HS

IDENTIFICATION AND FUNCTIONAL MAPPING OF THE PROTEIN PRODUCT OF THE V-REL ONCOGENE

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ABSTRACT

Reticuloendotheliosis virus, strain T (REV-T), is a defective avian retrovirus which causes a rapid fatal leukemia in susceptible birds. The expression of the viral oncogene, *v-rel*, is responsible for the oncogenicity of this virus. Initially, I produced antisera to synthetic peptides and to a v-*rel* related polypeptide expressed in *E. coli*, which allowed the identification of a 58 kDalton protein as the protein product of the v-*rel* oncogene.

The v-*rel* protein, pp59$^{v\text{-}rel}$, was shown to be localized in the nucleus of infected chicken embryo fibroblasts (CEF) and the cytoplasm of transformed lymphoid cells. I have confirmed the nuclear location of pp59$^{v\text{-}rel}$ in CEF and have also shown that it localizes in the nucleus when expressed in the Cos1 monkey cell line. This indicated that pp59$^{v\text{-}rel}$ contains a nuclear localizing signal recognized in avian and mammalian cells.

Although the majority of the coding sequence of the *v-rel* oncogene is derived from the cellular *rel* sequence, the N- and C-terminal amino acids are coded for by remnants of the REV *env* gene. The resulting *v-rel* protein can be described as an *env-rel-(out of frame env)* fusion protein. I constructed terminal deletion mutants to determine the role that *env* sequences played in the transforming activity of v-*rel*. Deletions were designed to remove only sequences of *v-rel* derived from former *env* sequence. Additional deletions removed more substantial amounts of coding sequence. Introduction of deleted genes into an REV-T based retroviral vector permitted the transforming activities to be determined. Deletion analysis has indicated that the N-terminal coding region of v-*rel* is required for full functional activity. Deletion of as many as 100 C-

terminal amino acids did not abolish transforming activity.

In order to map internal functional domains, 12 linker insertion mutants were constructed. The determination of the transforming activity of these mutants confirms the functional importance of the N-terminal coding region of v-*rel*. An important functional role of the N-terminal portion of the *v-rel* product is consistent with the report that this region demonstrates some homology with the *dorsal* protein of *Drosophila*.

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DEDICATION

This thesis is dedicated to Amy

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ABBREVIATIONS

μg	microgram
μL	microlitre
AEV	avian erythroblastosis virus
ALSV	avian leukosis-sarcoma virus
ALV	avian leukosis virus
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate ptoluidine salt
BES	N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid
BH-RSV	Bryan strain of RSV
Bis	N'N'-Bis-methylene-acrylamide
BSA	bovine serum albumin
cAMP	adenosine 3',5'-cyclic monophosphate
CBMC	REV-T transformed chicken bone marrow cells
CEF	chicken embryo fibroblasts
CIP	calf intestinal phosphatase
CMGF	chicken myelomonocytic growth factor
CSC	chick spleen cells
CSV	chick syncytial virus
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate

dITP	deoxyinosine triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E	encapsidation signal
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
g	gram
GAP	GTPase activating protein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HIFCS	heat inactivated fetal calf serum
IFA	immunofluorescence assays
IPTG	isopropyl β-D-thio-galactoside
kDa	kilodaltons
kb	kilobases
kbp	kilobase pairs
kg	kilogram
KLH	keyhole limpet haemocyanin
L	litre
LTR	long terminal repeats
MEM	minimal essential medium
mg	milligram
mL	millilitre
MMC-1	an endogenous type C virus of the rhesus monkey

NBT	p-nitro blue tetrazolium chloride
ng	nanogram
NP	non-virus producer
pbs	primer binding site
PBS	phosphate buffered saline
PBS_I	phosphate buffered saline used for immunofluorescence assays
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PMSF	phenyl methylsulfonylfluoride
ppt	polypurine tract
QEF	quail embryo fibroblasts
R	repeat region
RAV	Rous associated virus
RE	Reticuloendotheliosis species
REV-A	competent reticuloendotheliosis associate virus
REV-T	defective transforming reticuloendotheliosis virus
REV	reticuloendotheliosis virus, strain T, original isolate
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RSV	Rous sarcoma virus
sa	splice acceptor
sd	splice donor
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSV	simian sarcoma virus
TBS	tris buffered saline

TBS_w	tris buffered saline used for Western blots
TPB	tryptose phosphate broth
Tris	tris(hydroxymethyl)aminomethane
ts	temperature sensitive
TTBS	TBS_w containing 0.05% (v/v) Tween-20

INTRODUCTION

1. A HISTORICAL REVIEW OF RNA TUMOUR VIRUSES

The foundations of RNA tumour virology began over 80 years ago with the demonstration that a chicken leukemia and subsequently a chicken sarcoma had viral etiologies (Weiss *et al.* 1982, Svoboda 1986, Karpas 1982).

In 1908, Ellerman and Bang (Karpas 1982, Weiss *et al.* 1982) reported that an experimental chicken leukemia could be transmitted by injecting the cell free filtrate of leukemia cells. They concluded that a viral etiology was associated with this leukemia. The full significance of this work was not appreciated at the time since leukemia was considered to be a disease of the blood forming tissues and was not recognized as a form of cancer. The discovery of the Rous sarcoma virus (RSV) as the causative agent of a sarcoma in chickens clearly established a viral etiology for this experimental cancer (Svoboda 1986). In 1911, Peyton Rous demonstrated that a cell free filtrate prepared from tumours that he had been passaging through his flock of Plymouth Rock chickens could induce tumours in young healthy chickens. Since Rous's discovery, investigators have isolated numerous other RNA tumour viruses capable of causing cancers in animals (Weiss *et al.* 1982).

In 1938, Keogh developed an assay for the quantitation of RSV (Weiss *et al.* 1982). Inoculation of choriallantoic membranes with RSV produced lesions or tumours which could be counted. A quantitative assay allowed the demonstration of a linear relationship between the amount of virus inoculated and the number of tumours observed. This linearity was the first evidence that a single RSV particle was able to transform a normal cell into a cancerous cell. The development of a focus assay for RSV on

chicken embryo fibroblasts (CEF) confirmed this linear relationship (Temin and Rubin 1958).

While the original isolate of RSV was reproducibly recovered from tumours or transformed CEF, a subsequent strain of RSV, the Bryan strain (BH-RSV), was able to cause tumours or transform cells without the production of further virus (Temin 1962). Superinfection of the resulting non-virus producing (NP) tumours or cells with other avian retroviruses allowed the rescue or recovery of RSV (Svoboda 1986). Hanafusa suggested that the transforming BH-RSV was defective for replication but was associated with a second replication competent, but non-transforming retrovirus (RAV)(Hanafusa *et al.* 1963). The NP tumours or cells were infected and transformed by the defective virus alone such that further viral replication in the absence of the helper virus was not possible. Superinfection with competent retroviruses permitted the recovery of the defective genome by providing all of the viral proteins required for replication. This discovery was particularly important in light of the fact that most of the highly oncogenic retroviruses known to date are defective for replication and require the presence of a helper virus for continued replication (Weiss *et al.* 1982).

The most significant developments in understanding the replication of RNA tumour viruses was the observation that bromodeoxyuridine, an inhibitor of DNA synthesis, and actinomycin D, an inhibitor of the cellular RNA polymerase, could inhibit RSV replication (Temin 1963, Bader 1965). These critical observations led Temin to propose, in 1964, that RNA tumour viruses replicate through a DNA intermediate, a theory which was consistent with his data, but ran against central dogma (Weiss *et al.* 1982). This hypothesis was further substantiated six years later with the

characterization of an RNA-directed DNA polymerase activity in mature particles of RSV (Temin and Baltimore 1972). This important discovery established the novel replication strategy of the RNA tumour virus as well as revolutionizing the methodology of molecular biology.

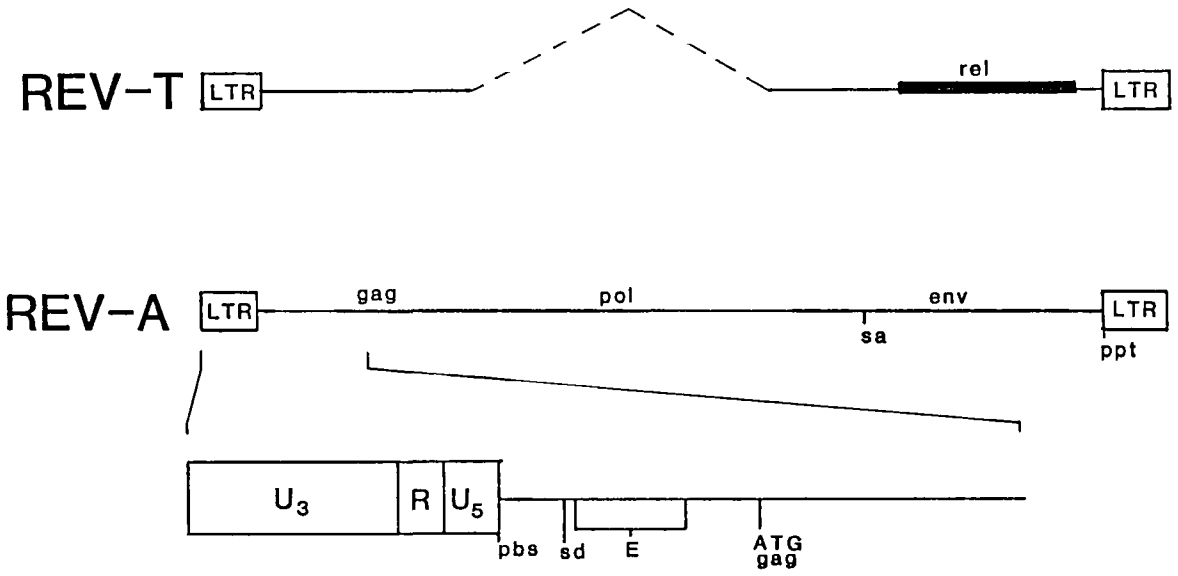
Although it was known that RSV carried the genetic information capable of inducing the transformation of susceptible cells, the nature or origin of this information was not clear. In 1976, Stehelin *et al.* showed that cDNA corresponding to the transforming gene of RSV hybridized to normal chicken DNA (Stehelin *et al.* 1976). This led to the eventual realization that viral oncogenes were derived from normal cellular genes.

2. REPLICATION OF RNA TUMOUR VIRUSES

a) Genome organization

Retroviruses are enveloped, positive-sense, single-stranded RNA viruses (Varmus 1988) that are unique in that each viral particle contains two genomic RNAs, physically associated with each other at their 5' ends. Viral genomic RNA is capped and polyadenylated similar to cellular messenger RNA. A short direct repeat is present at the immediate 5' end and at the 3' end, adjacent to the poly-A tail. Retroviruses, including defective transforming viruses, require the following *cis*-elements for replication (Fig. 1): 1. terminal repeat sequences (R), 2. transcriptional elements U_3 and U_5, 3. primer binding site for reverse transcription (pbs), 4. a polypurine tract (ppt) or site for initiation of the synthesis of positive sense DNA (second strand), 5. an encapsidation signal (E) to permit packaging of genomic RNA into virions, and 6. splice donor (sd) and splice acceptor (sa) sites for the proper splicing to generate subgenomic RNAs.

Figure 1. Genome organization of REV-T and REV-A.
The deletion of *gag-pol* sequences in REV-T is indicated by the dashed line. Cell-derived sequences, *rel*, represented by a thick black line replace *env* sequences present in REV-A. The location of the viral genes, *gag*, *pol* and *env*, the viral long terminal repeats (LTR), the *env* splice acceptor site (sa) and the polypurine tract (ppt) are indicated on the genomic map of REV-A. The unique regions of the LTRs (U_3 and U_5), the repeat region (R), the primer binding site (pbs), the *env* splice donor site (sd), the encapsidation signal (E) and the initiation codon for the *gag* gene are illustrated on the enlarged map of the 5' end of REV-A.


REV-T
LTR
rel
LTR
REV-A
LTR
gag
pol
env
sa
LTR
ppt
U3
R
U5
pbs
sd
E
ATG
gag

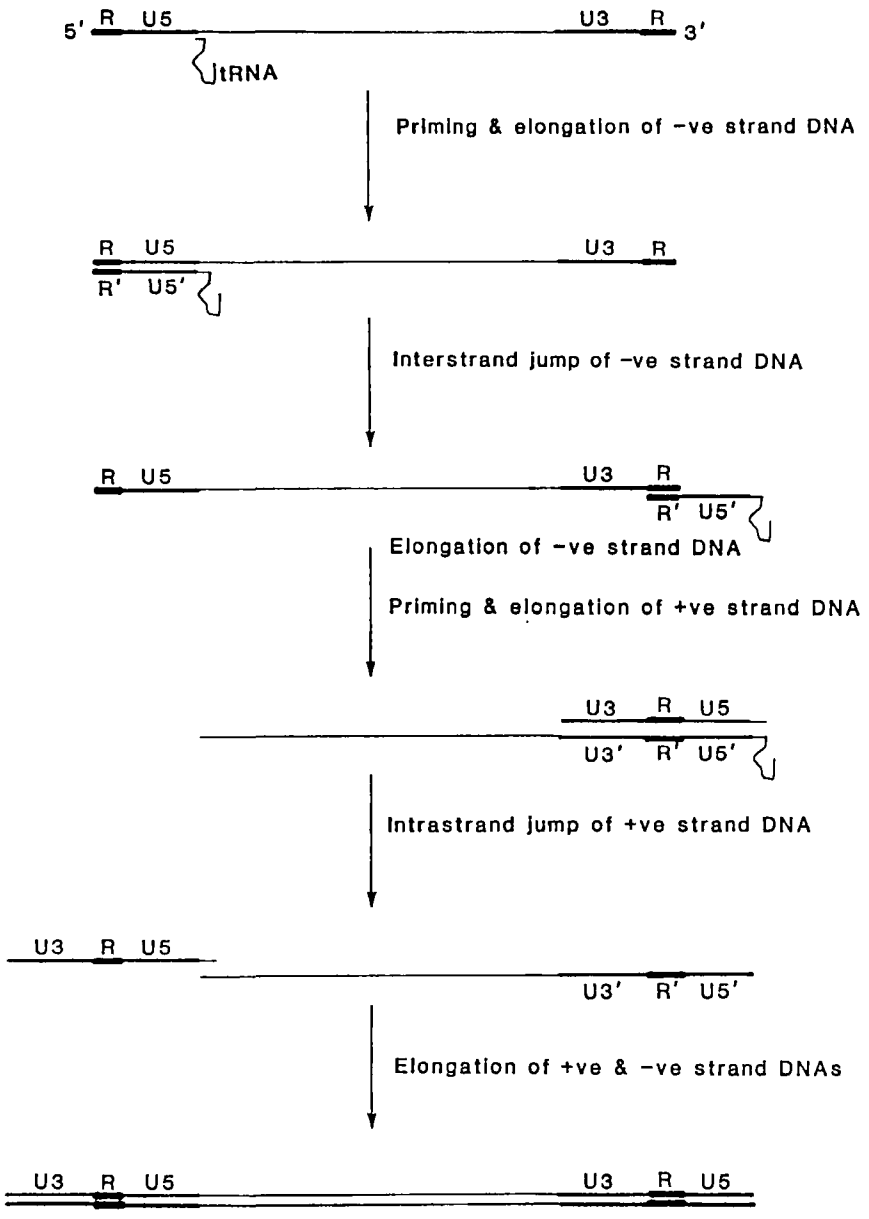
The genomic RNA of replication competent, non-transforming viruses can be defined into three coding regions: *gag, pol,* and *env* (Fig. 1). The *gag* gene encodes the capsid proteins, the *pol* gene encodes the protease, reverse transcriptase, and integrase enzymes and the *env* gene encodes the viral membrane glycoproteins. *gag* proteins are translated directly from genomic RNA. *pol* proteins are synthesized after proteolytic cleavage of a *gag-pol* polyprotein which is also translated from full length genomic RNA. The membrane glycoproteins are translated from a subgenomic *env* mRNA formed by splicing of the genomic RNA.

b) Replication

Retroviruses enter cells by receptor-mediated endocytosis which initially requires binding of viral surface glycoproteins to specific cell receptors (Varmus 1988). Following uncoating, the viral nucleoprotein complex is released and reverse transcription proceeds (Panganiban and Fiore 1988, Lobel and Goff 1985). Figure 2 outlines the major steps in the reverse transcription of a retroviral genome.

Priming of reverse transcription is by a specific tRNA annealed to the primer binding site (pbs) immediately 3' of the U_5 region of the genomic RNA. Reverse transcription from the primer binding site through the U_5 and repeat region (R) at the 5' end produces a short minus-strand DNA. In order for synthesis to continue, the nascent DNA chain complexed with the polymerase must reanneal to the 3' end of the genomic RNA. The complement of the repeat sequence (R') copied in the short minus-strand DNA allows the annealing of this DNA to the repeat sequence (R) located at the 3' end of the RNA, adjacent to the poly-A tail. Although the R region permits the specific transfer or jump of the polymerase/minus-strand DNA complex to the 3' R region, the actual

Figure 2. Reverse transcription of retrovirus RNA.
1. The viral reverse transcriptase initiates synthesis of the minus-strand DNA using the tRNA bound to the primer binding site (pbs) 2. After transcribing the 5' R region, the minus-strand DNA/polymerase complex "jumps" and resumes synthesis from the 3' R region of the second genomic RNA. 3. During elongation of the minus-strand DNA, the synthesis of plus-strand DNA is initiated by priming from the polypurine tract (ppt) on the minus-strand DNA. 4. Once the plus-strand DNA/polymerase complex has copied the pbs from the tRNA at the 5' end of the minus-strand DNA, it "jumps" to the pbs of the same minus-strand DNA and resumes synthesis. 5. Complete elongation of both plus- and minus-strand DNAs results in the linear proviral DNA with 2 LTRs.


5' R U5
U3 R 3'
tRNA
Priming & elongation of −ve strand DNA
R U5
U3 R
R' U5'
Interstrand jump of −ve strand DNA
R U5
U3 R
R' U5'
Elongation of −ve strand DNA
Priming & elongation of +ve strand DNA
U3 R U5
U3' R' U5'
Intrastrand jump of +ve strand DNA
U3 R U5
U3' R' U5'
Elongation of +ve & −ve strand DNAs
U3 R U5
U3 R U5

transfer can occur even if a portion of the R region has been copied and does not require that reverse transcription proceeds to the exact end of the viral RNA (Lobel and Goff 1985). The jump of the polymerase at this step appears to be to the 3' R region of the second genomic RNA and not within the same strand of RNA (Panganiban and Fiore 1988). This interstrand jump may explain the maintenance of the unusual diploid genome of retroviruses.

After transfer, the minus-strand DNA continues reverse transcription to the 5' end of the RNA. Shortly after the minus-strand DNA has copied the polypurine tract (ppt) near the 5' end of the genomic RNA, reverse transcription of the plus-strand DNA is initiated. Priming is from the polypurine tract using an RNA primer derived from partial RNase H degradation of the viral RNA, which occurs shortly after elongation of the minus-strand DNA (Olsen and Watson 1985). After elongation of the plus-strand DNA to the 5' end of the minus-strand DNA, the plus-strand DNA/polymerase complex jumps to the 3' end of the same elongated minus-strand DNA, guided by the annealing of the primer binding site sequence to the complement of the pbs present at the 3' end of the elongated minus-strand DNA. The end result after elongation of both strands is a double stranded DNA with long terminal repeats (LTR). The double stranded DNA then enters the nucleus where integration takes place.

In the nucleus, the linear viral DNA can circularize to form one or two LTR-containing structures. The two-LTR circle form was shown to be the precursor of integration for the spleen necrosis virus (Panganiban and Temin 1984a) however the linear form and not the two-LTR circle form was shown to be the precursor for murine retrovirus integration (Brown

et al. 1987, Ellis and Bernstein 1989). Integration involves the viral integrase enzyme (Panganiban and Temin 1984b) and results in the loss of several nucleotides from the ends of the viral DNA and the duplication of several nucleotides of host DNA sequence at the integration site. Although integration appears to be random, preferred targets appear to exist (Shih *et al.* 1988).

Once integrated, the cellular machinery uses the enhancer and promoter elements in the U_3 region, transcriptional stop and polyadenylation signals in the U_5 or R regions and RNA splice sites to synthesize genomic and subgenomic RNAs. The translation of viral polypeptides permits encapsidation of the genomic RNA containing the encapsidation signal (E) and the budding of the virus from the cell (Varmus 1988).

3. RETICULOENDOTHELIOSIS VIRUS, STRAIN T

a) Taxonomy

The discovery of a great number of different retroviruses in many different species led to the development of the classification system outlined by Fenner (1975). The family Retroviridae includes all RNA viruses which are associated with reverse transcriptase. Replication of the viral genome is through a DNA intermediate. Retroviridae is further divided into the subfamilies Oncovirinae, Lentivirinae and Spumavirinae based on the types of disease that they cause. The lentiviruses cause slow viral infections as typified in sheep by visna virus (Weiss *et al.* 1982) whereas spumaviruses persistently infect mammals without evident pathogenesis (Weiss *et al.* 1982). The RNA tumour viruses or Oncovirinae are classified into genera based on their morphology. Type B oncoviruses have electron-dense cores which are located eccentrically in the particle.

Precursor particles (type A) are observed in the cytoplasm of infected cells. Type C oncoviruses include most of the currently studied RNA tumour viruses and are characterized by centrally located electron-luscent cores. In addition, particles are not observed in the cytoplasm of infected cells. The type C oncoviruses are further divided into the mammalian type C subgenus and the avian type C subgenus. The avian type C subgenus is composed of two species: the avian leukosis-sarcoma viruses (ALSV) and the avian reticuloendotheliosis viruses. The avian reticuloendotheliosis virus consists of four strains: chick syncytial virus, duck infectious anemia virus, duck spleen necrosis virus and reticuloendotheliosis virus, strain T (REV). REV is the prototype of the reticuloendotheliosis viruses.

b) Origin

REV was isolated from turkeys in 1958 by Twiehaus and Robinson (Robinson and Twiehaus 1974). REV causes a rapidly fatal lymphoma in young chicks characterized by the appearance of neoplastic lesions in a wide variety of organs (Theilen *et al.* 1966). The oncogenic potential of REV is also demonstrable by its ability to transform *in vitro* spleen cells (Hoelzer *et al.* 1980), and chick (Franklin *et al* 1977) and quail (Hoelzer *et al.* 1979) embryo fibroblasts. An associated helper virus present in REV stocks is also pathogenic to chickens and turkeys and causes an acute runting disease.

REV is the only highly oncogenic member of the reticulo-endotheliosis (RE) species of viruses (Purchase and Witter 1975). The RE group has been shown to be distinct from the ALSV group of viruses. Morphological studies on REV revealed that the structure of mature particles resembled the mammalian leukemia-sarcoma viruses more than the ALSVs (Kang *et al.* 1975). Early experiments revealed that RE viruses

are unable to interfere with infection of CEF by RSV, and were also unable to complement various strains of RSV (Halpern *et al.* 1973). Serological comparison of the polymerase or reverse transcriptase proteins of these two groups detected only very weak cross reactivity (Mizutani and Temin 1973, Mizutani and Temin 1974). Significant cross hybridization between the two viruses was not observed (Kang and Temin 1973). In addition, unlike many of the ALSVs, RE viral sequences were largely absent from normal uninfected avian DNAs. Only 15% of the viral sequences appeared to be represented in host DNA (Kang and Temin 1974). These experiments taken together demonstrated that the closely related RE viruses are distinct from the ALSVs.

The observations that the RE viruses were highly pathogenic and hence not well adapted to their hosts and the close relatedness of the four members of the group suggested that the RE viruses appeared relatively recently in the avian species (Waite and Allen 1975). The polymerase of REV had a similar size and subunit structure (Mizutani and Temin 1974) as that of the mammalian retroviruses. As well, the divalent cation preference of REV and mammalian retrovirus reverse transcriptases was for manganese in contrast to the requirement for magnesium by the ALSVs (Kang 1975, Waite and Allen 1975). This information has led to the suggestion that the RE viruses may have originated from mammalian type C retroviruses (Kang *et al.* 1975, Waite and Allen 1975). Subsequently, in support of this suggestion, immunological relatedness and amino acid sequence homology was noted between the *gag* proteins of REV and MMC-1, an endogenous type C virus of the rhesus monkey (Oroszlan *et al.* 1981). In addition, cDNA from REV hybridized with DNA clones from

the *pol* and *gag* regions of the endogenous colobus virus, a closely related virus to MMC-1 (Rice *et al.* 1981).

c) The Biology of REV

As mentioned previously, REV causes a rapidly fatal lymphoma in young chicks characterized by the appearance of neoplastic lesions in a wide variety of organs (Theilen *et al.* 1966). *In vivo*, the target cell for transformation appeared to be an immature lymphoid cell (Beug *et al.* 1981). Beug *et al.* showed that transformed cells lacked markers specific for erythroid or myeloid cells, and did not synthesize any detectable levels of immunoglobulin. REV transformed spleen cells reacted strongly with sera reactive with B-cell surface determinants and weakly with sera reactive with T-cell surface determinants. This suggested that REV transforms early lymphoid cells, possibly early progenitors in the B-cell lineage. Lewis *et al.* (1981) obtained similar data, however they detected one clone of REV transformed spleen cells which expressed a μ immunoglobulin chain. *In vitro* transformations of cells recovered from different organs of young chicks indicated that the target cells for transformation by REV were present in the spleen and thymus, but were not present in detectable numbers in the Bursa.

d) Genome organization

As a genome, each virus particle of REV contains two copies of positive sense RNA molecules annealed at their 5' ends (Halpern *et al.* 1973, Bender *et al.* 1978). The presence of two copies of genomic RNA is typical for retroviruses and appears to be of functional importance for proviral DNA synthesis (Panganiban and Fiore 1988).

In vitro transformation of quail embryo fibroblasts (QEF) by REV followed two hit kinetics (Hoelzer *et al.* 1979). This indicated that REV

contained two viral species: an oncogenic virus (REV-T), defective for replication, and a competent helper virus (REV-A). In this same study, the helper virus (REV-A) was isolated and shown to be non-oncogenic, but pathogenic for chickens. When REV-A was inoculated into chickens, a severe runting disease was produced. As well, REV-A has been reported to cause immunosuppression in infected animals as detected by decreased *in vitro* blastogenic response of stimulated lymphocytes (Scofield and Bose, Jr. 1978, Rup *et al.* 1979).

The hypothesis that REV-T is defective for replication was supported by the isolation of transformed QEF (Hoelzer *et al.* 1979) or spleen cells (Hoelzer *et al.* 1980) which did not produce virus. REV-T could be recovered from these "non-producers" following superinfection with REV-A. Analysis of the genomic RNAs of REV, showed the presence of two RNA species of different sizes (Hoelzer *et al.* 1980, Breitman *et al.* 1980, Gonda *et al.* 1980). The genomic RNA of REV-A contained a single species which corresponded in size to the larger species of REV RNA. Characterization of these RNAs revealed that REV-T contained sequences not represented in the REV-A genome. Analysis of these sequences by RNase T1 mapping (Breitman *et al.* 1980) and cDNA hybridization (Wong and Lai 1981) indicated that such sequences were different from any of the other known transformation specific sequences. These unique sequences of REV-T, termed *rel* (Lewis *et al.* 1981), hybridized with avian DNA (Wong and Lai 1981, Chen *et al.* 1981).

The REV-T genome has been mapped in comparison with the REV-A genome by ordered oligonucleotide fingerprinting (Cohen *et al.* 1981), heteroduplex mapping (Hu *et al.* 1981) and restriction enzyme analysis of proviral DNA clones (Chen *et al.* 1981, Rice *et al.* 1982). Based on

restriction analysis of proviral DNA, the REV-A and REV-T genomes were estimated to be 8.3 kilobases (kb) and 5.5 kb in length, respectively. Comparisons of the proviral DNA of REV-A with REV-T indicated that REV-T has a large deletion of approximately 3 kb predominantly from the *pol* region and has a substitution of 1.5 kb in the *env* gene. The REV-A and REV-T genome organizations presented by Chen and Temin (1982) are shown in Figure 1.

Direct evidence that the substituted sequences in the *env* gene were involved in the transformation process came from Chen and Temin (1982). Deletions of *rel* sequences abolished *in vitro* transformation of spleen cells by the resulting virus. In addition to demonstrating the requirement for the v-*rel* oncogene, Chen and Temin (1982) also showed that the restoration of the deleted sequences from the *pol* region suppressed transformation. Thus at least two events were required for the generation of the oncogenic virus; the acquisition of the v-rel oncogene and the deletion of viral sequences in the *pol* gene.

The cellular counterpart to the v-*rel* oncogene of REV-T, c-*rel*, was cloned by Chen *et al.* (1983). Regions of c-*rel* homologous to v-*rel* span 25 kbp of the chicken DNA. This portion of the gene contained a minimum of 6 exons partly homologous to v-*rel* separated by 5 introns. The mRNA expressed by c-*rel* was 4.0 kb long and at its 3' end contained sequence information not represented in v-*rel*.

At the start of my Ph.D. program, very little was known about the v-*rel* gene or its gene product. The scope of this thesis is the investigation of the role of the v-*rel* oncogene in transformation. Before I introduce the specific objectives of this thesis, I would like to review mechanisms by which RNA tumour viruses activate cellular oncogenes, the

relevance of viral oncogenes to natural cancers and possible modes of action of the oncogene products.

4. ONCOGENES

a) Activation of oncogenes by retroviruses

RNA tumour viruses activate cellular proto-oncogenes in two ways: insertion of the retrovirus adjacent to an oncogene resident in the host cell genome or transduction of the oncogene into the viral genome.

Insertion of retroviral DNA into the host cell genome not only interrupts the local DNA sequence, but also inserts the relatively strong promoter and enhancer elements of the LTRs. The LTRs potentially could function to increase the transcription of nearby cellular genes through direct promoter activity of the viral LTR, or by activation of cellular promoters by the viral enhancer element residing in the LTR (Neil and Forrest 1987). Examples of each of these mechanisms are discussed below.

ALV induces B-cell lymphomas after a long latent period, but does not contain transforming genes (Hayward *et al.* 1981). Examination of a number of different lymphomas indicated that the ALV provirus inserted adjacent to the c-*myc* oncogene (Hayward *et al.* 1981). Increased expression of c-*myc* was due to expression of an new c-*myc* mRNA up to 30-100 fold higher than the normal c-*myc* mRNA found in normal tissues. The new mRNA contained both c-*myc* and viral sequences, a finding which was consistent with transcription originating from the viral LTR.

Normally, the 3' LTR of integrated proviruses does not act as a strong promoter, possibly due to transcriptional interference from the 5' LTR (Cullen *et al.* 1984). Analysis of the proviral DNA of ALV which had inserted adjacent to the c-*myc* oncogene in lymphomas showed that the proviruses suffered deletions and were defective (Cullen *et al.* 1984). The

deletions always removed the 5' LTR. The 3' LTR could then function as a strong promoter capable of driving high levels of expression of genes immediately 3' to the inserted provirus. Therefore, the activation of a cellular oncogene may require the insertion of the provirus adjacent to the specific gene and inactivation of the 5' LTR to permit efficient transcription from the 3' LTR.

REV-A has also been found to generate B-cell lymphomas. Although this system has not been as thoroughly studied, the REV-A provirus was shown to be integrated adjacent to c-*myc* in over 90% of the tumours examined (Noori-Daloii *et al.* 1981).

Thymic tumours induced by murine or feline leukemia viruses frequently are associated with integrated viruses upstream of c-*myc*. In contrast to the ALV induced B-cell lymphomas, the integrated provirus is positioned in an opposite transcriptional orientation with respect to c-*myc* (Neil and Forrest 1987). This arrangement juxtaposes the 5' LTR, including the U_3 enhancer element, adjacent to the c-*myc* gene. Activation of c-*myc* may be the effect of the viral enhancer on the c-*myc* promoter.

Cellular genes may be introduced into viral genomes by rare recombinational events with the integrated proviral DNA or possibly through rare events at the RNA level. Such rearrangements need not occur in one step but may occur over the lifetime of the infected cell since the proviral DNA normally resides permanently in the host cell genome. The random acquisition of cellular DNA would be expected only infrequently to introduce coding sequence situated properly in the virus to permit its expression. In addition, the insertion of sequence into the

retroviral DNA would frequently disrupt the viral coding sequences, making the viral progeny defective for replication.

The acquisition and activation of cellular oncogenes by the RNA tumour viruses gives a distinct pathogenicity to the virus; the ability to transform cells and cause cancers. This altered pathogenicity alone does not allow its continued propagation. The positive selection for the maintenance of these oncogenic viruses is largely applied by the research scientists with an interest in experimentally induced tumours. It is through the intervention of the scientist that virus is recovered from tumours and faithfully passaged in laboratories to maintain the oncogenic phenotype. Nevertheless, viral oncogenes have offered a model system for the study of oncogenesis which has attracted scientific interest due to the apparent simplicity. RNA tumour viruses introduce a single transforming gene which is then able to transform the appropriate cell. Natural human cancers are believed to be the result of a multistep process, likely to involve more than one gene (Klein and Klein 1985).

The different origins of viral induced and naturally occurring tumours bring into question the validity of the viral oncogene model. Yet despite the single step transformation and the rather artificial selection and maintenance of the oncogenic retroviruses, there is compelling evidence that they provide valuable insight into the initiation and maintenance of naturally occurring cancers.

b) Role of oncogenes in natural cancers

Approximately 22 viral oncogenes have been identified, each able to convert appropriate host cells to the transformed phenotype upon their introduction into the cells by DNA transfection or viral infection. One of the major goals of viral oncology has been to gain an understanding of

the transformation process mediated by highly oncogenic retroviruses. The application of the knowledge and tools derived from these studies may be relevant to the understanding of naturally occurring cancers. Since viral oncogenes present in the highly oncogenic retroviruses originated by transduction of normal cellular genes (proto-oncogenes) into the viral genome, it is possible that these proto-oncogenes can be activated by mutation in the cell to become active transforming genes without the aid of exogenous virus. Several approaches which have been utilized to delineate the role of oncogenes in the development of natural tumours are briefly described below.

The first method was based on the early work of Hill and Hillova (1972) who showed that CEF could be transformed by transfection with DNA from RSV transformed rat cells. This technique was applied to the search for oncogenes in various transformed cell lines and fresh tumours (Cooper and Lane 1984, Bishop 1987). The usual recipient cell line used for such transfection studies is the NIH 3T3 cell line. NIH 3T3 cells are much more efficient at integrating transfected DNAs than most other cell lines (Cooper and Lane 1984). The major limitation of this system is that the NIH 3T3 cells are not normal in that they are already immortalized. It is likely that a number of potential oncogenes which immortalize, but do not transform would not be detected in this assay. In addition, potential oncogenes may have target cell specificities which may preclude transformation of NIH 3T3 cells. Nevertheless, approximately 20% of cell lines and tumours tested contained oncogenes detected by this assay (Bishop 1987). Application of the transfection assay has detected transforming genes related to known viral oncogenes, as well as numerous other cellular oncogenes capable of transforming cells (Bishop 1987).

The availability of numerous viral and cellular oncogene probes has allowed direct testing of the expression levels of the various genes. Several research groups have looked for the inappropriate expression of cellular oncogenes in cells from fresh tumours and from cell lines derived from tumours (Slamon *et al.* 1984, Rothberg *et al.* 1984, Gallick *et al.* 1985). A frequent observation was that certain cellular oncogenes are expressed at higher than normal levels in these cells. The limitation of this approach is the uncertainty in correlating oncogene expression to a transformed phenotype in the absence of completely appropriate negative controls. In addition, this type of investigation detects only overexpressed oncogenes, but fails to detect expression of altered gene products.

Cytogenetic studies have identified chromosome rearrangements and DNA amplifications in tumour cells and their derived cell lines. Several types of tumours have been strongly linked to these type of events. Most notably, Burkitt's lymphoma has been linked to translocation of the c-*myc* oncogene (Alitalo *et al.* 1987) and chronic myelocytic leukaemia involved translocations of the c-*abl* proto-oncogene (Heisterkamp *et al.* 1983). Once again, it is the frequent occurrence of these events which supports their role in oncogenesis, although it has been difficult to verify causal relationships.

c) Functions of oncogenes

The viral oncogenes have been shown to be derived from cellular genes coding for growth factors, growth factor receptors, cytoplasmic tyrosine kinases, serine/threonine kinases, nuclear proteins of which some exhibit DNA binding activity, and nuclear proteins which behave as transcription factors (Cooper and Lane 1984, Heldin and Westermark 1984, Sporn and Roberts 1985, Bishop 1987). The study of the transforming

genes of RNA tumour viruses has allowed researchers to analyze a selected subset of genes whose cellular counterparts are apparently quite important in regulatory pathways for cell growth and may be involved in the generation of naturally occurring tumours. The important function that these genes appear to play in the regulation of cell growth and their association with natural tumours as described above is compelling evidence for their causal role in the generation and maintenance of naturally occurring tumours in humans as well as other mammals.

Approximately 22 viral oncogenes, and numerous cellular oncogenes have been described. Rather than attempting the formidable task of summarizing the complete literature on oncogenes, I have grouped them according to possible functions, and I have focused selectively on a small subset of investigations which suggest possible mechanisms of action of the viral or related cellular gene products.

d) V-*sis*: a growth factor

The v-*sis* oncogene of the simian sarcoma virus (SSV) codes for a 28 kDa protein ($p28^{sis}$) which forms disulfide linked homodimers. Intramolecular but not intermolecular disulfide linkages are required for biological activity (Giese *et al.* 1987) indicating that the dimer structure may not be functionally important. The coding information of v-*sis* was derived from viral *env* sequence and cell derived sequence. The 51 N-terminal amino acid residues are coded by the viral *env* sequence whereas the remaining 220 amino acids are derived from cell derived sequence (Devare *et al.* 1983). Interestingly, the viral *env* sequence is required for the transforming function exhibited by v-*sis* (Hannink and Donoghue 1984). The *env* sequence provides a signal sequence which presumably permits $p28^{sis}$ to enter the pathway for cell surface

expression or secretion. Transduction of v-*sis* into the SSV genome required not only correct in-frame integration for expression of the coding sequence, but also required a functional signal sequence which was provided by the viral *env* gene.

