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Deletions of N-Terminal Sequences of Polyoma Virus T-Antigens Reduce but Do Not Abolish Transformation of Rat Fibroblasts

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Polyoma virus transforms, upon infection or DNA transfection, nonpermissive Fisher rat fibroblasts. Cloned viral DNA was deleted of sequences around the *BglI* site at nucleotide 86 by Bal31 nuclease treatment and then recloned in *Escherichia coli*. The extent of deletion for each mutant was then determined by DNA sequencing. Deletions included the early transcription control signals; others stretched into the N-terminal coding sequences of the viral tumor antigens. The transformation efficiency of 16 mutants was tested by transfecting rat fibroblasts. Expression of the T antigens was analyzed by immunofluorescence detection after transfection of rat fibroblasts, mouse secondary embryo cells, and HeLa cells. We found that the absence of the early transcription control sequences (TATA and CAAT boxes) did not significantly alter the transformation capacity of the virus. On the other hand, deletion of the initiator methionine ATG codon or further into the coding sequences did abolish the transformation capacity in some mutants, whereas others maintained a reduced transforming activity, possibly by initiation of translation in a penultimate methionine.

Mouse cells are the natural host of polyoma virus. Infection of Fisher 3T3 (FR3T3) rat fibroblasts (20) or baby hamster kidney cells (16) by polyoma (Py) virus does not produce viral particles (24), but does result in a transformed phenotype, readily detectable by the absence of contact inhibition when the cells are grown on plastic and the appearance of multi-layer colonies. The virus expresses its three early genes only. These code for the large tumor (T) antigen, with an apparent molecular size of 95,000 daltons (d), the middle T antigen of 55,000 d, and the small t antigen of 22,000 d (21). It has been shown recently that viral sequences coding for middle T are sufficient for transformation of an established rat cell line (25). Initiation of transcription and the 5′ capped ends as well as the 3′ termini of the early mRNAs are identical, and only the splicing pattern varies (9, 22). Transcription is initiated at nucleotides (nt) 148 to 152 in a heterogeneous manner (8) and is controlled and promoted by noncoding sequences around the origin of DNA replication (8, 26; the numbering system for the Py DNA sequence adopted throughout this paper is the one proposed by Soeda et al. in reference 22).

We wanted to answer two questions, as follows. (i) What is the relative importance of noncoding control sequences, such as the Goldberg-Hogness box (M. Goldberg, Ph.D. thesis, Stanford University, Stanford, Calif., 1978) between nt 120 and 128, the CAAT box (2) between nt 61 and 69, and the binding site for large T antigen (4) between nt 49 and 64, in the efficiency of transformation? (ii) Will viral sequences transform FR3T3 in spite of deletions in the N-terminal coding sequences of the T antigens starting at nt 173?

To answer these questions, we deleted sequences of cloned Py DNA around the *BglI* site at nt 86 by Bal31 nuclease treatment and tested the transformation efficiencies of the altered viruses on FR3T3 cells (rat fibroblasts). The exact extent of the deletion for each cloned mutant was determined by DNA sequencing. The expression of the T antigens was also explored by immunofluorescence detection after transfection of replication-nonpermissive FR3T3 and HeLa cells as well as permissive secondary mouse embryo cells.

Our results demonstrate that viral transformation capacity could be conserved when different control elements were absent. Furthermore, deletions of N-terminal coding sequences greatly reduced but did not always abolish the transformation of FR3T3 by Py. The efficiency of such transformations is not directly linked to the extent of coding sequences deleted and may instead be related to a possible initiation of translation at an internal methionine.

MATERIALS AND METHODS

Materials. Restriction endonucleases were purchased from New England BioLabs, Bethesda Research Laboratories, or Boehringer Mannheim. Bal31 was from Bethesda Research Laboratories; calf intestine alkaline phosphatase and *Escherichia coli* DNA polymerase I-A fragment were from Boehringer. Lysozyme (grade I) was from Sigma, and pancreatic RNase I was obtained from Worthington. T4 DNA ligase and kinase were purified by standard procedures (19, 23). *Sal*I linkers were from Collaborative Research Inc., Waltham, Mass. [α-^{32}P]dTTP and [γ-^{32}P]ATP, both at a specific activity of 3,000 Ci/mmol, were obtained from Amersham Corp. Chemicals were all analytical grade or better and were purchased from Merck, BDH, or Sigma.

Cells. FR3T3 (Fisher rat fibroblasts; 20) were cultivated in Dulbecco modified Eagle minimal essential medium from GIBCO, supplemented with 7% fetal calf serum (GIBCO). Cells were plated at low densities (3×10^5 cells per 10-cm dish) and passaged every 3 days. Cells were discarded after 15 passages. Secondary mouse embryo cells (10 to 12-day embryos) were grown in Dulbecco modified Eagle minimum essential medium with 10% fetal calf serum. HeLa cells were grown in the same medium with 5% fetal calf serum.

