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Revertants and Partial Transformants of Rat Fibroblasts Infected with Fujinami Sarcoma Virus

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Fifteen revertants were isolated from three independent clones of rat fibroblasts transformed by Fujinami sarcoma virus (FSV). Three revertant clones resulted from the deletion of the one copy of the FSV provirus, and one encoded an enzymatically inactive, transformation-defective protein. The remaining revertant clones were characterized by a transcriptional block of the provirus. Digestion of chromosomal DNA with *Msp*I and *Hpa*II revealed that the FSV provirus was hypermethylated in these revertants, whereas proviral DNA of their spontaneous retransformants was hypomethylated. Furthermore, the revertants had lost DNase I-hypersensitive sites in and around the FSV provirus. The effect of transcriptional regulation of the FSV provirus was further analyzed in clones showing various degrees of phenotypic transformation. We quantitated v-*fps* mRNA levels in these cells by liquid hybridization and found that increasing levels of viral RNA correlated with a more pronounced transformed phenotype. These results suggest that transcription of FSV proviral DNA is under both viral and cellular control and that transformation by FSV is a function of the dosage of v-*fps* mRNA.

High expression of retroviral genes results from strong promoter activity associated with a specialized viral sequence, the long terminal repeat (LTR), present at both ends of the provirus (45). Insertion of an LTR in the vicinity of cellular genes can sometimes lead to their enhanced expression. Activation by an LTR of the c-*myc* gene was shown to result eventually in lymphomas in chickens infected with avian leukosis virus (20). Expression of retroviral genes may also be subject to cellular regulatory mechanisms, since retroviruses are maintained essentially as cellular elements, integrated at random in the host chromosome. Therefore, the extent of expression of a provirus should result from a constant interplay between these two modes of regulation, namely, the promoter activity of the LTR and the local cellular controlling elements. Very little is known about what determines the outcome of this interplay, but in several systems it was shown that hypermethylation of proviral DNA correlated with a transcriptional block of the provirus (7, 8, 12, 14–16, 21, 42). Thus, the strong promoter activity of the LTR can be superceded by local cellular controlling elements. However, many problems remain unresolved. For instance, certain chromatin domains may be more susceptible to such cellular regulatory events (due to, for instance, DNA conformation or nucleosome composition). In addition, these cellular signals may be able to modulate the promoter activity of the LTR, leading to various levels of expression of the retroviral genes in infected cells.

These problems are quite relevant to cell transformation by retroviruses. Several phenotypic revertants have been characterized by a transcriptional block of the provirus in infected cells (6, 11, 15, 33, 34). Although these studies confirmed that transformation resulted from the efficient transcription of the viral genome, they failed to provide information on the effect of low or intermediate levels of viral RNA on cell morphology. Also, it is not known whether transformation could occur in an all or none fashion or whether increasing amounts of viral RNA correlate with gradual morphological changes.

Fujinami sarcoma virus (FSV) (19, 23) proved to be useful in this kind of study, since we could obtain from FSV-transformed rat fibroblasts various revertants, of which many had resulted from a transcriptional block of the provirus. Furthermore, the isolation of partial transformants allowed us to demonstrate that in the case of FSV, different states of morphological transformation were the result of a differential dosage of the viral RNA and that the strong promoter activity of the LTR can be modulated by cellular controls.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts were cultured as described previously (17), except that Ham F-10 medium with 5% calf serum was used for all secondary cultures. Rat 3Y1 cells, transformed by FSV (27), were maintained in Dulbecco modified Eagle medium (GIBCO) containing 10% fetal calf serum.

Cloning of rat fibroblasts. Cultures to be cloned were trypsinized; single cells were picked under microscopic surveillance and placed into microtiter wells containing 0.1 ml of growth medium (10% fetal calf serum, Dulbecco modified Eagle medium). After 3 weeks, colonies were screened for morphology and transferred to 35-mm tissue culture dishes. Selected cultures were grown to mass culture and kept in the medium described above.

Treatment of rat fibroblasts with chemicals. Some of FSV-transformed 3Y1 cultures in growing conditions were treated with 10 μg of fluorodeoxyuridine (FUdR) per ml or 0.04 μg of colcemid per ml as described previously (31). Confluent

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cultures of FSV-transformed 3Y1 cells were treated with 1 mM hydroxyurea as described previously (46). In all cases, the treatment was done in growth medium. At the end of the treatment, cells were trypsinized and plated at low density. After 24 h, surviving cells were trypsinized and either cloned as described above or, in the case of hydroxyurea-treated cells, treated with FUdR as described above. Treatment with 5 or 15 μM 5-azacytine was carried out for 48 h. Thereafter, the cells were washed twice with growth medium and subcultured until they reached confluence. Cultures were fed with fresh medium every 3 days and examined for the appearance of foci.

Isotopic labeling of cells and preparation of cell extracts. Cultures were labeled with [^{35}S]methionine (New England Nuclear Corp.; >500 Ci/ml) or $^{32}P_i$ (New England Nuclear; carrier free) as described previously (27), except that labeling with $^{32}P_i$ was done in phosphate-free medium for 5 h with 3 mCi/ml. Labeled cultures were lysed in modified Nonidet P-40 buffer containing 0.1% 2-mercaptoethanol (27).

Immunoprecipitation and polyacrylamide gel electrophoresis. Serum from tumor-bearing rabbits (TBR serum) induced by the Schmidt-Ruppin D strain of Rous sarcoma virus, rabbit antiserum raised against Rous-associated virus 2 virion proteins (antivirion antiserum), and tumor-bearing rat antiserum raised against FSV-transformed 3Y1 cells (anti-FST antiserum) were obtained as described previously (13, 27). Immunoprecipitation, gel electrophoresis, and the protein kinase assay were performed by published procedures (27).

Preparation of DNA and RNA. Isolation of high-molecular-weight DNA was done as described previously (38). DNA concentration was measured by absorbance at 260 nm. Isolation of total cellular RNA was as follows. Cells were trypsinized, washed once, and centrifuged at 1,000 × *g* for 10 min. The cell pellet was lysed by the addition of 8 ml of guanidium-thiocyanate buffer (4 M guanidium-thiocyanate, 0.1 M sodium acetate [pH 5.0], 5 mM EDTA); this solution was layered on top of 4 ml of CsCl solution (0.84 g of CsCl per ml, 0.1 M sodium acetate [pH 5.0], 5 mM EDTA), and RNA was pelleted by centrifugation at 33,000 rpm for 15 h in an SW40 rotor. The RNA pellet was resuspended in 0.5 ml of TE buffer (40 mM Tris-acetate [pH 7.8], 5 mM sodium acetate, and 1 mM EDTA), and RNA was ethanol precipitated after the addition of sodium acetate to 0.2 M. The RNA concentration was calculated by measuring the absorbance of 260 nm.

