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Deletion Analysis of the Polyomavirus Late Promoter: Evidence for Both Positive and Negative Elements in the Absence of Early Proteins

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We have been interested in understanding more about the sequences that constitute the polyomavirus late promoter. Our approach has been to target specific deletions to the viral intergenic region by oligonucleotide-directed mutagenesis. Wild-type and mutant promoter cassettes with defined deletions were then inserted into a promoterless expression vector containing the bacterial chloramphenicol acetyltransferase (CAT) gene (*cat*). Plasmids were introduced into mouse NIH 3T3 cells by transfection, and promoter activities were assessed by quantitation of both CAT enzyme and *cat* mRNA levels. In this report, we present the results of experiments designed to map promoter elements which affect late transcription in the absence of early viral proteins and viral DNA replication. Using this approach, we mapped two major *cis*-acting elements (a positive and a negative one) which affect transcription in our transient expression system. The first, positive, element coincided with the enhancer A element, which is known to be important for early transcription and viral DNA replication. Removal of this element reduced late transcription by 50- to 100-fold. The second element was a negative one; removal of 89 base pairs that included two high-affinity large-T-antigen-binding sites just to the early side of the inverted repeat structure within the replication origin resulted in a 5- to 10-fold increase in late promoter activity. The implications of these findings for late promoter function and regulation are discussed.

Polyomavirus has been widely used as a model system for studying eucaryotic gene expression because of its relatively small genome and its limited number of genes whose expression is regulated both temporally and spatially. Early after infection of permissive mouse cells, the majority of the viral messages found in the cytoplasm are transcribed from the early strand of the viral genome and encode the three T antigen proteins; large T, middle T, and small t (63). Viral DNA replication is triggered by the binding of large T antigen to the viral genome at or near the bidirectional origin of DNA replication (13, 25, 54, 65). After the onset of DNA replication, the predominant viral cytoplasmic messages found are transcribed from the opposite, late strand of DNA and encode the three viral capsid proteins. These late cytoplasmic transcripts accumulate to high levels by 24 to 36 h postinfection.

The controlling elements for viral gene expression (the early and late promoters, origin of replication, and the enhancer region for the early promoter) appear to be located within a 500-base-pair (bp) region referred to as the regulatory region, which lies between the early and late transcription units (Fig. 1 and 2A). Within this regulatory region, several elements have been defined which are essential for DNA replication and transcription from the early promoter. The well-characterized early promoter contains typical eucaryotic promoter elements, such as a Goldberg-Hogness TATA box, a CAAT box sequence (8) upstream of the transcriptional start sites, and an enhancer region (16, 66). This enhancer region contains a number of sequences which are homologous to those found in other eucaryotic enhancers (3, 30, 69, 70).

The 240-bp enhancer region of polyomavirus has been divided into two separate enhancer domains, A and B (31) or α and β (49). Each enhancer domain can act independently of the other to stimulate transcription, and each displays a different cell type specificity. For example, in mouse fibroblast cells, the enhancer A element alone is three times more active than the enhancer B element. However, in embryonic carcinoma cells, in which the B enhancer shows the same activity as in fibroblasts, enhancer A is 3.5 times less active than in fibroblasts (31). These enhancers were further dissected into smaller independent elements that act more efficiently when positioned together to influence early transcription or viral DNA replication (47, 48, 60, 68). A number of factors have been described that bind to different sequences within the 240-bp enhancer region (6, 24, 34, 42, 50–53, 58) although the functional significance of these factor-enhancer interactions remains unclear at this time.

The sequence(s) comprising the polyomavirus late promoter is not yet known but is thought to be contained, at least in part, within the noncoding region between the early and late genes. The late promoter does not contain a TATA box sequence. In this regard it resembles promoters found for some known cellular housekeeping genes (56, 67). Another similar feature of these promoters is the presence of multiple transcriptional start sites (14, 39). Our laboratory strain of polyomavirus (59RA) contains a 31-bp duplication within the enhancer region, providing the virus with two major late start sites in addition to numerous minor start sites (14, 55; G. R. Adami and G. G. Carmichael, unpublished).

Kern et al. (38–40) have studied polyomavirus late promoter activity in both mouse and rat cells. Constructs containing the wild-type late promoter region or deletion mutant DNAs fused to the gene conferring neomycin resistance were introduced by transfection into rat cells. These chimeric plasmids were integrated into the rat genome, and late promoter function was assessed by Neo^r colony formation. The same polyomavirus wild-type and mutant sequences were then fused to the bacterial reporter gene for

