v-src induces clonal sarcomas and rapid metastasis following transduction with a replication-defective retrovirus

(oncogene/retroviral vector/tumor antigen/polymerase chain reaction/neoplasia)

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ABSTRACT  v-src is an effective carcinogen when expressed from Rous sarcoma virus (RSV) in vivo. Whereas RSV tumors require sustained oncogene expression, their growth is largely a balance between viral recruitment of tissues and host immune destruction of infected cells. We have therefore examined the tumorigenic potential of v-src in the absence of viral recruitment and viral antigen expression. v-src was introduced with high efficiency into chicken wing web tissues using replication-defective (rd) retroviral vectors. Clonal sarcomas were induced rapidly, and, furthermore, v-src potentiated metastatic progression in ~0.1%-1% of tumor clones with unexpectedly short latency. rd vectors proved effective not only in transducing v-src into tissues but also as insertional markers of tumor clonality. The rd vector present in most primary and metastatic tumors was a highly truncated form of RSV derived by viral transcription of spliced v-src mRNA; this vector should thus avoid viral recruitment and host anti-viral immune reaction through its complete lack of viral structural genes. Under such conditions v-src maintains strong carcinogenicity in vivo when restricted to clonal tumor growth and can confer rapid metastatic potential on a discrete subset of tumor clones.

The discovery of activated oncogenes transduced in animal retroviruses has provided considerable support for the genetic basis of cancer (for review, see ref. 1). A number of such oncogenes are now also implicated in the etiology of human tumors (for review, see ref. 2). The first oncogene to be described, v-src, was isolated in the avian retrovirus Rous sarcoma virus (RSV) and is a particularly potent carcinogen in culture and in vivo (3-6). Although v-src acts apparently in dominant fashion in cultured cells, its carcinogenic potential in vivo is more difficult to interpret using replicating oncogenic retroviruses. Whereas v-src expression is a prerequisite for RSV-mediated tumorigenesis, the contribution of the oncogene toward rapid tumor growth is unclear; growth of RSV tumors is largely dependent upon horizontal spread of the virus and the oncogene to neighboring tissues (7). Viral recruitment may enhance the malignancy of tumors by this process of oncogene dissemination, and the ability of neighboring tissues to limit or control tumor outgrowth may be subverted. This oncogenic virus model does not mirror the etiology of most naturally occurring neoplasms where clonal expansion is apparent. Another phenomenon peculiar to retroviral carcinogens such as RSV is the counteractive immune reaction of the host, thought to be directed against viral structural antigens (8). The cell-mediated immune response is implicated in the complete regression of certain RSV tumors.

There have been few studies of v-src oncogenesis following its transduction into tissues without attendant viral recruitment. Inoculation of birds with limiting dilutions of Bryan

strain RSV (a mixture of an env-defective transforming virus and helper virus) was shown to produce tumors containing no detectable replicating virus. With hindsight it is plausible these tumors were clonally derived from cells infected only with defective RSV (9). It was noted, however, that such "noninfective" tumors regressed at high frequency and many later evolved replication-competent viruses. Recent work presented by Fung et al. (10) demonstrated that tumors could be initiated after direct injection of v-src DNA into chicken tissues. These tumors had a clonal component, but again always regressed before progression was seen. Thus it has remained unresolved whether v-src is sufficient in vivo, in the absence of viral recruitment, to sustain full malignant progression or whether additional factors such as secondary oncogene activation are required.

In this study we used replication-defective (rd) retroviral vectors to introduce v-src directly and with high efficiency into tissues. These vectors were designed to preclude both viral recruitment and the expression of antigenic structural genes, facilitating direct analysis of v-src oncogenesis without these added complexities. Infectious rd viruses were obtained using an avian packaging cell line previously developed in our laboratory (11). v-src expression from one specific rd vector gave rise not only to aggressive, clonal primary tumors but also to selective, short-latency metastasis. This vector was present in tumor DNA as a highly truncated form of RSV, derived by reverse transcription of v-src mRNA. To our knowledge, the high transmission frequency and carcinogenicity of this vector are hitherto unreported. The de novo transduction of v-src into tissues was thus achieved readily with rd vectors, demonstrating the potential of this oncogene to induce progressing, clonal malignancy.

