The Embryonic Environment Strongly Attenuates v-*src* Oncogenesis in Mesenchymal and Epithelial Tissues, but Not in Endothelia

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Abstract. We demonstrate that the behavior of cells expressing v-*src*, a tyrosine kinase oncogene, differs profoundly between the embryonic and culture environments. V-*src* was introduced into avian embryo cells both in culture and in stage-24 embryo limbs, using replication-defective retroviral vectors. These vectors were used as single-hit, cellular markers to determine the environmental influences imposed by normal cells and tissues on clonal cell growth. The marker gene *lacZ* was coexpressed with v-*src* in order to locate the descendent cells. In culture, v-*src* induced rapid morphological transformation and anchorage-independent growth of embryo fibroblasts; the vectors were also tumorigenic in hatchling chickens. In contrast, most of the cell clones expressing v-*src* in the embryo grew normally without neoplasia. Expression of v-*src* vectors could be found in a wide range of cell types, demonstrating not only that neoplastic transformation is attenuated in ovo, but also that differentiation commitment in many lineages can be maintained concurrently with oncogene expression. Significantly, the embryonic control of cell growth could be perturbed by v-*src* under certain conditions. Rare, marked clones showed hyperplasia or dysplasia, and the primitive endothelium could succumb to rapid neoplasia; thus, these embryonic tissues are not inherently deficient in transformation factors. We propose that the environmental conditions imposed on cells in ovo are critical for the attenuation of neoplasia, while cultured cells lose this requisite environment.

It is widely accepted that proteins of the tyrosine kinase family play an important role in both carcinogenesis and embryonic development. The neoplastic transformation of cultured cells by activated tyrosine kinases is well established (for review see Bishop and Varmus, 1982), while critical roles for their endogenous counterparts in controlling embryonic events are suggested by their fetal expression, and evolutionary conservation (Cotton and Brugge, 1983; Hoffman et al., 1983; Levy et al., 1984; for review see Muller, 1986). The mechanisms of action of these proteins remain unclear, as do the reciprocal mechanisms for controlling their activities. Moreover, while the consequences of neoplastic and embryonic cell growth are quite different, it is known that traits such as migratory or invasive behavior, and continued growth at high cell density are common qualities (for review see Levine et al., 1984). Some of these traits may be related manifestations of tyrosine kinase action, seen under different cell and developmental conditions. One might therefore predict that the cellular response to tyrosine kinase activation would vary significantly depending upon the cell's environmental context. To support this, there are documented examples showing that oncogenesis by tyrosine kinases may be subject to embryo- and differentiation-induced modulation (Birek et al., 1988; Carmier and Samarut, 1986; Dolberg and Bissell, 1984; Milford and Duran-Reynals, 1943).

Our past work has shown that expression of Rous sarcoma virus (RSV)[1] is compatible with apparently normal myogenic development in ovo (Dolberg and Bissell, 1984; Howlett et al., 1988). RSV is normally a highly oncogenic retrovirus in cultured cells and in animals, expressing the v-*src* oncogene and its tyrosine kinase product $pp60^{v\text{-}src}$ (Brugge and Erikson, 1977; Martin, 1970; Rous, 1911; for reviews see Teich et al., 1982, and Bishop and Varmus, 1982). The lack of sarcoma response in ovo, despite expression of active $pp60^{v\text{-}src}$ (Howlett et al., 1988), suggests that v-*src*–embryo interactions are unusual. However, further embryonic studies with RSV have been hindered by problems of restricted tissue tropism, and lethal hemorrhagic disorders (Coates et al., 1968; Duran-Reynals, 1940; Howlett et al., 1987). A new approach is described which overcomes these problems, permitting us to study clonal cell responses to v-*src* under conditions in culture and in numerous tissues in ovo. We can now address whether embryo cells are intrinsically nonresponsive to v-*src* action, or whether the tissue environment in ovo imposes a dominant counteractive influence over neoplastic stimuli.

Replication-defective (rd) retrovirus vectors were used here to mark cells genetically, using the *lacZ* histochemical marker gene to trace their positional and behavioral fates. A

1. *Abbreviations used in this paper*: CEF, chicken embryo fibroblast; EFU, expression focus unit; rd, replication defective; RSV, Rous sarcoma virus.

critical advantage of rd vectors is their ability to restrict oncogene expression to individual cells and their direct descendents. Such cell clones, grown in intimate contact with other cells and tissues, would be subject to the normal regulative interactions prevailing in the environment. We have focused upon expressing v-*src* in cultured chicken embryo fibroblasts (CEF), and cells within the chicken forelimb between day 4 (embryonic stage 24; Hamburger and Hamilton, 1951) and day 14 of development. The limb was chosen as an apposite model of morphogenesis and cellular differentiation involving such cells as myoblasts, chondroblasts, and fibroblasts which are susceptible to transformation by v-*src* under culture conditions (Moss et al., 1979; Boettiger et al., 1983; Pacifici et al., 1977; Hinchliffe and Johnson, 1980). Our rd v-*src* vectors exhibited clear oncogenic properties in cultured embryo fibroblasts and in chickens, as expected. In contrast, cell clones expressing v-*src* in ovo exhibited normal growth regulation in many tissues, with a number of informative exceptions including the induction of endothelial neoplasia. These data suggest mechanisms through which the environment can influence the developmental and oncogenic behavior of cells, and may shed light on critical cellular determinants of v-*src* action.

Materials and Methods

Construction of rd Retroviral Vectors

Retroviral vector plasmid (prefix p) pCKlacI was derived from an avian erythroblastosis virus-based vector lacking the internal v-*erb*A and v-*erb*B genes (pCK, a gift of B. Vennstrom and K. Khazaie, European Molecular Biology Laboratory, Heidelberg). A 3-kb Bam HI fragment containing bacterial *lacZ* (obtained from pSP6-βgal, a gift from C. Cepko (Harvard Medical School), containing *lacZ* from pMC1871 [Price et al., 1987]) was inserted into the 5′ Bam HI site in residual *gag* sequences. The β-galactosidase protein encoded by *lacZ* is amino terminally fused to the first 52 amino acids of viral *gag*. pBlacsrc2 was derived from pA11, a proviral clone of a reverse-transcriptase defective B77 RSV (A gift of J. Wyke, Beatson Institute, Glasgow, UK; Levantis et al., 1986). Structural gene sequences between positions 630 and 6,983 bp (Prague C RSV reference sequence; Beveren et al., 1985) were removed, and the resulting plasmid (pA11-b) was digested with Bam HI (position 530 bp); the *lacZ* gene was inserted as above. Vector Bsrc2 is a subgenomic derivative of Blacsrc2, created by viral transmission from packaging cells of spliced v-*src* mRNA (Stoker and Sieweke, 1989). A molecular clone representing vector Bsrc2 was constructed from pA11-b and pAScrc7 (a subgenomic vector structurally similar to Bsrc2; Stoker and Sieweke, 1989): the SstI-NcoI fragment (259-7,127 bp of RSV) was removed from pA11-b and replaced with the 5′ SstI-NcoI segment from pASrc7 containing the fused splice junction. The resulting vector pBsrc2a is exactly representative of Bsrc2 except for a short segment derived from Rous-associated virus-1 (equivalent of 259-398 bp of RSV).

Cells, Virus Production, and Virus Assays

Viruses were obtained from the ASLV packaging cell line Q2bn after transfection with uncut vector plasmids (Stoker and Bissell, 1988). Virus was assayed using either the immunocytochemical expression focus unit (EFU) procedure (Stoker and Bissell, 1987), or histochemistry using 5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside (Xgal). CEFs (obtained as described in Stoker and Bissell, 1988; specific pathogen-free white leghorn embryos; Spafas, Inc., Norwich, CT) were infected after plating 4×10^5 cells per 35-mm plate. After 4 d the cells were fixed in 2% paraformaldehyde for 20 min, and washed in PBS/0.1% BSA/0.25% Triton X100, for 5 min. EFU assays were performed using monoclonal anti-pp60$^{v\text{-}src}$ as the primary antibody (JB327, a gift of J. Brugge, University of Pennsylvania, Philadelphia); the streptavidin-linked alkaline phosphatase (Zymed Laboratories, Inc., San Francisco, CA) was used at 1:2,000 dilution (Stoker and Bissell, 1987). Xgal-treatment of infected CEF and tissues was performed as described by Price and co-workers (1987). Viruses (prefix rd) rdBlacsrc2 and rdBsrc2 were transmitted concurrently from the packaging cells in a ratio of $\sim$3:2, respectively; Blacsrc2 was stable for *lacZ* and v-*src* coexpression; only 1-2% of the derived virus was phenotypically *lac*[+]*src*[−] (details of these quantitation analyses will be presented elsewhere; A. W. Stoker, manuscript in preparation). Before injection into embryos, rd viruses were concentrated in Centricon-30 microconcentrators (Amicon Corp., Danvers, MA) at 5000 *g* for 30 min in an SS-34 rotor (Sorvall Instruments Div., Newton, CT) yielding a 10-20-fold increase in active virus titer.

