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Cooperative Transforming Activities of *ras*, *myc*, and *src* Viral Oncogenes in Nonestablished Rat Adrenocortical Cells

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Early-passage rat adrenocortical cells were infected with Kirsten murine sarcoma virus and MMCV mouse *myc* virus, two retroviruses carrying the v-Ki-*ras* and v-*myc* oncogenes, respectively. Efficient morphological transformation required coinfection with the two viruses, was dependent on the presence of high serum concentrations, and was not immediately accompanied by growth in soft agar. The doubly infected cells coordinately acquired the capacity for anchorage- and serum-independent growth during passage in culture. The appearance of such highly transformed cells was correlated with the emergence of a dominant clone, as suggested by an analysis of retrovirus integration sites. These results indicate that the concerted expression of v-Ki-*ras* and v-*myc* could induce rapid morphological transformation of nonestablished adrenocortical cells but that an additional genetic or epigenetic event was required to permit full transformation by these two oncogenes. In contrast, v-*src*, introduced by retrovirus infection in conjunction with v-*myc*, rapidly induced serum- and anchorage-independent growth. Therefore, the $p60^{v\text{-}src}$ protein-tyrosine kinase, unlike $p21^{v\text{-}ras}$, is apparently not restricted in the induction of a highly transformed phenotype in adrenocortical cells. This system provides an in vitro model for the progressive transformation of epithelial cells by dominantly acting oncogenes.

The formation of cancer cells is proposed to involve a number of genetic and epigenetic changes, including activation of dominant oncogenes, loss of suppressor genes, and perturbations in hormonal regulation. Adrenocortical cells are derived from an adult hormone-producing epithelium and may be considered representative of mammalian cell types that give rise to the majority of adult solid tumors. As such, they represent a particularly interesting system to examine the steps required for neoplastic transformation.

Kirsten murine sarcoma virus (KiMSV), which contains the v-Ki-*ras* oncogene, is unable to fully transform rat adrenocortical cells immediately following infection, despite the abundant synthesis of $p21^{v\text{-}ras}$ (6, 8, 28). During subsequent long-term passaging, KiMSV-infected adrenocortical cells acquire a highly transformed phenotype, suggesting that at least one further genetic or epigenetic change is required for these cells to become permissive for complete transformation by the viral *ras* oncogene (6, 8, 28).

Rat adrenocortical cells in vitro phenotypically resemble immature, proliferating fibroblastic stem cells when cultured with high concentrations of serum but partially differentiate into slowly dividing, steroid-secreting epithelial cells when grown with low concentrations of serum (6–8). KiMSV-infected early-passage cells expressing high levels of viral $p21^{ras}$ respond phenotypically to changes in serum concentration in a fashion similar to uninfected adrenal cortex cells (6, 28). The appearance of serum-independent, transformed cells in KiMSV-infected rat adrenocortical cultures is closely associated with acquisition of anchorage-independent growth and tumorigenicity (7).

Some viral and cellular oncogenes, notably *myc*, can complement activated *ras* genes in inducing the neoplastic transformation of early-passage cells or primary cell types when either oncogene alone is essentially nontransforming (25, 26). *myc* and *ras* are considered to be representative of two classes of oncogenes that encode, respectively, nuclear or cytoplasmic proteins and cooperate in the transformation of primary cells (25, 26, 32, 35, 44).

We wished to know whether the coexpression of *myc* and *ras* oncogenes in adrenocortical cells would obviate the apparent requirement for further cellular events in the induction of neoplastic transformation by v-*ras* and whether the combination of these two genes would be sufficient to allow adrenocortical cells to grow without serum or in soft agar. Surprisingly, we found that coinfection of adrenocortical cells with viruses carrying v-Ki-*ras* and v-*myc* did not immediately induce anchorage- or serum-independent growth. These properties were acquired coordinately several passages after infection, suggesting that further cellular change, in addition to the expression of activated forms of *ras* and *myc*, was required before the cells could express a highly transformed phenotype. However, the v-*src* oncogene, in conjunction with v-*myc*, induced immediate and efficient conversion to a fully transformed phenotype.

MATERIALS AND METHODS

Cells and viruses. Rat-2 cells (47) were grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) in a 5% CO_2, humidified atmosphere at 37°C. Primary cultures of adrenocortical cells were established from 2- to 3-month-old Fischer rats essentially as previously described (40). The adrenal glands from each rat were minced and allowed to attach in a 60-mm-diameter tissue culture dish for 30 to 60 min at 37°C. The tissue explants were incubated in DMEM with 25% FBS. When the cells had grown to confluence after 10 to 14 days, the cultures

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TABLE 1. Viruses used for infection of adrenocortical cells

Virus	Source and reference	Oncogene	Long terminal repeat
MMCV	B. Vennström (48)	Avian v-*myc*	Mo-MLV and HaMSV[a]
KiMSV	Natural isolate (22)	v-Ki-*ras*	KiMSV
2-1	S. Anderson (3)	Avian v-*src*	4070

[a] HaMSV, Harvey murine sarcoma virus.

were trypsinized and frozen separately in liquid N_2 in DMEM with 20% FBS and 10% dimethyl sulfoxide until used. Cultures from individual rats were designated A through F.

The MMCV recombinant murine retrovirus containing the *myc* gene from the OK10 acutely oncogenic avian retrovirus (48, 49), and a KiMSV nonproducing NIH 3T3 line were kind gifts from B. Vennström and D. Lowy, respectively. MMCV and KiMSV were pseudotyped with Moloney murine leukemia virus (Mo-MLV) as high-titer viral stocks (1×10^6 to 2×10^6 CFU or focus-forming units, respectively, on Rat-2 cells). The 2-1 murine retrovirus containing the avian v-*src* gene (3), originally obtained from S. Anderson, was pseudotyped with the amphotropic retrovirus 4070 (16) and supplied as a high-titer stock (10^6 focus-forming units/ml) by K. Humphries. Viruses used in this study are summarized in Table 1.

Viral infections. Cells were seeded at 10^5 cells per 60-mm-diameter dish and were infected when approximately 50% confluent (5×10^5 cells per 60-mm-diameter dish). The virus stocks were brought to 4 μg of Polybrene per ml, and 0.5 ml was added to each 60-mm-diameter dish, giving a multiplicity of infection of approximately 2 to 3 (focus-forming units per cell). The cells were incubated for 1 h at 37°C. Fresh medium (4 ml) was added, and the cells were incubated overnight. The medium was then changed, and the cells were incubated 5 to 7 days before examination.

The efficiency of infection of rat adrenocortical cells by KiMSV was assessed by an infectious-center assay. Cultures of adrenal cells infected with KiMSV alone or with KiMSV and MMCV were trypsinized, and 1×10^2 to 1×10^4 cells from a single-cell suspension were added to dishes containing subconfluent Rat-2 cultures. Pretreatment of cells with 10 μg of mitomycin C per ml for 2 h did not affect the results. The cells were allowed to attach for 1 h, and the medium was then replaced with a 0.6% agar overlay in DMEM with 10% FBS. The numbers of foci were recorded after 5 days. KiMSV-infected Rat-2 cells formed colonies at efficiencies of 25 ± 5%. Since MMCV could not be measured directly on adrenocortical cells, the number of MMCV-infected adrenocortical cells was estimated by extrapolation from the relative titers of MMCV and KiMSV on Rat-2 cells.

