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Identification of a promoter component involved in positioning the 5′ termini of simian virus 40 early mRNAs

(transcription initiation/deletion mutants/cDNA sequence determination)

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ABSTRACT The 5′ termini of the principal early mRNAs produced in cells transformed by wild-type simian virus 40 lie 21–25 nucleotides downstream from an A-T-T-T-A-T sequence on the DNA template. The 5′ termini of early mRNAs produced by five origin-defective mutants containing deletions downstream from the A-T-T-T-A-T sequence and one viable mutant dl892 with a deletion starting 15 nucleotides upstream from this sequence were determined by a method involving synthesis, separation, and determination of the sequences of DNAs complementary to 5′ termini. Mutant dl892 produced early mRNAs with the same principal 5′ termini as wild-type virus; the origin-defective mutants produced mRNAs with principal 5′ termini shifted downstream by a distance equivalent to the length of the deleted DNA segment. These data suggest that a DNA sequence of 29 nucleotides, which includes the A-T-T-T-A-T sequence, contains a component(s) of a promoter for early transcription. This component functions in positioning the 5′ ends of the principal early mRNAs 21–25 nucleotides downstream from the A-T-T-T-A-T sequence and acts independently of these downstream sequences.

The early gene region of simian virus 40 (SV40) is a favorable system for studying eukaryotic gene transcription because the sequence of the viral DNA (1, 2) and structure of the early mRNAs (3, 4) are known and considerable information is available concerning the control of transcription (5–8). The early gene region spans a little over 2600 nucleotides and serves as a template for two early mRNAs (large and small T antigen mRNAs) that differ in their internal splices (3, 4). Both early mRNAs appear to have a number of 5′ termini, the principal ones lying at two adjacent sites (nucleotides 5235–5237 and 5230–5233‡) within the origin of replication of the viral genome (Fig. 1). Control over early transcription is exerted, at least in part, by large T antigen, which interacts with SV40 DNA at three sites within and overlapping the origin of replication (8) and autoregulates early RNA synthesis (5–7). Furthermore, a Goldberg–Hogness sequence (A-T-T-T-A-T) lies on the noncoding DNA strand 21 nucleotides upstream from the first of the principal 5′ termini of the early mRNAs (Fig. 1) and by analogy with other eukaryotic genes, it has been suggested that this sequence may constitute all or part of a promoter for early transcription (reviewed in ref. 10).

Among a number of important questions concerning control of early transcription, two concern the location and nature of the early transcriptional promoter(s) and the possibility that under specific conditions sites other than those at residues 5235–5237 and 5230–5233 can function as the 5′ termini of the early mRNAs. Gluzman *et al.* (11) have performed studies aimed at answering these questions by using a series of origin-defective viral mutants containing deletions either upstream from or including the templates for the principal 5′ termini of the early mRNAs (Fig. 1). Four mutants with deletions downstream from the A-T-T-T-A-T sequence were found to support early mRNA and T antigen synthesis in transformed rat cells (these mutants cannot carry out lytic infection), whereas an additional mutant with a large deletion including the A-T-T-T-A-T sequence failed to synthesize any early products. Furthermore, nuclease S1 studies on RNAs produced by two mutants having deletions of different sizes suggested that the 5′ termini of their early mRNAs lie approximately the same distance downstream from the A-T-T-T-A-T sequence rather than at a specific genomic site. In the present experiments we have carried studies with these mutants still further, inquiring into the specific sites used by each of the mutants for the 5′ termini of its early mRNAs. Our studies reveal the use of a wide variety of sites as 5′ termini of mutant early mRNAs; and we suggest that there is a measuring function from an upstream site that is operative in the positioning of the 5′ termini of both wild-type and mutant virus early mRNAs.

MATERIALS AND METHODS

Cells, Viruses, and DNAs. The origin-defective mutants 1-11, 3-20, 6-1, 6-17, and 8-4, constructed about the *Bgl* I site of SV40 DNA and characterized by Gluzman *et al.* (11, 12), and the viable deletion mutant dl892 (13), kindly provided by Tom Shenk, were used in these studies. The extent of the deletions in these mutants are shown in Fig. 1 and described in the text. The human line SV80, transformed by wild-type SV40, and rat fibroblasts, transformed by wild-type SV40 and the origin-defective mutants, were propagated as described (12). Vero African green monkey kidney cells were infected with wild-type SV40 and dl892 at a multiplicity of infection of 10–20 plaque-forming units/cell and grown in the presence of 20 μg of cytosine arabinoside per ml. Infected and transformed cells were lysed and RNA was extracted from the cytoplasmic fraction by standard methods (14). Poly(A)-containing terminal RNAs isolated on oligo(dT)-cellulose columns were used in all studies.