Molecular cloning and Bal31 nuclease treatment. Purified Py viral DNA (A2 strain) and pMK16* (5) DNA were linearized by *Bam*HI and ligated with T4 DNA ligase. The pMK16* vector was treated before ligation with alkaline phosphatase to prevent intramolecular circularization. The ligation mixture was used to transform $CaCl_2$-treated competent *E. coli* K-12 C600 bacteria as described elsewhere (11). DNA was purified from clones harboring a Py-containing hybrid plasmid by the slightly modified cleared-lysate technique (12) described elsewhere (11). After precipitation with 10% polyethylene glycol 6000 in the presence of 0.5 M NaCl and RNase I treatment, DNA was further purified by centrifugation in a cesium chloride-ethidium bromide density gradient.

An 80-μg sample of hybrid plasmid DNA (p2034) was digested with *Bgl*I restriction endonuclease, phenol extracted, and dialyzed against 5 mM Tris-hydrochloride (pH 8.0)–0.5 mM EDTA. After ethanol precipitation, DNA was dissolved in 250 μl of 20 mM Tris-hydrochloride (pH 8.0)–12.5 mM $MgCl_2$–12.5 mM $CaCl_2$–200 mM NaCl–1 mM disodium EDTA. The reaction was carried out at 37°C and was started by adding 1 μl (2 U) of Bal31 nuclease. Each minute, 12 μl of the reaction mixture was withdrawn, and the nuclease action in each sample was stopped by adding 4 μl of 0.1 M EDTA and heating for 5 min at 65°C. The amount of degradation was assayed by resriction analysis and gel electrophoresis, and DNA molecules shortened by 25 to 50 base pairs were saved. The Bal31-treated DNA was employed in two ways, as follows. (i) A 1-μg sample of this DNA was ligated with 1 U of T4 ligase for 48 h at 4°C; by labeling with 1 μCi of [γ-^{32}P]ATP and 2 mM ATP by T4 polynucleotide kinase as described elsewhere (11) before ligation, it was estimated that about 60% of the molecules were ligated. (ii) 5'-^{32}P-labeled *Sal*I linkers (50 times molar excess) were ligated to 1 μg of the DNA for 20 h at 4°C. After digestion for 4 h with 100 U of *Sal*I, plasmid DNA was separated from free linkers by Sephadex G-200 (Pharmacia) column filtration in 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA. After ethanol precipitation, the plasmid's *Sal*I cohesive ends were ligated with 0.1 U of T4 ligase for 4 h at 20°C, and the ligation mixture was used to transform *E. coli* K-12 C600 bacteria. DNA extracted from individual clones was submitted to digestion with restriction endonucleases.

Transfection and cellular transformation. Cells were transfected by plasmid DNA prepared as described above by the calcium phosphate technique (6). Briefly, 0.01 to 10 μg of DNA was phenol extracted, ethanol precipitated, and dissolved in 450 μl of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.05)–137 mM NaCl–5 mM KCl–5 mM dextrose–0.71 mM Na_2HPO_4. After the addition of 50 μl of 1.25 M $CaCl_2$, the precipitate was allowed to form for 20 min at room temperature and was then placed in a 6-cm dish seeded 24 h previously with 2.5×10^5 cells. After incubation for 4 to 24 h, the cells were washed twice with Tris-Dulbecco medium and covered with fresh medium. Alternatively, cells were incubated after the Tris-Dulbecco medium wash for 5 to 15 min with HEPES buffer containing 15% glycerol, then washed and covered with medium (18). Forty-eight hours after the calcium phosphate precipitate was added cells were trypsinized and transferred to a 75-cm^2 flask. After 10 or 11 days, cells were fixed with 90% ethanol and colored with Geimsa R stain. Apparent colonies were counted.

Electrophoresis systems. Analytical electrophoresis of DNA molecules was done on 1 to 2% horizontal agarose (Sigma, type II) slab gels. Preparative electrophoresis was performed with 6 to 8% acrylamide slab gels (30;1, acrylamide–*NN'*-methylenebisacrylamide). Both agarose and acrylamide gels were run in a 90 mM Tris-borate (pH 8.45)–2.5 mM EDTA electrophoresis buffer.

DNA 3'-end labeling and sequence determination. Two picomoles of plasmid DNA was cut by *Sal*I, *Bam*HI, and *Eco*RI restriction endonucleases. After phenol extraction and ethanol precipitation, the pellet was suspended in 10 mM Tris-hydrochloride (pH 7.6)–10 mM $MgCl_2$–50 mM NaCl–1 mM dithiothreitol, to which were added 40 μCi of [α-^{32}P]dTTP and 0.5 U of *E. coli* DNA polymerase I-A fragment. Incubation was at room temperature for 1 h. The ^{32}P-3'-end-labeled fragments were separated on a polyacrylamide gel and electroeluted as described elsewhere (11). Partial chemical degradation and analysis of the products on 6 and 20% thin acrylamide-urea gels were done by the procedure devised by Maxam and Gilbert (17). After electrophoresis, gels were submitted to autoradiography at −20°C on Kodak NS-2T or Fuji XR films.

