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Differential Susceptibility of Human Cells to Transformation by Murine and Avian Sarcoma Viruses (41441)

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Abstract. The susceptibility to Kirsten murine sarcoma virus (Ki-MSV) and Schmidt-Ruppin strain of Rous sarcoma virus (RSV-SR) transformation of cultured skin fibroblasts derived from hereditary adenomatosis of the colon and rectum (ACR), its Gardner's syndrome (GS) variant, and unrelated age and sex-matched controls was examined. Human skin fibroblasts from ACR and GS individuals were highly susceptible to transformation by Ki-MSV but not susceptible to RSV-SR transformation. However, the human osteosarcoma (HOS) clonal cells (TE-85 clone F-5), known to be sensitive to Ki-MSV transformation, could also be readily transformed by RSV-SR. The RSV-SR-transformed cells formed large cell aggregates and grew in this aggregate form when suspended in liquid growth medium above an agar base, formed colonies in soft agar with high efficiency, and grew to high saturation densities while the uninfected cells did not. The transformed cells produced tumors when transplanted subcutaneously into nude mice. The morphologically altered cells produced neither infectious virus nor type C virus particles. However, they contained RSV-RS complement-fixing antigen, a RSV transformation-specific protein ($p60^{src}$)-associated kinase activity, and a rescuable infectious virus.

Genetically determined adenomatosis of the colon and rectum (ACR) occurs in about one in 8000 people, manifesting itself in colonic neoplasia by the third decade of life, and is inherited as an autosomal dominant trait (1, 2). Gardner's syndrome, an ACR variant, includes benign tumors of soft tissue and bone (3). A number of cancer-associated *in vitro* expressions, such as abnormal growth properties, increased proteolytic activity, cell architecture, and increased susceptibility to transformation by RNA or DNA tumor viruses and chemical promoters have been described for cultured skin fibroblasts derived from ACR individuals (4). Pfeffer and Kopelovich (5) reported that human skin cultures from ACR individuals were considerably more susceptible to transformation by the Kirsten murine sarcoma virus (Ki-MSV) than were normal skin fibroblast cultures and they proposed that the Ki-MSV transformation assay might be a useful tool for the early detection of individuals genetically predisposed to colorectal cancer. We thought that this important finding should be carefully examined and further studied. We report herein confirmation that human skin fibroblasts from ACR and GS patients were highly susceptible to Ki-MSV transformation. In addition, we have studied the susceptibility of human skin fibroblasts from ACR or GS individuals and the human osteosarcoma (HOS) clonal cells to transformation by Rous sarcoma virus (RSV).

Materials and Methods. Human skin fibroblast cell strains derived from ACR individuals were obtained from Dr. Levy Kopelovich, Memorial Sloan–Kettering Cancer Center, New York. Human skin fibroblasts derived from GS individuals, as well as asymptomatic individuals from familial polyposis (FP/N) families were received as coded samples from Drs. M. B. Gardner and S. Rasheed, University of Southern California, School of Medicine, Los Angeles, California. These cell strains were found to be highly sensitive to transformation by Ki-MSV (5, 6). The human osteosarcoma (HOS) cell line TE-85, clone F-5 used in this study has been described in detail (7). The line had cytologic and karyologic characteristics like those of the parent tumor but its growth pattern is rela-

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tively regular and flat so that the transformed foci are readily recognized. This line was found to be highly susceptible to transformation by Ki-MSV (7).

