Replication-Dependent Transactivation of the Polyomavirus Late Promoter

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When a plasmid containing the wild-type polyomavirus intergenic regulatory region fused to the bacterial *cat* gene was introduced into mouse NIH 3T3 cells along with a plasmid coding for the early viral proteins (T antigens), chloramphenicol transacetylase enzyme activity and mRNA levels were increased about 10-fold over levels observed in the absence of early proteins. To investigate this transactivation phenomenon further, 11 specific deletion mutant derivatives of the wild-type parent plasmid were constructed and studied. One mutant (NA_L) with a minimal level of chloramphenicol transacetylase expression in the absence of T antigens was capable of being transactivated more than 40-fold. A number of other mutants, however, had little capacity for transactivation. Each of these mutants had in common a defect in large T-antigen-mediated DNA replication. Interestingly, one of the transactivation-defective mutants showed a basal late promoter activity fivefold higher than that of wild type and replicated in mouse cells in the absence of large T antigen. Subsequently, a small deletion abolishing viral DNA replication was introduced into those mutants capable of transactivation. The effect of the second deletion was to eliminate both replication and transactivation. Finally, wild-type and mutant constructs were transfected into Fisher rat F-111 cells in the presence or absence of early proteins. No transactivation or replication was ever observed in these cells. We concluded from these studies that the observed transactivation of the polyomavirus late promoter by one or more of the viral early proteins was due to either higher template concentration resulting from DNA replication or replication-associated changes in template conformation.

Polyomavirus gene expression is temporally regulated in a productive lytic cycle. Early after infection of permissive mouse cells, the predominant viral messages are from the "early" strand of the viral genome and encode the three T antigens: large T, middle T, and small t (62). During the early phase of infection, early messages predominate over late messages by a factor of 4 to 10 (41, 62). Large T antigen binds to the viral genome at specific sites and is required for the initiation of viral DNA replication (15, 27, 57, 63). After the onset of replication, messages from the "late" strand of the virus predominate and at late times can exceed early messages by a factor of 20 to 80 (41, 62). The two divergent viral transcription units are separated by an intergenic region thought to contain most or all of the elements responsible for their regulation.

Sequences important for early promoter function have been studied in a number of laboratories (19, 38, 53, 54, 64). As is typical of many eucaryotic RNA polymerase II promoters, the early promoter possesses canonical TATA and CAAT boxes (10). An enhancer region contains two major elements, A and B (38) or α and β (56), and several other minor elements (33, 53, 54, 65).

Several characteristics of the polyomavirus late promoter make it an interesting promoter to study. First, the late start sites are extremely heterogeneous and span more than 100 nucleotides (16, 24, 25, 35). Such heterogeneity, while also found in simian virus 40 (SV40), is highly unusual for polymerase II promoters. Second, the late promoter lacks a TATA box, CAAT box, or G+C-rich region, sequences often associated with the regulation of transcription initiation. Third, we (11) and others (7, 46) have shown that the

polyomavirus late promoter is active in the absence of viral early proteins and that DNA replication is not required for efficient late promoter function in transfected cells.

It has been reported that the polyomavirus late promoter is comprised of multiple elements mapped to the intergenic regulatory region (7, 11, 46-48). Recently, we have used deletion analysis to determine what sequences are required for basal late promoter activity (11). In agreement with work from other laboratories (7, 46, 48), certain sequences reported to influence early promoter function were also found to affect the late promoter. There was no one element which, when removed, totally abolished late promoter activity. This suggested that there are multiple *cis*-acting sequences that compose the polyomavirus late promoter. However, we defined two elements which strongly affected late promoter function in mouse cells in the absence of early proteins and viral DNA replication (11). The first was a 34-base-pair sequence (nucleotides [nt] 5122 to 5155; refer to reference 11 for numbering system) which, when removed, resulted in 20- to 100-fold-lower levels of chloramphenicol transacetylase (CAT)-specific mRNA. This element lies within the A enhancer of the early promoter (38). It is not yet known whether it acts as an enhancer for late transcription or functions as a directional promoter element. Others have also shown this region of the polyomavirus genome to be important for late promoter function (7, 46). The second major *cis*-acting element affecting late promoter function was contained in an 89-base-pair sequence located on the early side of the origin (nt 56 to 144). This element, when deleted, resulted in a 5- to 10-fold increase in late promoter activity (11).

The roles of early viral proteins and viral DNA replication in the course of a productive polyomavirus infection are not clear. Many other viruses encode gene products that regulate or stimulate the expression of a second set of viral

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genes. These include the E1A gene products of adenovirus (6), the immediate-early genes of herpes simplex virus (40), and the large T antigen of SV40 (2, 8, 32, 43). Although large T antigen of SV40 has been shown to stimulate SV40 late transcription (2, 8, 32, 43), several questions remain unanswered regarding the mechanism of this transactivation. Does SV40 large T antigen act directly on the viral genome to stimulate late transcription or does it induce some cellular factor(s) to function as a transcriptional activator? Does initiation of DNA replication have any influence on transactivation? Since SV40 and polyomavirus large T antigens share some homology at the protein level (60), one can hypothesize that these proteins transactivate their respective late promoters in a similar manner. Indeed, it has been reported that the polyomavirus late promoter can be modestly stimulated by early viral proteins (48, 49).

