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SV40 enhancer activation during retinoic acid-induced differentiation of F9 embryonal carcinoma cells

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The transient expression vector pSV_2CAT, which carries the bacterial chloramphenicol acetyl transferase (CAT) gene under the control of the SV40 early promoter, was used to transfect the murine embryonal carcinoma cell line F9 at various times during the retinoic acid-induced differentiation of these cells. Expression of the CAT gene under SV40 promoter control was found to increase markedly on F9 cell differentiation, measured relative to expression from the thymidine kinase promoter in the same cells. A series of constructs was prepared to identify the features of the SV40 early promoter required for transcription in differentiated and undifferentiated cells, as well as the factors limiting transcription in each case. The increased transcription seen on F9 cell differentiation was not observed when cells were transfected with molecules lacking a functional enhancer. It appears that as embryonal carcinoma cells differentiate, increased SV40 transcription results from enhancer sequence activation. In both differentiated and undifferentiated cell types the level of transcription was found to be limited by the availability and/or activity of cellular factors necessary for enhancer function.

Key words: CAT expression/embryonal carcinoma cells/enhancer sequence/SV40 promoter

Introduction

The F9 line of mouse embryonal carcinoma (EC) cells differentiates in monolayer culture in the presence of retinoic acid (RA) to produce cells with the characteristics of extra-embryonic parietal endoderm (Strickland and Mahdavi, 1978). The changes that take place during this differentiation closely mimic events of early mouse embryogenesis (Hogan *et al.*, 1983). The F9 cell system thus appears to provide a useful model for the study of regulatory events during development, and particularly for an investigation of gene activation/inactivation mechanisms.

Studies on the replication and transcription of viruses introduced into early embryos and their EC cell counterparts have provided a convenient way of investigating such regulatory mechanisms. While many viruses are infectious in EC stem cells, a small group shows reduced infectivity and gene expression compared with that seen in differentiated cell types. This group includes SV40, polyoma virus, Moloney murine leukemia virus, murine sarcoma virus and murine cytomegalovirus (Kelly and Boccara, 1976; Teich *et al.*, 1977; Swartzenruber *et al.*, 1977; Oldstone *et al.*, 1980; Fujimura *et al.*, 1981; Gautsch and Wilson, 1983; Huebner *et al.*, 1983; Niwa *et al.*, 1983).

Studies with polyoma virus have suggested that viral gene expression in EC stem cells is blocked at the level of transcription initiation (Dandolo *et al.*, 1983) and that this block can be relieved by mutations that affect the structures of early gene enhancer sequences (Vasseur *et al.*, 1983; Tanaka *et al.*, 1982; Sekikawa and Levine, 1981; Fujimura *et al.*, 1981). A defect in enhancer function also appears to explain the lack of Moloney murine leukemia virus transcription in undifferentiated EC cells (Linney *et al.*, 1984). For SV40, early gene expression may be blocked at transcriptional or at RNA processing steps in EC stem cells (Segal and Khoury, 1979; Huebner *et al.*, 1983; Nicolas and Berg, 1983). All but one of the SV40 studies involved expression in EC cells of the complete gene for the viral large T antigen, a protein which can itself considerably influence the properties of cells in which it is made and which also regulates its own transcription. The only study which has examined the activity of the SV40 early promoter attached to a marker gene demonstrated a clear difference in transcription of the marker gene in differentiated and undifferentiated cells (Nicolas and Berg, 1983).

We have extended this investigation of the SV40 early promoter to determine the mechanism which allows increased expression following F9 cell differentiation in the presence of RA. Using a transient expression system to assess SV40 early gene promoter activity (Gorman *et al.*, 1982), we have found a substantial increase in expression during cell differentiation in the presence of RA. This increase is dependent on the presence of a functional enhancer sequence and appears to be due to increased enhancer activity producing a rise in transcription level.

Results

Increased expression from the SV40 early promoter in differentiated F9 cells

In cells transfected with the plasmid pSV_2CAT, transcription across the chloramphenicol acetyltransferase (CAT) gene is driven by the SV40 early promoter/enhancer region (Gorman *et al.*, 1982). The level of CAT enzyme activity in cell extracts provides a measure of the level of transcription and hence of promoter activity. When undifferentiated F9 cells were transfected with pSV_2CAT DNA, a very low level of CAT activity was obtained (Figure 1). By contrast, the CAT activity in F9 cells transfected after RA treatment was much greater.

Is restricted expression from pSV_2CAT due to limited DNA uptake in F9 stem cells?