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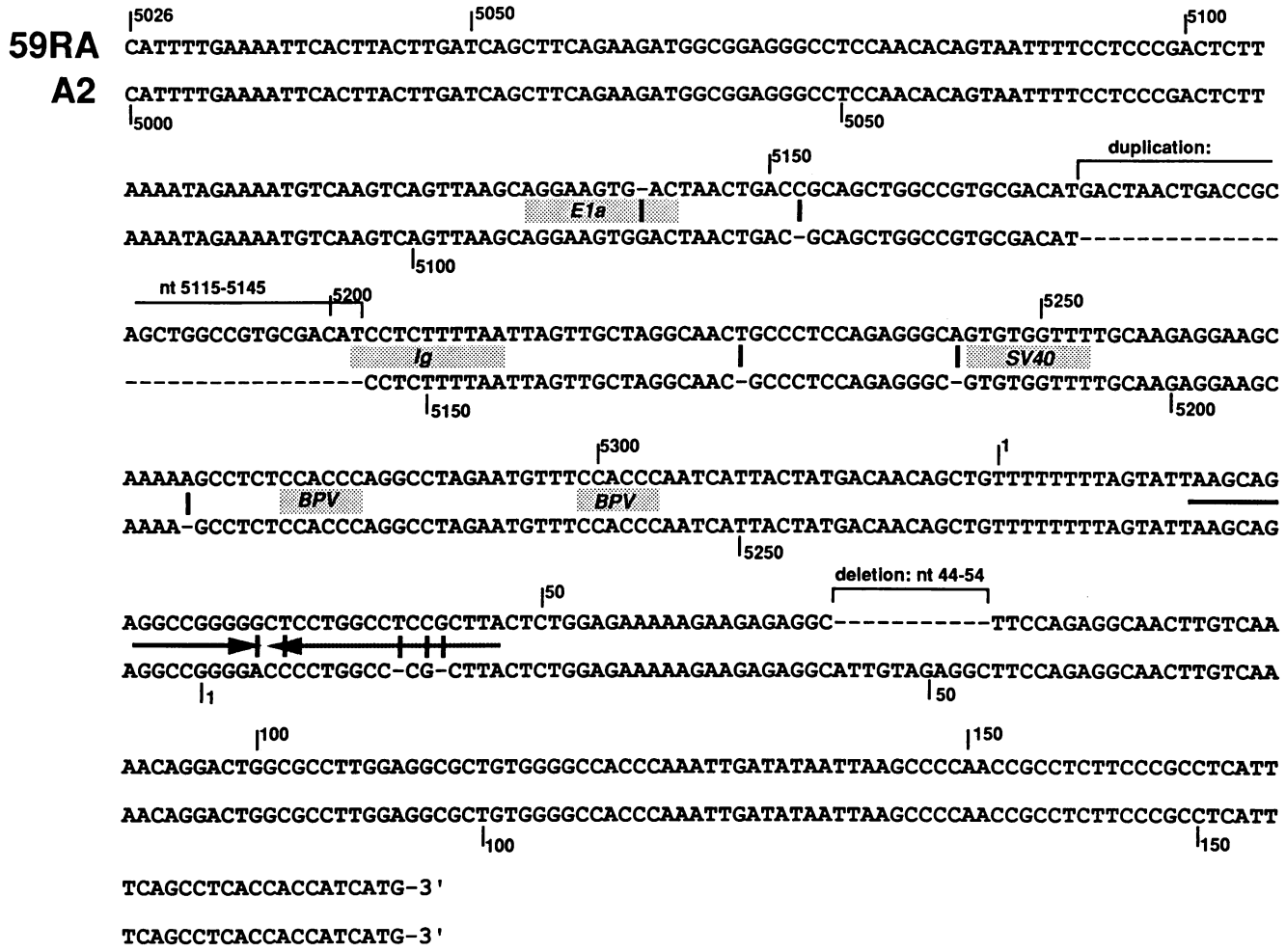

FIG. 1. Comparison of the intergenic control regions of polyomavirus strains 59RA and A2 at the nucleotide level. The corresponding nucleotide numbers are given for each strain. Shaded boxes indicate sequences with homologies to the core enhancer sequences of adenovirus type 5 E1A (30), SV40 (70), BPV (69), and immunoglobulin M (Ig) (3). Arrows represent the inverted repeat within the origin of replication. Dark vertical bars between the two sequences indicate base differences. The 31-bp duplication within the 59RA strain is shown along with the 11-bp deletion within the A2 strain.

chloramphenicol acetyltransferase (CAT) in order to assess transient promoter function in transfected mouse cells. The conclusion from these studies was that in both cell types, gene expression from the polyomavirus late promoter is dependent on the same viral regulatory elements as from the early promoter. However, their mutants contained either relatively large (>100 bp) or double deletions, making it difficult to define exactly which sequences were necessary for polyomavirus late promoter function.

Our laboratory has been interested in further defining the sequences necessary for the function and regulation of the polyomavirus late promoter. Our approach has been to use deletions to scan through the entire regulatory region by oligonucleotide-directed mutagenesis to remove small, defined blocks of sequence. We removed blocks of sequence that have been implicated by us or others as important in early enhancer function, origin function, or virus viability. In addition, we removed sequences homologous to those found to be crucial for the function of other enhancers. The effects of these deletions on late promoter function were assessed with a transient-expression system in which wild-type and mutant promoters were fused to the *cat* gene. Enzyme activity and mRNA levels were measured and correlated to promoter function. We present here the results of such assays in the absence of viral early proteins. We have defined two major *cis*-acting elements, one positive and one negative, which are involved in late promoter function in transfected mouse cells.

MATERIALS AND METHODS

Materials. All restriction enzymes, T4 DNA ligase, DNA polymerase large fragment (Klenow enzyme), T4 DNA polymerase, and polynucleotide kinase were from New England BioLabs and were used as suggested by the supplier. RNase T_2 was from Bethesda Research Laboratories. [α-^{32}P]dATP, [α-^{32}P]UTP, and [γ-^{32}P]ATP were from New England Nuclear Corp. [^{14}C]chloramphenicol (specific activity, 54 mCi/mmol) was from Amersham Corp.

Construction of plasmids pUC8-X, pKC-CAT, KBC-5, and pBS-CAT. Routine cloning techniques were those of Maniatis et al. (44).