To learn more about the function of polyomavirus early proteins in late transcription initiation, mouse NIH 3T3 cells were transfected with plasmids containing wild-type or mutant polyomavirus regulatory regions together with a plasmid that expresses all three early viral proteins. We found that the wild-type polyomavirus late promoter could be stimulated 5- to 10-fold by the presence of the early viral proteins. Although some mutant constructs could also be transactivated, others could not. Surprisingly, transactivation was invariably associated with DNA replication. Every mutant with a very low level of transactivation was also aberrant in large T-antigen-mediated DNA replication, and transactivation was not seen in a rat cell line that does not support polyomavirus DNA replication.

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 DNA ligase, DNA polymerase I, large fragment (Klenow enzyme), T4 DNA polymerase, and T4 polynucleotide kinase were from New England BioLabs and were used as suggested by the supplier. RNase T₂ was from Bethesda Research Laboratories, Inc. [α -³²P]dATP, [α -³²P]UTP, [γ -³²P]ATP, and avian myeloblastosis virus reverse transcriptase were from Dupont, NEN Research Products. [¹⁴C]chloramphenicol (54 mCi/mmol) was from Amersham Corp.

Construction of plasmids. Routine cloning techniques were performed by following protocols from Maniatis et al. (52). The oligonucleotide-directed mutagenesis protocol and the oligonucleotides used for mutagenesis have been described elsewhere (11). The 59RA strain of polyomavirus (23, 26) was used in all experiments.

pKC-CAT-WT_L and deletion mutants. The construction of wild-type and deletion polyomavirus promoter plasmids has been described previously (11).

Replication-incompetent plasmids. Deletion mutants NA_L, NB_L, 50_L, NA-50_L, 290P_L, Nstu_L, and PLM_L were used to create plasmids incapable of replicating by removing 8 additional bases, previously referred to as the Δ T deletion (11). All mutants were verified by sequencing, using the dideoxy method (59).

pPK. Plasmid pPK contains the entire polyomavirus genome (strain 59RA) cloned into pMK16* (28) at its unique *Bam*HI site (1).

Cell culture and transfections. Mouse NIH 3T3 cells were obtained from the American Type Culture Collection, and rat Fisher F-111 cells were obtained from T. Benjamin. The techniques for their propagation and transfection have been described elsewhere (11). Briefly, all transfections were

performed by using a modification of the calcium phosphate transfection procedure of Chen and Okayama (13). Cells were seeded at a concentration of 10⁶ cells per 150-mm plate in 10% bovine calf serum approximately 48 h prior to transfection. At 16 h prior to transfection, the cells were serum stimulated by the addition of fresh medium. The total amount of DNA per transfection was 40 μ g: 30 μ g of CAT plasmid and 10 μ g of salmon sperm DNA. Unlike the transfections done in 3T3 cells, the medium on the F-111 cells was changed 8 h after the initiation of transfection. Cotransfection experiments with pPK were done with 30 μ g of promoter plasmid, 6 μ g of pPK, and 4 μ g of salmon sperm DNA to maintain a constant amount of 40 μ g of DNA per 150-mm plate.

Harvesting of transfected cells for DNA, RNA, and CAT enzyme. Cells were harvested 44 to 48 h after initiation of transfection. Each plate was rinsed with 1 $\times$ phosphate-buffered saline-minus (without Mg²⁺ and Ca²⁺), and one-half of the plate was scraped for RNA, one-fourth was scraped for CAT enzyme extracts, and the last one-fourth was used to harvest episomal DNA, using the Hirt procedure (39) (used to normalize transfection efficiencies). The harvesting protocol has been described elsewhere (11).

CAT enzyme assays. Extracts were assayed by the procedure of Gorman et al. (29). The volume of extract used per assay was varied between different mutants to keep the enzyme concentration within the linear range of its activity. The values for enzyme activity were determined by counting the acetylated and unacetylated forms of [¹⁴C]chloramphenicol separated by thin-layer chromatography. Final CAT activity was expressed as microunits of CAT per microgram of protein. Protein concentrations were measured with a Bio-Rad protein assay kit. Only those values falling into the linear range of CAT enzyme activity (1 to 70% acetylation, determined by a standard curve analysis performed in substrate excess) were evaluated. Every plasmid was assayed in triplicate, using independent transfections.

