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CHARACTERIZATION OF TUMOR CELL HETEROGENEITY IN CLONAL SUBLINES FROM A SPONTANEOUS MURINE OSTEOSARCOMA

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<u>Abbreviations</u>: B-L-K-isoenzyme, bone-liver-kidney-isoenzyme; ALP, alkaline phosphatase; PBS, phosphate-buffered saline; D'MEM, Dulbecco's modified Eagle medium; PFA, paraformaldehyde; AKV, endogenous ecotropic provirus of AKR strain mice.

A B S T R A C T

The heterogeneous composition of a single primary tumor was studied using five clonal cell lines established from a spontaneous BALB/c mouse osteosarcoma. Four of these lines showed some similarities in morphology, in vitro growth properties, extracellular matrix production and osteogenic differentiation. They formed colonies with characteristic differences in size and morphology in soft agar and osteogenic sarcomas and metastases in syngeneic mice. Ultrastructurally cells in the transplant tumors showed marked osteogenic features. There were no osteoclast-like cells. The fifth cell line had somewhat different characteristics. All five lines expressed infectious endogenous murine leukemia viruses. Molecular analysis showed the presence of newly acquired proviral genomes integrated at different sites in the cellular DNA. Increased c-myc protooncogene expression was found in one cell line and c-fos expression at different levels in all lines. There was only very low expression of c-Ha-ras and no expression of c-Ki-ras and c-sis.
The results show the presence of distinct neoplastic subclones in a primary mouse osteosarcoma. Although the clones exhibited morphological, functional and molecular diversity they retained the basic pathogenic properties of the tumor from which they were derived.

I N T R O D U C T I O N

The expression of heterogeneous phenotypic traits is a well known characteristic of many human and non-human tumors (1). Distinct diversities emerge generally from unicellular carcinogenic events (2-5), which generate genetic instability, and progress via clonal evolution of different subpopulations within a given neoplasm (1, 6-10). Tumor cell heterogeneity becomes particularly evident in cell cultures established from primary tumors. It is embodied in differences in cell features including morphology, karyotype, attachment, plating efficiency, growth rates and cloning efficiency of the cells in soft agar, and the tumorigenic and metastatic potential of cells in syngeneic or immunosuppressed animals (11-14). A further basis of diversity may lie in the different expression of retroviral (15) and cellular oncogene sequences.
The majority of data on tumor cell characterization have been derived from studies of soft tissue tumors and leukemias (4, 12, 16-18). Relatively little is known about the degree of diversity in the composition of osteosarcomas (19, 20). Numerous reports have indicated that C-type retroviruses are associated with malignant disease in many animal species including humans (21) and the expression of endogenous retroviral sequences has been considered as a possible etiological event during carcinogenesis; particularly in human (22-24) and mouse osteosarcomas (25-28). The present study was aimed at characterizing distinct neoplastic cell clones derived from a spontaneous mouse osteosarcoma with the aim of addressing such questions as the presence of cellular heterogeneity in primary osteogenic tumors, the degree of cell transformation, and the presence of malignant phenotypes with respect to the expression of osteogenic marker molecules (29-31). In addition, the expression of cellular oncogenes associated with cell transformation and cell differentiation (32-40) was investigated, as well as the acquisition of additional retroviral sequences in the tumor cell DNAs. The results indicate that primary mouse osteosarcoma comprises a mixture of heterogeneous neoplastic cells which differ in a number of fundamental characteristics.

MATERIALS AND METHODS

Tumor tissue and cell cultures

Tumor tissue was derived from a spontaneous osteosarcoma of the distal femur of an 895-day-old female BALB/c mouse.
A primary cell culture (OS-50) was established by mincing the tumorous tissue and dissociating the cells with trypsin/EDTA solution (0.05%/0.02 EDTA in PBS). Single cells were seeded into cell culture dishes (Falcon, Oxnard, Ca.) and incubated with Dulbecco's Modified Eagle Medium (D'MEM) supplemented with 10% fetal calf serum (FCS) (Gibco, Karlsruhe, FGR), penicillin (50 U/ml) and streptomycin (50 mg/ml) (complete medium). The cell cultures were passaged four times. Cells from the 4th subculture were cloned by the endpoint dilution technique in 96-well microtiter plates. Twenty-eight single cell colonies were identified and cultivated further. Five of these clonal sublines, designated K 7, K 8, K 12, K 14, K 37, were selected on the basis of differences in morphology and enzyme activities and further cultured in complete medium in a humidified incubator in an atmosphere of 5% CO_2:95% air.

The plating efficiency was determined in duplicate cultures by seeding 500 cells/petri dish (100 mm) in 10 ml of complete medium and dividing the number of individual cell colonies by the number of seeded cells after a culture period of 14 days .

Cell growth and cell size were determined in a Coulter Counter Model ZM (Coulter Electr. Hialeah, Fla.) using Coulter Calibration Standard P.D.V.B. latex beads.
Cell morphology was studied in subconfluent cultures after fixation with ethanol and staining with Giemsa solution (10%, 20 min).

Cytoskeleton and extracellular matrix

Microfilaments were visualized in paraformaldehyde (PFA)-fixed (3.5% in PBS, 20 min, 4^{o} C) and Triton x-100-treated (0.2%, 10 min) cells. TRITC-labelled phalloidin (kindly provided by T. Wieland, MPI, Heidelberg, FRG) was added to cells at a concentration of 0.5 ug/ml. Twenty minutes later the cells were washed with distilled water and examined in a Zeiss fluorescence microscope. Fibronectin was labeled in PFA-fixed cells with an antibody raised in rabbits against human plasma fibronectin (Sigma) and FITC-labelled goat antirabbit IgG (Nordic, Tilbury, DK). Micrographs were taken using Kodak Tri-X-film.

Alkaline phosphatase (ALP) activity

Specific ALP activity was determined quantitatively in cell lysates, and enzyme-histochemically in paraformaldehyde-fixed cell cultures. Immunohistochemical detection of ALP was carried out in ethanol-fixed cell cultures using a polyclonal rabbit antibody to the bone-liver-kidney-specific isoenzyme of ALP (41) and horseradish peroxidase-labeled goat antirabbit IgG. 4-Chloro-1-naphthol (0.04% in 50 mM Tris, pH 7.6) was used as substrate. The procedures have been described in detail elsewhere (42).