Nucleic acid hybridization. Hybridization between $cDNA_{fps}$ and cellular RNA was carried out under conditions of moderate stringency (50°C in 30% [vol/vol] formamide, 0.45 M NaCl, 45 mM sodium citrate, 5 mM EDTA, 0.1% sodium dodecyl sulfate [SDS]), and the extent of hybridization was determined by S1 nuclease digestion (38). The number of copies of v-*fps* mRNA in transformed or phenotypically normal cells was calculated as described previously (39). We determined the amount of total RNA in 3Y1 fibroblasts to be 10 pg per cell.

DNase I treatment. Nuclei isolation and DNase I treatment were performed as described previously (29). Digestion was carried out at 37°C for 20 min, with final concentrations of DNase I ranging from 0 to 0.8 μg/ml. The nuclei suspension was adjusted to an optical density at 260 nm of 10 before digestion (or a nuclear concentration of DNA of approximately 0.5 mg/ml). After DNase I digestion, the samples were adjusted to 0.5 mg of pronase per ml and 0.5% SDS. DNA was extracted as described above.

Southern blot analysis. High-molecular-weight cellular DNA (10 to 15 μg) obtained from rat fibroblasts was digested with 20 to 30 U of several restriction endonucleases (New England Biolabs). DNA fragments were analyzed by separation in electrophoresis, blotting to nitrocellulose, and hybridization with various ^{32}P-labeled probes as described by Southern (26, 41).

Radioactive probes. Nick translation was performed as described by Maniatis et al. (26). The following probes were used. (i) FSV-representative probe (FSV_{rep}): total FSV DNA, purified from *Sac*I-digested λ-FSV-2 (40) was nick translated. (ii) *gag* probe: the *Eco*RI fragment of the λ-SRA clone (43) containing LTR and part of *gag* of Rous sarcoma virus was subcloned in pBR322 by F. Cross in this laboratory (clone pFC3). Purified *Eco*RI fragment DNA from this subclone was a gift from L.-H. Wang, also of this laboratory. It was digested with *Bam*HI, and the purified *Bam*HI-*Eco*RI fragment containing *gag*-specific sequences was nick translated. (iii) *Bam*HI-*Sac*II 3′ viral probe: FSV subclone pBR-F16 (40), containing LTR-*gag*, was digested with *Sac*II and then with *Bam*HI. The *Bam*HI-*Sac*II band was separated on a 5% polyacrylamide gel (acrylamide-bisacrylamide, 49:1) and used for nick translation. (iv) *Bam*HI-*Sac*I viral probe: pBR-F16 DNA was cut with *Bam*HI and then with *Sac*I. (v) *Sau*3A-*Hpa*II 5′ viral probe: pBR-F16 DNA was digested with *Bam*HI and then with *Hpa*II. The products were 5′-end labeled and separated on a 5% polyacrylamide gel. The *Bam*HI-*Hpa*II fragment (about 1 kilobase [kb] in length) was eluted and digested with *Sau*3A. The labeled 112-base pair *Sau*3A-*Hpa*II fragment was electroeluted and used for hybridization after heat denaturation (10 min at 95°C).

RESULTS

Isolation of revertant clones. 3Y1 rat fibroblasts were infected with the strain of FSV which codes for a 130-kilodalton protein (P130) (18, 27); 12 foci were isolated after 2 to 3 weeks, and transformed cells in each focus were cloned. In this process, among the several colonies derived from focus 5, two clones, 5G and 5H, showed intermediate levels of morphological transformation. These clones were treated with either colcemid or FUdR, a procedure which has been shown to improve the frequency of obtaining revertant cells (31). Treated cultures were subcloned, and 10 independent revertant clones were isolated from 5G and 5H, of which 4 were further characterized. As indicated in Fig. 1, all the revertants derived from focus 5 (including the six

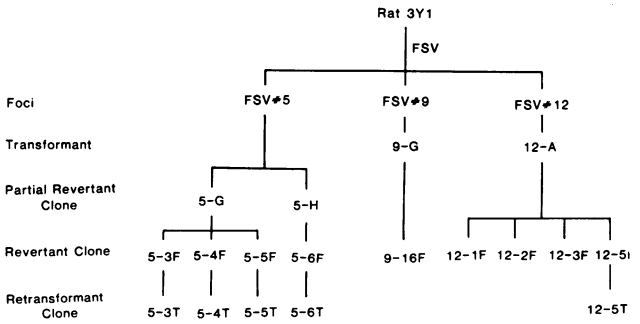

FIG. 1. Schematic representation of the derivation of various revertants and retransformants. Every step after the isolation of foci 5, 9, and 12 was preceded by cell cloning. From each single clone derived from single foci, subclones of morphologically flat revertant cells (designated F) were obtained. Retransformants derived from the revertants are designated T.

clones which were not extensively studied) yielded spontaneous retransformants with a frequency of about 10^{-6} to 10^{-7}.

Cells obtained from foci 9 and 12 did not give rise spontaneously to partial revertants. Transformants 9G and 12A were treated with hydroxyurea, a procedure known to select for quiescent cells in cultures maintained at high density (46). This selection was repeated twice, allowing time in between for surviving cells to reach confluence. Thereafter, cells were treated with FUdR and plated at low density to allow a first rapid screening of colonies. After screening an average of 5,000 to 10,000 cells from each clone, we isolated one colony from 9G and four from 12A which had the characteristic morphology of normal cells. Each colony was then subcloned, and representative flat subclones were selected for further experiments. Among these revertants, only one (clone 12-5F) retransformed, with a frequency similar to that observed in the focus 5 series; the other four revertants maintained their flat morphology even after 3 months of subculturing.

Figure 2 shows the typical morphology of revertants derived from focus 5 with their spontaneous retransformants. Revertants 5-5F and 5-6F displayed a morphology similar to that of uninfected 3Y1 cells, whereas the morphology of retransformants 5-4T, 5-5T, and 5-6T was radically different. Cells were round or spindle shaped, highly refractile, and characterized by a three- to fivefold increase in their saturation density.