METHODS

rd Virus Assays and Animal Inoculation. The establishment of retroviral packaging cells, the construction of the avian sarcoma and leukemia virus (ASLV)-based vector ASrcneo7, and the methods for rd virus production have been described (11). Virus titers were assayed using the neomycin-resistant (neo') QT6 colony assay (11) and the immunocytotoxic expression focus unit (efu) assay (ref. 12; anti-pp60v-src antibody JB327 was used, a gift of J. Brugge, Philadelphia). Virus titers were comparable using both assays within a 2-fold range. Virus was injected intramuscularly into the wing webs of hatchling chickens (specific-pathogen-free White leghorn; SPAFAS) in 50 or 100 μl of medium 199, using 25- or 20-gauge sterile needles, respectively. Chickens were sacrificed by lethal injection of T61 euthanasia solution (Hoechst Pharmaceuticals), and tumor tissues were removed

Abbreviations: rd, replication-defective; efu, expression focus unit(s); RSV, Rous sarcoma virus; PCR, polymerase chain reaction; CEF, chicken embryo fibroblasts; neo', neomycin resistant.

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and either frozen (see below) or processed for paraffin sectioning using standard procedures. The primary tumor tissues of birds were screened for replicating virus as follows: tumor tissue was finely chopped with a scalpel, dissociated using trypsin and collagenase for 1 hr at 37°C, and single cell suspensions were plated on 35-mm tissue culture plates. To test for the presence of infectious virus, supernatants were collected from healthy cultures after 2 days and placed onto fresh chicken embryo fibroblasts (CEF); after 4 days these CEF were processed for the efu assay using, in duplicate plates, primary monoclonal antibody to viral p19<sup>msw</sup> (a gift of D. Boetigger, Philadelphia) or pp60<sup>v-src</sup> antibody.

**Tumor DNA Analysis.** Tumors were removed after sacrifice and frozen immediately in liquid nitrogen. Tissues were crushed to a powder using a mortar and pestle (kept frozen throughout) and added to lysis buffer (0.1% SDS/10 mM Tris-HCl, pH 8.0/20 mM NaCl/100 μg of proteinase K per ml); this was followed by overnight incubation at 37°C. High molecular weight genomic DNA was isolated using standard procedures (13) and digested to completion with restriction enzyme HindIII or EcoRI. Digested DNA was separated using agarose gel electrophoresis and transferred onto nylon membranes [GeneScreenPlus (NEN) or Hybond N (Amersham)] using alkaline transfer as per manufacturers' recommended procedures. Hybridization with 35<sup>P</sup>-labeled probes was performed at 68°C overnight in 1% SDS/1 M NaCl/10% dextran sulfate/200 μg of sheared calf thymus DNA per ml (NEN procedures). Membranes were washed in 15 mM NaCl/1.5 mM sodium citrate/0.1% SDS at 68°C. Autoradiographs were made using Kodak X-Omat film. DNA probes were labeled with [35P]dCTP (Amersham) using the random primer procedure (14).

**Polymerase Chain Reaction (PCR) Amplification and DNA Sequencing.** High molecular weight tumor DNA was obtained as described above. One-tenth microgram of DNA was subjected to the PCR amplification procedure (15) using a programmable cyclic reactor (Ericomp, San Diego, CA). Fifty nanograms of each oligonucleotide were used, with 40 cycles of amplification using Taq polymerase (Thermus aquaticus DNA polymerase) (Perkin-Elmer/Cetus). The cycle parameters were as follows: denature 1 min at 95°C; anneal 1 min at 65°C; polymerize 2 min at 72°C. 20-Mer oligonucleotides (D. Koh, Lawrence Berkeley Laboratory; Applied Biosystems synthesizer) were based upon RSV sequences 9224–9243 base pairs (bp) and the complement of 7115–7134 bp (16). The PCR product was cut with EcoRI and BglI and subcloned into plasmid pSP72 (17). Double-stranded DNA sequencing was performed using an SP6 primer (Promega) and Sequenase procedures (United States Biochemicals) with dATP[35S].

**RESULTS**

**Tumor Induction by rd v-src Vector.** The rd vector used in this study was the avian sarcoma and leukemia virus-based ASsrcneo7. This vector does not encode any intact viral structural proteins and expresses v-src from a subgenomic RNA using RSV splice sites. Infectious rd virus stocks were obtained using vector packaging cells previously developed in our laboratory and contained minimal replicating viral contaminants (11). It has been found that rd virus derived from ASsrcneo7 could morphologically transform CEF in monolayer culture and sustain growth of these cells in suspension medium (A.W.S., unpublished data).