CAT mRNA assays. Total cellular RNA in 4.2 M guanidium thiocyanate solution was isolated by centrifugation through a 5.7 M cesium chloride cushion for 20 h at 110,000 $\times$ g (14). The protocol used to identify the levels of CAT RNA produced by each plasmid has been published elsewhere (11).

DNA replication assays. NIH 3T3 cells were seeded onto 100-mm plates at a concentration of 5 $\times$ 10⁵ cells per plate approximately 48 h prior to transfection. A total of 5 μ g of DNA was transfected onto each plate: 2 μ g of promoter plasmid DNA, 400 ng of pPK, and 2.6 μ g of salmon sperm DNA. Cells were harvested between 44 and 48 h after the initiation of transfection. Cells were rinsed with cold phosphate-buffered saline-minus, and then 1 ml of 1% Nonidet P-40 (in phosphate-buffered saline-minus) was added for 1 min, taking care not to jar the plates and detach the nuclei from the plates. This step was used to remove cell membranes, any cytoplasmic or extracellular debris, and non-nuclear DNA. Soluble cell debris was carefully aspirated off the plates, leaving the nuclei attached. Episomal DNA was then isolated by the method of Hirt (39). After centrifugation, drying, and suspension of the pellets in 200 μ l of 10 mM Tris chloride (pH 7.5)–10 mM NaCl–2 mM EDTA (TNE), the samples were treated with RNase A (5 μ g) for 30 min at 37°C and then with proteinase K (10 μ g) for 20 min at 22°C. The samples were then extracted once with phenol-chloroform and once with ether and ethanol precipitated. The pellets were suspended in 50 μ l of TE.

DNA isolated by the Hirt procedure (39) was digested with

*Xho*I and *Dpn*I, electrophoresed through a 0.8% agarose gel, and then transferred to Zeta-Probe membranes (Bio-Rad) in $10\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by the method of Southern (61). Membranes were briefly rinsed in $1\times$ SSC and air dried. These membranes were then prehybridized with 500 μ g of denatured salmon sperm DNA per ml at 65°C for 6 h and then hybridized in the same solution with a denatured probe prepared by the Feinberg and Vogelstein method, using random hexamers (22), at 65°C overnight. The probe was the entire *cat* gene, isolated from plasmid pKC-CAT by digestion with *Xho*I and *Pst*I. After hybridization, the membranes were rinsed briefly in $2\times$ SSC, then in $2\times$ SSC–0.1% sodium dodecyl sulfate (SDS) for 15 min at 50°C, and then in $1\times$ SSC–0.1% SDS for 15 min at 22°C. When additional washes were required, solutions of 0.5 $\times$ SSC–0.1% SDS and 0.1 $\times$ SSC–0.1% SDS were used for 15 min each at temperatures close to that used for the hybridization. The membranes were exposed to X-ray film for 6 h to 5 days with an intensifying screen.

Mapping 5' ends by primer extension. Primer extension analysis of the 5' ends of *cat*-specific transcripts was performed with a 65-base synthetic oligonucleotide complementary to the 5' end of the *cat* gene (the 3' end of the primer was complementary to the AUG codon of CAT). The procedure for mapping 5' termini of transcripts from the wild-type and mutant polyomavirus promoter plasmids was from Ausubel et al. (3). Briefly, the primer (500 ng) was labeled to high specific activity with 200 μ Ci of [γ - 32 P]ATP (6,000 Ci/mmol) and 4 U of T4 polynucleotide kinase. Total cellular RNA (50 μ g) harvested from cultures transfected with promoter plasmids and plasmid pPK was mixed, in a total volume of 100 μ l, with 10 μ l of 3 M sodium acetate (pH 5.2) and 4×10^5 cpm of labeled oligonucleotide. Samples were ethanol precipitated, and the pellets were suspended in 30 μ l of hybridization buffer {40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)], 400 mM NaCl, 1 mM EDTA, 80% deionized formamide} and incubated at 30°C overnight. A 170- μ l amount of 0.3 M sodium acetate (pH 5.2) was added, and samples were ethanol precipitated. Each pellet was suspended in a final volume of 25 μ l containing 3.5 μ l of a mix containing a 4 mM concentration of each of the four deoxynucleoside triphosphates, 25 mM Tris chloride (pH 8.0), 2.5 mM MgCl₂, 2.5 mM dithiothreitol, 25 mM KCl, 25 μ g of bovine serum albumin per ml, and 1.2 μ l (48 U) of RNasin (Promega). Some 33 U of avian myeloblastosis virus reverse transcriptase were added and incubated for 1 h at 42°C. An additional 11 U of avian myeloblastosis virus reverse transcriptase were added and incubation was continued for 1 h, after which 1 μ l of 0.5 M EDTA and 1 μ l of RNase A (1 mg/ml) were added for 30 min at 37°C. After this incubation, 20 μ g of glycogen and 100 μ l of 2.5 M NH₄ acetate were added and samples were extracted once with phenol-chloroform followed by ethanol precipitation. The dried pellets were suspended in 4 μ l of H₂O plus 4 μ l of loading dye, boiled for 3 min, chilled immediately, and loaded onto a denaturing 6% polyacrylamide gel.

