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Common Regulatory Elements Control Gene Expression from Polyoma Early and Late Promoters in Cells Transformed by Chimeric Plasmids

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In a previous report we showed that transcripts initiating from the late promoter of integrated polyoma plasmids could be detected at significant levels when neomycin resistance (*neo*) coding sequences were linked to this promoter. In this report we used chimeric plasmids that contain either a limited portion of the polyoma genome or deletions within the polyoma noncoding regulatory region to determine the sequence requirements for late promoter activity in this system. We observed no absolute requirement for either the polyoma early coding region or the origin of DNA replication for Neo^r colony formation. We were therefore able to independently assess the effects of deletions in the polyoma enhancer region on gene activity in both the early and late directions. We measured the ability of cells transfected with plasmids containing deletions in this region to form colonies in either semisolid or G418-containing medium under nonreplicative conditions. Our results indicate that either the *Pvu*II 4 fragment, which contains the simian virus 40 core enhancer sequence, or a region from nucleotides 5099 to 5142, which contains the adenovirus type 5 E1A core enhancer sequence, can be deleted without significantly affecting gene expression in either direction. However, a deletion of nucleotides 5099 to 5172 reduced activities to similar extents in both directions, and a plasmid containing a larger deletion of nucleotides 5055 to 5182 showed a further reduction in activity. Although having no effect by itself, a second origin region deletion of nucleotides 5246 to 127 when present in these mutant backgrounds caused either a further reduction or elimination, respectively, of both G418 and agar colony-forming ability, suggesting the presence of an additional common regulatory element within this region. A comparison of 5′ ends of *neo* transcripts present in cells transformed by these plasmids suggested that the reduction in activity was due to deletion of regulatory rather than structural elements of the late promoter. Our results indicate that the noncoding region of polyoma contains multiple complementing regulatory elements that control the level of both early and late gene expression.

The 5.3-kilobase (kb) genome of polyomavirus contains a noncoding region of approximately 460 base pairs (bp) that includes the viral origin of replication as well as sequences involved in the regulation of viral transcription. These sequences include an enhancer region that is required in *cis* for both early region gene expression and viral DNA replication (13, 41, 47, 55). The necessity of this enhancer region for replication is independent of the effects of this region on early region transcription since provision of the viral large T antigen in *trans* fails to induce DNA replication in mutants that lack this region (14).

The enhancer region is located within a 244-bp segment of polyomavirus DNA between the *Bcl*I and the *Pvu*II site at nucleotide 5262 (13). This region contains two nonoverlapping elements that have homology to two other viral enhancer elements (25). A core element found within the simian virus 40 (SV40) 72-bp repeats (2, 45) as well as in other viral enhancers is located within the *Pvu*II 4 fragment (25, 58). This fragment has per se only weak enhancer activity (25, 38, 57), but naturally occurring mutations which amplify this element (28, 50) (Fig. 1) confer on it much greater activity (25, 38). In contrast, the 120-bp *Bcl*I-*Pvu*II fragment has considerable enhancer activity that can be further increased by dimerization of these sequences (57). This 120-bp segment contains the region that has sequence homology to the adenovirus E1A enhancer (24). This region is duplicated in mutants of polyoma that are able to grow in PCC4 embryonal carcinoma cells, whereas the sequences containing the SV40 core enhancer element are deleted (33). In addition, sequence data of five mutant and wild-type strains of polyoma show that the region containing the E1A core enhancer element is often duplicated and mutated (49) (Fig. 1).

Like other enhancer elements, the 244-bp *Bcl*I-*Pvu*II fragment is capable of enhancing heterologous gene expression over large distances in an orientation-independent manner (13, 25, 46). The importance of these sequences in the control of polyoma early gene activity and viral DNA replication is clearly documented. However, it is not known whether late gene expression also depends on enhancers, since a significant level of viral late expression during lytic infection or in virus-transformed cells requires extrachromosomal virus DNA replication (16, 35, 53). Replication is dependent on the expression of the early gene for the viral large T antigen (3, 21, 53) and, as noted above, requires a functional enhancer element (14). Consequently, in these systems it cannot be determined whether the enhancer region has an independent effect on viral late gene expression.

The polyoma late promoter appears to be structurally different from other classical RNA polymerase II promoters such as the polyoma or SV40 early promoter. Late tran-

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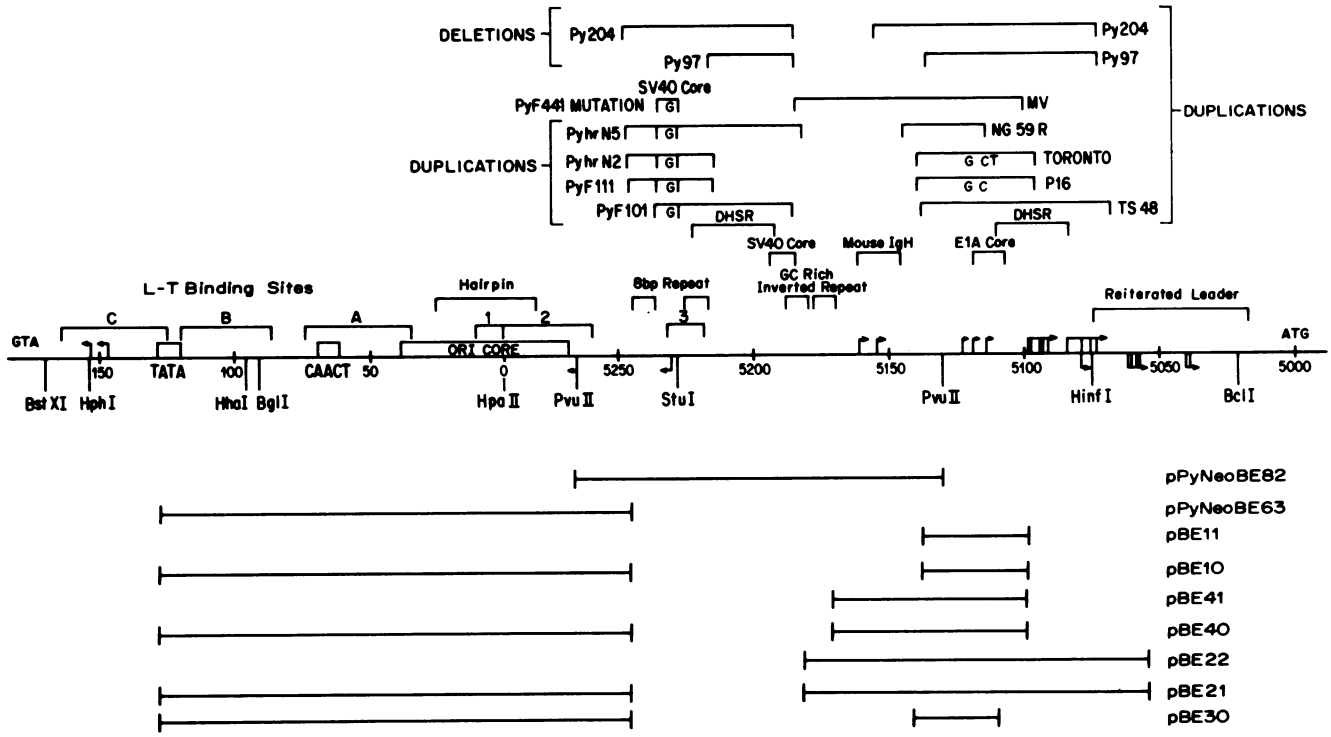