Analysis of the DNA sequence of v-*sis* indicated that the putative amino acid sequence was homologous to a portion of the primary sequence of platelet-derived growth factor (PDGF) (Doolittle *et al.* 1983, Waterfield *et al.* 1983). Cloning and sequencing of the human c-*sis* proto-oncogene identified it as the structural gene for one of the subunits of PDGF.

The transforming activity of $p28^{sis}$ is presumed to be the result of the uncontrolled expression of a potent mitogen. The level of action of this oncogene would therefore represent one of the earliest steps in the mitogenic pathway, at the level of ligand binding to growth factor receptors. This model may not require secretion of $p28^{sis}$ since the majority of the expressed protein is associated with the cell surface (Robbins *et al.* 1985). Less than 1% was detected as secreted in the culture medium. $p28^{sis}$ may still act in an autocrine manner by immediately binding to the appropriate growth factor receptors on the cells from which it is expressed. This could account for the cell surface localization of $p28^{sis}$. Conceivably, this binding could even occur in internal compartments of the secretory machinery.

Consistent with the model that inappropriately expressed growth factors can behave as oncogenes, the epidermal growth factor (EGF) gene was able to act as an oncogene when constitutively overexpressed in FR3T3 cells (Stern *et al* 1987). Also, the augmented secretion of basic fibroblast growth factor facilitated by the addition of a signal sequence caused cells to become transformed and tumorigenic (Rogelj *et al.* 1988).

e) Tyrosine kinases

A large number of viral oncogenes (*abl, erb-B, fes/fps, fgr, fms, kit, ros, src, yes*) code for proteins which possess tyrosine kinase activity (Heldin and Westermark 1984). All of the tyrosine kinases share some sequence homology indicating an evolutionary relationship (Hanks *et al.* 1988). Although related, the tyrosine kinases encoded by viral oncogenes can be membrane glycoproteins or cytoplasmic proteins often associated with the inner surface of the plasma membrane.

i. Oncogenes related to growth factor receptors

Three of the viral oncogenes (v-*erbB,* *v-fms* and v-*kit*) code for membrane glycoproteins possessing tyrosine kinase activity. The cellular proto-oncogenes c-*erbB* and c-*fms* have been identified as the genes for the EGF receptor (Downward *et al.* 1984) and the macrophage colony-stimulating factor receptor (Sherr *et al.* 1985), respectively. The c-*kit* proto-oncogene codes for a protein whose predicted structure closely resembles a growth factor receptor, although the actual ligand has not been identified (Yarden *et al.* 1987). I will limit my review to the v-*erbB* oncogene which has been studied most extensively.

The v-*erbB* oncogene is responsible for the transforming phenotype of the avian erythroblastosis virus (AEV). AEV induces erythroblastosis and sarcomas in susceptible chickens and transforms fibroblasts and erythroid cells *in vitro* (Graf *et al.* 1976). AEV also contains the v-*erbA* oncogene which alone is unable to transform cells but does potentiate the effects of the v-*erbB* oncogene (Frykberg *et al.* 1983). Erythroid cells transformed by v-*erbB* alone can spontaneously differentiate. Transformed erythroid cells can be maintained at an immature stage if v-*erbA* is also present (Frykberg *et al.* 1983).

Before discussing possible mechanisms of action of v-*erbB*, first I would like to describe the current model describing the peptide growth factor receptor action (Carpenter and Cohen 1984). Peptide growth factors mediate their effect by binding to specific receptors on the surface of the appropriate target cells. The binding of ligand activates a tyrosine kinase activity resident in the growth factor receptor. The biologically relevant substrates are not known, but probably represent second messengers which participate in the signal transduction from the cell surface to the nucleus. Ligand bound receptors are also internalized by receptor mediated endocytosis where they are likely degraded following fusion with lysosomal bodies leading to receptor down-modulation.

Comparison of the v-*erbB* and EGF receptor sequences reveals that v-*erbB* lacks the EGF receptor signal sequence, the extracellular ligand-binding domain (555 amino acids) and the 32 carboxyl-terminal amino acids (Ullrich *et al.* 1984). The absence of a ligand binding domain led to the proposal that the v-*erbB* protein was a permanently active tyrosine kinase unable to be regulated by external growth factors (Ullrich *et al.* 1984). Riedel *et al.* (1987) constructed a chimera between the v-*erbB* and EGF receptor genes which restored the N-terminal ligand binding domain. The resulting chimeric protein was able to bind EGF and subsequently undergo enhanced autophosphorylation activity. The growth of rat cells expressing the chimeric protein and the normal EGF receptor was inhibited by the addition of EGF. In addition, the chimeric gene retained a low level of transformation activity. This indicated that the oncogenic activation of the EGF receptor gene may involve deletion of sequences coding for the 32 C-terminal amino acids described above and

cannot be explained solely by the deletion of the ligand binding region of the gene.

The mode of action of the growth factor receptor type of oncogenes may still be the transduction of a mitogenic signal in the absence of external growth factor and perhaps the deficiency of a mechanism for receptor down-modulation. The generation of viral oncogenes from growth factor receptor genes would presumably require that the receptor activity becomes independent of growth factor. In addition, the transduced gene must retain sequences coding for signals which are required for cell surface expression of the protein product.

ii. V-*src*

Perhaps the most extensively studied viral oncogene is v-*src* (Wyke and Stoker 1987). Despite the wealth of information on v-*src*, to date there has been no conclusive experiments identifying the biologically relevant targets of this tyrosine kinase, although numerous protein and lipid substrates have been identified. As a result, I will not present information relating to possible substrates, but will present some of the information relating to the form and biochemical function of pp60$^{v\text{-}src}$, the protein product of the v-*src* gene.

pp60$^{v\text{-}src}$ contains an intrinsic tyrosine kinase activity that is required for transformation activity (Wyke and Stoker 1987). Mutation of the ATP-binding site abolishes kinase activity, transformation and tumorigenicity of pp60$^{v\text{-}src}$ (Snyder *et al.* 1985). pp60$^{v\text{-}src}$ is phosphorylated predominantly on a serine at amino acid position 17 and at a tyrosine at amino acid position 416 (Wyke and Stoker 1987), however phosphorylation of these sites is not essential for transformation (Cross and Hanafusa 1983).

pp60$^{v\text{-}src}$ is synthesized on cytosolic ribosomes. Shortly after, or perhaps concomitant with synthesis, the N-terminal methionine is removed and the N-terminal glycine acylated with myristic acid (Buss and Sefton 1985, Schultz *et al.* 1985). The signal for myristylation appears to be within the six N-terminal amino acids and was shown to be functional when added to a heterologous protein (Buss *et al.* 1988). Following translation, pp60$^{v\text{-}src}$ enters into a complex with two other cellular proteins of unknown function (Wyke and Stoker 1987). pp60$^{v\text{-}src}$ is transiently associated with this complex until the transforming protein becomes associated with the inner surface of the plasma membrane. The N-terminal myristic acid appears to function by localizing pp60$^{v\text{-}src}$ to the plasma membrane (Cross *et al.* 1984). Membrane localization of pp60$^{v\text{-}src}$ is necessary for transforming activity (Cross *et al.* 1984). Although mutants which have deleted N-terminal or membrane binding regions were unable to transform cells, they were able to stimulate cell proliferation (Calothy *et al.* 1987). This indicated that the mitogenic activity of pp60$^{v\text{-}src}$ is distinct from the transforming activity.

Recent efforts have sought to map more finely the functional regions of pp60$^{v\text{-}src}$ using linker insertion and deletion analysis (DeClue and Martin 1989, Wang and Parsons 1989). In addition, towards the understanding of cellular factors participating with pp60$^{v\text{-}src}$, a cellular clone was isolated which is temperature sensitive (ts) for transformation by *src* (Inoue *et al.* 1989). While the numerous studies of pp60$^{v\text{-}src}$ have increased our understanding of the important functional regions of the transforming protein, the mechanism of action is still unknown.

f) Serine-Threonine kinases

Very little is known about the mechanism of action of the viral oncogenes possessing serine/threonine kinase activity. The protein products of such oncogenes (v-*mil*, v-*raf*, v-*mos*) have been shown to be localized in the cytoplasm of transformed cells. Mutagenesis of the ATP binding site of v-*mil* abolished serine/threonine kinase activity as well as biological activity (Denhez *et al.* 1988). This indicated that the kinase activity probably mediates the oncogenic signal of the oncogene product. The proteins encoded by v-*mil* and v-*mos* have also been suggested to possess an ATP-dependent DNA and RNA binding activity (Bunte *et al.* 1983, Seth *et al.* 1987), although the relevance of such activities has not been evaluated.

g) The *ras* family of oncogenes

The oncogenes of the Harvey and Kirsten sarcoma viruses, *H-ras* and *K-ras* respectively, were derived from cellular *ras* genes which represent only part of a larger family of related genes. The *ras* genes have been of great interest because of the frequent detection of activated or oncogenic forms present in DNA from human tumours or cells lines (Finkel *et al.* 1984). Activation of *ras* involves mutations frequently at amino acid codon positions 12, 13 and 61, although other codons are sometimes involved (McCormick 1989).

The *ras* genes code for protein products (p21) which are localized on the inner surface of the plasma membrane and exhibit GTPase activity (McCormick 1989). p21 proteins are related to a larger family of proteins known as the G proteins (Hurley *et al.* 1984). G proteins transduce signals originating at the cell surface to generate second messengers inside the cell. G proteins exchange GTP for bound GDP following interaction with

activated or ligand bound cell surface receptors. The charged or GTP bound form of the G protein activates enzymes which generate increased levels of second messengers, such as cAMP. The intrinsic GTPase activity downregulates the charged form of the G proteins in the absence of further stimuli (Hurley *et al.* 1984, Lochrie *et al.* 1985).

Oncogenic forms of p21 often show reduced GTPase activity, although mutants have been constructed which maintain normal GTPase activity while retaining their ability to transform (Der *et al.* 1986). The membrane association of p21 is mediated by C-terminal sequences and was shown to be crucial for biological activity (Willumsen *et al.* 1984). GTP binding was found to be important for the transforming activity of p21, although one mutant was found which retained biological activity in the apparent absence of detectable GTP binding (Clanton *et al.* 1987).

The GTPase activity of normal p21 was shown to be enhanced dramatically *in vivo* through the interaction with a cytoplasmic protein, GTPase activating protein (GAP) (Trahey and McCormick 1987). In this same study, GAP was unable to stimulate GTPase activity of several oncogenic mutants of p21. As a result, the *in vivo* GTPase activity of p21 proteins was much less than that of the normal p21, although small or even no differences could be detected *in vitro* (McCormick 1989).

McCormick (1989) has proposed an attractive model for the signal transduction by *ras* p21 proteins. In normal cells, p21 exchanges bound GDP (p21-GDP) for GTP (p21-GTP) in response to receptor stimulation. GAP then binds p21-GTP and sends a signal simultaneously with the enhancement of the p21 GTPase activity. p21-GTP is converted to p21-GDP, GAP dissociates and the signal terminates. In transformed cells, the p21 proteins have low GTPase activity which is not enhanced by GAP

binding. p21 is thought to be bound to GTP at quite significant levels. p21-GTP binds GAP, which then mediates a signal. The inability of GAP to enhance the GTPase of p21 eliminates the normal down regulation of signal. The continued or prolonged association of p21 with GAP results in the uncontrolled signal transduction which ultimately results in cellular transformation.

McCormick's model is consistent with much of the data on *ras*, but will require substantial confirmation. A greater understanding of the structure-function relationships of the *ras* proteins will be facilitated by the recent structural determinations of the protein products of an oncogenic H-*ras* gene (Tong *et al.* 1989) and the normal H-ras gene (De Vos *et al.* 1988).

h) The nuclear oncogenes

A number of viral oncogenes (v-*fos*, v-*jun*, v-*myb*, v-*myb-ets*, v-*myc*) code for proteins which are localized in the nucleus of transformed cells and have been implicated in the replication or transcription of DNA. A considerable amount of information has been published on the related proto-oncogenes c-*myc*, c-*jun* and c-*fos*. I will discuss primarily the postulated roles of the cellular proto-oncogenes, while the mechanism of action of the viral oncogenes can only be imagined to be the result of the aberration of such normal activities.

i. C-*myc*

C-*myc* has been extensively studied as a result of its apparent activation and subsequent involvement in a number of cancers as discussed earlier. The exact function of c-*myc* is unknown, although a DNA binding activity has been demonstrated for the protein product (Persson and Leder 1984). In addition, several lines of evidence indicated

that it may be involved in DNA synthesis. Antibodies directed against c-*myc* were able to inhibit DNA polymerase activity in isolated nuclei (Studzinski *et al.* 1986). Heikkila *et al.* (1987) examined the phytohaemagglutinin stimulation of peripheral blood lymphocytes whose c-*myc* protein expression was inhibited by c-*myc* antisense oligonucleotides. Inhibition of c-*myc* expression prevented cells from undergoing detectable DNA replication. Cell progression from G_0 to G_1 was suggested to have occurred as evidenced by the stimulated expression of the interleukin 2 receptor and transferrin receptor genes and induction of greater ^{3}H-uridine incorporation. This implied that the expression of c-*myc* appears to be required for entry of cells into S phase. The ability of v-*myc* to transform cells may be related to a loss of control over an event involved in control of G_1 to S phase transitions.

ii. C-*jun* and c-*fos*

C-*jun* encodes a nuclear protein which shares homology with the DNA binding domain of the yeast transcription factor GCN4 (Vogt *et al.* 1987). C-*jun*, GCN4 and the eukaryotic transcription factor AP-1 bind the same specific DNA sequence (ATGA[G/C]TCAT)(Struhl 1987, Angel *et al.* 1987, Lee *et al.* 1987). Antisera raised against v-*jun* peptides reacted with a single major polypeptide of purified AP-1 preparations (Bohmann *et al.* 1987). Thus c-*jun* is at least one component of a mammalian transcription factor.

C-*fos* encodes a nuclear protein which was also able to bind specifically to the AP-1 consensus recognition sequence (Franza *et al.* 1988, Rauscher *et al.* 1988a). In addition, Curran *et al.* (1985) found c-*fos* and v-*fos* to be complexed with a 39,000 dalton cellular protein (p39). The identification of p39 as the protein product of c-*jun* (Rauscher *et al.* 1988b) established the relationship between the fos and jun proteins.

The interaction of the fos and jun proteins was shown to be dependent on the integrity of a heptad repeat of leucines, termed a "leucine zipper" present in both proteins (Kouzarides and Ziff 1988, Sassone-Corsi *et al.* 1988). The functional significance of the fos-jun interaction was revealed through studies of the DNA binding of jun protein alone or in combination with fos proteins (Halazonetis *et al.* 1988, Nakabeppu *et al.* 1988). *In vitro* translated jun proteins formed homodimers which exhibited specific DNA binding activity to the AP-1 consensus recognition sequence. In contrast, the *in vitro* translated fos protein did not assemble into homodimers and was not able to bind the AP-1 recognition sequence. Incubation of the two proteins together resulted in the formation of heterodimers which exhibited a greatly enhanced binding affinity to the AP-1 DNA site.

The transcription of the c-*jun* and c-*fos* proto-oncogenes can be stimulated by growth factors (Greenberg and Ziff 1984, Kruijer *et al.* 1984, Muller *et al.* 1984, Ryseck *et al.* 1988, Quantin and Breathnach 1988, Lamph *et al.* 1988) indicating that they do not represent simple factors which are constitutively expressed. The transcription of the related viral *jun* and *fos* genes under the control of viral LTRs in the absence of growth factor stimulation may explain their mode of action. In fact, the c-*fos* proto-oncogene can transform fibroblasts once the 3' untranslated sequences have been removed (Miller *et al.* 1984, Meijlink *et al.* 1985) permitting the transcription of a more stable c-*fos* mRNA (Lee *et al.* 1988).

i) Oncogene cooperation

Several oncogenic retroviruses carry two oncogenes which both participate in the transformation process. In the following three cases,

the additional oncogene is not fully oncogenic, but does significantly increase the potency of the primary viral oncogene.

i. V-*mil*

The MH2 virus carries the v-*mil* oncogene in addition to the v-myc oncogene (Jansen *et al.* 1983). As previously mentioned, the protein product of v-*mil* was shown to be a serine/threonine kinase (Denhez *et al.* 1988). The full transformation of macrophages by MH2 is mediated by both oncogenes. Macrophages transformed by v-*myc* alone are dependent on chicken myelomonocytic growth factor (cMGF). The dependency on cMGF is relieved upon the expression of v-*mil*. V-*mil* induces the expression of cMGF in macrophages transformed by v-*myc* resulting in an autocrine stimulation of cell growth (Weizsacker *et al.* 1986).

ii. V-*erbA*

The v-*erbA* oncogene, as described earlier, blocks the spontaneous differentiation of erythroblasts transformed by v-*erbB* (Fryberg *et al.* 1983). The mode of action of v-*erbA* may be somewhat unique among oncogenes given that the cellular homolog c-*erbA* codes for the thyroid hormone receptor (Sap *et al.* 1986, Weinberger *et al.* 1986). The v-*erbA* protein lacks the ligand binding domain of the receptor (Munoz *et al.* 1988) and appears to specifically suppress the transcription of at least one erythroid specific gene (Zenke *et al.* 1988). V-*erbA* may potentially act by inhibiting genes whose expression is required for the full differentiation of erythroblasts.

iii.V-ets

E26 is a defective avian retrovirus which expresses a nuclear *gag-myb-ets* fusion protein derived from the viral *gag* gene, the c-*myb* proto-oncogene and the c-*ets*-1 proto-oncogene (Leprince *et al.* 1983,

Nunn *et al.* 1984). Whereas viruses expressing only the v-*myb* oncogene transform cells of the myeloid lineage, E26 transforms both myeloid and erythroid cells (Radke *et al.* 1982). The isolation of *ts* mutants of the *ets* gene have permitted its role in the transformation to be examined (Golay *et al.* 1988). Briefly, it was shown that *ets* is responsible for the capability of E26 to transform erythroid cells and may modulate the phenotype of the transformed myeloid cells. The mode of action of *ets* is unknown although both v-*ets* and c-*ets* encode nuclear proteins which exhibit DNA binding activity (Ghysdael *et al.* 1986).

iv. Experimental cooperation between oncogenes

The deregulation of c-*myc* expression by chromosomal translocation and the amplification of cellular *myc* genes have been implicated in a number of natural cancers including Burkitt's lymphoma and certain neuroblastomas (Alitalo *et al.* 1987). Transgenic mice containing c-*myc* coupled to the immunoglobulin heavy-chain enhancer developed B-lymphoid tumours which were monoclonal in origin and developed spontaneously after variable incubation times (Adams *et al.* 1985, Harris *et al.* 1988). This indicated that expression of c-*myc* alone could not induce B-cell lymphomas, although it certainly predisposed animals to its eventual development. In contrast to bone marrow cells from normal mice, the infection of similar cells from the *myc*-transgenic mice with retroviruses carrying the v-H-*ras* or v-*raf* resulted in the isolation of cells which were highly proliferative, grew efficiently in soft agar and were tumourigenic in nude mice (Alexander *et al.* 1989). This is one example of two oncogenes cooperating to transform a cell which would not have been transformed by the introduction of either oncogene alone. C-*myc* and

mutant *ras* have previously been shown to cooperate in the transformation primary rat fibroblasts (Land *et al.* 1983).

j) Summary

The viral oncogenes code for proteins which function aberrantly in the signal pathways for cell growth. They are derived from cellular proto-oncogenes which encode growth factors, growth factor receptors, G proteins, tyrosine kinases, serine/threonine kinases and DNA binding proteins some of which are transcription factors. The full characterization of viral oncogenes and their cellular counterparts will provide valuable insight into the processes regulating cell growth and will permit a greater understanding of the mechanisms involved in the development and progression of neoplasia.

Characterization of newly discovered oncogenes may add significantly to the understanding of pathways involved in the control of cell growth. For this reason, I initiated studies directed at the characterization of the protein product of the v-*rel* oncogene. At the start of this Ph.D. program in September, 1983, the sequence of the v-*rel* oncogene had just been determined (H.M. Temin, personal communication), however, the protein product had not been identified. It was therefore of great interest to learn more about the protein product of this oncogene.

5. STATEMENT OF OBJECTIVES

a) To identify the product of the v-*rel* oncogene using antibodies generated against: *i*) synthetic peptides representing a segment of v-*rel* primary amino acid sequence based on available DNA sequence information, *ii*) v-*rel* proteins expressed in *E. coli*.

b) To determine the importance of both N- and C-terminal amino acids of v-*rel* in the transforming activity of the v-*rel* protein by studying proteins expressed from genes with deleted 5' or 3' ends.

c) To map important functional regions of the v-*rel* protein required for transformation by introducing linker insertion mutations throughout the gene.

MATERIALS AND METHODS

1. CELLS AND VIRUS

Cos1 cells were kindly provided by Dr. M. Schubert, NIH, Bethesda, MD. REV-T transformed chicken bone marrow cells (CBMC) were originally obtained from H.R. Bose, University of Texas at Austin. A proviral clone of REV-A, pSW253, referred to as pREV-A in this thesis was kindly provided by Dr. H.M. Temin, University of Wisconsin, Madison, WI. A proviral clone of REV-T, pREV-T, was obtained from the American Type Culture Collection, Rockville, MD.

a) Maintenance

All cells were grown at 37°C, 5% CO_2 in a humidified Shel-lab incubator (Sheldon Manufacturing Inc., Portland, OR) in media supplemented to 2mM glutamine. Media, media supplements, and trypsin were purchased from Gibco (Gibco/BRL, Life Technologies, Inc., Burlington, Ont.) unless otherwise mentioned. Centrifugation of cells was at 200 x g for 10 minutes at room temperature in a Sorvall GLC-2B clinical centrifuge (HL-4 rotor) unless otherwise indicated.

Cos1 cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 7.5% heat inactivated fetal calf serum (HIFCS). Chicken bone marrow cells (CBMC) transformed by reticuloendotheliosis virus, strain T (REV-T) (Franklin *et al.* 1974) or REV-T variants were grown in suspension culture in RPMI 1640, 10% HIFCS supplemented with penicillin-streptomycin (100U/mL penicillin G, 100μg/mL streptomycin sulphate) and anti-PPLO (60μg/mL tylocine).

Primary cultures of chicken embryo fibroblast (CEF) were prepared from 11 day embryos from S-SPF/COFAL/MAREK'S/gs/chf-39 eggs (SPAFAS, Inc., Norwich, CT). Briefly, embryos were sterilely removed

from eggs, decapitated, macerated by passing through a 10 mL syringe and incubated at 37°C for 30 minutes with stirring in TBS (137mM NaCl, 5mM KCl, 5.6mM glucose, 0.7mM Na_2HPO_4, 25mM Tris(hydroxymethyl)-aminomethane (Tris) pH 7.2), 0.1% trypsin-EDTA (1mM ethylene diamine tetra-acetic acid (EDTA)). The resulting suspension was filtered through 3-4 layers of sterile cheese cloth and the filtrate centrifuged to pellet cells. Cells were resuspended, counted and frozen away at a concentration of 10^6 cells/mL in RPMI 1640, 5% HIFCS, 10% tryptose phosphate broth (TPB), 15% dimethylsulfoxide (DMSO). Subsequent passages of CEF were maintained in RPMI 1640, 5% HIFCS, 10% TPB supplemented with penicillin-streptomycin (100U/mL penicillin G, 100μg/mL streptomycin sulphate) and anti-PPLO (60μg/mL tylocine).

b) Metabolic labeling of intracellular proteins

Cells were first starved of methionine for 1 hour in methionine free medium; Minimal essential medium (MEM), Eagle(modified) with Earle's salts without methionine (Flow Laboratories, Inc., McLean, Va.) for Cos1 cells and RPMI 1640 without methionine (Flow Laboratories) for CEF and CBMC. All media was supplemented with 5% dialyzed HIFCS. Starving medium was replaced with fresh methionine free medium containing L-[^{35}S]Methionine (Amersham Canada Ltd., Oakville, Ont.) at 50μCi/mL. Labeling was for 2-4 hours.

c) Transfections and electroporations

Cos1 cells were transfected by the method of Chen and Okayama (1987). Optimum tranfections were obtained when DNA was added to Cos1 cells 8 hours after splitting a confluent monolayer culture in 100mm dish at a ratio of 1:4. Briefly, 0.5 mL of 2xBBS (50mM N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES), 280mM NaCl, and 1.5 mM

Na_2HPO_4, pH 6.95) was added slowly to 10μg of DNA in 0.5 mL of 0.25M $CaCl_2$. After a 20 minute incubation at room temperature, the calcium phosphate-DNA solution was added dropwise to a 100mm plate of cells containing 10 mL of DMEM supplemented with 7.5% HIFCS. Plates were incubated overnight at 37°C in 3% CO_2 to allow precipitation of calcium phosphate-DNA complexes. The medium was removed, the cells washed twice with medium and then incubated under regular conditions.

CEF were transfected using the polybrene method (Kawai and Nishizawa 1984). CEF were seeded at $8x10^5$ cells/60mm plate 18 hours prior to transfection. Medium was removed and replaced with 1 mL of medium containing 30μg/mL polybrene (Aldrich Chemical Co., Inc., Milwaukee, Wis.), 5μg/mL pREV-T or related DNA and 1μg/mL of pSW253 (pREV-A). Transfected plates were incubated at 37°C, 5% CO_2 for 6 hours with occasional agitation. Transfection medium was then removed and replaced with medium containing 30% (v/v) DMSO. After 4 minutes of DMSO shock, monolayers were washed with medium and then returned to 37°C, 5% CO_2 with fresh medium.

Cos1 cells and CEF were electroporated using the BRL Cell-Porator (Gibco/BRL). Briefly, cells were trypsinized, pelleted to remove trypsin, washed in 40 mL of PBS (137mM NaCl, 8.1 mM Na_2HPO_4, 2.7mM KCl, 1.5mM KH_2PO_4, 0.9mM $CaCl_2$, 0.5mM $MgCl_2$) and then repelleted. Cells were resuspended in PBS at a concentration of $4x10^6$ cells/mL. One mL of cells was placed in each electroporation chamber, and DNA (usually 10μg) was added and mixed by inversion. Cells were electroporated at 300 volts (750 volts/cm) at a capacitance of 80μFarads. Cells were removed from the chamber and added to a 60mm plate containing 9 mL of the

appropriate growth medium. Plates were fluid changed following an overnight incubation at 37°C in 5% CO_2.

d) Virus production and titration

All REV-T and related viruses were recovered from CEF following electroporation or polybrene transfection of CEF. Transfected cells were fluid changed on day 5 or 6 following transfection and virus was harvested 24 hours later.

Titers of virus were determined by immunofluorescence assay (described later) of infected cells at 24-36 hours post infection.

e) *In vitro* transformations

Preparation of spleen cells and *in vitro* transformations were performed essentially according to Hoelzer *et al.* (1980) Spleens were aseptically removed from 1-3 week old leghorn chicks (Animal Disease Research Institute, Agriculture Canada, Ottawa) and bathed in RPMI 1640, 1% glutamine, 2% HIFCS. A pestle was used to force spleens through the steel mesh of a tea strainer while washing with the above medium. The resulting cellular suspension was passed through several layers of cheese cloth to remove aggregates. The filtrate containing spleen cells was pelleted at 400 x g for 5 minutes and cells were resuspended in the above medium at a concentration of 5 x 10^8 lymphoid cells/mL. Spleen cells (5 x 10^7) were infected with 2 mL of virus in the presence of 2 μg/mL polybrene for 1 hour at 37°C. After infection, spleen cells were pelleted at 400 x g for 5 minutes and the supernatant fluid removed. Infected cells were resuspended in 5 mL of agar (RPMI 1640, 15% HIFCS, 1% beef embryo extract, 1% heat inactivated chicken serum, 100U/mL penicillin G, 100μg/mL streptomycin sulphate, 60μg/mL tylocine and 0.3% noble agar)

and poured on to 60mm tissue culture plates. Transformed cells which are able to form colonies in the agar were scored 7-10 days after infection.

f) Extraction of RNA and Hirt DNA from Cos1 cells.

One plate of transfected Cos1 cells was washed twice with PBS and then scraped into PBS. Cells were pelleted then resuspended in 1 mL of TNE (10mM Tris pH 8.0, 100mM NaCl, 1mM EDTA), 1% NP-40 at 4°C and transferred into a microcentrifuge tube. After lysis for 15 minutes at 4°C, nuclei were pelleted by spinning for 1 minute in a microcentrifuge (IEC micro-MB microcentrifuge). The supernatant was made 50mM EDTA, 400μg/mL proteinase K and 1% sodium dodecyl sulphate (SDS) and incubated at 37°C for 30 minutes. This mixture was extracted two times with phenol:chloroform:isoamyl alcohol (25:24:1) and two times with chloroform:isoamyl alcohol (24:1). The extracted sample was made 0.3M sodium acetate and precipitated with 2.5 volumes of ethanol to precipitate the RNA. *In vitro* translation of RNA was done using a rabbit reticulocyte lysate kit (Promega Biotech, Madison, Wis.) following directions provided by the manufacturer.

Hirt DNA was extracted from nuclei using a modification of the Hirt method (Hirt 1967). One mL of lysis buffer (10mM Tris pH 7.5, 10mM EDTA and 0.6% SDS) was added to the nuclei pelleted in the procedure described above. After an incubation at room temperature for 20 minutes, the solution was made 1M NaCl and incubated overnight at 4°C. Following microcentrifugation for 15 minutes at 4°C, the supernatant was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). Hirt DNA was precipitated by the addition of 2.5 volumes of ethanol. The sample was treated with

ribonuclease A (RNase A)(100μg/mL) at 37°C for 30 minutes to degrade RNA.

2. ELECTROPHORESIS

a) SDS polyacrylamide gel electrophoresis

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)(Laemmli 1970) on 10% slab gels using the Bio-Rad Protean II or mini-Protean II systems (Bio-Rad Laboratories, Rockville Centre, N.Y.). Gels were made with a 10% running gel (10% acrylamide, 0.13% N'N'-Bis-methylene-acrylamide (Bis), 0.1% SDS, 0.75M Tris pH 8.8) and 5% stacking gel (5% acrylamide, 0.07% Bis, 0.1% SDS, 63mM Tris pH 6.8) and were electrophoresed in 200mM glycine, 25mM Tris and 0.1% SDS. Prior to electrophoresis, protein samples were denatured by heating at 95°C for 5-10 minutes in SDS-PAGE sample buffer (62.5mM Tris pH 6.8, 4% SDS, 0.4% dithiothreitol (DTT), 0.02% Bromophenol blue, 2% glycerol) used directly or diluted twofold. Gels were electrophoresed for approximately 1100 volt-hours (Bio-Rad Protean II) or at 200 volts (Bio-Rad Mini-Protean II) until the bromophenol blue reached the bottom of the gel.

b) Electrophoresis of DNA

Electrophoresis of DNA was on agarose or polyacrylamide gels as described (Maniatis *et al.* 1982). Briefly, DNA was electrophoresed in horizontal 0.6-1.5% agarose gels buffered with TBE (90mM Tris, 90mM boric acid, 2.5mM EDTA). Resolution of fragments smaller than 300 base pairs was attained using vertical 8% polyacrylamide gels buffered with TBE. Oligonucleotides were separated on 20% polyacrylamide gels.

c) Electrophoresis of RNA

Prior to electrophoresis, RNA was denatured by incubating at 60°C for 15 minutes in 10mM sodium phosphate pH 7.0 (6.1mM Na_2HPO_4, 3.9mM

NaH_2PO_4), 0.9M (6%) glyoxal and 50% DMSO (McMaster and Carmichael 1977). DNA fragments, used as markers, were also denatured by this method.

Denatured RNA was electrophoresed on 1.5% agarose gels buffered with 10mM sodium phosphate pH 7.0. Buffer was recirculated from anode to cathode to maintain proper pH throughout the gel during electrophoresis.

3. MOLECULAR CLONING AND RELATED TECHNIQUES

Maintenance of bacterial strains, general techniques for the handling and manipulation of DNA and for the preparation of necessary media and reagents are thoroughly discussed by Maniatis *et al.* (1982). It is assumed that these methods were used unless otherwise specified.

Medium speed and ultracentrifugations were done in Beckman J2-21 or L8-55M centrifuges (Beckman Instruments (Canada) Inc., Mississauga, Ont.), respectively, using the rotors indicated.

E. coli strains RR1, HB101 and JM101 were kindly provided by Dr. L. Visentin, NRC Canada, Ottawa. The following list indicates the source of all plasmids required for this work: pBR 322, pUC18 and 19 (kindly provided by Dr. L. Visentin), M13mp10 (kindly provided by Dr. K. Dimmock, University of Ottawa), M13mp19 (New England BioLabs, Beverly, Mass.), pJC119 (kindly provided by Dr. R. Lazzarini, NIH, Bethesda, MD), pSVL and pCH110 (Pharmacia (Canada) Ltd., Dorval, Que.), pSVL-F (kindly provided by M.-J. Côté, University of Ottawa) and pREV-T Hp/S 3' (kindly provided by Dr.H.M. Temin, University of Wisconsin, Madison, WI).

a) Preparation of Competent *E. coli*

Bacteria were rendered competent for the uptake of plasmid DNA by treatment with $CaCl_2$. First, 10 mL of an overnight culture of bacteria

grown in 2YT (1.6% tryptone, 1% yeast extract, 0.5% NaCl) without shaking were inoculated into 100 mL of P-medium (15.9mM K_2HPO_4, 6.3mM KH_2PO_4, 15mM $(NH_4)_2SO_4$, 10mM $MgSO_4$, 1.8μM $FeSO_4$, 1% Casamino acids and 0.25% glucose) and cells were grown to an OD_{600} of 0.4. as measured in a Beckman Du-8B spectrophotometer. Bacteria were pelleted at 6,000 x g for 10 minutes in a JA-10 rotor. After washing in 100 mL of 10mM NaCl at 4°C, cells were repelleted. Bacteria were resuspended in 100 mL of 50mM $CaCl_2$ and incubated at 4°C for 15 minutes. Finally, bacteria were pelleted and resuspended in 10 mL of 50mM $CaCl_2$, 16% (v/v) glycerol, aliquotted and quickly frozen in a dry ice/ethanol bath before being stored at -80°C. The transformation efficiency of the resulting cells was 5 x 10^6 to 5 x 10^7 colonies/μg of plasmid DNA.

b) Transformation of *E. coli*

Transformations were performed essentially according to the method of Hanahan (1983) with several modifications. Competent *E. coli* were thawed slowly on ice. DNA in no more than 10μL was mixed with 200μL of competent cells in 17mm x 100mm polypropylene tubes and incubated at 4°C for 30 minutes. The mixture was heated in a 42°C water bath for 90 seconds and then returned to ice for several minutes. 800μL of LB (1% bacto-tryptone, 0.5% yeast extract, 1% NaCl) broth was added and tubes agitated (200 rpm) at 37°C for 1 hour. Cells were then spread onto LB plates containing ampicillin (50μg/mL) and incubated overnight at 37°C.

Alternatively, when low efficiency transformations were acceptable, the method of Golub was used for the quick introduction of plasmid into *E. coli* (Golub 1988).

c) Purification of Plasmid DNA

Large scale purification of plasmid DNA (maxipreparation) was done essentially according to the method of Birnboim and Doly (1979) as modified and presented by Maniatis *et al.* (1982). Several modifications were made to adapt the protocol to available rotors. Bacteria were pelleted at 6,000 x g in a JA-10 rotor. The bacterial pellet from 500 mL of culture was resuspended in 7.5 mL of solution 1 (50mM glucose, 25mM Tris pH 8.0, 10mM EDTA, 5mg/mL lysozyme). 15 mL of solution 2 (0.2M NaOH, 1% SDS) and finally 12 mL of 5M potassium acetate (pH 4.8) were added before centrifugation at 72,000 x g in a Beckman SW28 rotor for 20 minutes. Pelleting of DNA following isopropanol precipitation was at 12,100 x g for 30 minutes in a JA-20 rotor. Samples prepared for cesium chloride gradients were precleared of precipitated proteins by centrifuging at 12,100 x g for 10 minutes prior to centrifugation to equilibrium in a VTi 65 rotor at 290,000 x g for at least 10 hours.

Small scale (minipreparations) purification of plasmid DNA for screening of recombinant plasmids was an adaptation of the above method as described by Maniatis *et al* (1982). The procedure was modified to include a chloroform:isoamyl alcohol (24:1) extraction following the recommended phenol:chloroform:isoamyl alcohol (25:24:1) extraction.

d) Purification of DNA from gels

DNA gels were stained briefly with ethidium bromide (0.5μg/mL) and observed with long wave ultraviolet light to permit precise excision of DNA fragments for purification. Gel slices were electroeluted using the IBI Model UEA electroeluter (International Biotechnologies Canada Inc., Toronto, Ont.) at 100 volts for 45 minutes. Eluted DNA was extracted

with butanol saturated with H_2O to remove ethidium bromide and was then precipitated with 2.5 volumes of ethanol.