RESULTS

Late promoter deletion mutants in the presence of viral early proteins. In a recent report, we described the effects of a number of specific deletions on polyomavirus late promoter activity in a transient expression assay (11). That study, however, addressed only basal late promoter activity. Here, in an effort to understand the regulation of late

promoter activity in a productive viral infection, we examined the effects of early viral proteins and DNA replication on expression from these constructs. WT_L and deletion mutants (Fig. 1) were cotransfected into NIH 3T3 cells with and without plasmid pPK (1), which expresses the three viral early proteins. Cell extracts were assayed for the amount of CAT enzyme produced as an indication of late promoter function. The wild-type promoter consistently showed an increase of about 10-fold in the presence of the early proteins (Table 1). This level of transactivation was independent of the amount of transfected wild-type plasmid (data not shown). Expression from the NA_L deletion mutant was stimulated approximately 40-fold in the presence of the early proteins, and mutant 50_L was stimulated about 20-fold. Even after its dramatic transactivation, however, mutant NA_L was defective in late promoter function relative to wild-type levels (Table 1). Mutants PLM_L and NB_L were transactivated to about the same extent as the wild type. Mutants NA-50_L, NA-NB_L, Δ T_L, and Nori_L had similar values regardless of the presence or absence of the early proteins. The levels of transactivation observed for mutants 290P_L and E89_L were significantly lower than the wild-type level.

To verify that these results were due to transcriptional effects, the levels of CAT-specific RNA were examined by using an RNase protection assay with a riboprobe complementary to the 3' half of the CAT message. Figure 2 is an example of a typical experiment performed in the presence and absence of the three early viral proteins. The WT_L and NA_L promoter plasmids showed a stimulatory effect on the late promoter in the presence of the early proteins as observed by the increase in CAT RNA levels. These increases corresponded well with those observed with CAT assays. Mutants NA-NB_L, Δ T_L, and E89_L showed no detectable increase in CAT RNA, also correlating well with the CAT enzyme data provided earlier.

5'-End analysis. Primer extension experiments were performed to determine whether the same viral startsites were being used by wild-type and the NA_L and E89_L late promoter constructs. Mutant NA_L was chosen because of its high level of apparent transactivation, and mutant E89_L was chosen because of its higher than wild-type activity in the absence of early proteins and its ability to replicate in the absence of large T antigen (see below). Figure 3 shows that mutant E89_L uses the same major start sites as the wild-type late promoter. These sites mapped to nt 5082 $\pm$ 2, 5110 $\pm$ 2, 5189 $\pm$ 2, and 5222 $\pm$ 2. Transcripts from the transactivated mutant NA_L promoter also showed most of the same start-sites as the wild type. The nt 5110 and 5222 start sites of mutant NA_L, however, were less prominent than for the wild type, and the nt 5189 start site was offset by 34 base pairs, due to the NA deletion.

Replication assays. It has been shown by using various replication assays that viruses lacking the core origin sequence (17, 42, 50, 56), the eight consecutive T residues adjacent to the origin (18, 50; G. Aparicio-Ozores and G. Carmichael, unpublished results), or both the enhancer A and B elements (20, 33, 50, 56) are incapable of replicating. Several of the deletion mutants that were not transactivated also contained deletions of sequences thought to be important for DNA replication. Therefore, we performed replication assays to determine which mutants were defective for DNA synthesis. A 2- μ g portion of each deletion-containing plasmid was transfected into NIH 3T3 cells along with 400 ng of plasmid pPK. Figure 4A shows the result of a typical assay with DNA obtained from Hirt extractions and digested with *Xho*I and *Dpn*I. The presence of a high-molecular-

FIG. 1. Polyomavirus regulatory region and mutants used in this study. (A) Intergenic regulatory region of polyomavirus, from restriction sites *Bcl*I to *Hph*I. The black boxes indicate core sequence elements, the enhancer A and B elements of the early promoter, the origin of replication, and the early promoter TATA box. Bars beneath the regulatory region represent homologies to enhancer sequences from the adenovirus type 5 E1A (34), immunoglobulin M (4), SV40 (67), bovine papillomavirus (66), and the three high-affinity large T antigen-binding sites (15, 27, 57). The direction of late transcription is also shown. The dashed line reflects the numerous late start sites, and the large dots indicate the major late start sites found in our laboratory (Fig. 3). The box just downstream of the 5' ends of late transcripts is the late leader exon. (B) Wild-type and mutant polyomavirus promoter cassettes. Solid lines indicate wild-type sequences, while gaps represent deleted sequences. The numbers appearing within the gaps reflect the nucleotides missing, using the numbering system of reference 11. The ends of each promoter cassette contain *Xho*I linkers, and the cassettes were cloned into pKC-CAT (11).

