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Regulation of Polyoma Virus Transcription in Murine Embryonal Carcinoma Cells

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Undifferentiated murine embryonal carcinoma (EC) cells are resistant to infection with wild-type polyoma virus. The block appears to be located at the transcriptional level. Polyoma host range mutants capable of expressing early and late functions in EC cells have been isolated. The modifications responsible for the phenotype of these mutants are localized in the noncoding region of polyoma DNA genome, containing regulatory sequences for replication and transcription. We compared the 5′ termini of early and late mRNAs of wild-type polyoma and mutant viruses in EC cells and in permissive cells. Our results show that wild-type mRNA is normally spliced in EC cells but present at a very low level. The sequence modifications of the mutant viruses lead to a 100-fold increase in the production of mRNA in these cells, but the major 5′ termini of early and late mRNAs are identical to those in wild-type-infected 3T6 cells.

Murine embryonal carcinoma (EC) cells, the stem cells of teratocarcinoma, are resistant to infection with polyoma virus (Py). Differentiated teratocarcinoma-derived cell lines, however, are susceptible to infection by the virus (4, 21). Similarly, expression of early antigens by the simian papovavirus simian virus 4 occurs in differentiated derivatives but not in undifferentiated EC lines. The resistance of EC cells to these viruses involves a block after adsorption and penetration but before T antigen expression. One of our laboratories (13, 14, 24) has isolated and characterized two classes of Py mutants which can express both early and late functions in the nullipotent EC cell line F9, in the pluripotent PCC4 Aza cell line, or in both. The mutants able to express in F9 cells were designated PyEC F9 viruses, whereas those able to overcome the block only in PCC4 Aza cells were designated PyEC PCC4. A number of further PyEC F9 mutants have also been described by other laboratories (9, 19). The difference in the ability of wild-type Py and PyEC PCC4 mutants to express in PCC4 cells is a temperature-sensitive one. Both grow in PCC4 cells at 31°C, but only the mutants express early and late functions efficiently at 37°C. By contrast, wild-type Py does not express in F9 cells at any temperature tested (6). DNA sequence determination has identified the alterations responsible for the phenotypes of both classes of mutants. All of the changes are localized in the portion of the genome between the late coding sequences and the origin of viral DNA replication (68 to 70 map units; nucleotide 5,150 to 5,250, according to the numbering system used by Soeda et al. [20]). These changes involve, among the several known mutants, duplications, deletions, and point mutations. Various hypotheses have been considered to explain the block in viral gene expression which occurs in EC cell lines and how it is removed by the mutations (13, 18). In this paper, we show that wild-type virus produces exceedingly low levels of viral mRNA after infection of EC cell lines. This suggested that the block could be at the transcriptional level. We therefore designed further experiments to quantitate and characterize the viral mRNAs produced after infection of EC cells with PyEC mutants. We were particularly interested in determining whether the DNA sequence alterations in the mutants, all of which lie within the noncoding sequences between the early and the late region, created new viral early or late "promoters" which would cause the production of mRNA species with novel 5′ termini (2, 7). Our results clearly demonstrate that the mutants synthesize viral mRNAs in EC cells more efficiently than the wild-type virus. However, the effect is essentially a quantitative one, since the major early and late region mRNAs have 5′ termini identical to those produced at far lower efficiency by the wild-type virus. The mutant PyEC PCC4-97 does produce early and late

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region mRNAs with novel 5′ termini mapping in the altered DNA sequence, but these are minor species present at similar relative abundancies after infection of either PCC4 or fibroblast cell lines. We have therefore eliminated one possible explanation for the mutant phenotype. An alternative suggested by observations (23) made subsequent to the completion of this study will be discussed.

MATERIALS AND METHODS

Virus, cells, and mRNAs. Polyoma virus strains A_2, PyEC PCC4-97 (14), and PyEC F9-1 (13) were used to infect 3T6 cells at 20 PFU per cell or PCC4 Aza (17) and F9 cells (1) at 200 PFU per cell.

Cells were harvested at 48 h after infection for EC cells and at 30 h for 3T6 cells. Cytoplasmic polyadenylated mRNA was prepared as described by Favaloro et al. (8).

S1 nuclease gel mapping. The method of Berk and Sharp (3) as modified by Weaver and Weissmann (25) was used. The annealing temperature was 30°C, the annealing time was 12 to 16 h, and the unhybridized DNA was digested with S1 nuclease for 2 h at 12°C. S1-resistant products were fractionated on 5, 8, and 12% urea polyacrylamide gels (0.3 mm by 23 cm by 43 cm) after denaturation in the formamide buffer used for DNA sequencing (16). Size markers used were 5′ ^{32}P-labeled Py DNA *Dde*I fragments.

