Competition studies with repressors and activators of viral enhancer function in F9 mouse embryonal carcinoma cells

M.J. Sleigh, T.J. Lockett, J. Kelly and D. Lewy

CSIRO Division of Molecular Biology, PO Box 184, North Ryde, NSW 2113, Australia

Received January 21, 1987; Revised and Accepted April 27, 1987

ABSTRACT

DNA competition studies have been used to investigate the presence of a repressor of viral enhancer function in F9 mouse embryonal carcinoma cells. The complete polyoma virus enhancer region, cotransfected into F9 cells with the SV40 promoter/enhancer attached to a chloramphenicol acetyl transferase marker gene, induced a small increase in pSV2CAT expression. This can be explained by preferential but weak binding by polyoma sequences of a molecule repressing pSV2CAT transcription. Repressor activity substantially disappeared when the cells were induced to differentiate by retinoic acid. Repressor binding was localised to one half of the polyoma enhancer, but was lost on further fragmentation of this region. It appears that multiple sequence elements may be required for repressor binding and that these are at least partially separable from the complement of elements binding enhancer activating molecules.

INTRODUCTION

Cells of the F9 mouse embryonal carcinoma (EC) line differentiate in response to treatment with retinoic acid, with or without additional cyclic AMP (1). In monolayer culture, product cells resemble extraembryonic parietal endoderm, a tissue produced from multipotent inner cell mass cells at about the time of implantation during mouse embryo development in vivo (2).

A characteristic of this differentiation step, both in vivo and in F9 cells in vitro, is the new synthesis of extracellular matrix and other proteins. At about the same time, the cells become permissive for expression of a range of viral genes. This activation of expression of genes from viruses including SV40, polyoma, Moloney murine leukemia virus, and cytomegalovirus has been used in several laboratories to investigate gene regulation mechanisms in differentiating F9 cells (3-10).

It is now evident that the block to viral gene expression in F9 and other EC stem cells is at the transcription step, and is due to partial or complete lack of function of viral transcriptional enhancer sequences.
Some evidence suggests the presence in EC cells of one or more molecules which repress enhancer activity. An adenovirus Ela-like activity has been reported in undifferentiated but not differentiated EC cells (16). A product of the adenovirus Ela region is known to repress transcription from the SV40 early gene promoter, apparently via an interaction with the enhancer sequences (17,18). More recently, Gorman et al. (14) reported that in undifferentiated F9 cells, the association of enhancers with viral gene promoters caused a decrease in transcription compared with that from the same promoters lacking enhancers. In addition, they found stimulation of transcription from the murine sarcoma virus promoter/enhancer when the Rous sarcoma virus (RSV) long terminal repeat sequence was present in the same cells, suggesting that the RSV sequence was preferentially sequestering a factor in F9 cells which was acting to repress enhancer function.

We have previously reported that the SV40 enhancer retains a small amount of activity in undifferentiated EC cells. Competition studies suggest that in both differentiated and undifferentiated F9 cells, the supply of enhancer activating molecules limits the amount of transcription seen from the SV40 early promoter/enhancer in transient assays (15). Thus a repressor molecule, if present, possibly acts to block the consequences of activator molecule binding, rather than the binding itself. This may suggest that activating and repressing molecules are not in direct competition, but bind to different DNA sequences, as described for the interferon gene enhancer (19). Alternatively, the repressor may represent a modified but inactive form of the activator, in which case binding of activator and repressor to the same DNA sequences might be expected.

