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Two Novel Variants of the v-*src* Oncogene Isolated from Low

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Four different transformed cell lines were isolated as a result of independent infection of primary hamster fibroblasts by Rous sarcoma virus (RSV SR-D stocks). These lines differ by the level of their spontaneous metastatic activity: HET-SR-1, HET-SR-8, and HET-SR-10 cell lines induced 70–200 metastatic nodules in the lung and/or lymph nodes of inoculated animals (high metastatic lines, HM). Metastatic activity was not identified after injection of HET-SR cells (low metastatic line, LM). All cell lines contained one copy of integrated and expressed intact RSV provirus. The difference in the amount of v-*src* protein in cell lines was not correlated with their metastatic potential *in vivo.* Complete v-*src*HM and v-*src*LM genes were cloned from corresponding gene libraries and sequenced. In the unique region of both v-*src* isoforms a GC-rich insert of 60 nucleotides (20 a.a.) was found. The presence of this insert explains the unusual apparent molecular weight of protein encoded by v-*src*HM and v-*src*LM: 62 kDa. Both genes had 10 identical amino acid changes when compared to the known RSV SR-D v-*src* sequence. v-*src*HM and v-*src*LM differ by several amino acid changes. Most of them are localized in the unique domain and the extreme carboxy-terminal region of the oncoprotein. Both v-*src* variants and chimeric v-*src* with mutually substituted parts were subcloned in a retroviral vector and introduced into avian neuroretina cells. Significant differences in the morphology of transformed neuroretina cells were associated with the mutations in the carboxy-terminal region of the v-*src* oncogene. Low metastatic HET-SR cells transfected with v-*src*HM and the chimeric gene v-*src*-LH remarkably increased their metastatic potential. In contrast, this effect was not observed when the same cells were transfected with v-*src*LM and the chimeric v-*src*HL gene. Specific changes in the distribution of fibronectin matrix typical for high metastatic cells were found in the lines transfected with v-*src*HM. © 1996 Academic Press, Inc.

INTRODUCTION

The transforming gene (v-*src*) of Rous sarcoma virus (RSV) encodes a 60-kDa phosphoprotein (pp60^{src}) that exhibits tyrosine-specific protein kinase activity essential for transformation (reviewed in Jove and Hanafusa, 1987). The v-*src* gene is derived from the cellular protooncogene c-*src* (Stehelin *et al.,* 1976). The *src* protein is structured in several functional domains (reviewed in Parsons and Weber, 1989; Koegel and Courtneige, 1991). At its N terminus p60^{src} contains sequences specific for myristoylation, allowing anchoring to the cell membrane. The amino acid sequence downstream from the membrane binding region, named unique domain (residues 18–84), is specific in each tyrosine kinases of the *src* family. This region is thought to interact with specific cellular substrates (Parsons and Weber, 1989). After residue 84, two homology domains are found that present sequence similarity with a number of proteins involved in signal transduction pathways (Pawson, 1988; Koch *et al.,* 1991). They were designated domains SH3 (residues 86–140) and SH2 (residues 141–260). The most conserved region of proteins of the *src* family is the catalytic domain (residues 265–516), which shares a high degree of homology with other tyrosine kinases and to a lesser extent with serine/threonine kinases. Mutations in the catalytic domain usually alter the kinase activity and the transforming potential of the v-*src* protein (Jove and Hanafusa, 1987; Parsons and Weber, 1989). A major difference is observed between the carboxy ends of c-*src* and v-*src* proteins. The last 19 amino acids of the c-*src* protein include a tyrosine residue at position 527, the phosphorylation of which downregulates its kinase activity. In v-*src* these 19 amino acids are replaced by a tail of 12 amino acids which is present in all known RSV strains (Dutta *et al.,* 1985). v-*Src* induces major phenotypic changes which are also generally observed in cells transformed by various agents (Jove and Hanafusa, 1987). Transformed cells have an altered morphology, proliferate under conditions in which normal cells do not, and acquire anchorage-independent growth capacity.

During the recent years remarkable progress has been made toward understanding of the mechanisms of *src*

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gene function and its role in cell transformation (reviewed in Jove and Hanafusa, 1987; Parsons and Weber, 1989; Taylor and Shalloway, 1993). Much less is known about the role of the *src* gene in the process of metastasis formation. It has been shown that transformation by v-*src* induces the metastatic phenotype (Egan *et al.,* 1987; Stoker and Sieweke, 1989). Interestingly, *in vitro* RSV transformed cells, even in the absence of selection *in vivo,* possess all properties necessary for maximal expression of metastatic activity (Deichman *et al.,* 1989).

We previously reported the isolation of four different transformed cell lines named HET-SR, HET-SR-1, HET-SR-8, and HET-SR-10, as a result of independent infection of primary Syrian hamster fibroblasts with different stocks of Schmidt–Ruppin RSV-D (SR RSV-D) from the Russian Cancer Research Center virus collection. All lines had a typically transformed phenotype and were highly tumorigenic in syngenic hamsters. However, remarkable differences were found in the spontaneous metastatic activity of transformed cells: within 2 months after subcutaneous injection of HET-SR-1, HET-SR-8, and HET-SR-10 cells, between 70 and 200 metastatic nodules appear in the lung and/or other organs of inoculated hamsters. These cell lines were designated as highly metastatic (HM). On the other hand, metastatic nodules were not usually identified after inoculation with HET-SR cells (low metastatic line, LM) (Deichman *et al.,* 1992). All cell lines contained one copy of integrated RSV provirus and expressed comparable levels of *src*-specific tyrosine kinase activity (Brashishkite *et al.,* 1989; Topol *et al.,* 1993; and Shtutman, unpublished results). The apparent molecular weight of v-*src* protein in both HM and LM types of cells was slightly higher than usual: 62 kDa (Topol *et al.,* 1993).

