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Control region for adenovirus *VA* RNA transcription

(deletion mapping/*in vitro* transcription/RNA polymerase III)

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ABSTRACT **Plasmids containing the *VA* RNA genes of adenovirus are faithfully transcribed by a crude cytoplasmic extract containing DNA-dependent RNA polymerase III [Wu, G.-J. (1978) *Proc. Natl. Acad. Sci. USA* 75, 2175–2179]. By subjecting these DNA templates to *in vitro* site-directed mutagenesis with a novel enzyme of *Pseudomonas* and recloning in pBR322, we have constructed an ordered series of deletions which affect the *in vitro* transcription of the major RNA polymerase III viral product, VA_I RNA. Three regions that are required for specific synthesis of VA_I RNA can be defined. One, inside the gene at nucleotides +10 to +76, affects the transcription in an all-or-none fashion. Transcription is initiated on plasmid sequences that replace up to 10 nucleotides downstream from the 5′ end of the gene. Variants with deletions past nucleotide +15 do not support the transcription of VA_I RNA. Removal of 3′-end sequences downstream from +76 allows correct initiation. A second region, upstream from the initiation site, affects the exact alignment of the first nucleotide of the transcript [Thimmapaya, B., Jones, N. & Shenk, T. (1979) *Cell* 18, 947–959]. A third region, downstream from +76, encodes signals for termination of transcription, and new signals were brought in with other viral DNA sequences. Transcription competition experiments indicate that the primary site for binding of a transcriptional regulation factor is located between nucleotides +55 and +70 and suggest that the control region is bifunctional. An internal control region for VA_I RNA, approximately 60 bases long and 11 bases downstream from the 5′ end of the gene, can be defined.**

The development of soluble cell-free extracts in which specific genes are selectively and accurately transcribed has greatly facilitated our understanding of the molecular basis of control in prokaryotes (1) and eukaryotes (2–6). Faithful *in vitro* transcription systems were first developed for RNA polymerase III products, like the adenovirus encoded 5.5S RNA (*VA* RNA) by Wu (2) and later modified (4) or the *Xenopus* 5S genes (3, 7). Appropriate templates in large amounts and with high purity can now be obtained by recombinant DNA techniques. These templates can be manipulated to determine the DNA signals required for control of accurate initiation or termination of transcription.

Recently it was shown that, for the 5S genes of *Xenopus laevis*, a region internal to the gene was required for specific initiation of transcription mediated by oocyte cell extracts (8, 9). A purified factor required for accurate initiation strongly bound to this region of the gene (10) and might participate in the temporal control of 5S transcription *in vivo* because it also binds to the 5S RNA gene product (10, 11). A different set of factors seems to be required for adenovirus *VA* transcription (10) and it was therefore of interest to establish whether a similar internal control region could be identified for this RNA polymerase III transcribed gene (12).

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The adenovirus *VA* gene region contains at least two different *VA* genes, with different nucleotide sequences (13–16), called VA_I and VA_{II}. In the VA_I region (where RNA is produced in largest amounts) there are two initiation sites, the major G start (17, 18) and a minor A start three nucleotides upstream (18–20). The VA_I RNAs arising from these start sites are 156 (VA_{IG}) and 159 (VA_{IA}) nucleotides in length, respectively. In addition, a class of longer molecules (VA_{200}), resulting from read-through at the first VA_I RNA termination site, can be detected *in vitro* and *in vivo* (16). The VA_{II} gene is located about 98 nucleotides downstream from the VA_I gene, has a different although related DNA sequence, and is also transcribed by RNA polymerase III (15, 16, 21).

Using the DNA sequence data from this region of the genome (17, 18, 21–23) and appropriate restriction enzymes, we have constructed plasmids that allow us to define the DNA signals required for initiation and termination of VA_I RNA transcription. We have found an internal control region for VA_I transcription initiation located close to the 5′ end of the respective gene, as determined by an ordered set of deletions constructed by using the nuclease BAL 31.