Oligonucleotides separated on polyacrylamide gels were visualized by ultraviolet shadow casting. Slices of polyacrylamide were transferred into elution buffer (0.5M ammonium acetate, 1mM EDTA, pH 8.0) and incubated overnight at 37°C. Gel slices were pelleted and elution buffer removed. Gel slices were washed one more time with elution buffer. Combined buffers were precipitated with 2.5 volumes of ethanol using 20μg of glycogen (Boehringer Mannheim, Mannheim, West Germany) as carrier.

e) Manipulation and modification of DNA

All DNA modifying enzymes were purchased from Pharmacia, New England Biolabs or Boehringer Mannheim. All methods were performed essentially according to techniques described by Maniatis *et al.* (1982). Only those techniques which have been modified are discussed below.

Generally, DNA was precipitated at 4°C for 15 minutes prior to microcentrifugation for 15 minutes at 4°C. Glycogen (20μg) was added as a carrier for the precipitation of small quantities of DNA or for the precipitation of oligonucleotides or linkers.

Restriction enzyme digestions were performed at 37°C for 1-2 hours using buffers recommended by the manufacturer. Partial digests were performed by reducing the length of digestion usually to less than 15 minutes. Partial digests of DNA with restriction enzymes which cut frequently were controlled using ethidium bromide (Parker *et al.* 1977). Briefly, restriction enzyme digestions were performed in the presence of ethidium bromide to partially inhibit cutting. Suitable concentrations of ethidium bromide, usually 10-100 μg/mL, were determined empirically for each experiment to provide adequate control over the reaction.

5' terminal phosphates were removed with calf intestinal phosphatase (CIP). 1 μg of DNA in restriction enzyme digestion buffer was treated with 2-5 unit of CIP for 15 minutes at 37°C and then continued for 15 minutes at 56°C. Fresh enzyme was added and the treatment repeated. CIP was inactivated by heating at 65°C for 10 minutes after adjusting the reaction buffer to 20mM EDTA, 0.5% SDS.

Ligations were performed in ligation buffer (50 mM Tris pH 7.6, 10 mM $MgCl_2$, 1mM DTT, 1mM ATP and 5% polyethylene glycol (PEG) 8000). Generally, ligations containing up to 200ng of vector DNA and up to a 10 fold molar excess of insert fragment were performed in 20μL of ligation buffer for 4 hours at room temperature.

Klenow treatment of insert or vector DNA (0.1-1μg) to generate blunt ends for subsequent ligation was done in 10μL of ligation buffer without PEG with 100 μM of each dNTP and 1U of Klenow enzyme at 37°C for 30 minutes. Klenow enzyme was heat inactivated at 65°C for 5 minutes prior to the addition of ligase in 10μL of ligation buffer.

f) Nick Translation of DNA

Plasmid or purified fragment DNA was nick translated as described (Maniatis *et al.* 1982). Reactions were performed not in the nick translation buffer described, but in ligation buffer (without PEG) containing 2μCi/μL or 2.5μM α-[^{32}P]dATP (800Ci/mmol, Amersham), 0.1mM dCTP, dGTP and dTTP. Reactions were terminated by the addition of EDTA to a final concentration of 25 mM. Labeled DNA was separated from unincorporated label on spin columns (Maniatis *et al.* 1982).

g) Colony hybridizations

Bacterial colonies were grown directly on Colony/plaque Screen (NEN, DuPont Canada, Inc., Lachine Que.) membranes overlayed on LB plates

containing ampicillin. Bacteria were lysed and fixed to the membrane following instructions provided by the manufacturer.

Membranes containing adherent DNA were prehybridized for a minimum of 6 hours in 1M NaCl, 1% SDS and 10% dextran sulphate at 65°C. Denatured probe (10ng/mL final concentration) and calf thymus DNA (100μg/mL final concentration) were added and hybridization allowed to proceed at 65°C for at least 12 hours. Membranes were washed twice at room temperature for 5 minutes with 2 x SSC, twice at 65°C for 30 minutes in 2 x SSC (0.3M NaCl, 30mM Na_3citrate) 1% SDS and finally twice at room temperature in 0.1 x SSC for 30 minutes. Membranes were thoroughly dried then exposed to Cronex 4L film (Picker International Canada Inc., Ottawa, Ont.) for several hours, as required.

h) Southern blots

DNA was transferred from agarose gels to Genescreen (NEN) by electroblotting (BioRad Transblot apparatus). Prior to transfer, DNA was denatured in the gel by incubating in 0.2N NaOH, 0.6M NaCl for 30 minutes at room temperature. After rinsing 3 times in H_2O, gels were neutralized in 3 changes of blotting buffer, 25mM sodium phosphate pH 6.5 (17.1mM NaH_2PO_4, 7.9mM Na_2HPO_4), at room temperature for 1 hour. Genescreen membranes were presoaked for 20 minutes prior to treatment. After blotting, membranes were dried and then baked under vacuum at 80°C for 3 hours. Hybridization was as described for colony hybridizations.

i) Northern blots

Glyoxal denatured RNA was transferred from agarose gels to Genescreen membrane using the BioRad Electroblot apparatus. Gels were not pretreated prior to transfer. Genescreen membranes were treated as

described for Southern blots. Hybridizations were also performed in the same manner as described for colony hybridizations.

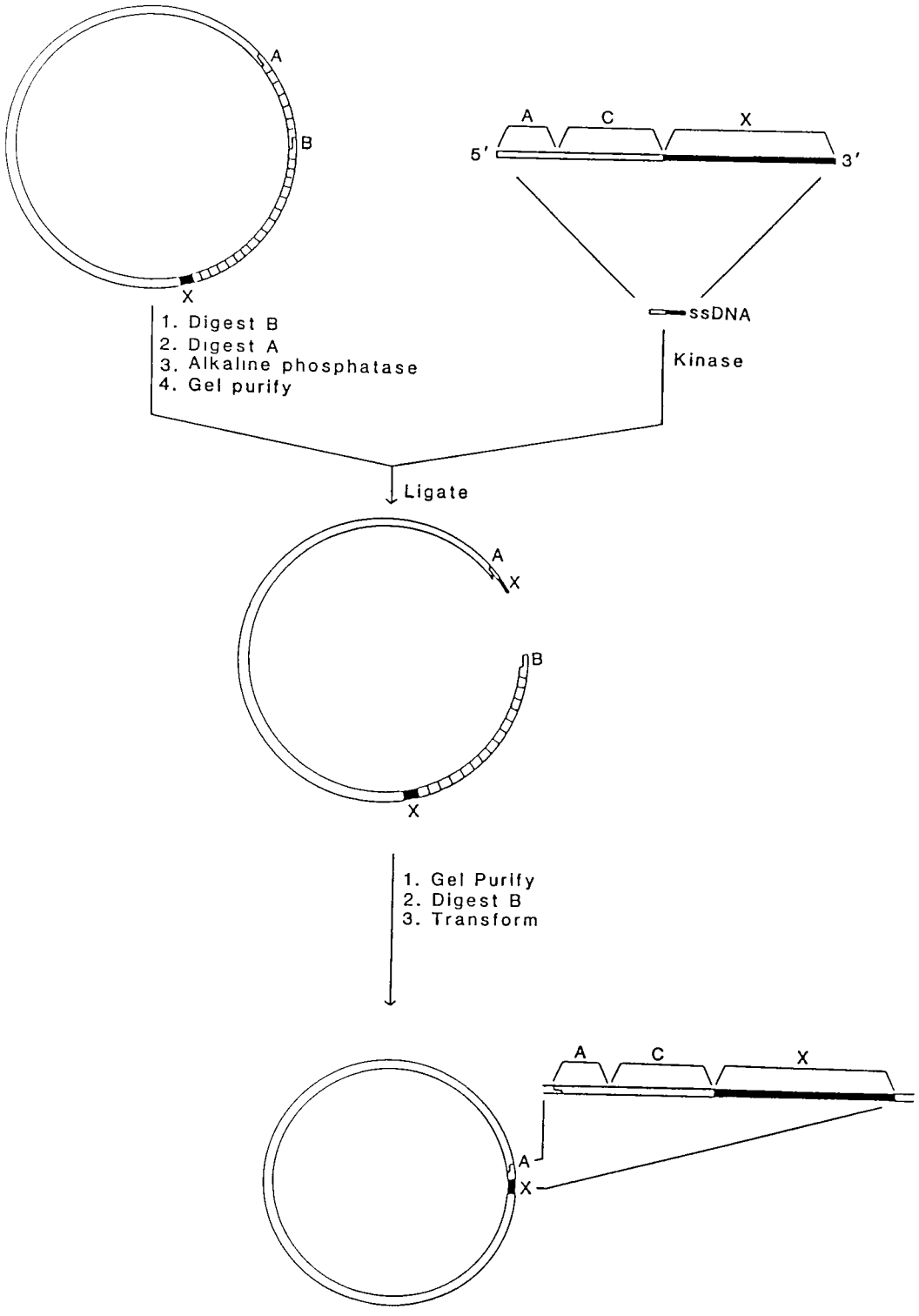
j) Crossover linker mutagenesis

Crossover linker mutagenesis (Sung *et al.* 1986) was performed using either single or double stranded oligonucleotides. Presented below are the general techniques used for crossover linker mutagenesis, while a description of the specific deletions will be discussed in the results section.

For single stranded oligonucleotides (Fig. 3), plasmid DNA was cleaved with two unique restriction enzymes (A+B). The crossover oligonucleotide was designed to have 5' sequences capable of annealing to the 3' overhang created by the first restriction enzyme (A), followed by sequence (C) designed to introduce restriction sites or add new sequences adjacent to the deletion. Finally, the 12 bases at the 3' end of the oligonucleotide (X) were homologous to the sequence in the gene immediately adjacent to the sequences desired to be deleted. After treatment of 1μg of the restriction cut plasmid DNA with alkaline phosphatase, a 500-1000 molar excess of the gel purified, kinased oligonucleotide was added and ligated overnight at 16°C. The T4 polynucleotide kinase was inactivated by heating at 65°C for 10 minutes prior to ligation. The ligation mix was separated on 0.7% agarose gels and the linear plasmid band electroeluted. After purification, the eluted DNA was recut with the enzyme "B" and then transformed into competent *E. coli* RR1. Colonies were screened by minipreparations to identify clones containing the desired deletion.

When double stranded oligonucleotides were used for mutagenesis, in addition to an oligonucleotide with the features described above, a second

Figure 3. Crossover linker mutagenesis: single stranded oligonucleotides.
The sequence of the oligonucleotide has been defined into 3 regions: The region "A" is homologous to the sequence of the 5' overhang created by restriction enzyme "A", the region "C" represents optional sequence which can be introduced into the oligonucleotide to introduce new restriction enzyme sites, initiation codons or stop codons, and the region X is homologous to the DNA immediately adjacent to the sequence to be deleted and provides the homology required for the "crossover" event to occur.


A
B
X
1. Digest B
2. Digest A
3. Alkaline phosphatase
4. Gel purify
A
C
X
5′
3′
ssDNA
Kinase
Ligate
A
X
B
X
1. Gel Purify
2. Digest B
3. Transform
A
C
X
A
X

shorter homologous oligonucleotide was used (Fig. 4). Oligonucleotides were prepared by gel purification, and kinasing of the first oligonucleotide. The second oligonucleotide was added to the first during heat inactivation of the kinase enzyme at 65°C, and allowed to anneal during cooling. Plasmid DNA cleaved at the restriction site "A" was ligated with a 500 molar excess of prepared oligonucleotides. The ligation mix was run on a 0.7% agarose gel and the linear band eluted and purified. After cleavage with enzyme "B", the DNA was transformed into *E. coli* RR1. Clones containing the deletion were identified after restriction enzyme analysis of minipreparations of the plasmid DNA.

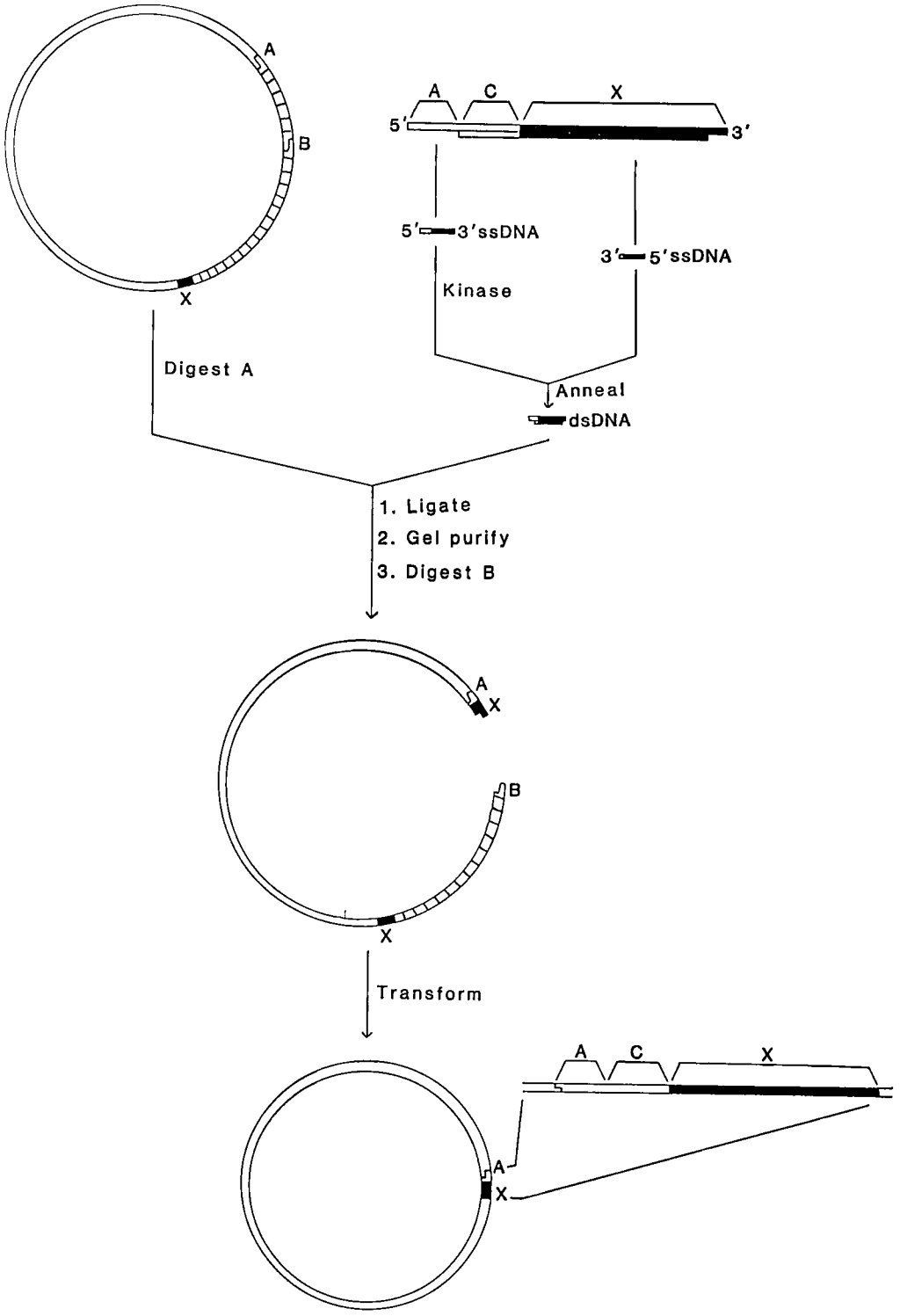
k) DNA Sequencing

Clones were sequenced by the dideoxy method (Sanger *et al.* 1977), as described below, after subcloning into M13 vectors mp10 or mp19 or directly using Sequenase (United States Biochemical Corporation, Cleveland, Ohio) following the protocol recommended by the supplier.

The replicative form of M13mp vectors was purified using the same method as for the large scale plasmid preparation following overnight culture of infected JM101 in 2YT. Subcloning into M13mp vectors utilized similar techniques. Techniques for the transformation of JM101 and the identification of recombinant plaques are described by Messing (1983). Orientations of clones were determined by the C-test (Messing, 1983) or by analysis of replicative form DNAs following restriction enzyme analysis of DNA purified using the standard plasmid minipreparation method.

Purification of single stranded phage DNA was from 9-12 hour cultures of M13 plaques inoculated directly into 5 mL of 2YT. Medium was centrifuged at 12,100 x g for 10 minutes to pellet bacterial cells. Supernatants were made 4% in PEG, 250mM NaCl. Phage was precipitated

Figure 4. Crossover linker mutagenesis: double stranded oligonucleotides.
The sequence of the oligonucleotide has been defined into 3 regions: The region "A" is homologous to the sequence of the 5' overhang created by restriction enzyme "A", the region "C" represents optional sequence which can be introduced into the oligonucleotide to introduce new restriction enzyme sites, initiation codons or stop codons, and the region X is homologous to the DNA immediately adjacent to the sequence to be deleted and provides the homology required for the "crossover" event to occur.


A
B
X
A
C
X
5'
3'
5'
3'ssDNA
3'
5'ssDNA
Kinase
Digest A
Anneal
dsDNA
1. Ligate
2. Gel purify
3. Digest B
A
X
B
X
Transform
A
C
X
A
X

at 4°C for 1 hour then collected by centrifugation at 12,100 x g for 30 minutes. Pellets were dissolved in 0.4 mL of TE (10mM Tris pH 8.0, 1mM EDTA), extracted once with phenol, twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). One half volume of ammonium acetate was added before precipitation with ethanol. Samples were reprecipitated before use in sequencing reactions.

1 μg of M13 template and 20 ng of sequencing primer were annealed in 9 μL of 10mM Tris pH 8.0, 10mM $MgCl_2$ by denaturing at 90°C for 2 minutes, incubating at 55°C for 10 minutes and then allowing the mixture to cool slowly to 42°C. Approximately 1/4 of the annealed primer-template was incubated in each of 4 different reaction mixes. All reaction mixes were 7mM Tris pH8.0, 5mM $MgCl_2$ and 3.2μM α-[^{35}S]-dATP (400Ci/mmol, Amersham) and 0.1U/μL of Klenow enzyme. More specifically, A reaction mix was 12μM ddATP, 25μM dCTP, 25μM dGTP, and 25μM dTTP, C reaction mix was 40μM ddCTP, 3.4μM dCTP, 35μM dGTP and 35μM dTTP, G reaction mix was 200μM ddGTP, 3.4μM dGTP, 35μM dCTP and 35μM dTTP and finally T reaction mix was 600μM ddTTP, 3.4μM dTTP, 35μM dCTP and 35μM dGTP. Reactions proceeded at 42°C for 20 minutes. The sequencing reactions were chased by the addition of each of the 4 dNTPs to a final concentration of 0.3mM followed by a further 20 minute incubation at 42°C. All reactions were terminated by the addition of an equal volume of sample buffer (10mM EDTA, 90% formamide, 0.3% bromophenol blue, 0.3% xylene cyanol, 0.3% orange G).

Sequencing reactions were electrophoresed using the IBI model STS 45 Thermoplate Sequencing Apparatus on 0.2mm 6% polyacrylamide gels (5.7% acrylamide, 0.3% bis, 8M urea in 1 x TBE). Electrophoresis was at 50 watts constant power until the xylene cyanol reached the bottom of the

gel (for sequences greater than 180 nucleotides from the primer) or until the bromophenol blue reached the bottom of the gel (sequences less than 180 nucleotides from the primer). After electrophoresis, gels were lifted onto Whatman 3MM paper and dried in a Bio-Rad model 583 gel drier. Sequences were read from Cronex 4 film after an overnight exposure.

l) Expression and Purification of a v-*rel/lacZ* Hybrid Protein

An overnight culture of *E. coli* (JM101) transformed by plasmid pLR-PE was used to inoculate 4 litres of 2YT containing 50μg/mL of ampicillin and 120μg/mL of isopropyl β-D-thio-galactoside (IPTG). Cultures were grown for 4 hours with agitation at 37°C. Bacterial pellets were recovered by centrifugation at 6,000 x g for 10 minutes at 4°C and then resuspended in 20 mL of ice cold French press buffer (50mM Tris pH 8.0, 0.5mM EDTA, 200μg/mL DNase 1, 2mg/mL lysozyme). After a 10 minute incubation at 4°C, the cell suspension was French pressed 3 times at 15,000 psi.(1 inch diameter cell, French Pressure Cell Press, American Instrument Company). Precooling of the French Press chamber at 4°C and collection of lysed samples on ice prevented excessive heating of the lysates. Lysates were adjusted to 0.4M NaCl and 5mM $MgCl_2$ and incubated at 4°C for 30 minutes. After 30 minutes, samples were centrifuged for 20 minutes at 11,000 x g at 4°C. The resulting pellets consisted of two distinct layers; the upper layer was white in colour and was removed and discarded by vigourous washing by pipette with pellet wash buffer (10mM Tris pH8.0, 1 mM EDTA); the bottom layer was brown in colour and was resuspended in 200μL of SDS-PAGE sample buffer. Samples were stored at 4°C. Prior to electrophoresis samples were heated at 95°C for 5 minutes and centrifuged for 5 minutes in a microcentrifuge to remove debris.

Preparative electophoresis was performed using the Bio-Rad Protean 2 Dual Slab gel system. Samples were separated by SDS-PAGE using 1.5mm thick 10% polyacrylamide gels and preparative wells. Electrophoresis was carried out at a maximum of 40mA per gel or for a total of 1100 volt x hours. After electrophoresis, gels were rinsed briefly in distilled water at 4°C and then stained in 0.25M KCl at 4°C for approximately 5 minutes (Hager and Burgess 1980). When necessary, background was reduced by washing gels in distilled water at 4°C. Appropriate protein bands were excised from the gel, and soaked in distilled water at 37°C for 15-30 minutes or until no further staining could be seen. Protein was electroluted from the gel (Jacobs and Clad 1986) using the Elutrap (Schleicher and Schuel, Keene, New Hampshire) electroeluter following the manufacturer's directions. Electroelution was done at 200 volts for 4 hours with cooling or at 100 volts for at least 8 hours. The same apparatus was used for electroconcentration of protein samples.

The electroeluted protein was dialyzed in Sartorius SM 132 00 collodion bags (Canlab, Pointe Claire, Que.) for 24 hours against several changes of TNE buffer. Protein was precipitated by the addition of 5 volumes of acetone and overnight incubation at -20°C. Protein was pelleted for 30 minutes at 4°C in a microcentrifuge (Eppendorf centrifuge 5414 or 5415). Pellets were air dried and then resuspended in PBS. Approximate quantities of protein were determined by visual comparison of the intensity of Coomassie blue staining of the v-*rel/lacZ* protein with known quantities of molecular weight standard proteins after SDS-PAGE .

V-*rel/lacZ* fusion protein was labeled with ^{125}I using Bolton Hunter reagent [^{125}I] kit (ICN Biomedicals Canada, Inc., Montreal, Quebec)(Bolton and Hunter 1973). Approximately 20 μg of protein was applied to a G-25

Sephadex column prepared in H_2O. Fractions containing the protein as determined by spectrophotometry were concentrated by lyophilization in a Savant Speed Vac Concentrator. The concentrated sample was reapplied to a second G-25 column prepared in H_2O, and the fractions containing the protein pooled and lyophilized. Protein was solubilized in 0.05M sodium borate pH 8.5. Labeling was performed following all precautions suggested by the manufacturer. Briefly, benzene containing the Bolton-Hunter reagent (1mCi) was evaporated under a stream of nitrogen. Upon drying, 20μg of protein (1μg/μL) in sodium borate buffer was added and allowed to react for one hour at 4°C. Labeling was terminated by the addition of 100μg of glycine (1mg/mL) followed by a 15 minute incubation at 4°C. Labeled protein was separated from unincorporated Bolton-Hunter reagent on a Sephadex G-10 column prepared in H_2O. The approximate specific activity of the purified protein was 1.5 x 10^7 cpm/μg as measured on a Beckman Gamma 5500 counter.

4. PREPARATION OF ANTI-*REL* ANTIBODIES

a) Immunization with synthetic peptides.

Synthetic peptides (commercially prepared by Drs. A. Huang and J.D. Young, Division of Infectious Diseases, Children's Hospital, Harvard Medical School) were first covalently linked to the protein carrier, Keyhole limpet haemocyanin (KLH, Calbiochem, La Jolla, California) by the glutaraldehyde method (Richardson *et al.* 1985). KLH (18mg/mL in saturated ammonium sulphate) was diluted 10 fold in PBS and dialyzed for 48hrs at 4°C with PBS. Two mL of O.65% glutaraldehyde in PBS was added dropwise to 3 mL of PBS containing 5mg of dialyzed KLH and 5mg of synthetic peptide. The reaction was allowed to proceed for 1hr at room temperature with rotation and was terminated by the addition of 1 mmol

(0.18g) of L-lysine. Rabbits were immunized with 1mg of peptide-KLH in PBS mixed with an equal volume of Freund's complete adjuvant. Antigen was administered subcutaneously at five sites on the back of 4-5 month old male white New Zealand rabbits (Richardson *et al.* 1985). Subsequent boosts were performed at 3 week intervals as described above but antigen was emulsified in Freund's incomplete adjuvant. Sera obtained at 10 days following immunizations were monitored for ability to react with the v-*rel* protein in Western blot assays.

b) Immunization with v-*rel/lacZ* fusion proteins

Gel purified v-*rel/lacZ* protein (20-40 μg) in PBS was emulsified in an equal volume of Freund's complete adjuvant for primary immunization or Freund's incomplete adjuvant for subsequent immunization. Antigen was administered intramuscularly in the leg and boosts were performed at 3 week intervals using alternating legs for immunization. Sera collected 10 days following immunizations were monitored for their ability immunoprecipitate (described below) ^{125}I-v-*rel/lacZ* protein .

c) Collection of sera

All rabbits were bled from the ear immediately prior to primary immunization and on the tenth day following primary or subsequent immunizations. Total bleeds of the rabbits at the completion of immunization were by heart puncture after sedation of rabbits with Innovar-Vet (O.22 mL/kg). Sacrifice of the rabbit was ensured by cervical dislocation after collection of blood. Sedation, heart puncture and cervical dislocation were performed by trained animal care technicians.

Whole blood was incubated at 37°C to allow complete clotting and spun at 400 x g for 10 minutes to remove cells and clots from the sera. All sera were aliquotted and stored at -80°C.

d) Affinity purification of v-*rel* antibodies

Initially, serum was dialyzed against 0.02M Tris pH8.0, 0.028M NaCl, 0.02% NaN_3. Dialyzed serum was then passed through a DEAE Affi-Gel Blue column (BioRad) following the procedure suggested by the manufacturer. The effluent was saved for further purification.

Synthetic peptide was coupled to CNBr-activated Sepharose 4B (Pharmacia) following the method recommended by the supplier. The resulting peptide-Sepharose beads were added to the effluent from the DEAE Affi-Gel Blue column and rotated overnight at 4°C. Beads were then placed into a column and washed extensively with 0.02M Tris pH8.0, 0.028M NaCl. Antibodies were eluted with 0.1M glycine pH2.5 and neutralized with Tris base prior to overnight dialysis against PBS. Dialyzed antibodies were used directly for immunodetection assays.

5. IMMUNOLOGICAL DETECTION

a) Western Blots

Cells were washed with PBS and then lysed directly by the addition of SDS-PAGE sample buffer to the cell monolayers. Lysates were collected directly into microcentrifuge tubes and boiled for 10 minutes prior to electrophoresis. Proteins were separated by 10% SDS-PAGE on the Bio-Rad Mini Protean 2 gel system. Electrophoresis was carried out at 200 volts. Following electrophoresis, proteins were transferred to nitrocellulose membrane using the Bio-rad Mini Trans-blot assembly. Transfer was carried out at 100 volts for 40 minutes in 25mM Tris, 192mM glycine, 20%(v/v) methanol, pH 8.3 (Burnette 1981).

Immunological detection was made using the Bio-Rad Immuno-blot GAR-AP assay kit and all steps were carried out at room temperature with agitation. Briefly, membranes were washed for 10 minutes in TBS_W

(20mM Tris, 500 mM NaCl, pH 7.5) and then blocked in TBS_W containing 3% gelatin for 30 minutes. Two 5 minute washes in TBS_W containing 0.05% (v/v) Tween-20 (TTBS) preceded an overnight incubation with v-*rel* antiserum, 1:500 in TTBS, 1% gelatin. The first antibody solution was removed and after two washes, 5 minutes each in TTBS, the membrane was incubated for 1 hour with goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad), 1:3000 in TTBS, 1% gelatin. Following two washes, 5 minutes each in TTBS and one wash in TBS_W, the membrane was developed in carbonate buffer (0.1M $NaHCO_3$, 1mM $MgCl_2$, pH 9.8) containing 0.30 mg of NBT (p-nitro blue tetrazolium chloride, Bio-Rad) and 0.15 mg of BCIP (5-bromo-4-chloro-3-indolyl phosphate ptoluidine salt, Bio-Rad). The colour reaction was terminated by washing the membrane with distilled water.

b) Dot blot immunoassays

Dot blot immunoassays were performed by direct application of protein samples to nitrocellulose. After drying, immunological detection was made using the Bio-Rad Immuno-blot GAR-AP assay kit as described for Western blotting.

c) Immunoprecipitation

All manipulations were performed at 4°C unless otherwise indicated. CBMC or Cos1 cells, labeled with L-[^{35}S]Methionine were lysed in RIPA buffer (50mM Tris pH 7.2, 150 mM NaCl, 0.1% SDS, 1% tritonX-100, 1% Na deoxycholate) containing 1mM benzamidine HCl and 1mM PMSF (Phenyl methylsulfonylfluoride). CEF were lysed in 20mM Tris pH 7.4, 5mM $MgCl_2$, 0.1M NaCl, 1% NP-40 and 0.5% SDS. Cell lysates were rotated overnight with 5mg of protein A-Sepharose CL-4B (Pharmacia) and 15μL of v-*rel* antiserum. Following overnight incubation, immune complexes bound to the

protein A sepharose beads were washed 5 times with RIPA buffer. Samples were prepared for electrophoresis by adding 30μL of SDS-PAGE sample buffer and heating at 95°C for 5 minutes. Samples were separated on 10% polyacrylamide gels containing SDS. After electrophoresis, gels were fixed in 30% methanol, 10% acetic acid for 30 minutes and then prepared for fluorography with Amplify (Amersham). Gels were dried using a Bio-Rad model 583 gel dryer and exposed to Cronex 4L film at -80°C until proper exposures were obtained.

d) Immunofluorescence assay

CEF were seeded on Flow multitest slides (Flow Laboratories) and then infected 24-36 hours later. Immunofluorescence assays (IFA) were done at 24 hours post infection. Cos1 cells were seeded directly on to the IFA slides following electroporation of expression plasmids. Immunofluorescence assays were performed on Cos1 cells at 36-48 hours post electroporation. IFA slides were washed for 5 minutes in PBS_I (8.1mM Na_2HPO_4, 1.9mM NaH_2PO_4, 137mM NaCl and 2.7mM KCl pH 7.5) prior to fixation in 100% acetone at -20°C for 5 minutes. Slides were dried and then washed for 5 minutes in PBS_I. V-*rel* antiserum, 1:250 in PBS_I, was added to each well of the slides and incubated for 1 hour at 37°C or overnight at 4°C in a humidified chamber. Following incubation with the first antibody, the slides were washed 3 times, 5 minutes each in PBS_I. Donkey anti-rabbit IgG linked with fluorescein (Amersham), 1:20 in PBS_I was added to each well and incubated for 1 hour at 37°C. Slides were then washed 3 times, 5 minutes each in PBS_I. After drying, number 1 coverslips were mounted with glycerol/PBS_I pH 8.0. Samples were viewed using a Leitz Laborlux K Fluorescent microscope. Colour photographs were taken using Scotch 640T 35mm slide film (3M Canada, Toronto).

RESULTS

1. IDENTIFICATION OF THE V-*REL* GENE PRODUCT

In order to identify the v-*rel* protein, specific antiserum was required which would be reactive with the putative v-*rel* gene product. The first approach was to produce antiserum against a synthetic peptide whose sequence was the same as that of a stretch of amino acids encoded by the v-*rel* nucleotide sequence.

a) Production of antiserum to synthetic peptides

The synthetic peptide, NH_2-Asp-Glu-Glu-Asp-Pro-Ser-Gly-Asn-Lys-Ala-Lys-Arg-COOH, which represents amino acid residues 290-301 of the putative *rel* protein (See appendix 1), based on DNA sequence data (Stephens *et al.* 1983, Wilhelmsen *et al.* 1984), was conjugated to KLH prior to immunization of rabbits. The conditions for covalent attachment of the synthetic peptide to KLH were first tested with bovine serum albumin (BSA). A densitometry scan of BSA conjugated with the synthetic peptide and of BSA alone separated by SDS-PAGE and stained with Coomassie blue (Fig. 5) demonstrated that covalent attachment of synthetic peptide had occurred.

After 4 months of immunization with the peptide-KLH conjugate, following the schedule previously outlined in the Materials and Methods section, rabbit serum was tested for reactivity with peptide conjugated to BSA in a dot blot immunoassay (Fig. 6A). Total serum and affinity purified antibodies were able to bind specifically to the peptide-BSA conjugate but not to BSA (Fig. 6A). When used in a Western blot assay, the peptide antiserum detected a protein with an apparent size of 55 kilodaltons (kDa) in REV-T transformed cells (CBMC) but not in

Figure 5. Densitometry analysis of a BSA-peptide conjugate separated on SDS-PAGE.
BSA (1μg) and BSA-peptide conjugate (1μg) were separated on 10% polyacrylamide gels containing SDS and stained with Coomassie blue R-250. The densitometric tracings were obtained using a Beckman Du8 spectrophotometer at 500nm. The dashed line represents the BSA sample and the solid line represents the BSA-peptide conjugate. The indicated molecular weight scale was based on the mobility of BSA and phosporylase B standards.

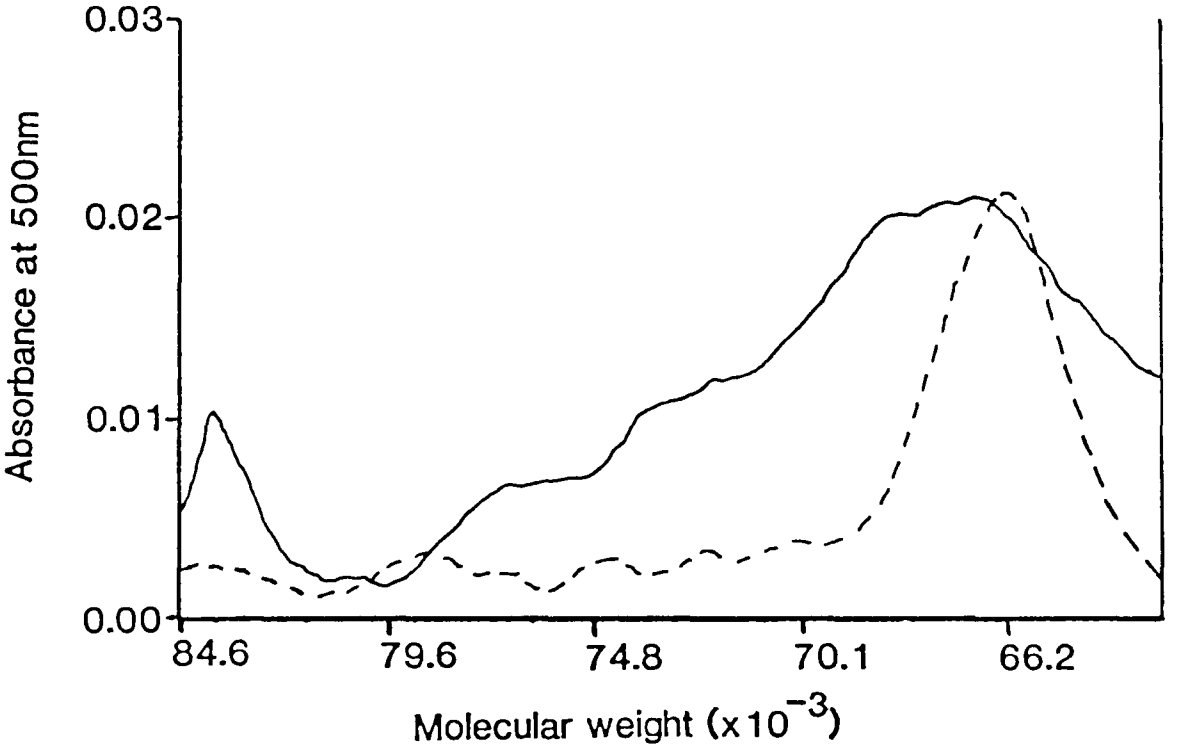
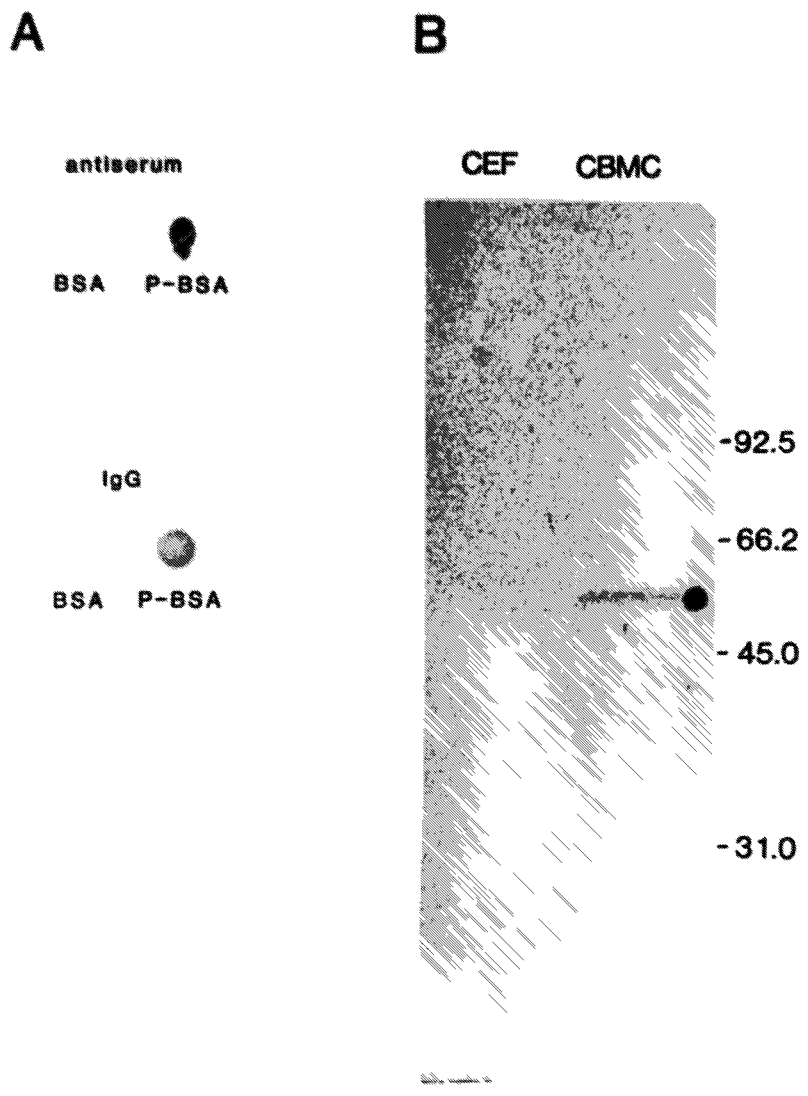

0.03
0.02
0.01
0.00
Absorbance at 500nm
84.6
79.6
74.8
70.1
66.2
Molecular weight (x10^{-3})

Figure 6. Specificity of v-*rel peptide antiserum.*
*A) 1*μg of BSA and 1μg of BSA-peptide (P-BSA) were spotted on nitrocellulose. Immunodetection was with crude peptide antiserum or with affinity purified antibodies using the Bio-Rad Immuno-blot GAR-AP assay kit.
B) Lysates from CEF and REV-T infected CBMC were separated on 12% polyacrylamide gels. Gels were subjected to Western blot analysis using peptide antiserum. A dot indicates the v-*rel* protein product. The position and size (kilodaltons) of standard prestained protein markers are shown. The standard proteins were phosphorylase b (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), and chymotrypsinogen (31.0 kDa).


