Multiple Domains in the Polyomavirus B Enhancer Are Required for Productive Infection of F9 Embryonal Carcinoma Cells

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A point mutation at nucleotide 5258 in the B enhancer of the polyomavirus host range mutant F441 leads to productive infection of F9 embryonal carcinoma cells, which are refractory to infection by wild-type polyomavirus. Specific oligonucleotides were used to construct mutations in two other potentially important domains within the B enhancer of F441 DNA. One of these domains is the binding site for a factor present in nuclear extracts of F9 cells, and the other is a region that has sequence similarity to putative core sequences observed in a number of different viral enhancers. Mutation within either of these two domains, even in the presence of the F441 mutation, was detrimental to polyomavirus enhancer activity in F9 cells, as determined by both transfection and infection assays.

The activity of the polyomavirus enhancer depends upon the differentiation state of the host cell. Mouse embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas, are refractory to infection by wild-type polyomavirus (32), probably because of the relative inactivity of the wild-type enhancer in these cells (16, 23). Differentiation of EC cells leads to cell types that are permissive for wild-type infection (11, 31). Host range mutants of polyomavirus, designated PyEC mutants, have been isolated and characterized (9, 20, 28). These mutants always have DNA sequence changes within the viral enhancer. The 245-base-pair enhancer of the polyomavirus genome can be divided into two fragments, designated A and B, that can function relatively independently as enhancers in mouse fibroblasts (6, 16, 25). However, the A and B enhancers do not seem to be equivalent for expression in EC cells. For example, PyEC mutants isolated from infection of undifferentiated F9 EC cells always have point mutations and tandem duplications of sequences in the B enhancer. PyEC mutants isolated from infection of PCC4 cells have deletions of sequences in the B enhancer and duplications of sequences in the A enhancer. Interestingly, PyEC mutants isolated from infection of PCC4 cells do not infect F9 cells, and conversely, mutants isolated from infection of F9 cells do not infect PCC4 cells (19, 28). These properties of PyEC mutants suggest that different domains within the polyomavirus enhancer are important for determining the cell specificity of the virus and that sequence alterations within the enhancer result in enhancer motifs that may be responding to the qualitative and quantitative spectra of regulatory factors that exist within a particular cell type.

The critical mutation for productive infection of F9 cells is an A · T to G · C transition at nucleotide (nt) 5258 of the polyomavirus A3 genome (9, 28). This mutation, located within the B enhancer of the PyEC mutant F441, is absolutely required for productive infection of F9 cells. Only the G · C nucleotide pair at position 5258, and not the three other possible sequences, is functional in F9 cells (33). Although the F441 mutation is necessary for infection of F9 cells, it is not known whether other regions within the B

enhancer are also required for productive infection of F9 cells. We have mutagenized two other putative domains within the B enhancer to determine the importance of these domains for infection of F9 cells. One domain is the binding site for a nuclear factor present in F9 cells (nuclear-factor-binding site [NFBS]) (2, 8, 26); the other has a sequence that resembles the consensus sequence of a putative core element first observed in the simian virus 40 (SV40) enhancer (36). Mutation at either of these sites, within the context of the F441 mutation, was detrimental to productive infection of F9 cells.

MATERIALS AND METHODS

Cell cultures and virus infections. Methods for growth and virus infection of F9 and 3T6 cell lines were as described previously (9). F9 cells were induced to differentiate in the presence of 1 μ M retinoic acid (30) as described previously (11). Infected cells were analyzed for viral early gene expression by measurement of immunofluorescence of tumor (T) antigens (9). Viral DNA in infected cultures was assayed by blot hybridization as described previously (10). The multiplicity of infection (MOI) and time after infection of assays for individual experiments are indicated below.

Plasmids. The *Bam*HI-*Sac*I fragment of F441 DNA was cloned into the corresponding sites within the polylinker of M13mp19, and the single-stranded phage DNA of the resulting construct was used as the substrate for oligonucleotide-directed mutagenesis. Mutations were introduced back into the entire polyomavirus genome by ligation of the mutated *Bam*HI-*Bgl*II fragment from the replicative-form DNA of M13mp19 constructs with the 4.5-kilobase *Bgl*II-*Bam*HI fragment of wild-type polyomavirus DNA as described previously (9).

