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Structural Requirements of Adenovirus VAI RNA for Its Translation Enhancement Function

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Recently, by genetic and biochemical approaches, it has been shown that adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection. To understand the nucleotide sequences and the domains of the VAI RNA that are responsible for the role of VAI RNA in enhancement of translation, a mutational analysis of the VAI gene was undertaken. Deletion, substitution, and insertion mutations covering most of the nucleotide sequences of VAI RNA were introduced into the VAI gene at the plasmid level. These mutant genes were then reintroduced into the virus, and growth properties of the mutant viruses were studied. The majority of the mutants retained normal or nearly normal levels of biological function. Mutations in the region between +43 and +53 and between +107 and the 3′ end of the gene resulted in a considerable loss of activity. These mutants, however, grew significantly better than did an adenovirus type 5 mutant lacking both functional VAI and VAII genes, indicating that they retain a portion of their activity. Because no one mutation was able to completely abolish the function, we suggest that the VAI RNA may have multiple functional sites for its translation modulation function. These multiple sites may be short oligonucleotide sequences that may interact with cellular or viral components or both during translation.

Adenoviruses synthesize large amounts of two low-molecular-weight RNAs designated VAI and VAII (20, 23). The genes coding for these two RNAs are located between 29.0 and 31.0 map units (m.u.) on the adenoviral chromosome. Nucleotide sequences of these RNAs have been determined (1, 19, 25). The virus-associated (VA) RNA genes are transcribed by RNA polymerase III (23, 31). An intragenic transcription control region for the VAI gene has been identified, and it maps between +10 and +69 (4, 8, 12) (the numbering here and throughout this article is relative to the starting point of the VAI-G RNA). In the infected cells, a cellular phosphoprotein of 45,000 molecular weight has been shown to be associated with VA RNAs, and the nucleoprotein complex is recognized by the anti-La class of lupus sera (9, 13, 14).

Constructed mutants have been used to probe the function of VA RNAs. An adenovirus type 5 (Ad5) mutant which fails to synthesize the minor VAII species grows normally in human cells. A mutant which fails to synthesize the major VAI species, however, grows more slowly than its parent, apparently because the VAI RNA is essential for the efficient initiation of translation of viral mRNAs synthesized at late times after infection (21, 26). We have undertaken mutational analysis of the VAI RNA gene to define the nucleotide sequences and structural features of this RNA that are necessary for it to function as an enhancer of translation. A series of Ad5 mutants were constructed in which the mutations were introduced into defined regions of the VAI gene. Growth properties of these mutants were studied to correlate the mutational alterations with the biological function. The majority of the mutants retained normal or nearly normal levels of biological function; a few of the mutants showed a partial loss of activity. None of the mutants were completely defective for VAI function, suggesting that multiple sequences in the VAI gene may be involved in its modulation of translation. In this paper, we report the construction and characterization of these adenovirus mutants.

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MATERIALS AND METHODS

Cells, viruses, and plasmids. The 293 cell line (Ad5-transformed human embryo cell line [10] and HeLa cells (obtained from T. Shenk, Princeton University, Princeton, N.J.) were maintained in Dulbecco modified minimal essential medium containing 10% calf serum. Mutant *dl*704 is an Ad5 variant which has its DNA segment, 29.0 to 31.0 m.u., replaced by corresponding adenovirus type 2 (Ad2) DNA fragment. The VAII RNA gene of this variant does not transcribe, owing to a 17-base-pair (bp) intragenic deletion (26). *dl*704 has single *Xba*I and *Eco*RI sites at 29.0 and 30 m.u., respectively. *dl*703 is identical to *dl*704, except that it has a functional VAII gene. The construction of these two variants has been described in an earlier report (5). A mutant lacking functional VAI and VAII genes (*dl-sub*720) was constructed as follows. A variant of *dl*703 was constructed first in which the 230-bp fragment between *Xba*I and *Eco*RI was replaced by an Ad2 DNA segment, 434 bp in length, from 88.3 to 89.7 m.u. This mutant is VAI$^-$/VAII$^+$. The *Eco*RI B fragment of this variant was ligated to *Eco*RI A fragment of *dl*704 to generate a mutant lacking VAI and VAII genes (VAI$^-$/VAII$^-$). The *Eco*RI sites in these two variants were located in the spacer segment between the two VA genes. DNA plaque assays were done on 293 cells, and viral plaque assays were done on HeLa cells as described earlier (26).

