Biological Activities of Oligonucleotides Spanning the F9 Point Mutation within the Enhancer Region of Polyomavirus DNA

MASANOBU SATAKE, KAZUHIRO FURUKAWA, AND YOSHIKI ITO*

Department of Tumor Virology, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606, Japan

Received 7 May 1987/Accepted 12 October 1987

A mutant of polyomavirus, F441, selected to grow in undifferentiated mouse F9 embryonal carcinoma cells, carries a single-base change in the enhancer region at nucleotide (nt) 5233 of the viral genome. Enhancers of most of the F9 mutants have a duplicated segment of viral DNA encompassing nt 5233. The minimum duplicated segment of all the known F9 mutants is from nt 5218 to nt 5239. We prepared oligonucleotides spanning the sequence from nt 5218 through nt 5239 of the genome of the wild type and F441 and examined the biological activities of the oligonucleotides by a transient assay of chloramphenicol acetyltransferase (CAT) gene expression in F9 cells. The oligonucleotide harboring the F441 mutation was shown to increase *cat* gene expression in F9 cells when linked at an upstream position in both orientations. When dimerized at an upstream position, the F441 oligonucleotide showed even higher *cat* gene expression enhancing activity. In contrast, no such effects were observed with the oligonucleotide of the wild-type sequence. In addition, the F441 oligonucleotide, but not the wild-type sequence, could inhibit the activity of whole enhancer fragment of F441 when cotransfected into F9 cells in excess amounts. On the basis of the results obtained, we suggest that the segment of F441 enhancer encompassing the point mutation contains a target for a cellular factor(s) which acts in a positive manner to increase the transcription of a gene in undifferentiated mouse F9 cells.

Wild-type polyomavirus does not grow in mouse cell lines representative of early stages of mouse development (2, 38). Examples include the embryonal carcinoma cell lines F9 and PCC4 (18) and trophoblast cell lines such as Tr/C57BL/6 (39). When such cells are induced to differentiate, they become permissive to the virus growth. Mutants of polyomavirus capable of growing in these undifferentiated cells can be isolated from cells chronically infected with wild-type virus. It has been shown that the mutations are localized to the enhancer region of the viral genome (13, 20, 21, 34, 39). In more recent reports, the enhancer region was shown to undergo changes in a differentiation-dependent manner when the virus was adapted to grow in Friend leukemia cells before and after differentiation (8, 9) and in neuroblastoma cells at various states of differentiation (27). These observations suggest that analysis of mutated enhancers may help disclose the mechanisms by which expression of cellular genes is regulated as cells differentiate (1, 17, 24, 25).

Alterations observed in the enhancers of embryonal carcinoma and trophoblast mutants include point mutation, deletions, duplications, sequence rearrangements, or combinations of some of these changes (summarized in references 1 and 40). The meaning of these genetic changes was not clear until recently. As the analysis of the enhancer region of polyomavirus reveals that there are multiple redundant functional elements (17, 29, 43) and that some sequences are homologous to the enhancer cores of other viral or cellular genes, it is now possible to address specific questions concerning the nature of the changes seen in the enhancers of embryonal carcinoma and trophoblast mutants (reviewed in reference 1). In the case of F9 mutants isolated to grow in F9 cells, the simplest change found in the enhancer region is an A-to-G transition at nucleotide (nt) 5233 seen in F441 (13) or F9-1 (21) (Fig. 1). Many other F9 mutants, including F101 and F111 (13; Fig. 1) and *hrN2* and *hrN5* (34), have dupli-

cation of the region spanning this nt 5233 in addition to the A-to-G point mutation. A minimum border of the segment of viral DNA present in the duplicated region in all the F9 mutants reported so far is from nt 5218 to nt 5239 (Fig. 1).

It has been suggested that a cellular factor(s) present in F9 cells recognizes the enhancer of Moloney murine sarcoma virus and inhibits its function (15). Likewise, the enhancer of wild-type polyomavirus may be negatively regulated in F9 cells. Negative regulation of the polyomavirus enhancer by the adenovirus E1a gene, a transforming gene of the virus (5), has also been shown to occur. Interestingly, a mutation from A to G at nt 5233 not only allows polyomavirus enhancer to function in F9 cells but also renders it insensitive to the negative effect induced by the adenovirus E1a protein (16). These results suggest that the adenovirus E1a gene induces, in differentiated cells, a negative regulatory factor(s) which is similar to the one normally expressed in F9 cells and that an alteration of the nucleotide at position 5233 can abrogate the negative effect. Possibly this site is a binding site for a negative regulator present in F9 cells, and the point mutation from A to G results in the elimination of this binding site. Alternatively, the alteration could create a binding site for a positive-acting factor which is able to overcome the repression normally occurring in F9 cells.

To address this question, we synthesized the oligonucleotides representing a segment of polyomavirus enhancer from nt 5218 to nt 5239, spanning the nt 5233 of either A or G (Fig. 1), and examined their biological effects. The results presented in this communication indicate that the short segment of viral DNA around the point mutation stimulates the expression of linked gene in *cis* in F9 cells when the nucleotide at 5233 is G. Since a dimer of this element is more efficient in stimulating expression and the segment also competes for factors to reduce expression, we suggest that the point mutation makes the segment a binding site for a positively functioning factor(s), rather than destroying a binding site for a negatively regulating factor(s).

