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Spontaneous Conversion of Nontransformed Avian Sarcoma Virus-Infected Rat Cells to the Transformed Phenotype

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Normal rat kidney (NRK) fibroblasts were infected with the Schmidt-Ruppin strain (SR-D) of avian sarcoma virus (ASV) and cloned 20 h after infection without selection for the transformed phenotype. Most infected clones initially exhibited the flat, nontransformed morphology that is characteristic of uninfected NRK cells. In long-term culture, however, the majority of the SR-D NRK clones began segregating typical ASV-transformed cells. Transforming ASV could be rescued by fusion with chicken embryo fibroblasts from most of the infected clones tested. Three predominantly flat, independently infected clones were further analyzed by subcloning 8 to 10 weeks after infection. Most flat progeny subclones derived at random from two of these "parental" SR-D NRK clonal lines did not yield virus upon fusion with chicken embryo fibroblasts, although a nondefective transforming ASV was repeatedly recovered from the parental clones. This observation suggested that most, but not all, daughter cells in these SR-D NRK clones lost the ASV provirus after cloning. The progeny of the third independent parental cell clone, c17, gave rise to both flat and transformed subclones that carried ASV. In this case, ASV recovery by fusion and transfection from the progeny subclones was equally efficient regardless of the transformation phenotype of the cells. The 60,000-dalton phosphoprotein product of the ASV *src* gene was, however, expressed at high level only in the transformed variants. The results of a Luria-Delbruck fluctuation analysis and of Newcombe's respreading test indicated that the event leading to the spontaneous conversion to the transformed state occurred at random in dividing cultures of these flat ASV NRK cells at a rate predicted for somatic mutation.

Avian sarcoma viruses (ASV) of various strains can induce sarcomas after injection into newborn mammals and are capable of transforming mammalian fibroblasts in vitro (44, 45). ASV-transformed mammalian cells, like avian fibroblasts transformed by ASV, become rounded, tend to overgrow in grapelike clusters, and express a whole array of transformation-related properties. The maintenance of the transformed state requires the continuous expression of the viral transforming gene, *src* (51): mammalian cells transformed by mutants of ASV which are temperature sensitive for transformation revert to the normal phenotype when shifted to the nonpermissive temperature (12, 25), and all ASV-transformed mammalian cells analyzed so far express the 60,000-dalton phosphoprotein product of the *src* gene, $pp60^{src}$ (9, 10, 15, 34, 36; H. Oppermann, manuscript in preparation).

Mammalian cells are, in general, nonpermissive for ASV replication. However, ASV-infected mammalian cells usually contain all the viral genetic information necessary for the production of complete infectious virus. ASV can be recovered from such cells by fusion with susceptible avian fibroblasts in the absence of any exogenous or endogenous helper virus (46). Transfection with high-molecular-weight DNA isolated from these cells leads to transformation and infectious virus production in chicken cell cultures (28), and an integrated ASV provirus can be detected in infected mammalian cells by molecular hybridization with labeled ASV-specific nucleic acid transcripts (29, 49).

Focus-forming titers of ASV are usually at least 10^2 times lower in mammalian cells than in chicken cells, even for such mammalotropic strains as the Bratislava 77 (B77) ASV, subgroup C, or Schmidt-Ruppin ASV, subgroup D (SR-D) (3, 22). The low efficiency of transformation does not seem to be caused by the absence of cellular receptors for ASV of the subgroups C and D (7) or by restriction of ASV DNA synthesis early after infection (49).

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Boettiger (6) was able to isolate clones of B77 ASV-infected rat kidney cells which retained their normal phenotype. These cells contained rescuable ASV which could not be distinguished from the parental B77 virus used in the initial infection. Such "silent" ASV infection appeared to be a common mode of interaction between the virus and the heterologous mammalian host cell. It was also noted that a few of the ASV-infected, originally nontransformed normal rat kidney (NRK) clones would eventually convert to the transformed phenotype (late transformants; 6). These observations indicated that the introduction of the transforming gene of ASV into the cell is, by itself, not sufficient for establishing the transformed state.

We have isolated randomly selected clones of NRK cells (21) after infection with SR-D ASV. These clones are isogenic; i.e., they were derived from a single clone of NRK cells infected with recloned virus, yet each infected clone stems from an independent infectious event. Most of the initially flat, nontransformed clones possessed the capacity to give rise to cell variants which expressed the morphological phenotype typical of ASV-transformed NRK cells. We found that in two infected clones, the lack of transformation in most individual cells present in the clonal population was associated with the absence of ASV detectable by rescue. The descendants of another single ASV-infected NRK cell comprised a mixture of flat and transformed cells, both of which carried ASV. In the present communication, we describe (i) the transformation and virus-related properties of these morphological variants, and (ii) a quantitative analysis of the process of spontaneous conversion to the transformed state.

MATERIALS AND METHODS

Cells and viruses. Primary cultures of chicken embryo cells, susceptible to all subgroups of avian leukosis-sarcoma viruses except E (type C/E), chick helper factor negative (52), were prepared from 9-day-old embryos (SPAFAS Inc., Norwich, Conn.) and cultured according to established procedures (50). Chicken cells used in transfection experiments were derived from a 12-day-old SPAFAS embryo, grown in liquid growth medium (50) supplemented with 3% fetal calf serum, and passaged every 3 to 4 days. These cells were frozen at the tertiary stage and used in infectious DNA assays between the sixth and ninth passage.

Recloned NRK cells were obtained from V. Klement and grown as described (21). ASV-infected NRK cells and revived cultures of uninfected NRK cells were kept in Ham F10 medium (92%) with heat-inactivated calf serum (4%), heat-inactivated fetal calf serum (1%), freshly added 200 mM L-glutamine (1%), and 2.8% sodium bicarbonate (2%) (NRK growth medium). Cloning of all NRK cells was carried out in Ham F10 medium with 10% heat-inactivated fetal calf serum, 10% tryptose phosphate broth, glutamine, and bicarbonate. All infected NRK cells were frozen and revived once before use in quantitative assays.

The Schmidt-Ruppin strain of Rous sarcoma virus has been classified as subgroup D (22). The SR-D stock used in this study was focus cloned and passaged once at a low multiplicity of infection in secondary chicken cells. The focus-forming titer in secondary chicken cells and in NRK cells was determined as described (29, 50) with minor modifications: the NRK cells were seeded at a lower density (2×10^5/60-mm dish) in the presence of 10 μg of Polybrene per ml and infected immediately. Foci of transformed cells were counted on day 7 in chicken cultures and on day 14 in NRK cells. The focus-forming titer of our laboratory SR-D stock was about 200- to 500-fold lower in NRK cells than in secondary chicken fibroblasts. This was a stable property of the virus stock throughout several cycles of single-focus cloning.