Plasmid pA5-113 contains a 230-bp DNA fragment from the *Hin*dII site to the *Eco*RI site of *dl*704 (29.0 to 30.0 m.u.) cloned between the *Pvu*II and *Eco*RI sites of pBR322. Plasmids were propagated in *Escherichia coli* HB101.

Mutagenesis of VAI gene. Substitution and insertion mutations located in the 5′ half of the gene were constructed with *Hin*dIII linkers as described in an earlier report (4). Deletion mutations in the 3′ half of the molecule were obtained by the D-loop mutagenesis procedure (11, 22). A 53-bp deletion in

the VAI gene of *dl*711 was introduced by deleting a 53-bp *Taq*I fragment from +62 to +116. Precise mutational alterations in all of these mutants were determined by DNA sequence analysis (17). The VAI-VAII hybrid gene in *dl*721 was constructed as follows. A DNA segment containing Ad2 DNA sequences between *Xba*I and *Hin*dIII sites (29.0 to 31.0 m.u.) was cloned into a derivative of pBR322. This plasmid was digested partially with *Taq*I and religated; HB101 was transformed with the ligated sample. A plasmid with a deletion of DNA sequences from +115 of VAI gene to +61 of VAII gene was identified. The DNA fragment from this plasmid coding for the hybrid gene (*Xba*I-*Eco*RI) was reintroduced between *Xba*I and *Eco*RI sites of *dl*704.

RNA analysis. HeLa cells were infected with various mutants at a multiplicity of 20 PFU per cell and immediately labeled with $^{32}P_i$ for 24 h. At the end of the labeling period, cytoplasmic RNA was prepared by treating the cells with Nonidet P-40 as described earlier (26). The labeled total cytoplasmic RNA was subjected to an 8% denaturing polyacrylamide gel containing 8 M urea. Electrophoresis was performed in a Tris-borate buffer system. For quantitation of RNA accumulated in the cytoplasm, at 15 h after infection, ^{32}P-labeled cytoplasmic RNAs isolated from the mutant-infected cells were electrophoresed as described above, and the appropriate bands were excised from the gels and their radioactivity was quantitated by Cerenkov counting. For fingerprint analyses, gel-purified RNA samples were subjected to the T_1 oligonucleotide mapping procedure described by Barrell (2) with thin-layer homochromatography on Cel-PEI300 plates (Brinkmann Instruments Inc.) for the second dimension. 5'-Terminal nucleotides of *sub*706 VAI RNA were identified by digesting the ^{32}P-labeled RNA with T_1, T_2, and pancreatic RNases and analyzing the digest by PEI-cellulose chromatography as described earlier (25).

The restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase and *Hin*dIII linkers were purchased from New England Biolabs. All of the enzymes were used as recommended by the supplier.

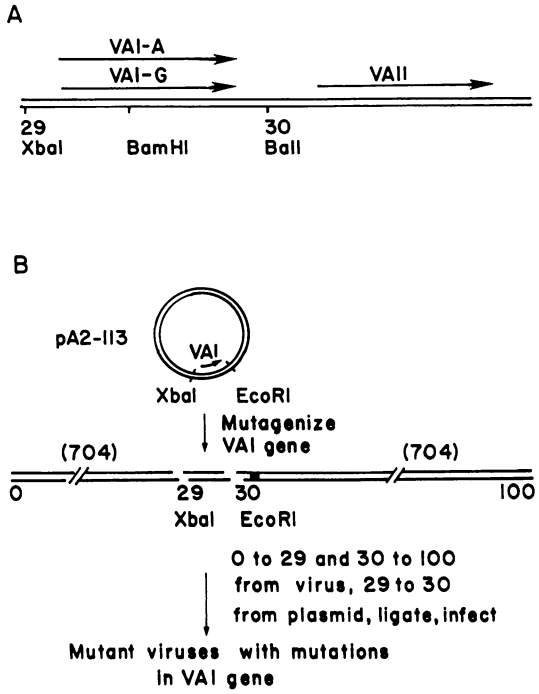

FIG. 1. (A) Location of the two VA genes on the Ad2 or Ad5 chromosome. The restriction sites are positioned on the Ad2 physical map. (B) Diagram of the strategy used to construct Ad5 mutants with mutations in VAI gene. The solid band depicts a deletion mutation in the coding region of VAII gene. See text for the construction of pA2-113. The *Xba*I and *Eco*RI sites of the plasmid pA2-113 correspond to the *Xba*I and *Eco*RI sites at 29.0 and 30.0 m.u., respectively, of *dl*704. The procedures to mutagenize VAI gene are described in the text.