Analysis of cDNAs. The 5′ termini of the early mRNAs produced by wild-type and mutant SV40 were identified by synthesis of DNAs complementary to the 5′ termini of these RNAs, separation of cDNAs on 8% (wt/vol) polyacrylamide/7 M urea gels, and sequence and size analyses of individual cDNAs. Methods for isolating SV40 restriction fragments used as cDNA synthetic primers, labeling of their 5′ termini with [γ-^{32}P]ATP, hybridization of DNA fragments to cytoplasmic RNAs, and synthesis and separation of cDNAs have all been described (15, 16). Two restriction fragments (A and B) were used as cDNA synthetic primers. Primer A was obtained by redigesting *Eco*RII-G with *Alu* I and spans nucleotides 5092–5127; primer B is *Hinf*-

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Abbreviation: SV40, simian virus 40.

‡ Nucleotide numbering in this paper follows the BBB system (9). However, Fig. 1 also includes the numbering system of Reddy *et al.* (2) used previously (3).

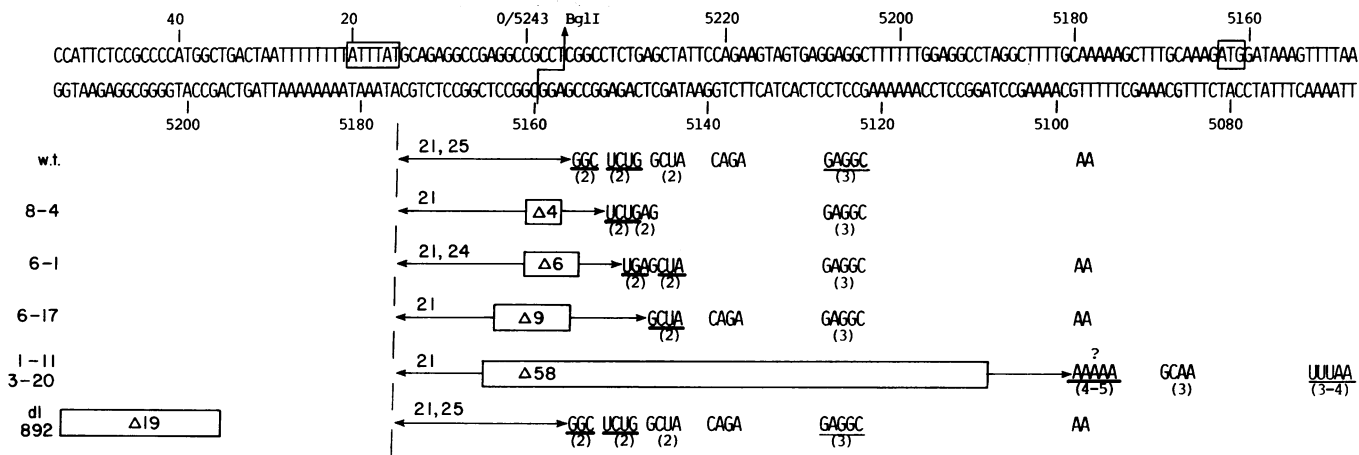

FIG. 1. Diagram of the region of SV40 DNA coding for the 5′ termini of the early viral mRNAs. Transcription occurs on the lower strand from left to right. On the upper strand a Goldberg–Hogness sequence and the AUG sequence for initiation of translation of large T antigen are indicated. Nucleotides are numbered over the upper strand according to the BBB convention (9) and beneath the lower strand according to Reddy *et al.* (2). In the lower portion of the figure are shown the deletions in the five origin-defective and dl892 mutants used in the present study and the RNA sequences within which the 5′ termini of the early viral mRNAs fall. Heavy and light lines under certain sequences indicate major termini and principal minor termini, respectively; nonunderlined sequences indicate sites of relatively scarce 5′ termini. Numbers in parentheses indicate number of cDNA 3′ termini and probably number of mRNA 5′ termini identified within the given stretches of nucleotides; where no numbers are given under sequences, only one mRNA 5′ terminus has been identified. Lines with terminal arrowheads indicate number of nucleotides from the Goldberg–Hogness sequence to templates for principal 5′ termini of each virus. An adenine residue has been found at position 5177 in the DNA of the origin-defective mutants (11); however, a guanine residue has been found in this position in mutant 8-4 and 1-11 early mRNAs. The boundaries of deletions are (inclusive): 5190–4 for mutants 1-11 and 3-20, 5239–5242 for mutant 8-4, 5237–5242 for mutant 6-1, 5238–3 for mutant 6-17, and 35–53 for mutant dl892.