A biochemical analysis of the revertants we isolated was performed at the DNA, RNA, and protein levels. Figure 3 shows the results of the DNA analysis of various transformants, revertants, and retransformants. High-molecular-weight DNA from each cell clone was digested with *Hin*dIII and run on an agarose gel. After transfer to nitrocellulose paper, the DNA fragments were hybridized to a probe representing the whole FSV provirus (FSV_{rep}). Since *Hin*dIII does not cut inside the FSV provirus (40), each hybridized fragment will be specific for a given integration site of FSV in the host chromosome. This analysis revealed that foci 5, 9, and 12 had only one copy of FSV integrated at a different site for each clone. Three revertant clones (9-16F, 12-1F, and 12-3F) had lost the entire FSV provirus. Since these clones represented a simple case of reversion, they were not studied further. However, each of them was susceptible to transformation after superinfection with FSV. All other revertants shared the same *Hin*dIII fragment characteristic of each parental clone. In addition, FSV sequences in the revertants showed no gross rearrangement as judged by digestion with *Bam*HI, an enzyme that cuts three times within the provirus (data not shown). The integration site of the spontaneous retransformants was identical to that of the revertants from which they had arisen.

Revertant clone 12-2F. Among the revertants that had kept a copy of the FSV provirus, clone 12-2F had the unique property of not giving rise to spontaneous retransformants. When a cell extract of 12-2F, labeled in vivo with [^{35}S]methionine, was immunoprecipitated with anti-FST (Fig. 4, lane

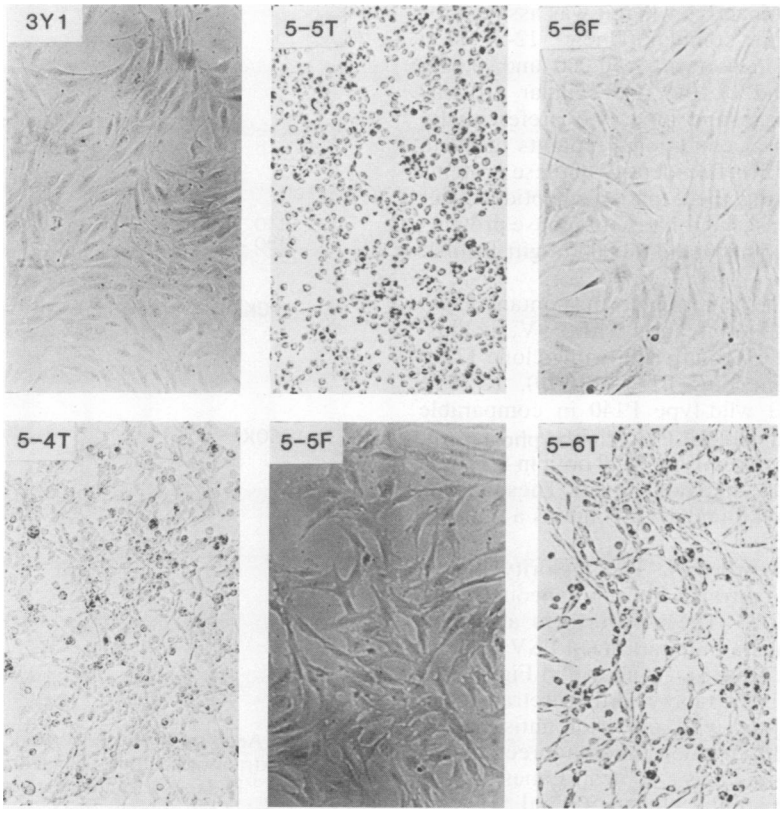

FIG. 2. Morphology of FSV-infected rat fibroblasts in revertant and retransformant cultures. 3Y1, Uninfected rat fibroblasts; 5-5F and 5-6F, revertant clones of 3Y1 cells originally transformed by FSV; 5-4T, 5-5T, and 5-6T, spontaneous retransformants of reverted clones 5-4F, 5-5F, and 5-6F.

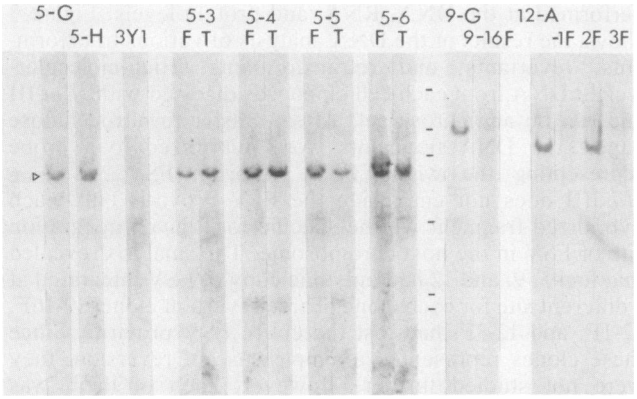

FIG. 3. FSV-specific DNA restriction fragments in FSV-infected clones. High-molecular-weight DNA from various clones was digested with the restriction enzyme *Hin*dIII. DNA fragments were separated on a 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized with an FSV_{rep} probe. The identity of each clone is indicated above each lane. The lineage of these clones can be seen in Fig. 1. Results obtained with 3Y1 cells and clones of the focus 5 series and those obtained with clones of the focus 9 and 12 series were derived from two different experiments. Markers obtained after digestion of λ phage with *Hin*dIII are indicated (23.3, 9.5, 6.4, 4.2, 2.2, and 1.8 kb).

a), a protein of 130,000 daltons (or slightly less) was detected. This protein (12-2F P130) was expressed at a level similar to that of wild-type P130 in an FSV-transformed 3Y1 culture (data not shown). 12-2F P130 was slightly underphosphorylated and lacked phosphotyrosine in vivo (lane c). Furthermore, it was enzymatically inactive when it was assayed for kinase activity in the immune complex (lane e). 12-2F P130 was found to be associated in vivo with a 50,000- and 90,000-dalton protein (lanes a and c), the two cellular proteins previously shown by Brugge and others to preferentially bind transforming proteins of conditional mutants of avian sarcoma viruses (1, 4, 5, 9, 24). The identity of these proteins was confirmed by comparing these phosphopeptides after partial digestion with protease V8 to those of mouse proteins with molecular weights of 50,000 and 90,000 (a gift from J. Brugge) (data not shown).

