1986

Structure And Function Of The Src Gene Of Rous Sarcoma Virus And The Human Cellular Src Gene

Stephen Kent Anderson

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STRUCTURE AND FUNCTION OF THE *SRC* GENE OF ROUS SARCOMA VIRUS AND THE HUMAN CELLULAR *SRC* GENE

by

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Submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Ontario

July, 1986

ISBN 0-315-33014-7

THE UNIVERSITY OF WESTERN ONTARIO
FACULTY OF GRADUATE STUDIES

CERTIFICATE OF EXAMINATION

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Stephen Kent Anderson

entitled
Structure and function of the src gene of Rous sarcoma virus and the human cellular src gene.

is accepted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

Date September 5/86

Chairman of Examining Board

ABSTRACT

The Rous sarcoma virus (RSV) *src* gene (v-*src*) is one of the most extensively studied oncogenes, yet the mechanism by which it transforms virus-infected cells remains unsolved. The study of mutants of RSV provides considerable information on the function of the v-*src* protein ($p60^{v\text{-}src}$).

Characterization of the chicken cellular counterpart of the v-*src* gene (c-*src*) has provided clues as to the alterations required to produce an oncogene from a normal cellular gene (proto-oncogene). Characterization of the human c-*src* gene will be useful for future studies aimed at detecting possible human c-*src* alterations associated with human neoplastic disorders.

The nucleotide sequence of the 3′ three-quarters of a molecularly cloned human c-*src* gene was determined. This region of the c-*src* gene encodes the tyrosine kinase domain of $p60^{c\text{-}src}$ and corresponds to exons 4 through 12 of the chicken c-*src* gene. Human c-*src* is very strongly conserved with respect to the chicken c-*src* gene; 90% nucleotide homology and 98% amino acid homology exists between human and chicken c-*src*. The exon sizes and the locations of the exon-intron boundaries are identical in human and chicken c-*src*. Sequences within introns are not conserved, and most of the introns of human c-*src* are much larger than the corresponding chicken c-*src* introns.

The strong amino acid conservation of this region of $p60^{c\text{-}src}$ of species as divergent as humans and chickens

suggests that this portion of $p60^{c\text{-}\underline{src}}$ specifies one or more functional domains that are of great importance to some aspect of normal cellular growth or differentiation.

The nature of the lesions in three fusiform mutants (W0101, W0201, and tsST529) of the Schmidt-Ruppin strain of RSV was determined by molecular cloning and DNA sequencing. W0101, W0201, and tsST529 all contained an in frame deletion in the v-src region coding for amino acids 116-140 of $p60^{\underline{src}}$. The deleted segment is flanked by consensus splice donor and acceptor sequences and contains an appropriately positioned branchpoint acceptor consensus sequence, suggesting that the deletion occurred through an aberrant RNA splicing event. These results suggest that a protein domain within the N-terminal 1/3 of $p60^{v\text{-}\underline{src}}$ is important for controlling morphological parameters of transformation in RSV-infected cells.

ST529 also contained a point mutation which results in the substitution of lysine for glutamic acid at position 93. This lesion is adjacent to an amino-terminal tyrosine which may possess a functionally important role in affecting $p60^{\underline{src}}$ kinase activity and/or function. Both of the lesions present in ST529 contribute to the temperature-sensitive phenotype of this mutant as demonstrated by the generation of non-ts ST529/wtRSV hybrids containing either the deletion or point mutation of ST529. This result demonstrates the importance of N-terminal sequences in controlling the kinase activity of $p60^{v\text{-}\underline{src}}$.

ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. D.J. Fujita for his support and enthusiasm throughout the course of this research. I thank Dr. E. Ball, Dr. G. Mackie, and Dr. K. Ebisuzaki for their continued interest and advice. I am indebted to Dr. A. Ridgway and Dr. A. Tanaka for their assistance and expert advice. I would also like to thank all the members of the Cancer Research Laboratory who provided an enjoyable working environment. I gratefully acknowledge the support of the National Cancer Institute of Canada. Special thanks to my parents and to my wife, Brenda, for their support and encouragement.

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ABBREVIATIONS

bp	- base pairs
cpm	- counts per minute
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
kb	- kilo base pairs
kd	- kilo daltons
ng	- nanogram
PIPES	- piperazine-N,N'-bis(2-ethanesulfonic acid)
RSV	- Rous sarcoma virus
TCA	- trichloro-acetic acid
ts	- temperature-sensitive
ug	- microgram
ul	- microliter
wt	- wild-type

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE RSV SRC GENE

The *src* gene of Rous sarcoma virus is the prototypical oncogene. Peyton Rous' discovery in 1911 of a filterable extract of a chicken tumor that could induce tumors when injected into healthy chickens (Rous, 1911) was not given much credibility at the time, but the study of oncogenic transformation by Rous sarcoma virus and other retroviruses harboring oncogenes has now shed considerable light on the mechanisms involved in the production of neoplasms (Bishop, 1983).

Rous sarcoma virus contains at least four genes with the order 5'-*gag*-*pol*-*env*-*src*-poly(A)-3' in the genomic RNA (Duesberg *et al*, 1976; Joho *et al*, 1976). Three of these genes are involved in viral replication. The fourth gene, *src*, is not required for viral replication, but is the sole gene implicated in the process of oncogenic transformation in RSV infected cells as demonstrated by numerous genetic studies (Toyoshima and Vogt, 1969; Martin, 1970; Kawai and Hanafusa, 1971; Vogt, 1971; Biggs *et al*, 1973). The product of the *src* gene is a 60kd phosphoprotein named $p60^{src}$, which was originally identified as a transformation-specific antigen present in avian sarcoma virus-transformed cells (Brugge and Erikson, 1977; Brugge *et al*, 1978; Jay *et al*, 1978; Purchio *et al*, 1978). Enzymatically active $p60^{src}$ has been synthesized by *in vitro* translation of the 3' one-third of Rous sarcoma virus RNA (Purchio *et al*, 1977; Beemon and Hunter, 1978; Erikson *et al*, 1978). The amino acid

sequence of the viral src protein was deduced from the nucleotide sequence of cloned proviral DNA (Czernilofsky et al, 1980; Delorbe et al, 1980; Takeya and Hanafusa, 1982; Schwartz et al, 1983). These sequences indicated that the viral src gene is able to code for a protein containing 526 amino acids with a molecular weight of approximately 60,000, which is in agreement with the size of the tumor antigen detected in Rous sarcoma virus infected cells.

The subcellular localization of $p60^{v-src}$ is predominately in the region of the plasma membrane. Immunofluorescence microscopy studies have shown that $p60^{v-src}$ is particularly concentrated near cell-cell junctions (Rohrschneider, 1979; Krueger et al, 1980b) and adhesion plaques (Rohrschneider, 1980). Some diffuse cytoplasmic fluorescence has been reported, and one group has shown an association of $p60^{v-src}$ with the cytoskeleton (Burr et al, 1980). The results of immunocytochemical electron microscopy are in agreemeat with the plasma membrane localization of the src protein (Willingham et al, 1979). This study showed that $p60^{v-src}$ was concentrated at gap junctions. Subcellular fractionation has also demonstrated the localization of $p60^{v-src}$ predominantly within the cellular membrane fraction (Courtneidge et al, 1980; Krueger et al, 1980b). In these studies, $p60^{v-src}$ behaves as an integral membrane protein. An unusual nuclear envelope and juxtanuclear reticular membrane localization of the v-src protein has been reported in RSV-transformed rat

cells (Krueger et al, 1980a). The src proteins in these cells are altered in their amino-terminus, and this alteration may prevent the translocation of p60^{src} to the cytoplasmic membrane (Garber et al, 1982).

P60^{src} was shown to possess a cyclic AMP-independent protein kinase activity by the ability of crude and purified preparations of p60^{src} to phosphorylate the IgG heavy chain in an immuno-precipitation reaction as well as various protein substrates in vitro (Collett and Erikson, 1978; Levinson et al, 1978; Erikson et al, 1979a; Erikson et al, 1979b; Maness et al, 1979). Eventually it was discovered that p60^{src} possessed an unusual protein kinase activity specific for tyrosine residues (Collett et al, 1980; Hunter and Sefton, 1980; Levinson et al, 1980).

The tyrosine kinase activity of p60^{src} was first described by Hunter and Sefton (1980), and since this was perceived as an uncommon form of protein modification, it provided an opportunity to look for targets of the p60^{src} kinase in cells transformed by RSV. Phosphotyrosine is present in normal cells at a level of less than 0.1% of the phosphorylated amino acids. Phosphoserine comprises the majority (90%) with phosphothreonine accounting for 5 to 10% of cellular phosphoamino acids. Cells transformed by RSV show a ten-fold increase in the level of cellular phosphotyrosine, and several proteins that show an increase in phosphotyrosine levels have been investigated with the hope of finding components of the cellular machinery

participating in the process of oncogenic transformation. This approach is justified by the observation that tyrosine phosphorylation of cellular proteins is strongly correlated with transformation mediated by the src protein as shown by Sefton and collaborators (1980b). Cellular phosphotyrosine levels are not elevated in cells infected with temperature-sensitive mutants of the RSV src gene when the cells are grown at the restrictive temperature. Shifting of these cells to the permissive temperature results in a rapid increase in cellular phosphotyrosine followed by morphological transformation. These results suggest that phosphorylation of cellular proteins by p60src is important for the production of the transformed phenotype.

Two of the suggested targets of p60src are peripheral membrane proteins, which is in agreement with the cell-membrane localization of p60src (Rohrschneider, 1979; Willingham et al, 1979; Rohrschneider, 1980). A 36,000 molecular weight protein (p36) is heavily phosphorylated upon transformation by RSV (Erikson and Erikson, 1980; Radke et al, 1980) and p36 has been shown to be present in monomer and dimer forms as a component of the terminal web found on the inner surface of some epithelial cells (Erikson et al, 1984; Gerke and Weber, 1984). Increased tyrosine phosphorylation of a human protein related to p36 was observed in A431 cells responding to EGF treatment (Hunter and Cooper, 1981). Vinculin, a 130kd protein localized in adhesion plaques, contains 10-fold more phosphotyrosine in

RSV transformed cells, but only approximately 1% of isolated vinculin molecules contain phosphotyrosine (Sefton et al, 1981). Adhesion plaques are involved in anchoring actin-containing microfilaments to the plasma membrane as well as the attachment of cells to the substratum. P60src has been found to be localized in adhesion plaques by indirect immunofluorescence (Rohrschneider, 1980). Since vinculin is also present diffusely in the cytoplasm, it may be that only the subpopulation of vinculin present in adhesion plaques is phosphorylated by p60src, and phosphorylation of vinculin may be able to alter the anchoring of microfilaments thereby causing a change in cellular morphology.

A potential cytoplasmic target of p60src is the 50kd phosphoprotein involved in a tri-molecular complex with p60src and the p90 heat-shock protein (Hunter and Sefton, 1980; Brugge et al, 1981; Oppermann et al, 1981a). P50 is phosphorylated on serine residues in normal cells, but becomes phosphorylated on tyrosine after infection with RSV (Brugge and Darrow, 1982). The complex between p50, p90, and p60src has been implicated in the transport of p60src to the cell membrane (Courtneidge and Bishop, 1982; Brugge et al, 1983). Another possible cytoplasmic target of the p60src tyrosine kinase is a p42 protein of unknown function which has also been shown to be phosphorylated on tyrosine when cells are treated with various mitogenic agents (Gilmore and Martin, 1983; Cooper et al, 1984). In addition, three

non-rate determining enzymes of glycolysis have been shown to possess increased phosphotyrosine levels in RSV-transformed cells (Cooper et al, 1983b).

In order to investigate the relevance of the phosphorylation of the presumed targets of p60*src*, Cooper et al (1983a) attempted to correlate phosphorylation of suggested targets with the production of transformation-related parameters in a series of partially transforming mutants of RSV. There was no correlation between the phosphorylation of the p36, p42, or an additional p28 cytoplasmic target with the production of morphological changes in mutant-infected cells. The phosphorylation of p36, p42, or the total phosphotyrosine content of cells correlated well with the production of plasminogen activator, and the phosphorylation of p28 and p42 correlated well with increased hexose transport. However, even when good correlations were observed, significant exceptions were noted. The investigators suggest that some of the presumed targets of p60*src* phosphorylated on tyrosine residues may not be causally related to the expression of transformation-induced properties.

The possibility that the potential targets of the p60*src* kinase which have been identified are a reflection of the high level of *src* expression in RSV-transformed cells was suggested by experiments performed by Jakobovits et al (1984), in which the RSV *src* gene was linked to a regulatable promoter. Transformation of cells was achelved

with levels of p60^{src} 10-fold lower than the level of p60^{src} in RSV-transformed cells. At this lower level of src expression, no phosphorylation of the p36 protein was detected. Since p36 is thought to be a major target of the src protein, it may be that the phosphorylated targets detected in RSV-transformed cells are merely a result of the high level of src expression, and if there are targets of p60^{src} directly involved in the process of oncogenic transformation, they may not be easily detected by current methods.

Further studies on the structure of p60^{src} have revealed several covalent modifications important for transforming activity. The amino-terminus of p60^{src} contains myristic acid (Buss and Sefton, 1984). The importance of this modification for membrane association and transforming activity of p60^{src} has been elegantly demonstrated by Hanafusa and co-workers (Cross et al,1984; Garber et al, 1985). By constructing various N-terminal deletions of p60^{src}, these investigators showed that the first ten amino acids are required for myristlyation and membrane association. Viral p60^{src} is phosphorylated on serine and tyrosine residues, and several groups have investigated the effect of these modifications on p60^{src} activity. The serine residue at position 17 in viral p60^{src} is phosphorylated by a cyclic AMP-stimulated protein kinase, and the tyrosine at position 416 is phosphorylated in a cyclic AMP-independent fashion (Collett et al, 1979a; Hunter and Sefton, 1980;

Smart et al, 1981; Patchinsky et al, 1982). Both of these residues have been removed by in vitro mutagenesis (Cross and Hanafusa, 1983; Snyder et al, 1983), and the mutant proteins retain transforming ability, although the deletion of tyrosine 416 generates a mutant which takes longer to produce transformed cells after transfection of the in vitro construct (Cross and Hanafusa, 1983). This lag was not observed when tyrosine 416 was substituted by phenylalanine (Snyder et al, 1983). These experiments suggest that the major sites of phosphorylation detected in viral $p60^{src}$ may not be essential for transforming ability.

Recent reports have demonstrated an association between amino-terminal tyrosine phosphorylation of $p60^{v-src}$ and increased kinase activity of the src protein (Collett et al, 1983; Purchio et al, 1983). When immunoaffinity purified $p60^{v-src}$ was incubated with ATP/Mg^{2+} and subsequently repurified on hexylamine-agarose, a significant increase in the activity of the enzyme resulted. An 18-fold increase in the tyrosine phosphorylation of the 36 kd putative target protein was observed. The more active form of the enzyme possessed several new sites of tyrosine phosphorylation. The vast majority of the new phosphorylation occurred on amino-terminal tyrosine residues that were not precisely localized. The investigators concluded that amino-terminal tyrosine phosphorylation is able to activate $p60^{v-src}$.

As a result of the enormous effort put into the investigation of viral oncogenes, we have been made aware of

the fact that *src* is just one member of a large family of tyrosine kinase oncogenes (Cooper and Hunter, 1981a; Hunter, 1984). A stretch of approximately 250 amino acids within the carboxy-terminal half of $p60^{src}$ is known to be the protein kinase domain. This has been substantiated by the demonstration of tyrosine kinase activity in a carboxy-terminal fragment generated by proteolytic cleavage of $p60^{v\text{-}src}$ (Levinson *et al*, 1981). A very homologous domain of roughly the same length is found in each of the other six viral oncogenes shown to have tyrosine-specific protein kinase activity (*yes*, *fgr*, *abl*, *fps*, *fes*, and *ros*). One of the two oncogenes carried by avian erythroblastosis virus, the *erb*-B gene is also related to this tyrosine kinase family of oncogenes. Recent work on the EGF receptor has demonstrated a striking homology between the receptor and the *erb*-B oncogene. In fact, *erb*-B appears to be a truncated form of the EGF receptor, containing the coding region for the internal catalytic domain and the transmembrane segment but not the EGF-binding domain (Hunter, 1984). Since the EGF receptor has an associated tyrosine kinase activity, could it be that the tyrosine kinase family of oncogenes is somehow able to deliver a signal for the cell to divide in the absence of the usual growth-promoting signals? Further investigations on the functions of the normal cellular counterparts of viral tyrosine kinase oncogenes is required to provide a definitive answer.

1.2 CELLULAR SRC GENES

The discovery in 1976 by Stehelin et al of src-related sequences in normal uninfected cells, paved the way for future studies on the role of cellular proto-oncogenes in cell growth and their possible involvement in the production of naturally occurring neoplasms. Spector et al (1978c) found cellular src sequences in all vertebrates which were examined. Src-specific mRNA has been detected in uninfected chicken cells (Spector et al, 1978a; Spector et al, 1978b). A src-like gene from Drosophila has been cloned and characterized, and shows 40% amino acid homology with viral src (Shilo and Weinberg, 1981; Hoffman et al, 1983; Hoffman-Falk et al, 1983; Simon et al, 1983; Simon et al, 1985). The amino acid sequence of this Drosophila src protein is equally related to the viral src and yes genes, so it is possible that a single ancestral gene has diversified into c-src and c-yes since the divergence of Chordates and Arthropods. A 60,000 molecular weight cellular src protein antigenically related to viral p60src was detected in uninfected vertebrate cells (Collett et al, 1978; Oppermann et al, 1979), suggesting that the virus has acquired and is capable of expressing the entire gene. A tyrosine kinase activity was immuno-precipitated from Drosophila cells with antibody reactive with viral src, (Simon et al, 1983), but the protein responsible for this activity was not determined. Similarly, tyrosine kinase activity was detected in extracts from the freshwater sponge

Spongilla lacustris by immunoprecipitation with anti-v-src antibody, but not from yeast or tetrahymena. This represents the most distant phylogenetic extension of src tyrosine kinase functional homology (Barnekow and Schartl, 1984).

To further address the mechanism of acquisition and generation of transforming ability of the src gene carried by Rous sarcoma virus, the cellular src gene of the natural host of RSV has been cloned and characterized (Parker et al, 1981; Shalloway et al, 1981; Takeya et al, 1981; Swanstrom et al, 1983; Takeya and Hanafusa, 1983). The chicken c-src gene is composed of twelve exon regions corresponding to the coding sequence and 5' untranslated region of the viral src gene. The boundaries of the v-src gene suggest that the c-src gene was captured by the virus through recombination at the DNA level on both sides of the c-src gene. Interestingly, the predicted carboxy terminus of the chicken c-src gene differs from that of the v-src gene. The last 19 amino acids of $p60^{c-src}$ have been replaced by 12 unrelated amino acids in the v-src protein. In addition, there are several isolated amino acid changes throughout the viral src protein (Takeya and Hanafusa, 1983).

Since the viral src gene is altered relative to the cellular src gene, the importance of these alterations for the transforming activity of p60v-src have been investigated by several groups (Hanafusa et al, 1984; Iba et al, 1984; Parker et al, 1984; Shalloway et al, 1984). In all studies performed to date, the cellular src gene has been unable to

efficiently transform primary fibroblasts, even when expressed at high levels. Substitution of the viral carboxy terminus for the cellular src carboxy terminus generates a hybrid protein capable of inducing oncogenic transformation. This and other experiments showing the possible involvement of other v-src specific amino acid changes in the oncogenic activity of p60v-src (Iba et al, 1984) suggest that the activity of $p60^{c-src}$ is tightly regulated in normal cells and structural changes in the src protein are required to activate its oncogenic potential.

The cellular src protein is phosphorylated on serine and tyrosine residues. The serine residue at position 17 that is phosphorylated in $p60^{v-src}$, is also phosphorylated in $p60^{c-src}$ (Collett et al, 1979b; Karess and Hanafusa, 1981). Phosphorylation of tyrosine 416 of cellular p60src does not occur in vivo, but another C-terminal tyrosine at amino acid 527, is the major site of tyrosine phosphorylation (Collett et al, 1979b; Karess and Hanafusa, 1981; Smart et al, 1981; Cooper et al, 1986). Courtneidge (1985) has proposed that $p60^{c-src}$ activity is negatively regulated in normal cells by tyrosine phosphorylation in the C-terminus of the c-src protein. Treatment of cells with the tumor promoters, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and teleocidin, or synthetic diacylglycerol, results in the phosphorylation of a serine at position 12 in both v-src and c-src proteins (Gould et al, 1985). The phosphorylation is mediated by protein kinase C, and essentially all p60src

molecules are phosphorylated at serine 12 after treatment of cells with agents known to activate protein kinase C. The investigators were unable to detect any increase in p60^{src} kinase activity associated with this modification (Goeld et al, 1985).

Amino-terminal phosphorylation of the cellular src protein has recently been shown to be associated with increased tyrosine kinase activity. Increased activity of p60^{c-src} was detected in rat cells infected with polyoma virus. C-src protein molecules associated with the polyoma middle-sized tumor antigen were shown to possess an elevated tyrosine kinase activity associated with a novel site of tyrosine phosphorylation in the amino-terminal half of p60^{c-src} (Yonemoto et al, 1985). Amino-terminal tyrosine phosphorylation of the human c-src gene product has been associated with increased tyrosine kinase activity in human neuroblastomas (Bolen et al, 1985).

Cellular src is detected only at a very low level in most normal cells. A search for a normal cell exhibiting an increased level of c-src expression was conducted with the intention of finding out more about the normal function of the src protein. Cotton and Brugge conducted a study of src expression in developing chicken embryos. The src gene was found to be transiently expressed in the developing brain, suggesting that p60^{c-src} plays a role in neuronal development (Cotton and Brugge, 1983). Similar results were reported by Maness and co-workers in the developing chick

neural retina where p60*src* was localized in developing retinal neurons by immunocytochemical staining using anti-v-*src* antisera (Sorge *et al*, 1984). *In situ* hybridization experiments using the *Drosophila* gene most closely related to v-*src*, demonstrated the association of *src* gene expression with developing neural tissue and muscle (Simon *et al*, 1985). *Src* gene expression was correlated with differentiation but not with cellular division and growth of neurons. The association of *src* with neural tissue has been strengthened further by the discovery of increased *src* kinase activity in human neuroblastomas (Bolen *et al*, 1985).