All the cell strains were grown and maintained in Eagle's minimal essential medium (EMEM) with 15% fetal bovine serum (FBS), 2 m*M* glutamine, 1% nonessential amino acid, and 50 mμg of gentamicin/ml (EMEM + 15% FBS). The Ki-MSV stock [supernatant fluids from a Ki-MSV-transformed NRK cell line, No. 58967 (8)] was kindly supplied by Dr. V. Klement, University of Southern California, Los Angeles, California. Twenty-four-hour culture fluid harvests of 58967 cells yielded 3×10^5 focus-forming units (FFU) of Ki-MSV/ml when assayed in NRK cells. The Schmidt-Ruppin strain of RSV (RSV-SR, originally from Dr. P. Vogt, 1969) was propagated in primary chick embryo fibroblasts (CEF) from resistance-inducing factor (RIF)-free eggs and had a titer of 7.5×10^4 focus-forming units (FFU)/ml when assayed on RIF-free DEAE–dextran-treated CEF (25 μg/ml for 30 min). Focus formation by Ki-MSV was assayed on the DEAE–dextran-pretreated cultures as described previously (7). Within 24 hr after cell plating (3×10^5 cells/25-cm^2 Petri dish) cultures were treated for one hour with 4 ml of 25 μg/ml of DEAE–dextran (Sigma Chemical, molecular weight 2×10^6 daltons). These were washed once with EMEM and inoculated with 0.4 ml of 10-fold serial dilutions of virus stock. Medium was changed twice weekly and cultures were observed for focus formation at 7 days for NRK cells and at 14 days for human skin fibroblasts. Focus formation by RSV was also assayed on DEAE–dextran-treated cultures (25 μg/ml for 30 min) inoculated at 2×10^5 cells/petri dish 24 hr prior to infection. A combination of the following procedures was used to detect the replication of the virus in culture: (a) test for CF antigen reactive with RSV-SR antiserum; (b) examination by electron microscopy for the presence of virus particle; and (c) assay for supernatant viral RNA-dependent DNA polymerase. CF tests were carried out by microtiter technique described for tumor antigen studies (9). Titers were recorded as reciprocals of the highest dilution given, 3+ to 4+ fixation of 1.8 units of complement. RSV-SR antibody was obtained from hamsters bearing RSV-SR tumors.

Formation of cellular aggregates by normal and transformed cells was tested by the method described by Steuer *et al.* (9). Freshly trypsinized viable cells (2×10^5) were seeded into 35-mm plastic dishes containing a 2-ml agar base layer (0.9% Difco agar in growth medium with 20% FBS). The dishes were incubated at 37° under 5% CO_2/95% air. Viable cells were counted daily for 4 days.

For the soft agar assay cells were suspended in 0.36% agar medium (EMEM + 20% FBS + antibiotics). This suspension was layered onto a 0.9% agar base layer at concentration of 10^4-10^5 cells per 35-mm dish. Dishes were incubated in a humidified atmosphere (5% CO_2) at 37°. After 2 weeks colonies greater than 0.125 mm in diameter were counted. Results are expressed as percentage plating efficiency (%PE = number of colonies $\times$ 100/number of cells plated).

A genome-rescue experiment was done by cocultivation of RSV-SR-transformed human cells (1×10^5) and CEF cells (5×10^5). Supernatants from these cultures taken from the ninth day after infection were assayed for virus on CEF cells.

NIH nude mice were inoculated subcutaneously with 5×10^6 freshly trypsinized cells in order to determine cell tumorigenicity.

Immune complexes formed between a RSV transformation-specific $p60^{src}$ from cell extracts of transformed and untransformed human cells and immune IgG from tumor-bearing rabbits (TBR) were incubated in the presence of [r-^{32}P]ATP. Immunoprecipitation was conducted according to the procedures of Collett and Erikson (10) and Richert *et al.* (11).