RESULTS

Construction of mutant viruses. Our strategy to construct mutant adenoviruses with deletion, substitution, and insertion mutations in the VAI gene is shown in Fig. 1. First, we cloned a 230-bp DNA segment coding for the VAI RNA from Ad5 variant *dl*704 into a pBR322 derivative (see above and below). Mutations were introduced into this VAI gene at the plasmid level by site-directed mutagenesis procedures (see above). Then, the mutant VAI genes were reintroduced into *dl*704. Mutant *dl*704 has a 17-bp deletion in the transcription control region of VAII gene which prevents transcription of its VAII gene (26). It also contains a single *Xba*I site at 29.0 m.u. and a single *Eco*RI site at 30 m.u. With these two restriction sites, a 230-bp fragment coding for VAI gene can be selectively excised, and in its place, the mutant VAI genes can be introduced. Because the VAII gene of *dl*704 is not transcribed, the growth of the reconstructed viruses is totally dependent on the added mutant VAI gene. This allowed us to correlate the induced mutational alterations with the biological function. All of the mutant viruses described in this report were of *dl*704 background, except *dl*711. This mutant VAI gene was introduced into a variant designated *dl*703, which is identical to *dl*704 except that its VAII gene is transcribed normally. Both *dl*704 and *dl*703 grow to wild-type (WT) levels (5).

Mutations in the 5' half of the gene. A *Bam*HI site is located at +72 of the Ad2 VAI gene. For the sake of convenience, in this report, we divide the DNA sequences of the VAI gene preceding the *Bam*HI site as the 5' half and from *Bam*HI to the 3' end as the 3' half. A DNA sequence block from +10 to +69 forms the intragenic transcription control region of VAI gene (8, 12). Earlier, it was thought the mutations in this block would abolish transcription and, hence, that the role of these sequences in biological functions could not be studied. However, we have recently shown that the mutagenesis of DNA sequences between +10 and +18 (box A) and +54 to +69 (box B) abolishes transcription in vitro (4). Transcription of the genes whose sequences were mutated between +18 and +54 by substitution or insertion of foreign DNA sequences would occur at normal or nearly normal levels. These results allowed us to study mutant VAI genes whose transcription was not greatly affected by loss of essential promoter elements.

The following mutant VAI genes with mutations in the 5' half of the gene were reintroduced into the virus: *sub*706, nucleotide sequences +1 to +10 substituted, except at +3 and +4; *dl-sub*707, nucleotide sequences +19 and +20 and +22 to +25 substituted and a deletion of a single bp at +26; *in*708, with an insertion of eight nucleotides after +26; *dl-sub*709, with nucleotide sequences +44 to +46 and +49 to +51 substituted and a single bp deletion at +52 and *in*710, with a 15-bp insertion after +53. Substitution and insertion mutations in these mutant genes all have *Hin*dIII linker sequences. Construction of these mutant genes has been described in an earlier report (4). All of these mutant genes transcribe at a good rate in an in vitro assay. The location of

these mutations in the nucleotide sequence of VAI RNA is shown in Fig. 2.

