Fonseca, 1996). The reasons for these differences from possible predictions based upon the observed distributions of splicing factors in HeLa cells treated with drugs that inhibit transcription are not well understood. These studies contain data important for future models of pre-mRNA processing within the context of nuclear architecture.

4.5 Implications of characterisation of the 1B4 antigen and future research directions

I have clearly demonstrated that the 1B4 antigen is a component of the nuclear matrix, which is a structure known to be very important for organising essential nuclear

processes. The presence of 1B4 in several cell lines derived from different species indicates that the function of this antigen is not species-specific, and thus likely to be related to a conserved nuclear function. The colocalisation of 1B4 with the known splicing factor SC35 through the cell cycle and in HeLa cells treated with drugs that inhibit transcription suggests that the function of the 1B4 antigen in the cell is related to pre-mRNA processing. The 1B4 antigen cannot be SC35, as the 1B4 antibody detects a 100 kDa protein in mitotic cell extracts under denaturing conditions, while SC35 has a weight of 35 kDa. The lack of a signal with the 1B4 antibody in interphase cells is believed to be a result of the denaturing conditions used. I have attempted to perform immunopreciptitations (under non-denaturing conditions), though this procedure is complicated by the IgE subtype of the 1B4 antibody. My observations of the 1B4 antigen, SC35, Pol IIo (identified by the CC-3 antibody), and snRNPs in L6E9 cells and murine splenocytes are interesting in that the distributions of 1B4 and SC35 differ. My results support other studies which have indicated that cells undergoing complex physiological processes may show unpredictable distributions of splicing factors. However, they give little insight to the problem of determining a function of the 1B4 antigen. My supervisor, Dr. M. Paulin-Levasseur, is presently collaborating with Dr. B. Chabot of the Université de Sherbrooke to determine whether the 1B4 antibody can inhibit the splicing reaction. If it is found that the splicing reaction is not inhibited by the 1B4 antibody, then it is probable that the nuclear matrix character of the 1B4 antigen is related to a structural role in binding active splicing components to the nuclear matrix.

Another approach to characterising the 1B4 antigen is the ongoing work to clone this protein. A complete description of my work with a cDNA identified by the 1B4

antibody is contained in Appendix II. The putative zinc finger motifs identified in this cDNA sequence are interesting because RNA-binding zinc-finger proteins have been found to participate in RNA processing (Barabino *et al.*, 1997), and to be associated with the nuclear matrix (Grondin *et al.*, 1996). However, proof that the 1B4 antibody identifies a protein corresponding to this cDNA has not been obtained. The next step in this line of research will be to raise antibodies to fusion proteins generated from this cDNA and check that the antigen they label colocalises with the antigen labelled with the 1B4 antibody by immmunofluorescence. A cDNA known to encode to the 1B4 antigen would also be invaluable in the design of studies to characterise the function of this protein.

APPENDIX I

Figure 1. The distribution of the 1B4 antigen in various cell types. Cells were labelled with the 1B4 antibody (a,d,g,j,m,p), and counterstained with Hoescht 33258 (b,e,h,k,n,q). Phase contrast micrographs are also shown (c,f,i,l,o,r). Cell types shown include undifferentiated HL60 cells (a-c), HL60 cells induced to differentiate into macrophages (d-f), wheat spheroblasts (g-i), 3T3 mouse fibroblasts (j-l), PTK_2 kangaroo rat kidney cells (m-o), and undifferentiated P19 mouse embryonal carcinoma cells (p-r). Bar = 5.4 mm.