* Corresponding author.

FIG. 1. Duplication of the region encompassing the site of the F9 point mutation in the genomes of F9 mutants. The thick solid line represents the polyomavirus genome, and the sequence numbering system is according to Soeda et al. (35), as modified by Tyndall et al. (41). The nucleotide at 5233 is converted from A to G in F441 (13) and F9-1 (20). In the mutants F101 and F111 (13), a stretch of DNA segment containing the mutation at nt 5233 is duplicated in tandem as shown.

MATERIALS AND METHODS

Polyomavirus enhancer fragments. Plaque-purified wild-type A2 strain and mutant F441 (obtained from E. Linney; 13) of polyomavirus were propagated in primary baby mouse kidney cell culture. Mouse 3T6 cells were inoculated with these stock viruses, and viral DNA was extracted by the Hirt procedure. Form I DNA was isolated by equilibrium centrifugations through CsCl-ethidium bromide. After cleavage with *Bam*HI, viral DNA was cloned at the *Bam*HI site of pBR322. Polyomavirus DNA fragments encompassing from the *Bam*HI site (nt 4632) to the *Pvu*II site (nt 5265) were isolated from cloned DNA, and a *Bam*HI linker was attached to the *Pvu*II site. This 630-base-pair (bp) fragment was subcloned at the *Bam*HI site of pUC13. Nucleotide sequencing of subcloned enhancer fragments by the chain termination method using oligonucleotide primer and dideoxynucleosides (32) confirmed the authenticity of the fragments.

Oligonucleotides. Oligodeoxyribonucleotides 28 bases long were a gift from Y. Teranishi, Mitsubishi-Kasei Co., Tokyo. They were purified by polyacrylamide gel electrophoresis and desalted through a Sephadex G-25 column, and their 5' ends were phosphorylated by T4 kinase. On annealing, they gave rise to double-stranded nucleotide sequences corresponding to nt 5218 through nt 5239 of the polyomavirus genome of the wild-type A2 strain or the F441 mutant (see Fig. 2B). The oligonucleotides were made in such a way that both ends generated cohesive ends as *Bam*HI sites after annealing. Authenticity and orientation of the cloned oligonucleotides in a vector were confirmed by nucleotide sequencing.

Vectors. pA₁₀CAT₂ (23) was used as a vector for insertion of polyomavirus enhancer fragments and oligonucleotides. pSVpNEO was a derivative vector constructed in this laboratory from pA₁₀CAT₂ and pSV₂NEO (36) in the following way. From pA₁₀CAT₂, the *Hind*III-to-*Bam*HI fragment containing pBR322 sequence and simian virus 40 (SV40) early promoter was purified. A *Bgl*II-to-*Bam*HI fragment, harboring the neomycin resistance gene and the SV40 poly(A) addition signal, came from pSV₂NEO. After conversion of the above *Bgl*II site to a *Hind*III site, two fragments were

ligated with T4 DNA ligase, giving rise to a new construct, pSVpNEO, in which the *Bgl*II site that remained was unique as in the case of pA₁₀CAT₂ and the bacterial promoter element for the neomycin gene was deleted. This pSVpNEO vector was used as a backbone vector to construct a series of competitor plasmids.

DNA transfection and CAT assay. F9 cells (obtained from F. Kelly, Pasteur Institute, Paris) and NIH 3T3 cells were cultivated in Dulbecco modified Eagle minimum essential medium supplemented with 10% fetal calf serum. Cells were seeded at a density of 10⁶ cells per 10-cm-diameter plate. After overnight incubation, they were transfected with plasmid DNA preparations as form I DNA by calcium-phosphate coprecipitation methods (46). Five hours later, cells were shocked for 60 s by overlaying 15% (vol/vol) glycerol in the culture medium and were then rinsed carefully with fresh medium. In some cases, cells were further incubated overnight in the presence of 1 mM sodium butyrate in the medium to increase the overall expression levels. At 48 h after transfection, cells were harvested, and cell extracts adjusted to a total of 100 μ l were prepared. Chloramphenicol acetyltransferase (CAT) enzyme activity accumulated in transfected cells was assayed according to the standard procedure of Gorman et al. (14). CAT activity recovered was indicated as the percentage of acetylated form in relation to the sum of both the acetylated and nonacetylated forms of chloramphenicol. Individual conditions for the incubation of cell extracts with [¹⁴C]chloramphenicol (concentration, 2.1 mM; specific activity, 47.3 mCi/mmol; New England Nuclear Corp.) are described in the figure legends. For transfecting differentiated F9 cells, 5 $\times$ 10⁵ cells were seeded in a 10-cm-diameter plate and treated with 1 μ M retinoic acid (Sigma Chemical Co.). On day 4 of successive treatment, cells were transfected with plasmid DNA preparations as described above.