Mutations in the 3′ half of the gene. The remaining mutant genes that we studied are all deletion mutants in the 3′ half of the gene. Because these deletions do not affect the intragenic promoter sequences, they are expected to transcribe normally. We have constructed and characterized eight mutant viruses in this category, and the VAI genes of these viruses have deletions of 6 to 53 bp. The viruses are: *dl*712, deletion of 7 bp from +69 to +77; *dl*713, deletion of 6 bp from +72 to +79; *dl*714, deletion of 22 bp from +90 to +113; *dl*715, deletion of 15 bp from +92 to +108; *dl*716, 17-bp deletion from +107 to +125; *dl-sub*717, deletion of 23 bp from +128 to +152; and *dl*718, deletion of 12 bp from +147 to +160 (nucleotides preceding and following the deletion are numbered). The mutant *dl-sub*717 also has 2 bp substituted at +126 and +127. This substitution probably occurred during construction and propagation in *E. coli*. Mutant *dl*711 is a deletion mutant which lost 53 bp from +62 to +116. The mutant VAI gene in this case was reintroduced into a *dl*703 variant which transcribed VAII RNA in normal amounts (5). Mutant *dl*721 is a deletion mutant in which a DNA segment from +115 of VAI gene to +61 of VAII gene is deleted (see above). As a result, this mutant synthesizes a hybrid RNA with nucleotide sequences from VAI and VAII gene. This mutant is of *dl*704 background.

Synthesis of altered VAI RNAs in infected cells. To analyze the RNAs transcribed from the mutant VAI genes, ^{32}P-labeled cytoplasmic RNAs of the mutant-infected cells were analyzed on 8% denaturing polyacrylamide gels (see above). An autoradiogram showing the VAI RNAs synthesized by these mutants is presented in Fig. 3. The VAI RNAs

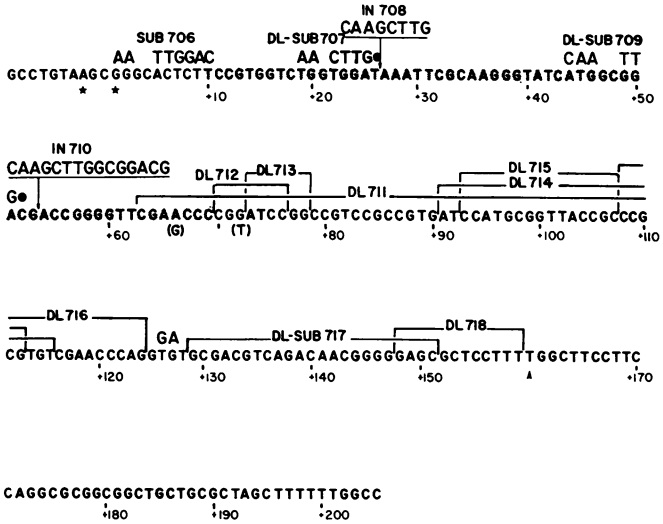

FIG. 2. Nucleotide sequences of the Ad2 genome coding for the VAI gene and the location of the deletion, insertion, and substitution mutations. The single-stranded sequence shown here reads as the sense strand for the VAI RNA. The nucleotide sequences shown in the shaded region are the intragenic promoter of the VAI gene. The nucleotide sequences shown in bold letters above the genome DNA sequence substitute for the corresponding nucleotides in the genome DNA sequence in the respective mutants. The nucleotide sequences shown above the genome DNA sequence and which are underscored are insertion mutations. Nucleotides shown in parentheses below the genome DNA sequence are the differences found in Ad5 DNA sequence. Symbols: ★, initiation points for the VAI gene; ▲, termination; and ●, deletion.