Preparation of single-stranded 5′ ^{32}P-labeled DNA fragments. The hybridization probes used to map early region mRNAs were the E DNA strand of the *Hin*fI DNA fragment (labeled at nucleotide 388 and extending to 5,076), of the *Bcl*I + *Bgl*I DNA fragment (labeled at nucleotide 90 and extending to 5,025) (Fig. 1), and of the *Hin*fI DNA fragment (labeled at nucleotide 963 and extending to 439). Late-region mRNA 5′ ends were mapped with the L DNA strand of the *Bcl*I + *Bgl*I DNA fragment labeled at nucleotide 5,021 and extending to 93 (Fig. 1). The methods used for 5′ ^{32}P labeling and purification of the separated DNA strands have been described previously (12).

RESULTS

The DNA sequence alterations of two PyEC mutants (Py PCC4-97 and Py F9-1) are shown in Fig. 1 in relation to the viral transcription map. The Py PCC4 mutants contain a duplication associated with a deletion: in Py PCC4-97, the duplicated sequence extends from nucleotide 5,072 to 5,135, starting at nucleotide 5,185, and the deletion extends from nucleotide 5,185 to 5,215. In addition, 15 nucleotides are inserted between residues 5,135 and 5,216, 12 of which originate also from the duplicated region (5,096 to 5,100 and 5,097 to 5,104). The final result of the rearrangements is an increase of 48 base pairs over the size of wild-type Py (14). The Py F9 mutant used in the experiment was Py F9-1, of which the only modifications are a transition of adenine to guanine at nucleotide 5,233 and the insertion of a thymine at nucleotide 5,173 and an adenine at nucleotide 5,185 and 5,208 (13). The sequence modifications of all the EC mutants described are located in the region between the *Bcl*I restriction site (nucleotide 5,021) and the *Pvu*II restriction site (nucleotide 5,262) (Fig. 1). This region has been determined to be involved in specific and efficient in vivo transcription of the Py early region (11).

Expression of early mRNA in EC cells. Preliminary investigation of the expression of wild-type Py in EC cells had suggested that very little viral RNA is synthesized (M. Vasseur, personal communication), but that the mRNA produced is normally spliced and polyadenylated (F. Kelly and R. Kamen, unpublished data). To confirm these observations, we hybridized cytoplasmic polyadenylated RNA prepared from virally infected cells to the E DNA strand of a 5′ ^{32}P-labeled DNA fragment spanning the 3′ intron-exon junction of the early region (see Fig. 1). The known sequence of the three spliced early region mRNAs allowed us to predict the S1-resistant products of the lengths indicated in Fig. 2B. Small T and middle T mRNAs have the same splice donor, and large T and small T mRNAs have the same splice acceptor. One would therefore expect two S1-resistant products, 152 and 166 nucleotides long. However, as shown in Fig. 2B, the three nucleotides located upstream from the splice acceptor of large T are complementary to three nucleotides of the splice donor. Thus, a third S1-resistant product of 169 nucleotides is obtained. The expected products were detected with mRNA prepared from wild-type Py-infected 3T6 cells (Fig. 2A, track 1) or from F9 cells infected with the mutant Py F9-1 (Fig. 2A, track 5). By contrast, using the same assay, almost no early mRNA was detected in PCC4 or F9 cells infected by wild-type virus (Fig. 2A, tracks 2 and 3) or in F9 cells infected with the Py PCC4-97 mutant (Fig. 2A, track 4). Inspection of the original autoradiograms revealed that the expected S1-resistant products, produced by normally spliced mRNA, were indeed present in the apparently negative tracks, but at such a reduced level (more than 100-fold) that they were not visible after reproduction. We conclude that wild-type Py is unable to efficiently synthesize early mRNA in EC cells.

Localization of the 5′ termini of Py early mRNAs in PCC4 and F9 cells. In 3T6 cells infected with wild-type Py, the major 5′ termini of early mRNAs, as determined by S1 nuclease gel mapping (12), are located at nucleotides 145 ± 2 and 155 ± 2, 31 nucleotides downstream from a sequence (nucleotides 120 to 127) closely related to the Hogness-Goldberg TATA box consensus (Fig. 1 and 3). At late times during infection, relatively abundant molecules with apparent 5′ termini at multiple points upstream of these principal sites are detected between

nucleotides 5,200 and 14 (Fig. 3, linear map and tracks 8 and 9). The 5′ termini of Py early region mRNAs extracted from PCC4 cells infected with the Py PCC4-97 mutant were localized on the viral genome by high-resolution nuclease S1 mapping. The RNAs were hybridized to the single-stranded 5′ ^{32}P-labeled *Hin*fI restriction fragment. The map position of the E DNA strand probe labeled at nucleotide 388 is indicated in Fig. 3. The major 5′ termini between nucleotides 145 and 155 and the minor 5′ termini located upstream from the latter are present in the same relative ratio in Py PCC4-97-infected PCC4 cells (Fig. 3, track 1) and in 3T6 cells infected with this mutant (Fig. 3, tracks 2 and 3) or with wild-type Py (Fig. 3, tracks 8 and 9). The band designated X is an artifact caused by incomplete nuclease digestion. As described (12), no products corresponding to this band were seen by primer extension studies. Additional minor S1-resistant products were, however, detected in mRNAs from cells infected with the Py PCC4-97 mutant. In mRNAs extracted from F9 cells infected with the Py F9-1 mutant (Fig. 3, tracks 6 and 7), only the major 5′ termini were detected. A longer exposure might allow detection of minor bands; however, the amount of viral mRNA in all teratocarcinoma cells is very low, as will be discussed below.