Isolation of single-cell SR-D-infected NRK clones. NRK cells (2×10^5) were seeded in a 100-mm dish in the presence of 10 μg of Polybrene per ml and infected immediately with 8×10^5 focus-forming units (FFU) of SR-D (as assayed in secondary chicken fibroblasts). The infected cells were trypsinized 20 h postinfection, the absence of cell clumps was verified under a microscope, and the single-cell suspension was cloned in 96-well cluster dishes at a seeding multiplicity of 0.5 cells/well. Uninfected NRK cells were cloned under identical conditions as a control. The medium was changed every 4 days, and cell colonies were counted and their morphology was recorded 14 days after cloning. The distribution of cell colonies in wells was random (Poisson distribution verified by a χ^2 test). Two transformed and 20 flat clones were selected at random from wells containing a single colony and subcultured. Care was taken to transfer the flat NRK clones before the cultures reached confluency. At transfer, however, parallel cultures were plated, and one was kept without transfer for at least a week to monitor spontaneous transformation.

Infectious center rescue assay. The assay was based on a previously described procedure for quantification of ASV rescue by Sendai virus-mediated fusion with chicken fibroblasts (5), but using polyethylene glycol as the fusing agent (20). ASV-infected NRK cells were seeded at 2×10^6 cells per 100-mm dish. Three days later, the cultures were treated with 10 μg of mitomycin C per ml for 2 h to prevent further cell division, washed once with medium, incubated for 30 to 60 min in mitomycin-free medium, and plated at 10-fold dilutions on freshly seeded secondary chicken fibroblasts (10^6/60-mm dish). The next day, the mixed cultures were treated with polyethylene glycol (Carbowax 1500, Fisher Scientific Co., Pittsburgh, Pa.; 48%, wt/vol, in F10 medium) for 2 min, and the medium was changed immediately. The dishes were overlaid with agar overlay medium (48) 18 to 24 h later and overlaid again every 3 to 4 days. Foci of transformed chicken cells, indicating the location of infectious centers (= heterokaryons formed between the ASV-infected NRK cells and the chicken indicator cells; 46), were counted 10 days later. Efficiency of the mitomycin treatment was monitored by control mixed cultures in which the polyethylene glycol fusion step was omitted. The number of infectious centers was directly

proportional to the number of ASV NRK cells seeded in the fused cultures. The rescue efficiency (= the number of infectious centers formed per 10^6 fused ASV NRK cells) was a stable, reproducible property of each individual infected NRK cell clone. A similarly modified rescue assay has been described by others (41).

DNA extraction and transfection. Confluent cell cultures were washed two times with Tris-buffered saline (50), detached with Puck EDTA-saline (8.0 g of NaCl, 0.4 g of KCl, 1.0 g of glucose, 0.35 g of $NaHCO_3$, and 0.2 g of EDTA tetrasodium salt per 1000 ml), and pelleted. The DNA was extracted according to Fritsch and Temin (24). DNA concentration was determined from the absorbance at 260 nm (A_{260}) of a dilute sample. The A_{260}/A_{280} ratio was equal to or greater than 1.8 in all DNA preparations.

Infectivity of DNA extracted from ASV-infected cells was assayed by the calcium precipitate technique of Graham and van der Eb (26). The amount of infectious DNA in a given sample was quantitated either by endpoint dilution (19, 24) or by direct focus formation under agar overlay (1). In the latter method, triplicate chicken cell cultures were inoculated with the DNA-calcium precipitate, treated 4 h later with 33% dimethyl sulfoxide (DMSO) for 4 min (43), and overlaid the next day. DMSO treatment of the transfected cultures enhances the number of transformed chicken cell foci 5- to 15-fold (N. G. Copeland and G. M. Cooper, personal communication; L. P. Turek and P. Luciw, unpublished observation).

Transformation parameters. The proportion of focus-forming cells in a given cell population was quantified by seeding 2×10^5 cells per 60-mm dish alone, or 10^4, 10^3, and 10^2 cells together with 2×10^5 uninfected NRK cells. Foci of typical ASV-transformed cells were counted 10 to 14 days later. The number of foci was directly proportional to the number of ASV-infected cells seeded. Anchorage-independent growth in semisolid agar medium was tested as described (12). Saturation density values were also obtained as described (12). The cultures were media changed daily with either NRK growth medium containing 4% calf and 1% fetal calf serum (standard serum) or with NRK growth medium containing 3% calf serum alone (low serum). Plateau values were reached after 6 to 8 days with standard serum medium and after 8 to 10 days with low-serum medium.

Detection of the *src* gene product. The methods and reagents used were recently described (39). In brief, the cells were seeded in 24-well cluster dishes and labeled for 4 h with either 100 μCi of [^{35}S]methionine (specific activity, 1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) in 250 μl of methionine-free medium per well or with 250 μCi of [^{32}P]orthophosphate (ICN, Irvine, Calif.) in 250 μl of phosphate-free medium per well after a 3-h incubation in phosphate-free medium. Cells were lysed in 0.5 ml of cold lysis buffer per well (100 mM NaCl, 50 mM sodium phosphate, pH 7.2, 1 mM disodium EDTA, 1 mg of bovine serum albumin per ml, and 1% Nonidet P-40), and all following steps were carried out in the cold. The lysates were preadsorbed with a portion of staphylococcus immunoadsorbent (31, 32) and clarified at 12,000 × *g* for 3 min in an Eppendorf microcentrifuge. NaCl concentration in the lysates was adjusted to 0.5 M. Appropriate rabbit serum was added at 4 μl per sample for 30 min. Immune complexes were collected with bacterial immunoadsorbent, washed, and dissolved as described (39). The proteins were then analyzed by polyacrylamide gel electrophoresis (33). Dried gels were exposed to Kodak X-O-Mat X-ray film for autoradiography. Partial digestion with staphylococcal protease V8 in the presence of sodium dodecyl sulfate (13) was performed on eluates of slices excised from the dried gels as described (W. Levinson, H. Oppermann, L. Lewintow, and J. M. Bishop, Biochim. Biophys. Acta, in press). Autoradiographic efficiency was enhanced by the use of intensifying screens, prefogged film, and exposure at −70°C (48).

