A. ANALYSIS OF THE HUMAN C-SRC LOCUS

B. EXAMINATION OF GENETIC HOMOLOGY BETWEEN MAREK'S DISEASE VIRUS AND HERPESVIRUS OF TURKEYS

By

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ABSTRACT

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Structural analyses of the human c-src locus are described in part A of the thesis. Both the endogenous chicken c-src and the retroviral v-src genes have been extensively characterized; the studies presented here were initiated to provide a detailed analysis of the human homolog of these genes. The human c-src locus was examined by detailed restriction mapping and hybridization to v-src probes. Nine exons were identified spanning a 20kb region. Strong homology to v-src was observed, although certain exons were found to be somewhat more divergent. Analysis of human c-src/chicken c-src heteroduplexes supported the hybridization results. In addition, six regions containing human Alu repeat family sequences were identified.

The nucleotide sequence of exons 2 and 4-12 was obtained. Comparison with chicken c-src and retroviral v-src demonstrated a strong sequence conservation within exons 4-12, with the majority of base substitutions silent. The human c-src retains the chicken c-src 3' terminus, rather than the v-src divergent 3' terminus, and also

retains the precise intron-exon junctions of chicken c-src. Exon 2 has undergone a rearrangement involving both insertions and deletions. These studies revealed a strong conservation of amino acid sequence in the carboxy three-fourths of the protein, the region containing enzymatic activity, with alterations in the amino-terminal portion that is involved in attachment to the plasma membrane.

The degree of genetic homology between Marek's disease virus (MDV) and herpesvirus of turkeys (HVT) is examined in part B of the thesis. Despite their strong antigenic relationship, previous studies suggested the two viruses share only limited (1-5%) homology. The goals of this study were two-fold: first, both plasmid and phage MDV clone banks were constructed to facilitate studies of the MDV genome; second, less stringent hybridization conditions combined with improved hybridization kinetics were used to reexamine the sequence homology between MDV and HVT. Cross-hybridizations using the total viral DNA indicated the two viruses are closely related: sequences sharing 70-80% homology were detected over 90-95% of the viral genomes. These results were confirmed using individual cloned MDV DNA fragments.

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LIST OF ABBREVIATIONS

APS	ammoniumpersulfate
BSA	bovine serum albumin
dNTP	deoxyribonucleotide triphosphate
DNase	deoxyribonuclease
ddNTP	dideoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	(ethylenedinitriol)-tetraacetic acid, disodium
kb	kilobase
µCi	micro Curie
PEG	polyethylene glycol
RNase	ribonuclease
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
TES	2-([tris-(hydroxymethyl)methyl]amino)ethanesulfonic acid
Tris	tris(hydroxymethyl)aminoethane

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW (part A)

INTRODUCTION

One of the most extensively studied retroviral oncogenes is the transforming gene of Rous sarcoma virus (RSV), v-src. The v-src gene encodes a tyrosine phosphorylating protein kinase, $pp60^{v\text{-}src}$. In normal cells, tyrosine phosphorylation is a relatively minor modification, accounting for only 0.01% of the phosphorylated amino acids. However, infection with RSV results in an increase in total cellular phosphotyrosine, as a number of specific cellular proteins, as well as $pp60^{v\text{-}src}$ itself, become phosphorylated. Although the mechanism by which v-src transforms cells has been the focus of numerous studies, the actual alterations induced by $pp60^{v\text{-}src}$ which lead to transformation have yet to be elucidated.

Normal cellular sequences related to v-src (termed c-src) have been found in a wide variety of distantly related species. DNA hybridizations in liquid demonstrated these c-src sequences share extensive nucleotide homology with the viral src gene, and antisera raised against $pp60^{v\text{-}src}$ is also capable of immunoprecipitating the normal cellular protein, $pp60^{c\text{-}src}$. Like its viral counterpart, $pp60^{c\text{-}src}$ possesses tyrosine kinase activity.

The function of the normal cellular src gene is not yet known. Elevated levels of c-src expression have been found in adult brain and neural tissue and the level of c-src message is particularly high in these tissues during embryogenesis, suggesting a possible role of c-src

in the development of nervous tissue. The high degree of homology between the c-src sequences of widely divergent species indicates a strong selection against mutation, which implies $pp60^{c\text{-}src}$ has an essential function for which substantial alterations within the protein can not be tolerated. Elucidation of the normal function of c-src should provide insight into the v-src induced alterations leading to transformation.

Of the endogenous cellular c-src genes, only the chicken locus has been extensively studied. The amino acid sequence of the chicken $pp60^{c\text{-}src}$ protein is nearly identical to that of $pp60^{v\text{-}src}$, with a few amino acid changes scattered throughout the protein. However, 19 different residues in $pp60^{c\text{-}src}$ have been substituted for the carboxy-terminal 12 amino acids of $pp60^{v\text{-}src}$. Although the data are not yet conclusive, evidence suggests this region plays a major role in determining the oncogenic potential of the src gene.

The main focus of the studies presented here is the detailed analysis of another c-src gene, the human c-src locus. The structural studies are described in Chapter 2 and the nucleotide sequence is presented in Chapter 3. The human c-src locus is compared with both the chicken c-src and the retroviral v-src genes. It is hoped further analyses of both c-src and v-src gene expression will lead to an understanding of the function of $pp60^{c\text{-}src}$ in normal cells as well as an understanding of the changes elicited by $pp60^{v\text{-}src}$ that lead to transformation.

LITERATURE REVIEW

The protein product of the v-src gene was initially identified as a non-structural transformation-specific antigen present in avian sarcoma virus (ASV)-transformed cells (11,13,90): it is a 60,000 dalton phosphoprotein, $pp60^{v\text{-}src}$. $pp60^{v\text{-}src}$ can be synthesized *in vitro* by translation of the 3' third of ASV viral RNA (90,91), corresponding to the region identified by genetic analyses as the viral transforming gene (9,60,75,135). This is the only viral gene required for transformation; direct injection of v-src DNA into chickens will induce tumors (44).

A. Enzymatic Activity of $pp60^{v\text{-}src}$

A unique feature of $pp60^{v\text{-}src}$ is the ability to phosphorylate tyrosine. This protein kinase activity can be easily assayed *in vitro*; extracts of transformed cells will phosphorylate the immunoglobulin heavy chain, while extracts from uninfected cells can not (10,21). Analysis of temperature-sensitive transformation mutants has confirmed the existence of this enzymatic activity (70). Protein kinase activity can be detected in immunoprecipitates of cells infected with the mutant tsNY68 when the cells are grown at the permissive temperature but not in immunoprecipitates from cells grown at the non-permissive temperature, although the level of $pp60^{v\text{-}src}$ is similar at both temperatures and the protein stability is unaffected by growth temperature.

In vitro assays further demonstrated the thermolability of the protein kinase activity of $pp60^{v\text{-}src}$ obtained from temperature-sensitive mutants (71). $pp60^{v\text{-}src}$ was prepared by immunoadsorption from cells infected with either wild-type Schmidt-Rupin D strain of ASV (SR-D ASV) or tsNY68 that had been grown at the permissive temperature, the extracts were incubated at 41.5°C (non-permissive temperature) for various intervals, then assayed for the ability to phosphorylate immunoglobulin. Kinase activity from the tsNY68-infected cells was inactivated 15-fold more rapidly than was the activity from SR-D ASV-infected cells. Several other mutants (tsLA90, BK5 and tsGI251) have been found to possess extremely labile kinase activity (105).

The relationship between the kinase activity of $pp60^{v\text{-}src}$ and the transformed phenotype has been investigated using a number of partial transformation mutants (3). Cells infected with two of the three mutants assayed (CU2 and tsCU11) contain levels of $pp60^{v\text{-}src}$ similar to that found in SR-A (Schmidt-Rupin A strain of ASV)-infected cells. However, the specific activity of the kinase activity of these mutant $pp60^{v\text{-}src}$ proteins is less than half that of wild-type $pp60^{v\text{-}src}$. Cells infected with the third mutant, CU12 have a much lower level of $pp60^{v\text{-}src}$, yet the protein exhibits slightly higher specific activity. The close correlation between enzyme activity and transformation observed with both the temperature-sensitive mutants and the partial transformation mutants strongly suggests the kinase activity plays an essential role in transformation.

In addition to possessing kinase activity, $pp60^{v\text{-}src}$ is itself phosphorylated at two residues, an amino-terminal serine, which can be phosphorylated by a cellular cyclic AMP-stimulated protein kinase (19), and a carboxy-terminal tyrosine residue that is phosphorylated in a cyclic AMP-independent reaction (19,22,43). $pp60^{v\text{-}src}$ phosphorylated *in vivo* appears to contain twice as much phosphoserine as phosphotyrosine (108). Additional minor sites of phosphorylation are also present (19,29,87,116). It has been suggested that $pp60^{v\text{-}src}$ is autophosphorylated since incubation of highly purified $pp60^{v\text{-}src}$ with [^{32}P-γ]ATP results in phosphorylation of the protein *in vitro* (22,89).

The major phosphotyrosine of $pp60^{v\text{-}src}$ is residue 416 (87,116). Analysis of several other retroviral transforming proteins (p90 of Y73 and p105 of PRC II, both strains of ASV; p80 of ESV, Esh sarcoma virus; p140 of FSV, Fujinami sarcoma virus; p85 of ST-FeSV, Snyder-Theilin strain of feline sarcoma virus; p120 of Ableson virus) (81,87) has revealed two common features: the phosphotyrosine is located 7 residues to the carboxy-terminal side of a basic amino acid and 4 or 5 residues to the carboxy-terminal side of a glutamic acid residue. Other proteins phosphorylated by $pp60^{v\text{-}src}$ ($p36^{cell}$, $p50^{cell}$ and the immunoglobulin heavy chain) show similar structural features: a basic amino acid is located 7 residues to the amino-terminal side of the phosphotyrosine, with one or more acidic residues between. These features may therefore be important in substrate recognition of the $pp60^{v\text{-}src}$ protein kinase.

The phosphotyrosine residue in $pp60^{v\text{-}src}$ was initially thought to be essential for transformation because a good correlation

was observed beween phosphorylation at tyrosine 416 and transformation (19,70). When tsNY68-infected cells are grown at the non-permissive temperature, the level of phosphotyrosine in $pp60^{v\text{-}src}$ is greatly reduced while the level of phosphoserine remains unaffected. However, using site-specific mutagenesis at either the serine 17 (119) or the tyrosine 416 (29,119) it has been conclusively demonstrated that neither phosphorylation is required. Mutants lacking serine 17 are identical to wild-type SR-A in all characteristics assayed. The biochemical properties of mutants lacking tyrosine 416 are likewise similar to those of wild-type SR-A except the mutants are slower in inducing foci and tumor formation requires a longer latent period. These results lead to the conclusion that neither residue is required for transformation, but tyrosine 416 might facilitate transformation by affecting the kinetics. In support of these findings, $pp60^{v\text{-}src}$ synthesized in bacteria lacks phosphorylation yet retains kinase activity (44,77).

B. Subcellular Localization of $pp60^{v\text{-}src}$

Immunofluorescence microscopy studies suggest $pp60^{v\text{-}src}$ is associated with the plasma membrane and is particularly concentrated near cell-cell junctions (64,95) and adhesion plaques (96) and may be associated with the cytoskeleton (14). Similarly, immunochemical electron microscopy techniques have been used to localize $pp60^{v\text{-}src}$ to the inner surface of the plasma membrane: the protein is concentrated at gap junctions (139). Analysis of *in vitro* phosphotransferase activity in cellular components obtained after cell fractionation (27,65) substantiates these findings: kinase activity is primarily found in the membrane fraction of SR-A infected cells.

Pulse-chase experiments demonstrated $pp60^{v\text{-}src}$ is synthesized on soluble polyribosomes and rapidly becomes associated with the cell membrane (69). Immediately after synthesis, $pp60^{v\text{-}src}$ can be found in the soluble fraction, yet within 5 minutes virtually all of the protein has become membrane-associated. These analyses further demonstrated that $pp60^{v\text{-}src}$ is not a cleavage product of a larger precursor since both the soluble and membrane-bound forms have the same mobility in SDS gels. $pp60^{v\text{-}src}$ can be removed from the membrane only by treatment with non-ionic detergents (27) suggesting the protein is inserted into the plasma membrane. In SR-D-infected rat (SR-RK and RR1022) and goat (Pc1) cells, $pp60^{v\text{-}src}$ appears to be associated with the nuclear, rather than cytoplasmic, membrane, and can also be found in the juxtanuclear reticular membranes (46,64,95). This altered distribution is stably inherited: these proteins have undergone alterations in the amino-terminus, which may be responsible for the different subcellular location (46).

Limited proteolysis was performed to identify the membrane-bound portion of $pp60^{v\text{-}src}$ (69). Results indicate the amino-terminal region (approximately one-fourth of the molecule) is associated with the inner cell membrane, while the carboxy-terminal two-thirds extends into the cytoplasm. Kinase activity is retained in the cytoplasmic domain: the carboxy-terminal fragment obtained from chymotrypsin digestion (approximately half of the molecule) is capable of functioning in an <u>in vitro</u> phosphotransferase assay.

At least two cellular proteins, $pp89^{cell}$ and $pp50^{cell}$, form a complex with cytoplasmic $pp60^{v\text{-}src}$ (12,26). The proteins are tightly bound to $pp60^{v\text{-}src}$ since the complex can be

immunoprecipitated with antisera specific for $pp60^{v\text{-}src}$. Sedimentation analysis suggests the complex contains a single molecule of $pp60^{v\text{-}src}$, $pp89^{cell}$ and $pp50^{cell}$ (12). The $pp60^{v\text{-}src}$ protein within the complex is phosphorylated only at serine and lacks tyrosine kinase activity (12,26). The two cellular proteins are not associated with the active membrane-bound form of $pp60^{v\text{-}src}$ (12).

The defect of one temperature-sensitive mutant, tsNY68, appears to involve the association of $pp60^{v\text{-}src}$ with the plasma membrane (12,26,83). At the non-permissive temperature 90-95% of the protein is found in the soluble fraction, associated with $pp89^{cell}$ and $pp50^{cell}$, and is not phosphorylated at tyrosine (19). The minor fraction of membrane-bound $pp60^{v\text{-}src}$ in these cells also lacks phosphotyrosine. After a shift down to the permissive temperature, cytoplasmic levels of $pp60^{v\text{-}src}$ decrease as the protein become associated with the membrane. In the converse experiment, shifting cells from the permissive to the non-permissive temperature, 70% of the membrane-bound $pp60^{v\text{-}src}$ becomes soluble and is found associated with the two cellular proteins $pp89^{cell}$ and $pp50^{cell}$. These results indicate the association of $pp60^{v\text{-}src}$ with the membrane is reversible, and the released protein is capable of re-forming complexes with $pp89^{cell}$ and $pp50^{cell}$.

The normal functions of $pp89^{cell}$ and $pp50^{cell}$ are unknown, however, $pp89^{cell}$ has been identified as a heat-shock protein (85): synthesis of $pp89^{cell}$ is greatly enchanced after treatment of normal chicken cells with a variety of chemical agents or by incubation at 45°C for 3 hours. Tryptic peptide maps of the

heat-shock protein and the $pp60^{v\text{-}src}$-associated protein are identical, strongly indicating they are the same protein. Nevertheless, antisera raised against the heat-shock protein is unable to precipitate the $pp60^{v\text{-}src}$ complex from ASV-infected cells, suggesting the protein conformations are different.

C. Cellular Proteins Phosphorylated by $pp60^{v\text{-}src}$

The ability of $pp60^{v\text{-}src}$ to phosphorylate tyrosine residues of normal cellular proteins may play a critical role in the transformation process. In normal cells, phosphotyrosine is present in minor amounts, constituting approximately 0.01-0.03% of the phosphorylated amino acid residues, with phosphoserine and phosphothreonine comprising the majority (>90% and 5-10%, respectively) (107). ASV infection results in a 6-10 fold increase in phosphotyrosine residues found in total cell protein (54,92,107). Phosphorylation of the $pp60^{v\text{-}src}$ protein itself accounts for approximately 20% of this increase (107), thus the increase in tyrosine phosphorylation of cellular proteins is around 5-6 fold.

Tyrosine phosphorylation of cellular proteins has been found to correlate quite well with the transformed phenotype. Cells infected with the transformation-defective mutant tdSR-D do not possess elevated levels of phosphotyrosine (54). The correlation between transformation and the level of phosphotyrosine in total cell proteins has been further substantiated using the temperature-sensitive mutant tsLA29 (107). Cellular levels of phosphotyrosine are not elevated in cells infected with tsLA29 grown at the non-permissive temperature, but are greatly increased when the cells are grown at the permissive temperature. When the cells are initially grown at the non-permissive temperature, then shifted down to the permissive temperature, the level

of phosphotyrosine increases rapidly: within 60 minutes after the temperature shift, the phosphotyrosine level increases to 60% of that present in cells grown at the permissive temperature. The converse results are obtained when cells are initially grown at the permissive temperature and shifted up to the non-permissive temperature: the amount of phosphotyrosine decreases to the level found in uninfected cells within 60 minutes after the shift. These findings suggest phosphorylation of specific cellular proteins may be essential for transformation.

To investigate the effect of $pp60^{v\text{-}src}$ on the synthesis and phosphorylation of individual cellular polypeptides, total cell proteins were analyzed on two-dimensional polyacrylamide gels (93). After labeling with ^{35}S-methionine, only a low percentage (2.5%) of the normal cell proteins show increases in labeling intensity after Prague A (Pr-A) or SR-A infection. Similarly, only 1.7% show decreases in labeling intensity. Thus, viral infection appears to produce only minor change in the levels of the majority of cellular proteins. When the cells are labeled with ^{32}P-orthophosphate, approximately 6% of the cellular proteins exhibit dectectable changes in relative labeling intensity. Phosphorylation of the $pp60^{v\text{-}src}$ protein is readily observed; in addition, phosphorylation of one cellular protein, $p36^{cell}$, is markedly increased. Since this protein appears to be a major substrate of $pp60^{v\text{-}src}$ *in vivo* accounting for approximately 15% of the total phosphotyrosine (7), it has been studied in some detail.

Kinetics of phosphorylation of $p36^{cell}$ were examined using the temperature-sensitive mutant tsLA29 (93). At the non-permissive

temperature phosphorylation of $p36^{cell}$ is barely detectable. Phosphorylation can be detected within 20 minutes after a shift down to the permissive temperature, and protein synthesis is not required for the phosphorylation to occur (94). The protein is phosphorylated at both serine and tyrosine (92), yet the charge difference between the phosphorylated and non-phosphorylated species suggests the phosphorylated $p36^{cell}$ consists of two distinct populations, one phosphorylated at a serine residue and the other at a tyrosine residue. $p36^{cell}$ isolated from normal cells is an efficient substrate for phosphorylation by $pp60^{v\text{-}src}$ in vitro (40), with tyrosine, but not serine, phosphorylated. Although the function of $p36^{cell}$ is unknown, the protein has been localized to the cytoskeletal framework in both normal and ASV-transformed cells (16,23,28), and both monomer and dimer forms are present (41).

Analyses of the phosphorylation of $p36^{cell}$ in cells infected with temperature-sensitive mutants (RSV-B77 Rat 1 cells and tsLA24-infected rat cells) suggests a high correlation between phosphorylation of $p36^{cell}$ and transformation (16). At the non-permissive temperature, $p36^{cell}$ is not phosphorylated; at the permissive temperature it is. Furthermore, an increase in $p36^{cell}$ phosphorylation can be detected within 1-2 hours after a shift from the non-permissive to the permissive temperature.

Despite this good correlation observed with the temperature-sensitive mutants, analysis of partial revertant mutants indicates phosphorylation of $p36^{cell}$ is not sufficient for transformation. Phosphorylation of $p36^{cell}$ was examined in fully transformed (1T), partial revertant (866-R5C) and full revertant

(866-4) ASV-transformed vole cell lines (80); the partial revertant had regained normal cellular morphology yet was capable of growing in soft agar and inducing tumors in nude mice. $p36^{cell}$ in lines 1T and 866-R5C is phosphorylated to a similar extent. Phosphorylation of $p36^{cell}$ in line 866-4 is greatly reduced. These results suggest phosphorylation of $p36^{cell}$ is not associated with morphological changes in transformed cells, yet might play a role in the ability to grow in soft agar and tumorigenicity. The correlation between phosphorylation of $p36^{cell}$ and a number of transformation-specific characteristics has been examined using a battery of 8 partial transformation mutants (24). Phosphorylation of $p36^{cell}$ correlates well only with the production of plasminogen activator, but not with changes in adhesiveness, hexose transport or colony formation in soft agar, nor is there a clear correlation between phosphorylation of $p36^{cell}$ and the ability of infected cells to form tumors in nude mice (57). In addition, the phosphorylation occurs only when total cellular phosphotyrosine levels are high, suggesting the protein may be a low-affinity substrate for $pp60^{v\text{-}src}$. Thus, phosphorylation of $p36^{cell}$ could be non-essential for transformation.

A number of other cellular proteins have been identified as substrates for $pp60^{v\text{-}src}$ *in vivo*, although the increase in phosphotyrosine in these molecules is somewhat less than that observed in $p36^{cell}$. Vinculin, a protein associated with the cytoplasmic side of adhesion plaques, which anchor actin-containing microfilaments to the plasma membrane and attach cells to the substratum, was identified as an *in vivo* substrate of $pp60^{v\text{-}src}$ (104). The

levels of phosphotyrosine in vinculin are 8-fold higher in ASV-infected cells than in uninfected cells. Nevertheless, only 1% of the vinculin molecules in transformed cells actually contain phosphotyrosine, so the biological significance of this phosphorylation remains unclear. In addition, vinculin is not phosphorylated in PRCII-infected cells (104). Other cytoskeletal proteins (filamin, myosin heavy chains, α-actinin and vimentin) are not phosphorylated by $pp60^{v\text{-}src}$ *in vivo*.

Two cytoplasmic proteins were identified as *in vivo* substrates of $pp60^{v\text{-}src}$, $p46^{cell}$ and $p28^{cell}$ (23,24). A series of partial transformation mutants were assayed for levels of phosphorylation of both $p46^{cell}$ and $p28^{cell}$ (24). Phosphotyrosine levels of both molecules correlate well with increased hexose transport. Phosphorylation of $p46^{cell}$ also correlates with the production of plasminogen activator. It was concluded that phosphorylation of either $p46^{cell}$ or $p28^{cell}$ is not sufficient for transformation but may be involved in expression of a particular transformation-specific phenotype. Alternatively, phosphorylation of either of these cellular proteins could be unrelated to transformation. These results would imply that phosphorylation of other cellular proteins, which appear as minor substrates by two-dimensional gel analysis, are the critical proteins necessary for transformation.