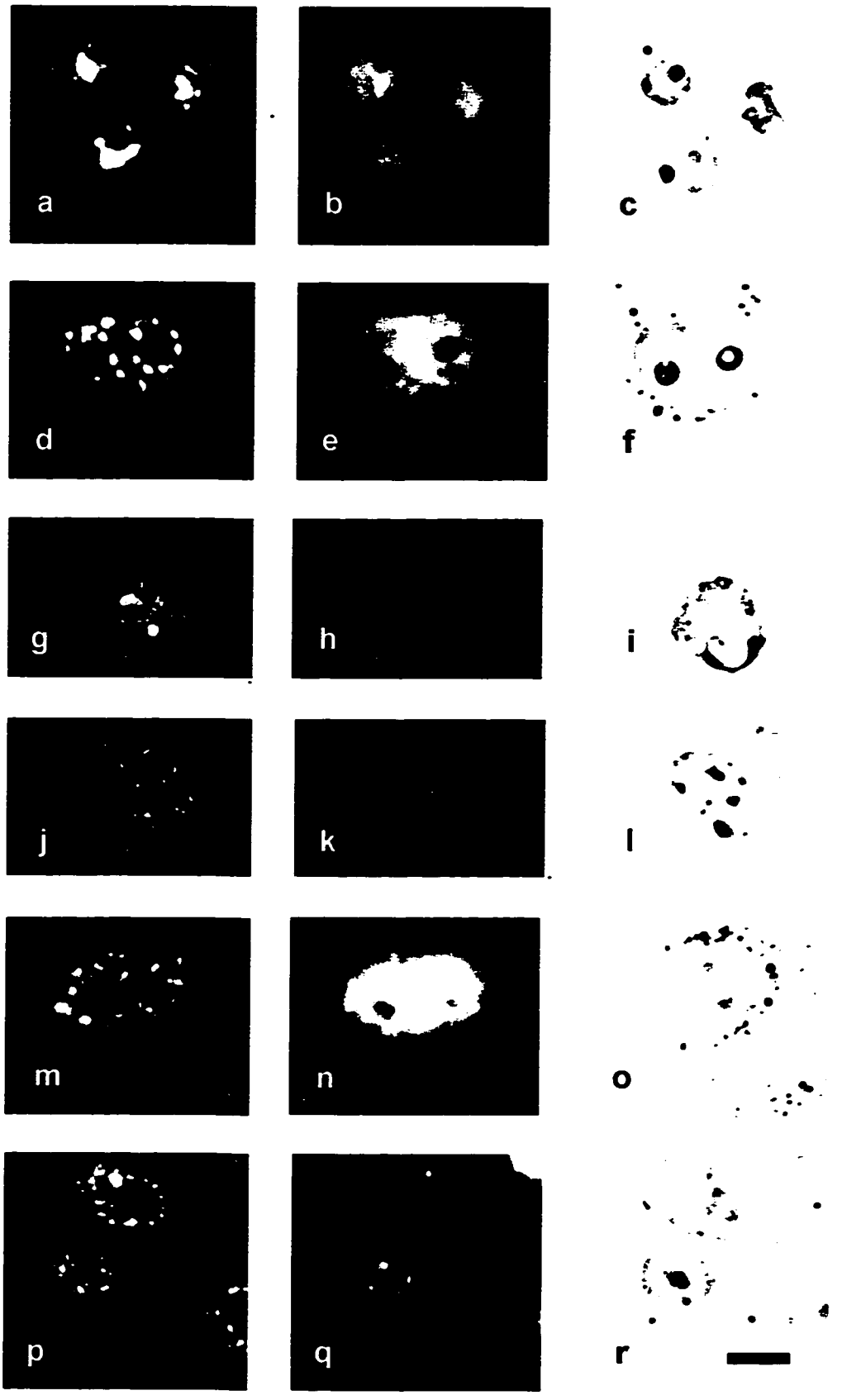
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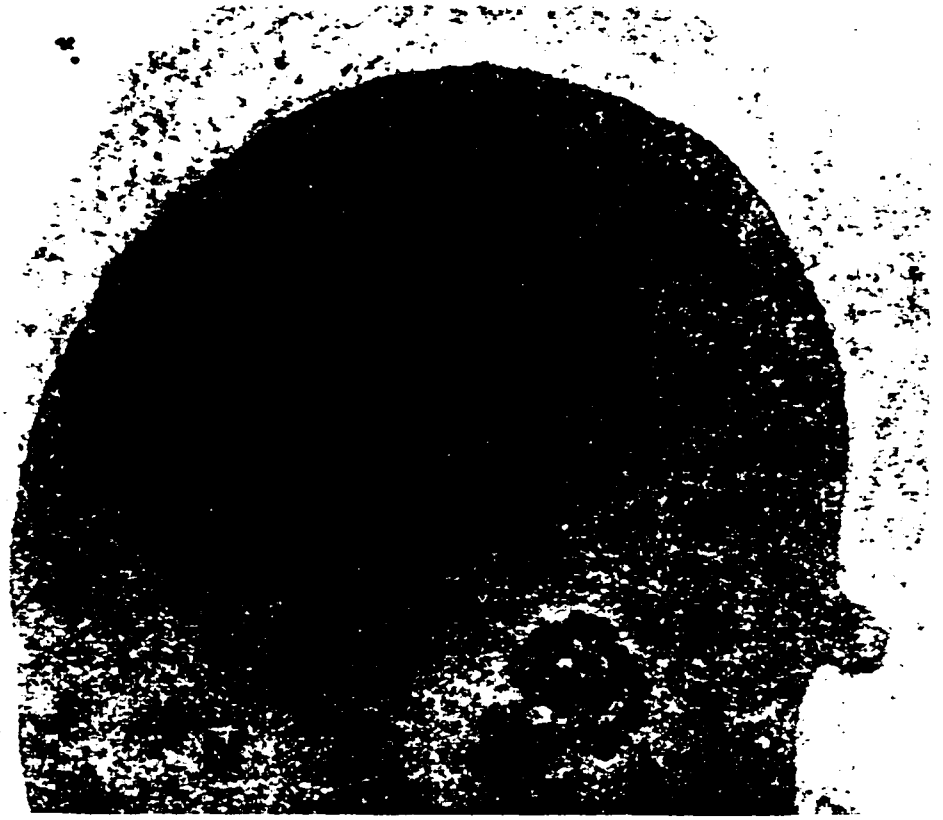
Figure 2. Electron micrograph showing the distribution of the 1B4 antigen in immunoperoxidase-labelled HeLa cells; prepared by Martha Julien.