Radiolabeling of cells. Cells were grown until they were 50 to 80% confluent and were then washed once with either Tris saline (for labeling with [^{35}S]methionine) or phosphate-free saline (for labeling with $^{32}P_i$). The cells were then incubated with 150 μCi of [^{35}S]methionine per ml (800 to 1,000 Ci/mmol; Amersham Corp.) in methionine-free DMEM with 3% FBS for 4 h or with 300 to 500 μCi of $^{32}P_i$ per ml (ICN Pharmaceuticals Inc.) in phosphate-free DMEM with 3% FBS for 8 to 12 h. After the cells were labeled, they were scraped into 1 ml (final volume) of lysis buffer and centrifuged at 35,000 × *g* for 30 min. For immunoprecipitations with the anti-p21^{ras} monoclonal antibody Y13-259 (from M. Furth [14]), the lysis buffer used consisted of 100 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris hydrochloride (pH 7.5), 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium dodecyl sulfate (SDS), and 1 mM leupeptin.

Immunoprecipitation. The clarified cell lysates were incubated on ice with 1 to 5 μl of the appropriate antibody for 60 min. The immunoprecipitations with Y13-259 were then incubated for a further 60 min on ice with 50 μl of 10% (vol/vol) *Staphylococcus aureus* (IgGsorb, The Enzyme Center) coated with rabbit anti-rat immunoglobulin G. The immune complex was then washed five to six times with the lysis buffer. The immunoprecipitations of ^{32}P-labeled cell lysates were then washed in a solution of 1 M NaCl, 10 mM Tris hydrochloride (pH 8.0), and 0.1% (vol/vol) Nonidet P-40. The immunoprecipitated material was released by incubation in SDS gel sample buffer at 37°C for 10 min, and the samples were prepared for electrophoresis by heating at 100°C for 3 min. The Y13-259 immunoprecipitates were analyzed on a SDS-polyacrylamide gel (12.5% polyacrylamide) (stock acrylamide/bisacrylamide, 29.2:0.8). All gels were fixed, stained for molecular weight markers, dried, and exposed to XAR-5 film (Kodak) at −80°C with an intensifying screen.

Immune complex kinase assay. A 100-mm-diameter dish of each cloned line was lysed at 4°C in 1 ml of modified RIPA buffer (0.15 M NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 10 mM sodium phosphate [pH 7.0], 2 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM leupeptin, 50 mM NaF). The lysate was clarified at 35,000 × *g* for 30 min, and 0.1 ml of the clarified supernatant was immunoprecipitated, using the anti-p60^{src} monoclonal antibody 327 (27) and *S. aureus* coated with rabbit anti-mouse immunoglobulin. The immunoprecipitate was collected by centrifugation through a pad of 10% sucrose in RIPA and then washed three times with the modified RIPA and once in 0.1 M NaCl–10 mM PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 7.0). The immunoprecipitates were suspended in 10 μl of a solution consisting of 20 mM PIPES (pH 7.0), 10 mM $MnCl_2$, 5 μCi of [γ-^{32}P]ATP (~3,000 Ci/mmol, Amersham), and 10 μg of acid-treated rabbit muscle enolase (Sigma Chemical Co.) (11) and incubated at 30°C for 10 min. The reactions were stopped by adding an equal volume of twofold-concentrated SDS gel sample buffer and heating at 90 to 100°C. The samples were then analyzed on a SDS-polyacrylamide gel (12.5% polyacrylamide).

Southern hybridization. Genomic DNA (10 μg) was digested with a threefold excess of *Bam*HI or *Hin*dIII (Bethesda Research Laboratories) for 2 h, phenol extracted and ethanol precipitated. The digested DNA was then made up in Tris borate-EDTA, and the samples were electrophoresed in a 0.75% (wt/vol) agarose submarine gel. The gel was then treated, and the DNA was transferred by capillary blotting to Hybond-N (Amersham) nylon membranes as recommended by the manufacturer. The DNA was fixed to the membrane by UV irradiation, washed at 65°C for 60 min in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% (wt/vol) SDS, and then prehybridized at 65°C for 4 to 8 h in a solution of 6× SSC, 5× Denhardt solution, 0.5% (wt/vol) SDS, and 20 μg of sonicated, denatured salmon sperm DNA per ml. The avian v-*myc* probe was prepared from a 1.5-kilobase (kb) fragment from pMC38 (a gift of J. M. Bishop [49]) containing the MC29 v-*myc* gene. The fragment was labeled by nick translation with [α-^{32}P]dCTP (3,000 Ci/mmol; Dupont, NEN Research Products). Hybridization was performed for 24 h at 65°C in the same buffer as that used for prehybridization. The membrane was then washed once in 2× SSC–0.1% (wt/vol) SDS at 65°C for 30 min, twice

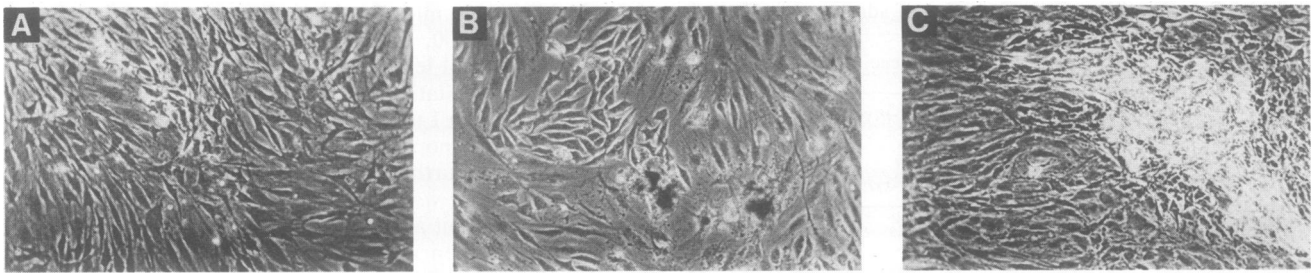

FIG. 1. Focus formation in infected, early-passage rat adrenocortical cells. Rat adrenocortical cells frozen at the end of primary passage were thawed and passaged once before infection. The cells were infected with KiMSV (A), MMCV (B), or KiMSV and MMCV (C). The cells were maintained in DMEM with 25% FBS until examination. The photomicrographs were taken 7 days after infection. Magnification, ×72.

in 2× SSC–0.5% (wt/vol) SDS at 65°C for 30 min, and once in 0.1× SSC at 64°C for 10 min. After the membrane was air dried, it was exposed to XAR-5 film with an intensifying screen at −80°C.