A more quantitative, although less qualitative approach has been used to analyze phosphotyrosine content in total cellular proteins (7). Phosphoproteins from SR-A infected cells were compared to those isolated from uninfected cells by SDS-polyacrylamide gel electrophoresis. The gels were then sectioned into 10 slices and each slice subjected to phosphoamino acid analysis. Phosphotyrosine is

found in all size classes in uninfected cells, ranging from 0.1 to 0.15% of the phosphoamino acids per fraction. In proteins from SR-A-infected cells, phosphotyrosine is likewise found in all fractions, but the levels are much higher than in uninfected cells, ranging from nearly 0.2% to 0.8%. Two broad peaks at approximately 35,000 daltons and 60,000 daltons are probably largely due to $p36^{cell}$ and $pp60^{v\text{-}src}$. These data demonstrate that a relatively large number of cellular proteins are phosphorylated in infected cells, more than are discernable by two-dimensional gel electrophoresis.

Cellular proteins critical for transformation remain to be identified: it is likely phosphorylation of more than one cellular protein is required for full expression of the transformed phenotype. Partial transformation mutants manifest different degrees of expression of transformation-specific characterisitics (3,24), and can not readily be arranged in a heirarchy of transforming potential. Furthermore, certain transformation-associated changes, for instance the loss of fibronectin, do not correlate with tumorigenicity (57). Analysis of temperature-sensitive mutants has also suggested that $pp60^{v\text{-}src}$ must interact with a number of cellular proteins to express the fully transformed phenotype (138). When infected cells were assayed for the expression of several transformation parameters after growth at temperatures intermediate between the permissive and non-permissive temperatures, 4 of the 5 mutants analyzed could be roughly ranked with respect to the severity of the mutation. The fifth mutant examined, tsGI251, is highly abnormal; changes in biochemical parameters are similar to those induced by the other mutants, yet temperature has an opposite effect on expression of growth-related properties. In

contrast to infection with most temperature sensitive mutants, tsGI251-infected cells form colonies in soft agar more efficiently at higher, rather than lower temperatures. Biochemical parameters and growth characteristics can therefore be clearly distinguished.

Thus, a number of studies suggest that the pleiotropic effects of $pp60^{v\text{-}src}$ may actually be brought about by the interaction of the protein with several normal cellular proteins, rather than a single substrate. Mutants have been described which elicit certain transformation-associated characteristics but not others; which of the many cellular changes are essential for tumorigenicity remains to be determined. It is clear that some of the cellular alterations associated with transformation are not required for tumor development; whether expression of these characteristics enchances the tumorigenic potential has not yet been elucidated.

It has been proposed that the v-src gene actually encodes two proteins read in overlapping reading frames (74). Evidence for the existence of a second protein rests primarily on analysis of the ASV-transformed rat cell line B31, the revertant line 000 and the retransformed line 000*. A single base pair mutation in line 000 causes a frameshift, which is compensated for by a 242 base pair duplication in line 000*. Examination of aberrant proteins synthesized in these two cell lines suggests initiation can occur at an internal AUG. The nucleotide sequence implies initiation at this internal site should result in the synthesis of a 7,000 dalton protein in the parental B31 line. Nevertheless, this small protein has not yet been identified, nor has any function been assigned to it. If the

7,000 dalton protein does exist, it is possible that the protein is responsible for one or more of the many cellular changes associated with transformation.

D. Structural Features of the v-src and c-src Loci

Complete nucleotide sequence of the v-src locus of both SR-A and Prague C (Pr-C) has been obtained (30,31,103). The v-src coding sequence is nearly 1.6kb in length, encoding 526 amino acids; the sequence of Pr-C differs from that of SR-A at 42 nucleotides, resulting in 22 amino acid changes.

Several interesting features have been observed in the regions flanking v-src (103). The v-src gene is flanked by direct repeats, slightly longer than 120 base pairs. Only one copy of the sequence is found in ALV, which lacks src sequences. A second sequence, termed E, is located upstream from v-src in SR-A, immediately after the direct repeat. In Pr-C, this sequence is located downstream from v-src, before the second direct repeat. Short (11 nucleotide) direct repeats (boundary repeats) flank the E region, suggesting E may actually be an insertion sequence. E itself is capable of forming a large hairpin structure.

Sequences homologous to the v-src coding region were detected in a variety of avian species using v-src specific cDNA as a probe under stringent conditions (125). Thermal stability of the v-src/avian DNA hybrids correlates strongly with phylogenetic distances between the species. Calculations indicate only 3-4% base mismatch between v-src and the endogenous chicken src (c-src) sequences. Endogenous src sequences from more distantly related avian species are only slightly less homologous.

Although under highly stringent conditions v-src related sequences cannot be detected in non-avian species (125), v-src will anneal to DNA from a variety of vertebrate species when the hybridization conditions are somewhat relaxed (124). Homology of these distantly related species to v-src is extremely high, with an average of only 8-10% base mismatch. Under relaxed conditions, v-src will also hybridize to Drosophila DNA (53,113). Hybridization kinetics suggest the c-src sequences in most species are present at a maximum of 5-10 copies per cell. The high degree of homology over wide phylogenetic distances suggests the c-src gene may have a critical, indispensable function, providing selective pressure for the conservation of nucleotide sequence.

Genomic clones spanning the chicken c-src locus have been obtained (86,110,128, 129); v-src related sequences are distributed over a region approximately 8kb in length. Initially 6 introns could be observed in chicken c-src/v-src heteroduplexes (86,110,129), 5 smaller introns have been identified by direct sequence analysis (128). The clones appear to contain the major chicken c-src locus since restriction maps of the cloned regions precisely predict the sizes of fragments detected in Southern blots of chicken DNA when v-src is used as probe (110,129). In addition, a second locus bearing homology to the 5' portion of v-src may also be present (86).

Direct comparison of the nucleotide sequence of chicken c-src and v-src (SR-A) reveals a number of interesting features (128). With the exception of the 3'-terminus, the sequences are highly homologous, with only 18 nucleotide changes producing 8 amino acid alterations. The major difference between the two proteins is found at the 3' terminus,

where 19 completely different amino acids in $pp60^{c\text{-}src}$ have been substituted for the the carboxy-terminal 12 amino acids of $pp60^{v\text{-}src}$. A 39 base pair sequence corresponding to the 3' terminus of v-src is located approximately 900 base pairs downstream from the termination codon of chicken c-src.

Sequence homology between chicken c-src and v-src extends approximately 90 nucleotides upstream from the v-src initiation codon (128). The sequences diverge immediately upstream from a splice acceptor site present in the RSV genome. Neither the E sequence nor the direct repeats flanking v-src are found flanking c-src. Finally, all chicken c-src splice donor and acceptor sites retain the GT-AG consensus sequence.

RNA hybridizing to v-src DNA has been found in uninfected chicken cells (123), with an estimated 1-4 copies per cell, suggesting the c-src protein is present at low levels in normal cells. The chicken c-src mRNA has a sedimentation coefficient of 28-30S, thus is approximately 6kb long; it is polyadenylated and associated with polyribosomes (122). Levels of c-src mRNA are identical in sparse and densely cultured cells, and are not affected by serum starvation (123), therefore c-src expression does not appear to correlate with cell proliferation.

E. Analysis of $pp60^{c\text{-}src}$

Serum obtained from tumor-bearing animals is able to precipitate a protein antigenically related to $pp60^{v\text{-}src}$ from uninfected avian cells (18,20,84,97,106) termed $pp60^{c\text{-}src}$. Partial proteolysis of $pp60^{c\text{-}src}$ revealed the normal cellular protein is structurally very similar to $pp60^{v\text{-}src}$ (ASV), but not identical. In particular, the carboxy-terminal fragment produced by V8 protease digestion is slightly larger in $pp60^{c\text{-}src}$ than in $pp60^{v\text{-}src}$.

This fragment contains the divergent carboxy terminus, which the nucleotide sequence predicted should be 7 amino acids longer in $pp60^{c\text{-}src}$ (128). Several other differences between the two proteins have been identified by tryptic peptide analysis (106). Like the viral protein, $pp60^{c\text{-}src}$ is phosphorylated at both serine and tyrosine residues (20). However, the phosphopeptide maps are completely different (18), indicating the residues phosphorylated in $pp60^{c\text{-}src}$ are not identical to those in $pp60^{v\text{-}src}$.

The level of $pp60^{c\text{-}src}$ in normal cells is estimated to be 30-100 fold lower than the levels of $pp60^{v\text{-}src}$ in SR-A transformed cells (18,84). Although $pp60^{c\text{-}src}$ immunoprecipitated with certain sera does not exhibit protein kinase activity (18,97), phosphotransferase activity can be demonstrated when other antisera are used (20,84,97). Similar enzymatic activities suggest $pp60^{c\text{-}src}$ and $pp60^{v\text{-}src}$ are functionally similar.

Sera from tumor-bearing animals can also precipitate a $pp60^{v\text{-}src}$-related protein from a variety of avian cells (18), as well as mammalian (20,84,97,106) and frog cells (97), but not from Drosophila cells (97,84). Partial proteolytic digests (20,84,97) and tryptic peptide mapping (106) demonstrated the different src proteins are nearly identical to $pp60^{c\text{-}src}$. This high degree of amino acid conservation is in complete agreement with the strong nucleotide homology observed among the divergent species (124). As with chicken $pp60^{c\text{-}src}$, the src proteins from other species also exhibit protein kinase activity (20,97).

Two forms of $pp60^{c\text{-}src}$ are present in human fibroblasts and human epidermal carcinoma cells, with molecular weights of 59,000

daltons and 60,000 daltons (111). The two forms are differentially phosphorylated: phosphorylation of the 59,000 dalton form is almost exclusively on the amino-terminal serine, while the 60,000 dalton form is phosphorylated predominantly on the carboxyterminal tyrosine, with minor serine phosphorylation. Two-dimensional chymotryptic analysis demonstrated the two human $pp60^{c\text{-}src}$ forms share several common peptides. However, significant differences are present. It has been suggested the two forms arise from two distinct human c-src loci or they are generated by alternate splicing of the same initial transcript.

Although most sera are unable to precipitate a src-related protein from Drosophila or other lower organisms (84,97), one serum was obtained which could precipitate a tyrosine kinase activity from a wide variety of species, including mammals, birds, fish, insects and sponges (100). A 60,000 dalton phosphoprotein similar to $pp60^{v\text{-}src}$ could be detected by immunoprecipitation from all tissues that could be labeled with ^{32}P-orthophosphate. The protein was capable of phosphorylating immunoglobulin in vitro, with phosphorylation specific for tyrosine. In all species examined, the highest level of phosphotransferase activity was detected in brain and nervous tissue. No kinase activity could be detected in immunoprecipitates of unicellular organisms or plants.

Expression of c-src in normal brain and nervous tissue was confirmed by analysis of various tissues from chicken embryos (25). Lysates of brain and neural retina tissues have 8-10 fold higher levels of tyrosine kinase activity than do other tissues. $pp60^{c\text{-}src}$ concentration remains low until day 6 of embryogenesis, when it rapidly

reaching a plateau at day 9. Slightly before hatching, the level drops to that found in the adult tissue. In retinal neurons, $pp60^{c\text{-}src}$ is localized within cell bodies and processes of ganglion neurons, bodies of amacrine cells and processes of rods and cones (120). In these cells, levels of $pp60^{c\text{-}src}$ are particularly elevated during developmental stage 35, at the time when cell-cell contacts between neurons are established.

The demonstration of a $p60^{v\text{-}src}$-related tyrosine kinase activity in phylogenetically distant species and the strong conservation of amino acid sequence in organisms as different as birds and mammals implies $pp60^{c\text{-}src}$ serves an extremely important function in normal cells. Elevation of c-src expression in the brain and neural tissue during development suggests a role in the differentiation of nervous tissue. Nevertheless, the actual function of $pp60^{c\text{-}src}$ remains unknown.

F. Other Members of the src Family

Comparison of the nucleotide sequence of v-src with sequences of other retroviral oncogenes revealed v-src belongs to a large family. Related sequences include abl (from Ableson virus; 48,53), erbB (from avian erythroblastosis virus; 88, 140), fes (from feline sarcoma virus; 50), fps (from Fujinami sarcoma virus; 112), mos (from Moloney murine sarcoma virus; 134) and yes (from Y73 strain of ASV; 61). Homologous sequences are clustered in the 3' half of v-src, with homology particularly strong in the regions flanking the codon for Tyr 416. Most similar to src is yes (61), with 82% amino acid homology beginning around residue 80 of v-src. At the 3' terminus, the yes sequence shares homology with c-src, rather than v-src. In addition, a

Drosophila clone hybridizing to both v-src and v-abl has been obtained (53). Several members of the src family (abl, fes, fps and yes) exhibit tyrosine kinase activity.

Also related to src is the catalytic chain of bovine cyclic AMP-dependent protein kinase (4). Homologous sequences are localized in the carboxy-terminal half of v-src, suggesting this protein is also a member of the src family.

In addition, several peptides derived from the human epidermal growth factor (EGF) receptor are similar to regions of v-erbB (39). One function of the EGF receptor is the ability to phosphorylate tyrosine, thus the EGF receptor may be another member of the src family.

G. v-src/c-src Hybrids

The specific differences between v-src and c-src that determine tumorigenic potential are not clear. Transformation-defective mutants containing large deletions in the v-src gene have been observed to recombine with endogenous c-src sequences generating fully transforming revertants, rASVs (59,136,137) which contain a chimeric src gene. The ability to generate rASVs is directly proportional to the amount of v-src sequence remaining in the viral genome. Partial proteolytic digestion patterns confirm the $pp60^{src}$ proteins encoded by rASV isolates are hybrids of $pp60^{c\text{-}src}$ and $pp60^{v\text{-}src}$ (59,126). Peptides common to either $pp60^{v\text{-}src}$ or $pp60^{c\text{-}src}$ have been observed by tryptic peptide mapping (58) and differences between RNase T1-resistant oligonucleotides have been observed by fingerprint analysis (136,137). The extent of v-src or c-src derived sequence is different for each rASV isolate; the amino-terminal region is

particularly plastic, capable of tolerating large variations in size (58). Three rASV src genes analyzed in detail (157, 1441 and 1702) appear to have the same 3' terminus (less than 20% of the coding sequence) derived from v-src, the remainder from c-src (58). The $pp60^{src}$ isolated from rASV-infected cells retains tyrosine kinase activity (59) and is phosphorylated at both serine and tyrosine (58). The phosphotyrosine residue is identical to that found on $pp60^{v\text{-}src}$, not $pp60^{c\text{-}src}$. Direct nucleotide sequencing of rASV1441 revealed the 5' recombination occurred upstream from the initiation codon and the second crossover site was slightly upstream from the $pp60^{v\text{-}src}$ phosphotyrosine residue 416 (126,127).

Despite the fact that src sequences in rASVs are primarily derived from endogenous c-src, the viruses resemble SR-A in transformation potential. Two possibilities have been suggested: either $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ are identical in function, but overexpression of $pp60^{v\text{-}src}$ is sufficient to cause transformation or the two proteins recognize different intracellular targets, with the altered substrate specificity of $pp60^{v\text{-}src}$ resulting in transformation.

To elucidate the factors contributing to oncogenicity, chimeric v-src/c-src molecules have been constructed and assayed for the ability to transform fibroblasts in culture. Results from the hybrids in plasmid vectors (109) are somewhat different from those in vectors designed to allow for virus production (51).

Constructions containing the chicken c-src gene in an expression vector were unable to transform NIH3T3 cells, while similar constructs containing v-src transformed with high efficiency (109). Src

expression and kinase activity in both cultures were identical, and cells transfected with either construct showed similar increases in total phosphotyrosine. The results suggest functional differences exist between $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$, overexpression of $pp60^{c\text{-}src}$ and the resultant increase in the level of phosphotyrosine is not sufficient for transformation.

Chimeric v-src/c-src hybrids were also constructed and assayed for the ability to transform NIH3T3 cells (109). A plasmid containing the 5' portion of v-src and the 3' terminus of c-src was able to induce only a small number of foci. The reverse construction, containing the 5' portion of c-src and the 3' terminus of v-src transformed efficiently. A final construct, containing v-src sequences with the carboxy-terminal 9 amino acids replaced by 9 random amino acids, also transformed efficiently. Results from these chimeric constructions indicate the carboxy terminus is responsible for the functional differences of $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$, and suggest the carboxy terminus of $pp60^{c\text{-}src}$ actually inhibits transformation.

Chimeric constructs which will result in the production of infectious virus (51) produced somewhat different results upon their transfection into chicken embryo fibroblasts. Constructs containing sequences derived solely from c-src produced limited morphological transformation, with small, compact colonies that were highly sensitive to culture conditions. Virus recovered from the media was also weakly transforming; it was suggested these weakly transforming viruses may contain a mutated c-src gene.

Both hybrid constructs containing c-src with the v-src 3' terminus and those containing v-src with the c-src 3' terminus transformed with

equal efficiency, and high-titer transforming virus could be obtained from the culture media. These results suggest the transforming potential is dependent upon interactions of the amino and carboxy portions of the protein, rather than residing solely in the carboxy terminus. These results are different from those obtained with the plasmid constructs in NIH3T3 cells (109), and may reflect differences between the primary chicken cells and the established mouse cell line or subtle differences in the constructs themselves, or could be a function of the ability to generate infectious virus.

Thus, while the 3' terminus of the src gene appears to play a major role in determining the oncogenic potential, other factors may also be required.

H. Involvement of Proto-oncogenes in Human Cancers

A number of other human counterparts of retroviral oncogenes (proto-oncogenes) have been identified; most of the analyses have focused on the role of these genes in human cancers. At least two proto-oncogenes have been found at the breakpoint of chromosomal translocations associated with specific neoplasias, others have been identified by their ability to transform NIH3T3 cells. Proposed mechanisms of activation include both mutation and over-expression due to promotion, action of enhancers, amplification or deregulation. The involvement of c-myc, c-ras (Ha, Ki and N) and c-abl in tumorigenesis has been examined in some detail (myc is the transforming gene of MC29, Ha-ras of the Harvey murine sarcoma virus, Ki-ras of the Kirsten murine sarcoma virus and N-ras a human gene with sequences related to v-ras).

In the majority of Burkitt's lymphoma cells, c-myc has undergone rearrangements (1,6,32,33,132). Three specific translocations to

immunoglobulin loci have been observed, translocation involving the IgK locus on chromosome 2, translocation to the IgH locus on chromosome 14, and translocation to the Igλ locus on chromosome 22. In translocations involving either chromosome 14 or 22, the breakpoint occurs near or within the 5' end of the c-myc gene (6,132), thus the c-myc promoters may or may not be lost. The altered c-myc is usually overexpressed (42,49) and the ratio of utilization of the two promoters (if present) is frequently changed, with the Burkitt tumor cells favoring P1, normal cells favoring P2 (133). In most cases, the c-myc protein itself does not appear to be altered (6). It has been proposed that the translocation disrupts the 5' regulatory sequences (68,133), thus activation of c-myc involves deregulation. The role of c-myc in translocations involving chromosome 2 is not quite clear since the translocation actually occurs distal to the c-myc gene (33).

Members of the human c-ras family have been implicated in several carcinomas of epithelial origin: activation of Ha-ras in bladder (37) carcinomas, activation of Ki-ras in lung (36,37) and colon (36,76) carcinomas and activation of N-ras in neuroblastomas (114,115). Elevated expression of N-ras has also been observed in bone marrow cells from a patient with acute myeloblastic leukemia (46). A single amino acid change has been found sufficient to activate both the Ha-ras gene (15,131) and the N-ras gene (130). Amplification of Ki-ras has been observed in several tumor cell lines (76).

The c-abl gene has been implicated in the Philadelphia chromosome translocation associated with chronic myelogenous leukemia (5,35). C-abl is located at the breakpoint in at least one cell line (52), however, there is no evidence of increased expression.

Involvement of other proto-oncogenes in human neoplasia is not well documented. Amplification of N-myc has been observed in neuroblastomas (2,63,101,102), and in small cell lung cancers (72), with the amplified sequences located in double minutes or homogeneously staining regions. The Blym gene isolated from Burkitt's lymphoma cells will transform NIH3T3 cells (38). At least one human T-cell leukemia virus (HTLV)-transformed cell line has elevated levels of c-sis expression (17; sis is the transforming gene of simian sarcoma virus). A cDNA clone containing the 3' portion of the c-sis message isolated from these cells was capable of transforming NIH3T3 cells. Elevated levels of c-src message have been found in a few sarcomas and mammary carcinomas (55).

Evidence for the involvement of other c-onc genes in neoplasia is circumstantial. c-mos is located near the breakpoint of translocations common in the M2 type of acute nonlymphocytic leukemia (ANLL), c-fes near the breakpoint of translocations common in the M3 type of ANLL (141). The chromosomal region containing c-myb (myb is the transforming gene of avian myeloblastosis virus) is deleted in some cases of actue lymphocytic leukemia.

A number of recent studies have suggested transformation is a multi-step process involving alteration of at least two or more cellular functions (66,82,98). Two distinct complementation groups have been identified. The first is involved in immortalization and can be assayed by the establishment of primary cells in tissue culture. This group includes myc, polyoma large T antigen and adenovirus E1a. The second complementation group is implicated in morphological changes and anchorage independence, and includes Ha-ras, N-ras and polyoma

middle T antigen. A third complementation group may be involved in determining the tumorigenic/metastatic potential of transformed cells.

While the involvement of proto-oncogenes in neoplasia is becoming clearer, little is known of the roles of these genes in normal cells. Analysis of these proteins in transformed cells may shed light on their normal function. Likewise, elucidation of the normal role of proto-oncogenes should provide valuable insight into the biochemical changes leading to neoplasia.

CHAPTER II

STRUCTURAL ANALYSIS OF THE HUMAN C-SRC LOCUS

CHAPTER II

MATERIALS AND METHODS

A. Materials

Restriction enzymes were purchased from Bethesda Research Laboratories, Boehringer Mannheim or Promega Biotec. EcoRI was a gift from Dr. A. Revzin. DNA polymerase I was from New England Nuclear; ligase from Promega Biotec; proteinase K and calf intestine alkaline phosphatase from Boehringer Mannheim; DNase, RNase and pronase were from Sigma. [α-^{32}p]dNTPs were purchased from Amersham. Ampicillin, tetracycline chloramphenicol, lysozyme, salmon sperm DNA and yeast RNA were obtained from Sigma. All materials for electron microscopy were purchased from Pelco; cytochrome c was from Calbiochem.