A difficulty in using transient expression systems to compare gene promoter activity in different cell types is the possibility that the cell types do not take up DNA equally during transfection. For this reason, a common approach has been to co-transfect cells with the construct of interest, and with a vector expressing a second marker gene from a promoter which appears to show relatively constant expression from one cell type to another. Expression from the promoter of interest can then be assessed relative to that of the control vector in each cell type (see, for example, Herbomel *et al.*, 1984).

From a vector in which β-galactosidase is expressed under the control of the SV40 early gene promoter (pSVgal), we prepared ptkgal, in which the SV40 promoter is replaced by the promoter

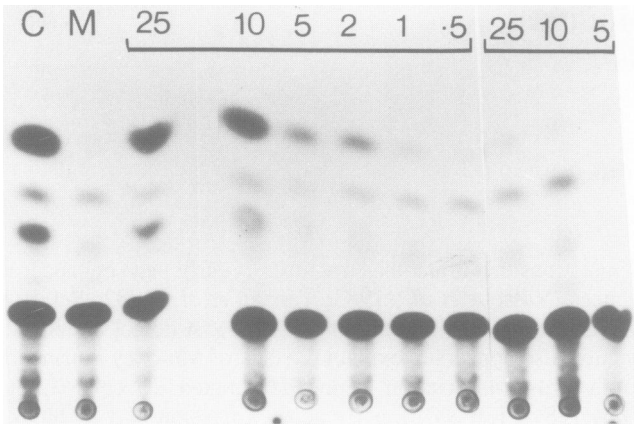

Fig. 1. CAT expression from the SV40 early gene promoter in F9 stem and differentiated cells transfected with pSV_2CAT. Acetylated reaction products (upper spots) were separated from unreacted [^{14}C]chloramphenicol (lower spot) by t.l.c. to determine CAT enzyme levels in extracts of transfected cells. C = conversion from 0.01 units of CAT enzyme (P-L Biochemicals); M = mock-transfected F9 cells. The next group shows CAT activity in F9 cells treated with RA for 6 days (at the time of harvest) transfected with various amounts (25 – 0.5 μg) of pSV_2CAT DNA. The last three samples are from F9 cells transfected with 25 – 5 μg pSV_2CAT.

region from the herpes simplex virus thymidine kinase gene (see Materials and methods and Figure 8). Neither pSVgal nor ptkgal expresses high levels of β-galactosidase in transfected cells, but satisfactory measurements of β-galactosidase activity were obtained with long (generally overnight) incubations of reaction mixtures, carried out as described by Miller (1972).

In an initial experiment, β-galactosidase activity was measured in cells transfected with 5 μg of either pSVgal or ptkgal. Results for mock-transfected cells were also determined, and deducted from values obtained.

A comparison of ptkgal expression in L^-a^- cells, F9 stem cells or differentiated F9 cells suggested that the different cell types differed in their relative abilities to take up and express DNA during transfection. In this experiment, relative expression of β-galactosidase in the three cell types was 4:1:2.

In L^-a^- cells, an 8:1 ratio of expression from pSVgal and ptkgal was observed. In F9 stem cells the ratio was 2.5:1, but in differentiated cells the ratio rose to 33:1. These results confirm a real increase in SV40 early gene promoter activity in differentiating F9 cells, independent of any change in receptivity to transfecting DNA.

Figure 2 shows the results obtained when different amounts of pSV_2CAT were co-transfected with 5 μg ptkgal into differentiated and undifferentiated F9 cells and L^-a^- cells. The upper panel shows the uncorrected results for CAT activity in cell extracts, and the lower panel shows results adjusted for the relative levels of β-galactosidase activity in different cell extracts. Under the transfection conditions described, variation in galactosidase activity from one extract to another of the same cell type was quite small. Thus co-transfection of cells with ptkgal was principally useful in normalising for DNA uptake between cell types.

The results confirm that the SV40 early gene promoter functions much more effectively in F9 cells after differentiation than before. Maximal CAT expression was obtained when 10 – 20 μg pSV_2CAT DNA was used for transfection.

