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ORIGINAL ARTICLE

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Tumor cells induced by the v-*src* oncogene are heterogeneous for expression of markers of mesenchyme differentiation

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Abstract The observation that v-*src*-induced tumors contain tumor cells of differing morphology, notably fibroblastoid or polygonal, raised the question as to whether the tumor cells are also heterogeneous with respect to expression of markers of cellular differentiation. Of the markers tested here, consistent reactivity for tumor tissue was noted only for antibody probes reactive to muscle actin (HHF35, αsm-1) or to procollagen type I (SP1. D8); for any given tumor, whether induced by v-*src* DNA or by Rous sarcoma virus, each of these markers was found only in a subpopulation of tumor cells. The observation of marker heterogeneity in the one v-*src* DNA-induced tumor examined here that typed as monoclonal suggests that v-*src*-induced transformation is consonant with a degree of plasticity in the phenotypes of the clonal progeny of a single transformant.

Key words v-*src* oncogene · Rous sarcoma virus
Fibrohistiocytic tumour · Immunohistochemistry
Southern blotting

Introduction

One approach to define the differentiation potential of tumor cells is to characterize their histology and immunohistochemistry in the resultant tumor mass. For the case of v-*src*-mediated transformation, the tumors induced by infection with Rous sarcoma virus (RSV), which is the naturally occurring vector of the v-*src* oncogene, have been frequently classified as fibrosarcomas (Loomis and Pratt 1956; Stewart et al. 1959). This classification denotes the presence of a tumor cell population which is exclusively fibroblastoid in morphology and uniform for the expression of collagen to the exclusion of other markers of mesenchymal differentiation (Enzinger and Weiss 1988). In the absence of a significant histopathological heterogeneity among the tumor cells, a diagnosis of fibrosarcoma implies that v-*src*-transformed cells are committed to a single end-point of differentiation, if not to a single pathway of differentiation.

One difficulty inherent in the interpretation of the histology of RSV-induced tumors relates to the usual polyclonality of these tumors. This polyclonality, which is a consequence of a continued process of virus-mediated recruitment of new tumor cells, complicates any correlation of a given differentiation phenotype with the expansion of a single clone. This complication is lessened by the use for tumor induction of DNA fragments that contain v-*src*, but are deleted for viral replication-specific sequences, as these fragments induce pauci- or monoclonal tumors in chickens at the wing-web site of inoculation (Fung et al. 1983). In fact, our previous observations that v-*src* DNA-induced tumors comprise transformed cells of differing morphologies (Halpern et al. 1990) suggested that a classification of fibrosarcoma is inaccurate. The present study was therefore undertaken to reevaluate the histopathology of v-*src*-induced tumors.

Materials and methods

Line SC chickens (Hyline, Dallas Center, Iowa) were inoculated subcutaneously in the wing web at 1–2 days post-hatch with either: (1) 10^5 focus-forming units of BH-RSV(RAV-2), an avian sarcoma virus pseudotype in which the defective Bryan high-titer strain of RSV is complemented by the Rous-associated virus-2 leukosis virus); (2) 20 μg of the pJDΔ11 construct (Stoltzfus et al. 1987), after restriction with *Hin*dIII to free the v-*src*(+) insert from the cloning vector; or (3) 20 μg of the pRL$^{v\text{-}src}$ construct (Halpern et al. 1990), after restriction with *Xho*I and *Eco*RI. Since similar results were obtained with either the pJDΔ11 or pRL$^{v\text{-}src}$ construct, each will hereafter be referred to as v-*src* DNA.

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Chickens were killed at various intervals post-inoculation. Tumor tissue from the chickens was excised and processed both for microscopic examination and for expression of markers of cell differentiation; in addition, seven v-*src* DNA-induced primary tumors were also examined by Southern blotting to determine their clonality, i.e., the number of sites of residence of v-*src* DNA in each tumor.