One approach to investigating such questions has been through competition studies carried out in vitro (20) or in vivo (21,22). Previous results (14) have suggested that competing DNA sequences could be used to derepress viral enhancer function in F9 cells, presumably via sequestration of a DNA-binding repressor molecule. We have used a range of DNA sequences competing in transfected F9 cells with a marker gene driven by the SV40 promoter/enhancer region. These studies have provided further evidence for repression of viral enhancer function in undifferentiated (but not differentiated) F9 EC cells and have suggested that different complements of DNA sequence elements may be involved in mediating the repression and enhancement of viral gene transcription.
MATERIALS AND METHODS

Cell lines and culture methods

The F9 cell line used for this study (OTF963) (23) was obtained from Dr. A. Levine, and was maintained on gelatin-coated dishes at 37°C in a humidified atmosphere of 7.5% CO₂. Differentiation of the cells was initiated by addition to the medium of 5 x 10⁻⁷ M all-trans retinoic acid (Sigma Chemical Company). The fibroblast line Lᵃ⁻ 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 (50μg/ml). All other lines were maintained in DMEM + 10% heat inactivated fetal calf serum.

Transfection procedure

Cells were plated to reach a density of approximately 10⁶ per 10cm dish at the time of transfection. Transfection of DNA into cells was carried out by the calcium phosphate method (24). Sixteen hours after addition of the DNA precipitate to the plates, cells were washed twice in PBS containing 0.5mM 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 and then resuspended in 0.2 ml of 0.25M 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 (25).

Enzyme Assays

β-galactosidase activity was measured in cell extracts as described (26). CAT activity was assayed in cell extracts that had been heated at 65°C for 10 min and then centrifuged for a further 5 min. This treatment removed an acetyl coenzyme A-degrading activity but did not affect the CAT enzyme. Assays were carried out using a rapid, non-chromatographic procedure which measures transfer of ¹⁴C-labelled acetyl groups from acetyl coenzyme A to unlabelled chloramphenicol (27).

DNA used for transfection

Plasmids were propagated in E.coli RR1. DNA for transfection was isolated by alkaline lysis of cells, with separation of supercoiled forms by two rounds of cesium chloride/ethidium bromide centrifugation (28). Enzymes used in constructions were obtained from New England Biolabs, and were used according to the suppliers' recommended conditions.
PSV2CAT (25) was obtained from Dr. E. Dennis. The construction and use of ptkgal (β-galactosidase gene coding sequences transcribed from the herpes virus thymidine kinase promoter) has been described previously (15).

pSVEN (15) contained one complete and one partial copy of the SV40 enhancer cloned into pBR322. pMoe contained a synthetic oligonucleotide equivalent of one of the 72 base pair repeats constituting the enhancer of Moloney murine sarcoma virus (29). The fragment (the promoter proximal repeat) was synthesised as two 45-mers with complementary overlapping sequences at the centre of the fragment. The two oligomers (kindly synthesised by Dr. G. Both) were annealed and extended to form a complete double stranded molecule (28). This was cleaved at an EcoRI site included in the molecule next to MoMSV-derived sequences, and cloned into EcoRI and Smal-cut pUC18.

pIgE contained the Immunoglobulin heavy chain gene enhancer (30) cloned into SalI- and HindIII-cleaved pUC18. The 313bp insert was derived by HindIII and XhoI digestion of pPyigE, kindly supplied by Dr. J. de Villiers.

Complete and partial polyoma enhancer sequences for cloning into pUC18 were derived from a series of deletion mutants described elsewhere (31) and kindly supplied by Dr. G. Veldman. The constructs prepared are described in Table 1 and Figure 3. An additional construct (pPyF12) containing three copies of the insert present in pPyF2 was obtained by EcoRI-PvuII digestion of pPyF7, blunt ending of the 495bp fragment using Klenow DNA polymerase (28) and ligation into Smal-cut pUC18.

RESULTS

Design of competition experiments

The aim of this series of experiments was to determine whether competing DNA sequences present in the same cell as a transcribing gene would preferentially sequester activating or repressing molecules binding to enhancer elements. The effect observed would be a net decrease or increase, respectively, in expression of the transcribing gene.