FIG. 1. The polyoma control region and the location of deletions present in plasmids used in this study. Shown are the locations of the large T antigen major and minor binding sites, TATA and CCAAT boxes for the early coding region, and minimal sequences constituting an origin of replication (10, 29, 30), as well as a 57-bp reiterated leader sequence generated by splicing of multimeric transcripts during lytic infection (18, 31, 36). Some of the structural features of the region are illustrated, including an area that can form a hairpin loop, an 8-bp direct repeat, and a GC-rich inverted repeat (14, 39, 51). The DNase hypersensitive region (DHSR [26]) and regions having homology to a core sequence present in the SV40 enhancer and adenovirus E1A enhancer elements are also indicated, as well as a region having homology to the mouse immunoglobulin heavy-chain enhancer element (24, 25, 58). Also shown are regions duplicated, deleted, and mutated in naturally occurring variants of polyomavirus (49) or in variants capable of growth in F9 (20, 50) or PCC4 (33) embryonal carcinoma cells. Mutated bases are indicated on the early strand. Locations of 5′ termini of polyoma early (32) and late-lytic (11, 54) transcripts are indicated by arrows above the line. Locations of additional 5′ termini or *tk* or *neo* late direction transcripts observed after transfection with chimeric plasmids (35) as well as the approximate locations of late-early transcripts (17) are indicated by the arrows below the line. Regions deleted in plasmids are indicated by the black line(s) to the left of the plasmid name. The nucleotide numbering system is according to Soeda et al (51).

scripts have heterogeneous initiation sites (11, 35, 54) that are not preceded by the canonical TATA (M. Goldberg, Ph.D. thesis, Stanford University, Stanford, Calif., 1978) or CCAAT (15) boxes that do form part of the polyoma early transcription unit (29, 30). We considered it therefore of interest to determine whether the same sequences within the regulatory region regulate the levels of both early and late transcription.

In a previous report we described a system in which coding sequences for neomycin resistance (*neo*) or herpes simplex virus thymidine kinase (*tk*) coding sequences are linked to the polyoma late promoter. We observed that when polyoma late coding sequences were replaced with these sequences, transcripts initiating from the late promoter could be detected in the absence of extrachromosomal replication in rat cells containing integrated copies of these chimeric plasmids (35). In this study we describe experiments performed with various deletions of these plasmids in an attempt to determine the viral sequences that are required for transcription in the late direction to occur. We found that transcription can occur in the absence of the polyoma early region and that a functional viral origin of replication is also unnecessary. This allowed us to independently assess the effects of deletions in the enhancer region on the expression of both the late and early transcriptional units. Our results indicate that the late polyoma promoter consists of a number of transcriptional elements and that deletions in the noncoding regulatory region affect early and late gene expression to approximately the same extent.

MATERIALS AND METHODS

Cells and cell culture. Rat F2408 fibroblasts were maintained in Dulbecco modified Eagle medium containing 10% (vol/vol) calf serum. The EL-2 cell line has been described in a previous publication from this laboratory (37). Details of the procedures used for transfection and selection for growth in G418-containing medium (8, 52) or growth in semisolid medium have been previously described (35).

Plasmid isolation and construction. Plasmid DNA was prepared by the alkaline lysis method and CsCl-ethidium bromide gradient centrifugation (42). Plasmids pPyNeoAE-2 and pB32 have been described previously (12, 35, 48). pAE2E1 was prepared by digestion of pPyNeoAE-2 with *Eco*RI and elution of the 6.1-kb fragment, followed by ligation and transformation of HB101. This plasmid therefore no longer contained the C-terminal portion of the polyoma large T coding region. pPyNeoBE63 was constructed by ligation of the 1.65-kb *Bcl*I-*Eco*RI fragment of pB32, which contains a 173-bp origin deletion and a portion

of the polyoma early coding region, to the *Bcl*I-*Eco*RI fragment of pPyNeoAE-2. (Both plasmids were grown in the *dam*$^-$ *dcm*$^-$ bacterial host 1255.) pB2E63 was constructed by digestion of pPyNeoBE63 with *Eco*RI, followed by treatment with calf intestinal phosphatase and ligation to the 3.5-kb *Eco*RI fragment of pPyNeoAE-2 that contains the C-terminal portion of the polyoma large T antigen. pPyNeoBE82 was constructed in a manner analogous to that described for pPyNeoBE63 by using the 1.7-kb *Bcl*I-*Eco*RI fragment of plasmid A2420P obtained from C. Tyndall. This plasmid contains a deletion of the *Pvu*II 4 fragment of polyoma.