Tumor tissue recovered from chickens at necropsy was fixed with cold ethanol and subsequently processed by the method of Sainte-Marie (1962). For microscopic examination of tissue, sections were stained with hematoxylin and eosin. Immunoperoxidase assays to resolve markers of cell differentiation were carried out as previously described (England et al. 1991), utilizing appropriate dilutions of primary antibodies (see below), followed by biotinylated anti-rabbit IgG or anti-mouse IgG, and then avidin DH-biotinylated horseradish peroxidase H complex (Vector Laboratories, Burlingame, Calif.). Peroxidase activity was visualized with diaminobenzidine / hydrogen peroxide. Normal rabbit serum or unrelated mouse monoclonal antibodies (mAb) of the same IgG subclass as the specifically reactive primary antibodies were used to define background reactivity.

The primary antiserum used was rabbit anti-chicken α-keratin serum (O'Guin et al. 1982) obtained from Dr. Loren Knapp (University of South Carolina, Columbia, S.C.). The following mouse mAb were used: anti-human myocardial actin (HHF35; Tsukada et al. 1987; Enzo Diagnostics, New York, N.Y.); anti-α smooth muscle actin (α sm−1; Skalli et al. 1986; Sigma, St. Louis, Mo.); anti-sheep procollagen type I amino terminal extension peptide (SP1. D8; Foellmer et al. 1983); anti-chicken gizzard desmin D3; Danto and Fischman 1984); anti-chicken pectoralis myosin (MF20; Bader et al. 1982); anti-chicken zeugmatin (mAb20; Maher et al. 1985); anti-bovine titin (9 D10; Wang and Greaser 1985); anti-newt 102 kDa skeletal muscle marker (12/101; Kinter and Brockes 1984; the latter all obtained from Developmental studies hybridoma bank, University of Iowa, Iowa City, Iowa). Unrelated mAb: anti-human mouse monoclonal IgG1 and anti-human mouse monoclonal IgG2a were obtained from Dr. Georgio Trinchieri, The Wistar Institute, Philadelphia, Pa.).

For Southern blot analysis DNA was isolated from tumor tissue and red blood cell nuclei as previously described (Halpern and McMahon 1987). The methods of restriction of DNA and of Southern blot hybridization have also been described (Halpern and McMahon 1987). The *src* DNA probe (American Type Culture Collection, Rockville, Md.) used for the detection of both v-*src* and c-*src*-positive restriction fragments is a subclone of the pSRA-2 construct (DeLorbe et al. 1980), consisting of the *Pvu*II internal fragment of the v-*src* gene of the subgroup A strain of Schmidt-Ruppin avian sarcoma virus (Halpern et al. 1989). Individual sites of residence of v-*src* DNA were resolved by restriction of genomic DNA with either a zero-cutter (*Sac*I) or a one-cutter (*Bgl*II) for v-*src*. The *Pvu*II internal fragment of the v-*src* used as probe (Halpern et al. 1989) recognizes only the portion of v-*src* downstream from the single *Bgl*II site in v-*src*. Southern blot analysis of red blood cell DNA from the same chicken was used to identify restriction fragments containing c-*src*. Histological sections were prepared from tumor tissue adjacent to the mass used for the Southern blot analysis.

Results

As an initial step in analysis, we examined the morphology of the tumor cells in v-*src*-induced wing-web masses arising in line SC chickens. The SC host was chosen because our previous results had shown that tumor growth induced in the SC host by v-*src* DNA inoculation of newly hatched chicks is characterized by an interval of 5–7 weeks of rapid tumor expansion (within this interval, 30–70% of the chickens die with disseminated tumors) and for the survivors, a much longer period of apparent tumor stasis or very gradual regression (Halpern et al. 1990, 1991). The use of the SC host, therefore, facilitated the histological analysis of tumor tissue at different stages of tumor growth. Tumor cells were identified in histological sections on the basis of a nuclear morphology characterized by nuclear enlargement, irregular nuclear membranes, and irregular nucleoli.