A
antiserum
BSA
P-BSA
IgG
BSA
P-BSA
B
CEF
CBMC
-92.5
-66.2
-45.0
-31.0

uninfected CEF (Fig. 6B). Preimmune serum did not react with proteins in parallel assays (data not shown). This 55 kDa protein was identified as the gene product of the v-rel oncogene. Attempts to immunoprecipitate the v-*rel* protein from transformed cells were unsuccessful using crude antiserum or affinity purified antibody. Peptide antiserum also failed to react positively in indirect immunofluorescence assays (IFA) with CBMC. The peptide antiserum was only useful for the detection of the v-*rel* protein by Western blot assays.

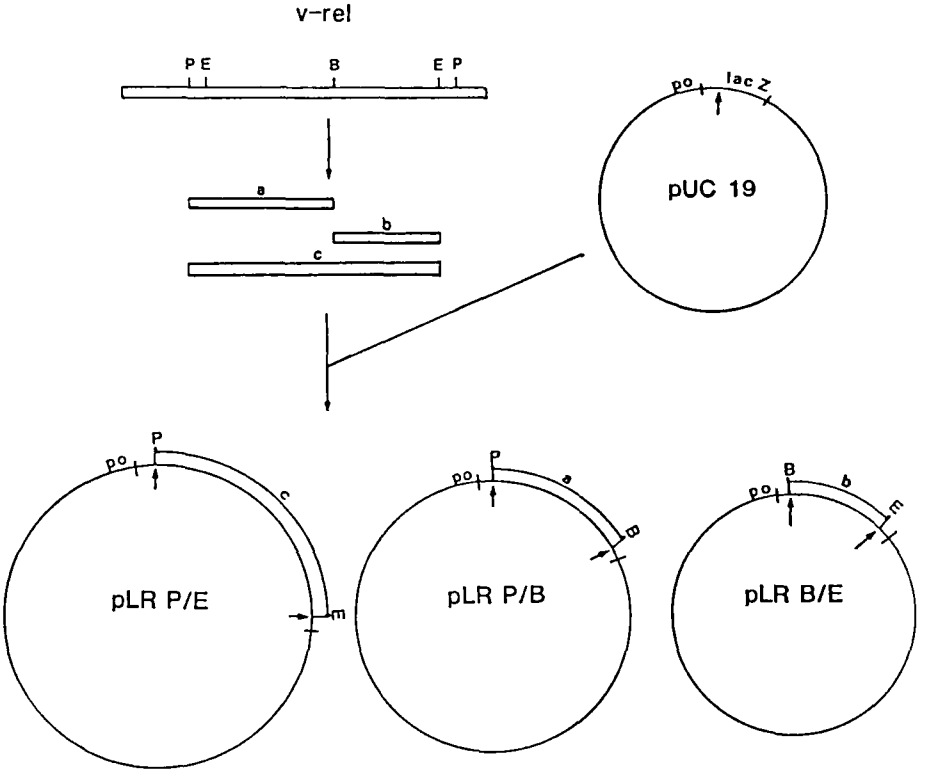
b) Production of antiserum to v-*rel/lacZ* fusion proteins

In order to carry out the characterization of the v-*rel* protein, immunoprecipitating v-*rel* antiserum was needed. Plasmids which expressed v-*rel* sequences fused to sequence from the *lacZ* region of pUC19 were constructed. One of the expressed fusion proteins was used as an antigen for the production of immunoprecipitating v-*rel* antiserum.

i. Construction of expression plasmids

Restriction fragments Pst1(277)-EcoRI(1308), Pst1(277)-BamHI(876) and BamHI(876)-EcoRI(1308) (See appendix 1 for the position of restriction sites) were purified from pSVL-REL (described later). The Pst1-EcoRI fragment was isolated in several steps due to the requirement for incomplete EcoRI digestion. Partial EcoRI digestion of pSVL-REL permitted the purification of an EcoRI fragment containing vector sequence and 5' v-*rel* sequence up to the EcoRI site at position 1308. Complete Pst1 digestion of this fragment permitted the isolation and purification of the Pst1-EcoRI DNA fragment. The Pst1-BamHI and BamHI-EcoRI fragments were purified directly following complete digestion of pSVL-REL with the appropriate enzymes. The purified fragments were cloned into the multiple cloning site of pUC19 (Fig. 7) to generate the

Figure 7. Construction of *E. coli* expression plasmids.
The Pst1-EcoRI (c), BamHI-EcoRI (b) and Pst1-BamHI (a) fragments of v-*rel* were purified and ligated into the multiple cloning site of pUC19. Inserts were in frame with *lacZ*.


v-rel
P E
B
E P
a
b
c
po
lac Z
pUC 19
P
po
c
m
pLR P/E
P
po
a
m
pLR P/B
B
po
b
m
pLR B/E

expression plasmids pLR P/E, pLR P/B and pLR B/E as indicated. *E. coli* transformed by these plasmids were examined directly for the expression of the fusion proteins.

ii. Expression in *E. coli*

Cell lysates of *E. coli* transformed with pLR P/E, pLR P/B, pLR B/E or pUC19 grown in the presence or absence of IPTG were centrifuged, and the proteins in the pellet separated by SDS-PAGE and stained with Coomassie blue (Fig. 8). Cultures harboring plasmids pLR P/E and pLR P/B expressed appropriately sized proteins when induced with IPTG. Detectable expression of novel proteins from pLR B/E and pUC19 was not observed after IPTG induction. The novel protein expressed from pLR P/E was used to prepare specific v-*rel* antiserum as described below.

iii.V-rel/lacZ antiserum

Gel-purified protein expressed from pLR P/E (Fig. 8) was used to immunize rabbits. The resulting antiserum (B-1) was able to react with the v-*rel* protein in immunoprecipitation assays (Figs. 9A and 9C) and in Western blot assays (Fig. 9B). While Western blots (Fig. 9B) and immunoprecipitations of infected CEF lysates (Fig. 9C) revealed one major protein band detected by B-1 serum, several proteins in addition to v-*rel* were present in immunoprecipitations of CBMC lysates (Fig. 9A). In addition, the use of molecular weight markers which were not prestained in Figures 9A and 9C, permitted the apparent molecular weight of the v-*rel* protein to be more accurately determined as 58,000.

The peptide antiserum, useful in Western blots only, and the B-1 or v-*rel*/lacZ antiserum, useful in Western blots and immunoprecipitations, were used for the immunological detection of the v-*rel* and v-*rel* mutant proteins in all subsequent experiments.

Figure 8. Expression of v-*rel/lacZ* fusion proteins in *E. coli*.
E. coli JM101 transformed with pUC19, pLR B/E, pLR P/B or pLR P/E were grown in 2YT broth in the presence (+) or absence (-) of IPTG. Cells were lysed in a French press and total insoluble proteins were run on 10% SDS-PAGE and stained with Coomassie blue. V-*rel/lacZ* fusion proteins are marked with arrows. Electroeluted fusion protein (Ag) is shown. The position and size (kilodaltons) of standard protein markers are shown. The 21.5 kDa protein standard was lactalbumin.

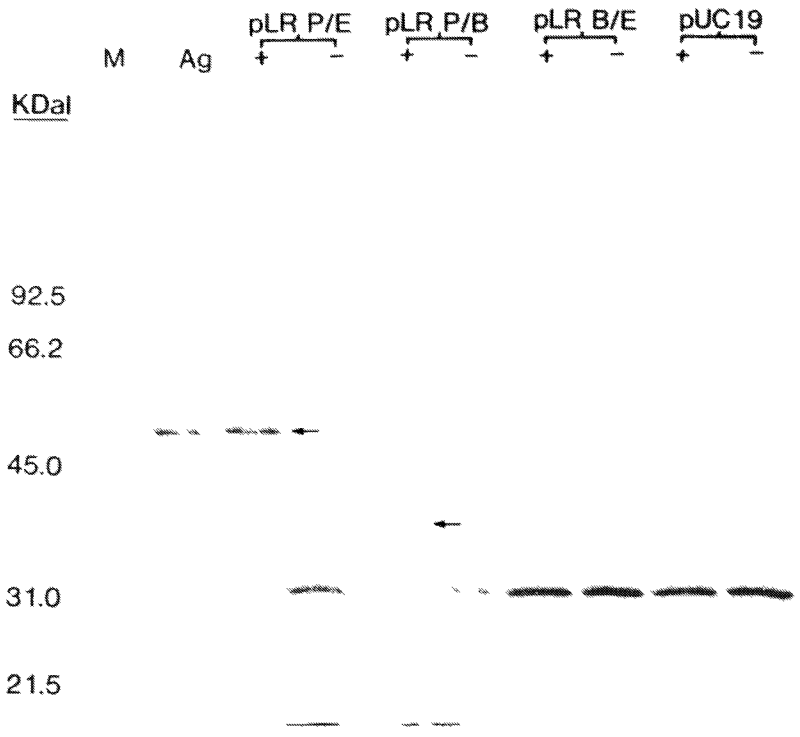
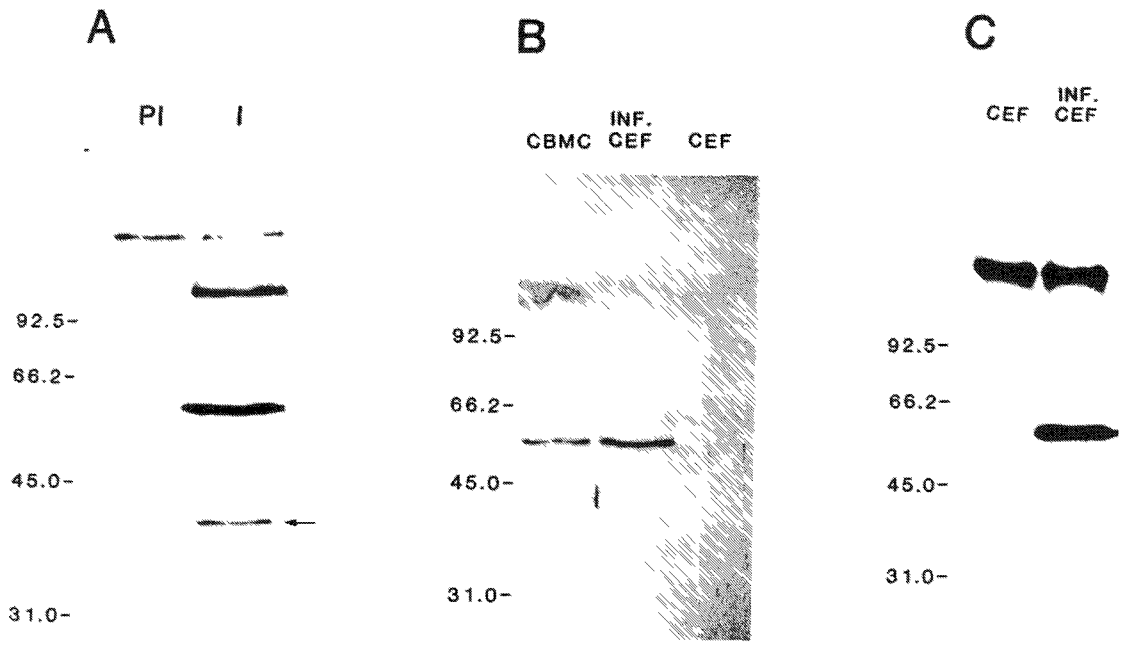

pLR P/E
pLR P/B
pLR B/E
pUC19
M
Ag
+
−
+
−
+
−
+
−
KDal
92.5
66.2
45.0
31.0
21.5

Figure 9. Detection of v-*rel* with v-*rel/lacZ* antiserum.
A) Preimmune serum (PI) or antiserum to the v-*rel/lacZ* fusion protein (I) was used to immunoprecipitate the v-*rel* protein from REV-T transformed CBMC labeled with ^{35}[S]-methionine for 2 hours. Immunoprecipitates were electrophoresed on 10% polyacrylamide gels containing SDS and subjected to fluorography. The position and size (kilodaltons) of standard protein markers are shown. The arrow indicates a 40 kDa protein which co-precipitates with v-*rel*.
B) Lysates of CEF, CEF infected with REV-T (Inf. CEF) and CBMC were separated on 10% SDS-PAGE and subjected to Western blot analysis using antiserum to the v-*rel/lacZ* fusion protein. The position and size (kilodaltons) of standard prestained protein markers are shown.
C) V-*rel-lacZ* antiserum was used to immunoprecipitate lysates from CEF and infected CEF labeled with ^{35}[S]-methionine for 2 hours. Immunoprecipitates were electrophoresed on 10% polyacrylamide gels containing SDS and subjected to fluorography.


A
PI
I
92.5-
66.2-
45.0-
31.0-
B
CBMC
INF. CEF
CEF
92.5-
66.2-
45.0-
31.0-
I
C
CEF
INF. CEF
92.5-
66.2-
45.0-
31.0-

In summary, antiserum raised against a synthetic peptide representing amino acids 290-301 of the putative v-*rel* protein permitted the identification of a protein with apparent molecular weight of 55K present in CBMC transformed by REV-T (Fig. 6B). This protein, which we have identified as the protein product of the v-*rel* oncogene, was not detected in normal uninfected CEF. Due to the inability of this peptide serum to immunoprecipitate the v-*rel* protein product, portions of the v-*rel* sequence were expressed in *E. coli* (Fig. 8). The protein expressed from plasmid pLR/PE was gel purified and used to immunize rabbits. The resulting antiserum reacted with the v-*rel* protein in Western blot and immunoprecipitation assays (Fig. 9). The apparent molecular weight of the v-*rel* protein was 58,000. Immunoprecipitation of the v-*rel* protein from CBMC resulted in the co-precipitation of several other cellular proteins (Fig. 9A). Simek and Rice (1988b) and Tung *et al.* (1988) also reported the co-precipitation of several cellular proteins with the v-*rel* protein in transformed lymphoid cells.

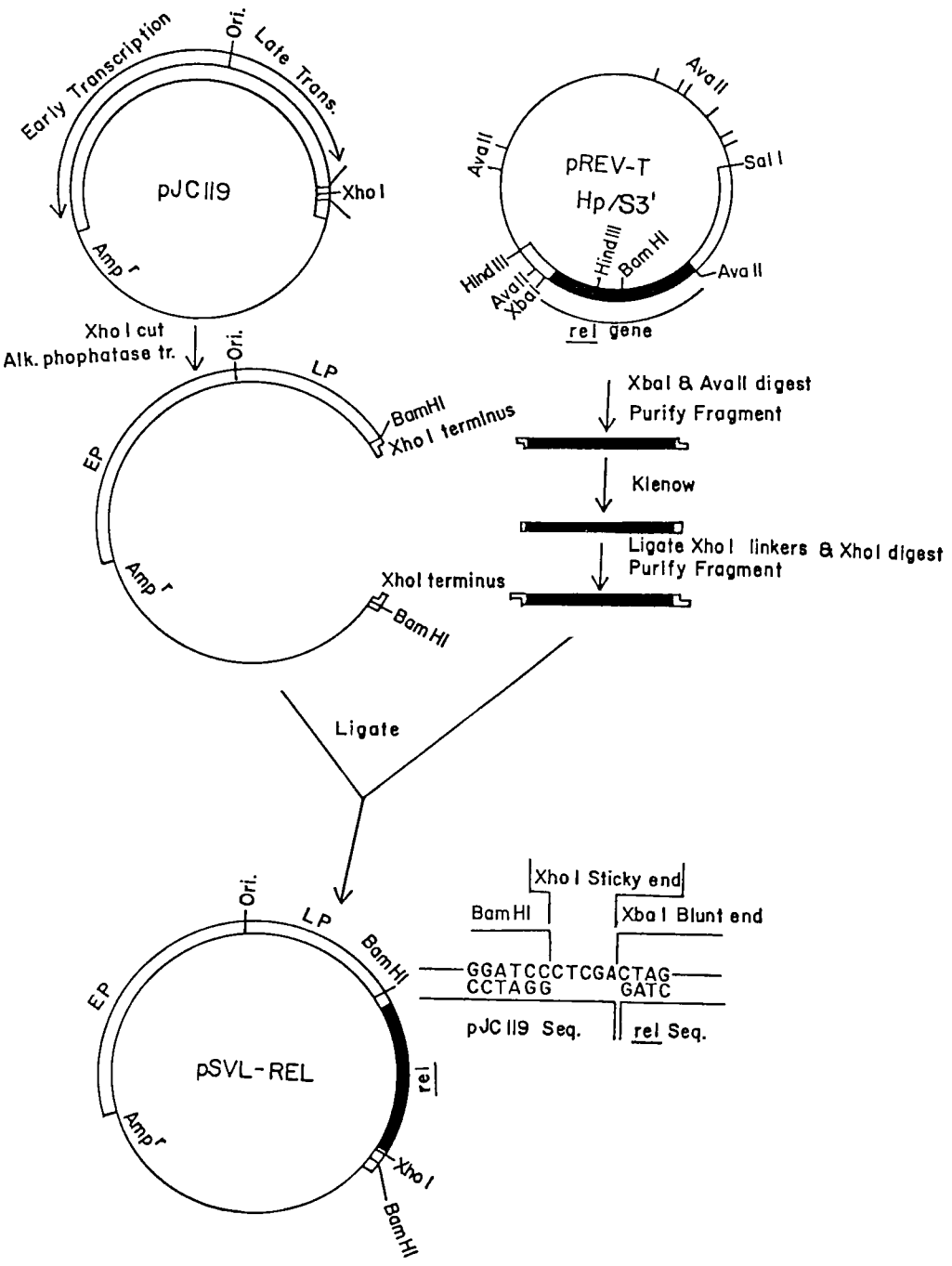
2. EXPRESSION OF V-*REL* IN COS1 CELLS

The v-*rel* oncogene was placed under the control of the late promoter of SV40 in plasmid pJC119 (Sprague *et al.* 1983) to permit its expression in Cos1 cells. The expression of v-*rel* in this transient system was to provide a model for further studies of v-*rel* mutants.

The XbaI(-27)-AvaII(1534) fragment (See appendix 1) containing the v-*rel* oncogene was purified from pREV-T Hp/S 3' and cloned into pJC119 using XhoI linkers as illustrated (Fig. 10). The resulting plasmid, pSVL-REL was used directly for expression of the v-*rel* protein.

Transfection of Cos1 cells with pJC119 or pSVL-Rel was monitored by Southern blot assay of Hirt DNA and Northern blot assay of total

Figure 10. Construction of pSVL-REL.
The AvaII fragment of pREV-T Hp/S3' was subcloned into the XhoI site of pJC119 to generate pSVL-REL. The junction between the 5' end of the v-*rel* gene and pJC119 was sequenced, and the sequence is shown.


Early Transcription
Ori.
Late Trans.
pJC119
Xho I
Amp r
pREV-T
Hp/S3'
Ava II
Sal I
Hind III
Bam HI
Xba I
rel gene
Xho I cut
Alk. phophatase tr.
LP
EP
Bam HI
Xho I terminus
Xba I & Ava II digest
Purify Fragment
Klenow
Ligate Xho I linkers & Xho I digest
Purify Fragment
Ligate
pSVL-REL
rel
Xho I Sticky end
Xba I Blunt end
GGATCCCTCGACTAG
CCTAGG
GATC
pJC 119 Seq.
rel Seq.

cytoplasmic RNA extracted from cells 48 hours after transfection. Hirt DNA was detected using nick translated pSVL-REL as probe. Hirt DNA replicated in the Cos1 cells was differentiated from the original transfected DNA by examining the methylation state of the purified Hirt DNA. Hirt DNA was resistant to restriction enzyme digestion with Dpn1, which cleaves only methylated recognition sites and sensitive to Mbo1 digestion, which cleaves only non-methylated recognition sites (Fig. 11A).

A single species of RNA, approximately 2100 bases in length was detected after hybridization of total cellular RNA with a ^{32}P-labeled *rel* gene fragment (Fig. 11B). This RNA species was not detected in total cytoplasmic RNA isolated from non-transfected Cos1 cells.

In vitro translation of total cytoplasmic RNA from Cos1 cells transfected with pSVL-REL resulted in the synthesis of a unique product with a molecular weight of approximately 55,000 which was not present in the *in vitro* translation products of RNA from non-transfected Cos1 cells (Fig. 12). Detection of the v-*rel* protein expressed directly in Cos1 cells was facilitated by the development of the specific v-*rel* peptide antiserum described above. Transfected Cos1 cell lysates were screened for the v-*rel* protein by Western blot assay (Fig. 13). A faint band was detected in Cos1 cells transfected with pSVL-REL which was not present in Cos1 cells transfected with pJC119 alone. The identified protein had an apparent molecular weight of 55,000 and migrated with the same mobility as the v-*rel* protein expressed in CBMC.

The molecular weight standards in figures 12 and 13 have slightly different molecular weight designations than the other figures. The same proteins were used as standards for these experiments, however, the estimated molecular weights for BSA, ovalbumin, chymotrypsinogen and

Figure 11. Analysis of Hirt DNA and cytoplasmic RNA from transfected Cos1 cells.
A) Hirt DNA prepared from Cos1 cells transfected with pSVL-REL or pJC119 was restricted with the indicated enzymes and hybridized in a Southern blot with ^{32}P-labeled pSVL-REL as a probe.
B) 8, 4 and 2 μg of RNA and 8μg of RNase-treated RNA from Cos1 cells transfected with pSVL-REL and 8μg of RNA from Cos1 cells were analyzed by Northern blotting using a ^{32}P-labeled v-*rel* gene probe. Plasmids containing v-*rel* were digested with various restriction enzymes, and the DNA denatured with glyoxal to generate ssDNA markers.

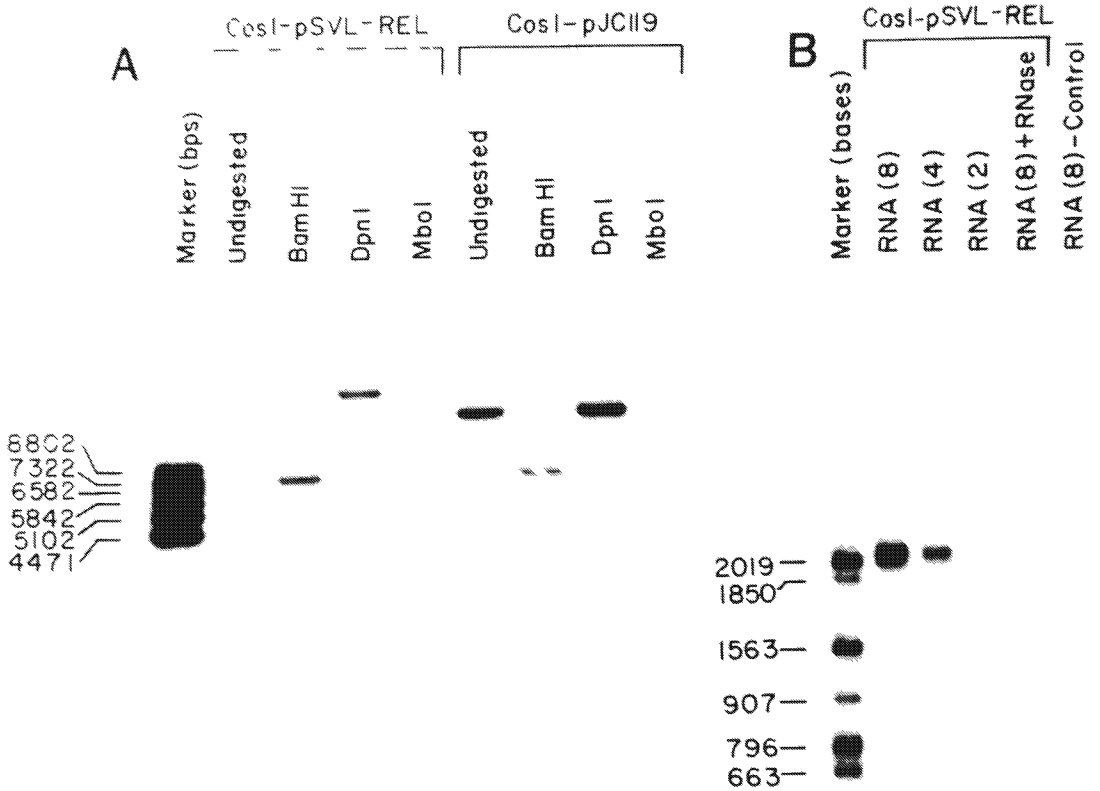

A
CosI-pSVL-REL
CosI-pJC119
Marker (bps)
Undigested
BamHI
DpnI
MboI
Undigested
BamHI
DpnI
MboI
8802
7322
6582
5842
5102
4471
B
CosI-pSVL-REL
Marker (bases)
RNA (8)
RNA (4)
RNA (2)
RNA (8)+RNase
RNA (8)-Control
2019
1850
1563
907
796
663

Figure 12. ***In vitro*** translation products of total RNA from transfected Cos1 cells.
In vitro translations were performed in rabbit reticulocyte lysates by the addition of 0μg of RNA (-mRNA), 1μg of Brome Mosaic virus RNA (BMV), or 10, 5, or 2.5μg of RNA from Cos1 cells or Cos1 cells transfected with pSVL-REL. Products were separated by SDS-PAGE on 15% polyacrylamide gels and subjected to fluorography to visualize ^{35}S-methionine labeled ***in vitro*** translation products. The position and size (kilodaltons) of standard prestained protein markers are shown. The additional 200 kDa protein standard was myosin.

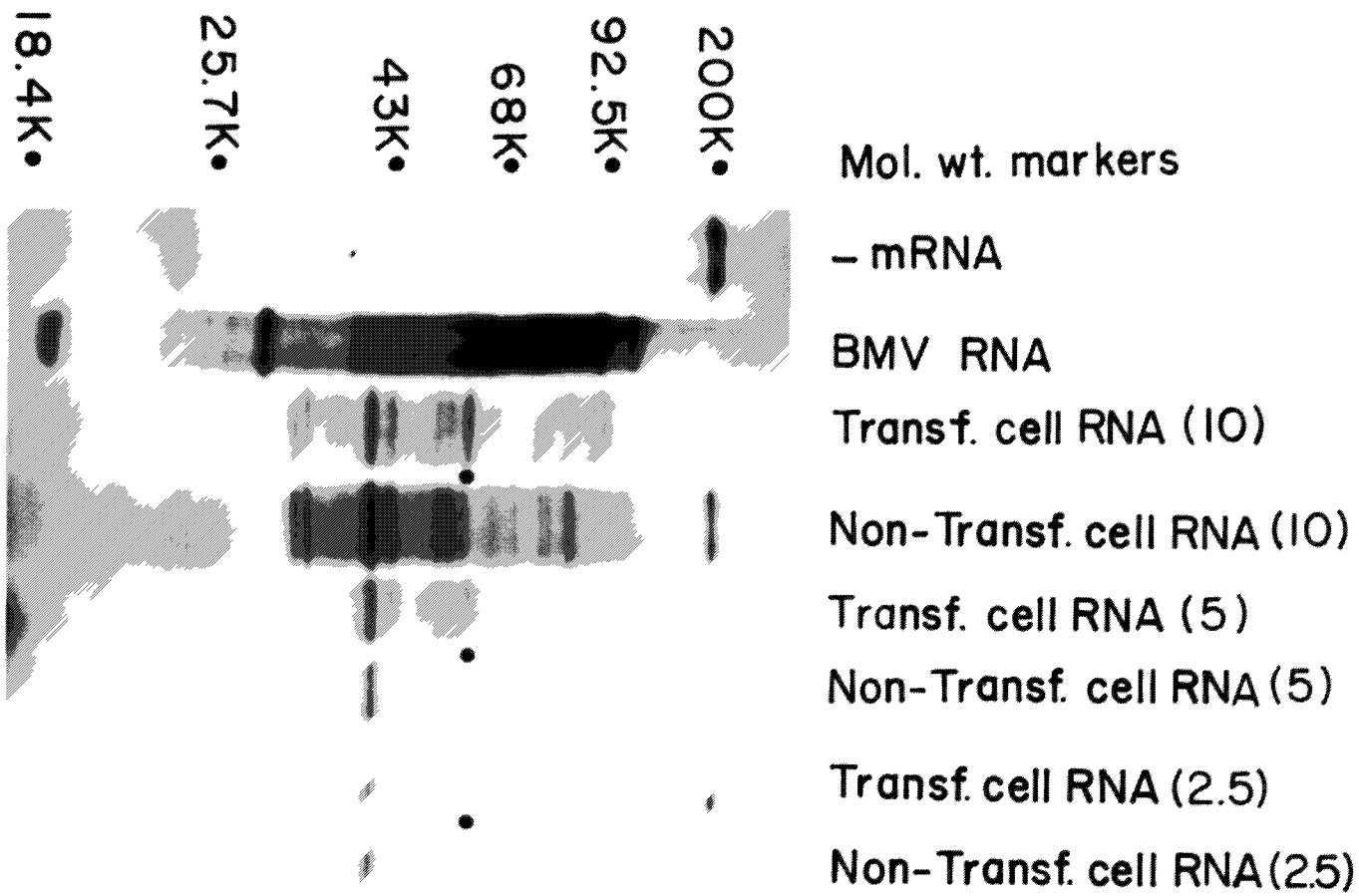

18.4K•
25.7K•
43K•
68K•
92.5K•
200K•
Mol. wt. markers
– mRNA
BMV RNA
Transf. cell RNA (10)
Non-Transf. cell RNA (10)
Transf. cell RNA (5)
Non-Transf. cell RNA (5)
Transf. cell RNA (2.5)
Non-Transf. cell RNA (2.5)

Figure 13. Identification of the *rel* gene product expressed in Cos1 cells.
Lysates from CEF, CMBC, and Cos1 cells transfected with pJC119 or pSVL-REL were separated by SDS-PAGE on 12% polyacrylamide gels and subjected to Western blot analysis using peptide antiserum. The position of the v-*rel* proteins are indicated with dots. The position and size (kilodaltons) of standard prestained protein markers are shown. The additional 12.3 kDa protein standard was lysozyme.

Mol. wt. markers
Cos I-pJC 119
Cos I-pSVL-REL
CBMC
CEF
200K
92.5K
68K
43K
25.7K
18.4K
12.3K

ovalbumin were slightly different at the time of the preparation of these two figures.

In conclusion, the expression of the v-*rel* gene in Cos1 cells resulted in the synthesis of a protein product with the same mobility as the protein detected in CBMC (Fig. 13). This indicated that the Cos1 expression system might be useful for the screening of proteins expressed from v-*rel* genes which had been altered by mutagenesis.

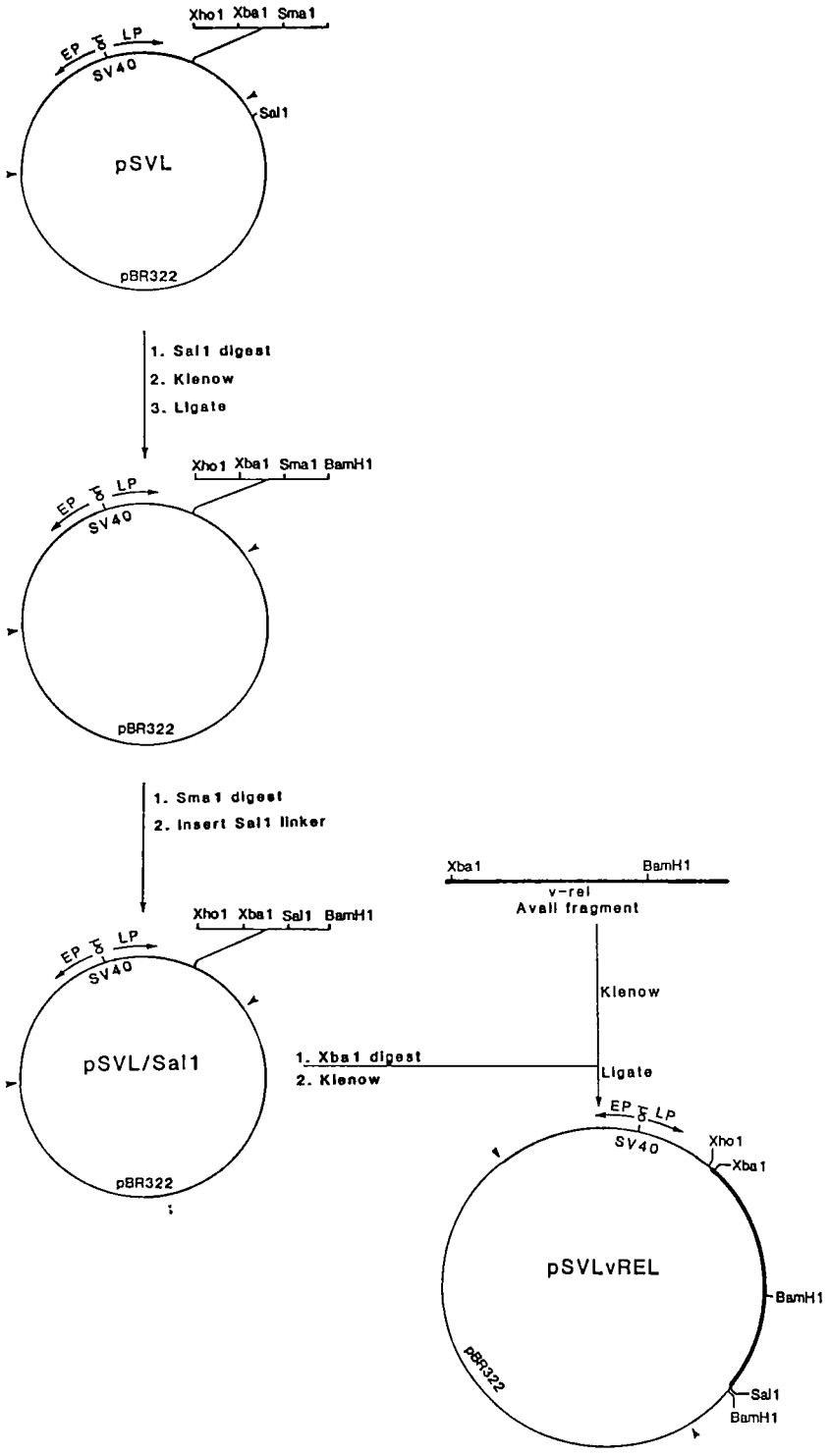
3. DELETION MUTAGENESIS OF THE V-*REL* GENE

a) Construction of deletion mutants

Reports of the nucleotide sequence of v-*rel* (Stephens *et al.* 1983, Wilhelmsen *et al.* 1984) revealed that the N-terminal 12 amino acids and C-terminal 19 amino acids are coded for by sequence derived from the remnants of the viral *env* gene. In order to test the functional importance of these terminal sequences, they were deleted as described below. In addition, more substantial deletions were made at each termini.

Deletion mutagenesis was performed on the v-*rel* gene in a modified plasmid related to pJC119 (Sprague *et al* 1983). pSVL, a derivative of pJC119, was further modified by removal of a unique SalI site by recirculization of pSVL after digestion with SalI and treatment with Klenow enzyme (Fig. 14). A SalI linker (pGGTCGACC) was inserted into the SmaI site of the resulting clone to generate plasmid pSVL/SalI. The AvaII (-44 to 1534) fragment of pREV-T Hp/S 3' containing the v-*rel* gene (see Fig. 10) was treated with Klenow and ligated into XbaI digested, Klenow treated pSVL/SalI (Fig. 14). The resulting clone, pSVLvREL, was used for subsequent mutagenesis of the 5' end of the v-*rel* gene.