Plasmids pBE10, pBE21, pBE30, and pBE40 were constructed as follows. pB32 was partially digested with *Pvu*II, the 8.0-kb fragment containing single-cut linear molecules was eluted, and portions were digested with BAL 31 nuclease for increasing lengths of time. The digested fragment was then ligated, and the ligation mix was used to transform HB101. Colonies were then screened for the absence of a *Pvu*II site at nucleotide 5129, and the approximate size of the BAL 31-generated deletion was determined by polyacrylamide gel electrophoresis after digestion with appropriate restriction enzymes. Four plasmids were chosen for further analysis. They were used to transform the *dam*$^-$ *dcm*$^-$ 1255 bacterial host, and the 1.5- to 1.6-kb *Bcl*I-*Eco*RI fragment was ligated to the 4.4-kb *Bcl*I-*Eco*RI fragment of pPyNeoAE-2. The precise locations of the deletions present in these plasmids were determined by Maxam and Gilbert sequencing (43) using fragments 5′-end labeled at the *Bcl*I site with [γ-^{32}P]ATP (7,000 Ci/mmol). These plasmids therefore contained double deletions within the polyoma control region: a deletion of varying length approximately centered around the *Pvu*II site at nucleotide 5129 and the pB32 origin deletion that spans from nucleotides 5246 to 127. This latter deletion was replaced by substitution of the mutant 1.5-kb *Stu*I-*Eco*RI fragment with the 1.6-kb origin-containing wild-type fragment from pPyNeoAE-2 to generate plasmids pBE11, pBE22, and pBE41. The origin-containing wild-type plasmid pBE102 was constructed in a similar manner by replacing the *Stu*I-*Eco*RI fragment of pPyNeoBE63.

pBgNeo3 was constructed in the following manner. An 813-bp *Bam*HI-*Bgl*II fragment was prepared from pCB7, a plasmid described in a previous study (35). This fragment spans the polyoma origin of replication from nucleotides 4632 to 153 and also contains a *Bgl*II linker added at the polyoma *Hph*I site at nucleotide 153. This fragment was inserted into a pML bacterial vector (40) via a *Bgl*II linker inserted at the *Bam*HI site of pML, giving rise to plasmid pBgOri-3 in which the *Eco*RI site of pML is to the early side of the polyoma origin. The *dam*$^-$ *dcm*$^-$ host 1255 was transformed with pBgOri-3, and a 0.8-kb *Bcl*I-to-*Eco*RI fragment that spans the polyoma origin was ligated to the 4.4-kb *Bcl*I-*Eco*RI *neo*-containing fragment of pPyNeoAE-2 described above.

RNA extraction and S1 analysis. Total polyadenylated [poly(A)$^+$] RNA was isolated by using the guanidium isothiocyanate extraction method, followed by oligodeoxythymidylate chromatography. S1 analysis of 5′ ends (6, 56) was performed with a *Taq*I-*Hph*I fragment of plasmid pBE40 5′-end labeled at the *Taq*I site by using [γ-^{32}P]ATP (7,000 Ci/mmol). Details of these procedures have been described previously (35).

RESULTS

Polyoma late promoter activity in the absence of early coding sequences and a functional origin of replication. In a previous report we showed that polyoma late transcription can occur from integrated templates in rat cells stably transformed by chimeric plasmids containing *neo* or *tk* coding sequences linked to the polyoma late promoter at nucleotide 5022 (35). In that report we utilized plasmids that contained a complete early coding region of the *tsA* strain of polyomavirus as well as the viral origin of replication. Stable TK$^+$ or Neo^r transformants were obtained when our experiments were carried out at 39°C, the nonpermissive temperature for the thermolabile large T antigen that is required for extrachromosomal viral DNA replication (19, 27, 28). This indicated that there was no absolute requirement for large T antigen or viral replication for late transcription to occur.

To confirm these conclusions and to begin defining the sequences in the polyoma noncoding regulatory region that might be required for late transcription, we constructed plasmids either devoid of any early sequences or with deletions of the origin of replication (Fig. 2). Since the frequency of colony formation should be a function of the level of gene expression (5, 44), we tested the ability of rat F2408 fibroblasts to form Neo^r colonies after being transfected with these plasmids. All of these plasmids contain *neo* coding sequences linked to the polyoma late promoter at the *Bcl*I site.

pBgNeo3 contains no early coding sequences, yet cells transfected with this plasmid are still capable of forming colonies in G418 (Table 1), thereby indicating no absolute requirement for viral early proteins for late transcription. pB2E63 contains a deletion from nucleotides 5246 to 127 and, therefore, lacks the core sequences of the viral origin of replication. The deletion derives from the pB32 polyoma plasmid, which is unable to replicate even when supplied in *trans* with large T antigen (12). Rat cells transfected with pB2E63 plasmid are also able to form Neo^r colonies with high frequency, indicating that a functional origin of replication is also not required for transcription in the late direction in this system.

Effect of noncoding region deletions on polyoma early and late gene expression. The results presented above indicated that a deletion of the entire early coding region did not affect the level of expression of *neo* linked to the polyoma late promoter. This result allowed us to assess whether deletions in the noncoding region of polyoma could independently affect transcription in either direction or whether deletions that affected early region activity would also affect transcription in the late direction.