The differences in metastatic potential of HM and LM cells could be due either to modifications of some cellular factors or to changes in the structure and properties of the v-*src* oncogene. The latter supposition was based on the presence of subtle differences between HM and LM cell lines in restriction analysis of the v-*src* genes and V8 protease digestion patterns of v-*src* proteins (Topol *et al.,* 1993). Therefore, we molecularly cloned and sequenced the v-*src* genes from both types of cells. We found, that the v-*src* genes in both HM and LM cells had significant structural changes that were not observed in the alleles of the v-*src* gene so far described. The proteins encoded by the new variants differed from SR-D $p60^{src}$ essentially by the presence of a 20-amino-acid insert in the unique domain and by several amino acid mutations mostly located in the unique domain and in the extreme carboxy-terminal coding region. In addition, some point mutations were observed between v-*src*HM and v-*src*LM. Retroviral vectors carrying v-*src*HM and v-*src*LM caused distinguishable morphological cell transformation and changes of the metastatic activity *in vivo.* We also obtained data suggesting that these distinct biological activities were associated with modifications in the C-terminal regions of the proteins.

RESULTS

Nucleotide sequences of v-*src*HM and v-*src*LM

High-molecular-weight DNA from hamster HET-SR and HM HET-SR-8 cells, transformed by different stocks of SR RSV-D (SR-D), were used to prepare genomic libraries in λgt10 after *Eco*RI digestion. v-*src* clones containing the 3.1-kb DNA fragment of RSV provirus (3′ end of *env*–v-*src*–U3 region of LTR) were isolated from both libraries. These inserts were subcloned in $pBSKS^+$ (Bluescript) plasmid and sequenced on both strands. The complete nucleotide sequences of v-*src*HM and v-*src*LM are shown in Fig. 1 and compared with the sequence of v-*src* from SR RSV-D (Reddy *et al.,* 1990). The main difference between v-*src*HM, v-*src*LM, and all other known *src* genes is the existence, in the unique region of both v-*src* variants, of a GC-rich (86%) insert of 60 nucleotides (Figs. 1 and 2). No stop codons were found in the three reading frames of the insert. Its sequence included short direct and inverted repeats on both ends and a potential site for phosphorylation by protein kinase C in the middle (Fig. 2). It shared 74% homology with part of the 5′ untranslated region of the *bcr* gene (Hariharan and Adams, 1987). We do not exclude that this apparent homology is due to the high GC content in both sequences. When we used these sequences as a probe, they appeared to be homologous to chicken genomic DNA but not to several mammalian DNAs (data not shown). Therefore, these inserted sequences are likely to be of chicken origin and might be due to recombination with cellular DNA during long-term passaging of SR-D virus stocks by tumor transplantation in chicken. This insertion explains the unusual apparent molecular weight (62 kDa) of the protein encoded by v-*src*HM and v-*src*LM in transformed cells (Topol *et al.,* 1993).

In comparison with v-*src* SR-D, 24- and 27-nucleotide changes, respectively, were identified in v-*src*HM and v-*src*LM. Mutations were equally allocated in the different regions of the genes except the myristoylation domain (positions 1–15), which had no nucleotide alteration in both v-*src* variants (Fig. 1). Sixteen nucleotide differences that were identical in both v-*src*HM and v-*src*LM were observed. Among these, 6 mutations were silent but the 10 others caused a change in the amino acid sequence. These substitutions are E62 (*src* SR-D) to G82, G77 → R97, E159 → G179, Q318 → R338, D348 → N368, D368 → A388, V399 → E419, I426 → A446, A434 → G454, and M461 → V481. In 4 of 10 cases (positions 82, 338, 388, and 481 of v-*src*HM and v-*src*LM), these substitutions restored the amino acid sequence of the chicken c-*src* protein (Figs. 3 and 4).

The differences between the primary structure of v-*src*HM and v-*src*LM are of particular interest because

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SR-D  ATGGGGAGTAGCAAGAGCAAGCCTAAGGACCCCAGCCAGCGCCGGCGCAGCCTGGAGCCACCCGACAGCACCCACCACGGGGGATTCCCAGCCTCGCAGA  100
HM/LM ****************************************************************************************************

SR-D  CCCCCAACAAGACA----------------------------------------------------------GCAGCCCCCGACACGCACCGCACCCC  140
HM    ************GGCGCCGCTCACCGCCGCGCCCCGCAGCTCGCGCCGCCCGCCGGCTTCTCAGCACCGCGCC**********************  200
LM    ************GGCGCCGCTCACCGCCGCGCCCCGCAGCTCGCGCCGCCCGCCGGCTTCTCAGCACCGCGCC**G*********************  200

SR-D  CAGCCGCTCCTTCGGGACCGTGGCCACCGAGCCCAAGCTCTTCGAGGACTTCAACACTTCTGACACCGTTACGTCGCCGCAGCGTGCCGGGGCACTGGCT  240
HM    ***************G**********************C***G*****************************************A**********  300
LM    **************************A*****************G*****************************************A**A********  300

SR-D  GGCGGCGTCACCACTTTCGTGGCTCTCTACGACTACGAGTCCTGGATTGAAACGGACTTGTCCTTCAAGAAAGGAGAACGCCTGCAGATTGTCAACAACA  340
HM/LM ****************************************************************************************************  400

SR-D  CGGAAGGTAACTGGTGGCTGGCTCATTCCGTGACTACAGGACAGACGGGCTACATCCCCAGTAACTATGTCGCGCCCTCAGACTCCATCCAGGCTGAAGA  440
HM/LM ********************************************************T*******************************************  500

SR-D  GTGGTACTTTGGGAAGATCACTCGTCGGGAGTCCGAGCGGCTGCTGCTCAACCCCGAAAACCCCCGGGGAACCTTCTTGGTCCGGGAGAGCGAGACGACA  540
HM    ************************************G*****************T*********************************************  600
LM    ************************************G*****************************A*********************************  600

SR-D  AAAGGTGCCTATTGCCTCTCCGTTTCTGACTTTGACAACGCCAAGGGGCTCAATGTGAAGCACTACAAGATCCGCAAGCTGGACAGCGGCGGCTTCTACA  640
HM/LM *************************************************A**********************************************  700