Vectors pAOPCATT and pXOPCATT (see Fig. 2) were constructed by multiple cloning (24) into vector pUC18NX, a derivative of pUC18 in which an *Xho*I linker had been introduced at the *Nar*I site. The 200-base-pair polyomavirus fragment spanning from the *Mbo*I site at nt 2784 to the *Hinc*II site at nt 2986 and containing the polyadenylation signals was cloned into the compatible *Bam*HI and *Sma*I sites of pUC18NX to produce pT-3. A polyomavirus DNA fragment (AOP) spanning from the *Bcl*II site at nt 5047 to the *Bst*XI site at nt 183 and containing the A enhancer, the origin of replication, and the early promoter was inserted by use of

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TABLE 1. Transfection assays for B enhancer activity

Insert	CAT activity ^a in:			
	F9 cells with:		3T6 cells with:	
	pX plasmid	pA plasmid	pX plasmid	pA plasmid
None	0.33 (1.0)	0.86 (1.0)	0.75 (1.0)	15.46 (1.0)
A enhancer ^b	0.69 (2.1 ± 0.6)	NA	25.63 (34.2 ± 12.1)	NA
Wild type	0.44 (1.3 ± 0.4)	9.74 (11.3 ± 8.0)	3.74 (5.0 ± 2.3)	19.17 (1.2 ± 0.1)
F441	2.62 (7.9 ± 2.7)	21.81 (25.4 ± 16.7)	15.68 (20.9 ± 7.2)	23.81 (1.5 ± 0.1)
BstEII/F441	0.86 (2.6 ± 1.0)	6.57 (7.6 ± 3.5)	5.42 (7.2 ± 2.9)	29.22 (1.9 ± 0.3)
NruI/F441	1.05 (3.2 ± 1.9)	7.19 (8.4 ± 5.2)	5.84 (7.8 ± 4.9)	24.43 (1.6 ± 0.2)

^a The CAT activities represent the percent transfer of radioactivity from [¹⁴C]acetyl coenzyme A to chloramphenicol in 2 h as assayed at 37°C by the procedure of Sleigh (29) as described in Materials and Methods. The numbers in parentheses represent CAT activities ± standard deviations normalized to the activity of the vector without an insert.

^b Values are for pAOPCATT assayed concurrently with the pX series of plasmids. NA, Not applicable.

linkers between the *Xho*I and *Hind*III sites of pT-3 to produce pAOPT. The polyomavirus AOP fragment lacked the B enhancer through deletion of the *Pvu*II-4 fragment with insertion of an *Sma*I linker at the site of deletion. The *Hind*III-*Bam*HI fragment of pSV0-cat (12) containing the chloramphenicol acetyltransferase (*cat*) gene was cloned into the corresponding sites of pAOPT to produce pAOPCATT. pXOPCATT was constructed by removing the *Xho*I-*Sma*I A enhancer fragment from pAOPCATT and inserting a synthetic polylinker which reconstructed the *Xho*I and *Sma*I sites and introduced unique *Bgl*II and *Eco*RV sites. The *Pvu*II-4 fragments (B enhancer) of appropriate wild-type and mutant polyomavirus DNAs were cloned by blunt-end ligation into the *Sma*I sites of pXOPCATT and pAOPCATT, respectively, to obtain the pX and pA series of constructs. All constructs used in these studies had the polyomavirus DNA fragments arranged in the natural orientation with respect to each other. The reading frame of the *cat* gene with respect to the polyomavirus fragments in these constructs was the same as that of the polyomavirus early genes in the viral DNA.

Site-directed mutagenesis. Mutagenesis was done by the two-primer method of Zoller and Smith (38) with the M13mp19 construct mentioned above and appropriate oligonucleotides as described elsewhere (33).

Transient transfection assays. F9 and 3T6 cells were transfected with calcium phosphate precipitates of plasmid DNAs by a modification (33) of the method of Chen and Okayama (4). Preparation and assay of cell extracts for CAT enzyme

activity were performed by the method of Sleigh (29) with minor modifications (33). There was some variation in CAT activities from experiment to experiment, but the relative activities among different constructs were reproducible. For CAT activities shown in Table 1, all data within one column were obtained from transfections performed at the same time and repeated at least twice. Thus, it is valid to compare the CAT activities within one column in Table 1 but not between different columns.