The investigation of the mechanism of the production of neoplasms by viral oncogenes has turned out to be an investigation of the role of normal cellular proteins in the control of cellular growth and development. Oncogene research has already converged with the growth factor and growth factor receptor fields due to the discovered identity of several oncogenes and certain growth factors and receptors (e.g. PDGF/*sis*, Waterfield *et al*, 1983; EGF receptor/*erb*-B, Downward *et al*, 1984; CSF-1 receptor/*fms*, Sherr *et al*, 1985). Each time the normal cellular function of an oncogene is identified, we obtain a more complete understanding of the control of cellular growth and the loss of control that can lead to neoplasm.

1.3 OBJECTIVES

Our laboratory has been primarily interested in the structure and function of the *src* gene in both the viral and cellular form. Our intention has been to gain as much information on the normal as well as the oncogenic function of these proteins. We have molecularly cloned and determined the DNA sequence of the human cellular *src* gene. The characterization of the *src* gene in humans will constitute the foundation for future studies aimed at detecting possible alterations of cellular *src* in human neoplastic disorders, as well as studies on the structure and function of this protein in normal human growth and development. The characterization of human cellular *src* may also shed some light on the reported detection of two forms of the *src* protein in human cells (Shealy and Erikson, 1981). Since the cellular *src* gene of chickens has already been characterized, it allows us to make observations on the evolutionary conservation of this gene between two relatively distant species.

The second line of investigation we have pursued involves the characterization of fusiform mutants of RSV. Four mutants of this type are in use in our laboratory; WO101, WO201, WO401 and ST529 (Fujita *et al*, 1981a). These mutants have been shown to possess an unlinked pattern of transformation parameters, giving rise to cells which have the growth properties of transformed cells but exhibit an unusual elongated spindle shape in contrast to the usual

rounded cell morphology of RSV-transformed cells. Previous studies in our laboratory have shown that all of these mutants harbor amino-terminal deletions in p60^{src} (Fujita et al, 1981a). Molecular cloning and sequencing of the proviral DNA of these mutants will help to define regions of the src protein which are involved in producing some of the morphological changes induced in cells transformed by RSV.

The ST529 mutant is also a temperature-sensitive mutant (Fujita et al, 1981b). The kinase activity of the src gene product is inhibited at the restrictive temperature, and ST529 infected cells behave as normal cells under these conditions. By identifying the src lesion(s) responsible for the temperature-sensitive behaviour it may be possible to propose a mechanism for the temperature-sensitivity of the kinase activity.

CHAPTER 2

MOLECULAR CLONING AND DNA SEQUENCING OF THE HUMAN CELLULAR SRC GENE

2.1 INTRODUCTION

Although considerable information has been obtained regarding the RSV v-*src* and the chicken c-*src* genes and their protein products, relatively little is known about the human c-*src* gene. A human p60*src* phosphoprotein has been detected by immunoprecipitation of human cell extracts with anti-v-*src* antiserum obtained from rabbits bearing tumors induced by RSV (Oppermann *et al*, 1979; Rohrschneider *et al*, 1979; Sefton *et al*, 1980a). Our laboratory has isolated molecular clones of the human cellular *src* gene from a human genomic DNA library (Lawn *et al*, 1978). One of the clones isolated (λS3H) spanned 16 kilobases of human DNA, and contained sequences capable of hybridizing to most of the viral *src* gene, and was chosen for further characterization. After extensive characterization of this clone, it was discovered that sequences coding for the carboxy-terminus but not the amino-terminus of *src* were present. Screening of the lambda library for clones containing additional 5' sequences resulted in the isolation of a clone (λS11H) which possessed an additional coding exon containing the start codon of the human *src* gene. To verify that the lambda clones contained fragments of human DNA originating from the same locus, lambda clones S3H AND S11H were digested with 7 different restriction endonucleases and subjected to Southern blotting with v-*src* probes. The presence of many identical *src*-hybridizing bands demonstrated the overlapping nature of these clones. Additional Southern hybridizations

were performed using human placental DNA, and the HeLa and WI-38 human cell lines to prove that the lambda clones represented an authentic locus present in human genomic DNA. The cloning and DNA sequencing of the human *src* amino-terminal coding sequence was performed by Akio Tanaka in our laboratory. Initial screening of the human library was conducted by Joe Baar, and restriction enzyme mapping of lambda clones was performed by Janet Radul. Fine structure restriction enzyme mapping of λS3H and λS11H was performed by Carol Gibbs in the laboratory of H.-J. Kung, and this proved to be very helpful for planning the strategies used for DNA sequencing. Since the coding sequence of human *src* is split by many large introns, a DNA sequencing strategy was employed that allowed the selection of regions containing exons of human *src*. Large DNA fragments capable of hybridizing to the viral *src* gene were digested with restriction enzymes that cut DNA frequently and the resulting fragments were subcloned into the M13mp8 vector (Messing, 1982). Individual subclones were selected for sequencing by their ability to hybridize to the viral *src* gene.

During the investigation of the human *src* gene, we became aware of a possible second human c-*src* locus located on human chromosome 1 (Parker *et al*, 1985). This locus possesses a more distant homology to the v-*src* gene, and has been recently shown to be the human equivalent of the v-*fgr* gene (Nishizawa *et al*, 1986). The human c-*src* locus we have

investigated is located on human chromosome 20, and represents the human gene with the greatest homology to v-*src*.

Our laboratory has successfully cloned and sequenced the entire coding region of the human *src* gene. Human c-*src* spans 20 kb of cellular DNA and the coding sequence is interrupted by 10 intron segments. This chapter will describe the isolation of human c-*src* genomic clones, the DNA sequencing of the 3' three-quarters of the human c-*src* coding sequence, and the surprising homology of this gene to the evolutionarily distant chicken c-*src* gene.

2.2 MATERIALS AND METHODS

2.2.1 CHEMICALS

The following radiochemicals were purchased from New England Nuclear Corp.: [alpha-^{32}P]dATP (800.0 Ci/mmol); [alpha-^{32}P]dCTP (3000.0 Ci/mmol); [alpha-^{35}S]dATP (500 Ci/mmol). Ultra-pure urea was from Schwarz/Mann. Acrylamide was from BDH Chemicals Ltd. TEMED and APS were from Bio-Rad Laboratories. IPTG and X-Gal were from BRL (Bethesda Research Laboratories Inc.). Dextran sulfate was from Pharmacia. Other chemicals were purchased from Sigma Chemical Co., J.T. Baker, and Fischer.

2.2.2 BACTERIAL, PLASMID, AND VIRUS STRAINS

A lambda charon4A human fetal liver library was obtained from Fritsch (Lawn et al, 1978). The phage host bacteria was DP50supF, and required NZCY-DT (10 grams NZ amine, 5 grams yeast extract, 5 grams NaCl, 2 grams $MgCl_2$, 0.1 gram diaminopimelic acid, 0.05 gram thymidine per liter) for growth. Plasmid subclones of human src in PBR322 were provided by A. Tanaka and A. Ridgway in our lab. M13mp8 r.f. and the JM103 host bacterium was provided by Dr. D.T. Denhardt. JM103 was grown in SOB medium (20 grams Bacto-tryptone, 5 grams yeast extract, 0.5 gram NaCl per liter, plus 20ml of 1M $MgSO_4$ sterilized separately).

2.2.3 PREPARATION OF RADIOLABELED VIRAL SRC DNA PROBE

A 3.1 kb EcoRI fragment of RSV containing the entire viral src gene obtained from W. DeLorbe (DeLorbe et al,

1980) was nick translated in the presence of 50 to 100 microcuries of [alpha-^{32}P]dCTP using a modification of the procedure of Rigby et al (1977). The reaction was performed in a volume of 20 to 30 ul containing 0.1 ug probe DNA, 50 mM Tris-HCl pH 7.2, 10 mM $MgSO_4$, 1 mM dithiothreitol, 500 ug per ml bovine serum albumin, 0.1 mM of each of dATP, dTTP, and dGTP. 0.5 microliters of DNase I (0.1 ug per ml) was added followed by 5 units of DNA polymerase I. After incubation for one hour at 15 degrees C, the reaction was stopped by the addition of EDTA to 25 mM and the volume was adjusted to 100 ul. The unincorporated nucleotides were removed with a "spun column" (next section) and TCA precipitable counts were measured. Total acid precipitable cpm varied between 10 and 50 million cpm per 0.1 ug of DNA. Poor labelling usually indicated impure DNA.

2.2.4 SPUN COLUMN PROCEDURE FOR REMOVAL OF FREE NUCLEOTIDES

This procedure is from Maniatis et al (1982), and can be used to remove over 80% of the free nucleotides present after nick-translation. The bottom of a 1 ml disposable syringe was plugged with a small amount of sterile glass wool, and the column was filled with G-50 equilibrated with TE (pH 8.0). The column was placed in a 15 ml centrifuge tube and spun briefly at 1500g in a clinical centrifuge to pack the column. The column was refilled and packed until the column volume was 0.9 to 1.0 ml. 100 ul of TE was added and the column was spun for 4 minutes. The DNA sample was added in a volume of 100 ul and the column was spun at 1500g

for 4 minutes with a 1.5 ml eppendorf tube placed underneath to collect the sample.

2.2.5 SCREENING OF THE HUMAN GENOMIC LIBRARY

1 ml of an overnight culture of DP50supF was innoculated into 20 mls of NZCY-DT and grown to an OD_{600nm} of approximately 1.0. Up to 20,000 plaques per 100 mm plate or 60,000 per 150 mm plate were screened using the plaque transfer method of Benton and Davis (1977). Briefly, 0.1 ml of an appropriate dilution of phage was mixed with 0.2 ml of bacteria and the phage were allowed to absorb to the bacteria for 15 minutes. 3 to 9 mls of soft agar (NZCY-DT + 7.0 grams per liter agar) at 45 degrees C was added, quickly mixed and poured onto plates containing hard agar (NZCY-DT + 15.0 grams per liter agar). After overnight incubation at 37 degrees, the plates were cooled to 4 degrees C and labelled nitrocellulose filters (Schleicher & Schuell, Inc.) were placed onto the lawn of bacterial cells and left in place for one to two minutes. While in contact with the plate, the filter was marked assymetrically with a 20 gauge needle attached to an ink-filled syringe. The filter was then carefully removed and placed into denaturing solution (1.5 M NaCl, 0.5 M NaOH) for one minute and then transferred into neutralizing solution (0.5 M Tris-HCl pH 8, 3M NaCl) for one to five minutes and then air dried. The process was repeated in order to make additional replicas, however, the nitrocellulose filter was left on the plate for two to three minutes to allow absorption of more phage. After air-drying,

the filters were baked for two hours at 80 degrees C in a vacuum oven.

Filters were hybridized using a modification of the procedure of Wahl et al (1979). Prehybridization was for four to eight hours at 42 degrees in a solution containing 50% formamide, 5X SSC (1X SSC is 0.15 M NaCl and 0.015 M trisodium citrate), 1X Denhardts' (0.2 mg/ml of each of ficoll, polyvinylpyrrolidone, and bovine serum albumin), 20 mM disodium phosphate, 10% dextran sulfate, and 100 ug/ml denatured E. coli. total nucleic acid. After prehybridization, up to 10 million acid precipitable cpm of denatured probe was added, and hybridization was allowed to proceed overnight at 42 degrees in a sealed plastic bag. After hybridization, the filters were washed at least five times in 2X SSC-0.1% SDS followed by two 30 minute washes in a large volume of 1X SSC-0.1% SDS at 42 degrees and then a single wash in 0.2X SSC for 30 minutes at room temperature. The filters were air dried, then exposed to XAR-5 film (Kodak) for one to three days. Films were aligned with the filters and the positions of alignment holes on the filters were marked onto the film with a pen. Duplicate filters were compared, and signals present on both filters were aligned with the master plate and the agar surrounding the signal location was cut out and placed into .1 ml of phage buffer (10 mM Tris-pH 7, 10 mM $MgCl_2$). By replating at a lower density (<1000 phage per plate), and subsequent rescreening, the hybridizing phage were purified to homogeneity.

2.2.6 LARGE SCALE PREPARATION OF LAMBDA DNA

4×10^{10} bacterial cells were resuspended in 10 mls of phage buffer, mixed with 8×10^{8} phage particles and incubated at 37 degrees C for 15 minutes. The phage and bacteria were then innoculated into 1 liter of NZCY-DT and incubated at 37 degrees C for 8 hours with vigorous shaking. 1 ml of chloroform was added and the flask was incubated for another 15 minutes. A 10 minute centrifugation at 5,000 rpm was performed to pellet unlysed bacteria and debris. DNase I and RNase A were added to the supernate at a concentration of 10 ug/ml each. The supernate was spun at 16,000 rpm in a T19 rotor for 90 minutes at 4 degrees C. The resulting pellet was resuspended in 4 mls of 10 mM Tris-HCl pH 8, 25 mM EDTA. The solution was placed at 68 degrees C for 10 minutes, SDS was added to 0.2%, proteinase K was added to a final concentration of 200 ug/ml. After incubation at 68 degrees C for 30 minutes, the phage DNA was extracted one time with phenol, followed by a phenol-chloroform extraction, and a final chloroform extraction. The DNA was precipitated by the addition of 2 volumes of ethanol without added salt, gently pelleted for 10 seconds at 5,000 rpm, washed with 70% ethanol, dried under vacuum, and resuspended in 1-2 mls of TE pH 7.5. This method allowed the isolation of approximately 1 ug of lambda DNA per ml of lytic culture. The purified DNA was easily digested by restriction endonucleases.

2.2.7 RESTRICTION ENDONUCLEASE DIGESTION OF DNA

Restriction endonucleases were purchased from New England Biolabs, BRL, or Boehringer-Mannheim. All restriction enzyme digestions of DNA were done in the buffers recommended by the supplier and at a enzyme unit to DNA ratio of 2 to 4 units to 1 microgram of DNA in a 2 to 4 hour digestion at 37 degrees. Digested DNAs were run on agarose (BRL) gels (0.7 to 1.5 % in TAE with 1 microgram per ml ethidium bromide) and stained DNA bands were visualized by illumination with ultraviolet light to verify that complete digestion had occurred. Hind III digested lambda DNA (BRL) was used for molecular weight standards.

2.2.8 ELECTROELUTION OF DNA FRAGMENTS

All DNA fragments of human src used for subcloning were obtained by digestion of a plasmid subclone and electroelution of the desired fragment from agarose gels as follows. The desired DNA band was localized on the gel using a long-wavelength ultraviolet lamp. The region of the gel containing the band was cut out and placed in a dialysis tube filled with TAE buffer. The DNA was eluted from the gel slice at 100 volts for one hour, at which time the DNA was stuck tightly to the dialysis membrane and the gel slice was removed. The current was reversed momentarily to free the DNA and the solution was removed from the dialysis bag, extracted with butanol several times followed by phenol-chloroform extraction and ethanol precipitation in the presence of 2.5 M ammonium acetate.

2.2.9 SUBCLONING OF THE HUMAN *SRC* GENE INTO M13MP8

DNA fragments which had been previously shown to contain sequences homologous to viral *src* by hybridization studies (Gibbs *et al.*, 1985), were digested with Alu I and/or Hae III restriction endonucleases which generated blunt-ended fragments that were subsequently ligated into the blunt-ended Sma I cleaved M13mp8. The restriction enzyme was inactivated by heating or removed by phenol/chloroform extraction and ethanol precipitation if the enzyme could not be inactivated by heating (Hae III). A 0.6 kb Bam HI fragment containing the carboxy-terminal coding region was cloned directly into the Bam HI site of M13mp8. Pst I and Pst I/Pvu II digestions were performed on a 2.8 kb Bam HI fragment, and the resulting fragments were inserted into the appropriate sites in M13mp8. Ligations were performed at 15 degrees for 4 to 8 hours in 10 to 20 ul of ligation buffer (66 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, and 1 mM ATP) with 2 units T4 DNA ligase (BRL). 50 to 500 ng of ligated DNA was then used to transform JM103 bacterial cells made competent by treatment with 50 mM $CaCl_2$ for 20 minutes on ice (Mandel and Higa, 1970). After incubation for 40 minutes on ice, the mixture was heat-shocked at 42 degrees for two minutes and 0.2 ml of log-phase JM103 (made by growing a 1/100 dilution of an overnight culture for 1-2 hours) were added. After the addition of 10 ul of 0.1 M IPTG and 50 ul of X-Gal (BRL), the cells were mixed with 3 mls of soft agar and spread on 100 mm LB plates. Overnight incubation at 37

degrees C allowed visualization of approximately equal numbers of clear (containing DNA inserts) and blue-coloured plaques (religated vector).

2.2.10 PREPARATION OF SINGLE-STRANDED M13 TEMPLATE DNA

Individual well isolated plaques were picked and placed into 2 mls of exponentially growing JM103 (see previous section), and grown for 6 to 12 hours at 37 degrees C with constant agitation. The bacteria were pelleted by a 10 minute centrifugation, and 1.2 mls of supernate was removed and mixed with 0.3 mls of 2.5 M NaCl- 25% PEG in a 1.5 ml eppendorf tube. The mixture was left at room temperature for 15 minutes, put on ice for 5 minutes, then centrifuged for 15 minutes at 4 degrees C. The resulting pellet was carefully drained using microcapillary tubes and resuspended in 0.4 ml of 10 mM Tris-HCl. pH 8, 1 mM EDTA (TE pH 8), 0.5% sarkosyl. The viral DNA was then reprecipitated by adding 0.1 ml of a freshly made solution containing 2.5 M NaCl, 25% PEG, 0.5% sarkosyl, using the method described above. The pellet was resuspended in 0.1 ml of TE-sarkosyl and phenol/chloroform extracted twice followed by a single chloroform extraction. The DNA was precipitated by the addition of ammonium acetate to 2.5 M plus two volumes of 95% ethanol, placed at -70 degrees for 30 minutes, and centrifuged for 15 minutes. The purified DNA pellet was rinsed with 70% ethanol, dried under vacuum, and resuspended in 20 ul of TE pH 7.5.

2.2.11 HYBRIDIZATION OF M13 SUBCLONES TO V-SRC PROBES

2 ul of virus-containing supernatant (see previous section) from each subclone was spotted onto nitrocellulose filters (Schleicher & Schuell) and the filters were treated and hybridized to radiolabelled src probe as described in section 2.2.5. Most of the hybridizing clones could be detected by exposure of filters to Kodak XAR 5 film for 6 hours with a Dupont Cronex intensifying screen.

2.2.12 DNA SEQUENCING OF M13 SUBCLONES

The procedures used for DNA sequencing are modifications of the method described by Sanger et al (1977) for sequencing using [alpha-^{32}P]dATP, or the method of Biggin et al (1983) when [alpha-^{35}S]dATP was used. The template was annealed to a 17 nucleotide primer (New England Biolabs) in a 0.5 ml Eppendorf tube containing 5 ul (1-2 ug) template, 2 ul (5ng) primer, 1.5 ul reaction buffer (100 mM Tris pH 8, 50 mM $MgCl_2$) and 4 ul water. The tube was sealed and placed into a 15 ml test tube in boiling water. After 5 minutes, the test tube was removed and allowed to cool to room temperature. 1 ul of radiolabelled dATP was added, then 0.5 ul of DNA polymerase Klenow fragment (5 units/ul, BRL), and 3.0 ul aliquots were added to each of the four reaction tubes containing 1 ul ddNTP (0.1 to 2 uM) and 1 ul dNTP mix (10 uM of the inhibited nucleotide and 150 uM of the other three). The reaction was incubated for 15 minutes at 30 degrees C (20 minutes if [^{35}S]dATP was used), and then 1 ul of "chase mix" (0.5 mM of each dNTP) was added to each tube

and the reaction was continued for an additional 15 minutes. The reaction was terminated by the addition of 10 ul of 95% formamide, 10 mM EDTA containing 0.2% bromphenol blue and 0.2% xylene cyanol. The samples were boiled for 5 minutes and put on ice before loading onto sequencing gels.

Sequencing gels (40 cm long by 0.3 cm thick) of 8% acrylamide, 0.4% Bis-acrylamide, 8 M urea, were made up and run in 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA. These gels were used to sequence up to 250 nucleotides from the M13 primer. Sequence over 500 nucleotides from the primer could be obtained by using 80 cm gels run in a buffer containing 135 mM Tris, 45 mM boric acid, 2.5 mM EDTA (Anderson, 1981). The gels were preheated to 50 degrees before the DNA sample was loaded. Gels were run at an average current of 25 milliamperes for times ranging from 2 hours for short gels to 24 hours for long gels. A metal plate was attached to the front of the glass plate to avoid uneven heating of the gel. If [^{32}P] labeling was used, the gels were covered with cellophane and exposed to Kodak XAR-5 film at -70 degrees overnight. When [^{35}S] label was used, the gel was fixed in 10% acetic acid for 10 minutes, rinsed for 10 minutes in water, and dried on a gel dryer. The dried gel was then exposed directly to the film at room temperature for 1 to 2 days. Sequences obtained were compared with the v*src* gene using a sequence comparision program designed for the Apple II computer (Larson and Messing, 1982).

2.3 RESULTS

2.3.1 STRUCTURE OF THE HUMAN CELLULAR *SRC* GENE

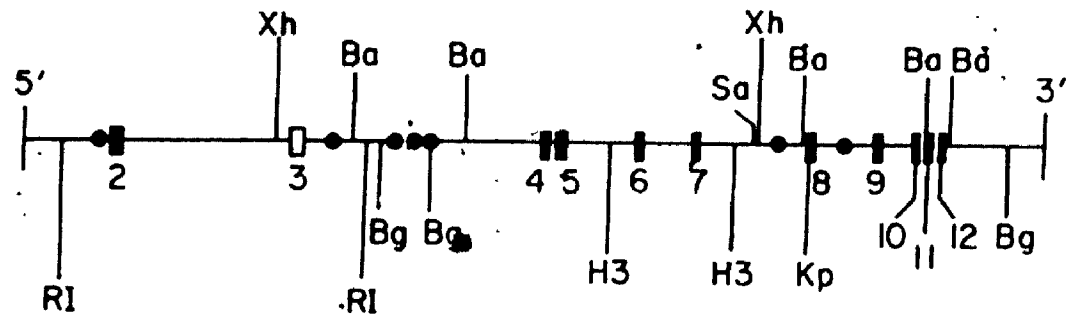
Approximately one million recombinant bacteriophage from a lambda Charon 4A human genomic library were screened with a radiolabelled viral *src* DNA probe as described in materials and methods. A total of 10 recombinant clones containing human cellular *src* sequences were isolated. Four of these clones, designated S3H, S4H, S5H, and S11H, were used for a restriction enzyme mapping study. Fine structure restriction enzyme mapping was performed on S3H and S11H. Exon-containing regions of the human *src* gene were localized by combining restriction enzyme mapping with Southern blotting (Southern, 1975) analysis using either total v-*src* probes or probes specific for certain regions of v-*src*. Figure 1 shows the restriction map of human cellular *src* with the exon locations indicated and the regions of the gene represented by the lambda recombinant phage isolated.