weight band indicates the presence of replicated DNA. As expected, the WT_L promoter plasmid was capable of replicating, as was the NA_L plasmid. The three plasmids previously assumed to be incapable of replicating, ΔT_L, Nori_L, and NA-NB_L, in fact had no replicated plasmid band. Mutant 290P_L showed very low levels of replication. The construct containing the E89 deletion (and which lacks two of three high-affinity large T antigen-binding sites) was capable of replicating. This result was unexpected because this mutant lacks sequences proximal to the palindrome from the early proximal boundary of the origin, which have been reported to be important for replication (42). Upon further experimentation, this plasmid was found to replicate even in the absence of any early viral proteins (Fig. 4B). This result was confirmed in four independent experiments and is discussed below.

Mutants rendering the promoter plasmids replication incompetent. Because of our findings correlating transactivation with the ability of the viral DNA to replicate, additional experiments were performed to substantiate these results.

Regulatory region deletion mutants capable of being transactivated were coupled with a second deletion, ΔT, rendering them unable to replicate. Since the ΔT_L mutant, in the absence of early proteins, had approximately 70% of WT_L promoter activity (11), we assumed that the removal of these eight T's from other mutants would have little effect on their late promoters. The results of CAT assays with these constructs are shown in Table 2. In the absence of early viral proteins, the double mutants had late promoter activities similar to those found for the single deletions. However, these replication-minus promoter plasmids showed no evidence of any stimulatory effect provided by the early proteins. Of specific interest were the results from the double mutant containing both the NA and ΔT deletions. Whereas the NA_L mutant was transactivated approximately 40-fold in the presence of the early proteins, when this mutation was coupled to the ΔT deletion, no stimulatory effect was seen. Thus, the observed transactivation of the polyomavirus late promoter by early viral proteins again correlated closely with viral DNA replication.

TABLE 1. CAT activity in NIH 3T3 cells

Construct	% Wild type $\pm$ SE (no. of expt) ^a		Fold increase ^b	Replication ^c
	- Early proteins	+ Early proteins ^d		
WT _L	100	1,100 $\pm$ 100 (51)	11	+
PLM _L	39 $\pm$ 4 (13)	312 $\pm$ 70 (11)	8	ND
NA _L	3.5 $\pm$ 0.6 (14)	147 $\pm$ 35 (17)	42	+
50 _L	57 $\pm$ 11 (13)	969 $\pm$ 23 (13)	17	+
NB _L	86 $\pm$ 18 (13)	1,032 $\pm$ 34 (14)	12	ND
290P _L	75 $\pm$ 10 (18)	300 $\pm$ 60 (18)	4	$\pm$
NA-50 _L	2 $\pm$ 0.7 (4)	6 $\pm$ 1.6 (8)	— ^e	ND
NA-NB _L	2.4 $\pm$ 1 (4)	6 $\pm$ 1.2 (12)	— ^e	—
Nst _L	17 $\pm$ 7 (4)	102 $\pm$ 29 (4)	6	ND
Δ T _L	67 $\pm$ 21 (4)	107 $\pm$ 47 (4)	1.6	—
Nori _L	68 $\pm$ 9 (12)	116 $\pm$ 7 (15)	1.7	—
E89 _L	493 $\pm$ 115 (12)	1,479 $\pm$ 345 (11)	3	+

^a Transfections and CAT assays were performed as described in Materials and Methods. Basal late promoter activity (values in the absence of early proteins) is expressed as percent wild type.

^b Fold increase over basal activities of the same construct.

^c Replication was assayed as described in Materials and Methods. ND, Not done.

^d Mean value relative to wild-type-minus early proteins.

^e Since CAT activities for these mutants were always close to background, the values should be considered approximate only and as such are not reliable multipliers for determining the fold increase in the presence of T antigens.

Wild-type and deletion mutants transfected into rat cells. In an additional approach to study the relationship between replication and late promoter transactivation, we performed cotransfection experiments with rat F-111 cells. These cells are unable to support DNA replication of the 59RA strain of polyomavirus (31; A. Roome and G. Carmichael, unpublished results). The same panel of regulatory-region mutants was transfected into F-111 cells in the presence or absence of the early proteins. The wild-type level of CAT activity was

the same for both NIH 3T3 and F-111 cells (data not shown), indicating similar promoter strength in the two cell types. However, there were some notable differences between NIH 3T3 and F-111 cells in basal late promoter function (Table 3). Whereas the NA_L mutant showed a similar reduction in CAT expression when compared with the wild type in both cell types, there was no enhancement seen with the E89_L mutant in F-111 cells. The modest increase over wild type in expression from the 290P_L construct was a consistent finding in the F-111 cells, while in NIH 3T3 cells it was consistently at or below the wild-type level. These results suggest that in these cell types the late viral promoter elements may differ. A more detailed analysis of the polyomavirus late promoter in rat cells will be published elsewhere.