The transcribing gene used is that coding for the bacterial enzyme chloramphenicol acetyl transferase (CAT), with transcription driven by the SV40 early promoter and enhancer region (pSV2CAT; 25). In addition to pSV2CAT, transfected cells received 5µg ptkgal to act as an internal control for efficiency of transfection. Cells receiving large amounts of DNA during transfection showed some depression in endogenous
Table 1 Derivation of cloned polyoma enhancer fragments

<table>
<thead>
<tr>
<th>Cloned fragment in pUC18</th>
<th>Parent plasmid\textsuperscript{a}</th>
<th>Digestion to generate fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPyF1</td>
<td>B5137</td>
<td>EcoRI + PvuII</td>
</tr>
<tr>
<td>pPyF2</td>
<td>pPyCAT</td>
<td>EcoRI + PvuII</td>
</tr>
<tr>
<td>pPyF3</td>
<td>B5183</td>
<td>EcoRI + PvuII</td>
</tr>
<tr>
<td>pPyF4</td>
<td>P5179dB</td>
<td>EcoRI + PvuII</td>
</tr>
<tr>
<td>pPyF5</td>
<td>pPyCAT</td>
<td>EcoRI + HaeIII</td>
</tr>
<tr>
<td>pPyF7</td>
<td>pPyCAT</td>
<td>EcoRI + partial PvuII</td>
</tr>
<tr>
<td>pPyF9</td>
<td>pPyF2</td>
<td>Sau3A(blunt ended)\textsuperscript{b} + EcoRI</td>
</tr>
<tr>
<td>pPyF10</td>
<td>P5179dB</td>
<td>HpaII(blunt ended)\textsuperscript{b} + EcoRI</td>
</tr>
<tr>
<td>pPyF11</td>
<td>pPyF2</td>
<td>HaeIII(blunt ended)\textsuperscript{c} + EcoRI</td>
</tr>
</tbody>
</table>

\textsuperscript{a} parent plasmids (other than pPyF2 which was generated as described above) had structures as outlined previously (31).

\textsuperscript{b} Sau3A and HpaII-cut ends were converted to blunt end cuts using Klenow DNA polymerase I (28).

\textsuperscript{c} HaeIII-cut ends were converted to blunt end cuts using mung bean nuclease (Boehringer Mannheim) according to the suppliers' instructions.

\(\beta\)-galactosidase expression but this effect appeared to be independent of the nature of DNA introduced (Henderson & Sleigh, unpublished results). Thus "mock transfected" samples for determination of background levels of \(\beta\)-galactosidase received pUC18 DNA (23.3\microgram per plate) but not ptkgal or pSV\textsubscript{2}CAT.

The third component of transfection was the competing enhancer sequence cloned into pBR322 or pUC18. In practice, DNA precipitates were prepared containing increasing amounts of the competing molecule, progressively replacing an equivalent amount of pUC18 DNA.

Under the conditions of cell growth and transfection used in these studies, maximal transient expression is reached when approx. 10-20\microgram of pSV\textsubscript{2}CAT DNA is used to transfect differentiated or undifferentiated F9 cells. Availability of enhancer-binding factors is a limiting factor for transcription in both cell types. At these levels, transcription factors binding to the SV40 early promoter are still in excess in F9 cells but approaching full utilisation in differentiated F9 cells (15).
Table 2  Competition between pSV2CAT and pPyF7