To localize more accurately the additional minor 5′ termini of mRNAs extracted from Py PCC4-97-infected cells, we hybridized a shorter DNA probe (the *Bcl*I + *Bgl*I E DNA strand, labeled at nucleotide 90, see Fig. 4) to increasing

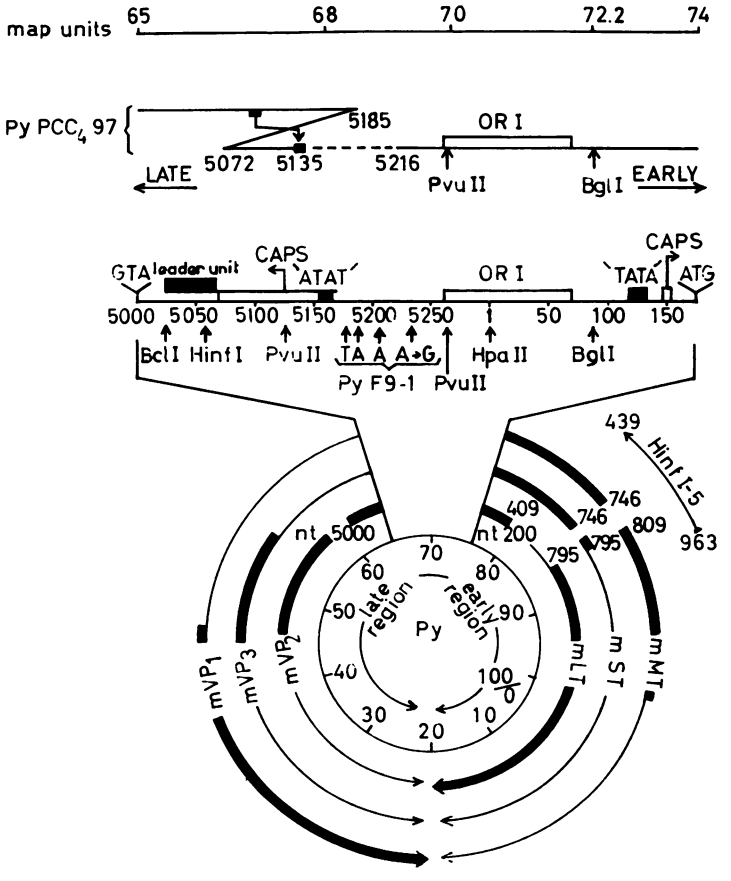
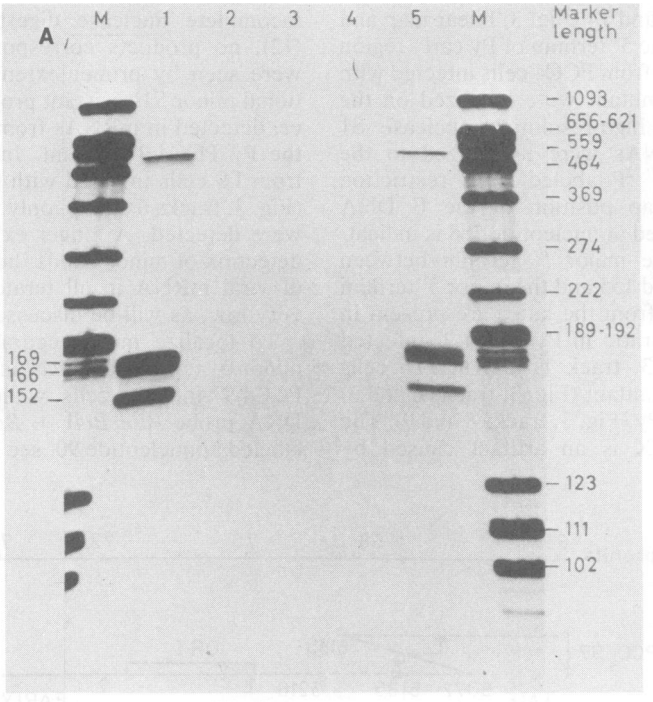


FIG. 1. Polyoma DNA origin region. The sequence modifications responsible for the EC mutant phenotypes are located within the 500 nucleotides spanning the replication region. Coordinates are given in map units (top line) and in nucleotide numbers (bottom line). Location of mRNA cap sites, sequences related to the TATA box consensus, and the translational initiation codons are shown on the bottom line. Leader unit indicates the position of the sequences tandemly repeated near the 5′ termini of late mRNAs. ORI indicates the palindromic region required for DNA replication. The tandemly repeated sequence of Py PCC4-97 and the base pair insertions and transition of the Py F9-1 mutant are shown on the bottom line. The restriction sites correspond to the fragments used for the nuclease S1 mapping experiments.