RESULTS

Effect of F441 or wild-type oligonucleotides as competitors on F441 enhancer activity. A cloned DNA fragment encompassing the *Bam*HI site (nt 4632) through the *Pvu*II site (nt 5265) of the viral genome was used as an enhancer throughout the present studies (see the map of polyomavirus enhancer in Fig. 2A). Although a viral DNA fragment from the *Bcl*I site at nt 5021 to the *Pvu*II site at nt 5265 is usually used as a polyomavirus enhancer (10, 11), we used this 630-bp *Bam*HI-*Pvu*II fragment as an enhancer, since a part of an enhancer activity may be also present at the late side of the *Bcl*I site (29). Wild-type and F441 enhancer fragments were inserted in a sense orientation into a pA₁₀CAT₂ vector upstream of a *cat* gene driven by the SV40 early promoter (Fig. 2C), generating pWEN · CAT and pF441EN · CAT, respectively. Plasmid DNAs were transfected to cells, and induced CAT activities were measured. We usually transfected each plasmid DNA to cells in triplicate dishes, and CAT activities recovered from cell extracts were shown as an average of three dishes. In addition, this one-round transfection experiment was always repeated at least one more time, and the reproducibility of results was confirmed. When pF441EN · CAT and pWEN · CAT were transfected into F9 cells, the former induced roughly 10 times higher CAT activity than the latter (Table 1). A 3- to 10-fold difference in CAT activity between F441 and the wild-type enhancer was observed in a range of amounts of DNA tested from 2 through 20 μ g, at which point the expressed CAT activity reached a plateau. In contrast, both pWEN · CAT

FIG. 2. Schematic representation of the polyomavirus enhancer region. The thin solid line represents the polyomavirus genome on which several restriction sites and their sequence numbers (35, 41) are shown. The thick solid line extending from the *Bam*HI site (nt 4632) through the *Pvu*II site (nt 5265) represents the DNA fragment used as an enhancer in this study. The origin of viral DNA replication is indicated as ori, and cap sites for mRNA of the early (19) and late (7) genes are shown by the open boxes with the direction of transcription indicated. Enhancer elements A, B, C, and D, defined by Veldman et al. (43), are shown at the top. Sequence stretches found to be duplicated frequently in various polyoma F9 mutants are expanded in the bottom portion of the panel. The nucleotide at 5233 (asterisk) is A for the wild-type (W) A2 strain and G for F441 and F9-1 mutants (13, 20). The sequence CCACCCA underlined is homologous to the core of bovine papillomavirus enhancer (26). (B) Nucleotide sequence of synthesized oligonucleotides. The oligonucleotide covers the virus genome from nt 5216 to nt 5239. The ends of oligonucleotides were made so as to generate cohesive *Bam*HI sites. The nucleotide at 5233 (asterisk) is the point where the base change from A (wild type) to G (F441, F9-1) is observed. (C) Structure of CAT constructs. pA₁₀CAT₂ is a backbone vector in which the *cat* gene is driven by the SV40 early promoter (21-bp repeats and TATA box). The poly(A) addition signal is from the segment of the SV40 early gene. Into the unique *Bgl*III site, the polyomavirus enhancer fragment of nt 4632 through nt 5265 was inserted, generating the pWEN · CAT construct, bearing the wild-type enhancer, and the pF441EN · CAT construct, bearing the F441 enhancer. A cross in the enhancer fragment indicates the point mutation at nt 5233 in the F441 sequence.

and pF441EN · CAT attained approximately the same levels of CAT activity in NIH 3T3 cells, a differentiated mouse cell line (Table 1).

The oligonucleotides representing polyomavirus enhancer sequence from nt 5218 to nt 5239 with either A or G at nt 5233 were synthesized with *Bam*HI sites at both ends (Fig. 2B). This segment of DNA was chosen because it is frequently duplicated in F9 mutants, suggesting that the duplicated segment may represent a functional unit. In the original polyomavirus enhancer region, one copy each of the heptanucleotide sequence which constitutes the core sequence of the bovine papillomavirus enhancer (26) is present at both sides near nt 5233. The oligonucleotides contain only one copy of the motif at the late side of nt 5233.

We examined whether the oligonucleotide could compete with pF441EN · CAT and thus affect the level of induced

CAT activity. It has been suggested that an enhancer element must be linked in *cis* to a promoter element to achieve an efficient competition in vivo when cotransfected with a test gene controlled by that enhancer (6, 33, 45). Thus, we prepared competitors by inserting the oligonucleotide or the enhancer fragment at an upstream position of pSVpNEO (Fig. 3C). Since the backbone vector, pSVpNEO, was constructed to possess the same SV40 early promoter element as pA₁₀CAT₂, the resulting competitor genes are no different from the test gene in terms of the structural feature of the enhancer-promoter complex (compare Fig. 2C and 3C). The only difference is the presence of either a *cat* gene or a neomycin resistance gene. A constant amount (2 μ g) of a test gene, carried on plasmid pF441EN · CAT, was cotransfected into F9 cells with increasing amounts of a competitor, and CAT activity was measured. The total

TABLE 1. Comparison of relative CAT activities induced by wild-type and F441 oligonucleotides in F9, differentiated F9, and NIH 3T3 cells