RESULTS

Characterization of NRK clones selected at random shortly after infection with SR-D ASV. We derived single-cell clones of NRK cells shortly after infection with 4 FFU (as measured in permissive chicken cells) of SR-D ASV per cell (6). The experimental design and genealogy of the SR-D NRK clones are schematically depicted in Fig. 1. Since the focus-forming titers of our SR-D stock were repeatedly 200- to 500-fold lower in NRK cells than in chicken fibroblasts, the proportion of clones that

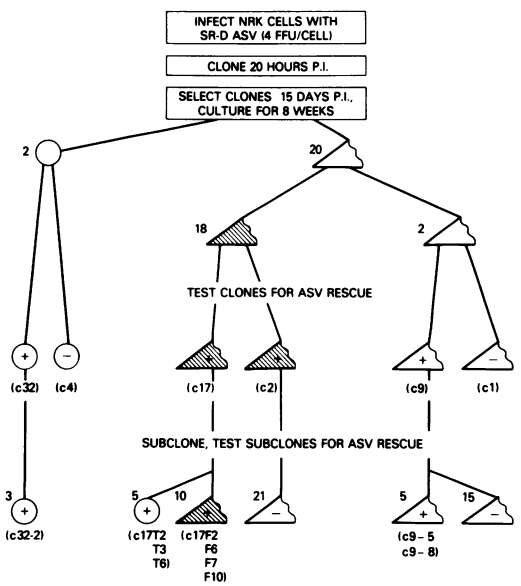

FIG. 1. *Experimental design and genealogy of SR-D ASV-infected NRK cells. Symbols denote the morphology of ASV-transformed (circles) and nontransformed (triangles) infected clones. Initially nontransformed clones which spontaneously segregated ASV-transformed cell variants (converting clones) are shown as shaded triangles. The signs inside the symbols refer to results of ASV rescue experiments. The number of clones falling into each category precedes each symbol. Designation of those clones which were analyzed further are given in parentheses.*

would carry ASV was expected to be relatively small. Only about 5% of the primary colonies of infected cells consisted of refractile fusiform or round cells piling up at the colony center. The remaining cell colonies were composed of flat cells in a monolayer and were quite similar in appearance to colonies formed by uninfected control NRK cells.

Two transformed and 20 flat cell colonies were selected at random 14 days after cloning and subcultured. The cellular morphology of representative flat and transformed clones is shown in Fig. 2. In some of the originally flat clonal cultures, typical round refractile ASV-transformed cells began to appear 2 weeks after the first passage. These cells were initially undetectable in the sparse stock cultures, yet formed prominent foci of transformation in parallel dishes which were kept at confluency after each transfer (Fig. 2c). The number of clones which began shedding the transformed variants (i.e., which were converting to the transformed phenotype) increased as a function of time in culture: by 8 weeks postinfection, 18 of the 20 originally flat SR-D NRK clones contained at least one or more typical ASV-transformed cell foci (Fig. 3). These clones will be referred to as converting clones. Only two of the flat clones showed no background transformation at this time, even when the cells were kept for up to 3 weeks at confluency. No transformation was observed in two clones derived from the uninfected control NRK cells.

The presence of nondefective ASV in the infected clones was confirmed 6 to 9 weeks postinfection by rescue of ASV in the infectious center rescue assay. Twelve of the 18 converting clones were tested; all 12 were positive for infectious transforming ASV. The efficiency of rescue varied widely from clone to clone (see Table 2), in agreement with Boettiger's findings (6). One of the two nonconverting clones, SR-D NRK c9, also contained a rescuable nondefective transforming ASV. The other nonconverting clone (c1) derived from the culture of SR-D-infected NRK cells was repeatedly found to be negative for ASV rescue, and was assumed to be the only uninfected clone in the set. Interestingly, only one of the two initially transformed clones, c32, contained rescuable virus. The other transformed clone, SR-D NRK c4, was repeatedly rescue negative in fusion experiments with both

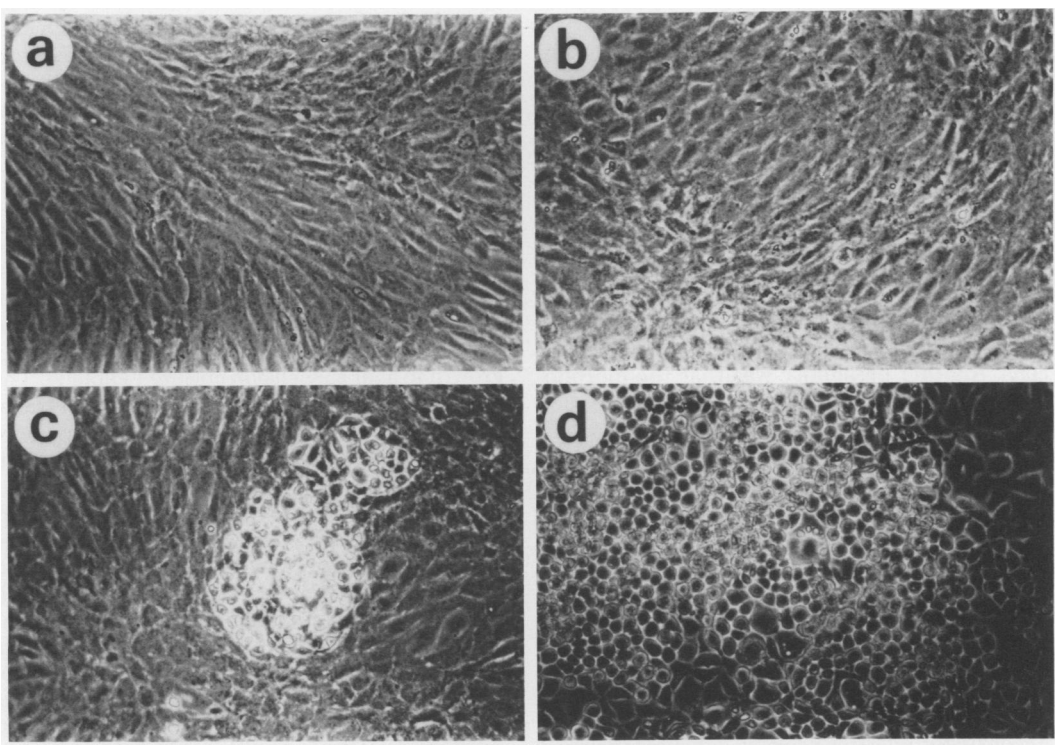

FIG. 2. *Cellular morphology of representative flat and transformed single cell-derived clones of SR-D-infected NRK cells: (a) uninfected NRK cells; (b) flat clone SR-D NRK c9 cells; (c) a predominantly flat culture of clone SR-D NRK c17 cells with a focus of spontaneous conversion to the ASV-transformed phenotype; (d) transformed clone SR-D NRK c32. Phase contrast, ×100.*

