Effect of Silencer on Polyomavirus DNA Replication

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We have cloned the cellular sequence termed box DNA from the enhancer region of polyomavirus F9 mutant fPyF9. Box DNA functions as a negative transcriptional element (silencer) in undifferentiated F9 cells but not in differentiated L cells. Plasmid DNAs containing the origin and enhancer of polyomavirus were used to measure simultaneously transcriptional and replication activities in transfected cells. DNA replication activity was significantly reduced under conditions in which the silencer was able to reduce enhancer activity in F9 cells. On the other hand, when the silencer could not repress enhancer activity in MOP-8 cells, which are mouse NIH 3T3 cells producing polyomavirus T antigen constitutively, replication activity was still intact. The silencer itself had no effect on DNA replication or transcription in either type of cells. Furthermore, the insertion of a 6-base oligonucleotide within a consensus sequence of box DNA abolished the repressive effect of the silencer on DNA replication and enhancer activities. These results suggest that enhancer factors, interacting with silencer factors, may be closely associated with the mechanism of replication.

Gene expression in eucaryotic cells is believed to be controlled by trans-acting factors and a cis-acting transcriptional element such as the promoter, enhancer, or silencer (33). Recent studies on the β-globulin gene (1) and the simian virus 40 and adenovirus genes (11) suggest that DNA replication plays an important role in transcriptional activation. Activations of DNA replication and transcription may be closely related. An interesting question is how the transient breakdown of higher-order chromatin structure brought about by DNA replication can affect transcription.

The silencer, the negative regulatory element originally discovered at the MAT locus in Saccharomyces cerevisiae (6), is known to be an opposite counterpart of the enhancer. The S. cerevisiae silencer has multiple functions, including autonomous replication and centromerelike segregation functions (23). Furthermore, transcriptional repression by the silencer is closely associated with repression of autonomous replication (1). This fact shows that transcriptional control by the silencer, like that by the enhancer, may require DNA replication. Therefore, the interactions between silencer and enhancer are thought to regulate both RNA transcription and DNA replication.

Virus systems have provided good tools for studying RNA transcription and DNA replication. In the transcriptional machinery of simian virus 40, sequence-specific trans-acting factors such as the TATA box factor (18), Sp1 and Sp2 in promoter sequences (1), and AP1, AP2, AP3, and AP4 in enhancer sequences (25) have been identified. In addition, the simian virus 40 ori-core sequence responds to transcriptional functions (8–10, 19, 21, 27). Polyomavirus (PyV) also offers a good model system for studying the correlation between transcriptional activation and DNA replication. An enhancer element, consisting of six GGGCGG boxes, is required for PyV ori-dependent DNA replication (12, 28, 37).

We have cloned the PyV mutant fPyF9, which is capable of replicating in an episome in undifferentiated F9 cells (2). fPyF9 contains three copies of cellular sequences in the enhancer region; the consensus sequence of the inserts, GCATTCCATGTTGTCAAAAAG, is termed box DNA (2). Box DNA has been found to function as a negative regulatory element (silencer) in undifferentiated cells by an enhancerlike mechanism (3).

Here we describe the interaction of enhancer and silencer in DNA replication. A chimeric plasmid containing enhancer, silencer, and ori repressed transcriptional enhancer activity as well as PyV DNA replication. The results suggest that the interaction of silencer and enhancer plays an important role in regulating transcription and DNA replication.