B. Media

BHI media is 37 g brain heart infusion per liter. LB is 10 g tryptone; 5 g NaCl; 5 g yeast extract per liter. M9 is 6 g Na_2HPO_4; 3 g KH_2PO_4; 0.5 g NaCl; 1 g NH_4Cl per liter, with 25 ml 20% glucose, 25 ml 20% casamino acids, 1 ml 2% thiamine HCl and 1 ml 1M $MgCl_2$ added after autoclaving. NZCYM is 10 g NZamine; 5 g NaCl; 5 g yeast extract; 1 g casamino acids per liter, with 10 ml 1M $MgSO_4$ added after autoclaving. All media was prepared in twice-distilled H_2O. Plates were made with 1-1.5% agar; top agar contained 0.7% agar.

C. Isolation of Bacteriophage DNA

Phage were grown in the host bacteria K802 or K803; phage were isolated by PEG precipitation followed by centrifugation through a glycerol step gradient (73). Liter cultures were grown in NZCYM media at 37°C until lysis was evident; 3 ml of chloroform was added and the cells incubated for an additional 15 min. The preparation was centrifuged for 10 min at 8K. RNase and DNase were added to the supernatent to a final concentration of 1 μg/ml each and the lysate incubated at 4°C for 60 min. 58.44 g of NaCl and 100 g of PEG were added and the preparation incubated at 4°C for another 60 min. The precipitate was spun at 8K for 10 min. The pellet was resuspended in 5 ml TM (50 mM Tris-HCl pH 8.0; 10 mM $MgSO_4$) and extracted with an equal volume of chloroform. The phage solution was layered over a 5%/40% glycerol step gradient and spun at 35K for 60 min at 4°C. The pellet was resuspended in 2 ml SM (0.1 M NaCl; 10 mM $MgSO_4$; 50 mM Tris-HCl pH 7.5; 0.01% gelatin), RNase was added to 50 μg/ml and DNase added to 1 μg/ml. The solution was incubated at 37°C for 30 min. 5X STEP buffer (0.5% SDS; 50 mM Tris-HCl pH 7.5; 0.4 M EDTA; 1 mg/ml proteinase K) was added to one-fifth the final volume and the solution incubated at 50°C for 15 min. The DNA was extracted once with phenol: chloroform (1:1) and once with chloroform and dialyzed against 20 mM Tris-HCl pH 8.1; 0.1 mM EDTA.

D. Isolation of Plasmid DNA

Bacteria was grown at 37°C in M9 media in the presence of ampicillin (50 μg/ml) or tetracycline (15 μg/ml) until reaching an OD_{600} of 0.6. 150 mg dry chloramphenicol was added per liter and the culture incubated at 37°C for 16 hrs. Cells were chilled on ice

for 5 min, then centrifuged at 5K for 10 min. The pellet was resuspended in 40 ml 10 mM Tris-HCl; 1 mM EDTA pH 8.0 and recentrifuged at 7K for 10 min. The cell pellet was resuspended in lysozyme buffer (50 mM glucose; 10 mM EDTA; 25 mM Tris-HCl pH 8.0), 8 mg dry lysozyme was added and the suspension incubated for 30 min at 4°C. 8 ml of 0.2 N NaOH; 1% SDS was added, the sample was vortexed and incubated at 4°C for 5 min. 6 ml of 3 M NaAc pH 4.8 was added, mixed gently, and the suspension incubated at 4°C for 60 min. The sample was centrifuged at 15K for 20 min. Two volumes of ethanol were added to the supernatent and the nucleic acid precipitated at -20°C overnight. DNA (and RNA) was precipitated by centrifugation at 7K for 40 min. The pellet was resuspended in 5 ml 0.2 M NaCl and precipitated with ethanol a second time. The sample was again spun at 7K for 40 min. The pellet was resuspended in 15 ml 150 mM Tris-HCl; 1 mM EDTA pH 7.4, 16 g of cesium chloride and 0.75 ml of 10 mg/ml ethidium bromide were added and the sample centrifuged at 22°C, 37K for 48 hrs. The plasmid band was collected, extracted with butanol and dialyzed against 10 mM Tris-HCl; 0.1 mM EDTA pH 7.4.

E. Isolation of Genomic DNA

Cells were washed twice in cold phosphate buffered saline (PBS), then resuspended in 10 ml PBS. SDS was added to 1% and pronase added to 0.5 mg/ml. The suspension was incubated at 37°C for 2 hrs. DNA was gently extracted twice with phenol, once with phenol: chloroform (1:1) and once with chloroform, then dialyzed against 10 mM Tris-HCl; 0.1 mM EDTA pH 7.4.

F. Restriction Enzyme Digestion

Digestions were performed according to manufacturer's specifications.

G. Agarose Gel Electrophoresis and Southern Transfer

DNAs were electrophoresed through 0.5-2.0% agarose gels in TBE buffer (89 mM Tris-HCl; 89 mM boric acid; 2.5 mM EDTA pH 8.3). After electrophoresis, gels were stained with 0.5 µg/ml ethidum bromide for 20 min, then visualized and photographed under ultraviolet illumination. Gels were treated with 0.25 M HCl for 15 min, rinsed with distilled H_2O,incubated in denaturation buffer (1.5 M NaCl; 0.5 M NaOH) for 60 min, then in neutralization buffer (3M NaCl; 0.5 M Tris-HCl pH 7.4) for 120 min. DNA was transfered to nitrocellulose by the standard Southern blotting procedure (121), using 10 x SSC (20 x SSC is 3 M NaCl; 0.3 M sodium citrate pH 7.2). Transfer was carried out for 18 hrs for cloned DNA, 48 hrs for genomic DNA. After transfer, filters were air-dried and vacuum-baked for 2 hrs at 80°C.

H. Isolation of DNA Fragments from Agarose Gels

Large-scale digestions of DNA were electrophoresed through agarose as described above. The segment containing the desired DNA band was cut out of the gel and placed within dialysis tubing containing a minimal amount of TBE buffer. The DNA was electroeluted into the buffer. After electrophoresis, the agarose gel slice was gently removed from the dialysis tubing. The DNA was concentrated by passage over an Elutip-d column (Schleicher and Schuell). The DNA was then precipitated with ethanol.

I. Ligation

All vectors with staggered ends were treated with phosphatase prior to ligation. After digestion, 1 M Tris-HCl pH 9.0 was added to a concentration of 0.1 M and the sample heated at 68°C for 10 min. Calf intestine alkaline phosphatase was added, followed by incubation at 37°C for 60 min. Vector DNA was extracted with phenol:chloroform (1:1) and precipitated with ethanol. Ligations were performed following manufacturer's specifications. Ligations involving staggered ends were performed at 4°C, those involving blunt ends were performed at room temperature. All ligations were incubated overnight.

J. Bacterial Transformation

The host bacteria used was E. coli strain HB101. Cells were grown in 200 ml LB to an OD_{600} of 0.3, chilled on ice for 5 min and centrifuged at 5K for 10 min. The cell pellet was resuspended in 20 ml cold buffer A (1.4 g $MnCl_2 \cdot 4H_2O$; 0.33 g NaAc; 0.44 g $CaCl_2 \cdot 2H_2O$ per 100 ml). Cells were incubated for 20 min on ice, centrifuged at 5K for 10 min, and resuspended in 6.7 ml cold buffer A. Ligated DNA was mixed with 0.2 ml competent cells and incubated on ice for 60 min. Cells were then heated at 37°C for 2 min and plated on BHI media containing ampicillin or tetracycline.

K. Nick Translation

Nick translations were performed following standard protocols (73), using all four radioactive dNTPs. 100 µCi (10 µl) [α-^{32}P]dNTP, 2 µl 10X buffer (10X buffer is 0.5 M Tris-HCl pH 7.2; 0.2 M $MgSO_4$; 1 mM DTT; 0.5 mg/ml BSA) and 0.5-1.0 µg DNA were mixed together and distilled H_2O added to a final volume of 18.5 µl. A 1 mg/ml solution of DNase 1 was diluted 10^4-fold in cold distilled

water; 1 µl of the diluted DNase 1 was added to the nick translation mixture. One unit of E. coli DNA polymerase I was added and the sample incubated at 16°C for 60 min. Unincorporated nucleotides were removed by chromatography through a Sephadex G-50 column. The labeled DNA was precipitated with ethanol. Short DNA fragments (less than 500 base pairs) were ligated prior to nick translation to increase the efficiency of incorporation.

L. Hybridization

Hybridizations were performed at 42°C in 1 M NaCl; 10 mM Tris-HCl pH 7.4; 1 x Denhardts' solution (100X Denhardt's solution is 2% polyvinylpyrrolidone; 2% ficoll; 2% BSA); 45% formamide; 100 µg/ml sheared, single-stranded salmon sperm DNA; 100 µg/ml yeast RNA. After hybridization, filters were washed in 0.2 x SSC; 0.2% SDS at 50°C (conventional) or 64°C (high stringency). These conditions are described in detail in Chapter 5.

M. Elution of Probes from Nitrocellulose

Probes were eluted from nitrocellulose filters by washing the blots in 0.1 M NaOH, followed by neutralization with 3 M NaCl; 0.5 M Tris-HCl pH 7.4. All filters were then exposed to X-ray film to insure complete removal of probe. If treated gently, the filters could be subjected to 8 rounds of hybridization before finally disintegrating. Only slight loss of filter-bound DNA was observed.

N. Heteroduplex Analysis

A modified renaturation protocol was used (34). Briefly, 250 ng of each DNA species were gently mixed together in a final volume of 25 µl containing 0.1 N NaOH; 20 mM EDTA, and incubated at room temperature for 10 min. 5.5 µl of 1 M TES was added by gently mixing, then 2.5 µl

of distilled H_2O and 22 µl (relaxed) or 27 µl (stringent) deionized formamide were added. DNAs were allowed to renature for 60 min at room temperature. The samples were dialyzed overnight in 10 mM Tris-HCl; 1 mM EDTA pH 7.8 at 4°C. The Kleinschmidt formamide protocol (62) was used to spread the samples. Several formamide concentrations were assayed for stability of the heteroduplexed regions. A formamide concentration of 40% in the hypophase, 10% in the hyperphase was found to be optimal, although some variation was noted from experiment to experiment. Generally, the heteroduplexes were not stable at higher formamide concentrations, and tangling was a problem at lower concentrations.

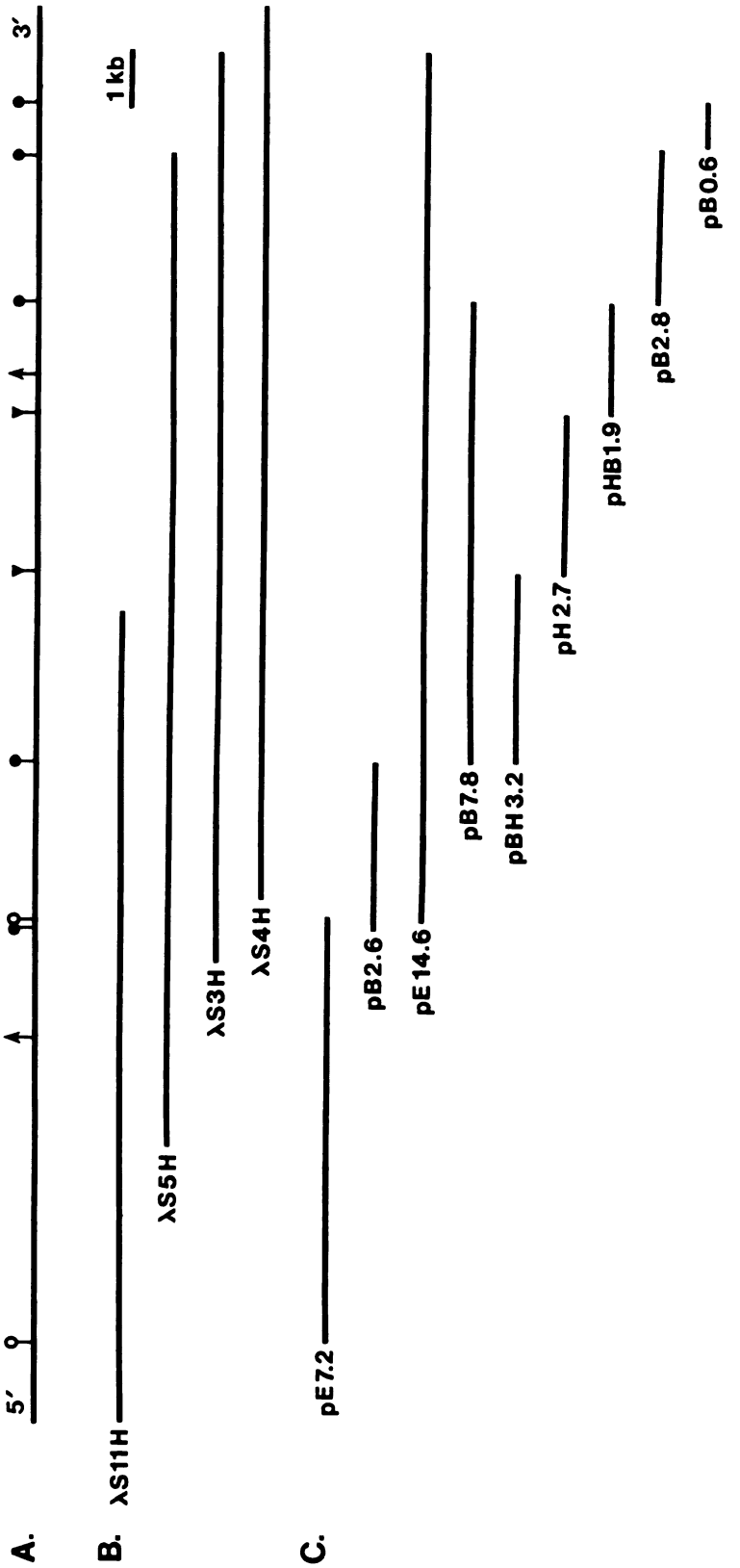
RESULTS

A. Isolation and Preliminary Analysis of Human c-src Clones

To obtain genomic clones spanning the human c-src locus, a human genomic library (67) was screened with the 3.1kb Eco RI fragment of cloned RSV-SRA-2 (D. Fujita and S. Anderson). This clone contains the entire 1.6kb v-src coding region, as well as flanking sequences, which do not hybridize to human DNA. Ten clones containing human c-src sequences were obtained; four of these clones, λS3H, λS4H, λS5H and λS11H were examined in detail. A preliminary restriction enzyme map of the cloned region is shown in Figure 1A; the inserts from the four phage clones are illustrated in Figure 1B.

Initial restriction mapping of the lambda clones demonstrated the inserts overlap extensively, together spanning a region approximately 24kb in lenth. The genomic library was found to be severely biased:

Figure 1. Clones spanning the human c-src locus. A. Preliminary restriction enzyme map of the human c-src locus. B. Phage clones isolated from the human fetal liver library. C. Fragments subcloned into plasmid vectors. o, Eco RI; ●, BamHI; ▼, HindIII; A, XhoI.


A.
5′
3′
1 kb
B.
λS11H
λS5H
λS3H
λS4H
C.
pE7.2
pB2.6
pE14.6
pB7.8
pBH3.2
pH2.7
pHB1.9
pB2.8
pB0.6

other clones isolated overlap with λS3H and λS4H, rather than with λS11H. Southern hybridizations using the 3.1kb v-src probe indicated that the human c-src sequences are widely distributed within the cloned region. Sequences homologous to v-src were detected in the 7.2kb EcoRI fragment as well as the 7.8 , 2.8 and 0.6kb BamHI fragments.

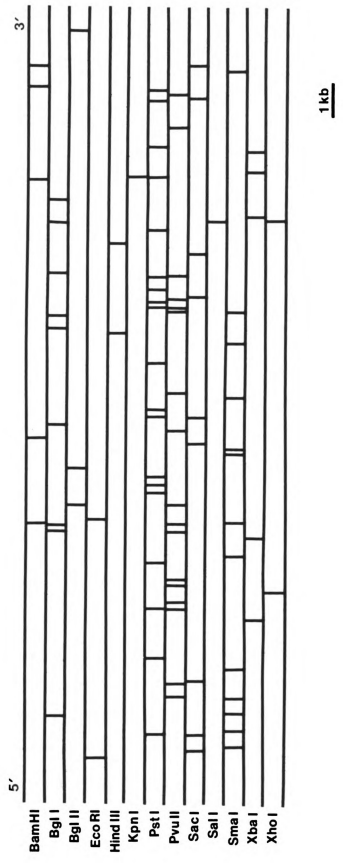
This distribution of v-src related sequences over a region larger than 10kb suggested the human c-src gene contained several introns. To localize the exons more precisely, the human c-src locus was extensively mapped and analyzed by hybridization to a battery of region-specific v-src probes.

Restriction mapping was facilitated by subcloning specific fragments into plasmid vectors: the subclones that were constructed are shown in Figure 1C. Inserts from the subclones were gel-purified away from the plasmid vector and subjected to single and double restriction enzyme digestions; the DNAs were then electrophoresed through 1.0-1.8% agarose gels. After staining with ethidium bromide, bands as short as 50-75 base pairs in length could usually be detected. The restriction enzyme map was constructed by logical deduction from the fragment sizes generated by the various restriction enzyme combinations. The majority of sites were later confirmed by partial digestions of end-labeled DNA (118) (A. Tanaka, personal communication). A summary of the mapping data is presented in Figure 2. It should be noted the 5' 1.0kb and the 3' 0.8kb have not been subcloned and therefore certain enzyme sites have not been mapped within these regions.

B. Localization of Exons

Hybridizations with v-src subgenomic probes were performed to identify narrowly-defined regions homologous to v-src. Five

Figure 2. Detailed restriction enzyme map of the human c-src locus. The 5' 1.0kb and 3' 0.8kb have not been mapped to completion with PstI, PvuII, SacI and SmaI.

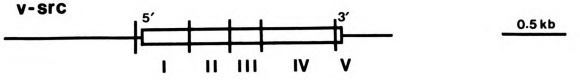

5′
3′
BamHI
Bgl I
Bgl II
EcoRI
HindIII
Kpn I
Pst I
Pvu II
Sac I
Sal I
Sma I
Xba I
Xho I
1 kb

region-specific v-src probes were constructed by subcloning the v-src PstI fragments into the pBR322 plasmid vector (Figure 3). Southern blots containing digests of the various human c-src subclones were analyzed by successive hybridization to each of the 5 v-src subgenomic probes.

Typical hybridization results are illustrated in Figure 3. In this example, a series of double digestions were performed on the 14.6kb EcoRI fragment, which had been gel-purified from the pE14.6 plasmid (see Figure 1). This fragment extends from the second EcoRI site to an aritificial EcoRI site (generated during construction of the library by addition of synthetic EcoRI linkers) present at the 3' end of the λS3H insert. The ethidium bromide stained gel is shown in panel A. The blot was hybridized sequentially to v-src probes I-V. Probes I and V did not hybridize to this fragment. Hybridizations to probes II, III and IV are shown in panels B-D. Each probe detects a specific set of fragments, although some of the fragments hybridize to both II and III or to III and IV.

Similar analyses were performed using a variety of restriction enzymes and other human c-src fragments. Specific fragments hybridizing to the v-src probes were aligned on the restriction enzyme map, allowing delineation of those regions homologous to v-src, presumably the human c-src exons. Figure 4 summarizes the exon-intron map obtained from the hybridization analyses. A minimum of 9 distinct regions bearing homology to v-src can be identified, with the homologous sequences distributed over a region 20kb in length. The exons have been numbered by correspondence to the chicken c-src exons, based on heteroduplex and sequence analysis to be discussed in detail

Figure 3. Hybridizations using the v-src subclones. The 3.1kb EcoRI fragment of cloned RSV-SRA-2 is illustrated, the box depicts the v-src coding region. PstI sites are indicated by vertical lines; fragments used as probes are labeled I-V. A. Ethidium bromide stain of restriction digests of the 14.6kb EcoRI fragment containing the 3' portion of the human c-src locus. B. Hybridization to probe II. C. Hybridization to probe III. D. Hybridization to probe IV. E. Hybridization to the BLUR8 probe. Digestions are as follows: (1), SacI; (2), SacI + BamHI; (3), SacI + BglII; (4), SacI + HindIII; (5), SacI + KpnI; (6), SacI + SalI; (7), SacI + XbaI; (8), SacI + XhoI; (9), SacI + SmaI. Molecular weights are given in kb, sizes were calculated from a standard of HindIII-digested lambda DNA and HaeII-digested pBR322 DNA.