To test the carcinogenicity of this vector in vivo, rd virus was injected intramuscularly into the wing webs of 1.5- to 5-day-old chickens. Initial tests using inocula of 30–3000 viral efu produced palpable tumors within 7–8 days at 100% efficiency. Tumors arose locally as numerous discrete nodules, thereafter coalescing during rapid growth (Fig. 1A). rd tumors grew aggressively to >3 cm in diameter within 3–4 weeks, by which time many animals were moribund and were sacrificed. rd tumor growth was sustained until the time of sacrifice, and tumor regression was not observed. Histological analysis of rd primary tumors revealed their coalescing, nodular structure, and the histopathology of fibrous tissue sarcomas (Fig. 1B).

Post-mortem analysis of rd tumor-bearing birds revealed secondary tumors in the liver in ~25% of cases. Secondary tumors were rarely seen in other organs; only in one bird was a large tumor load found in liver and lungs. The liver tumors had grown to diameters of 2–6 mm in the time between virus inoculation and sacrifice, 18 days in one case. These secondary tumors must therefore have arisen with very short latency. The secondary tumors were histologically similar to the primary sarcomas, showing clear invasion of normal liver tissues (Fig. 1C).

**rd Tumors Are Clonal in Origin.** rd tumors were examined for evidence of viral recruitment of tissues, using two different approaches. In the first approach, rapidly growing tumors were explanted into culture and the supernatants of these were screened for release of infectious virus. The sensitive efu procedure was used to screen for replicating helper viruses and v-src-expressing viruses (12), and none were detected.

![Fig. 1. Pathology of tumors induced by rd v-src vectors. (A) Primary tumor in the wing web of a 21-day-old chicken, 16 days after inoculation with 10⁷ efu of rd virus. The tumor mass represents the coalescence of multiple, discrete tumor nodules, with other nodules remaining separated (arrow). (Scale bar = 1 cm.) (B-D) Hematoxylin/eosin-stained paraffin sections of primary tumor tissue (B), secondary tumor in the liver (C), and unaffected liver tissue (D). (Scale bars = 0.1 mm.) The sarcoma character of tumor tissues can be seen in B and C, and the invasive character of the tumor is seen in C, where hepatocyte tissue is surrounded by infiltrating tumor (arrowheads).](image-url)
detected in 24 of 30 explants (80%). The helper virus detected in the remaining 20% of cases was most likely to have been introduced as a low-level contaminant from the packaging cells. The moderate variations in growth rate seen between rd tumors did not correlate with their virus-producing status. Unexpectedly, at high inocula (>1000 efu) the growth rates of replicating virus-free rd tumors were comparable to, or even exceeded, the growth rate of RSV tumors of comparable inocula.

In the second approach, the rd vector insertion patterns were examined in tumor DNAs: nonrecruitment tumors should be genetically clonal with respect to such insertions. Tumor DNA was cut with HindIII, an enzyme that does not cleave the vector. At high inocula the tumors consisted of many coalesced nodules, and accordingly their DNAs contained multiple, discrete insertions (not shown). At such inocula this insertion pattern could not be distinguished easily from the multiclonality seen in certain early-stage RSV tumors (Fig. 2A, lane wt). The virus inoculum was therefore reduced to the lowest point at which tumors arose, and the clonality of these tumors was examined. Injection of 5 viral efu gave a tumor incidence of ~90% within 21 days, and single nodules were obtained predominantly. Further dilutions of 10- and 100-fold did not produce tumors in this time period. The low-inoculum tumors could sustain growth for at least 5 weeks, reaching diameters of >3 cm before sacrifice of the animals. The genomic DNA of discrete, low-inoculum tumor nodules was analyzed after HindIII digestion, revealing that each contained only one detectable proviral insertion fragment (Fig. 2A). This is a strong indication of their clonal derivation.

The v-src hybridization signal was reproducibly below that of the endogenous c-src hybridization in many of these tumors (Fig. 2A). The vector is present in only single copies per cell, however, and the presence of blood and other tissues lacking vector insertions within tumors would further exaggerate the c-src component. Indeed, it was noted in explants of rd tumors that significant numbers of adherent, pp60v-src-negative cells were isolated together with the tumor cells themselves. (A.W.S., unpublished observations). It is probable that many of these adherent cells were of endothelial or macrophage origin, although this has yet to be determined.