As shown in Fig. 1, for v-*src* DNA-induced tumor tissue recovered at the expansion phase of growth, the tumor cells included fibroblastoid as well as polyglonal forms. This observed pleomorphism (polygonal and fibroblastoid) is at variance with the monomorphism of human fibrosarcomas, which comprise only fibroblastoid elements. In addition, it is implicitly discordant with a classification of the v-*src* DNA-induced tumors as fibrosarcoma, prompting an examination of RSV-induced tumors. As also shown in Fig. 1, a similar histological pleomorphism was observed under conditions of inoculation of 1–2-day-old SC chickens with a replication-competent strain of RSV; this latter observation indicated that the morphological pleomorphism extends to the natural viral tropism which, in contrast to DNA transfection, is based on the interaction of the viral glycoprotein and a cognate cellular receptor. Although the observed pleomorphism of both v-*src* DNA-induced or RSV-induced tumors suggested that a classification of v-*src*-induced avian tumors as fibrosarcomas is inaccurate, the cavil remained that sectioning of tumor cells in random alignment might artifactually contribute to an appearance of pleomorphism.

However, two additional sets of observations provided further evidence against a diagnosis of fibrosarcoma. The first comprised histological observations showing that some of the polygonal cells, present in the v-*src* DNA-induced tumor tissue recovered at the static or regressive phase of growth, were considerably larger than the bulk of the polygonal forms observed under conditions of progressive tumor growth; these larger cells were further distinguishable on the basis of abundant eosinophilic cytoplasm and prominent nucleoli (Fig. 1C). Given the much greater diameter of these cells relative to the short axis of the fibroblastoid cells, it is difficult to reconcile their presence with a sectioning artifact. Tumor cells of comparable morphology were not observed under the conditions of the rapid, progressive growth of the RSV-induced tumors which resulted in death 2–3 weeks post-inoculation.

The second line of evidence against a diagnosis of fibrosarcoma derived from assays (Table 1) undertaken to characterize the tumor cells with respect to their expression of particular markers of cell differentiation. Epithelial and mesenchymal markers were examined because, on the one hand, the polygonal morphology of a significant subpopulation of the v-*src*-induced tumor cells is similar to the morphology of carcinoma cells; on the other hand, the pleomorphism of the v-*src*-induced tumor cells (which mimics the pleomorphism of non-fibrosarcomatoid, mesenchymal human tumors) raised