Soft Agar and Tumor Assays

CEFs infected with viruses were seeded in soft agar (0.4% final concentration in medium 199 supplemented with 10% tryptose phosphate, 4% calf serum, 1% chicken serum). After incubation at 37°C for 9-14 d, the intact agar layer was fixed in 4% paraformaldehyde for 1 h, then treated with Xgal as above. Tumorigenicity was assayed by injecting virus intramuscularly into the wing webs of day 5 hatchling chickens (White leghorn; Spafas, Inc.). Tumor nodules were removed and fixed in 4% paraformaldehyde for 1 h, followed by a 1-h wash in PBS, 0.1 M glycine, 0.25% Triton X100. Tissues were treated with Xgal overnight.

Limb Bud Injection and Whole Mount Histochemistry

Virus was injected into day 4 limb buds (embryonic stage 24; Hamburger and Hamilton, 1951) as described previously (Dolberg and Bissell, 1984). Up to 500 viruses were injected per limb in a volume range of 0.1-0.5 μl. After further incubation, embryos were killed and limbs were fixed in 4% paraformaldehyde for 30 min (day 5-7 embryos) or 45-60 min (day 11-14 embryos), followed by washing in PBS, 0.1 M glycine, 0.25% Triton X100 for 30 min. Whole limbs were reacted with Xgal overnight at room temperature. Tissues being prepared for immunofluorescence were not reacted with Xgal before cryosectioning.

Cryosectioning and Immunofluorescence

Tissues with or without prior XgaI staining were equilibrated to 15% followed by 30% sucrose in PBS before embedding in OCT compound (Miles Laboratories, Inc., Elkhart, IN) and freezing in liquid nitrogen. Frozen sections (4-5 μm) were made using a Leitz Kryostat 1720, and collected on gelatin-coated slides. Sections were stained histologically using Diff-Quik (American Scientific Products Div., McGaw Park, IL). For immunofluorescence, serial sections were used from Xgal untreated tissues: sections were washed for 30 min in PBS, 0.5% Triton, 0.1% BSA, followed by the primary anti-pp60$^{v\text{-}src}$ antibody (v-*src* (Ab-1) [affinity-purified form of JB327], 1:10 dilution; Oncogene Science, Manhasset, NY) added for 2 h at room temperature in PBS, 0.25% Triton, 1% BSA. After washing in PBS, 0.1% BSA, 0.25% Triton for 1 h, the secondary antibody (biotinylated antimouse; Amersham Corp., Arlington Heights, IL, used at 1:50 dilution) was added for 1 h. After washing as above, the tertiary affinity layer (Streptavidin-conjugated Texas red, 1:100 dilution; Amersham Corp.) was added for 45 min, followed by overnight washing at 4°C. Sections were mounted in buffered glycerol with p-phenylenediamine (Sigma Chemical Co., St. Louis, MO), and photomicrographs were taken using a Zeiss Photomicroscope III and Ektachrome film (Eastman Kodak Co., Rochester, NY); cibachromes were printed by ZIBA Color Lab. (Berkeley, CA). Serial sections of those used for immunofluorescence were reacted with Xgal overnight.

Expression Assays for pp60$^{v\text{-}src}$

35-mm plates containing 4×10^5 quail QT6 cells were transfected with circular plasmid using the calcium phosphate procedure (Graham and Van der Eb, 1973). Either 0.4 or 0.8 μg of vector DNA was transfected per plate together with either 3.6 or 3.2 μg of pUC19 carrier DNA, respectively. Quadruplicate plates were used per experiment. After 20 h, a pair of plates from each set was placed with methionine-free medium 199 for 1 h, then metabolically labeled for 3 h with [^{35}S]methionine (Amersham Corp.). Cells were lysed in RIPA buffer and pp60^{src} was immunoprecipitated using excess monoclonal 327 or a monoclonal raised against residues 2-17 of pp60$^{v\text{-}src}$ (Hybridoma 203-7D10; National Cancer Institute/Biological Carcinogenesis Branch repository, Bethesda, MD). After polyacrylamide gel electrophoresis, the proteins were visualized using fluorography and preflashed film (X-Omat; Eastman Kodak Co.); intensities of pp60$^{v\text{-}src}$ bands were measured using scanning densitometry (Ultrascan densitometer/Integrator; Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The remaining pairs of plates were processed for Hirt DNA extraction as described in Arrigo et al. (1988). Hirt DNA was digested to completion with restriction

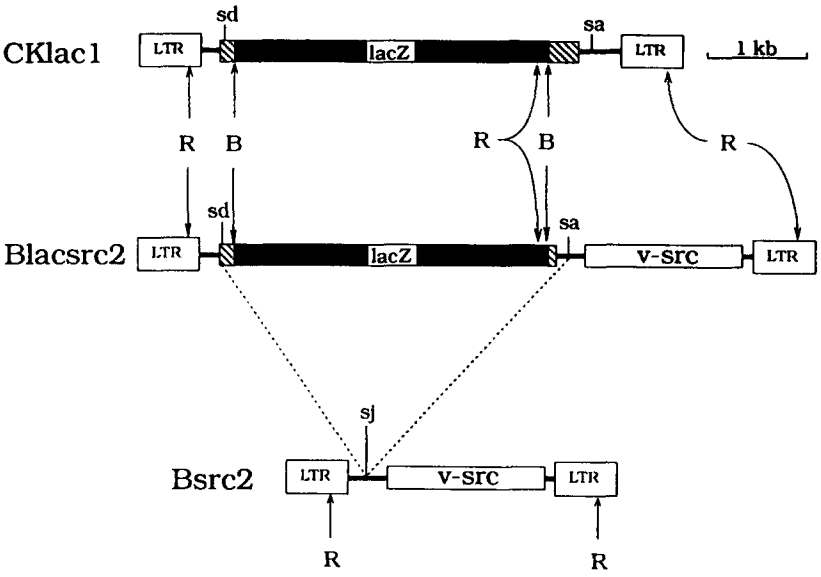


Figure 1. Proviral structures of rd vectors. Procedures for the construction of recombinant vectors are described in Materials and Methods. CKlacl is the control, nononcogenic vector. Blacsrc2 and Bsrc2 are derived from the unspliced and spliced RNAs, respectively, expressed from pBlacsrc2 in the packaging cells (loss of intron sequence is indicated by *dashed lines*); both rdBlacsrc2 and rdBsrc2 are released concurrently from these cells. *sa*, *sd*, and *sj*, represent splice acceptor, donor, and junction, respectively. *LTR* represents viral long terminal repeat. Hatched boxes represent residual structural gene sequences. Restriction sites (*E*) Eco RI; (*B*) Bam HI.

enzyme Eco RI, and subjected to agarose gel electrophoresis. DNA was transferred to nylon membranes (Hybond-N plus; Amersham Corp.), and hybridized to a 600 bp PstI v-*src* probe (8,054 to 8,666 bp of RSV) labeled by random priming with [^{32}P]dCTP (Feinberg and Vogelstein, 1983). Hybridized DNA was quantitated by scintillation counting.

Results

Construction and Characterization of rd Vectors

rd retroviral vectors were constructed to provide an efficient means of gene transduction both into cultured cells and tissues in the embryo (Fig. 1). An important characteristic of these vectors was their ability to express the histochemical marker gene *lacZ*, encoding bacterial β-galactosidase; vector Blacsrc2 was also designed to coexpress the oncogene v-*src*. Infectious retroviruses were obtained after transfection of plasmid vector DNA into retroviral packaging cells (Stoker and Bissell, 1988). Significantly, expression of vector Blacsrc2 in these cells caused the release of not only rdBlacsrc2, but also its subgenomic derivative virus, rdBsrc2 (Fig. 1) (Stoker and Sieweke, 1989). Thus, it should be noted that in all studies using these viruses, a mixed population of rdBlacsrc2 and rdBsrc2 is used. The two vectors infect cells independently, and these cells can be identified by their differential expression of *lacZ* and pp60$^{v\text{-}src}$ (see below and Materials and Methods). The titers of rdCKlacl, rdBlacsrc2, and rdBsrc2 ranged from 10^4–5×10^4/ml before concentration; titers up to 10^6/ml were obtained after concentration.