MATERIALS AND METHODS

DNA Templates. Restriction enzyme fragments were prepared from whole adenovirus 2 (Ad2) DNA purified on CsCl density gradients as described (16) and inserted into plasmid pBR322. We constructed a wild-type clone containing the Ad2 sequences from the *Sal* I site (coordinate 26.9) to the *Hin*dIII site (coordinate 31.5) on the physical Ad2 map which goes from 0 to 100. dl 5′ +70 (deletion of all sequences upstream from nucleotide +70) was created by partial *Bam*HI digestion and religation of a recombinant containing the Ad2 *Sal* fragment C (coordinates 26.9–45.9). The 3′-end deletion in dl 3′ +76 was generated by *Bam*HI cleavage and religation of a plasmid carrying the Ad2 *Bgl* II fragment A (coordinates 24.8–45.3) in the *Bam*HI site of the vector.

Digestions with nuclease BAL 31 (Bethesda Research Laboratories, Gaithersburg, MD) (24) were at 20°C in 100 μl containing 20 μg of DNA and 10 units of BAL 31. *Sal* I linkers (Collaborative Research, Waltham, MA) were phosphorylated with T4 polynucleotide kinase (P-L Biochemicals) (25) and added to BAL 31-treated termini with T4 DNA ligase (20 units/ml; P-L Biochemicals) in a 5- to 10-μl reaction mixture containing 0.25 and 10 μM 5′ ends of plasmid DNA and phosphorylated *Sal* I octamer, respectively. Incubations were at 12.5°C for at least 16 hr in 50 mM Tris, pH 7.6/10 mM $MgCl_2$/10 mM dithiothreitol/0.1 mM ATP. The DNA fragment obtained after *Hin*dIII and *Sal* I digestions was eluted from an 8% polyacrylamide gel and inserted into *Sal* I/*Hin*dIII-digested pBR322 plasmid. Transfections of competent HB101 *Escherichia coli* with the ligated DNAs were performed as described

Abbreviation: Ad2, adenovirus 2.

(26). Sequence analysis was performed by two methods as described (25, 27).

Transcription. Cytoplasmic extracts from exponentially growing suspensions of HeLa cells were prepared as described (2, 4). DNAs were incubated at 15–60 μg/ml with 600 μM unlabled ATP, CTP, and UTP and 25 μM [α-^{32}P] GTP (ICN; 15–25 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and 20 μl of extract at 30°C for 1 hr. After extractions with phenol/$CHCl_3$/isoamyl alcohol, 25:24:1 (vol/vol), and ethanol precipitation, the samples were dissolved in 90% formamide/5% glycerol and run on 0.4-mm-thick 6–8% Tris borate/urea sequencing gels (25) at 800 V. Autoradiography of the gels was at −70°C in the presence of Lightning-Plus DuPont screens with Kodak RP-1 film. RNA-containing bands were excised, eluted at 37°C in 0.5 M ammonium acetate containing 10 μg of tRNA per ml, and subjected to nucleotide pattern analysis (28). Analysis of 5′-end triphosphate was on polyethyleneimine-cellulose of either T2 or P1 nuclease-digested RNA eluted from gels as described (21, 29) followed by autoradiography. In this case, the transcription reactions contained all four ribonucleotide [α-^{32}P]triphosphates at 50 μM.

RESULTS

The strategy for the preparation of the 5′-end-deleted VA_I genes required a *VA*-containing plasmid with single *Xba* I and *Bam*HI sites (Fig. 1). These are located 30 nucleotides upstream and 70 downstream, respectively, from the initiation site for VA_I RNA transcription. An Ad2 *Sal* I/*Hin*dIII recombinant plasmid was linearized by cleavage with *Xba* I followed by controlled digestion with BAL 31. This nuclease from *Pseudomonas* digests single-stranded DNA rapidly but digests double-stranded DNA at a much slower and controllable rate (24). The constant digestion rate at 20°C, as monitored by the changes in molecular weight of a DNA fragment (results now shown), was approximately 22 base pairs per min per end. The smaller deletions were generated by 1- to 2-min BAL 31 digestions; the larger ones were from 2- to 4-min digestions. Flush ends that are left on the DNA after BAL 31 treatment do not religate by themselves efficiently under conditions normally required for the construction of recombinant plasmids (unpublished data). However, synthetic DNA linkers can be joined to these ends with almost 100% efficiency.