MATERIALS AND METHODS

Construction of CAT plasmids. The nucleotide-numbering scheme for PyV proposed by Soeda et al. (35) was used. All plasmids used in this study are depicted schematically in Fig. 1. To construct pUC-CAT, the chloramphenicol acetyltransferase (CAT) fragment was obtained by digestion of pSV2CAT (15) with HindIII and BamHI, and the BamHI site was changed to a HindIII site by use of Klenow fragment, followed by ligation of the HindIII linker. The treated CAT fragment was inserted into the HindIII site of pUC19. The parental plasmid, pPyNRS-CAT(+), was constructed by introduction of a PyNRS fragment into pUC-CAT. The PyNRS fragment spans from the BclI site (nucleotide [nt] 5021) to the HphI site (nt 162) of PyhrN2, which is a PyV host-range mutant capable of replicating in undifferentiated F9 cells (34). The PyhrN2 DNA has the same sequences as does the wild type (wt) in the region containing the origin, promoter, and RNA starting points. Both ends of the PyNRS fragment were rendered blunt with Klenow fragment and T4 polynucleotide polymerase, added with the PsI linker at both ends, and cloned into the PsI site of pUC-CAT.

pPyNRS-CAT(−) was constructed as follows. pPyNRS-CAT(+) was cleaved with SmaI and partially with HindIII, and the end of the isolated PyNRS-CAT gene was blunted, added with SalI linker, and cloned into the SalI site of pUC19. For construction of pOri-CAT, after digestion of

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input DNA and the DpmI-resistant newly replicated DNA (24). The single BamHI site was located within the poly- 
l linker region of the test plasmid. The digested DNAs were 
fractionated by 1% agarose gel, blotted onto nitrocellulose 
filters (36), and hybridized with a 32P-labeled HindIII frag- 
ment of pUC-CAT in buffer containing 3 x SSC (SSC is 0.15 
M NaCl plus 0.015 M sodium citrate) x Denhardt solution, 
50 mM Tris (pH 7.5), 20 μg of salmon sperm DNA per ml, 1 
mM EDTA, and 50% formamide at 42°C. The filters were 
dried, washed, and autoradiographed. 

Transfection of F9-28 cells was performed by the calcium 
phosphate precipitation method (16). Briefly, F9-28 cells 
were plated at a density of 2 x 107/100-mm-diameter dish, 
and 20 μg of DNA was left on the cells for 30 min at room 
temperature in buffer (1% dextran, 140 mM NaCl, 5 mM 
KCl, 1 mM Na2HPO4, 20 mM N-2-hydroxyethylpiperazine-
N’-2-ethanesulfonic acid [HEPES; pH 7.05]), during which 
time the cells were shaken every 10 min. The cells were 
refed with 5 ml of fresh medium and incubated for 5 h. After 
the transfection mixture was removed, 4 ml of 15% glycerol 
was added to the medium. At 1, 2, or 3 days posttransfection, 
DNAs were extracted and analyzed as described above.

**CAT assay.** Transfection of F9-28 and MOP-8 cells was 
performed by the calcium phosphate precipitation method as 
described above. In the case of MOP-8 cells, 104 cells were 
transfected with 5 μg of DNA. At 48 h after transfection, 
the cells were harvested with a rubber policeman, suspended 
in 200 μl of 0.25 M Tris hydrochloride (pH 7.8), and 
dispersed by three cycles of freeze-thawing, followed by sonication. 
Concentrations of protein in the extracts were determined by 
the Bradford method (5) to normalize the reaction. For F9-28 
cells, 2 x 106 cells were transfected with 20 μg of DNA; 
other procedures were as described above for MOP-8 cells.

CAT assays were carried out as described by Gorman et al. (15). Typically, the reaction mixture contained 0.15 μCi 
of dichloroacetyl-1,2-[14C]chloramphenicol, 7 mM acetyl 
coenzyme A, 250 mM Tris hydrochloride buffer (pH 7.8), 
and 25 to 50 μl of cell extract. Reaction mixtures were 
natated at 37°C for 2 to 4 h and extracted with cold ethyl 
acetate; the organic phase was removed and dried. The 
residue was dissolved in 15 μl of ethyl acetate, spotted on a 
thin-layer chromatography plate, chromatographed in buffer 
containing chloroform-methanol (95:5), and autoradiographed. 
Densities of the spots corresponding to the acetylated 
and nonacylated forms were quantitated by computer 
analysis with an image-analyzing program (MICRONOVA 
version 2.09). CAT activity was calculated as acetylated/ 
total chloramphenicol; values varied within approximately 
20% among three independent transfection experiments. 
Generally, 25 μl of extract from MOP-8 cells transfected 
with pPyNRS-CAT (positive control) converted approximately 
40% of the chloramphenicol to the acetylated form 
after 2 h of incubation. The same 40% conversion in F9-28 
cells required 50 μl of pPyNRS-CAT-transfected cell extract 
and 4 h of incubation.