SR-D  TCACCTCACGCACACAGTTCAGCAGCCTGCAGCAGCTGGTGGCCTACTACTCCAAACATGCTGATGGCTTGTGCCACCGCCTGACCAACGTCTGCCCCAC  740
HM/LM ****************************************************************************************************  800

SR-D  GTCCAAGCCCCAGACCCAGGGACTCGCCAAGGACGCGTGGGAAATCCCCCGGGAGTCGCTGCGGCTGGAGGTGAAGCTGGGGCAGGGCTGCTTTGGAGAG  840
HM    *************************************************T**********************************************  900
LM    ****************************************************************************************************  900

SR-D  GTCTGGATGGGGACCTGGAACGGCACCACCAGAGTGGCCATAAAGACTCTGAAGCCCGGCACCATGTCCCCGGAGGCCTTCCTGCAGGAAGCCCAAGTGA  940
HM/LM ****************************************************************************************************1000

SR-D  TGAAGAAGCTCCAGCATGAGAAGCTGGTTCAACTGTACGCAGTCGTGTCGGAAGAGCCCATCTACATCGTCATTGAGTACATGAGCAAGGGGAGCCTCCT 1040
HM/LM ***********G************************************G*********************************************** 1100

SR-D  GGATTTCCTGAAGGGAGAGATGGGCAAGTACCTGCGGCTGCCACAGCTCGTTGATATGGCTGATCAGATTGCATCCGGCATGGCCTATGTGGAGAGGATG 1140
HM/LM *A**************************************************C********************************************* 1200

SR-D  AACTACGTGCACCGAGACCTGCGGGCGGCCAACATCCTGGTGGGGGAGAACCTGGTGTGCAAGGTGGCTGACTTTGGGCTGGCACGCCTCATCGAGGACA 1240
HM/LM ****************************************************A*************************G**************** 1300

SR-D  ACGAGTACACAGCACGGCAAGGTGCCAAGTTCCCCATCAAGTGGACAGCCCCCGAGGCAGCCCTCTATGGCCGGTTCACCATCAAGTCGGATGTCTGGTC 1340
HM    ************************************GC***************T*******G************************************ 1400
LM    ************************************GC***************T*******G***G*****************************T***** 1400

SR-D  CTTCGGCATCCTGCTGACTGAGCTGACCACCAAGGGCCGGATGCCATACCCAGGGATGGGCAACGGGGAGGTGCTGGACCGGGTGGAGAGGGGCTACCGC 1440
HM    *************************************T***G************************************************************ 1500
LM    *************************************G************************************************************ 1500

SR-D  ATGCCCTGCCCGCCCGAGTGCCCCGAGTCGCTGCATGACCTTATGTGCCAGTGCTGGCGGAGGGACCCTGAGGAGCGGCCCACTTTTGAGTACCTGCAGG 1540
HM    ****************************************************************T*********************************** 1600
LM    ****************************A*************************************A*********************************** 1600

SR-D  CCCAGCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAG                                                              1581
HM    ********************A*******************                                                              1641
LM    ****************************C---**********                                                              1638
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FIG. 1. Nucleotide sequence of v-*src*HM and v-*src*LM in comparison with v-*src* SR-D (Reddy *et al.,* 1990). Single nucleotide mutations in the v-*src* of SRHM and SRLM are indicated by boldface characters. The sequences of v-*src*HM and v-*src*LM will appear in the EMBL data base under Accession Nos. X84073 and X84074.

they might be responsible for the distinct metastatic capacities of transformed cells. There were 18 nucleotide differences between v-*src*HM and LM, resulting in eight amino acid differences (Figs. 1 and 3). Four amino acid changes were localized in the unique domain of the *src* gene: position 73, N (v-*src*LM); position 81 L (v-*src*HM) → F (v-*src*LM); and position 98 A (v-*src*HM) → A (v-*src*HM) → T (v-*src*LM); position 76, T (v-*src*HM) → T (v-*src*LM).

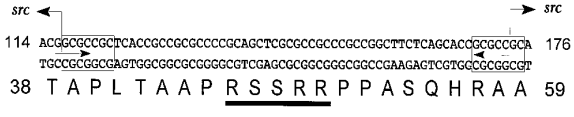

FIG. 2. Structure of the 60-nucleotide insert in the unique region of v-*src*HM and v-*src*LM. Direct repeats on both ends are indicated by rectangles. Inverted repeats are indicated by arrows. A potential site for phosphorylation by PK-C in the deduced amino acid sequence is underlined.

One mutation was found in the C-terminal region of the kinase domain: position 522, D (v-*src*HM) → E (v-*src*LM). The other amino acid differences between HM and LM isoforms of v-*src* were identified in the carboxy-terminal tail that is characteristic of all v-*src* oncogenes (Fig. 3). These amino acid replacements were in position 541, M (v-*src*HM) → V (v-*src*LM); in position 543, E (v-*src*HM) → A (v-*src*LM); and in position 544 of v-*src*LM the absence of valine was caused by a deletion of 3 nucleotides (Figs. 1 and 3). The mutations inside the C-terminal tail were unexpected, because this region was shown to be well conserved in all v-*src* genes from the main strains of RSV. Among the eight residue changes between v-*src*HM and v-*src*LM, four mutations represent substitutions of amino acids pertaining to the same group in the mutation matrix of Dayhoff and are not expected to affect the function of the *Src* protein (positions 76, 81, 522, 541). Therefore, the amino acid differences that are likely to be re-