Immunofluorescence assays. Immunofluorescence detection of T-antigen-positive cells was done 60 to 72 h after transfection with plasmid DNA as described above, according to techniques described elsewhere (27).

RESULTS

Formation of Py deletion mutants by Bal31 nuclease treatment. A2 strain Py virus DNA extracted from infected secondary mouse embryo cells by the Hirt technique (7) was further

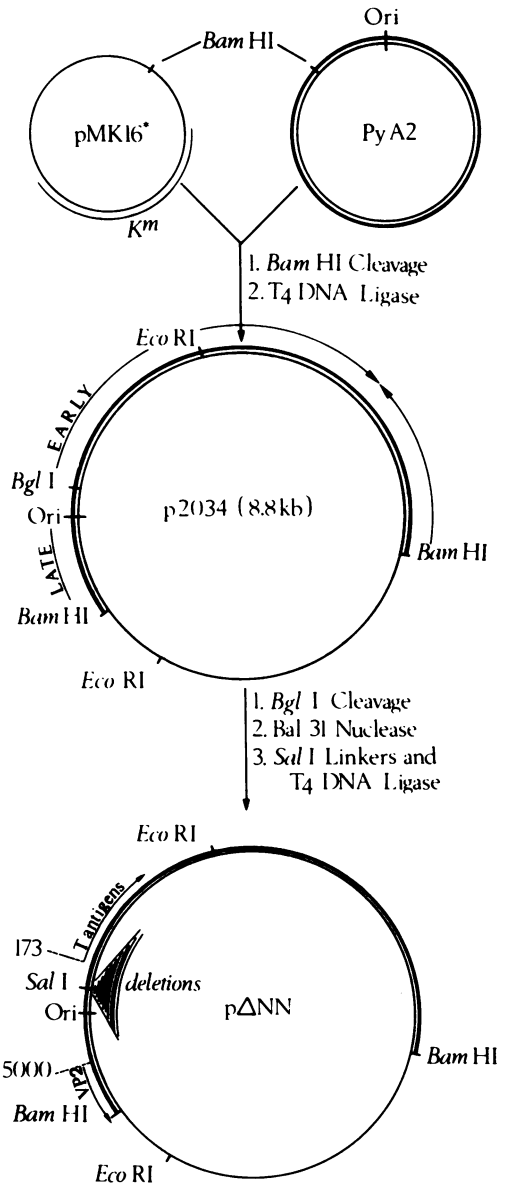

FIG. 1. Construction of Py mutants by Bal31 nuclease digestion. Linearized Py DNA and pMK16* were ligated through their unique *Bam*HI site. After transformation of *E. coli*, one clone harboring a hybrid plasmid (p2034) was chosen. Plasmid DNA was extracted and linearized by its unique *Bgl*I site. After Bal31 nuclease digestion, *Sal*I linkers were ligated with T4 DNA ligase, and after elimination of the free linkers by column filtration, the plasmids were recircularized by ligase and used to transform bacteria. DNA extracted from individual clones was analyzed, sequenced, and used in transformation and immunofluorescence assays on various cell lines. The *Bam*HI, *Bgl*I, *Sal*I, and *Eco*RI sites of the different plasmids, the origin of DNA replication of Py (Ori), the sense of transcription, and the early and late regions of Py are indicated.

purified by centrifugation in cesium chloride-ethidium bromide density gradients. We chose to clone the viral DNA with pMK16* as a vector since this plasmid is devoid of *Bgl*I sites (5). The entire Py genome was inserted into the plasmid in the L orientation (the Py origin of DNA replication is near the *Eco*RI site of pMK16*; see also reference 11). This hybrid plasmid (p2034), used in all the experiments described in this paper, was exensively examined to assure its correspondence to the viral Py DNA by restriction analysis with *Hpa*II, *Pvu*II, and *Taq*I endonucleases followed by agarose gel electrophoresis (results not shown).

After linearization of p2034 with *Bgl*I (see Fig. 1), it was submitted to Bal31 nuclease digestion. Surprisingly, Bal31 digested the plasmid DNA at a much slower rate than expected. Agarose gel electrophoresis showed that the Bal31 digestion progressed four or five times faster into the AT-rich early region than towards the GC-rich origin of DNA replication (results are not shown, but see reference 22 for the nucleotide sequences in this region). The enzyme seemed to be arrested in GC-rich clusters of the DNA and generally stopped at a GC base pair. In 16 mutants for which the exact extent of deletion was determined by DNA sequencing, Bal31 was found to have stopped its action 27 times at a GC base pair and only 5 times at an AT base pair (see Fig. 2). These results might solely reflect that the high salt concentration in the digestion buffer (0.2 M NaCl) impairs separation at a GC base pair and thus hinders the enzyme's action. Bal31-digested DNA was recloned in *E. coli* to obtain well-characterized deletions. Insertion of *Sal*I linkers in the deletion plasmids before cloning was performed to facilitate the sequence determination and the use of this deleted Py DNA in other vector systems (B. Bourachot et al., Eur. Mol. Biol. Organ. J., in press).