Plasmid DNA transfected	Relative CAT activity		
	F9 cells	Differentiated F9 cells	NIH 3T3 cells
pA ₁₀ CAT ₂	1.0	1.0	1.0
pWS · CAT	1.7	1.2	1.6
pF441S · CAT	4.3	3.5	1.9
pWA · CAT	1.3	1.6	1.4
pF441A · CAT	4.1	4.4	1.8
pCAT · WS	1.6	1.3	1.8
pCAT · F441S	2.0	2.4	2.0
p(WA) ₂ · CAT	0.5	1.4	1.8
p(F441A) ₂ · CAT	21	15	2.9
pWEN · CAT	4.0	9.0	25
pF441EN · CAT	46	28	27

amount of transfected DNA was adjusted to 30 µg by supplementing with a carrier DNA on a backbone vector, pSVpNEO, which lacks the enhancer element. When the ratio of a test gene to a competitor lay within a range of 1:5, very little competition was detected. However, when the ratio was increased to 1:12, reduction of CAT activity was reproducibly observed. In F9 cells the CAT activity was decreased to 27% by cotransfecting with a 12-fold excess of a competitor, pF441 · NEO, harboring the F441 oligonucleotide (Fig. 3B). pW · NEO containing the wild-type oligonucleotide inhibited the CAT activity only slightly. In these experiments we assumed the CAT activity obtained by cotransfecting pF441EN · CAT with pSVpNEO to be 100%. The F441 oligonucleotide, but not the wild-type oligonucleotide, when present in excess amount, could compete efficiently with the function of the full-length F441 enhancer (Fig. 3B).

As controls, we also performed the competition experiment using the whole enhancer fragment as a competitor at the test gene, with a competitor ratio of 1:12. Competition with the wild-type enhancer reduced CAT activity to 44%, whereas competition with the F441 enhancer reduced CAT activity to 15% (Fig. 3A).

The results of *in vivo* competition experiments suggest that the F441 oligonucleotide contains a target sequence for a cellular factor and that the factor seems to be a positively functioning one, since removal of the factor in the competition assay resulted in the decrease of CAT activity.

F441, but not wild-type oligonucleotide, can enhance gene expression when linked in *cis*. Since the competition experiments described above suggested the involvement of a positive regulatory factor in the F441 enhancer, we tested directly whether the oligonucleotide itself has the ability to enhance gene expression. The wild-type or F441 oligonucleotide was inserted at an upstream position (*Bgl*II site) or downstream position (*Bam*HI site) in the vector pA₁₀CAT₂ in a sense or an antisense orientation. Each construct was transfected into F9 cells, and CAT activity was measured. The constructs containing the F441 oligonucleotide at an upstream position, in either orientation (pF441S · CAT and pF441A · CAT), showed roughly three times higher CAT activities than the constructs containing the wild-type oligonucleotide (pWS · CAT and pWA · CAT) or a backbone plasmid (pA₁₀CAT₂) (Fig. 4). The results indicate that the oligonucleotide containing the F441 mutation acts as though it has a mini-enhancer-like activity in F9 cells. The oligonucleotide with the F441 mutation placed downstream from the

cat gene (pCAT · F441S) did not induce significant levels of CAT activity. Reproducibility of the results was vigorously checked, particularly in these experiments, by using two different plasmid DNA preparations in several independent transfection experiments. It must be noted here that the CAT activities we could detect with the pF441S · CAT or pF441A · CAT constructs were relatively low: from less than 5% (Fig. 4) to about 10% (Table 1), depending on the experiments, of the activity induced by the pF441EN · CAT plasmid. Our findings that the oligonucleotides could activate the *cat* gene only weakly even with the F441 mutant sequence may seem to be inconsistent with the evidence that they interfered with the *cat* gene expression to a much greater extent in a competition assay (Fig. 3). It is conceivable, however, that the oligonucleotides have sequences necessary for a cellular factor to bind but that the binding of the factor to the sequences is not necessarily sufficient to fully activate the linked gene. It is also worth mentioning that the very short segment of the sequence, even though it represents the most important region within the enhancer, is known to function poorly by itself as an enhancer and that it works much better in conjunction with the adjacent sequences (17).

We have also evaluated the consequences of duplication of the oligonucleotide with respect to its ability to stimulate a linked promoter. The wild-type or F441 oligonucleotide was inserted as a dimer in a head-to-tail linkage at an upstream position (*Bgl*II site) of pA₁₀CAT₂. Constructs bearing the dimer in an antisense orientation were selected and compared with ones carrying the monomer of oligonucleotides in the same orientation. A duplication of F441 oligonucleotide in a CAT construct, p(F441A)₂ · CAT, further strengthened the expression of the *cat* gene in F9 cells, thus mimicking the situation seen in various F9 mutants (Fig. 5). On the other hand, wild-type oligonucleotide, when inserted as a dimer [p(WA)₂ · CAT], did not significantly alter the final CAT activity under our experimental conditions.

cat gene expression driven by the oligonucleotide was also assayed in differentiated F9 cells. Retinoic acid induces F9 cells to differentiate (37). On day 4 after the addition of retinoic acid, when morphological changes of the cells became evident in more than 90% of cells, the cells were transfected with DNA from each of the CAT constructs. The monomer (pF441S · CAT and pF441A · CAT) and the dimer [p(F441A)₂ · CAT] of F441 oligonucleotide induced a significantly higher level of CAT activity than the respective wild-type oligonucleotides (Table 1). We transfected all the constructs tested so far into all three of the cell lines in duplicate at the same time with the same DNA preparations and measured the CAT activity relative to that obtained by the control plasmid, pA₁₀CAT₂ (Table 1). *cat* gene expression was also tested in differentiated mouse fibroblasts (NIH 3T3 cells; Table 1). When monomers of oligonucleotides were compared, no significant differences could be observed between the wild-type (pWS · CAT and pWA · CAT) and F441 (pF441S · CAT and pF441A · CAT) oligonucleotides, as in the case of wild-type (pWEN · CAT) and F441 (pF441EN · CAT) enhancer fragments. However, p(F441A)₂ · CAT, harboring the dimer of the F441 oligonucleotide, induced a significantly higher level of CAT activity. Thus, the F441 oligonucleotide functions positively and increases gene expression in differentiated F9 and NIH 3T3 cells as well as in undifferentiated F9 cells, suggesting a similar mechanism involved in both cell types. Our result is consistent with the report by Herbolmel et al. (17), which