The above data demonstrate therefore that most rd neoplasms were not viral recruitment tumors but had a high degree of tumor cell clonality. Individual rd virus infections could rapidly inoculate this clonal cell population.

Secondary rd Tumors Are Metastases. Liver tumors were isolated from two birds whose primary neoplasms were free of replicating virus in explant assays. HindIII digestion of the secondary tumor DNA revealed a discrete insertion fragment in each, once more indicative of their clonal origin (Fig. 2C). In only one sample was a second, discrete fragment also seen (lane 8). Such clonality data did not unequivocally classify secondary tumors as metastases; it was formally possible that liver tissues were infected directly by virus escaping into the blood during inoculation. Common vector insertion sites in both primary and secondary tumors may have been difficult to locate given the expected multiclonal nature of the former (inocula used 25 and 1000 efu). An alternative means was used therefore to determine the metastatic origins of secondary tumors: examination of the HindIII-cut DNA revealed that all liver tumors had common insertion fragments, identical in size within each bird but differing between the birds. This is compelling evidence that secondary tumors were clonally related within each group, arising as metastases from select subpopulations in the primary tumors. The second fragment in Fig. 2C, lane 8, is likely to represent a second metastatic clone within a mixed liver tumor.

An approximate value of the metastatic rate can be calculated given (i) the virus inoculum used in each bird, (ii) 25% of birds had metastases, and (iii) the assumption that metastases arose from only one or two tumor clones per bird. From such calculations it was estimated that 0.1%-1% of primary tumor subclones metastasized. The short latency period within which such metastases arose is reemphasized.

A Highly Truncated rd Vector Is Present in Tumors. HindIII vector fragments in certain rd tumors were below the minimal size limit to contain intact ASrcneo7. Tumor DNA was therefore cleaved with EcoRI to determine the internal structure of integrated vectors. Whereas an internal 3.0-kb EcoRI fragment was expected from ASrcneo7 (see Fig. 4), we instead observed a 2.7-kb fragment in most tumors (Figs. 1B and 3), and larger fragments were seen in a few instances (Fig. 3, lanes 5 and 8). HindIII digestion of the same DNA had shown each sample to contain from one to at least five discrete insertion sites; yet the 3.0-kb EcoRI fragment was not detectably represented in these tumors. Furthermore, the two groups of metastatic tumors examined also contained the shorter, 2.7-kb EcoRI fragment (not shown; the second insertion in Fig. 2C, lane 8, gave a 4.5-kb EcoRI fragment,
Fig. 3. Presence of truncated vectors in rd tumor DNA. A Southern analysis of DNA from control cells and 10 rd tumors is shown. Tumor DNA is representative of only a small specimen taken from each tumor. DNA was digested with EcoRI to release internal vector fragments containing v-src. The 3.0-kb EcoRI fragment seen in control lane 11 is not seen in rd tumor DNA; instead, a 2.7-kb fragment is observed in most cases with a low incidence of larger, aberrant fragments (lanes 5 and 8). Viral inocula used to induce rd tumors are as follows: lanes 1-3, 30 efu; lanes 4-6, 300 efu; lanes 7-10, 3000 efu. Lane 11, QT6 cell line DNA harboring a provirus (ASrcneo6, ref. 11) with the 3.0-kb EcoRI fragment of RSV. Lane 12, uninfected CEF. Hybridization to endogenous c-src sequences is indicated; the quail QT6 c-src fragment is larger than the chicken counterpart.

indicating a rearranged vector). Although ASrcneo7 could not be found in rd tumors, this vector was indeed present in virus stocks. This was evidenced by the high neo' viral titers found in culture (11). In addition, preliminary studies have shown the 3.0- and 2.7-kb EcoRI fragments in DNA from rd virus-infected CEF (A.W.S., unpublished data).

A comparison between the map of ASrcneo7 and the 2.7-kb EcoRI fragment suggested that subgenomic v-src mRNA derived from ASrcneo7 was being virally transmitted; this had also been suggested by our inability to detect neo-related intron sequences in tumor DNAs. To examine this directly, a proviral fragment spanning the putative splice junction was isolated from tumor DNA using the PCR procedure (15). The PCR products made from several tumor DNAs were of the predicted size of 540–550 bp, and a representative nucleotide sequence was subsequently obtained (Fig. 4). The splice junction was located, aligning perfectly with that predicted from RSV DNA sequences (16). This subgenomic form of vector ASrcneo7, named ASrc7, is structurally identical to reverse-transcribed RSV v-src mRNA. From the minimum rd virus inocula that gave rise to tumors, it was estimated that ASrc7 had been released at a titer of at least $2 \times 10^4$ per ml from the packaging cells.