For the experiments described, the *Sal* I linker was joined directly to the BAL 31-treated termini by using T4 DNA ligase. Cleavage with *Sal* I at the attached linker and at another restriction enzyme site flanking the VA genes, *Hin*dIII in this case, generated "sticky-ended" deleted DNA fragments that were easily inserted in pBR322 in only one orientation. These recombinants were then used to transform the HB101 strain of *E. coli*. We have been able to obtain more than 2000 colonies of 5′-end-deleted VA_I DNA in a single experiment using this procedure. Individual variant DNAs were then purified on CsCl/ethidium bromide density gradients.

Taking advantage of the strategically located *Sal* I and *Bam*HI sites in any given clone, it was possible to screen rapidly for a set of variant clones which represented the desired range of deletions. The *Sal* I/*Bam*HI-cut DNA fragments were analyzed on a sequencing gel with appropriate size markers, and the extent of BAL 31-generated deletions could be estimated. Alternatively, the *Bam*HI fragments were cleaved at an *Hha* I site located in the 5′-flanking pBR322 DNA and the resulting virus–pBR322 fragment was subjected to sequence analysis as described (25, 27). The endpoints of several deletions and their DNA sequence are indicated in Fig. 1. In some cases the first or second nucleotide brought in by the linker matches the sequence of the parental viral DNA, in which case the deletion

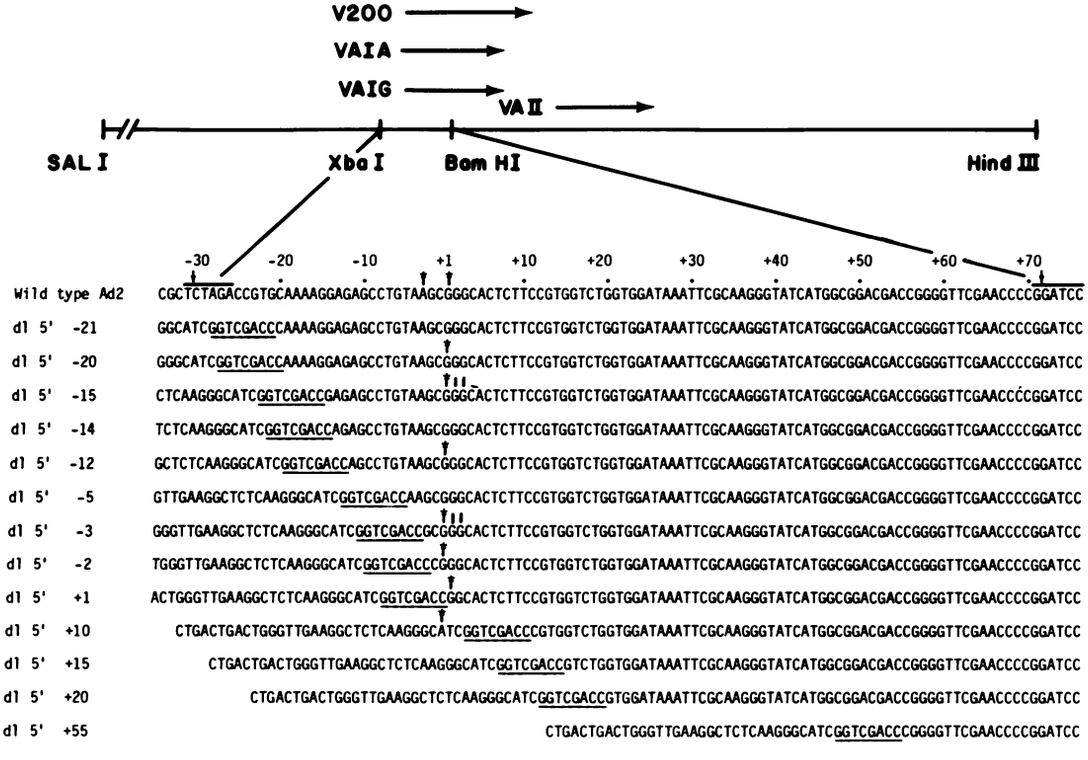

FIG. 1. Deletion mutants at the VA_I gene. The region of the viral genome with its restriction enzyme sites is diagrammed above the DNA sequence of the region between the *Bam*HI and *Xba* I sites of the noncoding strand (18). The underlined sequences indicate those portions corresponding to the *Sal* I linker; to the left are the sequences of pBR322 that agree with those published by Sutcliffe (30) and to the right are the sequences of Ad2 that agree with those in the literature (18, 21, 22). Asterisks and vertical lines indicate initiation sites.

has been named starting from the first base pair that differs from the virus parental sequence.