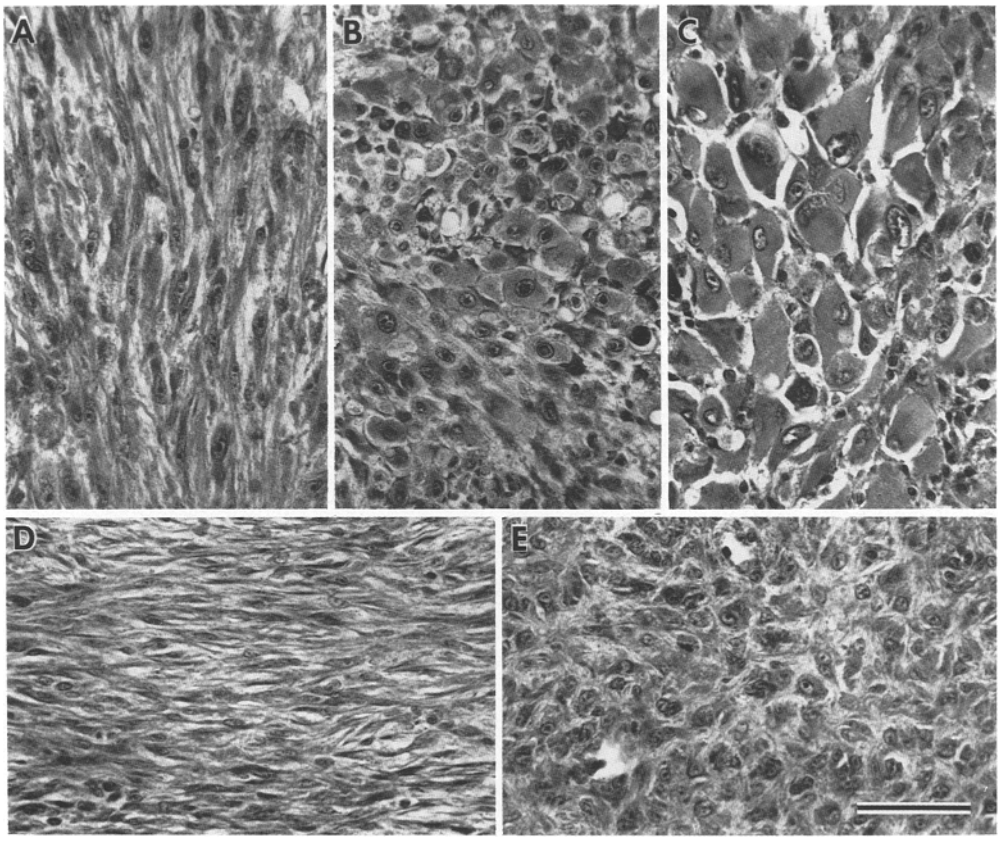

Fig. 1A–E Representative histology of v-*src*-induced wing-web tumor tissue. **A, B** A v-*src* DNA-induced tumor recovered 4 weeks post-inoculation. **C** A v-*src* DNA-induced tumor recovered 6 weeks post-inoculation. **D, E** An RSV-induced tumor recovered 2 weeks post-inoculation. Sections stained with hematoxylin and eosin. *Bar* represents 50 μm

Table 1 Markers of mesenchymal differentiation in v-*src*-induced tumors

Antigen	Antibody	Cell reactivity in assays of normal chicken tissue	Reactivity for wing-web tumor cells[a] (inducing agent)		Presence of analogous constituent in human tumors	
			Rous sarcoma virus (%)	v-*src* DNA (%)	Fibrosarcoma (1)	Malignant fibrous histiocytoma
Cytokeratin	Anti-chicken α keratin	Epithelial and mesothelial cells	2/10 (0.01–0.02)	2/9 (0.005–0.01)	–	+ (Rare tumor cells; Lawson et al. 1987; Weiss et al. 1988)
Procollagen	SP1.D8	Fibroblasts	26/27 (10–60)	21/21 (15–80)	+[b]	+[b] (Enzinger and Weiss 1988; Roholl et al. 1988)
Muscle-specific actin	HHF35	Smooth and striated muscle cells	25/28 (5–60)	18/21 (15–80)	–	+ (Roholl et al. 1990)
Smooth muscle actin α	αsm-1	Smooth muscle cells	25/26 (2–60)	18/20 (10–50)		+ (Roholl et al. 1990)
Desmin	D3	Smooth and striated muscle cells	0/9[c]	0/11	–	+(Rare tumors; Roholl et al. 1990
Sarcomere myosin[d]	MF-20	Striated muscle cells	0/6[c]	0/9	–[e]	–[e] (Enzinger and Weiss 1988)

[a] The ratios indicate the number of chickens exhibiting marker-positive tumor tissue to the total number of tumor-bearing chickens tested; the numbers in parentheses indicate the approximate range of the percent-reactive tumor cells in the positive tumors

[b] Extracellular collagen detected by light and electron microscopy

[c] As noted with Rous sarcoma virus (RSV)-induced tumors, but not with v-*src* DNA-induced tumors, a few scattered cells with atypical nuclei scored positive for markers of striated muscle differentiation (desmin, sarcomere myosin, zeugmatin, titin, and 102-kDa skeletal muscle marker). These cells were localized to areas of skeletal muscle which had been invaded and disrupted by tumor cells. Because these cells, like the invading tumor cells, expressed p27 (the major nucleocapsid antigen of RSV) we assume that they were infected with exogenous retrovirus. It is not known at present whether these cells represent a rare subtype of tumor cell or alternatively retrovirus-infected regenerating myofibers

[d] Tumor cells also did not react with monoclonal antibodies raised against: zeugmatin, titin, and 102-kDa skeletal muscle marker