A

B

APPENDIX II Attempt to isolate a cDNA corresponding to the 1B4 antigen

i Introduction

It is advantageous to obtain DNA sequence information early in the characterisation of a potentially novel protein. DNA sequence information is also very useful as a basis of functional studies. As the 1B4 antibody had previously been used to screen a cDNA expression library, my objective was to characterise the clones and verify that their DNA sequence coded for the 1B4 antigen. This would involve using the cloned sequences to produce a fusion protein which would be used to raise a polyclonal antibody in rabbits. To be sure that the identified DNA sequence coded for the 1B4 antigen, this polyclonal antibody would have to identify a protein of the same molecular weight in mitotic cells. Ideally it would also determine a molecular weight in interphase cells. Double-labelling immunofluorescence using the monoclonal 1B4 antibody and the polyclonal serum to the fusion protein should show perfect colocalisation. This project has not been completed; this appendix describes the progress made thus far.

ii Plaque purification

M. Paulin-Levasseur and I. Skerjanck used the 1B4 antibody to screen a cDNA expression library constructed in the vector λgt22A, which is a derivative of λgt11 (Young and Davis, 1983). I plaque-purified phage from eight original stocks using standard media and methods (Sambrook *et al.*, 1989). Ampicillin was added to all media both to prevent contamination and to ensure that the host bacterium, *Escherichia coli* Y1090, maintained the pMC9 plasmid. This plasmid carries genes for production of the *lac* repressor (to

prevent the synthesis of potentially toxic fusion proteins), as well as the gene for ampicillin resistance. I chose two of the original eight phage isolates which were consistently detected with the 1B4 antibody for further study.

Plating bacteria (*E. coli* Y1090) were prepared by growing a culture in LB broth containing 0.2% maltose from a single picked colony. After an 18 h incubation at 37°C with moderate agitation (250 cycles/min), the turbid broth was centrifuged at 4000 *g*, 4°C. The cell pellet was resuspended in SM buffer, which is a standard medium used to store phage (Sambrook *et al.*, 1989). The absorbance of the pellet in SM buffer (using SM buffer as a calibration blank) at 600 nm was used to calculate the titre of bacteria, given that an optical density of 2 at 600 nm corresponds to 1.6×10^9 cells/ml. When necessary, the titre was adjusted to 1.6×10^9 cells/ml by dilution with SM buffer. This preparation of plating bacteria was stored at 4°C for up to 3 weeks.