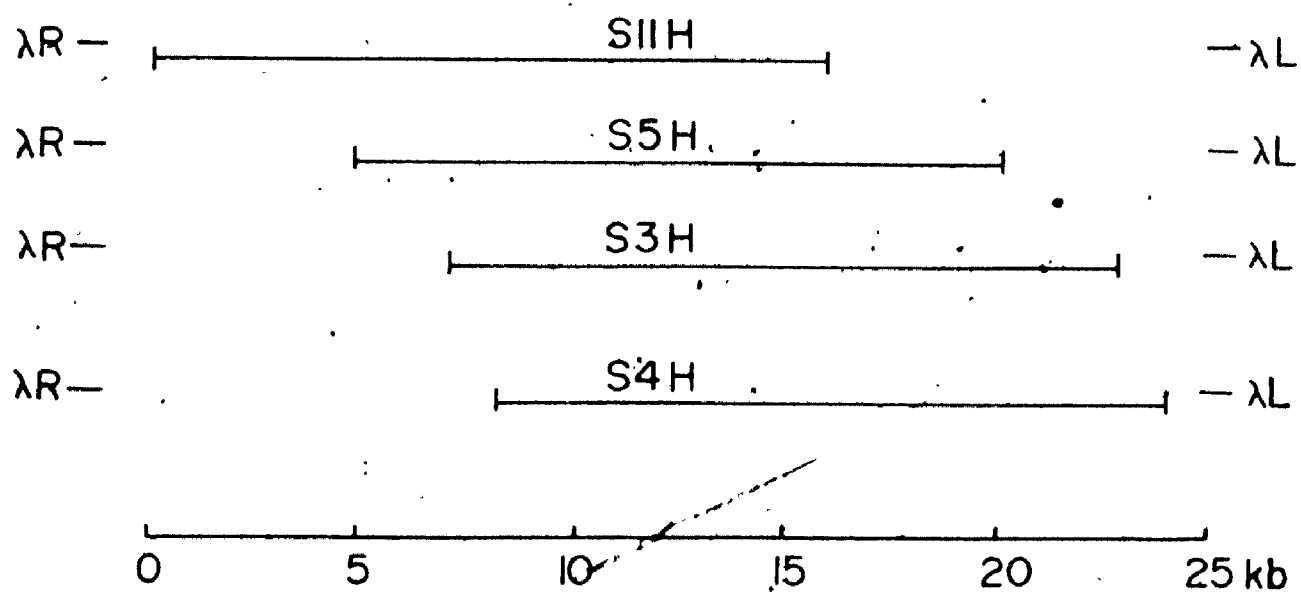
2.3.2 SEQUENCING STRATEGY FOR HUMAN *SRC*

Since the human *src* locus spans some 20 kb of cellular DNA and is split by many large introns, some larger than 2 kb, we adopted a sequencing strategy that would avoid sequencing the large amount of DNA that did not code for the human *src* protein. Specific restriction endonuclease fragments that had been shown in Southern blotting studies to contain regions homologous to v-*src* (Gibbs *et al*, 1985), were subcloned into M13mp8, and subclones were selected for

Figure 1. Restriction map of the human c-*src* gene. The topography of the human c-*src* locus is shown with the regions represented by lambda phage clones indicated below. Abbreviations and symbols: RI, Eco RI; Xh, Xho I; Ba, Bam HI; Bg, Bgl II; H3, Hind III; Sa, Sal I; Kp, Kpn I; ■, exons regions representing human c-*src* coding sequences; ●, Alu family repeated sequence; λR- lambda Ch4A right arm; λL- lambda Ch4A left arm. From Gibbs *et al*, 1985.

λ Clones of Human c-src



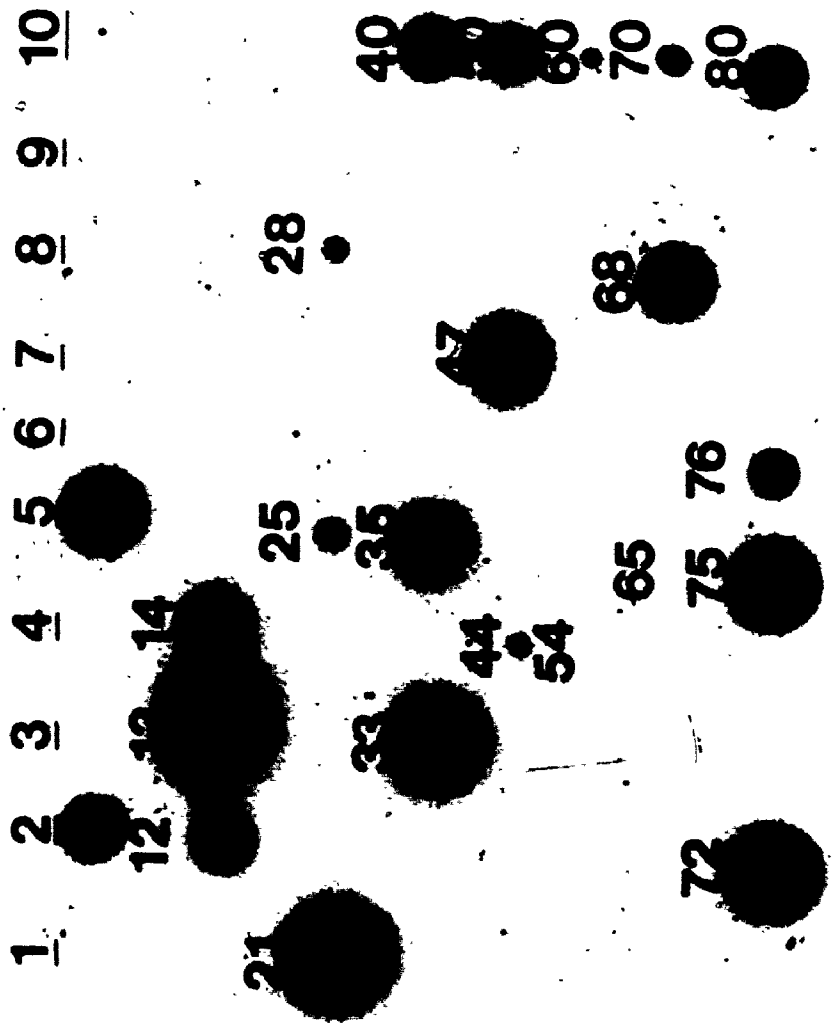


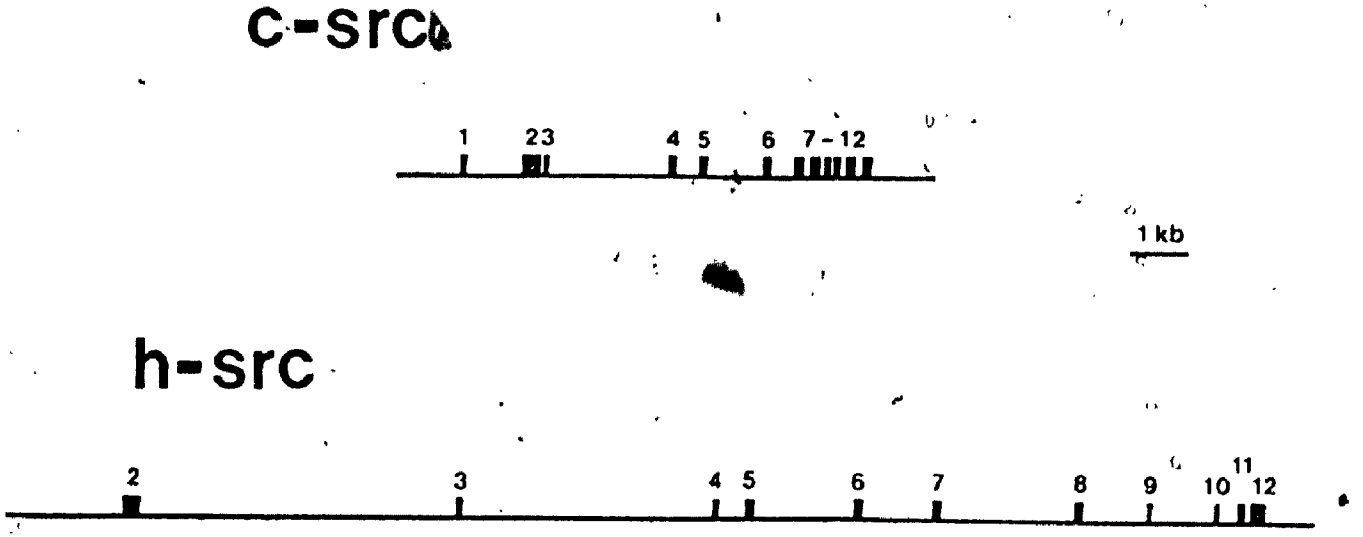
sequencing by hybridization to v-src in "dot blot" assays (see section 2.2.11). A sample "dot blot" assay is shown in Figure 2. The DNA fragments used for subcloning were: a 3.9 kb Bam HI to Hind III fragment containing exons 4 and 5 (see Fig. 1; the rationale for numbering human src exons will be explained in the discussion section); a 2.9 kb Hind III fragment containing exons 6 and 7; a 2.8 kb Bam HI segment containing exon 8, 9, 10, and part of 11; a 600 bp Bam HI fragment containing the rest of exon 11 and exon 12 (the carboxy-terminal coding region of human src). The actual regions and the strands (5' to 3' or 3' to 5' strand) sequenced are illustrated in Figure 3. Any ambiguities in the sequence determined from one strand were resolved by the sequence read from the other strand.

2.3.3 ACCURACY OF THE SEQUENCING PROCEDURE

We are confident of the sequences determined using the dideoxy method for several reasons: (i) the sequences read from gels were, in general, unambiguous; (ii) any region where there was any doubt as to the sequence obtained was clarified by the sequence read from the other strand; (iii) the three carboxy-terminal exons of human c-src have been sequenced by another group using the Maxam and Gilbert method of DNA sequencing (Parker et al, 1985), and the sequence determined corresponds exactly to the sequence I have determined for this region; (iv) I have participated in sequencing the B gene of bacteriophage Mu using the same method, and the sequence determined agrees with the sequence

Figure 2. Dot blot assay. A representative dot blot is shown. 80 Hae III subclones of the 2.9 kb Hind III fragment containing exons 6 and 7 (see Figure 1) were tested using the procedure described in the text. The faintly hybridizing spots (e.g. 25), represent subclones containing a 7 bp Hae III fragment within exon 7.


1
2
3
4
5
6
7
8
9
10
12
14
21
25
28
33
35
40
44
47
54
60
65
68
70
72
75
76
80


c-src
1
23
4
5
6
7-12
1 kb
h-src
2
3
4
5
6
7
8
9
10
11
12

DNA SEQUENCING STRATEGY

of this gene derived by the Maxam and Gilbert sequencing procedure (Miller et al, 1984). For illustrative purposes, Figure. 4 shows two representative 80 cm DNA sequencing gels. Note the much clearer and finer DNA bands in the upper region of the gel in which [^{35}S]dATP was used. The clearer bands are due to the lower energy of [^{35}S] beta emissions, and the sharper pattern allows more nucleotides to be read from each gel. Finer bands than those shown in Figure 4 could be obtained by loading less DNA onto the gel, but this resulted in an increased exposure time of the autoradiogram which was inconvenient. Also, the safety of the procedure is increased due to the lower energy of the radioactive emissions (Biggin et al, 1983).

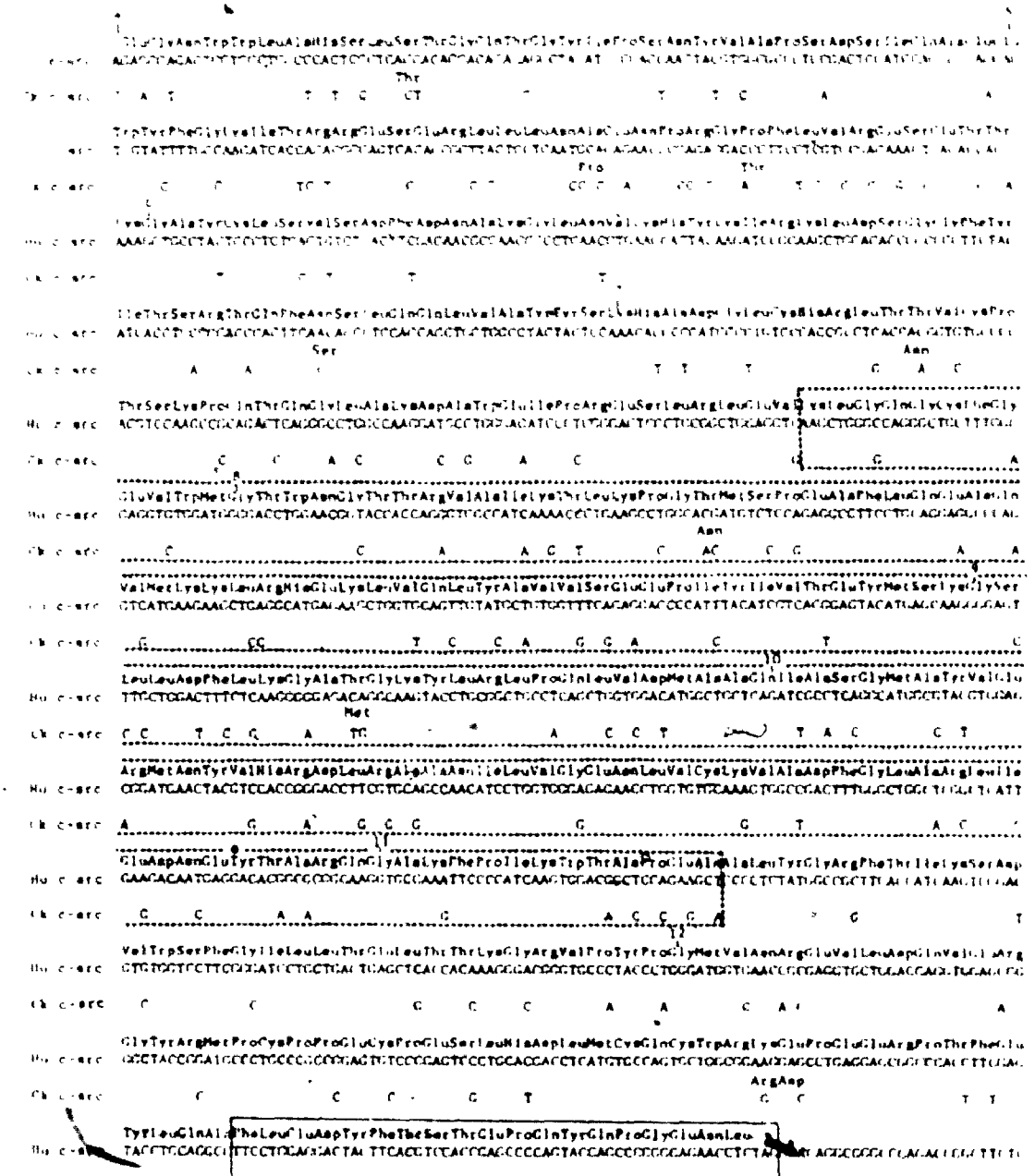
2.3.4 NUCLEOTIDE SEQUENCE OF THE 3' THREE-QUARTERS OF HUMAN SRC AND THE DEDUCED AMINO ACID SEQUENCE

This section describes the nucleotide sequence of the nine exon regions of human c-src that correspond to nucleotides 341 to 1542 of the v-src coding region (Czernilofsky et al, 1980; Takeya and Hanafusa, 1982) and are predicted to code for the carboxy-terminal three-quarters of human p60^{src}. The predicted nucleotide sequence of mature human src mRNA and the deduced amino acid sequence for this region of the human src protein are presented in Figure 5. Human c-src is compared with the chicken cellular src gene using the exon and nucleotide numbering scheme of chicken c-src since the number and location of introns in the human c-src gene was found to be

Figure 4. Sample 80 cm sequencing gels. The autoradiogram on the left represents a sequence ladder generated using [^{32}p]dATP. The autoradiogram on the right is a sequence generated with [^{35}S]dATP. The nucleotide represented by several of the bands is indicated at the bottom of the autoradiogram. Numbers indicate the size of the single-stranded DNA fragments separated. Detailed procedures are described in the text.

300
300

Figure 5. Coding sequence of human c-*src* exons 4 to 12. The nucleotide sequence and the deduced amino acid sequence of the 3′ three-quarters of the human c-*src* coding sequence is compared with the homologous chicken c-*src* exons. Only nucleotides or amino acids that differ from those of human c-*src* are shown for chicken c-*src*. The beginning of each c-*src* exon is indicated by a numbered vertical line. The sequence enclosed by the solid lines is the carboxy-terminal region of c-*src* that is different from v-*src*. The dotted lines enclose the tyrosine kinase domain that is conserved among several tyrosine kinase oncogenes. Numbers beside the sequence represent the nucleotide number of the *src* coding sequence. The numbers in brackets represent the amino acid number of p60*src*.



identical to that of chicken c-*src* (Takeya and Hanafusa, 1983). Nucleotide and amino acid residues of chicken *src* are indicated only where they differ from the human *src* gene. There is considerable nucleotide conservation of cellular *src* between the two species, since 87% (1100 of 1258 residues) of the nucleotides are homologous. The majority of the nucleotide substitutions are phenotypically silent changes in the third position of each codon, resulting in no amino acid substitutions, and therefore only a 2% divergence exists at the amino acid level. Furthermore, all nine of the amino acid changes that were observed within this region of p60*src* are conservative substitutions as defined by the criteria of French and Robson (1983). Interestingly, both the molecular size and the relative hydrophobicity of the amino acid residues are conserved in seven of the nine changes. The tyrosine residue at position 416 which is phosphorylated in viral p60*src* *in vivo* and in chicken p60*src* *in vitro* (Oppermann *et al*, 1979; Smart *et al*, 1981) has also been conserved in the human *src* gene, as has lysine 295, which has been implicated as an ATP-binding site in viral p60*src* (Kamps *et al*, 1984).

Of particular significance is the sequence homology at the 3' ends of the human and chicken cellular *src* genes. The predicted amino acid sequence of the human p60*src* carboxy terminus is identical to that of the chicken *src* protein but different from that of RSV p60*src*. Takeya and Hanafusa (1983) have shown that the last 19 amino acids predicted to

be the chicken cellular src carboxy-terminus have been substituted by twelve unrelated amino acids in the viral src protein. The v-src specific 3' coding region is thought to have been captured by RSV through a recombinational event involving a region 1 kb downstream from the normal chicken cellular src termination codon. Our nucleotide sequencing result predicting the identical amino acid sequence at the carboxy-termini of the human and chicken p60src molecules supports this model. However, we have no evidence at this time suggesting the existence of an analogous "v-src-like" sequence downstream from the carboxy-terminal coding region of human src; hybridization analyses with a v-src probe have failed to detect such a sequence within a region extending 2 kb downstream from the termination codon of human src.

2.3.5 EXON AND INTRON ORGANIZATION OF HUMAN CELLULAR SRC

The sequence data demonstrates that the human src exon sizes and intron-exon junctions of exons 4 through 12 are identical to those of the chicken src gene (Takeya and Hanafusa, 1983; Figure 5; Table 1). Sequences of the predicted human src splice sites bordering each exon are listed in Table 1. All of the putative splice acceptor signals are similar to the consensus sequence YNYYYNCAG/, and the presumed splice donor signals conform to the /GTNAG consensus sequence, strongly suggesting that these regions are indeed splice junctions. The chicken cellular src gene spans 6 kb of cellular DNA (Takeya and Hanafusa, 1983), whereas the human gene covers over 20 kb. This discrepancy

TABLE I

SPLICE DONOR AND SPLICE ACCEPTOR SEQUENCES OF HUMAN c-SRC[a]

EXON		INTRON (SA)	EXON SIZE (BASE PAIRS)[b]	INTRON (SD)
4	H	CCTGCTCAG AGAGGGAGA	....99....	GGCTGAGGA GTTAG
	C	TCTTG G A T		A A T
5	H	CCCCCAG GTGGTATTT	...104....	CCACGAAAG GTACG
	C	A C		G A GAT
6	H	GCCCCGCAG GTGCCTACT	...153....	ACTACTCCA GTGAG
	C	TA T T		T
7	H	CCTCCTCAG AACACGCCG	...156....	TGTGGATGG GTAAG
	C	TGTGCT T T		C
8	H	CCTCAACAG GGACCTGGA	...180....	TGAGCAAGG GTGAG
	C	GTG TGT		
9	H	TCTGCCCAG GGAGTTTGC	....77....	GCTGCTCAG GTGAG
	C	CT A CC C		G
10	H	CTGCAG ATCGCCTCA	...154....	CGCGGCAAG GTGGG
	C	CA T A C		A T
11	H	TTCCTGCAG GTGCCAAAT	...132....	CCTACCCTG GTAAG
	C	CC GCC G		A A G
12	H	CTGCCACAG GGATGGTGA	...(209...TAG)	
	C	T T C		

a The nucleotide sequences of human (H) splice acceptors (SA) and splice donors (SD) are compared with the chicken (C) splice junctions. Only nuceotide differences are shown for chicken c-src.

b Exon sequences at the splice junctions are shown.

is due to the much larger average size of human introns (Table 2; Figure 6).

In contrast to the extensive nucleotide homology found within human and chicken cellular src exons, sequences within the introns have not been conserved. This finding is supported by several lines of evidence: (1) the proposed intron regions do not form heteroduplexes that are visible by electron microscopy, even under low-stringency spreading conditions, in contrast to the exon regions (C. Gibbs, personal communication); only one of the corresponding introns has a similar size (Table 2); the nucleotide sequence of human src intron #11 was compared to chicken intron #11, and no regions of homology were discovered (Takeya and Hanafusa, personal communication).