**RESULTS**

*Isolation and characterization of F9-28 cells.* F9-28 cells (F9 
cells carrying pPyF9 at high copy number) were obtained by 
subcloning from F9 cells to yield a high level of T antigen. 
Therefore, F9-28 cells should be useful for our purposes 
as MOP-8 cells, which are NIH 3T3 cells producing T 
tant. To determine the replication efficiencies of the CAT 
plasmids pOri-CAT, pB(+)-PyNRS-CAT, and pPyNRS--
CAT(+) after Southern blot analyses, a probe specific to the CAT gene was used to differentiate between replicated pPyF9 and plasmid-transfected F9-28 cells, since the CAT gene is contained in all of the test plasmids but not in pPyF9.

Box DNA silencer represses DNA replication activated by the enhancer. The replication efficiencies of the CAT plasmids pOri-CAT, pB(+)PyNRS-CAT, and pPyNRS-CAT(+) were measured after transfection of DNAs into MOP-8 and F9-28 cells, both of which constitutively express PyV T antigen. At various days after transfection, low-molecular-weight DNA was extracted by the Hirt method (20), cut with BamHI and DpnI to digest methylated input DNA, and subjected to Southern analysis (Fig. 2). pOri-CAT was shorter by 241 base pairs than pPyNRS-CAT(+) and pPyNRS-CAT(-). Replication activities of DNAs were compared with the density of the linear-form DNA. DpnI-resistant linearized fragments were observed in two CAT plasmids, pPyNRS-CAT and pPyNRS-CAT containing box DNA [pB(+)PyNRS-CAT] but not in pOri-CAT, which was deleted in the enhancer region (BclI-PvuII fragment; Fig. 1) (Fig. 2). The CAT plasmid containing the PyhrN2 enhancer, pPyNRS-CAT(+), replicated well in both MOP-8 and F9-28 cells, showing that the PyhrN2 enhancer could significantly stimulate ori-dependent DNA replication in both types of cells. The wt PyV enhancer was capable of stimulating replication only in MOP-8 cells, as previously reported (data not shown). The CAT plasmid containing box DNA, pB(+)PyNRS-CAT, replicated at lower levels during 3 days than did box DNA-less pPyNRS-CAT in F9-28 cells. However, in MOP-8 cells, the levels of replication of both plasmids were similar even in the presence of box DNA, suggesting that DNA replication activation by the enhancer is inhibited by box DNA silencer only when the silencer functions in F9 cells.

Transcription and DNA replication repressed by the silencer. To test the extent of repression by the silencer in both transcription and DNA replication activities were measured by using the CAT plasmids pOri-CAT, pPyNRS-CAT, and pPyNRS-CAT. DNA replication activity was determined by Southern blot analysis, and transcriptional activity was assayed by measuring CAT expression in cell extracts. High levels of DNA replication were correlated with strong transcriptional activity [pPyNRS(+)CAT and pPyNRS(-)CAT in F9 cells; all plasmids except pOri-CAT and pB-Ori-CAT in MOP-8 cells; Fig. 3]. On the other hand, lower levels of both activities [pB(+)PyNRS-CAT, pB(-)PyNRS-CAT, pPyNRS-B(+)-CAT, and pPyNRS-B(-)-CAT in F9 cells] were dependent on the effect of box DNA silencer in the CAT plasmids tested. These results suggest that the presence of box DNA has a direct negative effect on promoter and ori-core se-