Retroviral expression

The expression of viral protein and infectious virus particles was examined as described elsewhere (42).

Soft agar test

Colony formation of the cells in slim-solid soft agar medium was determined as described in detail by Lloyd et al. (43).

Tumorigenicity assay

Tumorigenicity of the 5 clonal cell culture sublines was studied in newborn and 6-8 week-old BALB/c mice. 4×10^5, 10^6 and 2×10^6 cells, respectively, were injected subcutaneously in the dorsal region. The animals were checked on 6 days of the week. Mice were killed and autopsied when tumors were detected macroscopically or at the end of the observation period. Tumors and organs were processed for microscopic examination by standard techniques. In addition a transplant line (T 79) was established from the primary osteosarcoma in BALB/c mice by serial i.m. injections of minced tumor tissue.

Electron microscopy

Cell cultures of the clonal sublines, and tumor tissue derived from animals injected with cells of the clonal sublines, were fixed in cacodylate-buffered glutaraldehyde (3%) followed by chrome-osmium, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate, and examined in a Zeiss EM10CR electron microscope.

DNA analysis

High molecular weight DNAs were extracted from cells of the clonal sublines, from transplant tumor tissue (T 79) and from BALB/c embryonic tissue (E9) according to standard procedures. Briefly, the specimens were frozen and homogenized in liquid nitrogen, treated with pancreatic RNase and proteinase K and extracted with phenol:chloroform:isoamylalcohol. After ethanol precipitation and dialysis against 10 mM Tris-HCl
1 mM EDTA, the DNAs were digested to completion with an excess of the indicated restriction endonucleases. The DNA fragments were separated on 0,8% agarose gels, transferred to nitrocellulose filters as described (44), and hybridized under stringent conditions with a ^{32}P-labelled probe (a 400 SmaI-SmaI fragment, coordinates 6,6 - 7,0 kbp of Akv, specific for ecotropic mouse provirus, kindly provided by Dr. U. Rapp, Bethesda, MD..

RNA analysis

RNA was extracted by the guanidinium isothiocyanate method and purified by sedimentation through a CsCl cushion according to Chirgwin et al. (45). Slot blot analysis was carried out with a Minifold II filtration device (Schleicher and Schüll, Dassel, FRG) using Biodyne A nylon membranes (Pall, Glen Cove, N.Y.). Total RNA was denatured (60 ^{o}C, 15 min., 0.9 M NaCl, 0.09 M Na-citrate, 7.4% formaldehyde) and 20 μl aliquots were applied to the filtration device under low pressure. Blotting, immobilization, hybridization and dehybridization were carried out as described previously (46). The protooncogene probes and experimental details are given in

Schön et al. (46). The v-Ha-ras probe (pHaSV-B59), representing a 420 bp SmaI-SalI fragment (47), was kindly provided by Dr. R. Müller, EMBL, Heidelberg. The amount of transferred mRNA was estimated by hybridization of the filters with ^{32}P-labelled (2.5x10^{8}cpm/ug) human actin sequences (p Hac69) a gift from Dr. D. Gallwitz, Marburg (48). The intensity of hybridization signals was quantified with an ULTROSCAN XL laser densitometer (LKB), programmed to provide automatic peak integration.

R E S U L T S

Primary Tumor

A tumor with a diameter of 8 mm was observed macroscopically in the right thigh of an 894 day-old female BALB/c mouse. The X-ray of the distal end of the right femur (Fig. 1a) showed destruction of bone tissue by an irregular, dense tumor. An additional spread of the tumor was visible at the proximal end of the femur.

Histologically the tumor had the typical appearance of an osteosarcoma, with some richly cellular areas (Fig. 1b) and some areas showing formation of bone by pleomorphic osteoblast-like cells (Fig.1c). The marrow cavity of the femur was completely filled with tumor tissue. The tumor invaded the surrounding muscle and connective tissue at both ends (but preferentially at the distal end). In addition numerous nodules (diameter 2 to 7 mm) of metastatic osteosarcoma were found in the lung.

Osteosarcoma cell cultures

Osteosarcoma tissue was removed aseptically and cut into small pieces approximately 1 mm in diameter. Repeated trypsinization yielded single cell suspensions which were seeded at a concentration of 5x10^{4} cells/cm^{2} in complete medium.

A confluent cell layer formed within 10 days and exhibited heterogeneous morphology. Most of the cells were mononucleated and multipolar. The cultures were interspersed with elongated bipolar cells and a few bi- and multinucleated cells with a

large cytoplasm which accumulated in compact areas of confluent cultures. The cell cultures were split in a 1:10 ratio and further cultivated until the 4th passage.
All passage-4 cells were positive for retroviral core protein p30 expression and cell-free supernatant from the cell culture was infectious for both NIH 3T3 mouse fibroblast cells and CCL-64 mink epithelial cells, indicating production of infectious ecotropic and xenotropic murine leukemia virus particles.

Clonal sublines

Passage-4 cells were cloned by end point dilution in 96-well tissue culture cluster plates. Thirty-six single cell colonies were identified, isolated from individual wells, and grown to petri dish level. 5 clones, designated K 7, K 8, K 12, K 14 and K 37, representative of the different cell types in the original cell culture were selected.
The K 7 culture contained mainly fibroblast-like and spindle shaped elongated cells, which exhibited criss-cross growth of cytoplasmic extensions after reaching confluency. K 8 cells showed predominantly epitheloid morphology with an apparent inhibition of cell division after reaching confluence. K 12 cultures consisted of large epitheloid and fibroblast-like cells which aggregated in compact areas of the cell culture. K 14 cells were morphologically similar to those of K 12, but were markedly smaller in both size and volume (in the spheric state after trypsinization). The K 37 culture contained large multipolar cells which developed stellate cytoplasmic extensions after prolonged time in culture. The cells showed inhibition of cell division at an early subconfluent state.

Plating efficiency and cell growth

The plating efficiency (P.E.) differed greatly in the clonal sublines. The highest P.E. was found in K 7 cells (43.3 %). K 8, K 12 and K 14 cells had a smaller P.E. with values of 19.8 %, 15.5 % and 23.9 %, respectively, and K 37 cells had a P.E. of only 9.2 % (Tab. 1). Similarly, cell growth in the K 7 culture exceeded that in the three clonal sublines K 8, K 12 and K 14, all of which had a similar proliferation rate. Growth of K 37 cells was significantly reduced in comparison to that of the other four sublines. The data are shown in Fig. 2.