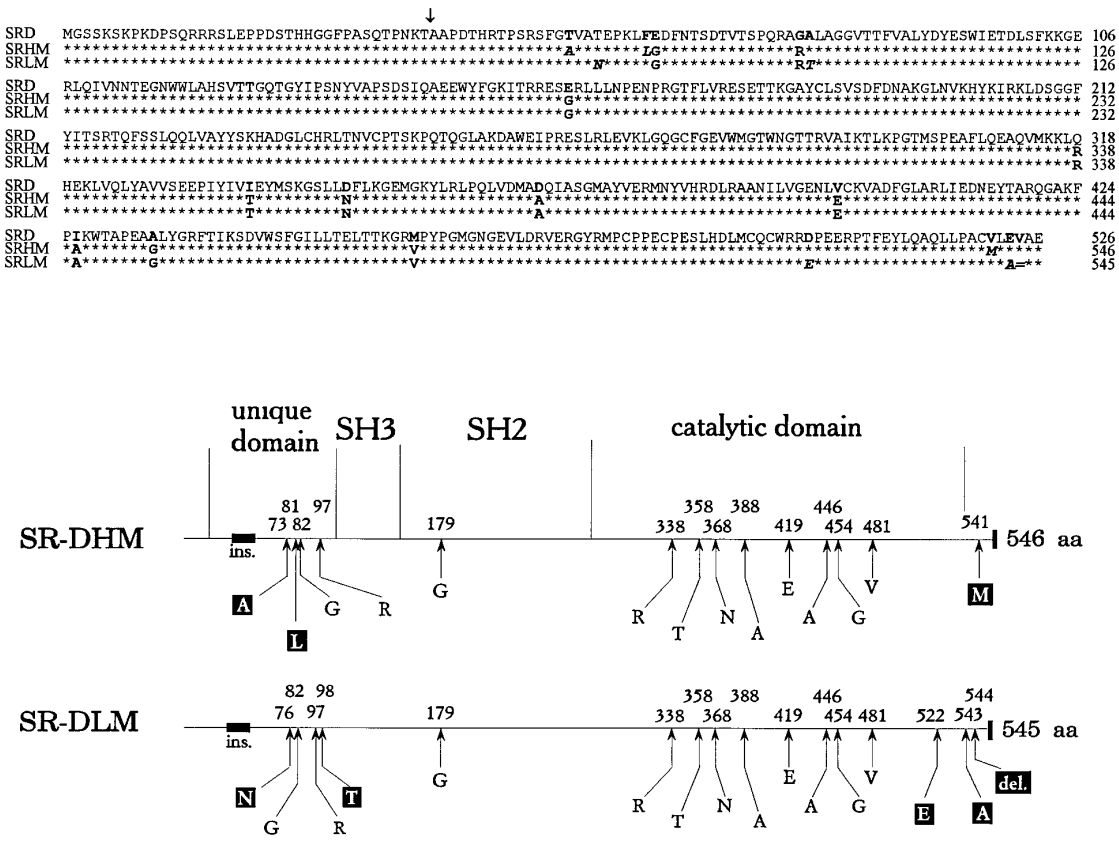

FIG. 3. Comparison of amino acid sequences of SR-D v-*src* (Reddy *et al.,* 1990) with v-*src*HM and v-*src*LM and schematic representation of amino acid mutations of v-*src* proteins in SRHM and SRLM. Top: the site of the 20-amino-acid insertion within v-*src*HM and v-*src*LM is indicated by an arrow. Bottom: mutations which differentiate SRHM from SRLM are indicated in white letters with a black background.

sponsible for the metastatic status of the tumor cells were two alanine–threonine substitutions at residues 73 and 98, a glutamic acid–alanine replacement at residue 543, and the deletion of valine at position 544.

Plasmid constructs expressing v-*src*HM, v-*src*LM, and chimeric v-*src* genes

To analyze the biological properties of these new v-*src*HM and v-*src*LM variants, retrovirus-based plasmids carrying these oncogenes were constructed. For this purpose we used a retroviral vector, pIC10*Neo,* derived from the IC10 virus (Felder *et al.,* 1994).

The cloned *Eco*RI v-*src*-containing fragments from both types of RSV proviruses were digested by *Mae*I and 2.3-kb fragments containing the coding sequence of v-*src* and its splice-acceptor site were isolated. After addition of an *Xba*I linker, v-*src*HM and v-*src*LM were cloned in the *Xba*I site of pIC10*Neo.* The resulting plasmids were named pICHM and pICLM, respectively.

To identify the specific region of v-*src*HM and v-*src*LM that is associated with the gain or loss of metastatic ability, chimeric genes containing reciprocally substituted 5′ and 3′ moieties of *src* were constructed. The strategy was similar to that used earlier (Jove *et al.,* 1986; Dezélée *et al.,* 1992): since an *Mlu*I site is located in the middle of the v-*src* coding sequence and another is present 112 base pairs downstream of the v-*src* stop codon, digestion of pICHM or pICLM plasmids by this enzyme yields two fragments: the smallest (920 bp) contains the 3′ moiety of the gene, whereas the largest (7.6 kb) contains the 5′ moiety. By ligation of the appropriate fragments the following chimeric genes were constructed: 5′*src*HM–3′*src*LM (v-*src*HL), and 5′*src*LM–3′*src*HM (v-*src*LH). The corresponding plasmids were named pICHL and pICLH, respectively.

All four plasmids (pICHM, pICLM, pICHL, and pICLH) were transfected into a packaging cell line (Kundry), producing helper-free nonreplicative retrovirus with a C-subgroup envelope (Cosset *et al.,* 1990, 1992). We thus obtained stocks of retrovirus infectious for both avian and mammalian cells, carrying v-*src*HM, v-*src*LM, and the chimeric genes v-*src*LH and v-*src*HL.

Properties of v-*src*HM and v-*src*LM genes

Plasmids and derived virus stocks expressing v-*src*HM, v-*src*LM, and the chimeric v-*src* genes were used to study the morphological transformation of neuroretina cells, the metastatic activity of transformed cells, and the structure of the fibronectin matrix.