Transforming Properties of rd Vectors in Culture

To compare the behavior of cells expressing v-*src* under culture and embryonic conditions, the vectors Blacsrc2 and Bsrc2 were first introduced into CEF in monolayer culture. CEFs were infected with rd vectors and after 4 d the cells were treated with Xgal to visualize cells expressing Blacsrc2 or CKlacl, followed by the immunocytochemical EFU assay to visualize those cells expressing pp60$^{v\text{-}src}$ from Bsrc2 (see Materials and Methods). In this period the majority of cells expressing Blacsrc2 had formed distinct, well-transformed foci; the same being true for Bsrc2 (Fig. 2, *A*, *B*, and *F*). CEF infected with rdCKlacl remained morphologically indistinguishable from uninfected cells (Fig. 2 *E*). The second, and more stringent parameter of carcinogenicity tested, was cell growth in suspension media (Shin et al., 1975). After seeding in soft agar, CEF infected with rdBlacsrc2 and rdBsrc2 grew and formed compact colonies, whereas mock-infected CEF failed to grow. Approximately 75% of colonies reacted uniformly with Xgal, indicating that Blacsrc2 induced suspension growth (Fig. 2 *C*); the remaining colonies were devoid of Xgal stain showing that Bsrc2 induces similar growth (Fig. 2 *G*). Significantly, the ratios of colonies induced by Blacsrc2 and Bsrc2 in agar were close to the frequencies of the two rd viruses (3:2); both vectors thus sustained suspension growth with similar efficiency. Furthermore, no significant differences were seen in the morphology or average sizes of colonies induced by RSV, Blacsrc2, or Bsrc2. Under culture conditions, therefore, these vectors are highly oncogenic.

Oncogenicity of v-src Vectors in Hatchling Chickens

As a further test of v-*src* vector carcinogenicity, rd viruses were injected intramuscularly into the wing webs of hatchling chickens. Tumor nodules were palpable 7 to 8 d after inoculation, and these could grow rapidly for several weeks (Fig. 2 *H*). Tumors formed in nearly 100% of injected chickens, with the lower limit of inoculation being approximately one to two viruses (Fig. 2 *H*). Tissues removed 14–16 d after inoculation were stained with Xgal, revealing that focal growths of Xgal reactive cells, 0.5–4 mm in diameter, were present (Fig. 2 *D*). The Blacsrc2-induced growths showed characteristics of fibrosarcomas by histology (data not shown). 80–90% of tumor nodules did not react with Xgal, and Southern analysis of tumor DNA showed that Bsrc2 was invariably present in these (not shown). Both Blacsrc2 and Bsrc2 were therefore carcinogenic in hatchling chicken tissues, although the subgenomic vector Bsrc2 was more aggressive in this tumor assay. This is in agreement with recent tumor studies performed with related rd vectors (Stoker and Sieweke, 1989). It is important to note that in

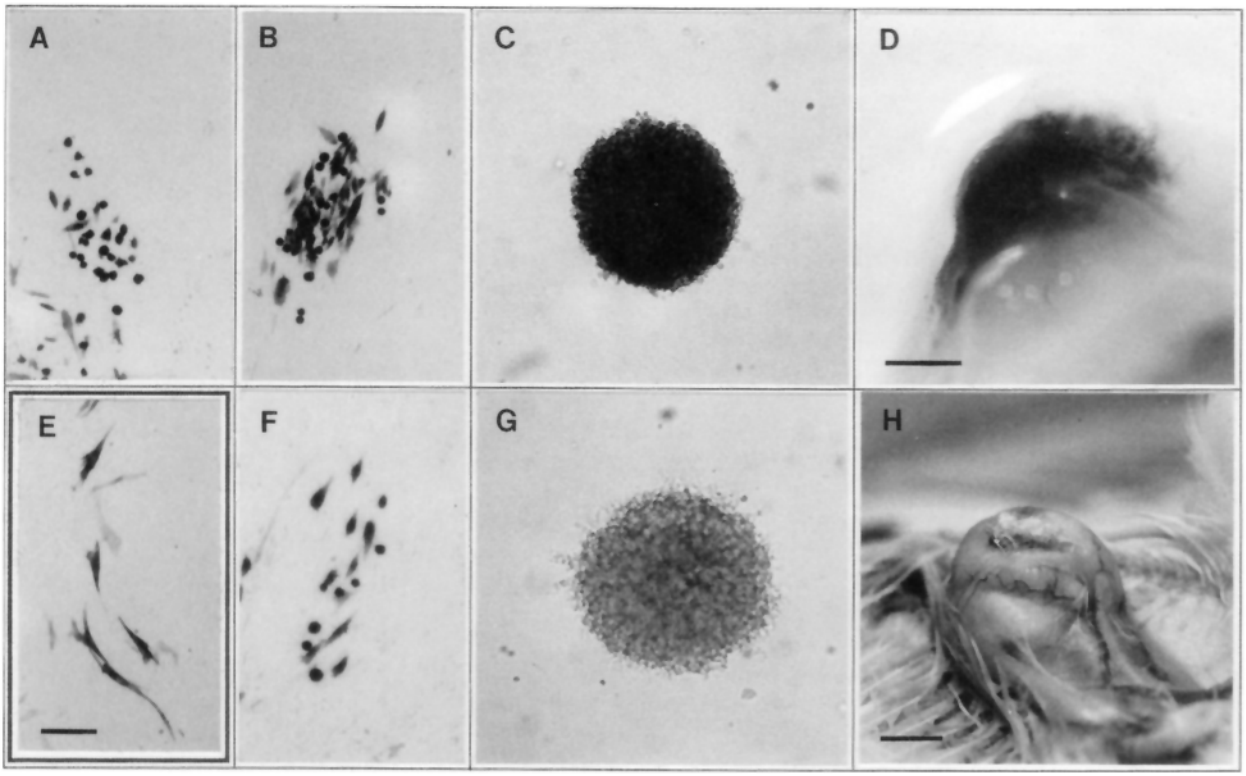


Figure 2. Oncogenic properties of rd vectors in culture and hatched chickens. (*A* and *B*) Transformed CEF foci expressing Blacsrc2 and stained with Xgal 4 d after infection. The background is a monolayer of uninfected, Xgal nonreactive CEF. (*C*) Soft agar colony of CEF expressing Blacsrc2 14 d after seeding. Cells were stained intensely blue (appearing dark) with Xgal. (*D*) Small tumor nodule removed 14 d after inoculation of wing tissue with rdBlacsrc2; the Xgal-stained tumor tissue is seen in whole mount. (*E*) Nontransformed CEF expressing CKlacl and stained with Xgal in monolayer culture 4 d after infection. (*F*) Transformed CEF focus expressing Bsrc2, immunochemically stained for $pp60^{v\text{-}src}$ using the EFU assay (Stoker and Bissell, 1987). The cell monolayer had received prior treatment with Xgal and the cells shown were nonreactive. (*G*) Soft agar colony of CEF expressing Bsrc2; nonreactive with Xgal. (*H*) Wing web tumor induced by rdBsrc2, growing at 2 mo after inoculation; approximately two viruses had been injected. This tumor was clonal, containing a single proviral copy of Bsrc2 (A. W. Stoker, unpublished data). Bars: (*D*) 0.4 mm; (*A–G* same scale) 0.1 mm; (*H*) 1 cm.

hatched chickens the target cells for v-*src* carcinogenesis are as yet undefined.

Cell Lineage Marking In Ovo with a Control rd Vector

Having determined the neoplastic response of cultured cells to v-*src*, the comparative response of embryo limb cells in ovo was examined. The first stage of this analysis involved injecting stage 24 limb primordia with the control virus rdCKlacl, to determine both the range of infectable progenitor cell types, and their normal growth characteristics. Limbs were allowed to develop after injection and were next examined at days 11–14 of development. To detect the descendents of infected progenitor cells, limbs were treated in whole mount with Xgal. Marked cells were located readily in this way (Fig. 3). 1–20 clusters of marked cells were observed in each limb, occurring predominantly in the lower arm and wrist regions. Histologically, these clusters were usually of a single cell type, thus, although strict clonality could not be proven it was considered probable in most cases. Marked cell clones were located predominantly within mesenchymal tissues, including the dermis, loose connective tissues, perichondrium, periosteum, and cartilage (Fig. 3, *C* and *D*). CKlacl-expressing cells were also located within skeletal muscle blocks, although here the possible cell types which were marked, e.g., myotubes, myogenic satellite cells, or the perimesium, were not determined unequivocally. Epidermal clones were observed in both the inner, basal layers, and more frequently in the outer, peridermal layers (Fig. 3, *A* and *B*). All marked clones were incorporated into the normal cytostructure of tissues. These data demonstrate the utility of this clonal cell marking technique, and in particular the ability of rd viruses to infect progenitors of, and be expressed within, a broad range of cell lineages in the embryo.