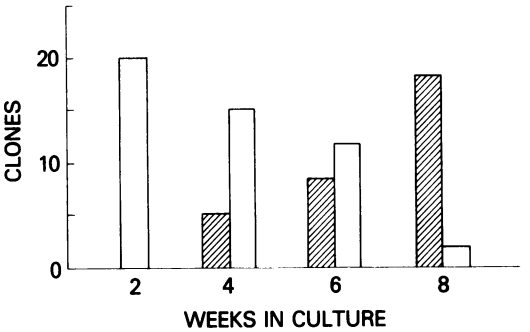

FIG. 3. *Conversion of nontransformed SR-D NRK cell clones to the ASV-transformed phenotype as a function of time in culture. Twenty single cell-derived flat SR-D-infected NRK clones were passaged for 8 weeks and observed for the spontaneous appearance of ASV-transformed variants for 8 weeks post-infection. Open bars, number of infected clones free of spontaneously transformed variants; shaded bars, number of clones containing one or more foci of typical ASV-transformed cells.*

uninfected and helper virus-infected chicken cells, even though it produced the 60,000-dalton phosphoprotein product of the ASV *src* gene. These transformed cells thus resembled the nonrescuable clones that were recently shown to harbor a defective ASV provirus (29).

In general, it was easier to rescue the virus from clones with a higher proportion of transformed cells than from predominantly flat clonal cultures (see below). A regulatory mechanism in the flat infected cells which would influence the overall expression of viral genes, including the *src* gene, could limit the availability of viral proteins and virion RNA, and thus restrict infectious virus production in the heterokaryon (5, 6). This model would predict that subclones derived at random from such lines would retain the characteristic low rescuability of the parent cells. Another possibility was that the morphologically flat cells in a given clonal population have lost ASV before or after integration.

To distinguish between these two possibilities, we derived single-cell subclones of one transformed and three mostly flat independent parental SR-D NRK clones and tested them for ASV rescue. The isolation of subclones was carried out at 8 to 10 weeks after infection, i.e., at a time when any proviral copies of ASV were expected to be stably integrated in the cellular genome. It is known that individual transformed subclones of an established SR-D NRK line retain the ASV provirus in its original insertion site (29). Accordingly, all three progeny subclones derived from the initially transformed parental SR-D NRK clone c32 were transformed and harbored rescuable ASV (Fig. 1). Different rescue results were obtained with the flat, randomly selected progeny subclones of two of the three flat parental SR-D NRK clones: 15 of 20 progeny subclones of the nonconverting clone c9, and all 21 flat progeny subclones randomly derived from the converting clone c2, were found to be ASV negative in fusion experiments. Continuously maintained cultures of the parental clones contained rescuable transforming ASV both at the time of subclone isolation and at the time their progeny subclones were tested in fusion experiments, 14 to 16 weeks postinfection. These results suggest that many individual progeny cells of these two ASV-infected parental clones may have lost the ASV genome between the initial cloning and the isolation of progeny subclones, i.e., between 20 h and 8 weeks after infection.

The third flat parental clone analyzed, SR-D NRK c17, yielded only ASV-positive flat subclones (Fig. 1). In these flat cells, the absence of morphological transformation had to be caused by some other mechanism than proviral loss. This parental clone and its subclones were subjected to further analysis.

Properties of flat and spontaneously transformed progeny subclones of a single flat SR-D-infected rat cell. Both flat and spontaneously transformed cells were present among the progeny of a single SR-D-infected NRK cell in the converting clone c17. To obtain transformed subclones of c17 for further studies, five independent foci of transformed cells that appeared spontaneously in separate confluent c17 cultures were aspirated with a capillary pipette, recloned, and found to be virus positive after fusion with chicken fibroblasts (Fig. 1). These focus-derived c17 subclones expressed several additional transformation-related parameters besides their morphology, such as density-independent focus formation on confluent sheets of uninfected NRK cells and colony growth in agar suspension culture (Table 1). The difference in saturation densities between transformed and nontransformed cells became more prominent in medium with lower serum concentration.

Despite the clear-cut difference in cell morphology and three other growth parameters related to transformation, the flat and transformed sibling subclones of SR-D NRK c17 contained an ASV proviral complement that was indistinguishable in rescue or transfection experiments (Table 2). ASV recovery from most of the subclones was equally efficient in the infectious center rescue assay. However, the transformed subclone c17T3 repeatedly showed about 10-fold higher, and the flat subclone c17F7 approximately 10-fold lower, efficiency of infectious center formation than the parental c17 cells and the other subclones tested. A difference of several

orders of magnitude was observed in the ease of ASV rescue from the independently infected parental clones.

Two different approaches were used to titrate the specific infectivity of cellular DNA extracted from several representative parental SR-D NRK clones and from c17 subclones: endpoint dilution (19) and direct focus formation (1). The latter

TABLE 1. *Transformation parameters of parental SR-D NRK clones and their subclones*

Clone	Morphology	Focus formation[a]	Agar colony formation[b]	Saturation density ($\times 10^{-5}$ cells/cm^2)	
				Standard serum[c]	Low serum[d]
Parental clones					
c32	Transformed	370,000	250,000	3.3	3.2
c2	Converting	400	260	2.1	ND
c9	Flat	<100	<2	1.7	ND
c17	Converting	100	200	1.9	0.66
Subclones					
c17F2	Flat	<100	10	1.7	0.64
c17F6	Flat	<100	8	1.3	0.58
c17T3	Transformed	230,000	210,000	1.4	1.3
c17T6	Transformed	420,000	230,000	2.0	2.0
c32-2	Transformed	260,000	170,000	3.2	4.0
Uninfected NRK	Flat	<100	<2	1.5	0.76

[a] Number of focus-forming cells/10^6 cells seeded at 10-fold dilutions together with 2×10^5 uninfected NRK cells.

[b] Number of colony-forming cells/10^6 cells seeded at 10-fold dilutions in 0.35% agar suspension culture.