A/*Hin*dIII, spanning positions 5136–5171. Size analyses of cDNAs were carried out on thin (0.5-mm) polyacrylamide gels and sequence analyses by the method of Maxam and Gilbert (17). Despite the sensitivity and refinement of these methods, it is often difficult to read sequences to the terminal nucleotide. For this reason, we have indicated the location of 5′ termini within stretches of two or more nucleotides.

RESULTS

Gel electrophoretic patterns of cDNAs transcribed on the 5′ termini of polyadenylylated cytoplasmic RNAs obtained from cells transformed by wild-type SV40 and the origin-defective mutants are shown in Fig. 2 *a* and *b*. The number of individual species included within specific cDNA bands, their termini, and the localizations of early mRNA 5′ termini, deduced from localizations of cDNA termini, are shown schematically on the SV40 genome in Fig. 1. Two different primers, A and B, were used to locate the 5′ termini of the early viral mRNAs. The improved resolution of cDNA bands in Fig. 2*b* compared to that in Fig. 2*a* is a result of two factors: (*i*) use of primer B, which maps 44 nucleotides closer than primer A to the 5′ ends of the mRNAs (primer A was used for cDNA synthesis shown in Fig. 2*a* to ensure that 5′ termini of shorter than usual mRNAs would be detected); and (*ii*) electrophoresis of cDNAs on a thin analytical polyacrylamide gel (0.5 mm in contrast to 2 mm).

The SV80 transformed line has served as a standard in all our prior studies on the structure of wild-type early viral-specific mRNAs. It shows the same cDNA pattern on the standard separation gel (Fig. 2*a*) as previously reported (3, 4): two principal cDNAs (nos. 1 and 2) of about equal abundance, a minor cDNA (no. 5) accounting for about 5–8% of total cDNAs as determined by densitometry, and other minor cDNAs both longer and shorter than the two principal species. However, examination of the SV80 cDNAs applied in a very fine band to a thin gel (Fig. 2*b*) reveals that cDNA no. 1 is composed of two separate cDNAs (nos. 1a and 1b) and cDNA no. 2 also appears to be a doublet (nos. 2a and 2b). Because only certain cDNAs resolve into multiple species on thin gels, we believe that the early mRNAs from SV80 cells and other transformed lines probably have three or four rather than two principal 5′ termini. Recent sequence analyses indicate that cDNAs 1a and 1b terminate between residues 5235 and 5237 whereas cDNAs 2a and 2b terminate between residues 5230 and 5233 (data not shown). In addition, on thin-gel electrophoresis (Fig. 2*b*), cDNA no. 5 appears to consist of three bands (nos. 5a, 5b, and 5c), all of about equal abundance. Both sequence analysis and gel position indicate that the components of cDNA no. 5 terminate between residues 5204 and 5208. The significance of the minor cDNAs, which are shorter than the two principal cDNAs, has been uncertain; however, data presented below suggest that most, if not all, mark the 5′ termini of minor RNA species *in vivo*. The longer cDNAs contain in most instances only viral sequences.

The rat fibroblast line transformed by wild-type SV40 contains viral RNAs that give the same principal cDNAs as the SV80 cell line. This conclusion is based upon both coelectrophoresis of cDNAs (Fig. 2) and sequence analysis and is in keeping with our prior finding that the principal 5′ termini of the early mRNAs in a wide variety of transformed lines lie at the same genomic loci (3, 4). Although not visible in Fig. 2*a*, cytoplasmic RNAs isolated from the transformed rat cell line also serve as templates for a number of additional cDNAs, most of which comigrate with minor SV80 cDNAs.