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DNA for transfection</th>
<th>CAT assays</th>
<th>β-gal assays</th>
<th>CAT/dgal</th>
<th>Relative expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>L^-a^-</td>
<td>pUC 18</td>
<td>266</td>
<td>0.227</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>pSV2CAT</td>
<td>15,221</td>
<td>0.440</td>
<td>71,460</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>pSV2CAT+F7,1:1</td>
<td>13,261</td>
<td>0.580</td>
<td>35,552</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>pSV2CAT+F7,1:2</td>
<td>10,654</td>
<td>0.510</td>
<td>37,646</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>pSV2CAT+F7,1:4</td>
<td>10,604</td>
<td>0.740</td>
<td>20,670</td>
<td>29</td>
</tr>
<tr>
<td>F9</td>
<td>pUC 18</td>
<td>302</td>
<td>0.138</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>pSV2CAT</td>
<td>930</td>
<td>0.158</td>
<td>31,400</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>pSV2CAT+F7,1:1</td>
<td>850</td>
<td>0.167</td>
<td>18,896</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>pSV2CAT+F7,1:2</td>
<td>1,894</td>
<td>0.175</td>
<td>51,189</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>pSV2CAT+F7,1:4</td>
<td>1,810</td>
<td>0.165</td>
<td>67,037</td>
<td>213</td>
</tr>
<tr>
<td>F9+RA</td>
<td>pUC 18</td>
<td>298</td>
<td>0.240</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>pSV2CAT</td>
<td>35,226</td>
<td>0.344</td>
<td>338,711</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>pSV2CAT+F7,1:1</td>
<td>23,654</td>
<td>0.296</td>
<td>422,392</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td>pSV2CAT+F7,1:2</td>
<td>31,512</td>
<td>0.352</td>
<td>281,357</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>pSV2CAT+F7,1:4</td>
<td>32,265</td>
<td>0.368</td>
<td>252,070</td>
<td>74</td>
</tr>
</tbody>
</table>

a 23.3 μg DNA was used for transfections, consisting of 5 μg pSV2CAT and 5 μg ptkgal where appropriate, pPyF7 in the ratio shown to pSV2CAT, and the balance pUC18 DNA.

b cpm in 10^-6 acetylichloramphenicol after 60 min. assay of approximately 30 μl cell lysate prepared as described in Methods. 0.01 units of purified CAT enzyme (P.L.Biochemicals) yields approx. 20,000 cpm under these conditions.

c β-galactosidase assays were carried out on 0.1 ml aliquots of cell lysate, using overnight assays as described in Methods.

d Calculated after subtraction of mock-transfected blanks.

e Results in the presence of competitor calculated relative to those for pSV2CAT alone.

Competition experiments described here were carried out at 5 μg pSV2CAT per 10 cm plate of cells. This was the minimal level that could be used to provide CAT expression that could be measured reliably in F9 cell extracts (15). However it did impose some limitations on the ratio of competing to transcribing molecules that could be achieved. Using the methods of cell culture and transfection described here, DNA uptake capacity appeared to be limited to a maximum of around 25 μg DNA per 10 cm dish of cells (Results not shown). For this reason maximum competitor to gene molecular ratio used in initial experiments have been 4:1. An additional construct containing multiple copies of the most effective competitor fragment has also been prepared, providing a competitor:gene ratio of 12:1. The transcribing gene, pSV2CAT, contained multiple regulatory elements within two 72bp repeats in the enhancer region (32, 33) while the competing
Fig. 1 Effect of competing enhancer sequences on pSV2CAT expression. F9 cells (○), F9 cells treated with RA for 4 days at the time of transfection (●) or L2a cells (■) were transfected with 5 µg pSV2CAT, 5 µg ptkgal and 13.3 µg pUC18 DNA, progressively replaced by pSVEN (SV), pPy77 (Pv), pN0E (No) or pIgE (Ig). Two days later CAT and β-galactosidase activities in the cells were measured as described in Methods. Results were calculated as described for Table 2, and are expressed for each point relative to the normalised CAT expression obtained from cells receiving pSV2CAT and pUC18 DNA but no competing enhancer.

The competitor ratio is the number of competing enhancer molecules introduced to the cells relative to each pair of 72bp repeat sequences on pSV2CAT. Results shown are averaged from three independent experiments.

molecules also contained several regulatory sequence elements (Veldman et al., 1985). Thus the exact ratio of activator or repressor binding sequences in competing compared with transcribing DNA in each competition experiment is difficult to define any more exactly.