Sequence analysis of the cloned deletion mutants. As outlined in the previous section, the plasmids contained a *Sal*I linker inserted in the middle of the deletion. Each plasmid was digested with *Sal*I, *Bam*HI, and *Eco*RI, and the *Sal*I cohesive ends were specifically labeled at their 3′ ends with [α-^{32}P]dTTP in the presence of *E. coli* DNA polymerase I-A fragment. pΔ52 was digested with *Pst*I and *Sal*I before labeling because it lacked the *Bam*HI and *Eco*RI sites of pMK16* (see Fig. 1). After acrylamide gel electrophoresis, the two *Sal*I 3′-labeled fragments were electroeluted and sequenced by the partial chemical degradation technique of Maxam and Gilbert (17). Sequences of about 200 nt around the *Sal*I site were established, and the results for the 16 plasmids analyzed are summarized in Fig. 2. These sequences, except for the difference due to deletions themselves, were identical to

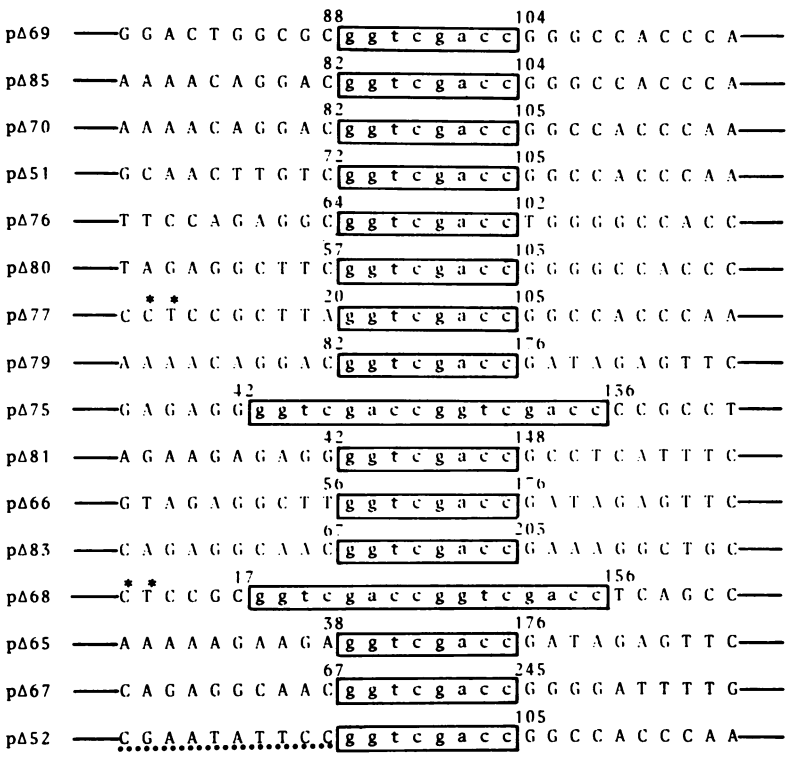

FIG. 2. Sequence of the deletions in different mutants. The sequences on the borders of the Bal31-generated deletions, as determined by the Maxam and Gilbert technique (17), are shown in both directions from the inserted *Sal*I linker, which is boxed. pΔ75 and pΔ68 possess two *Sal*I linkers. The numbering system is that of Soeda et al. (22). The asterisks show nucleotides in addition to those found in the previously published sequence of wild-type A2 Py (22).

the one published for A2 Py by Soeda et al. (22) with the following exceptions: (i) nt 5 was found to be guanine and not adenine; (ii) two additional nucleotides, a cytosine and a thymine, were inserted between nt 13 and 14. A deletion of 1,200 nt was observed in pΔ52. This deletion proceeded towards the late region of Py, including all the Py sequences up to the *Bam*HI site at nt 4,632 and about 450 nt into pMK16* (see Fig. 1 and 2). This deletion was much larger than the mean deletion observed in the other 15 mutants; the explanation might simply reside in the removal by Bal31 of a fragment starting at a single-strand nick on pMK16*.

To estimate the number of *Sal*I linkers inserted, we measured the rate of cleavage by *Sal*I of the different plasmids. Appearance of linear pΔ75 and pΔ68 was twofold slower than that observed for the other 14 plasmids. Since the insertion of two linkers should have created a supplementary CCGG *Hpa*II site, these two plasmids were digested with *Hpa*II. After analysis by electrophoresis on a 2.5% agarose gel, an additional *Hpa*II site was effectively observed within the Py *Hpa*II-5 fragment (in which the *Sal*I linker was inserted).