Figure 14. Construction of pSVLvREL.
The AvaII fragment of pREV-T Hp/S3' containing the v-*rel* oncogene was cloned into the SalI site of pSVL/SalI. The direction of transcription from the late promoter (LP) and early promoter (EP) of SV40 is shown.


Xho1 Xba1 Sma1
EP
LP
SV40
Sal1
pSVL
pBR322
1. Sal1 digest
2. Klenow
3. Ligate
Xho1 Xba1 Sma1 BamH1
pBR322
1. Sma1 digest
2. Insert Sal1 linker
Xba1
BamH1
v-rel
AvaII fragment
Xho1 Xba1 Sal1 BamH1
pSVL/Sal1
pBR322
Klenow
1. Xba1 digest
2. Klenow
Ligate
Xho1
Xba1
pSVLvREL
BamH1
Sal1
BamH1
pBR322

Oligonucleotides rcl1, rcl3, rcl4, and rcl5 and the partially double stranded linker rcl2/rcl2b were used for crossover linker mutagenesis to delete sequences indicated in Table 1 and Figure 15 (See appendix 2 for oligonucleotide sequence). Deletions at the 5' end of v-*rel* were accompanied by the insertion of new initiation codons and likewise termination codons were introduced immediately adjacent to the site of 3' deletions. Oligonucleotides rcl1 and rcl3 were ligated to pSVLvREL digested with XhoI and XbaI and linker rcl2/rcl2b was ligated to pSVLvREL digested with XhoI for crossover linker mutagenesis as described in the Materials and Methods section. It should be noted that ligation of the oligonucleotides on to the XhoI site completed the XhoI recognition sequence resulting in the maintenance of this XhoI site in the final clones.

pSVLREL, generated by deletion of 5' noncoding sequences using oligonucleotide rcl1, was further modified to create a convenient vector for deletion mutagenesis of the 3' end of v-*rel*. A 1.8 kb SalI/XhoI DNA fragment from plasmid pSVL-F was introduced into the SalI site of pSVLREL (Fig. 16). The rationale for this construction was to insert additional restriction sites 3' to v-*rel*, while maintaining the single SalI site. The resulting plasmid, pSVL-Rel/F was used for mutagenesis of the 3' end of the v-*rel* gene. Oligonucleotides rcl4 and rcl5 were ligated to pSVL-Rel/F which had been digested with SalI and XbaI for crossover linker mutagenesis. Ligation of these oligonucleotides on to the SalI site failed to complete the SalI recognition site such that this site was not present in the final clones.

Table 1

CROSSOVER LINKER OLIGONUCLEOTIDES

Linker	Sequences Deleted[a]	Mutant
rcl1	5'-NC[b]	REL
rcl2/rcl2b	5'-NC, Δ1-33	ΔN11
rcl3	5'-NC, Δ1-297	ΔN99
rcl4	3'-NC, Δ1452-1509	ΔC19
rcl5	3'-NC, Δ1209-1509	ΔC100

[a]The nucleotide postion of deletions are indicated. Nucleotide 1 is the adenine of the v-*rel* initiation codon and nucleotide 1509 is the guanosine immediately preceeding the v-*rel* termination codon.

[b]NC, noncoding sequence.

Figure 15. Deletion mutants of v-*rel*.
Noncoding sequence is indicated by heavy black lines. The diagonal striped region represents 5' *env* sequence and the vertical striped region represents 3' *env* sequence. The nucleotide position of each deletion is marked on the map of the wild-type v-*rel* gene.

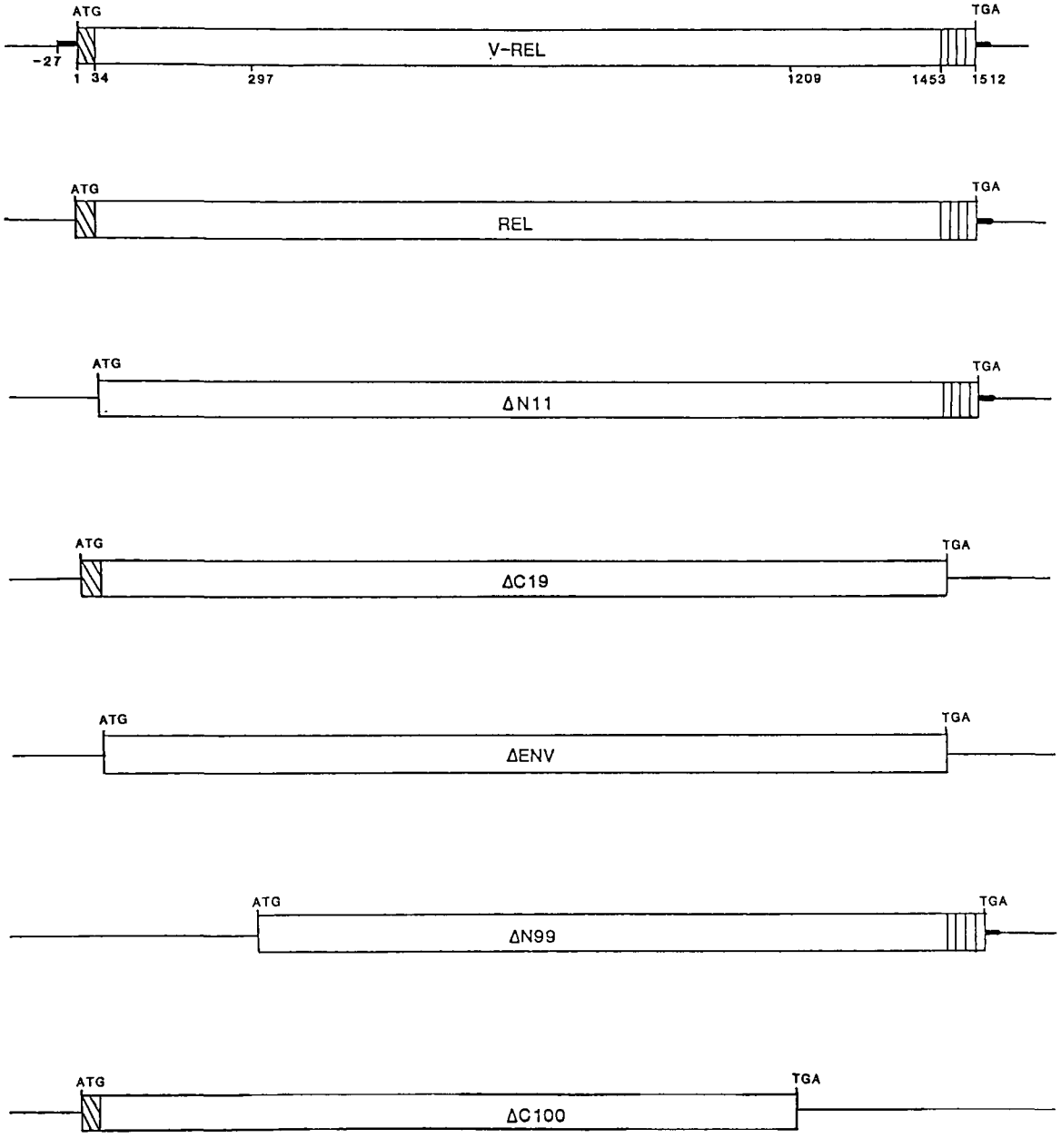
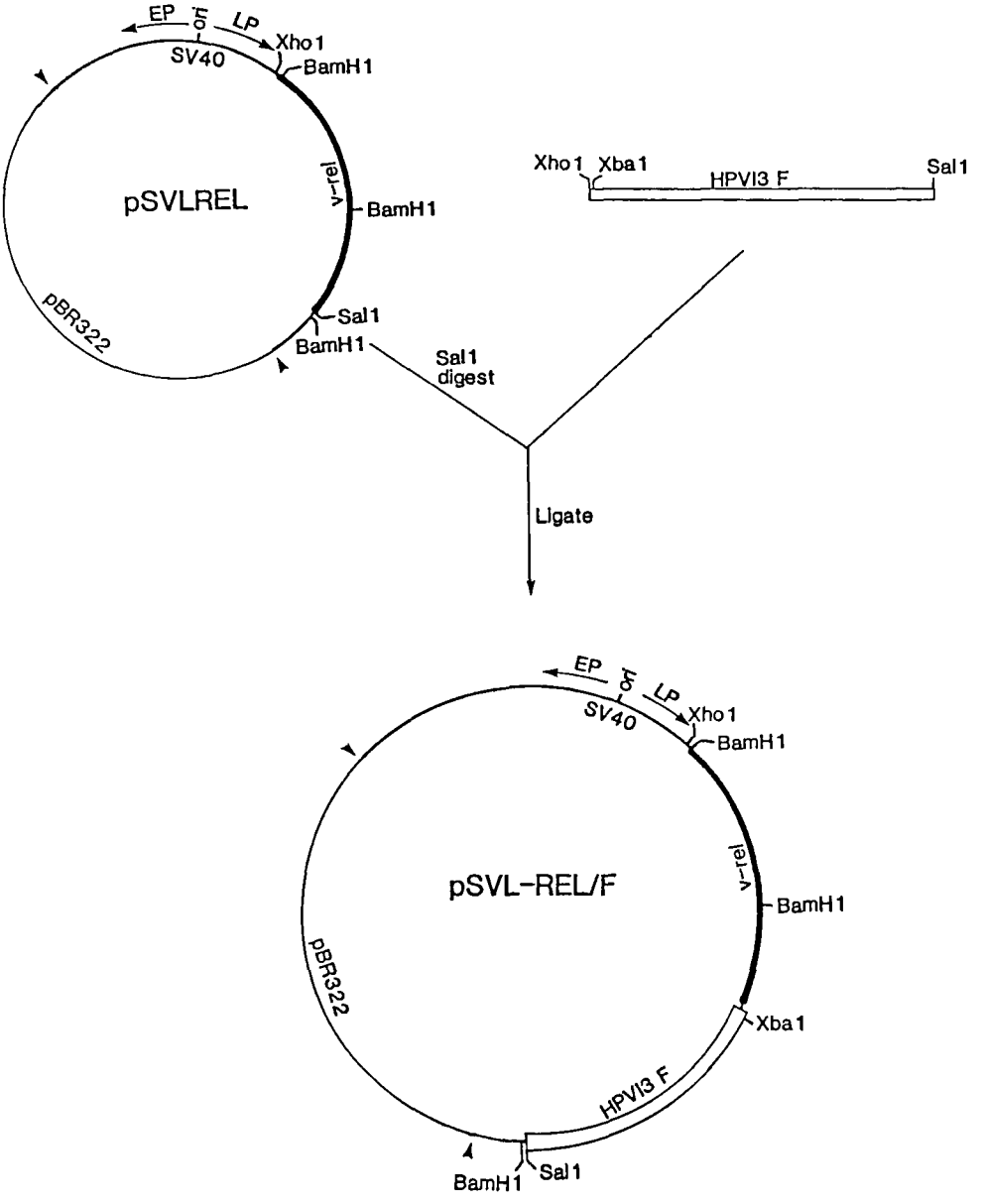

ATG
TGA
V-REL
-27
1
34
297
1209
1453
1512
ATG
TGA
REL
ATG
TGA
ΔN11
ATG
TGA
ΔC19
ATG
TGA
ΔENV
ATG
TGA
ΔN99
ATG
TGA
ΔC100

Figure 16. Construction of pSVL-REL/F.
A XhoI-SalI fragment containing the Human Parainfluenza type 3 F gene (HPIV3 F) was cloned into the SalI site of pSVLREL. The direction of transcription from the late promoter (LP) and early promoter (EP) of SV40 is shown.


EP
LP
Xho1
SV40
BamH1
pSVLREL
v-rel
BamH1
pBR322
Sal1
BamH1
Xho1
Xba1
HPVI3 F
Sal1
Sal1
digest
Ligate
EP
LP
Xho1
SV40
BamH1
pSVL-REL/F
v-rel
BamH1
pBR322
Xba1
HPVI3 F
BamH1
Sal1

The deleted v-*rel* genes resulting from the crossover linker mutagenesis are depicted in Figure 15. The construct labeled ΔENV will be described later.

b) Expression of deletion mutants in Cos1 cells

The integrity of the coding regions of the REL, ΔN99, ΔC19 and ΔC100 mutants was initially tested by direct expression of the genes in Cos1 cells. Cos1 cells were transfected with the various constructs, labeled with ^{35}[S]-methionine, and at 48 hours post-transfection the lysates were immunoprecipitated with B-1 antiserum. Analysis of the immunoprecipitated proteins by SDS-PAGE and fluorography indicated that the deletion mutants expressed appropriately sized proteins in Cos1 cells (Fig. 17). In addition, all mutants including ΔN11 had the predicted sequences at the site of deletion as determined by the sequencing of the appropriate M13mp19 subclones.

The subcellular location of the v-*rel* protein and the various mutants expressed in Cos1 cells was determined by indirect immunofluorescence using B-1 antiserum. Fluorescent staining indicated that the v-*rel* protein and the gene products of the described mutants were located in the nucleus of transfected Cos1 cells (Fig. 18). The level of expression of the mutants varied from cell to cell, however no gross differences in expression levels were noted.

c) Construction of a retroviral cloning vector

In order to test the transforming capabilities of the various v-*rel* mutants, an REV-T based retroviral vector was synthesized (Figs. 19, 20 and 21).

The v-*rel* oncogene was deleted from pREV-T Hp/S 3', a plasmid which contains sequence from the 3' end of REV-T (Chen and Temin

Figure 17. Expression of v-*rel* deletion mutants in Cos1 cells.
Cos1 cells transfected with pSVLvREL (v-rel), pSVLREL (Rel), pSVLΔN99 (N99), pSVLΔC19 (C19) and pSVLΔC100 (C100) were labeled with ^{35}[S]-methionine for 4 hours. Cell lysates were immunoprecipitated with B-1 antiserum and electrophoresed on 10% polyacrylamide gels containing SDS. Gels were subjected to fluorography. The position and size (kilodaltons) of standard protein markers are shown.

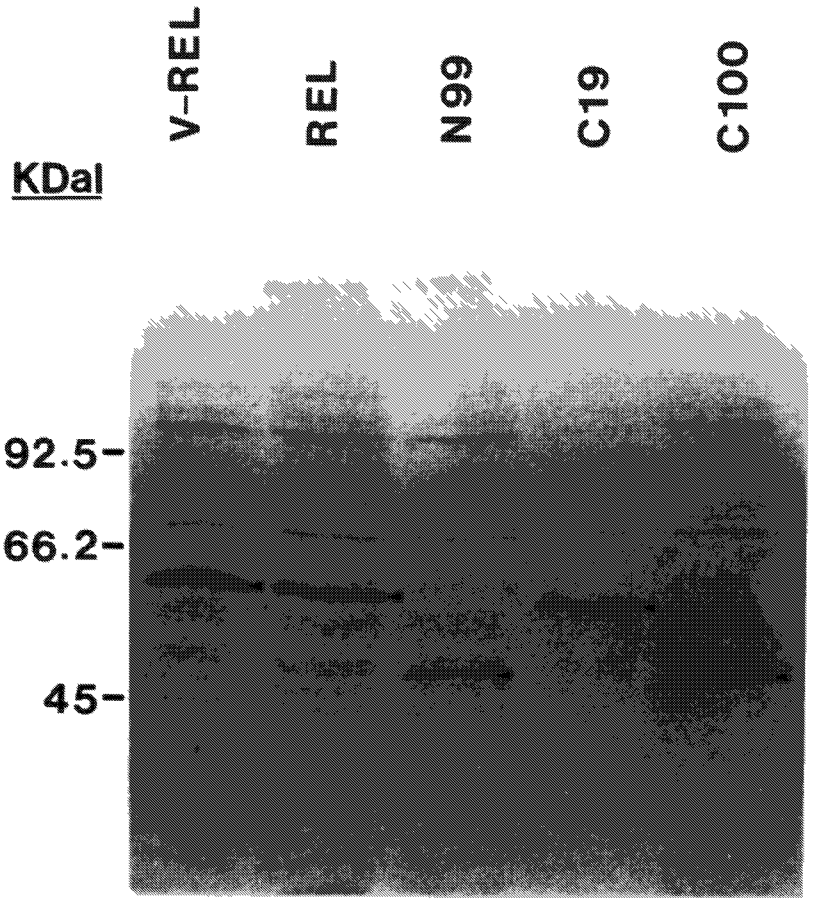

V-REL
REL
N99
C19
C100
KDal
92.5
66.2
45

Figure 18. Immunofluorescence of Cos1 cells electroporated with v-*rel* deletion mutants.
Cos1 cells electroporated with pSVL, pSVLREL, pSVL-ΔN11, pSVL-ΔN99, pSVL-ΔENV, pSVL-ΔC19 and pSVL-ΔC100 were examined in immunofluorescence assays using B-1 serum 48 hours after electroporation. The white bar in the bottom right panel represents 20μm.

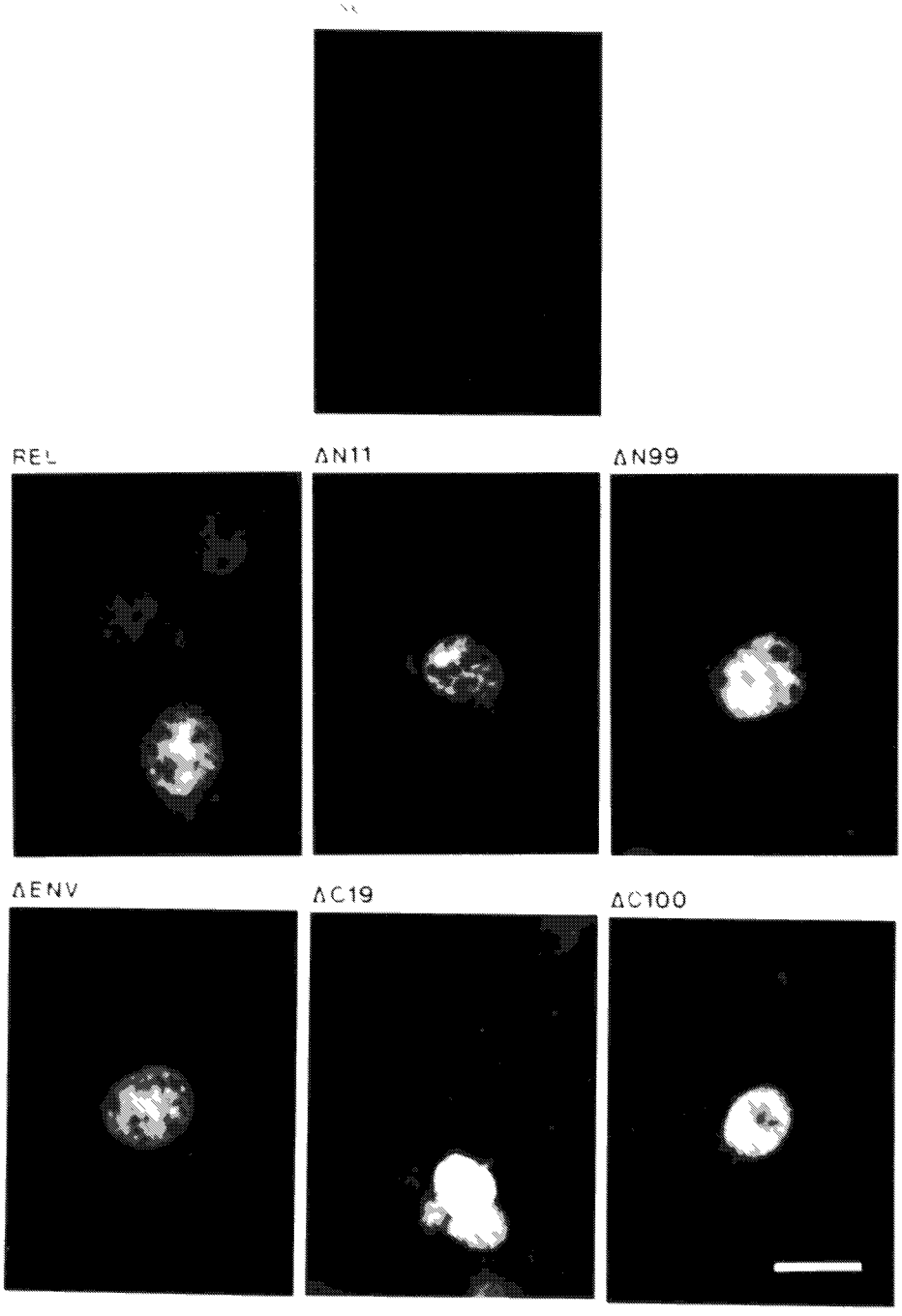

REL
ΔN11
ΔN99
ΔENV
ΔC19
ΔC100

Figure 19. Construction of pREV-3'ΔR.
Insertion of XhoI linkers at the 5' and 3' ends of v-*rel* permitted the deletion of v-*rel* and the creation of a XhoI cloning site. Restriction sites are H (HindIII), Xba (XbaI), N (NruI), S (SalI) and X (XhoI).

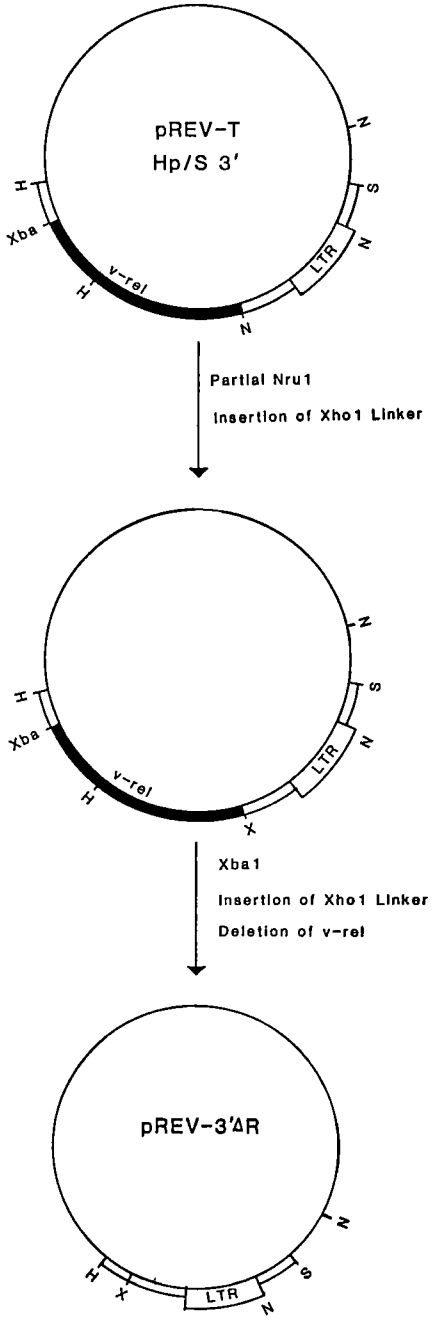

pREV-T
Hp/S 3'
N
S
N
LTR
N
H
Xba
H
v-rel
Partial Nru1
Insertion of Xho1 Linker
N
S
N
LTR
X
H
Xba
H
v-rel
Xba1
Insertion of Xho1 Linker
Deletion of v-rel
pREV-3'ΔR
N
S
N
LTR
X
H

Figure 20. Construction of pREV-ΔR.
5' REV-T sequences from pREV-T, a circularly permuted clone of REV-T, were added to pREV-3'ΔR. Restriction sites are H (HindIII), X (XhoI) and S (SalI).

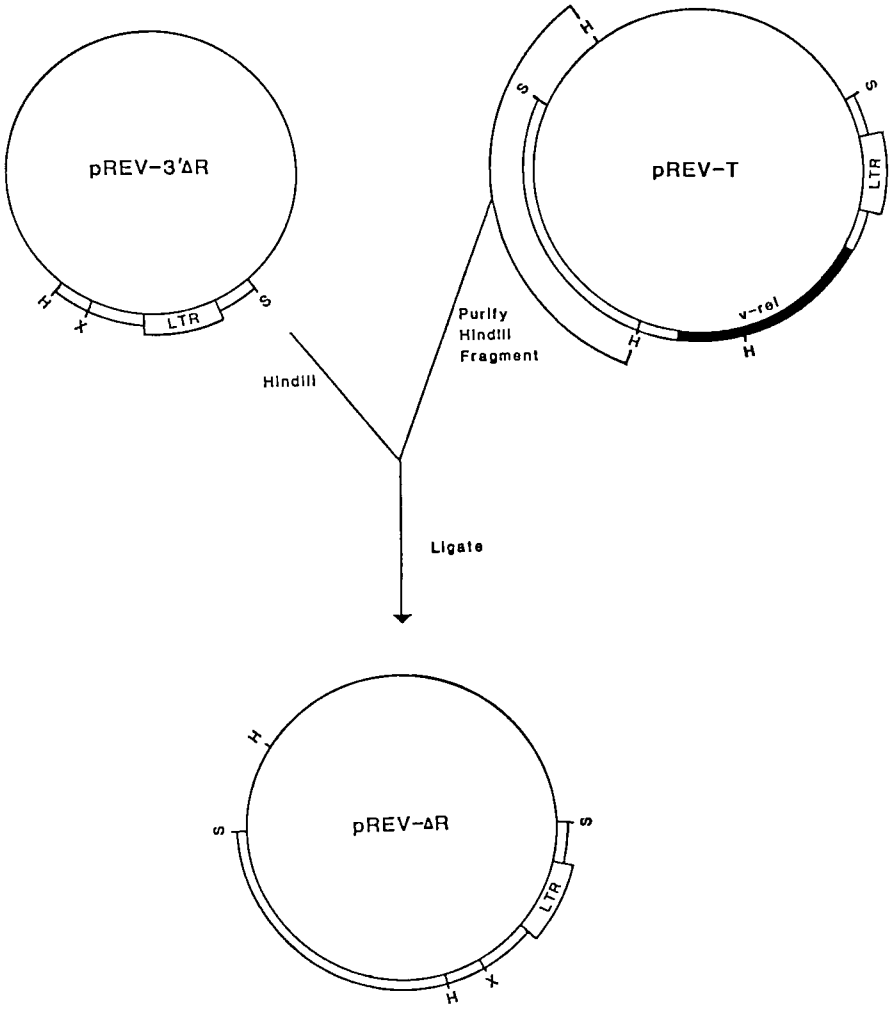
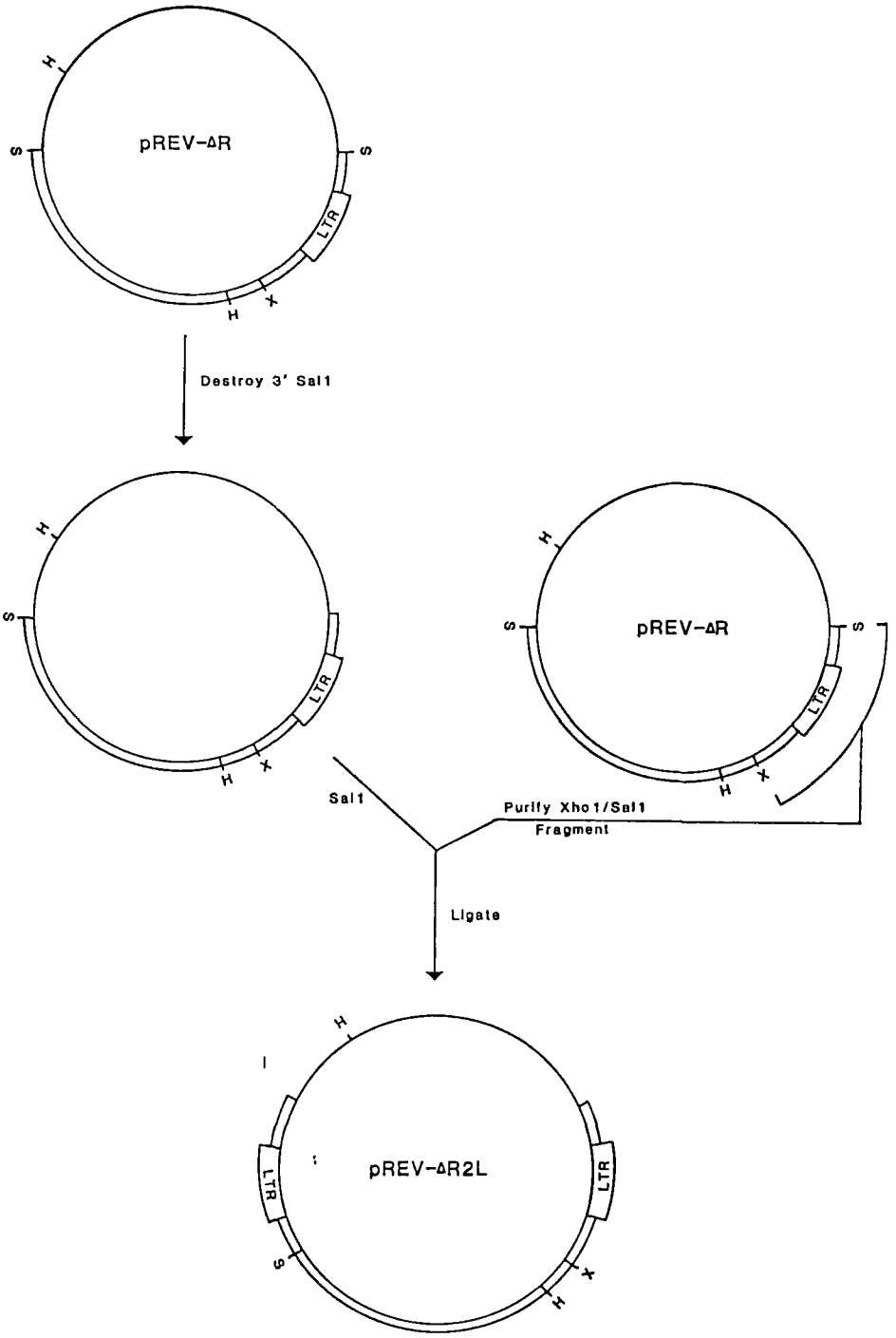

pREV-3'ΔR
H
X
LTR
S
HindIII
pREV-T
H
S
S
LTR
v-rel
H
H
Purify
HindIII
Fragment
Ligate
pREV-ΔR
H
S
S
LTR
H
X

Figure 21. Construction of pREV-ΔR2L.
pREV-ΔR was modified by the destruction of the SalI site adjacent to the LTR. The XhoI-SalI fragment from pREV-ΔR containing the viral LTR was inserted into the remaining SalI site of the modified pREV-ΔR to generated pREV-ΔR2L. Restriction sites are H (HindIII), X (XhoI) and S (SalI).


pREV-ΔR
H
S
S
LTR
H
X
Destroy 3' Sal1
H
S
LTR
H
X
pREV-ΔR
H
S
S
LTR
H
X
Sal1
Purify Xho1/Sal1
Fragment
Ligate
H
pREV-ΔR2L
LTR
LTR
S
H
X

1982). pREV-T Hp/S 3' was partially cut with NruI and a XhoI linker was inserted (Fig. 19). This construction inserted a XhoI site 32 nucleotides 3' to the v-*rel* termination codon. A second XhoI linker was inserted into the XbaI site located 27 nucleotides 5' to the v-*rel* initiation codon. The linker was inserted after treatment of the XbaI cleaved DNA with Klenow. The XhoI restriction digestion used to remove excess linkers during this step resulted in cleavage of the XhoI site inserted immediately 3' to the v-rel gene (Fig. 19). The plasmid recovered after ligation and transformation (pREV-3'ΔR) contained a unique XhoI cloning site spanning a deletion which removed the entire v-*rel* coding sequence including 27 nucleotides of 5' and 32 nucleotides of 3' noncoding sequences.

pREV-3'ΔR contains the viral LTR and sequences from the 3' end of the virus. The circularly permuted clone of REV-T, pREV-T, was used to provide 5' viral sequences not present in pREV-3'ΔR. A HindIII fragment of pREV-T, containing pBR322 and REV-T 5' sequences, was inserted into the HindIII site of pREV-3'ΔR (Fig. 20). Plasmid pREV-ΔR, contains all REV-T viral sequences with the exception of the deleted v-*rel* region.

In order to avoid the inconvenience of using a circularly permuted clone as a retroviral vector, a second LTR was inserted into pREV-ΔR (Fig. 21). In order to facilitate this construction, pREV-ΔR was partially digested with SalI, treated with Klenow and religated. A clone was selected which had lost the SalI restriction site adjacent to the LTR, but had maintained the other SalI site (Fig. 21). The XhoI-SalI fragment of pREV-ΔR containing the viral LTR was purified and subcloned into the remaining SalI site of the clone described above to generate pREV-ΔR2L (Fig. 21).

A comparison of pREV-ΔR2L and a linear clone of REV-T is shown in Figure 22. All viral sequence has been maintained in pREV-ΔR2L with exception to the deleted v-*rel* region.

In order to test the viability of pREV-ΔR2L as a retroviral vector, the β-galactosidase gene derived from pCH110 (Hall *et al.* 1983) was subcloned into the XhoI cloning site. CEF were electroporated with pREV-LACZ and pREV-A (pSW253) and cytoplasmic lysates harvested at 4 days. In addition, supernatant tissue culture fluid from the electroporated cells was harvested at 4 days and used to infect fresh CEF. Cytoplasmic lysates from electroporated cells and lysates from infected cells at four days post-infection were assayed for β-galactosidase activity by visual inspection of substrate colour development after overnight incubation. β-galactosidase was detected in electroporated and infected CEF but not in uninfected CEF (Data not shown), indicating that the vector pREV-ΔR2L was able to express RNA which could be packaged into infectious virus in the presence of REV-A.

Allthough pREV-ΔR2L was able to function as a retroviral vector for the production of infectious virus, it was not clear whether it would express at levels comparable to the wild type virus. For this reason, the expression of v-*rel* in pREV-ΔR2L was compared with that of wild type plasmids.

The v-*rel* gene, with 5' non-coding sequences deleted, was excised from pSVLREL by digestion with XhoI and SalI and inserted into the XhoI cloning site of pREV-ΔR2L to generate pREV-REL. The level of expression of v-*rel* from pREV-REL was compared in a Western blot assay to that of pREV-T, a circularly permuted clone of REV-T and to pREV-2LTR, whose construction is outlined in Figure 23. Since pREV-T was

Figure 22. Comparison of proviral DNA of REV-T with REV-ΔR2L.
The v-*rel* sequences are represented by the heavy black line and LTRs are shown as boxes. Restriction sites are H (HindIII), Xba (XbaI), S (SalI) and X (XhoI).

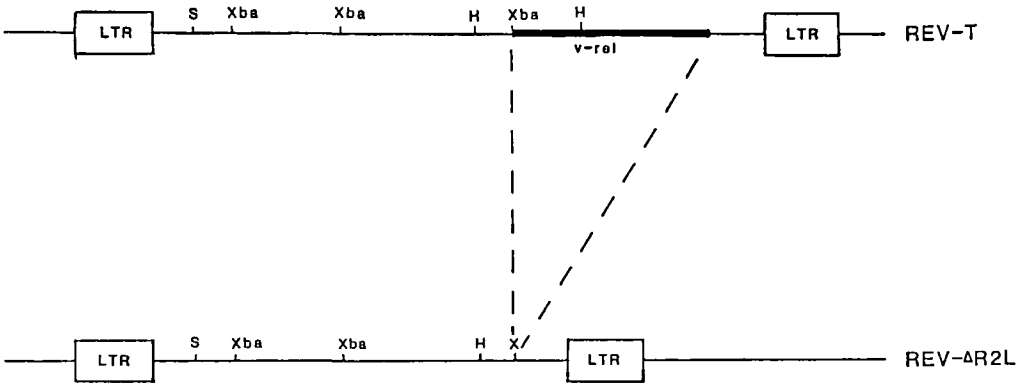
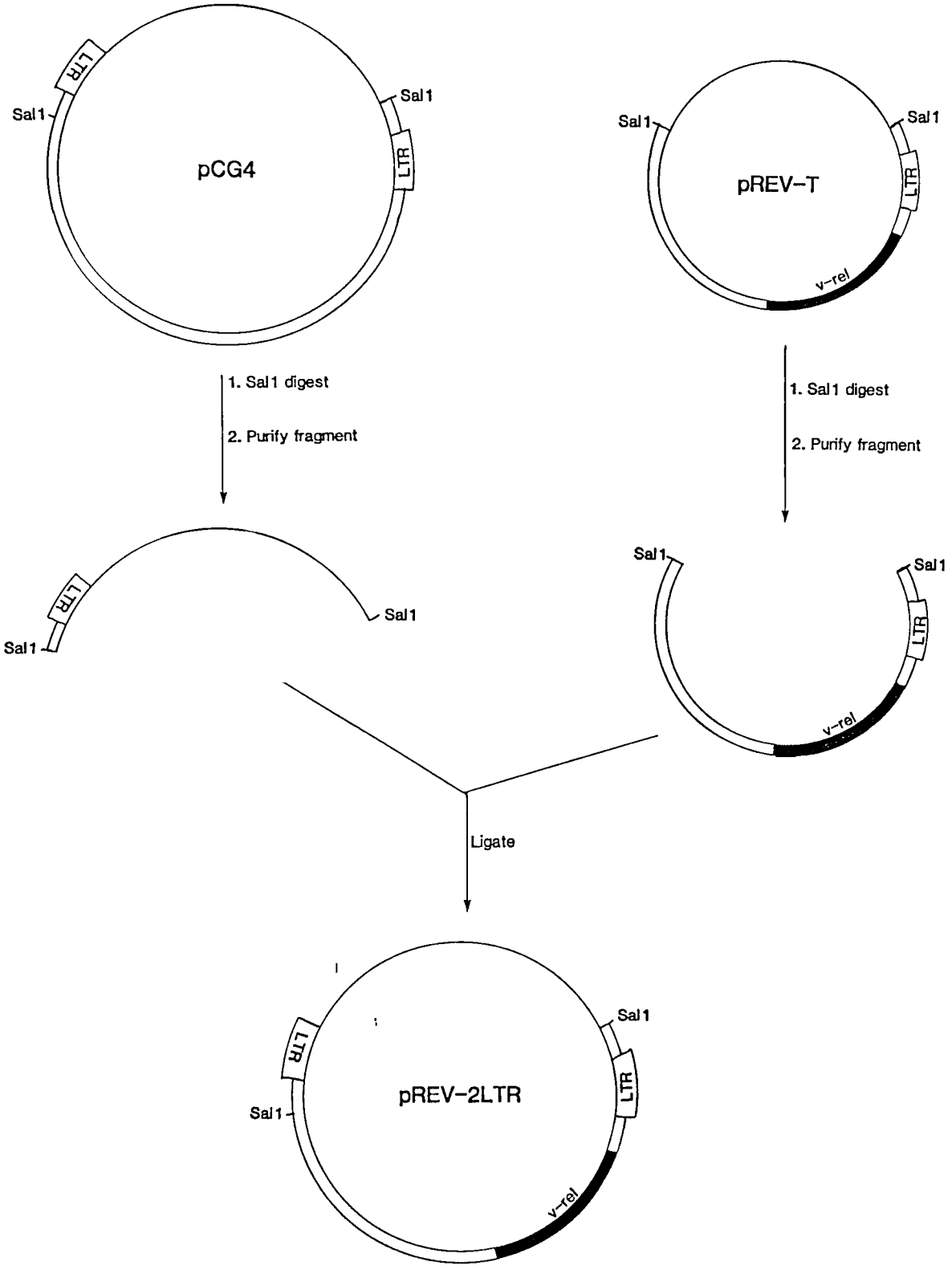

LTR
S
Xba
Xba
H
Xba
H
v-rel
LTR
REV-T
LTR
S
Xba
Xba
H
X
LTR
REV-ΔR2L

Figure 23. Construction of pREV-2LTR.
The entire REV-T sequence from pREV-T was cloned downstream of the LTR of pCG4, a spleen necrosis viral clone. The SalI fragment of pCG4, representing the majority of the viral sequences was deleted in this construction.


pCG4
Sal1
LTR
Sal1
LTR
1. Sal1 digest
2. Purify fragment
pREV-T
Sal1
LTR
Sal1
v-rel
1. Sal1 digest
2. Purify fragment
Sal1
LTR
Sal1
Sal1
LTR
Sal1
v-rel
Ligate
pREV-2LTR
Sal1
LTR
Sal1
LTR
v-rel

circularly permuted, and contained only one LTR, it was digested with SalI and ligated to generate circular forms which would permit transcription of viral RNAs. Lysates from CEF, CEF electroporated with pREV-A, pREV-REL + pREV-A, pREV-2LTR + pREV-A and pREV-T/SalI + pREV-A were harvested 4 days after electroporation. The level of expression of v-*rel* was similar for all three vectors (Fig. 24) as estimated from Western blots.