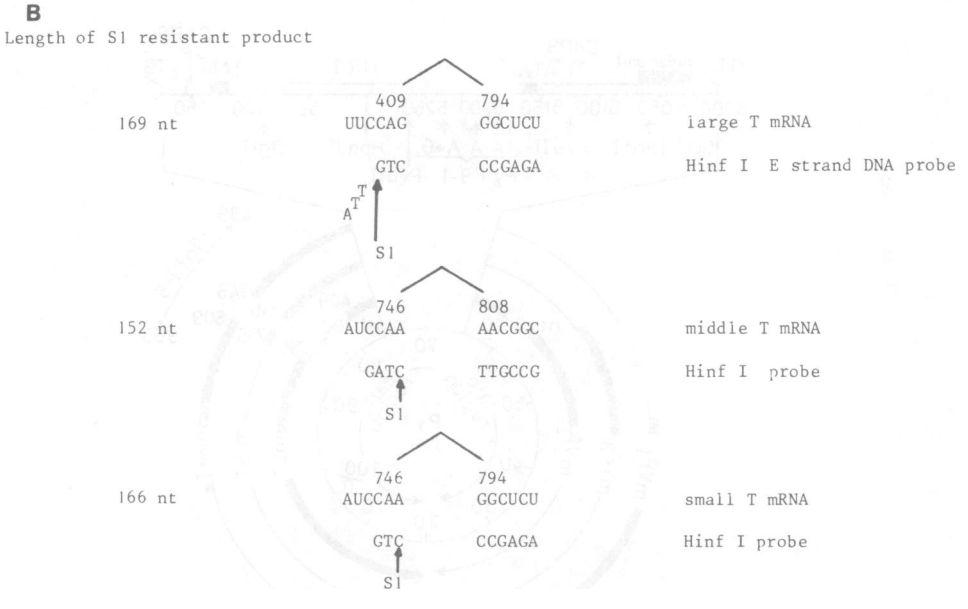


FIG. 2. Splicing of early mRNAs in 3T6 and EC cells. (A) Polyacrylamide urea gel (8%) fractionation of S1 nuclease-resistant 5′ ^{32}P-labeled DNA products resulting from the hybridization of various RNA samples from Py-infected 3T6 and EC cells to the *Hin*fI E DNA strand probe (0.5 to 2 μCi per pmol of 5′ end) extending from nucleotide 963 to 440 of wild-type Py DNA. RNA samples used were: wild-type Py mRNA from 3T6 cells (track 1), PCC4 cells (track 2), and F9 cells (track 3) and Py PCC4-97 mRNA (track 4) and Py F9-1 mRNA (track 5) from F9 cells. The same amount (30 μg) of cytoplasmic polyadenylated mRNA was used in all hybridizations. The marker lengths of the 5′ ^{32}P-labeled *Dde*I fragments of Py DNA are indicated to the right of the gel. The length of the S1-resistant products is indicated on the left. (B) Expected lengths (in nucleotides [nt]) of the S1-resistant products resulting from the hybridization of the three early cytoplasmic polyadenylated mRNAs to the *Hin*fI E DNA strand probe.