Before screening, the titre of a stored plaque was determined by plating serial dilutions upon a lawn of *E. coli* Y1090, so that a dilution that would yield approximately 20-200 plaques could be selected for screening. To plate phage upon a lawn of bacteria, 100 µl phage stock with 100 µl plating bacteria were incubated in a 10 ml test tube at 37°C for 15 min. After setting 6 µl of 50 mg/ml ampicillin (Sigma) at the upper inside edge of the tube, 3 ml of molten (47°C) agarose (0.7% w/v in LB broth) were added to the tube. The tube was vortexed to mix before the contents were poured quickly over a 90 mm plate of LB agar containing 75 mg/ml ampicillin. The overlay was allowed to set 3-5 minutes before being incubated inverted overnight at 37°C.

For screening, titred phage stocks were plated on a lawn of *E. coli* Y1090, and the

overlay incubated inverted for 3.5 h at 42°C. Dry nitrocellulose filters that had previously been soaked in 10 mM isopropylthio-ß-D-galactoside (IPTG, Sigma) were laid over the plates. These were incubated inverted for another 10-12 h at 37°C.

Fusion proteins expressed by the phage upon induction with IPTG were detected with the 1B4 antibody by Western blotting as described in the Materials and Methods. Plaques producing fusion proteins detected by the 1B4 antibody were identified by aligning the blot with the original agar plate. These were isolated from the plate with a Pasteur pipette and stored in SM buffer containing formaldehyde at 4°C. These newly isolated stocks were stored for several hours to allow the phage to elute from the plaque before being used in a subsequent round of screening.

iii Phage DNA extraction

Phage DNA was prepared from λgt22A using a the protocol of Dr. D. Johnson. Briefly, the procedure involves growing a large number of phage under conditions that promote lysis, isolating the phage particles from the bacterial debris, and finally destroying the phage capsules to harvest linear phage DNA.

An 18 h culture of Y1090 *E. coli* in 0.5 ml of LB broth was inoculated with titred phage stock in SM buffer at a multiplicity of infection of 0.025. After incubating at 37°C for 20 min, 100 µl of this culture was used to inoculate 50 mL of prewarmed LB broth in a 250 mL Erlenmeyer flask, and shaken continuously at 37°C. Lysis (identified by the presence of debris at the bottom of the flask) usually occurred within 6 hours. To complete the lysis step, 1.0 ml chloroform was added to a lysed flask, which was shaken and allowed

to sit for 20 minutes at room temperature.

To isolate the phage, solid sodium chloride was added to a concentration of 1.0 M, and the solution centrifuged at 6000 rpm for 10 minutes at 4°C to remove debris. Solid polyethylene glycol 6000 (BDH) was added to a final concentration of 8% (w/v) and dissolved before setting on ice for 20 minutes to precipitate the phage. To recover the phage, this solution was centrifuged at 6000 rpm for 10 minutes at 4°C.

To purify the phage DNA, the phage pellet suspended in 4 ml of SM buffer was incubated at 37°C for 20 minutes in the presence of 50 μg/ml (final concentration) DNase, 50 μg/ml RNase, and 0.5X ONE-PHOR-ALL buffer (100 mM Tris acetate, 100 mM magnesium acetate, 500 mM potassium acetate). Then 2.0 ml of chloroform were added, vortexed to mix, and centrifuged at 5000 rpm for 10 minutes at room temperature. The aqueous phase was isolated and incubated at 65°C for 20 minutes in the presence of 25 mM EDTA, 0.5% SDS, and 100 μg/ml proteinase K. Phage DNA was extracted sequentially with phenol/chloroform twice and then once with chloroform alone. Phage DNA was precipitated in 0.75 M ammonium acetate in ethanol at -80°C for 1 h. After rinsing the concentrated DNA with ethanol, and resuspending in TE buffer (10 mM Tris, 1 mM EDTA), it was further purified by an overnight treatment with 20% PEG, 1.0 M NaCl.

iv Restriction digest of phage DNA

The cloned cDNA could be isolated from phage DNA by sequential digests with *SalI* and *NotI* restriction enzymes. The DNA fragments isolated from these clones were

determined to have lengths of 1.2 kb and 2.3 kb by agarose gel electrophoresis. The DNA inserts were cut from the agarose gel and purified from the agarose by a procedure involving the use of Sephaglas beads (Pharmacia, Baie d'Urfé, PQ).