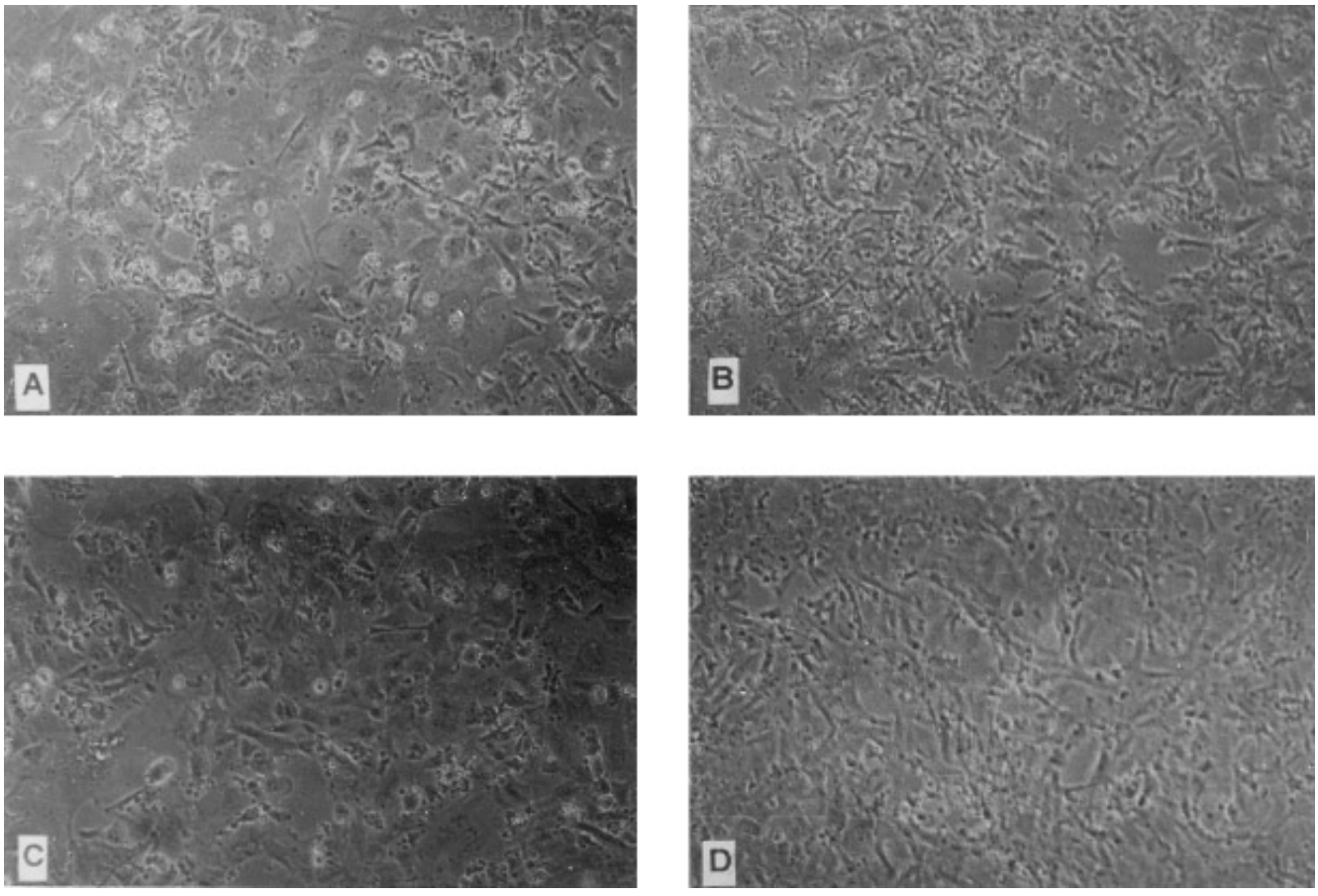

FIG. 4. Morphology of avian neuroretina cells infected with viruses carrying v-*src*HM, v-*src*LM, and chimeric variants of these genes. (A) v-*src*HM-infected NR cells; (B) v-*src*LM-infected NR cells; (C) v-*src*LH-infected NR cells; (D) v-*src*HL-infected NR cells.

Morphological transformation of neuroretina cells. We previously showed that infection of avian embryonic NR cells with RSV results in transformation and in acquisition of sustained proliferation capacity (Pessac and Calothy, 1974). The mitogenic activity of RSV requires continuous expression of a functional $p60^{v\text{-}src}$ (Calothy *et al.,* 1980). These cells proved useful in analyzing v-*src* variants that retain mitogenic capacity but display reduced transforming properties and are therefore suitable to identify distinct morphological phenotypes (Calothy *et al.,* 1980). To analyze the effects of the amino acid substitutions localized in the unique and the C-terminal regions of v-*src*HM and v-*src*LM, retroviruses carrying v-*src*HM, v-*src*LM, and chimeric v-*src*LH and v-*src*HL were used for infection of chicken neuroretina cells. All four viral variants expressed mitogenic activity and transformed infected cells. However, NR cells infected by v-*src*HM and v-*src*LH viruses had similar morphology, with the presence of abundant rounded morphologically transformed cells (Figs. 4A and 4C). In contrast, cells infected by v-*src*LM and v-*src*HL viruses displayed a less transformed phenotype. The majority of cells had an elongated shape and almost no rounded cells were visible (Figs. 4B and 4D). This result suggested that the differences in biological properties of v-*src*HM and v-*src*LM isoforms were associated with structural changes in the C-terminal region of the protein.

Metastatic activity. To compare the metastatic activity of the various v-*src* genes, pICHM, pICLM, pICHL, and pICLH vectors were transfected in the HET-SR LM transformed cells. Several G418-resistant colonies from each transfected cell culture were selected. We identified the presence of 2 to 10 copies of introduced plasmids in the different cell clones by restriction mapping of the integrated vector (data not shown). Only cells carrying exogenous intact v-*src* genes were used in subsequent metastatic assays *in vivo.*

The cells were injected in syngenic Syrian hamsters (15–20 animals for each cell line). The results of analysis of pICHM-transfected cells (HETHM-11, HETHM-13), pICLM-transfected cells (HETLM-7, HETLM-14), pICHL (HETHL-22), and pICLH (HETLH-25) are presented in Fig. 6, in comparison with the parental low and high metastatic cells. The tumorigenic activity of all transfected cells was high and corresponded to that of parental cells (Deichman *et al.,* 1992). Cells transfected with v-*src*HM remarkably increased their metastatic potential since almost half of inoculated animals presented more than 50