This procedure for the *in vitro* generation of deleted DNA molecules offers several advantages over other protocols: (*a*) the size of the deletions can be regulated by the extent of BAL 31 digestion; (*b*) a gaussian distribution of deletion termini is generated for each incubation time point (these are so closely spaced around the expected median that they can be obtained for each base pair around the region of interest); (*c*) the sizing step allows preliminary selection of the desired deletions, which reduces the screening effort; and (*d*) the use of DNA linkers provides precise mileposts for the deletion analysis, the extent of which can be accurately determined. The estimates of the deletions obtained by sizing (dl 5′, −27; dl 5′, −21; dl 5′, −15; dl 5′, −14; dl 5′, −12; dl 5′, −5; dl 5′, −3; dl 5′, −2; dl 5′, +55) agreed very well with the values obtained by direct DNA sequencing (dl 5′, −20; dl 5′, +1; dl 5′, +10; dl 5′, +15; dl 5′, +20, and wild type).

The purified plasmids were incubated with the crude cell extract prepared as described (2, 4). The *in vitro* transcripts were analyzed in an 8% polyacrylamide/urea gel (25) run at room temperature to allow separation of VA_I from VA_{II} RNAs (Fig. 2) (these two RNAs comigrate if the electrophoresis temperature is above the melting point of VA_{II} RNA). The VA_I and VA_{II} RNAs synthesized in this system have the same electrophoretic mobilities as the products synthesized *in vivo*. Furthermore, the sequence of the VA_I RNA synthesized *in vitro* is identical to that of the *in vivo* product (4) (Fig. 3*A*). Not only do deletions upstream from the initiation site at +1 allow VA_I and VA_{II} transcription to occur, but deletions inside the gene up to position +10 also support transcription of a product that comigrates with VA_I RNA (Fig. 2). Because, in the dl 5′, +10 template, some of the viral sequences are replaced by plasmid and linker sequences, the *VA* RNA made from this template should differ in its sequence from the wild-type VA_I RNA.

Fig. 3*D* shows the pattern of gel-purified VA_I RNA made with the dl 5′, +10 plasmid as template. The spot corresponding to nucleotides 4–13 [C-A-C-U-C-U-U-C-C-G (Fig. 1)] is not present; the spot corresponding to nucleotides 25–33 remains unaltered. The dl 5′, +10 DNA sequence, which agrees with published reports (18, 21, 22, 30), predicts the replacement of this 9-nucleotide RNase T1 product by a new 5-nucleotide product, A-C-C-C-G, probably the one indicated by the arrow in Fig. 3*D*. Therefore, transcription of VA_I dl 5′, +10 is in fact initiated on the plasmid DNA template. In contrast, alterations upstream from the inititation site [dl 5′, −12 (Fig. 3*C*)] do not affect the mobility of the 5′-end-related oligonucleotide located between +4 and +13.

To establish the exact site of initiation of the VA_I RNA transcripts on the plasmid sequences, we determined the 5′ nucleotide. The VA_I RNA made in the presence of all four ribonucleotide [α-^{32}P]triphosphates was purified on polyacrylamide gels, digested with the nuclease P1, and run on thin-layer polyethyleneimine plates with appropriate markers (results not shown). Transcription of the wild-type VA_I plasmid gave 95% of the triphosphate as GTP. Initiation also occurred with GTP for most of the mutants as indicated by a vertical bar and an asterisk in Fig. 1. The minor bands below VA_I analyzed for dl 5′, −15 and dl 5′, −3 were initiated with GTP, suggesting that either other Gs are used or the RNAs differ at their 3′ ends. In the case of dl 5′, +1, the G used is displaced one nucleotide with reference to the parental sequence, indicating that the purine preference prevails over exact nucleotide alignment. Thin-layer analysis of the nuclease P1-digested RNA transcribed from dl 5′, +10 showed initiation with A (Fig. 1), as predicted from the electrophoretic migration (Fig. 2).