[e] Markers of striated muscle differentiation

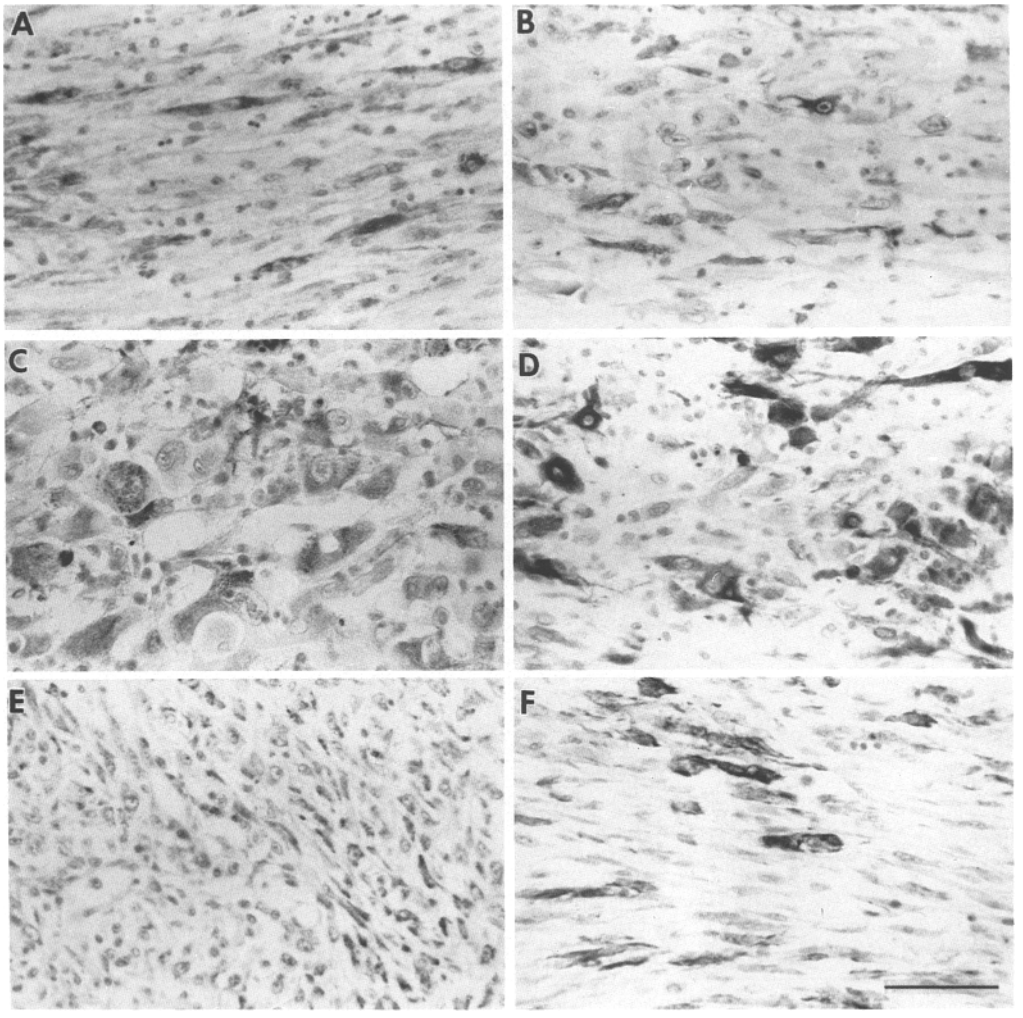

Fig. 2A–F Immunoperoxidase assays of v-*src*-induced wing-web tumor tissue, as based on the use of monoclonal antibodies to markers of mesenchymal differentiation. The two panels in each row represent sections from the same tissue sample; those on the left show reactivity with anti-procollagen type I; those on the right show reactivity with anti-α smooth muscle actin. **A, B** Av-*src* DNA-induced tumor recovered 4 weeks post-inoculation. **C, D** A v-*src* DNA-induced tumor recovered 6 weeks post-inoculation. **E, F** An RSV-induced tumor recovered 2 weeks post-inoculation. All sections were counterstained with hematoxylin. The *arrows* indicate immunoperoxidase-negative cells. *Bar* represents 50 μm

the possibility that the avian tumors were of a mesenchymal lineage other than fibrosarcoma.