RESULTS

Figure 1 shows the sequences of the two mutants constructed for this study by oligonucleotide-directed mutagenesis. These two mutants were made to introduce unique restriction sites at two potentially important regions within the B enhancer. These two regions are the NFBS (2, 8, 26) and a region having sequence similarity to a putative core sequences present in several viral enhancers, including that of SV40 (SV40 core) (36). A *Bst*EII site (GGTNACC) was constructed at nts 5191 to 5197 in the NFBS, and an *Nru*I site (TCGCGA) was introduced near the SV40 core-like sequences at nts 5221 to 5226. Both of the mutants were constructed in F441 backgrounds.

The *Pvu*II-4 fragments of these mutants as well as those of the wild type and F441 were assayed for the enhancement of *cat* expression in F9 and 3T6 cells with the enhancer trap vectors pXOPCATT and pAOPCATT. Both vectors, whose structures are shown in Fig. 2, contain a bacterial *cat* gene

FIG. 1. Nucleotide sequences of polyomavirus enhancer mutants. The A and B enhancers of polyomavirus DNA with *Bcl*I and *Pvu*II restriction sites (with the corresponding nucleotide numbers in parentheses) are shown. The locations of sequences similar to putative core sequences in the enhancers of the adenovirus E1A gene (14) and the SV40 early promoter (36), of the NFBS (2, 8, 26), and of the F441 mutation are indicated. The sequence shown corresponds to nts 5181 to 5260 of the early strand of wild-type polyomavirus A3 DNA (5). The NFBS and SV40 core sequences are underlined. Sequence changes in the mutants, F441, BstEII/F441, and NruI/F441, are shown.

FIG. 2. Structures of *cat* vectors pAOPCATT and pXOPCATT. The broken lines indicate pUC18NX sequences. pAOPCATT contains the A enhancer (A), the replication origin (O), and the early promoter (P) of polyomavirus linked to a bacterial *cat* gene (CAT) and followed by the polyomavirus polyadenylation signal (T). pXOPCATT was derived from pAOPCATT by deletion of the A enhancer and insertion of a synthetic polylinker into the site of the deletion. Several restriction sites are shown. Restriction sites destroyed during the construction of these plasmids are shown in parentheses. The top map represents the nucleotide numbers and restriction sites of polyomavirus (Py) DNA relevant to the plasmid constructs. The B enhancers of appropriate polyomavirus DNAs were inserted by blunt-end ligation of the corresponding *PvuII*-4 fragments into the *SmaI* sites of pXOPCATT or pAOPCATT to obtain the pX or pA series of constructs, respectively.

driven by the polyomavirus early promoter. Because they lack the B enhancer through deletion of the *PvuII*-4 fragment of polyomavirus DNA, both vectors are enhancer dependent for the expression of CAT activity in F9 cells. Neither vector has functional T-antigen-coding sequences, so they do not replicate in transfected cells unless a functional T antigen is provided in *trans* (data not shown). *PvuII*-4 fragments of appropriate polyomavirus DNAs were blunt-end ligated into the *SmaI* site of each vector to analyze the effects of the B enhancer on *cat* expression by these vectors in the absence (pX series) or in the presence (pA series) of the A enhancer.

Table 1 shows the CAT activities in F9 and 3T6 cell extracts prepared 48 h after transfection with these plasmids. The effects of the mutations are most evident in the results of transfection with the pX series of plasmids. The F441 mutation activated the wild-type B enhancer in both F9 and 3T6 cells. Introduction of either the *BstEII* or the *NruI* mutation into the F441 B enhancer lowered its activity in both F9 and 3T6 cells. For the pX series of plasmids, the reduced activity of the B enhancer resulting from these mutations was still higher than the activity of the wild-type B enhancer in both of these cell lines. A similar response was seen for the pA series of plasmids in F9 cells. In the case of the pA series of plasmids in 3T6 cells, the high activity of the polyomavirus A enhancer in these cells masked much of the effects resulting from these mutations in the B enhancer.

To ensure that the effects of the *BstEII* and *NruI* mutations on CAT activities observed in these plasmid constructs were reflecting relevant biological properties, we constructed complete polyomavirus genomes having these mu-

tations and propagated viral stocks in 3T6 cells. Because the A enhancer was not altered in these mutants, they grew efficiently in 3T6 cells. This was not the case for F9 cells. Table 2 shows polyomavirus early gene expression measured by immunofluorescence of viral T antigens. It was clear that both the *BstEII*/F441 and *NruI*/F441 mutants expressed very low levels of T antigen in F9 cells, as compared with F441. The levels of T antigen expressed by these two mutants in F9 cells were similar to the level expressed by wild-type polyomavirus in F9 cells and were at least 30-fold lower than the level expressed in F441-infected F9 cells. Although all four viruses expressed T antigen efficiently in 3T6 cells, the *NruI*/F441 mutant consistently showed lower levels of T-antigen expression than did the other three viruses.