Although clone 12-2F failed to retransform spontaneously, it can be transformed by superinfection with FSV, as illustrated by clone 12-2ST1, derived after infecting clone 12-2F with the strain of FSV encoding P140 (18, 19). 12-2ST1 expresses 12-2F P130 and wild-type P140 in comparable amounts (Fig. 4, lane b), and 12-2F P130 can be phosphorylated in *trans* on tyrosine by wild-type P140 both in vivo and in vitro (Fig. 4, lanes d and f; data not shown). These results show that 12-2F is not a cell mutant but contains a mutated provirus.

Transcriptional block of provirus. The majority of the revertants had the ability to retransform spontaneously with a frequency of 10^{-6} to 10^{-7}. These revertants and their retransformants had retained an unrearranged FSV genome at the same integration site (Fig. 3). As shown in Fig. 5, ^{35}S-labeled cell extracts of the revertants and their retransformants were immunoprecipitated with antivirion antiserum to test for the presence of P130. In lane a, immunoprecipitation of uninfected 3Y1 cells is shown as a control. Lanes b and c illustrate the case of the parental clones 5G and 5H from which the revertants were derived; they show low or intermediate levels of P130. The revertant clones 5-5F and 5-6F (lanes d and f) expressed no or very little P130, whereas the retransformants 5-5T and 5-6T (lanes e and g) showed high levels of P130. Similar results were obtained when the level of P130 synthesis was measured by the in vitro kinase assay (kinase panel in Fig. 5).

The amount of v-*fps* mRNA in the revertants and in the retransformants was checked by liquid hybridization. RNA was extracted and hybridized to a labeled $cDNA_{fps}$ probe to various C_rt values. As shown in Fig. 6, no *fps* mRNA was detected in uninfected 3Y1 cells or in three revertants (5-3F, 5-4F, and 5-5F), even at the highest C_rt value. Revertant 5-6F, which showed a detectable amount of P130, expressed about three to five copies of viral RNA per cell. This contrasted sharply with the levels found for the retransformants, which ranged from 60 to 100 copies of RNA per cell.

Methylation of FSV proviral DNA. In many systems, an inverse correlation between DNA methylation and gene expression has been observed (35). DNA of actively transcribed genes was found to be hypomethylated, whereas untranscribed genes had their DNA hypermethylated. To check whether this correlation was also observed with these revertants and their retransformants, we digested total DNA isolated from these clones with the isoschizomers *Hpa*II and *Msp*I. These enzymes recognize the same sequence, CCGG, but when the internal C of this sequence is methylated, only *Msp*I is able to cut (48). Figure 7 shows the results obtained with two pairs of revertants and their retransformants, 5-5F, 5-5T and 5-6F, 5-6T. Digestion of genomic DNAs with *Msp*I generated four identifiable fragments after hybridization with an FSV_{rep} probe. Prediction of the *Msp*I-*Hpa*II sites in FSV from the nucleotide sequence (37) allowed us to assign these as 0.7- and 0.4-kb fragments derived from the *fps* sequence and the 5′ and 3′ junction fragments (Fig. 7). All

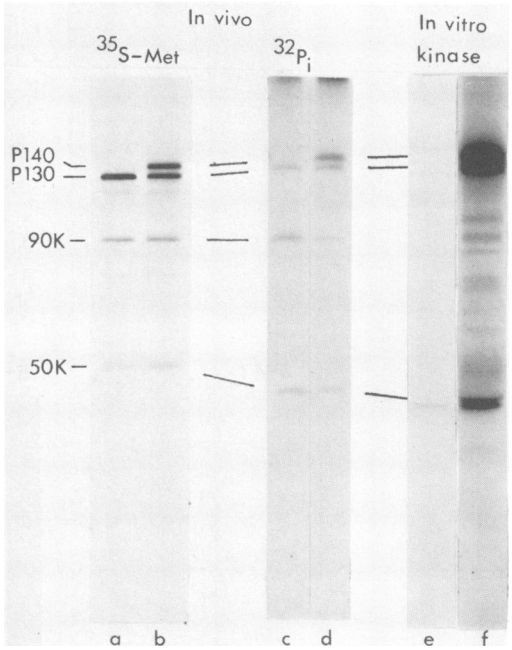

FIG. 4. Analysis of the FSV viral protein complex in clone 12-2F. Cell extracts of cultures labeled with [^{35}S]methionine (lanes a and b) and $^{32}P_i$ (lanes c and d) were immunoprecipitated with anti-FST; unlabeled cell extracts (lanes e and f) were assayed for protein kinase activity after immunoprecipitation with anti-FST. Proteins were separated by SDS-polyacrylamide gel electrophoresis (8.5%). Lanes a, c, and e, clone 12-2F; lanes b, d, and f, clone 12-2ST1.

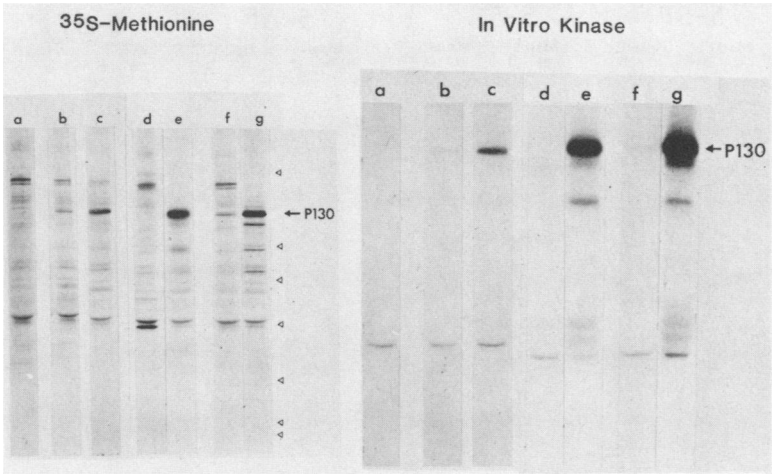

FIG. 5. P130 levels in clones derived from partial transformants 5G and 5H. P130 was detected after either immunoprecipitation of [^{35}S]methionine-labeled cell extracts with antivirion antiserum ([^{35}S]methionine panel) or in vitro autophosphorylation of P130 in the immune complex obtained after immunoprecipitation of unlabeled cell extracts with antivirion antiserum (in vitro kinase panel). Proteins were separated as described for Fig. 4. Lane a, 3Y1 cells; lane b, clone 5G; lane c, clone 5H; lane d, clone 5-5F; lane e, clone 5-5T; lane f, clone 5-6F; lane g, clone 5-6T.