Alkaline phosphatase activity

ALP activity, which indicates osteogenic differentiation in osteoblast-like cells (31, 41), was demonstrated enzyme histochemically in situ in PFA-fixed confluent cell cultures. The results are shown in Fig. 3 and Table 1. The enzyme activity was distributed irregularly across the cultures. A particularly high activity was seen in densely packed cells in compact areas of K 7 and K 12, and also in part in K 14, cell cultures. K 8 cells showed a more evenly distributed ALP activity with sharply defined patches of cells exhibiting different levels of enzyme activity. In all cells with high ALP activity the enzyme was preferentially located along the cell membranes. In K 37 cells ALP activity was only noted in small amounts in epitheloid cells. Quantitative analysis of specific ALP activity showed a high enzyme activity in K 8, K 12 and K 14 cells of between 4.4 U/mg and 4.9 U/mg protein. Smaller activities of 1.6 U/mg protein and 0.8 U/mg protein were found in K 7 cells and K 37 cells, respectively. A similar pattern of ALP activity was found in 3 week-old soft agar cell colonies (data not shown).

Immunohistochemical labeling of ALP with polyclonal antibody to the bone-liver-kidney-specific ALP isoenzyme confirmed the data obtained by the enzyme activity assay and provided further evidence for the osteogenic origin of the cells (Fig. 3).

Cytoskeleton and extracellular matrix

The fluorescent patterns of the cytoskeleton showed partial accumulation, and whorl-like structures of various sizes, of actin microfilaments on the dorsal surface in K 7 and K 37 cells and to a lesser extent in K 8 cells. Remnants of long actin-containing sheaths, together with diffuse matrices of actin-containing material, were noted in all sublines. A rather pronounced expression of irregularly oriented actin microfilaments, together with submembraneous condensations of brightly fluorescing material, was noted in K 14 cells. K 37 cells showed predominantly ruffles and flower-like aggregates of polymerized structures, preferentially located in the pseudopod-like cytoplasmic branches (Fig. 3).

Immunofluorescent staining of fibronectin revealed a strongly labeled fibril network extending across the entire culture of K 7 and K 14 cells. K 12 cells showed a lower expression of fibronectin fibrils, restricted to intercellular spaces, and K 8 cells a fibrillar pattern together with patchy aggregates of fibril-related fluorescence. K 37 cells had only small spots of cell-associated fibronectin and no intercellular fibril network (Fig. 3).

Soft agar growth and tumorigenicity

Cells from all five clonal sublines gave rise to cell colonies in semisolid agar medium. However the size and morphology of the colonies varied considerably. After 14 days in culture K 7 and K 8 cells developed small compact colonies with little variation in size, K 12 and K 14 cells large compact colonies, and K 37 cells large, loosely packed colonies. None of the soft agar colonies formed by these cells reached the size of those observed after seeding OS-5 cells, a permanent cell line established from a radiation-induced mouse osteosarcoma which was used as a control (49).

In a first in vivo experiment newborn BALB/c mice were injected subcutaneously with 10^6 cells from the clonal sublines which had been scraped off from the culture dish with a rubber policeman. Four of the five clonal sublines formed osteosarcomas, and very occasionally spindle-cell sarcomas, with a mean latent period of 39 to 67 days after injection and a mean diameter of approximately 6x10 mm (Table 2). K 7 and K 14 cells showed a similar time of appearance of the tumors (6-9 weeks) and cumulative tumor incidence. In contrast, K 8 and K 12 cells formed slow-growing tumors (Fig. 4). K 37 cells treated in the same way as cells from the other four clones did not form detectable tumors within the observation period of 84 days.

In order to study the tumorigenicity of K 37 cells further, the cells were trypsinized and $2x10^6$ or $4x10^5$ cells, injected into 30 6 to 8 week-old mice. At the end of the observation period of 58 days K 37 cells had formed polymorphic sarcomas with an incidence of 71% (20/28) after injection of $2x10^6$ cells, or 62% (18/29) after injection of $4x10^5$ cells; the mean diameter of the tumors was 19x13 mm and 12x9 mm, respectively (Table 2).

Metastatic nodules of osteosarcomas were found in the lungs of at least some animals in all the groups in which tumors were formed. The incidence of metastases, however, varied greatly between 100% in K 7-injected mice and 12% in K 14-injected mice (Table 2).

<u>Electron Microscopy</u>

The ultrastructural appearance of cells in the different cell cultures was very similar. Cells were loosely associated, occasionally forming close groups. Nuclei were mostly irregular in outline with varying amounts of marginated condensed chromatin and medium prominent nucleoli. The cell cytoplasm contained a number of mitochondria, a fair amount of often slightly dilated rough endoplasmic reticulum, and varying numbers of secondary lysosomes, some with recognizable remnants of C-type virus particles. All cell cultures contained large numbers of, often atypical, C-type virus particles, with the exception of K 37 cultures in which particles were considerably fewer and all of classic type.

The transplant tumors from K 7, K 8, K 12 and K 14 were also very similar with marked osteogenic features. Transplant tumors from K 37 were not investigated. The cells had the appearance of osteoblasts or osteocytes, with spindle-shaped irregular nuclei containing varying amounts of marginated and clumped condensed chromatin and medium prominent nucleoli. Cytoplasm was often scanty containing a high proportion of

dilated rough endoplasm reticulum, together with mitochondria, occasional lyosomes and lipid vacuoles and, very rarely, glycogen. Cells were surrounded by large amounts of osteoid and mineralized bone substance often in the form of lacunae. All tumors contained large numbers of, often atypical, virus particles (Fig. 5).
There were no osteoclasts or osteoclast-like cells in any of the cell cultures or transplants.