In F-111 cells, cotransfection with pPK did not result in transactivation of the wild-type late promoter or any of the mutants tested (Table 3). Since our strain of polyomavirus lacks the ability to replicate in rat cells, these results are again consistent with the conclusion that transactivation of the late promoter is dependent on the viral DNA replication.

DISCUSSION

We report here that increased transcription from *cat* constructs containing the polyomavirus late promoter was observed when they were transfected into mouse NIH 3T3 cells concomitantly with a plasmid that supplied the three viral early proteins in *trans*. The level of this transactivation was about 10-fold for the wild-type late promoter and varied considerably for different mutant constructs. Some mutants (e.g., NA_L and 50_L) had apparent transactivation levels much higher than those of the wild type, while a number of mutants (e.g., NA-NB_L, Δ T_L, Nori_L, and E89_L) had virtually no increase in the levels of CAT enzyme activity or CAT

FIG. 2. RNase protection measuring the levels of polyomavirus-CAT RNA transcripts in the absence and presence of the viral early proteins. Each reaction used 50 μ g of total cellular RNA hybridized to an RNA probe complementary to the 3' half of the CAT RNA transcripts. The top half shows the vector, pBS-CAT (11). An antisense radiolabeled probe was constructed from pBS-CAT, which contains the *cat* gene cloned downstream of the T3 RNA polymerase promoter. The *cat*-specific riboprobe contained 20 bases at its 5' end which are not complementary to CAT mRNA and which arise from the pBS-CAT vector. These hybrids were subjected to RNase T₂ treatment, denatured, and loaded onto a denaturing polyacrylamide gel. Following electrophoresis and autoradiography, a protected RNA band of 412 bases is seen.

FIG. 3. Analysis of the 5' termini of polyomavirus-CAT transcripts. Total cellular RNA was isolated from mouse cells transfected with WT_L, NA_L, or E89_L DNA and pPK. Total cellular RNA (50 µg) was hybridized to a 5'-end-labeled oligonucleotide, which was extended with reverse transcriptase as described in Materials and Methods. The denatured DNA products were electrophoresed through a denaturing polyacrylamide gel. The sequencing panel on the right shows WT_L DNA sequenced with the same oligonucleotide as a primer. The four major 5' termini are indicated by the solid arrows. The stippled arrow indicates one 5' end from the NA_L mutant which has been shifted due to a 34-base-pair deletion in this construct.

mRNA in the presence of early proteins. Of particular interest was the fact that, although the NA_L, NB_L, and 50_L mutants each could be transactivated quite well, the double mutants, NA-50_L and NA-NB_L, could not be transactivated significantly. At least for mutants NA_L and E89_L, the same major 5' ends as wild type were found on transcripts from their respective late promoters.

One straightforward way to interpret our results would be to postulate that the late promoter, though active in the absence of early viral proteins, can be stimulated via a transcriptional activator protein. One or more of the T antigens could serve directly in this capacity or indirectly by inducing or activating a cellular protein(s). In this scenario, deletion mutants which fail to show increased levels of expression should define the factor binding site(s) responsible for transactivation. However, we believe our results make this interpretation improbable for several reasons. A number of mutants, with nonoverlapping deletions, were not transactivated. Mutants NA-50_L and NA-NB_L lack important enhancer domains which have been shown to be important for both the early promoter and DNA replication (20, 33, 38, 50, 53, 55, 56, 65). Mutant 290P_L lacks the sequences between the *Pvu*II sites (nt 5156 to 5324), which include much of the enhancer region. Mutants with this same *Pvu*II deletion (in other strains) have been described, and they

appear to be competent for, but reduced in, early gene expression and DNA replication (50, 64). Mutants Nori_L and ΔT_L lack elements of the core origin of replication (17, 36, 37, 42, 51, 56, 58, 63, 64). Mutant E89_L lacks sequences adjacent to the origin palindrome on the early side, including two of the three high-affinity binding sites for large T antigen. This region is involved in autoregulation of early gene expression, and one large T-antigen-binding site is known to be required for DNA replication (5, 15, 21, 27, 42, 57). Since so many different deletions abolished transactivation, it is unlikely that all of these regions can act as target sites for transcriptional activation of the late promoter.

Since all mutants with defects in transactivation contained lesions that might also affect DNA replication, we examined the DNA replication competence of these mutants. The results indicated that, with the single exception of E89_L, all mutants that had only low levels of transactivation also had little or no replication in the presence of large T antigen. Thus, transactivation of polyomavirus late promoter constructs by viral early proteins is strongly correlated with replication, or replication competence (Table 1).