![Figure 2](image1.png)  
**FIG. 2.** Time course experiment showing the effect of box DNA silencer on enhancer-activated DNA replication in F9-28 (A) and MOP-8 (B) cells. A 1- or 20-µg amount of DNA was transfected into MOP-8 or F9-28 cells, respectively, and extracted by the Hirt procedure (20) at 1, 2, and 3 days after transfection; one-fourth of the DNA obtained was digested with BamHI and DpnI and subjected to Southern blot analysis, using a probe plasmid pB-Ori-CAT (Ori) pPyNRS-CAT (PyNRS), or pB(+)PyNRS-CAT [B(+)PyNRS]. In lane M, 100 copies of pPyNRS-CAT(+) linearized with BamHI per cell (►) was used as a copy marker.

![Figure 3](image2.png)  
**FIG. 3.** Repression of DNA replication by box DNA (A) in parallel with the reduction in CAT activity (B). Transient replication assays were carried out as described in the legend to Fig. 2. In the CAT assays, 5 or 20 µg of CAT plasmid DNA was transfected into F9-28 (top panels) or MOP-8 (bottom panels) cells, respectively, and cell extracts were prepared at 48 h after transfection; 25 µl of MOP-8 extract was incubated at 37°C for 2 h, and 50 µl of F9-28 extract was incubated at 37°C for 4 h. Acetylated and nonacetylated forms of chloramphenicol were then separated by thin-layer chromatography. Plasmids used for transfection are shown above the lanes, abbreviated as in the legend to Fig. 2. ▶, Position of BamHI-linearized pPyNRS-CAT (lane M).
Polyomavirus silencer and enhancer

sequences in the ori fragment (PvuII [nt 5262]-HphI [nt 162]), repressing both activities. There was little if any difference between pOri-CAT and pB(+)Ori-CAT in either DNA replication or transcriptional activity in F9 and MOP8 cells, suggesting that the sequences within promoter and ori-core may not be affected by the presence of box DNA.

Box DNA silencer represses DNA replication by an enhancerlike mechanism. The monomer of box DNA was inserted into the BamHI site of pPyNRS-CAT(+) and pPyNRS-CAT(--), and the resultant pB(+)PyNRS-CAT, pB(--PyNRS-CAT, pPyNRS-CAT-B(+), or pPyNRS-CAT-B(--) was used to examine the effect of position and orientation of box DNA on silencing action (Fig. 3). Results showed that the efficiency of PyhrN2 DNA replication decreased independently of orientation [compare pB(+)PyNRS-CAT with pB(--)PyNRS-CAT and pPyNRS-B(+)CAT with pPyNRS-B(--)-CAT] and position [compare pB(+)PyNRS-CAT with pPyNRS-B(+)CAT and pB(--)

PyNRS-CAT with pNRS-B(--)-CAT] of box DNA relative to the promoter.

Mutation analysis of box DNA. To confirm the repression of DNA replication by box DNA silencer, mutation analysis was carried out. An Xhol linker (CTCGAG) was inserted in the box DNA of the CAT plasmids used in the experiments described above. The results of CAT activity were consistent with the results presented in the accompanying paper (3), which show that the consensus sequence of box DNA is important for silencer action. When the transcriptional effect of box DNA was inhibited by the mutation at the center of the consensus sequence of box DNA, M1 (GCATTCCTC...)}
TABLE 1. Repressive activity of box DNA in transcription and DNA replication

<table>
<thead>
<tr>
<th>CAT plasmidb</th>
<th>Regulatory elementa</th>
<th>Relative activity</th>
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<td>Sil</td>
<td>Enh</td>
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<td>pUC-CAT</td>
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<td>pOri-CAT</td>
<td>+</td>
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<tr>
<td>pPyNRS-CAT(+)</td>
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<td>pM3-PyNRS-CAT</td>
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<tr>
<td>pPyNRS-CAT(-)</td>
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<tr>
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<td>pPyNRS-CAT-M3</td>
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a. All plasmids were constructed as described in Materials and Methods.
b. +, Presence of a regulatory element, from upstream to downstream of the CAT gene. Abbreviations: Sil, silencer; Enh, enhancer; ori, origin of DNA replication; CAT, chloramphenicol acetyltransferase.

c. Transient replication assays were performed as described in Materials and Methods. The results of Fig. 3 and 5 are summarized. All values are expressed relative to that of the positive control plasmid, pPyNRS-CAT(+)(++)+. Significant lower activity than that of the positive control; +, lowest level of activity.

d. Determined as described in Materials and Methods. The results of Fig. 3 and 4 are summarized. Relative values were obtained by considering the activity of pPyNRS-CAT(+), the positive control plasmid, as 100.