To address this question, we used the plasmids depicted in Fig. 1 and 3. Again, these plasmids contain a *neo* gene linked to the polyoma late promoter at the polyoma *Bcl*I site at nucleotide 5022. All plasmids containing noncoding region deletions were linked to the *neo Bcl*I site (4). Two different wild-type control plasmids were utilized. One plasmid, pAE2E1, contained the polyoma sequences linked to the *neo Bgl*II site located 5 bp upstream of the *neo Bcl*I site. The other plasmid, pBE102, contained the polyoma and *neo* sequences linked at their respective *Bcl*I sites. No appreciable differences in the number of Neo^r or agar colonies were observed between these two plasmids in transfection experiments. The plasmids contain a polyoma early polyadenylation signal downstream of the *neo* coding sequences and the bacterial vector pML (40) from the *Bam*HI-to-*Eco*RI site in the same orientation. All plasmids contain a portion of the polyoma early coding region extending to the *Eco*RI site and, therefore, lack the C-terminal portion of the large T antigen but contain the coding sequences for the polyoma middle T antigen as well as the alternate polyadenylation

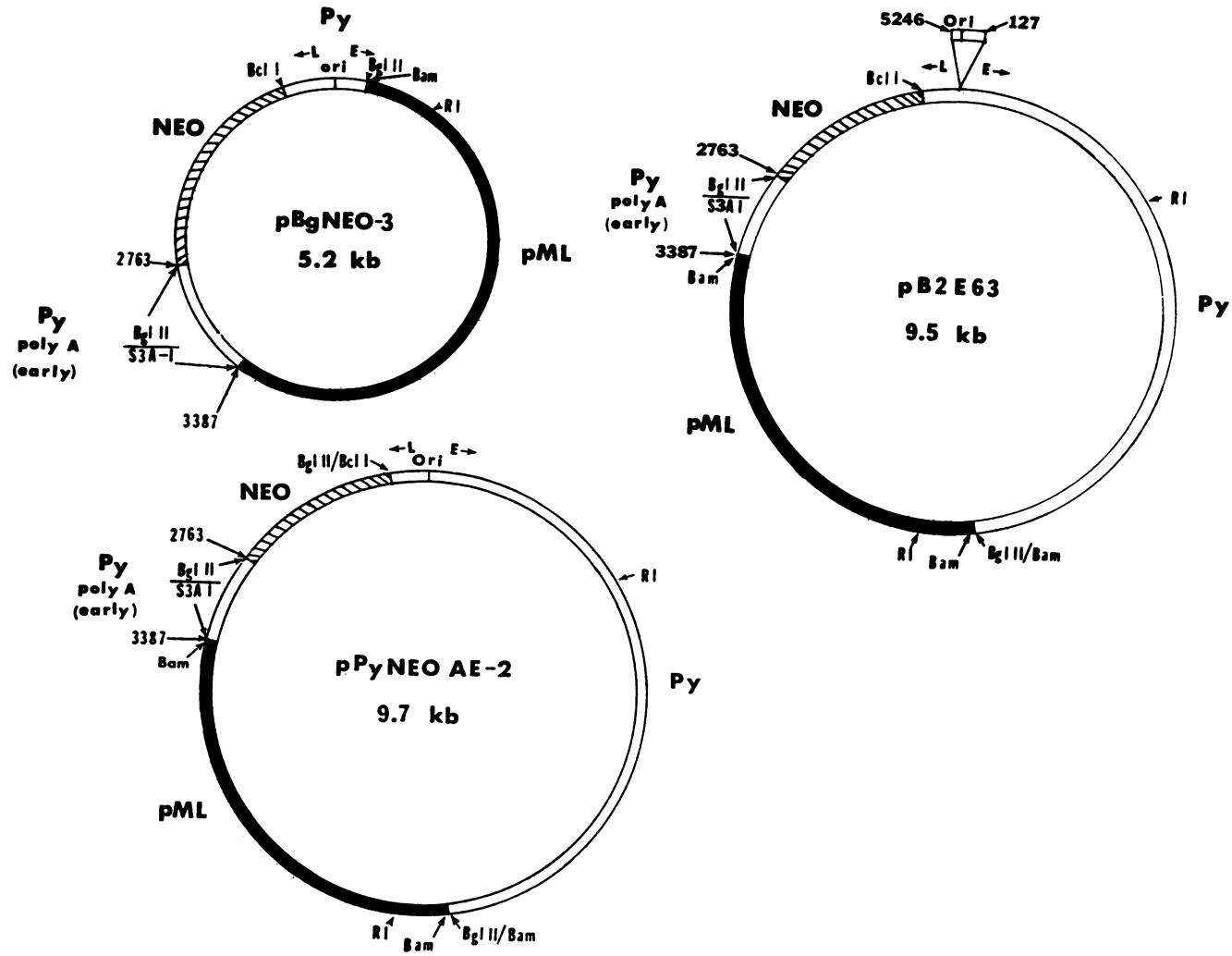

FIG. 2. Plasmids used to determine that late promoter activity does not require either a functional origin of replication or a polyoma early coding region. pBgNeo3 contains a segment of polyoma from nucleotide 153 through the origin of replication to nucleotide 5022. pB2E63 contains a deletion from nucleotides 5246 to 127 that spans the polyoma origin of replication. This plasmid contains the complete early coding region of *tsA* polyoma. pPyNeoAE-2 has been described in a previous publication (35).

signal at 99 map units (16, 51). Since the polyoma middle T antigen is sufficient for transformation in continuous cell lines, we could assess the level of early region activity with these plasmids independent of possible replicative effects caused by the presence of a functional large T antigen by scoring for the ability of cells to form colonies in agar. Similarly, the level of late region activity was measured by scoring the number of Neo^r colonies obtained using the same transfected cells.

TABLE 1. Late promoter activity in plasmids lacking early coding sequences or a functional origin of replication[a]

Plasmid	Polyoma origin	*tsA* early region	Frequency of colony formation in G418 ($\times 10^{-5}$)
pPyNeoAE-2	+	+	145.8
pBgNeo3	+	−	147.8
pB2E63	−	+	125.6
None	−	−	0

[a] Transfection was done using 2.0 μg of intact plasmid DNA per 100-mm dish. Selection was performed in G418 (250 μg/ml) at 39°C.

Our initial experiments were performed with a plasmid that contained a deletion of the *Pvu*II 4 fragment from nucleotides 5130 to 5265 and with four plasmids that contained a double deletion in the noncoding region. A deletion of the polyoma origin of replication from nucleotides 5246 to 127 was common to all four plasmids. In addition, the four plasmids contained a deletion of variable length that was generated by cleavage of the parental plasmid at the *Pvu*II site at 5130 and digestion with BAL 31 nuclease as described above.