TABLE II

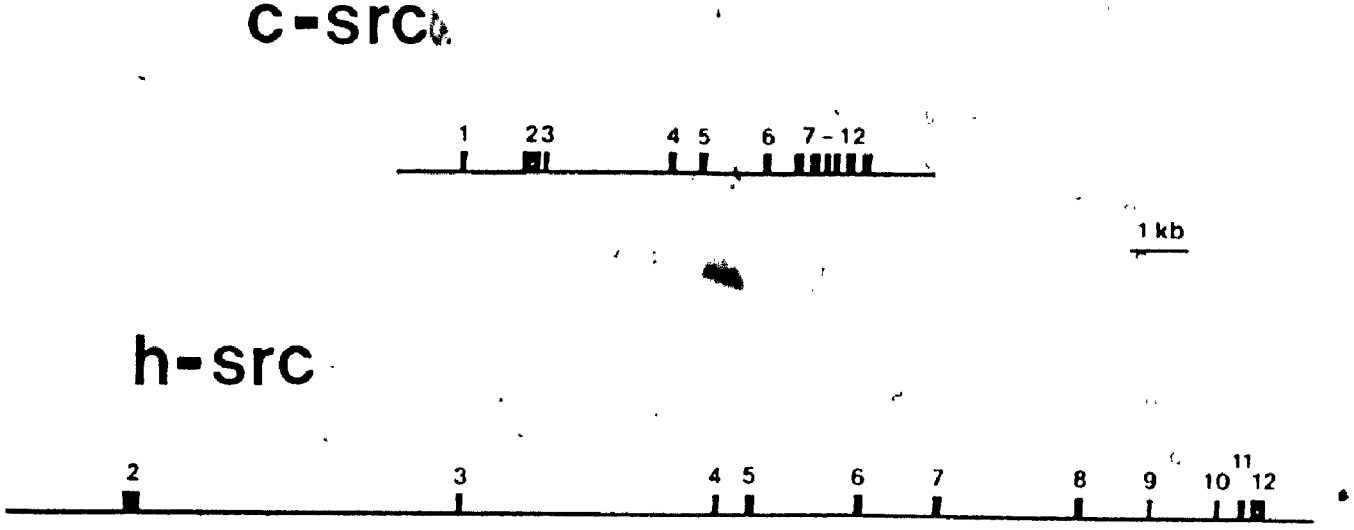
INTRON SIZE IN THE C-SRC GENES OF HUMAN AND CHICKEN

INTRON[b]	INTRON SIZE (BASE PAIRS)[a]	
	HUMAN C-SRC	CHICKEN C-SRC
4	350	390
5	1750	1010
6	1200	350
7	2300	85
8	1600	78
9	780	61
10	160	118
11	280	79

a Human intron sizes are estimates from restriction enzyme mapping and DNA sequencing information. Chicken intron sizes are from Takeya and Hanafusa (1983).

b Introns are numbered here with the same number as the exon located immediately 5' of the intron.

Figure 6. Comparison of the structural organization of the human and chicken c-*src* genes. The structure of the human and chicken c-*src* genes are drawn to scale, showing a similar spacing of exon units, but much larger introns in the human c-*src* gene.


c-src
1
23
4 5
6
7-12
1 kb
h-src
2
3
4 5
6
7
8
9
10
11
12

2.4 DISCUSSION

The human cellular src gene displays the typical intron-exon structure characteristic of eucaryotic cellular genes. Human src spans some 20 kb of DNA, but contains only 1.6 kb of coding sequences. To limit the amount of non-coding sequence obtained, we specifically sequenced the regions surrounding the coding exons which represent the information carried into the mature mRNA after processing of primary RNA transcripts. Exon regions were selected by hybridization to v-src probes and their identity as true exons of human src was confirmed by the presence of a continuous open reading frame coding for amino acids homologous to v-src, and the existence of splice donor and acceptor sequences at appropriate junction locations where the homology to v-src terminated. In the midst of this study, the sequence of the chicken cellular src gene was published (Takeya and Hanafusa, 1983). Exons of human src have been numbered by analogy to corresponding exons of the chicken src gene, which exhibits the same general structural organization as human src. Human src exons 4 through 12 have been found to correspond precisely to the homologous chicken src exons with respect to exon size and location of exon-intron boundaries. However, most of the human introns are much larger (Table 2), which accounts for the larger size of the human src locus (20 kb) when compared with the chicken gene (6 kb; Figure 6).

The coding region of the cellular *src* gene is very highly conserved in chickens and humans, exhibiting 87% nucleotide homology, which probably reflects strong functional constraints on p60^{src} evolution, as well as a similar pattern of codon usage in vertebrates (Hastings and Emerson, 1983). On the amino acid level, 98% homology exists between their products in the corresponding region encompassing amino acids 115 to 533, and the nine amino acid changes observed in this region can be interpreted as conservative substitutions (French and Robson, 1983). In contrast to the highly conserved nature of the cellular *src* gene, the human c-*myc* gene shares much less amino acid homology (72%) with the chicken c-*myc* gene, and contains several large deletions and insertions relative to chicken c-*myc* (Colby *et al*, 1983; Watson *et al*, 1983).

It has been suggested that the p60^{src} protein contains at least two functional domains: a domain in the carboxy-terminal half, containing tyrosine kinase activity, and one or more domains in the amino-terminal half which may affect membrane binding, cell morphology, and tumorigenicity (Fujita *et al*, 1981a; Levinson *et al*, 1981; Cross *et al*, 1984; Parsons *et al*, 1984; Stoker *et al*, 1984). The region of human c-*src* described here encompasses the tyrosine kinase domain which is highly homologous to the corresponding region of chicken c-*src*. The domain of p60^{src} specified by this region also exhibits considerable homology to regions within several oncogene products, including those

of the abl, fes, fps, and yes genes. They are all apparently members of a gene family coding for proteins that possess a tyrosine kinase activity (Hampe et al, 1982; Goffen et al, 1983; Hunter, 1984). In contrast to the strong homology of the tyrosine kinase domain, the DNA sequence of the human c-src amino terminus (exon 2) has been determined, and it contains a region of non-homology which may reflect a species-specific alteration in p60^{src} structure and function (A. Tanaka, personal communication).

Although we have not performed an exhaustive analysis of the structural features of human p60^{src} as predicted by our sequencing results, we have noticed that several regions thought to be of functional importance in viral and/or cellular src have been conserved in human src. For example, the tyrosine 416 residue and adjacent amino acids are totally conserved in human p60^{src}; this tyrosine is known to be phosphorylated in viral src molecules in vivo and chicken p60^{c-src} in vitro, although it is apparently not phosphorylated on chicken p60^{c-src} in vivo (Oppermann et al, 1979; Smart et al, 1981). It is not known at this time whether or not this site is phosphorylated on human p60^{src} molecules. Lysine 295, a residue involved in ATP binding of the viral src protein (Kamps et al, 1984), and neighbouring amino acids have been conserved in both the human and chicken cellular src proteins.

A close visual examination of the amino acid sequence revealed the presence of a region within p60^{src} that shares

strong homology to regions that are highly conserved in several regulatory proteins that have DNA binding activity, including the repressor and cro proteins of bacteriophage lambda and 434, and the Mu phage B transposition protein (Sauer et al, 1982; Ohlendorf et al, 1983; Miller et al, 1984). This region is centered around glycine 288 in human p60src and includes the accepted consensus sequence residues:

Gly(284)-X-X-X-Gly(288)-X-X-X-X-X-Ile(294).

Several known procaryotic DNA binding proteins contain a characteristic structural motif consisting of a DNA binding domain containing two alpha-helical regions separated by a reverse turn occurring at the central glycine of the consensus sequence (Sauer et al, 1982; Ohlendorf et al, 1983). A computerized secondary structure prediction (courtesy of G. Cnaconas) indicates that the sequence of amino acids in this region is consistent with this structure. For illustrative purposes, Figure 7 provides a comparison between this region of the human src protein and the DNA binding domains of six known procaryotic DNA binding proteins that possess additional homology to this region of the c-src protein. Of the 22 amino acids in this region of c-src, 13 can be found in an identical position in one of the DNA binding proteins shown. Of the nine non-homologous residues, 6 can be assigned to the same conservative group (classified by molecular size and hydrophobicity) as the residues observed in the procaryotic DNA binding proteins.

Figure 7. p60^{src} contains a DNA-binding consensus sequence. The region of the src protein described in the text, is compared with DNA binding regions of procaryotic regulatory proteins (Sauer et al, 1982; Ohlendorf et al, 1983). Residues marked by a heavy bar are consensus residues found in a large number of DNA binding regions examined. Human c-src amino acids that match those in corresponding regions of certain procaryotic DNA binding domains are underlined, as are the matching residues.

pp60^{src}-human	Phe-Gly-Glu-Val-Trp-Met-Gly-Thr-Trp-Asn-Gly-Thr-Thr-Arg-Val-Ala-Ile-Lys-Thr-Leu-Lys-Pro
λ Cro	Phe-Gly-Gln-Thr-Lys-Thr-Ala-Lys-Asp-Leu-Gly-Val-Tyr-Gln-Ser-Ala-Ile-Asn-Lys-Ala-Ile-His
P22 repressor	Ile-Arg-Gln-Ala-Ala-Leu-Gly-Lys-Met-Val-Gly-Val-Ser-Asn-Val-Ala-Ile-Ser-Gln-Trp-Glu-Arg
434 repressor	Leu-Asn-Gln-Ala-Glu-Leu-Ala-Gln-Lys-Val-Gly-Thr-Thr-Gln-Gln-Ser-Ile-Glu-Gln-Leu-Glu-Asn
CAP	Ile-Thr-Arg-Gln-Glu-Ile-Gly-Gln-Ile-Val-Gly-Cys-Ser-Arg-Glu-Thr-Val-Gly-Arg-Ile-Leu-Lys
λ cII	Leu-Gly-Thr-Glu-Lys-Thr-Ala-Glu-Ala-Val-Gly-Val-Asp-Lys-Ser-Gln-Ile-Ser-Arg-Trp-Lys-Arg
434 Cro	Met-Thr-Gln-Thr-Glu-Leu-Ala-Thr-Lys-Ala-Gly-Val-Lys-Gln-Gln-Ser-Ile-Gln-Leu-Ile-Glu-Ala

Two of the exceptions are tryptophane residues at positions 282 and 286 of p60^{src} which are residues with a small molecular size in the DNA binding proteins. The only other exception is the presence of an asparagine at position 287 which is normally a hydrophobic residue.

The possible DNA binding region of human c-*src* is conserved in chicken and RSV p60^{src} (Czernilofsky *et al*, 1980; Takeya and Hanafusa, 1983). We do not not the significance of this result, nor do we know if this region can indeed function as a DNA binding region. Such an activity is highly speculative, since the majority of p60^{src} molecules are localized at the inner surface of the cellular plasma membrane (Willingham *et al*, 1979), and a DNA binding activity has not been demonstrated for the *src* protein. A putative DNA binding sequence has been reported for the product of the avian myeloblastosis virus *myb* gene, which is known to be localized in the cell nucleus (Lipsick *et al*, 1984). It is possible that the presence of a DNA binding-like sequence in this region of the *src* protein is merely a reflection of the ATP binding domain which overlaps with this region in viral p60^{src} (Kamps *et al*, 1984). However, it should be noted that bovine cAMP dependent protein kinase does not contain the DNA binding consensus, although it possesses conserved residues thought to be important for ATP binding (Bark[illegible] and Dayhoff, 1982; Kamps *et al*, 1984).

The last 19 amino acids of the predicted human p60*src* protein are identical to those of the chicken carboxy terminus. In contrast, these 19 amino acids are replaced by 12 unrelated amino acids in the RSV *src* protein. This finding lends further support to the hypothesis that the 3' terminus of v-*src* was acquired by a recombinational event involving the capture of a region downstream from the normal chicken c-*src* carboxy terminus (Takeya and Hanafusa, 1983). It is perhaps also possible that the p60$^{v\text{-}src}$ carboxy-terminal region was derived through an alternative splicing mechanism. However, this possibility seems less likely, since a consensus splice donor sequence is not present in this region and the nucleotide homology between the human and chicken c-*src* genes extends up to, but not beyond, the proposed termination codon for each gene, suggesting that this region signals an authentic p60*src* carboxy terminus (Fig. 4).

Experiments designed to test the transforming ability of chicken c-*src* indicate that c-*src* is not capable of inducing cellular transformation (Hanafusa *et al*, 1984; Iba *et al*, 1984; Parker *et al*, 1984; Shalloway *et al*, 1984). However, chimeric forms of chicken c-*src* and RSV v-*src* that contain the v-*src* carboxy-terminal coding region are capable of causing efficient transformation of infected cells. In addition, recent evidence suggests that alterations in other chicken c-*src* regions may result in transforming activity (Iba *et al*, 1984). These results suggest, by analogy, that

certain alterations of the human *src* gene might affect its functional activity and/or its ability to transform appropriate cells. At this time, the involvement of *src* in the development or progression of specific human neoplastic disorders has not been clearly documented. However, the level of expression of *src* mRNA has been reported to be elevated in some human leukemias (Rosson and Tereba, 1983; Slamon *et al*, 1984), and increased $p60^{src}$ kinase activity has been found in human neuroblastomas (Bolen *et al*, 1985).

In conclusion, we have demonstrated that the nucleotide sequence and exon structure of the 3' three-quarters of the human c-*src* gene are highly conserved relative to that of chicken c-*src*. The conservative nature of the amino acid changes observed suggests that the three-dimensional structure of this region of human and chicken $p60^{src}$ is nearly identical. This finding is consistent with the suggestion that $p60^{src}$ has an important role in cellular function and possibly plays a tissue specific role in development or differentiation (Gonda *et al*, 1982; Mueller *et al*, 1982; Cotton and Brugge, 1983; Sorge *et al*, 1984).

CHAPTER 3

CLONING AND CHARACTERIZATION OF FUSIFORM MUTANTS OF ROUS SARCOMA VIRUS

3.1 INTRODUCTION

Chicken embryo fibroblasts infected with wild-type (wt) RSV, develop a typical rounded transformed cell morphology within several days. In contrast, cells transformed by fusiform mutants of RSV exhibit an elongated spindle shape. This fusiform or morphf phenotype was first described by Temin (1960). Cells transformed by some fusiform mutants have been shown to display an unlinked pattern of transformation parameters (Anderson et al, 1981; Fujita et al, 1981b). Such cells expressed many typical transformation parameters such as increased hexose uptake, increased level of plasminogen activator, density independent growth, and in some cases, tumorigenicity in vivo. However, cells transformed by these viruses also retained high levels of fibronectin and cell adhesiveness, and exhibited less disruption of actin containing microfilaments (Fujita et al, 1981b). Several fusiform mutants have been isolated and characterized (Temin, 1960; Yoshii and Vogt, 1970; Anderson et al, 1981; Fujita et al, 1981a; Fujita et al, 1981b; Oppermann et al, 1981; Kitamura and Yoshida, 1983), but the exact molecular nature of the genomic lesions involved had been determined for only one of the naturally occurring mutants (Kitamura and Yoshida, 1983). RSV mutants containing various amino-terminal alterations in $p60^{v\text{-}src}$ have been generated by in vitro mutagenesis (Cross et al, 1984; Hughes et al, 1984; Cross et al, 1985) and some of these mutants are reported as causing

a fusiform type morphology of infected cells. These mutants indicate alterations that may cause the fusiform phenotype, provided that no additional mutations have occurred after transfection of *in vitro* constructs.

The occurrence of fusiform mutants, and the unlinked pattern of transformation parameters reported for some of them, suggests that the *src* gene exerts its effects by acting on more than one substrate. Analysis of the genomic alterations in these mutants should provide clues concerning a possible functional domain within $p60^{src}$ responsible for some of the morphological changes observed in cells transformed by RSV.

This chapter presents the results obtained from a molecular cloning and DNA sequencing analysis of the lesions present in the v-*src* gene of three fusiform mutants: WO101, WO201, and tsST529 (Fujita *et al*, 1981a). Characterization of the *src* proteins of these mutants by partial proteolytic cleavage, had revealed the presence of *src* proteins of 55 or 56 kd that were lacking regions present in the amino terminal one-quarter of wild-type $p60^{v-src}$ (Fujita *et al*, 1981a). Analysis of tyrosine phosphorylation of the two major peripheral protein targets of $p60^{v-src}$, the 36 kd target, found in epithelial cell brush borders (Gerke and Weber, 1984), or the 130 kd vinculin target, found in adhesion plaques (Sefton *et al*, 1981), revealed no obvious differences in the level of phosphorylation between wild-type and fusiform mutants (Bechberger and Fujita,

unpublished observation). However, unlike some other mutants described as having a fusiform morphology, WO101 and WO201 were found to be highly tumorigenic in chickens, and their src proteins were found to be associated with the cell membrane fraction at levels comparable to wild-type RSV (Bechberger and Fujita, unpublished results).

The objective of this study was to determine the nature of the v-src lesions reponsible for the production of the fusiform phenotype in these mutants in an attempt to define a specific region of the v-src gene responsible for the morphological differences observed between wild-type and fusiform RSV-transformed chicken fibroblasts. We have cloned and sequenced supercoiled DNA intermediates of the WO101, WO201 and ST529 mutant src genes; the observed alterations help define more precisely a region of the src gene that may be important in the induction of morphological changes by the src protein. The results suggest that aberrant RNA splicing events, followed by reverse transcription, can serve as a mechanism to cause stable deletions in a retroviral genome.

3.2 MATERIALS AND METHODS

3.2.1 GROWTH MEDIA

Chicken embryo fibroblasts were grown in Dulbeccos media (Gibco) supplemented with 10% TPB, 5% calf serum (Gibco) and antibiotics. Virus-transformed cells were grown in media containing 0.5% dimethyl sulfoxide.

3.2.2 CELLS AND VIRUSES

Wild-type SR-A was obtained originally from H. Hanafusa through W.S. and H. Robinson. Isolation of mutants has been described previously (Fujita *et al*, 1981a; Fujita *et al*, 1981b). Chicken embryo fibroblasts were prepared from day ten embryos (Spafas), as described previously (Fujita *et al*, 1981b).

3.2.3 MOLECULAR CLONING OF MUTANT VIRAL DNAS

Chicken embryo fibroblasts were seeded onto five 150mm plates at a density of approximately 10^7 cells per plate and infected at a multiplicity of 2 to 4. Cellular DNA was harvested at 18-24 hours after infection from Hirt supernatant fractions of cell extracts (Hirt, 1967), which were greatly enriched in viral DNA supercoils. Southern blotting (Southern, 1975) using a v-*src* probe (Delorbe *et al*, 1980) was performed with all preparations to ensure the presence of viral DNA and to help predict the cloning efficiency. The DNA preparation was cleaved with Sac I,

which cleaves circular viral DNA at a unique site (Delorbe et al, 1980), and then ligated into the Sac I site of lambda gtWES (Leder et al, 1977). A typical ligation contained 2ug of Sac I cleaved λgtWES and 1 ug of Sac I cleaved Hirt supernatant DNA in a total volume of 20 ul. Ligations were for 4-12 hours at room temperature (see section 2.2.9 for conditions). The recombinant lambda DNA was packaged in vitro as described below. The λgtWES library was screened with a DNA probe made from the pSRA-2 molecular clone (Delorbe et al, 1980; 2.2.3), and positive clones were isolated at a frequency of 1/5,000 to 1/80,000 plaques plated. The method used for screening lambda plaques is described in chapter 2 (section 2.2.5).

3.2.4 IN VITRO PACKAGING OF LAMBDA DNA

The procedure used for packaging of lambda DNA was a modification of the method of Hohn et al(1979). Briefly, the two temperature-sensitive lysogens, BHB2690 and BHB2688 were streaked on LB plates and individual colonies were tested for inducibility at 42 degrees C. The prehead donor strain, BHB2690 was used to innoculate 500ml of NZ media to an OD_{600nm} of 0.1. The culture was then grown at 32 degrees C until an OD_{600nm} of 0.3 was acheived. The culture was induced at 45 degrees C for 15 minutes, then grown at 37 degrees C for three hours. The cells were collected by centrifugation for 10 minutes at 5000 rpm at 4 degrees C. The same procedure was repeated for the packaging protein

donor strain BHB2688, except that three 500 ml cultures were prepared. The BHB2690 cells were resuspended in 3.6 mls of cold sonication buffer (20 mM Tris pH 8, 1 mM EDTA, 3 mM $MgCl_2$, 5 mM beta-mercaptoethanol). The solution was sonicated on ice until the solution cleared, and then centrifuged at 4 degrees C for 10 minutes at 10,000 rpm. The supernatant was collected, an equal volume of cold sonication buffer was added, and 1/6 volume of packaging buffer was added (6 mM Tris pH 8, 50 mM spermidine, 50 mM putrescine, 20 mM $MgCl_2$, 30 mM ATP, 30 mM beta-mercaptoethanol). 15 ul of the resulting mixture was aliquoted into precooled Eppendorf tubes and frozen in liquid nitrogen. The BHB 2688 cells were resuspended in 3 ml cold sucrose solution (10% sucrose, 50 mM Tris pH 7.5), split into 6 Eppendorf tubes, and 25 ul of lysozyme solution (2 mg/ml lysozyme in 0.25 M Tris pH 8) was added to each tube and the tubes were frozen in liquid nitrogen. The tubes were thawed, pooled, and then 150 ul of packaging buffer was added. 10 ul aliquots were frozen in Eppendorf tubes, and stored at -70 degrees C.

1 ug of ligated λgtWES DNA was added to the prehead donor lysate and the packaging lysate was added. After 1 hour, 1 ml of lambda storage buffer was added (10 mM Tris pH 7.5, 10 mM $MgSO_4$), and the recombinant phage was titered on DP50 sup F.

3.2.5 SEQUENCING OF MUTANT VIRAL DNAS

The 3.0 kilobase (kb) Eco RI fragment containing the v-*src* gene, or the entire 9.4 kb Sac I fragment, was purified from lambda clones carrying mutant RSV genomes by gel electrophoresis (see 2.2.8), digested with Pst I or Alu I and subcloned into M13mp18 (Messing, 1983) for dideoxy sequencing. Sequencing was performed using [alpha-^{35}S]dATP as described in chapter 2 (Anderson *et al*, 1985).

3.2.6 ANALYSIS OF CELLULAR RNA IN VIRUS-INFECTED CELLS

(i) T1 RNASE/RNASE A MAPPING: A 345 base pair (bp) Pst I fragment of pSRA-2 encompassing the region found to be deleted in the mutants (see Figure 11) was ligated into the Pst I site of the pSP 64 vector (Melton *et al*, 1984). HB101 bacterial cells made competent by the $CaCl_2$ method (Mandel and Higa, 1970) were transformed with the ligated DNA, and ampicillin (50 ug/ml) resistant colonies were examined for the presence of the plasmid and insert by small scale isolation of plasmid DNA (Maniatis *et al*, 1982) and restriction enzyme digestion with Hinc II which cleaves once within the vector and once within the insert. Plasmid clones containing the Pst I fragment inserted in the reverse (antisense) orientation were selected. Large-scale isolation of plasmid DNA was performed using an alkaline lysis method followed by CsCl buoyant density centrifugation (Maniatis *et al*, 1982). The plasmid DNA was cleaved with Hinc II, which cleaves the Pst I fragment 12 nucleotides from the 3' end of the non-coding strand, and transcription with SP 6 RNA

polymerase was performed with [alpha-^{32}P]GTP (New England Nuclear) using the conditions recommmended by the supplier (Promega Biotec). The resulting 345 base transcript was hybridized to cellular RNA extracted from virus-infected cells using the guanidinium isothiocyanate-cesium chloride method (Maniatis et al, 1982), and digested with T1 RNAse and RNAse A using the conditions recommmended by the supplier (Promega Biotec). The resulting fragments were run on sequencing gels, and exposures of less than 6 hours to Kodak XAR-5 film were sufficient to visualize major bands.