Results. In the Ki-MSV-infected cultures focus areas of transformation were observed in most instances at the end of the first week. Foci were characterized by refractile spindle-shaped and round cells which grew on top of the monolayer (Fig. 1B). The number of foci did not increase for

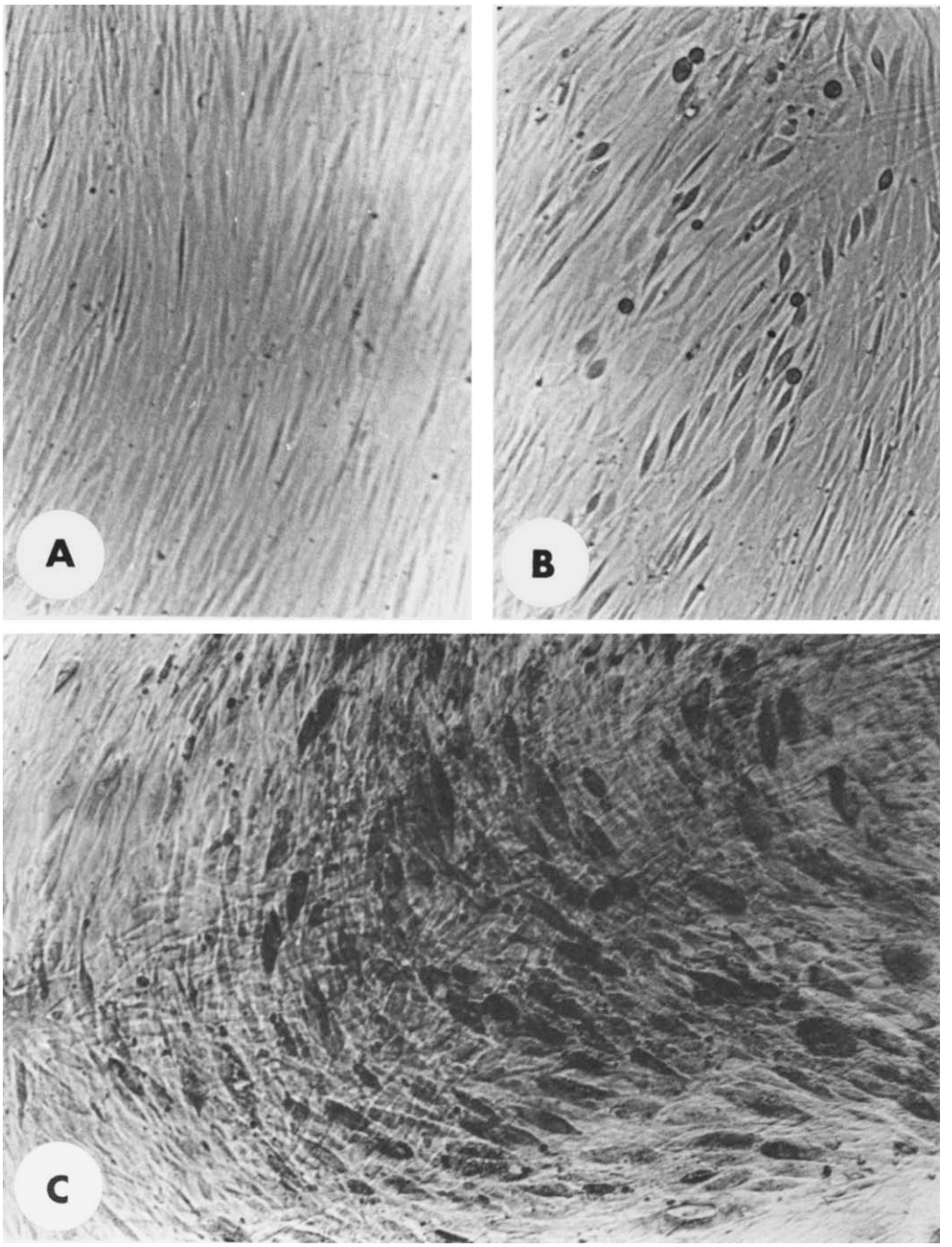


FIG. 1. Morphological alteration induced by Ki-MSV in human skin fibroblasts. (A) Uninfected human skin fibroblasts 12 days after plating, unstained. ×72. (B) Focus of refractile spindle-shaped cells mixed with round cells in human skin fibroblasts 12 days after infection, unstained. ×72. (C) A more advanced focus area seen at 17 days on human skin fibroblasts. Note a pronounced proliferative effect with multilayered cell growth. Large cytoplasmic vacuoles were also seen. Giemsa, ×120.