For pUC8-X, plasmid pUC8 DNA was cleaved at its unique *Eco*RI site and its ends were filled in with T4 DNA

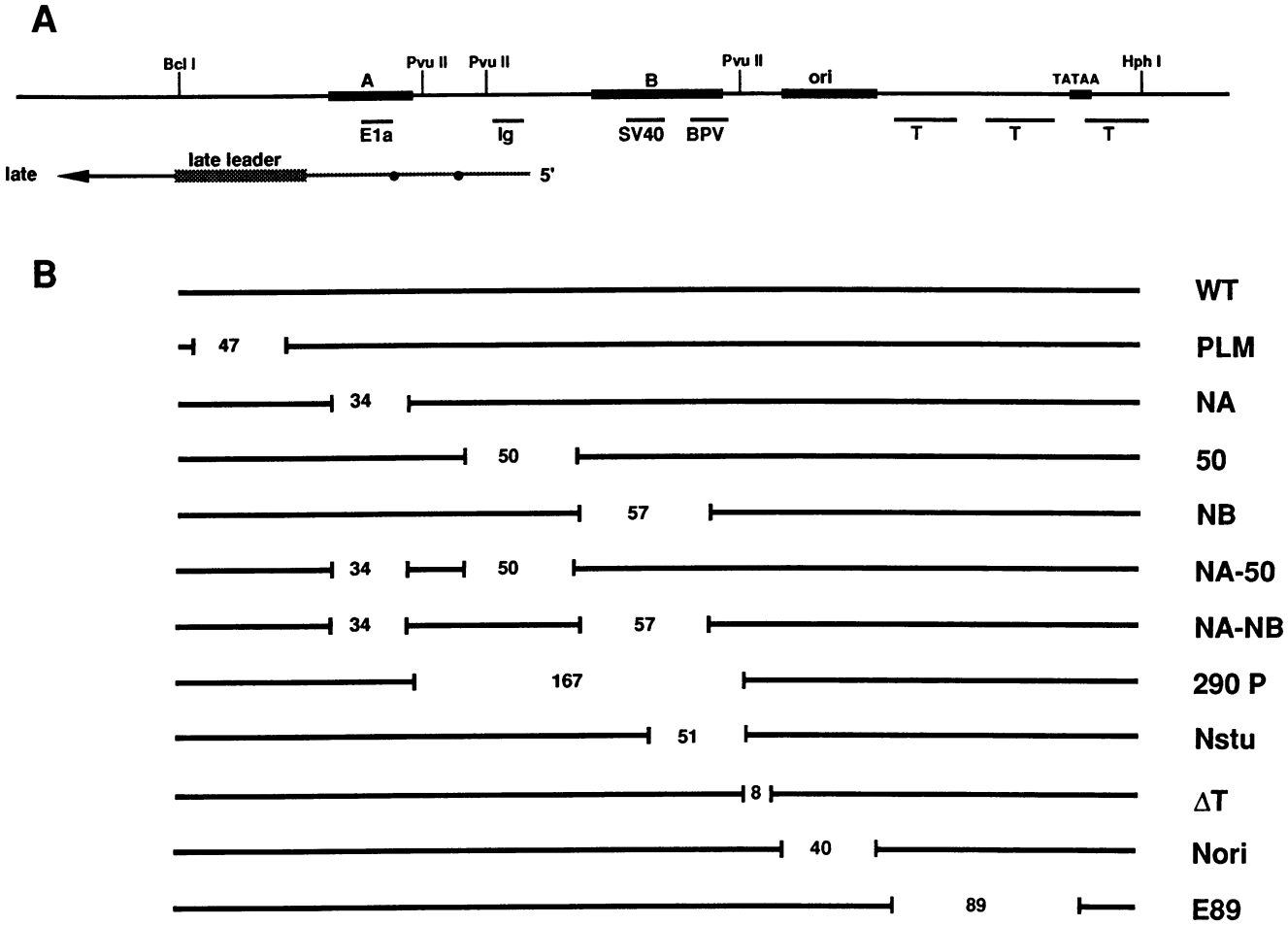

FIG. 2. Polyomavirus regulatory region and mutants used in this study. (A) The intergenic regulatory region of polyomavirus, from restriction sites *Bcl*I to *Hph*I. Other restriction sites of interest are the *Pvu*II sites. The black boxes indicate core sequence elements, the enhancer A and B elements 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 (30), immunoglobulin M (3), SV40 (70), BPV (69), and the three high-affinity large-T-antigen-binding sites (13, 30). The direction of late transcription is also shown. The two dots indicate the two major late start sites of strain 59RA (unpublished), and the line through them reflects the numerous minor start sites. 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, and gaps represent deleted sequences. The numbers appearing within the gaps reflect the number of nucleotides missing. To the ends of each promoter cassette were added *Xho*I linkers, and the cassettes were cloned into pKC-CAT (see Materials and Methods) to measure promoter activity.

polymerase. Eight-base *Xho*I linkers (CCTCGAGG) were ligated to these blunt ends, reconstructing the *Eco*RI sites bordering the *Xho*I site. The insertion of these bases maintains the *lacZ* coding region within the plasmid, resulting in blue colonies when grown in the presence of the chromogenic substrate *O*-nitrophenyl-β-D-galactopyranoside.

For pKC-CAT, the 237-bp *Bam*HI-*Bcl*I simian virus 40 (SV40) fragment (nucleotides [nt] 2451 to 2688) containing an efficient 3′-processing/polyadenylation site was inserted via *Hin*dIII linkers into the unique *Hin*dIII site of pUC8-X. This plasmid was linearized with *Sal*I, and the ends were filled in as described above. Plasmid pBR325 was cut with *Taq*I, a 776-bp fragment containing the *cat* gene was isolated, and its ends were filled in and then ligated into the *Sal*I site of pUC8-X. A clone was chosen that contained the AUG site of the *cat* gene proximal to the *Xho*I site and distal to the SV40 poly(A) addition site. This plasmid is a promoterless *cat* plasmid possessing several unique cloning sites (*Xho*I, *Sma*I, *Xma*I, *Bam*HI) upstream of the *cat* gene (see Fig. 3). The SV40 polyadenylation site used by the *cat* transcripts is the same as that for late SV40 transcripts. We have found that it is unnecessary to include splice sites in our construct to obtain high levels of CAT expression.