(ii) S1 MAPPING: The 345 bp Pst I fragment was dephosphorylated with calf intestinal phosphatase, end labeled with polynucleotide kinase in the presence of [gamma-^{32}P]ATP (Maniatis et al, 1982), cleaved with Hinc II, and used for S1 mapping (Berk and Sharp, 1977) of infected cell RNA. Cellular RNA was hybridized to denatured probe DNA for 4 hours at 45 degrees C in 80% formamide containing 40 mM PIPES (pH 6.7), 0.4 M NaCl, 1 mM EDTA. For S1 digestion, the reaction was adjusted to 400 ul with S1 buffer (0.03 M sodium acetate [pH 4.5], 0.25 M NaCl, 1 mM $ZnSO_4$, 5% glycerol, 20 ug/ml denatured calf thymus DNA), and 40 units of S1 nuclease (Calbiochem) was added. Samples were incubated at 45 degrees C for 1 hour and then ethanol precipitated with 5 ug of tRNA as carrier. The pellet was resuspended in 10 ul of 80% formamide-1 mM EDTA-0.1% xylene cyanol, and heated at 90 degrees C for 2 min. 5 ul of each sample was run on a sequencing gel (see Chapter 2).

3.2.7 TREATMENT OF CELLS WITH AGENTS THAT AFFECT CELLULAR METHYLATION

Chicken cells infected with wt RSV were treated with agents that affect methylation of nucleic acid and then total cellular RNA was extracted and the utilization of the presumed splice acceptor sequence was studied using S1 analysis as described above. Methylation of nucleic acid was inhibited by treating cells for 12 hours with 40 mM cycloleucine (Sigma) in minimal essential media (Gibco) containing 10 mM methionine. Methylation of cytosine residues was specifically inhibited by treatment with 5 uM 5-azacytidine for 24 hours (Taylor et al, 1984). Concentrations of the methylation inhibitors were selected based on previously published studies in which chicken cells were treated with these agents (Stoltzfus and Dane, 1982; Leshin, 1985). The effect of non-specific methylation was investigated by treating cells with 40 mM cycloleucine and 1 ug/ml dimethyl sulfate for 12 hours.

3.3 RESULTS

3.3.1 CLONING AND SEQUENCING OF MUTANT VIRAL DNAS

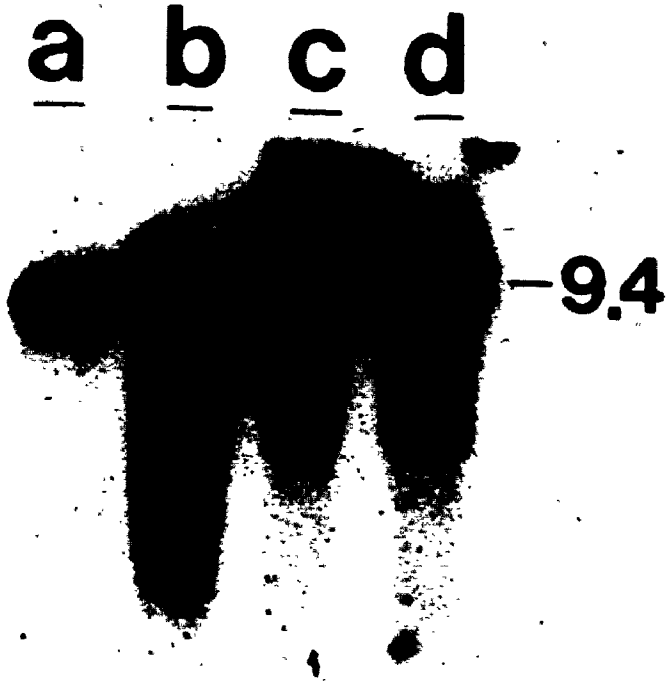
Southern blotting experiments performed on Hirt supernatant DNA from virus infected cells allowed an estimate of the frequency of the desired viral DNA in the preparation, and indicated the number of recombinant λgtWES phage required to ensure isolation of a clone containing RSV DNA. Figures 8 and 9 show the results of the Southern blotting experiments performed on W0101 and ST529 Hirt supernatant DNA. W0101 and W0201 Hirt supernatant DNAs contained a large circular DNA molecule (18 kb) which appeared to be a dimer containing two complete RSV genomes in opposite orientations (Figure 8). The predicted frequency of viral DNA in the ST529 preparation (Figure 9) agreed with the actual frequency of mutant isolation observed (approximately 1 of 5,000 plaques screened).

Enrichment of viral circular DNA molecules by Hirt fractionation allowed the isolation of recombinant clones of the mutant viruses at an average frequency of 1/10,000 lambda plaques plated. Use of an acid phenol procedure to enrich for supercoiled DNA molecules (Delorbe et al, 1980), did not increase the cloning efficiency, possibly due to a large amount of relaxed circular molecules in the DNA preparation. The DNA from each Hirt supernatant fraction was cleaved with Sac I and ligated into Sac I cleaved λgtWES vector DNA (Leder et al, 1977), which had been derived from

Figure 8. Southern blot of WO101 Hirt supernate DNA . 10 ug of Hirt supernatant DNA from WO101 cells was digested with appropriate restriction enzymes, electrophoresed on 0.7% agarose, transferred to nitrocellulose, and hybridized to v-src probe as described in the text. An autoradiogram of the nitrocellulose filter is shown. Lane (a) is undigested, lane (b) is digested with Sac I, and lane (c) is digested with Eco RI. The numbers on the right represent the sizes of the DNA bands (kb).

Figure 11. Sequencing strategy for fusiform mutants. The region of the RSV genome containing the v-*src* gene is shown. The arrows indicate the direction and extent of sequence obtained. Abbreviations and symbols: t.s., location of the temperature-sensitivity associated lesion in ST529; S.A., splice acceptor used to generate *src* subgenomic mRNA; del., location of the deletion present in all three mutants; ATG, start codon of the *src* gene; TAG, stop codon of the *src* gene; P, Pst I; A, Alu I; P*, Pst I site altered in W0101 and W0201.

Figure 9. Southern blot of ST529 Hirt supernate DNA. Lanes (a) and (b) contain 0.1 and 1.0 ng of the W0101 9.4 Kb Sac I fragment respectively. Lane (c) is 10 ug of undigested ST529 Hirt supernatant DNA. Lane (d) is 10 ug of Sac I digested ST529 Hirt supernatant DNA.


a b c d
9.4

a λgtWES variant (sgtWES) lacking the 1.1 kb "stuffer" fragment, which was constructed by packaging religated Sac I cleaved λgtWES. The recombinant lambda gtWES libraries constructed contained from 100,000 to 500,000 recombinant clones. The results of typical ligation and packaging experiments are shown in Table 3.

Recombinant phage were screened with a genomic SR-A DNA probe (Benton and Davis, 1977; Delorbe et al, 1980), and up to 10 independent clones were isolated for each viral mutant analyzed. Five independent λgtWES recombinant clones representing each of WO101, WO201 and ST529 were analyzed, and all were found to harbour a 9.4 kb insert of proviral DNA. These 9.4 kb inserts all contained a 3.0 kb Eco RI fragment encompassing the v-src gene (Figure 10). This fragment was slightly smaller in size than the corresponding 3.1 kb fragment derived from wild-type RSV SR-A DNA (Delorbe et al, 1980).

The DNA sequence of the entire src gene from the splice acceptor used for the production of subgenomic src mRNA to the termination codon of $p60^{v-src}$ was determined for all three mutants analyzed. Pst I or Alu I subclones of the 3.0 kb Eco RI fragment of RSV were constructed in M13mp18. Dideoxy sequencing was performed, and the regions sequenced are shown in Figure 11. The sequence of the 5' end of the gag gene, including the splice donor used for the generation of subgenomic src mRNA was determined as well, and corresponds exactly to the published sequence from this

TABLE III

ACTIVITY OF LAMBDA PACKAGING EXTRACTS

DNA PACKAGED[a]	PFU/ml[b]
NO DNA	0
wt lambda	2×10^8
lambda gtWES	5×10^7
religated Sac I cut lambda gtWES	1×10^7
Sac I gtWES + ST529 DNA	5×10^5
Sac I gtWES + WO101 DNA	1×10^5
Sac I gtWES + WO201 DNA	4×10^5

a DNA was packaged *in vitro* as described in the text.

b PFU/ml = plaque forming units per ml when the phage was titered on DP50 supF.

Figure 10. Restriction analysis of lambda clones. Restriction enzyme analysis of four putative ST529 lambda clones isolated from \gtWES recombinant libraries as described in the text. 1 ug of lambda DNA was digested with Eco RI and analyzed on a 0.7% agarose gel. Lanes (a), (b), and (c) contain the restriction pattern of actual ST529 virus clones. Lane (d) is a clone which possesses a 9.4 kb Sac I insert in the orientation opposite to that of the clones in lanes (a) and (b). The lower band in lanes (a) and (b) is the 3.0 kb Eco RI fragment containing the src gene. Lane (c) is a randomly selected lambda clone that was purified.

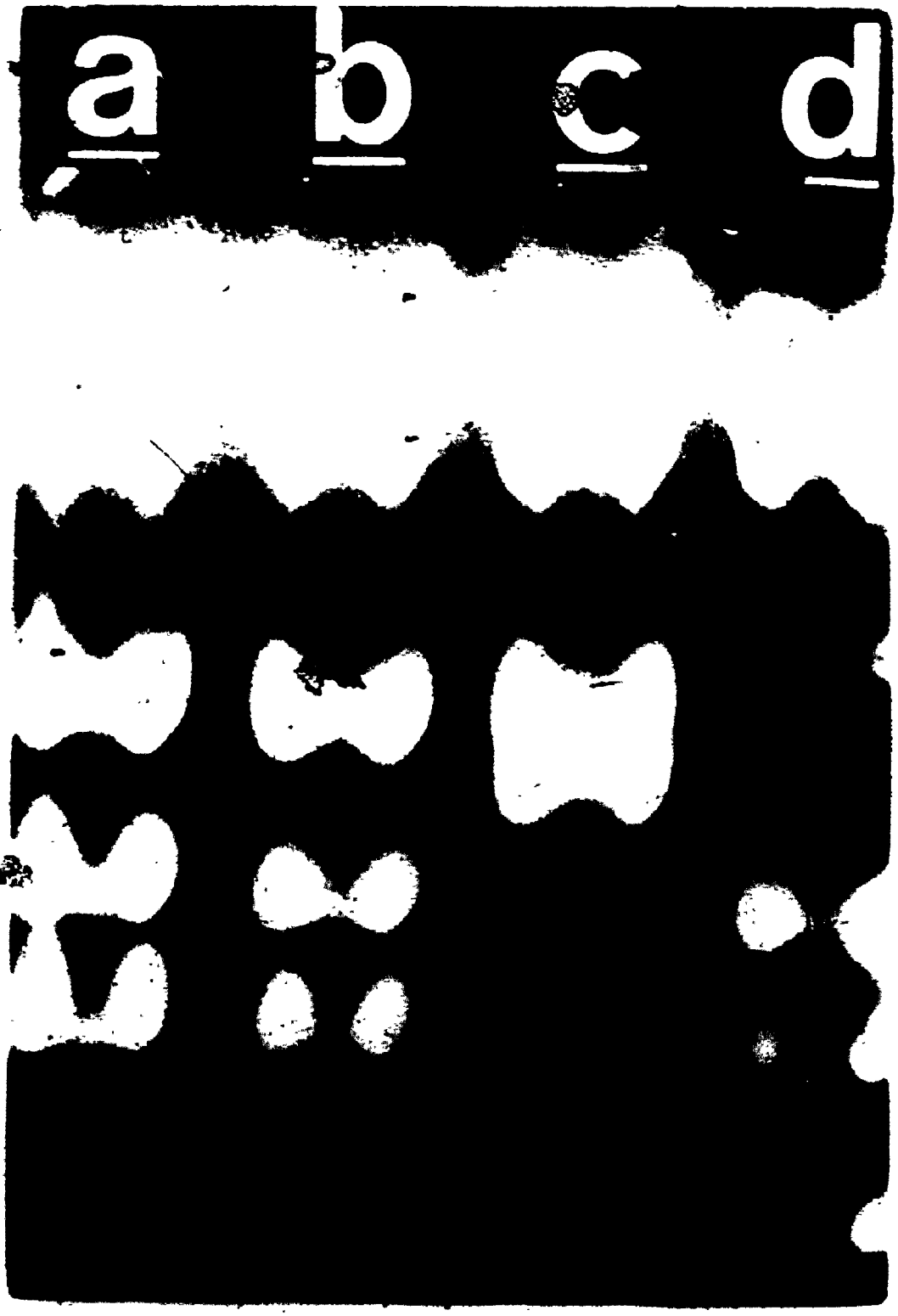
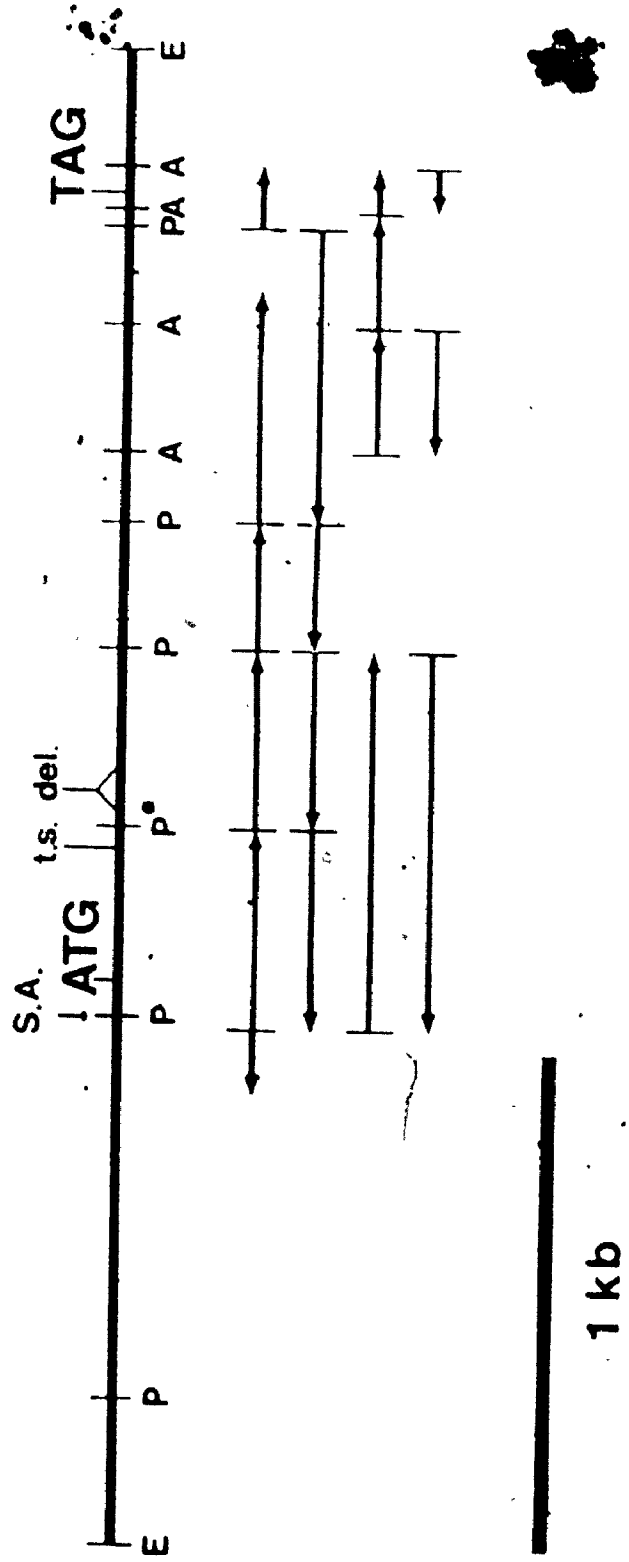

a
b
c
d

Figure 11. Sequencing strategy for fusiform mutants. The region of the RSV genome containing the v-*src* gene is shown. The arrows indicate the direction and extent of sequence obtained. Abbreviations and symbols: t.s., location of the temperature-sensitivity associated lesion in ST529; S.A., splice acceptor used to generate *src* subgenomic mRNA; del., location of the deletion present in all three mutants; ATG, start codon of the *src* gene; TAG, stop codon of the *src* gene; P, Pst I; A, Alu I; P*, Pst I site altered in W0101 and W0201.


TAG
E
A
PA
A
A
P
P
P
t.s. del.
ATG
S.A.
P
P
E
1 kb

region of SR-A (Swanstrom et al, 1982; Figure 12). Sequences of Pst I or Alu I fragments obtained from independent lambda clones of the same viral mutant corresponded exactly, and indicated that the alterations that were found did not arise from minority components of the viral mutant population.

3.3.2 WO101

Sequence analysis of the viral DNA of the WO101 mutant revealed a deletion of 75 nucleotides in the 5' region of the src gene: Figure 13A shows the boundaries of the deletion observed in this mutant. The deletion removes nucleotides 347 to 422 (amino acids 116-140) of the src coding region, which generates an in frame deletion of 25 amino acids yet does not alter the amino acids flanking the deletion. The loss of 25 amino acids agrees with a previous report from our laboratory that these mutants lacked 3-4 kd of protein in the N-terminal one-quarter of $p60^{v-src}$ (Fujita et al, 1981a). Except for the deletion, and a nearby silent single base substitution at position 322, (C to T; Figure 19), the nucleotide sequence of the entire 1.7 kb src region is identical to that previously reported for the src gene of SR-A (Czernilofsky et al, 1980; Takeya and Hanafusa, 1982). Figure 14 is a sequencing gel which shows the alterations present in WO101.

The deletion present in this mutant is very unusual, since consensus RNA splice donor and splice acceptor sequences, (underlined in Figure 13A) are found at the ends of the deleted segment. The potential splice acceptor

Figure 12. Splice donor and acceptor used for src mRNA. (A) The sequence surrounding the 5' splice donor of RSV is shown. The amino acids of the amino terminus of the gag gene product are shown. The splice donor used for the production of subgenomic RSV mRNAs is underlined. (B) The sequence surrounding the splice acceptor used for the production of src subgenomic mRNA is shown. The amino acids of the $p60^{v-src}$ amino terminus are shown. The splice acceptor signal is underlined.

A

MetGluAlaValIleLysValIleSerSerAlaCysLysTheTyrCysGlyLysIleSerPro

GGATCAAGCATGGAAGCCGTCATTAAGGTGATTTCGACCGCGTGTAAAACCTATTGCGGCAAAATCTCTCCT

(SD)

B

CTTTTGTCTGTGCTGCAGGAGCTGAGCTGACTCTACGTAGTGGCCTCAGCTACCACTGTTGCCATGGGTAG

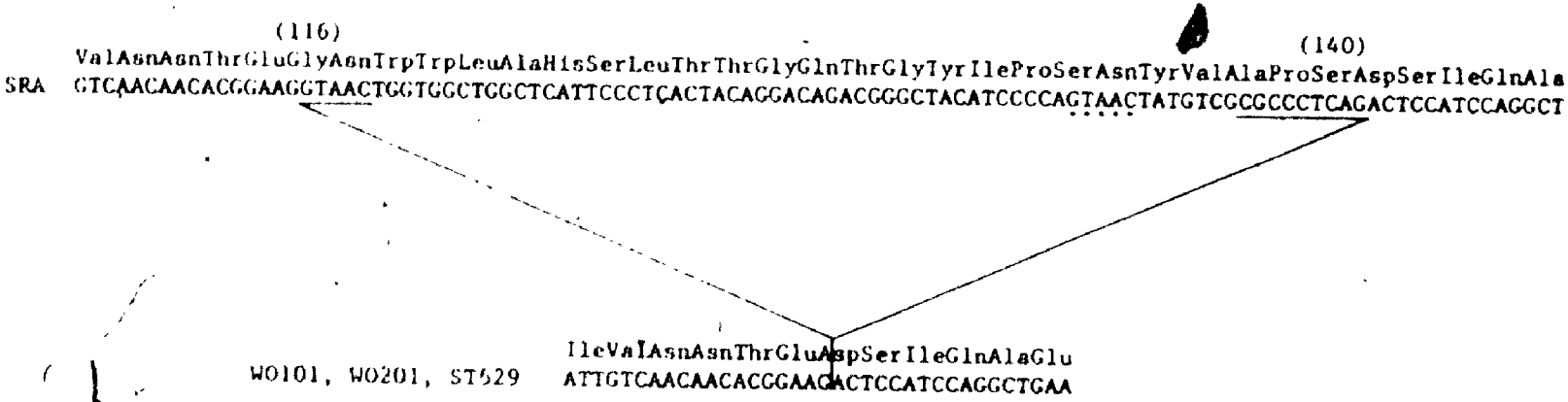
(SA)

MetGlySerSerLysSerLysProLysAspProSerGlnArgArgArg

CTGGGACGTGCAGCCCACCACCATGGGAGTAGCAAGACCAAGCCTAAGGACCCCAGCCAGCGCCGCCGCCAG

Figure 13. The region deleted in the mutant viral DNAs resembles a eucaryotic intron. (A) Region of v-src deleted in fusiform mutants. Bracketed numbers indicate amino acid number of $p60^{v\text{-}src}$ (Takeya and Hanafusa, 1982). The underlined sequences indicate the proposed splice donor and acceptor sequences. The dotted underlining represents the proposed branchpoint acceptor sequence. (B) Proposed RNA lariat formation within the intron-like region. The splice donor sequence is shown looped onto the branchpoint acceptor sequence (Reed and Maniatis, 1985), with positions of possible base-pairing indicated by vertical lines. The dotted line shows the predicted 2′ to 5′ phosphodiester bond formed during pre-mRNA splicing.