2 weeks, but after that time in certain strains a pronounced proliferative effect with multilayered cell growth was noted (Fig. 1C). Most of the cells were fusiform in shape and many contained large cytoplasmic vacuoles (Fig. 1C). The number of transformed foci was counted at 14 days; the same virus preparation produced foci more rapidly on NRK cells and here foci were counted at 7 days. In some cultures, the foci of transformed cells were diffuse, however, the foci were countable with careful examination. The infection of sensitive human skin fibroblast cultures with serially diluted virus stock resulted in a titration curve characteristic of the one-hit type. The same type of transformation was observed in the high and low susceptibility cultures.

All the transformed human skin fibroblast cultures were found to be Ki-MSV producers and contained murine leukemia virus p30 antigen. Tumors were produced by some of the morphologically altered human skin fibroblast cultures when inoculated into nude athymic mice. However, the tumors produced in nude mice were always characterized karylogically as "mouse cells," not as "human cells". Most of the transformed human skin fibroblast cultures had limited lifespans, but certain strains of transformed cultures had prolonged lifespans. After 20 transfers over the course of more than a year morphologically transformed cells could still be seen.

Four strains of skin fibroblasts from ACR individuals (provided by Dr. Kopelovich) and eight human skin fibroblasts from GS patients and unrelated normal individuals (provided by Drs. Gardner and Rasheed) were tested for their Ki-MSV and RSV transformation efficiency (Table I). The fibroblasts from four ACR and five GS patients were transformed at 10^{-3} to 10^{-4} dilutions, whereas fibroblasts from unrelated normal individuals showed transformation only at 10^{-1} or 10^{-2} dilutions of the Ki-MSV stock. Thus skin fibroblasts taken from ACR and GS patients were 10- to 100-fold more susceptible to transformation than were normal skin fibroblasts as reported previously (5, 6). As described previously (7) the human osteosarcoma (HOS) clonal line (TE-85 clone F-5) was highly susceptible to Ki-MSV transformation. The HOS (TE-85 clone F-5) line was also sensitive to

TABLE I. SUSCEPTIBILITY OF HUMAN SKIN FIBROBLASTS FROM INDIVIDUALS GENETICALLY PREDISPOSED TO COLORECTAL CANCER TO TRANSFORMATION BY Ki-MSV[a] OR RSV[b]

Group	Cell strain	Subculture level	Age/sex	Transformation	
				Ki-MSV	RSV
ACR[c], symptomatic	VF-2	p-11	31/M	$10^{-3.5}$	10^{0}
	AF	p-14	17/M	10^{-3}	10^{0}
	MED-4	p-15	17/m	$10^{-3.5}$	10^{0}
ACR, asymptomatic	PF	p-15	19/M	10^{-4}	10^{0}
GS[d]	HT-3247	p-15	20/M	10^{-4}	10^{0}
	HT-3252	p-12	28/F	10^{-4}	10^{0}
	HT-3253	p-24	11/M	$10^{-3.5}$	10^{0}
	HT-3255	p-16	33/F	$10^{-3.5}$	10^{0}
	HT-3256	p-17	56/F	10^{-3}	10^{0}
FN/N[e]	HT-3258	p-13	22/F	10^{-2}	10^{0}
	HT-3259	p-10	14/M	10^{-1}	10^{0}
Normal[f]	HT-3152	p-14	55/M	10^{-1}	10^{0}
	HT-3153	p-10	61/F	10^{-2}	10^{0}
Human osteosarcoma	HOS	p-25	13/F	$10^{-3.5}$	$10^{-2.5}$

[a] Virus titer was 3×10^5 FFU/ml in NRK cells.
[b] Virus titer was 7.5×10^4 FFU/ml in chick embryo fibroblasts.
[c] Adenomatosis of the rectum and colon.
[d] Gardner's syndrome.
[e] Familial polyposis of colon, asymptomatic family member.
[f] Normal, unrelated to familial cancer.