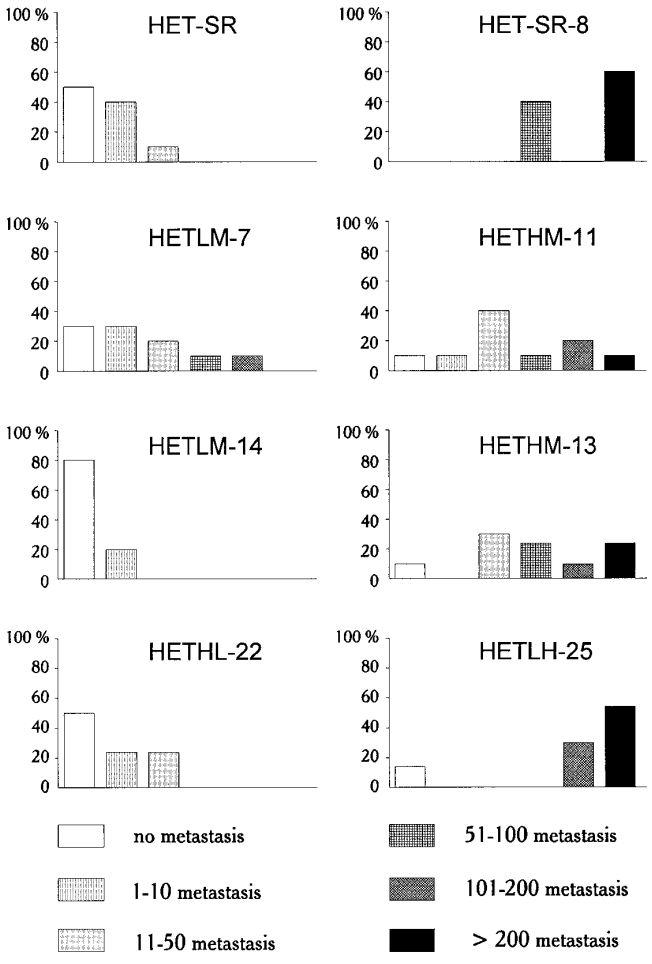

FIG. 5. Metastatic activity *in vivo* of HET-SR cells transfected with v-*src*HM, v-*src*LM genes, and chimeric genes v-*src*LH and v-*src*HL (see text).

metastatic nodules in the lungs. Moreover, hamsters with 100–200 metastases were found in 30–35% of the cases. Only 10–20% of the animals injected with HETHM-11 and HETHM-13 had less than 10 metastatic nodules in the lungs (Fig. 5). In contrast, in 60% of HETLM-7-injected hamsters, the number of metastatic nodules was less than 10, and 50 or more nodules were found in only 20% of the cases. In the second v-*src*LM-transfected cell line (HETLM-14) metastatic activity was very low (Fig. 5). Several other cell lines carrying v-*src*HM and v-*src*LM genes demonstrated a similar pattern of potential metastatic activity *in vivo* (data not shown). A cell line transfected with the chimeric v-*src*HL gene (HETHL-22) had a rather low metastatic potential in contrast to a cell line (HETLH-25) transfected with the chimeric gene v-*src*LH. This result suggests that the changes in the C-terminal moiety of v-*src*HM protein are likely responsible for the high metastatic phenotype. The level of metastatic activity of transfected cells was not correlated to the number of integrated v-*src*HM or v-*src*LM copies (data not shown).

Structure of the fibronectin matrix. The degradation of extracellular fibronectin, a major extracellular adhesion protein, is believed to play an important role in cell migration through the extracellular matrix during tumor invasion (Liotta, 1986; Stetler-Stevenson *et al.,* 1993). Usually, fibronectin is lost from the cell surface after transformation with RSV. Disappearance of fibronectin from the extracellular matrix is probably due to proteases, activated as a result of $p60^{src}$ kinase effects on the plasma membrane at the cell/substartum contact sites (Chen *et al.,* 1984, 1985). The structure of the fibronectin matrix was examined in HET-SR cells containing transfected v-*src*HM and v-*src*LM genes. Indirect immunofluorescent staining using rabbit polyclonal antibodies showed that all clones hardly formed some fibronectin fibers in sparse culture. However, significant differences in the fibronectin matrix formed by cells in dense cultures were observed. In monolayer cultures of nontransfected low metastatic HET-SR cells, we observed the presence of a substantial amount of thin fibers organized in a nonregular loose network under most of the cells (Fig. 6A). Similarly, in HETLM-7 and HETLM-14 clones short fibers and patches formed separated small networks under approximately half of the cells (Figs. 6C and 6D). In comparison, in cultures of HETHM-11 and HETHM-13, fibronectin structure appeared as short fibers and patches under a few cells (Figs. 6E and 6F). A similar picture was observed in the parental high metastatic HET-SR-8 cells (Fig. 6B).

Thus, the differences in fibronectin distribution and *in vivo* metastatic activity appear to be related to the introduced genes: v-srcHM-transfected cells acquired the metastatic potential and lost fibronectin matrix, whereas v-*src*LM-transfected cells did not undergo significant changes in fibronectin organization.

DISCUSSION

We have cloned two novel variants of the v-*src* oncogene and studied their possible involvement in metastasis. Several amino acid mutations found only in v-*src*HM and v-*src*LM were characterized in the different domains of these v-*src* proteins. The most unusual structural alteration is the existence of an insert of 60 nucleotides within the unique region of these genes. This insert had no significant effect on the tumorigenic activity of the transformed cells and does not seem to be associated with the difference in the metastatic potential since it was present in both v-*src*HM and v-*src*LM. This result is in agreement with a previous report showing that linker insertions within the unique domain of *src* gene do not alter the phenotypic transformation of the cells (DeClue and Martin, 1989).