Acquisition of new proviruses

The EcoRl pattern of the DNA from all specimens hybridized with an ecotropic specific virus probe showed a common band of 19.5 kb. This band represented the ecotropic endogenous provirus of the BALB/c mouse as shown by the single ecotropic provirus-containing fragment found in genomic DNA from the BALB/c embryo E9. Various additional provirus-containing fragments were found in the different sublines and also in the DNA from a transplant tumor line established from the original osteosarcoma, indicating the occurrence of additional provirus integration events at independent sites in the different sublines and the transplanted tumor (Fig. 6). The DNA from K 12 and K 37 cells showed a similar pattern of virus integration after EcoRI digestion. In order to characterize viral integration in these two cell lines further, the DNA was digested with a second restriction enzyme. SacI-digested DNAs from K 12 and K 37 cells also showed similar virus integration patterns (data not shown) indicating that these two cell lines have additional proviral sequences integrated at the same sites.

Protooncogene expression

Protooncogene expression in the five clonal sublines from the spontaneous osteosarcoma is shown in Fig. 7. C-myc expression was very low in K 7 and K 8 cells, a moderate expression was found in K 12 and K 14 cells and a high c-myc expression in K 37 cells. All clonal sublines showed expression of c-fos oncogene. K 7, K 12 and K 37 cells expressed similar levels of c-fos, K 14 cells a clearly higher expression, and K 8 cells the highest expression exceeding that of K 14 cells by a factor of 2. Expression analysis revealed very low Ha-ras signals. Expression of c-sis and c-Ki-ras was not detected.

D I S C U S S I O N

The experiments described here demonstrate the heterogeneity of clonal cell cultures isolated and established from a primary mouse osteogenic sarcoma. The five cell clones which were investigated in detail were selected from 28 cell clones on the basis of differences in morphological appearance and the presence or absence of alkaline phosphatase expression. The expression of this enzyme, regarded as a hallmark molecule for osteogenic cells (31), is closely associated with an epitheloid-polygonal shape of both primary (30, 42, 50, 51) and immortalized (52-55) osteoblast-like cells in culture. The neoplastic cells in clones K 7 and K 14 had the morphological properties of transformed cells as well as high ALP levels, indicating the maintenance of differentiated, functions in the transformed state. The result of immunohistochemical labeling of the cells with the specific antibody to the B-L-K-isoenzyme of ALP was consistent with that obtained by enzyme histochemistry and suggests nidation of the mature enzyme in the cell membrane as found in normal differentiated osteoblasts in culture (42, 55). The production of collagen fibres, shown representatively for K 14 cells, and the formation of differentiated osteogenic tumors in syngeneic mice, further support the identification of the cells as neoplastic osteogenic cells. The K 37 cells, which lacked significant ALP activity, formed undifferentiated polymorphic sarcomas. Thus the expression of osteogenic traits in vitro, indicated by ALP as a marker, mirrored the different properties of the osteosarcoma cells in vivo.

One of the primary effects resulting from transformation by an oncogenic virus is the abrogation of expression of differentiated phenotypes. This has been shown for virus-transformed myoblasts (56, 57), melanoblasts (58, 59) chondroblasts (60, 61) and fibroblasts (62, 63). Osteogenic tumors and cell lines established therefrom appear to provide an exception to this rule. Such cell lines are capable of expressing a variety of differentiated functions together with the morphologically and functionally transformed phenotype (53). The general validity of this observation was confirmed by the results described here. In addition to relatively high levels of ALP activity, four of the five clonal cell lines formed a considerable amount of fibronectin. These data contrast with previous findings in which decreased levels of fibronectin were observed in cell cultures transformed by oncogenic viruses or derived from tumors (64-66), and particularly low fibronection levels were found in metastatic tumor cells (67), indicating the decreased adherence of transformed cells to their surrounding substrate (68). Our findings are a further indication of the special properties of transformed bone cells which retain the ability to produce large amounts of collagenous and non-collagenous pericellular matrix proteins.

Partial depolymerization, and dot-like organization of the cell cytoskeleton, was a consistent pattern observed in the five subclones. These findings confirm the close association between acquisition of the neoplastic phenotype and actin-depolymerization and loss of actin-containing sheaths, which has been observed in transformed mouse and chicken fibroblasts (67, 69-71).

Taken together the results describing morphological and functional parameters in the different cultures indicate the presence of neoplastic clones which show osteogenic differentiation, but with a wide variability in the extent to which this is expressed. The observations are consistent with the stem cell concept of tumor initiation (3, 72) in which the neoplastic event is considered to take place in a committed osteogenic stem cell.

Recently we reported the presence of additional retroviral sequences in radiation-induced osteosarcomas, and the absence of such sequences in non-tumorous tissue, suggesting a critical involvement of retroviruses in tumorigenesis (28). It is known that the integration of additional viral DNA into the cellular genome can directly damage genes (73, 74) or bring them under the control of powerful regulatory elements present in the viral genome (75). Southern analysis revealed the presence of additional proviral sequences in the clonal sublines. In addition there was a high expression of infectious virus particles in both the cell lines and the tumors which they formed. The enhancer and promoter containing sequences newly integrated in the neoplastic clonal cell lines, might play an initiating or supporting role in the generation of genetic instability during the "self renewal" period (2, 6). Equally they could render the cells increasingly susceptible to proliferation-inducing signals in the course of tumor progression. (21, 76). A third possibility, insertion of viral promoters near cellular protooncogenes (77-79), seems unlikely: enhanced expression of c-myc was only found in K 37, of c-fos only in K 8 and K 12 cells. Further, new fos-containing fragments were not observed in the restriction enzyme analysis of the DNA from the five clones (data not shown). Although this does not rule out the insertion of viral promoters near cellular oncogenes other than those investigated here, enhanced expression of a host DNA-coded oncogene by retrovirus promoter insertion does not appear to be the predominant mode of action of retroviruses in mouse osteosarcomagenesis.

Conflicting results were obtained in the in vivo assay with respect to the tumor incidence in syngeneic mice. When K 37 cells were scraped off from the culture dish and injected subcutaneously, they did not form tumors. In contrast, after dissociation of entire cells with trypsin, tumor growth was observed in 78% to 93% of the injected animals. This discrepancy was very likely due to damage of the particularly large K 37 cells (Table 1) during the scraping procedure resulting in the reduction of the number of viable cells in the inoculum by more than 90%. As shown by the proliferation kinetics of the cells in culture, K 37 cells grew significantly more slowly than those of the other subclones, even though a logarithmic growth rate was observed until day 8 of the culture. These data are consistent with the idea that a threshold number of cells in the inoculum is necessary to initiate tumor growth, a requirement which may have not been fulfilled in the first tumorigenicity assay.