RSV transformation; however, none of 13 skin fibroblasts tested was sensitive to RSV transformation (Table I).

Human cells can be transformed morphologically *in vitro* by RSV (12–14). However, in contrast to cell lines derived from other mammalian species, human cells transformed by RSV are difficult to maintain since with serial cultivation the proportion of morphologically altered cells decreases or disappears. Thus, RSV-transformed human cells had not heretofore been established as continuous cell lines (15, 16) and had not also been characterized. Therefore, the RSV-transformed HOS lines were further characterized (Table II). Approximately 12 to 14 days after infection, foci began to appear in the RSV-infected HOS cultures (Fig. 2B). The foci began to pile up focally forming small projections and releasing round cells from foci (Fig. 2C). The cells grew in chains or islets. Transformed cells also grew in suspension above the cells attached to the bottom of the flasks when the cultures reached the confluent state. After one transfer, quite distinct foci (Fig. 2D) were seen in the infected cultures but none were observed in the uninfected controls (Fig. 2A). Transformed foci consisted of a mixture of refractile round cells with vacuolated cytoplasm, refractile spindle-shaped cells in an irregular growth pattern and a few vacuolated giant cells (Fig. 2E). The vacuolated cells (Fig. 2E) showing enlarged nuclei with an abnormally large multinucleolus could not be detected immediately in the new monolayer but they appeared in the culture within 3 to 4 days after subcultivation. The comparative susceptibility of human cells and CEF cells to focus formation by RSV-SR showed that the transformation efficiency of the RSV-SR in human cells was about 300-fold lower than that obtained in CEF cells.

A number of foci-derived lines were established. Two lines, designated RSV 1 HOS and RSV 5 HOS, were further characterized (Table II). Saturation densities of transformed lines were higher than in the control, untransformed line. When suspended above an agar base, the transformed cells formed larger cell aggregates than those formed by normal HOS cells. Viable cell counts of trypsinized aggregates on 4 consecutive days indicated that control, untransformed cells underwent a significant decline in a number of

TABLE II. PROPERTIES OF RSV-TRANSFORMED HUMAN CELL LINES

Properties	RSV 1 HOS cells	RSV 5 HOS cells	HOS cells
Morphology	Transformed	Transformed	Flat
Saturation density[a] ($\times 10^5$/cm^2)	4.8	4.2	1.7
Cell aggregates[b]			
Size	Medium	Medium	Small
Viability of cells ($\times 10^5$)	4.4	2.2	1.5
Plating efficiency (%) in soft agar	11.0	2.4	0.2
CF titers of RSV antigen	1:4	1:2	<2
Reverse transcriptase[c]	Negative	Negative	Negative
Virus particles	Negative	Negative	Negative
Rescuable infectious RSV[d]	Present	Present	None
Tumorigenicity[e]	Positive	Positive	Negative

[a] Maximum number of cells obtained after initial planting with 5×10^3 cells/cm^2 and then incubating at 36° under conditions where growth medium was changed every 3 days.

[b] Cell aggregates formed after 4 days with an agar static system. Viability of cell aggregates determined by 4 days after planting 2×10^5 cells per plate initially.

[c] RNA-dependent DNA polymerase activity was measured by incorporation of [^{3}H]thymidine triphosphate into acid-precipitable materials in 100× concentrated supernatant of culture (7).

[d] RSV-transformed human cells (1×10^5) were cocultivated with 5×10^5 cells of chick embryo fibroblasts. Supernatants from these cultures were taken at 9 days, were passed through an 0.4-μm HA filter, inoculated into chick embryo fibroblasts and examined for foci.