For KBC-5, polyomavirus DNA was cut with *Dpn*I and *Hph*I, and a 451-bp fragment containing the early and late promoters (nt 5047 to 170 in our numbering system) was isolated. The ends of this fragment were filled in with Klenow enzyme, and 8-base *Xho*I linkers were added. This fragment was then inserted into M13mp8-X (11) at its unique *Xho*I site. The resulting recombinant, KBC-5, provided single-stranded DNA for oligonucleotide-directed mutagenesis (see below). For the construction of pKC-CAT WT_L, which contains the wild-type viral late promoter directing CAT expression, the polyomavirus fragment of KBC-5 was excised with *Xho*I and ligated into pKC-CAT. All subsequent deletion mutants were created by either oligonucleotide-directed mutagenesis or restriction cleavage on KBC-5, and sequences were verified. These promoter deletion

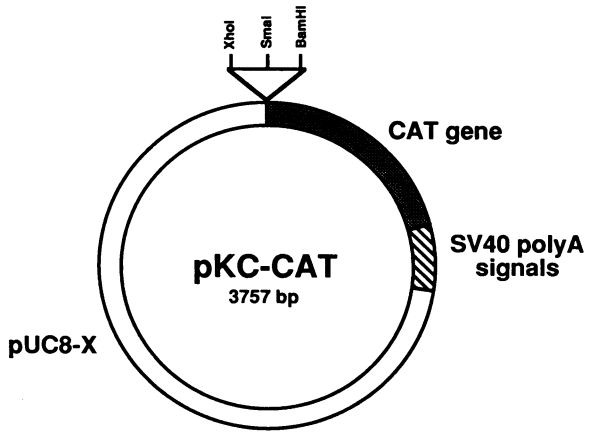

FIG. 3. Promoterless expression vector pKC-CAT. A 776-bp *Taq*I fragment from pBR325 containing the *cat* gene (stippled box) was inserted into the multiple cloning site of pUC8-X (see Materials and Methods) at the *Sal*I site. The 237-bp *Bam*HI-*Bcl*I fragment from SV40, carrying the 3'-processing and polyadenylation (polyA) signals (slashed box), was cloned into the *Hin*dIII site downstream of the *cat* gene. Upstream from the *cat* gene are unique *Xho*I, *Sma*I, *Xma*I, and *Bam*HI restriction sites into which promoter cassettes can be inserted. Polyomavirus promoter cassettes (Fig. 2B) were all cloned into the *Xho*I site so that the late promoter drives expression from the *cat* gene.

cassettes were then cloned into the pKC-CAT vector (Fig. 3) in the late orientation.

pBS-CAT contains the *cat* gene (a 776-bp *Taq*I fragment from pBR325) cloned into the Bluescribe vector pBS$^+$ (Stratagene) at its unique *Sal*I site. pBS-CAT was used to create radiolabeled riboprobes needed to measure the levels of *cat* mRNA.

Oligonucleotide-directed mutagenesis procedure for the construction of deletion mutants. The oligonucleotides used in this study all ranged from 22 to 30 bases and were made by using a Biosearch Cyclone DNA synthesizer. They were constructed to anneal to the single-stranded DNA containing the wild-type polyomavirus promoter fragment in KBC-5, resulting in the appearance of a mismatched loop (containing the sequences to be deleted. The mutagenesis procedure used was based on the method of Eckstein and co-workers (61, 62) and used as a kit supplied by Amersham. KBC-5 single-stranded DNA (4 pmol) was annealed to a 20-fold molar excess of oligonucleotide, and heteroduplexes were formed by using the Klenow enzyme and T4 DNA ligase. The heteroduplex was then separated from the remaining single-stranded DNA by filtration through nitrocellulose and transformed into UT481 cells [*met thy* Δ(*lac-pro*) r_m^- *supD* Tn*10*(F' *traD36 proAB lacI*^{q} *lacZ36* ΔM15 amber]. Plaque hybridizations were performed with 5'-end-labeled oligonucleotides (the same oligonucleotides used in the mutagenesis) as radioactive probes to screen for positive deletion mutants. Plaque lifts, using nitrocellulose (Schleicher & Schuell), were prehybridized in 0.8 M NaCl–40 mM Tris chloride (pH 8.0)–8× Denhardt solution–0.8 mM EDTA–0.08% $NaPP_i$–0.08% sodium dodecyl sulfate (SDS)–150 μg of denatured salmon sperm DNA per ml for 4 h at 60°C. Labeled probe (20 pmol) was added, and hybridization was allowed to occur for 24 h at 60°C. Filters were rinsed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.2% SDS at 60°C for 15 min and then with 1× SSC–0.1% SDS for 15 min at 60°C. Positive plaques were revealed by autoradiography. All mutants were sequence verified by the dideoxy method (57).

Mutant $290P_L$ was made by cloning the wild-type polyomavirus fragment from KBC-5 into the *Sal*I site of pL3 (64), because this vector does not contain any *Pvu*II sites. This clone was then cleaved with *Pvu*II and religated, resulting in the excision of 167 bp from the polyomavirus fragment. This promoter cassette was then cloned back into M13mp8-X and used in an identical manner as the other deletion mutant cassettes.

Preparation of plasmid DNA. pKC-CAT WT_L and mutants were grown in JM83 [*ara* Δ(*lac-proAB*) *rpsL* (φ80 *lacZ* DM15)] and isolated by the lysozyme-alkaline lysis procedure (5). The DNA was purified further by two successive centrifugations through CsCl-ethidium bromide equilibrium density gradients. After the extraction of ethidium bromide, the DNA was dialyzed against a 1,000-fold volume of sterile 10 mM Tris chloride (pH 7.5)–1 mM EDTA (TE buffer), with one change of buffer at 4°C.