The clonal sublines showed marked differences in their metastatic potential. However, these data could not be attributed to particular functional properties of the cells, such as plating efficiency, matrix production or degree of osteogenic differentiation, or to the expression of a distinct cellular oncogene. We have recently shown for several osteosarcoma cell lines, including the OS-50 cell line from which the above subclones were selected, that migration towards fibroblast-conditioned medium and invasion through a reconstituted basement membrane can be inhibited by human leucocyte and mouse fibroblast interferon (80, 81). We are presently studying the response of the above cell clones to various inhibitors of tumor cell migration, and their invasion of basement membranes.
In summary the results show the heterogeneity of cell clones derived from a single primary tumor. Such osteogenic neoplastic clones provide a further means of studying the influence and importance of various factors on and for tumorigenesis.

A C K N O W L E D G E M E N T S

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F I G U R E   L E G E N D S

Fig. 1

X-ray and histological appearance of the original spontaneous osteosarcoma in an 894 day-old mouse.
a, X-ray of the right femur.
Destruction of bone and spread of dense tumor tissue is shown at the distal, and to a lesser extent the proximal, end of the femur.
b, Section through a richly cellular part of the osteosarcoma with numerous mitotic figures.
c, Section through a different area of the same tumor showing bone formation by pleomorphic osteoblast-like cells.

Fig. 2.

Growth curves of clonal sublines established from the mouse osteosarcoma. The cells were seeded into 2x2 cluster plates at a density of 2×10^4 cells/well. Cell growth was determined on days 1, 3, 5, and 8 post-seeding. △, K 7; ▽, K 8; □, K 12; ■, K 14; ◇, K 37.
Mean of quadruplicate determinations ± standard deviation.

Fig. 3.

Actin microfilaments, extracellular matrix-fibronectin and alkaline phosphatase in clonal sublines from a mouse osteosarcoma.
a-d, K 7; e-h, K 8; i-m K 12; n-r, K 14; s-v, K 37.
a, e, i, r, s, fluorescence micrographs of TRITC-phalloidin-labeled actin microfilaments. (x400).
b, f, k, o, t, immunofluorescence micrographs of fibronectin (FN). Subconfluent cells were treated with anti-FN followed by FITC-conjugated goat antirabbit IgG. (x400).
c, g, l, p, u, light micrographs of immunohistochemically labeled alkaline phosphatase. The cells were treated with polyclonal rabbit antibody to the bone-liver-kidney-specific ALP isoenzyme followed by incubation with peroxidase-coupled goat antirabbit IgG. 1-chloro-4-naphthol was used as substrate. Note the membrane-associated localization of the reaction product in the labeled cells. (x250).
d, h, m, r, v, enzyme histochemical detection of alkaline phosphatase activity in formaldehyde-fixed cells. The cells were incubated in naphthol-AS-MX-phosphate and fast blue BB salt solution for 30 min. Note the prevailing membraneous localization of the reaction product in ALP-positive cells. (x125).

Fig. 4.

Cumulative tumor incidence of clonal sublines established from a mouse osteosarcoma. Newborn BALB/c mice were injected with $2x10^6$ cells subcutaneously. Animals were killed when tumors were detected macroscopically and tumor tissue was processed for histological examination. ■, K 7; ▼, K 8; △, K 12; ◆, K 14.

Fig. 5.

K 14 transplant tumor. Section through an osteocyte surrounded by osteoid and mineralized bone matrix. Large numbers of pleomorphic C-type virus paricles lie adjacent to the cell and trapped within osteoid.
Inset: virus particle budding from the cell surface.
Bar 2 μm.

Fig. 6.

Newly acquired ecotropic proviral sequences in 5 clonal sublines established from a spontaneous mouse osteosarcoma and in the osteosarcoma tumor transplant line. DNA from the clonal sublines K 7, K 8, K 12, K 14, K 37, from the tumor transplant T79, and from a normal BALB/c mouse embryo (E9) was digested with the restriction endonuclease EcoRI and the electrophoretically separated DNA fragments hybridized to an ecotropic specific probe. There is a 19.5 kb fragment representing the ecotropic germline provirus of BALB/c strain mice, in all DNAs (arrows). Several additional bands indicative of newly acquired proviral sequences are seen in the clonal sublines and in the DNA of the osteosarcoma tumor transplant.

Fig. 7.

Slot blot analysis of c-myc, c-fos, c-sis, c-Ki-ras and c-Ha-ras protooncogene expression in 5 clonal sublines (see Fig. 6 legend). K 7, K 8, K 12, K 14, K 37, total RNA from clonal sublines; C, control oncogene DNA; L, mouse liver DNA. Left panel, equal amounts of total RNA were hybridized with the oncogene probes; right panel, same as left, after dehybridization and hybridization with a human actin probe. The amount of cellular RNA was the same in all the samples assayed as shown by hybridization of the same filters with the specific human actin probe.

Table 1

In vitro characteristics of clonal sublines from spontaneous mouse osteosarcoma

Cell line	Plating Efficiency (%)	Growth in Soft Agar	ALP Activity (U/mg Protein)	Mean Cell Diameter (μ)[a]
K 7	63.3	+	1.6 ± 0.3	12.8
K 8	19.8	+	4.6 ± 0.7	13.1
K 12	15.5	+	4.4 ± 1.0	14.5
K 14	23.9	+	4.9 ± 1.1	12.6
K 37	9.2	+	0.8 ± 0.2	17.2