[c] Plateau value for cultures of 5×10^5 cells/60-mm dish fed NRK growth medium containing 4% calf and 1% fetal calf serum daily.

[d] Plateau value for cultures of 5×10^5 cells/60-mm dish fed NRK growth medium containing 3% calf serum daily. ND, Not done.

TABLE 2. *ASV recovery from parental SR-D NRK clones and their subclones by fusion and transfection*

Clone	Cell morphology	RSV rescue[a]	Transfection[b]	
			Endpoint dilution[c]	Focus formation[d]
Parental clones				
c32	Transformed	34,000	3.4	1.5
c2	Converting	4	ND	ND
c9	Flat	37	<0.2[e]	0.07
c17	Converting	1,100	0.6	0.3
Subclones				
c17F2	Flat	800	0.7	0.5
c17F6	Flat	1,500	0.5	0.4[e]
c17F7	Flat	100	ND	ND
c17F10	Flat	900	ND	ND
c17T2	Transformed	1,400	ND	ND
c17T3	Transformed	9,600	0.8	0.3
c17T6	Transformed	1,200	0.8	0.4

[a] Mitomycin C-treated SR-D NRK cells were plated at 10-fold dilutions together with secondary chicken embryo cells, and the mixed cultures were exposed to polyethylene glycol the next day. The fused cultures were overlaid 16 to 20 h later, and foci of ASV-producing transformed chicken cells (indicating the location of ASV-producing heterokaryons which formed between the rat and chicken cells) were enumerated after 10 days. The rescue efficiency is expressed as the number of infectious centers/10^6 SR-D NRK cells.

[b] Specific infectivity of high-molecular-weight cellular DNA preparations was determined by the calcium precipitate method of Graham and van der Eb (26). ND, not done.

[c] Triplicate cultures of chicken cells were inoculated with twofold dilutions of cell DNA. The DNA concentration was adjusted to 10 μg/ml with salmon sperm DNA before calcium precipitation. The 50% infectious dose (= one infectious unit) was determined after testing the harvests of transformation-negative recipient cultures on fresh chicken cells (19). Specific infectivity is expressed in infectious units per microgram of DNA.

[d] Triplicate cultures of chicken cells were infected with 5 μg of cell DNA/dish and treated with 33% DMSO in *N*-2-hydroxyethylpiperazine-*N*'-2-ethansulfonic acid (HEPES)-buffered saline (43) 4 h later. Foci of transformed chicken cells were scored under agar overlay 10 days later. Specific infectivity is expressed in focus-forming units per microgram of DNA (1).

[e] One of three recipient cultures inoculated with 5 μg of DNA/dish was transformation positive in two independent experiments.

method was rendered more sensitive by the inclusion of DMSO treatment (44). In both cases, the specific infectivity of the parental clone c17 DNA and of the subclone DNA preparations was approximately equivalent regardless of the different transformation phenotype (Table 2). Infectious DNA titers varied between the individual parental SR-D NRK clones and reflected the ease at which ASV could be rescued from these cells by fusion with chicken cells. The differences in relative DNA infectivity were reproducibly much smaller than the differences in efficiency of ASV recovery from the parental SR-D NRK clones by fusion with chicken cells.

Expression of the *src* gene product of ASV. ASV-transformed cells of both avian and mammalian origin contain a specific phosphoprotein with an apparent molecular weight of 60,000 ($pp60^{src}$) that has been shown to be the product of the ASV *src* gene (9, 10, 36). This protein is absent in morphological revertants of SR-D-transformed hamster cells (9, 36), yet an apparently altered $pp60^{src}$ is synthesized in revertants of field vole cells transformed by SR-D (15, 34). To determine whether the morphologically normal ASV-infected NRK cells expressed $pp60^{src}$, the proteins of uninfected NRK cells and selected SR-D NRK cells were labeled with [^{35}S]methionine. Cell lysates were prepared, immunoprecipitated with serum from a tumor-bearing rabbit, and resolved on a 7.5% polyacrylamide slab gel (Fig. 4). The three morphologically transformed variants of c17 (lanes h, i, and j) synthesized ASV $pp60^{src}$ in quantities comparable to those found in the initially transformed clone c32 cells which were included in the experiment as a positive control (lane b). In addition, all the transformed cells contained detectable levels of the *gag* precursor polyprotein, $Pr76^{gag}$ (23). The identity of both viral $Pr76^{gag}$ and SR-D $pp60^{src}$ was confirmed by one-dimensional peptide mapping (not shown). Two ASV-positive subclones of the nonconverting parental clone c9 (lanes c and d) as well as the uninfected NRK control (lane a) contained no detectable $pp60^{src}$ or $Pr76^{gag}$. By contrast, the converting flat clone c17 and its flat subclones were found to synthesize $pp60^{src}$ at a low level (lanes e, f, and g).

Uninfected rat cells contain low levels of a 60,000-dalton phosphoprotein that shares some antigenic determinants with the SR-D-coded $pp60^{src}$. This protein is thought to be the translational product of an endogenous rat gene homologous to the *src* sequence of ASV (39). The two proteins can be distinguished by one-dimensional peptide mapping after partial hydrolysis with staphylococcal V8 protease (13). A characteristic marker of the endogenous pp60 is the mobility of the carboxy-terminal V8 fragment. It has an apparent M_r of 27,000, whereas the ASV-coded $pp60^{src}$ yields an analogous fragment with an M_r of approximately 24,000 (16; Oppermann et al., in preparation). To compare the concentration of the SR-D $pp60^{src}$ seen in the flat c17 cells and their flat subclones with the endogenous rat pp60 levels, a rabbit serum reacting with both SR-D $pp60^{src}$ and rat pp60 (39) was used to precipitate [^{32}P]phosphoric acid-labeled cellular proteins of the representative cell lines shown in Fig. 4. In this case, all the morphologically flat NRK cells, including the uninfected control, contained traces of a 60,000-dalton phosphoprotein. The origin of these proteins was then determined by V8 protease digestion, followed by polyacrylamide gel analysis of the resulting peptides (Fig. 5). Uninfected NRK control (lane b) and the two nonconverting SR-D NRK c9 subclones (lanes c and d) contained only endogenous rat pp60 (lane a). The c17 clone and its flat subclones produced low levels of both rat pp60 and SR-D-directed $pp60^{src}$ (lanes e, f, and g). The V8 peptides of the latter were indistinguishable from those present at high concen-