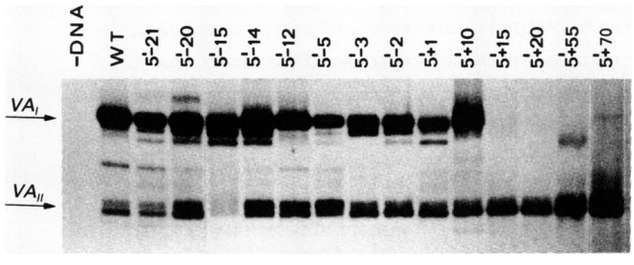

FIG. 2. *In vitro* transcription of deletion-substitution DNAs. RNA extracted from *in vitro* transcription reactions performed in the presence of the different DNA deletion templates described in Fig. 1 was analyzed on 8% polyacrylamide/8 M urea gels. Positions of VA_I and VA_{II} RNAs are indicated.

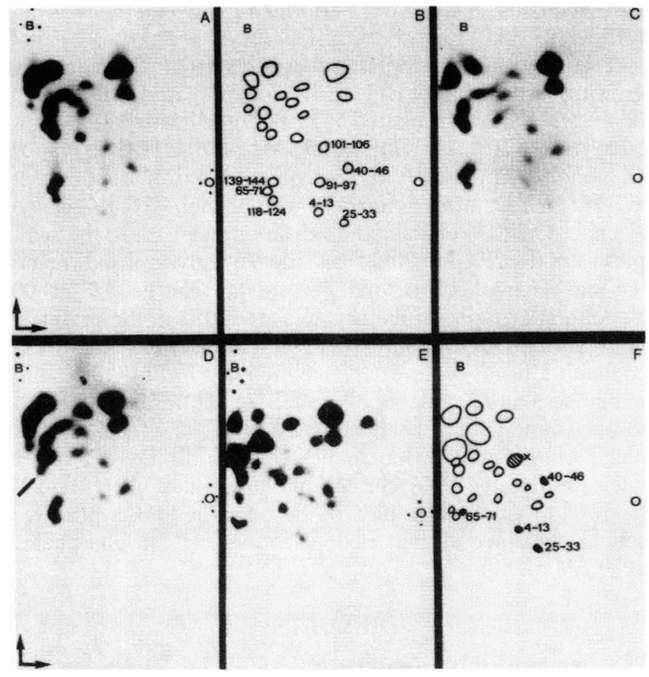

FIG. 3. Nucleotide digestion pattern analysis of VA_I RNA made by deletion-substitution mutants. The VA_I RNAs were made in transcription reactions using [α-^{32}P]GTP purified, concentrated, digested with RNase T1, and subjected to two-dimensional analysis as described (28). Cellulose acetate electrophoresis was from left to right; and homochromatography on DEAE-cellulose plates was from bottom to top. (*A*) *In vitro* transcribed parental plasmid. (*B*) Schematic tracing of spots in *A*. The numbering of the spots corresponds to their position in the DNA sequence in Fig. 1. (*C*) VA_I RNA made from dl 5′, −12. (*D*) VA_I RNA made from dl 5′, +10. (*E* and *F*) Pattern and tracing of the two pooled major bands made from dl 3′, +76 (see Fig. 4). The spots that correspond to those of wild-type VA_I (major oligonucleotides only) are solid in *E*.

Similarly, we tried to determine the limit of the control region at its 3′ end. Replacement of sequences downstream from the *Bam*HI site with sequences of Ad2 starting at 42 map units was performed by direct digestion at the *Bam*HI site of a *Sal* I fragment C Ad2 plasmid and confirmed by appropriate restriction enzyme analysis. This purified plasmid, when analyzed in the *in vitro* system, resulted in transcripts roughly 400 and

650 nucleotides long (Fig. 4). Although the pattern was considerably more complex than in the case of VA_I RNA (Fig. 3A), oligonucleotides corresponding to the 5′ half of the wild-type VA_I RNA—such as 4–14, 26–35, 40–47, and 65–71—were present in their normal positions whereas oligonucleotides 91–97, 118–124, and 139–144 were absent or showed altered mobilities (Fig. 3 *E* and *F*). The spot marked X in Fig. 3*F* coincides with the VA_I RNA spot at position 101–105 but the lack of sequence data for the substituted region of the Ad2 genome does not allow us to exclude that a similar T1 RNase product arises from the new sequences downstream from the *Bam*HI site. This suggests that the DNA sequences downstream from nucleotide +76 play no role in determining the site for initiation of VA_I RNA transcription, although a DNA signal required for correct termination is encoded in this region of the DNA. The replacement of the viral DNA with novel sequences provides new termination signals which explain the longer transcripts.