other *Msp*I fragments are smaller and were not detected by Southern analysis. When *Hpa*II was used, only the DNAs from the retransformants gave the expected internal 0.7- and 0.4-kb fragments, results consistent with the hypomethylation of their proviral DNAs. DNAs of revertants yielded high-molecular-weight fragments which were not well resolved, indicating a higher degree of methylation of the FSV provirus and of the cellular flanking sequences. Revertant 5-6F, which expressed a small but detectable amount of viral RNA, was characterized by DNA fragments of intermediate size which were absent in clone 5-5F. This lesser degree of DNA methylation may correlate with the weak expression of viral RNA observed in this clone. It was of interest that the 5′ junction fragment, which was identified by its ability to hybridize to an *Sau*3A-*Hpa*II 5′ virus-specific probe (Fig. 7), was larger in *Hpa*II lanes than in *Msp*I lanes, both for flat and transformed cells, indicating that the first 5′ CCGG sequence in flanking DNA was methylated not only in revertants but also in retransformants. This suggests that only a small region limited to the proviral DNA is hypomethylated in transformed cells.

The frequency of spontaneous retransformation is rather low in these clones (10^{-6} to 10^{-7}). To determine whether we could increase this frequency, we treated the clones with 5-azacytidine, a cytosine analog which cannot be methylated at the 5 position. We observed a moderate increase (three- to fourfold) in the frequency of retransformation (Table 1), suggesting that retransformation was linked to demethylation of FSV proviral DNA.

Chromatin structure of FSV proviral DNA. Actively transcribed genes have been shown to be more susceptible to DNase I digestion and to possess DNase I-hypersensitive sites, whereas untranscribed genes are relatively resistant even to high concentrations of DNase I and do not contain hypersensitive sites (49, 50). To test whether the relationship between DNase I sensitivity and gene activity was also applicable to FSV provirus, we isolated nuclei from the revertant clone 5-3F and its retransformant 5-3T and treated them with increasing concentrations of DNase I. DNA was isolated and digested with *Eco*RI or *Hin*dIII, which do not cut the FSV provirus, and with *Bam*HI, which cuts three times in the provirus. The digested DNAs were subjected to a Southern analysis using an FSV_{rep} probe. The *Eco*RI panel in Fig. 8 shows that the FSV provirus of the revertant clone 5-3F is quite resistant to DNase I digestion. No specific subbands were evident, even at the highest DNase I concentration. In the retransformant 5-3T, the provirus was susceptible to DNase I digestion, and three subbands of increasing intensity with increasing DNase I concentrations were detected. Results with *Hin*dIII confirmed the presence of hypersensitive sites in the provirus of 5-3T, and in this case two subbands were observed. The fuzzy subband, denoted

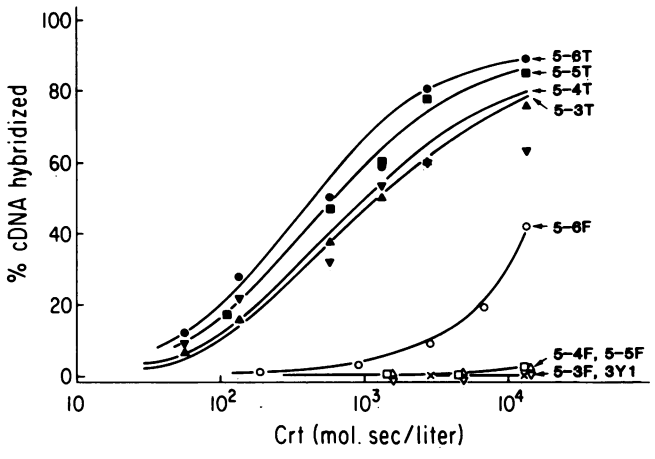

FIG. 6. Hybridization of $cDNA_{fps}$ to cellular RNA in revertants and retransformants. Total cellular RNA was extracted from uninfected cells and the indicated revertants and retransformants. Various amounts (0.32 to 100 μg) of cellular RNA were incubated with 500 cpm of ^{3}H-labeled $cDNA_{fps}$ in 8 to 16 μl of hybridization solution. The reaction was carried out for 100 h under conditions of moderate stringency (50°C; 30% formamide, 0.45 M NaCl, 45 mM sodium citrate, 5 mM EDTA, 0.1% SDS). The extent of hybridization was measured by S1 nuclease digestion.

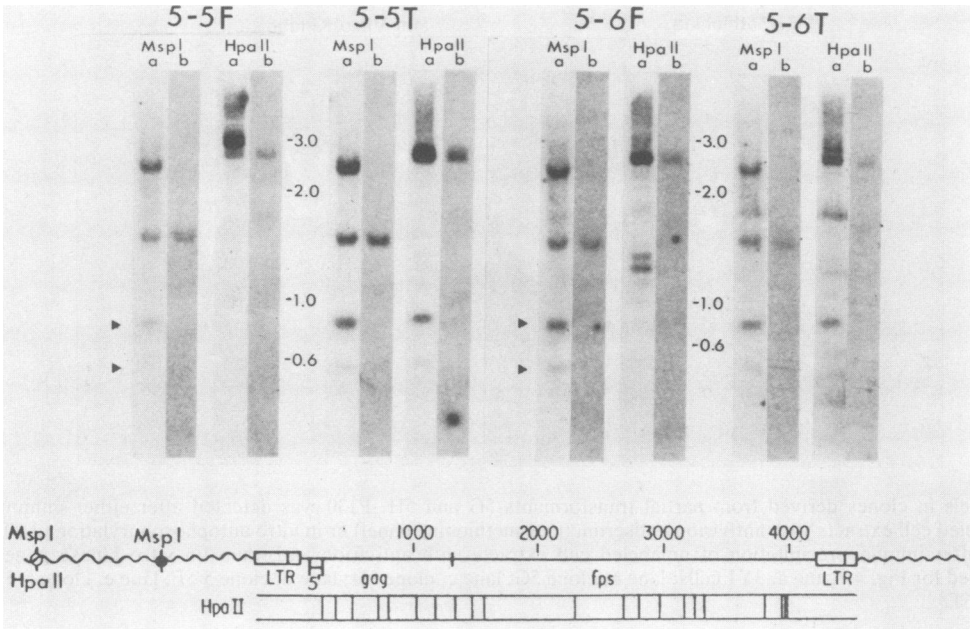

FIG. 7. DNA methylation of FSV proviral DNA in revertants and retransformants. High-molecular-weight DNA of clones 5-5F, 5-5T, 5-6F, and 5-6T were digested with *Msp*I or *Hpa*II (as indicated above each lane), separated on a 1.5% agarose gel, transferred to nitrocellulose paper, and hybridized to an FSV_{rep} probe (lanes a); the hybridized probe was removed by washing the filter at 65°C in low salt buffer, and the blot was rehybridized to the *Sau*3A-*Hpa*II 5′-specific probe (labeled 5′ in diagram) (lanes b). A schematic diagram of FSV provirus is given, with *Hpa*II (or *Msp*I) sites indicated by vertical bars. The *Msp*I and *Hpa*II sites indicated in the 5′ flanking region are unmethylated (○) or methylated (●) in transformed cells (cellular sequences shown by wavy lines are not drawn to scale).