Colony formation in soft agar. The bottom agar layer was composed of 1× DMEM, 5× minimal essential medium vitamins, 0.6% agarose, penicillin, streptomycin, and either 25% FBS or 5% horse serum (HS) or calf serum as required for the assay. The top agar contained 1× DMEM plus antibiotics, 5× minimal essential medium vitamins, 0.35% agarose, either 25% FBS or 5% HS or calf serum, and exogenous growth factors as necessary. A total of 10^4, 3×10^4, or 10^5 cells in 0.1 to 0.2 ml were suspended in 2 ml of the top agar mix for each 60-mm-diameter dish. The soft agar was covered with DMEM and serum, with or without exogenous growth factors, and incubated for 2 to 3 weeks.

RESULTS

Induction of foci and a transformed morphology by KiMSV and MMCV. Rat adrenocortical cell lines maintained in high concentrations of serum were infected in passage 2 with KiMSV alone, with the recombinant murine retrovirus MMCV that carries an avian v-*myc* gene (48), or with KiMSV and MMCV together. The morphological responses of the cells to these infections are shown in Fig. 1. Both KiMSV and MMCV independently produced foci of altered cells but only at a frequency of 0.01 to 0.1% of the cells actually infected (measured in infectious-center assays for KiMSV and estimated for MMCV as described in Materials and Methods). Cells in foci induced by MMCV infection had an epithelioid morphology and were more polygonal in shape than normal. This is similar to changes described in a NRK cell line expressing elevated levels of c-*myc* (42). Foci of altered cells that were apparent after KiMSV infection had an elongated, refractile morphology. Coinfection with KiMSV and MMCV resulted in the formation of a greater number of foci (Table 2) containing cells that were more rounded and refractile than with either virus alone (Fig. 1). The predicted number of coinfected cells, an estimate based on infectious-center assays of singly infected cultures, was somewhat less (25 to 50%) than the number of foci seen in the coinfected cultures. These results indicated that the simultaneous introduction of the two oncogenes v-*myc* and v-Ki-*ras* induced efficient focus formation in early-passage adrenocortical cells cultured in high concentrations of serum, when either gene alone was very inefficient.

Phenotypic changes induced by coinfection with KiMSV and MMCV require a high concentration of serum or epidermal growth factor (EGF). Focus formation is induced with low efficiency in early-passage adrenocortical cells by KiMSV and requires the presence of 10 to 25% FBS (6). If KiMSV-infected cultures are transferred to low concentrations of serum (1 to 5% HS), the morphologically transformed cells revert to a normal epithelial morphology. Since the expression of high levels of *myc* has been associated with a reduction in the requirement of serum for growth (5, 21), cultures of coinfected cells were examined for their ability to grow and maintain a transformed morphology in low concentrations of serum (5% HS).

Subculturing the KiMSV-and-MMCV coinfected cultures into 5% HS resulted in a reversion of the transformed cells to a normal phenotype, while parallel cells grown in 25% FBS continued to display a highly transformed morphology (Fig. 2). Conversely, the addition of FBS (final concentration, 25%) to coinfected cells that had been grown in 5% HS converted the morphologically normal cells to a transformed phenotype characteristic of the coinfected cells maintained in 25% FBS throughout (data not shown). Thus, a factor(s) present in FBS is required for early-passage adrenocortical cells to be permissive for efficient morphological transformation induced by coordinate expression of v-*myc* and v-Ki-*ras*.

To investigate the effect of growth factors on the phenotype of KiMSV-and-MMCV-infected cells, cultures infected with KiMSV, MMCV, or KiMSV and MMCV were plated and maintained in 5% HS until they had reached confluence. Purified growth factors (EGF, platelet-derived growth factor, fibroblast growth factor, and insulinlike growth factor II) were then added in the presence of 5% HS. Of these growth factors, only EGF induced a transformed morphology in the KiMSV-and-MMCV-infected cultures similar to that seen in response to the addition of high concentrations of FBS (data not shown). Other growth factors were without effect on KiMSV-and-MMCV-infected cells. These results suggest that EGF or an EGF-like activity might be the active

TABLE 2. Focus formation by rat adrenocortical cells after infection with acutely oncogenic retroviruses

Cell line	No. of foci/dish[a]	
	KiMSV and MMCV	KiMSV
A	22.5	0
B	5.5	0
C	12	0
E	101	6
F	15.5	1

[a] Rat adrenocortical cells were infected at passage 2 either with KiMSV alone or with both MMCV and KiMSV. Foci appeared 3 to 5 days after infection, and the number of foci per 60-mm-diameter dish were counted 5 to 7 days after infection.

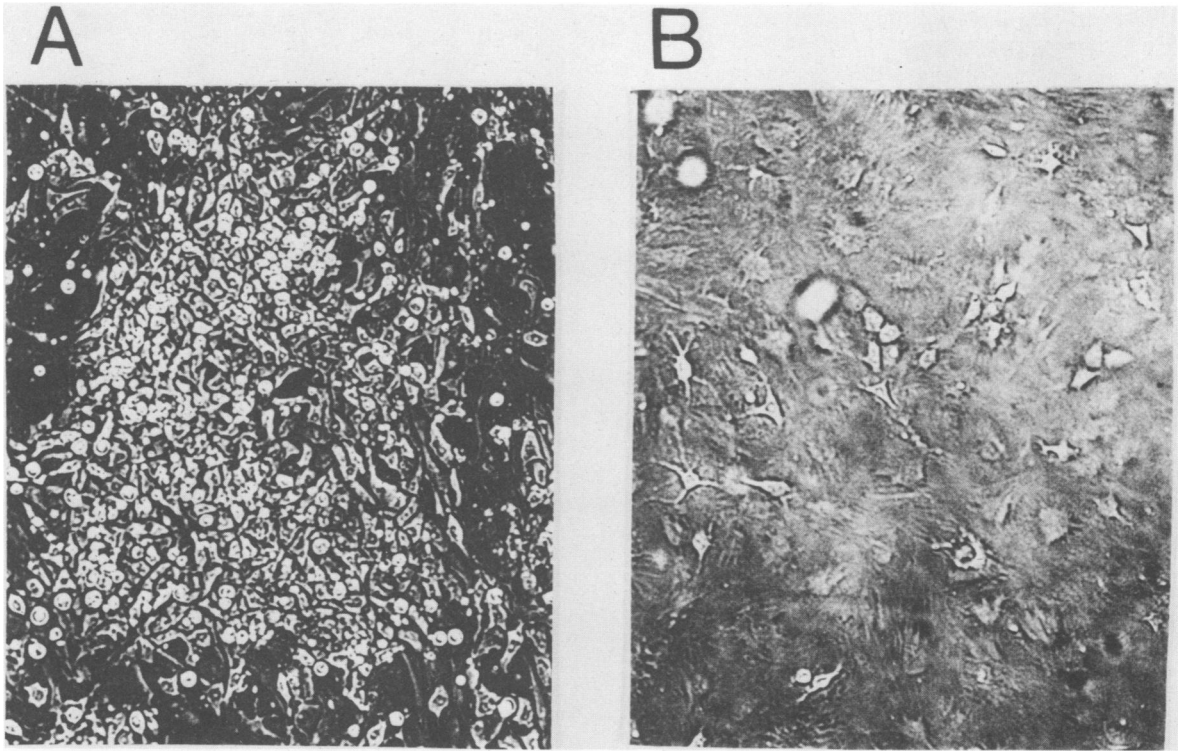

FIG. 2. Serum sensitivity of the transformed morphology induced by KiMSV-and-MMCV coinfection of rat adrenocortical cells. Three passages after infection with KiMSV and MMCV, the cells were plated in DMEM with 25% FBS and after 24 h were either shifted into DMEM with 3% HS (B) or maintained in DMEM with 25% FBS (A). The photomicrographs were taken 7 days later. Magnification, ×40.

component of FBS required for *ras*-and-*myc*-induced morphological transformation of adrenocortical cells.