A


(116)
(140)
ValAsnAsnThrGluGlyAsnTrpTrpLeuAlaHisSerLeuThrThrGlyGlnThrGlyTyrIleProSerAsnTyrValAlaProSerAspSerIleGlnAla
SRA GTCAACAACACGGAAGGTAACTGGTGGCTGGCTCATTCCCTCACTACAGGACAGACGGGCTACATCCCCAGTAACTATGTCGCGCCCTCAGACTCCATCCAGGCT
IleValAsnAsnThrGluAspSerIleGlnAlaGlu
WO101, WO201, ST529 ATTGTCAACAACACCGAAGACTCCATCCAGGCTGAA

B

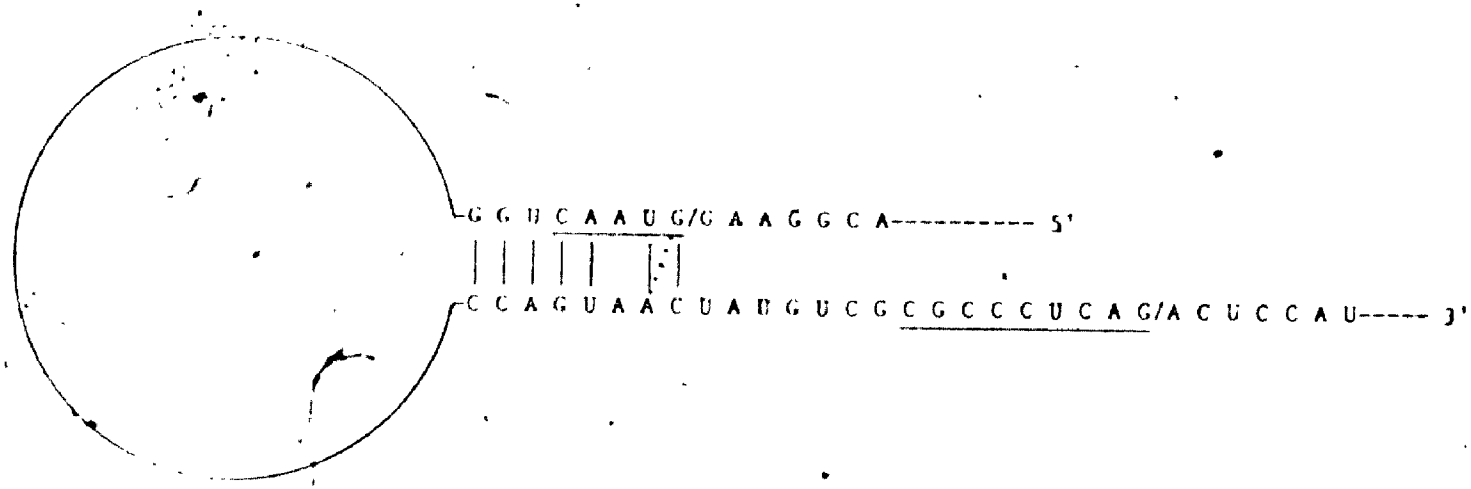
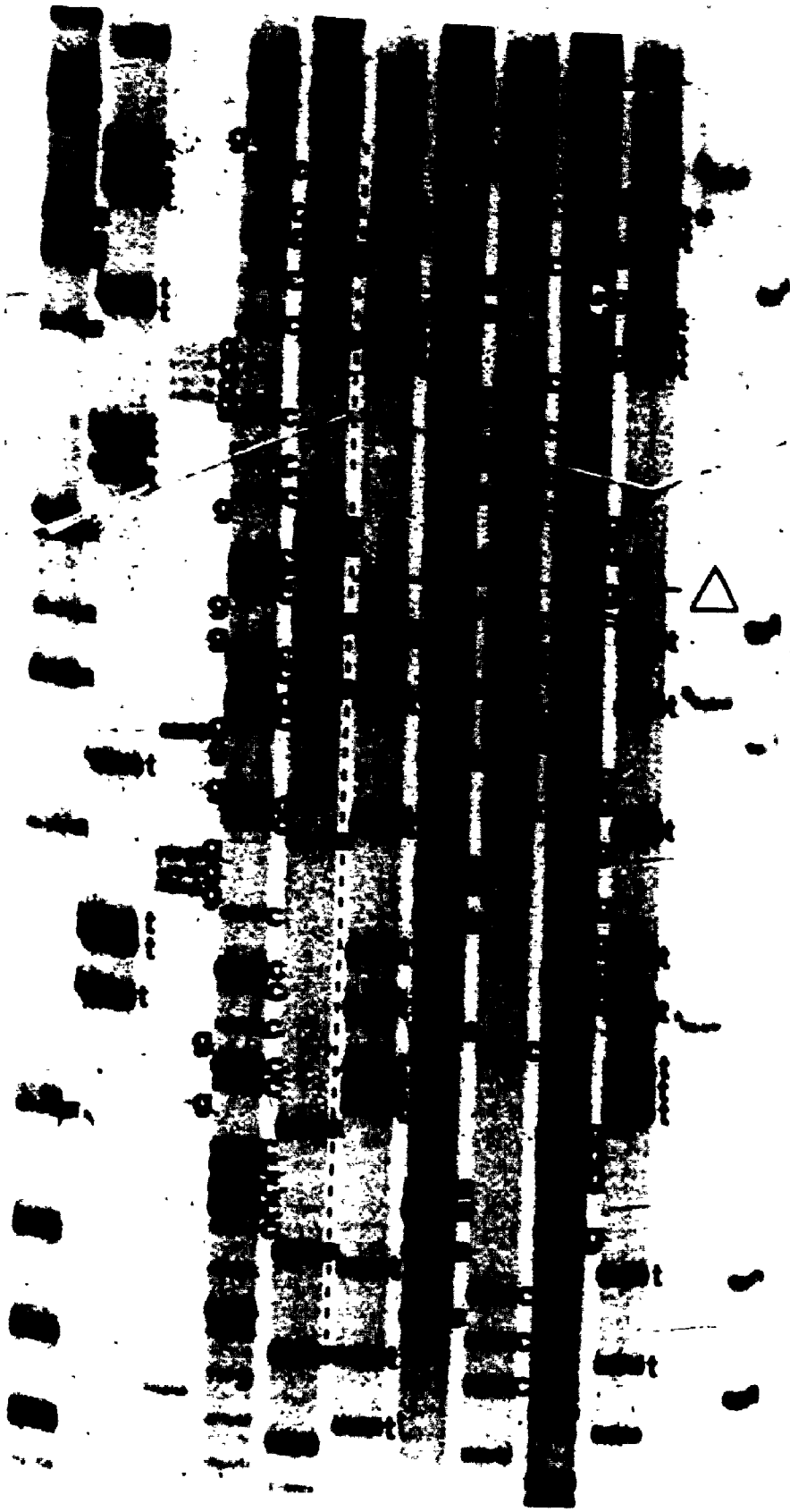

G G U C A A U G/G A A G G C A---------- 5'
C C A G U A A C U A U G U C G C G C C C U C A G/A C U C C A U----- 3'

Figure 14. Sequence gel of W0101. The sequence of the region of W0101 containing the alterations described in the text is shown. The single base pair change (C to T) is indicated by a *. The position of the deletion is indicated by the line ending with △.



conforms exactly to the accepted consensus (YNYYYNCAG) sequence (Sharp, 1981), and the splice donor (GTNAC) differs from the consensus (GTNAG) (Sharp, 1981) only at the fifth position which is C in wt SR-A (Takeya and Hanafusa, 1982). A sequence similar to the consensus sequence for branchpoint formation (PyXPyTPuAPy; Reed and Maniatis, 1985) is located 18 nucleotides upstream from the proposed splice acceptor site. Interestingly, this region is an inverted repeat of the splice donor-like sequence of this cryptic intron, and pairing of these complementary sequences might assist branchpoint formation and splicing (Figure 13B).

The dinucleotide AG preceding the splice donor sequence and contained within the splice acceptor sequence is the only repeat flanking this deletion, so it is unlikely that homologous recombination generated this mutation. Non-homologous recombination has been shown to occur at relatively high frequency through topoisomerase I mediated events (Bullock et al, 1986), and a preferred cleavage site (AAG) exists at the 5' junction of this deletion. However, since there is no site at the 3' junction, and very few DNA copies of RSV are present in the cell, the frequency of such an event would be expected to be much lower than the observed frequency of mutant isolation (ca. 0.2% of foci examined; I. Nedic and D. Fujita, unpublished observation).

3.3.3 W0201.

The sequence of the entire 1.7 kb *src* region of W0201 was identical to that determined for W0101. The exact

correspondence of the W0101 and W0201 mutant src genes could be explained by three possible mechanisms: i) the observed deletion could have occurred in two independent events, and the silent single base change at position 322 could have pre-existed in the wild-type viral stock used for the isolation of the mutants; ii) the deletion mutant may have been present in the wt stock before it was split for mutant isolation; iii) cross-contamination might have occurred during virus propagation. These alternatives are considered further in the discussion section.

3.3.4 ST529

ST529 also contained the same deletion of 75 nucleotides observed in W0101 and W0201, but does not contain the C to T transition at position 322. The base subsitution present in W0101 and W0201 destroys a Pst I endonuclease site in wt RSV proviral DNA, and this Pst I site is intact in ST529 DNA. In addition to the same deletion found in W0101 and W0201, ST529 contains a G to A substitution at nucleotide 277 of src, causing the substitution of lysine for glutamic acid at residue 93 of p60src (Figure 19). A section of a sequencing gel containing the ST529 point mutation is shown in Figure 15. This alteration is presumably responsible for the temperature sensitivity of the ST529 src gene product, as it imparts a considerable change in charge (+2) at amino acid 93 of

Figure 15. Sequence of a second alteration in ST529. The sequence of ST529 viral DNA surrounding the G to A transition at nucleotide 277 of the v-src gene is shown. The altered nucleotide is indicated by a *.

p60v-*src*. The ts nature of this mutant will be discussed further in the next chapter.

3.3.5 ANALYSIS OF *SRC* RNA IN RSV-INFECTED CELLS

The analysis of cellular RNA from infected cells was performed to confirm the existence of the deletion observed in cloned viral DNA, and to investigate whether or not the alternative splice donor or acceptor signals implicated in the mutant deletions are used at a detectable level in wt RSV RNA. The state of RNA in virus infected cells was examined through RNAse mapping experiments using a uniformly [alpha-^{32}P]GTP labeled 348 nucleotide RNA probe specific for the region involved in the mutant deletions. Figure 16 shows the result of hybridizing the 348 nucleotide radiolabeled RNA probe (containing 333 bases complementary to the region of v-*src* involved in the deletions) to mutant and wild-type infected cell RNA followed by digestion with RNase A and RNase T1. Chicken embryo fibroblasts which had been transfected with the pSRA-2 molecular clone (Delorbe *et al*, 1980) of RSV were used as a source of wild-type RSV RNA to avoid the possibility of mutations accumulating in passaged wt RSV stocks. The probe protected the expected 245 nucleotide RNA fragment in the W0101, W0201, and W0529 infected cell RNA (lanes b, c, and e), demonstrating the existence of the observed deletion in viral RNA. Wild-type infected cellular RNA protected the expected 333 nucleotide fragment (lanes a and g), with no additional bands at the

Figure 16. T1 RNAse/RNAse A mapping of viral RNA. 10ug of cellular RNA was hybridized with a [alpha-^{32}P]GTP labelled 348 base RNA probe overnight at 45 degrees C and digested with T1 and RNase A. Cellular RNA was from chicken embryo fibroblasts infected with the following viruses: lanes (a) and (g), wild-type SRA; lane (b), WO101; lane (c), WO201; lane (d), uninfected; lane (e), ST529. Lane (f) is a marker.


a b
60
40

positions of the splice acceptor or donor signals flanking the deletions observed in the mutants studied. Uninfected cell RNA (lane d) showed only a faint 348 nucleotide RNA band, resulting from hybridization of the probe to vector DNA present in the hybridization mixture. Bands which appeared upon longer exposure of gels, corresponded to positions containing several radiolabeled GTP residues in the probe and could be seen in lanes containing no wild-type RNA.

In order to overcome the background problems associated with probe degradation in the T1-RNase A mapping experiments, standard S1 mapping (Berk and Sharp, 1977) of viral RNA was performed using the 845 bp Pst I fragment end labeled and digested with Hinc II to produce a 336 base pair fragment labeled at the 3′ end only. The results of the S1 mapping experiment are shown in Figure 17. The expected protected fragment of 245 nucleotides is observed with ST529 infected cell RNA (lane b). A protected fragment of the same size is detected at a low level (approximately 0.5%) in wild-type RSV (pSRA-2) infected cellular RNA (lane c). This band is not detected in the control lane containing no wild-type RNA (lane a) even if 100,000 cpm of probe was used, so it is not an artifact due to probe degradation. If 100,000 cpm of probe was hybridized to uninfected cell RNA, no bands were visible following S1 digestion (data not shown). These results suggest that the splice acceptor

Figure 17. S1 mapping of the proposed splice acceptor. S1 mapping of wild type RSV-infected cellular RNA. 20ug of cellular RNA was hybridized with a 336 base-pair, 3' end-labelled Hinc II- Pst I fragment spanning the deleted region (see Fig. 1). Lane (a) is a control showing degradation of probe alone. (a) contains 1000 cpm of probe. Lanes (b) and (c) contain 100,000 cpm hybridized to cellular RNA and digested with S1. Lane (b) is cellular RNA from ST529-infected cells, lane (c) contains wild-type (SRA-2) infected cell RNA.

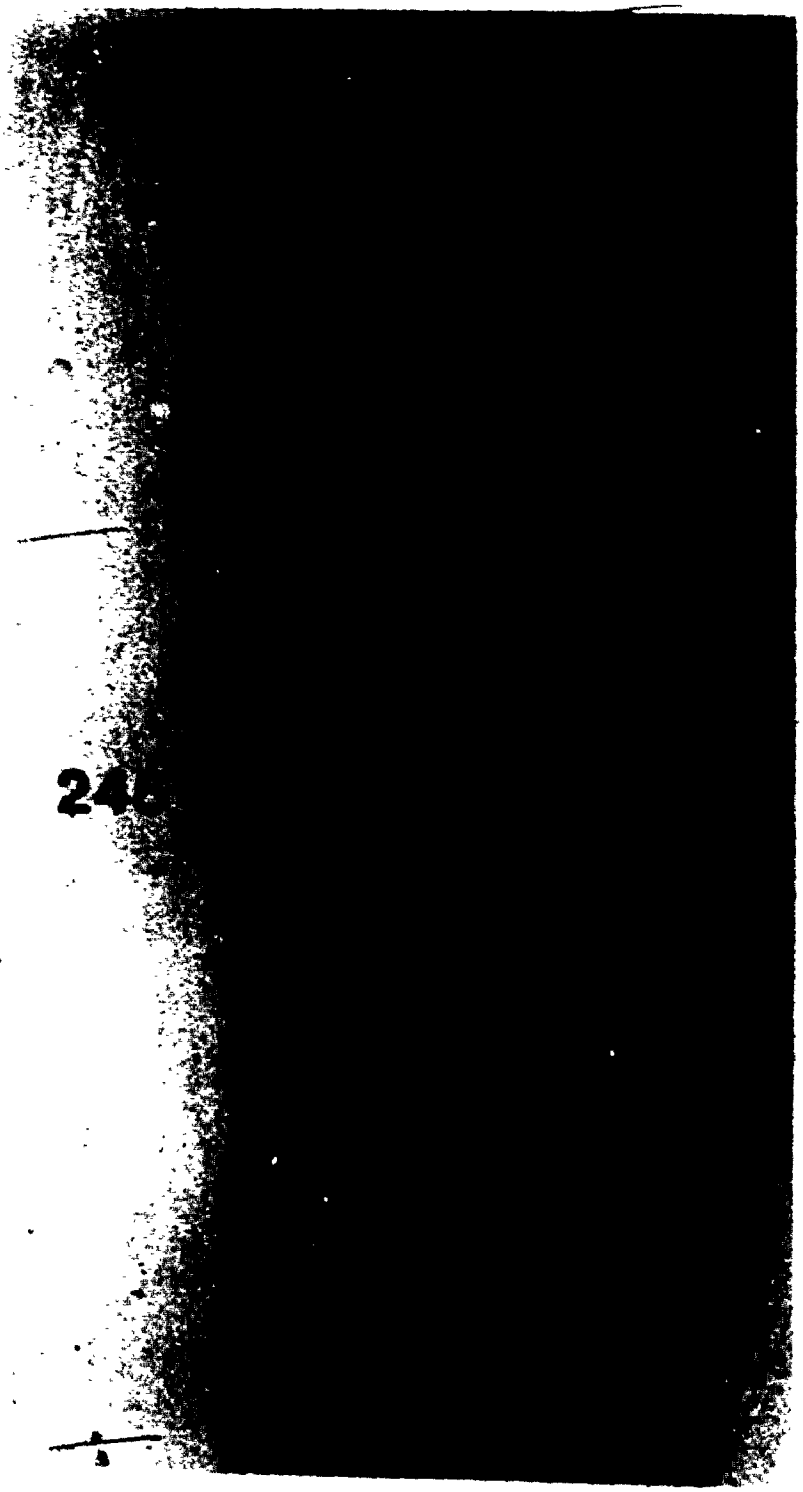

245

sequence described here is used at a low level in wt RSV-infected cells.

3.3.6 EFFECT OF RNA METHYLATION ON SPLICING

Cells infected with wt RSV were subjected to treatments which affect methylation of nucleic acid, cellular RNA was isolated, and S1 mapping was performed as described in the previous section. The relative intensity of the labeled DNA band representing cleavage of wt RSV RNA at the proposed splice acceptor site was compared to the intensity of the full-length DNA band by performing densitometer scans on various exposures of the autoradiogram shown in Figure 18, as well as cutting out bands from the gel and measuring the radioactivity. Within experimental error, no significant effect on the use of the proposed splice acceptor sequence was observed when wt RSV-infected cells were treated with 5 mM 5-azacytidine (lane b). Cycloleucine treatment caused a five-fold reduction in the level of cleavage of RSV RNA at the proposed splice acceptor site (lane d). Treatment of infected cells with cycloleucine and dimethylsulfate resulted in the same five-fold reduction (lane e).

Figure 18. Effect of methylation on RNA splicing. Chicken embryo fibroblasts infected with wt RSV were subjected to various treatments and S1 mapping was performed on cellular RNA. Lane (b) is untreated. Lane (c) is 5-azacytidine treated. Lane (d) is cycloleucine treated. Lane (e) is cycloleucine plus dimethylsulfate treated. Lane (a) is ST529-infected cellular RNA.


24

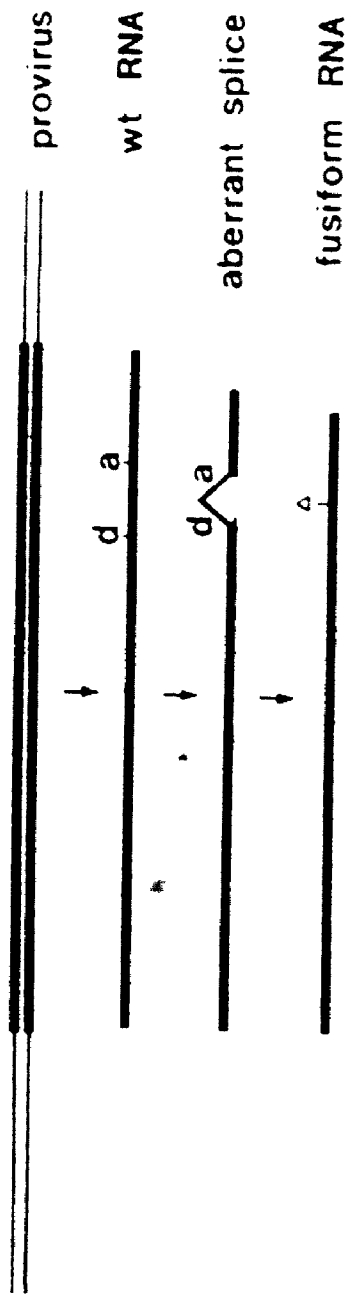
3.4 DISCUSSION

3.4.1 GENETICALLY STABLE DELETIONS MEDIATED BY ABERRANT RNA SPLICING EVENTS

The unusual deletion observed in the WO101, WO201, and ST529 mutants is flanked by potential splice donor and acceptor sequences. The 5' and 3' junctions of the deletion are in the exact positions expected if an aberrant RNA splicing event was involved in the generation of these mutants. Figure 19 diagrams the predicted mechanism of mutant production from wild-type infected cells. The cryptic intron was presumably spliced out of genome-length viral RNA by a relatively rare splicing event and packaged to produce a mutant virus particle, which would then infect other cells. Reverse transcription of the viral RNA would result in proviral DNA harboring this deletion. Subsequent transcription would result in the stable transmission of this lesion to progeny virus.

To our knowledge, this is the first indication of a deletion in the coding region of a eucaryotic viral genome which was likely to have arisen through a mechanism involving RNA splicing and reverse transcription. It is also possible that a point mutation occurred at the DNA level to produce a splice donor sequence that would exactly match the consensus sequence. However, this would probably not increase the level of this splicing event since splice donors which do not match the consensus sequence at the

Figure 19. Mechanism of mutant production. Genome-length viral RNA (wt RNA) is aberrantly spliced at the site of the deletion to produce a mutant RNA genome which is subsequently packaged to produce a fusiform viral particle. d: splice donor; a: splice acceptor; Δ: deletion in RNA.


provirus
wt RNA
aberrant splice
fusiform RNA
d
a
d
a

fourth or fifth position exist in the chicken cellular src gene (Takeya and Hanafusa, 1983), in which they are efficiently used.

Recent reports have demonstrated the involvement of intron sequences in lariat formation during pre-mRNA splicing (Reed and Maniatis, 1985). A sequence similar to the consensus sequence for branchpoint formation (PyXPyTPuAPy) can be found 18 nucleotides upstream from the proposed splice acceptor, which is the same spacing observed in the human alpha globin IVS 2 and rat insulin IVS 1 (Reed and Maniatis, 1985). We have noticed that the consensus sequence for branchpoint formation resembles the inverse complement of the splice donor consensus sequence. If the proposed splice donor sequence of this cryptic intron is compared with the inverse complement of the suggested branchpoint sequence, a 7 of 8 nucleotide match is found. Interestingly, these two sequences differ from their respective consensus sequences at a single position, but the two differing residues complement each other and contribute to the match found between these two consensus sequences. If base-pairing occurred between these two sequences in viral RNA, it might assist in branchpoint formation and splicing of this cryptic intron, since this would bring the G residue at the beginning of the intron close to the A residue in the branchpoint acceptor consensus sequence (Figure 13B). The close proximity of these two residues might enhance the

formation of the 2′ to 5′ phosphodiester bond of the branched RNA structure.