by an open arrowhead (Fig. 8, *Hin*dIII panel), seemed to be present in both 5-3F and 5-3T proviral DNAs and may represent c-*fps*/*fes* (which can be detected in 3Y1 cells if we use an *fps*-specific probe). Digestion of total DNA with *Bam*HI did not reveal any specific subband after hybridization with FSV_{rep}, even in the case of clone 5-3T (Fig. 8).

To understand this last result, we mapped the DNase I-hypersensitive sites. First, the *Eco*RI, *Hin*dIII, and *Bam*HI sites were mapped in the cellular flanking regions of the FSV provirus. Total DNA was digested with *Eco*RI and *Bam*HI or *Hin*dIII and *Bam*HI, and the DNA fragments were hybridized to a 5′ or 3′ virus-specific probe (see above). Second, the blot shown in the *Eco*RI panel was rehybridized with a *gag*-specific probe, which recognized the *gag* region 3′ to the first *Bam*HI site in the provirus. In addition to the 11-kb *Eco*RI fragment, we detected the 4.6-kb subband. The 6.5- and 3.7-kb subbands did not hybridize to that probe (data not shown). Third, the blot obtained after *Bam*HI digestion was rehybridized to a 3′ virus-specific probe. In addition to the expected 4.7-kb junction fragment, we detected a faint subband at 2.6 kb (data not shown). This subband was only seen at higher concentrations of DNase I in 5-3T proviral DNA. By combining these results and the size of the different subbands observed after *Eco*RI and *Hin*dIII digestion, we mapped three DNase I-hypersensitive sites in the chromatin region containing the FSV provirus of retransformant 5-3T, as shown in the diagram in Fig. 8. One DNase I site coincides with a *Bam*HI in proviral DNA (within a few nucleotides). The 3′ DNase I site generates a *Bam*HI-DNase I subband which exactly comigrates with the internal

TABLE 1. Properties of 3Y1-FSV clones

Type of clone	No. of clones examined	Provirus copies per cell	FSV mRNA copies per cell[a]	P130 per cell	Protein kinase activity	DNA methylation	Retransformation by 5-azacytidine	Superinfection by FSV
Original transformants	3	1	60–100	+++	+++			
Partial revertants	3	1	10–30	+	+			
Flat revertants	3	0	0	−	−		No	Yes
	1	1	60–100	+++	−		No	Yes
	11	1	<3	−	−	High	Yes	Yes
Retransformants	11	1	60–100	+++	+++	Low		

[a] Copies of FSV RNA were calculated from the data presented in Fig. 6.