DNAs complementary to the 5′ termini of RNAs obtained from cells transformed by mutants 8-4, 6-1, and 6-17 (with deletions of four, six, and nine base pairs, respectively) show electrophoretic patterns that are different from but related to those of wild-type virus and of each other (Fig. 2 *a* and *b*). The electrophoretic pattern of mutant 8-4 reveals a single principal cDNA comigrating with wild-type cDNA no. 2 and a minor cDNA (no. 6) that migrates slightly faster than wild-type cDNA no. 5 and is less abundant. On thin-gel electrophoresis, it is apparent that cDNA no. 2 is composed of one abundant rela-

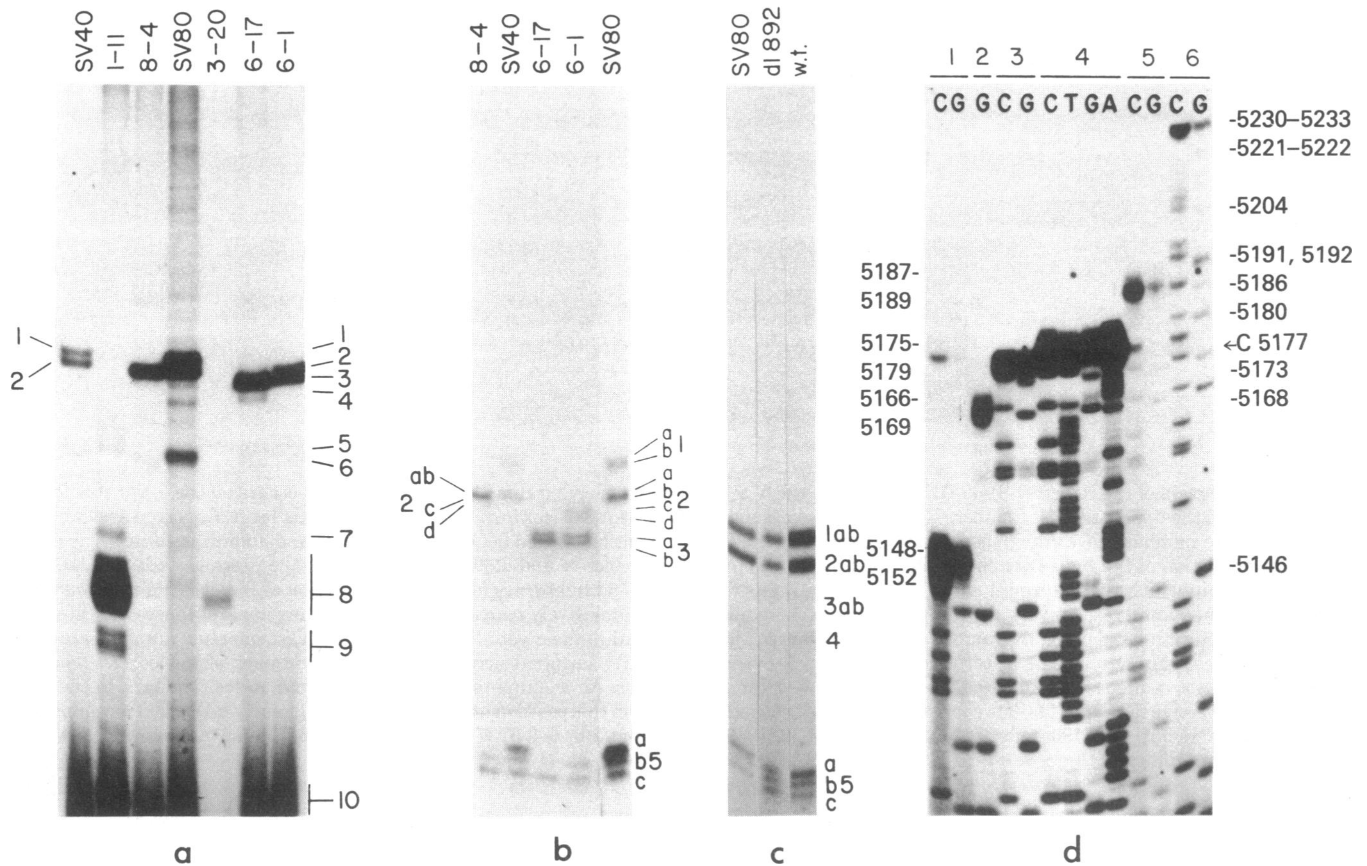

FIG. 2. (*a–c*) Electrophoresis of DNAs complementary to the 5′ termini of viral mRNAs on standard (2.0-mm *a* and *c*) and thin (0.5-mm *b*) 8% polyacrylamide/7 M urea gels. Sources of early mRNAs in *a* and *b* were the SV80 transformed human line and rat cells transformed by DNA of wild-type SV40 and the origin-defective mutants 1-11, 8-4, 3-20, 6-17, and 6-1. Sources of mRNAs in *c* were Vero cells infected with wild-type SV40 and dl892 for 24 hr in the presence of 20 μg of cytosine arabinoside per ml. Primer A, spanning residues 5092–5127, was used for synthesis of cDNAs shown in *a* and *c*; primer B, spanning nucleotides 5136–5171, was used for synthesis of cDNAs shown in *b*. Numbers in margins or adjacent to cDNA bands indicate cDNAs referred to in text. The autoradiogram in *b* consists of two portions spliced together; the lower portion has been developed for an extended period of time to bring out minor gel bands. (*d*) Maxam–Gilbert (17) sequence analyses of the indicated cDNA bands removed and eluted from the gel shown in *a*. Sequences are read from the bottom upwards, corresponding to progression on the cDNA in a 5′-to-3′ direction and on the mRNA template in a 3′-to-5′ direction. Columns 1–5 represent sequences of mutant 1-11 bands from *a*: 1 corresponds to band no. 10, 2 to band no. 9, 3 to band no. 8 (lower part), 4 to band no. 8 (upper part), and 5 to band no. 7. Column 6 represents sequences of band no. 2 of mutant 8-4.

tively broad cDNA band (nos. 2a and 2b) comigrating with wild-type cDNAs nos. 2a and 2b and two scarce cDNAs (nos. 2c and 2d) migrating slightly faster and that cDNA no. 6 consists of three cDNAs coelectrophoresing with cDNAs nos. 5a, 5b, and 5c. As noted, cDNAs nos. 2a and 2b terminate between residues 5230 and 5233; from their electrophoretic positions, cDNAs nos. 2c and 2d terminate between residues 5228 and 5231 and cDNAs nos. 5a, 5b, and 5c terminate between residues 5204 and 5208.