Acquisition of serum-independent and anchorage-independent growth. Anchorage-independent growth was used as an in vitro parameter to monitor the expression of a fully transformed phenotype (7, 10, 18). Early-passage cultures of KiMSV-and-MMCV-infected cultures were unable to form colonies in soft agar, despite the presence of serum-dependent foci in the monolayer. Four to six passages after infection, cells whose transformed morphology was maintained in the presence of low concentrations of serum appeared in the coinfected cultures. The onset of reduced requirements for serum was coincident with the appearance of anchorage-independent growth (Table 3). The acquired ability to form colonies in soft agar was independent of serum concentration. The addition of exogenous growth factors in the presence of high or low concentrations of serum did not stimulate colony formation in soft agar before the cells became serum independent (data not shown), even though EGF was sufficient to elicit a transformed morphology. Neither of the singly infected lines formed any colonies in soft agar during the course of these experiments. These results indicate that the *ras* and *myc* oncogenes have cooperative effects in inducing adrenocortical cell transformation but alone are not sufficient for the acute transformation of the early-passage adrenocortical cells as measured by serum- and anchorage-independent growth.

Presence and expression of viral oncogenes in transformed adrenocortical lines. Transformation of adrenocortical cells was greatly enhanced by infection with both *ras*- and *myc*-containing viruses. To determine the actual origin of the transformed cells and to investigate whether they contained and expressed both viral oncogenes, clonal lines were established by isolating soft agar colonies from the fully transformed, coinfected cultures. These cloned cell lines were examined for viral $p21^{ras}$ by immunoprecipitation with anti-$p21^{ras}$ antibody from lysates of cells metabolically labeled with $^{32}P_i$. This procedure specifically identifies the v-Ki-*ras* gene product, as an amino acid substitution at residue 59 allows the viral $p21^{ras}$ to act as a phosphoacceptor, while the cellular $p21^{ras}$ does not (37, 38). Of 20 lines examined, 19 contained viral $p21^{ras}$; representative results are shown in Fig. 3.

Of the lines examined for synthesis of the v-*ras* product, 16 were also probed for the presence of the v-*myc* gene by

TABLE 3. Serum-independent and anchorage-independent growth in KiMSV-and-MMCV-infected cells

Cell line	Passage no. for growth[a]	
	Serum independent[b]	Anchorage independent[c]
A	5	6
C	4	4
E	4	4
F	6	6

[a] Passage numbers required for serum- or anchorage-independent growth following KiMSV and MMCV injection. The adrenocortical cultures infected with either KiMSV or MMCV alone did not form colonies in soft agar during the course of the experiment. Infected cells from cell line A were not checked for growth in agar at passage 5.

[b] Serum-independent growth was assayed at each passage after infection with KiMSV and MMCV by switching the medium from 25% FBS to 5% HS after the cells had atached.

[c] Anchorage-independent growth was measured at the end of each passage by plating 10^5 cells in soft agar in a 60-mm-diameter dish and culturing for 2 to 3 weeks.

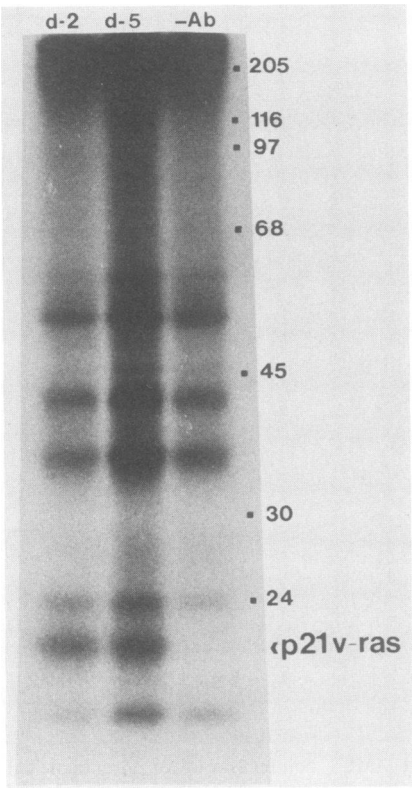

FIG. 3. $p21^{ras}$ expression in KiMSV-and-MMCV-infected rat adrenocortical cells. Transformed cell lines (d-2 and d-5) from the KiMSV-and-MMCV-infected cultures were derived from colonies in soft agar. Cells were labeled with $^{32}P_i$, lysed, and immunoprecipitated with Y13-259 anti-$p21^{ras}$ monoclonal antibody. Cell line d-5 was treated without the primary anti-*ras* antibody (−Ab). Immunoprecipitates were analyzed by electrophoresis through a SDS-polyacrylamide gel (12.5% polyacrylamide), dried, and exposed to XAR-5 film for 5 days at room temperature.