v-src
5′
3′
0.5 kb
I
II
III
IV
V

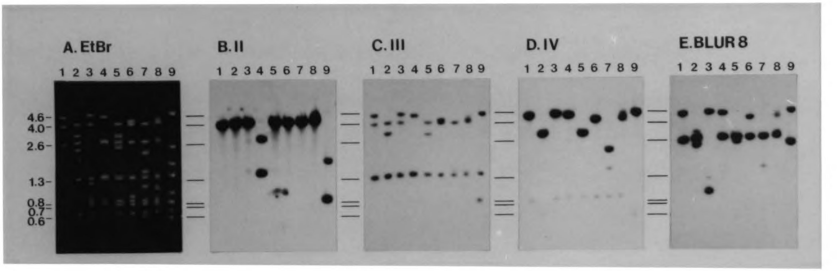
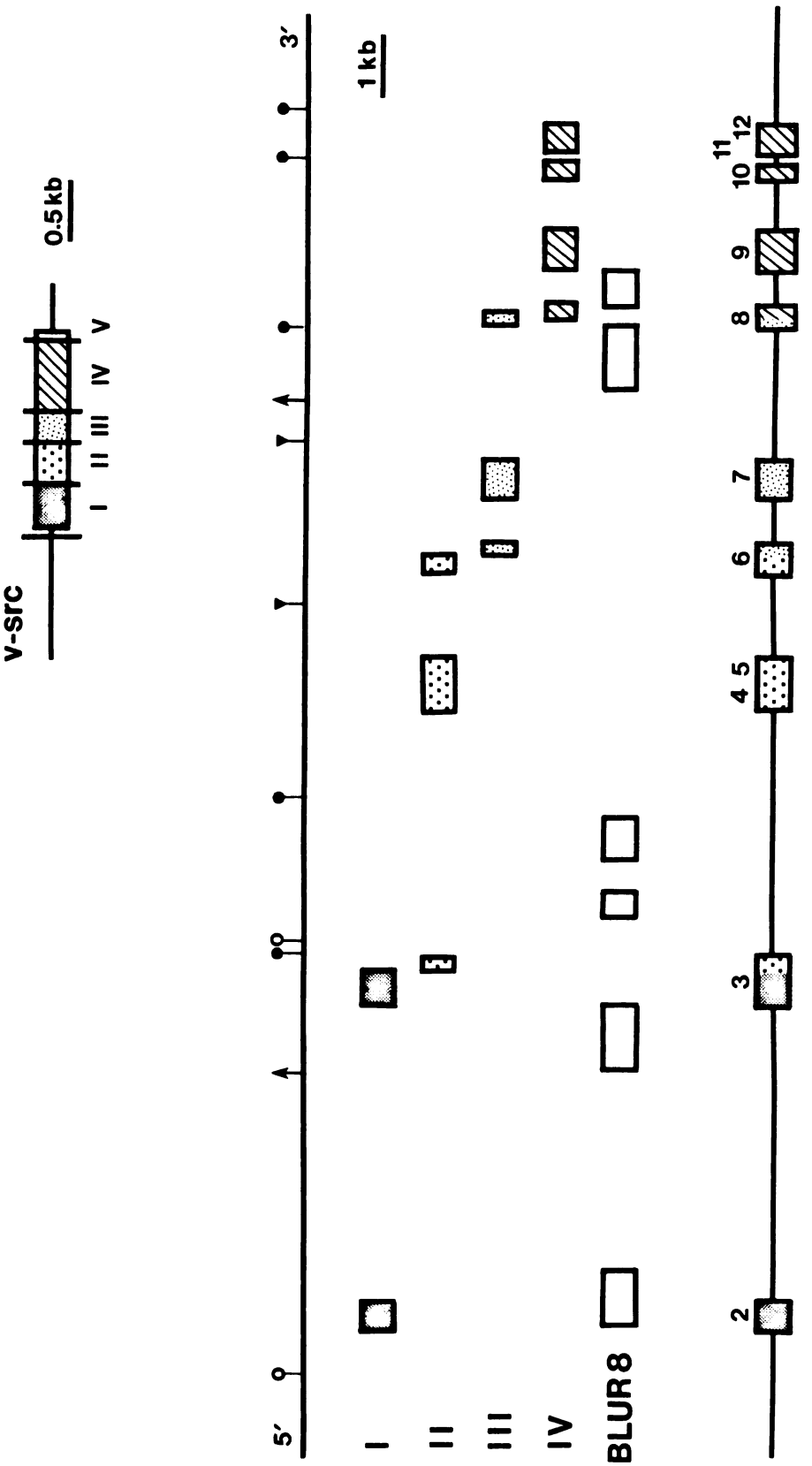

A. EtBr
B. II
C. III
D. IV
E. BLUR 8
1 2 3 4 5 6 7 8 9
4.6
4.0
2.6
1.3
0.8
0.7
0.6

Figure 4. Localization of human c-src exons. Regions hybridizing to v-src probes I-IV are depicted beneath the restriction map of the human c-src locus. The v-src map illustrates the PstI probes used. A summary of the hybridization data is shown in the exon map at the bottom of the figure. Exons are indicated by open boxes and are numbered according to correspondence to the chicken c-src exons. Regions hybridizing to the BLUR8 probe are also indicated. ●, Bam HI; ○, Eco RI; ▼, Hind III; ▲, XhoI.


v-src
0.5 kb
I II III IV V
5'
3'
1 kb
I
II
III
IV
BLUR8
2
3
4 5
6
7
8
9
10
11
12

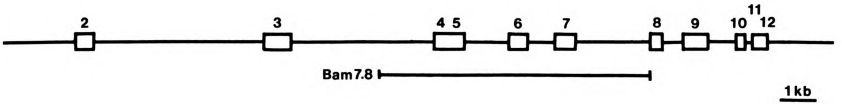
below. Of particular interest is probe V, which contains the divergent 3' terminus of the v-src gene. This probe did not hybridize to the human c-src clones analyzed here.

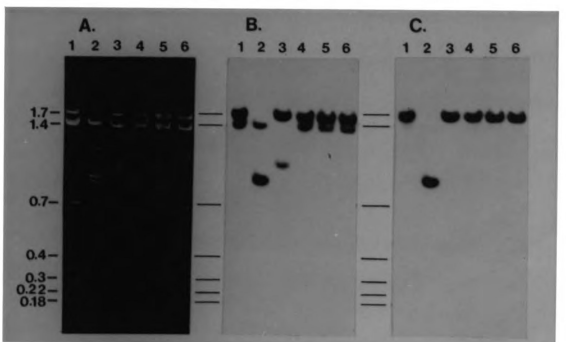
The placement of exon 3 remains tentative. With the exception of the 3' 17 base pairs, which correspond to the 5' end of probe II, the chicken c-src exon 3 is primarily homologous to probe I. Probe I has a high GC content (66%) and will bind non-specifically to several fragments, complicating the hybridization analyses. The region labeled exon 3 in Figure 4 hybridizes somewhat more intensely than other fragments, but the bands are not stable under stringent washing conditions (see below). It is possible these bands are an aritifact due to the GC content; an alternative location of exon 3 is downstream from, but very close to, exon 2. In this location, exon 2 and exon 3 would not be distinguished by the restriction enzymes used.

Somewhat weaker hybridization was observed for several of the exons (3,4,5 and 9); in these regions, hybridization to v-src is not stable under stringent conditions. For example, when a Southern blot containing restriction digests of the 7.8kb BamHI fragment (gel-purified from the pB7.8 plasmid, see Figure 1) is hybridized to probe II, two bands can be detected in each lane (Figure 5). These bands correspond to exons 4 + 5 and 6. After washing under stringent conditions, only one band, corresponding to exon 6, remains in each lane. Similar results were obtained for exons 3 and 9. It can be estimated from the reaction conditions (see Chapter 5 for a detailed explanation) that these exons share less than 90% sequence homology with v-src.

C. <u>Human c-src and Chicken c-src Heteroduplex Analysis</u>

Figure 5. Differential hybridization under different stringencies. Digests of the 7.8kb BamHI fragment was hybridized to probe II under conventional and stringent conditions. A. Ethidium bromide stain. B. Hybridization to probe II under conventional conditions. C. Hybridization to probe II under stringent conditions. Digestions are as follows: (1), PstI; (2), PstI + Hind III; (3), PstI + PvuII; (4), PstI + SacI; (5), PstI + SalI; (6), PstI + XhoI. Molecular weights are given in kb, sizes were calculated from a standard of HindIII-digested lambda DNA and HaeII-digested pBR322 DNA.


2
3
4 5
6
7
8
9
10
11
12
Bam7.8
1kb


A.
B.
C.
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6
1.7
1.4
0.7
0.4
0.3
0.22
0.18

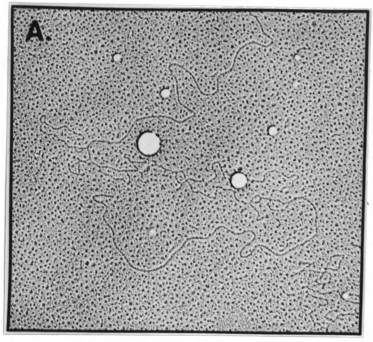
Heteroduplexes of human c-src and chicken c-src lambda clones were examined by electron microscopy (Figure 6). In heteroduplexes of λS3H, which contains human c-src exons 4-12, and the chicken c-src clone λ46D, which contains chicken c-src exons 1-8, four double-stranded regions are consistently observed. Size measurements indicate these regions correspond to exons 4 and 6-8 of chicken c-src. The large loop between exon 8 and the right arm of the lambda vector contains the 3' portion of the λS3H insert, including exons 9-12, which are not present in λ46D. Heteroduplex at exon 5 was found only in two of the molecules examined. Under the most stringent renaturation and spreading conditions, only heteroduplex at exon 8 remained stable.

Attempts to form heteroduplexes at exons 9-12 were unsuccessful due to technical reasons. The inserts of the human c-src clones and the available chicken c-src clone containing the 3' portion of the gene (λ54E) are in the opposite orientations. During the renaturation step, the vector arms rapidly anneal, leaving the inserts in reverse orientations and therefore unable to form heteroduplexes.

D. Hybridization of v-src to Genomic DNA

To verify the authenticity of the human c-src clones, Southern blots of normal human DNA (line NF812, provided by D. Fry) were probed with the v-src 3.1kb EcoRI fragment (Figure 7). Based on the restriction enzyme map of the cloned region, BamHI digestion (lane A) should yield 7.8, 2.8 and 0.6kb fragments, as well as a 5'-specific fragment that extends upstream and is larger than 7.2kb. Bands corresponding to the 7.8, 2.8 and 0.6kb fragments can be detected. In addition, three other bands of 10.5, 9.2 and 6.7kb hybridize to the

Figure 6. Heteroduplex between human c-src and chicken c-src clones. A. Electron micrograph of a heteroduplex between λS3H and λ46D. B. Schematic representation of the introns and exons of the heteroduplex in A.


A.

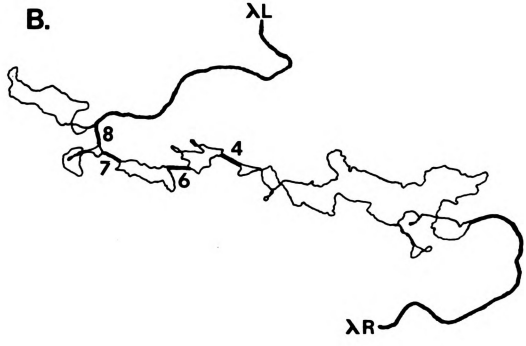
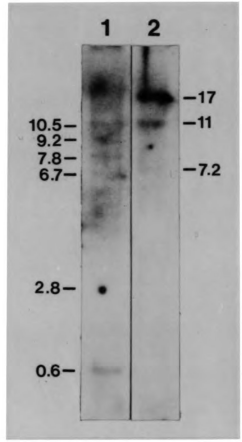

B.
λL
8
7
6
4
λR

Figure 7. Hybridization of v-src to human genomic DNA. (1) BamHI and (2) EcoRI digests of human genomic DNA were probed with the 3.1kb EcoRI fragment containing the entire v-src coding region. Sizes of the hybridizing fragments (difficult to see in the photographic reproduction) are indicated. Molecular weights were calculated from a standard of HindIII digested lambda DNA.


1
2
17
11
7.2
10.5
9.2
7.8
6.7
2.8
0.6

v-src probe; either the 10.5 or the 9.2kb fragment presumably contains the 5' end of the c-src locus present in the clones analyzed here.

Digestion of human DNA with EcoRI (Figure 7, lane B) should produce a 7.2kb fragment and a fragment larger than 14.6kb that contains the 3' sequences and extends downstream. Both a large fragment of approximately 17kb and a 7.2kb band are detected with the v-src probe. In addition, an 11kb fragment also hybridizes.

When the v-src Pst1 subclones (see Figure 3) are used to probe genomic DNA, faint bands can be seen in addition to the bands predicted by the retriction enzyme map (D. Fujita, personal communication). These additional bands suggest the presence of other v-src related sequences within the human genome.

E. Localization of Human Alu Repeat Sequences

Use of the human c-src clones to probe human genomic DNA results in a smear, strongly suggesting repeat sequences are present within the lambda inserts. To identify the location of these sequences, the BLUR8 plasmid (56), which contains one member of the human Alu repeat family was used as a probe. As an example, hybridization to the 14.6kb Eco RI fragment is shown in Figure 3E. Altogether, 6 regions containing human Alu repeat sequences were localized within the human c-src locus (Figure 4).

DISCUSSION

Four overlapping clones encompassing the human c-src locus have been examined in detail. Together the cloned inserts span a region 24kb in length (Figure 1); v-src related sequences have been localized

within these clones to a region 20kb in length. Thus the human c-src locus is far larger than the corresponding chicken c-src locus, which is approximately 8kb in length (86,110,128,129).

By combining extensive restriction enzyme mapping with hybridization to region-specific v-src probes, 9 specific regions homologous to v-src have been identified (Figure 4). These regions have been numbered 2-12, based on correspondence with the chicken c-src exons observed in heteroduplex analysis as well as direct nucleotide sequence comparison, which will be presented in Chapter 3. In addition, use of the v-src probes has allowed the orientation to be determined as indicated in Figure 4. It is assumed the homologous regions contain exon sequences, while the regions that do not hybridize to v-src are introns, although this has not been conclusively demonstrated.

The location of exon 3 has not yet been unequivocally determined. Assignment of this exon to the position indicated in Figure 4 is based solely on hybridization to probe I. However, due to a high GC content (66%), probe I hybridizes weakly to numerous fragments throughout the v-src locus, although hybridization is more intense at the region labeled exon 3 in Figure 4. Nevertheless, it is possible the hybridization attributed to exon 3 is an artifact of this non-specific binding. In addition, hybridization in this region is not stable under stringent washing conditions. This phenomenon has also been observed for other exons, however, notably 4, 5 and 9, and could be the result of nucleotide sequence divergence. An alternative location for exon 3 is close to the 3' end of exon 2, with no pertinent restriction enzyme sites between the exons. In this position, exons 2 and 3 would not be

distinguished by the restriction mapping and hybridization procedures utilized. A third possibility, the nucleotide sequence of exon 3 might be so divergent that it does not hybridize to v-src probe I, is also consistent with the data.

Both the heteroduplex and hybridization analyses suggest certain regions of the human c-src coding sequence are somewhat less homologous than others. In particular, hybridizations at exons 3 (if identified correctly), 4, 5 and 9 are not stable under stringent conditions (see Figure 5), with an estimated 10-20% base mismatch in these regions. Heteroduplex analysis indicates exon 5 is highly divergent; few hybrid molecules contained heteroduplex at exon 5.

The inability of probe V to hybridize to the human c-src clones is particularly intriguing. This probe is specific for the divergent 3' terminus of v-src. It is not present at the 3' terminus of the chicken c-src gene, instead, this sequence is located approximately 900 base pairs downstream from the chicken c-src termination codon (128). Unfortunately, the human c-src clones only extend about 800 base pairs downstream from the termination site. It is possible sequences corresponding to the divergent terminus are located further downstream. Clearly, genomic clones extending further downstream should be examined for the presence of this sequence.

The size fragments obtained in genomic blots of normal human DNA using the v-src probe suggests the lambda clones are indeed authentic and have not undergone any obvious gross rearrangements during the cloning procedure. All bands predicted by the restriction enzyme map can be detected. However, in addition to the predicted bands, other bands can also be seen. Although the intensity of these bands is simi-

lar to that of the predicted bands when the 3.1kb v-src fragment is used as probe (Figure 7), the extra bands only hybridize weakly when the v-src PstI subclones are used as probe (D. Fujita, personal communication). The reason for the intensity difference is not clear, but may be a result of the different stringencies of the hybridization conditions used. Some of the extra bands are smaller than the predicted bands and therefore can not be explained by incomplete digestion of the DNA. In addition, the extra bands have been observed in DNA from three human cell lines (NF812, HT1080 and T044, provided by D. Fry) as well as in DNA extracted from human placenta (D. Fujita, personal communication). The presence of additional bands suggests the existence of a second c-src locus or src-related sequence within the human genome. It may be that the two human c-src loci encode the two forms of $pp60^{c\text{-}src}$ observed in human cells (111). Analysis of these src-related sequences should prove to be very interesting.

Finally, six regions containing human Alu repeat family sequences have been identified within the human c-src locus. Generally, these sequences are spaced approximately 2-3kb apart (56), thus there is nothing abnormal about the distribution within the c-src locus. Although the function of the repeat sequences is unknown, it has been suggested they serve as origins of DNA replication (56).

CHAPTER III

NUCLEOTIDE SEQUENCE ANALYSIS OF THE HUMAN C-SRC LOCUS

CHAPTER III

MATERIALS AND METHODS

Digestions, isolation of DNA fragments from agarose gels, and ligations were described in Chapter 2.

A. Materials

Klenow polymerase was purchased from Boehringer Mannheim. Replicative forms of MP8 and MP9 (79) and dideoxyNTPs were obtained from P-L Biochemicals. The pentadecamer universal primer was from New England Biolabs. DeoxyNTPs were obtained from Sigma. Isopropylthio-β-D-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were from Bethesda Research Laboratories.

B. Media

YT media is 8 g tyroptone; 5 g NaCl; 5 g yeast extract per liter. 2xYT is double this concentration.

C. M13 Transformation

E. coli strain JM103 was transformed by standard protocols (78). Host cells were grown in 100 ml YT media to an OD_{600} of 0.3-0.4. Cells were centrifuged at 6K for 5 min, resuspended in 50 ml cold 50 mM $CaCl_2$ and chilled on ice for 20 min. Cells were recentrifuged and resuspended in 10 ml cold 50 mM $CaCl_2$. Ligated DNA was mixed with 0.3 ml competent cells and the mixture incubated on ice for 40 min. The cells were heat-shocked for 2 min at 42°C. 10 µl 0.1 M IPTG, 50 µl

2% X-Gal and 0.2 ml exponentially growing JM103 cells were added and the bacteria plated out on YT agar.

D. In situ Screening of M13 Plaques

Plates containing 100-300 plaques were screened by *in situ* hybridization. Nitrocellulose filters were gently placed on the plates and allowed to adsorb for 30 sec. Filters were then immersed in denaturation buffer (0.1 N NaOH; 1.5 M NaCl) for 5 min, then transferred to neutralization buffer (3 M NaCl; 0.5 M Tris-HCl pH 7.0) for 10 min. Filters were dried and baked in a vacuum oven at 80°C for 2 hrs.

E. M13 Dot Blot Assay

A saturated JM103 culture was diluted 1:100 into 2xYT media. Clear plaques were transferred into 2 ml of the diluted JM103 culture and incubated at 37°C for 6-8 hrs. 20 µl of the supernatent was spotted onto nitrocellulose. The filters were submerged in denaturation buffer (0.1 N NaOH; 1.5 M NaCl) for 5 min, then transferred to neutralization buffer (3 M NaCl; 0.5 M Tris-HCl pH 7.0) for 10 min. Filters were air-dried and vacuum-baked for 2 hrs at 80°C.

F. Hybridization

Hybridizations were performed as described in Chapter 2, except the hybridization buffer contained 25% formamide and the washings were conducted at 36°C.

G. Template Isolation

2 ml cultures were spun in a Brinkman microfuge for 10 min. 1.2 ml of the supernatent was transferred to an Eppendorf tube containing 0.3 ml 20% PEG; 2.5 M NaCl. The sample was mixed well and incubated at

room temperature for 30 min. The phage were pelleted by a 10 min centrifugation in the microfuge and resuspended in 160 µl TES (20 mM Tris-HCl pH 7.5; 10 mM NaCl; 0.1 mM EDTA). The DNA was extracted with 160 µl phenol: chloroform (1:1); 140 µl of the aqueous phase was then reextracted with 140 µl phenol: chloroform. 120 µl was removed, 12.5 µl 3 M NaAc pH 5.2 and 340 µl 95% ethanol were added and the mixture left at -20°C overnight. Prior to the annealing reaction, the samples were spun 10 min in the microfuge and resuspended in 20 µl TES.

H. Dideoxy Sequencing Reaction

To anneal the primer to the template, 7.5 µl template, 1.5 µl 10X Hin (60 mM Tris-HCl pH 7.5; 60 mM $MgCl_2$; 10 mM DTT; 0.5 M NaCl) and 0.2 pmole primer were mixed and the volume brought to 13.5 µl with distilled H_2O. The mixture was heated at 98°C for 3 min, incubated to 65°C for 30 min and chilled on ice for 5 min. Samples were used immediately or frozen at this point.

Several modifications of the dideoxy reaction procedure (78,99) were made in an attempt to develop a reliable protocol using all four labeled [α-^{32}P]dNTPs. 1 µl [α-^{32}P]dNTPs (10 µCi) and 0.5u Klenow polymerase were mixed with the primed template and the sample incubated at room temperature for 10 min. 3 µl was then transferred to each of 4 tubes (containing 1 µl of the N° and 1 µl of the ddN solutions described below) for the C, T, A and G reactions. Samples were incubated at 37°C for 30 min, or occasionally at higher temperatures when strong secondary structure was a problem. 1 µl of chase solution (0.125 mM each dCTP, dTTP, dATP and dGTP) was added and the solutions incubated at room temperature for 15 min. Finally, 10 µl of stop dye (0.3% xylene cyanol; 0.3% bromphenol blue; 99% formamide;

10 mM EDTA pH 7.5) was added. Prior to electrophoresis, the samples were heated at 98°C for 3 min and chilled on ice.

Optimal concentration of dNTPs and ddNTPs in the reactions were determined in the following manner. Each sequencing reaction should contain an excess of the other three dNTPs, with the dNTP of the reaction in limiting quantities. The extent of elongation and the degree of termination are dependent upon the dNTP:ddNTP ratio. Initially, the dNTP present in the [α-^{32}P]dNTPs was used as the limiting factor. These reactions proved to be fairly reproducible, but occasionally the concentration of one of the dNTPS in the isotope preparation was observed to be low, causing reactions to terminate prematurely due to the decrease in the dNTP:ddNTP ratio. Conditions were therefore developed that would not rely so heavily on the dNTP concentration in the [α-^{32}P]dNTP preparation. Briefly, the N° solutions contained 1 μl 0.5 mM dNTP (limiting), 15 μl of each of the other three dNTPs (0.5 mM each, in excess), 20 μl 10X Hin buffer and 14 μl distilled H_2O. Concentrations of the ddNTPs were determined empirically and could be adjusted for early or late termination. These conditions, although not yet tested extensively, have thus far proven to yield consistent results: 400-500 bases can be read on a good gel.

I. Sequencing Gel Electrophoresis

Both 8% acrylamide gels and 6% buffer gradient gels were used. The stock solutions of the 8% gel consisted of 420 g urea; 200 ml 40% acrylamide (deionized); 4 g bis-acrylamide; 100 ml 10XTBE (10X TBE is 162 g Tris base; 27.5 g boric aid; 9.3 g EDTA per liter) per liter. The stock solution was degassed for 30 min and stored at 4°C. Each 85 X 17.8 cm gel required 100 ml of stock solution, 1 ml 10% APS, and 30 μl TEMED.