Table 2 shows a summary of a number of experiments in which the results are presented as the proportion of colonies obtained with the mutant plasmids compared to those obtained with the control plasmids. In agreement with the results presented above with plasmid pB2E63, deletion of the polyoma replication origin again had no effect on either Neo^r or agar colony-forming ability when present in a plasmid containing a truncated early coding region. Deletion of the polyoma *Pvu*II 4 fragment also had no appreciable effect on either the level of Neo^r colonies or colony formation in agar. As seen in Fig. 1, this region contains an 8-bp repeat with homology to the bovine papillomavirus activator

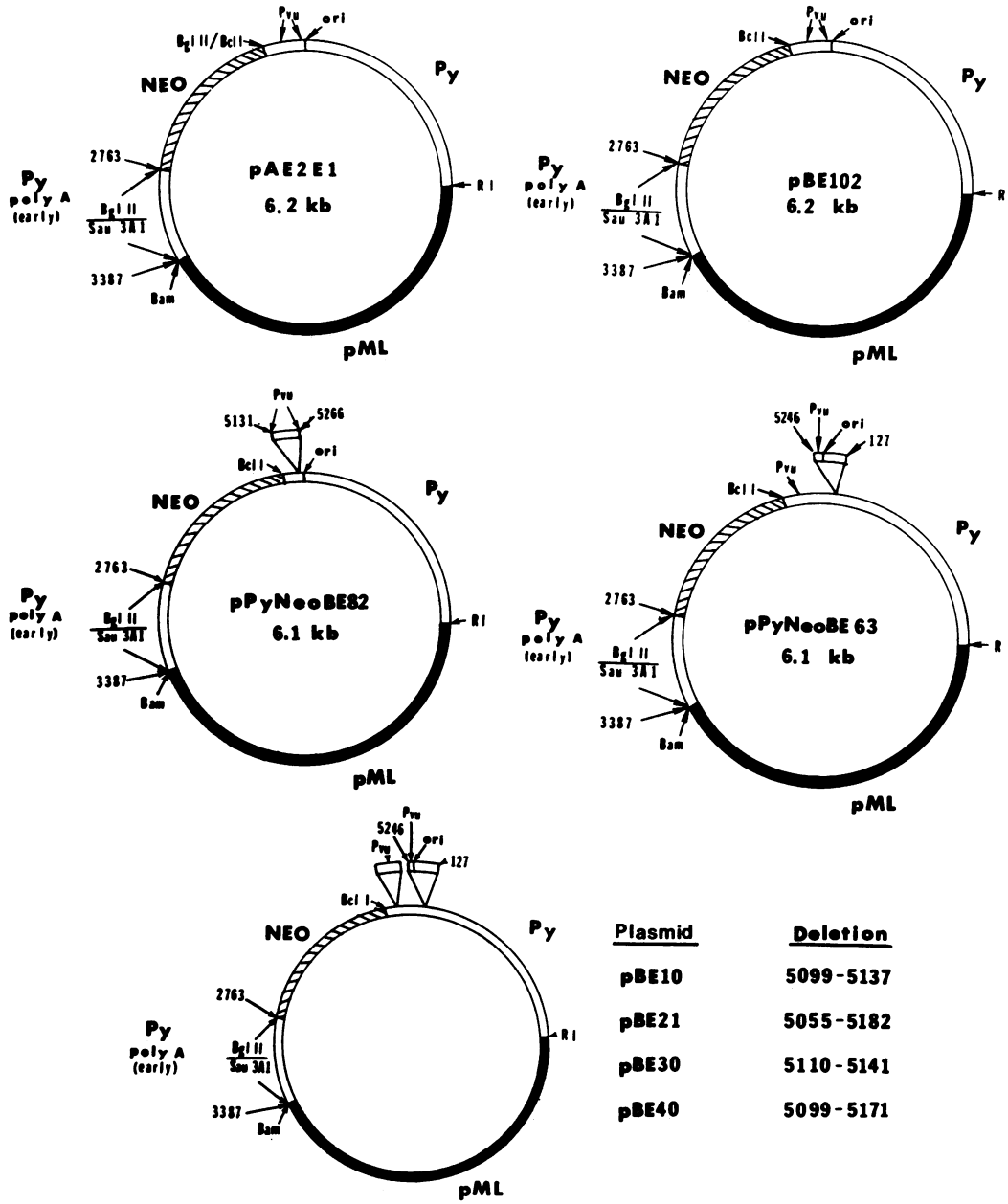

FIG. 3. Plasmids used to determine the polyoma sequence requirements for late promoter activity. Plasmids pAE2E1 and pBE102 are wild type in the noncoding regulatory region of polyoma and differ only in the linkage of the *neo* coding sequences to the late promoter. pPyNeoBE82 contains a deletion of the *Pvu*II 4 fragment of polyoma. pPyNeoBE63 contains a deletion of nucleotides 5246 to 127 and thus is deleted of the polyoma *ori*-region. This same deletion is also present in plasmids pBE10, pBE21, pBE30, and pBE40. These plasmids also have a second deletion that was generated by BAL 31 digestion at the polyoma *Pvu*II site at nucleotide 5130. The deleted sequences are indicated.

element (39) as well as a region having homology to the SV40 core enhancer element (25, 58). Our transformation results in rat cells agree with data recently published that indicate no effect of deletion of this region on transformation ability provided that other transcriptional elements important in early gene expression are present (41, 46). Although deletion of this fragment has been shown to eliminate the ability of recircularized viral DNA to form viable plaques when mouse embryo cells are transfected (55), the results of our *neo* transfection experiments indicate that these sequences are not essential for late transcription in our system.

Two of our plasmids containing double deletions also showed little change in the level of both Neo^r or agar colonies when compared to the plasmids we used as wild-type controls. The deletions in these plasmids (pBE10 and pBE30) either completely or partially eliminate, respectively, a stretch of polyoma sequences having homology to the E1A enhancer element as well as several other viral enhancer elements (24, 25).