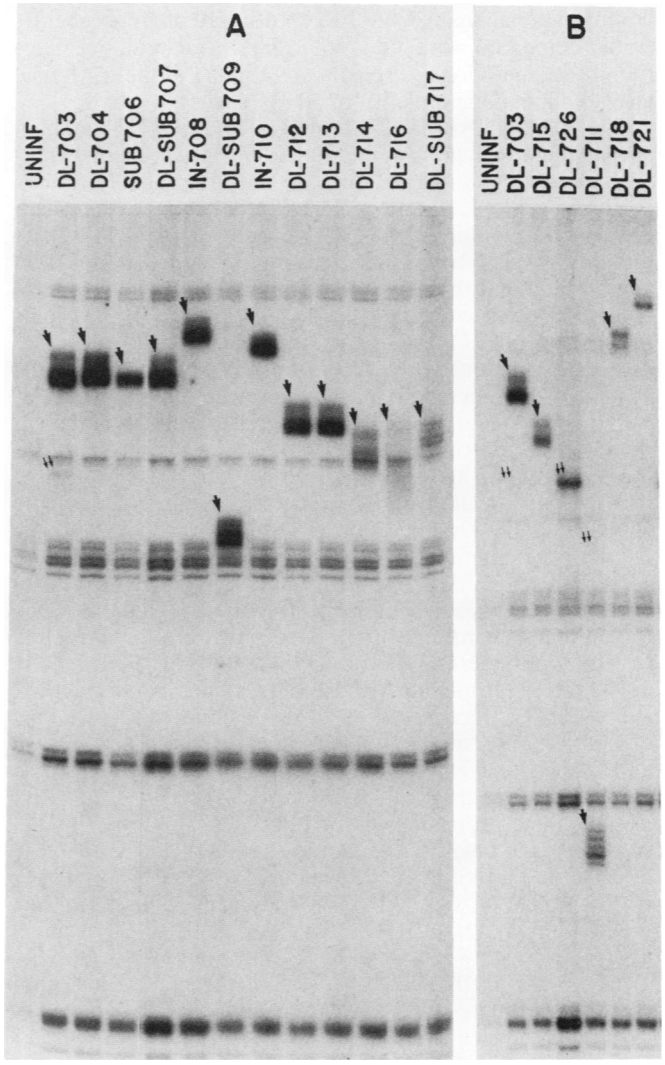

FIG. 3. Analysis of low-molecular-weight RNAs synthesized by *dl*703, *dl*704, and various Ad5 mutants with VAI-specific mutations. ^{32}P-labeled total cytoplasmic RNAs isolated from mutant-infected cells were electrophoresed on an 8% polyacrylamide–8 M urea gel (0.4 mm thick; 600 V in a Tris-borate buffer) and autoradiographed. (A) and (B) were results obtained from separate experiments. The single and double arrows show the positions of VAI or VAII RNAs, respectively. The top 4 cm and the bottom 6 cm of the autoradiograms are not shown.

synthesized by *dl*704 or *dl*703 are comparable to that of WT (5). Three of the mutants, *sub*706, *dl-sub*707, and *dl-sub*709, have their VAI RNAs identical or almost identical in size to that of *dl*704 (Fig. 3A; *dl-sub*707 and *dl-sub*709 have lost only a single nucleotide). Initiation of VAI RNA in the case of *sub*706 appears to be affected. Analysis of the 5′ end of this RNA (25) showed that the initiation occurred with two closely located adenine residues (results not shown). Although the VAI RNA of *dl-sub*709 is comparable in size to WT VAI RNA, it migrates much faster in the gel (Fig. 3A). We believe that this is due to a conformational change in the molecule. A T_1 oligonucleotide map shows all of the predicted T_1 spots corresponding to the nucleotide sequence of this RNA (see below). Mutants *in*708 and *in*710 have 8 and 15 bp insertions, respectively, in their VAI genes. As a result, the VAI RNAs of these mutants migrate at a slower rate compared with that of WT. Again, probably owing to

conformational change, VAI RNA of *in*710 migrates slightly faster compared with that of *in*708. In general, we have found that mutations in region +40 to +53 of the VAI gene lead to anomalous mobility of RNA in gels.

The VAI RNAs of *dl*712, *dl*713, *dl*714, *dl*716, and *dl-sub*717 migrate in gels in accordance with their size, whereas *dl*715 VAI RNAs migrate slightly slower, probably for the aforesaid reason. Mutant *dl*711 has a deletion of 53 nucleotides in the 3′ half of the VAI gene (+62 to +116). The mobility of this RNA is commensurate with its length, and it can be seen that this mutant synthesizes normal amounts of VAII RNAs as compared with *dl*703 (Fig. 3B). *dl*711 has VAII RNA of Ad2 origin. Ad5 and Ad2 VAII RNAs (*dl*703 and *dl*711, respectively) migrated differently in our gel system. Also included in this gel was *dl*726, which is $VAI^-/VAII^+$ (Fig. 3B). This mutant has a deletion of 7 bp in the B-block control sequence of the intragenic promoter of the VAI gene. This mutation prevents the transcription of the VAI gene but leads to enhanced transcription of the VAII gene (5a, 12, 29).