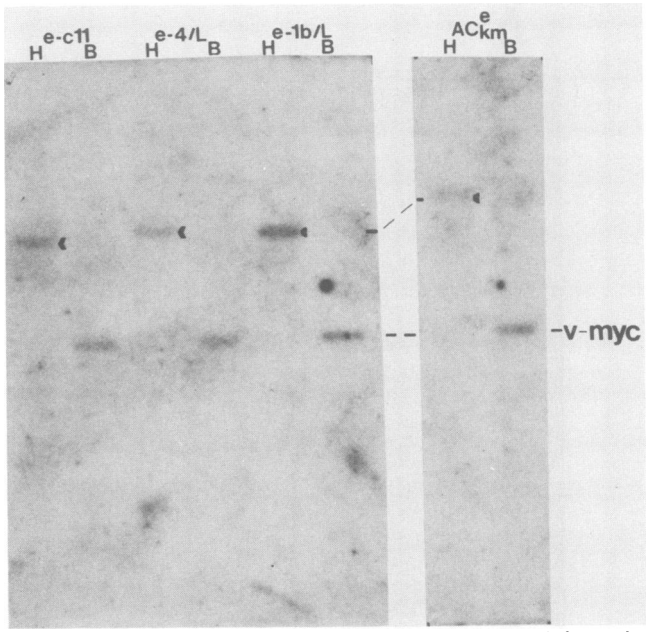

FIG. 4. Southern analysis of genomic DNA derived from the KiMSV-and-MMCV-transformed rat adrenocortical cultures. AC^e_{km} genomic DNA was extracted from the KiMSV-and-MMCV-infected, uncloned adrenocortical line E after the culture had become serum independent for growth. DNA was also isolated from lines obtained from soft agar colonies (e-C11, e-4/L, and e-1b/L) derived from the AC^e_{km} culture. Genomic DNA (10 μg) from each line was digested with *Hin*dIII (H) or *Bam*HI (B). The blots were probed with a v-*myc* fragment specific for the avian *myc* gene contained in MMCV under the hybridization conditions used. *Bam*HI digestion released the v-*myc* gene in a 2.5-kb fragment (indicated as v-*myc* to the right of the gel). *Hin*dIII cut the provirus once outside the v-*myc* gene, which produced a virus-cell junction fragment of greater than 3.5 kb (indicated by an arrowhead).

Southern blotting. Genomic DNA was digested with *Bam*HI, which liberates a 2.5-kb fragment containing the v-*myc* gene from the MMCV provirus, and analyzed with a v-*myc* probe. All 16 lines contained the avian v-*myc* gene identified as a 2.5-kb band; representative results are shown in Fig. 4. Five cloned coinfected cell lines were also examined for expression of the v-*myc* gene product by immunoprecipitation. All five lines expressed the avian v-*myc* product, which was distinguished from the endogenous murine c-*myc* protein on the basis of size (data not shown). The presence of v-*myc* in all 16 lines probed and the expression of viral $p21^{ras}$ in 19 of 20 lines examined strongly suggests that the increased efficiency and rapidity of transformation to anchorage-independent growth displayed by coinfected adrenocortical cultures requires the coexpression of v-*myc* and v-Ki-*ras* oncogenes in the same cell.

The latent period required for the appearance of serum- and anchorage-independent growth in the KiMSV-and-MMCV-infected adrenocortical cultures suggested that a further genetic or epigenetic change in these cells was necessary to allow transformation. If this were the case, the serum-independent cultures might have arisen from the clonal expansion of one cell or a small number of cells that had undergone this additional event and thereby acquired a fully transformed phenotype.

To determine whether the highly transformed mass cultures of KiMSV-and-MMCV-infected, serum-independent cells had arisen from a specific clone, we used the integration site of MMCV as a marker. Three separate KiMSV-and-MMCV-infected cultures were passaged until the cells were serum independent and fully transformed. The genomic DNA from each of these three cultures was then isolated, digested with *Hin*dIII, which cuts once within the MMCV provirus (48), and analyzed by Southern blotting. The probe employed recognizes only the 3′ MMCV fragment, whose mobility will be dependent on linked cellular sequences. In the *Hin*dIII digest of genomic DNA obtained from each of the three uncloned cultures examined, a single band hybridizing with the v-*myc* probe was observed, indicating that a single dominant cell clone with a MMCV proviral integration made up the bulk of transformed cells in each culture. Data are shown for one such culture (designated E) (Fig. 4, AC^e_{km}). The other two cultures examined showed single bands of different mobilities in the *Hin*dIII digests of genomic DNA (data not shown).

Culture E was also seeded into soft agar, and three resulting transformed colonies (e-C11, e-4/L, and e-1b/L) were isolated and examined in a similar fashion by Southern blotting (Fig. 4). Two of these cloned lines (e-4/L and e-1b/L) contained a *Hin*dIII fragment of the same size as that of the parental culture (AC^e_{km}), suggesting that they were derived from the dominant clone. The third clone (e-c11) gave a *Hin*dIII band of a different size, indicating that the original uncloned culture contained transformed cells that arose from a separate viral integration event. Three coinfected trans-

TABLE 4. Focus formation induced by 2-1 *src* in virus-infected adrenocortical cells

Cell line and virus infection	No. of foci[a]
Cell line A	
2-1	25
2-1 and KiMSV	23
2-1 and MMCV	111
Cell line B	
2-1	9
2-1 and KiMSV	6
2-1 and MMCV	28

[a] Foci were counted after 5 days. The morphology of the foci induced by 2-1 was readily distinguishable from that induced by KiMSV or MMCV. Approximately 1% of cells (5×10^3 cells per dish) were infected with 2-1, as judged by infectious-center assays.

formed cultures (AC^a_{km}, AC^c_{km}, and AC^e_{km}, described above) and a total of 16 cloned lines derived from these cultures have been examined. The cloned lines fell into two classes. Eight lines (one of two AC^a_{km}, four of eight AC^c_{km}, and three of six AC^e_{km}) contained a v-*myc* *Hin*dIII fragment with a similar mobility to that in the uncloned cultures from which they were derived. The rest of the cloned lines had v-*myc* *Hin*dIII fragments distinct from those of the uncloned cultures. These results suggest that clonal overgrowth may be a general phenomenon associated with the expression of a fully transformed phenotype in the KiMSV-and-MMCV-infected rat adrenocortical cells.

These data support the hypothesis that serum-independent cells capable of anchorage-independent growth arose in KiMSV-and-MMCV-infected cultures as a result of an additional low-frequency genetic or epigenetic cellular change. These experiments used nonestablished cells derived from the adult rat adrenal cortex as a model for transformation. Since it was possible that contaminating cells not derived from the adrenal cortex were present and susceptible to transformation, eight of the transformed lines cloned from soft agar were assayed for their ability to metabolize a precursor of the steroid biosynthetic pathway to an intermediate progesterone, a specific marker for adrenocortical cells. The cells were incubated with [^{3}H]pregnenolone as a precursor of the steroid biosynthetic pathway and assayed for the production of progesterone by a radioimmunoassay. At least six of the eight lines produced steroid intermediates and positively confirmed the adrenocortical origin of these transformed cultures (results not shown).

v-*src* and v-*myc* cooperate in the rapid transformation of early-passage adrenocortical cells. Previous work has demonstrated that v-*src* alone can efficiently induce transformation of early-passage Syrian hamster embryo cells (15), unlike *ras*, which required a cooperating *myc* gene (33). To test the transforming activity of v-*src* in adrenocortical cells, uninfected adrenocortical cells or cultures in passage 2 after infection with MMCV(Mo-MLV) or KiMSV(Mo-MLV), were infected (or superinfected) with the 2-1 v-*src*-containing murine retrovirus pseudotyped with the 4070 murine amphotropic virus. In each case, foci of refractile, morphologically transformed cells were observed, although with higher frequency in MMCV-infected cells than with KiMSV-infected or uninfected cells (Table 4). Following two passages, cell cultures coinfected with MMCV and 2-1 virus appeared completely overgrown with morphologically transformed cells (Fig. 5a). Cultures coinfected with KiMSV and 2-1 viruses exhibited only a few transformed foci in passage 2 after superinfection (Fig. 5b), and cells infected with