Although the central core of the catalytic domain is highly conserved in c-*src* and different v-*src* genes, several coding and silent mutations were found in v-*src*HM and v-*src*LM, within regions 382 to 419 and 427 to 460

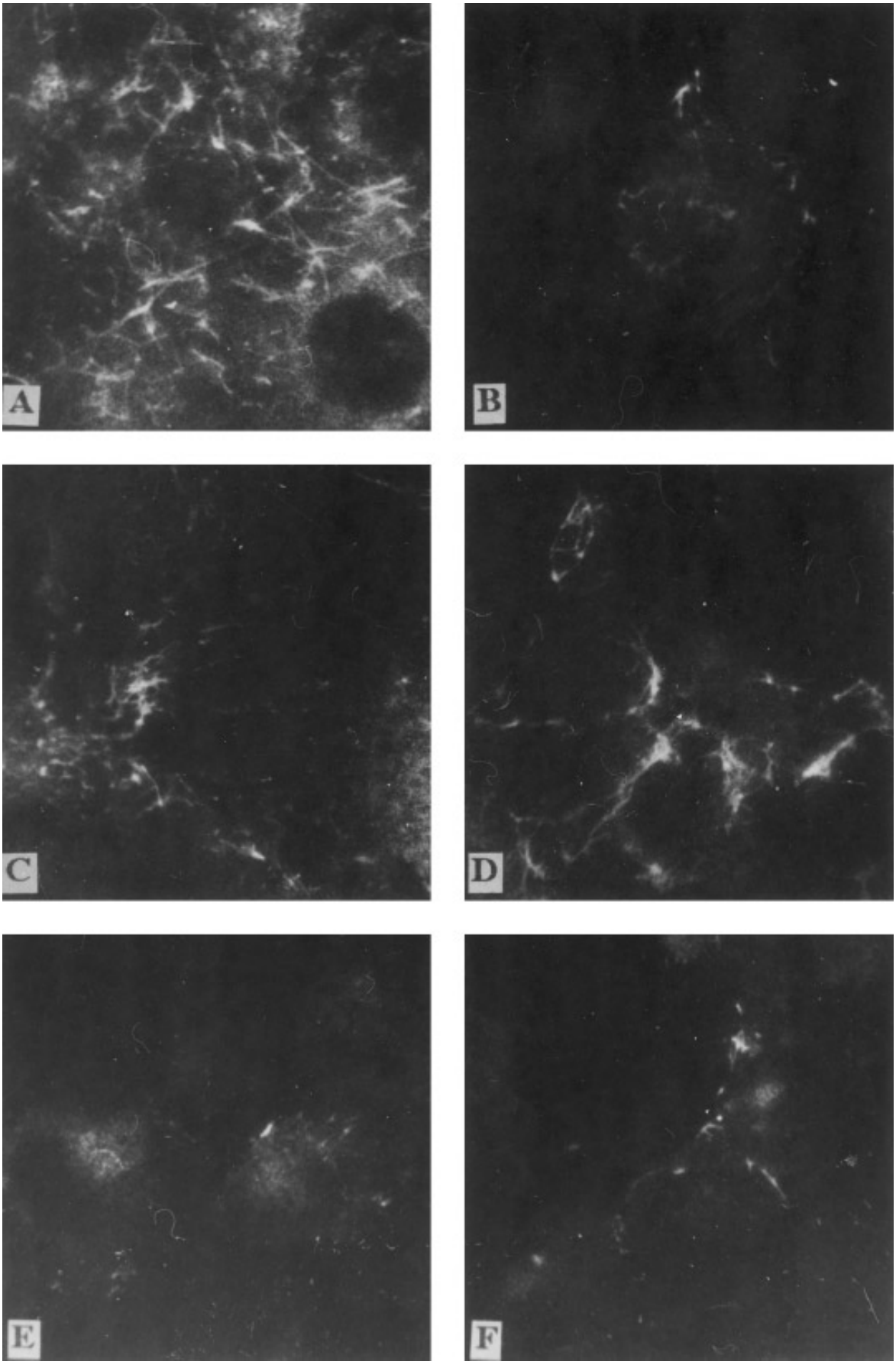

FIG. 6. Structure of fibronectin matrix of HET-SR cells transfected with v-*src*HM and v-*src*LM genes. (A) HET-SR cells; (B) HET-SR-8 cells; (C) HETLM-7 cells; (D) HETLM-14 cells; (E) HETHM-11 cells; (F) HETHM-13 cells.

(Fig. 3). These amino acid replacements (residues 419 and 454 in v-*src*HM and v-*src*LM corresponding to residues 399 and 434 in all other *src* genes) showed in contrast to previous reports (Parsons and Weber, 1989; Reddy *et al.,* 1990) that the intactness of this area is not absolutely required for v-*src* gene activity. In three instances, the mutations in *src*HM or v-*src*LM were identical to those found in v-*src* from the Bryan strain of RSV: A → G at position 217 (v-*src*HM and LM), A → G at position 536, and a paired transition AT → GC at positions 1337–1338 (Fig. 1).

Our main result is the identification of naturally occurring highly tumorigenic variants of the v-*src* oncogene which apparently induced different levels of metastatic activity of the corresponding transformed cells. The demonstration that expression of v-*src* from HM cell lines could change the phenotype of LM cells to that of HM showed that specific structures of the described genes may be responsible for the acquisition or loss of metastatic potential. V-*src*HM and v-*src*LM differ in several amino acid replacements, mostly localized within the unique domain and in the carboxy-terminal region of the oncoprotein. The distinctions in the metastatic potential and the type of morphological transformation of NR cells, induced by chimeric variants of v-*src*HM and v-*src*LM, indicate that mutations in the C-terminal part of the gene are likely to be responsible for the differences in their transforming properties. Three amino acid changes and one deletion in v-*src*LM are located in this area. Two of them are in the very end of the carboxy-terminal region, which is not present in the cellular *src* protein, but is found in all v-*src* proteins, including v-*src*HM. Although it was earlier shown that changes in carboxy-terminal amino acids of v-*src* do not affect the kinase activity or cell transformation (Yaciuk and Shalloway, 1986), our results suggest that it is likely that the differences between v-*src*HM and v-*src*LM in this site are responsible for the differences in the metastatic activity. Among these, the exchanges of E522 (v-*src*LM) for D and V541 (v-*src*LM) for M are unlikely to modify the structure of the v-*src* protein because they belong to the same groups of conformational homology. For this reason we undertook the mutagenesis of v-*src*LM by the change of A543 in E, the reinsertion of V544, or both changes. We are currently investigating the transforming properties of these mutants.