Transformation assays with characterized Py deletion plasmids. The 16 sequenced Py deletion plasmids were tested for their transformation efficiency on FR3T3. Transfection of the cells with 1 μg of A2 Py viral DNA, p2034, or the Py deletion plasmids was done as described in Materials and Methods. The results obtained are summaried in Table 1; The number of transformants counted when p2034 was used was taken as 100% transformation efficiency.

FR3T3 fibroblasts were transfected with increasing amounts of p2034 DNA from 0.01 to 10 μg. The number of transformants generated was colinear with the quantity of DNA employed up to roughly 0.1 μg, but not for larger amounts. A further 100-fold increase of the input DNA only tripled the number of transformants obtained. In all experiments described in this paper, no carrier DNA was added in the transfection experiments. We estimated that 0.1 to 0.2% of the FR3T3 cells were effectively transformed by the wild-type A2 Py-containing plasmid.

TABLE 1. Transformation efficiency of cloned Py deletion mutants[a]

Mutant	Transformation efficiency (%)[b]	Amplification factor[c]	Size of foci[d]
p2034	100	3	Big
Py A2	55	3	Big
pΔ69	21	3	Big
pΔ85	20	2.5	Big
pΔ70	18	3.5	Big
pΔ51	21	2	Medium
pΔ76	18	3.5	Medium
pΔ80	21	3.5	Big
pΔ77	29	4	Very big
pΔ79	0		
pΔ75	21	3.5	Big
pΔ81	23	3	Big
pΔ66	0		
pΔ83	0		
pΔ68	20	2.5	Medium
pΔ65	4	2	Small
pΔ67	0.5	3.5	Very small
pΔ52	2	6	Big

[a] A 1-μg sample of plasmid DNA was used to transfect 5×10^5 FR3T3 cells in a 6-cm petri dish. Cells were transferred to a 75-cm^2 flask after 2 days and were examined for transformation 8 to 9 days later.

[b] Values were normalized to that of the nondeleted Py-harboring plasmid p2034 in non-glycerol-treated cells.

[c] Augmentation in number of transformants observed when transfection was followed by a 5-min treatment with 15% glycerol.

[d] Overall size of the transformed foci in comparison with the size observed 10 to 11 days after transfection with the p2034 transformants.

The conditions of the experiment were varied by using 0.1 or 10 μg of DNA in transfections or by incubating the cells with calcium phosphate-DNA precipitates for 4 to 24 h. However, these changes did not modify the relative transformation efficiencies of the different DNAs used. With pΔ83, one to two transformants were observed when 10 μg of DNA were used; none were seen at lower amounts. No transformants were ever observed with pΔ66 and pΔ79. Viral Py DNA had only 60% of the efficiency observed when Py was inserted in pMK16* (p2034). The transformation process of the cell implies the integration of the transfected DNA into the host's chromosomes (24). The probability of integration in the 1.6-kilobase-pair middle T-antigen coding sequences is higher for the 5.3-kilobase-pair Py DNA than for the 8.8 kilobase pairs of p2034. Furthermore, it is unknown whether FR3T3 cells are completely nonpermissive to Py infection, and a complementary explanation might be a limited expression of Py viral DNA in FR3T3, which impairs with the transformation processes, but which is not possible when Py is integrated in a plasmid.

When transformation was followed by a 15% glycerol treatment of 5 to 15 min, we observed a net augmentation of two to six times in the number of transformants visualized (see Table 1). This factor varied from one plasmid to another but was constant for a given plasmid from one experiment to another. Parker and Stark (18) have shown that a glycerol treatment after DNA transfection increased the number of simian virus 40 lysis foci observed on permissive cells. The simplest interpretation might be that glycerol enhances the uptake of the membrane-bound calcium phosphate crystals by modifying the membrane structure.

Transformation with heterogeneous stocks of Py deletion plasmids. To examine further the correlation between the overall size of deletion and the transformation efficiency, we devised the following experiment. DNA taken after different times of the Bal31 digestion was restricted by *Pst*I and electrophoresed on an agarose gel to measure the size of mean deletions towards the early region downstream from the *Bgl*I site. Mean deletions upstream towards the origin of DNA replication were harder to estimate because of a lack in judicious restriction sites, but as stated above these deletions were rarely longer than 70 nt at the time points when the Bal31 nuclease digestion was analyzed, probably because of the high GC content at the origin of DNA replication.