FIG. 3. Competition of CAT activity under the control of F441 enhancer in F9 cells by wild-type and F441 enhancer fragments or oligonucleotides. (A) Competition by the polyomavirus enhancer fragment, *Bam*HI-*Pvu*II. A 2- μ g sample of pF441EN·CAT DNA was cotransfected into F9 cells with 24 μ g of DNA from pSVpNEO, pWEN·NEO harboring the wild-type enhancer fragment, or pF441EN·NEO harboring the F441 enhancer fragment. Whole-cell extracts (100 μ l) were incubated with 7 μ M [14 C]chloramphenicol at 37°C for 120 min. Acetylated and nonacetylated forms were separated by thin-layer chromatography as described in Materials and Methods. (B) Competition by the oligonucleotides. A 2- μ g sample of DNA from the pF441EN·CAT construct was cotransfected into F9 cells with 25 μ g of DNA from pSVpNEO, pW·NEO harboring the wild-type oligonucleotide, or pF441·NEO harboring the F441 oligonucleotide. CAT activity assay was as described above. (C) Structure of competitor constructs. pSVpNEO is a backbone vector which has the same SV40 early promoter (21-bp repeats and TATA box) and poly(A) addition signal as pA₁₀CAT₂ (Fig. 2C) but harbors a neomycin resistance gene instead of a *cat* gene. Into the unique *Bgl*II site, various competitor sequences were inserted. For pWEN·NEO or pF441EN·NEO constructs, polyomavirus enhancer fragments (nt 4632 through nt 5265) of wild-type or F441, respectively, were inserted. For pW·NEO or pF441·NEO, oligonucleotides of wild-type or F441, respectively, were inserted. A cross in the enhancer fragment or the oligonucleotide indicates the point mutation at nt 5233.

showed that the *Pvu*II-4 fragment (*Pvu*II at nt 5128 through *Pvu*II at nt 5265) containing G at nt 5233 works better both in undifferentiated (PCC3) and differentiated (3T6) cells than the one containing A at that position.

DISCUSSION

The results presented in this communication clearly demonstrated that the F441, but not the wild-type, oligonucleotide sequence can function in a positive way to increase gene expression in F9 cells. The described characteristics of F441 oligonucleotide are consistent with the phenotype of F9 mutants. Also the minimum essential domain to be crucial for F9 mutant enhancer is contained within the oligonucleotide as the segment from nt 5218 to 5239.

It is generally accepted that multiple cellular factors interact with the polyomavirus enhancer to ensure their activity (4, 12, 30, 31). Now that the F441 oligonucleotide has been shown (this study) to enhance gene expression in F9 cells, the question would be what kind of cellular factors are involved in the activation mechanism. It has been a favored view recently that a point mutation eliminates the binding site for a negatively acting factor which is speculated to be present in F9 cells and to repress the enhancer activity of wild-type sequence (16). However, it would be difficult to explain with a mere removal of a repressor our results showing that duplication of the F441 oligonucleotide resulted in much higher CAT activity and that the F441 oligonucleotide could compete more efficiently with the F441 enhancer

FIG. 4. Effects on CAT activity of wild-type and F441 oligonucleotides in F9 cells when linked to the *cat* gene in *cis*. The oligonucleotide of wild-type or F441 sequence was inserted into the *Bgl*II or *Bam*HI site of the pA₁₀CAT₂ vector (Fig. 2C), as follows. pWS-CAT, Wild-type oligonucleotide inserted at the *Bgl*II site in the sense orientation; pF441S-CAT, F441 oligonucleotide inserted at the *Bgl*II site in the sense orientation; pWA-CAT, wild-type oligonucleotide inserted at the *Bgl*II site in the antisense orientation; pF441A-CAT, F441 oligonucleotide inserted at the *Bgl*II site in the antisense orientation; pCAT-WS, wild-type oligonucleotide inserted at the *Bam*HI site in the sense orientation; pCAT-F441S, F441 oligonucleotide inserted at the *Bam*HI site in the sense orientation. A 5- μ g sample of DNA from each construct was transfected into F9 cells. Whole-cell extracts (100 μ l) were incubated with 3.5 μ M [¹⁴C]chloramphenicol at 37°C for 120 min. Transfection was done in triplicate for each construct, and the average CAT activity is shown as a percentage in the lower half of the figure. The thin-layer chromatogram of one representative experiment is shown at the top.