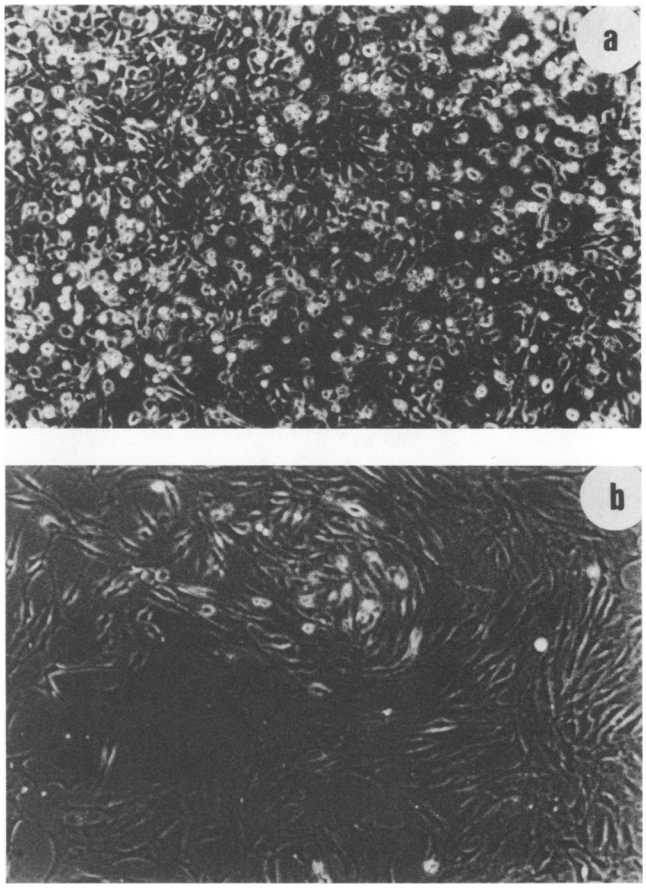

FIG. 5. Morphology of rat adrenocortical cells infected with MMCV and 2-1 (a) or with KiMSV and 2-1 (b). Cultures of rat adrenocortical cells infected with either KiMSV or MMCV were passaged twice and then superinfected with a high-titer stock of 2-1 (10^6 focus-forming units per ml). The cultures were maintained in DMEM with 25% FBS and passaged twice before phase-contrast photomicrographs were taken. Panel a is representative of the culture, while panel b was chosen to include one focus in the culture, as well as cells expressing a normal morphology. Magnification, ×140.

MMCV alone exhibited little evidence of morphological change. Similar results were obtained with four independent adrenocortical cultures, each obtained from different animals, suggesting that v-*src* cooperates effectively with v-*myc* in the induction of morphological transformation.

To assess the effects of v-*src* on serum requirements and anchorage dependence, cultures were passaged into low concentrations of serum or were plated in soft agar. All cultures infected with 2-1 retained morphologically transformed cells when passaged into low concentrations of serum immediately following infection. 2-1-and-MMCV-infected cultures formed large colonies at an efficiency of 10 to 20%, with an overall colony-forming efficiency of 30 to 40%, when seeded in soft agar two passages after superinfection (Fig. 6a), while only a low percentage (3 to 5%) of 2-1-infected or 2-1-and-KiMSV-infected cells divided in agar to form very small colonies (Fig. 6c). Parallel cultures infected with MMCV or KiMSV alone did not form colonies in agar. 2-1-and-MMCV-infected cells formed soft agar colonies with equivalent efficiencies in high or low concentrations of serum when plated at 1.5×10^4 cells per ml. When these cells were plated at a lower density (0.5×10^4 cells per

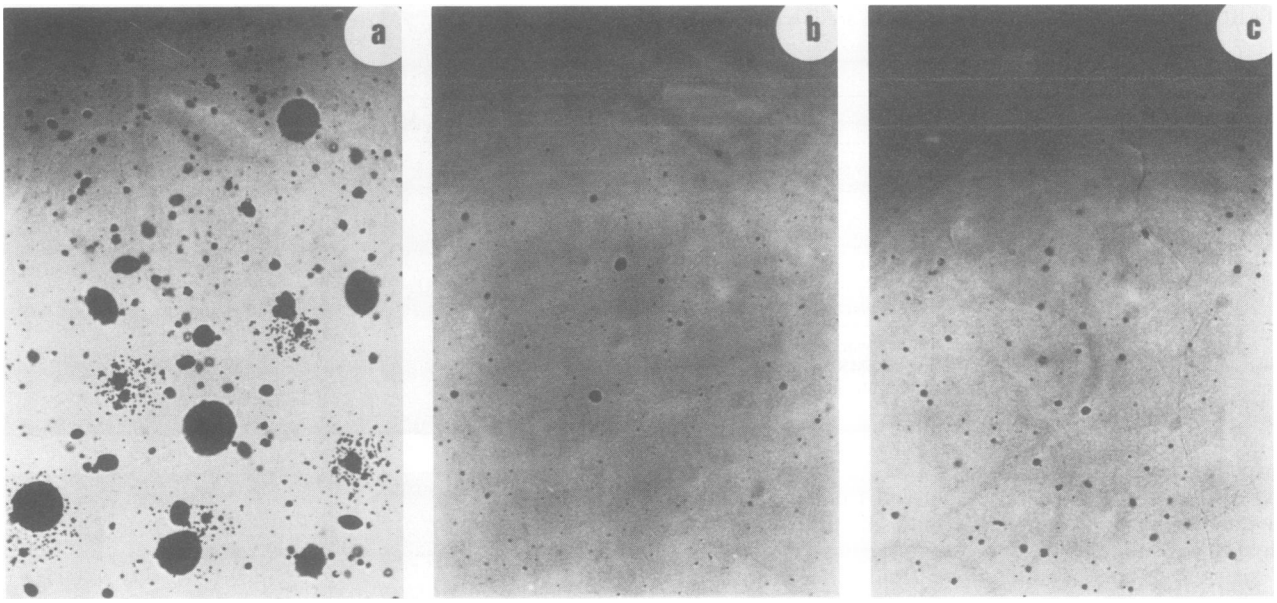

FIG. 6. Colony formation in soft agar by adrenocortical cultures superinfected with 2-1. The KiMSV- and MMCV-infected cultures superinfected with 2-1 were passaged twice and then assayed for anchorage-independent growth. The cells were suspended at 3×10^4 or 10^4 cells in 2 ml of 0.35% agarose–DMEM–5% FBS in a 35-mm-diameter well, as described in Materials and Methods, and incubated for 16 days. (a) MMCV-and-2-1-infected adrenocortical cells seeded at 3×10^4 cells per well; (b) MMCV-and-2-1-infected adrenocortical cells seeded at 10^4 cells per well; (c) KiMSV-and-2-1-infected adrenocortical cells seeded at 3×10^4 cells per well. The photomicrographs were taken with regular optics. Magnification, ×25.

ml) and in low concentrations of serum, the efficiency and size of colony formation showed a large, nonlinear decrease relative to the number of cells assayed (Fig. 6b and Table 5). Addition of 20% FBS to cells plated at the lower density restored the efficiency of colony formation. The density dependence of colony formation at a low serum concentration is suggestive of autocrine stimulation.