The retroviral vector, pREV-ΔR2L, was able to express v-*rel* at levels comparable to other vectors, and formed infectious virus when co-transfected with helper virus DNA.

d) Expression and transforming activity of v-*rel* deletion mutants

The deletion mutants of v-*rel* were subcloned into vector pREV-ΔR2L, for the production of virus which could be tested for transforming activity.

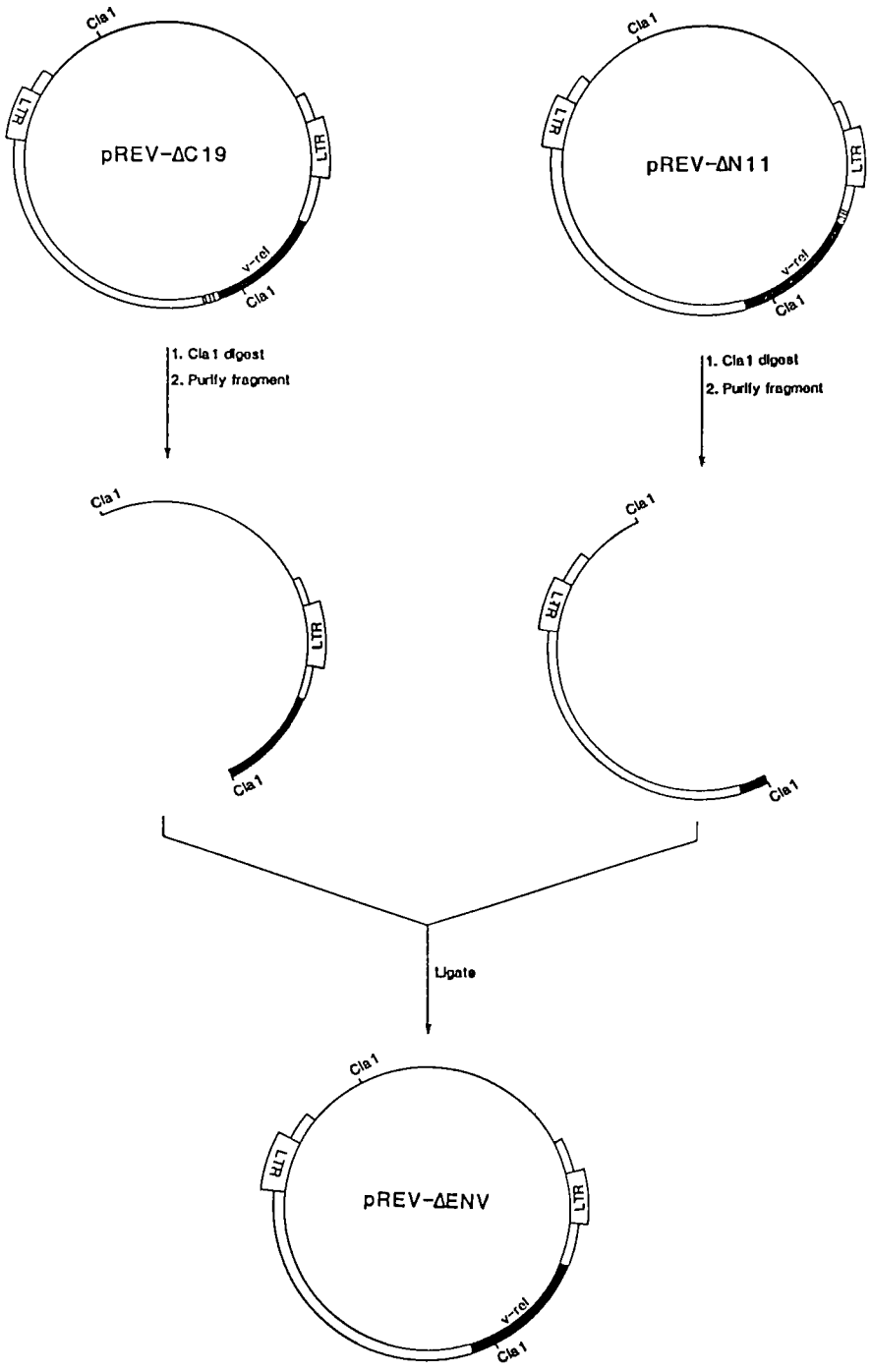
Plasmids pSVLΔN11 and pSVLΔN99 were digested with XhoI and SalI and the v-*rel* DNA fragments were purified and subcloned into the XhoI site of pREV-ΔR2L to generate pREV-ΔN11 and pREV-ΔN99, respectively. Plasmids pSVLΔC19 and pSVLΔC100 were digested partially with BamHI, and the v-*rel* DNA fragments were purified and subcloned into the XhoI site of pREV-ΔR2L to generate pREV-ΔC19 and pREV-ΔC100. All subclones were identified by colony hybridization and restriction enzyme analysis to ensure proper orientation of the v-*rel* inserts. A new mutant, ΔENV, shown earlier in Figure 15, was constructed by combining the 5' region of v-*rel* from pREV-ΔN11 with the 3' region of v-*rel* from pREV-ΔC19 as shown in Figure 25.

pREV clones were transfected into CEF with pREV-A DNA for the production of virus. Western blots of lysates from CEF infected with the

Figure 24. Expression of v-*rel* in CEF.
Lysates of CEF, or CEF electroporated with pREV-A (pREV-A), pREV-REL + pREV-A (pREV-REL), pREV-2LTR + pREV-A (pREV-2LTR) or pREV-T/SalI + pREV-A (pREV-T/SalI) were subjected to Western blot analysis using B-1 antiserum. The position and size (kilodaltons) of standard prestained protein markers are shown.

CEF
pREV-A
pREV-REL
pREV-2LTR
pREV-T/Sal1
KDal
-92.5
-66.2
-45
-31

Figure 25. Construction of pREV-ΔENV.
The ClaI fragment of pREV-ΔC19 containing the C-terminal deletion of v-*rel* was ligated to the ClaI fragment of pREV-ΔN11 containing the N-terminal deletion of v-*rel* to generate pREV-ΔENV. The solid bar represents cell derived *rel* sequence, and the striped bar represents *env* derived sequences.


Cla1
LTR
pREV-ΔC19
v-rel
Cla1
1. Cla1 digest
2. Purify fragment
Cla1
LTR
Cla1
pREV-ΔN11
v-rel
Cla1
1. Cla1 digest
2. Purify fragment
Cla1
LTR
Cla1
Cla1
LTR
Cla1
Ligate
Cla1
LTR
pREV-ΔENV
LTR
v-rel
Cla1

various viruses showed the expression of proteins by each mutant (Fig. 26). Indirect immunofluorescence assays detected v-*rel* proteins in the nucleus of infected cells (Fig. 27).

Virus recovered from transfected CEF was used directly for *in vitro* transformation assays with chick spleen cells (CSC). The number of transformed colonies appearing in the soft agar was determined and the results are summarized in Table 2.

Generally, viruses which transformed, produced in excess of 100 colonies on each 60mm plate. In such cases, the number of colonies was scored as >100. Average relative titers for non-transforming or weakly transforming viruses were determined by IFA and are expressed as percentages of the titer obtained for REV-REL. Exact counts of fluorescent nuclei were made only when initial visual inspection of samples indicated a decreased titer. Negative and positive control plates for *in vitro* transformations are shown in Figure 28.

Transformed colonies were picked and grown in RPMI 1640 supplemented with 10% FCS. Generally, most transformants established by infection with REV-REL, REV-ΔC19 or REV-ΔC100 continued to grow in liquid cultures. REV-ΔN11 transformants formed diffuse colonies in agar and often failed to grow in liquid culture. One transformant grew enough to permit examination of protein expression by Western blot analysis, but subsequently failed to undergo further proliferation. Lysates of equivalent numbers of transformed CSC were screened for the expression of v-*rel* proteins by Western blot analysis (Fig. 29A). All transformed CSC expressed the appropriately sized proteins.

To verify that the size of proteins expressed in the CSC was the same as those expressed in infected CEF, samples were co-electrophoresed

Figure 26. Expression of v-*rel* deletion mutants in CEF.
Proteins in the lysates of CEF infected with REV-ΔR2L (ΔR2L), REV-REL (REL), REV-ΔN11 (ΔN11), REV-ΔN99 (ΔN99), REV-ΔENV (ΔENV), REV-ΔC19 (ΔC19) and REV-ΔC100 (ΔC100) were separated on 10% polyacrylamide gels containing SDS and were subjected to Western blot assay with B-1 antiserum. The position and size (kilodaltons) of standard prestained protein markers are shown.

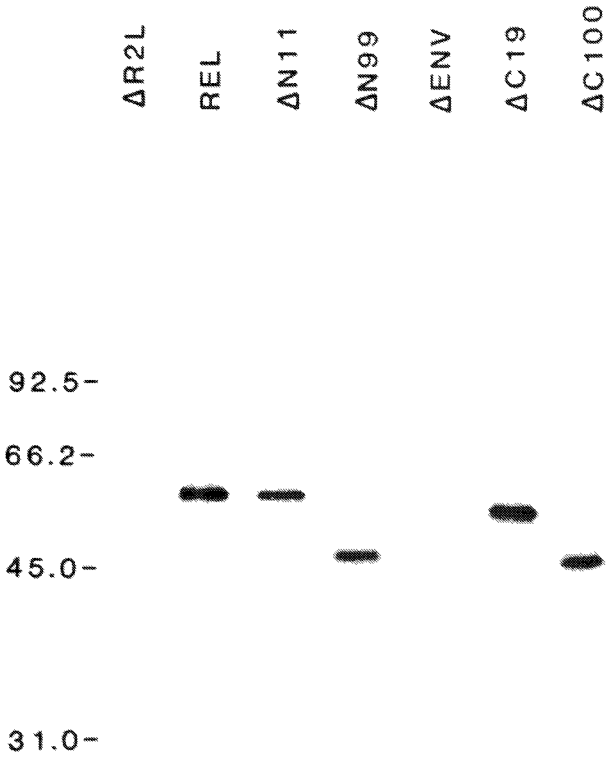

ΔR2L
REL
ΔN11
ΔN99
ΔENV
ΔC19
ΔC100
92.5-
66.2-
45.0-
31.0-

Figure 27. Immunofluorescence of CEF infected with REV-REL and v-*rel* deletion mutants. Immunofluorescence assays were done with B-1 antiserum on CEF, and CEF 24 hours after infection with REV-REL, REV-ΔN11, REV-ΔN99, REV-ΔENV, REV-ΔC19 and REV-ΔC100. The white bar in the bottom right panel represents 20μm.

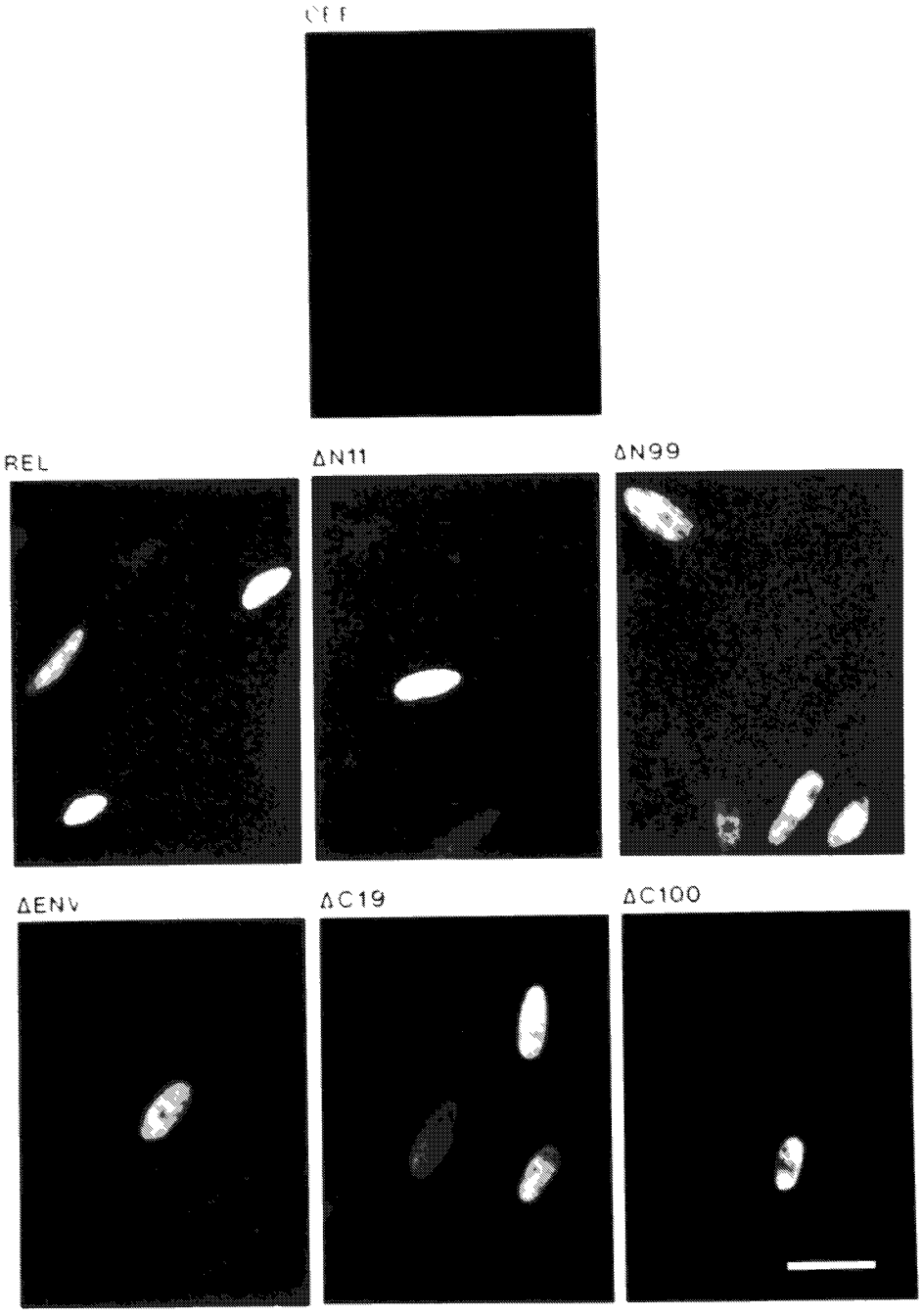

CEF
REL
ΔN11
ΔN99
ΔENv
ΔC19
ΔC100

Table 2

TRANSFORMATION OF CSC BY V-*REL* DELETION MUTANTS

Mutant	# of Assays	Average Number of Colonies	Titer of Virus: Average Number of Fluorescent Nuclei[a]
ΔR2L	24	0	nd[b]
REL	9	>100	28.0 (100)
ΔN11	25	0.3	16.5 (59)
ΔENV	18	0	5.4 (19)
ΔC19	9	>100	nd
ΔC100	15	3.7	nc (100)[c]

[a]The averages are of 12 experiments. Percentages relative to the wild type control (REL) are indicated in parentheses.

[b]nd, not determined.

[c]nc, Fluorescent nuclei were not counted. Approximate titer based on visual comparison with REV-REL.

Figure 28. ***In vitro*** transformed chicken spleen cells.
A) Colonies of chick spleen cells (CSC) 10 days after infection and transformation by REV-REL.
B) A control plate representing CSC 10 days after infection by REV-ΔR2L.

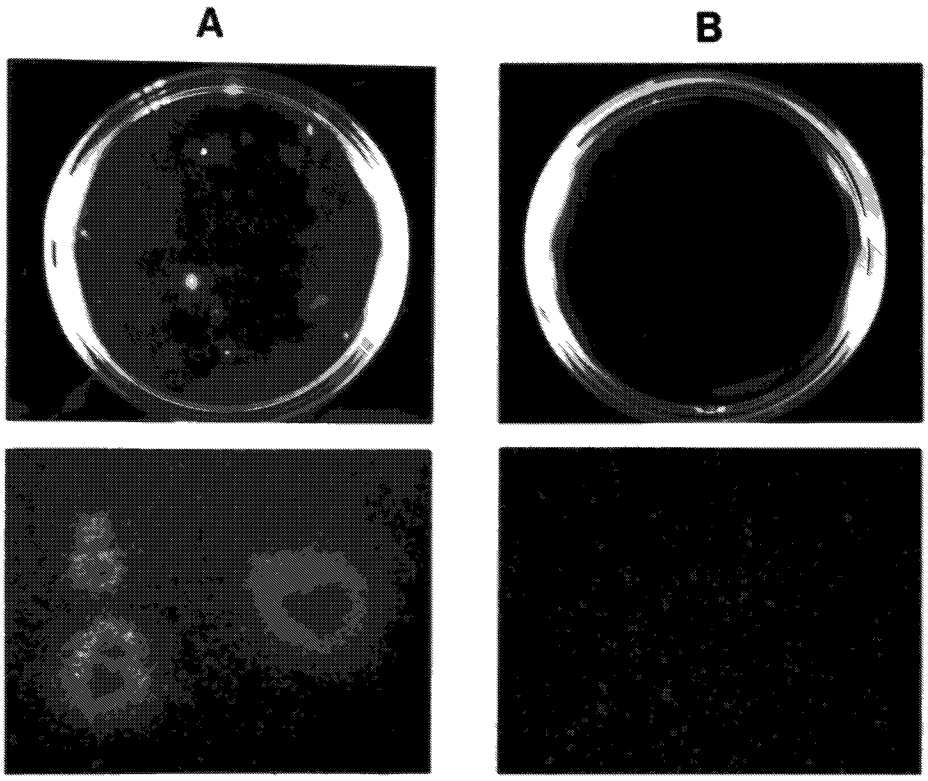
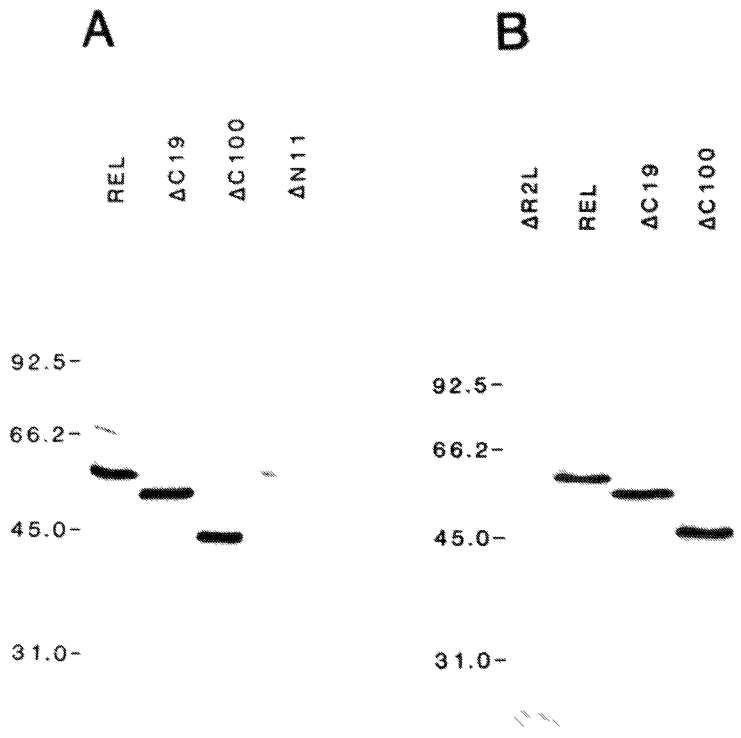

A
B

Figure 29. Expression of v-*rel* deletion mutants in transformed CSC.
A) Lysates of CSC transformed by REV-REL (REL), REV-ΔC19 (ΔC19), REV-ΔC100 (ΔC100) and REV-ΔN11 (ΔN11) were separated on 10% polyacrylamide gels containing SDS and were subjected to Western blot assay with B-1 antiserum.
B) Lysates from the transformed cells were mixed with lysates from the appropriately infected CEF and co-electrophoresed prior to Western blot analysis. The CEF and transformed CSC lysates contained approximately equivalent amounts of v-*rel* protein as determined by Western blot analysis of individual lysates. The position and size (kilodaltons) of standard prestained protein markers are shown for each panel.


A
REL
ΔC19
ΔC100
ΔN11
92.5-
66.2-
45.0-
31.0-
B
ΔR2L
REL
ΔC19
ΔC100
92.5-
66.2-
45.0-
31.0-

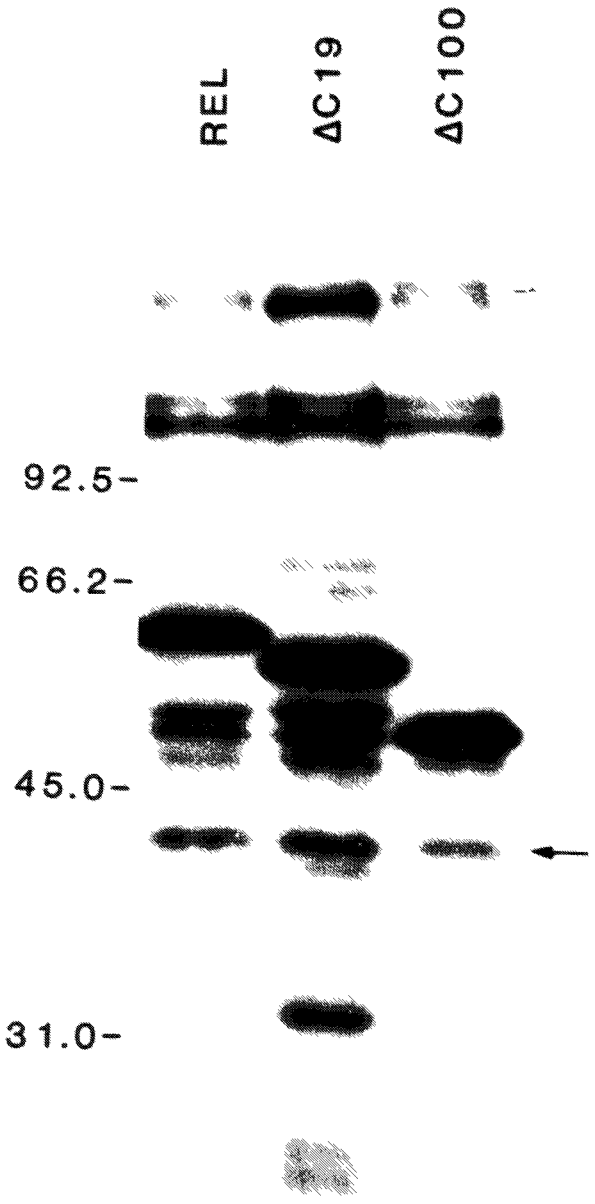
(Fig. 29B). V-*rel* proteins expressed in the transformed CSC co-migrated with their counterparts expressed in CEF. This analysis could not be extended to REV-ΔN11 due to the lack of sufficient sample. Immunoprecipitation of ^{35}S-labeled protein from transformed CSC is presented in Figure 30. Immunoprecipitation of v-*rel*, ΔC19 and ΔC100 proteins resulted in the co-precipitation of several other proteins in addition to v-*rel* (Fig. 30).

In summary, deletions were made to the N- and C-terminal coding sequences of v-*rel* to determine the importance of these sequences in transformation (Fig. 15). Deletions were made using the technique of crossover linker mutagenesis. The resulting clones expressed the appropriately sized proteins in Cos1 cells (Fig. 17). The expressed proteins were all transported to the nucleus of electroporated Cos1 cells (Fig. 18).

A retroviral vector, pREV-ΔR2L, was constructed to permit the expression of the deletion mutants in CEF and to allow the recovery of REV virus carrying the mutant genomes. The resulting viruses, REV-REL, REV-ΔN11, REV-ΔN99, REV-ΔENV, REV-ΔC100 and REV-ΔC19 expressed proteins of the predicted size (Fig. 26) which were transported to the nucleus of CEF (Fig. 27). Only viruses REV-REL, REV-ΔC19 and REV-ΔC100 were able to fully immortalize lymphoid cells in a spleen cell transformation assay (Table 2). All other viruses failed to generate colonies in this assay with the exception of REV-ΔN11 which appeared to produce colonies of cells which were not able to be propogated extensively in liquid culture.

Cell lines derived from the transformed colonies all expressed the appropriately sized proteins (Fig. 29). Finally, on the basis of an immunoprecipitation assay, the altered v-*rel* proteins expressed in the

Figure 30. Immunoprecipitation of v-*rel* deletion mutants from CSC.
CSC transformed by REV-REL (REL), REV-ΔC19 (ΔC19) and REV-ΔC100 (ΔC100) were labeled with ^{35}[S]-methionine for 2 hours. Cell lysates were immunoprecipitated with B-1 antiserum and electrophoresed on 10% polyacrylamide gels containing SDS. Gels were subjected to fluorography. The position and size (kilodaltons) of standard protein markers are shown. An arrow identifies a 40 kilodalton protein which coprecipitates with the v-*rel* protein.


REL
ΔC19
ΔC100
92.5-
66.2-
45.0-
31.0-

various cell lines co-precipitated the same set of cellular proteins as disussed previously (Fig. 30). This indicated that deletions of C-terminal coding sequences did not interfere with the ability of the resulting protein products to bind with these cellular proteins.

4. LINKER INSERTION MUTAGENESIS OF V-*REL*

a) Construction of mutants

In order to map functional regions throughout the v-*rel* gene, linkers were inserted which maintained the v-*rel* reading frame, but added 4 new amino acids. The disruption of functional regions by linker insertions would be expected to abolish transforming activity.

The v-*rel* oncogene, with the 5' noncoding sequences deleted, was removed from pSVLREL by a partial BamHI digestion. This fragment was inserted into pUC19 and a clone was selected (pUC-v-rel) which had the 5' end of v-*rel* adjacent to the SalI site of the multiple cloning site of pUC19. The v-*rel* gene could be excised from pUC-v-rel after SalI digestion, by cleavage of the SalI site in the pUC19 multiple cloning site (5' to v-*rel*) and cleavage of the SalI site (originally from pSVL/SalI) located between the 3' end of v-*rel* and the BamHI site.

pUC-v-rel was partially digested using controlled time courses with BamHI, EcoRI, FspI, HindII, HindIII or XmnI. Partial digests controlled with ethidium bromide were made with PvuII or RsaI. 12-mer XhoI linkers (CCGCTCGAGCGG) were inserted into gel-purified linear size plasmids cut with FspI, HindII, XmnI, PvuII or RsaI. Gel-purified linear size plasmids cut with BamHI, EcoRI or HindIII were made blunt-ended by treatment with Klenow prior to insertion of 8-mer XhoI linkers (CCTCGAGG). Clones were selected which had incorporated XhoI linkers into the v-*rel* coding region without the deletion of sequence.

PvuII linker insertion mutants were not isolated due to the strong preference for cleavage at the 2 sites located in pUC19 sequence. In one experiment, 30 linker insert mutants were all found to contain XhoI linkers inserted into the PvuII sites of pUC19. In order to avoid this problem, pBR322 was first modified by destroying the PvuII site by insertion of an XbaI linker. The v-*rel* gene was removed from pUC-v-rel by SalI digestion and inserted into the SalI site of the modified pBR322 to generate plasmid pBR-REL. In a similar fashion, linker insertion mutants were derived after partial PvuII digestion of pBR-REL. In addition, a third RsaI linker insertion mutant was isolated.

Table 3 lists the linker insertion mutants derived using these approaches. The clones have been named according to the position of the first amino acid in the predicted protein sequence which differs from that of the wild type v-*rel* protein sequence.

b) Expression and transforming activity of v-*rel* linker insertion mutants

In order to test the biological activity of each mutant, they were transferred into the retroviral vector, pREV-ΔR2L. Each linker insertion mutant and the wild type v-*rel* gene were excised from their respective plasmids by SalI digestion and subcloned into pREV-ΔR2L. The plasmid resulting from the insertion of the wild type v-*rel* gene into pREV-ΔR2L was named pREV-LREL to differentiate it from the slightly different pREV-REL whose origin was described earlier.

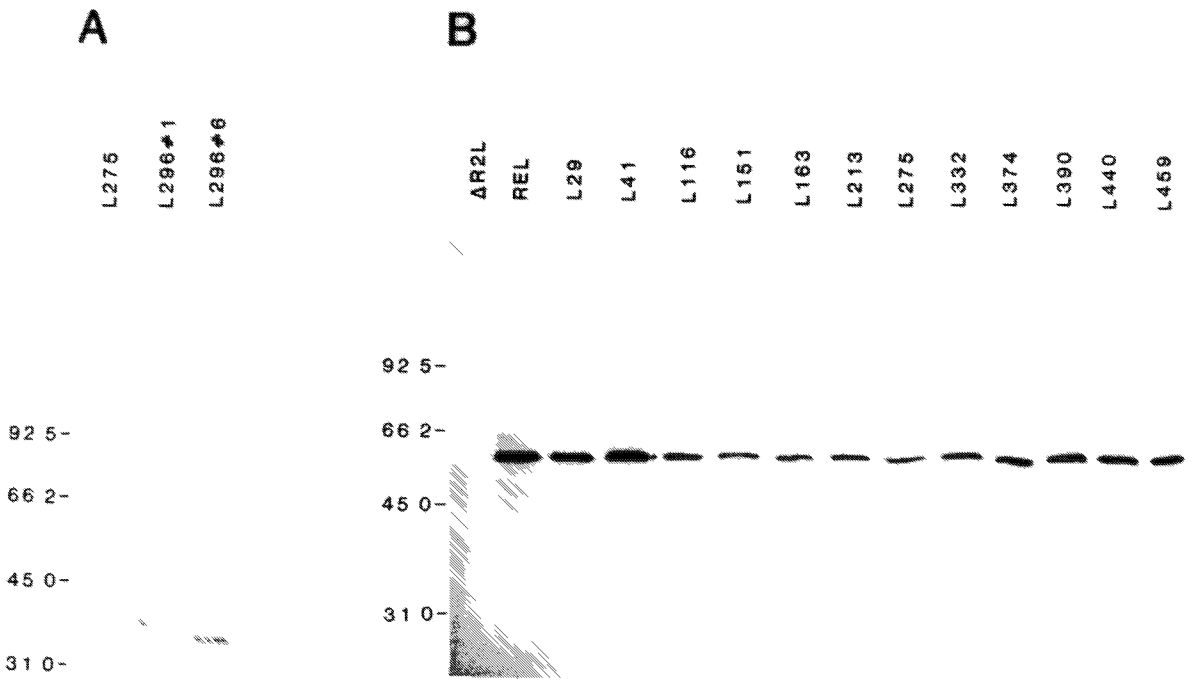
pREV linker insertion clones were co-transfected into CEF with pREV-A and virus was recovered. Lysates of infected CEF were screened for v-*rel* protein expression by Western blot assay. Two independent linker insertion mutants of pREV-L296 expressed truncated proteins (Fig. 31A). These mutants were not used for subsequent studies. The remaining

Table 3

LINKER INSERTION MUTANTS OF V-*REL*

Enzyme	Site	Vector	Name
RsaI	84	pUC-v-rel	L29
PvuII	118	pBR-REL	L41
EcoRI	341	pUC-v-rel	L116
RsaI	451	pBR-REL	L151
HindIII	482	pUC-v-rel	L163
XmnI	635	pUC-v-rel	L213
XmnI	819	pUC-v-rel	L275
BamHI	876	pUC-v-rel	L296
HindII	993	pUC-v-rel	L332
FspI	1118	pUC-v-rel	L374
PvuII	1167	pBR-REL	L390
EcoRI	1308	pUC-v-rel	L440
PvuII	1369	pBR-REL	L459

Figure 31. Expression of v-*rel* linker insertion mutants in CEF.
Lysates of CEF infected with the indicate REV viruses were separated on 10% polyacrylamide gels containing SDS and were subjected to Western blot assay with B-1 antiserum. The position and size (kilodaltons) of standard prestained protein markers are shown. Panel A shows the truncated proteins expressed by L296 mutants. Panel B shows the expression of all linker insertion mutants used for further experiments.


A
L275
L296#1
L296#6
92 5-
66 2-
45 0-
31 0-
B
ΔR2L
REL
L29
L41
L116
L151
L163
L213
L275
L332
L374
L390
L440
L459
92 5-
66 2-
45 0-
31 0-

mutants all expressed proteins similar in size to the wild type virus (Fig. 31B).

The regions of linker insertion of the mutants were sequenced directly using rel-specific primers (See appendix 2). Sequencing was done using Sequenase with the replacement of dGTP by dITP to avoid compression problems generated by the palindromic sequence of the inserted linkers. All sequences were as expected for the insertion of a single linker. A summary of the linker insertion mutants is presented in Figure 32.

Indirect immunofluorescence assays showed similar nuclear localization of the v-*rel* proteins (Fig. 33) as observed earlier (Fig. 27). One mutant, 116, reproducibly showed a light diffuse cytoplasmic staining in addition to the heavier nuclear staining. The weak cytoplasmic staining is not readily seen in Figure 33 after photographic reproduction.

Virus recovered from transfected CEF was also tested for the ability to transform CSC. The results of the *in vitro* transformations are presented in Table 4. The titers of all viruses were judged to be the same as wild type level as determined by immunofluorescence. Briefly, N-terminal linker insertion mutants all failed to transform CSC, whereas all C-terminal linker insertion mutants beginning with pREV-L332 were positive for transformation.

The expression of v-*rel* proteins in the transformed cells was demonstrated by Western blot analysis (Fig. 34a). V-*rel* proteins expressed in transformed CSC were co-electrophoresed with their counterparts expressed in CEF prior to Western blot analysis (Fig. 34a). Proteins expressed in either cell appeared to have the same mobility. Immunoprecipitation of v-*rel*, L332, L374, L390, L440 and L459 proteins

Figure 32. Linker insertion mutants of *v-rel.*
The restriction site, the amino acid position and the sequence of amino acids inserted by the various linker insertion mutants are shown.