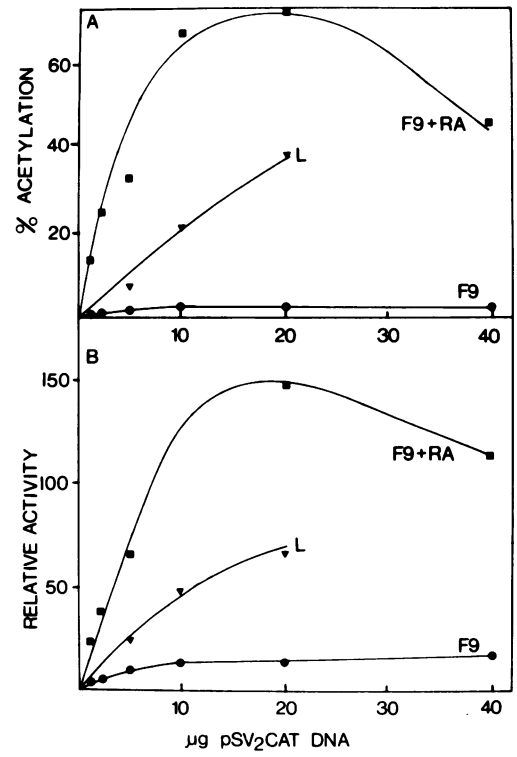

Fig. 2. CAT expression for a range of input pSV_2CAT levels. Results show CAT expression directed by the SV40 early gene promoter in L^-a^- cells, F9 stem cells and F9 cells treated with RA for 4 days at the time of transfection. Each plate of cells was transfected with 5 μg ptkgal and varying amounts of pSV_2CAT DNA. Results show percent conversion of [^{14}C]chloramphenicol to acetylated forms by one quarter of the sonicate (50 μl) from each 9 cm dish of cells. **Panel A:** Absolute CAT values obtained. **Panel B:** CAT expression relative to β-galactosidase expression in the same cell extracts. Each CAT assay result was divided by the absorbance at 440 nm obtained when 50 μl of the corresponding cell extract was assayed for β-galactosidase activity as described by Miller (1972). Galactosidase results were first corrected for endogenous levels of enzyme activity measured by assay of extracts from mock-transfected cells.

The timing of increased CAT expression during F9 cell differentiation

Our studies of the F9 cell system as a model developmental process have indicated that new protein and mRNA species appear not at a single time but at various stages following RA addition to the undifferentiated cells (Lockett and Sleigh, unpublished results). We asked at what stage increased CAT expression from the SV40 early promoter appeared, by carrying out transfections on cells at various stages in the differentiation process. Cell cultures were set up on successive days to provide RA treatments of from 0 to 7 days. Two days prior to completion of RA treatment, cells were transfected with 10 μg of pSV_2CAT. The results (Figure 3) suggest that increased SV40 promoter activity does not occur in these cells for at least 3 days after the differentiation process is initiated.

Relationship of SV40 promoter activation to appearance of particular cell types during differentiation

Hogan *et al.* (1983) reported that F9 cell differentiation induced by RA proceeds first through a state resembling the primitive endoderm of the embryo. Full development of the parietal endoderm cell type occurs only in the presence of added cyclic AMP, to which the cells become receptive following RA treatment. The

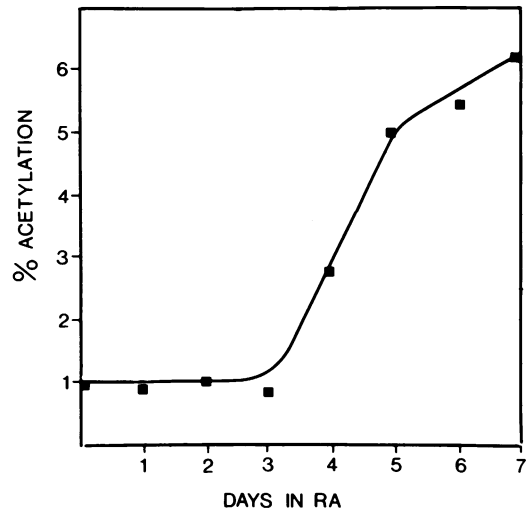

Fig. 3. Time course of pSV_2CAT expression during F9 cell differentiation. Plates of cells were set up on successive days with RA (5×10^{-7} M). All plates were transfected on the same day with 10 μg pSV_2CAT, 42 h prior to harvest. The results show CAT activity obtained with 20 μl of sonicate from cells exposed to RA for the period indicated at the time of cell harvest.

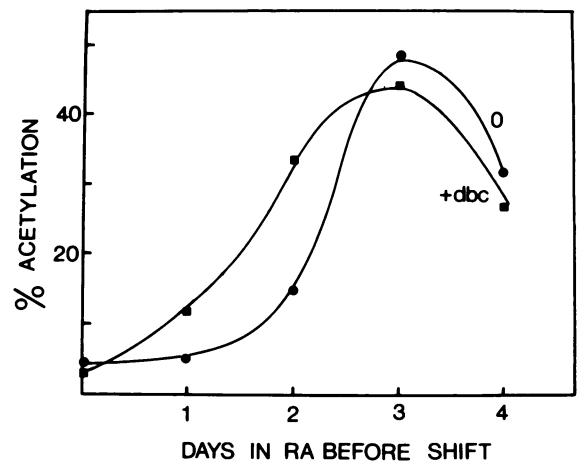

Fig. 4. Effect of dibutyryl cAMP on SV40 promoter utilisation during F9 cell differentiation. All F9 cell samples were set up in RA 4 days before transfection with 10 μg pSV_2CAT. Cells were harvested 42 h after transfection for assessment of pSV_2CAT expression. After 0, 1, 2, 3 or 4 days in RA, cells were shifted from RA-containing medium into medium containing either no additives (●) or 1 mM dibutyryl cAMP (■).

effect of added cyclic AMP on SV40 early gene promoter activation was examined and results are shown in Figure 4.