To determine whether expression of vector-encoded genes initiated soon after their introduction in ovo, rdCKlacl expression was examined 9 and 19 h after injection of the virus. By 9 h, individual Xgal-reactive cells could be seen in the limb appendage, and by 19 h marked cells were distinguishable in both ectodermal and mesenchymal compartments, some already present as sister-paired cells (data not shown). rd vectors thus act as generalized markers of embryo cells with rapid onset of expression.

Normal Embryonic Cell Growth with Concurrent Expression of v-src

rd v-*src* vectors were introduced into day 4 embryo limbs as described for CKlacl above. Cell clones expressing the vectors were later detected through their reactivity with Xgal

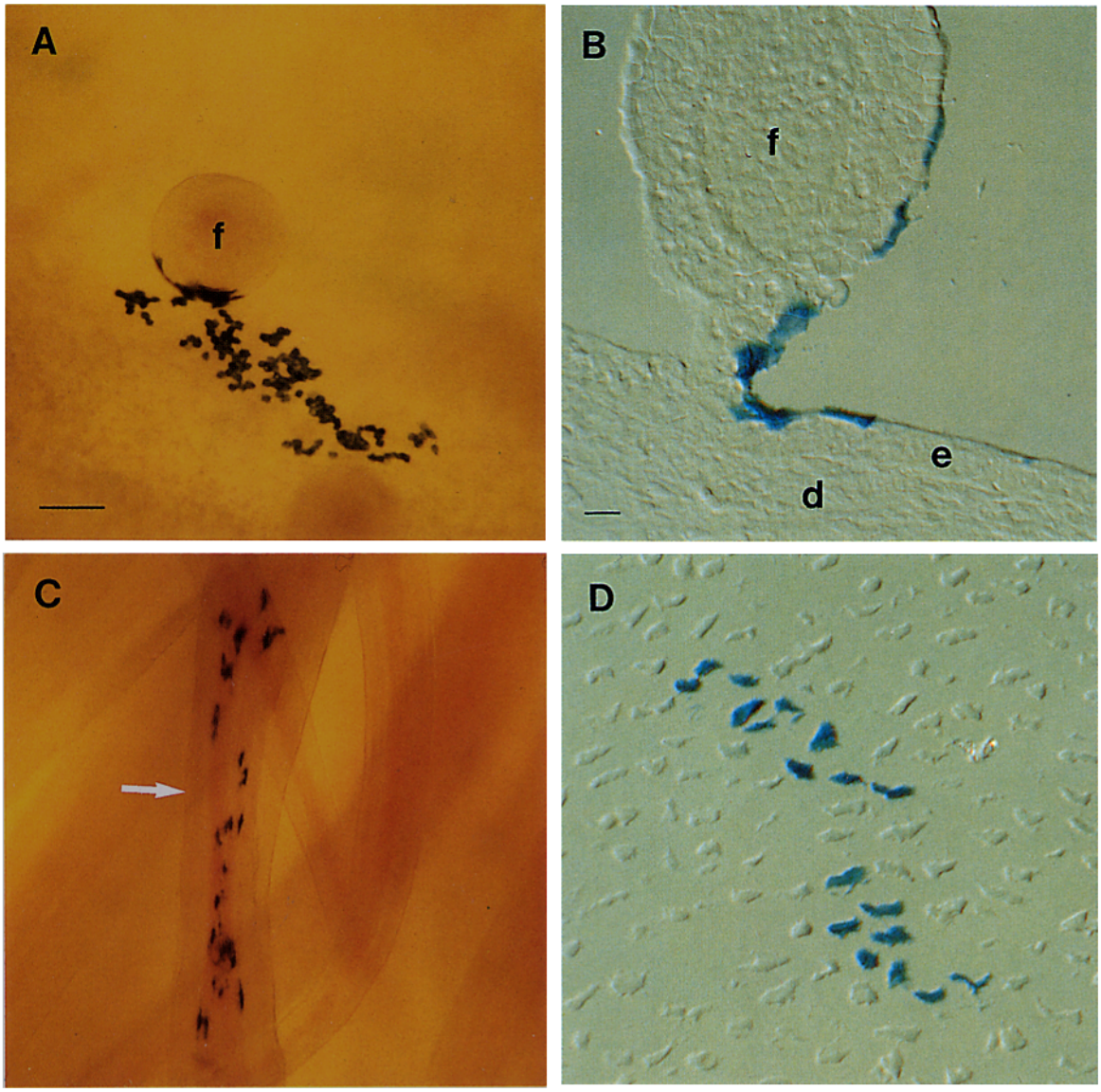
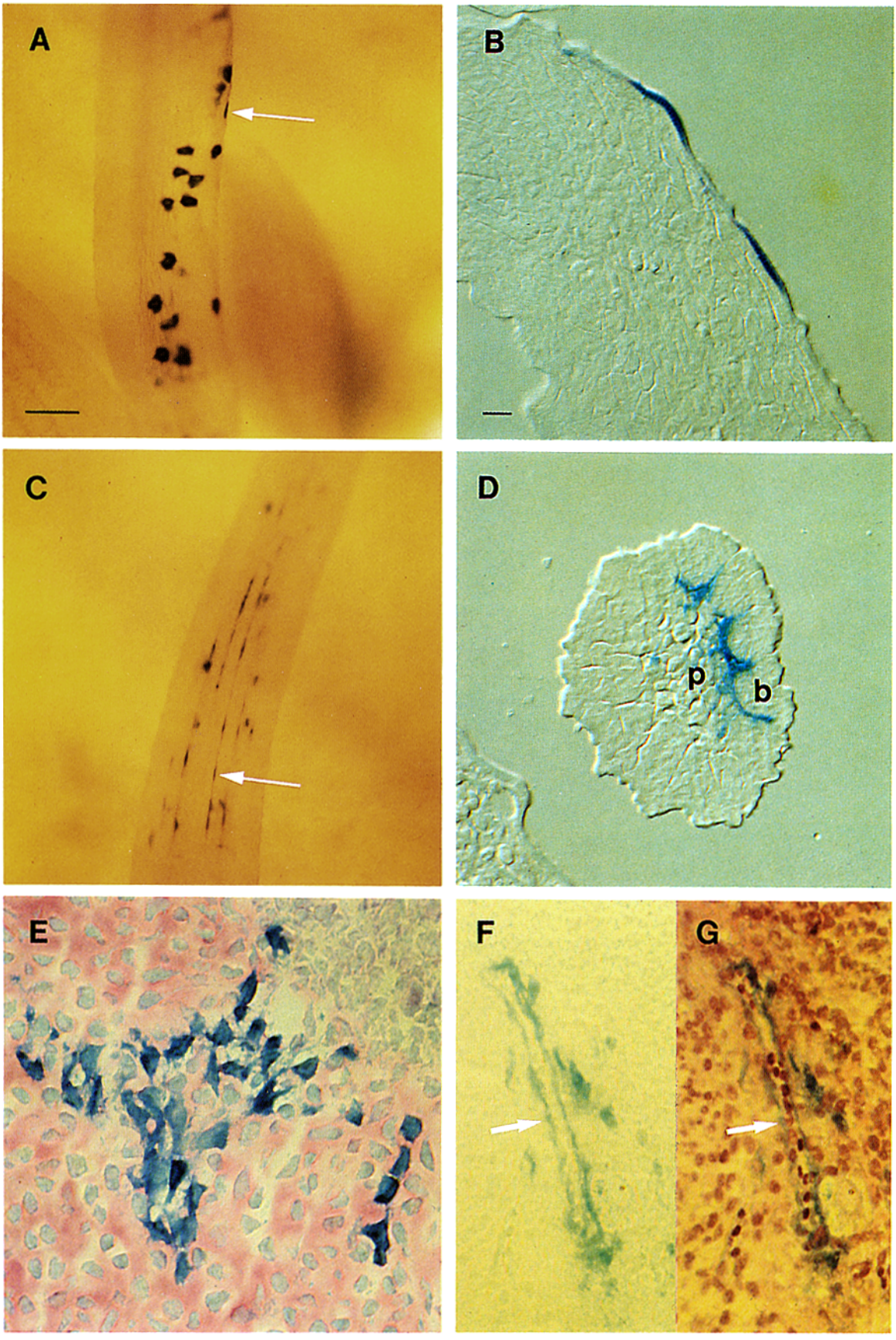