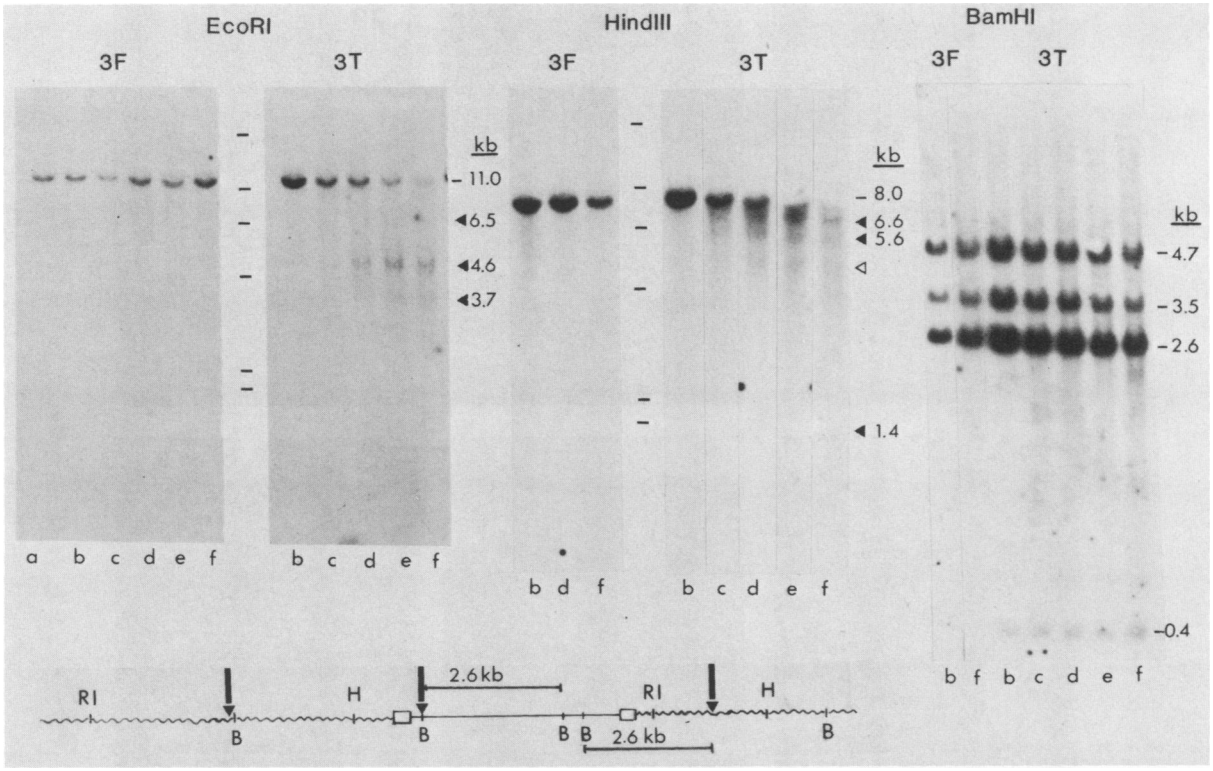

FIG. 8. DNase I hypersensitivity of proviral DNA in clones 5-3F and 5-3T. Nuclei from clones 5-3F and 5-3T were treated with increasing amounts of DNase I. DNA was extracted and cut with various enzymes. DNA digests were separated on a 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized to an FSV_{rep} probe. (a) Control untreated 5-3F DNA. 5-3F DNA was treated with DNase I, 0 μg/ml (b), 0.1 μg/ml (c), 0.2 μg/ml (d), 0.4 μg/ml (e), and 0.8 μg/ml (f). Solid arrowheads, DNase I-specific subbands, open arrowhead, DNase I nonspecific subband. Size markers are included in *Eco*RI and *Hin*dIII panels (λ DNA digested with *Hin*dIII, 23.3, 9.5, 6.4, 4.2, 2.2, and 1.8 kb). The schematic diagram shows the DNase I-hypersensitive sites in clone 5-3T indicated by arrows. FSV provirus is shown by a solid bar flanked by LTR (open boxes) and cellular flanking sequences (wavy lines). RI, *Eco*RI sites; B, *Bam*HI site; H, *Hin*dIII sites. The 2.6-kb comigrating fragments, the internal *Bam*HI FSV fragment, and the *Bam*HI-DNase I (3′ site) junction fragment are indicated.

*Bam*HI-*Bam*HI 2.6-kb fragment; this is one of the reasons for the absence of a new band in *Bam*HI fragments after DNase I digestion. In addition, the 5′ DNase I site lies just upstream of the *Bam*HI site present in the 5′ flanking sequence, and therefore this *Bam*HI junction fragment would not be shortened by DNase I digestion. In revertant clone 5-3F, we did not detect any DNase I-hypersensitive sites within a region covering 14 kb and containing the FSV provirus.

Intermediate transformants. In the process of screening revertants, we isolated three partial revertants (clones 5-G, 5-H, and 12-S), which showed intermediate morphological transformation and expressed intermediate levels of viral RNA and P130 protein (Table 2; data not shown). Clone 5-6F, although classified as a revertant, also showed subtle changes in the appearance of the cell surface compared with 3Y1 cells. (This difference, which is difficult to see in Fig. 2, may be correlated with differences in the level of FSV RNA shown in Fig. 6.) To confirm these observations, we decided to include a larger number of clones. To this end, 3Y1 cells were infected with FSV. Selected foci differing in their extent of morphological transformation were cloned and grown to mass culture, examples of which can be seen in

TABLE 2. Level of v-*fps* mRNA in FSV-infected rat cells showing various extents of phenotypic transformation[a]

Clone	Transformed phenotype	RNA content (copies/cell)
3Y1	−	0
5G	±	7
5H	+	19
21A	±	11
22G	++	28
22J	+	17
23I	++	15
23J	±	10
25A	+	51
26B	++++	125
26G	+++	80
28A	±	23
29B	++	46
29E	++++	80
30E	++	54
31B	+	20

[a] Total cellular RNA was extracted from uninfected 3Y1 cells or 3Y1 cell clones infected with FSV. The RNA content was determined by hybridization with $cDNA_{fps}$ as described in the legend to Fig. 6.

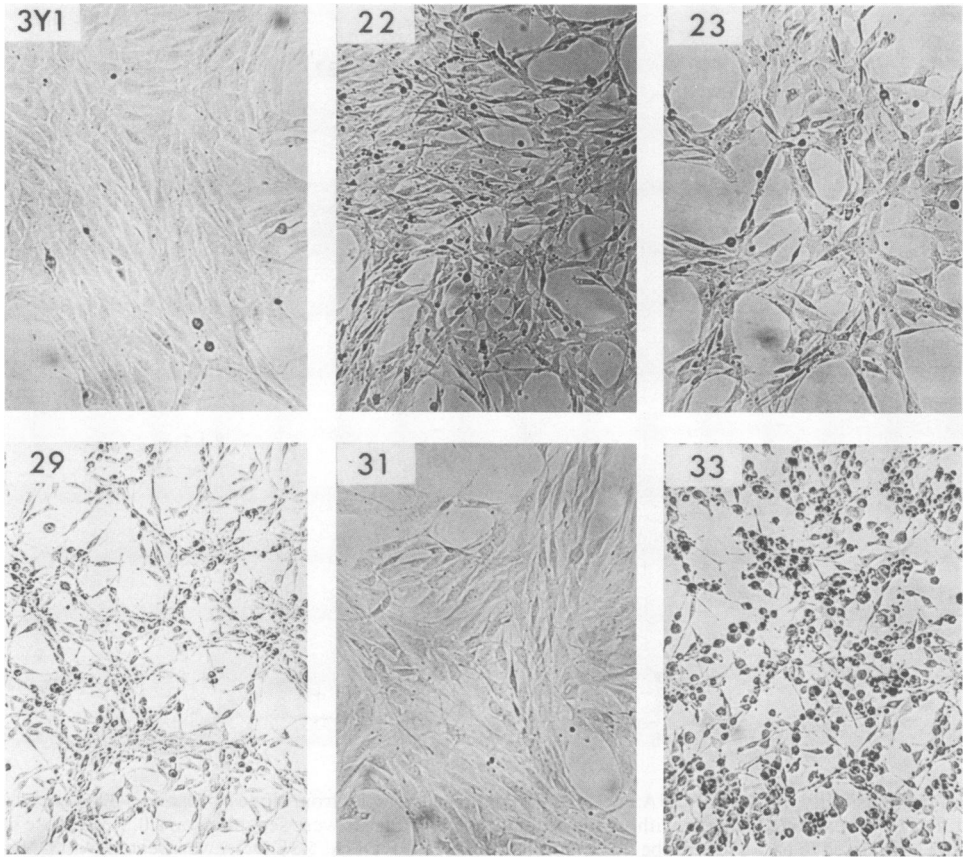

FIG. 9. Variations in morphology of FSV-infected clones. 3Y1 cells were infected with FSV. Foci showing variable extent of phenotypic transformation were selected, and cells derived from each independent focus were cloned. The photomicrographs show uninfected rat fibroblasts (3Y1) and individual FSV-infected clones derived from foci 22, 23, 29, 31, and 33.