To obtain evidence for expression of the 2-1 v-*src* gene, we cloned three soft agar colonies from 2-1-and-MMCV-infected cells. These lines (designated a-1, e-1, and e-3) all expressed elevated levels of catalytically active $p60^{src}$ relative to a KiMSV-and-MMCV-infected line, consistent with expression of the v-*src* gene (Fig. 7). In addition, Southern analysis of genomic DNAs from the same 2-1-and-MMCV-transformed cells revealed the presence of the *Bam*HI 2.5-kb v-*myc* proviral DNA fragment (Fig. 8). Thus, the anchorage-independent colonies arising after 2-1 superinfection of MMCV-infected cells appear to be doubly infected.

TABLE 5. Anchorage-independent growth of 2-1-and-MMCV-infected adrenocortical cells[a]

Cell line	No. of cells assayed	No. of colonies
A	3×10^4	183
	1×10^4	2
C	3×10^4	261
	1×10^4	21
E	3×10^4	77
	1×10^4	5

[a] Transformed cultures two passages after 2-1 superinfection of MMCV-infected adrenocortical cells were plated in 2 ml of 0.35% agarose–DMEM–5% FBS in a 60-mm-diameter dish with the cell numbers as noted. Macroscopic colonies were counted after 3 weeks.

DISCUSSION

These data suggest that the in vitro transformation of nonestablished rat adrenocortical cells involves several steps. Transformation was monitored first by focus formation in monolayer cultures and later by more stringent criteria, anchorage- and serum-independent growth. Previous work has correlated the appearance of these latter properties with the acquisition of a highly tumorigenic phenotype in vivo (7, 10, 18). Coinfection of early-passage cells with KiMSV and MMCV indicated that v-*ras* and v-*myc* could jointly mediate the rapid and efficient induction of a transformed morphology; in contrast, only a very low percentage of cells infected with KiMSV or MMCV alone showed any morphological change. v-Ki-*ras* and v-*myc* could cooperate to produce focus formation, but the cells within these foci were still dependent on high concentrations of serum for maintenance of a transformed morphology and were unable to grow in soft agar.

The requirement of early-passage KiMSV-and-MMCV-infected adrenocortical cells for high concentrations of serum to express a transformed morphology indicates that their phenotype can be readily modulated by environmental conditions. Cell lines infected with Harvey murine sarcoma virus or KiMSV produce transforming growth factors (particularly transforming growth factor α [TGF-α] and TGF-β) (4) that induce a transformed phenotype in corresponding uninfected cells (9, 19, 20, 34). TGF-α is related to EGF (9, 13, 30) and exerts its effect through the EGF-receptor (9, 12, 46). The ability of EGF to specifically substitute for FBS in the induction of morphological alterations in the KiMSV-and-MMCV-infected adrenocortical cells suggests that the early-passage coinfected cells were either not producing TGF-α or were not responsive to the levels produced.

It has been shown that EGF treatment of NRK cells overexpressing *myc* results in anchorage-independent

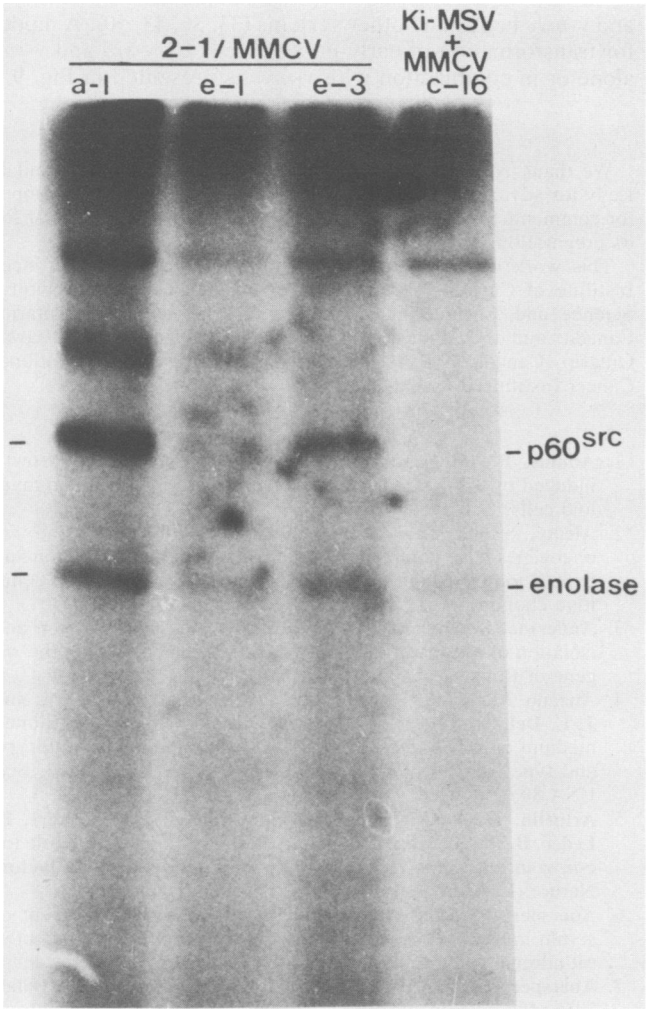

FIG. 7. $p60^{src}$ kinase activity in MMCV-and-2-1-infected adrenocortical cultures. Three cell lines (a-1, e-1, and e-3) coinfected with MMCV and 2-1 virus (2-1/MMCV) were isolated as soft agar colonies and expanded. The lines were plated into 100-mm-diameter dishes, and when the cultures were 50% confluent, the cells were lysed, immunoprecipitated with the anti-$p60^{src}$ antibody 327, introduced into a kinase reaction mixture containing the substrate enolase, and analyzed on a SDS-polyacrylamide gel (12.5% polyacrylamide). A KiMSV-and-MMCV-transformed line (c-16) served as a control for endogenous $p60^{c\text{-}src}$ (indicated by $p60^{src}$).