The buffer gradient gel specifications were modified from the standard protocol (8) to decrease the spacing between bands. The top solution consisted of 6% acrylamide (38:2 acrylamide: bis-acrylamide); 0.5 X TBE; 8 M urea. The bottom solution contained 6% acrylamide (38:2 acrylamide: bis-acrylamide); 2.5 X TBE; 8 M urea; 10% sucrose and enough bromphenol blue to turn the solution dark blue. Both solutions were degassed 30 min and stored at 4°C. To pour the gels, 20 ml of bottom and 20 ml of top solution were placed in separate beakers. 200 µl of 10% APS was added to each solution. 5 µl TEMED was added to the bottom solution; 10 µl was added to the top. The solutions were then mixed well. 15 ml of the top solution was drawn up into a 25 ml pipet. 15 ml of the bottom solution was carefully drawn up into the same pipet. The solutions were gently mixed by allowing a few air bubbles to pass up the pipet. The mixture was then poured between the glass plates. The gradient was immediately overlaid with top acrylamide (60 ml top solution; 600 µl 10% APS; 15 µl TEMED). Gels were run with 0.5 X TBE in the upper reservoir and 2.5 X TBE in the lower reservoir.

RESULTS

A. Isolation of M13 Clones

The human c-src exons were sequenced by the dideoxy method using an M13 selection protocol. Briefly, a restriction fragment containing the exon of interest was gel-purified, digested with an enzyme compatible with the M13 vectors (AluI, HaeIII, RsaI and Sau3A were used), and the resulting fragments subcloned into the MP8 or MP9 vectors (79). Phage from clear plaques were grown in 2 ml cultures for 6-8 hrs. M13 clones harboring inserts containing v-src related

sequences were identified by dot blot hybridization using the v-src PstI subclones as probe. Alternatively, a modified in situ hybridization procedure was used. However, this technique was found to be less sensitive and could not distinguish clones containing only a short stretch (less than 30 nucleotides) of v-src related sequence. All hybridizations were performed under relaxed conditions in order that short v-src related sequences could be detected.

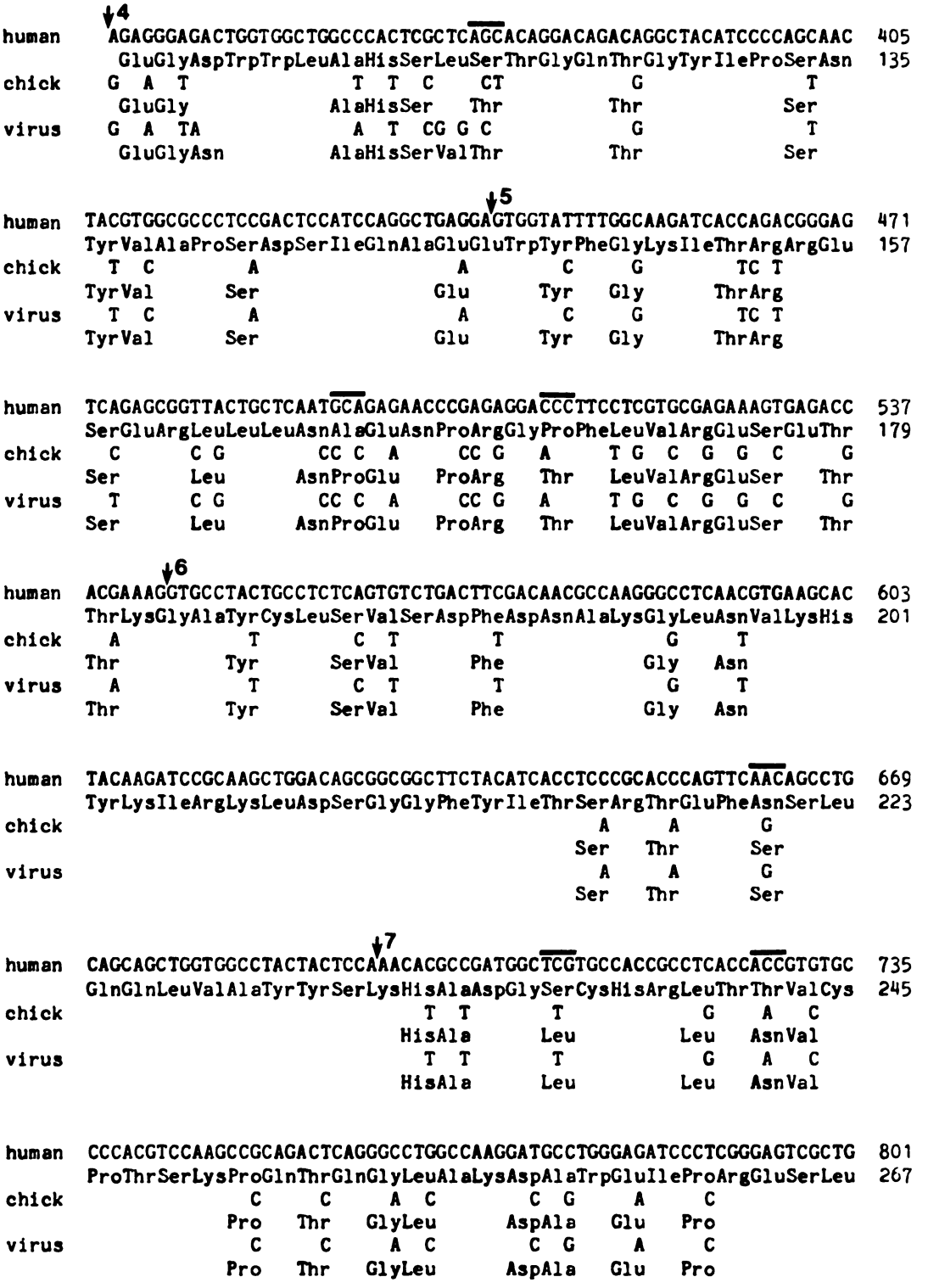
The M13 clones were sequenced by a modified dideoxy protocol (see materials and methods). Initially, clones positive in the dot blot assay were screened by performing only the G reaction. This rapid screen allowed the identification of identical inserts and provided an estimate of the amount of template DNA recovered as well as an indication of the background level.

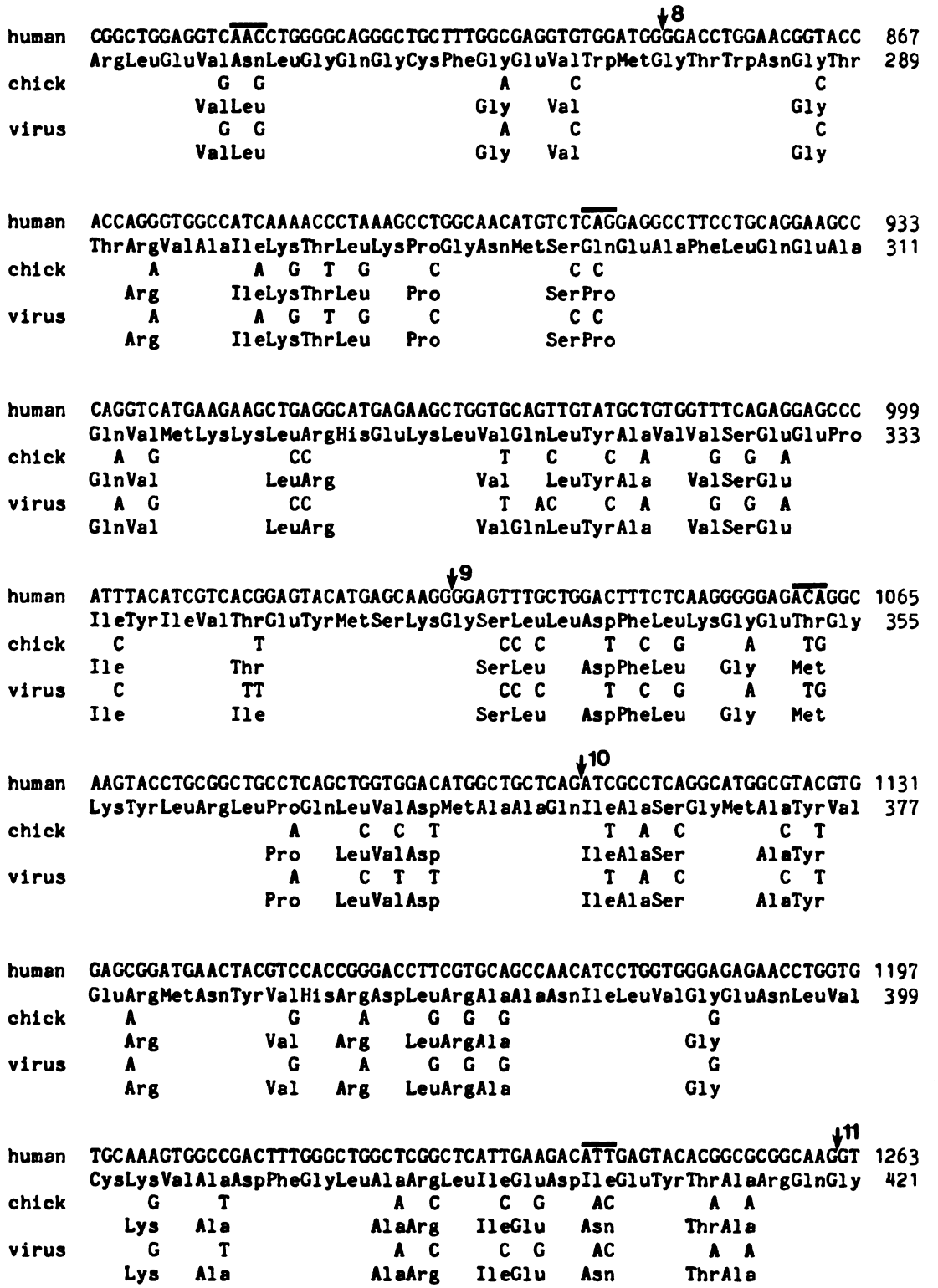
B. Comparison of the Human c-src Sequence with v-src and Chicken c-src Sequences.

All sequences were obtained by reading both strands and, if possible, by analysis of overlapping clones. Src-related sequences were identified by visual analysis or with computer assistance. The human c-src sequence has been determined for exons 2 and 4-12, exon 3 has not been sequenced. A comparison of the human c-src, chicken c-src and SR-A v-src nucleotide and deduced amino acid sequences is presented in Figure 1. Although a number of nucleotide changes are present, the major portion of the amino acid sequence is very similar, with only a few scattered substitutions. The human c-src retains the chicken c-src 3' terminus, rather than the divergent v-src terminus (128).

The sequence of exon 2 appears to have undergone a rearrangement in the human locus (Figure 2). The 5' one-third and 3' one-third are

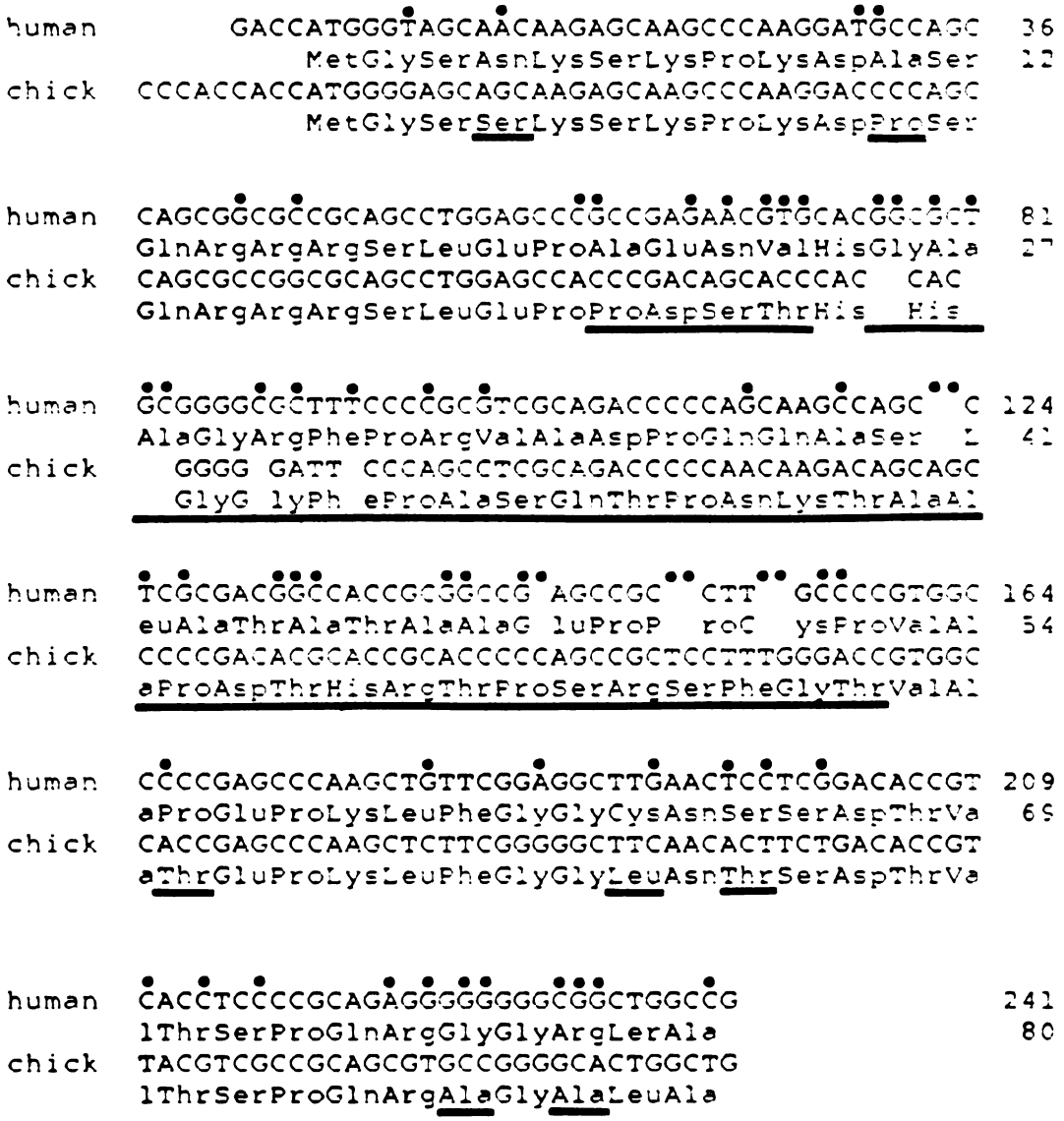
Figure 1. Nucleotide sequence of the human c-src coding region. The nucleotide sequence and deduced amino acid sequence of exons 4-12 are compared between the human and chicken c-src and SR-A v-src genes. The chicken c-src and SR-A v-src sequences (128) are given only where different from the human c-src sequence. Exons are denoted by arrows, divergent amino acids are indicated by bars. Numbering of nucleotides and amino acids follow reference 128. Portions of the human c-src sequence were obtained by S. Anderson and A. Tanaka.


4
human AGAGGGAGACTGGTGGCTGGCCCACTCGCTCAGCACAGGACAGACAGGCTACATCCCCAGCAAC 405
GluGlyAspTrpTrpLeuAlaHisSerLeuSerThrGlyGlnThrGlyTyrIleProSerAsn 135
chick G A T T T C CT G T
GluGly AlaHisSer Thr Thr Ser
virus G A TA A T CG G C G T
GluGlyAsn AlaHisSerValThr Thr Ser
5
human TACGTGGCGCCCTCCGACTCCATCCAGGCTGAGGAGTGGTATTTTGGCAAGATCACCAGACGGGAG 471
TyrValAlaProSerAspSerIleGlnAlaGluGluTrpTyrPheGlyLysIleThrArgArgGlu 157
chick T C A A C G TC T
TyrVal Ser Glu Tyr Gly ThrArg
virus T C A A C G TC T
TyrVal Ser Glu Tyr Gly ThrArg
human TCAGAGCGGTTACTGCTCAATGCAGAGAACCCGAGAGGACCCTTCCTCGTGCGAGAAAGTGAGACC 537
SerGluArgLeuLeuLeuAsnAlaGluAsnProArgGlyProPheLeuValArgGluSerGluThr 179
chick C C G CC C A CC G A T G C G G C G
Ser Leu AsnProGlu ProArg Thr LeuValArgGluSer Thr
virus T C G CC C A CC G A T G C G G C G
Ser Leu AsnProGlu ProArg Thr LeuValArgGluSer Thr
6
human ACGAAAGGTGCCTACTGCCTCTCAGTGTCTGACTTCGACAACGCCAAGGGCCTCAACGTGAAGCAC 603
ThrLysGlyAlaTyrCysLeuSerValSerAspPheAspAsnAlaLysGlyLeuAsnValLysHis 201
chick A T C T T G T
Thr Tyr SerVal Phe Gly Asn
virus A T C T T G T
Thr Tyr SerVal Phe Gly Asn
human TACAAGATCCGCAAGCTGGACAGCGGCGGCTTCTACATCACCTCCCGCACCCAGTTCAACAGCCTG 669
TyrLysIleArgLysLeuAspSerGlyGlyPheTyrIleThrSerArgThrGluPheAsnSerLeu 223
chick A A G
Ser Thr Ser
virus A A G
Ser Thr Ser
7
human CAGCAGCTGGTGGCCTACTACTCCAAACACGCCGATGGCTCGTGCCACCGCCTCACCACCGTGTGC 735
GlnGlnLeuValAlaTyrTyrSerLysHisAlaAspGlySerCysHisArgLeuThrThrValCys 245
chick T T T G A C
HisAla Leu Leu AsnVal
virus T T T G A C
HisAla Leu Leu AsnVal
human CCCACGTCCAAGCCGCAGACTCAGGGCCTGGCCAAGGATGCCTGGGAGATCCCTCGGGAGTCGCTG 801
ProThrSerLysProGlnThrGlnGlyLeuAlaLysAspAlaTrpGluIleProArgGluSerLeu 267
chick C C A C C G A C
Pro Thr GlyLeu AspAla Glu Pro
virus C C A C C G A C
Pro Thr GlyLeu AspAla Glu Pro


8
human CGGCTGGAGGTCAACCTGGGGCAGGGCTGCTTTGGCGAGGTGTGGATGGGGACCTGGAACGGTACC 867
ArgLeuGluValAsnLeuGlyGlnGlyCysPheGlyGluValTrpMetGlyThrTrpAsnGlyThr 289
chick G G A C C
ValLeu Gly Val Gly
virus G G A C C
ValLeu Gly Val Gly
human ACCAGGGTGGCCATCAAAACCCTAAAGCCTGGCAACATGTCTCAGGAGGCCTTCCTGCAGGAAGCC 933
ThrArgValAlaIleLysThrLeuLysProGlyAsnMetSerGlnGluAlaPheLeuGlnGluAla 311
chick A A G T G C C C
Arg IleLysThrLeu Pro SerPro
virus A A G T G C C C
Arg IleLysThrLeu Pro SerPro
human CAGGTCATGAAGAAGCTGAGGCATGAGAAGCTGGTGCAGTTGTATGCTGTGGTTTCAGAGGAGCCC 999
GlnValMetLysLysLeuArgHisGluLysLeuValGlnLeuTyrAlaValValSerGluGluPro 333
chick A G CC T C C A G G A
GlnVal LeuArg Val LeuTyrAla ValSerGlu
virus A G CC T AC C A G G A
GlnVal LeuArg ValGlnLeuTyrAla ValSerGlu
9
human ATTTACATCGTCACGGAGTACATGAGCAAGGGGAGTTTGCTGGACTTTCTCAAGGGGGAGACAGGC 1065
IleTyrIleValThrGluTyrMetSerLysGlySerLeuLeuAspPheLeuLysGlyGluThrGly 355
chick C T CC C T C G A TG
Ile Thr SerLeu AspPheLeu Gly Met
virus C TT CC C T C G A TG
Ile Ile SerLeu AspPheLeu Gly Met
10
human AAGTACCTGCGGCTGCCTCAGCTGGTGGACATGGCTGCTCAGATCGCCTCAGGCATGGCGTACGTG 1131
LysTyrLeuArgLeuProGlnLeuValAspMetAlaAlaGlnIleAlaSerGlyMetAlaTyrVal 377
chick A C C T T A C C T
Pro LeuValAsp IleAlaSer AlaTyr
virus A C T T T A C C T
Pro LeuValAsp IleAlaSer AlaTyr
human GAGCGGATGAACTACGTCCACCGGGACCTTCGTGCAGCCAACATCCTGGTGGGAGAGAACCTGGTG 1197
GluArgMetAsnTyrValHisArgAspLeuArgAlaAlaAsnIleLeuValGlyGluAsnLeuVal 399
chick A G A G G G G
Arg Val Arg LeuArgAla Gly
virus A G A G G G G
Arg Val Arg LeuArgAla Gly
11
human TGCAAAGTGGCCGACTTTGGGCTGGCTCGGCTCATTGAAGACATTGAGTACACGGCGCGGCAAGGT 1263
CysLysValAlaAspPheGlyLeuAlaArgLeuIleGluAspIleGluTyrThrAlaArgGlnGly 421
chick G T A C C G AC A A
Lys Ala AlaArg IleGlu Asn ThrAla
virus G T A C C G AC A A
Lys Ala AlaArg IleGlu Asn ThrAla

```
human  GCCAAATTCCCCATCAAGTGGACGGCTCCAGAAGCTGCCCTCTATGGCCGCTTCACCATCAAGTCG 1329
       AlaLysPheProIleLysTrpThrAlaProGluAlaAlaLeuTyrGlyArgPheThrIleLysSer  443
chick      G                A  C  C  G  A             G
         Lys               ThrAlaProGluAla           Arg
virus      G                  A  C  C  G  A             G
         Lys               ThrAlaProGluAla           Arg

                                                                    ↓12
human  GACGTGTGGTCCTTCGGGATCCTGCTGACTGAGCTCACCACAAAGGGACGGGTGCCCTACCCTGGG 1395
       AspValTrpSerPheGlyIleLeuLeuThrGluLeuThrThrLysGlyArgValProTyrProGly  465
chick   T  C          C                 G     C     C        A     A
       AspVal         Gly               Leu   Thr   Gly      Pro   Pro
virus   T  C          C                 G     C     C        A     A
       AspVal         Gly               Leu   Thr   Gly      Pro   Pro

human  ATGGTGAACCGCGAGGTGCTGGACCAGGTGGAGCGGGGCTACCGGATGCCCTGCCCGCCGGAGTGT 1461
       MetValAsnArgGluValLeuAspGlnValGluArgGlyTyrArgMetProCysProProGluCys  487
chick      C   A G                      A          C              C     C
         Val   Arg                      Arg       Arg           Pro   Cys
virus      GC  G G            G         A          C              C     C
         Gly   Gly           Arg        Arg       Arg           Pro   Cys

human  CCCGAGTCCCTGCACGACCTCATGTGCCAGTGCTGGCGGAAGGAGCCTGAGGAGCGGCCCACCTTC 1527
       ProGluSerLeuHisAspLeuMetCysGlnCysTrpArgLysGluProGluGluArgProThrPhe  509
chick        G     T                           G   C                 T  T
           Ser   His                          ArgAsp               ThrPhe
virus        G     T     T                     G   C                 T  T
           Ser   His   Leu                    ArgAsp               ThrPhe

human  GAGTACCTGCAGGCCTTCCTGGAGGACTACTTCACGTCCACCGAGCCCCAGTACCAGCCCGGGGAG 1593
       GluTyrLeuGlnAlaPheLeuGluAspTyrPheThrSerThrGluProGlnTyrGlnProGlyGlu  531
chick                                    C  G  A                   T  A
                                       ThrSerThr                ProGly
virus                CAGCTGCTTCCTGCTTGTGTGTTGGAGGTCGCTGAGTAG
                     GlnLeuLeuProAlaCysValLeuGluValAlaGlu

human  AACCTCTAG                                                        1599
       AsnLeu                                                           533
chick      A
         Leu
```