TABLE 2. T-antigen expression in infected cells

Virus	T-antigen immunofluorescence ^a in:	
	F9 cells	3T6 cells
Wild type	0.045 (1.00)	77.2 (1.00)
F441	1.350 (30.00)	74.0 (0.96)
<i>BstEII</i> /F441	0.060 (1.33)	73.1 (0.95)
<i>NruI</i> /F441	0.006 (0.13)	23.2 (0.30)

^a Values represent the percentage of cells positive for T-antigen immunofluorescence. Numbers in parentheses represent values normalized to a wild-type infection for each cell line. F9 cells were infected at an MOI of 200 PFU per cell and assayed for T antigen 48 h postinfection; 3T6 cells were infected at an MOI of 20 PFU per cell and assayed 24 h postinfection.

FIG. 3. Viral DNA replication in infected cells. Cultures of F9 cells (A), F9 cells induced to differentiate with retinoic acid (B), and 3T6 cells (C) were infected with the indicated viruses, and viral DNAs from infected cells were analyzed by blot hybridization. F9 cultures were infected at an MOI of 50 PFU per cell and assayed 72 h postinfection, differentiated F9 cells were infected at an MOI of approximately 1 to 4 PFU per cell and assayed 72 h postinfection, and 3T6 cells were infected at an MOI of 1 PFU per cell and assayed 48 h postinfection. WT, Wild type.

Figure 3 shows the amounts of viral DNA in infected cells as measured by blot hybridization. These levels of viral DNA closely paralleled the levels of T antigen measured by immunofluorescence. In the case of F9 cells (Fig. 3A), infection with wild-type polyomavirus resulted in very low levels of viral DNA, as would be expected. F441 infection of F9 cells resulted in about 50-fold more viral DNA than did wild-type infection. The *BstEII* and *NruI* mutations both reduced the level of viral DNA in F9 cells, as compared with F441. The amount of viral DNA in *BstEII*/F441-infected F9 cells was about 10% the amount in F441-infected F9 cells, while that in *NruI*/F441-infected F9 cells was about the same as that in wild-type-infected cells. For comparison, Fig. 3A also shows results for F101-infected F9 cells. F101, which has two copies of the F441 mutation because of a tandem duplication of B enhancer sequences (9), accumulated about fivefold more viral DNA than did F441 in F9 cells. This result is consistent with previous observations that F101 is 5 to 10 times more efficient than F441 in infecting F9 cells (9).

Figure 3B shows the levels of viral DNA found in F9 cells induced to differentiate in the presence of retinoic acid (30) and then infected with the polyomavirus mutants. It is clear that differentiation of F9 cells resulted in efficient replication of all four viruses. Similar results were observed for 3T6 cells infected with these viruses (Fig. 3C). Although all four viruses replicated efficiently in 3T6 cells, the *NruI*/F441 mutant showed lower levels of replication than did the other three viruses, paralleling the lower efficiency of T-antigen expression by the *NruI*/F441 mutant in 3T6 cells (Table 2).

DISCUSSION

Other work from our laboratory has shown that the F441 mutation at nt 5258 is absolutely required for polyomavirus infection of F9 cells (33). The results of the present experiments indicate that at least one other domain within the B enhancer, defined by the *BstEII* and *NruI* mutations, is required in conjunction with the domain defined by the F441 mutation for enhancer activity in F9 cells. That the F441 mutation lies in an enhancer domain distinct from that defined by the *BstEII* and *NruI* mutations is suggested by the

fact that PyEC mutants selected for growth on F9 cells usually have tandem duplications of B enhancer sequences encompassing and including the F441 mutation (9, 28), but these duplications usually do not include the region of the B enhancer mutagenized here. The domain(s) defined by the *BstEII* and *NruI* mutations lies within the region of the B enhancer defined by deletion analyses to be important for B enhancer function in mouse fibroblasts with regard to the enhancement of both transcription and replication (1, 13, 35). In contrast, deletion of sequences encompassing nt 5258 is not severely detrimental for B enhancer activity in mouse fibroblasts. Similarly, viable mutants with deletions of nt 5258 also have been selected on PCC4 EC cells (20).