Characteristics of the CAT Assay method

The assay method used here for CAT enzyme activity in cell extracts (27) differs in several respects from the chromatographic assay used commonly (25). Because enzyme activity inactivating acetylCoA is removed by heating cell extracts at 65°C, the assay remains linear over several
Nucleic Acids Research

hours (Henderson and Sleigh, unpublished results). The specific activity of the acetylCoA can be varied to increase the sensitivity of assay of low activity samples. Thirdly, the assay quantitates reaction product rapidly and directly, increasing both the consistency of results and the ease with which multiple cell samples can be assayed and duplicate assays carried out. These characteristics have been very important in this study, where activities have often been low, and it has been necessary to carry out repeat experiments and duplicate cell treatments within experiments to confirm the significance of quite small variations in enzyme activity.

Effect of various competing enhancer sequences on transient expression of pSV2CAT

Cloned enhancer sequences from the immunoglobulin heavy chain gene (pIgE), Moloney murine sarcoma virus, (pMoE) polyoma virus (pPyF7) and SV40 (pSVE7) were tested in competition experiments with pSV2CAT to determine whether they would influence its transcription rate by net removal of either enhancer-activating or enhancer-repressing molecules. The results from one such experiment, carried out in Ltk−, F9 and differentiated F9 cells, are shown in Table 2. Relative pSV2CAT expression in the three lines before and after correction for β galactosidase expression remained approximately constant from one experiment to another although absolute values obtained varied somewhat. Each experiment reported was carried out at least three times to confirm the consistency of results obtained.

Figure 1 summarises the results from these multiple experiments for the four different competing enhancers listed above. Inhibition of CAT expression was produced in all cell types by the enhancer from Moloney sarcoma virus (MoSV) and by the SV40 enhancer itself. The immunoglobulin enhancer, which would not be expected to be active in any of these cell types, reduced CAT expression in L cells, but not in the other cell types. The complete polyoma enhancer region depressed pSV2 CAT expression in L and differentiated F9 cells, but in F9 stem cells, a small but reproducible stimulation of pSV2CAT expression was seen (Figure 1). This result is consistent with preferential binding by the polyoma enhancer of a repressor molecule present in undifferentiated F9 cells, but not in their differentiated progeny or in L cells.

Localization of the repressor-binding sequences within the polyoma virus enhancer

The enhancer sequences required for polyoma virus replication and transcription have been characterised in several laboratories (11,31,34).
Effect of competing polyoma enhancer fragments on pSV2CAT expression. Experiments were carried out and results calculated and expressed exactly as described for Figure 1. Competing polyoma enhancer sequences were the entire enhancer region (pPyF7) or the A and B regions (11; pPyF2 and pPyF1 respectively). (●) F9 cells, (○) F9 + RA cells, (■) L512 cells.

Results shown are the average of those obtained in three independent experiments for each competing fragment.

Sequences contributing to enhancer function are spread over the region from nucleotides 5020-5270 with contributions from several different sequence elements within this region. In fibroblasts the "A" region (bases 5020-5130) is the most active (11). In PCC3 embryonal carcinoma cells, the "A" region is relatively inactive, with a small amount of residual enhancer function in the "B" region (bases 5130-5270). This suggests that a polyoma enhancer repressing molecule present in F9 cells may bind predominantly to the A region, with activating molecules binding to both A and B. When the cloned A and B regions were introduced into cells in competition with pSV2CAT, all of the ability of the complete polyoma enhancer to stimulate pSV2CAT expression appeared to reside in the A region (pPyF2 – Figure 2). This is consistent with binding by the polyoma A region of a molecule repressing pSV2CAT expression in F9 but not in other cells.

To further localise activator- and repressor-binding sequences, other fractions of the polyoma enhancer region (Figure 3) were tested as
Structure of polyomavirus enhancer fragments used in competition studies. The derivation of the fragments is described in Table I. All fragments ended at an EcoRI site corresponding to nucleotide 4635 of polyoma (a BamHI site in the original virus). Termi of other sequences contained in the fragments are shown. Unnumbered termini are at the BclI site (nucleotide 5021) or the PvuII sites at 5130 or 5267. Nucleotide numbering is as in reference 31.