fragment than could the wild-type oligonucleotide. In addition, when the F441 oligonucleotide was used as a dimer in *in vivo* competition experiments, it inhibited better than the respective monomer (unpublished data). Thus, it would be simplest to infer that the mutation created a binding site or increased the affinity for a positive regulatory factor(s). Indeed, we examined in our recent report (22) the binding of this putative cellular factor to the wild-type and F441 oligonucleotides used in this study. By mobility shift assay and footprinting techniques, a cellular factor(s) present in F9 cells was found to recognize the F441 but not the wild-type oligonucleotide. These results and results presented in this

communication together favor the notion that the mutation creates a binding site for, or strengthens the affinity to, a positively functioning factor(s). In relation to this notion, we note that the mutation at nt 5233 from A to G creates a new sequence, (5232)T-G-G-A-A-T-G(5238), which looks similar to the consensus sequence of the SV40 enhancer core, G-T-G-G-A/T-A/T-A/T-G (44). A mobility shift assay by Kovesdi et al. (22) also demonstrated that a cellular factor(s) binding to the F441 oligonucleotide is not confined to undifferentiated F9 cells but seems to be present in a variety of tissues including differentiation-induced F9 cells and NIH 3T3 cells. This result is also compatible with the finding in this report that the F441 oligonucleotide can induce *cat* gene expression more strongly than the wild-type oligonucleotide in differentiated F9 and NIH 3T3 cells.

Using a replication assay of transfected DNA, Veldman and others (43) have located four distinct functional regions (A through D) within the enhancer from the *Bcl*I (nt 5021) to the *Pvu*II site (nt 5265) (see Fig. 2A). Their analysis did not record any activity within the region that we examined in our present studies. Since they used a wild-type sequence and

FIG. 5. Effect of dimerization of the oligonucleotides on CAT activity when linked to the *cat* gene in *cis*. Wild-type or F441 oligonucleotide was inserted into the *Bgl*II site of pA₁₀CAT₂ as a head-to-tail dimer in an antisense orientation, producing the p(WA)₂-CAT and p(F441A)₂-CAT constructs, respectively. A 5- μ g sample of DNA from each construct was transfected into F9 cells. Whole-cell extracts (100 μ l) were incubated with 3.5 μ M [¹⁴C]chloramphenicol for 120 min at 37°C. The transfection was done in triplicate, and the average CAT activity is shown as a percentage in the lower portion of the figure. At the top, a thin-layer chromatogram of one representative experiment is shown.

we found that the wild-type oligonucleotide had only very low, if any, enhancing activity, it may not be surprising that they did not find any biological activities in this region represented by our oligonucleotides. Polyomavirus enhancer is required for viral DNA replication (43). It has been shown that this enhancer is responsible for tissue-specific DNA replication in F9 (13), Friend leukemia (9), and neuroblastoma (27) cells. Thus, it would be interesting to see whether the F441 oligonucleotide is more effective than the wild-type oligonucleotide when tested in DNA replication assay. These studies, in combination with the present one, may eventually explain the better growth of F441 mutant virus than of wild-type virus in F9 cells.

Böhnlein and others (3) performed a functional analysis of the region near the F441 (F9-1) mutation. They showed that removal of 40 bp (from nt 5225 through nt 5265) at the 3' end of the F441 (F9-1) *PvuII*-4 fragment resulted in a drastic drop of enhancing activity when measured in Ltk⁻ or NIH 3T3 cells. In this deleted 40-bp sequence the site of point mutation (nt 5233) was contained. On the basis of this observation, they claimed that the 3' boundary of enhancing activity of F441 (F9-1) *PvuII*-4 fragment is between nt 5225 and nt 5265. Another interesting observation made by Böhnlein and others (3) was that murine sarcoma virus enhancer fragment was able to activate gene expression in Ltk⁻ and F9 cells only when it was linked to the segment from nt 5207 to 163 of F441 (F9-1), but not when linked to the segment from nt 5241 to 163. The latter segment lacked the site of F441 point mutation. Since the segment of F441 (F9-1) enhancer between nt 5207 and 5265 functioned both as a part of the enhancer and a part of the promoter, they named this 60-bp segment the upstream promoter element (3).

Now, in light of the findings presented in this paper and our recent report (22), it is most likely that the decrease of enhancer-promoter activity observed by employing various deletions is due to the removal of a factor-binding site which functions in a positive way to increase gene expression. It must also be mentioned that Böhnlein and others (3) did not test the corresponding wild-type sequence in most of their experiments. Thus, it is not clear from their studies whether the segment of wild-type sequence has any enhancer or promoter activity. Since we demonstrated that the wild-type oligonucleotide had only a very low ability to enhance gene expression compared with the F441 oligonucleotide, the term "upstream promoter element" that they proposed is applicable only in the case of the F441 mutation at the present.

The plasmid pA₁₀CAT₂ (23) that we used in the present studies does not contain a majority of the SV40 enhancer sequence but retains a remnant of the 72-bp enhancer element that is the P motif defined by Zenke et al. (47). Thus, it is possible that the oligonucleotide used contained an enhancer-promoter subelement which in combination with this P motif formed an effective enhancer. This might account for the observation that the oligonucleotides augmented *cat* gene expression only when placed upstream of the *cat* gene (Fig. 4).

The conclusion from the present studies is consistent with the fact that F9 mutants carrying the F441 mutation can grow in F9 cells. However, one point is left unexplained, related to the case of some F9 mutants which have duplications of the oligonucleotide region without the point mutation of F441 (42). Under our experimental conditions, it was difficult to discern any significant difference between the wild-type oligonucleotide and its dimer in ability to increase gene expression in F9 cells. It may be necessary to prepare

trimers or tetramers of the wild-type oligonucleotide to test this point.