The importance of replication for transactivation was underscored by an analysis of mutants containing the ΔT deletion, which abolished replication with little effect on basal late promoter activity. None of the ΔT-containing mutants showed significantly higher late promoter activity in the presence of early viral proteins. An especially striking result was that mutant NA_L had >40-fold-higher activity in the presence of early proteins, while the NA-ΔT_L double mutant had virtually the same activity in the presence or absence of early proteins. The ΔT region probably does not define a binding site for a transcriptional activator, because other mutations that blocked DNA replication also eliminated transactivation.

Interestingly, mutants NA_L and 50_L had higher than wild-type levels of stimulation when assayed in the presence of early viral proteins. It is possible that in these mutants DNA replication, or some replication-associated change in template conformation, can at least partially overcome the late promoter defects. Alternatively, in the presence of T antigen(s) other sequence elements may become important for late promoter function.

Although it failed to show significant transactivation by viral early proteins, mutant E89_L proved to have interesting replication properties. This mutant scored positively in repeated replication assays, but this replication was not dependent on large T antigen. We have repeated this assay four times, always with the same result. Thus, mutant E89_L appears to contain a large T-antigen-independent replication origin, which would explain why it expressed higher levels of CAT activity than wild type in the absence of early proteins in mouse NIH 3T3 cells and why it failed to display significantly higher activity in the presence of early proteins. This observation, though surprising (it lacks origin proximal sequences thought to be essential for replication), is not unprecedented. Katinka and Yaniv (42) constructed several mutants very similar to E89_L and reported that they appeared to replicate in the absence of detectable large T antigen. Our laboratory is continuing to study the T-antigen-independent replication of mutant E89_L.

As another approach to the question of polyomavirus late promoter transactivation, we transfected many of our original panel of constructs into Fisher rat F-111 cells, in the presence or absence of early proteins. Our strain of polyomavirus is unable to replicate in these cells. The results (Table 3) clearly indicated that, while the late promoter was

FIG. 4. DNA replication assays of wild-type and mutant plasmids. (A) The indicated DNAs (2 μ g) were cotransfected with pPK (400 ng) into mouse NIH 3T3 cells. Low-molecular-weight DNA was isolated and cut with *Xho*I and *Dpn*I. The digested DNAs were electrophoresed through agarose, transferred to Zeta probe membranes, and hybridized with a probe for the *cat* gene (see Materials and Methods). The high-molecular-weight bands (exposure time, 48 h) represent unmethylated replicated DNA, while the lower bands (exposure time, 2 h) represent methylated unreplicated input DNA. (B) Replication assays were done as in panel A, except in the indicated lanes plasmid pPK was not cotransfected into the cells. Mutant E89_L was unique among the constructs used in that it always showed a replicated band in the absence of large T antigen.

active in these cells in the absence of T antigens, there was no evidence of transactivation. Although our results with F-111 cells are consistent with the idea that DNA replication is required for late promoter transactivation, the possibility exists that the absence of transactivation in rat cells could be due to reasons other than an inability to replicate.

Is the observed transactivation of the polyomavirus late promoter due only to an increase in template copy number resulting from DNA replication? This is an attractive explanation for the increased levels of CAT enzyme activity and RNA that we see, but we cannot at this time rule out certain other possibilities. For example, our transfection method results in large amounts of apparently inert plasmid DNA entering cells (Fig. 3). It is therefore impossible to correct our CAT assay results for levels of DNA replication as we cannot estimate the fraction of input molecules that are transcriptionally active or that are competent to replicate. It is also possible that DNA replication, per se, is not as

important for the transactivation phenomenon as is the competence to replicate or a change in chromatin structure associated with replication. Viral DNA molecules which are associated with replication. Viral DNA molecules which are associated with viral or cellular proteins or both in replication-competent complexes might be more transcriptionally active than molecules not associated with factors involved in DNA replication. Cereghini and Yaniv (12) have reported that there are replication-associated structural changes in the origin-promoter enhancer region of polyomavirus. Also, a recent report from Kelly et al. (45) presented evidence that transactivation of the SV40 late promoter by large T antigen occurs on replicated, or replicating, DNA molecules. In their studies, transactivation was not observed on replication-defective templates. This conclusion differs, however, from the work of several other laboratories (9, 44). In addition, Grass et al. (30) have recently reported that DNA replication

TABLE 2. CAT activity in NIH 3T3 cells^a

Construct	% Wild type $\pm$ SE (– early proteins)	Fold increase $\pm$ SE (+ early proteins)
WT _L	100	11 $\pm$ 1 (51)
PLM-ΔT _L	14 $\pm$ 3 (3)	1.3 $\pm$ 0.2 (3)
NA-ΔT _L	13 $\pm$ 2 (12)	2.5 $\pm$ 1 (6)
50-ΔT _L	21 $\pm$ 4 (3)	0.8 $\pm$ 0.1 (3)
NB-ΔT _L	36 $\pm$ 14 (6)	1.4 $\pm$ 0.2 (4)
290P-ΔT _L	26 $\pm$ 7 (3)	0.7 $\pm$ 0.4 (3)
NA-50-ΔT _L	1.3 $\pm$ 0.2 (3)	0.9 $\pm$ 0.2 (3)
Nstu-ΔT _L	25 $\pm$ 4 (3)	1.2 $\pm$ 0.1 (3)
ΔT _L	67 $\pm$ 21 (4)	1.6 $\pm$ 0.7 (4)

^a Data are presented as in Table 1. Values in parentheses indicate number of independent experiments performed.