Figure 3. Whole mount and cryosection analyses of cells expressing CKlacl in day 12 embryo limbs. Vector CKlacl was introduced into limb buds by injection of rd virus at stage 24 of development. Whole mount staining of injected limbs at day 12 of development shows the blue Xgal reaction product in expressing cells (*A* and *C*). The detailed cytostructure of expressing tissues is seen in cryosections (*B* and *D*; Nomarski optics). (*A*) CKlacl-marked clone of epidermal cells extending onto a feather germ (*f*). (*B*) A 5-μm section of the tissue in *A*, showing the peridermal location of marked cells. *e*, Epidermis; *d*, dermis; *f*, feather germ. (*C*) CKlacl-marked cells within the mesenchymal core of a feather filament (*white arrow*). (*D*) CKlacl-marked clone of cells embedded in cartilage tissue. Bars: (*A* and *C* same scale) 100 μm; (*B* and *D* same scale) 10 μm.

and expression of pp60$^{v\text{-}src}$. Most information was gained from cells expressing Blacsrc2, due to the ease with which numerous independent clones could be identified in both whole mount and sectioned tissues; over 130 such clones were identified. At day 12–14 of development Xgal-reactive cells were located within connective tissues, skeletal muscle regions, capillary endothelium, perichondrium, periosteum, cartilage, and ectoderm (Fig. 4). The range of mesenchymal tissues was similar to that seen with CKlacl, indicating that Blacsrc2 did not significantly alter the developmental fate or viability of diverse lineages. With few exceptions the histology and clone sizes of cells expressing Blacsrc2 were comparable to those seen using the control vector. Clear examples of this regulated clonal growth were seen in the periderm (of ectodermal origin) (Fig. 4, *A* and *B*; comparable clone seen in Fig. 3, *A* and *B*), and the mesenchymal core of feather filaments (Fig. 4, *C* and *D*; comparable clone seen in Fig. 3 *C*). Numerous examples of normal cartilage clones embedded in collagenous matrix were found (Fig. 4 *E*; comparable clone seen in Fig. 3 *D*), in addition to endothelial cells within capillaries (Fig. 4, *F* and *G*).

The coexpression of v-*src* from Blacsrc2 in ovo was confirmed using indirect immunofluorescence analysis of the oncoprotein pp60$^{v\text{-}src}$. As the available procedures precluded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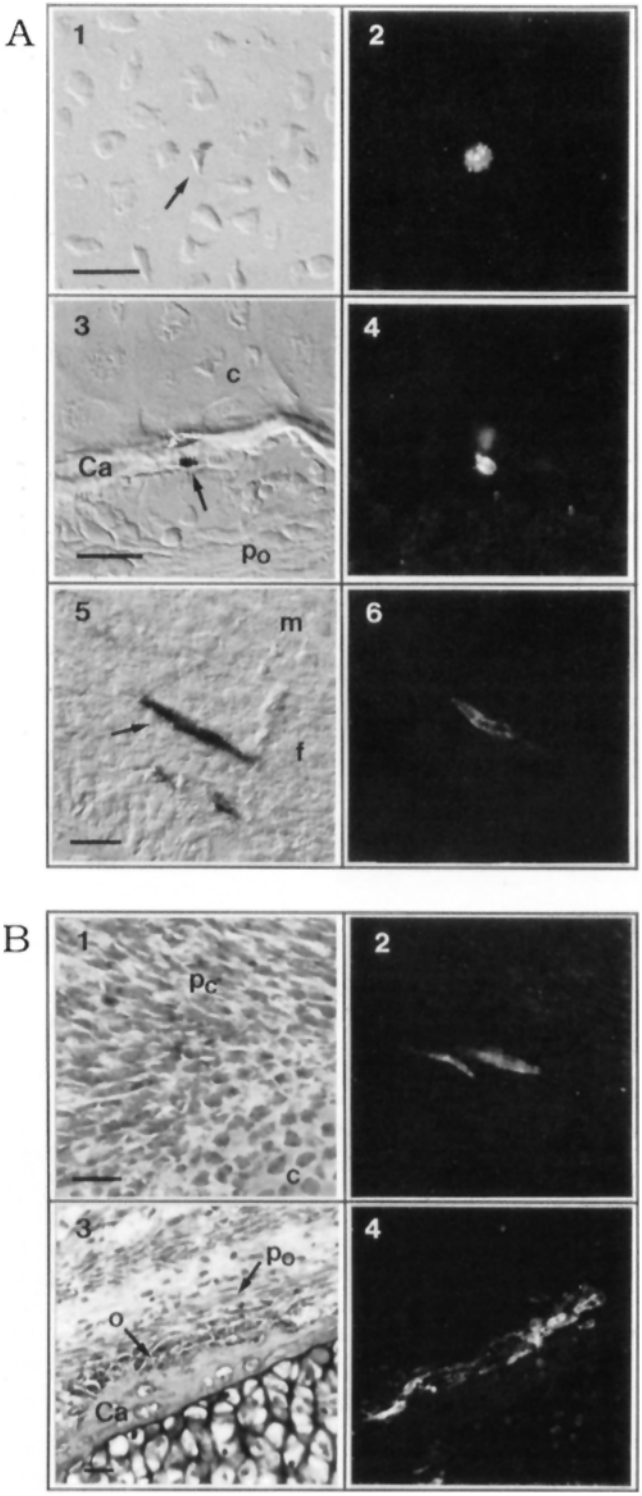

codetection of βgal and pp60$^{v\text{-}src}$ in the same frozen sections, serial sectioning was performed on Xgal-untreated tissues instead, followed by screens for β-gal and pp60$^{v\text{-}src}$ in neighboring sections. Approximately 75% of the Xgal reactive clones clearly coexpressed pp60$^{v\text{-}src}$, either in the same cell when seen in an adjacent section (Fig. 5, *A1* and *A2*), or in neighboring cells of the same marked clone (Fig. 5, *A3–6*). pp60$^{v\text{-}src}$ localization was apparent in both the plasma membrane and cytoplasm of cells, and again all tissues expressing pp60$^{v\text{-}src}$ were histologically normal. In the remaining 25% of clones pp60$^{v\text{-}src}$ expression was faint or undetectable using immunofluorescence. No specific antibody staining was detected in uninfected tissues.

In these same immunofluorescence studies, cell clones expressing vector Bsrc2 were also identified. These were located through their expression of pp60$^{v\text{-}src}$ and complete nonreactivity with Xgal. To date these have been identified within the perichondrium, periosteum/osteoblast tissues, and muscle (Fig. 5 *B*), although this list of cell types is unlikely to be exclusive. As with Blacsrc2, these examples of Bsrc2 expression again coincided with normal clonal histology in the context of their surrounding tissues.

Conditional Disturbance of Cell Growth In Ovo Caused by v-src

The predominant response of embryonic tissues to v-*src* appeared to be that of normal growth regulation. Unless the embryonic environment were absolutely refractory to v-*src* oncogenesis, one would expect to see some exceptions to this growth control under specific circumstances. Two examples of clones marked with Blacsrc2 were identified, one epider-

Figure 5. Expression of pp60$^{v\text{-}src}$ in histologically normal tissues of day 12 limbs. (*A*) Coexpression of *lacZ* and pp60$^{v\text{-}src}$ in Blacsrc2-marked cell clones. Injected limbs were sectioned at day 12 and serial sections were either treated with Xgal or processed for immunofluorescence. (*1*, *3*, and *5*; Nomarski optics) Xgal-treated frozen sections showing the marked cells in center field (*arrows*). (*2*, *4*, and *6*) Indirect immunofluorescence detection of pp60$^{v\text{-}src}$. (*1* and *2*) The same marked cell is seen in serial sections in cartilage tissue. (*3* and *4*) Sections (nonserial) of osteoblast/osteocyte-containing tissue showing nearby cells of one marked clone, reacting with Xgal (*3*) and expressing pp60$^{v\text{-}src}$ (*4*). (*5* and *6*) Adjacent cells of a single marked clone within fibrous tissue (*f*) next to a muscle primordium (*m*). (*B*) Expression of pp60$^{v\text{-}src}$ in Bsrc2-marked cells. (*1* and *3*) Diff-Quik–stained sections of day 12 limb tissues. *c*, Cartilage; *Pc*, perichondrium; *Po*, periosteum; *o*, osteoblasts; *Ca*, calcifying matrix. (*2* and *4*) Indirect immunofluorescence detection of pp60$^{v\text{-}src}$ in sections directly adjacent to those in *1* and *3*, respectively; the tissues were not reactive with Xgal. Bars, 20 μm.