Do the *BstEII* and *NruI* mutations lie within a single element of the B enhancer or do they define two distinct elements? Several studies suggest that these two mutations lie in distinct enhancer elements. The deletion analyses performed by Veldman et al. (35) suggested two possible elements, designated B and C, which happen to coincide with the regions around the *NruI* and *BstEII* mutations, respectively. Furthermore, these two regions of the B enhancer can bind to at least two distinct nuclear factors in vitro. Element C (*BstEII*) corresponds to the binding site for a nuclear factor initially identified in F9 cells but also present in various other cell lines (2, 8, 26). Element B (*NruI*) is bound by EBP-20, a nuclear factor isolated from rat liver that binds to similar sequences in several viral enhancers (18). Thus, there may be three separate elements within the polyomavirus B enhancer, all of which must be functional to ensure enhancer activity in F9 cells. These elements are defined by the F441, *NruI*, and *BstEII* mutations. It may be significant that EBP-20, while showing one primary binding site in the polyomavirus enhancer as determined by DNase I footprinting, shows two secondary footprints in addition to the primary one at higher concentrations (18). The primary footprint lies in element B (*NruI*), while the two secondary footprints lie in element C (*BstEII*) and the region adjacent to the site of the F441 mutation, indicating that these three potential elements in the B enhancer may somehow interact with each other in the presence of an appropriate protein factor(s).

The present experiments indicate that the *BstEII* and *NruI* mutations affect both T-antigen expression and viral DNA replication (Table 2 and Fig. 3). This effect could be due to the dependence of viral DNA replication on either a functional T antigen or a functional enhancer or both. Mixed-infection experiments (data not shown) verified that these two mutations directly affect viral DNA replication in F9 cells, as the two mutants were not rescued by F441. This result is similar to the previous observation that wild-type polyomavirus is not rescued by F441 during a mixed infection of F9 cells (10). Thus, these two mutations both affect enhancer activity with regard to DNA replication as well as transcription. As has been suggested previously (7), the same enhancer elements of polyomavirus may be involved in both transcription and DNA replication, perhaps implying that some cellular factors may have dual roles in the enhancement of these two processes.

It has been suggested that F9 cells have a negative regulatory factor, perhaps having some functional similarity to the adenovirus E1A gene products (17), which represses the activity of the wild-type polyomavirus enhancer (3, 34). It has been reported that the F441 point mutation is not sensitive to negative regulation by either the E1A gene products (15) or the E1A-like activity in F9 cells (27). Recent observations that at least one nuclear factor can bind pref-

erentially to the F441 sequence over the wild-type sequence (21, 37) suggest that, even though negative regulation may be relevant, the F441 mutation must also have an active role in enhancer activation in F9 cells. This suggestion is consistent with the observation that the F441 mutation appears to increase the activity of the B enhancer in both F9 and 3T6 cells. Furthermore, we have observed that only the F441 sequence, and not the three other possible sequences, at nt 5258 can activate polyomavirus enhancer function in F9 cells (33).

Present results suggest that the A enhancer has different roles in 3T6 and F9 cells. The apparent superiority of the A enhancer over the B enhancer in 3T6 cells is in agreement with the results of Herbolme et al. (16) but not with those of Bohnlein et al. (1). The F441 mutation appears to increase the activity of the B enhancer in both F9 and 3T6 cells, and our results support the hypothesis presented by Herbolme et al. (16) that the F441 mutation activates the polyomavirus genome in F9 cells by creating an active enhancer motif that can function in these cells. The F441 enhancer motif, however, is not active in PCC4 EC cells, as polyomavirus mutants isolated from F9 cells do not infect PCC4 EC cells (19, 28).

Activation of the wild-type polyomavirus genome during differentiation of F9 cells may occur by a separate mechanism not related to activation at the F441 site. Perhaps relevant to this is the observation by Kryszke et al. (22) that the intranuclear levels of a factor binding to the E1A-like core sequence in the A enhancer (PEA1) increase during differentiation of F9 cells. This increase may result in activation of the A enhancer during F9 cell differentiation but, even if this is so, there probably are other events taking place because the wild-type B enhancer also appears to be activated during F9 cell differentiation (23). It appears that very specific combinations of *cis*-acting regulatory elements are required for enhancer function in a particular cell type. The particular combination of *cis* elements of the polyomavirus enhancer that are active within a specific cell type may correspond to recognition sites for potential *trans*-acting regulatory factors in the cell.

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