v Attempted subcloning of 1.3 kb and 2.3 kb clones into pBluescript

Larger quantities of DNA than can be obtained by phage DNA extraction protocols are required for DNA sequencing. I attempted to subclone the 1.3 kb and 2.3 kb inserts into the Bluescript plasmid (Stratagene, La Jolla, CA). pBluescript contains a polycloning site containing *Sal*I and *Not*I sites flanked by bacteriophage T7 and T3 promoters that can be used to generate single-stranded RNA. Unfortunately, it was later found by Dr. D. Johnson that the *Not*I polylinker site was damaged. Thus it is not surprising that numerous attempts to subclone the fragments failed. Given that subcloning into pBluescript was not completed within a reasonable period of time, the objectives of this research were re-directed towards the cell biology-based approach described in the main body of this document.

vi Attempted validation of 1.3 kb and 2.3 kb clones

Recombinant lysogen was prepared using a standard protocol (Huynh, Young, and Davis, 1985). It was not recognised by the 1B4 antibody in Western blots. However, control experiments designed to verify that the 1B4 antigen/ß-galactosidase fusion protein

was expressed were unsuccessful. The available antibody to ß-galactosidase appeared to bind the phage protein extract non-specifically.

vii Polymerase chain reaction (PCR) amplification of the 1.3 and 2.3 kb clones

The phage vector λgt22A is a derivative of λgt11, and primers flanking the λgt11 polylinker sites were successfully used to amplify the 1.3 and 2.3 kb clones. One microlitre of DNA was amplified in a 50 µl reaction volume containing 1X Vent buffer (New England Biolabs), 2.5 mM $MgSO_4$, 0.2 mM dNTPs, 60 µM each of forward and reverse primers, and one unit Vent polymerase (New England Biolabs). The PCR program required one cycle (95°C for 5 min, 55°C for 45 s, 72°C for 2 min), 30 cycles (95°C for 45 s, 55°C for 45 s, 72°C for 2 min), and one cycle (95°LC for 45 s, 55°C for 45 s, and 72°C for 5 min), before resting at 4°C.

viii Characterisation of 1.3 kb and 2.3 kb Clones by Southern and Northern Blotting

Some preliminary characterisation of the 1.3 and 2.3 kb clones was performed concomitant with the attempts to subclone the 1.3 and 2.3 kb clones into pBluescript.

To determine whether the 1.3 kb and 2.3 kb clones might overlap, a Southern blot was performed to see if they would hybridise to each other. Using self-hybridisation as a positive control, it was found that the 1.3 kb and 2.3 kb clones did not hybridise to each other.

To create probes, PCR products were labelled with ^{32}P (Amersham) using a

multiprime labelling technique. DNA fragments (7 μl volume) to be labelled were boiled for 2-5 minutes to denature them, and chilled before adding 6 μl 5X random primer extension buffer, 6 μl 5X deoxynucleotide mixture, and 10 μl of 10 mCi/ml α^{32}P-dCTP. The reaction was started by adding 1 μl Klenow enzyme, and allowed to proceed for 30-60 minutes at room temperature. After stopping the reactions, the samples were run over Sephadex spin columns to remove unbound α^{32}P-dCTP.

Northern and genomic Southern blots were desirable as evidence that the clones represented mRNA sequences transcribed from a sequence on the genome. Neither of these were obtained during the period of time in which I was attempting to subclone them. I prepared samples of total mRNA and genomic DNA from P19 murine embryonal carcinoma cells using standard protocols (Sambrook *et al.*, 1989; Isolation of total RNA from mammalian cells, pp. 7.6-7.9; Isolation of DNA from mammalian cells: Protocol I, pp. 9.16-9.19). Difficulty in obtaining a signal by Northern blotting is not uncommon, as rare mRNAs will not produce a signal strong enough to be visible

Information later obtained by sequencing the 2.3 kb clone indicated that it would be unlikely that using the entire clone as a probe would detect a single-copy gene. There is a part of a mouse retroposon (L1) within the non-coding 3' region. The L1 part of the probe hybridised to the large number of copies of L1 present in the genome. Some L1 sequences are transcribed into RNA. This would account for smears rather than than single distinct signals in the blots I performed.

ix Sequencing of Fragments of PCR-amplified Clones

The 1.3 kb and 2.3 kb clones were divided into smaller sections which were then subcloned into pGEM4Z. Overlapping sections were used to align the sequences. The 1.3 kb cDNA was found to be homologous to a cysteine protease, and was not studied further. Two sections of the 2.3 kb cDNA sequence (Figure 1) show homology to zinc finger proteins identified through a BLAST search (Altschul *et al.*, 1990). Only the search results showing the highest degree of homology are shown (Figures 2 and 3). Several other zinc finger proteins were identified in this search. The amino acid homology comparison was obtained using a BLAST search of a non-redundant SwissProt sequence database (Altschul *et al.*, 1990; Gish *et al.*, 1993). These findings are preliminary results.