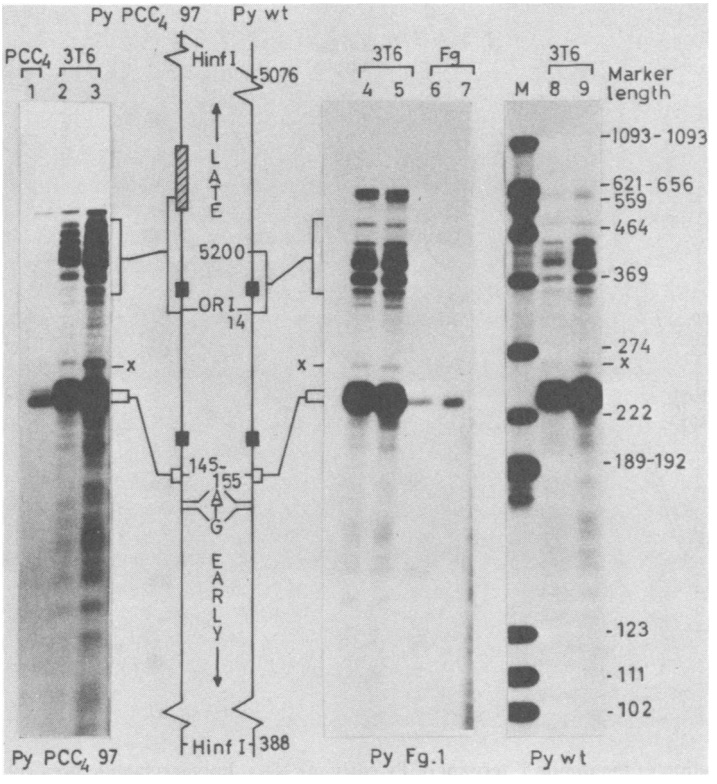


FIG. 3. Localization of 5′ termini of Py early mRNAs by S1 nuclease gel mapping. Polyacrylamide urea gel (5%) fractionation of S1 nuclease-resistant 5′ ^{32}P labeled DNA products resulting from the hybridization of various RNA samples from Py-infected mouse 3T6 cells and EC cells to the *Hin*fI E DNA strand probe (0.5 to 2 μCi/pmol), extending from nucleotide 388 to 5077 as shown on the linear map. RNA samples were the following: Py PCC4-97 cytoplasmic polyadenylated mRNA from PCC4 cells (track 1, 20 μg per annealing) and from 3T6 cells (tracks 2 and 3, 2 and 10 μg); Py F9-1 cytoplasmic mRNA from F9 cells (tracks 6 and 7, 10 and 40 μg); and wild-type (wt) Py cytoplasmic mRNA from 3T6 cells (tracks 8 and 9, 2 and 10 μg). Each RNA sample was hybridized to its homologous DNA probe. Track M is 5′ ^{32}P-labeled *Dde*I fragments of Py DNA used as size markers. Marker lengths are indicated to the right of the gel. The length of the S1-resistant products was used to deduce the positions of the 5′ termini with respect to the viral sequence, as shown on the linear map. Sequences related to the TATA box consensus are indicated by black boxes. The hatched box represents the duplicated region in Py PCC4-97 DNA.

concentrations of RNA extracted from wild-type Py- or Py PCC4-97-infected 3T6 cells. Two additional S1-resistant products detected in the latter (compare tracks 4 and 5 and tracks 9 and 10, Fig. 4) are localized in the duplicated sequence of the Py PCC4-97 mutant DNA. The possible significance of these additional minor S1-resistant products will be discussed below.

Localization of the 5′ termini of Py late mRNAs in PCC4 and F9 cells. As described previously (8, 15), late viral mRNAs consist of a 5′ nontranslated leader structure joined to the 3′ body of the coding sequences corresponding to the capsid proteins. The leader structure comprises a variable number of exact head-to-tail tandem repeats of the 57-nucleotide sequence (between nucleotide 5,020 and 5,076) and is thought to result from repeated splicing within the giant tandem transcripts of the entire viral genome, which are the nuclear precursors of late mRNAs (22).

The capped 5′ termini of late-region mRNAs are highly heterogeneous, with 15 different purine cap sites (5) localized in the 94-base pair region from nucleotide 5,075 to 5,168 (Fig. 1) proximal to the leader unit. An A-T-rich tract (nucleotide 5,158 to 5,150) is included within this region, but only one of the many mRNA 5′ termini (at nucleotide 5,129 ± 2) is at the correct distance from this possible TATA box. A series of minor 5′ termini are also detected between nucleotide 5,150 and 5,170 (Fig. 5, tracks 1 and 2).