The question arises, however, as to why the proposed splicing event does not occur at a high rate in normal wild-type RSV-infected cells. Other than the existence of consensus splice donor, splice acceptor, and branchpoint acceptor sequences, the requirements for splicing are not completely understood (Sharp, 1985), so there is possibly some requirement for splicing that this pseudo-intron lacks, and the splice which produced this deletion was a relatively rare mistake. Since the region deleted from the virus contains several characteristics of a eucaryotic intron and there is no evidence of homologous recombination having occurred, we conclude that this deletion was generated by an unusual splicing event which is presumably a low frequency "mistake" of the RNA splicing machinery. The consensus sequences discussed above cannot be the only factors controlling the splicing of this intron-like unit.

A recent report has demonstrated the importance of RNA secondary structure in controlling the use of splice sites (Solnick, 1986). This study showed that exons were not efficiently spliced from pre-mRNAs if the exon was placed in the loop of a hairpin structure created by inserting 100 bp inverted repeats into the flanking intron sequences. This study indicates the importance of RNA secondary structure in controlling the utilization of splice donor and acceptor sequences <u>in vivo</u>. It is unlikely that the 8 bp inverted

repeat located within the cryptic intron described here is preventing splicing from occurring. Secondary structure formed by the association of these inverted repeats might actually enhance splicing as discussed above.

We have noticed that the splice donor sequence involved in the fusiform deletions is 5 bp downstream from the exon 3/exon 4 junction in the human and chicken cellular src genes. If exon sequences are important in the selection of splice donor and splice acceptor sites, the utilization of this splice donor sequence may be due to its close proximity to an actual 5' exon boundary.

Another study has suggested a requirement of methylated residues in viral RNA for splicing to occur in the RSV system (Stoltzfus and Dane, 1982). Several N^6-methyladenosine (m^6A) residues are present in the 3' half of the RSV genome. The production of subgenomic mRNAs was shown to be inhibited in the presence of an agent that decreases DNA and RNA methylation (cycloleucine). It has been proposed that m^6A may be a positive signal for branch formation during pre-mRNA splicing (Zeitlin and Efstratiadis, 1984). In this regard, the consensus sequences surrounding the A residue involved in branchpoint formation (PuAPY; Sharp, 1985) resemble the consensus residues surrounding sites of RSV RNA methylation (PuAC; Kane and Beemon, 1985). Several of the sites of m^6A RNA methylation have been localized in RSV RNA (Kane and Beemon, 1985), and two sites of RNA methylation are in close proximity to the

cryptic intron described in this chapter (50 and 60 bp 5′ to the proposed splice donor sequence). In contrast to the models of m^6A function discussed above, Kane and Beemon (1985) have suggested that m^6A negatively regulates the utilization of potential splice acceptor sequences.

In order to investigate the possibility of methylation controlling the splicing of this cryptic intron, wt RSV-infected cells were treated with agents that affect methylation of nucleic acid. The five-fold reduction of cleavage occurring at the proposed splice acceptor site observed in the presence of 40 mM cycloleucine was similar to the three to four-fold reduction in the production of RSV subgenomic RNAs reported by Stoltzfus and Dane (1982). Inhibition of cytosine methylation by 5-azacytidine did not produce any significant effect on the utilization of the splice acceptor sequence, suggesting that it is the reduction of m^6A residues that is affecting the level of utilization of the splice acceptor sequence. The inhibition of cleavage at the proposed splice acceptor site in the presence of cycloleucine supports the hypothesis that this sequence is indeed capable of functioning as a splice acceptor. Since treatment with cycloleucine has a detrimental effect on the level of splicing occurring at the splice acceptor site within the cryptic intron, the low level of utilization of this site in wt RSV RNA may not be due to a lack of m^6A residues near the splice acceptor site. However, there may be a low level of methylation occurring

at the A residue within the branchpoint acceptor consensus sequence which is located 18 bp 5′ to the splice acceptor. This A residue is also within an AAC sequence which is a potential site of RNA methylation (Kane and Beemon, 1985). If methylation of this A residue is required for splicing, methylation at this site may be occurring in 0.5% of the RNA molecules, resulting in the 0.5% level of splicing detected at this site. The lower splicing efficiency in the presence of cycloleucine could also be explained by a general reduction in splicing efficiency due to the inhibition of m^6G capping of pre-mRNAs.

Alternatively, it is possible that a specific cellular environment may control this unusual RNA splicing event. A particular state of cellular differentiation may be required for this splicing event to occur. Since chicken embryo fibroblast preparations are of a heterogeneous nature, the low frequency of utilization of the splice acceptor sequence detected in wt RSV-infected chicken embryo fibroblasts may be due to the low frequency presence of a particular differentiated cell type that is capable of performing this splicing event. Perhaps certain cells types are able to control their morphology by regulating the frequency of occurence of similar splicing events. The splice donor and acceptor sequences involved in the splicing of this cryptic intron are present in the chicken c-*src* gene, which suggests that this event could occur in RNA transcripts of the c-*src* gene. However, it is not possible to extend theories

regarding the possible splicing of this cryptic intron to the human c-*src* gene, since neither the splice donor or acceptor sequence is intact in human c-*src* (Figure 5).

The frequency of occurrence of this splicing event is of particular interest, since three of four fusiform mutants isolated from the same wild type stock contained identical deletions. On the basis of current evidence, we are not able to distinguish whether the deletion observed in the three morph^f mutants described here arose through three independent events, or if this particular deletion was present in the original wild-type stock used for mutant isolation. Utilization of the splice acceptor sequence in wild-type RSV-infected cells as deduced from S1 mapping of wt RSV-infected cellular RNA (Figure 17), was at a level similar to the frequency of mutant isolation, and supports the possibility of identical deletions arising from independent events. ST529 was isolated by D. Fujita in the laboratory of W.S and H. Robinson several years prior to the isolation of the WO101 and WO201 mutants (Fujita *et al*, 1981b), and contains a point mutation relative to wt RSV that is different from the point mutation present in WO101 and WO201, supporting the possibility that the deletion in the ST529 virus arose independently of the WO101 and WO201 deletions.

3.4.2 DETECTION OF CLEAVAGE OF WT RSV RNA AT THE PROPOSED SPLICE ACCEPTOR SITE

T1-RNase A mapping demonstrated the presence of the deletions observed at the DNA level in the viral RNA of the mutants. No protected fragments corresponding to the utilization of the splice donor or splice acceptor sequences were detected above the background produced by probe degradation.

When S1 nuclease mapping was performed, the background created by probe degradation was low enough to allow detection of a protected fragment corresponding to cleavage of the RNA at the proposed splice acceptor site. The intensity of this band was approximately 0.5% of the full-length wt RSV RNA-protected DNA band. These results suggest that the splice acceptor sequence described here may be used at a low level in wild-type RSV infected cells. The frequency of splicing of the cryptic intron can not be inferred by observing the frequency at which either the splice donor or acceptor site is used. However, the frequency of splicing of this intron must be less than 0.5 percent. This frequency is similar to the unusually high frequency of mutant isolation (approximately 0.2% of foci examined; I. Nedic and D. Fujita, unpublished observation). It therefore appears that the event which created the deletions observed in the mutants was a low frequency, aberrant RNA splicing event. The introduction of a stop codon into the intron-like segment in wt RSV would allow selection of fusiform revertants that had spliced out this

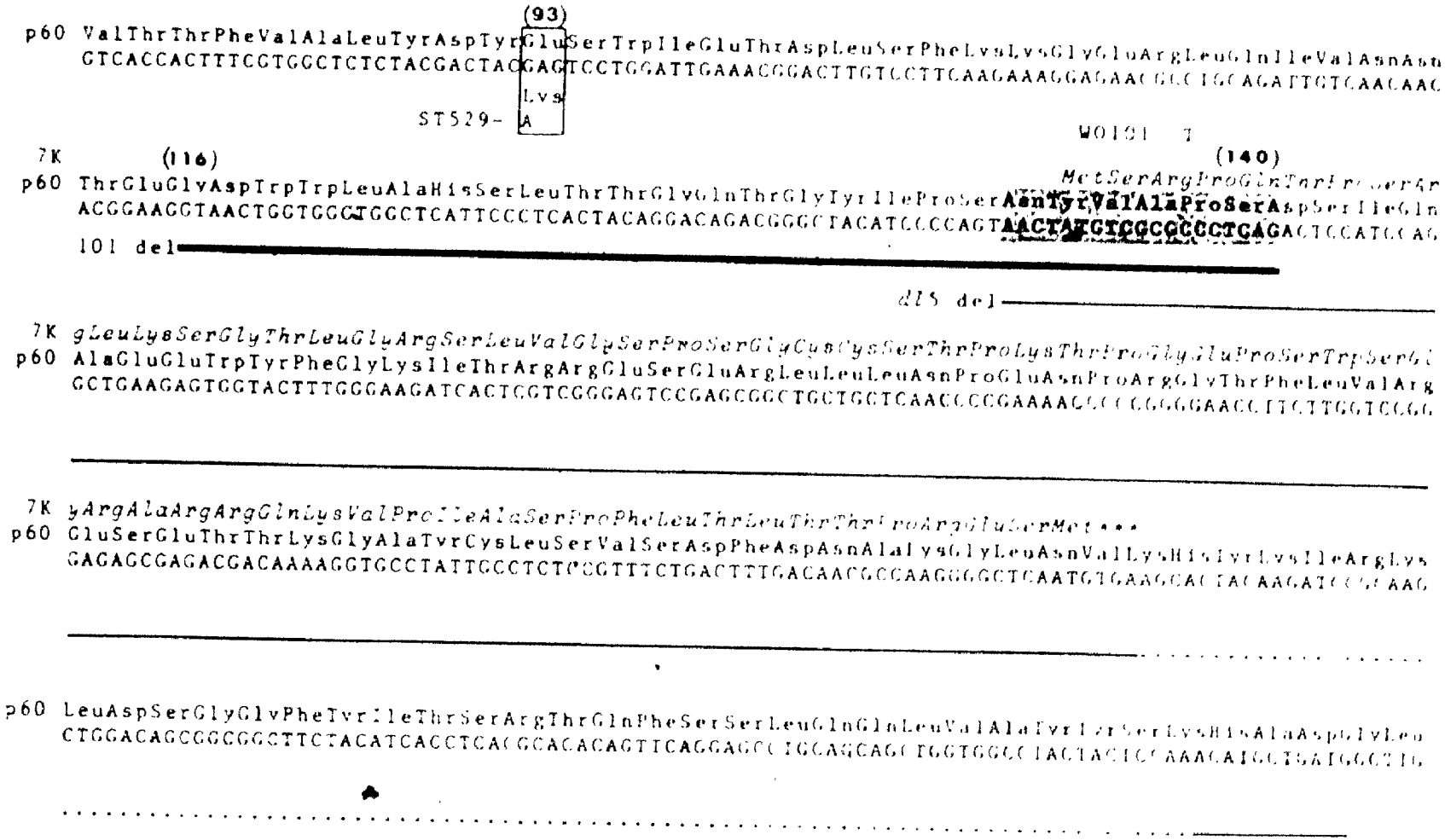
region and might allow quantitation of this proposed splicing event.

3.4.3 A COMMON REGION OF V-*SRC* IS DELETED IN SEVERAL FUSIFORM MUTANT VIRUSES

Another fusiform mutant that has been molecularly characterized by DNA sequencing is dl 5, a Pr-C mutant described by Kitamura and Yoshida (1983). Fig. 20 diagrams the deletion of WO101 and compares it with the dl 5 deletion. Dl 5 contains two deletions; the first deletion of 196 nucleotides (65 a.a.) causes a frameshift in *src* which continues for 33 amino acids before the correct reading frame is restored by an additional deletion of 11 nucleotides. The lesion identified in WO101, WO201, and ST529 mutants described here creates a less drastic alteration in the *src* protein, deleting only 25 amino acids with no changes in the remaining amino acids of $p60^{v-src}$. However, the deletion overlaps with a small portion of the deletion defined in dl 5, suggesting that the region defined by this overlap is important for the production of the fusiform morphology. It is important to note that the fusiform-associated deletions do not extend into the kinase domain of $p60^{v-src}$, which is consistent with the previously reported kinase activity of these mutants (Fujita *et al*, 1981a; Kitamura and Yoshida, 1983).

The region in or near the overlap may be important in regulating the activity or substrate specificity of the *src*

Figure 20. A small region of overlap of deletions present in fusiform mutants. The sequence shown is of SR-A (Takeya and Hanafusa, 1982). The amino acids in italics are those of the proposed internal open reading frame. The shaded region is the region of overlap which contains the start codon of the open reading frame (24). The deletion identified in W0101, W0201, and ST529 is indicated by the heavy underlining. The lighter underlining indicates the regions deleted in the dl 5 mutant (19). The dotted underlining represents the region of missense amino acids in dl 5. Additional alterations present in ST529 and W0101/W0201 are also indicated below the wild-type sequence.


(93)
p60 ValThrThrPheValAlaLeuTyrAspTyrGluSerTrpIleGluThrAspLeuSerPheLysLysGlyGluArgLeuGlnIleValAsnAsn
GTCACCACTTTCGTGGCTCTCTACGACTACGAGTCCTGGATTGAAACGGACTTGTCTTCAAGAAAGGAGAACGCCTGCAGATTGTCAACAAC
Lys
ST529- A
7K (116)
p60 ThrGluGlyAspTrpTrpLeuAlaHisSerLeuThrThrGlyGlnThrGlyTyrIleProSerAsnTyrValAlaProSerAspSerIleGln
ACGGAAGGTAACTGGTGGCTGGCTCATTCCCTCACTACAGGACAGACGGGCTACATCCCCAGTAACTATGTCGCCCCCTCAGACTCCATCCAG
101 del
7K gLeuLysSerGlyThrLeuGlyArgSerLeuValGlySerProSerGlyCysCysSerThrProLysThrProGlyGluProSerTrpSerGl
p60 AlaGluGluTrpTyrPheGlyLysIleThrArgArgGluSerGluArgLeuLeuLeuAsnProGluAsnProArgGlyThrPheLeuValArg
GCTGAAGAGTGGTACTTTGGGAAGATCACTCGTCGGGAGTCCGAGCGGCTGCTGCTCAACCCCGAAAACCCCCGGGGAACCTTCTTGGTCCGG
7K yArgAlaArgArgGlnLysValProIleAlaSerProPheLeuThrLeuThrThrProArgGluSerMet***
p60 GluSerGluThrThrLysGlyAlaTyrCysLeuSerValSerAspPheAspAsnAlaLysGlyLeuAsnValLysHisTyrLysIleArgLys
GAGAGCGAGACGACAAAAGGTGCCTATTGCCTCTCCGTTTCTGACTTTGACAACGCCAAGGGGCTCAATGTGAAGCACTACAAGATCCGCAAG
p60 LeuAspSerGlyGlyPheTyrIleThrSerArgThrGlnPheSerSerLeuGlnGlnLeuValAlaTyrTyrSerLysHisAlaAspGlyLeu
CTGGACAGCGGCGGCTTCTACATCACCTCACGCACACAGTTCAGGAGCCTGCAGCAGCTGGTGGCCTACTACTCCAAACATGCTGATGGCCTG

protein itself, possibly resulting in an alteration in phosphorylation of one or more targets affecting the morphological changes in transformed cells. In this regard, two mutants of Rous sarcoma virus that were created by *in vitro* mutagenesis (NY310 and NY311) possess deletions that overlap with this region and are reported as causing fusiform transformed-cell morphology (Cross *et al*, 1985; Figure 21). Of particular interest is the presence of both a tyrosine at position 136 and a serine residue at position 140 in the region of overlap between the W0101 and d15 deletions (Figure 20). Mutations replacing either of these residues might have an effect on the morphology of transformed cells if phosphorylation of these residues is important.

A particularly interesting observation is that the overlap region of W0101 and d1 5 deletions contains the start codon and several possible coding residues of an open reading frame sufficient to code for a small 7kd protein from the -1 reading frame within the *src* mRNA in infected cells, as shown by the amino acid sequence in italics above the normal coding sequence of $p60^{v\text{-}src}$ in Figure 5. The shaded region is the region of overlap which contains the start codon of the internal open reading frame. The ability of the *src* gene to produce two proteins was suggested by a study of mutant 000 and its revertant 000# by Mardon and Varmus (1983). The 7kd protein itself was not detected in this study, but its existence was implied by the apparent

use of the internal AUG of the open reading frame. The results found with the fusiform mutants suggests that the 7kd open reading frame might possibly be involved in producing some of the morphological alterations observed in cells transformed by RSV, since the two deletions identified overlap at the initiation codon of the internal open reading frame. However, it is difficult to reconcile this possibility with the following observations: 1. The sequence of wild-type Pr-C determined by Kitamura and Yoshida (1983) reveals a stop codon immediately following the ATG of the 7Kd internal open reading frame. 2. The 000* revertant is not capable of producing the 7kd peptide, and there is no report of abnormal morpholoigy in this transformed revertant (Mardon and Varmus, 1983; Oppermann et al, 1981). 3. The deletion of amino acids 173-227 of $p60^{v-src}$ (tsCH119) removes over half of the open reading frame, but does not cause fusiform morphology in mutant-infected cells (Bryant and Parsons, 1982; Figure 21). Also, a comparison of the open reading frame between chicken and human c-*src* demonstrates a lack of evolutionary conservation of the proposed product, suggesting that it does not play an important role in normal cell growth and development (A. Tanaka, unpublished observation).

3.4.4 A PUTATIVE DOMAIN OF $P60^{v-SRC}$ CONTROLLING TRANSFORMED-CELL MORPHOLOGY

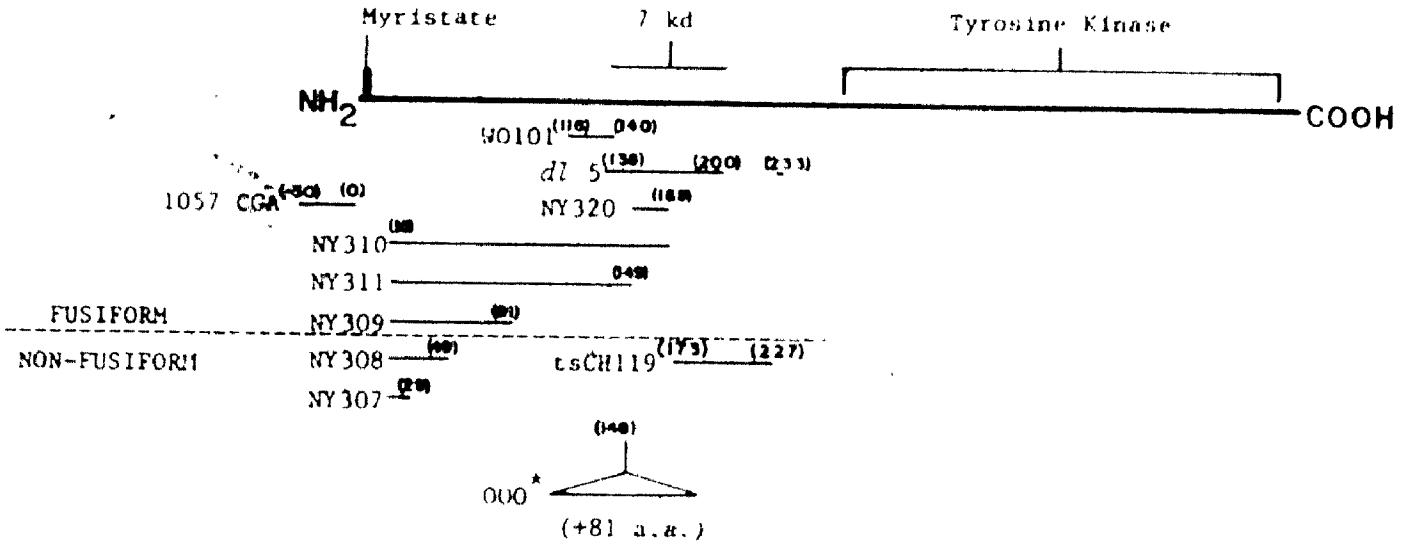
The results obtained with the fusiform mutants suggest that the region of the *src* gene near the initiator of a 7 kd internal open reading frame is important in the control of the morphology of cells transformed by Rous sarcoma virus. It is probable that the region of overlap of the fusiform-associated deletions discussed above is of importance in the function of $p60^{v-src}$ itself. For example, this region of $p60^{v-src}$ may be part of a protein domain that influences the specificity or extent of phosphorylation of one or more cellular target proteins responsible for some of the morphological changes in RSV-transformed cells, and disruption of this domain by the observed deletions may prevent the *src* protein from acting on substrates which normally lead to these morphological changes.

The possibility of additional amino-terminal sequences contributing to a fusiform-related domain is suggested if one considers two *in vitro* generated mutants (NY309, 1057CGA) that do not involve this region but alter regions closer to the amino-terminus, and are reported as causing a "fusiform-like" morphology (Cross *et al*, 1984; Hughes *et al*, 1984; Figure 21). If the phenotype of these mutants is indeed fusiform, it suggests that other regions of the primary amino acid sequence of $p60^{v-src}$ contribute to the fusiform-related domain. Alternatively, many different alterations capable of affecting the size or conformation of the amino-terminus of $p60^{v-src}$ may be sufficient to cause

altered phosphorylation of a target important for morphological transformation of cells infected with RSV.

Figure 21 is a summary of several deletion mutants of the amino-terminal region of $p60^{v-\underline{src}}$ that have been characterized. Analysis of the overlaps of these deletions suggests that a limited region of $p60^{v-\underline{src}}$ is involved in fusiform mutants, since all of the fusiform mutant deletions affect the region of $p60^{v-\underline{src}}$ spanning amino acids 50 to 169, and deletions of amino acids 15 to 49 (NY308), or 173 to 227 (tsCH119) do not cause fusiform morphology. Therefore, this region may contain all the primary amino acid sequences that comprise a domain responsible for selecting targets of the src protein involved in producing morphological alterations in RSV-transformed cells. The involvement of amino acids 149 to 169 in the generation of fusiform mutants is in doubt, since the insertion of 30 amino acids at position 148 (000*) does not create a fusiform mutant (Mardon and Varmus, 1983) and the mutants containing deletions up to amino acid 169 (NY310, NY320) are reported as generating fusiform-type morphology after several passages of virus-infected cells, and the viruses are somewhat defective in focus formation (Cross et al, 1985). The generation of a fusiform mutant by the addition of 30 amino acids at the N-terminus of $p60^{v-\underline{src}}$ (1057CGA; Hughes et al, 1984) can only be explained by an altered conformation of the protein due to the addition of these extra amino acids. However, this observation does not refute

Figure 21. Summary of $p60^{v\text{-}src}$ N-terminal alterations. The origin of each mutant is described in the text. The numbers in brackets indicate the amino acid number at the beginning and end of each deletion, or the position of an insertion. Alterations below the dotted line are those mutants which are capable of producing rounded virus-transformed cells.