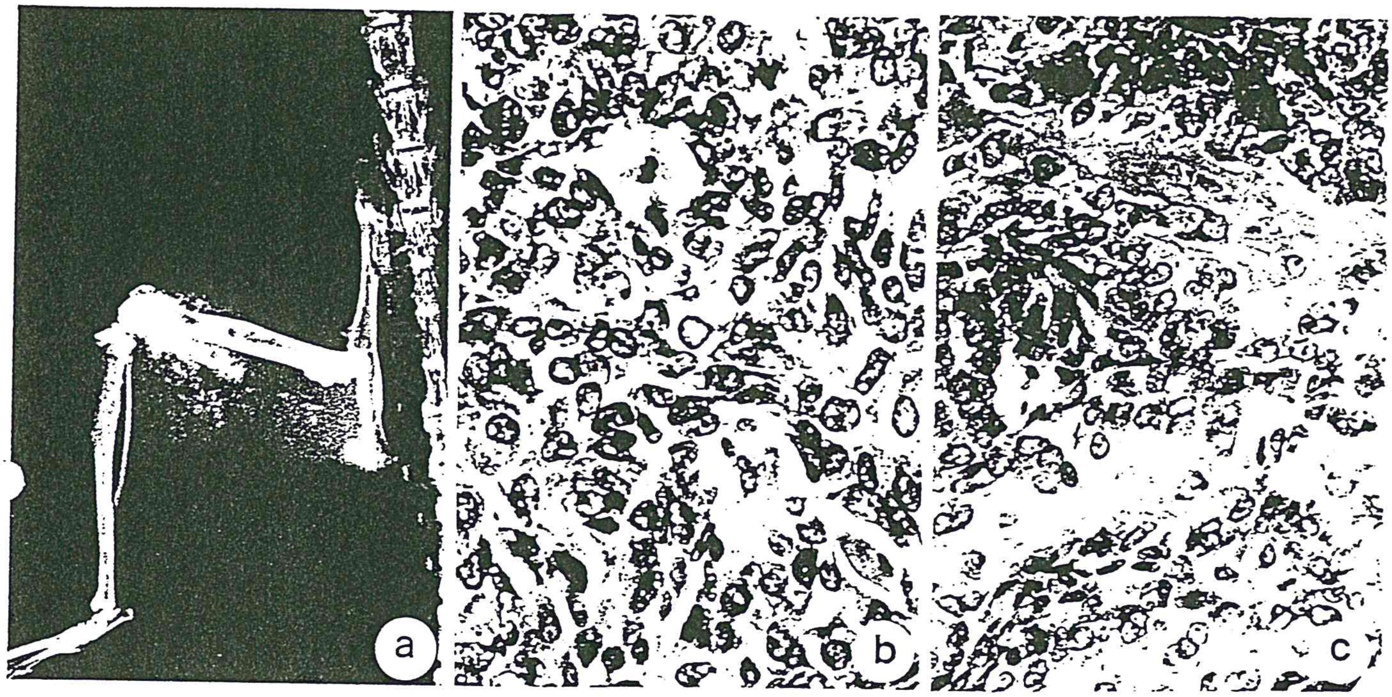
a, the diameter was determined in the spheric state of the cells after trypsinization.

Table 2

Tumorigenicity and metastastatic potential of clonal sublines from spontaneous osteosarcoma in BALB/c mice

Cell line	Age of Recipient	No. of Cells Inoculated[a]	Tumor Incidence	Metastases	Observation Period (days)	Mean Latent Period (days)	Mean Tumor Size (mm)
K 7	newborn	10^6	11/11	11/11	50	45	10x7
K 8	newborn	10^6	9/9	8/9	76	67	10x7
K 12	newborn	10^6	5/9	3/9	79	65	10x8
K 14	newborn	10^6	7/8	1/8	73	39	9x5
K 37	newborn	10^6	0/10	0/10	84		
K 37	6-8 weeks	$2x10^6$	22/28	20/28	58	28	19x13
		$4x10^5$	27/29	18/29	58	30	12x 9

a, newborn animals were injected with cells scraped off from the culture dish.
adult animals were injected with trypsinized cells.