Cytoplasmic polyadenylated mRNAs extract-

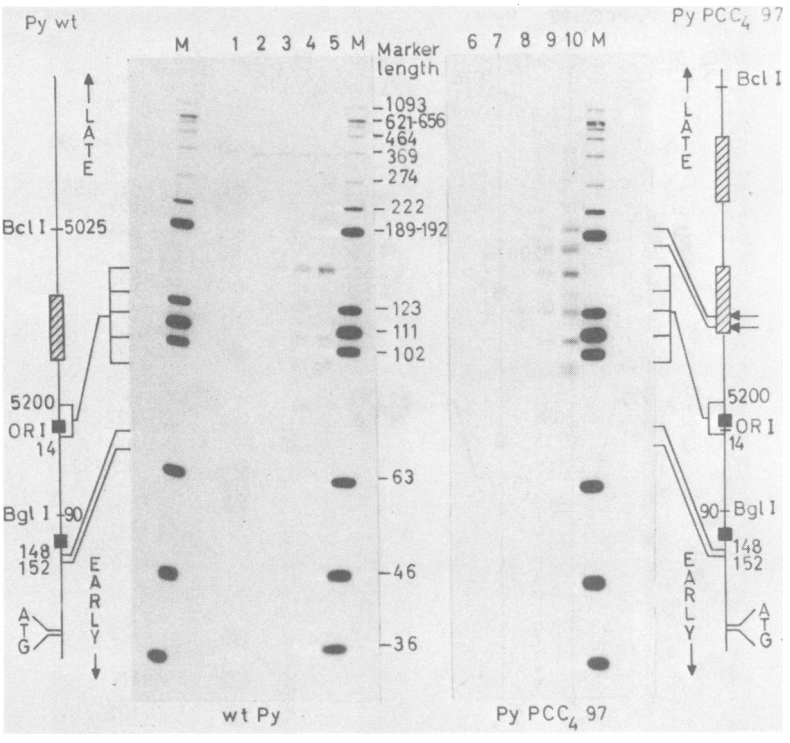


FIG. 4. Localization of the minor 5′ termini of Py early mRNAs. Polyacrylamide urea gel (8%) fractionation of S1 nuclease-resistant 5′ ^{32}P-labeled DNA products resulting from the hybridization of RNA samples from Py-infected mouse 3T6 cells to the *Bcl*I + *Bgl*I E DNA strand fragment from wild-type (wt) Py DNA or from Py PCC4-97 DNA. Tracks 1 to 5 are increasing concentrations, from left to right, of wild-type cytoplasmic polyadenylated mRNA, and tracks 6 to 10 are increasing concentrations of Py PCC4-97 mRNA. Each RNA sample was hybridized to its homologous probe. Alignment of S1-resistant products with the size marker track M (5′ ^{32}P-labeled *Dde*I fragments of Py DNA) was used to determine the positions of the minor 5′ termini. These positions are indicated on the linear maps corresponding to each virus. The sequences related to the TATA box consensus in this region are indicated by black boxes. The sequence which is duplicated in the Py PCC4-97 mutant is indicated by a hatched box, and the arrows correspond to the two additional minor 5′ ends found in these mRNAs.

ed from Py PCC4-97-infected PCC4 cells were hybridized to the homologous *Bcl*I + *Bgl*I L DNA strand probe (labeled at nucleotide 5,021) (Fig. 5, track 8). Major and minor S1-resistant products are present in the same relative ratio as in 3T6 cells infected with the same mutant (Fig. 5, tracks 5 and 6) or with wild-type Py (Fig. 5, tracks 1 and 2). However, in 3T6 cells, at high concentrations of RNA, two additional minor 5′ termini are detected.

To precisely determine their position, we hybridized the same homologous DNA probes with increasing concentrations of mRNA extracted from 3T6 cells infected with wild-type Py or with Py PCC4-97 mutant and separated the S1-resistant products on a 12% urea polyacrylamide gel (Fig. 6). As the concentration of RNA increased, we observed a displacement due to favored hybridization with longer mRNA species compared to hybridization with shorter ones (compare tracks 1 and 6 and 7 and 12, Fig. 6). The two additional 5′ termini detected in 3T6 cells infected with the Py PCC4-97 mutant can be precisely located in the duplicated region of the DNA sequence of this mutant. One of these 5′ termini corresponds to the sequence ACAGT, from which, in wild-type Py DNA originate the major capped 5′ termini.

Temperature effect on transcription of wild-type Py in PCC4 cells. We have previously shown that restriction of Py expression in PCC4 cells, but not in F9 cells, can be abolished if infection is carried out at 31°C (6). Analysis by S1 nuclease mapping (Fig. 5) of late polyadenylated cytoplasmic mRNA extracted from wild-type Py-infected PCC4 cells confirmed this observation: the amounts of late mRNA detected in PCC4 cells infected at 31°C with wild-type Py (track 3, Fig. 5) and in PCC4 cells infected at 31 or 37°C with Py PCC4-97 are similar (tracks 7