DISCUSSION

The carcinogenic behavior of v-src when transduced within RSV is complicated by viral recruitment phenomena and antiviral immunity. In the present study we have reduced this complexity by introducing v-src into tissues with rd retroviral vectors. The sarcomas arising after individual v-src trans-
rapid, clonal tumorigenesis. V-src not only sustained primary tumor growth but also rapid progression to metastasis. Vector insertion analysis of secondary liver tumors provided genetic evidence of their metastatic origin. An estimated 0.1%–1% of v-src transductions led to this early progression, a selectivity that in itself is not surprising given the rarity of metastatic progression in naturally occurring tumors. The ease of inducing numerous, independent tumor clones with rd vectors, may have been critical in observing these low-frequency metastases. We remain faced, however, with the surprisingly short latency period of these metastatic tumors. This may indicate that some direct impetus is provided by v-src for progression to occur. For instance, it is possible that cells with a predisposition to migration or tumor progression steps were occasionally targeted by the v-src vector. An alternative explanation could be that rare vector integrations led to insertional mutations in either host or vector DNAs. Detailed characterization of these metastatic tumors should lead to a greater understanding of these selective events in vivo and the role that v-src played in these processes.

The extreme carcinogenicity of ASrc7 is not clearly understood at present. We have recently completed studies with a different rd v-src vector and have found again that spliced forms of the vector, structurally similar to ASrc7, are the more carcinogenic in vivo (unpublished data). This suggests that unique structural or expression characteristics of the subgenomic vector are responsible for its oncogenicity. First, the aggressive nature of primary rd tumor growth may reflect in part the lack of immune reaction to viral antigens: unlike RSV, Asr7 does not encode viral structural genes and should not be subject to the cell-mediated immune reaction against viral antigens (8). Escape from antiviral immunity may also increase the ability of rd tumor cells to metastasize. A second factor that may enhance the oncogenicity of ASrc7 is its lack of introns: given the inefficient splicing of RSV-derived RNAs, an intronless vector is likely to sustain relatively increased levels of v-src mRNA and thus the oncoprotein. Whether this indeed occurs and is a factor in the aggressive tumorigenicity awaits determination. The frequent transmission of the subgenomic vector from packaging cells was also unexpected. Our data now show in fact that v-src mRNA contains sufficient viral packaging information in cis for efficient transmission. This is surprising in view of the low level of subgenomic viral RNAs normally transmitted from retrovirus-infected cells (refs. 18 and 19; for review, see ref. 20). The unusual circumstances within packaging cells where full-length viral genomes are not competing for encapsidation may increase subgenomic RNA incorporation into viral particles. Past reports show that direct injection of v-src DNA (10), or low inocula of Bryan RSV (9), leads to regressing tumors without malignant progression. The use of efficient rd expression vectors now shows not only that regression is avoidable but also that progression can occur. Our current data suggest these are optimal conditions for v-src oncogenesis in such tissues, whereas other modes of oncogene expression are more subject to host restrictions. Our laboratory has previously defined other in vivo assays in which v-src oncogenesis is restricted. First, RSV tumor-bearing birds can often remain free of secondary tumors although systemically infected with oncogenic virus. Distal tumors can be induced effectively in such chickens by local wounding and associated wound factors (21, 22). This suggests that disturbances in tissue homeostasis can provide a suitable microenvironment within which tumor initiation can occur. In the second example, expression of RSV or v-src alone in a range of avian embryonic tissues has been shown to be insufficient to induce neoplasia (refs. 23 and 24; A.W.S., unpublished data); developmental regulation can thus be superimposed upon oncogenic responses in vivo. The degree of v-src oncogenicity is thus contingent upon its mode of expression and the complex influence of host tissue environments.

To conclude, we have demonstrated the utility of rd vectors for oncogene transduction in vivo and as genetic markers of solid tumor clonality. These vectors clearly offer an effective approach to dissect the factors governing induction and restriction of oncogenesis in the animal. We provide compelling evidence that v-src induces clonal neoplasia in the absence of viral recruitment, and, moreover, under the same conditions this oncogene initiates selective and rapid malignant progression.

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