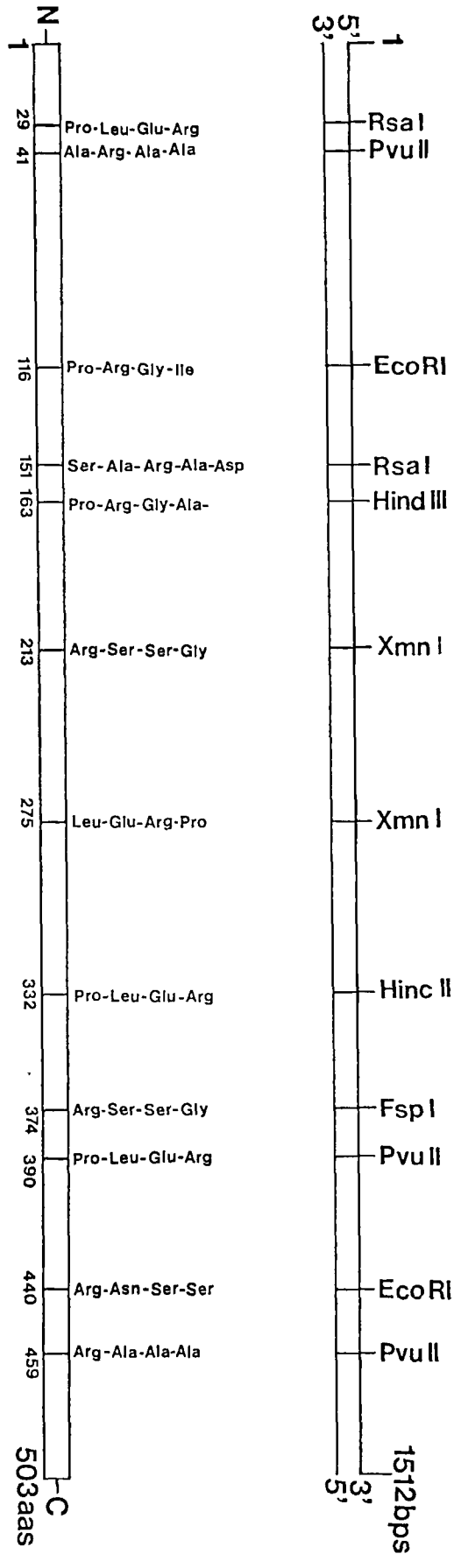

N
1
29
41
116
151
163
213
275
332
374
390
440
459
C
503aas
Pro-Leu-Glu-Arg
Ala-Arg-Ala-Ala
Pro-Arg-Gly-Ile
Ser-Ala-Arg-Ala-Asp
Pro-Arg-Gly-Ala-
Arg-Ser-Ser-Gly
Leu-Glu-Arg-Pro
Pro-Leu-Glu-Arg
Arg-Ser-Ser-Gly
Pro-Leu-Glu-Arg
Arg-Asn-Ser-Ser
Arg-Ala-Ala-Ala
5'
3'
1
RsaI
PvuII
EcoRI
RsaI
HindIII
XmnI
XmnI
HincII
FspI
PvuII
EcoRI
PvuII
3'
5'
1512bps

Figure 33. Immunofluorescence of CEF infected with REV-REL and v-*rel* linker insertion mutants.
Immunofluorescence assays were done with B-1 antiserum on CEF, and CEF 24 hours after infection with the indicated virus. The white bar in the bottom right panel represents 20μm.

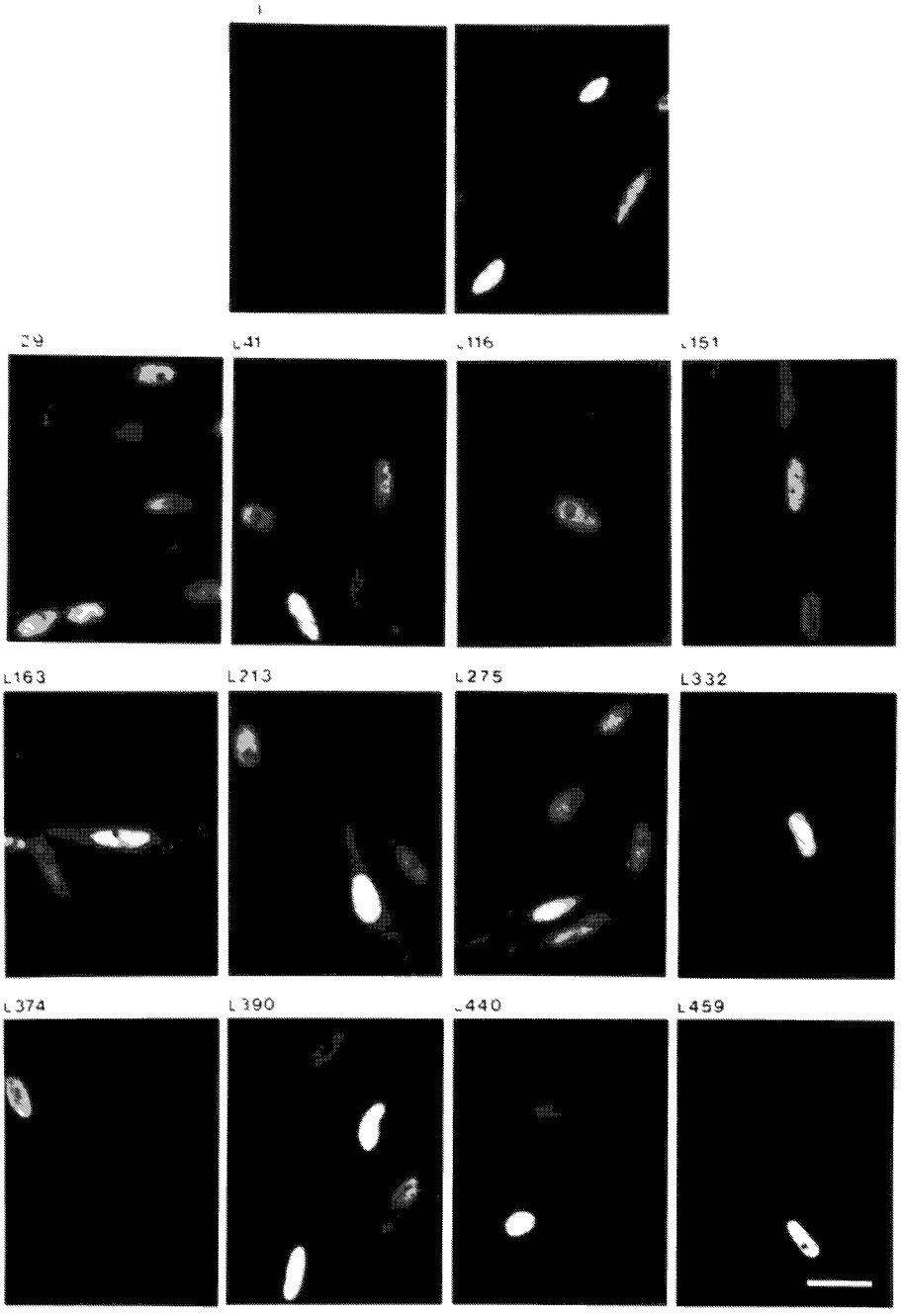
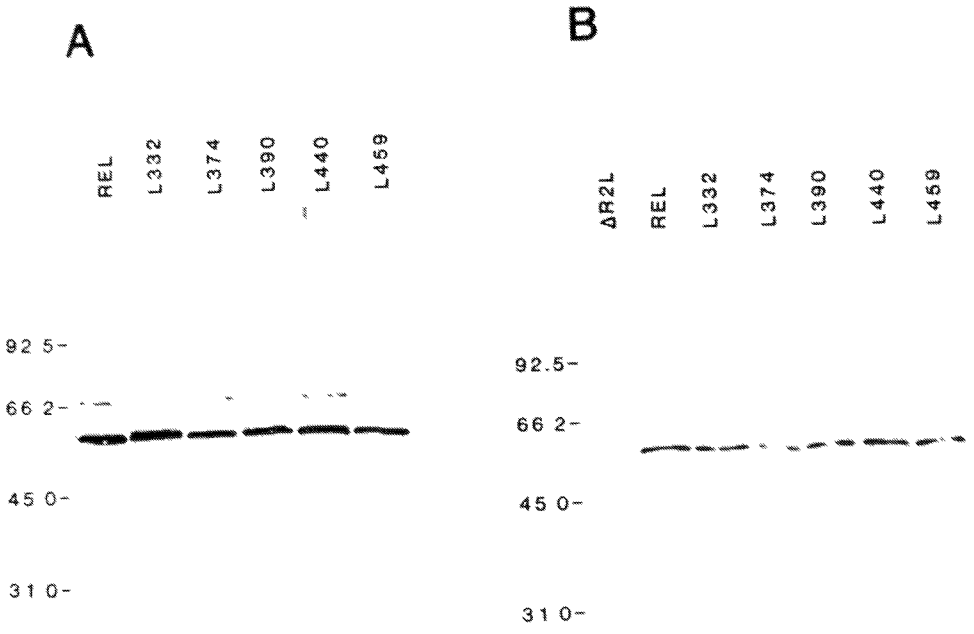

29
L41
L116
L151
L163
L213
L275
L332
L374
L390
L440
L459

Table 4

TRANSFORMATION OF CSC BY V-*REL* LINKER INSERTION MUTANTS

Mutant	# of Assays	Average # of Colonies
ΔR2L	24	0
LREL	12	>100
L29	11	0
L41	9	0
L116	10	0
L151	9	0
L163	9	0
L213	9	0
L275	9	0
L332	9	>100
L374	9	>100
L390	9	>100
L440	9	>100
L459	9	>100

Figure 34. Expression of v-*rel* linker insertion mutants in transformed CSC.
A) Lysates of CSC transformed by the indicated viruses were separated on 10% polyacrylamide gels containing SDS and were subjected to Western blot assay with B-1 antiserum.
B) Lysates from the transformed cells were mixed with lysates from the appropriately infected CEF and co-electrophoresed prior to Western blot analysis. The CEF and transformed CSC lysates contained approximately equivalent amounts of v-*rel* protein as determined by Western blot analysis of individual lysates. The position and size (kilodaltons) of standard prestained protein markers are shown for each panel.


A
REL
L332
L374
L390
L440
L459
92 5-
66 2-
45 0-
31 0-
B
ΔR2L
REL
L332
L374
L390
L440
L459
92.5-
66 2-
45 0-
31 0-

resulted in the co-precipitation of several other proteins in addition to v-*rel* (Fig. 35).

In summary, Xho1 linkers were inserted into the v-*rel* gene to generate 12 linker insertion mutants (Fig. 32). Subcloning of the mutant genes into the vector pREV-ΔR2L permitted the recovery of virus for further studies. Infection of CEF with the resulting viruses resulted in the expression of wild type sized proteins for all of the linker insertion mutants (Fig. 31). The protein products of all of the mutant genes were transported to the nucleus of CEF (Fig. 33). Determination of the transforming potential of each mutant (Table 4) indicated that changes to the N-terminus (amino acids 29-275) abolished transforming activity, whereas changes to the C-terminus (amino acids 332-459) did not alter this activity.

Cell lines established from mutants REV-L332, REV-L374, REV-L390, REV-L440 and REV-L459 all expressed wild type sized v-*rel* proteins (Fig. 34). Immunoprecipitation analysis (Fig. 35) indicated that all of the transforming mutants expressed v-*rel* proteins which were able to bind and co-precipitate the same set of cellular proteins as observed for the wild type.

All of the results of the transformations for deletion and linker insertion mutants are summarized in Table 5.

Figure 35. Immunoprecipitation of v-*rel* linker insertion mutants from CSC.
CSC transformed by the indicated viruses were labeled with ^{35}[S]-methionine for 2 hours. Cell lysates were immunoprecipitated with B-1 antiserum and electrophoresed on 10% polyacrylamide gels containing SDS. Gels were subjected to fluorography. The position and size (kilodaltons) of standard protein markers are shown. An arrow identifies a 40 kilodalton protein which coprecipitates with the v-*rel* protein.

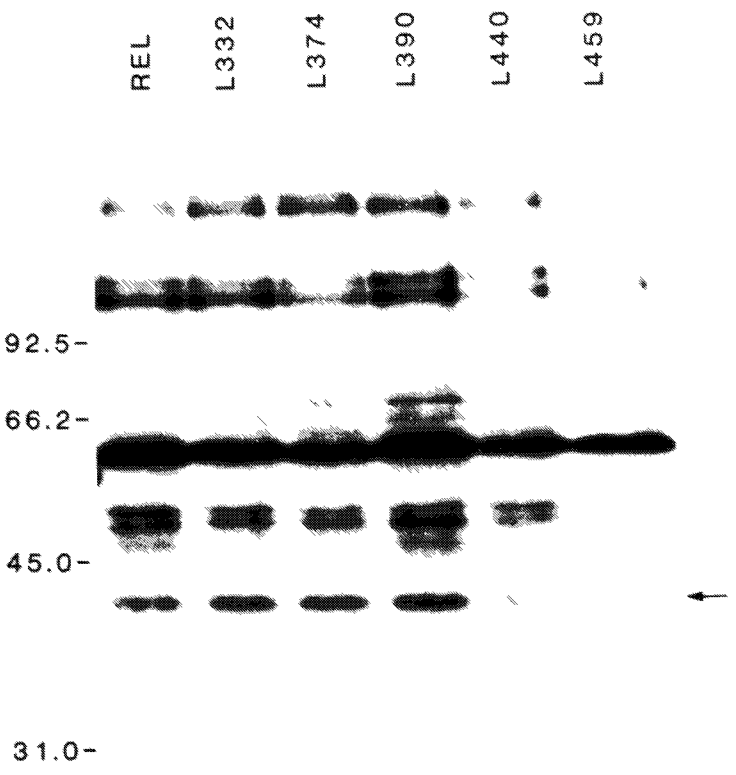

REL
L332
L374
L390
L440
L459
92.5-
66.2-
45.0-
31.0-

TABLE 5

SUMMARY OF V-REL DELETION AND LINKER INSERTION MUTANTS

Mutant	CEF Cellular Location	Expression Level in CEF	Transforming Activity
ΔR2L	-	-	-
REL	nucleus	wt[a]	+++++
ΔN11	nucleus	reduced	+/- [b]
ΔN99	nucleus	wt	-
ΔENV	nucleus	reduced	-
ΔC19	nucleus	wt	+++++
ΔC100	nucleus	wt	+
L29	nucleus	wt	-
L41	nucleus	wt	-
L116	nucleus	wt	-
L151	nucleus	wt	-
L163	nucleus	wt	-
L213	nucleus	wt	-
L275	nucleus	wt	-
L332	nucleus	wt	+++++
L374	nucleus	wt	+++++
L390	nucleus	wt	+++++
L440	nucleus	wt	+++++
L459	nucleus	wt	+++++

[a]wt, expression of proteins was at wild type levels.

[b]+/-, transformed colonies were observed, but they could not be propogated.

DISCUSSION

Understanding the ways in which viral oncogenes transform normal cells may reveal the mechanisms which underlie the development of human cancers. As well, the identification of several of the viral oncogene products as components of the mitogenic pathways indicates that the study of viral oncogenes may advance our knowledge of basic events involved in signal transduction and cell growth.

The v-*rel* oncogene was interesting because it transformed immature lymphoid cells and showed no significant homology to other viral oncogenes. V-*rel* may mediate transformation of cells through mechanisms different from the other viral oncogenes. The primary objectives of my research were to identify the protein product of v-*rel* and create a series of mutants which may allow a better understanding of its function.

I have arranged this discussion to deal with some of the technical aspects of the experiments in sections 1, 2 and 3, and to discuss the results which advance our knowledge of the v-*rel* oncogene in sections 4, 5 and 6.

1. IDENTIFICATION OF THE V-REL GENE PRODUCT

a) Use of peptide antiserum

The selection of the peptide used for immunization was limited to amino acid sequences present in hydrophilic portions of the predicted protein sequence. The sequence chosen was extremely hydrophilic and contained an internal proline residue. The rationale for such a selection was to optimize the chances that the resulting antiserum would react with the v-*rel* protein. The assumption was that the peptide sequence would be externally situated on the native protein due to its hydrophilic character. While peptide antiserum was able to react with the denatured v-*rel*

protein in Western blot assays (Fig. 6), it was unable to immunoprecipitate or react in immunofluorescence assays. Affinity purified antibody was also unable to react in immunoprecipitation assays. The most trivial explanation for this reactivity pattern is based on protein conformation. Antibodies which recognize the peptide may be unable to recognize this amino acid sequence in the fully folded protein despite the criteria discussed above for the selection of the peptide. Denaturation of the protein following treatment with SDS during Western blot analysis may permit the interaction of the antibodies with the proper epitope. Other possibilities include technical problems which could have resulted from either low affinity antibodies, low titers of antibody or a combination of these two factors.

The peptide antiserum did permit the identification of the v-*rel* protein product expressed in chicken bone marrow cells transformed by REV-T (Fig. 6)(Garson and Kang 1986). The apparent size of this product, 55 kilodaltons is in close agreement with the predicted 56 kilodalton size of the putative v-*rel* gene product derived from the nucleic acid sequence (Stephens *et al.* 1983, Wilhelmsen *et al.* 1984). This molecular weight estimation was based on a comparison of the mobility of the v-*rel* protein with the mobility of prestained protein standards.

b) Use of v-*rel-lacZ* antiserum

The limitations of the peptide antiserum required that alternate antiserum be prepared. The purification of eukaryotic polypeptides expressed in *E. coli* is often simplified by their frequent insolubility in bacteria (Marston 1986). The insertion of v-*rel* coding sequence in-frame with the *lacZ* gene of pUC19 permitted the expression of proteins which were found to be in the insoluble fraction of lysed *E. coli* (Fig.8). The

fusion protein expressed from pLR-P/E often represented as much as 40% of the protein present in crude pellets from lysed cells. The v-*rel/lacZ* fusion protein selected for immunization was expressed from plasmid pLR-PE, which contained 69% of the total coding information of v-*rel*, representing amino acids 92-437 of the predicted v-*rel* protein.

The v-*rel/lacZ*, or B-1 antiserum reacted well with the v-*rel* protein in Western blots and immunoprecipitations (Fig.9). A more accurate determination of the size of the v-*rel* protein was made after immunoprecipitation. Previously, the size of the v-*rel* protein was based on comparison with the mobility of prestained molecular weight markers. Comparison of the mobility of the v-*rel* protein after immunoprecipitation with the mobility of untreated protein molecular weight markers suggested an apparent size of 58 kDa. Similarly, others have estimated the v-*rel* protein to have a molecular size of 57 kDa (Herzog and Bose 1986) and 59 kDa (Rice *et al.* 1986, Gilmore and Temin 1986). In addition, the v-*rel* protein product was reported to be phosphorylated on serine residues (Rice *et al.* 1986, Gilmore and Temin 1986). Other post-translational modifications were not detected (Rice *et al.* 1986). The protein product of the v-*rel* oncogene has been designated $pp59^{v\text{-}rel}$ (Gilmore and Temin 1986). I have used this designation throughout my thesis.

2. EXPRESSION OF V-REL IN COS1 CELLS

Cos1 cells constitutively express the large T antigen of SV40 permitting the replication of SV40 based vectors and the transcription from their late promoters (Gluzman 1981). The expression vectors used in this study were pJC119 and the related plasmid pSVL. pJC119 has sequences derived from the bacterial plasmid pML2 allowing its replication in *E. coli* and sequences from a late-region deletion mutant of SV40

permitting its amplification in mammalian cells in the presence of the large T antigen of SV40 (Sprague *et al.* 1983). The Xho1 cloning site of pJC119 (Fig. 10) or the multiple cloning site of pSVL (Fig. 14) span a deletion which removes the majority of the VP1 gene including the translation initiation codon, however, sites required for correct transcriptional initiation, splicing and polyadenylation of the late transcripts are retained (Sprague *et al.* 1983). Translation initiation and termination signals are provided by the foreign genes cloned into the Xho1 or multiple cloning sites. Foreign gene expression is transient and can be detected usually 1-4 days following transfection (Subramani and Southern 1983).

The v-*rel* oncogene was expressed in Cos1 cells to establish a model for further study of the v-*rel* protein. *In vitro* mutagenesis of the v-*rel* gene directly in the vectors pJC119 or pSVL permitted the expression of the altered genes and detection of their protein products 48 hours following transfection of the plasmids. This rapid screening permitted initial confirmation of the fidelity of the mutagenesis and offered the possibility of biochemical characterization of the expressed proteins.

Cos1 cells transfected with pJC119 or pSVL-REL were analyzed for proper replication and transcription of the plasmids. DNA transfected into the Cos1 cells was differentiated from DNA replicated after transfection on the basis of its sensitivity to cleavage by restriction enzymes Dpn1 and Mbo1 (Rio *et al.* 1985). Dpn1 can only cleave DNA which is methylated on the adenine base of its recognition site (Geier and Modrich 1979). Such methylation occurs in *E. coli* as a consequence of the *dam* methylation system (Marinus and Morris 1973, Geier and Modrich 1979), but does not occur in mammalian cells. Mbo1 cleaves DNA which has not

been methylated (Gelinas *et al.* 1977), and is able to cleave DNA replicated in mammalian cells but not DNA replicated in *E. coli* which have an active *dam* methylase. Southern blot analysis of Hirt DNA transfected with pJC119 and pSVL-REL and treated with restriction enzymes Mbo1 and Dpn1 revealed that the plasmid DNA detected was DNA which had been replicated in the Cos1 cells (Fig. 11).

Northern blot analysis indicated that a single mRNA containing v-*rel* sequence was synthesized in the Cos1 cells transfected with pSVL-REL. Transcription of the v-*rel* gene from the late promoter in pSVL-REL would be predicted to produce a transcript containing approximately 400 bases derived from the leader region (Subramani and Southern 1983) spliced to 1550 bases of v-*rel* sequence followed by 1-2 hundred bases of the poly-A tail. The single species of RNA detected by Northern blot analysis had a size of 2100 bases, consistent with the predicted size for the properly spliced message from pSVL-REL (Fig. 11). The ability of this RNA to translate was tested in an *in vitro* translation system. A translation product of appropriate size present only in translations of total RNA from Cos1 cells transfected with pSVL-REL was tentatively identified as the v-*rel* protein (Fig. 12). Since immunoprecipitating antiserum was not available at this time, the immunological identification of this protein as the v-*rel* translation product could not be made.

Expression of v-*rel* in Cos1 cells was confirmed by Western blot analysis of transfected cell lysates using peptide antiserum (Fig. 13). The protein product expressed from pSVL-REL was similar in size to pp59$^{v\text{-}rel}$ expressed in the transformed CBMC.

3. MUTAGENESIS OF V-REL

a) Crossover linker mutagenesis

i. Technical considerations

The general method for crossover linker mutagenesis requires ligation of the oligonucleotide on to the plasmid DNA followed by transformation into *E. coli*. A number of potential problems were initially encountered with the application of this method to delete regions of the v-*rel* gene. I observed that it was of critical importance to prevent more than one oligonucleotide from ligating to the vector DNA.

Oligonucleotides used for deletion of 5' v-*rel* sequence were synthesized to insert BamH1 sites 5' to the deletion. When plasmid pSVLvREL was digested with Xho1 alone or if the second digest, Xba1, was incomplete, the oligonucleotides could ligate to both Xho1 termini of the linear DNA. Transformation of this material resulted in the efficient production of clones which had inserted BamH1 sites without the deletion of the desired region. It appeared that the palindromic sequence of the BamH1 sites present in the crossover oligonucleotides allowed the two linkers to anneal and stabilize a circular DNA for transformation of *E. coli*. A similar event was observed to occur when 3' end deletions were attempted even when the crossover oligonucleotides did not code for restriction enzyme sites. In these instances, it was possible that the Sal1 termini to which the oligonucleotides were attached managed to anneal. The use of double stranded linkers did not alleviate this problem.

There appeared to be two ways of avoiding these problems: the use of double digested vector DNA, or the use of lower ratios of oligonucleotide to vector DNA to reduce the chances of two oligonucleotides attaching to the single digested vector DNA (D.W. Yoo,

personal communication). The methods described in the Materials and Methods section were designed to ensure that the plasmid DNA was completely digested with two different enzymes and hence each plasmid molecule carried only a single oligonucleotide prior to transformation into *E. coli*.

ii. Design of the deletions

Two deletions were made to directly test the functional contribution of viral *env* sequence to v-*rel*. These deletions removed the 11 N-terminal amino acids (ΔN11) or the 19 C-terminal amino acids (ΔC19). Although the first 12 amino acids are coded by the *env* gene, I chose to delete only 11 to place a G nucleotide at position +4 of ΔN11. The context of the resulting initiation codon for ΔN11 was that suggested by Kozak to be ideal for translation (Kozak 1986), namely, ACCATGG. All deletion clones had initiation codons in this context.

Two additional deletion mutants were constructed which coded for proteins with 99 N-terminal amino acids deleted (ΔN99) or 100 C-terminal amino acids deleted (ΔC100). These mutants were constructed as reference points for any further deletion studies that might be undertaken.

Mutants ΔN99, ΔC19, ΔC100 and the wild type gene expressed proteins of appropriate size in Cos1 cells (Fig.17). Mutants ΔN11 and ΔENV were not available for this analysis, but their expression was detected by IFA in conjunction with the other mutants (Fig. 18).

b) Linker insertion mutagenesis

One interesting technical aspect of the linker insertion mutagenesis was the biased cleavage pattern observed for several of the restriction enzymes. Whether partial digests were controlled in a time course or by inclusion of ethidium bromide in the digestion buffer, preferential

insertion of linkers into certain cleavage sites was observed. An extreme example of this was observed for PvuII. Unfortunately, cleavage of the two PvuII sites present in pUC19 occurred very efficiently, whereas cleavage of any of the four sites in v-*rel* was not observed. This type of biased cleavage has been reported (Thomas and David 1975, Forsblum *et al.* 1976) and can effectively prevent the recovery of certain desired linker insertion mutants.

3. CONSTRUCTION OF A RETROVIRAL VECTOR

The transforming function of REV-T can be demonstrated by the *in vitro* transformation of CSC. The target cell for transformation by REV-T represents only 17 out of 10^6 lymphoid cells in the spleen (Lewis *et al.* 1981). Therefore, the only practical method of introducing mutants of the v-*rel* gene into such a limited number of target cells is by viral infection.

Packaging cell lines have been described for REV-T, but the titer of virus recovered after transfection of replication defective viral DNAs was 2×10^3 or less (Watanabe and Temin 1983). Given the low number of target cells in the spleen cell population, the viral titers derived from the helper cell line would be too low to ensure cell transformation. In order to avoid this problem, I constructed a retroviral vector which upon co-transfection with competent helper virus DNA into CEF would produce virus of sufficient titers to infect and transform the appropriate target cells.

The primary consideration in the construction of a retroviral vector is to maintain the required *cis* acting sequences for replication and transcription including the LTR, pbs, ppt, sd, sa, and E sites. The level of expression of the introduced mutated genes must also be at a level comparable to the wild type virus. Viruses which express v-*rel* at 5% of

the level of the wild type were unable to efficiently transform CSC (Miller and Temin 1986).

In order to maintain all required *cis*-elements, the v-*rel* oncogene including 27 nucleotides of 5' and 32 nucleotides of 3' noncoding sequences was excised, and all other viral sequences retained. The only *cis* acting sequences adjacent to v-*rel* are the splice acceptor (sa) site and the polypurine tract. The sa site for REV-T was found to be 260bp 5' to the v-*rel* initiation codon (Miller and Temin 1986). The ppt is located 12bp 3' of the *env* gene of REV-A, and based on available sequence data (H.M. Temin, personal communication), it is at least 100bp 3' to the v-*rel* termination codon. Thus the deletion of REV-T sequences to generate cloning vector pREV-ΔR2L did not remove any of the required *cis*-elements for viral replication and transcription (Figs. 19, 20, 21, 22). Genes inserted into the Xho1 cloning site of pREV-ΔR2L would be expressed from a subgenomic RNA.

4. EXPRESSION OF V-REL MUTANTS IN CEF

All v-*rel* mutants expressed at relatively the same level after infection of CEF with the exception of ΔN11 and ΔENV (Fig. 26). The levels of expression of ΔENV and ΔN11 were usually lower than the wild type. The 5' viral sequences of ΔENV were derived from ΔN11 during its construction (Fig. 25). To rule out the possibility that a rearrangement or mutation of viral sequences important for viral replication or transcription was present in pREV-ΔN11 and had been transferred into pREV-ΔENV, these clones were reconstructed independently of each other. The resulting plasmids also expressed lower levels of the pp59$^{v\text{-}rel}$ (data not shown). This suggests that the reduced titers of virus derived from these

plasmids was not due to a mutation in the viral sequences required for replication.

The reduced level of expression of v-*rel* by ΔN11 and ΔENV viruses was accompanied by reduced titers of virus following transfection of CEF with their corresponding DNAs along with helper virus DNA (Table 2). The approximate level of expression within infected cells was estimated by immunofluorescence. The virus REV-ΔN11 expressed at levels comparable to or perhaps only slightly lower than the wild type virus. REV-ΔENV, however, expressed at a lower level than the wild type virus. The reduced expression of ΔN11 and ΔENV as seen in Western blots may be in part a consequence of the lower viral titers. One explanation for these results would be that the transfections were inefficient due to the use of poor quality plasmid DNA. This possibility was unlikely since several different preparations of plasmid DNA all gave lower v-*rel* expression, whereas virtually all other preparations of plasmid for other v-*rel* mutants expressed at wild type levels following transfection (Figs. 26, 31). It is possible that the deletion of the N-terminal sequences created genomic RNAs which were less efficiently transcribed, more labile or inefficiently packaged into virus. Interestingly, Sylla and Temin (1986) found that a deletion of the N-terminus of v-*rel* also resulted in slightly lower titers of virus, although the level of expression in individual cells as judged by immunofluorescence was not reduced.

The reduced level of expression of ΔENV in infected cells as determined by immunofluorescence may be the result of decreased stability of the expressed protein or RNA, or reduced transcription. In addition, because of the reduced cellular expression of ΔENV, the

estimated titers (Table 2) may have been underestimated, since very weakly fluorescent nuclei were difficult to count.

The size of the proteins expressed by the deletion mutants of v-*rel* were approximately as predicted by the sequence (Fig. 26). ΔN11 expressed a protein which migrated only slightly lower than pp59$^{v\text{-}rel}$ and was predicted to have a molecular weight approximately 1,200 less than the wild type. ΔC19 expressed a protein which was of reduced apparent molecular weight. The predicted reduction in molecular weight from pp59$^{v\text{-}rel}$ was approximately 2,000. While it is difficult to experimentally measure such small reductions of molecular weight by SDS-PAGE analysis, it appears clear that the mobility of the ΔN11 product did not reduce as substantially as expected when compared with the product of ΔC19. Likewise, the predicted molecular weights of ΔN99 and ΔC100 are 44,616 and 45,402, respectively. The expressed product of ΔC100 in CEF (Fig. 26) or in Cos1 cells (Fig. 17) did appear to migrate faster than the product of ΔN99. While these mobility changes could be due to changes in amino acid composition, or due to structural features maintained after SDS-denaturation, it is also possible that the loss of amino acid sequence could involve the loss of phosphorylation sites resulting in further unpredicted mobility changes.

All linker insertion mutants expressed proteins which appeared to have the same mobility as pp59$^{v\text{-}rel}$ (Fig. 31).

5. IMMUNOLOGICAL DETECTION OF pp59$^{v\text{-}rel}$

a) Western blots and Immunoprecipitation

B-1 antiserum identified a single prominent band in CEF infected with REV-REL (Figs. 9, 26). Screening of transformed lymphoid cells by Western blot assay revealed one major band and a second minor band

with an apparent molecular weight of 68 kDa (Fig. 29a). This second band was not evident in the less developed Western blot presented in Figure 9. This second band may represent the c-*rel* protein. The c-*rel* protein, p68$^{c\text{-}rel}$, was recently identified as a 68 kDa protein (Simek and Rice 1988a). The expression of rel-specific RNA has been shown to be highest in avian hematopoietic tissues (Herzog *et al.* 1986) and the quantity of c-*rel* protein expressed was higher in lymphoid cells than in CEF (Simek and Rice 1988a). This may explain why this 68 kDa protein was detected in the transformed lymphoid cells and not in CEF.

A single specific band was seen in the autoradiogram of the immunoprecipitate of infected CEF (Fig. 9c). In contrast, the same sera immunoprecipitated multiple bands from the transformed CBMC (Fig. 9a). In addition to pp59$^{v\text{-}rel}$, a 40 kDa protein (p40) indicated with an arrow, and a larger 120 kDa protein were also immunoprecipitated (p120). Preimmune serum failed to precipitate the same proteins. These additional proteins are either associated with pp59$^{v\text{-}rel}$ and co-precipitate or cross react with the B-1 antiserum. Recently, the 40 kDa protein (Simek and Rice 1988b, Tung *et al.* 1988) and several other proteins (Simek and Rice 1988b) were shown to be associated with pp59$^{v\text{-}rel}$ in a 400 kDa complex. In addition to p40, Simek and Rice (1988b) reported that the c-*rel* protein, p68, a 115 kDa protein (p115) and a 124 kDa (p124) protein formed a complex with pp59$^{v\text{-}rel}$. p115 and p124 were not always well resolved in their experiments and sometimes appeared as a single band. Similarly, I observed both a single band (p120) (Fig. 9a) or a doublet in different experiments (Figs. 30 and 35). It will be interesting to determine whether the association of pp59$^{v\text{-}rel}$ with these other proteins is of functional importance.

b) pp59$^{v\text{-}rel}$, a protein kinase?

Several reports have suggested that pp59$^{v\text{-}rel}$ may be associated with a protein kinase (Rice *et al.* 1986, Walro *et al.* 1987). I have not been able to demonstrate specific kinase activity in immunoprecipitation complexes with B-1 antiserum (data not shown) using v-*rel* expressed in Cos1 cells or in transformed lymphoid cells. It is not clear why I have been unable to demonstrate an associated kinase activity, however, the major reagent which differs between this laboratory and that of the other groups is the source of antiserum. It remains a possibility that the B-1 antiserum inhibits the putative pp59$^{v\text{-}rel}$ associated kinase activity. Recently M. Park of our laboratory detected protein kinase activity in preparations of pp59$^{v\text{-}rel}$ immunoprecipitated from nuclei of *Spodoptera frugiperda* cells expressing pp59$^{v\text{-}rel}$ (personal communication). It still remains to be determined if this activity is intrinsic to pp59$^{v\text{-}rel}$, or is present in associated proteins.

If pp59$^{v\text{-}rel}$ is a protein kinase, it would be unique among the protein kinase oncogenes products in that it has no demonstrated homology with the well conserved catalytic domain of protein kinases (Stephens *et al.* 1983). The v-*rel* oncogene does not encode a consensus sequence for ATP-binding (Stephens *et al* 1983). The conclusion from this is that either pp59$^{v\text{-}rel}$ is not a protein kinase, or it is an unusual kinase, unrelated to the large family of previously identified kinases (Hanks *et al.* 1988). One must bear in mind, however, that although a number of protein kinases with related catalytic domains have been identified, their identification is often based on nucleic acid homology with known protein kinases (Hanks *et al.* 1988). This method of identification may be very efficient, but it may bias our knowledge of

protein kinases. There may be a large number of previously uncharacterized protein kinases unrelated to the currently recognized family.

c) Subcellular location of v-*rel*

The v-*rel* protein was localized in the nucleus of CEF infected with REV-REL (Fig. 27) and Cos1 cells transfected with pSVLREL (Fig. 18). The location of all deletion mutants expressed in CEF or Cos1 cells was nuclear (Figs. 18, 27). The expression of all linker insertion mutants in CEF was also nuclear (Fig. 33), although mutant L116 also showed light cytoplasmic staining. The cellular location of pp59$^{v\text{-}rel}$ was found to be cytoplasmic in transformed lymphoid cells (Simek *et al.* 1986, Gilmore and Temin 1986). The nuclear localization of pp59$^{v\text{-}rel}$ in Cos1 cells demonstrates that v-*rel* encodes a dominant acting nuclear localizing sequence which can function in avian and mammalian cells. The cytoplasmic location of pp59$^{v\text{-}rel}$ in transformed lymphoid cells may be explained if the nuclear localizing signals are not recognized in these cells, or if the association of pp59$^{v\text{-}rel}$ with several other cytoplasmic proteins maintains a cytoplasmic location, perhaps by masking the transport signal.

Gilmore and Temin (1988) identified a nuclear localizing signal between amino acids 274 and 318 of pp59$^{v\text{-}rel}$. The putative nuclear localizing core sequence consisted largely of basic amino acids, similar to other reported nuclear localizing signals (Gilmore and Temin 1988). A fusion protein of chloramphenicol acetyl transferase, v-*rel* (including the putative nuclear localizing signal) and β-galactosidase was expressed in the nucleus of both CEF and transformed lymphoid cells (Gilmore and Temin 1988). This indicated that the v-*rel* nuclear localizing sequence can

direct heterologous proteins to the nucleus of CEF and the transformed lymphoid cells. Since the v-*rel* nuclear localizing signal functions in the lymphoid cells, the retention of pp59$^{v\text{-}rel}$ in the cytoplasm may be the result of the masking of this signal by the associated proteins.

Gilmore and Temin (1988) also suggested that v-*rel* may encode secondary nuclear localizing signals. They showed that the deletion of the identified nuclear localizing signal did not abolish nuclear localization of pp59$^{v\text{-}rel}$, but resulted in cytoplasmic and nuclear immunofluorescent staining. Mutant L116 presented a similar pattern of immunofluorescent staining. It is possible that this linker insertion either modulated the activity of the identified nuclear localizing signal or it disrupted a secondary nuclear localizing signal.

6. TRANSFORMING ACTIVITY OF V-REL MUTANTS

The transformation assays were performed under conditions to maximize the detection of transforming virus. In this assay, the target cells are limiting. A 25-fold dilution of standard virus, REV-REL, transformed >12 target cells indicating that the potential transforming activity of the undiluted wild type virus in each experiment was >300 colony forming units. As a result of these kinetics, small differences between the transforming activity of the mutants with the wild type would not be detected. Since a minimum of 9 independent experiments were carried out for each mutant, theoretically, a virus transforming at 0.4% of the wild type would generate approximately 10 transformed colonies over the 9 experiments.