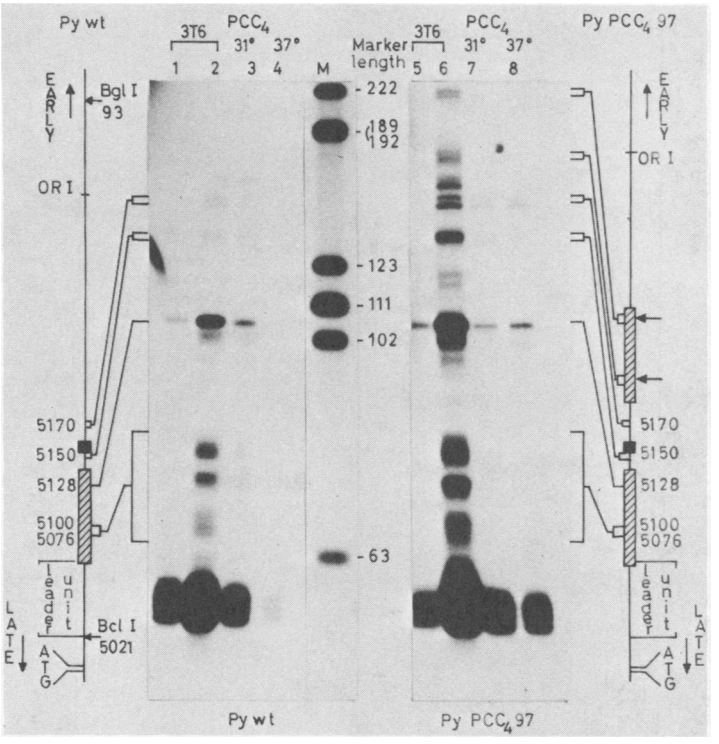


FIG. 5. Localization of the 5′ termini of Py late mRNAs by S1 nuclease gel mapping. Polyacrylamide urea gel (8%) fractionation of S1 nuclease-resistant 5′ ^{32}P labeled DNA products resulting from the hybridization of various RNA samples from Py-infected 3T6 and PCC4 cells to the *BclI* + *BglI* L DNA strand fragment from wild-type (wt) Py DNA or from Py PCC4-97 DNA. The RNA samples used were wild-type Py cytoplasmic polyadenylated mRNA from 3T6 cells (tracks 1 and 2, 0.05 and 0.5 μg) and from PCC4 cells (track 3 at 31°C and track 4 at 37°C, 10 μg per annealing) and Py PCC4-97 mRNA from 3T6 cells (tracks 5 and 6, 0.05 and 0.5 μg) and from PCC4 cells (track 7 at 31°C and track 8 at 37°C, 10 μg per annealing). The homologous DNA probe was used in each case. The size marker (track M) is 5′ ^{32}P-labeled *DdeI* fragments of Py DNA with marker lengths indicated in the center. The sequence related to the TATA box consensus is indicated by a black box. The positions of the 5′ termini are indicated on the linear maps. The arrows indicate the additional 5′ termini located in the duplicated sequence of Py PCC4-97.

and 8, Fig. 5). By contrast, only trace amounts of late mRNAs are present when PCC4 cells are infected at 37°C with wild-type Py (track 4, Fig. 5). The 5′ termini detected in PCC4 mRNAs at 31°C are not markedly different from the 5′ termini detected in wild-type Py-infected 3T6 cells. Late mRNAs are therefore synthesized with the same efficiency in PCC4 cells infected with wild-type Py at 31°C and with the Py PCC4-97 mutant at 37°C; lowering the temperature abolishes the block of viral expression in PCC4 cells.

DISCUSSION

The expression of papovaviruses (simian virus 40 and Py virus) in undifferentiated EC cells appears to be blocked at the transcriptional level. Segal et al. (18) have shown that the viral RNA from simian virus 40-infected EC cells, present in very low amounts, is unspliced, suggesting that the regulation of transcription of this virus is located at the level of splicing of the early messengers. F. Kelly and R. Kamen (unpublished data) have shown that after infection with wild-type Py viral mRNA is present at a very low level but is normally spliced. We therefore investigated the regulation of transcription at the level of initiation, using the Py mutants capable of expressing early and late functions in EC cells. Since all PyEC mutants isolated so far are modified in the same region, located on the late side of the junction between *HpaII* fragments 3 and 5, in the noncoding region, containing regulatory sequences for replication as well as for transcription, we compared the 5′ termini of early and late mRNAs in EC cells and in permissive 3T6 cells infected with the PyEC mutants.