Fig. 9. These cultures displayed different degrees of morphological transformation, ranging from an almost normal appearance to a fully transformed one. Although somewhat subjective, cell shape, refractiveness, and cell density at confluence served as the basis to assess the extent of transformation in the various clones. The amount of viral RNA expressed in these cultures was then determined by liquid hybridization. As shown in Table 2, the levels of v-*fps* mRNA varied from 10 to 125 copies per cell. Although some scatter in the data was observed in a few cases (e.g., clone 25A), the amount of viral RNA correlated well with the degree of morphological transformation. The discrepancies in some clones may be explained by the fact that the phenotype of some clones was not indefinitely stable and tended to evolve toward a more transformed state. Both RNA quantitation and morphology assignment represent an average over 10^8 to 10^9 cells derived from a single cell.

DISCUSSION

Several mechanisms have been proposed to explain how cells originally transformed by retroviruses reverted to phenotypically normal cells (6, 10, 22, 33, 47). Most of these mechanisms were also involved in phenotypic reversion of rat fibroblasts transformed by FSV. Table 1 summarizes the types of revertant cell we analyzed. Fifteen independent clones were isolated from three original foci. Three were characterized by the loss of the FSV provirus; one resulted from a mutated provirus which encoded an enzymatically inactive P130 (12-2F). The majority (11 clones) failed to express FSV mRNA, even though a functional proviral DNA was present, as evidenced by their retransformation. Of these 11 clones, 10 were derived from focus 5, whereas only one (at much lower frequency) was obtained from focus 12, and none from 9. Although the data are limited, we believe that the incidence of getting transcriptionally inactive proviruses in 3Y1 cells may be influenced by the integration site.

The possibility that any of the revertants we isolated had arisen from a cellular mutation was ruled out, since all of them could be retransformed after superinfection with FSV (Table 1). This is not surprising if cellular revertants result from a mutation affecting simultaneously a pair of cellular alleles involved at some stage of the oncogenic process, making it an extremely rare event.

Excision of provirus. Although it is conceptually the simplest case of reversion, very little is known mechanistically about how the loss of the provirus occurs. Speculations about structural similarities between proviruses and transposable elements (for a review, see reference 44) allow a mechanism in which precise excision can be achieved by homologous recombination between the terminal repeats of the LTRs. However, it has been reported that partial deletion of the retroviral genome can occur after integration (11, 47), indicating that the loss of viral sequences need not result from recombination between LTRs. Only deletions of the

complete provirus were observed in this study; whether some cellular flanking sequences, or even the whole chromosome, have been lost remains to be determined.

Provirus mutation. Clone 12-2F expressed a transformation-defective protein which failed to autophosphorylate in vivo and in vitro. This was not the consequence of a conditional temperature-sensitive lesion, since incubation of 12-2F cells at 32°C for several days had no effect on their morphology or on the properties of 12-2F P130. Furthermore, the failure of 12-2F P130 to autophosphorylate in vitro was not due to an impaired phosphate-accepting capacity, since it was phosphorylated in vitro by wild-type P140. Although the exact nature of the defect of P130 in clone 12-2F is still unclear, we believe that FSV 12-2F provirus has suffered some small deletion, since even after 3 months of constant passings, no spontaneous retransformant has been observed, making a point mutation, as the defect in 12-2F provirus, very unlikely.

Alteration in transcription. An inverse correlation between gene activity and methylation has been described in several systems (see references 35 and 50 for a review), including retroviruses (7, 8, 12, 14–16, 21, 42). This correlation, however, has not been absolute (30). In some cases the lack of transcription of a gene correlated better with the presence of certain methylated domains, especially in the 5′ region of that gene, than with the overall extent of methylation (25, 28, 36). Surprisingly, we found that the retransformants expressed a high level of v-*fps* mRNA, even though they had retained a 5′ junction fragment hypermethylated (Fig. 7). In this case, hypomethylation seemed to be restricted only to the proviral DNA. However, in clones derived from focus 5, the first CCGG site is about 1 kb upstream of the 5′ LTR; therefore, we cannot rule out the possibility that the key region for regulation of transcription by methylation lies between this site and the LTR. Alternatively, since we only tested methylation sites recognized by *Hpa*II-*Msp*I, our analysis may have overlooked some important regulatory sites in the 5′ flanking region of the provirus. Finally, methylation of DNA affects only localized regions of the host genome, since superinfection with P140-encoding FSV caused retransformation of these cells with the same efficiency as with uninfected 3Y1 cells. Retransformation occurred without the concomitant reexpression of P130, the product of the resident provirus before superinfection.

As in cellular genes, the expression of retroviral genomes can be correlated with the conformation of these sequences in chromatin (7, 8, 16, 42). In retransformants derived from revertants of cells originally transformed with avian sarcoma virus B77, Chiswell et al. described the presence of two new hypersensitive sites in the B77 provirus (7). In particular, one site was mapped in the LTR, confirming an observation made by Groudine et al., who located the hypersensitive sites of the transcriptionally active ev-3 avian endogenous retrovirus in the LTRs (16). We detected three sites located within and around proviral DNA in the retransformant but none in the LTRs of FSV. Two sites lay in the flanking sequences, whereas one was located in the *gag* region (around nucleotide 535 [37]) of the provirus. We did not detect any hypersensitive sites in the revertant, and since the retransformant was derived from the former, hypersensitive sites were generated de novo. It would be interesting to determine whether the same hypersensitive sites were present in the original focus 5.

Dosage of v-*fps* mRNA. The different types of revertants are not unique to FSV. Similar mechanisms have been implicated in the generation of revertants isolated from mammalian cells infected with feline sarcoma virus (2, 11, 15, 33), Rous sarcoma virus, B77, and avian erythroblastosis virus (3, 6, 10, 32, 34, 47). In our case, the relative ease of isolating partial transformants allowed us to further define the requirements for FSV transformation. We observed that a moderate expression of viral *fps* mRNA correlated with low to intermediate states of morphological transformation. This argues for a model in which FSV transformation is a function of the dosage of v-*fps* mRNA (33) and that it is not an all or none phenomenon.

Expression of retroviral genes is under the influence of the strong promoter activity of the LTR. However, as we have observed in revertants and partial transformants, this expression can be significantly altered by cellular signals. Exactly how these signals influence or relate to transcriptional activity is still a matter of speculation. However, the partial transformants should prove well suited to the analysis of the nature of these cellular signals; in particular, the state of methylation and chromatin structure of the FSV provirus can be studied in these cells and related to the level of v-*fps* mRNA.

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