Initial experiments, involving an anti-chicken α-keratin-based immunoperoxidase assay of histological sections, were undertaken to examine the expression of cytokeratin; a ubiquitous marker for normal epithelial and mesothelial cells as well as for carcinomas and diffuse mesothelioma (in contrast, sparsely scattered cytokeratin-positive cells have been observed in certain types of mesenchyme-based human tumors; Larson et al. 1987; Weiss et al. 1988). As tabulated in Table 1, the avian tumors were largely cytokeratin-negative, although a small percentage (10–20%) of tumors, as reported previously (England et al. 1991), contain rare (0.01%) cytokeratin-positive cells. In addition, although a number of antibody probes of mesenchymal markers were also nonreactive, probes to other mesenchymal markers (notably muscle-specific actins and type I procollagen) were reactive with extensive and (as determined with serial sections) at least in part, non-overlapping areas of the v-*src*-induced tumors (Table 1, Fig. 2); expression of each of the positive markers was detected in both fibroblastoid and polygonal tumor cells. Since cells expressing one or both of these markers represented the majority, and in some individual tumors the bulk of the tumor mass, we concluded that the v-*src*-induced tumors were mesenchymal in origin. As human fibrosarcomas do not express markers specific for muscle differentiation, the presence of such markers in the v-*src*-induced tumors further shows that the v-*src*-induced tumors are distinguishable from fibrosarcoma (Fig. 2).

The observation that tumor cells in individual v-*src*-induced tumors are heterogeneous with respect to morphology and marker expression in turn raises the question of whether this heterogeneity arises under conditions of a single or multiple clonality. Given the absence of a methodology to directly determine the clonal relatedness of individual tumor cells in fixed tissue sections, one approach to address this question would be to attempt to correlate the clonal heterogeneity of bulk tumor tissue, with the histology of representative samples removed from this tissue. Although the presence of multiple clones would leave the question unresolved, the recovery of a monoclonal tumor exhibiting marker heterogeneity would suggest that this heterogeneity arises in the course of the expansion of a single clone.

This approach was used here, with analysis of clonality based on Southern blotting to determine the number

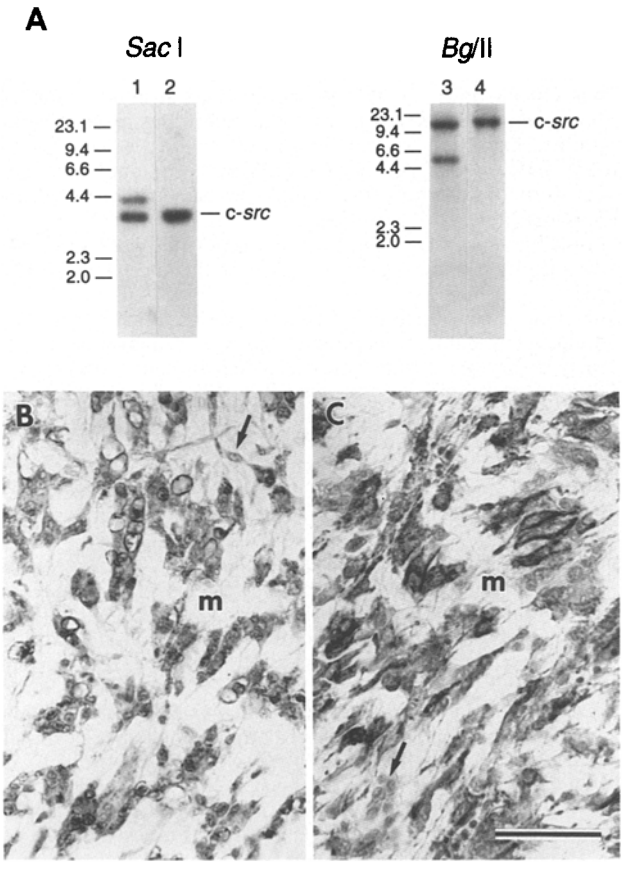

Fig. 3A–C Representative histology of a v-*src* DNA-induced wing-web tumor that was recovered 7 weeks post-inoculation. This tumor exhibited a predominant v-*src*-specific restriction fragment. **A** Southern blot analysis of bulk tumor DNA (*lanes* 1 *and* 3) or red blood cell DNA (*lanes* 2 *and* 4) from the same chicken; conditions of restriction with a zero-cutter (*Sac*I) or a one-cutter (*Bgl*II) for v-*src*. **B** Immunoperoxidase assay based on the use of anti-procollagen type 1. **C** Immunoperoxidase assay based on the use of anti-α smooth muscle actin. The histological sections were prepared from tumor tissue adjacent to the mass used for the Southern blot analysis. *m* indicates myxoid extracellular matrix material surrounding tumor cells. The *arrows* indicate immunoperoxidase-negative cells. *Bar* represents 50 μm