Also marked on the parent polyoma sequence is the region for the start of late transcription (bar and arrow) and the A and B regions of the polyoma enhancer (11).

Competitors with pSV2CAT. These results (Figure 4) demonstrate that no fragment smaller than the complete A region (pPyF2) stimulated pSV2CAT expression. However, a comparison of the results for undifferentiated and differentiated F9 cells suggests that pPyF6 and pPyF11 may retain some ability to bind repressor molecules. Several subsections of the A region were effective in binding activator molecules, as evidenced by reduced pSV2CAT expression.

**cAMP requirement for disappearance of the repressor effect**

Figure 5 summarises the competition results obtained for each fragment tested, at the maximum level of competitor used. This demonstrates the relative effect of competing molecules in the 3 different cell types. Interestingly, the reductions in pSV2CAT expression induced by molecules which have net repressor-binding activity in F9 stem cells (pPyF7 and pPyF2) were not as great in differentiated F9 cells as those seen in L
Effect of subtractions of the polyoma virus enhancer on transient expression of pSV2CAT. Experiments were carried out and results calculated and expressed exactly as described for Figure 1. Sequences contained within competing molecules are described in Figure 3. Results shown are the average of those obtained in 2-4 independent experiments for each competing fragment. (●) F9 cells, (O) F9 + RA cells, (■) L"a" cells. However some subsections of the A region which showed no net repressor binding in F9 stem cells produced a greater reduction in pSV2CAT expression in the differentiated cells than that seen in L cells. One explanation for this is that the disappearance of the enhancer-binding repressor is not complete even after 6 days exposure of F9 cells to retinoic acid.

It has been suggested (2, 35, 36) that treatment of F9 cells with cAMP in addition to RA is required for full differentiation to parietal endoderm-type cells. In previous experiments on SV40 enhancer function, we found that enhancer activation was similar in F9 cells treated with RA alone (at 5x10⁻⁷M), or RA + 1mM cAMP (15).

In competition experiments using pPyF2 and pPyF7, inhibition of pSV2CAT expression was similar in F9 cells treated only with RA (5x10⁻⁷M), or with RA (5x10⁻⁷M) + dibutyryl cAMP (1mM) (results not shown). Thus additional treatment with cAMP does not appear to lead to more complete disappearance of the repressor effect than in cells treated with 5x10⁻⁷M RA alone.
**Fig. 5**

Effects of competing molecules on transient expression of pSV2-CAT. The figure compares polyoma sequences contained in competing molecules on the left hand side with the percent change in pSV2-CAT expression seen at the maximal level of competitor tested (4 competing molecules to each pSV2-CAT molecule). For each competitor, bars above the baseline represent stimulation of pSV2-CAT expression. Those below the base line show inhibition. For each competing fragment, effectiveness as an enhancer in a DNA replication assay (31) is shown, relative to wild type (pPyF7 - 100%).

**Effect of higher competitor:gene ratios on pSV2-CAT expression**

To determine whether higher levels of the polyoma "A" region could further stimulate pSV2-CAT expression in F9 cells, pPyF12, containing three tandem copies of this region was prepared and tested in competition experiments. The results are shown in Figure 6. This experiment confirmed stimulation of pSV2-CAT expression in F9 but not F9+RA cells at low (3-4:1) competitor:gene ratios. However, as the amount of competitor was increased, suppression of pSV2-CAT expression was observed. This can be explained by simultaneous removal of enhancer activating molecules by the
Fig. 6  Competition between high levels of polyoma enhancer region "A" and pSV2CAT in transient assays.

Experiments were carried out and results calculated as described for Figure 1. The competing plasmid, pPyF12, consisted of pUC18 containing three head-to-tail copies of the "A" region insert from pPyP2. ( ) F9 cells, ( ) F9+RA cells. Results shown are the average of those from two transfection experiments.

competing plasmid. This results in a rather narrow "window" in which removal of repressor predominates in determining levels of pSV2CAT expression.