Cell culture and transfections. Mouse NIH 3T3 cells (American Type Culture Collection) were propagated in Dulbecco modified Eagle medium containing 5% bovine calf serum (GIBCO Laboratories) plus L-glutamine and penicillin and streptomycin at 37°C in 5% CO_2. For transfections, cells were seeded at a density of 15×10^5/150-mm plate in 10% calf serum approximately 24 h prior to transfection. Four hours prior to transfection, the cells were serum stimulated by the addition of fresh medium. A 30-μg amount of plasmid DNA was used in each transfection along with 10 μg of salmon sperm DNA as a carrier. All transfections were performed by a modification of the calcium-phosphate transfection procedure of Chen and Okayama (9). This technique involves forming a $CaPO_4$ coprecipitate of plasmid DNA in BES [*N*,*N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid] buffer. The DNAs were mixed with $CaCl_2$ to a final concentration of 0.25 M. Equal volumes of DNA-$CaCl_2$ and 2× BBS (BES-buffered saline), pH 6.95, were mixed in the presence of bubbled air. The precipitate was allowed to incubate at 22°C for 15 min and was then added dropwise to the cells. The cultures were then incubated at 37°C in 5% CO_2. The medium was changed 20 h later. We achieved a significantly higher transfection efficiency from this procedure than from the $CaPO_4$-HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) procedure of Wigler et al. (71) (data not shown).

Harvesting of transfected cells for DNA, RNA, and CAT enzyme. Cells were harvested 44 to 48 h after the initiation of transfection. Each plate was rinsed with 1× phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+}. Cells from half of the plate were scraped into medium (without serum), and the cells were pelleted by centrifugation at $1,000 \times g$ for 3 min, suspended in 2 ml of 4.2 M guanidine thiocyanate, and frozen at −20°C. Cells from one-quarter of the plate were scraped, pelleted, and suspended in 1 ml of ice-cold 40 mM Tris chloride (pH 7.5)–150 mM NaCl–1 mM EDTA. These cells were then repelleted, drained thoroughly, and suspended in 100 μl of ice-cold 0.25 M Tris chloride, pH 6.8. They were then lysed by four cycles of freeze-thawing. Cell membranes were removed by spinning in a microfuge for 12 min at 4°C. The supernatants were immediately frozen at −70°C and stored until use. Cells from the remaining one-quarter plate were harvested for episomal DNA by the Hirt lysis procedure (32). These DNAs were used to normalize transfection efficiencies.

CAT enzyme assays. The extracts containing the CAT enzyme were assayed by the procedure of Gorman et al.

(26). Amounts of extracts used varied between different mutants to keep the enzyme concentration within its linear range of activity (this was determined in control experiments with purified enzyme obtained from Pharmacia; data not shown). The values for enzyme activity were determined by scraping and counting the acetylated and unacetylated forms of [^{14}C]chloramphenicol from the silica gel thin-layer chromatography plates. Final CAT activity was expressed as microunits of CAT per microgram of protein. Protein concentrations were measured with a Bio-Rad Laboratories protein assay kit. Only those values falling into the linear range of CAT enzyme activity (from 1 to 70% acetylation, determined by a standard curve analysis performed in substrate excess) were evaluated. Every plasmid was assayed in triplicate with independent transfection reactions.

CAT mRNA assay. 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 × *g* (10). When small volumes of material were processed, an alternative centrifugation procedure was used. This involved layering the RNA extracts over a cesium chloride cushion of 0.5 ml and centrifuging for 3.5 h at 40,000 rpm (100,000 × *g*) and 20°C in a Beckman TL100 ultracentrifuge with a TLS55 rotor. The pelleted RNAs were drained well and suspended in 300 μl of 10 mM Tris chloride (pH 7.0)–1 mM EDTA (pH 7.0)–0.2% SDS and ethanol precipitated. RNAs were suspended in 100 μl of TE buffer, and 5 μl of each was observed on an agarose gel stained with 30 μg of acridine orange per ml (46) to ensure that there was no DNA contamination. RNAs were hybridized at 70°C for 2 min and then at 55°C for at least 16 h to a riboprobe spanning the 3′ half of the *cat* gene. This riboprobe was created by T3 RNA polymerase transcription of pBS-CAT cut with *Msp*I and labeled with [α-^{32}P]UTP. This antisense probe (434 bases) has 20 additional bases at its 5′ end that do not hybridize to the isolated *cat* mRNA. RNA hybrids were digested with 80 U of RNase T_2 per ml for 2 h at 34°C and electrophoresed on an 8% polyacrylamide–7 M urea gel (modification of Hart et al. [27]). Following electrophoresis, the gel was fixed in 10% acetic acid–10% methanol for 15 min, dried under vacuum, and exposed to Kodak XAR5 X-ray film at −70°C with a Du Pont Cronex intensifying screen. Results were quantified by densitometric scanning of autoradiograms with a Bio-Rad model 620 video densitometer.

RESULTS

Our strain of polyomavirus, 59RA (19, 20), contains a duplication of 31 bp within the enhancer region (55) as well as other base changes from the sequence published for the A2 strain (59). A comparison of the sequences of the regulatory regions of A2 and 59RA is shown in Fig. 1, along with the numbering system we have adopted for our viral strain.

We chose a 451-bp region of the polyomavirus genome (the *Dpn*I-*Hph*I fragment, nt 5047 to 170) to begin our studies of the late promoter. Landmarks of this fragment are shown in Fig. 2A. We hypothesized, based on our work and that of others, that the major elements of the late promoter would be contained within this regulatory region.

Polyomavirus late promoter mutants. Figure 2B shows the mutants used in this study. Table 1 lists the bases deleted in each mutant, and a brief description of each follows.

PLM_L is missing 47 of the 57 bases which constitute the "late leader" exon. Multiple, tandem copies of the late leader exon are found at the 5′ ends of all late viral messages. Leader multiplicity results from splicing of leader exons to one another in giant, multigenome-length late primary transcripts (1, 64).