Cells were treated with RA for different times, and then shifted to medium with no RA and with or without added dibutyryl cAMP. All cells were transfected with pSV_2CAT 4 days after RA treatment was initiated, and harvested for CAT assay 2 days later. In cells given only a short (1 – 2 day) treatment with RA, higher CAT expression was seen subsequently in cells incubated with dibutyryl cAMP than in cells grown in medium with no additives. However, maximal CAT expression was seen in cells treated with RA for 3 days, whether they were incubated subsequently in the presence or absence of dibutyryl cAMP (Figure 4). Thus it appears that cAMP treatment is not an absolute requirement for the progression of F9 cells to a type which is permissive for SV40 expression.

Transfection experiments were also carried out with embryo-derived parietal and visceral endoderm, tissues thought to arise from the inner cell mass via a primitive endoderm intermediate during embryo development (Hogan *et al.*, 1983). The tissues were dissected from 12.5 day mouse embryos and then dissociated to form cell monolayers. Both cell types showed high levels of transient CAT expression after transfection with pSV_2CAT (Hogan and Sleigh, unpublished results). Thus SV40 promoter activation appears to occur during formation of both visceral and parietal endoderm cells during embryogenesis. It is possible that activation occurs at the stage of primitive endoderm formation, prior to the final commitment of cells to either differentiation pathway.

The results in Figure 4, and others not shown, demonstrated that levels of CAT expression declined if RA treatment of F9 cells was continued during the period of transfection. This appeared to be associated with reduced growth rate or death in the cell population. Thus in all transfection experiments other than that shown in Figure 3, RA treatment was discontinued when the cell medium was changed immediately prior to transfection.

What features of the SV40 early gene promoter affect CAT expression in F9 cells?

The SV40 early gene promoter has been extensively studied at both the structural and functional level. In addition to a TATA-like box which is required for correct transcription initiation, six GC-rich repeats are found 50 – 115 bp before the start site for transcription. These determine the levels at which transcription occurs (Benoist and Chambon, 1981; Fromm and Berg, 1982; Hansen and Sharp, 1983). Further upstream are perfect 72-bp repeats with enhancer function (Gruss *et al.*, 1981). To assess the relative importance of these features in determining the level of CAT expression in F9 and RA-treated F9 cells, we prepared pSV_2CAT derivatives from which one or both enhancer sequences had been deleted. These plasmids, called pSVEndel and pSVEnless, respectively, were identical to pSV(Sph)CAT and pSV_1CAT described by Gorman *et al.* (1982). Results of transfection experiments using these two plasmids are summarised in Figure 5. Removal of a single enhancer sequence from the promoter had little effect on CAT expression at high levels of input DNA, when CAT expression was approaching its maximal level. However, at levels of DNA of 10 μg and less, CAT expression from pSVEndel was significantly less than that seen using pSV_2CAT in both cell types.

Removal of both enhancer sequences from the promoter region (pSVEnless) reduced CAT expression to a very low level which was similar for both differentiated and undifferentiated cells (note the difference in scale on the ordinate for the two panels of Figure 5). The presence of enhancers produced only a 3-fold increase in CAT expression in F9 cells, but a 20- to 30-fold increase in RA-treated cells. This suggests that the increased CAT expression from pSV_2CAT during F9 cell differentiation is mediated through the enhancer sequences.

What factors limit CAT expression in F9 and RA-treated F9 cells?

Experiments with type 2 eukaryotic promoters have established that, in addition to RNA polymerase II, cellular components including a TATA sequence-binding protein and, in the case of the SV40 early gene promoter, 21-bp binding protein, are required for transcription to occur (Dynan and Tjian, 1983). In addition, cell type-specific proteins required for enhancer activity have been proposed (Khoury and Gruss, 1983; Scholer and Gruss, 1984). To determine whether any of these components was limiting the rate of transcription from the SV40 early promoter in F9 and RA-treated F9 cells, we carried out competition experiments with appropriate parts of the promoter sequence.