Finally, it is necessary to ask the original question again, why does wild type not grow in F9 cells? If the function of the wild-type enhancer is inhibited by a negative regulatory factor and the F441 mutation creates a binding site for a positive regulator to override the effect of negative regulator, it is important to find out this putative negative regulator protein and its binding site. Such a site could be located distantly from nt 5233. Since the region where duplication often occurs is the binding site for a positive regulatory factor, the region where deletions occur frequently is an obvious candidate for a negative factor binding site. There are several regions reported which are often deleted in mutant enhancers (21, 27, 28, 39). Examination of this hypothesis and an attempt to locate the binding sites for a negative regulator will be the subject of future studies.

ACKNOWLEDGMENTS

We thank Y. Teranishi (Laboratory of Biochemistry, Research Institute, Mitsubishi-Kasei Kogyo Co., Tokyo, Japan) for the synthesis of oligonucleotides, and G. Pavlakakis and B. Ferber (Frederick Cancer Research Facility, Frederick, Md.) for plasmids pA10CAT2 and pSV2neo and for technical advice on CAT assay.

This work was supported by a Grant-in-Aid for Scientific Research (Subject no. 60480172) from the Ministry of Education, Science and Culture, Japan.

LITERATURE CITED

- Amati, P. 1985. Polyoma regulatory region: a potential probe for mouse cell differentiation. *Cell* 43:561-562.
- Boccaro, M., and F. Kelly. 1978. Expression of polyoma virus in heterokaryons between embryonal carcinoma cells and differentiated cells. *Virology* 90:147-150.
- Böhnlein, E., K. Chowdhury, and P. Gruss. 1985. Functional analysis of the regulatory region of polyoma mutant F9-1 DNA. *Nucleic Acids Res.* 13:4789-4809.
- Böhnlein, E., and P. Gruss. 1986. Interaction of distinct nuclear proteins with sequences controlling the expression of polyomavirus early genes. *Mol. Cell. Biol.* 6:1401-1411.
- Borrelli, E., R. Hen, and P. Chambon. 1984. Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. *Nature (London)* 312:608-612.
- Brady, J., M. R. Loeken, and G. Khoury. 1985. Interaction between two transcriptional control sequences required for tumor-antigen-mediated simian virus 40 late gene expression. *Proc. Natl. Acad. Sci. USA* 82:7299-7303.
- Cowie, A., C. Tyndall, and R. Kamen. 1981. Sequences at the capped 5'-ends of polyoma virus late region mRNAs: an example of extreme terminal heterogeneity. *Nucleic Acids Res.* 9:6305-6322.
- Delli Bovi, P., V. De Simone, R. Giordano, and P. Amati. 1984. Polyomavirus growth and persistence in Friend erythroleukemia cells. *J. Virol.* 49:566-571.
- De Simone, V., G. La Mantia, L. Lania, and P. Amati. 1985. Polyomavirus mutation that confers a cell-specific *cis* advantage for viral DNA replication. *Mol. Cell. Biol.* 5:2142-2146.
- de Villiers, J., L. Olson, C. Tyndall, and W. Schaffner. 1982. Transcriptional "enhancers" from SV40 and polyoma virus show a cell type preference. *Nucleic Acids Res.* 10:7965-7976.
- de Villiers, J., and W. Schaffner. 1981. A small segment of polyoma virus DNA enhances the expression of a cloned β -globin gene over a distance of 1400 base pairs. *Nucleic Acids Res.* 9:6251-6264.
- Fujimura, F. K. 1986. Nuclear activity from F9 embryonal carcinoma cells binding specifically to the enhancers of wild type polyoma virus and PyEC mutant DNAs. *Nucleic Acids Res.* 14:2845-2861.
- Fujimura, F. K., P. L. Deininger, T. Friedman, and E. Linney. 1981. Mutation near the polyoma DNA replication origin per-