cDNAs transcribed on mutant 6-1 RNAs include two major and one minor species when electrophoresed under standard conditions. The most abundant cDNA (no. 3) comigrates with a minor wild-type cDNA just ahead of cDNA no. 2 (Fig. 2*a*), but it is, in fact, a doublet (nos. 3a and 3b) when electrophoresis is carried out on the thin gel. The electrophoretic position suggests that the termini of the cDNA no. 3 doublet lie four and five nucleotides downstream from the termini of cDNAs nos. 2a and 2b (i.e., between residues 5225 and 5228, a location confirmed by direct sequence analysis). The second major cDNA of mutant 6-1 appears to comigrate with wild-type cDNA no. 2 on standard electrophoresis, but on thin-gel analysis it comigrates as a doublet with cDNAs nos. 2c and 2d of mutant 8-4. The minor cDNA of mutant 6-1 (no. 6) comigrates with cDNAs nos. 5a, 5b, and 5c on thin-gel electrophoresis. For both mutants 8-4 and 6-1, cDNAs 5b and 5c are more abundant than cDNA 5a.

On standard gel electrophoresis, the cDNAs copied on mutant 6-17 RNAs include a single principal species in the position of cDNA no. 3, a minor species (cDNA no. 4) comigrating with a minor cDNA copied on wild-type RNA, and a very scarce cDNA comigrating with the aforementioned cDNA no. 6. On thin-gel electrophoresis, cDNA no. 3 resolves into a doublet comigrating with cDNAs nos. 3a and 3b of mutant 6-1 whereas cDNA no. 6 resolves into the cDNA triplet nos. 5a, 5b, and 5c. Like the aforementioned mutants and in contrast to wild-type virus, 5b and 5c are more abundant than 5a. cDNA no. 4, which is barely visible on the thin gel, has a 5′ terminus between residues 5218 and 5221, as determined by sequence analysis (data not shown).

Mutants 1-11 and 3-20 were independently derived but contain an identical deletion of 58 nucleotides spanning residues 5–5189 (Fig. 1). cDNAs copied on the 5′ ends of their early RNAs give identical patterns on standard separation gels; the patterns shown in Fig. 2*a* differ only in a quantitative sense. The cDNA band designated no. 8 represents approximately 90% of the cDNAs copied on the early mRNAs of these mutants.