For the following experiment, we used 0.1 μg of populations with mean early deletions of up to 300 nt. After ligation, FR3T3 cells were transfected and the number of transformants was counted. Figure 3 summarizes the results of this experiment. We defined 100% efficiency as the number of foci obtained with 0.05 μg of p2034. Efficiency dropped as bigger deletions were present in the population. With only small deletions of about 25 nt, it was already down to 30 to 50% of p2034, very similar to what was observed in the preceding section for individual deletion plasmids. Efficiency was down to 1.5% when mean deletions attained 100 nt. Surprisingly, when deletion stocks were taken from even longer Bal31 digestion times (125 to 150 nt removed), the number of transformants not only did not drop further but increased to about 3%. Even longer digestion times (250 to 300 nt) reduced the number of transformants to an efficiency of 0.1 to 0.2%. The increase in transformation efficiency for deletions of 125 to 150 nt suggests that a feature other than the size of deletion may be involved and corroborates the results obtained with the individual cloned Py deletion plasmids described above: pΔ67 with a

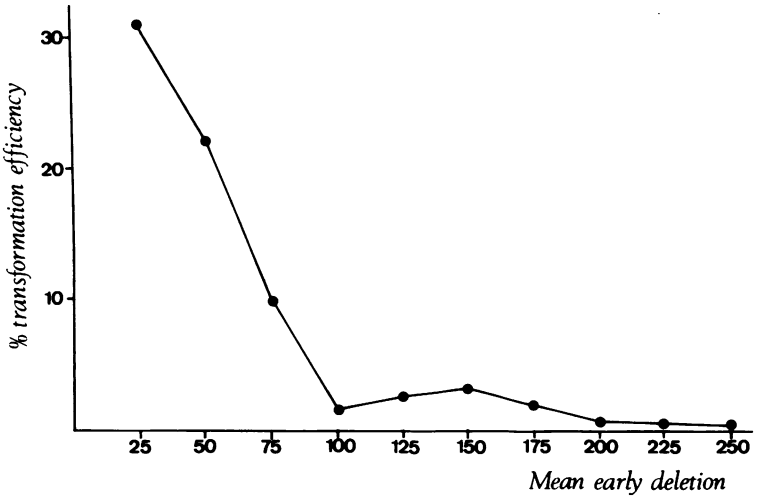

FIG. 3. Effect of deletion size on transformation of FR3T3 cells by Py. FR3T3 cells were transfected with 0.1 μg of DNA. Cloned Py DNA (p2034) was digested with *BglI* and submitted to Bal31 nuclease treatment. Mean deletions towards the early region, as the Bal31 digestion proceeded, were estimated with *PstI* cleavage, followed by 2% agarose gel electrophoresis. After ligation, the heterogeneous deletion stock was transfected onto FR3T3. The percentage of transformants is to be compared to that obtained with 0.05 μg of undeleted p2034. Points on the graph are the average of three independent transfection experiments. The results for a given point varied by less than 5% and were thus not beyond experimental error.

151-nt deletion from the *BglI* site preserves some of its transforming capacity, whereas other plasmids such as pΔ83, pΔ79, and pΔ66, with respective deletions of 110, 83, and 83 nt, did not.

Immunofluorescence detection of T antigens. Three cell lines were used for detection by immunofluorescence of the T antigens after transfection: replication-permissive secondary mouse embryo cells and the nonpermissive FR3T3 and human HeLa cell lines. No glycerol treatment was included after transfection with 1 μg of plasmid DNA because HeLa cells proved to be extremely sensitive to glycerol shock. Immunofluorescence detection of T-antigen expression was done 60 to 72 h after transfection. Assays at earlier times gave poor or no results even with pure Py viral DNA (Table 2). The percentage of T-antigen-positive cells with FR3T3 or HeLa cells generally reflected the transformation efficiency of the different Py deletion plasmids described above. T-antigen expression of the deletion mutants was nearly zero on secondary mouse embryo cells. Also, only 0.1% of secondary embryo cells expressed T antigen when transfected with cloned Py DNA, in contrast to 20% of positive cells when viral DNA was used. Cloned viral DNA was excised from its pMK16* vector by *BamHI* cleavage, which does not interrupt the coding sequences of the early region and permits the recircularization of the Py genome in the cell (unpublished data). However, release of the Py DNA in this manner did not increase the low percentage of T-antigen-positive cells. This phenomenon has also been observed with other Py-harboring plasmids in mouse 3T6 fibroblasts (unpublished data) and might suggest that modifications of the DNA in *E. coli* K-12 C600 bacteria, such as methylations, inhibit the expression of the Py early region in mouse cells, but not in FR3T3 or human HeLa cells. It is worth noting that with pΔ67, 1 cell out of roughly 2×10^4 cells was T-antigen positive. This means that there was some expression of T antigen detectable by the moderately sensitive immunofluorescence technique, in spite of a 71-nt deletion in the N-terminal coding sequences of the three T antigens.