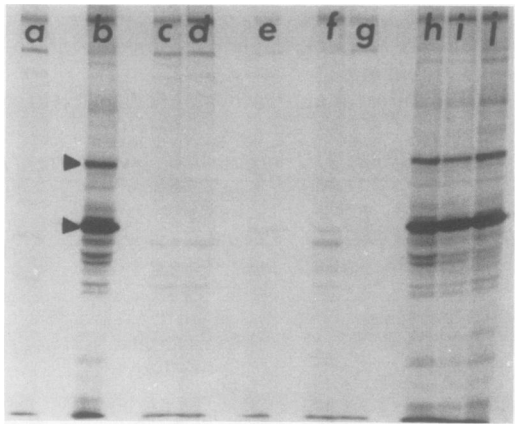

FIG. 4. *Analysis of ASV-coded proteins synthesized by flat and transformed SR-D NRK cell clones. Cells were labeled with [^{35}S]methionine, and cell lysates were prepared and immunoprecipitated with serum of an SR-D tumor-bearing rabbit. The serum used reacts with both viral $pp60^{src}$ (lower arrow) as well as $Pr76^{gag}$ (upper arrow). The proteins were resolved on a 7.5% polyacrylamide slab gel and visualized by autoradiography. Several slots on the gels were left without samples to prevent cross-contamination of lanes. Lanes represent: (a) uninfected NRK cells; (b) SR-D NRK clone c32; (c, d) flat, nonconverting ASV rescue-positive subclones c9-5 and c9-8; (e–g) flat, converting parental clone c17 and its flat subclones c17F2 and c17F6; (h–j) transformed subclones c17T2, c17T3, and c17T6.*

tration in the transformed subclones of c17 (lanes h, i, and j) and clone c32 (lane k). The difference in morphological phenotype between the flat and transformed subclones of SR-D NRK c17 was thus accompanied by a marked quantitative difference in pp60^{src} concentration.

Origin of converted variants. In principle, the morphological phenotype of the flat and transformed progeny cells of clone c17 could have been permanently established relatively early after infection. In this case, the phenotype of the flat and transformed variants would remain stable after a single cycle of recloning. All the phenotypically transformed clones and subclones remained apparently stably transformed throughout this study (morphological reversion occurring at a low frequency would have escaped detection). On the other hand, the originally flat subclones of clone c17 began to segregate transformed variants in long-term culture. The conversion was also observed in second-generation single-cell reclones randomly selected from the c17F2 and c17F6 subclones. Thus, the tendency to shed transformed variants at a low frequency, rather than the flat phenotype per se, was the genetically stable characteristic of SR-D NRK c17 cells.

The high fluctuation in the number of transformants observed among parallel independent subclones suggested that the converted variants expressing the ASV-transformed phenotype and the pp60^{src} of SR-D arose spontaneously, at random, in the phenotypically untransformed cultures passaged at high dilution. Since the transformants were usually not detectable in subconfluent cultures, the alternative possibility existed that the variant phenotype was induced by incubation at confluency or in agar suspension.

We performed the fluctuation test of Luria and Delbruck (37) in order to distinguish between these two possibilities. If the conversion event could be induced in any individual cell by incubation at confluency, all c17 cultures seeded at the same density and incubated under identical conditions would contain approximately the same number of transformants. If the conversion occurred at random in the course of cell growth, however, the converted cells would multiply before the cultures were assayed for their presence. The number of transformed variants would then fluctuate considerably among parallel cultures grown independently for a longer period of time.

The variation in the number of transformed cells observed in replicate cultures from the same c17F6 cell stock could be explained by random distribution (sampling error) with a probability $P = 0.25$ (Table 3). The fluctuation in the numbers of transformants among the parallel cultures grown up from small inocula was, however, too high to fit Poisson distribution ($P < 0.005$). This observation was inconsistent with the possibility that each c17F6 cell had an equal probability of converting to the transformed phenotype at confluency, and indicated that at least some conversion events must have occurred earlier in the course of cell growth.

To confirm this conclusion, we carried out the respreading experiment of Newcombe (38) and used a mixture of a small number of the ASV-transformed subclone c17T6 cells with uninfected NRK cells as a quantitative control (Table 4). A series of parallel cultures was grown for approximately six to seven cell generations without transfer. At this point, the cells in alternate dishes were trypsinized and replated ("respread"). Foci of transformed cells were then counted in the intact and the "respread" cultures after a week at confluency, i.e., 10 days post-transfer. In the undisturbed cultures, the progeny of a single transformed variant was expected to grow up in loco, and thus form a single focus. If some of the transformed variants arose one or more cell generations before transfer, each progeny transformed cell would give rise to a new focus in the "respread" cultures. On the other hand, had the variant phenotype been induced

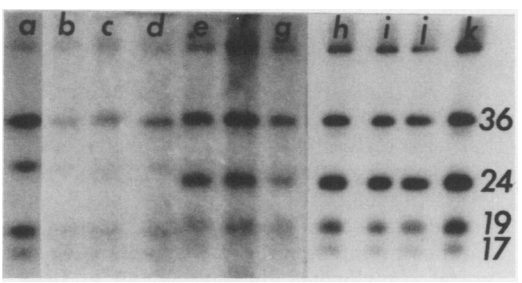

FIG. 5. *One-dimensional peptide maps of pp60 present in uninfected and SR-D-infected NRK cells. Cellular proteins labeled with [^{32}P]phosphate were precipitated with an antiserum reacting with both viral pp60^{src} and endogenous rat pp60. The 60,000-dalton regions of a 7.5% polyacrylamide gel similar to the one in Fig. 4 were excised, eluted, digested with 200 ng of V8 protease, and resolved on a 7.5% polyacrylamide gel. Approximate molecular weights of the resulting fragments are indicated to the right. Digest of a preparative sample of endogenous NRK pp60 is included in lane a. Other lanes correspond to: (b) uninfected NRK; (c, d) nonconverting subclones c9-5 and c9-8; (e) flat, converting clone c17; (f, g) flat subclones c17F2 and C17F6; (h) transformed clone c32; (i–k) transformed variant subclones c17T2, c17T3, and c17T6. Lanes a through g were exposed with screens at −70°C for 14 days. Lanes h through k were exposed without screen for 24 h at room temperature.*

TABLE 3. *Fluctuation analysis of the conversion rate in SR-D NRK c17F6*[a]