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Figs 1a, b, c

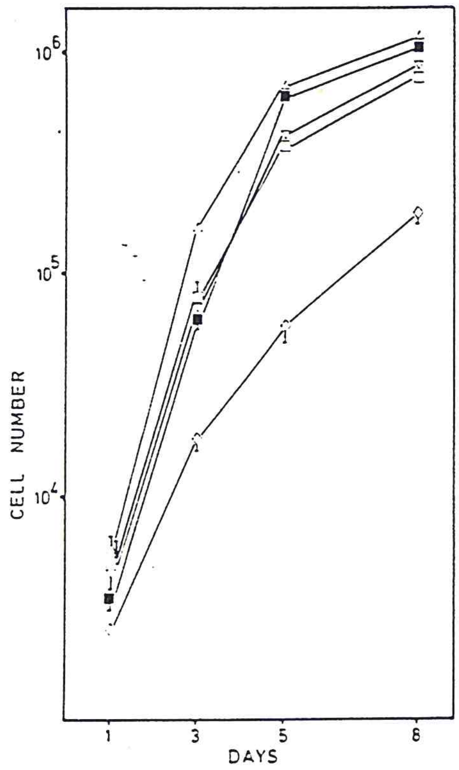


Fig 2

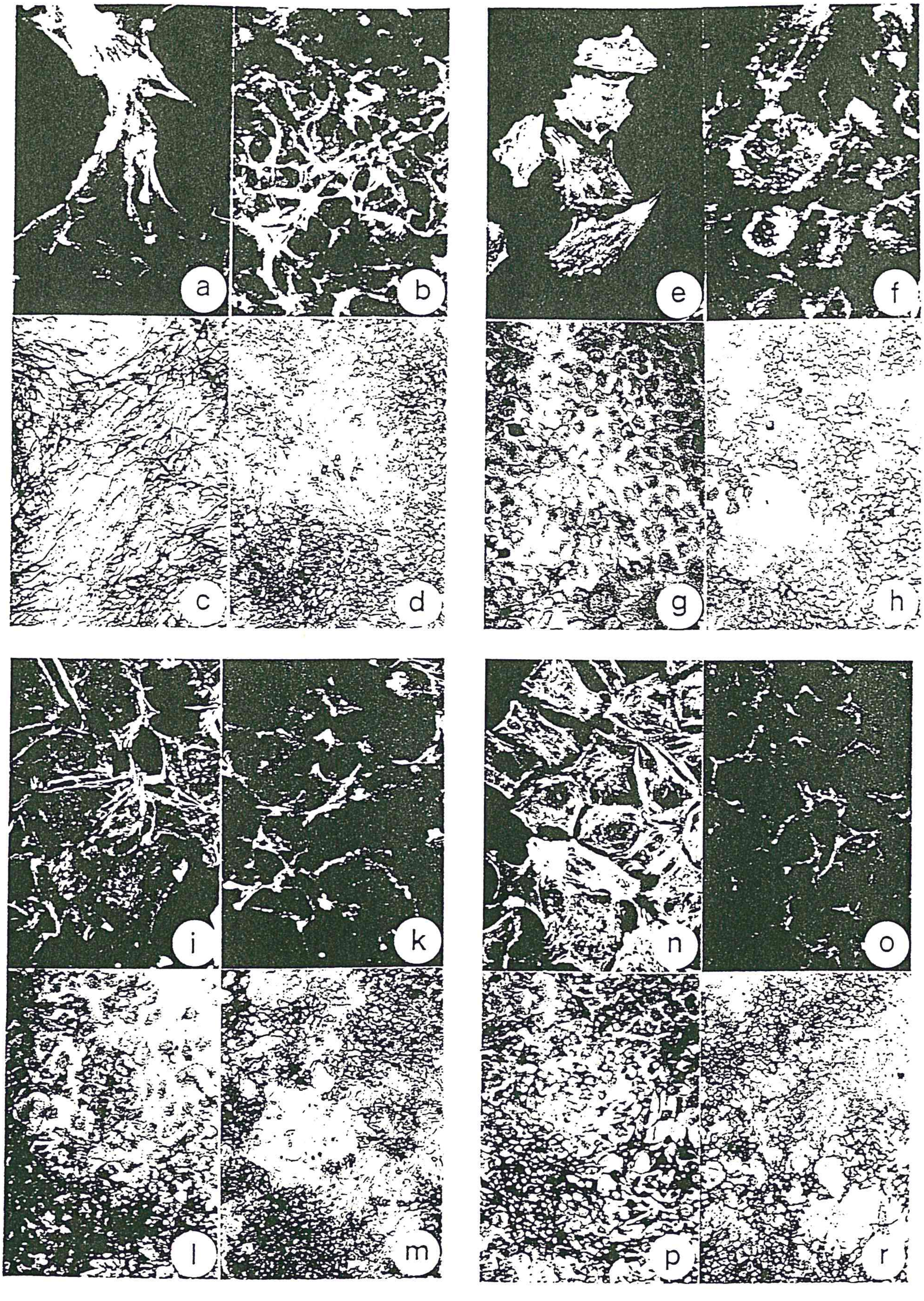
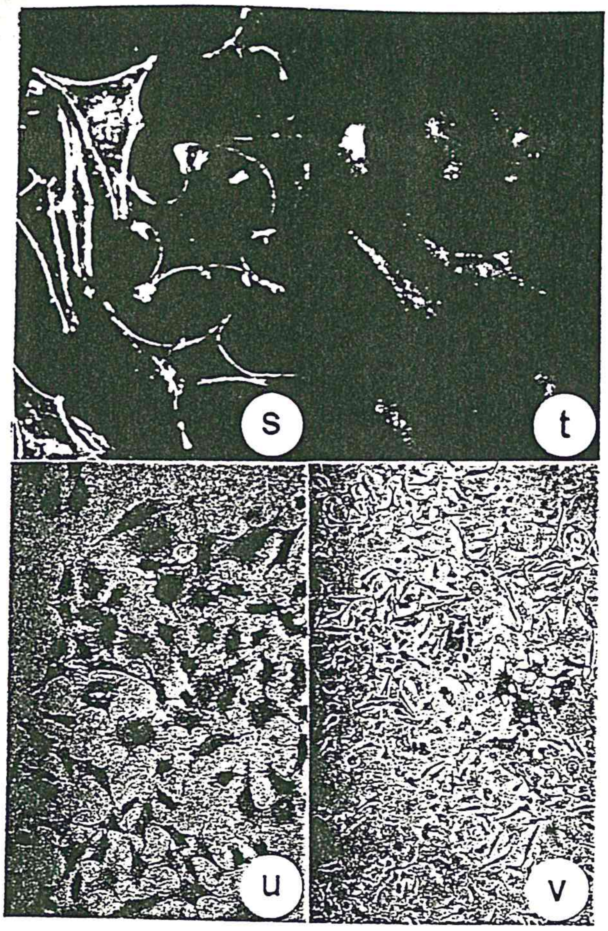


Fig 3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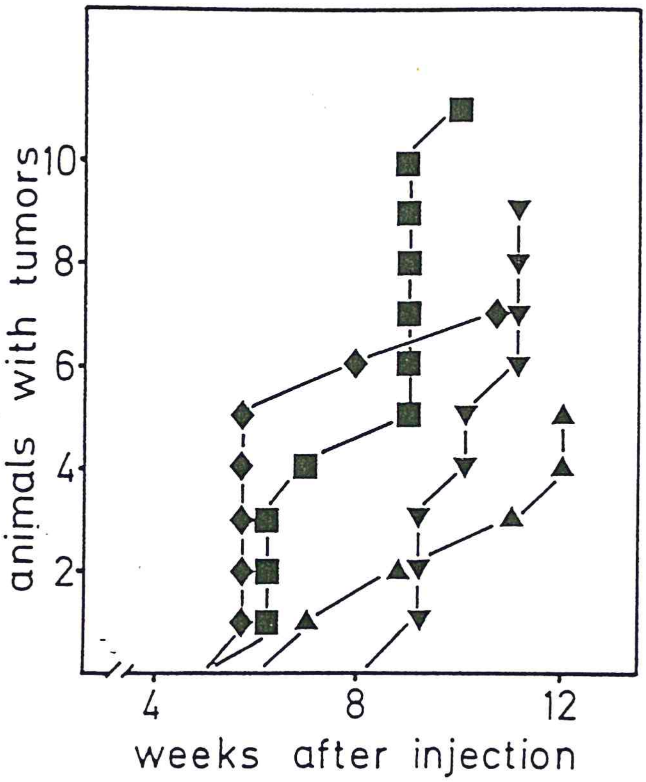