The titers of the various viruses also determined the number of transformed colonies expected from each experiment. Initially, the titers of all viruses were roughly estimated by IFA through visual comparison

with wild type controls. All viruses except REV-ΔN11 and REV-ΔENV had titers similar to REV-REL. Additional *in vitro* transformation experiments were performed for these samples to improve the chances for the recovery of potential transformed colonies. The transforming titer of REV-ΔC100 was approximately 1% of REV-REL. The increased numbers of experiments for virus ΔN11 allowed its transforming titer to be estimated at approximately 0.2% of REV-REL. Although no transformed colonies were detected for ΔENV, the reduced titers observed for this virus would only enable a transforming titer greater than 0.1% to be detected in the spleen cell transformation assay.

a) Deletion mutants of v-*rel*

The N-terminal but not the C-terminal *env*-derived sequences appear to be an important region of v-*rel* which influences transforming function (Table 3). The sensitivity of the immediate N-terminus to change is probably the result of structural alterations rather than the deletion of important enzyme active sites. Gilmore and Temin (1988) reported that the addition of several different amino acid sequences to the N-terminus of v-*rel* also abolished transforming function. Prediction of the secondary structure for the v-*rel* amino acid sequence by Garnier's method (Garnier 1978) using the software package PC/Gene, 5.11 (Intelligenetics, Mountain View, CA)(See appendix 3) revealed an α-helix comprised of 9 amino acids at the immediate N-terminus. Perhaps a helix at the N-terminus fulfills a structural requirement of pp59$^{v\text{-}rel}$.

While the N-terminal *env* sequences of v-*rel* are important for full transforming function, they are not required for partial transformation. The virus, REV-ΔN11, did transform cells at a much reduced level, although the colonies which have been recovered cannot be propagated

indefinitely. Western blot analysis of the transformed cells that were recovered revealed a band of appropriate size for the protein product of ΔN11, however other bands were evident (Fig. 29). The unusual Western blot pattern may be explained by partial degradation of the cell proteins due to the metabolic inactivity of the cells. At the time of preparation of the ΔN11 lysates for Western blots, ΔN11 transformed lymphoid cells labelled with [^{35}S]-methionine failed to synthesize any proteins reactive with B-1 antiserum. Even after lengthy exposures, no background radioactivity was detected suggesting that the cells failed to efficiently incorporate the labeled amino acid.

A virus with a similar phenotype to REV-ΔN11 was reported by Gilmore and Temin (1988). The virus which had v-*rel* amino acids 332-389 deleted induced transformed cells which they were unable to propagate. These v-*rel* mutants appear to be unable to completely immortalize target cells as observed by the wild type gene.

Although the precise deletion of the N-terminal *env*-derived sequences has not been reported, alterations of the N-terminus have generally resulted in nontransforming virus (Sylla and Temin 1986, Gilmore and Temin 1988). The replacement of the N-terminal v-*rel* sequences with the corresponding c-*rel* sequences abolished transformation (Sylla and Temin 1986). This replacement effectively deleted the 12 *env*-derived N-terminal amino acids and the following 17 *rel* encoded amino acids and inserted a methionine replacing the threonine at position 29 of v-*rel* (Wilhelmsen *et al.* 1984). Although this methionine behaved as an initiation codon in their experiment, it has not been demonstrated that this is the natural initiation codon for the c-*rel* protein product. The transformation activity of ΔN11 indicates that the N-terminal *env* amino acids alone are critical

for the full activity of v-*rel*. The v-*sis*, v-*fps* and v-*abl* oncogenes also have N-terminal sequences encoded by viral genes which appeared to be important for full transforming activity (Hannink and Donoghue 1984, Stone *et al.* 1984, Pryes *et al.* 1983).

One might have expected a low number of partially transformed colonies following infection of CSC with REV-ΔENV, however, the lower titer of this virus may have prevented their detection. The deletion of as many as 100 C-terminal amino acids did not prevent transformation. REV-ΔC100 transformed at approximately 1% the level of REV-REL. The resulting transformed cells, however, were fully immortalized and grew at a similar rate to REV-REL transformed cells.

There is no indication why REV-ΔC100 gave lower numbers of transformed colonies. The efficiency of transformation was high enough that it is unlikely that secondary mutations of cellular genes or of ΔC110 were required for the establishment of a fully immortalized and transformed cell. It is not likely that an increased threshold of expression was required for transformation by ΔC100 since the level of expression of the ΔC100 protein in the transformed cells was similar to the level of expression of pp59$^{v\text{-}rel}$. It is possible that the target cell population was more narrowly defined for REV-ΔC100.

The target cell population for transformation by REV-T may include lymphoid cells at several early stages of B-cell development. The normal target cell population is thought to be early lymphoid cells, possibly committed to B-cell development (Beug *et al.* 1981, Lewis *et al.* 1981). More recent studies have indicated that the *in vivo* targets for transformation may be influenced by the pathogenicity of the helper virus, REV-A (Barth and Humphries 1988a, 1988b). The immunosuppression

and bursal atrophy induced by infection with REV-A may eliminate more differentiated B-cell targets (Barth and Humphries 1988a). The use of the related chick syncytial virus (CSV), which does not demonstrate the above noted pathogenicity, to co-infect with REV-T resulted in the isolation of transformed cells which express IgM (Barth and Humphries 1988b). *In vitro* transformed CSC were also shown to have undergone different stages of B-cell development (Chen *et al.* 1988). Some clones had the germ line configuration of immunoglobulin genes representing a very early class of lymphoid cells, whereas others had notable immunoglobulin gene rearrangements. Assuming that such rearrangements had occurred prior to immortalization by v-*rel*, the potential target cells for transformation by REV-T may extend over several developmental stages of early B-cells. With respect to REV-ΔC100, it would be of interest to determine whether this virus is also able to transform the same range of target cells.

The association of $pp59^{v\text{-}rel}$ with several other cellular proteins may be of functional importance. Immunoprecipitation of v-*rel* proteins expressed in CSC transformed by REV-ΔC19 and REV-ΔC100 resulted in the co-precipitation of the same set of proteins (Fig. 30). While this does not demonstrate a functional role for the binding with these proteins, it at least does not contradict their possible role in transformation. The identity of the prominent 32 kDa protein co-precipitated from CSC transformed by ΔC19 (Fig. 30) is unknown, however, the failure to detect this by Western blot assay indicates that it is probably not a truncated product of a rearranged v-*rel* gene.

b) Linker insertion mutants of v-*rel*

Before discussing the results of the *in vitro* transformations with

the linker insertion mutants, I would like to address the limitations of this type of analysis.

The primary goal of linker insertion mutation studies is to map regions of a protein which are important for its function. It is certainly conceptually easy to understand that the insertion of several amino acids into an active site of a protein will abolish its function. It is more difficult to determine what structural alterations are induced by the insertion of several amino acids. One concern with linker insertion mutations is that they may eliminate the activity of a protein by inducing structural changes at a great distance from the true functional regions of the protein.

The results of a number of recent linker insertion mutagenesis studies (Giguerre *et al.* 1986, Ng and Privalsky 1986, Privalsky *et al.* 1988, DeClue and Martin 1989) indicated that linker insertion mutations generally have a relatively local effect on protein function. Linker insertion mutants which inhibited protein function were clustered predominantly in the kinase domain of v-*src* (DeClue and Martin 1989), in the DNA binding region of v-*erb*A (Privalsky *et al.* 1988), in the kinase domain of v-*erb*B (Ng and Privalsky 1986) and in 4 domains of the human glucocorticoid receptor, which function in DNA binding, steroid binding and stimulation of transcription (Giguere *et al.* 1986).

The application of linker insertion mutagenesis to v-*rel* clearly revealed that the N-terminus, unlike the C-terminus is sensitive to change (Table 4). This result corresponds with the terminal deletion analysis, which also showed that change to the N-terminus and not the C-terminus eliminated the transforming activity of v-*rel*. None of the linker insertion mutants demonstrated temperature sensitivity for transformation at 33°C,

37°C or 41°C. All mutants expressed the v-*rel* proteins in the nucleus of CEF (Fig. 33), although the mutant REV-L116 also showed a low level of diffuse cytoplasmic immunofluorescent staining.

As discussed earlier, pp59$^{v\text{-}rel}$ is complexed with several other cellular proteins in transformed cells. All of the linker insertion mutants which transformed lymphoid cells, expressed products which appeared to precipitate the same set of proteins (Fig. 34). Once again, I would like to point out that this does not demonstrate that the proteins complexed with pp59$^{v\text{-}rel}$ are important for mediating transformation, but it does not contradict this model.

Secondary structure analysis was performed on the amino acid sequence of v-*rel* (see appendix 3) and all linker insertion mutants (data not shown) by the method of Garnier (1978). Briefly, linker insertion mutants L116, L151, L213, and L459 introduce amino acids into regions of v-*rel* which are predicted to have a minimum of 10 consecutive amino acids in an α-helical conformation. L151 and L459 insert amino acids which conserve the predicted helix, whereas L116 inserts a turn and L213 inserts an extended conformation at the N-terminal end of their respective helices. Linker insertion mutants L163, L332, L374 and L390 insert amino acids into regions of v-*rel* which are predicted to have a minimum of 5 amino acids in an extended conformation. Linker insertion mutants L29, L41, L275 and L440 insert amino acids into regions of v-*rel* which do not have 5 consecutive amino acids either in α-helical or extended conformations.

Based on these predictions, linker insertion mutants in areas of probable structure have transforming (L332, L374, L390, L459) and nontransforming (L116, L151, L163, L213) phenotypes. Linker insertion

mutants in areas without uniform structure also have transforming (L440) and nontransforming (L29, L41, L275) phenotypes. Thus, no clear correlation exits between the predicted structure at the site of linker insertion with the transforming potential of the altered genes. It is possible, however, that some of the helical regions in the N-terminal portion of v-*rel* have important functional roles which are sensitive to alteration. It should also be noted that 69% of all of the amino acid residues predicted to be in a helical conformation are in the N-terminal half of v-*rel*. The higher level of structure may be required for functions mediated by the N-terminus of pp59$^{v\text{-}rel}$.

Gilmore and Temin (1988) constructed deletion mutants of v-*rel* to study function. Out of 8 mutants with substantial portions of v-*rel* coding sequence deleted, only two mutants which had deletions in the C-terminus maintained a transforming phenotype. Deletion of v-*rel* sequence coding for amino acids 332-389, as discussed earlier, resulted in a virus which was able to transform CSC, but unable to fully immortalize (Gilmore and Temin 1988). As well, mutant ΔC100, with sequence coding for amino acids 403-503 deleted, was able to fully transform CSC. The sequence deleted from these two clones covers the C-terminus from amino acids 332 to 503 with exception to amino acids 390-402. This further supports the results of the linker insertion mutagenesis which identifies functional regions in v-*rel* in the N-terminus, but not in the C-terminus.

The v-*rel* nucleotide sequence has been analyzed to identify potentially important functional regions by comparison with consensus sequences and with the nucleotide sequences of other genes. Two potential serine phosphorylation sites at amino acid positions 275 and 304 were identified by sequence analysis (Stephens *et al.* 1983). Although v-*rel*

does not encode a typical ATP-binding site (-Gly-X-Gly-X-X-Gly-) (Stephens *et al.* 1983), it does encode a similar sequence at amino acids 89-95 (-Gly-X-Gly-X-X-X-Gly-X-). It is not known whether this site functions in the binding of ATP. Interestingly, all of these sites are localized in the N-terminal portion of v-*rel* which has been shown by linker insertion mutatagenesis to be of functional importance.

Genes and in certain cases transcripts homologous to v-*rel* have been identified in human, rat, mouse, cat and salmon (Chen *et al.* 1981, Brownell *et al.* 1985, Brownell *et al* 1986, Brownell *et al.* 1987, Brownell *et al.* 1988a, Brownell *et al.* 1988b). In particular, human DNA clones have been characterized which are homologous to portions of v-*rel*. Human c-*rel* sequences corresponding to amino acids 187-293 were detected, but not to the remaining portions of v-*rel*. The human c-*rel* amino acid sequence was 87% similar to the corresponding v-*rel* sequence. It appeared that only this portion of v-*rel* was well conserved in human.

The v-*rel* protein has been demonstrated to share homology with the *fnr* protein of bacteria (Chen *et al.* 1986) and the *dorsal* protein of *Drosophila* (Steward 1987). The *fnr* protein is a transcriptional regulatory protein which modulates the transcriptional activity of genes involved in the anaerobic electron transport chain (Shaw and Guest 1982). The level of homology between v-*rel* amino acids 122-241 and *fnr* amino acids 64-188 was low, but was sufficient to suggest biological significance (Chen *et al.* 1986). Recently, Gelinas and Temin (1988) reported that v-*rel* transactivates the transcription of certain promoters when co-transfected into specific cell lines. This putative function of v-*rel* corresponds well to its homology with *fnr*.

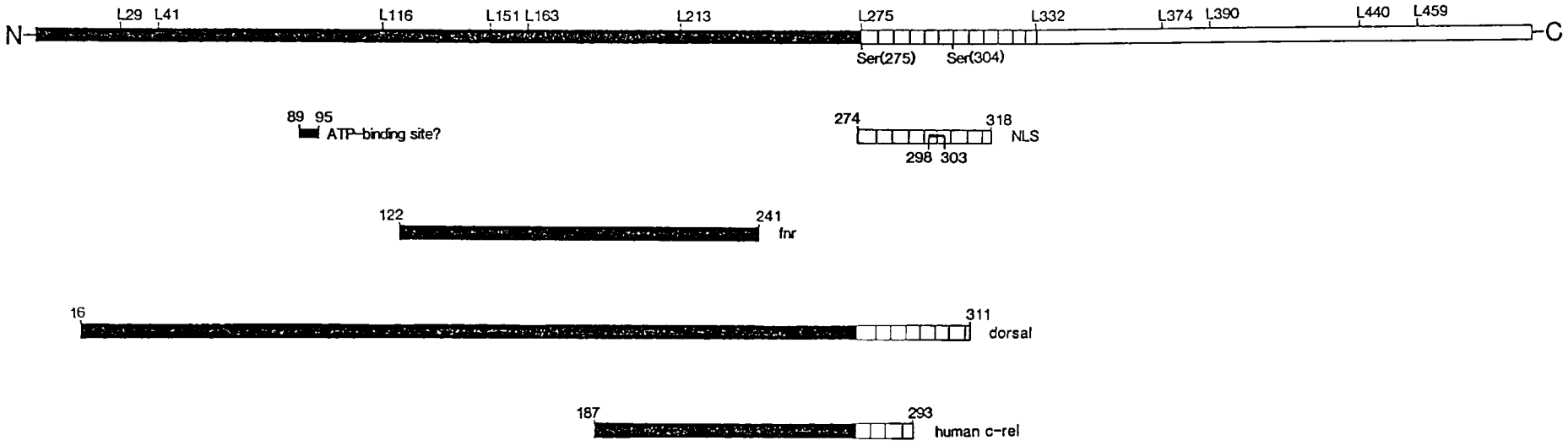
The similarity between v-*rel* amino acid residues 16-311 with amino acid residues 42-341 of the *dorsal* protein of *Drosophila* is approximately 47% (Steward 1987). If all conservative amino acid changes are scored as unchanged in this determination, the overall similarity would reach 80%. The C-terminus of the *dorsal* protein (amino acids 342-677) did not show any homology with v-*rel*. *Dorsal* is one of eleven genes which are required for proper development of dorsal-ventral polarity in the *Drosophila* embryo (Anderson *et al.* 1985a, 1985b). *Dorsal* is a maternal effect locus, in that the genotype of the mother determines this aspect of development (Anderson *et al.* 1985a). The *dorsal* protein is active between 1.25 and 2.5 hours post-fertilization (Steward 1987). The exact mode of action of *dorsal* is unknown, but likely involves the direct or indirect regulation of the gene expression required for the development of correct dorsal-ventral polarity in the *Drosophila* embryo. The protein product of v-*rel* is further implicated as a potentiator of gene expression through its homology with *dorsal*.

The potential phosphorylation sites, ATP-binding sites, nuclear localizing signal and regions of v-*rel* homologous to human c-*rel*, *fnr*, and *dorsal* are mapped with respect to the amino acid sequence of v-*rel* and the positions of linker insertion mutants in Figure 36. Immediately, it can be seen that the regions of v-*rel* conserved in the human c-*rel* and the homologous regions identified in *fnr* and *dorsal* map within the region of v-*rel* identified by linker insertion mutagenesis to be of functional importance.

7. CONCLUSION

In conclusion, I have identified the protein product of the v-*rel* oncogene as a 58 kDa protein which localizes in the nucleus of CEF and

Figure 36. A functional map of v-*rel*.
The v-*rel* protein is depicted at top with the position of all linker insertion mutants shown. Two serine residues which are potential phosphorylation sites are marked. Below, the regions of v-*rel* which have been identified as a potential ATP-binding site, and a nuclear localizing signal (NLS) or have been reported to be homologous to *fnr*, *dorsal* or human c-*rel* are shown. The small black bar in the NLS represents a putative core sequence. The large solid bar represents the region of v-*rel* shown by linker insertion mutagenesis to be of functional importance. The striped bar represents the sequence between the non-transforming mutant L275 and transforming mutant L332.


N
C
L29
L41
L116
L151
L163
L213
L275
L332
L374
L390
L440
L459
Ser(275)
Ser(304)
89
95
ATP-binding site?
274
318
NLS
298
303
122
241
fnr
16
311
dorsal
187
293
human c-rel

Cos1 cells. The N-terminal 11 amino acids coded for by *env* sequence are required for the full transforming activity of v-*rel*. The C-terminal 100 amino acids are not essential for the transforming activity. The N-terminus of v-*rel* was identified by linker insertion mapping to be important for transforming activity. The homology of this region with genes present in other species supports the hypothesis that the functional domain of v-*rel* is localized in the N-terminus.

8. FUTURE EXPERIMENTS

Despite the studies described in this thesis and reported by others, a clear biochemical function for v-*rel* has not been assigned. I have been unable to detect a specific kinase activity for v-*rel*, and considerable controversy still exists over the nature of the associated kinase activity (Gimore and Temin 1986). Ultimately, the authors who suggest that v-*rel* is a protein kinase must demonstrate that appropriate mutants fail to undergo autophosphorylation in kinase assays and that the loss of kinase activity correlates with the loss of the transforming phenotype.

V-*rel* has been reported to be a transactivator of specific promoters in certain cell types (Gelinas and Temin 1988). The relevance of this activity in the transformed lymphoid cells is not clear, nor is it clear how directly or indirectly this effect is mediated by v-*rel*. Further testing of the series of mutants that I have constructed may reveal whether a strong correlation exists between transactivation and transformation.

The cellular location of action of $pp59^{v\text{-}rel}$ is not known. Although $pp59^{v\text{-}rel}$ resides in the cytoplasm of transformed cells, mutants which carry the SV40 large T antigen nuclear localizing signal transform CSC and express in the nucleus (Gilmore and Temin 1988). Mutation of the putative core sequences for nuclear localization of $pp59^{v\text{-}rel}$ in CEF did

not prevent the resulting virus from transforming CSC (Gilmore and Temin 1988). In CEF this altered protein showed cytoplasmic and nuclear staining indicating that other sequences were also involved in the nuclear targeting of v-*rel*. Clearly, further information is required before the site of action of pp59$^{v\text{-}rel}$ is defined.

Although I presented the transformation of CSC by REV-T, it has also been reported that it can transform CEF (Franklin *et al* 1977, Hoelzer *et al.* 1979). The demonstration of REV-T induced transformation of CEF has been controversial and difficult to differentiate from simple cytopathic effects resulting from infection with the helper virus REV-A (Gilmore and Temin 1986). Recently, the conditions and criteria for CEF transformation by REV-T have been reported in detail by Moore and Bose (1988). Interestingly, pp59$^{v\text{-}rel}$ was expressed in the cytoplasm of transformed CEF and co-precipitated a 40 kDa protein. The transformation of CSC and CEF may not be the result of identical function of pp59$^{v\text{-}rel}$. CEF transformation could be tested for all of the deletion and linker insertion mutants to see whether a direct correlation exists between CSC and CEF transformation.

The association of pp59$^{v\text{-}rel}$ with several other proteins in transformed lymphoid cells (Simek and Rice 1988b, Tung *et al.* 1988) may also be of functional significance. I have shown that all of the mutants which transform, co-precipitate the same set of cellular proteins. A more interesting question is whether the mutants which fail to transform also bind these proteins. If binding of pp59$^{v\text{-}rel}$ with these cellular proteins is important for transformation, then mutations in possible binding sites would also abolish transformation. Mapping of the binding site(s) on

pp59$^{v\text{-}rel}$ for the cellular proteins p40 and p120 would allow the importance of this binding to be evaluated.

The determination of the binding of various non-transforming mutants to p40 and p120 would be most relevant if carried out in CSC transformed by pp59$^{v\text{-}rel}$. Superinfection of non-virus producing transformed CSC with various mutants would allow the expression of non-transforming mutants in the appropriate lymphoid cell. The association of the protein being tested with the p40, p120, pp59$^{v\text{-}rel}$ complex could initially be tested by determining in a Western blot assay if the test protein resides in the 400 kDa fraction of the cell lysate after size fractionation. As well, if the test protein binds within the complex it should also be co-precipitated with the p40 protein using antisera prepared against p40.

In order to do these experiments, one must be able to differentiate between the v-*rel* protein which initially transformed the CSC and the v-*rel* mutant being tested. Deletion mutants could be differentiated from pp59$^{v\text{-}rel}$ on the basis of size. Mutants which are of similar size to pp59$^{v\text{-}rel}$, could be tested in transformed cell lines established with REV-ΔC19 or REV-ΔC100. Once again, the test proteins could be differentiated from the transforming protein on the basis of size.

Ultimately, the binding site(s) for v-*rel* could be mapped and perhaps the role of p40 and p120 in the transformation process determined. It would also be interesting to determine the subcellular location of mutants which fail to bind p40 and p120. A nuclear location may support the suggestion that the binding of the cellular proteins is responsible for the retention of pp59$^{v\text{-}rel}$ in the cytoplasm of transformed lymphoid cells.

I have created a number of mutants which have allowed me to map the functional region of v-*rel*. It is clear that the continued study of these and other mutants may add further to the understanding of v-*rel* function.

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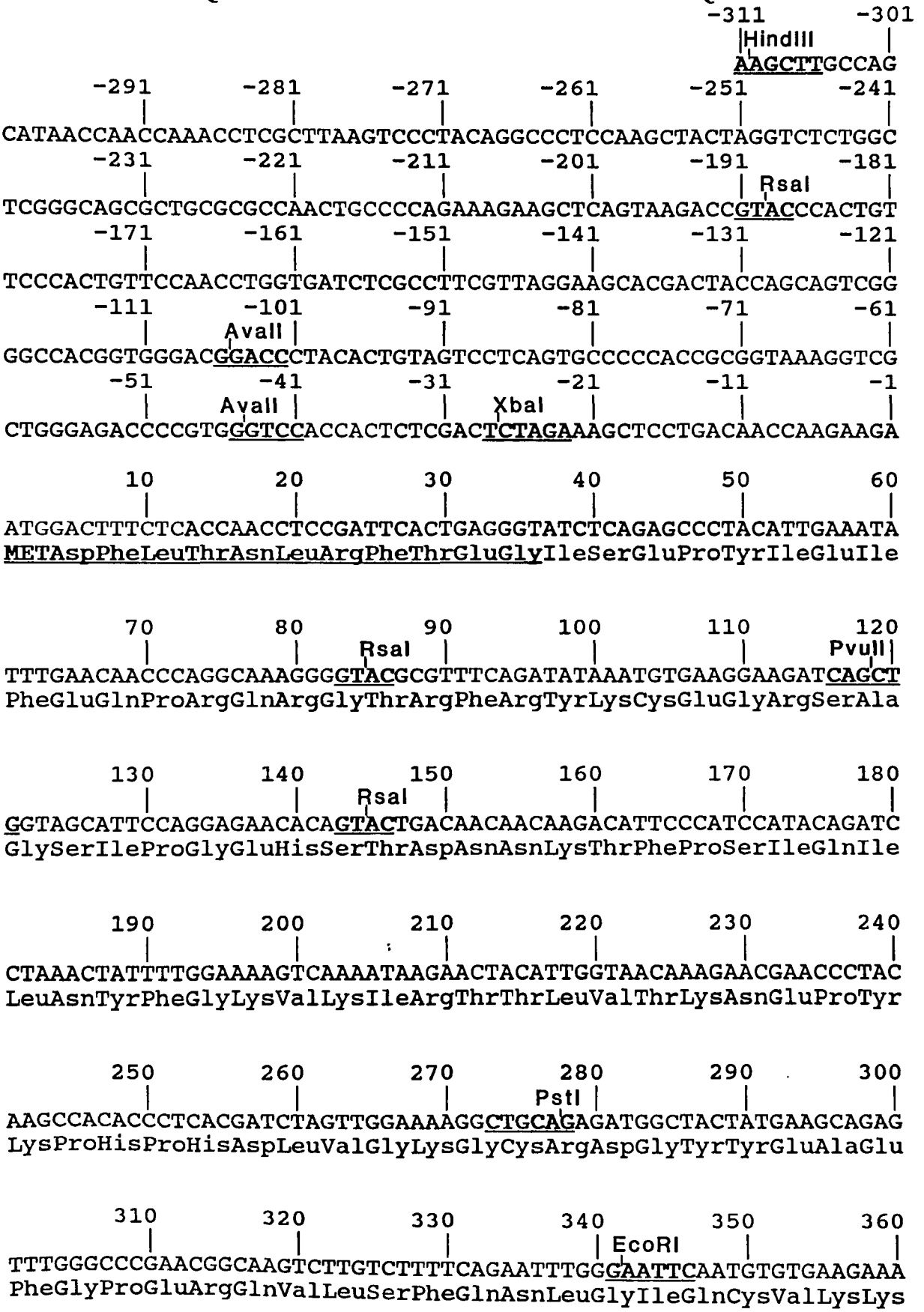
APPENDIX 1

NUCLEOTIDE SEQUENCE AND PREDICTED AMINO ACID SEQUENCE OF V-REL



```
        370         380         390         400         410         420
         |           |           |           |           |           |
AAAGACCTGAAAGAATCAATTTCTTTGCGAATCTCAAAGAAAATCAATCCCTTTAATGTG
LysAspLeuLysGluSerIleSerLeuArgIleSerLysLysIleAsnProPheAsnVal

        430         440         450         460         470         480
         |           |    ClaI   | RsaI      |           |           |
CCTGAGGAACAGTTGCATAACATCGATGAGTACGATCTCAACGTTGTCCGCCTCTGTTTC
ProGluGluGlnLeuHisAsnIleAspGluTyrAspLeuAsnValValArgLeuCysPhe

        490         500         510         520         530         540
 HindIII |          |           |           |           |           |
CAAGCTTTCCTCCCTGATGAACATGGCAACTACACATTGGCTCTTCCTCCTTTGATTTCC
GlnAlaPheLeuProAspGluHisGlyAsnTyrThrLeuAlaLeuProProLeuIleSer

        550         560         570         580         590         600
         |           |           |           |           |           |
AACCCAATCTATGACAACAGAGCTCCCAACACGGCAGAACTGAGGATTTGTCGTGTGAAT
AsnProIleTyrAspAsnArgAlaProAsnThrAlaGluLeuArgIleCysArgValAsn

        610         620         630         640         650         660
         |           |           |    XmnI   |           |           |
AAGAACTGTGGAAGTGTAAAGGGAGGAGATGAAATTTTTCTTCTGTGTGACAAAGTTCAA
LysAsnCysGlySerValLysGlyGlyAspGluIlePheLeuLeuCysAspLysValGln

        670         680         690         700         710         720
         |           |           |           |           |           |
AAAGATGACATAGAGGTCAGATTTGTCTTGGGCAACTGGGAGGCAAAGGGCTCCTTCTCC
LysAspAspIleGluValArgPheValLeuGlyAsnTrpGluAlaLysGlySerPheSer

        730         740         750         760         770         780
         |           |           |           |           |           |
CAAGCTGATGTTCATCGCCAGGTCGCAATTGTATTTAGAACACCGCCGTTCCTCGGAGAC
GlnAlaAspValHisArgGlnValAlaIleValPheArgThrProProPheLeuGlyAsp

        790         800         810         820         830         840
         |           |           |           |           |           |
                                          XmnI
ATCACAGAACCCATCACGGTGAAGATGCAGTTACGAAGGCCTTCAGACCAGGCAGTCAGT
IleThrGluProIleThrValLysMetGlnLeuArgArgProSerAspGlnAlaValSer

        850         860         870         880         890         900
         |           |           |           |           |           |
                                      BamHI
GAACCAGTGGATTTCAGATATTTACCAGATGAAGAGGATCCGTCTGGCAACAAAGCAAAA
GluProValAspPheArgTyrLeuProAspGluGluAspProSerGlyAsnLysAlaLys
```

```
       910        920        930        940        950        960
                                                  PvuII
AGGCAAAGATCAACACTGGCTTGGCAAAAACCCATACAGGACTGCGGATCAGCTGTGACA
ArgGlnArgSerThrLeuAlaTrpGlnLysProIleGlnAspCysGlySerAlaValThr

       970        980        990       1000       1010       1020
                                    HincII
GAGAGGCCAAAAGCGGCTCCTATCCCCACTGTCAACCCTGAAGGAAAGCTGAAGAAAGAA
GluArgProLysAlaAlaProIleProThrValAsnProGluGlyLysLeuLysLysGlu

      1030       1040       1050       1060       1070       1080
CCAAATATGTTTTCACCTACGCTGATGCTGCCTGGGCTAGGAACACTGAGCTCCAGTCAG
ProAsnMetPheSerProThrLeuMetLeuProGlyLeuGlyThrLeuSerSerSerGln

      1090       1100       1110       1120       1130       1140
  RsaI                                 FspI
ATGTACCCTGCATGCTGCCAGATGCCCACCCAGCCTGCGCAGCTTGGCCCTGGGAAGCAG
MetTyrProAlaCysCysGlnMetProThrGlnProAlaGlnLeuGlyProGlyLysGln

      1150       1160       1170       1180       1190       1200
                          PvuII RsaI
GACACACTCCATTCCTGCTGGCAGCAGCTGTACAGCCCCTCCCCTTCAGCCAGCAGCCTG
AspThrLeuHisSerCysTrpGlnGlnLeuTyrSerProSerProSerAlaSerSerLeu

      1210       1220       1230       1240       1250       1260
CTCAGCTTGCACTCACACAGCAGCTTCACAGCGGAAGTGCCTCAGCCTGGTGCTCAGGGC
LeuSerLeuHisSerHisSerSerPheThrAlaGluValProGlnProGlyAlaGlnGly

      1270       1280       1290       1300       1310       1320
                                              EcoRI
AGTAGCTCTCTCCCGGCCTATAACCCACTGAACTGGCCTGATGAGAAGAATTCCAGTTTT
SerSerSerLeuProAlaTyrAsnProLeuAsnTrpProAspGluLysAsnSerSerPhe

      1330       1340       1350       1360       1370       1380
                                         PvuII      PstI
TACAGGAATTTTGGCAACACACATGGGATGGGAGCAGCGTTGGTGTCAGCTGCAGGCATG
TyrArgAsnPheGlyAsnThrHisGlyMetGlyAlaAlaLeuValSerAlaAlaGlyMet

      1390       1400       1410       1420       1430       1440
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GlnSerValSerSerSerSerIleValGlnGlyThrHisGlnAlaSerAlaThrThrAla
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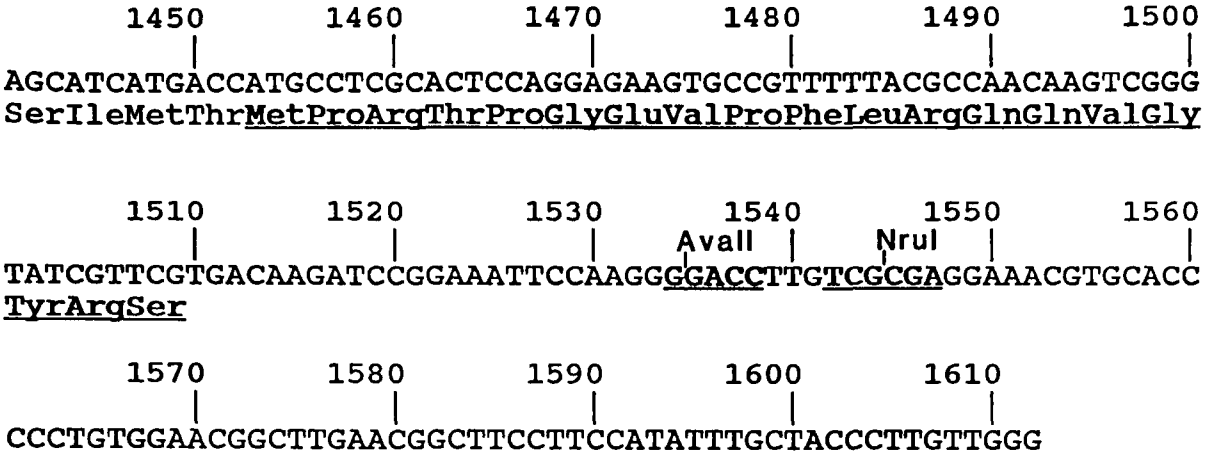
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SerIleMetThrMetProArgThrProGlyGluValProPheLeuArgGlnGlnValGly
1510 1520 1530 1540 1550 1560
AvaII
NruI
TATCGTTCGTGACAAGATCCGGAAATTCCAAGGGGACCTTGTCGCGAGGAAACGTGCACC
TyrArgSer
1570 1580 1590 1600 1610
CCCTGTGGAACGGCTTGAACGGCTTCCTTCCATATTTGCTACCCTTGTTGGG

APPENDIX 2

OLIGONUCLEOTIDE SEQUENCES

Crossover Linker primers

rcl1	5'-TCG AGG ATC CAC CAT GGA CTT TCT-3'
rcl2	5'-TCG AGG ATC CAC CAT GGG TAT CTC AGA G-3'
rcl2b	5'-GAG ATA CCC ATG GTG GAT CC-3'
rcl3	5'-TCG AGG ATC CAC CAT GGA GTT TGG GCC C-3'
rcl4	5'-TCG AGT CAG GTC ATG ATG CT-3'
rcl5	5'-TCG AGT CAC AAG CTG AGC AG-3'

Primers for sequencing of linker insertion mutants

REL-1	5'- AAG ACT TGC CGT TCG GGC CC-3'
REL-2	5'-GAA AAG GCT GCA GAG ATG GC-3'
REL-3	5'-GCA GAA CTG AGG ATT TGT CG-3'
REL-4	5'-AAG ATC AAC ACT GGC TTG GC-3'
REL-5	5'-CTC CCC TTC AGC CAG CAG CC-3'

APPENDIX 3

PREDICTED SECONDARY STRUCTURE OF THE V-*REL* PROTEIN

Symbols used in the semi-graphical representation:
Helical conformation: X Extended conformation: -
Turn conformation: > Coil conformation: *

PREDICTION FOR V-REL:

```
        10        20        30        40        50
         |         |         |         |         |
MDFLTNLRFTEGISEPYIEIFEQPRQRGTRFRYKCEGRSAGSIPGEHSTD
XXXXXXXXX**>****XX--XXX*>>>>>----->>>>>>***----*>>
XXXXXXXXX**>****XX--XXX*>>>>>----->>>>>>***----*>>
        60        70        80        90       100
         |         |         |         |         |
NNKTFPSIQILNYFGKVKIRTTLVTKNEPYKPHPHDLVGKGCRDGYYEAE
>>>*--------->>>--------->**>>**------>----------X
>>>*--------->>>--------->**>>**------>----------X
       110       120       130       140       150
         |         |         |         |         |
FGPERQVLSFQNLGIQCVKKKDLKESISLRISKKINPFNVPEEQLHNIDE
XXXXXX---->>>XXXXXXXXXXXXXXXXX->>******XXXXXXXXXX
XXXXXX---->>>XXXXXXXXXXXXXXXXX->>******XXXXXXXXXX
       160       170       180       190       200
         |         |         |         |         |
YDLNVVRLCFQAFLPDEHGNYTLALPPLISNPIYDNRAPNTAELRICRVN
XXXXX---------X>>>>>-------------->>-***XXXXX---->
XXXXX---------X>>>>>-------------->>-***XXXXX---->
       210       220       230       240       250
         |         |         |         |         |
KNCGSVKGGDEIFLLCDKVQKDDIEVRFVLGNWEAKGSFSQADVHRQVAI
>>>>--*>>XXXXXXXXXXXXXXX----XX*XXXX>>**XXXXXXX----
>>>>--*>>XXXXXXXXXXXXXXX----XX*XXXX>>**XXXXXXX----
       260       270       280       290       300
         |         |         |         |         |
VFRTPPFLGDITEPITVKMQLRRPSDQAVSEPVDFRYLPDEEDPSGNKAK
----*>>**----XXXX------>>**----------**>XXXXXXXXX
----*>>**----XXXX------>>**----------**>XXXXXXXXX
       310       320       330       340       350
         |         |         |         |         |
RQRSTLAWQKPIQDCGSAVTERPKAAPIPTVNPEGKLKKEPNMFSPTLML
-----*>>----->>>------>>*-------XXXXXXX-X>X-------
-----*>>----->>>------>>*-------XXXXXXX-X>X-------
       360       370       380       390       400
         |         |         |         |         |
PGLGTLSSSQMYPACCQMPTQPAQLGPGKQDTLHSCWQQLYSPSPSASSL
---*--->>>>>-->----------**>>----->>-----*****----
---*--->>>>>-->----------**>>----->>-----*****----
```

```
      410       420       430       440       450
        |         |         |         |         |
LSLHSHSSFTAEVPQPGAQGSSSLPAYNPLNWPDEKNSSFYRNFGNTHGM
---X****>----***>>**-----><-*>***>>>>>--->>>>****-
---X****>----***>>**-----><-*>***>>>>>--->>>>****-
      460       470       480       490       500
        |         |         |         |         |
GAALVSAAGMQSVSSSSIVQGTHQASATTASIMTMPRTPGEVPFLRQQVG
XXXXXXXXXX---**-------**--XXXX------***>--------->
XXXXXXXXXX---**-------**--XXXX------***>--------->

YRS
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