The results shown in Table 2 are from experiments in which 2 μg of plasmid DNA was used for transfection. However, no effect of the origin deletion, the *Pvu*II 4

TABLE 2. Effect of polyoma control region deletions on the ability of recombinant plasmids to induce growth in agar or G418 resistance

Plasmid	Deletion	Relative efficiency[a]	
		Agar	G418
pAE2E1	None	1.0	1.0
pPyNeoBE63	5246–127	1.30 (2)	1.12 (3)
pPyNeoBE82	5131–5266	0.72 (3)	0.93 (4)
pBE10	5246–127 + 5099–5137	0.89 (2)	1.17 (4)
pBE40	5246–127 + 5099–5171	0.13 (3)	0.23 (4)
pBE21	5246–127 + 5055–5182	<0.01 (2)	<0.01 (4)
pBE30	5246–127 + 5110–5141	0.85 (2)	0.93 (3)

[a] Combined results indicating the average obtained from the number of independent determinations indicated by the number in parentheses. Transfections were done with 2.0 μg of plasmid DNA per 100-mm dish. Selection for G418 resistance was carried out at 39°C using 250 μg of G418 per ml.

fragment deletion, or the pBE10 or pBE30 double deletions was detected in experiments in which 0.1 μg of plasmid DNA was used, indicating that this result is not related to the high multiplicity of input plasmid DNA (see Table 4; unpublished data).

More extensive deletions around the *Pvu*II site at nucleotide 5130 in the origin-minus background did, however, affect to an equal extent the activity in both the early and late directions. pBE40 contains a deletion having the same 3′ end with respect to late transcription as pBE10 at nucleotide 5099, but the deletion extends 5′ an additional 33 bp to nucleotide 5171. The effect of this additional deletion is a reduction of Neo^r and agar colony-forming ability to approximately 20 and 10% of wild-type levels, respectively. This suggests the presence of a transcriptional element located between or near nucleotides 5137 and 5172 that affects both early and late transcription. pBE21 contains a larger deletion extending from nucleotides 5055 to 5182. In the origin-minus background, this deletion completely abolishes both early and late activity (Table 2). These data therefore indicate that late transcription also requires *cis* regulatory elements and that the sequences important for this function are the same for both early and late transcription.

An alternative explanation of the data presented above, however, is that the effect on late transcription is not due to the deletion of regulatory sequences but rather to the deletion of structural elements of the late transcriptional unit, such as the major cap sites used in the late *neo* plasmids or elements that fix the position of these cap sites. The further reduction in activity of pBE21 compared to pBE40 could be either the result of the extension of the deletion from nucleotides 5171 to 5182 or a reflection of the extension of the deletion in both directions. The abolishment of late gene expression could be due solely to the deletion of the region between nucleotides 5099 and 5055. This latter segment contains two regions utilized as transcription initiation points for *neo* transcripts (35) (Fig. 1).

To resolve these possibilities, we used S1 nuclease to map the 5′ ends (6, 56) of the *neo* transcripts in several Neo^r cell lines isolated after transfection with either the wild-type, pBE10, or pBE40 plasmid (Fig. 4). No differences were found among the RNAs extracted from these cell lines that would be consistent with the possibility that the reduced ability of the pBE40 plasmid to transform cells to a Neo^r phenotype is due to a lack of utilization of proper late mRNA cap sites. The locations of these start sites are shown diagrammatically in Fig. 1 and 4 and are in agreement with the positions used by other constructs containing *tk* or *neo* genes linked to the polyoma late promoter. As described in a previous publication, these positions are similar or identical to those used during polyoma lytic infection (35). In the case of the pBE21 deletion, which physically removes some of the major *neo* cap sites, this type of experiment would not have been informative. A different set of experiments, however, indicated that the presence of the *neo* cap sites upstream of nucleotide 5055 is not necessary for Neo^r colony formation.