DISCUSSION

Enhancer regions from both viral and cellular genes have been shown to consist of multiple sequence elements which may have independent, overlapping or equivalent functions in regulation of gene activity in different cell types (32, 33; reviewed in 37). Specificity of enhancer action may be modulated by changing complements of sequence elements in different enhancers (33). In addition, different cell types appear to vary in the range and/or activity of enhancer-binding molecules (presumably proteins) that they possess. Such molecules may exert a positive or negative effect on expression of associated genes (for example, reference 22).

Results reported here, and previously (14, 38) suggest that in F9 embryonal carcinoma cells, negative regulatory factors binding to specific, enhancer-associated DNA sequence elements influence the level at which viral genes are expressed. After cell differentiation, this repressive effect is substantially reduced.

If disappearance of repressor molecules were the principal cause of enhancer activation during cell differentiation, then removal of the
repressor by binding to competing DNA in undifferentiated cells should increase the enhancer effect to a level approaching that seen in differentiated cells. As seen in Figure 5, no such increase was achieved. The maximum stimulation of pSV2CAT expression by competing DNA was 50-80%, while full restoration of enhancer activity should increase expression to at least ten times the level seen in undifferentiated cells (15).

In part this could be due to limitations imposed by the experimental design. As demonstrated previously (15), enhancer activating molecules become limiting for transcription when 10-15μg pSV2CAT is transfected into differentiated or undifferentiated F9 cells. Since the competing polyoma DNA molecules that bound repressor also bound activators, as seen for the results for L-α- and differentiated F9 cells, then the maximum stimulation of transcription could be limited to two- to three-fold.

That this level was not always achieved may reflect the relative affinities of enhancer elements and regions for DNA binding molecules. It has previously been reported that the SV40 and polyoma enhancer regions do not compete strongly with each other for enhancer activating factors (17, 20, 39) and this is confirmed by the relatively poor competitive effects reported here. SV40, polyoma and other viral enhancer regions may also sequester different subsets of the total complement of factors (activators or repressors) available in a particular cell type (20, 32, 33, 40). Thus, despite the strong suppression of the MoMSV enhancer in F9 cells (13, 14), the MoMSV enhancer segment described here did not show net repressor binding in competition with pSV2CAT. On the other hand, up to 75-fold stimulation of expression from the MoMSV promoter/enhancer by RSV sequences has been described (14), suggesting that these sequences have a very high affinity for the repressor molecule(s) blocking MoMSV expression in F9 cells.

Since the polyoma sequences described in this report are themselves strongly suppressed in F9 cells, but appear to have low affinity for the SV40 enhancer repressor, the possibility arises that more than one repressing factor is present. If so, then the polyoma sequences shown here to be important in binding the SV40 repressor may be different from those of primary importance for repression of the function of the polyoma enhancer itself in EC cells.

An important result from the competition experiments of Figures 2, 4 and 5 is that binding sequences for repressing and activating molecules are partially separable (compare results for pPyF2 and pPyF4). Deletion of
nucleotides 5113-5130 from pPyF2, the most effective repressor binder in F9 cells, produced pPyF11, which showed less net repressor binding in F9 cells as well as a small decrease in net activator binding in differentiated cells. This suggests that not only is this sequence element very important for polyoma enhancer function, as suggested previously (31), but it also contributes to the binding of the SV40 repressor. However its interaction with other sequence elements is clearly different for the two functions. In the absence of other "A" region sequences this element failed to bind repressor molecules but was an effective binder of activator molecules and produced high enhancer activity (pPyF4 and pPyF10 of Figure 5 and reference 31). Evidently the 5113-5130 element in combination with other elements of the "A" region is required for binding of the SV40 repressor, while the same sequences in combination with the "B" region are optimal for activator binding and enhancer activity.