Two other mutants whose VAI RNA became larger are *dl*718 and *dl*721. Mutant *dl*718 has a 12-bp deletion, from +147 to +160. Four or more consecutive adenine residues on the template strand form a part of the termination signal for RNA polymerase III (6). Transcription of VAI gene is presumed to terminate with uracil residues between +157 and +160 (7). Because of the 12-bp deletion which overlaps this termination signal, transcription of the VAI gene in *dl*718 proceeds downstream and presumably terminates with another uracil-rich segment between +195 and +201 (Fig. 2). Our fingerprint analysis of this RNA supports this assumption (see below). The size of the VAI RNA in the case of *dl*718 is, therefore, ca. 188 nucleotides, and the behavior of this RNA on our gel is in agreement with the above prediction (Fig. 3B). The mutant VAI RNA of *dl*721 has a hybrid gene between VAI and VAII (described earlier). Transcription in this case is initiated from a VAI initiation site and terminates with a VAII-specific termination signal. The predicted size of the transcript for this gene is about 217 nucleotides, which corresponds to its mobility on the gel (Fig. 3B).

T_1 oligonucleotide map analysis of the mutant VAI RNAs. We have further confirmed the mutational alterations of the VAI transcripts by subjecting the ^{32}P-labeled, gel-purified VAI RNAs to two dimensional fingerprint analysis. Figure 4A to K shows the T_1 map of the WT and mutant VAI RNAs. Nucleotide sequence spots 1 through 9 have been identified earlier (4; see legend to Fig. 4 for details). The T_1 spot corresponding to sequence 4 to 13 is missing from the T_1 map of *sub*706, in agreement with the mutation (Fig. 4B). Similarly, spot 2 of the T_1 map of *dl-sub*707 is missing, and in its place, a new spot (spot X) appears (Fig. 4C). The position of this spot in the map is consistent with the sequence AAAUUCG which is a result of the mutation. A new T_1 spot also appears in the place of spot 3 in the T_1 map specific to *dl-sub*709 (Fig. 4D), because of the substitution of sequences between +43 and +53 in the VAI gene of this mutant. The position of the new spot in this map is consistent with the nucleotide sequence UAUCCAAG.

The T_1 maps of the VAI RNAs of *dl*714, *dl*716, and *dl-sub*717 shown in Fig. 4E, F, and G, respectively, are in complete agreement with the mutations (*dl*714, deletion from +90 to +113, missing T_1 spots 4 and 5; *dl*716, deletion from +107 to +125, missing T_1 spot 7; *dl-sub*717, deletion from +128 to +152, missing T_1 spot 8). The mutant *dl*718 has a 12-bp deletion from +147 to +160. As stated earlier, transcription of the VAI gene in this case presumably terminates downstream, between +195 and +202. Nucleotide sequences downstream from +160 to +201 should generate an 11-nucleotide-long T_1 product with a sequence CUUCCUUCCAG if the transcription terminates between +195 and +202. The T_1 map of this mutant RNA shows a large new T_1 product whose position is consistent with the sequence described above (Fig. 4H). Figure 4I shows the T_1 map of the transcript synthesized from the hybrid gene 5′ VAI/3′ VAII (*dl*721). T_1 spots 7 and 8 of VAI RNA are missing in the T_1 map of the VAI RNA transcribed by *dl*721, in agreement with the deletion of VAI sequences from +115 to +160. Instead, this T_1 map shows four new large T_1 spots corresponding to spots 12, 13, 14, and 15 of VAII RNA (Fig. 4J). The nucleotide sequences of the spots 12, 13, 14, and 15 of Ad2 VAII RNA are likely to correspond to nucleotides +128 to +140, +141 to +146, +118 to +123, and +101 to +108, respectively, which are derived from the nucleotide sequences of the 3′ half of the VAII RNA. Figure 4K shows the T_1 map of the VAI RNA of mutant *dl*711 which has a deletion of 53 bp from +62 to +116. Accordingly, T_1 spots 4, 5, and 6 are missing from this map. The results presented above uniformly confirm the mutational alterations present in the VAI genes of the mutant viruses that are described.