of sites of residence (invariably haploid) of v-*src* DNA in an individual tumor, i.e., restriction of genomic DNA with a zero- or one-cutter for the v-*src* plasmid insert. Of the seven individual v-*src* DNA-induced primary (wing-web) tumors examined here, all of which showed morphological and marker heterogeneity, only one (Fig. 3) exhibited a single v-*src*-specific band that comprised approximately 50% of the intensity of the band of c-*src* (diploid), a restriction pattern indicative of the presence of a single clone. The fact that this tumor (Fig. 3) exhibited the gamut of histological phenotypes described above argues that at least some of the observed heterogeneity arose in the course of the expansion of the predominant clone scored on the Southern blot. (Although it is presently ambiguous, it is not excluded that some or all of the six other tumors were also monoclonal, given that multiple v-*src*-specific restriction fragments on a Southern blot are consistent not only with the possibility of multiple clones but also with the possibility of a single clone that was subject to multiple integration events.) While results based on this methodology cannot be considered definitive, the marker heterogeneity in the one demonstrably monoclonal tumor is suggestive that the clonal expansion of a v-*src*-induced transformant can give rise to progeny expressing distinct phenotypes.

Discussion

Independently of whether their induction is by transfection with oncogenic DNA fragments or by infection with RSV, v-*src*-induced tumors exhibit a common histopathology which is characterized by tumor cell pleomorphism and heterogeneity for expression of markers of mesenchymal differentiation. In a tumor induced by infection of a susceptible host with an exogenous retrovirus, such as RSV, the infection of a particular cell type is dependent on the recognition by the virus-encoded glycoprotein of a cognate cell receptor, and therefore a characteristic tumor histopathology could reasonably be ascribed to the tissue tropism of the virus. However, the unrelatedness of the mechanisms of virus glycoprotein-mediated penetration and of DNA uptake argues that in the case of RSV infection, there is a restriction of tumor cell phenotype post virus penetration and, therefore, that this phenotype is determined by the product of the v-*src* oncogene and not by the viral envelope glycoprotein.

The histopathology of the v-*src*-induced tumors differs from that of fibrosarcoma but is remarkably similar to that of a separate class of human mesenchyme-based tumors, collectively designated as fibrohistiocytic tumors (the immunohistochemical properties of malignant fibrous histiocytoma are summarized in Table 1). At present, the basis of the heterogeneity of marker expression in cells of fibrohistiocytic tumors is not understood. The system of tumor induction by v-*src* DNA transfection, which exemplifies the phenomenon of tumor cell heterogeneity in the context of the progression of an oligoclonal (or occasionally a monoclonal) tumor, may provide a model for the analysis of differentiation plasticity in soft tissue tumors.

Although several clinico-pathological features of human fibrohistiocytic tumors, such as depth of invasion, tumor size and tumor location, have been related to prognosis, no strict correlation has been found between tumor histology and the aggressiveness of tumor growth (Enzinger and Weiss 1988). This lack of correlation is consistent with the possibility that differences in tumor growth patterns may be dependent on the status of host resistance factors rather than on differences in the properties of the tumor cells themselves. In the case of v-*src* DNA-induced tumor formation, the genetics of the host, as exerted at the level of the relative strength of the tumor immune response, is a central determinant of the tumor growth pattern (Halpern et al. 1991; Wisner

et al. 1991). Work is currently in progress to determine whether the v-*src*-induced tumor cells present in a rapidly regressing tumor mass, one arising in a line of chickens (Halpern et al. 1991) genetically distinct from that utilized in this study, exhibit the same histopathology as described here for the tumor cells from a progressing mass.

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