Two sequence elements homologous to the SV40 enhancer are found in the "A" region. These lie between nucleotides 5110-5120 and 5030-5040 of polyoma (31), the elements being related to GT element II and Sph element II (33) of the SV40 enhancer respectively. A requirement for both of these elements to effect repressor binding by polyoma sequences would be consistent with the results described here.

Differential interactions of repressors and activators with DNA elements may have significant implications for attempts to isolate activating and repressing molecules using in vitro DNA binding methods. However they do not clarify the relationship between repressors and activators, and how this changes as F9 cells differentiate. The results may be explained either by distinct molecules interacting either directly or indirectly with different sequences in the enhancer, or by related molecules binding to the same sequence but interacting differently with neighbouring sequence elements. Studies on binding of nuclear proteins to polyoma enhancer sequences have not yet clarified the situation. No differences in binding patterns have been reported when extracts from differentiated and undifferentiated F9 cells are compared (41).

Several laboratories have isolated mutants of polyoma virus which are able to grow in undifferentiated EC cells (reviewed in 42). The outstanding feature of these mutants, and of revertants of ineffective SV40 enhancer mutants assayed in other cell types (32), was that enhancer activity was restored either by duplication of existing sequence elements or by rearrangement (SV40) or mutation (polyoma) to create new ones. An
additional feature of the polyoma mutants, as pointed out previously (43), is that the different types of sequence elements making up the enhancer region have a different relative arrangement in the mutants compared with wild type virus. A particularly interesting example is the F9.1 mutant, where a point mutation creates a new enhancer core sequence in the B region of the polyoma enhancer. This mutant enhancer is more effective in all cell types and shows no evidence of repression in EC cells (12). It is also resistant to repression by the adenovirus E1A gene product (38). A simple explanation is that for polyoma virus the sequences around this point mutation are required for repressor binding, but that alone would not explain the more general increase in effectiveness of this enhancer. An alternative possibility is that not only has a new element important for binding activator molecules been created (explaining the general increase in effectiveness) but also that this is now differently placed with respect to sequences involved in enhancer repression in EC cells (nucleotides 5020-5130 from our experiments) thus reducing the effectiveness of bound repressor.

The results reported here suggest some additional strategies for generating enhancers that would escape binding by the SV40 repressor in EC cells. In particular, removal of nucleotides 5040-5100 of polyoma should remove repressor binding. Such a deleted enhancer retains activator binding activity (see pPyF4 and pPyF10 in Figure 5) and had undiminished enhancer activity in a DNA replication assay (31). One report describes the creation of a hybrid enhancer from the origin-distal half of the SV40 enhancer and nucleotides 5090-5137 of polyoma (33). This enhancer, which our results would predict should largely lack repressor binding sequences in the polyoma segment, was functional in both EC and HeLa cells. The naturally occurring polyoma mutant PyPLC78 similarly lacks sequences from the BclI site to nucleotide 5096 (see Figure 3) in the A region of the polyoma enhancer. This mutant had high enhancer activity in 3T3 cells and was less suppressed than the wild type virus in PCC3 EC cells (11).

However in this virus there were also other rearrangements, deletions and mutations, presumably to restore signals needed for initiation of late transcription, which normally occurs within the deleted region. If similar repressor molecules act on both SV40 and polyoma virus enhancers in F9 cells, then an unrearranged equivalent of this enhancer region, i.e. containing nucleotides 5110-5270, should have equal activity in
differentiated and undifferentiated F9 cells. Experiments to test this hypothesis are in progress.

ACKNOWLEDGEMENTS

The authors are grateful to Drs. G. Veldman, J. de Villiers and R. Kamen for helpful discussions and supply of polyoma DNA, to Dr. G. Both for synthesis of oligonucleotides and to Drs. P. Jennings and P. Molloy for critical review of the manuscript. The work has been supported in part by a grant to MJS from the Australian Meat Research Committee.

REFERENCES