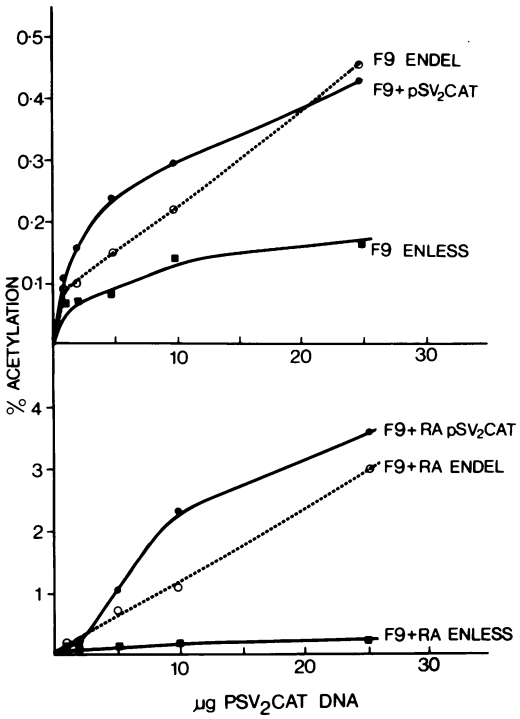

Fig. 5. CAT activity in F9 and RA-treated cells transfected with DNA containing 0, 1 or 2 enhancer sequences. The lower panel shows F9 cells treated with RA for 4 days and then transfected with different levels of pSV_2CAT (two enhancers – ●), pSVEndel (one enhancer – ○) or pSVEnless (no enhancers – ■). The upper panel shows, on a different scale, parallel experiments with F9 cells.

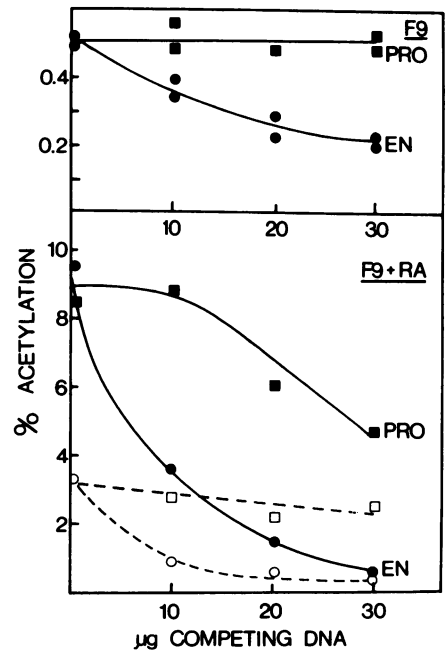

Fig. 6. Effect on SV40 early promoter usage of competing enhancer and promoter sequences. F9 cells untreated or differentiated by treatment with RA for 4 days were transfected with 15 μg (closed symbols) or 5 μg (open symbols) pSV_2CAT DNA, together with 30 μg pBR322 DNA. CAT activity in these cells, harvested 2 days later, gave the values for zero competing DNA. For other cell samples, the pBR322 DNA was progressively replaced by pSVPRO or pSVEN DNA. All results are from duplicate plates of cells, transfected and assayed separately. However, only the average values are shown in the lower panel for the sake of clarity.

The plasmids used in competition experiments are described in Figure 7 and in Materials and methods. pSVEN contains one complete copy of the early gene enhancer sequence cloned into pBR322. pSVPRO contains the rest of the promoter, including an incomplete enhancer sequence, the complete 21-bp repeat region, the TATA box and the start site for transcription.

Figure 6 summarises the results of competition experiments using these two constructs. Differentiated or undifferentiated F9 cells were co-transfected with 15 μg pSV_2CAT (a level giving close to maximal CAT expression for both cell types) and 30 μg pBR322 DNA. The plasmid DNA was progressively replaced by equal amounts of pSVPRO or pSVEN, and the level of CAT enzyme was measured in transfected cell extracts. The results show that pSVEN was effective in reducing the level of transcription from pSV_2CAT, in both differentiated and undifferentiated cells. Reduced CAT expression in the presence of excess pSVPRO was seen only in differentiated F9 cells, where pSV_2-CAT transcription was proceeding much more extensively than in undifferentiated cells.

Similarly, when differentiated F9 cells were transfected with amounts of pSV_2CAT (5 μg) below those producing maximal CAT expression (Figure 2), little competition by pSVPRO was observed. However pSVEN was still an effective competitor molecule in these cells. The results suggest that, except at very high rates of transcription, expression from the SV40 early gene promoter is largely limited by the availability of enhancer-binding factors, or of other factors required for the complete process of enhancer action.

Discussion

Restricted expression of a range of viruses has been described in both early mouse embryo cells and undifferentiated EC cells. For polyoma virus and Moloney leukemia virus, the low level of transcription observed has been attributed to relative inactivity of the viral enhancer sequences (Herbomel *et al.*, 1984; Linney and Donerly, 1983; Linney *et al.*, 1984). The results reported here suggest that marker gene expression driven by the SV40 early gene promoter in F9 EC cells is low largely for the same reason.