Growth of viruses reconstructed with VAI mutations. To determine how mutations on the VAI gene affected biological functions, each mutant was assayed for ability to grow on HeLa cells. Comparison of the yield of the mutant virus with that of its parent (*dl*704) indicates the level at which the mutant VAI RNA is able to function in the infected cells. Growth kinetics of these mutants are shown in Table 1 and Fig. 5. Mutant viruses with substitution and insertion mutations in the 5′ half of the VAI gene grew as well or nearly as well as did their parent (*sub*706 yielded a titer which was two-fifths of that of *dl*704, whereas growth of *dl-sub*707, *in*708, and *in*710 was comparable to that of *dl*704 [Table 1]). The only mutant which showed considerable loss of growth is *dl-sub*709 which yielded a titer which was one-ninth that of *dl*704 at the end of day 4 (Fig. 5A; the different VAI mutations and the yield of the mutant viruses are also summarized in Fig. 7). The above results suggest that long nucleotide sequences at the 5′ half of the molecule may not interact with viral or cellular components during translation. If such an interaction existed, these substitution and insertion mutations should have prevented it.

Deletion mutations in the 3′ half of the gene had a varied effect on its function. Three of the deletion mutants, *dl*712, *dl*713, and *dl*715, grew to the level of their parent *dl*704 with identical kinetics (Table 1). Growth was affected considerably when deletion mutations were introduced into the region between +107 and the 3′ end of the molecule. *dl*716, for example, which has a 17-bp deletion between +107 and +125, grew more slowly, yielding a titer which was one-seventh that of *dl*704, and *dl-sub*717 yielded a titer which was one-ninth that of *dl*704 (Fig. 5B and see Fig. 7). Mutant *dl*718, which synthesized larger VAI RNAs, grew more slowly with a titer 1/12 of that of *dl*704. Mutant *dl*721, which carried a hybrid gene between VAI and VAII, grew much more slowly and gave a titer 1/20 of that of *dl*704 (Fig. 5C and 7).

Mutant *dl*711, which has a 53-bp deletion in the VAI gene (described above), yielded a titer about one-half that of *dl*703. This finding indicates that the mutant VAI gene of *dl*711 is biologically functional. Because this mutant has a functional VAII gene, it was necessary to examine the extent to which normal levels of VAII RNA contributed to