TABLE 3. CAT activity in rat F-111 cells^a

Construct	% Wild type $\pm$ SE (– early proteins)	Fold increase $\pm$ SE (+ early proteins)
WT _L	100	0.4 $\pm$ 0.1 (8)
PLM _L	16 $\pm$ 3 (8)	ND
NA _L	8 $\pm$ 2 (17)	0.7 $\pm$ 0.2 (8)
50 _L	129 $\pm$ 31 (9)	ND
NB _L	95 $\pm$ 29 (8)	0.9 $\pm$ 0.2 (3)
290P _L	278 $\pm$ 59 (20)	ND
NA-50 _L	35 $\pm$ 8 (8)	ND
NA-NB _L	20 $\pm$ 5 (6)	0.8 $\pm$ 0.2 (3)
Nstu _L	59 $\pm$ 16 (6)	ND
ΔT _L	90 $\pm$ 21 (8)	0.5 $\pm$ 0.2 (3)
Nori _L	53 $\pm$ 16 (11)	ND
E89 _L	101 $\pm$ 35 (17)	1.2 $\pm$ 0.2 (5)

^a Data are presented as in Table 1. Values in parentheses indicate number of experiments performed. ND, Not done.

can exert a strong and perhaps direct effect on the activities of the SV40 and adenovirus major late promoters and that this effect can be dissociated from increased template copy number.

Finally, the formal possibility remains that one or more of the early viral proteins can, in fact, transactivate the polyomavirus late promoter in mouse cells independently of DNA replication. However, this transactivation must be modest and alone would not account for the dramatic accumulation of late-strand transcripts at late times in infection. The results reported here are consistent with other recent results from our laboratory which have indicated that the early-to-late switch in polyomavirus is not controlled at the level of transcription initiation (41).

Transactivation of the polyomavirus late promoter by large T antigen has also been studied by another laboratory (48, 49). Kern et al. (48) looked at the effect of large T antigen on expression from the polyomavirus late promoter in rat F2408 fibroblast cells. Transactivation from integrated plasmids was only observed when the basal promoter activity was greatly reduced, in a mutant similar to our mutant NA_L. In addition, an integrated origin-minus plasmid was not transactivated. The level of transactivation from a wild-type episomal plasmid containing the *cat* gene was about 5-fold, while a mutant with a defective late promoter was transactivated 23-fold. It is not clear whether or not a low level of replication occurred in these cells. Interestingly, we have also observed higher levels of transactivation by using mutants (e.g., NA_L and 50_L) with lower basal late promoter activities.

In a subsequent report, Kern et al. (49) tested a number of late promoter cassettes fused to the bacterial *cat* gene and transfected into both rat and mouse cells. Although their results differed in certain ways from ours, there were also differences in experimental design, including different transfection protocols, making meaningful comparisons difficult. Their cassettes differed from ours in two ways. First, their constructs were derived from a different viral strain. Compared to strain A2, strain 59RA has a number of base substitutions in the intergenic region (11, 26), as well as a mutation in the large T-antigen gene which results in a replication defect in rat F-111 cells (31). Second, the polyomavirus sequences used by Kern et al. included about one-half of the early coding region of the viral genome, making them capable of expressing small and middle T antigens and the amino-terminal half of large T antigen. Our plasmids lack any early virus-coding sequences or polyadenylation signals. When the wild-type construct of Kern et al. was cotransfected into mouse NIH 3T3 cells along with a plasmid which expressed functional large T antigen, a transactivation of almost 70-fold was observed. Two replication-negative derivatives were capable of being activated 20-fold by large T antigen, but only when much of the enhancer region was intact. Furthermore, removal of the high-affinity large T antigen-binding sites led these authors to conclude that these binding sites must be present to achieve a maximal level of transactivation from the late promoter. In that report, however, one replication-defective late promoter construct, pL1, had polyoma endpoints at about the same sites as our plasmids. Significantly, and in agreement with our findings reported here, pL1 showed little, if any, transactivation by large T antigen in two cotransfection experiments in NIH 3T3 cells. We are currently constructing late promoter cassettes containing more of the early coding region to assess, using our virus strain, the role of such sequences in late promoter regulation.

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