Figure 2. Nucleotide sequence of the rearranged exon 2. The nucleotide and deduced amino acid sequence of exon 2 is compared between the human and chicken c-src (128) genes. Sequences are aligned to maximize nucleotide homology. Divergent amino acids are underlined, nucleotide changes are indicated by ●. Numbering of nucleotides and amino acids follow reference 128. The sequence of the human c-src exon 2 was determined by A. Tanaka.


human GACCATGGGTAGCAACAAGAGCAAGCCCAAGGATGCCAGC 36
MetGlySerAsnLysSerLysProLysAspAlaSer 12
chick CCCACCACCATGGGGAGCAGCAAGAGCAAGCCCAAGGACCCCAGC
MetGlySerSerLysSerLysProLysAspProSer
human CAGCGGCGCCGCAGCCTGGAGCCCGCCGAGAACGTGCACGGCGCT 81
GlnArgArgArgSerLeuGluProAlaGluAsnValHisGlyAla 27
chick CAGCGCCGGCGCAGCCTGGAGCCACCCGACAGCACCCAC CAC
GlnArgArgArgSerLeuGluProProAspSerThrHis His
human GCGGGGCGCTTTCCCCGCGTCGCAGACCCCCAGCAAGCCAGC C 124
AlaGlyArgPheProArgValAlaAspProGlnGlnAlaSer L 41
chick GGGG GATT CCCAGCCTCGCAGACCCCCAACAAGACAGCAGC
GlyG lyPh eProAlaSerGlnThrProAsnLysThrAlaAl
human TCGCGACGGCCACCGCGGCCG AGCCGC CTT GCCCCGTGGC 164
euAlaThrAlaThrAlaAlaG luProP roC ysProValAl 54
chick CCCCGACACGCACCGCACCCCCAGCCGCTCCTTTGGGACCGTGGC
aProAspThrHisArgThrProSerArgSerPheGlyThrValAl
human CCCCGAGCCCAAGCTGTTCGGAGGCTTGAACTCCTCGGACACCGT 209
aProGluProLysLeuPheGlyGlyCysAsnSerSerAspThrVa 69
chick CACCGAGCCCAAGCTCTTCGGGGGCTTCAACACTTCTGACACCGT
aThrGluProLysLeuPheGlyGlyLeuAsnThrSerAspThrVa
human CACCTCCCCGCAGAGGGGGGGGCGGCTGGCCG 241
lThrSerProGlnArgGlyGlyArgLerAla 80
chick TACGTCGCCGCAGCGTGCCGGGGCACTGGCTG
lThrSerProGlnArgAlaGlyAlaLeuAla

closely homologous to the corresponding chicken c-src and SR-A v-src sequences, but in the central portion, the human c-src sequence has shifted, due to insertions and deletions. Nevertheless, the v-src initiation site has been conserved and the length of the coding sequence in exon 2 is the same in both the human and chicken loci.

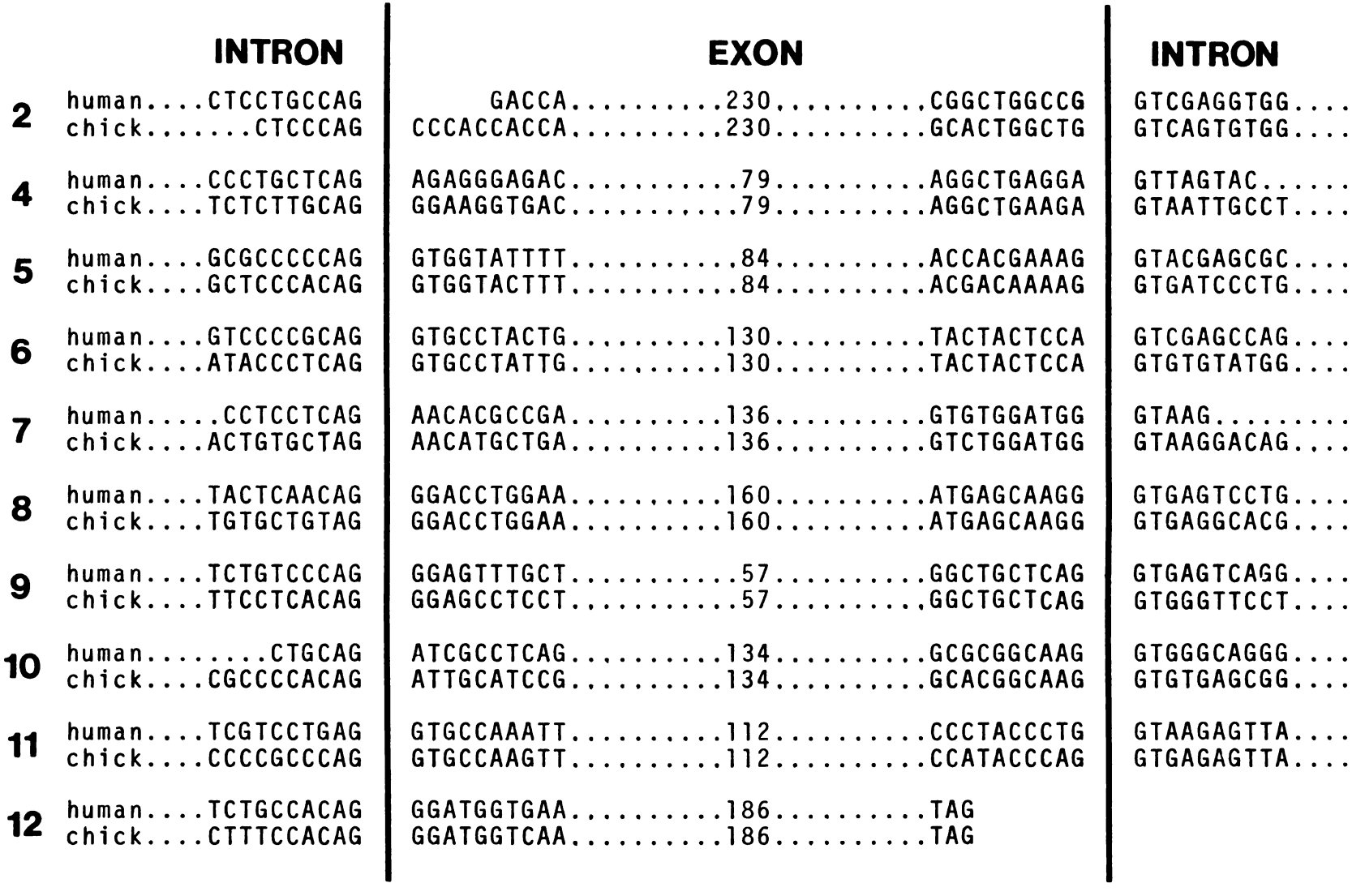
A comparison of the homology between the three src genes is presented in Table I. For the majority of exons, the nucleotide homology is fairly high, however, the homology within exon 5 is somewhat lower (76.9% between the human and chicken c-src genes), and exon 2 is quite divergent, as a result of the rearrangement. The deduced amino acid sequences (with the exception of exon 2) demonstrate a striking conservation. Excluding exon 2, the overall amino acid homology between human and chicken c-src is 97.1%. Similarly, amino acid homology between human c-src and v-src is quite high, 93.0%: nearly half of the different residues fall within the divergent carboxy terminus.

The sequence analysis has also demonstrated that the chicken c-src exon structure is conserved in the human c-src locus. The intron-exon boundaries are identical in the two genes (Table 2), however, the intron sequences are completely divergent and the intron sizes are not conserved. One difference can be noted at the 5' boundary of exon 2, where the chicken c-src exon is 5 base pairs longer than the corresponding human c-src exon. This difference is located in the non-coding region upstream from the initiation codon. Finally, all of the human c-src splice sites are consistent with the AG/GT consensus rule.

Table 1. Homology between the human and chicken c-src and SR-A v-src genes. Nucleotide and amino acid homology are calculated for each exon, with the homology at the 3' terminus of exon 12 calculated separately. Overall homology was calculated from exons 4-12, and does not include exon 2.

	CHICKEN c-src		SR-A v-src	
exon	nucleotide	amino acid	nucleotide	amino acid
2	78.0% (188/241)	55.0% (44/80)	76.8% (185/241)	53.8% (43/80)
4	85.9% (85/99)	96.9% (31/32)	83.8% (83/99)	90.6% (29/32)
5	76.9% (80/104)	94.1% (32/34)	76.9% (80/104)	94.1% (32/34)
6	94.0% (141/150)	98.0% (48/49)	94.0% (141/150)	98.0% (48/49)
7	88.5% (138/156)	94.1% (48/51)	88.5% (138/156)	94.1% (48/51)
8	87.8% (158/180)	98.3% (58/59)	86.7% (156/180)	96.6% (57/59)
9	83.1% (64/77)	96.0% (24/25)	81.8% (63/77)	96.0% (24/25)
10	85.7% (132/154)	98.0% (50/51)	85.7% (132/154)	98.0% (50/51)
11	88.6% (117/132)	100.0% (43/43)	88.6% (117/132)	100.0% (43/43)
12(466-514)	91.3% (136/149)	95.9% (47/49)	89.3% (133/149)	89.8% (44/49)
12(515-533)	89.5% (51/57)	100.0% (19/19)	27.8% (10/36)	8.3% (1/12)
total(4-12)	87.6% (1102/1258)	97.1% (407/419)	85.1% (1053/1237)	93.0% (383/412)

Table 2. Comparison of the intron-exon boundaries in the human and chicken c-src loci. Nucleotide sequences surrounding the putative splice sites are given.


INTRON
EXON
INTRON
2
human....CTCCTGCCAG
chick.....CTCCCAG
GACCA.....230.....CGGCTGGCCG
CCCACCACCA.....230.....GCACTGGCTG
GTCGAGGTGG....
GTCAGTGTGG....
4
human....CCCTGCTCAG
chick....TCTCTTGCAG
AGAGGGAGAC.....79.....AGGCTGAGGA
GGAAGGTGAC.....79.....AGGCTGAAGA
GTTAGTAC.....
GTAATTGCCT....
5
human....GCGCCCCCAG
chick....GCTCCCACAG
GTGGTATTTT.....84.....ACCACGAAAG
GTGGTACTTT.....84.....ACGACAAAAG
GTACGAGCGC....
GTGATCCCTG....
6
human....GTCCCCGCAG
chick....ATACCCTCAG
GTGCCTACTG.....130.....TACTACTCCA
GTGCCTATTG.....130.....TACTACTCCA
GTCGAGCCAG....
GTGTGTATGG....
7
human.....CCTCCTCAG
chick....ACTGTGCTAG
AACACGCCGA.....136.....GTGTGGATGG
AACATGCTGA.....136.....GTCTGGATGG
GTAAG.....
GTAAGGACAG....
8
human....TACTCAACAG
chick....TGTGCTGTAG
GGACCTGGAA.....160.....ATGAGCAAGG
GGACCTGGAA.....160.....ATGAGCAAGG
GTGAGTCCTG....
GTGAGGCACG....
9
human....TCTGTCCCAG
chick....TTCCTCACAG
GGAGTTTGCT.....57.....GGCTGCTCAG
GGAGCCTCCT.....57.....GGCTGCTCAG
GTGAGTCAGG....
GTGGGTTCCT....
10
human.....CTGCAG
chick....CGCCCCACAG
ATCGCCTCAG.....134.....GCGCGGCAAG
ATTGCATCCG.....134.....GCACGGCAAG
GTGGGCAGGG....
GTGTGAGCGG....
11
human....TCGTCCTGAG
chick....CCCCGCCCAG
GTGCCAAATT.....112.....CCCTACCCTG
GTGCCAAGTT.....112.....CCATACCCAG
GTAAGAGTTA....
GTGAGAGTTA....
12
human....TCTGCCACAG
chick....CTTTCCACAG
GGATGGTGAA.....186.....TAG
GGATGGTCAA.....186.....TAG

Sequences homologous to exon 3 have not been identified by sequence analysis. Over 900 M13 clones have been constructed spanning the 10kb region between exons 2 and 4. However, none of these clones hybridized strongly to probe I in the dot blot assay. Two regions are not represented within the M13 clone bank. The first is a 0.8kb fragment delineated by the second Bam H1 site and the downstream Sac1 site (see Chapter 2, Figure 2). This region did not even hybridize weakly to probe I, therefore it is unlikely exon 3 lies within this fragment, unless it is highly divergent. The second region not cloned into M13 centers around the first XhoI site, which was used in isolation of fragments prior to digestion for subcloning into M13. Since XhoI is incompatible with the M13 vector ends (BamHI and SmaI), the sequences immediately adjacent to the XhoI site are therefore not represented within the clone banks. If exon 3 is located in this region, it would imply that no AluI, HaeIII, RsaI or Sau3A (used to construct the M13 subclones) sites are located within exon 3 or between exon 3 and the Xhol site. Either construction of another set of clones or use of the chemical degradation method (117) to obtain the sequence around the XhoI site could determined if this is the location of exon 3.

DISCUSSION

Nucleotide sequence analysis has revealed a number of similarities between the human and chicken c-src loci. Within the two species, the sequence has been highly conserved, with the exception of the central portion of exon 2. Likewise, the exon structure has been conserved, while the size and sequence of the introns has not.

Although it has not been proven conclusively, it is likely the entire human c-src coding sequence resides within the cloned region. While it is possible that additional coding exons lie upstream or even within the "intron" sequences not found in v-src, analysis of the src proteins suggests this is highly unlikely. Results from partial proteolytic digestion indicate both the human and chicken $pp60^{c\text{-}src}$ proteins are very similar (111), and are closely related to $pp60^{v\text{-}src}$ (18,20,84,97,106). The major difference between the $pp60^{c\text{-}src}$ proteins and $pp60^{v\text{-}src}$ lies in the carboxy termini; no other size alterations have been observed, nor are major size differences found in the chimeric v-src/c-src proteins (51). By a similar reasoning, it is unlikely that the c-src coding sequences extend downstream from the putative termination site. Thus it can be concluded the extent of coding sequences within the three loci are the same, with the exception of the minor differences in the 3' termini.

In agreement with this is the observation that the 5' end of exon 2 is shorter in the human c-src locus than in the chicken locus, while all other intron-exon boundaries have been precisely conserved (Table 2). This is a region upstream from the initiation codon of v-src. As a non-coding region in the c-src genes, there may not be the strong pressure for sequence conservation that is found within the coding regions.

As would be expected, the human c-src retains the chicken c-src 3' terminus, rather than the divergent v-src 3' terminus (Figure 1), explaining the lack of hybridization to probe V described in Chapter 2. This is a region that has been implicated in determining the oncogenic potential of the src gene (104); it has been suggested the sequence of

the c-src terminus is actually inhibitory for transformation.

The sequence rearrangement found within exon 2 is rather unusual, appearing to involve both insertions and deletions. While it is possible this rearrangement is an artifact that arose during the genomic cloning, the sequence may indeed be correct. The size of the rearranged exon is identical to the corresponding chicken c-src exon 2, so a frameshift has not been introduced, nor can any termination codons be found within the rearranged region. In addition, $pp60^{v\text{-}src}$ will tolerate extensive alteration at the amino terminus without any effect on subcellular localization, enzymatic activity or transformation potential (58). Thus there may be less pressure for the conservation of amino acid sequence at the amino terminus. Another lambda clone should be obtained to confirm the rearrangement.

No sequences corresponding to the chicken c-src exon 1 have been detected in the human c-src clones. However, this is probably a non-coding exon in the chicken locus (128). As such, there would be no a priori reason to expect the nucleotide sequence would be highly conserved. Alternatively, sequences related to the chicken c-src exon 1 could be present, but located upstream from the region represented within the lambda clones. An overlapping clone extending further upstream should be examined for the presence of exon 1. A good cDNA clone would also be very helpful in analysis of the 5' untranslated region.

The inability to conclusively locate exon 3 is extremely puzzling. Hybridization results presented in Chapter 2 suggest two possible locations: either very close to exon 2 or toward the 3' end of the 7.2kb EcoRI fragment. If the latter position is correct, the hybridization results indicate the sequence is somewhat divergent

since under stringent conditions, hybridization to v-src is not stable in this region. However, no M13 subclones constructed over this region hybridized to probe I. Together, these results are consistent with three alternatives. First, exon 3 could be located adjacent to the XhoI site, and the sequences would therefore not be represented in the M13 clone bank. This location is relatively consistent with the mapping and hybridization data, assuming some error in measurement. Alternatively, exon 3 could contain a "poison" sequence that cannot be cloned into M13. If this is the case, exon 3 could be located close to exon 2, and therefore not be distinguishable by any of the restriction enzymes used. A "poison" sequence is also consistent with exon 3 localized in the 3' end of the 7.2kb EcoRI fragment, which hybridizes to probe I under relaxed conditions. Thirdly, the sequence of exon 3 could be so divergent that it does not hybridize to the v-src probe even under relaxed conditions. Analysis of a cDNA clone may be necessary to identify exon 3.

The high degree of sequence homology between the human c-src and both the chicken c-src and viral src genes is in close agreement with results of DNA hybridization in liquid, which suggested that the human and viral src sequences contain only 10% base mismatch (124). Direct nucleotide sequencing demonstrates the homology is only slightly lower, with 85.1% sequence conservation over the human c-src exons 4-12. Homology beween the human and chicken c-src coding sequences is slightly higher, with 87.6% base conservation over exons 4-12. The amino acid sequence in this region is extremely highly conserved, with 97.1% homology between the human and chicken c-src, 93.0% homology between the human and viral src. This high degree of homology is reflected in the similarity of the partial proteolytic digestion

products of the $pp60^{c\text{-}src}$ proteins (111) and in the ability of antisera raised against $pp60^{v\text{-}src}$ to precipitate both the human and chicken $pp60^{c\text{-}src}$ protein (18,20,84,97,106).

SUMMARY (part A)

The structural and sequence analysis of the human c-src locus presented here demonstrates the close similarity of the human and chicken c-src genes. Within exons 4-12, the intron-exon structure of the human c-src locus is virtually identical to that of the chicken c-src, with the two genes retaining the precise intron-exon junctions, as well as identical (putative) initiation and termination sites.

The location of exon 3 has not yet been conclusively demonstrated, nor has the sequence been determined. Examination of the downstream flanking region for the presence of the v-src divergent 3' terminus, and examination of the upstream region for possible homology to the chicken c-src exon 1 are also needed. A full-length cDNA clone would be extremely useful for these studies.

The conservation of amino acid sequence is particularly strong within exons 4-12, which includes the region required for tyrosine kinase activity (69). Exon 2 exhibits much lower amino acid homology; this is primarily due to the rearrangement within exon 2. However, this region of the molecule is involved in binding to the plasma membrane and therefore conservation of sequence may not be so critical. In fact, gross alterations within this region have been observed: the rASV isolates which have been analyzed contain numerous differences within the amino-terminal region (58), suggesting the amino terminus can tolerate substantial changes without affecting the protein function.

Athough obtained from fairly divergent species, the human and chicken c-src genes are remarkably similar. The nucleotide sequences share a high degree of homology, nearly 90% over exons 4-12, and the deduced amino acid sequences are practically identical. Such a strong sequence conservation, particularly in the portion of the molecule containing the enzymatic activity, suggests the $pp60^{c\text{-}src}$ protein has a critical, indispensable role. The identification of src-related sequences in a wide variety of species (53,113,124) lends strong support to this hypothesis. Nevertheless, the normal role of $pp60^{c\text{-}src}$ has not yet been elucidated.

Of particular interest are the two forms of $pp60^{c\text{-}src}$ observed in human cells (111). Because the two forms have similar but not identical two-dimensional chymotryptic peptide patterns, it was suggested they arose either from two distinct c-src loci or from alterntive splicing of the same initial transcript. Analysis of cDNA clones is needed to address the latter possibility. The observation of extra bands in genomic blots (Chapter 2, Figure 7) suggests the presence of a second c-src locus. However, under relatively stringent conditions, these additional bands are much fainter than the bands corresponding to the locus analyzed here. In addition, the 10 lambda clones obtained are all derived from the same locus (D. Fujita, personal communication). Thus the second locus is probably fairly divergent.

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CHAPTER IV

INTRODUCTION AND LITERATURE REVIEW (part B)

INTRODUCTION

Marek's disease virus (MDV) is a highly contagious avian herpesvirus that causes a malignant T-cell lymphoma known as Marek's disease (MD) in chickens. With the implementation of intensive poultry farming methods, MD has become a serious economic problem in the poultry industry. Control of this disease is generally effected by vaccination with the antigenically related herpesvirus of turkeys (HVT). HVT is apathogenic in chickens, producing mild subclinical lesions while eliciting a protective immune response against subsequent MDV infection. MD is therefore the first herpesvirus infection and the first neoplastic disease to be prevented by routine vaccination.

Elucidation of the mechanism of this protective immunity could be extremely useful in the development of effective vaccines against other herpesviruses as well as providing insight into the immunological defense mechanisms against neoplastic disease.

However, this system has not been exploited, primarily due to numerous technical difficulties. In cell culture, MDV remains highly cell-associated, with little infectious cell-free virus released into the media. The inability to obtain large quantities of virus-specific proteins has led to difficulties in obtaining mono-specific anitsera, which in turn has prohibited detailed analyses of most viral proteins. Only two MDV proteins, A and B antigen, have been characterized in any detail.

Genetic analyses have also been limited. Because of similar bouyant densities, the viral DNA can not be separated from cellular DNA. Only minimal quantities of MDV DNA can be obtained from virus particles released into the culture media. Thus, only a preliminary physical map is available and no genetic map has yet been constructed. These studies are also complicated by the large genome size (180kb for MDV, 150kb for HVT).