Interestingly, the apparent difference in expression of pSV_2-CAT between differentiated and undifferentiated F9 cells was reduced when results were expressed relative to the activity of a co-transfected marker gene (β-galactosidase transcribed from the herpes virus thymidine kinase promoter). This suggests that the stem cells have a lower inherent capacity to take up and express transfecting DNA. This was confirmed in independent experiments assessing the amount of transfecting DNA retained within cells at the time of harvest for transient expression measurements. In cells washed with EGTA to reduce residual surface-attached DNA, the relative amounts of plasmid DNA found by hybridisation in L^-a^- cells, F9 stem cells and differentiated F9 cells roughly paralleled the relative expression from ptkgal reported here (Sleigh, unpublished results). The reduced DNA uptake capacity of F9 stem cells may explain the low frequencies with which transformants are obtained when transfected EC stem cells are selected for marker gene expression (Pellicer *et al.*, 1980; Wagner and Mintz, 1982; Bucchini *et al.*, 1983; Nicolas and Berg, 1983).

The results in Figure 5 show that in F9 stem cells the enhancer sequences of the SV40 early gene promoter make only a small although positive contribution to the level of transcription from introduced pSV_2CAT. These findings are in agreement with those

of Nicolas and Berg (1983) and Herbomel *et al.* (1983). However, in differentiated F9 cells, it is evident that the enhancers are essential for the high level of expression observed. Thus, as has been described for polyoma and Moloney leukemia viruses, it appears that viral enhancer activation makes a major contribution to increased expression from the SV40 early gene promoter as EC cells differentiate.

As shown in Figure 3, increased pSV_2CAT expression was first seen in cells transfected 2 days after F9 cell differentiation was initiated. This was about the time at which cell morphology changed and other markers of the differentiated cell type (e.g., laminin) began to appear. Georges *et al.* (1982) found that expression of SV40 and polyoma virus antigens was initiated at the same time, and accompanied the first appearance of plasminogen activator in differentiating EC cells. It seems likely that there is a common mechanism involved in the activation of different viral enhancer sequences. The same mechanism may also be involved in mediating switches in the activity of endogenous genes during EC cell differentiation.

The ability of enhancer sequences to stimulate transcription from nearby promoters requires the presence of cell type-specific enhancer binding factors (Scholer and Gruss, 1984). Sassone-Corsi *et al.* (1985) demonstrated such a class of factors in HeLa cell extracts. These bound to both the 3′ and 5′ domains of the SV40 enhancer element and also interacted to varying degrees with other viral enhancer elements.

At the same time, negative regulation of enhancer activity has been reported (Borrelli *et al.*, 1984; Velcich and Ziff, 1985). A product of the adenovirus Ela region was found to repress transcription from the SV40 early gene promoter, apparently via interaction with the enhancer sequences. Imperiale *et al.* (1984) have described the presence of an Ela-like protein in undifferentiated but not differentiated EC cells. Thus either positive regulatory molecules alone, or positive and negative regulators acting together, may control the activity of viral enhancer sequences in EC cells.

The competition experiments shown in Figure 6 indicate that in differentiated F9 cells, which are efficiently transcribing from the SV40 early gene promoter, a cloned SV40 enhancer sequence very effectively competes with pSV_2CAT for positive regulatory factors, producing a sharp drop in the amount of CAT expression observed. The cloned enhancer sequence was a much more effective competitor than pSVPRO (Figures 6 and 7) which should sequester other transcription factors. Thus in differentiated cells the extent of transcription appears to be limited primarily by the availability of enhancer-activating factors. However, other transcriptional factors seem to be approaching full utilisation, also explaining the 'self-competition' by high levels of pSV_2CAT seen in Figure 2.

The highest levels of competing pSVEN DNA (30 μg), used in these experiments in conjunction with 15 μg pSV_2CAT, should provide 3.4 complete and an additional 3.4 partial competing enhancer sequences for every pair of CAT-associated sequences, while 30 μg pSVPRO provides 2.5 competing promoter regions for every CAT-associated promoter. The reduction in CAT expression by competing pSVEN seems greater than would be expected from distribution of a single activating factor equally over available binding sequences. This might be explained by cooperative binding of one or more factors to achieve enhancer activity.

Results with undifferentiated cells are somewhat less reliable than those for differentiated cells, because of the low levels of expression obtained. However the experiment shown in Figure 6, and others of the same type, revealed no competitive effect of cloned promoter sequences, presumably because transcription factors are in excess at such a low level of expression. The cloned enhancer sequence was much less effective as a competitor than in differentiated cells, but did produce a modest decrease in expression, to about the level seen in these cells in the absence of enhancer sequences (Figure 5).