In fact, this band is extremely broad, and examination of properly exposed autoradiograms and sequence analysis (Fig. 2*d*) of slices taken from within this band suggest that it is composed of four or five individual cDNAs with termini at residues 5175–5179. Two additional bands with downstream termini (nos. 9 and 10) account for approximately 10% of total cDNAs; two cDNAs (no. 7 and one comigrating with the aforementioned no. 5), with termini upstream from the major cDNAs, are also apparent. Whereas cDNA no. 7 appears to contain a single terminus between residues 5187 and 5189, band no. 9 consists of at least three cDNAs with termini in the region of residues 5166–5169 and band no. 10 includes three or four cDNAs with termini between residues 5148 and 5152 (Fig. 2*d*).

For the reasons discussed previously with regard to wild-type virus (3, 4) we believe that the termini of the major cDNAs of the mutants studied mark the sites of the 5′ termini of their principal early mRNAs. The remarkable specificity of the cDNA pattern for each of the mutants supports this contention and suggests that the termini of minor cDNAs mark the 5′ ends of minor mRNAs. It is striking that an increase in the size of the deletions in the mutants shifts the principal 5′ termini of the early mRNAs in a downstream direction (Fig. 1). Furthermore, for each mutant, the shift of the major 5′ terminus (or termini) downstream approximates the length of the deletion. It is also noteworthy that mutants 1-11 and 3-20 are able to compensate for deletion of residues 5225–5237, which code for the principal 5′ termini of the early mRNAs of wild-type virus and mutants 6-1, 6-17, and 8-4, by using a large number of downstream sites for the 5′ termini of their early mRNAs.

Because all five origin-defective mutants with deletions downstream from the A-T-T-T-A-T sequence produce principal mRNAs with 5′ termini downstream from those used by wild-type SV40, we inquired into the effect of a deletion upstream from the A-T-T-T-A-T sequence on the selection of 5′ termini. For this experiment we used the mutant dl892, with a deletion extending from residue 34 to residue 52 (Fig. 1). Because this mutant is viable, we compared patterns of DNAs complementary to the 5′ termini of dl892 and wild-type polyadenylylated cytoplasmic RNAs extracted from lytically infected cells. Fig. 2*c* reveals virtually identical cDNA patterns with abundant cDNAs nos. 1, 2, and 5 and less abundant cDNAs nos. 3 and 4. Thus, deletion of sequences a short distance upstream from the A-T-T-T-A-T sequence does not shift the 5′ termini of the principal early mRNAs.

Because of the altered genomic localizations of the 5′ termini of the early mRNAs of the origin-defective mutants, we also considered the possibility that the internal splices of the mutant early mRNAs might be altered. To investigate this possibility, we obtained cytoplasmic RNAs from cells transformed by mutant 1-11 (58-nucleotide deletion) and synthesized cDNAs by using a primer just downstream from the 3′ terminus of the two wild-type early splices. On sequence analysis, all cDNAs were found to contain only the two wild-type early splices (data not shown).

Finally, determination of the sequences of the cDNAs of mutants 8-4 and 1-11 revealed a cytosine residue instead of a thymidine residue at position 5177 (Fig. 2*d*). This thymidine-to-cytosine transition has been observed only in cDNAs synthesized from mRNAs extracted from transformed cells and not from lytically infected cells. Therefore, we believe that this transition is not an artifact of reverse transcription. Furthermore, this transition does not reflect a mutation in the original viral DNA used to transform the rat cells because the sequence of the cloned DNA was determined and found to contain a thymidine at this position (8). The mechanism of this phenomenon is unknown.

DISCUSSION

Two important findings have emerged from the present studies. First, we have shown that deletions of increasing size about the *Bgl* I site of SV40 DNA result in the progressive shift of the principal 5′ termini of the viral early mRNAs in a downstream direction. These shifts occur even when the original sequences coding for the wild-type cap sites are preserved (mutants 8-4, 6-1, and 6-17). If one uses the A-T-T-T-A-T sequence as a fixed point upstream from the principal 5′ ends, the most upstream major 5′ terminus of the early mRNAs of wild-type virus and each of the mutants studied lies 21–23 nucleotides downstream from this site; for wild-type virus and mutant 6-1, each with two sets of principal 5′ termini, the more downstream set of termini lies 24–28 nucleotides downstream from the A-T-T-T-A-T sequence. These data strongly suggest that a measuring function from a fixed point upstream from the *Bgl* I site is involved in fixing the location of the 5′ termini of the early SV40 messengers. Such a function is reminiscent of that suggested for generation of the 5′ terminus of *Xenopus* 5S RNA by RNA polymerase III, although in this case measurement occurs in an upstream direction from a fixed point within an intervening sequence of DNA (18). It is also in accord with the recent data of Grosschedl and Birnstiel (19) on localization of the 5′ termini of histone H_2A mRNAs.