Although there is a lack of detailed genetic information, protein analyses suggest MDV and HVT are closely related. The two viruses share a number of cross-reactive antigens: the majority of virus-specific polypeptides can be immunoprecipitated with the heterologous antisera. Furthermore, HVT will elicit a protective immune response directed against both viremia and tumor development.

Nevertheless, earlier studies indicated MDV and HVT share only minimal (1-5%) genetic homology. These estimates were in sharp contrast to the results from protein analyses and could not be easily reconciled with the immunological data. The primary goal of this study was to re-examine the homology between MDV and HVT using highly sensitive, well-defined experimental conditions. Secondary to this analysis, two clone banks of the MDV genome were constructed.

LITERATURE REVIEW

Marek's disease (MD) was originally described by Marek in 1907 (37) and for many years was thought to be related to the retrovirally-induced lymphoid leukosis. However, specific pathological and etiological differences suggested MD was a distinct disease. It was not until 1967 that a highly cell-associated herpesvirus, now known as Marek's disease virus (MDV), was identified as the causative agent of MD (11,43).

A. Background

Prior to 1950, only a few isolated outbreaks of MD occurred in the United States, but by the the 1960's the incidence of MD had risen drastically, and MD became the most serious economic threat to the poultry industry. This rapid rise in MD incidence paralleled the development and implementation of intensive poultry farming methods, which allow rapid spread of the virus throughout the flock. By 1969 an attenuated virus had been isolated by successive passage through cell culture (12): this attenuated strain was administered successfully as a vaccine. Later, both naturally occurring avirulent strains of MDV (47,57,71) and the herpesvirus of turkeys (HVT) (68) were isolated and shown to protect against the development of MD. Currently, HVT is the most common vaccine in use.

Isolates of MDV have been classified into three groups: serotype 1 consists of virulent and attenuated strains, apathogenic strains

comprise serotype 2, and all isolates of HVT are serotype 3.

B. Pathology

Two forms of MD have been described: the classical or neurotropic form produces neurological lesions that lead to progressive paralysis, while the rapid development of lymphoid tumors characterizes the acute viscerotropic form.

MDV is spread horizontally via an airborne route, with infection occurring through the respiratory tract. One to three days after exposure, virus is observed in the spleen, bursa of Fabricus and thymus. Infection in these organs peaks at 5 to 7 days, with the appearance of degenerative microscopic lesions. The tissue begins to atrophy and eventually becomes necrotic. A persistent cell-associated viremia develops that spreads to visceral organs and the skin, and T cell lymphomas will develop in visceral organs. Cell-free infectious virus is produced in the feather follicle epithelium. Virus is then released to the air along with dust and dander; infectious virus can usually be detected around two weeks post infection.

In the classical form of MD, extensive neural lesions develop as nerves become infiltrated with T-lymphocytes. These lesions lead to progressive paralysis and resultant muscular atrophy; the clinical symptoms depend upon which nerves are infected. Other symptoms include blindness, skin lesions and the development of visceral lymphomas. The course of disease is fairly prolonged and may last several weeks. Although some chickens do recover, infected birds usually succumb to starvation and dehydration because they are unable to reach the food and water; others are trampled to death.

The acute form of MD primarily affects young chickens. The onset is sudden, the mortality rate high, and the birds frequently do not develop obvious symptoms.

C. Vaccination and Immunity

Although the disease has not been completely eradicated, routine vaccination has led to the decreased incidence of MD. Prior to the development of vaccines, losses in infected flocks due to both death and condemnation often ranged up to 30%, and occasionally over 50% of the birds were condemned. It is estimated that vaccination has reduced losses by 90%; the HVT virus is approximately 80-100% effective against MDV challenge (44).

A major factor in the efficacy of vaccination is the susceptibility of the chicken line. Certain strains of chickens are highly susceptible to MD, others are more resistant; vaccination is more effective in less susceptible chicken lines. Genetic resistance within inbred lines probably involves only a few genes, since selection for resistance or susceptibility to MD can be achieved within 2 to 3 generations (13). Resistance appears to be associated with the B locus of the major histocompatibility complex (35) as well as the Ly-4 and Th-1 loci (34).

Other factors affecting the efficacy of vaccination are the presence of maternal antibodies (3,5), the length of time between vaccination and challenge, and the challenge dose (15). The virulence and oncogenicity of the challenge MDV strain are also quite important. In recent years, several highly virulent isolates of MDV have been reported (14,52,69); vaccines in current use are less effective in protecting against these virulent isolates (67).

In chickens, HVT will produce mild, sub-clinical lesions,

particularly transient lymphoproliferation in peripheral nerves. A persistent latent infection characteristic of herpesviruses will be established. Protection is directed against tumor development: MDV challenge produces a mild infection, but the birds do not develop lymphomas, and production of infectious virus is quite low.

Immunity to MDV involves both humoral and cellular responses. Cell-mediated responses are probably the most important since bursectomy, and therefore elimination of the B cell response, does not significantly affect the efficacy of vaccination (16). The significance of T cells has been confirmed by an in vitro chromium-release cytotoxicity test (48,53,54); macrophage and natural killer cells may also be involved.

Although the humoral response may not be essential, antibody production does offer some protection against MD. Antibodies generally appear 3 to 5 weeks after infection and can be detected by a variety of techniques including immunodiffusion, immunofluorescence and virus neutralization. Acquired antibodies are directed against cell-free virus as well as cell-associated virus. Passively administered antibodies are capable of protecting a significant proportion of birds (4,5). Likewise, maternal antibodies are somewhat beneficial. After MDV challenge, chicks with maternal antibodies have a longer incubation period, decreased virus titer and less severe lesions than chicks lacking maternal antibodies (5). Thus, while antibody production may not be essential, the humoral response does offer some protection against MDV infection, complementing the cell-mediated response.

D. Viral Antigens

Several cells lines have been established from MD tumors (26,6,42,45). In all lines the virus remains highly cell-associated:

production of infectious virus is very low and may be undetectable. Nevertheless, all cell lines will cause tumors when cells are injected into susceptible chickens. MDV can also be propagated as cell-associated virus in chick embryo fibroblasts (CEF) or duck embryo fibroblasts (DEF).

A number of antigens have been identified in MDV-infected cells. Three prominent bands visible in agar gel precipitation were called A, B and C (10). Of these, A has been the most extensively characterized: it is a glycoprotein with an apparent molecular weight of 61,000 to 65,000 daltons by SDS-polyacrylamide gel electrophoresis and is found primarily in the culture medium (18,32,33). B and C are found in extracts of infected cells and have not been as well characterized as A antigen (64). An intracellular antigen (IA) was found in both the nucleus and cytoplasm of infected cells (46,58), and a virus-specific membrane antigen (MA), which may be identical to A antigen, has also been identified (1,9,39). Additionally, a tumor specific antigen (MATSA) has been observed on the surface of transformed cells (45,70). SDS-polyacrylamide gel analysis of MDV virion proteins revealed 8 polypeptides, two of which are glycosylated and associated with the viral membrane (8,63).

Thirty five virus-specific polypeptides were detected in lysates of productively infected cells using high-resolution two-dimensional gel electrophoresis; 18 of these polypeptides are glycosylated (62). Many similarities can be found in the two-dimensional patterns of MDV- and HVT-specific polypeptides, although a number of differences in migration can also be noted (61). In addition, the 4 major virus-specific polypeptides found in cells infected with virulent, aviruent or attenuated MDV or the non-oncogenic HVT share common

antigenic determinants and can be immunoprecipitated with heterologous antisera.

Sensitive studies using antibodies purified by affinity chromatography revealed 46 [^{35}S]-methionine labelled MDV-specific polypeptides in productively infected CEF (23). Six of these polypeptides can also be detected in the culture medium, 7 are phosphorylated. Protein profiles from CEF infected with an attenuated MDV strain are very similar to those infected with virulent MDV, and most of the polypeptides can be precipitated with heterologous antisera, although a few minor differences can be observed. A 36,000 dalton phosphorylated protein has been found only in CEF infected with virulent MDV: it was suggested this protein may be involved in transformation. All 7 phosphoproteins can be immunoprecipitated with purified HVT antibody. Most of the [^{35}S]-methionine labeled polypeptides will cross-react with heterologous antisera, however several HVT glycoproteins can not be immunoprecipitated with MDV antisera (24). Monoclonal antibodies reacting with both MDV and HVT proteins have recently been described (25,55).

In vivo, viral-specific antigens are found in the feather follicle epithelium, at the site of productive virus replication. Low levels of viral antigens may occasionally be detected in the thymus, spleen and bursa of Fabricus. However, the presence of viral antigens in lymphoid tissue is not consistent, and no viral proteins have yet been shown to definitively correlate with transformation. Viral antigen expression in cell lines derived from MDV-induced lymphomas is variable. Most cell lines express certain viral antigens, but in some lines no viral-specific antigens can be detected.

E. Molecular Biology

Current knowledge of the regulation of MDV gene expression is minimal. In MDV-infected CEF, RNA hybridizes to 45% of the viral genome and all of the transcripts can be found associated with polyribosomes (56). RNA extracted from the MKT-I cell line hybridizes to only 12-14% of the viral genome yet only 60-70% of these RNA species are found in the polyribosome fraction. After treatment with 5-iodo-2-deoxyuridine (IUdR), which induces the synthesis of certain viral antigens but does not affect viral DNA synthesis, 42% of the genome is transcribed. Again, only 60-70% of these RNAs are found associated with polyribosomes. These data suggest the presence of a post-translational control mechanism that prevents certain RNA species from associating with polyribosomes during non-productive infection. This control mechanism is still operable after induction by IUdR, even though IUdR induces transcription of over 90% of the viral RNAs found in productively infected CEF.

Virus-specific RNA has also been detected in tumor tissue by *in situ* hybridization (49). However, only a portion of the MDV genome was used in these studies so a quantitative interpretation of the results could not be made.

Lymphoblastoid cell lines carry an estimated 15-90 copies of the MDV genome per cell (40,49). *In situ* hybridization using cloned MDV DNA fragments to probe lymphoid tumors and infiltrated nerves suggests the majority of both proliferating and infiltrating lymphoid cells contain virus DNA, with approximately 15 copies of the viral genome per cell (49). Up to 150 copies per cell have been observed in productively infected CEF.

The state of viral DNA within established cell lines is unclear: species corresponding to both integrated and free viral DNA have been observed in a number of cell lines (27) and the presence of covalently closed circular DNA has been detected in two lines (51,60). However, these analyses were performed by cesium chloride density gradient centrifugation, and interpretation of results has been difficult because the bouyant density of the MDV genome is close to that of chicken DNA. Sucrose gradient analysis of metaphase chromosomes from the MSB-I cell line indicated MDV DNA is associated with at least two chromosomes (22). No characteristic chromosomal aberrations have been observed in cells transformed by MDV.

In established cell lines, viral DNA replication is under cellular control. Phosphonoacetate, which specifically inhibits the MDV viral DNA polymerase, does not affect viral DNA replication (29,41). Furthermore, in transformed cell lines, viral DNA replicates only during early S phase (28).

The MDV genome is approximately 180kb with a GC content of 45% (38). HVT is approximately 150kb with a similar GC content. The structure of both viral genomes resembles that of herpes simplex virus (HSV), consisting of unique long (U_L) and unique short (U_S) regions flanked by inverted repeat sequences (IR_L/TR_L and IR_S/TR_S) (7). In MDV a small repeat is located within the U_L segment and the 3' end is heterogenous in length (17). Unlike HSV, the MDV U_L and U_S segments do not invert.

Although the physical map for the GA strain of MDV has been partially completed (17,30), maps of other MDV strains are not available, nor has the map for any HVT strain been constructed.

Restriction analysis of several MDV strains, including virulent, attenuated and apathogenic isolates, indicates most of the restriction sites are conserved; only a few minor differences can be detected between strains (19,26). However, one apathogenic isolate, HPRS24, has a markedly different restriction pattern (50). The restriction patterns of HVT DNA are completely different from those of MDV (19,26).

Cross-hybridization between MDV and HVT DNA has been assayed in several ways, including DNA/DNA reassociation (19,31,50) and Southern hybridization using either cRNA or nick-translated viral DNA as probe (26,31). In all cases, results indicated little homology exists between the two viruses, with an estimate of 1-5% sequence homology. Only 4 EcoRI fragments of MDV were found to hybridize to the HVT probe (31). DNA from one serotype 2 isolate (Cal-I) shares extensive homology with serotype 1 DNA (36), while another serotype 2 isolate (HPRS24) does not hybridize well to serotype I probes under the conditions used (50). Attenuated strains, as expected, share strong homology with their parent virulent strain (26,50).

F. Summary

MD is the only neoplastic disease, as well as the only herpesvirus-induced disease, for which an effective vaccine has been developed and is routinely used. MD therefore serves as a model for the development of vaccines both against viral-induced malignancies and against herpesvirus infections.

Despite this potential as a model system, little is known about the molecular biology of MDV. Primarily because of a highly cell-associated nature, the virus has proven to be extremely difficult to work with. Because the virus is spread from cell to cell,

identification of early, middle and late antigens, which have been extensively characterized in other herpesvirus systems, cannot be made. Furthermore, it has been virtually impossible to obtain purified preparations of individual viral antigens. Even the virion proteins have not been studied in detail since cells in culture release only minimal amounts of infectious virus. The application of monoclonal antibody technology to the MDV system should greatly facilitate analysis of MDV-specific proteins. Of particular importance will be the detailed analysis of those proteins which cross-react with HVT and the antigens involved in eliciting a protective immune response.

As with the viral proteins, analysis of the MDV genome has also been hampered by the cell-associated nature of the virus. Since only small amounts of infectious cell-free virus are produced, only minimal quantities of viral DNA can be isolated from virus particles released to the media. Unlike DNA from other herpesviruses, intracellular MDV DNA can not be separated from genomic DNA by density gradient centrifugation because the bouyant density of the viral DNA is very close to that of cellular DNA.

Nevertheless, analysis of the MDV genome is important for a number of reasons. First, critical examination of the nucleotide homology between MDV and HVT should clarify the nature of the close antigenic relationship of the two viruses. Second, construction of a physical map will greatly facilitate construction of a genetic map: localization of specific protein coding regions should be relatively straight-forward using cloned MDV DNA fragments in hybrid selection/*in vitro* translation assays. Third, an understanding of the genomic organization is critical for the identification of specific changes

involved in attenuation as well as characterizing the differences between virulent and avirulent isolates. These analyses should allow identification of the gene or genes involved in transformation and may aid in the understanding of the mechanism of MDV-induced neoplasia. Finally, a combination of genetic and protein analyses should lead to an understanding of the protective immune response elicited by HVT and should form a solid basis from which an effective vaccine could be designed.

CHAPTER V

CROSS-HYBRIDIZATION BETWEEN MDV AND HVT

CHAPTER 5

MATERIALS AND METHODS

Isolation of genomic, phage and plasmid DNAs, restriction enzyme digestion, agarose gel electrophoresis, Southern transfer, nick translation, ligation and bacterial transformation were described in Chapter 2.

A. Isolation of Viral DNA

Purified nucleocapsids were obtained from K. Nazerian. The solution was adjusted to 10 mM Tris-HCl pH 7.4; 0.1 mM EDTA and incubated with 50 µg/ml DNase I and 50 µg/ml RNase I at room temperature for 120 min. Nucleocapsids were disrupted by addition of one-half the volume of 3% sarcosyl; 75 mM Tris-HCl pH 9.0; 50 mM EDTA and the sample incubated at 55°C for 10 min. Pronase was added to 1 mg/ml followed by incubation at 37°C for 120 min. Viral DNA was gently extracted twice with phenol and once with chloroform and precipitated with ethanol.

B. Hybridizations and Washings

Hybridizations were performed under either conventional or relaxed conditions. Conventional conditions were used for colony screening with the MDV probe and for screening of the MSB-I phage library. Only relaxed conditions were used for cross-hybridizations. To increase the rate of association, dextran sulfate was incorporated into all

hybridization buffers (65). Also, the reactions were performed in minimal volumes. These modifications allowed hybridizations to reach to 3 x $Cot_{1/2}$ within 3-4 days.

Conventional hybridization conditions were performed in 50% formamide; 5x Denhardt's solution; 5x SSPE (20x SSPE is 3.6 M NaCl; 200 mM NaH_2PO_4, pH 7.4; 20 mM EDTA, pH 7.4); 0.1% SDS; 100 µg/ml sheared, single-stranded salmon sperm DNA; 100 µg/ml yeast RNA; 10% dextran sulfate at 42°C.

The washing procedure consisted of three 10 minute washes in 2x SSC; 0.1% SDS at room temperature followed by washing in 1x SSC; 0.1% SDS at 68°C for 60 minutes.

Low stringency hybridizations were performed in a buffer consisting of 1 M NaCl; 10 mM Tris-HCl pH 7.4; 25% formamide; 100 µg/ml sheared, single-stranded salmon sperm DNA; 100 µg/ml yeast RNA; 10% dextran sulfate at 42°C. Washings were in 0.2 x SSC; 0.2% SDS at 36°C (low stringency), 50°C (medium stringency), or 64°C (high stringency). These conditions were calculated to allow 30%, 20%, or 10% base mismatching, respectively, according to the following formulas (21):

$$Tm = 81.5 + 16.6 (\log M) + 0.41 (\% GC) - 0.72 (\% F)$$

$$\% mm = \frac{T_m - T_H}{1.4}$$

T_m = melting temperature

M = monovalent salt molarity

%GC = percent guanine plus cytosine

%F = percent formamide

T_H = hybridization temperature

%mm = percent base mismatch

C. In situ Colony Hybridization

Sterile nitrocellulose filters were placed on minimal agar plates containing ampicillin and tetracycline. Colonies were picked with toothpicks and spotted onto duplicate filters. To serve as a hybridization control, bacteria harboring the pBR328 vector were spotted onto each filter. Plates were incubated at 37°C until colonies were approximately 2mm in diameter. DNA was liberated from the bacteria and bound to the nitrocellulose by standard protocols (36). The filters were placed on a pool of 0.5 N NaOH for 3 min, blotted on Whattman paper, and placed on a second pool of 0.5 N NaOH for 3 min. Filters were blotted again, then placed on a pool of 1 M Tris-HCl pH 7.4 for 5 min. This neutralization step was repeated twice. Filters were air-dried and vaccum-baked at 80°C for 2 hrs.

D. Lambda Library Screening

The MSB-I genomic library (a gift of J. Casey) was screened using standard protocols (36). 3 X 10^5 phage were plated out on NZCYM in a 22 x 35 cm glass dish. After incubation the dish was cooled at 4°C for 60 min. A nitrocellulose filter was carefully layered on the agarose, left for 1 min, then gently peeled off. The filter was submerged in 1.5 M NaCl; 0.5 M NaOH for 1 min, then twice submerged in 1.5 M NaCl; 0.5 M Tris-HCl pH 8.0 for 5 min. The filter was rinsed in 2XSSPE and air-dried. Four replica filters were pulled. Filters were vacuum-baked for 2 hr at 80°C.

RESULTS

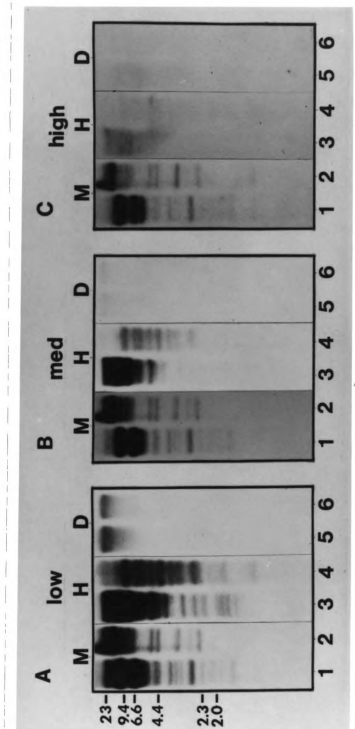
A. Cross-Hybridization Between MDV and HVT

Homology between the MDV and HVT genomes was examined by cross-

hybridizations performed under different stringencies. The conditions used were specifically designed to allow 30% (low stringency), 20% (medium stringency) or 10% (high stringency) base mismatch, thus an estimate of the degree of homology could be obtained. Dextran sulfate was included in all hybridization buffers when total viral DNA was used as probe, enabling the reactions to proceed to 3 x $Cot_{1/2}$ within 2 to 3 days.

Initially, hybridization patterns of MDV viral DNA to the MDV or HVT genomes present in infected DEF, which carry multiple copies of the respective viral genomes, were examined. Total cellular DNA was isolated from heavily infected DEF when the cells showed extensive cytopathic effect. DNA isolated from uninfected DEF was included as a negative control. DNAs were digested with EcoRI or HindIII, separated by agarose gel electrophoresis and transferred to nitrocellulose. MDV viral DNA isolated from nucleocapsids obtained from the supernatant of infected CEF was nick-translated and used as a probe under the low stringency conditions. As shown in Figure 1A, discrete bands corresponding to the MDV (lanes 1 and 2) or HVT (lanes 3 and 4) genomes could readily be detected in DNA from the virus-infected cells. The banding patterns correspond to the restriction fragments in ethidium bromide stained digests of the viral DNAs (26) and are characteristic for the respective genomes. As expected, all of the MDV-specific bands hybridized to the MDV probe. In addition, virtually all of the HVT fragments could be detected, indicating that homologous sequences exist over a substantial portion of the genome. In contrast, only a faint smear was observed in the lanes containing DNA from uninfected DEF (lanes 5 and 6), presumably due to cross-hybridization with

Fig. 1 Hybridization of MDV viral DNA to DNA from infected cells. Southern blots of total cellular DNA isolated from viral infected or uninfected DEF probed with MDV viral DNA under relaxed conditions. M, MDV-infected DEF; H, HVT-infected DEF; D, uninfected DEF; DNAs were digested with EcoRI (lanes 1, 3 and 5) or Hind III (lanes 2, 4 and 6). Blots were washed under low stringency (A), medium stringency (B) or high stringency (C) conditions. Molecular weight sizes are in kb.

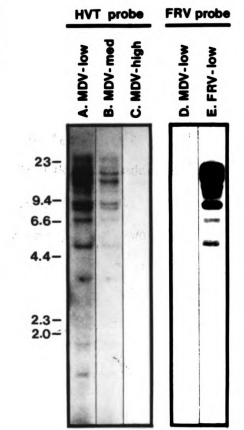

A low
B med
C high
M
H
D
1 2 3 4 5 6
23–
9.4–
6.6–
4.4–
2.3–
2.0–

contaminating CEF DNA sequences present in the probe: under more stringent conditions (see below) this smear was not detected (lanes 5 and 6 in Figure 1B and C).