To establish whether areas inside the control region could participate in some phase of transcription initiation, we performed the experiment summarized in Table 1. We analyzed the efficiency of transcription of dl 3′, +76, which gives distinct VA_I RNA products (see Fig. 4), in the presence of increasing amounts of each of our 5′ deletion mutants. We expect that, in the case shown in Table 1, with a molar DNA ratio of dl 5′ to dl 3′, +76 of 4:1, the level of the 400- and 650-nucleotide-long transcripts should be decreased to 20% of the value in the absence of competition. All deletions up to dl 5′, +55 could compete as efficiently as wild-type DNA for VA_I dl 3′, +76 transcription, although dl 5′, +70 did not. This means that, although the mutants dl 5′, +15, dl 5′, +20, and dl 5′, +55 are unable to be used as templates for transcription, some sequences that can bind a factor present in limiting amounts are retained in these deletions. These sequences are located between nucleotide +55 and +70.

Table 1. Competition of VA_I gene deletions for transcription of dl 3′, +76 DNA

Competing DNA	Transcription obtained, %				
	Exp. I	Exp. II	Exp. III	Exp. IV	Exp. V
Wild type	31	9.8	8.3		
dl 5′, −15	30.3	—	—		
dl 5′, +1	21.8	—	—		
dl 5′, +10	32.1	27.8	32.4		
dl 5′, +15	44.9	41.3	33.7		
dl 5′, +20	32.3	28.5	28.5		
dl 5′, +55	19.3	—	—	22.7	30.9
dl 5′, +70	75.1	90.7	80.9		

Reaction mixtures contained 0.3 μg of dl 3′, +76 DNA and 4 times the molar equivalent of the respective deletion DNA. Final DNA concentrations of 40 μg/ml were attained by adding appropriate amounts of pBR322. Incubations in volumes of 50 μl contained 10 μCi of [^{32}P]GTP. RNAs were extracted and analyzed as described for Fig. 2. Individual bands were excised from the gel and assayed by Cerenkov radiation. Values from five experiments are expressed as percentage of the value for the 400- and 650-nucleotide long transcripts obtained in the absence of competition (12,300 cpm in Exp. I, 5400 in II and III, 30,300 in IV, and 2800 in V). The level of VA_{II} RNA in these experiments was similar for deletions +10 through +55 (800–1000 cpm in II) and dl 5′, +70 (700 cpm in II) which essentially did not show detectable levels of competition.

DISCUSSION

The method described here is useful for generating deletions at unique restriction enzyme sites and therefore for manipulating the genetic structure of any gene. Analysis of a collection of these deletions and subsequent transcription revealed that, as with other RNA polymerase III-transcribed genes, a region internal to the gene is required for initiation of transcription. The RNA transcripts produced are initiated (dl 5′, +10) on plasmid sequences and a colinear plasmid–viral *VA* RNA is made, as demonstrated by 5′-end-triphosphate analysis and nucleotide pattern analysis. The boundaries of this internal control region are +11 and +75 nucleotides inside the transcribed region of the gene.

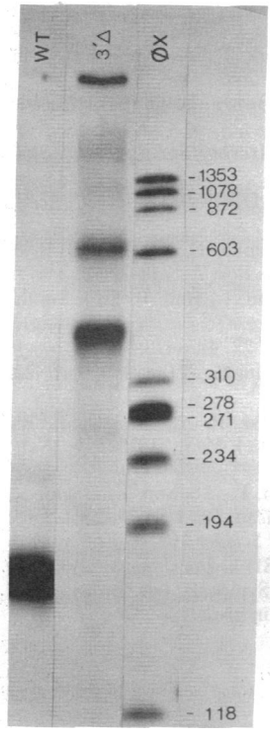

FIG. 4. Transcription of a 3′-end deletion. Autoradiogram from a transcription reaction with a deletion from the 3′ end of the gene. Lanes: WT, wild-type plasmid; 3′Δ, deletion-substitution from the 3′ end, starting at the *Bam*HI site at +76 (Fig. 1); ϕX, size markers from a *Hae* III digest of end-labeled ϕX DNA (lengths, in base pairs, are indicated).