The data obtained thus far will be very useful for further work with this project. Zinc fingers are known to be common in the DNA-binding domains of transcription factors (reviewed by Coleman, 1992). Zinc finger motifs contain cysteine and histidine residues separated by spacers of defined lengths. The pairs of cysteines and histidines are thought to participate in a tetrahedral zinc complex, with the 12-13-residue loop or "finger" portion forming the DNA-binding surface. Zinc finger motifs have also been found to bind RNA by factors involved in mammalian 3'-end processing (Barabino *et al.*, 1997). Thus, these results do not argue against the findings in the body of this thesis which state that the 1B4 antigen is likely to have a role in pre-mRNA processing. The portion of the cDNA which does not contain the zinc fingers should be used in future genomic Southern blots. To verify that this cDNA corresponds to the protein identified by the 1B4 antibody, antibodies will need to be raised against peptides derived from the cDNA sequence. Avoiding the zinc

finger sections of the sequence would likely be part of the strategy to express a protein containing the 1B4 epitope.

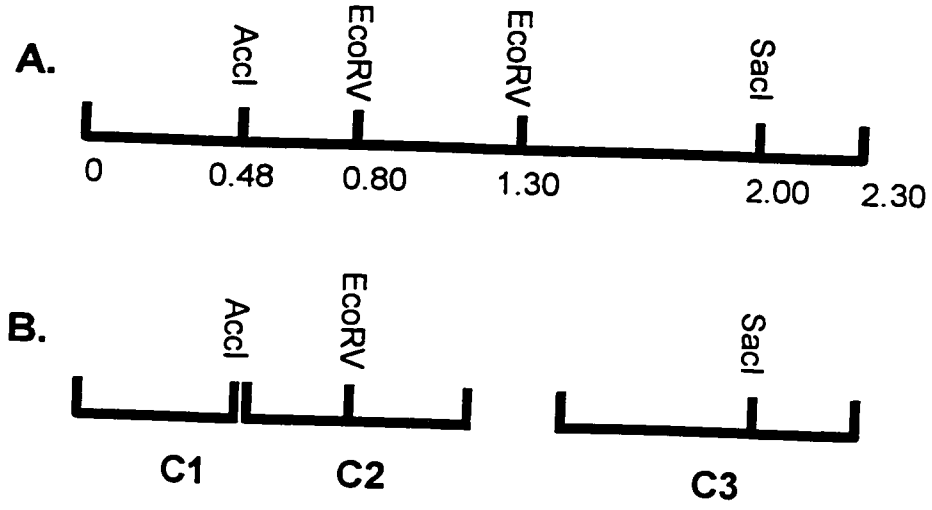


Figure 1. Diagram showing (A) the positions of restriction sites within the 2.3 kb clone, and (B) the positions of three contiguous elements within the 2.3 kb clone.

A.

```
                5'                                3'
ZNF125:     624 CAATTTTCATTGCAGGTATGTAAGT 648
                |  | |||||||||||||||||||| |
CT2:         15 CCAATTTCATTGCAGGTATGTAAAT 39

ZNF125:     649 AATCATAGTGGAGAAAAACTCTATG 673
                ||||||||||||||| ||||||||||
CT2:         40 AATCATAGTGGAGAGAAACTCTATG 64

ZNF125:     674 AATGTAATGAACGTTCTAAAGCCTT 698
                |||||||||||||||||||||||||
CT2:         65 AATGTAATGAACGTTCTAAAGCCTT 89

ZNF125:     699 TTTATGTCACAGTCATCTCCAAAGT 723
                || ||||| |||||||||||||| ||
CT2:         90 TTCATGTCCCAGTCATCTCCAATGT 114

ZNF125:     724 AATATAAGAAAGAACAAATTGGAGA 748
                 ||| |||||    | |       |||
CT2:        115 CATAAAAGAAGACAAATTGGAGAGA 139

ZNF125:     749 GAAAA 753
                 || |
CT2:        140 AAACA 144
```