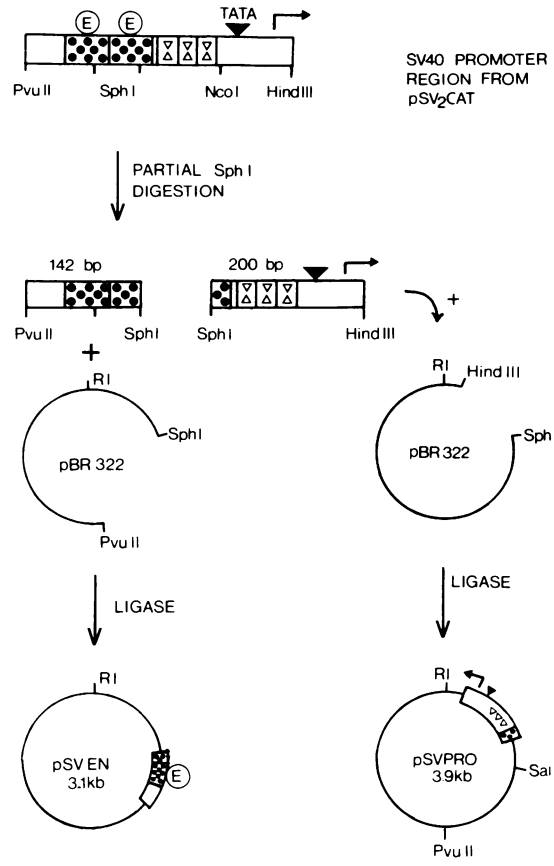

Fig. 7. Construction of plasmids containing fragments of the SV40 early promoter. The starting material was a 340-bp *Pvu*II/*Hind*III fragment isolated from pSV_2CAT, containing the SV40 early promoter and enhancers. Partial *Sph*I digestion yielded two fragments which were isolated after electrophoresis through low melting point agarose. These were cloned by ligation to restriction fragments of pBR322 containing complementary ends.

These results suggest that in F9 stem cells, as in differentiated cells, the level of pSV_2CAT expression is limited by the availability of enhancer-activating molecules. These might be present in undifferentiated cells at very low levels, or in a form where they are very ineffective in stimulating enhancer activity. On the other hand, the results do not rule out regulation of enhancer activity by a balance between activator and inhibitor molecules, an explanation which most satisfactorily accounts for the existence of polyoma mutants able to grow in EC cells as a result of slight alterations to their enhancer sequences (Vasseur *et al.*, 1983; Tanaka *et al.*, 1982; Sekikawa and Levine, 1981; Fujimura *et al.*, 1981; Linney and Donerly, 1984; Herbomel *et al.*, 1984). The results of Figures 5 and 6 would be consistent with the presence in F9 cells of a negative regulator with a somewhat lower affinity for the SV40 enhancer than that of activating molecules. This can be compared with the results obtained by Borrelli *et al.* (1984) using HeLa cell extracts as a source of activating molecules. In these experiments, an inhibitory molecule (an adenovirus Ela product) was sequestered preferentially by competing

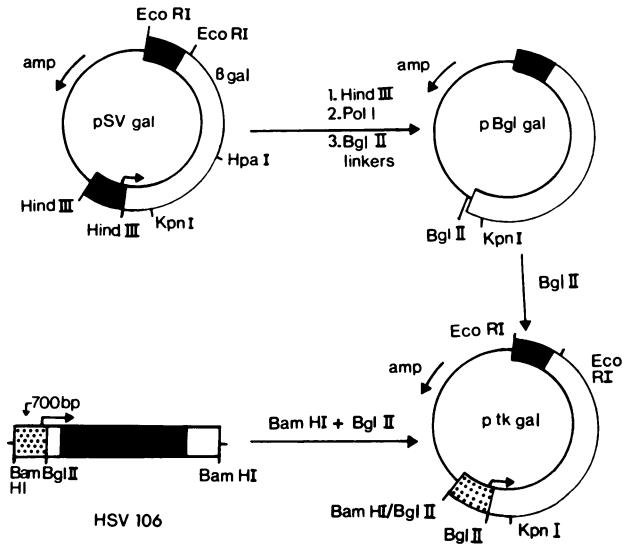

Fig. 8. Construction of β-galactosidase vectors. The starting plasmid, pSVgal (pCH1101-3-SVearly), is a derivative of pCH110 (Hall *et al.*, 1983). Deletion of the SV40 early promoter, on a 400-bp *Hind*III fragment, and addition of *Bgl*II synthetic linkers (see Materials and methods) generated pBglgal. A 700-bp *Bam*HI-*Bgl*II fragment containing the promoter region and transcriptional start from the herpes simplex virus thymidine kinase gene was isolated from the plasmid HSV106 (McKnight, 1980). This was inserted at the new *Bgl*II site in pBglgal to yield ptkgal.