Since the previous results had been obtained with plas-

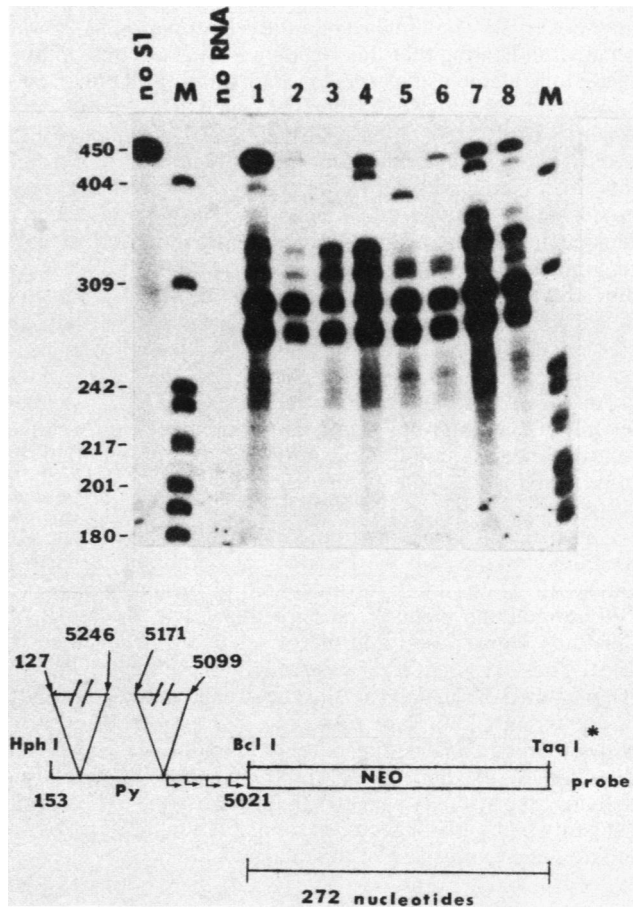

FIG. 4. S1 analysis of 5′ initiation sites of *neo* transcripts in cells transformed by plasmids containing deletions in the polyoma control region. Poly(A)$^+$ RNA (15 μg) was hybridized to 40,000 cpm of the 450-bp *Taq*I-*Hph*I fragment from plasmid pBE40. This fragment was end labeled at the *Taq*I site located in the *neo* coding sequences 272 bp downstream of the junction with the polyoma late promoter. The locations of the double deletions present in this fragment with respect to wild-type polyoma DNA are indicated. Hybridization was at 52°C for 3 h followed by digestion with S1 nuclease (200 U/ml) at 14°C for 2 h. Protected fragments were denatured and size fractionated on a 5% polyacrylamide–7 M urea denaturing gel. Lanes: 1 and 2, poly(A)$^+$ RNA from two Neo^r cell lines obtained after transfection with plasmid pBE40; 3 and 4, poly(A)$^+$ RNA from two Neo^r cell lines obtained after transfection with the wild-type plasmid pBE102; 5 and 6, poly(A)$^+$ RNA from two cell lines derived from agar colonies obtained after transfection with pBE10 that were subsequently found to be Neo^r; 7 and 8, poly(A)$^+$ RNA from cell lines derived from agar colonies obtained after transfection with pBE41 that were also found to be Neo^r. Arrows indicate the locations of the major initiation sites observed with the wild-type plasmid.

mids carrying two deletions, we wanted to assess whether deletion of the sequences between nucleotides 5246 and 127, which by itself had no effect, could have enhanced the effect of deletions around the *Pvu*II site. We therefore restored the wild-type sequences around the origin in the plasmids and determined their ability to transform cells to growth in agar or G418-containing medium. Replacement of the origin deletion from nucleotides 5246 to 127 in pBE40 to generate pBE41 resulted in an increase in ability to form colonies in both G418 and agar (Table 3). These results therefore suggest the presence of an additional transcriptional element located with the origin region that can affect both early and late transcription. However, the presence of this element does not restore activity to wild-type levels, and its effect is only observed when higher input levels of plasmid DNA are utilized, indicating that this region contains a transcriptional regulatory element that serves an auxiliary function.

A similar effect is seen when the origin deletion present in plasmid pBE21 is replaced to generate plasmid pBE22, leaving a single deletion from nucleotides 5055 to 5182. In this case, Neo^r colony-forming ability is restored to approximately 15% of wild-type levels. We tested the effect of replacement of the origin region deletion in pBE21 on early region activity using the EL-2 rat fibroblast cell line, which our laboratory has recently shown to be a more sensitive assay system for morphological transformation than the F2408 cell line (37). Even when this system was used, plasmid pBE21 did not yield colonies in agar above background levels (Table 4). However, plasmid pBE22 was able to produce a small but significant number of agar colonies following transfection, indicating that replacement of the origin region deletion affects early as well as late region activity.

Ten pBE22-transformed colonies were picked from agar medium, and the cells were tested for their ability to grow in G418. All 10 cell lines were capable of forming colonies in G418-containing medium, in agreement with the result obtained by transfection and direct selection for G418 resistance. These results therefore strengthen the hypothesis that the presence of the *neo* cap sites upstream of nucleotide 5055 is not essential for late *neo* gene expression. The partial restoration of transforming activity in both the early and late direction in plasmid pBE21 in the absence of the origin deletion also strongly suggests that the absence of regulatory elements is the major factor responsible for the suppression of late gene expression in this plasmid.

TABLE 3. Effect of replacement of origin region deletions in plasmids pBE10 and pBE40 on colony formation in agar and G418-containing medium

Plasmid	Deletion	Frequency of colony formation ($\times 10^{-5}$) in[a]:		
		Expt 1[b]		Expt 2[c]
		G418	Agar	G418
pAE2E1	None	49.6 (1.0)	18.6 (1.0)	12.3 (1.0)
pBE10	5246–127 + 5099–5137	41.8 (0.84)	15.8 (0.85)	15.4 (1.25)
pBE11	5099–5137	58.9 (1.19)	21.4 (1.15)	23.5 (1.91)
pBE40	5246–127 + 5099–5171	8.1 (0.16)	2.2 (0.12)	2.3 (0.19)
pBE41	5099–5171	23.4 (0.47)	4.8 (0.26)	2.3 (0.19)

[a] Numbers in parentheses indicate the relative efficiency.
[b] Transfection was done with 2.0 μg of intact plasmid DNA per 100-mm dish.
[c] Transfection was done with 0.1 μg of intact plasmid DNA per 100-mm dish.

TABLE 4. Effect of replacement of the origin deletion in plasmid pBE21 on colony formation in agar and G418-containing medium

Plasmid	Deletion	Frequency of colony formation ($\times 10^{-5}$) in[a]:	
		G418 (expt 1)[b]	Agar (expt 2)[c]
pBE102	None	126.1 (1.0)	245.8 (1.0)
pBE21	5246–127 + 5055–5182	0.2 (0.001)	<0.1 (<0.001)
pBE22	5055–5182	20.1 (0.16)	17.2 (0.07)

[a] Numbers in parentheses indicate the relative efficiency.
[b] Transfection into F2408 was done with 2.0 μg of intact plasmid DNA per 100-mm dish.
[c] Transfection into EL-2 was done with 2.0 μg of intact plasmid DNA per 100-mm dish.

DISCUSSION

In this report we attempted to define the polyoma sequence requirements for late promoter activity originating from integrated templates. In so doing we circumvented the necessity for viral DNA replication and, therefore, were able to assess the effects of deletions on late promoter activity independent of their effects on the early region. Our results demonstrate that like the polyoma early transcription unit, the late control region also consists of multiple genetic elements. In fact, the results obtained show that there is no one essential region from nucleotides 5055 to 127 that is absolutely required for either late or early region activity and point out the flexibility of this region with regard to the sequence requirements for functional promoter activity.