No. of transformed cells/culture	No. of cultures	
	Replicate	Parallel
0	6	16
1	6	2
2	6	2
3	3	1
5	1	
8		1
Mean	1.45	0.77
Variance + mean	1.11	4.11
χ^2	2.44	90.4
$P(\chi^2)$	0.25	<0.005
Conversion rate		$2.21 \pm 0.91 \times 10^{-7}$

[a] Twenty-two parallel cultures were started with approximately 100 viable c17F6 cells (as verified by colony counts 7 days later) and grown for 12 to 14 days with one transfer until the average number of cells reached 10^6. The cultures were then trypsinized and seeded at 5×10^5 cells/60-mm dish. Twenty-two replicate cultures were obtained by seeding 5×10^5 cells of a c17F6 stock culture/60-mm dish. The dishes were changed with growth medium containing 3% calf serum every 3 to 4 days, and foci of converted cells were counted 14 to 17 days later. The rate of spontaneous conversion (= probability of a conversion event per cell per generation) was calculated from the number of parallel cultures which contained no transformed cell variants by the P_o method (35).

with an equal low probability in any cell by incubation at confluency, the number of converted cells would be the same in the undisturbed and transferred cultures (or rather follow a Poisson distribution) since both sets of dishes contained approximately the same final number of cells (Table 4).

In the undisturbed dishes of both the c17F6 cells and the mixed controls, the number of foci was rather small. Its variation followed Poisson distribution, indicating that no significant spread of transformed cells from the primary foci took place without "respreading." In the transferred c17F6 cultures as well as in the mixed controls, however, the number of transformed cell clusters was much higher, and, in analogy to the Luria-Delbruck test, their fluctuation was too high to fit a Poisson distribution. This result proved that the variant transformed phenotype must have been determined in some cases one or more cell generations before transfer. Since, contrary to the mixed controls, none of the transformed variants was present in the initial inoculum (see legend to Table 4), the conversion event must have occurred in the subconfluent dividing cultures between seeding and transfer.

In the mixed controls, the number of focus-forming cells increased approximately at the same rate as the total number of cells between seeding and transfer. Thus the transformed variants did not show a selective growth advantage or disadvantage under the given conditions. In the converting c17F6 cultures, however, the increase in the mean number of variants was much lower, and also much closer to the number of cell generations which took place between seeding and "respreading," confirming that the conversion occurred at random as a function of time (42). The rate of conversion was estimated to be 2×10^{-7} conversion events per cell per generation in the c17F6 subclone by both the fluctuation analysis and the respreading test (Tables 3 and 4) and 1×10^{-7} to 3×10^{-7} in the c17F2 subclone (fluctuation test data not shown).

DISCUSSION

We derived single-cell clones of NRK cells infected with SR-D ASV at a multiplicity of 4 FFU/cell. In view of the repeatedly observed 200- to 500-fold-lower susceptibility of the NRK cells to transformation by SR-D ASV as compared with susceptibile chicken cells, the number of clones that would contain ASV was expected to be relatively low. Most such infected NRK clones were, however, found to consist of a mixture of nontransformed (flat) and typical round ASV-transformed cells by 8 weeks postinfection, even though all the clones had been subcultured from randomly selected single colonies of nontransformed cells in independent multititer wells. All 12 such converting clones that were tested by fusion with chicken embryo fibroblasts were found to harbor transforming ASV. Nineteen of a total number of 20 clones randomly selected 20 h postinfection were thus found to be infected by ASV, demonstrable by typical transformed morphology of at least a minority of the cells or by rescue of transforming ASV. The effective multiplicity of infection calculated from $P_0 = 1/20$ of a Poisson distribution was ~3.0 infectious virions per cell, and thus almost as high as in chicken cells.

Experiments with pseudotypes of vesicular stomatitis virus in the envelope of avian RNA tumor viruses demonstrate that SR-D ASV can enter mammalian cells as efficiently as chicken cells (7), and nucleic acid hybridization experiments detect comparably high levels of ASV DNA synthesis early after mammalian cell infection (49). The level of detectable ASV DNA tends to decrease rapidly within a few passages of such mass-infected mammalian cell cultures (S. H. Hughes, H. E. Varmus, and P. K. Vogt, personal communication). One possible explanation for the mixed morphology observed in

TABLE 4. *Newcombe respreading test*

Culture no.	No. of transformed cell foci in parallel cultures:			
	Mixture of uninfected NRK + transformed subclone c17T6[a]		Converting subclone c17F6[b]	
	Undisturbed	Transferred	Undisturbed	Transferred
1	0	106	0	2
2	1	154	1	0
3	1	0	1	9
4	2	0	0	10
5	0	468	0	0
6	1	0	2	20
7	0	135	4	0
8	4	0	1	0
9	1	0	2	0
10	0	0	1	4
11	0	142		
12	1	0		
Mean no. of transformed cells/culture	0.92	83.8	1.20	4.50
Variance ÷ mean	1.36	207	1.13	8.85
$P(\chi^2)$	0.1	<0.005	0.25	<0.005
Factor of cell no. increase		120×		100×
No. of cell generations		6.9		6.6
Factor of transformed cell no. increase		91.4		3.75
Final no. of cells			5.1×10^6	4.9×10^6
Conversion rate			2.35×10^{-7}	

[a] Twenty-four cultures were started with a mixed inoculum of 6 transformed c17T6 cells and 2×10^4 uninfected NRK cells per 60-mm dish. After 6 days, when the cells had been dividing for approximately 7 generations, every other culture was trypsinized, and the cells were resuspended, counted, and redistributed over the whole area of a new 60-mm dish ("transferred" cultures). The remaining 12 dishes were incubated further without transfer ("undisturbed" cultures). Foci of transformed cells were counted in both sets of dishes 10 days later, and two cultures containing no transformed foci were trypsinized to obtain the final cell number. The expected number of transformed cells per culture was determined as the mean number of foci in 10 additional cultures which received the same inoculum together with 2×10^5 uninfected NRK cells.