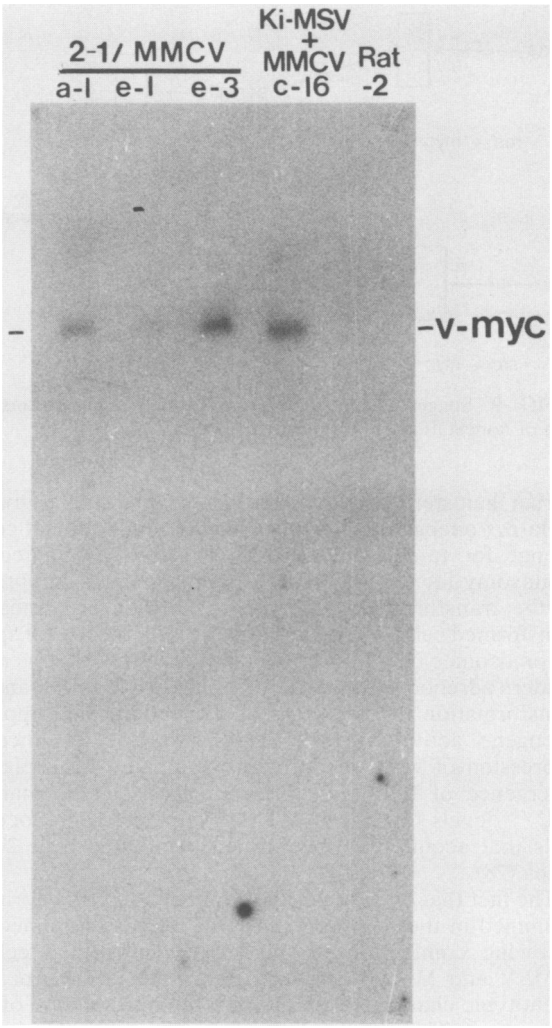

FIG. 8. Southern analysis for v-*myc* on adrenocortical cell lines infected with 2-1 and MMCV. Genomic DNA was prepared from three cloned MMCV-and-2-1-infected adrenocortical cell lines (a-1, e-1, and e-3), from a KiMSV-and-MMCV-infected adrenocortical line (c-16), and from Rat-2 fibroblasts. *Bam*HI fragments were separated on a 0.75% agarose gel, transferred, and hybridized with a v-*myc* probe. The blot was exposed to film with an intensifying screen at −80°C for 6 days.

growth (42). EGF did not induce either anchorage-independent growth or morphological alterations in the adrenocortical cultures infected with MMCV alone. These results imply that there is a restriction to *myc* activity in nonestablished adrenocortical cells, which is not present in the NRK cell line. Our data support the idea that the role of *myc* in cooperative transformation may not be restricted to the simple enhancement of TGF-α activity but may involve synergistic stimulation of other $p21^{ras}$-induced changes or *myc*-induced phenotypic alterations independent of *ras*. Indeed, transformation by an activated *ras* gene does not require autocrine stimulation by TGF-α, indicating that other pathways contribute to fibroblast transformation by *ras* (29). It should be emphasized that although the acquisition of anchorage-independent and serum-independent growth were coincident, the presence of 25% FBS or exogenous growth factors was insufficient to stimulate early-passage coinfected cells to grow in agar. This suggests that the change(s) that confers anchorage independence to KiMSV-and-MMCV-infected cells has a pleiotropic effect that includes, but is not limited to, serum independence. The detection of a single dominant clone in KiMSV-and-MMCV-infected cultures at the same time as the phenotypic expression of anchorage independence is consistent with the hypothesis that a further, rare cellular event was required for the progression of v-*myc*-and-vi-Ki-*ras*-expressing cells to a fully transformed phenotype. The requirement for a further step to complement *ras* and *myc* in the transformation of nonimmortalized cells has been described for hematopoietic cells, and in these cases is also associated with a reduction in serum dependence (36, 43, 50). The emergence of clonal or oligoclonal populations of transformed murine hematopoietic cells from cultures infected with v-*myc* retroviruses has been documented by analysis of viral integration sites (31).

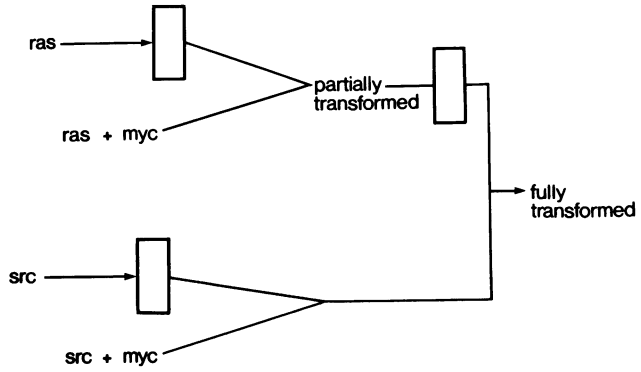

FIG. 9. Suggested pathways of oncogene-mediated transformation of nonestablished adrenocortical cells.

Syrian hamster embryo fibroblasts expressing v-*myc* and v-Ha-*ras* oncogenes may also require an additional cellular change for tumorigenicity (45), which is associated with monosomy for chromosome 15 (33). Similarly, suppression of the transformed phenotype in hybrids of normal and transformed cells is lost coordinately with the loss of specific chromosomes (23, 24, 41). It is possible that the event that renders adrenocortical cells fully permissive to *ras*-and-*myc* transformation involves loss of a function that suppresses oncogene activity in the early-passage cells. Increased expression of viral oncoproteins is unlikely to explain the emergence of fully transformed cells, as little change in $p21^{v\text{-}ras}$ levels were seen in KiMSV-infected adrenocortical cells that acquired a transformed phenotype over 27 passages (28).

The fact that only one of the parameters of transformation examined in these experiments was expressed immediately following coinfection of the rat adrenocortical cells by KiMSV and MMCV suggests that there is a hierarchy of phenotypic changes resulting from the introduction of v-*ras* and v-*myc*. The activated *ras* and *myc* oncogenes induced only limited changes during early passage of infected adrenocortical cells. However, the joint expression of v-*ras* and v-*myc* accelerated the development of full adrenocortical cell transformation when compared with v-*ras* acting alone. This system may therefore serve as a model with which to correlate in vitro phenotypic changes with preneoplastic and malignant states in vivo. Indeed, data obtained with mice containing both *myc* and *ras* transgenes indicates that coexpression of the two oncogenes in vivo reduces the latency of tumor formation but is not sufficient for immediate oncogenesis (39). These results reflect what we have seen in vitro.

In comparison with v-*ras*, v-*src* appears to be the more potent oncogene for adrenocortical cells. v-*src* alone can rapidly transform these nonestablished cells to serum and anchorage independence although with low efficiency. The greater number of foci, significantly more rapid overgrowth by morphologically transformed cells, and greater colony-forming ability of the 2-1-and-MMCV coinfected cultures indicates that v-*src* is able to cooperate with v-*myc* in the efficient transformation of adrenocortical cells. v-*src* is also able to transform nonestablished rat embryo fibroblasts more efficiently than is v-*ras* (17). Synergism between v-*src* and v-*myc* has been previously shown in transformation of avian chondroblasts (2) and hematopoietic cells (1).

Transformation of adrenocortical cells by v-*src* and v-*myc* therefore fits a two-step pathway and is not subject to the cellular suppression that inhibits full transformation by v-*ras* and v-*myc* in this and other systems (33, 36, 43, 50). A model for transformation of early-passage cells by v-*src* and v-*ras* alone or in combination with v-*myc* is presented in Fig. 9.

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