TABLE 1. Late-promoter mutants used in this study

Mutant	Nucleotides deleted[a]
WT_L	None
PLM_L	5053–5099
NA_L	5122–5155
50_L	5184–5232
NB_L	5233–5289
$NA50_L$	5122–5155, 5182–5231
$NA\text{-}NB_L$	5122–5155, 5233–5289
$290P_L$	5157–5323
$Nstu_L$	5270–5320
ΔT_L	1-8
$Nori_L$	12–51
$E89_L$	56–144

[a] The numbers are from our numbering system for strain 59RA (see Fig. 1).

NA_L is missing the core enhancer A sequences (31), which also show a strong homology to the core element of the adenovirus E1A enhancer (30).

50_L is missing much of the sequence between enhancer elements A and B. This region contains a sequence with homology to the immunoglobulin enhancer (3). This mutant also removes an A+T region that might function as a TATA-like element for positioning of one of the major late start sites.

NB_L is missing the core enhancer B element (31), which shows a strong homology to the SV40 core element found in the 72-bp repeats (70), and a sequence homologous to one shown to be important for the function of the bovine papillomavirus (BPV) enhancer located 3′ to a cluster of genes expressed early after infection (69).

$290P_L$ is missing the *Pvu*II-4 fragment of polyomavirus, which extends from the border of the A enhancer element through the B enhancer element.

$Nstu_L$ is missing one-half of enhancer B, lacking only the homology to the BPV enhancer and sequences immediately adjacent to the core origin of replication.

ΔT_L is missing a stretch of eight consecutive T's which are found on the late side of the origin, immediately adjacent to the region of dyad symmetry. This stretch of T's is known to be absolutely required for DNA replication, both for polyomavirus and for SV40 (15, 43; G. Aparicio-Ozores and G. G. Carmichael, unpublished data).

$Nori_L$ is missing 40 bp from the core origin of replication, including a 26-bp inverted repeat structure (59).

$E89_L$ is missing 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-binding site is known to be required for DNA replication (4, 13, 18, 25, 35, 54).

In addition, two double mutants were made: $NA\text{-}50_L$ and $NA\text{-}NB_L$.

Comparison of wild-type and mutant late promoters. (i) Comparison of CAT enzyme activities. Wild-type and mutant polyomavirus promoter cassettes were fused to the *cat* gene in the late orientation (Fig. 3) and introduced into NIH 3T3 cells by transfection, as described in Materials and Methods. pKC-CAT WT_L was the wild-type plasmid used in this study (the level of CAT enzyme produced by our WT_L plasmid is approximately three to four times lower than that from pSV2-CAT). The parent plasmid, pKC-CAT, lacks polyo-

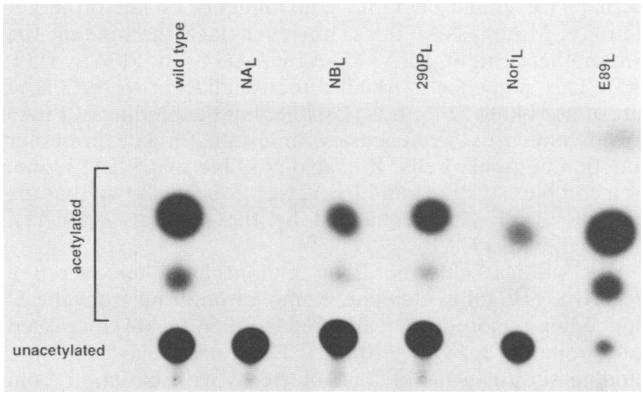

FIG. 4. CAT assays of late-promoter mutants compared with the wild type. The three different acetylated forms of [^{14}C]chloramphenicol ascend the chromatogram faster than the unacetylated form. Identical amounts of extract were used to measure activity. CAT activities for mutants NA$_L$ and E89$_L$ were not within the linear range of enzyme activity. These two assays were repeated with appropriate amounts of cell extracts in order to obtain reliable values.

mavirus sequences and was incapable of producing detectable CAT enzyme (data not shown). Typical results obtained in such assays of late promoter function with wild-type and mutant constructs are shown in Fig. 4. Table 2 shows quantitative results of CAT enzyme assays of all of the mutant late promoter sequences compared with the wild type. Each value was corrected for transfection efficiency (determined by electrophoretic analysis of plasmid DNA recovered from transfected cells), and each represents an average of three independent experiments. We have repeated such analyses of the wild-type and mutant constructs numerous times, each time obtaining values similar to those shown in Table 2. A number of interesting points can be made from these results.

First, most of the deletions had only modest effects on late promoter activity. Mutants PLM$_L$, 50$_L$, NB$_L$, 290P$_L$, Nstu$_L$, ΔT$_L$, and Nori$_L$ showed CAT activities of between 20 and 80% that of the wild type. Although CAT enzyme activities can show slight variabilities between assays, none of these mutants ever had activities as high as the wild type.

Second, three of the mutants, NA$_L$, NA-NB$_L$, and NA-50$_L$, showed a dramatic 50- to 100-fold decrease in CAT

TABLE 2. CAT activities of wild-type and mutant late promoters[a]

Mutant	CAT activity (10^3 U/μg of protein)	% of wild-type activity
WT$_L$ (wild type)	294.4	100
PLM$_L$	75.8	26
NA$_L$	3.5	1
50$_L$	65.0	22
NB$_L$	122.9	42
NA50$_L$	7.4	3
NA-NB$_L$	2.7	1
290P$_L$	231.5	79
Nstu$_L$	62.1	21
ΔT$_L$	245.8	83
Nori$_L$	83.2	28
E89$_L$	3,102.0	1,053

[a] The values for enzyme activity were determined as described in Materials and Methods. The percent acetylation for each assay was plotted against a standard curve of percent acetylation versus units of CAT enzyme.