Myristate
7 kd
Tyrosine Kinase
NH2
COOH
1057 CGA
NY310
NY311
FUSIFORM
NY309
NON-FUSIFORM
NY308
NY307
NY320
tsCH119
000*
(+81 a.a.)

the hypothesis that amino acids 50 to 169 specify a domain of the src protein required for complete morphological transformation of cells infected by Rous sarcoma virus. In order to more closely define the region of $p60^{v-src}$ involved in producing morphological alterations in RSV-transformed cells, it will be necessary to isolate additional RSV mutants that contain amino-terminal $p60^{v-src}$ deletions, but retain a normal round transformed-cell morphology. It is significant that the deletions involved in all of the fusiform-type mutants characterized do not extend into the kinase domain of $p60^{v-src}$, which is consistent with the expectation that an altered src protein still requires kinase activity to produce the partially transformed phenotype.

CHAPTER 4

TWO SEPARATE LESIONS ARE REQUIRED TO PRODUCE THE TEMPERATURE-SENSITIVE PHENOTYPE OF ST529

4.1 INTRODUCTION

Although the mechanism by which $p60^{v-src}$ transforms cells remains unsolved, the isolation and study of temperature-sensitive mutants of the *src* gene has lead to significant advances in knowledge regarding the function of the *src* protein in RSV-transformed cells. Studies involving ts *src* mutants have established the importance of the *src* gene product, its kinase activity, and the membrane localization of $p60^{v-src}$ in the maintenance of the transformed state (Toyoshima and Vogt, 1969; Martin, 1970; Kawai and Hanafusa, 1971; Garber and Hanafusa, 1983). Analysis of ts mutants has also revealed the existence of different functional domains within the $p60^{v-src}$ protein. A study by Stoker *et al* (1984) demonstrated that carboxy-terminal lesions affecting kinase activity, as well as amino-terminal lesions that have little or no effect on kinase activity, are capable of causing a ts transformation phenotype. The existence of ts lesions outside of the protein domain containing the kinase activity of $p60^{v-src}$, suggests that additional amino-terminal protein domains are responsible for the recognition of targets phosphorylated by the *src* protein, or for the modulation of the activity of $p60^{v-src}$.

The lesion in a mutant with ts $p60^{v-src}$ activity was recently characterized by molecular cloning and DNA sequencing (tsNY68; Nishizawa *et al*, 1985), and this mutant was shown to possess two carboxy-terminal alterations that

were both required to produce the ts transformation phenotype. The exact mechanism of the ts behaviour of this mutant is unknown. It has been reported that tsNY68 infected-cells maintained at the non-permissive temperature contain $p60^{v\text{-}\underline{src}}$ almost exclusively as a cytoplasmic protein bound to the p50 and p90 proteins implicated in the transport of $p60^{v\text{-}\underline{src}}$ to the plasma membrane. The carboxy terminus of the *src* protein is thought to be involved in forming the complex with p50 and p90, since antisera raised against the carboxy terminus of $p60^{\underline{src}}$ do not precipitate the complex as efficiently as does tumor-bearing rabbit serum (Sefton and Walter, 1982), and *in vitro* constructed *src* mutants with extensive N-terminal deletions are capable of transient complex formation. The inability of tsNY68 to transform cells at 41 degrees C could be due to either a decreased kinase activity or an altered protein structure resulting in the trapping of the *src* protein in the complex with p50 and p90.

The cloning and sequencing of two other ts mutants has been recently reported (Fincham and Wyke, 1986). The ts behaviour of each of LA24 and LA31 is due to a distinct single amino acid substitution near the carboxy terminus of the *src* protein.

A ts mutant has been generated by *in vitro* mutagenesis (tsCH119; Figure 20), and this mutant contains an amino-terminal deletion which is presumed to be responsible for the ts phenotype of cells infected with this mutant

(Bryant and Parson, 1982). The *src* protein from these cells exhibited only a 50% reduction in kinase activity when isolated from cells grown at 42 degrees C. This deletion may encompass an amino-terminal domain of $p60^{v-src}$ that is important for transforming ability but not essential for kinase activity.

This chapter describes the analysis of the lesions discovered in the ts fusiform mutant, ST529. Recombinant DNA techniques were used to separate the two lesions present in ST529 in order to determine if both were required to produce the ts behaviour of ST529-infected cells. Hybrid viral DNA molecules were transfected into chicken embryo fibroblasts, and the resulting phenotype was observed.

4.2, MATERIALS AND METHODS

4.2.1 CONSTRUCTION OF HYBRID VIRAL DNAS

The method used to construct the hybrid molecules required for this study took advantage of three conveniently located restriction enzyme sites which cleave the viral DNA into three fragments: (i) a Sac I to Sal I fragment containing the *gag*, *pol*, and part of the *env* gene (ii) a Sal I to Hinc II fragment containing the rest of the *env* gene and the beginning of the *src* gene which contained the single G to A nucleotide substitution in ST529 viral DNA. (iii) a Hinc II to Sac I fragment containing the rest of the *src* gene which harbored the 75 bp deletion in ST529 viral DNA. The 1.5 kb Sal I to Hinc II and the 2.3 kb Hinc II to Sac I fragments were obtained by digestion of viral DNA with all three enzymes and electroelution (as described in chapter 2) of the appropriately sized fragments. The 5.7 kb Sac I to Sal I fragment had to be obtained from a Sac I/Sal I digestion of viral DNA since Hinc II cleaves several times within this fragment. The three fragments were isolated from both ST529 and wt RSV cloned viral DNAs.

Equimolar amounts of each fragment were mixed with two molar equivalents of Sac I cleaved λgtWES DNA. The W0529 hybrid contained 5′ ST529 *src* sequences joined to 3′ wt *src*. W0501 contained wt 5′ *src* joined to 3′ ST529 *src* (Figure 21). The mixture was ligated for 4 hours at room temperature (see section 2.2.8 for conditions) and then packaged *in*

<u>vitro</u> (see section 3.2.4). The resulting phage stock was plated at low density and 6 plaques were picked and analyzed. The methods used for preparation of viral DNA and analytical agarose gel electrophoresis are described in chapter 2.

4.2.2 TRANSFECTION OF VIRAL DNA INTO CHICKEN EMBRYO FIBROBLASTS

The technique used was similar to the $CaCl_2$ transfection procedure described by Stowe and Wilkie (1976). 5 ug of Sac I cleaved lambda DNA containing a RSV viral DNA insert was added to 1 ml of HEPES-buffered saline (137 mM NaCl, 5 mM KCl, 7 mM Na_2HPO_4, 6 mM dextrose, 20 mM HEPES pH 7.05). The DNA was precipitated by the addition of 0.1 volume of 1.25 M $CaCl_2$ while bubbling N_2 through the solution. The precipitate was added to a 100 mm plate of chicken embryo fibroblasts which had been seeded at a density of 1 X 10^6 cells per plate the previous day. After 6 hours at 37 degrees, the medium was removed and the cell monolayers were treated with 1 ml of 30% dimethyl sulfoxide in HEPES-buffered saline for 4 minutes. The plates were washed with fresh medium and then maintained at 37 degrees C with the media changed daily.

4.2.3 FOCUS ASSAY OF RSV

Trypsinized chicken embryo fibroblasts were seeded at a density of 1 X 10^6 cells per 60 mm plate. Two hours after plating, 0.1 ml of diluted (10^{-1} to 10^{-6}) viral stock was

added to each plate. 12 hours after infection, the media was removed and replaced with 6 mls of agar overlay (2X Hams, 10% TPB, 5% fetal calf serum, 0.1% sodium bicarbonate, 0.5% dimethyl sulfoxide, 0.7% regular agar). 3-4 days after overlaying the cells, an extra 4 mls of agar overlay was added. Foci of transformed cells were counted at day 8.

4.3 RESULTS

4.3.1 LESIONS PRESENT IN ST529

The cloning and sequencing of ST529 viral DNA molecules is described in chapter 3. The position of the two ST529 alterations relative to the DNA sequence of wt RSV are shown in Figure 20. The 75 bp deletion and the association of this deletion with the production of the fusiform morphology is described in chapter 3. The additional G to A transition at nucleotide 277 of the src coding sequence was thought to be responsible for the ts transformation phenotype of ST529-infected cells. In order to investigate the possibility that the deletion present in ST529 contributes to the ts phenotype, hybrid molecules were constructed using ST529 and wt SR-A RSV DNA clones to allow the study of each lesion independently.

4.3.2 ISOLATION OF HYBRID VIRAL MOLECULES

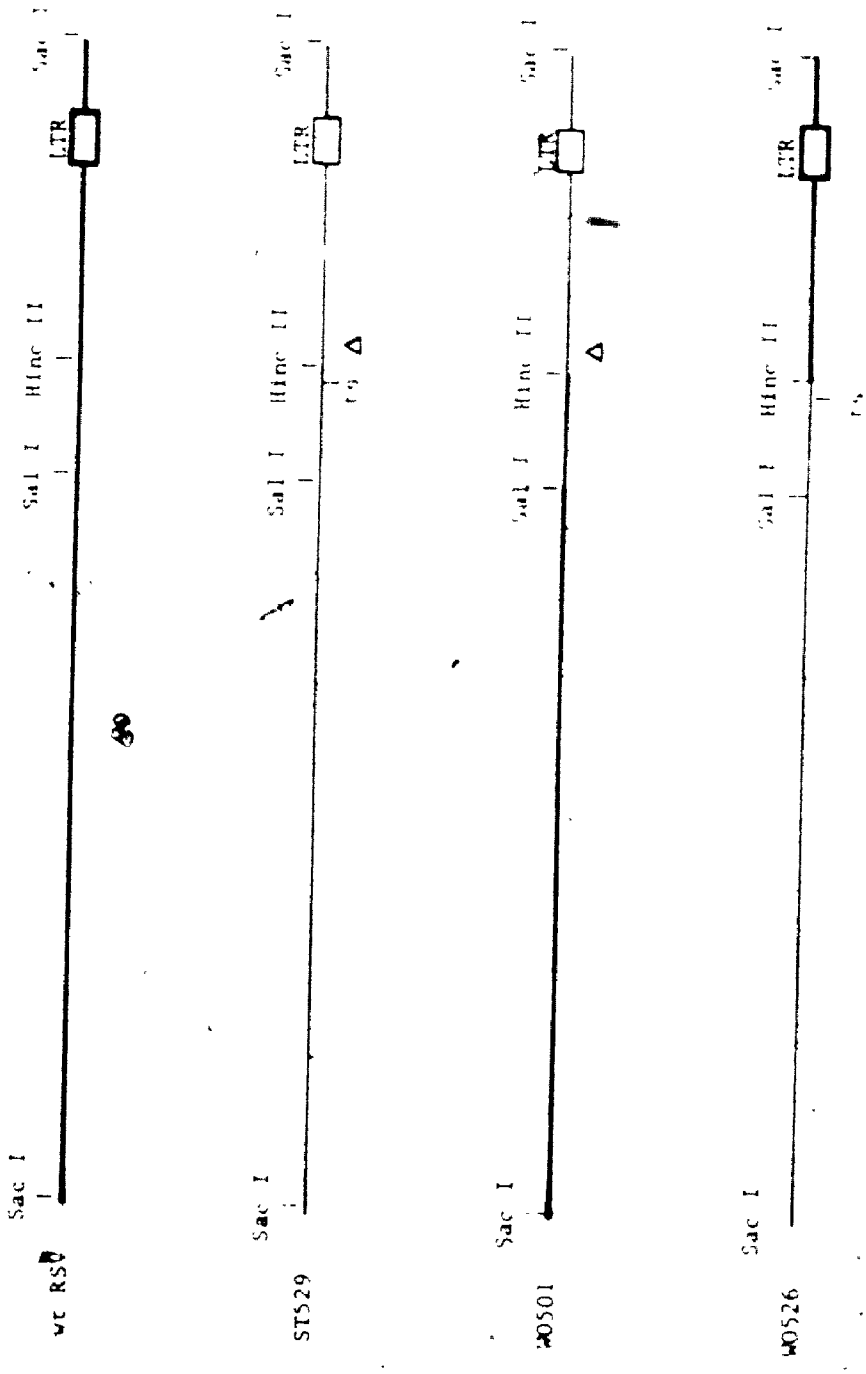
Recombinant λgtWES phage containing hybrid RSV molecules were generated by mixing and ligating appropriate combinations of the fragments isolated from wt and ST529 viral DNAs, and packaging the DNA *in vitro*. Phage plaques were picked at random, and the viral DNA was isolated and tested by restriction enzyme cleavage and agarose gel electrophoresis. Of the phage tested, at least half of the recombinants contained the desired 9.4 kb insert of viral DNA. λgtWES phage containing no Sac I insert comprised the

rest of the phage isolated. The identity of the recombinants with the structure of wt RSV DNA was confirmed by digestion with Eco RI, which yielded fragments of 3.7 kb, 3.1 kb, 2.0 kb, and 0.3 kb, corresponding to the pattern expected from a wt RSV molecule. The structure and name of the constructs is shown in Figure 22.

4.3.3 TRANSFECTION OF VIRAL DNA MOLECULES

Small foci of transformed cells were visible three days after transfection, and passage of the cells at this time resulted in plates of cells that were completely transformed. Media was collected at six days post-transfection, frozen at -70 degrees C, and kept as a viral stock. The viral stocks were titered in focus assays, and contained 1-2 X 10^7 focus-forming units per ml. Cells transfected with WO526 recombinant DNA had a rounded cell morphology identical to that of wt RSV-transformed cells. Transformation by WO526 was not ts, as confirmed by parallel focus assays performed at 37 and 42 degrees. Transfection with WO501 yielded cells with a fusiform transformed-cell morphology which were also non-ts. The ST529 molecular clone produced temperature-sensitive fusiform transformation of transfected cells.

Figure 22. Construction of hybrid wtRSV/ST529 DNA molecules. Wt RSV is indicated by the thick lines. ST529 is shown with thin lines. The positions of the deletion and the ts-associated alteration are indicated. The methods used to construct the WQ501 and WQ526 hybrids are described in the text. The major structural features of proviral DNA are shown, including the long terminal repeat (LTR). Restriction enzyme sites used for construction of hybrid molecules are shown.


LTR
Sac I
Sal I
Hind II
wt RSV
ST529
Δ
20501
WO526

4.4 DISCUSSION

In order to investigate whether or not the fusiform-related deletion in ST529 contributes to the ts nature of this mutant, hybrid *src* genes were constructed using ST529 and a wt RSV molecular clone. It was possible to separate the ts-associated lesion from the fusiform-associated lesion by making use of a Hinc II restriction enzyme site which was located between the two lesions in ST529 viral DNA (see Figure 22). The scheme used for construction took advantage of the packaging limits of λ DNA. A ligation reaction containing five different DNA fragments was performed, but only one possible combination of the five fragments could be efficiently ligated and packaged. This selection resulted in the isolation of only the hybrid molecules desired.

The biological activity of the hybrid viral DNA molecules was tested by transfection into chicken embryo fibroblasts, which generated foci producing viable virus particles and lead to the infection of the entire plate of cells. Table 4 shows the results of transfections with ST529, W0526, and W0501. The W0501 hybrid contained the 75 bp deletion at nucleotide 347 of the *src* gene coding sequence, and produced infected cells that were fusiform and non-ts as expected from the results obtained with the W0101 and W0201 fusiform mutants. The W0526 hybrid molecule that contained the single base change at nucleotide 277 of *src*, with no other alterations, produced transformed cells that

TABLE IV

PHENOTYPE OF HYBRID RSV MOLECULES

VIRUS	TRANSFORMED CELL MORPHOLOGY	TRANSFORMATION	
		37	41
SR-A (wt)	ROUNDED	+	+
ST529	FUSIFORM	+	-
WO501	FUSIFORM	+	+
WO526	ROUNDED	+	+

a Morphology of cells within foci was observed

b All viruses indicated as positive had a transforming titer of greater than $1X10^6$ focus forming units per ml (ffu/ml). At 41 degrees C, ST529 had a titer of approximately $1X10^3$ ffu/ml and is indicated as negative.

were surprisingly, non-ts and possessed the same morphology as wt RSV-transformed cells. The molecular clone of ST529 produced fusiform-transformed cells that were ts. These results indicate that the substitution of lysine for glutamic acid at amino acid 93 of p60v-*src* is not sufficient to produce a ts transformed-cell morphology, and the deletion of amino acids 116 to 140 contributes in some way to the ts nature of ST529-transformed chicken embryo fibroblasts.

The additional G to A substitution at nucleotide 277 of *src*, causes the substitution of lysine for glutamic acid at residue 93 of $p60^{v-src}$ (Figure 5). This alteration must contribute to the temperature-sensitive transformation phenotype of ST529 infected cells. The substitution of lysine for glutamic acid would produce a change in the local charge of $p60^{v-src}$ of +2, significantly modifying the electrostatic environment of this region of $p60^{src}$. Presumably this region could have some modulatory effect on $p60^{v-src}$ kinase activity, and the change in this region in the mutant might inhibit the kinase activity at elevated temperatures. The occurrence of a tyrosine residue adjacent to the mutation may have some significance in this respect, since N-terminal phosphorylation on tyrosine has been associated with increased $p60^{src}$ kinase specific activity by several investigators (Collett *et al*, 1983; Purchio *et al*, 1983; Bolen *et al*, 1985; Yonemoto *et al*, 1985). Tyrosine phosphorylation within the N-terminal half of $p60^{v-src}$ has

been observed in the presence of high ATP concentrations and results in elevated kinase activity (Collett *et al*, 1983; Purchio *et al*, 1983). Interestingly, phosphorylation of a tyrosine residue in the corresponding region of murine $p60^{c\text{-}src}$ has been shown to occur as a result of association with polyoma middle T antigen in polyoma virus transformed cell lysates. This phosphorylation event has been correlated with an increase in $p60^{c\text{-}src}$ kinase activity (Yonemoto *et al*, 1985). If the tyrosine residue at position 92 is a site of phosphorylation associated with increased kinase activity, the mutation in ST529 might conceivably inhibit phosphorylation of tyrosine 92 at elevated temperatures. Examination of residues surrounding sites of tyrosine phosphorylation in several proteins has shown that phosphorylated tyrosines are in close proximity to a glutamic acid residue (Patschinsky *et al*, 1982). The mutated residue adjacent to tyrosine 92 in ST529 is glutamic acid, supporting the hypothesis that tyrosine 92 is a functionally important site of N-terminal tyrosine phosphorylation.

Several other *in vivo* generated temperature-sensitive *src* mutants have been characterized by DNA sequencing. TsNY68 contains two carboxy-terminal alterations that are both required to produce the ts phenotype (Nishizawa *et al*, 1985). LA24 and LA31 (Fincham and Wyke, 1986) each contain a single amino acid change near the carboxy terminus that is responsible for temperature-sensitive $p60^{src}$ activity. The *in vitro* ts mutant generated by Bryant and Parsons (1982),

contains a deletion of amino acids 173-227, but does not have a fully ts kinase activity.

ST529 represents a different class of ts mutant, since it contains two N-terminal alterations, associated with a thermolabile $p60^{v-\underline{src}}$ kinase activity. Also, it should be noted that although the deletion present in ST529 can be solely responsible for fusiform morphology, the results obtained with hybrid viral molecules indicate that the deletion contributes in some way to the ts nature of this mutant.

SUMMARY

This work investigated the structure and function of the *src* gene from two different aspects: the normal cellular *src* gene and the oncogenic viral *src* gene. The structure of the normal non-transforming human cellular *src* gene was determined and compared with the published chicken cellular *src* gene structure (Takeya and Hanafusa, 1983). This study revealed a remarkable structural conservation of the cellular *src* gene between two evolutionarily distant species. The coding region of both human and chicken cellular *src* is divided into 11 exons and the sizes and boundaries of *src* exons are identical in both species. The human and chicken p60*src* predicted amino acid sequences are 98% homologous, suggesting an important, evolutionarily conserved function for the *src* protein.

A possible DNA binding domain within the *src* protein was described. The domain was detected by comparison of the human *src* protein with the consensus residues of known DNA binding domains. It is interesting to speculate that the *src* protein might be able to migrate into the nucleus to a small extent and bind to the control regions of genes whose expression is required for accelerated cell growth.

The study of the fusiform mutants of Rous sarcoma virus helped to define an amino-terminal region of the *src* protein that is likely to be involved in the interaction of the *src* protein with cellular targets that are important for the production of morphological changes in RSV-infected cells.

It is possible that the fusiform mutations destroy a domain of the *src* protein that is required for binding to vinculin, and phosphorylation of vinculin at a site required to produce morphological transformation is inhibited.

Analysis of the alterations in ST529 revealed two amino-terminal alterations that were required for the production of the ts phenotype. A region of overlap was found when the deletion observed in the W0101, W0201, and ST529 mutants was compared with the deletions in the d15 fusiform mutant described by Kitamura and Yoshida (1983). The region of overlap contained amino acids 135-140 of $p60^{v-src}$. This region presumably contains amino acid residues that are crucial to the recognition of $p60^{v-src}$ targets involved in the control of cellular morphology. We also noticed that the region of overlap contains the initiation codon of a previously described internal open reading frame within the *src* gene (Mardon and Varmus, 1983). However, there is no evidence linking disruption of the internal open reading frame with production of the fusiform morphology.

A close analysis of the region deleted in the W0101, W0201, and ST529 mutants showed that the deleted region had all the hallmarks of a typical eucaryotic intron. S1 mapping of wt RSV RNA indicated that the proposed splice acceptor was utilized at a low frequency in wt RNA. This is the first indication of a stable deletion in a eucaryotic viral genome arising through RNA splicing and reverse transcription.

The results obtained from the characterization of the cellular *src* gene assisted in the analysis of the v-*src* gene in two ways. First, the comparison of the human and chicken internal open reading frames showed a lack of evolutionary conservation, supporting the notion that this reading frame is not functionally important. Second, familiarity with splice donor and acceptor sequences gained from the analysis of cellular *src* insured recognition of the intron-like segment in v-*src*, as well as the proximity of the splice donor to the exon 3/4 junction of c-*src*.

Future work on human cellular *src* should be focussed on detecting the role of c-*src* alterations in human cancer or possible *in vitro* alterations that can lead to oncogenic activity of p60*src*.

The results obtained from the analysis of the fusiform mutants of RSV generates several new questions. *In vitro* mutagenesis can be performed to more precisely define amino acid residues important for the morphological transformation mediated by $p60^{v\text{-}src}$. The efficiency of splicing of the cryptic intron within v-*src* might be increased if appropriate *in vitro* mutations were generated. It would be interesting to look at v-*src* RNA in several different tissues to see if there is a tissue specific control of splicing of the cryptic intron.

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