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Fig. 4

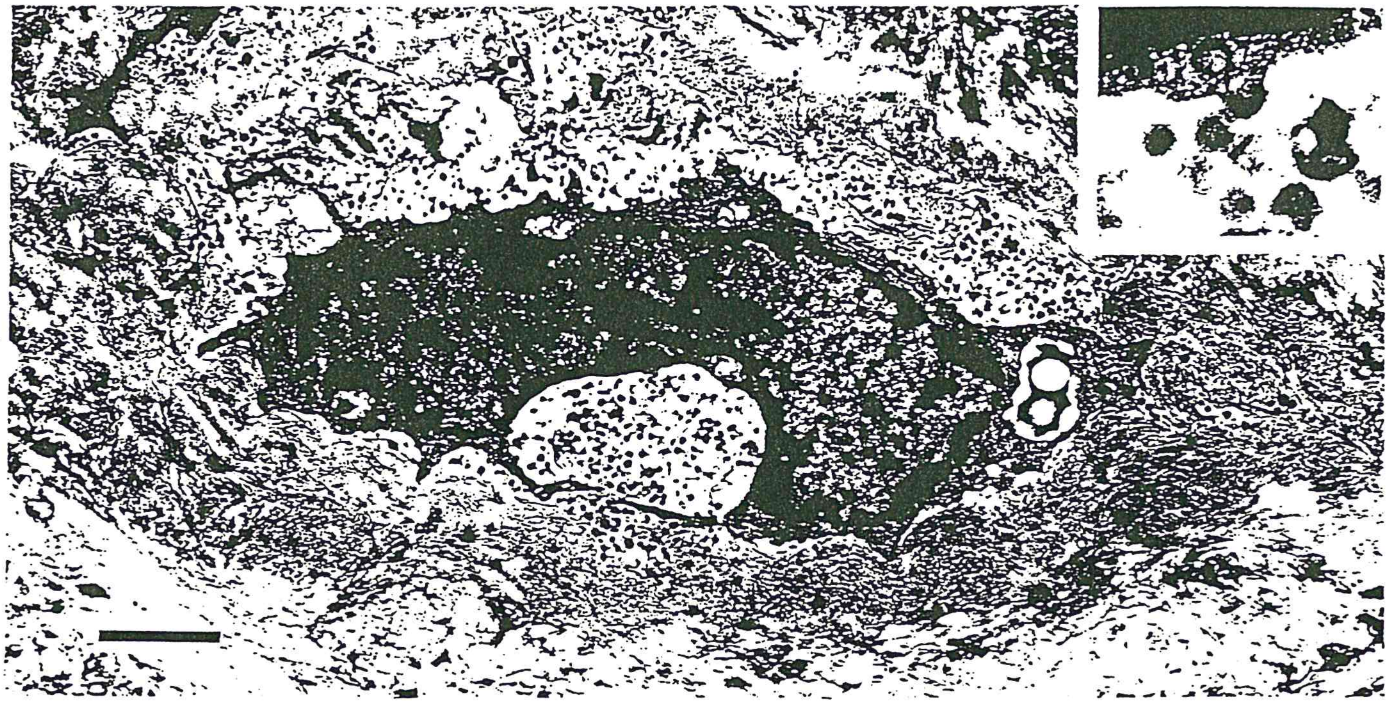

Fig 5

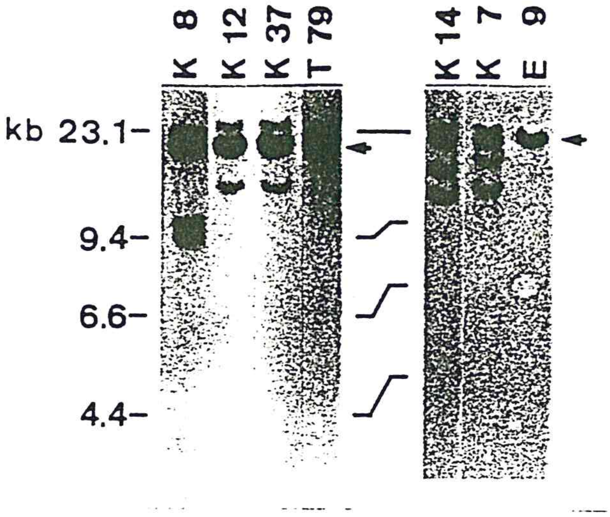
K 8
K 12
K 37
T 79
K 14
K 7
E 9
kb 23.1–
9.4–
6.6–
4.4–

Fig 6

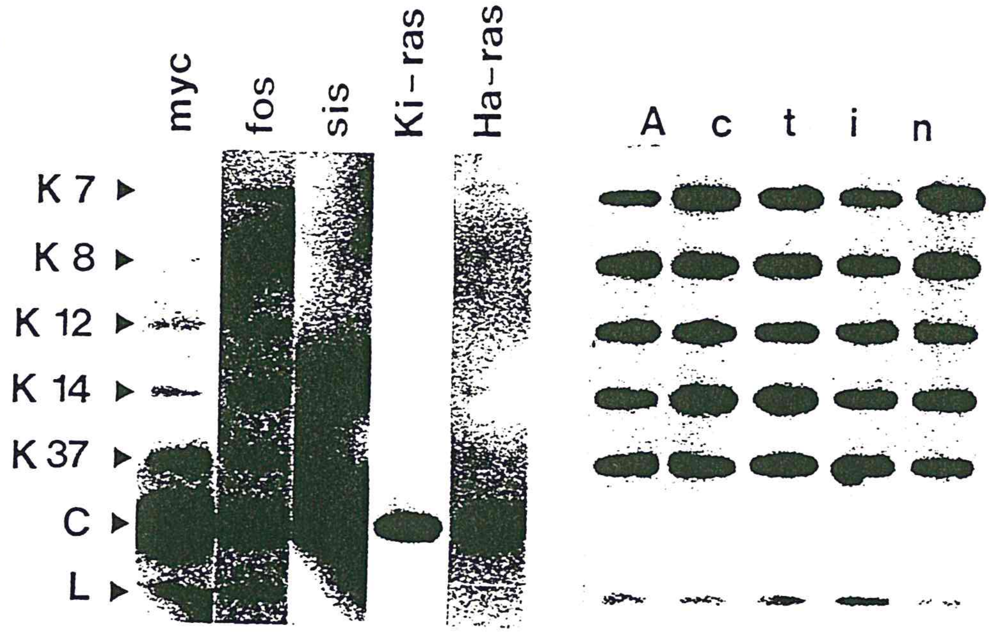
myc
fos
sis
Ki-ras
Ha-ras
A c t i n
K 7
K 8
K 12
K 14
K 37
C
L

Fig. 7