[e] Five million cells inoculated subcutaneously into each NIH nude mouse.

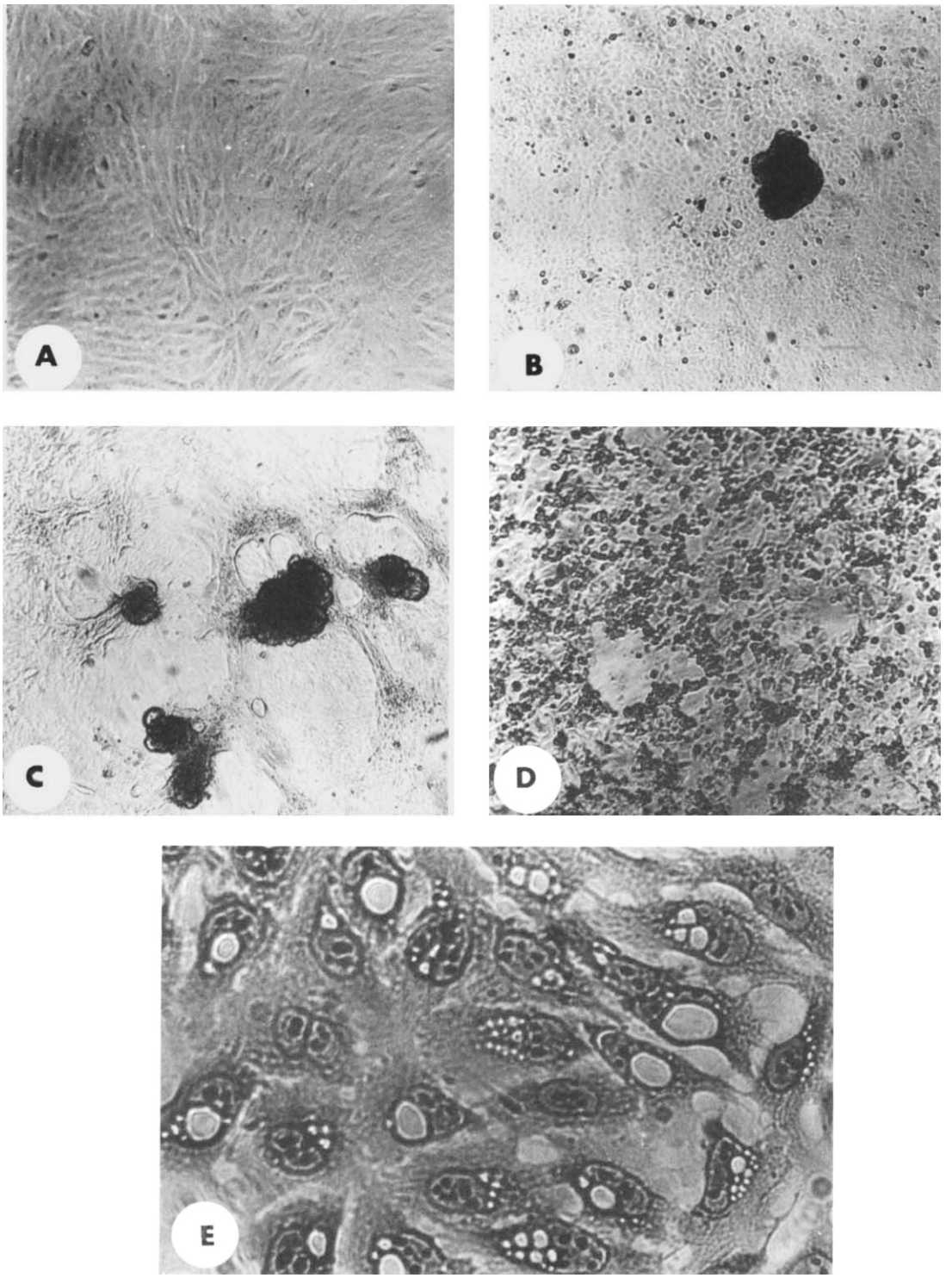
viable cells, whereas RSV-transformed HOS cells showed growth in the aggregate form. The plating efficiencies of transformed cells in soft agar were higher than those of control, untransformed cells. When inoculated into NIH nude athymic mice, RSV-transformed cells produced tumors within 2 weeks at the site of inoculation. The tumors were characterized histopathologically as poorly differentiated sarcomas. No tumors developed over a period of 60 days in a group of nude mice inoculated subcutaneously with the same number of cells from the control cultures.