SV40 enhancer sequences. The result was increased expression from the SV40 promoter.

The F9 EC cell system provides the means of investigating these possibilities further, for example by competition studies using different viral enhancers. It should also be possible to determine whether cellular genes changing in activity during F9 cell differentiation are regulated in a manner analogous or identical to that observed for viral genes.

Materials and methods

Cell lines and culture methods

The F9 cell line used for this study (OTF963) (Rosenstraus and Levine, 1979) was obtained from Dr A.Levine, and was maintained on gelatin-coated dishes at 37°C in a humidified atmosphere of 7.5% CO_2. Differentiation of the cells was initiated by addition to the medium of 5×10^{-7} M all-*trans* retinoic acid (Sigma Chemical Company). The fibroblast line L^-a^- which is thymidine kinase- and aprt-negative, was obtained from Columbia University via Dr K.Raphael. This was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and diaminopimelic acid (50 μg/ml). All other lines were maintained in DMEM + 10% heat-inactivated fetal calf serum.

Transfection procedure

Cells were plated to reach a density of $\sim 3 \times 10^6$ per 9 cm dish at the time of transfection. Transfection of DNA into cells was carried out by the calcium phosphate method of Wigler *et al.* (1977). Sixteen hours after addition of the DNA precipitate to the plates, cells were washed twice with phosphate-buffered saline (PBS) containing 0.5 mM EGTA (to remove loosely attached DNA) and then once in PBS before addition of fresh medium containing mycostatin. Twenty eight hours after the medium change, cells were washed twice in PBS, harvested by scraping and collected by centrifugation. The cell pellet was washed in PBS + 0.5 mM EGTA and then resuspended in 0.2 ml of 0.25 M Tris-HCl buffer, pH 7.8. Cell extracts were prepared by sonication and then centrifuged for 15 min in the cold in an Eppendorf microcentrifuge to remove cell debris.

Enzyme assays

Aliquots of cell supernatants were used for CAT assay as described by Gorman *et al.* (1982) except that the [^{14}C]chloramphenicol substrate was reduced to 0.2 μCi. Results were quantitated by determining the radioactivity in spots scraped from thin layer plates, and are expressed as the percentage of [^{14}C]chloramphenicol converted to acetylated forms. In most experiments, duplicate plates were transfected and assayed independently. β-galactosidase activity was measured in cell extracts as described by Miller (1972).

Plasmid constructions

Plasmids were propagated in *Escherichia coli* RR1. DNA for transfection was isolated by Triton X-100 or alkaline lysis of cells, with separation of supercoiled forms by two rounds of cesium chloride/ethidium bromide centrifugation (Maniatis *et al.*, 1982). Enzymes used in constructions were obtained from New England Biolabs, and were used according to the supplier's recommended conditions.

pSV_2CAT (Gorman *et al.*, 1982) was obtained from Dr E.Dennis. pSVEndel is identical to pSV_2CAT except for removal of one of the 72-bp repeat enhancer sequences of the SV40 promoter. This was achieved by *Sph*I digestion of pSV_2CAT and religation. pSVEnless (no complete enhancer sequences) was prepared by *Acc*I/*Sph*I digestion of pSV_2CAT, filling in of single-stranded ends with DNA polymerase I (Klenow) and religation (Maniatis *et al.*, 1982). pSVEndel and pSVEnless are identical to pSV(Sph)CAT and pSV_1CAT described by Gorman *et al.* (1982). Structures of the additional plasmids used for transfection are shown in Figures 7 and 8. pSVPRO and pSVEN contain the SV40 early promoter and enhancer regions, respectively, cloned into pBR322 (Figure 7).

pSVgal (pCH1101-3-SVearly) was obtained from Dr J.F.Nicolas. This is a derivative of pCH110 (Hall *et al.*, 1983) and contains the SV40 early gene promoter on a 400-bp *Hind*III fragment, directing transcription across the *E. coli* β-galactosidase gene. To generate a vector in which transcription of the β-galactosidase gene is controlled by a thymidine kinase promoter, the *Hind*III fragment was deleted, and the *Hind*III site converted to a *Bgl*II site by the use of synthetic linker molecules (pBglgal). A 700-bp *Bam*HI-*Bgl*II fragment containing the promoter region from the herpes simplex virus thymidine kinase gene was isolated from the plasmid HSV106 (McKnight, 1980) and inserted at the new *Bgl*II site to yield ptkgal (Figure 8).

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