Figure 4. Expression of Blacsrc2 in nonneoplastic limb tissues. Blacsrc2 was introduced into limb buds by injection of rd virus at stage 24 of development. (*A* and *B*) Cells expressing Blacsrc2 seen in the periderm (*p*) of a day 14 feather filament (*f*), in whole mount (*A*) and in cryosection (*B*; Nomarski optics). (*C* and *D*) Blacsrc2-marked cells in the mesenchymal pulp of a day 14 feather filament (*f*) in whole mount (*C*) and in cryosection (*D*; Nomarski optics). The epidermal lobes of barbule tissue (*b*) are clearly delineated. (*E*) Blacsrc2-marked cells in day 12 cartilage tissues, embedded within collagenous matrix (Diff-Quik counterstain). (*F* and *G*) Blacsrc2 expression in endothelial cells of a day 12 capillary (*arrowheads*), seen before (*F*) and after (*G*) Diff-Quik counterstaining. Bars: (*A* and *C* same scale) 100 μm; (*B* and *D–G* same scale) 10 μm.

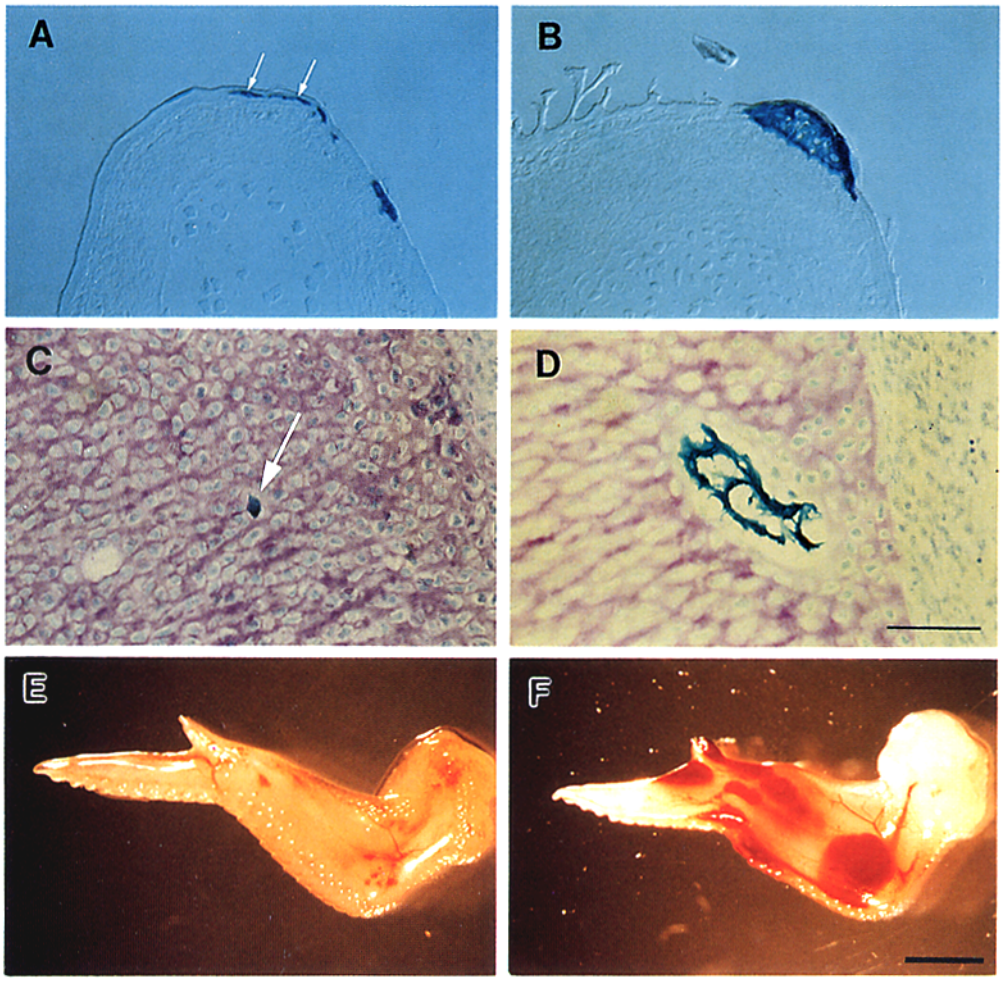

Figure 6. Growth disturbances by v-*src* in ovo. (*A–D*) Limb primordia were injected at stage 24 with rdBlacsrc2, and tissues were stained with Xgal at day 13 (*A* and *B*) or day 11 (*C* and *D*). (*A* and *B*) Nearby sections through a single epidermal clone of cells expressing Blacsrc2. Normal epidermal cells in the stratifying layer were seen in this clone (*white arrow, A*), as well as hyperplastic regions (*B*). (*C* and *D*) Nearby sections through a single chondrocytic clone marked with Blacsrc2. Normal cells in this clone were present in isolated lacunae (*arrow, C*), together with neighboring abnormal subpopulations in enlarged, matrix-depleted lacunae (*D*). (*E* and *F*) Whole mount day 11 limbs. (*A*) Uninjected normal limb. (*B*) Limb previously injected with rd v-*src* viruses at embryo stage 24, showing discrete vascular lesions in the lower arm and wrist regions. Bars: (*A–D*) 80 μm; (*E* and *F*) 0.25 cm.

mal and the other chondrocytic, in which part of the clone was normal and the rest clearly abnormal. The first example, an epidermal clone in the thumb, was identified as having regions of both normal stratifying epidermal cells (Fig. 6 *A*), and patches of localized hyperplasia or dysplasia (Fig. 6 *B*). A heterogeneous chondrocytic clone was also found which, similarly, consisted of both normal cells in isolated cartilage lacunae (Fig. 6 *C*), and groups of atypical cells occupying enlarged lacunae devoid of matrix (Fig. 6 *D*). Localized disturbance in cell growth and/or differentiation had therefore been induced by v-*src* in these rare instances during this period of embryonic development.

Two further kinds of growth disturbance were observed. Firstly, a minority of limbs (<20%) contained single localized regions of abnormal mesenchymal tissue (not reactive with Xgal; data not shown). These abnormalities did not conform to any consistent histological pattern and their tissues of origin could not be determined. In the second case, a frequent and highly consistent pattern of overt neoplasia was seen. Approximately 60% of limbs injected with v-*src* vectors contained macroscopic vascular lesions, first visible 5 d after injection (Fig. 6, *E* and *F*, and 7 *A*). Lesions were not observed in limbs injected with either media or rdCKlacl. Histologically, the lesions were blood-filled cysts lined with layers of normal and/or neoplastic (epithelioid) endothelial tissue (Fig. 7, *A* and *C*); neoplastic cells were frequently contiguous with normal endothelium and cysts were directly associated with invasive neoplastic tissue. The poorly differentiated nature of tumor tissue was confirmed by the low and often undetectable levels of the endothelial marker, Von Willebrand factor (data not shown). Histological studies suggest that growth of the endothelium became abnormal within 3–4 d of vector introduction, leading to cyst formation and tumor growth into interstitial spaces. The histopathology bore a resemblance to hemangioma or hemangiosarcoma, possibly representing the early embryonic counterpart of these neoplasms. The tumors were similar to those observed in transgenic mice strains carrying the polyoma middle T oncogene (Bautch et al., 1987; Williams et al., 1988).

The vascular lesion tissue showed extensive expression of $pp60^{v\text{-}src}$, confirming the direct involvement of the oncoprotein. $pp60^{v\text{-}src}$ exhibited prominent plasma membrane association in many tumor cells, forming a sharp interface with the underlying, $pp60^{v\text{-}src}$-negative stromal tissue (Fig. 7, *B* and *D*). Further immunofluorescence studies did not reveal any helper virus $p19^{gag}$ proteins in vascular lesions (data not shown). Interestingly, Xgal-reactive endothelial cells were rarely associated with vascular lesions, and only then in scattered patches of cells (not shown). Thus, although cells expressing Blacsrc2 may occasionally be involved in lesion etiology, the majority of the tumors were initiated by individual cell clones expressing Bsrc2. Having characterized the cause