The RSV-transformed lines were nonproducers. Assays for supernatant viral polymerase were negative. Culture fluids from these transformed cells were negative when tested for focus formation in RIF-free CEF. Examination of transformed cells by electron microscopy revealed no evidence of virus particles. However, the transformed cells contained RSV CF antigen and the RSV genome which was readily detected by cocultivation with RIF-free CEF. Supernatants from the cocultivation cultures taken at 9 days were filtered, inoculated into RIF-free CEF, and examined for the presence of foci. The rescued sarcoma virus produced foci readily in RIF-free CEF.

Previous studies have established that the viral *src* gene is responsible for malignant transformation by RSV (17, 18). This viral gene codes for a 60,000-dalton protein ($p60^{src}$) which has been found to possess an associated protein kinase activity (10, 19). In order to obtain biochemical evidence in support of our belief that the RSV/HOS cells are stably transformed by RSV, we have determined the presence of $p60^{src}$ kinase activity in extracts of RSV/HOS and HOS cells. Immune complexes formed between $p60^{src}$ from cell extracts and immune IgG from tumor-bearing rabbits (TBR) were incubated in the presence of [r-^{32}P]ATP. The phosphorylated products were then analyzed on a 7.5% Polyacrylamide–$NaDodSO_4$ gel with the appropriate molecular weight markers (M_r). Unlike extracts from HOS cells, which exhibited no immune complex kinase activity (Fig. 3, left lane), incubation of immune complexes formed in the presence of RSV/HOS extracts and $p60^{src}$ immune IgG resulted in the specific phosphorylation of the IgG heavy chain (Fig. 3, right lane), and to a lesser extent $p60^{src}$ itself as detected with a longer exposure. Replacement of immune IgG with control IgG gave no detectable phosphorylation (data not shown). By incubating a fixed amount of TBR serum with increasing amounts of extracts from either RSV/HOS or HOS cells, it could be estimated that endogenous $p60^{src}$ kinase activity in the untransformed HOS cells was either absent or was less than 1% of that found in the transformed RSV/HOS cells (Fig. 3). This suggests that $p60^{src}$ kinase activity is specifically induced in RSV/HOS cells in the process of transformation.

Discussion. The data obtained herein confirmed a previously reported finding (5, 6) that skin fibroblasts from individuals genetically predisposed to colorectal cancer were preferentially transformed by Ki-MSV. Relative susceptibility to transformation by Ki-MSV of cultured skin fibroblasts appears to clearly differentiate ACR patients from normal controls, but our findings do not establish that this skin transformation assay is specific for these mutant cells. The occasional occurrence of apparently normal individual displaying one or two phenotypic expressions of the ACR risk profile has previously been reported (4). Whether the occurrence of such individuals in the control population indicates persons at risk for cancer or is coincidental with the "risk profile" of the ACR patient remains to be established. Obviously the frequency with which such individuals occur in the general population should be determined. Miller and Rasheed have recently shown that skin fibroblasts from Huntington patients, an autosomal dominant disorder, were not sensitive to Ki-MSV (20).