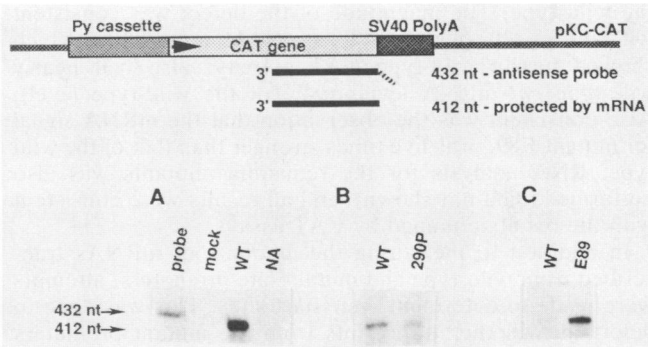

FIG. 5. RNase protection assay to measure levels of *cat*-specific transcripts. The top half of the figure shows the vector, pKC-CAT. An antisense radiolabeled probe was constructed from pBS-CAT, which contains the *cat* gene cloned downstream of the T3 RNA polymerase promoter (see Materials and Methods). 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. PolyA, Polyadenylation site. Total cellular RNA (25 μg) from cells transfected with wild-type and mutant polyomavirus (Py) promoter plasmids were hybridized to this probe. 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 was seen. (A) Probe alone (1:500 dilution), the protected RNA bands from an experiment with wild-type (WT) and mutant (NA) NA$_L$ RNAs, and RNA from mock-transfected cells. (B) Independent experiment with wild-type and mutant 290P$_L$ RNAs. (C) Comparison between wild-type and mutant E89$_L$ RNAs.

enzyme activity. These mutants all lack the enhancer A element, either alone (NA$_L$) or in conjunction with the enhancer B element (NA-NB$_L$) or with some of the sequences between the two elements (NA-50$_L$). We do not think that the enhancer B element or the sequences deleted in mutant 50$_L$ contribute greatly to this decrease in promoter activity, because singly deleting either of these sequences decreased CAT activity by only 2.5- to 4.5-fold (Table 2). In addition, mutant 290P$_L$, which lacks both the enhancer B element and the sequences deleted in mutant 50$_L$, showed only a 20% reduction in CAT activity.

Third, a surprising result was observed for the E89$_L$ mutant. This mutant exhibited late promoter activity which was 10-fold greater than that of the wild type. This mutant lacks two major large-T-antigen-binding sites, but why late promoter activity increased in the absence of these sites and in the absence of viral early proteins in this mutant is not yet clear. This point will be considered further in the Discussion.

(ii) Comparison of *cat* mRNA levels. Levels of *cat* mRNA were next measured to assure us that the decrease or increase in promoter activity demonstrated by the CAT enzyme data correlated with a decrease or increase in RNA transcription and was not due to posttranscriptional or translational effects. To do this, the *cat* gene was cloned into the vector pBS$^+$, which allowed us to generate an antisense RNA probe specific for *cat* mRNA (see Materials and Methods). Total-cell RNA was isolated from transfected cells and annealed to an excess of labeled riboprobe. Hybrids were then digested with RNase T$_2$, and protected species were resolved by polyacrylamide gel electrophoresis. Figure 5 shows the results of three such experiments. Mutant NA$_L$ exhibited a severe defect in *cat* mRNA accumulation compared with the wild type. Densitometry of autoradiograms of this experiment showed a 32-fold reduction in *cat*-specific mRNA for mutant NA$_L$ compared with

the wild type. The magnitude of the defect was consistent with the results of the CAT assays. Mutant $290P_L$, which showed nearly wild-type CAT activity, also had nearly wild-type *cat* mRNA levels (94% of the wild-type level). Also consistent was the observation that the mRNA signal for mutant $E89_L$ was five times stronger than that of the wild type. RNA analysis for the remaining mutants was also performed (data not shown), and all results were consistent with the results obtained by CAT assays.

In addition to measuring the amounts of mRNAs transcribed from wild-type and mutant late promoters, attempts were made to determine their start sites. This was done to determine whether transcripts from the mutant promoters initiate at the same sites as those from the wild-type promoter. Transfections were performed with pKC-CAT WT_L, pKC-CAT NA_L, and pKC-CAT $E89_L$ DNAs, cotransfected with a plasmid that encodes the three polyomavirus T antigens. In such experiments, there was a marked increase in the levels of *cat* mRNA produced compared with those performed in the absence of the early proteins (manuscript in preparation). Such cotransfections were necessary to observe significant levels of RNA initiating from all of these promoter constructs. In each case, the same four major start sites were observed (manuscript in preparation).

DISCUSSION

We have demonstrated here that multiple elements constitute the polyomavirus late promoter in transfected mouse NIH 3T3 cells. An important positive, *cis*-acting element lies within the enhancer region for the early promoter. By deleting 34 bp (nt 5122 to 5155) within enhancer element A (mutant NA_L), late promoter function was severely compromised (50- to 100-fold) as demonstrated by a decrease in CAT enzyme activity and *cat* mRNA production. A variety of other deletions within the viral regulatory region had modest (two- to fivefold) but reproducible effects on late promoter activity and may act to modulate the levels of late transcription. Finally, sequences which lie on the early side of the replication origin appeared to act in *cis* to negatively affect the late promoter in these cells. The removal of these sequences (mutant $E89_L$) resulted in a substantial increase in *cat* mRNA levels and protein accumulation. We have not yet determined whether any of the sequences we have identified as important for late promoter function act in an orientation- or distance-dependent fashion.

Due to the 31-bp duplication in the enhancer A region of strain 59RA, 16 of the 34 bases deleted in the NA_L mutant (nt 5140 to 5153) are still present. Because of this, it is possible that only nt 5122 to 5139 are responsible, in *cis*, for late promoter function. Alternatively, all 34 bp deleted in the NA_L mutant may actually be critical for late promoter function. It will be of interest to introduce the NA_L deletion into other strains of polyomavirus that do not have sequence duplications in this region.