The results we obtained also show that there is no absolute requirement for a functional origin of replication for late promoter activity in our system. This is similar to the situation found with SV40, in which no effect on the number of stable TK$^+$ transformants was observed when the minimal replication origin was deleted from plasmids containing a promoterless herpes simplex virus *tk* gene linked to the SV40 late promoter (22). However, a small 4-bp deletion in the replication origin caused a 10-fold reduction in the efficiency of SV40 late transcription when this mutant was microinjected into *Xenopus laevis* oocytes and compared to wild-type DNA in this nonpermissive system (9). It therefore remains possible that late transcription initiating from integrated and extrachromosomal templates may have different sequence requirements.

Our results also indicate that there is no absolute requirement for any of the three viral early proteins for late promoter activity from integrated genomes. However, this does not, by necessity, indicate that the expression of early proteins has no effect on late promoter activity. Although we did not observe any strong effect of the viral early proteins on plasmids containing intact regulatory regions when using the *neo* transformation system described here, we were able to detect a partial restoration of Neo^r colony-forming ability by the pBE21 plasmid when it was cotransfected with a plasmid that codes only for the polyoma large T antigen (unpublished data). This stimulation of late promoter activity by the polyoma large T antigen may be similar to the enhancement of SV40 late promoter activity by the SV40 large T antigen in the absence of viral DNA replication that has been reported by other laboratories (7, 23, 34). Additional experiments examining the effect of the polyoma large T antigen on late promoter function are currently in progress.

Irrespective of the influence of large T antigen on late promoter activity, our data clearly indicate that the expres-

sion of the polyoma late promoter is also dependent on regulatory enhancer elements and that these elements appear to be the same as those necessary for early transcription. Recent results indicate the presence within the polyoma control region of two nonoverlapping enhancer elements. A major element is located between nucleotides 5096 and 5129, while a second enhancer is located between nucleotides 5175 and 5229 (25). In agreement with the results of Mueller et al. (46) that are also based on a transformation assay, the results presented here indicate that either of these two regions can be deleted without affecting early gene activity, provided the other element is present. Our results also indicate that the situation is the same for late promoter activity as well.

We noticed no evidence of reduced gene expression in mutant pBE11 containing a deletion from 5099 to 5137. However, both early and late region activity were reduced when this deletion was extended in the early direction from nucleotides 5137 to 5171 in mutant pBE41. A further reduction in activity was observed when deletions were extended in the late direction from nucleotides 5099 to 5055 and in the early direction from nucleotides 5171 to 5182 in mutant pBE22. Most importantly, these deletions appear to affect both early and late region activity to a similar extent. Also noteworthy is the finding that complete elimination of both early and late activity was only observed with this second larger deletion in an origin-minus background that contains a second deletion from nucleotides 5246 to 127. Restoration of this origin region increased both early and late region activity, even though the origin deletion alone had no effect on either early or late activity.

It has recently been suggested that the polyoma control region consists of a mosaic of complementing transcriptional elements (25), and our results support this interpretation. Mueller et al. (46) describe an element between nucleotides 5277 and 89 that can substitute for another element located between 5130 and 5229 in bringing about expression from the polyoma early promoter. A more detailed analysis of the transcriptional element present within our origin region deletion that affects both early and late activity should reveal whether these elements are one and the same.

Herbomel et al. (25) have pointed out that the degree of sequence homology between the mouse immunoglobulin heavy-chain enhancer (1) and nucleotides 5145 to 5162 suggests the presence of an additional transcriptional element located within this region, and our results comparing pBE11 and pBE41 lend further support to this notion. The reduction in activity observed between these two plasmids could be due to an element located beween nucleotides 5137 and 5171. Alternatively, this reduction could also be due to the deletion of the sequences bordering the B enhancer element described by Herbomel et al. between nucleotides 5175 and 5229 (25), with the lack of these neighboring sequences between nucleotides 5137 and 5171 subsequently causing a reduced activity of this B element.

The deletion present in pBE21 and pBE22 includes all of the A element and extends into the borders defined by Herbomel et al. (25) for the minor B element, a fact which most likely accounts for the absence or extremely low levels of activity observed with these plasmids. In addition, these two plasmids delete nucleotides 5055 to 5099, which contain two of the three principal regions where *neo*-containing transcripts have previously been shown to initiate by S1 mapping (35), as well as much of the region that functions as a leader sequence during polyoma lytic infection (18, 31, 36). However, since Neo^r colonies are observed when the origin deletion present in pBE21 is replaced to give pBE22, it is unlikely that these factors can fully account for the complete absence of Neo^r colonies observed after transfection with pBE21. Moreover, a comparison of the 5′ initiation site of *neo* transcripts in pBE40- and pBE41-transformed cells by S1 analysis did not indicate that replacement of the origin region leads to the utilization of new major initiation sites (Fig. 4). It is therefore unlikely that the replacement of the origin provides a new late transcription initiation site in plasmid pBE22. A definitive answer to these questions must await S1 analysis of the 5′ *neo* termini present in RNA from cells transformed with pBE22 and testing of the effects of a wild-type enhancer element placed distal to the *neo* gene on the levels of *neo* transcription in this mutant.

Also unresolved is whether the sequences between nucleotides 5099 and 5055, which are deleted in the nonfunctional pBE21 plasmid, have any role in augmenting the levels of either early or late transcription. We observed with plasmids pBE11 and pBE30 that deletion of the region from nucleotides 5099 to 5141 had no effect on either early or late activity. However, Mueller et al. (46) report that extension of a 5′ unidirectional deletion mutant from nucleotides 5039 to 5131 results in an eightfold decrease in early region activity. In combination with our results, this would therefore implicate the region between nucleotides 5039 and 5099 as being functionally important. In addition, it has recently been shown that this region can also contribute to the degree of enhancement effected by the neighboring core enhancer A element (25). Since this region does contain *neo* cap sites, it is conceivable that it includes a *cis*-acting regulatory element for early transcription that also functions as a structural element for late transcription. Analysis of additional plasmids containing deletions of this region should be able to determine whether such an element with overlapping functions does indeed exist.

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