B.

```
HZF-3:      628 FHCRYVSNHSGEKLYECNERSKAFLCHSHLQSNIRK 735
                FHCRYV+NHSGEKLYECNERSKAF C SHLQ + R+
CT2:          7 FHCRYVNNHSGEKLYECNERSKAFSCPSHLQCHKRR 42
```

Figure 2. Alignment of the CT2 fragment with the highest ranking homologue found in a BLAST search. A) ZNF125 is a human genomic sequence (GenBank accession number S52508) showing 110/130 (84%) identities and 110/130 (84%) positives in comparison to CT2. B) The amino acid sequence of a portion of human zinc finger protein 125 (HZF-3; SwissProt number P35274) is shown compared to that of CT2. This comparison shows 29/36 identities (80%), with 32/36 positives (88%).

Figure 3. Alignment of the CT3 fragment with the highest ranking homologue found in a BLAST search. A) 5' end of the mouse murine interferon gamma gene (GenBank accession number M28381) shows 170/193 (88%) identities and 170/193 (88%) positives in a plus/plus strand base pair comparison to CT3. B) The amino acid sequence of a portion of human zinc finger protein 135 (SwissProt number P52742) is shown compared to that of CT3. This comparison shows 52/96 (54%) identities, and 65/96 (67%) positives. The cysteine and histidine amino acids of the zinc finger motifs are indicated in bold type.

A.

```
                5'                                        3'
Query1:   680 CCCTGTAGGCAGAACAACAATAGAAACTAA 709
              |||| ||||  ||| |||||||  ||||||
CT3:      213 CCCTATAGGAGGAAAAACAATATGAACTAA 242

Query1:   710 CCAGTACCCCCAGAGCTCATGTCTCTAGCT 739
              |||||||||||||||||| ||| |||||||
CT3:      243 CCAGTACCCCCAGAGCTCGTGTTTCTAGCT 272

Query1:   740 GCATATATAGCAGAAGATGGCCTAGTCTGC 769
              |||||||||||||||||||||||||||| ||
CT3:      273 GCATATATAGCAGAAGATGGCCTAGTCAGC 302

Query1:   770 CATCAGTGGAAAGAGAGGCCCATTGGTCAT 779
              ||||| ||| ||||||||||  ||||| |
CT3:      303 CATCAGTGGAAAGAGAGGCCCATTGGTCAT 332

Query1:   800 GCAAATTTTTTAAGCCCCAGTACAGGGGAA 829
              ||||| ||| || ||||||||||||||||
CT3:      333 GCAAACTTTATATGCCCCAGTACAGGGGAA 362

Query1:   830 TGCCAGGGCCCAGAAGTGGGAGTGGGTGGG 859
                ||||||||  ||||||||||  |||| ||
CT3:      363 CACCAGGGCCAAGAAGTGGGAGAGGGTAGG 392

Query1:   860 TAGGGGAGCAGTG 872
              ||||||||||| |
CT3:      393 TAGGGGAGCAGGG 405
```

B.

```
Query2:   153 KTFTSQNGLQYHKRTHTGEKPYECNQCGKA 242
              KTF+  + L  H+RTHTGEKPYEC+QCGKA
CT3:      256 KTFSHSSSLSQHERTHTGEKPYECSQCGKA 285

Query2:   243 FSQYIYLQYHKRTHTGEKPYKCHQCGQAFK 332
              F Q  +L  H+R HTGEKPY+C+ CG+AF
CT3:      286 FRQSTHLTQHQRIHTGEKPYECNDCGKAFS 315

Query2:   333 KCSHLQXHKRTHTGEKP*ECNW*SLCTSQSSP 428
                S L  H+R HTGEKP ECN      SQ +P
CT3:      316 HSSSLTKHQRIHTGEKPYECNQCGRAFSQLAP 347
```