[b] Twenty cultures of 2×10^4 converting c17F6 cells per dish were seeded and cultured according to the same protocol. Again, every other culture was trypsinized and respread after the cells reached near-confluency, and foci of transformed cells were counted 10 days later. An additional six dishes that were started with the same inoculum received 2×10^5 uninfected NRK cells each. None of these cultures contained transformed cell foci, indicating that the small inoculum of c17F6 cells contained less than 0.17 already transformed cells per culture at the time of seeding. The expected number of transformants per culture present at the outset (0.12) was determined from the number of foci in 10 replicate cultures of 5×10^5 c17F6 cells each. The conversion rate was calculated as: mean number of transformants per undisturbed culture/final number of cells per culture (42).

the parental single-cell SR-D NRK clones would thus be loss of the ASV genome from the flat progeny cells due to inefficient proviral integration in the heterologous host cell. The newly synthesized ASV provirus can be found integrated in cellular DNA within 12 h after infection (49), but some proviral DNA persists in an unintegrated state for a longer period of time (40). Since the SR-D NRK cells were cloned 20 h after infection, it is conceivable that a single infected cell could have undergone one or several divisions before the ASV provirus stably integrated in the genome of one daughter cell. The resulting clonal cell population would then become a mosaic of (i) transformed cells carrying ASV and (ii) flat uninfected cells. Loss of ASV provirus could also account for the large quantitative differences in the efficiency of ASV rescue from different parental clones (6). This model can explain the absence of rescuable ASV in all 21 randomly selected subclones of one parental clone, SR-D NRK c2, and in 15 of 20 subclones of another clone, c9, even though the parental clones (and in the case of SR-D NRK c9 also 5 of 20 subclones) were repeatedly positive for the presence of rescuable transforming ASV. It seems unlikely that a stably integrated provirus would be lost at such a high rate (29). Although the rescue method we used was quite sensitive (5, 6, 41), our observations do not ex-

clude the possibility that the rescue-negative subclones of c2 and c9 contain either a defective (29, 47) or a suppressed ASV. These results indicate, however, that single-cell clones derived early after retroviral infection may consist of a heterogeneous cell population in terms of proviral integration or expression. Analysis of the ASV provirus by nucleic acid hybridization will help to distinguish between these two possibilities.

Some subclones of SR-D NRK c9 and all subclones of c17 carried rescuable transforming ASV despite their flat morphology. In agreement with Boettiger's observations (6), these cells contained a nondefective ASV capable of transforming NRK cells, and were found to be as susceptible to transformation by either SR-D or by the Kirsten murine sarcoma virus as uninfected NRK cells (data not shown). Analysis of sibling flat and transformed subclones derived from the progeny of a single infected cell, SR-D NRK c17, revealed that only the morphologically transformed variants expressed additional parameters related to transformation. The viral *src* gene-coded phosphoprotein, pp60^{src} (9, 10, 36), was synthesized at a high level only in the transformed cells. It is not clear whether the small amounts of pp60^{src} observed in the flat subclones of c17 were produced by a minority of cell variants which arose in these lines or by a low-level expression of the *src* gene in all cells. This question could only be answered by immunofluorescence analysis at the single-cell level. The results of one-dimensional peptide mapping (13, 16, 39) showed that the c17 cells contain SR-D ASV that is capable of coding for an apparently unaltered *src* protein. The pp60^{src} concentrations in the flat SR-D NRK c17 derivatives were only slightly higher than the low levels of endogenous rat pp60, the putative protein product of *sarc*-related sequences present in rat cellular genome (39).

The proviral complement present in most subclones of SR-D NRK c17, regardless of their morphology, was indistinguishable in both quantitative rescue and transfection experiments. Thus, the proportion of cells yielding ASV as assayed in the infectious center rescue assay did not correlate with transformation as a parameter of ASV gene activity in the SR-D ASV-infected rat cells (5, 6). One flat subclone, c17F7, was reproducibly about 10-fold less rescuable, and one transformed subclone, c17T3, was approximately 10-fold more efficient in fusion rescue experiments than the parental clone 17 cells and the remaining subclones we have tested. It is not clear whether these two exceptions reflect a true regulatory phenomenon or some other mechanism, such as different efficacy of fusion with chicken cells.

The high-molecular-weight DNA ($\geq 3 \times 10^7$; P. Luciw and L. P. Turek, unpublished data) of representative flat and transformed c17 subclones was as infectious in chicken recipient cultures as the parental c17 cell DNA. We have also successfully demonstrated the infectivity of the DNA extracted from the flat parental clone c9 even though only about one-fourth of the individual cells contained rescuable ASV. The DNA preparations were not sheared before transfection; it is therefore unlikely that the ASV genome in the flat c17 and c9 cells is closely linked to a *cis*-acting host cell DNA element that would limit efficient transfection (11, 18). Since the specific infectivity of a given cell DNA is thought to reflect the number of nondefective retroviral genomes (4), both the flat and the transformed c17 subclones appeared to contain the same number of infectious transforming ASV genomes as the parental c17 clone cells by this criterion. However, since the effective multiplicity of infection used in the initial infection was relatively high, and the SR-D stock apparently contained viral mutants defective in virus replication functions (as seen in the transformed clone c4), it is conceivable that a provirus different from the biologically active ASV recovered by fusion and transfection could be responsible for conversion to transformation in SR-D NRK c17 cells and their derivatives.

The transformed c17 variants arose by conversion of flat, nontransformed c17 cells. The tendency of flat c17 cells to segregate transformed variants at a low frequency was a trait stable upon at least two successive cycles of single-cell subcloning. The conversion event occurred at random in dividing cultures without any apparent inducing influence. The rate of spontaneous conversion, obtained by two different experimental approaches (37, 38), resembled the rate of spontaneous mutation described for several enzyme-coding single gene loci in cultured mammalian cells, such as hypoxanthine phosphoribosyl transferase, adenine phosphoribosyl transferase, and aryl hydrocarbon hydroxylase (2, 14, 27, 30). Once the conversion occurred, the transformed phenotype was stable over at least several months in culture. The spontaneous conversion was associated with an increase in the synthesis of pp60^{src} from a low level, close to the level of endogenous rat pp60 expression, to concentrations found in cells transformed by SR-D ASV directly after infection.

These observations indicate that the spontaneous conversion to transformation in c17 cells

represents a genetically stable alteration in the interaction between a transformation-competent viral genome and a transformation-susceptible host cell. If the ASV provirus originally integrated in an unexpressed portion of the cellular genome (6), then its stable activation may possibly involve a rearrangement of the cellular DNA sequences adjacent to the integrated provirus, with or without a change in the site of integration itself or proviral amplification. These possibilities are amenable to experimental analysis by physical mapping of the proviral DNA present in the flat ASV-infected cells and in their transformed variants (8, 29). Collins et al. (17) have recently published data showing that an integrated ASV provirus is retained in its original insertion site in morphological revertants of ASV-transformed NRK cells and in their retransformants. It will be interesting to see whether the spontaneous conversion in c17 cells is accompanied by any detectable change at the proviral insertion site.

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