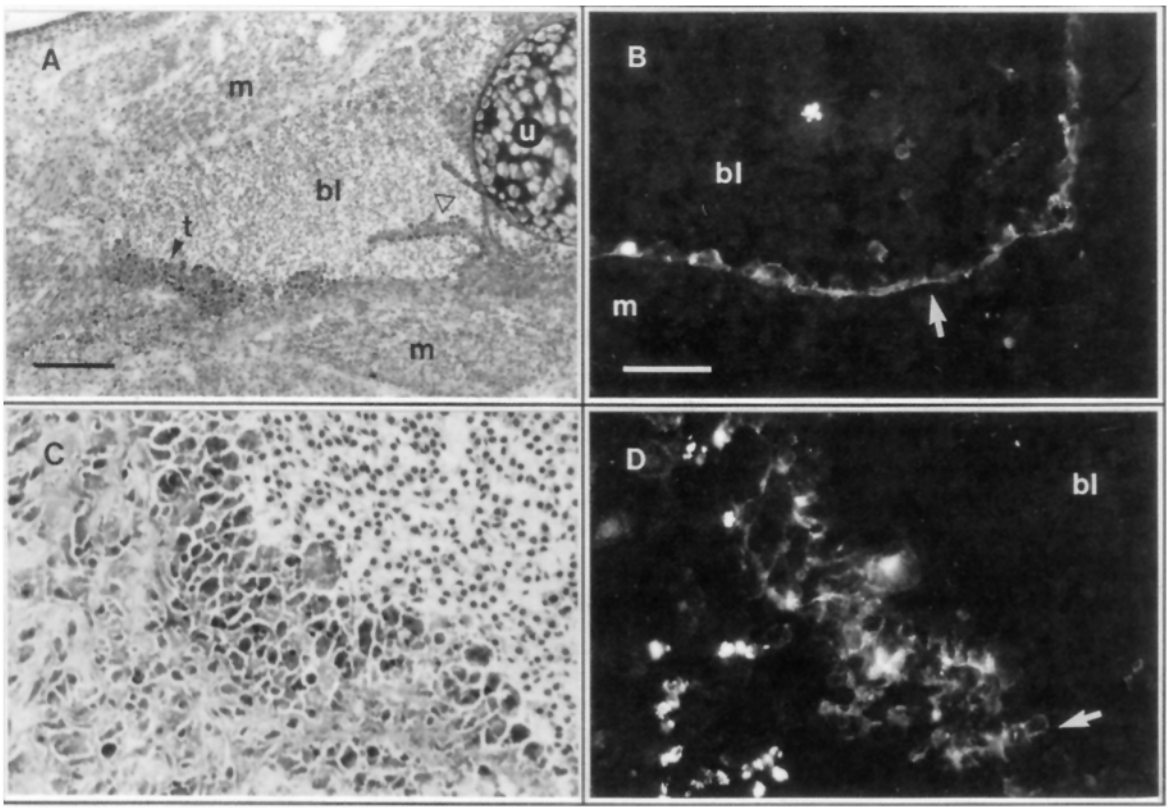

Figure 7. Histology and pp60$^{v\text{-}src}$ immunofluorescence analysis of vascular lesions. Histological analysis (*A* and *C*) and indirect immunofluorescence detection of pp60$^{v\text{-}src}$ (*B* and *D*) in a day 12 limb containing a cystic vascular lesion. The limb had been inoculated at stage 24 with rd v-*src* viruses. The tissues of this lesion were not reactive with Xgal. (*A*) Diff-Quik–stained section showing the blood-filled lesion (*bl*) lined by endothelial tumor cells (*t*), and spurs of tumor tissue (*open arrowhead*). The lesion is flanked by normal muscle primordia (*m*), and the ulna (*u*). (*B*) pp60$^{v\text{-}src}$ expression in endothelial tumor cells lining the lesion; cells are flanked by blood (*bl*) and a sharp interface (*arrow*) with pp60$^{v\text{-}src}$-negative muscle tissues (*m*). (*C*) Enlargement of tumor area similar to that marked as *t* in *A*. Basophilic tumor cells are seen flanking the erythrocyte-filled cyst. (*D*) Serial section adjacent to *C*, showing pp60$^{v\text{-}src}$ expression in the tumor tissue lining the lesion (*bl*). Strong pp60$^{v\text{-}src}$ signal is seen particularly at cell–cell interfaces (*arrow*). The lack of sharp interface with underlying tissues reflects their invasion by tumor cells (contrasts with noninvasive tissue in *B*). The bright spherical cells seen at lower left are granulocytes which nonspecifically bind the biotin-streptavidin reagents (irrespective of primary antibody used); such cells were often associated with tumor tissues. Bars: (*A*) 0.4 mm; (*B–D* same scale) 40 μm.

of these vascular cysts, it is now apparent that the hemorrhagic disease caused by RSV probably arises from a systemic form of this endothelial neoplasia (Duran-Reynals, 1940; Coates et al., 1968).

Differential Expression of pp60$^{v\text{-}src}$ as a Possible Determinant of Vascular Oncogenesis

One factor underlying the different tumorigenic potency of Blacsrc2 and Bsrc2 in endothelial cells, may be differential expression levels of pp60$^{v\text{-}src}$. To begin addressing this, expression of pp60$^{v\text{-}src}$ was assayed in transfected cultured cells, after a plasmid clone of vector Bsrc2 had been constructed (vector pBsrc2a; see Materials and Methods). In ovo, vector Bsrc2a gave rise to vascular tumors identical to those seen with Bsrc2 (A. W. Stoker, data not shown). Using pBlacsrc2 and pBsrc2a, the relative expression levels of pp60$^{v\text{-}src}$ were measured after transient expression in transfected quail QT6 cells (Fig. 8 *A*). In addition, our original clone of B77 RSV, pA11 (reverse transcriptase defective), was used for comparison. After correcting for low levels of coprecipitated pp60$^{c\text{-}src}$ (Fig. 8 *A*, lane QT6), the pp60$^{v\text{-}src}$ levels were normalized for transfection efficiency using the plasmid DNA levels in Hirt extracts from parallel cell plates (Fig. 8 *B*; see Materials and Methods).

The data reveal high expression levels of the oncoprotein from both rd vectors, with Bsrc2a expressing on average 50% more pp60$^{v\text{-}src}$ than Blacsrc2 (Fig. 8 *C*). The RSV clone, pA11, expressed two to three times more oncoprotein than either vector in these assays. The relative elevation in pp60$^{v\text{-}src}$ expression from Bsrc2(a), if reiterated under in ovo conditions, may explain in part why this vector, and not Blacsrc2, induces rapid endothelial neoplasia in ovo. Possible factors such as restricted splicing of Blacsrc2 RNA in endothelial cells, may further differentiate these vectors, and this is presently under study.

Discussion

We have examined the influence of cellular environment upon oncogenesis, after transducing v-*src* into cells both in culture and in the embryo. Our approach of using rd vectors has a number of significant benefits. Firstly, expression of the

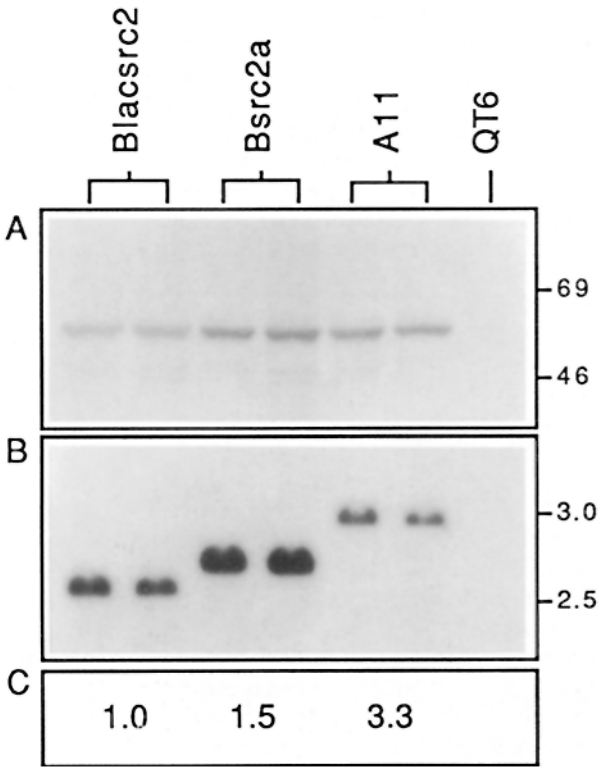

Figure 8. Relative pp60$^{v\text{-}src}$ expression from retroviral v-*src* vectors. (*A*) Fluorograph of a polyacrylamide gel containing immunoprecipitated pp60$^{v\text{-}src}$. Vector plasmids pBlacsrc2, pBsrc2a, and pA11 were transfected into QT6 cells and [^{35}S]methionine-labeled pp60$^{v\text{-}src}$ was immunoprecipitated with mAb. Lane *QT6* is a control sample of QT6 cells transfected with pUC19 only; a faint band of pp60$^{c\text{-}src}$ is present. Protein molecular masses in kD are at right. (*B*) Autoradiograph of a Southern transfer, containing Hirt DNA hybridized with a v-*src* probe. Hirt DNA was extracted from duplicate plates of QT6 cells to those used in *A*, and the DNA was subjected to Southern analysis after digestion with enzyme Eco RI. Molecular weights in kbp are at right. (*C*) The values represent relative pp60$^{v\text{-}src}$ expression levels from pBlacsrc2, pBsrc2a, and pA11. The amounts of pp60$^{v\text{-}src}$ in *A* were first normalized to the respective transfection efficiency calculated from the hybridization in *B* (see Materials and Methods). The value for Blacsrc2 is taken arbitrarily as 1.0. Each value is the average of two independent experiments.