- mits productive infection of F9 embryonal carcinoma cells. *Cell* 23:809-814.
14. Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044-1051.
 15. Gorman, C. M., P. W. Rigby, and D. P. Lane. 1985. Negative regulation of viral enhancers in undifferentiated embryonic stem cells. *Cell* 42:519-526.
 16. Hen, R., E. Borrelli, C. Fromental, P. Sassone-Corsi, and P. Chambon. 1986. A mutated polyoma virus enhancer which is active in undifferentiated embryonal carcinoma cells is not repressed by adenovirus-2 E1A products. *Nature (London)* 321:249-251.
 17. Herbolmel, P., B. Bourachot, and M. Yaniv. 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell* 39:653-662.
 18. Jacob, F. 1977. Mouse teratocarcinoma and embryonic antigens. *Immunol. Rev.* 33:3-32.
 19. Kamen, R., P. Jat, R. Treisman, J. Favaloro, and W. Folk. 1982. 5' Termini of polyoma virus early region transcripts synthesized *in vivo* by wild-type virus and viable deletion mutants. *J. Mol. Biol.* 159:189-224.
 20. Katinka, M., M. Vasseur, N. Montreau, M. Yaniv, and D. Blangy. 1981. Polyoma DNA sequences involved in control of viral gene expression in murine embryonal carcinoma cells. *Nature (London)* 290:720-722.
 21. Katinka, M., M. Yaniv, M. Vasseur, and D. Blangy. 1980. Expression of polyoma early functions in mouse embryonal carcinoma cells depends on sequence rearrangements in the beginning of the late region. *Cell* 20:393-399.
 22. Kovsed, I., M. Satake, K. Furukawa, R. Reichel, Y. Ito, and J. R. Nevins. 1987. A factor discriminating between the wild-type and a mutant polyomavirus enhancer. *Nature (London)* 328:87-89.
 23. Lamins, L., G. Khoury, C. Gorman, B. H. Howard, and P. Gruss. 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. *Proc. Natl. Acad. Sci. USA* 79:6453-6457.
 24. Linney, E., B. Davis, J. Overhauser, E. Chao, and H. Fan. 1984. Non-function of a Moloney murine leukaemia virus regulatory sequence in F9 embryonal carcinoma cells. *Nature (London)* 308:470-472.
 25. Linney, E., and S. Donerly. 1983. DNA fragments from F9 PyEC mutants increase expression of heterologous genes in transfected F9 cells. *Cell* 35:693-699.
 26. Lusky, M., L. Berg, H. Weiher, and M. Botchan. 1983. Bovine papilloma virus contains an activator of gene expression at the distal end of the early transcription unit. *Mol. Cell. Biol.* 3:1108-1122.
 27. Maione, R., C. Passananti, V. De Simone, P. Delli-Bovi, G. Augusti-Tocco, and P. Amati. 1985. Selection of mouse neuroblastoma cell-specific polyoma virus mutants with stage differentiative advantages of replication. *EMBO J.* 4:3215-3221.
 28. Melin, F., H. Pinon, C. Reiss, C. Kress, N. Montreau, and D. Blangy. 1985. Common features of polyomavirus mutants selected on PCC4 embryonal carcinoma cells. *EMBO J.* 4:1799-1803.
 29. Mueller, C. R., A.-M. Mes-Masson, M. Bouvier, and J. A. Hassell. 1984. Location of sequences in polyomavirus DNA that are required for early gene expression *in vivo* and *in vitro*. *Mol. Cell. Biol.* 4:2594-2609.
 30. Ostapchuk, P., J. F. X. Diffley, J. T. Bruder, B. Stillman, A. J. Levine, and P. Hearing. 1986. Interaction of a nuclear factor with the polyomavirus enhancer region. *Proc. Natl. Acad. Sci. USA* 83:8550-8554.
 31. Piette, J., and M. Yaniv. 1986. Molecular analysis of the interaction between an enhancer binding factor and its DNA target. *Nucleic Acids Res.* 14:9595-9611.
 32. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
 33. Schöler, H. R., and P. Gruss. 1984. Specific interaction between enhancer-containing molecules and cellular components. *Cell* 36:403-411.
 34. Sekikawa, K., and A. J. Levine. 1981. Isolation and characterization of polyoma host range mutants that replicate in nullipotent embryonal carcinoma cells. *Proc. Natl. Acad. Sci. USA* 78:1100-1104.
 35. Soeda, E., J. R. Arrand, N. Smolar, J. E. Walsch, and B. E. Griffin. 1980. Coding potential and regulatory signals of the polyoma virus genome. *Nature (London)* 283:445-453.
 36. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* 1:327-341.
 37. Strickland, S., and V. Mahdavi. 1978. The induction of differentiation in teratocarcinoma stem cells by retinoic acid. *Cell* 15:393-403.
 38. Swartzendruber, D. E., and J. M. Lehman. 1975. Neoplastic differentiation: interaction of simian virus 40 and polyoma virus with murine teratocarcinoma cells *in vitro*. *J. Cell Physiol.* 85:179-188.
 39. Tanaka, K., K. Chowdhury, K. S. S. Chang, M. Israel, and Y. Ito. 1982. Isolation and characterization of polyoma virus mutants which grow in murine embryonal carcinoma and trophoblast cells. *EMBO J.* 1:1521-1527.
 40. Tanaka, K., K. Chowdhury, L. T. Liang, K. S. S. Chang, M. Israel, and Y. Ito. 1983. Relationship between growth restriction to polyoma virus and stages of differentiation in embryonic cell lines, p. 295-305. *In* J. Silver, G. R. Martin, and S. Strickland (ed.), *Teratocarcinoma stem cells*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 41. Tyndall, C., G. L. Mantia, C. M. Thacker, J. Favaloro, and R. Kamen. 1981. A region of the polyoma virus genome between the replication origin and late protein coding sequences is required in cis for both early gene expression and viral DNA replication. *Nucleic Acids Res.* 9:6231-6250.
 42. Vasseur, M., M. Katinka, P. Herbolmel, M. Yaniv, and D. Blangy. 1982. Physical and biological features of polyoma virus mutants able to infect embryonal carcinoma cell lines. *J. Virol.* 43:800-808.
 43. Veldman, G. M., S. Lupton, and R. Kamen. 1985. Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. *Mol. Cell. Biol.* 5:649-659.
 44. Weiher, H., M. Konig, and P. Gruss. 1983. Multiple point mutations affecting the simian virus 40 enhancer. *Science* 219:626-631.
 45. Weintraub, H., P. F. Cheng, and K. Conrad. 1986. Expression of transfected DNA depends on DNA topology. *Cell* 46:115-122.
 46. Wigler, M., S. Pellicer, S. Silverstein, and R. Axel. 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell* 14:725-731.
 47. Zenke, M., T. Grundström, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon. 1986. Multiple sequence motifs are involved in SV40 enhancer function. *EMBO J.* 5:387-397.