A number of cellular proteins have been identified that bind to DNA sequences in the polyomavirus enhancer region (6, 34, 42, 50–53). No function has yet been assigned to any of these proteins. Piette and Yaniv (53) have identified two cellular proteins from NIH 3T6 nuclear extracts, PEA1 and PEA2, that interact with the polyomavirus enhancer A region. Their recognition sites are duplicated in strain 59RA. PEA1 binds to 5′-TGACTAA-3′ (nt 5140 to 5146 and nt 5171 to 5177) and PEA2 binds to 5′-TGACCGCAG-3′ (nt 5148 to 5156 and nt 5179 to 5187). Our mutant NA_L lacks only one of these binding sites for both PEA1 and PEA2, suggesting that perhaps PEA1 and PEA2 have no influence on late promoter activity. Mutant NA_L does, however, lack the binding site for another protein, PEA3 (5′-AGGAAG-3′, nt 5134 to 5139) (45). This sequence is found directly adjacent to the PEA1 site at nt 5140 to 5146. It is possible that the binding of PEA3 to polyomavirus sequences is important for late promoter function in mouse cells. It is also possible that PEA1 alone, or a complex of PEA1 and PEA2 (it has been shown that the binding of PEA2 is enhanced by the binding of PEA1), interacts with PEA3.

Our studies with the $E89_L$ mutant have uncovered a negative, *cis*-acting element. Removal of 89 bp from the 5′ noncoding region of the early genes (nt 56 to 144) increased late promoter activity ~10-fold. This region may contain a binding site for a factor that interferes with initiation from the late promoter. This putative repressor cannot be large T antigen, since our analyses were carried out in the absence of early viral proteins. However, large T antigen and a cellular factor(s) might compete for common binding sites. Another possibility is that removal of these sequences abolishes early promoter function, thus relieving promoter interference. We are in the process of testing this possibility.

Kern et al. (41) also studied wild-type and mutant polyomavirus late promoters fused to the *cat* gene and transfected into mouse cells. One of their promoter deletion mutants, pBE21CAT (a double mutant), showed a large decrease (>20-fold) in late promoter function. This mutant lacked sequences from within the late leader unit through the enhancer A segment and into the region sharing homology to the immunoglobulin enhancer. In addition, it lacked the palindromic origin and all sequences up to and including the early TATA box sequence. Constructs containing either of these deletions alone showed late promoter activities to be decreased by only two- to sixfold compared with the wild type. These data suggested that, like the early viral promoter, the polyomavirus late promoter is dependent on multiple *cis*-acting elements. At present we cannot reconcile the results of Kern et al. with our data, which show (i) a 50- to 100-fold reduction in late promoter activity by the removal of only 34 bp found within the enhancer A element and (ii) a 5- to 10-fold increase in late promoter activity in the absence of 89 bp within the early region of polyomavirus.

In an additional set of experiments, Kern et al. (38, 40) transformed rat F2408 cells with late promoter deletion mutants fused to a neomycin resistance gene. One plasmid used in that study, pBE11, lacks 38 bp (our numbering system, nt 5125 to 5163) and gave rise to the same number of $G418^r$-transformed colonies as the wild-type plasmid. The deletion in pBE11 is very similar to the deletion in our mutant NA_L (nt 5122 to 5155). The discrepancy between the results for pBE11 of Kern et al. with ours for NA_L may not be so puzzling. Our data came from transient-expression assays, while the transformation assay used by Kern et al. relied on integration of the plasmid, which occurs at various sites within the rat genome, of which some sites might be more transcriptionally active than others. A direct comparison between these two results is also difficult because of the different cell types and transfection methods used and the differences in the sequences of the mutants. We are currently performing experiments to determine what sequences are necessary for polyomavirus late promoter function in rat cells with a transient-expression system. Preliminary results suggest that the same mutants used in this study behave in somewhat different ways in rat cells (K. B. Cahill, A. J. Roome, and G. G. Carmichael, unpublished results). These studies will be reported elsewhere.

It should be noted that the late promoter of polyomavirus works well in transfection experiments, even in the absence of large T antigen (i.e., in the absence of viral DNA replication). This is an apparent contradiction to observations that the early transcripts account for >80% of the viral transcripts present early after infection but late transcripts are readily detectable only at late times (33, 63). Through the use of nuclear run-on assays, it has been shown in our laboratory that the polyomavirus late promoter is functional early after infection of NIH 3T6 cells (33). It has been proposed (33) that the lack of accumulation of late-strand transcripts at early times results from inefficient RNA splicing. It is not until later in infection that the late transcripts are spliced efficiently and accumulate rapidly.

Finally, like polyomavirus, SV40 also contains two transcription units (early and late), binding of its large T antigen to the genome triggers replication, and there are enhancer sequences used by its early promoter (for review, see reference 21). The late promoter consists of multiple elements. A number of studies have mapped both *cis*-acting positive and negative elements of the SV40 late promoter. These include the origin of replication (12), the 21-bp repeats (7, 28), and the 72-bp repeats (17, 22, 23, 29). However, this promoter is inefficient in the absence of large T antigen (7, 29, 36, 37). Some of the elements mapped for the SV40 late promoter require large T antigen for functioning when other specific sequences have been deleted (2, 36, 37). Although the polyomavirus late promoter does not require large T antigen for activity in transfected cells, early proteins have been reported to increase its activity (40, 41). We have also observed an increase in activity when cells are cotransfected with our late promoter plasmids and a plasmid that expresses the three viral early proteins. Our results in these studies on the transactivation of the late promoter by early proteins will be presented elsewhere (K. B. Cahill, A. J. Roome, and G. G. Carmichael, manuscript in preparation).

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