GAGCATTTGTTCATAAAG (Fig. 4), the level of DNA replication increased (Fig. 5). Insertion of an XhoI linker at the ends of consensus sequences (M2) and out-of-consensus sequences (M3) had a little and no effect on DNA replication and transcription, respectively. This effect of mutated box DNA on both activities was independent of position (pM1-PyNRS-CAT and pPyNRS-M1-CAT, for example). The results obtained (summarized in Table 1) indicate that box DNA is capable of repressing DNA replication as a result of the reduction in enhancer activity.

**DISCUSSION**

In this study, we showed that box DNA silencer represses PyV ori-dependent DNA replication by inhibition of transcriptional enhancer activity. For this experiment, the PyhrN2 enhancer (34) was selected for the following reasons: (i) the PyV enhancer contains multiple redundant sequence elements that activate both DNA replication and RNA transcription (12, 28). Therefore, the action of the silencer is understandable with respect to both DNA replication and transcription. (ii) The silencer of interest in this study functions in undifferentiated mouse F9 cells. The PyhrN2 enhancer is also necessary for PyV to replicate in F9 cells, whereas wt PyV cannot replicate in F9 cells. PyhrN2, the host-range mutant of PyV, has a point mutation (G to C at nt 5230) and duplication of the region spanning from nt 5181 to nt 5246 but has the same ori sequence as wt PyV (34). By using plasmids containing the silencer, PyhrN2 enhancer, and ori linked to the CAT gene, we investigated the interaction between box DNA silencer and PyhrN2 enhancer on ori-dependent DNA replication and CAT expression.

The PyV enhancer-ori fragment essential to DNA replication was inserted into the polylinker region of pUC-CAT. Almost all of the polylinker regions present in pPyNRS-CAT(+) and pPyNRS-CAT(−) were located upstream of the PyhrN2 enhancer and downstream of the CAT gene, respectively. Also, the PyhrN2 enhancer-ori fragment cloned in pBR322 as a control contained no polylinker region. Two kinds of CAT plasmids gave approximately similar levels of both DNA replication and transcriptional activities (Fig. 3), indicating that the location or presence of polylinker in the plasmid had no effect on either activity.

With PyV DNA replication cannot occur in F9 cells, since the enhancer is not activated in these cells (11, 38). Another question of interest is whether the wt PyV enhancer can activate ori-dependent DNA replication in F9-28 cells when T antigen is supplied. Results of our preliminary experiment, using a transient replication assay, indicated that the wt PyV enhancer was unable to activate DNA replication even though the ori sequence of wt PyV was the same as the PyhrN2 sequence (data not shown). It is possible that the factor(s) present in F9 cells that inhibits transcriptional activation by the enhancer also inactivates PyV ori-dependent DNA replication. Therefore, the factor identified as a silencer-binding protein (3) may be a candidate.

We have showed that an enhancer in the c-myc gene is capable of activating DNA replication according to its transcriptional activation (22). This enhancer appears to be functionally similar to the PyV enhancer. It has been reported that a silencer is present near or in an enhancer region, such as the long terminal repeat of human T-cell lymphotrophic virus type III (30), the β-interferon gene (14), the c-myc gene (29), and the p53 gene (4). Therefore, the interaction between silencer and enhancer may have an effect both on transcription and on DNA replication. Thus, PyV DNA replication may be a good model system for studying the mechanism of the interaction between the two.

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**LITERATURE CITED**


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