Recent analyses of the 5S RNA gene (8, 9) and tRNA genes (31, 32) have demonstrated that not only are the 5′- and 3′-flanking regions of the gene unnecessary for proper initiation of transcription but also that the DNA sequences required are probably internal to the gene. In the case of 5S RNA, a region from +46 to +80 is required for initiation. A protein, which functions as an essential cofactor, binds to an overlapping region of the gene between nucleotides +50 and +90 (10). In the case of tRNAs, the 3′ and 5′ halves of the genes were unable to function independently (32). A point mutation affecting transcription has been located at position +63 for yeast sup 4-tyr tRNA (33). It therefore seems that all genes transcribed by DNA-dependent RNA polymerase III contain internal control regions.

The novel feature of the VA_I RNA gene is the proximity of the control region to the 5′ side of the gene. The control region of Ad2 is located downstream from nucleotide +11 and upstream from +76. Similar results were recently obtained by Fowlkes and Shenk (34), who defined an adenovirus control region located between +6 and +69 (our numbering system).

The control signals encoding termination are located downstream from nucleotide +76 and probably consist of a string of adenosines which results in the 3′ stretch of uridines of the *VA* RNA. In the case of our 3′ deletion shown in Fig. 2 and others in which plasmid DNA sequence was replacing the normal 3′ sequences (results not shown), transcripts of abnormal sizes were generated.

With regard to the regions upstream from the gene and the control of transcription, a deletion located at −27 (22) affected the A initiations of VA_I RNA. Modifications of tRNA 5′-flanking sequences also affect the initiation site (31). In the case of the mutants described here, abnormal initiations were more fre-

quent for some of the 5′ deletions but the purine preference was conserved. For an alanine tRNA gene of *Bombyx morii*, it has been shown that, although heterologous systems allowed ready transcription of a dl 5′, −11, a homologous RNA polymerase III system was unable to respond to the deleted gene (35). We tried a similar approach by comparing the ability of extracts from uninfected and infected cells to transcribe the VA_I gene in some of our deletions. We tested dl 5′, −12 and dl 5′, +1 and obtained identical levels of VA_I RNA with infected or uninfected RNA polymerase III-containing extracts (unpublished data). It thus seems unlikely that a similar regulatory mechanism is operating in *VA* transcription. However, as shown in several cases for tRNA genes (31, 32, 35, 36) modifications in the 5′ region upstream from the initiation site do affect the rate of *in vitro* transcription (see also Fig. 2). The lower rate of transcription observed with dl 5′, −3, dl 5′, −5, and dl 5′, +1 was also observed in kinetic experiments (results not shown). Therefore, some control, probably of alignment of the initiation site and of transcription rate, is effective at the 5′-flanking region of the gene.

It has been shown that the transcriptional protein factor binding to the 5S control region can be separated chromatographically from the one required for VA_I RNA transcription (10). However, the same protein is required for initiation by VA_I and VA_{II} RNAs (34). A model can be proposed in which the factor is just bound to a selective region of DNA template, followed by binding of RNA polymerase III which recognizes both a protein site on the factor and a DNA site at the 5′ side of the control region. It is this second site that is affected by dl 5′, +15. After recognition of the second site, the RNA polymerase III extends to the initiation site, lines up with a purine at approximately the correct distance from the factor binding site, and initiates transcription. Support for this model comes from: (*i*) binding of 5S factor is slightly downstream of the internal control region as defined by deletion analysis; and (*ii*) the deletions +15, +20, and +55 which do not allow VA_I transcription still compete as efficiently as VA_I for this factor, but +70 does not (Table 1). This experiment defines the limits of the initial factor-binding region between nucleotides +55 and +70. It is not clear whether the nucleic acid sequences between the two regions are important for transcription initiation, but insertion of a 21-base-pair *lac* repressor (37) between the two regions of a tRNA gene does not affect transcription significantly.

The transcription of *VA* genes by uninfected cell extracts (2, 4), the sequence similarity between *VA* and tRNA for this region of the genome (34, 38), and the ability of *VA* and tRNA genes to compete for transcriptional factors (39) suggest some relationship between them. Whether this relationship is limited to common transcriptional factors or is a phylogenetic one has to be analyzed more carefully.

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