We previously reported that v-*src* proteins from high and low metastatic RSV transformed hamster cells displayed similar levels of autophosphorylating and transphosphorylating tyrosine kinase activity *in vitro* (Topol *et al.,* 1993). Also, no direct correlation between the amount of $p62^{src}$ protein and metastatic capacity was found in HET-SR-transfected cells (data not shown). According to preliminary analysis, the overall phosphotyrosine level in cellular proteins was approximately similar in all cell lines and independent of their metastatic potential (data not shown). Thus, no specific differences were found at the level of production or catalytic properties of v-*src*HM and v-*src*LM proteins, associated with the metastatic potential of transformed cells. However, this does not exclude that the structural changes between v-*src*HM and v-*src*LM, notably in the C-terminal region, may influence the metastatic properties by subtle modifications of the interactions of both genes with v-*src*-specific substrates or with components of the v-*src* signaling complex. These effects could be connected either with amino acid substitutions in the protein tail by themselves or in combination with mutations in other parts of the gene. Since wild-type RSV v-*src* was shown to be HM (Egan *et al.,* 1987; Stoker and Sieweke, 1989), it is possible that the 20-amino-acid insertion in the unique domain contributes to the LM phenotype, whereas additional differences between LM and HM would restore an HM capacity.

It is clear that an oncogene product per se does not directly mediate all the phenotypic changes required for metastatic behavior of tumor cells. A cascade of additional cellular factors are involved in this process. Identification of the peculiarities of protein interactions with the v-*src*HM and v-*src*LM products is necessary to understand the various biological effects of these genes in transformed cells.

MATERIALS AND METHODS

Cells

HET-SR, HET-SR-1, and HET-SR-8 are hamster cell lines transformed *in vitro* with different stocks of the Schmidt–Ruppin D strain of RSV from Russian Cancer Research Center viral collection (Deichman *et al.,* 1989). HET-SR was low metastatic, whereas HET-SR-1 and HET-SR-8 are highly metastatic (Deichman *et al.,* 1989, 1992). All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 100 mg/ml gentamycin at 37° in 5% CO_2 atmosphere.

Preparation and infection of neuroretinal (NR) cell cultures from 7-day-old chicken was previously described (Pessac and Calothy, 1974). They were maintained in Eagle's basal medium supplemented with 5% fetal calf serum.

Molecular cloning and DNA sequencing

High-molecular-weight cellular DNA was prepared from HET-SR and HET-SR-8 cell lines, digested with *Eco*RI, and subjected to 0.8% agarose gel electrophoresis. DNA fragments of 2.8–3.4 kb in size were recovered using the "Geneclean" technique (Bio101, La Jolla), ligated to purified *Eco*RI arms of λgt10 phage, and packaged *in vitro* by standard procedures (Maniatis *et al.,* 1982). Recombinant clones containing v-*src* sequences were selected by plaque hybridization with a ^{32}P-labeled *src*-specific *Pvu*II fragment (DeLorbe *et al.,* 1980). Several

clones from each gene library were further purified by three rounds of plaque purification. The 3.1 kb of RSV provirus containing the 3′ end of the *env*–v-*src*–U3 region of LTR from both libraries was subcloned into the *Eco*RI site of Bluescript (KS^+) plasmid ($pBSKS^+$). These clones were sequenced by the dideoxy-chain termination method using internal oligonucleotide primers (Sanger, 1981). All regions were sequenced at least twice on both DNA strands.

Retroviral vector construction

To prepare expression vectors carrying the cloned genes, we used a nonreplicative retroviral vector, pIC10-*Neo,* based on virus pIC10 (Felder *et al.,* 1994). Briefly, pIC10*Neo* contains the 5′ part of avian retroviral *gag* sequences with the *gag* splice donor, an *Xba*I site for cloning the gene of interest, introduced by addition of the 1062 New England Biolabs *Xba*I amber linker to the blunted *Xho*I site in *gag,* and the neomycin resistance gene (*Neo*) preceded by an AEV-derived splice acceptor. These regions are flanked by LTR from the avian IC10 virus, which derived from the RAV-1 LTR (Eychène *et al.,* 1989), and cloned in *Hind*III and *Sac*I sites of $pBSKS^+$. The strategy of *src* variant cloning is described under Results.

DNA transfection and recovery of infectious viruses

HET-SR cells were transfected by the calcium phosphate precipitation method (Wigler *et al.,* 1978). Briefly, the cells were plated 24 hr before transfection. Plasmid DNA was used for transfection of cells according to standard protocols. Precipitates were removed after 4 hr and the cells were dispersed and replated at 1:2 dilution. Cells expressing the *neo* gene were selected in medium containing 400 μg/ml G418.

To obtain retrovirus of the C-subgroup envelope carrying the different isoforms and chimeric variants of *src* gene, a recently described ALV-based packaging cell line (Kundry) producing helper-free avian viruses was used (Cosset *et al.,* 1990, 1992). DNAs from expression vectors were transfected into Kundry cells. Transfected cells were selected in medium containing 200 μg G418 per milliliter. Resistant colonies were isolated after 12–15 days. Fresh medium was added on confluent producer cells and 6–8 hr later virus samples were harvested and kept at −80°. Recovered viruses were then used to infect NR cells according to standard protocol (Pessac and Calothy, 1974).

Metastatic activity assay

Spontaneous metastatic activity of different cells was determined by examining autopsied Syrian hamsters at least 2 months after subcutaneous inoculation of about 10^4 cultured cells. The number of lung metastases was counted using a dissection microscope (Deichman *et al.,* 1992). Groups of 5 to 20 normal adult animals were used for each cell line.

Immunofluorescence study

For fibronectin detection indirect immunofluorescense assay with polyclonal antibodies to fibronectin was used (Ljubimov *et al.,* 1985). Extraction and fixation were performed as described (Bershadsky *et al.,* 1987). Cells were examined by photomicroscope 3 (Opton) 40× oil-immersion objective.

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