The second significant finding of the present studies is that, whereas mutations downstream from the A-T-T-T-A-T sequence result in a downstream shift in early mRNA 5′ termini, deletion of 19 nucleotides (residues 35–53) starting 13 nucleotides upstream from the A-T-T-T-A-T sequence (mutant dl892) does not alter the ability of the virus to make early mRNAs with the principal wild-type 5′ termini. This result indicates that the genomic site from which downstream measurement occurs lies between residues 6 (one nucleotide upstream from the 5′ terminus of the deletions in mutants 1-11 and 3-20) and 34 (one nucleotide downstream from the 3′ end of the deletion in dl892) (Fig. 1). Recent transcriptional studies *in vitro* (ref. 20, and unpublished data) suggest that the principal 5′ termini of the early mRNAs are derived by transcription initiation. If this is so, the span of 29 nucleotides between residues 6 and 34 including the Goldberg–Hogness A-T-T-T-A-T sequence would appear to contain a component of a transcriptional promoter which directs downstream positioning of the 5′ termini of the principal early mRNAs by RNA polymerase II. Recent information suggests that another component(s) of an SV40 early promoter is localized upstream from the A+T-rich region: a 72-base-pair repeat, located 88 nucleotides upstream from residue no. 15 of the A+T-rich region, is indispensable for synthesis of T antigen *in vivo* (C. Benoist and P. Chambon and P. Gruss and G. Khoury, personal communications), whereas the removal of the A+T-rich region and 11 nucleotides upstream from it (positions 5228–5242) do not affect the production of T antigen *in vivo* (21). Therefore, the early promoter of SV40 appears to consist of at least two separate components. In this sense this eukaryotic promoter resembles prokaryotic promoters, which are composed of two regions: a −35 or binding region and an A+T-rich Pribnow box (reviewed in refs. 22 and 23).

The majority of eukaryotic mRNAs contain one or two cap structures (usually purines) located about 25 nucleotides downstream from a single A+T-rich (Goldberg–Hogness) sequence (ref. 24 and references therein). In contrast, mRNAs coding for the structural proteins of SV40 and polyoma virus have heterogeneous 5′ ends (15, 25, 26); however, A+T-rich sequences are not found 25 nucleotides upstream from these termini. Our observation of heterogeneity in the major 5′ termini of early

SV40 mRNAs agrees with the finding of four to six major cap structures for SV40 mRNAs isolated from tsA-infected cells at the nonpermissive temperature (27, 28). Because an A+T-rich sequence is found upstream from these 5′ ends, two possible explanations for the observed heterogeneity can be proposed. First, because this A+T-rich sequence consists of 17 nucleotides (residues 15–31) rather than the six or seven A·T pairs found in the typical Goldberg–Hogness box, inaccuracy in the measuring function may be introduced, thus causing heterogeneity in the location of the 5′ termini. Second, once RNA polymerase has bound to the early promoter and downstream measurement has taken place, a certain degree of freedom may exist in the precise location of sites at which 5′ termini arise; presumably, DNA sequences at these sites would determine the precise position(s) at which 5′ termini are generated.

Fig. 2 *a* and *b* points out a downstream shift in the 5′ ends of minor mutant mRNAs at positions 5204–5208 which is definite but much less prominent than that observed for major 5′ ends. Because these 5′ termini are located about 45 nucleotides downstream from the Goldberg–Hogness sequence, the positioning function might have less influence on the location of these termini. It is also possible that the positioning of the minor 5′ termini may not be dependent on the Goldberg–Hogness box at residues 15–20, and it is of note that a stretch of A·T base pairs is found about 20 nucleotides upstream from each 5′ end. Another explanation for the lack of a large downstream shift of the minor 5′ termini lies in the fact that seven pyrimidines are present at positions 5198–5204 on the noncoding DNA strand. Because purines are preferentially capped (23), this stretch of pyrimidines might inhibit capping in this region.

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