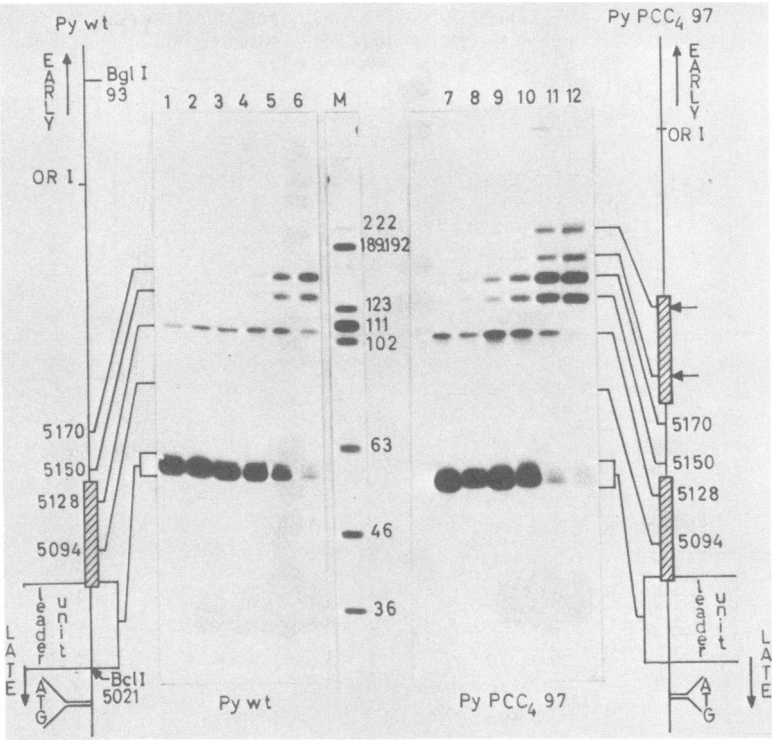


FIG. 6. Localization of the minor 5′ termini of Py late mRNAs. Polyacrylamide urea gel (12%) fractionation of S1 nuclease-resistant 5′ ^{32}P-labeled DNA products resulting from the hybridization of RNA samples from Py-infected mouse 3T6 cells to the *BclI* + *BglI* L strand DNA fragment from wild-type (wt) Py DNA or from Py PCC4-97 DNA. Tracks 1 to 6 are increasing concentrations, from left to right, of wild-type Py cytoplasmic polyadenylated mRNA, and tracks 7 to 12 are increasing concentrations of Py PCC4-97 cytoplasmic polyadenylated mRNA. Each RNA sample was hybridized to its homologous probe. Alignment of the S1-resistant products with the size marker track M (5′ ^{32}P-labeled *DdeI* fragment of Py DNA) was used to determine the positions of the 5′ minor termini. These positions are indicated on the linear maps corresponding to each virus. The sequence which is duplicated in the Py PCC4-97 mutant is indicated by a hatched box, and the arrows correspond to the two additional minor 5′ ends found in these mRNAs.

Our results have shown that the major 5′ termini of early mRNAs are identical in all cell lines (PCC4, F9, and 3T6). However, after infection with the Py PCC4-97 mutant, two additional S1-resistant products appear, located in the duplicated sequence of this mutant.

These two additional products could be the result of what was defined previously as a shadow (12) due to RNA-RNA annealing between the minor long early mRNAs and the 5′ portions of complementary late-region transcripts. These results therefore indicate the presence of mRNA species with 5′ termini extending further within the late region of the genome. These 5′ termini are, however, minor species. In fact, the relative proportion of the major early 5′ termini is similar in EC cells and in 3T6 cells infected by the mutants or by wild-type Py.

The late messenger RNA 5′ termini are again identical in both cell lines (PCC4 and 3T6). However, two additional 5′ termini are detected after infection by mutant Py PCC4-97. These two minor 5′ termini are located in the duplicated region, and one in particular can be located on the sequence TCAGT, which is the sequence present at the major capped 5′ termini of late mRNA at nucleotides 5,091, 5,093, and 5,094. This sequence is also the center of the DNase I-hypersensitive site in wild-type Py DNA (10).

In PCC4 cells infected with wild-type virus, only trace amounts of mRNAs were detected as compared to wild-type-infected 3T6 cells (approximately 1,000-fold decrease). Still, both early and late polyadenylated cytoplasmic mRNAs are normally processed, thus confirming the results of F. Kelly and R. Kamen. The sequence modification of PyEC mutants leads in EC cells to at least a 100-fold increase in the production of mRNA. However, this level of RNA remains lower than in infected 3T6 cells, thus suggesting

that the mutation does not completely abolish the restriction of viral expression in EC cells at 37°C.

Furthermore, the block at the transcriptional level in PCC4 cells is thermosensitive since mRNAs are detected at the same level in cells infected at 31°C with either the Py PCC4 mutant or wild-type Py, thus confirming our previous observations (6).

The experiments that have been described in this paper show that the selection of the 5′ termini in EC cells is not drastically modified, although some minor RNA species were detected in cells infected with the mutants containing the duplication. This implies that the transcriptional block is located at an earlier step than the choice of the 5′ termini by the RNA polymerase.

Since S1 mapping detects only steady-state levels of mRNA, one cannot exclude completely a very rapid turnover of primary transcripts in EC cells infected with wild-type Py.

The region located between the *BclI* site at nucleotide 5,021 and the *PvuII* site at nucleotide 5,262 has been determined as a DNase I-hypersensitive region of viral chromatin. Tyndall et al. (23) have shown that this region (region A) is required for early gene expression and therefore might be involved in some recognition signal for the RNA polymerase.

In all Py PCC4 mutants, the G · C-rich palindrome between nucleotide 5,173 and 5,188, allowing a stable secondary structure, is conserved. Furthermore, the mutations of the Py F9 mutants, inserting two base pairs in this region, increases the stability of this structure (13). This observation, in addition to the thermosensitive block in PCC4 cells, strongly suggests the interaction of this sequence of DNA with a protein (cellular or viral) involved in a regulatory recognition signal for the RNA polymerase.

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