It should be noted that the Ki-MSV-transformed human skin fibroblasts were virus producers and were difficult to maintain since with serial cultivation the proportion of altered cells decreased or disappeared. Thus, Ki-MSV-transformed human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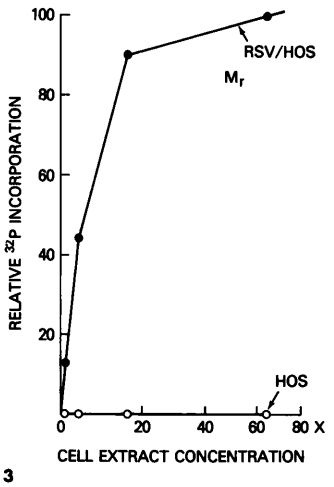

FIG. 3. Detection of immune complex kinase activity in extracts of normal and RSV-transformed HOS cells. Increasing concentrations of extract from HOS or RSV/HOS cells (1× = 35 μg of protein) were incubated with 1 μl of TBR serum for 45 min at 4°. Immunoprecipitates formed in the presence of *S. aureus* were washed twice with RIPA buffer and once with the kinase assay buffer (20 m*M* Tris–HCl, pH 7.2, 5 m*M* $MnCl_2$), before incubation for 10 min at 30° with 25 μl of the assay buffer containing 0.4 μ*M* [γ-^{32}P]ATP (1000 Ci/mmole). The pellets were then washed twice with RIPA buffer, and the immune complexes were eluted from the *S. aureus* by heating at 95° for 2 min in 20 μl $NaDodSO_4$–gel buffer. Supernatant fluids were then analyzed on a 7.5% polyacrylamide–$NaDodSO_4$ gel and the autoradiogram of the dried gel was scanned in a LKB soft laser densitometer. The relative incorporations of 32P in the IgG heavy chain at different cell extract concentrations were plotted. The insert shows the autoradiogram displaying the phosphorylated products from 140 μg of proteins from extracts of HOS cells (left) and RSV/HOS cells (right). The molecular weight markers (M_r) used were phosphorylase *b* (92,500), bovine serum albumin (69,000), ovalbumin (46,000), and carbonic anhydrase (30,000).

skin fibroblasts failed to become established lines, had limited lifespans, and were unable to produce tumors when transplanted into nude mice.

The human osteosarcoma clonal cells infected with RSV-SR had the following properties: (a) altered morphology, (b) increased growth rate, (c) higher efficiency of colony formation in soft agar medium, (d) formation of larger cell aggregates and growth in this aggregate form above an agar base, and (e) tumorigenicity in nude mice. The transformed cells produced neither RNA-dependent DNA polymerase nor type C virus particles; however, the transformed cells contained RSV-SR CF antigen and a rescuable infectious virus. The transformed cells also contained a RSV transformation-specific protein $p60^{src}$-associated kinase activity. Thus we have demonstrated the transformation *in vitro* by RSV-SR of human cells from a continuous cell line. To our knowledge, this is the first documentation of the establishment of RSV-transformed human cell lines. These cell lines should be useful in studying the interaction of RSV and human cells.

As shown in this study, it is interesting to note that ACR-derived skin fibroblasts are sensitive to Ki-MSV transformation but not to RSV transformation. These results indicate that each type of virus requires its own specific cell surface receptor for adsorption and infection. It has been known that absence or defects in the receptor sites resulted in a loss of virus adsorption and virus infection (22) and release of virus from the membrane may also be modified by host cell protein (23).

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FIG. 2. Transformed foci induced by RSV-SR in human osteosarcoma (HOS) clonal (TE-85 clone F-5) cells. (A) Uninfected HOS cells. ×72. (B) Round foci induced by RSV-SR in HOS cells on Day 12 after infection. ×72. (C) Round foci gradually increased in size and began to be released from the projection-like budding. ×72. (D) Typical foci of transformed cells. ×72. (E) Multi-nucleated, vacuolated giant cells were also seen in some foci. Nuclei showed also clearly enlarged nucleoli. Giemsa stain, ×120.

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