oncogene was restricted to independent clones of cells in integral association with normal cells and tissues, thereby providing unperturbed conditions for environmental regulation. This may be a more accurate approach than is available, for example, in murine transgenic models where the environment is potentially disturbed by transgene expression in entire tissues. A second advantage is that vectors can carry a marker gene together with the oncogene, facilitating the precise localization of oncogene-expressing cells and resolution of their clonal growth behavior. The third advantage demonstrated here is the ability of rd vectors to mark a wide range of cell lineages, contrasting with the apparent myogenic tropism of replication-competent RSV (Howlett et al., 1987); the prominent expression of RSV in muscle probably reflects preferential viral spread in the tissue. A final benefit of using rd vectors is the prevention of systemic viral disease. Specifically, the lethal hemorrhagic disease of RSV is avoided, significantly increasing embryo longevity. A potential drawback in this and other in vivo model systems is the uncertainty of transgene expression levels. Although immunodetection of transgene products is sensitive, it is not straightforward to quantify. Given this caveat, however, we believe that pp60$^{v\text{-}src}$ expression levels in ovo differ little from those found in cultured cells; no reproducible differences have been observed at the immunofluorescence level (A. W. Stoker, unpublished observations), and rd vector expression is similar both in ovo and in culture, as judged by *lacZ* expression.

By analyzing more than 100 independent transductions of v-*src* into a range of tissue progenitors, we gained broad insight into diverse environmental influences, and the possible effects of v-*src* on differentiation patterns. Our data show v-*src* having little influence over the differentiation commitment or growth patterns of several lineages in the limb during the period examined, unlike the rapid neoplastic transformation of cultured CEF. Similar transformation was seen in culture after introducing rd v-*src* vectors into differentiated embryo tendon cells (A. W. Stoker and R. Schwarz, unpublished observations; see also Schwarz et al., 1978). Using RSV, others have demonstrated previously that v-*src* causes rapid loss of growth regulation and differentiation characteristics in other types of cultured embryo cell (Boettiger et al., 1983; Ephrussi and Temin, 1960; Moss et al., 1979; Pacifici et al., 1977; Pessac and Calothy, 1974). There could be several explanations for the embryo–culture disparity. Cells are frequently altered biochemically and behaviorally upon their transfer to culture, and there can be weakened maintenance of the differentiated state (Lee et al., 1984; for reviews see Bissell, 1981, and Bisell and Hall, 1987). Such changes may predispose cultured cells to perturbation by oncogenes. Interestingly, differentiated "revertant" myotubes can occasionally arise from RSV-transformed myoblasts, suggesting that differentiation may override v-*src* oncogenesis in culture under certain conditions (Tato et al., 1983).

Cells in culture may aberrantly express effectors of v-*src* action or cofactors required for transformation, whereas in ovo they do not. The expression of one pp60$^{v\text{-}src}$ substrate, p36, or calpactin, is indeed restricted in the embryo limb, but is expressed rapidly upon placing limb cells in culture (Carter et al., 1986). Although p36 has no known role in cell transformation, its expression may be indicative of more critical targets which are induced upon culturing. Recent work in our laboratory has also implicated transforming growth factor β as a cofactor in RSV tumorigenesis in chickens (Sieweke et al., 1990); it is plausible that similar factors may exhibit differential expression in the embryo, affecting oncogenic permissiveness. Our present data nevertheless demonstrate that latent permissiveness towards cell transformation does exist in the embryo (Figs. 6 and 7). This is seen clearly in the embryonic endothelium, where overt neoplasia can be induced, and also in less frequent growth abnormalities of cartilage and epithelia. Thus, an inherent deficiency of transformation cofactors in ovo is now considered unlikely in several tissues; our data instead strongly implicate some aspect(s) of the embryonic tissue environment in attenuating oncogenesis.

The environmental influences governing cell growth, differentiation, and morphogenesis during limb development

must be tightly regulated, and may be themselves involved in resisting v-*src* neoplasia. The extracellular matrix is implicated in maintaining differentiated function in cells (Blum et al., 1987; Bissell et al., 1987; Li et al., 1987; for review see Bissell and Hall, 1987), and in governing many processes during development (for reviews see Hay, 1984; Thiery et al., 1989). Interestingly, significant differences have recently been identified in the levels and organization of extracellular matrix components of cells in vivo and those cultured on plastic (Streuli and Bissell, 1990). This may contribute to environmentally-conditioned cellular responses to oncogene expression. A second factor integral to embryonic processes is junctional communication between cells (Fraser, 1985). Significantly, gap junction communication with normal cells is also known to suppress tumor cell growth both in vivo and in culture (Dotto et al., 1988; La Rocca et al., 1989; Mehta et al., 1986; Podesta et al., 1984; Stoker et al., 1966). Little evidence for similar suppression of cells expressing pp60$^{v\text{-}src}$ has been found to date, however (Bignami et al., 1988; La Rocca, 1989; Weiss, 1970), and detailed embryonic studies have not been reported with this oncogene. With this rd vector model we show that cells expressing v-*src* can be subject to potential suppressive influences in tissues, the basis of which can now be examined.

What intracellular mechanisms could translate the environmental state into abrogation of, or tolerance to v-*src* action? Cellular tyrosine kinases such as c-*src* and c-*abl* are expressed at elevated levels in several tissues during embryogenesis, and are presumably biochemically active (Cotton and Brugge, 1983; Levy et al., 1984; Muller, 1986). It is likely, therefore, that kinase signal pathways are also active, and that concurrent expression of specific tyrosine protein phosphatases occurs (for reviews see Hunter, 1989; Lau et al., 1989; Tonks and Charbonneau, 1989). Tyrosine phosphorylation signal pathways that are active specifically in the embryo, may tolerate v-*src* action without disturbance to cell behavior. Relatedly, phosphatases which may be critical regulators in embryonic tyrosine phosphorylation pathways, could interfere with the potentially disruptive kinase action of pp60$^{v\text{-}src}$. However, both the signal pathways and the expression of phosphatases may be disrupted upon culturing embryonic cells, allowing the oncogene to subvert cell growth control.

The endothelial neoplasia caused by v-*src*, and the endotheliomas induced by polyoma middle T oncogene, are strikingly similar (Bautch et al., 1987; Williams et al., 1988). These endothelial neoplasias may arise by a related mechanism, since one prerequisite for middle T oncogenesis is its ability to bind and activate the pp60$^{c\text{-}src}$ tyrosine kinase (Bolen et al., 1984; Courtneidge and Smith, 1984). Furthermore, the similarity between rd vascular tumors and the lethal hemorrhagic disease caused by RSV, signifies their common origin as endothelial neoplasms. This sensitivity of the endothelium towards neoplasia indicates that it may harbor specific cofactors for transformation, or its unique vascular environment predisposes it to oncogenesis. Of interest are the elevated endogenous phosphotyrosine levels found in endothelial tissues of normal embryo limb (Howlett et al., 1988; Takata and Singer, 1988). The differing endothelial tumorigenicity of Blacsrc2 and Bsrc2 may arise from differential levels of pp60$^{v\text{-}src}$ expression, but this remains to be fully explained.

Finally, the rare growth disturbances caused by Blacsrc2 in ovo are informative. The heterogeneity within certain clones, resulting in both normal and aberrant subpopulations, suggests that secondary events in certain cells occur. These are more likely to be epigenetic changes than primary genetic mutations in this time period (7–9 d of growth). Changes such as increases in pp60$^{v\text{-}src}$ expression, or reduced tolerance to existing oncoprotein levels, would remain consistent with there normally being a high basal resistance to growth perturbation.

In summary, we have established that v-src expression in diverse lineages does not lead to neoplasia in ovo, and that these cells can maintain their commitment towards differentiation. This contrasts with cells under culture conditions where v-src oncogenesis occurs readily. An apparent exception is seen in embryonic endothelia, which can be subject to rapid, v-*src*-induced neoplasia in ovo. Although the general resistance to v-*src* oncogenesis in ovo is not fully understood, our data suggest that it is largely due to the unique cellular environment of the embryo. We have proposed several feasible mechanisms, and our new model system will be of value in testing these. Such studies will enhance our understanding of the cellular microenvironment as a controlling influence over carcinogenesis and embryo development, and will shed light on the requisite cofactors for v-*src*-induced neoplasia.

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