To further examine the degree of homology between the MDV and HVT genomes, the filters of Figure 1A were rewashed under the medium stringency conditions designed to allow 20% base mismatch (Figure 1B). This resulted in an overall decrease in intensity of the HVT bands (lanes 3 and 4) relative to the intensity of the MDV bands (lanes 1 and 2). The filters were then washed under the high stringency conditions designed to allow 10% base mismatch (Figure 1C). Bands in the HVT lanes (3 and 4) were extremely faint while bands in the MDV lanes (1 and 2) remained intense. These data indicate that MDV and HVT hybrids are not stable under conditions that only allow less than 10-20% base mismatch. The approximate overall degree of homology between the two genomes is estimated to be between 70 to 80%.

The reciprocol hybridization was then performed: nick-translated HVT viral DNA was hybridized to a Southern blot containing Bam HI-digested MDV viral DNA (Figure 2). Results were in complete agreement with those observed in Figure 1. Under low stringency (lane A), strong hybridization beween HVT and MDV was observed, with most of the MDV fragments cross-hybridizing. Upon washing at medium stringency, the bands became fainter (lane B); with further washing under high stringency, bands could barely be detected (lane 3). These data substantiate the results of Figure 1, providing additional evidence that related sequences extend throughout the viral genomes, with the level of homology around 70-80%.

Fig. 2 Hybridization of HVT viral DNA to MDV viral DNA. A-C. BamHI-digested MDV viral DNA probed with HVT viral DNA under relaxed conditions. The Southern blot was successively washed under the low, medium, and high stringency conditions used in Fig. 1. D. Bam HI-digested MDV viral DNA and E. EcoRI-digested FRV viral DNA probed with FRV viral DNA under the low stringency conditions. Molecular weight sizes are in kb.


HVT probe
FRV probe
A. MDV-low
B. MDV-med
C. MDV-high
D. MDV-low
E. FRV-low
23
9.4
6.6
4.4
2.3
2.0

A negative control was included to demonstrate the hybridization specificity. Feline rhinotracheitis virus (FRV) is a herpesvirus with a GC content identical to that of MDV (38,66). FRV viral DNA (provided by R. Maes and P. Rota) was nick-translated and hybridized to a Southern blot of Bam HI-digested MDV viral DNA under the low stringency conditions used in Figure 2 lane A (Figure 2 lane D). No bands were apparent, even after long exposures. In contrast, when the same FRV probe was hybridized to digest of FRV viral DNA, the characteristic FRV band were readily detected (Figure 2 lane E). These results demonstrate the hybridization conditions used are indeed specific for homologous sequences; the homology detected in the cross-hybridization experiments is not an artifact due to a similar GC content.

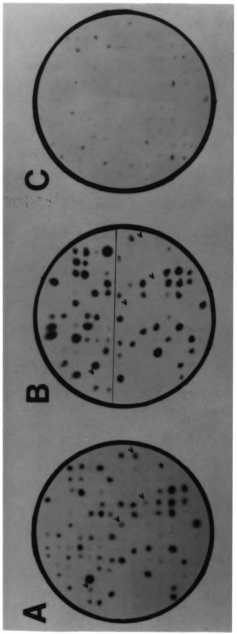
B. Cross-Hybridization Utilizing Cloned MDV Fragments

To firmly estalish the genetic homology between MDV and HVT, individual cloned MDV DNA fragments were used as hybridization substrates. Both a plasmid and a phage genomic library were constructed; the cloning and cross-hybridization results will be presented first for the plasmid library and then for the phage library.

1. Construction of the MDV plasmid library

MDV viral DNA was isolated from nucleocapsids obtained from the supernatant of infected DEF. EcoRI fragments were ligated into the plasmid vector pBR328 and transformed into the host bacteria HB101. The colonies showing the correct drug resistance were initially screened by *in situ* colony hybridization using MDV viral DNA as probe to identify those plasmids bearing MDV inserts (Figure 3A). Over 300 colonies, approximately 45%, scored positive with the MDV probe. Gel analysis of DNA from these clones (performed by D. Bauer) revealed the

Fig. 3 *In situ* colony hybridization analysis of MDV plasmid clones. A. Filter was probed with MDV viral DNA under conventional conditions. B. Duplicate filter probed with HVT viral DNA under relaxed conditions and washed under low stringency. C. Triplicate filter probed with HVT viral DNA under conditions used by Lee, *et al*. (7). Arrows indicate clones hybridizing to MDV but not HVT.


A
B
C

presence of over 20 different size classes, with inserts ranging from 0.5 to 15kb.

2. Cross-hybridization utilizing MDV plasmid clones

As an initial rapid screen, clones containing MDV inserts that bear homology to HVT were identified by *in situ* colony hybridization using HVT viral DNA as a probe under the low stringency conditions described above (Figure 3B). The HVT probe hybridized to the majority (approximately 95%) of the clones containing MDV-specific inserts. However, a few exceptions (indicated by arrows) were noted. In no case did a clone hybridize to the HVT probe but not the MDV probe. These results are consistent with the extensive homology between the two viral genomes observed in Southern hybridizations of total viral DNA.

In situ colony hybridization was repeated using the more stringent conditions described by Lee, *et al.* (31) (Figure 3C). Hybridization signals were extremely weak, with a very low signal-to-noise ratio, and only a few clones scored positive with the HVT probe. Nevertheless, those that did give a positive signal were also positive under the more sensitive conditions used in Figure 3B. The differences in the sensitivity of the hybridization conditions helps account for the discrepancy between the results previously reported (19,26,31,50) and those obtained in these studies.

In situ colony hybridization cannot be used quantitatively since the signal intensity is dependent upon a number of uncontrollable factors. Therefore, individual cloned MDV fragments were subjected to Southern blot analysis. Plasmid DNAs were digested with EcoRI, electrophoresed through agarose and transferred to nitrocellulose. The

ethidium bromide stain of 14 representative clones is shown in Figure 4A (clones L and M each contain two inserts). The filter was hybridized to nick-translated MDV viral DNA (Figure 4B); as expected, all inserts hybridized to this probe, confirming the MDV origin of the inserts. Hybridization to the smaller band in lane M is not obvious in the photograph but can be seen in the original autoradiogram.

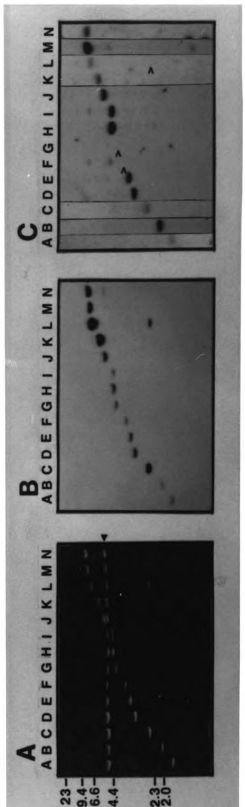
When a duplicate blot was hybridized to the HVT viral DNA probe under the low stringency conditions, 13 of the 16 inserts were positive (Figure 4C). The pBR328 vector bands did not hybridize, indicating the hybridization is specific for the viral DNAs. These data substantiate the findings obtained using total viral DNA as a hybridization substrate: regions homologous to HVT extend over a large portion of the MDV genome. However, the presence of fragments that do not hybridize to HVT indicates that divergent regions do exist. Furthermore, the obvious intensity differences among the different fragments suggests certain regions share more extensive homology than do others.

When the plasmid clones were used individually to probe MDV- or HVT-infected DEF, each clone detected only a few bands (I. Sithole, personal communication). These results conclusively demonstrate the observed cross-hybridization is not due to the presence of homologous repetitive sequences.

3. Construction of the MDV phage library

Due to the relative difficulty in obtaining sufficient quantities of MDV viral DNA for the construction of an MDV lambda library, an MSB-I genomic library in the λL47 vector (provided by J. Casey) was used to obtain phage containing MDV-specific inserts. MSB-I is a

Fig. 4 Southern hybridization analysis of MDV plasmid clones. Plasmids containing MDV inserts were digested with EcoRI and electrophoresed through 0.7% agarose. A. Ethidium bromide staining pattern. B. Hybridization to MDV viral DNA. C. Hybridization to HVT viral DNA under relaxed conditions. Clones L and M contain multiple inserts. The pBR328 vector band is indicated by ◄, inserts that do not hybridize to HVT are indicated by >. Molecular weight sizes are in kb.


A
A B C D E F G H I J K L M N
23
9.4
6.6
4.4
2.3
2.0
B
A B C D E F G H I J K L M N
C
A B C D E F G H I J K L M N

lymphoblastoid cell line derived from a splenic lymphoma of an MDV-infected chicken (2), and contains an estimated 15-90 copies of the MDV genome per cell (22,40). The library was screened with two probes: the first was nick-translated MDV viral DNA and the second consisted of a pool of 8 MDV-positive plasmid clones. A total of 14 plaques were detected with the MDV viral DNA probe, 7 of these also hybridized to the plasmid pool.

Preliminary restriction enzyme maps of 7 of the clones have been constructed (Figure 5). Initial restriction mapping suggests only two of the inserts (2 and 10) overlap extensively; the analyses are not yet complete enough for other overlaps to be identified. However, hybridization of individual clones to DNA from MDV-infected cells suggests additional overlaps do exist (Y. Naidu, personal communication). It is estimated that 50% of the MDV genome is represented in these clones.

The low number of clones obtained is a direct result of the inherent difficulties involved when using total viral DNA as a probe. The complexity of the viral DNA (180kb) combined with the large volume required during hybridization resulted in unfavorable hybridization kinetics: hybridizations could not be carried out to completion. Thus, the signals obtained with the total MDV viral DNA probe were extremely weak. However, rescreening with other plasmid clones or with specific fragments from the existing lambda inserts should produce additional phage clones containing the remainder of the MDV genome.

4. Cross-hybridization utilizing MDV phage clones

Cross-hybridization of the MDV lambda clones to HVT viral DNA was examined by Southern blot analysis of the phage DNAs. Figure 6A shows

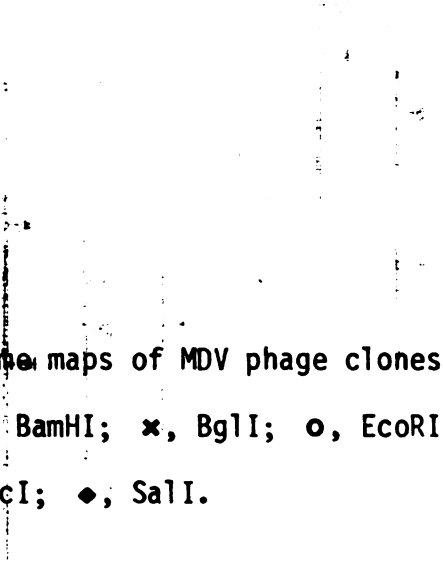


Fig. 5 Restriction enzyme maps of MDV phage clones. Symbols are as follows: ●, BamHI; ✖, BglI; o, EcoRI; ▼, HindIII; ○, KpnI; ▲, SacI; ◆, SalI.

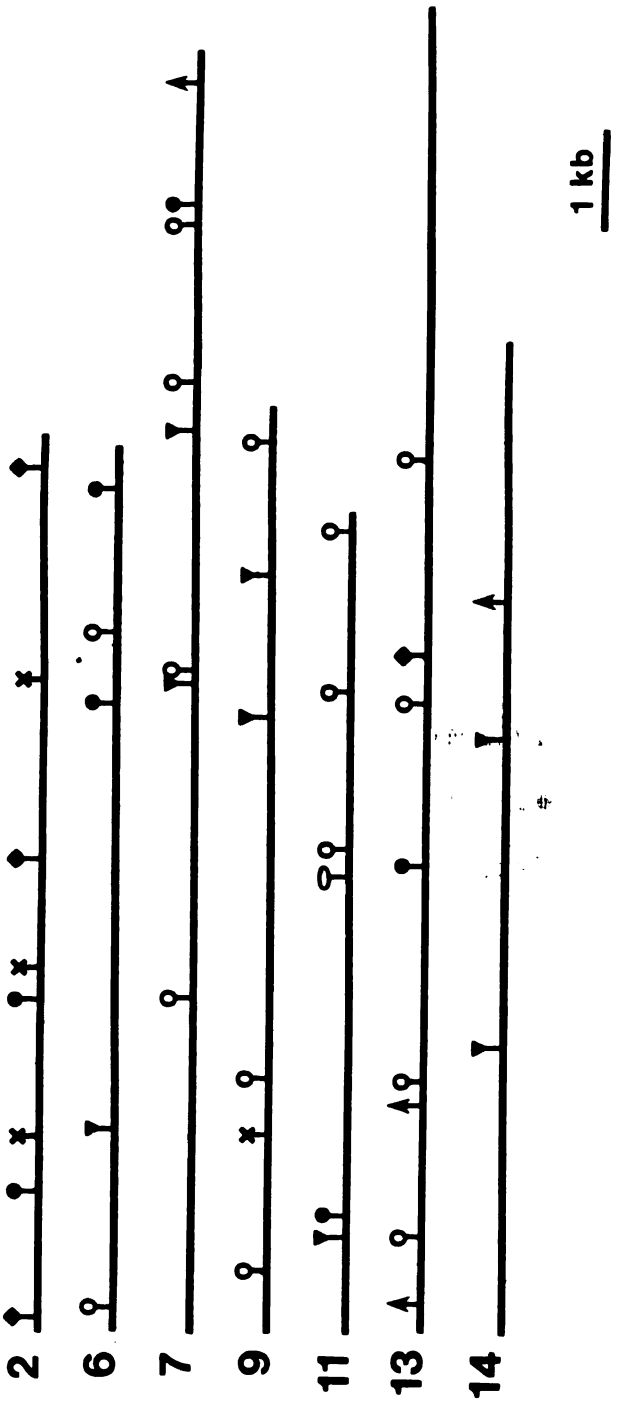
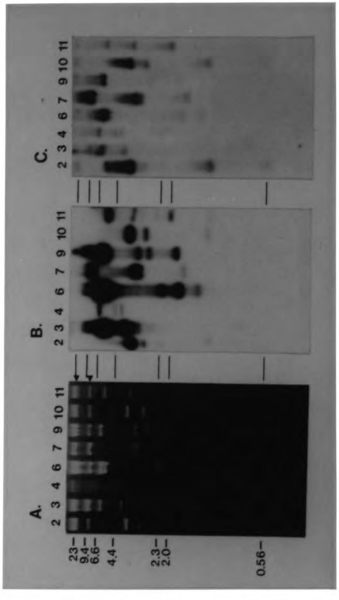

2
6
7
9
11
13
14
1 kb

Fig. 6 Southern hybridization analysis of MDV phage clones. Phage DNA containing MDV inserts were digested with EcoRI and electrophoresed through 0.7% agarose. A. Ethidum bromide staining pattern. B. Hybridization to MDV viral DNA. C. Hybridization to HVT viral DNA under relaxed conditions. The lambda arms are indicated by ◄. Molecular weights sizes are in kb.


A.
B.
C.
2 3 4 6 7 9 10 11
23
9.4
6.6
4.4
2.3
2.0
0.56

the ethidium bromide staining pattern of EcoRI digests of 8 of the phage clones; the two largest bands in each lane contain the lambda arms. All fragments derived from the inserts hybridized to the MDV viral DNA probe (Figure 6B), while the lambda arms did not. This hybridization confirms the inserts are MDV-specific.

Under the low stringency conditions, a duplicate blot was hybridized the HVT viral DNA probe (Figue 6C). In agreement with the results obtained with the plasmid clones, the majority of insert fragments cross-hybridized with the HVT probe. Again, relative intensity differences among the fragments suggests homology is more extensive in certain regions.

DISCUSSION

A herpesvirus-induced malignant lymphoma of chickens, MD is effectively prevented by vaccination with the non-oncogenic virus, HVT. Antigenically, the two viruses appear to be closely related. Over 40 MDV-specific polypeptides can be detected in productively infected cells; the majority of these proteins can be immunoprecipitated with antisera raised against HVT (18,23,24,64). In addition, a number of monoclonal antibodies developed against various MDV antigens will cross-react with HVT proteins (25,55). Despite this close immunological relationship between the two viruses, cross-hybridization analyses suggested the viral genomes share only 1-5% homology (19,26,31,50). This conflict has been addressed in these studies.

The difficulties involved in isolation of viral DNA have proven to be a major deterrent in analysis of the MDV and HVT genomes, thus the degree of homology between the viral DNAs has not been examined in

detail. To avoid the problems inherent with the use of purified viral DNA, two MDV clone banks have been constructed, the first in a plasmid vector and the second in a lambda phage vector. Altogether, it is estimated over 90% of the MDV genome is represented in those two libraries. These cloned fragments have greatly facilitated cross-hybridization analyses and should be extremely useful for a variety of other studies.

The homology between the MDV and HVT genomes has been reexamined using highly sensitive hybridization conditions: contrary to earlier reports, results indicate the two viruses share extensive homology. Under conditions allowing 30% base mismatch, virtually all of the HVT viral DNA restriction fragments can be detected with total MDV viral DNA as probe (Figure 1). The converse is also true: the majority of MDV restriction fragments hybridize to the HVT viral DNA probe (Figure 2). This extensive cross-hybridization was confirmed using cloned MDV fragments. 90% of the MDV fragments in the plasmid library hybridized to the HVT probe (Figure 4). Likewise, the majority of inserts in the MDV phage library could be detected with the HVT probe (Figure 6).

This data unequivocally demonstrate the two viruses share extensive homology. The lack of cross-hybridization between MDV and FRV, an antigenically unrelated herpesvirus with a GC content identical to that of MDV and HVT, clearly indicates the hybridization conditions are highly specific. Since no cross-hybridization between MDV and FRV could be detected under the low stringency conditions, it is unlikely the bands observed in the MDV/HVT cross-hybridizations are an artifactual result of non-specific trapping of probe which might occur under relaxed conditions. In addition, viral DNAs did not hybridize to DEF DNA, nor to the lambda or plasmid vector DNAs.

Under conditions allowing 30% base mismatch, homology extending over 90-95% of the MDV genome could readily be detected with the HVT probe. These MDV/HVT hybrids are less stable under conditions of 20% base mismatch, and most have dissociated under conditions allowing only 10% base mismatch. Thus a 20-30% sequence divergence between the two viral genomes is indicated, with homologous sequences extending throughout the MDV and HVT genomes.

These results readily explain the lack of homology reported by other investigators (19,26,31,50). Previous cross-hybridizations had been conducted under conditions approximating 15-20% base mismatch, and as shown here, most MDV/HVT hybrids are unstable below 20% base mismatch, although readily detected under less stringent conditions. In addition, it is likely hybridizations conducted by earlier investigators were not carried out to completion. Due to the complexity of the viral genomes (150-180kb), the DNAs follow relatively slow reassociation kinetics; under conventional hybridization conditions a lengthy incubation period is usually required to complete such a reaction (36). To overcome this problem, in these experiments dextran sulfate was included in all hybridization buffers when total viral DNAs were used as probe. This compound has been shown to increase the rate of reassociation ten-fold (65), thus allowing hybridizations to proceed to completion within 2-3 days.

Direct comparison of the hybridization conditions used here with those used previously (31) demonstrates the striking difference in sensitivity between the two methods (Figure 3, B and C). Thus the quite different conclusion reached by earlier investigators (19,26,31,50) can be accounted for by the sensitivities of the

conditions used. Of primary importance is the lowered stringency used here; improved reassociation kinetics probably play a minor role. While these studies were in progress, two other groups initiated projects similar to those presented here (20,59); their results confirm the extensive homology described in this report.

Substantial genetic homology is consistent with the strong immunological relationship between MDV and HVT. Homologous sequences located throughout most of the viral genomes could easily accomodate the number of virus-specific polypeptides in MDV- or HVT-infected cells which can be immunoprecipitated with heterologous antisera (18,23,24,64). The estimated degree of mismatch, 20-30%, is sufficient to explain the minor differences observed between a number of the corresponding viral proteins (18,61), as well as the observation that certain antigens do not cross-react as strongly as others (23) and monoclonal antibodies have been obtained that are specific for either MDV or HVT (25,55). This level of homology also explains the different restriction enzyme patterns of the two viruses (19,26). Finally, the presence of regions that do not cross-hybridize suggests unique or highly divergent proteins may also exist, again consistent with the finding that a few viral-specific polypeptides do not cross-react with the heterologous antisera (23).

Thus the previous conflicting data obtained from protein and DNA analyses of MDV and HVT have been clarified. The estimate of 70-80% sequence homology extending throughout 90-95% of the viral genomes is in complete agreement with the strong antigenic similarities between the viruses, yet would not have been detected under stringent hybridization conditions. These results suggest the efficacy of the

HVT vaccine could involve a number of viral antigens, although certain proteins are much more abundant and may play the major role in eliciting a protective immune response.

SUMMARY (part B)

MD is the only neoplastic disease which can be effectively controlled by routine vaccination; the commonly used HVT vaccine elicits a strong immune response that protects chickens against MDV-induced lymphoma. Elucidation of the mechanism of this protection will aid in the understanding of the nature of defense mechanisms against viral-induced malignancies. Such information will be valuable for the development of vaccines against other viral-induced neoplasias as well as vaccines effective against other herpesviruses. Despite this potential as a model system, little progress has been made toward understanding the nature of protection afforded by the HVT vaccine, primarily because of a number of inherent difficulties within the MDV system.

Results presented here offer significant technical and conceptual contributions to the field. The availability of MDV genomic libraries, both the phage and plasmid clone banks, provides a source for obtaining unlimited amounts of viral DNA, thereby circumventing the problems encountered during purification of total viral DNA, which have hampered analyses of the MDV genome until now. These genomic clones will greatly facilitate the construction of both physical and genetic maps, and in conjunction with protein analyses should provide a foundation for detailed examination of antigens involved in the protective immune response as well as those involved in

transformation. Such analyses have already been initiated. One plasmid clone, M, is capable of selecting message for A antigen (R. Isfort, personal communication), and therefore the insert contains part or all of the A antigen coding sequence.

The finding of substantial homology between MDV and HVT DNA, 70-80% homology extending over 90-95% of the viral genomes, suggests the viruses share a number of antigenetically related, although not identical proteins. These results are in complete agreement with the close immunological relationship exhibited by the two viruses and clarify earlier reports that seemed to indicate MDV and HVT share little genetic homology. Immunity to MDV is likely to involve a number of viral antigens, and it is probable that protection entails responses directed against the various stages of viral infection.

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