DISCUSSION

We have constructed in vitro a series of Py deletion mutants around the *BglI* site at nt 86 by digesting Py DNA inserted into pMK16* with nuclease Bal31. In general, deletions reduced but did not abolish transformation. Small deletions of 17 nt (pΔ69) decreased transformation efficiency as tested on FR3T3 cells (rat fibroblasts) by a factor of 5. This effect could not be attributed to the deletion per se since the influence of the inserted 8-base-pair *SalI* linker was not estimated in our experiments. However, when transformation was carried out with heterogeneous deletion stocks containing no *SalI* linker, small deletions (25 to 50 nt) showed a

TABLE 2. Immunofluorescence detection of T-antigen-positive cells after transfection[a]

Mutant	Secondary embryo cells (%)	FR3T3 (%)	HeLa (%)
Py A2 DNA	20	15	23
p2034	100 (0.1)	100 (6)	100 (22)
pΔ69	~5	10	8
pΔ85	~5	12	10
pΔ70	~5	11	10
pΔ51	~5	15	10
pΔ76	~5	10	5
pΔ80	~5	12	5
pΔ77	~5	40	45
pΔ79	0	0	0
pΔ75	~5	20	25
pΔ81	0	30	25
pΔ66	0	0	0
pΔ83	0	0	0
pΔ68	0	25	22
pΔ65	0	<1	<1
pΔ67	0	0	0
pΔ52	0	<1	<1

[a] At 60 to 72 h after calcium phosphate transfection of 10^5 FR3T3 or HeLa cells with 1 μg of plasmid, the cells were fixed in acetone-methanol (2:1 ratio) and monitored for T-antigen-positive cells by immunofluorescence (27). The numbers in parentheses given for p2034 indicate the percentage of positive cells. Percentages of the positive cells obtained with the other plasmids are normalized to the values obtained with p2034 on the respective cell line (taken as 100%). The percentage of positive cells after calcium phosphate transfection with pure viral Py DNA is also given. On secondary mouse embryo cells, very few positive cells could be detected (1 of 2×10^4 to 8×10^4 cells) for eight plasmids (pΔ69, pΔ85, pΔ70, pΔ51, pΔ76, pΔ80, pΔ77, and pΔ75), and none were observed for the other eight (pΔ79, pΔ81, pΔ66, pΔ83, pΔ68, pΔ65, pΔ67, and pΔ52). This can be compared with 1 positive cell out of 10^3 to 2×10^3 cells observed with wild-type Py-harboring p2034 when secondary embryo cells were transfected.

similar three to fourfold decrease in transformation efficiency.

Figure 4 outlines the deletions with respect to the known transcription control signals present in the early region of Py: the TATA box (Goldberg, thesis) at nt 120, the CAAT box (2) at nt 61, and the large T-antigen binding site (4) between nt 49 and 64. Deletions removing the CAAT box at nt 61, either partially (pΔ76) or totally (pΔ77 and pΔ80), did not further decrease transformation efficiency from levels obtained with pΔ69. Since the CAAT box and the large T-antigen binding site are overlapping (Fig. 4), we could not determine more than the conjugated effect of their removal. In the case of pΔ77, in which the whole large T-antigen binding site was lost, the transformation efficiency was somewhat increased in comparison to pΔ69 and the transformants were much bigger. This could be attributed to a loss of repression by large T antigen of the T-antigen transcription (3), thus enhancing the production of Py early proteins necessary for transformation. Removal of the TATA box at nt 120 along with the CAAT box (pΔ75 and pΔ81), or further deletions removing the major cap sites for early transcription at nt 150 ± 2 (pΔ68), did not further affect the transformation efficiency. Jat et al. (8) have shown that deletion of the TATA box decreased the rate of in vitro transcription in HeLa cell extracts by 20-fold, while preserving the normal cap sites. In their study, deletions from nt 145 and downstream resulted in heterogeneous initiation sites in vitro, but the virus was viable.

We have shown that in pΔ52, all the noncoding sequences downstream from the *BglI* site towards the late region were deleted and resulted in a 50-fold decrease in its transformation efficiency. This result is in agreement with the observations of Tyndall et al. (26), who demonstrated that sequences on both sides of the *PvuII* site on the late side of the origin of DNA replication (nt 5,128) were necessary for the viability of the virus and that their absence decreased transformation efficiency 30-fold. These findings illustrate that sequences more than 300 nt upstream from the major cap site are important for the control of early transcription.

We have recently characterized a series of Py virus mutants which, unlike wild-type Py, can grow in mouse embryonal carcinoma PCC4 and F9 cell lines (10, 11, 26). All of these mutants had sequence variations (rearrangements and point mutations) in the noncoding sequences on the late side of the origin of DNA replication, with no modification in the early side sequences. These modifications permit the expression of the early functions otherwise blocked in wild-type Py (1) in embryonal cells. This again emphasizes the importance of certain noncoding late sequences for viral early expression and development.