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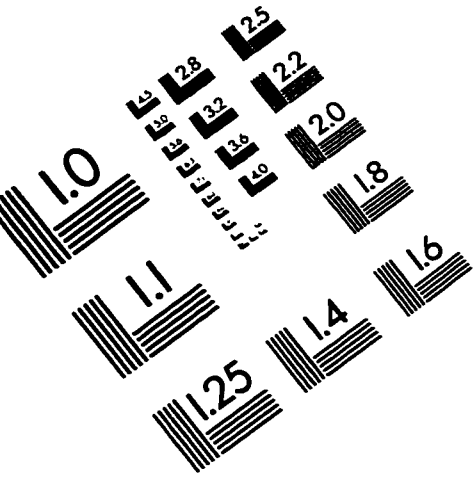
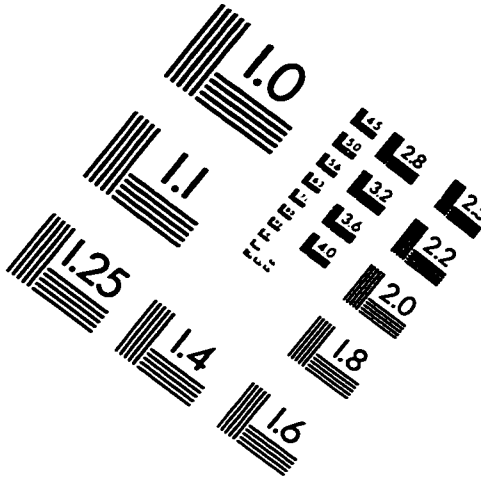
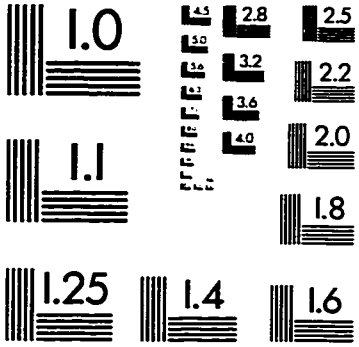
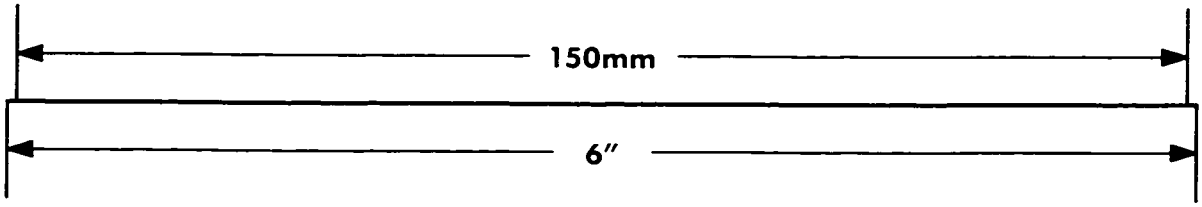
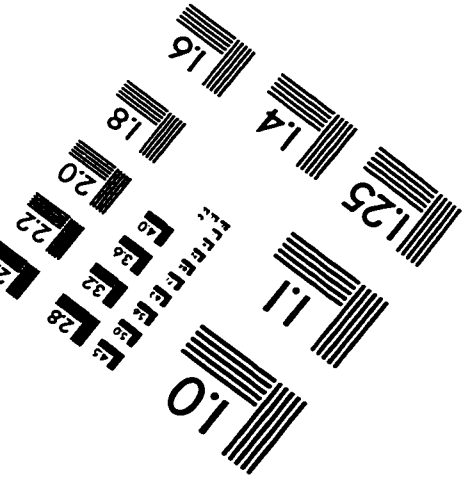
IMAGE EVALUATION TEST TARGET (QA-3)

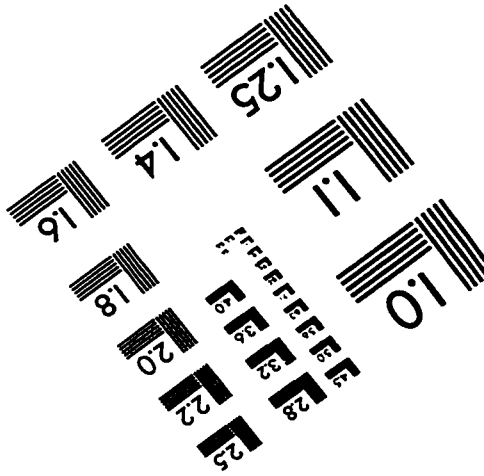












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