Five deletions in mutants of the present series extended into the early coding sequences. Three of those mutants were completely negative for transformation, as would be expected. Surprisingly, the other two, pΔ65 with a deletion including the 3 nt coding for the T-antigen ATG initiator codon, and pΔ67 with a 71-nt deletion into the coding sequences, were still positive in the transformation assay. Two alternative mechanisms can permit the expression of these deletion mutants. First, initiation of translation could occur at the second ATG codon found at position 260. Second, transcription initiation inside the coding sequences of T antigen has been observed in vivo and in vitro at nt 300 ± 2 (8). This position is preceded by a CAAT and a

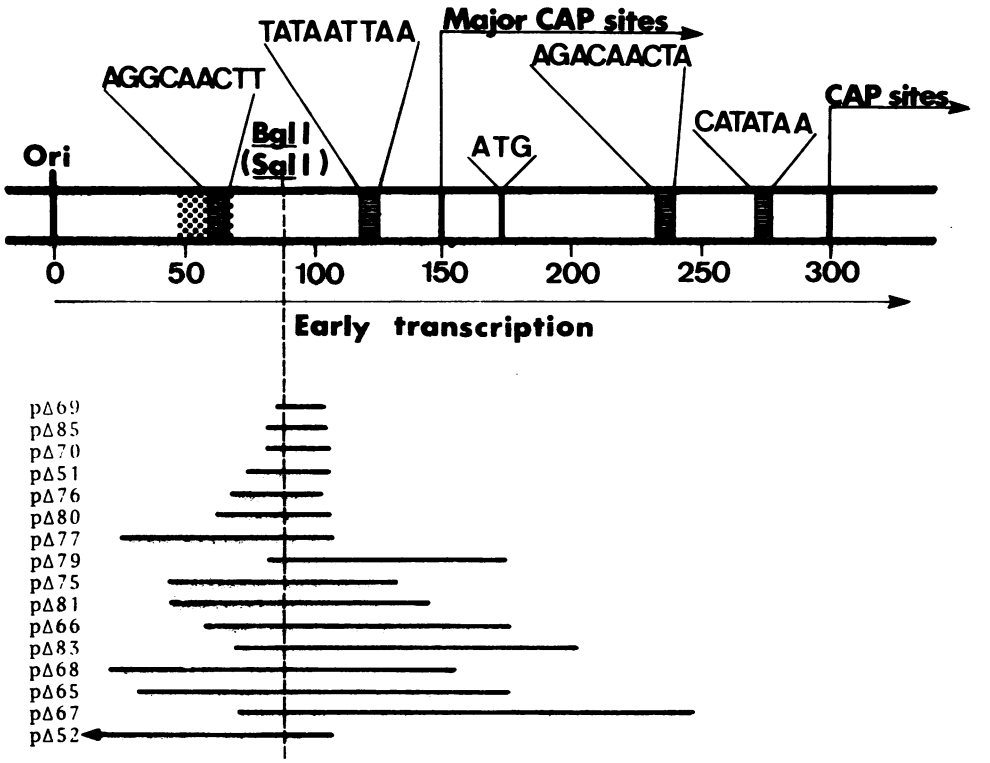

FIG. 4. Deletions in the Py genomes around the *BglI* site in relation to the biological map of the early region. Locations of the CAAT (CAAC at nt 61 and 233) and TATA (nt 120 and 270) boxes and the origin of DNA replication (Ori) at the *HpaII* site are indicated. These and the major and minor cap sites of early transcription were compiled from Jat et al. (8). The large T-antigen binding site at nt 49 to 64 (4) is indicated by the dotted area. The *BglI* site, which in deletion mutants is replaced by a *SalI* linker, and the ATG initiation codon of the T antigens at nt 173 are also marked. The numbering system is that of Soeda et al. (22).

TATA box (Fig. 4), but was disregarded since no biological role could be attributed to it. However, there is an ATG codon at nt 317, 15 to 19 nt downstream from this minor cap site. This is approximately the same distance (21 to 25 nt) as that observed between the major cap site and the normal ATG start codon. Both alternative mechanisms implicate an initiation at an internal methionine, possibly according to the model proposed by Kozak (13–15). In this "fix and slide" model for the initiation of translation, the ribosome 40S particle binds to the 5′ extremity of the mRNA and then slides to the nearest ATG and combines with the 60S particles; the mature 80S ribosome then permits translation. Kozak et al. likewise predicted shortened T antigens and that the N-terminal sequences of the mT-antigen are partly dispensable for transformation of FR3T3 cells. Effectively, transformation-defective Py hrt mutants (21) have deletions and mutations far downstream from the N-terminal sequences and away from the sequences common to the three T antigens.

Another curious observation was that two mutants, pΔ66 and pΔ79, which had the deletion up to nt 176 as for the transformation-positive pΔ65 (Fig. 2), did not transform FR3T3 cells. Again, whereas pΔ67 with a deletion up to nt 245 is transformation positive, pΔ83, comprising a shorter deletion (up to nt 203), is transformation defective. We have no explanation for this observation. However, if transcription starts at a site upstream from these deletions, the size and sequence composition of the transcripts may determine their stability or translation efficiency. We are now isolating FR3T3 transformants with these deletion mutants to map the early mRNAs and to study the synthesis of truncated T-antigens.

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