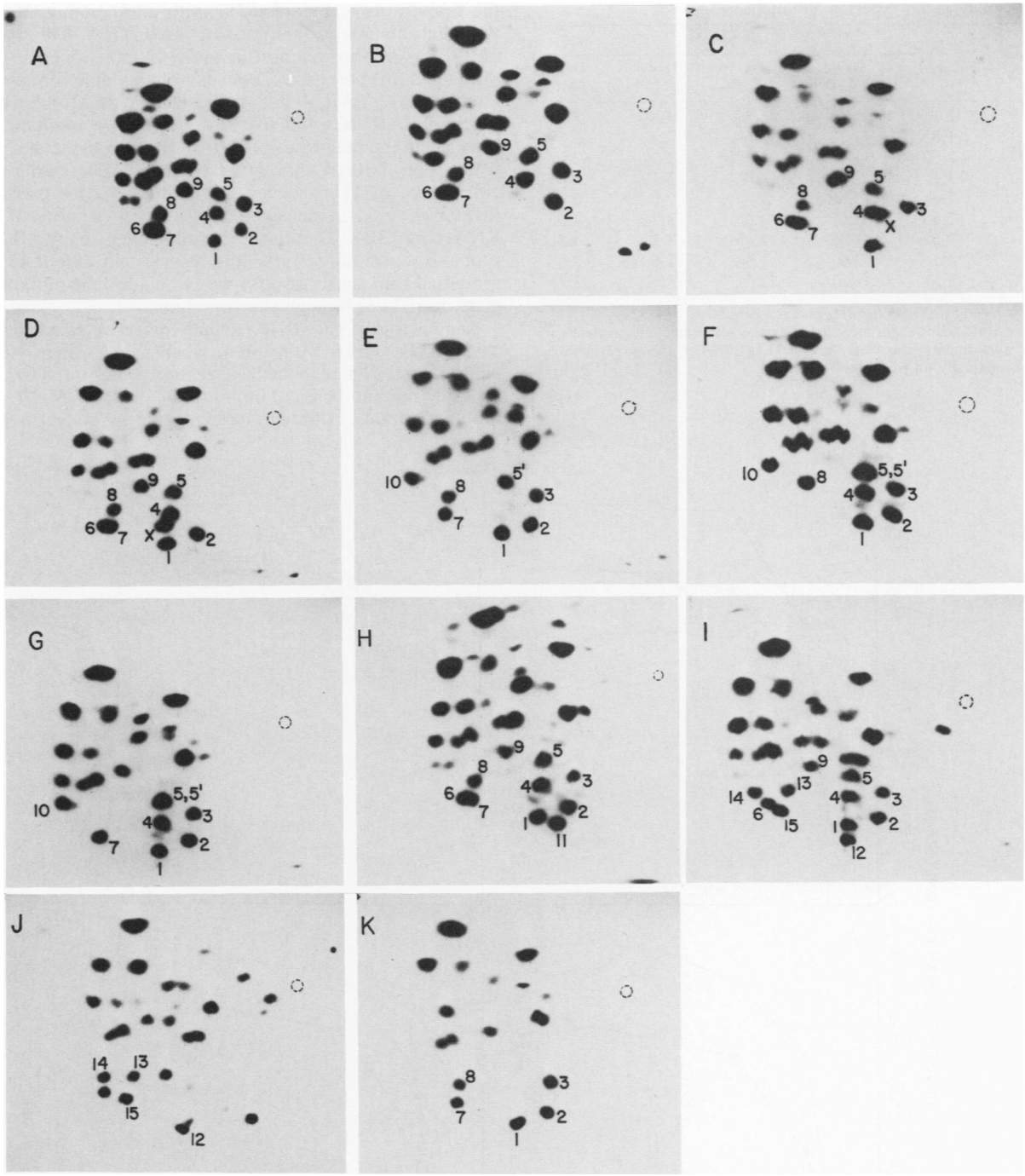

FIG. 4. T_1 oligonucleotide map of the VAI RNAs synthesized by the Ad5 mutants. Approximately 10,000 to 20,000 cpm of the gel-purified RNA was digested with RNase T_1 and subjected to electrophoresis for the first dimension (from left to right) and homochromatography for the second dimension (from bottom to top). The nucleotide sequences of the spots 1 through 9 are as follows: spot 1, nucleotides 4 to 13; spot 2, nucleotides 25 to 33; spot 3, nucleotides 40 to 46; spot 4, nucleotides 91 to 97; spot 5, nucleotides 101 to 106; spot 6, nucleotides 65 to 71; spot 7, nucleotides 118 to 124; spot 8, nucleotides 139 to 144, and spot 9, nucleotides 73 to 77. The mutants described in these experiments have either Ad2 or Ad5 VAI genes. Because there are two differences in the nucleotide sequences between Ad2 and Ad5 VAI genes (residue +66 is adenine in Ad2 and guanine in Ad5, whereas residue +72 is guanine in Ad2 and thymine in Ad5), spots 6 and 9 of Ad2 VAI RNA do not appear in Ad5 VAI RNA. Instead, spot 9 migrates with spot 5 and appears in 2 M quantities designated 5 and 5′ (UUACCG, nucleotides 101 to 106 and UAUCCG, nucleotides 72 to 77, respectively). The nucleotide sequence of spot 6 of the Ad2 map is changed from AACCCCG to CCCCG in the Ad5 T_1 map. Therefore, a new spot (spot 10) appears in the Ad5 T_1 map. The T_1 map of WT VAI RNA shown in (A) is of Ad2 origin. Mutants *sub*706, *dl-sub*707, *dl-sub*709, *dl*718, and *dl*721 have Ad2 VAI genes, whereas mutants *dl*714, *dl*716, and *dl-sub*717 have Ad5 VAI genes. ○, Position of the orange dye; the green dye reached to the top of the PEI-cellulose plate. (A) WT, (B) *dl*706, (C) *dl-sub*707, (D) *dl-sub*709, (E) *dl*714, (F) *dl*716, (G) *dl-sub*717, (H) *dl*718, (I) *dl*721, (J) Ad5 VAII RNA, and (K) *dl*711. T_1 maps for the remaining mutants are not shown.

TABLE 1. Growth kinetics of adenovirus mutants with VAI-specific mutations[a]

Virus	Yield of virus at:				
	Day 1 ($\times 10^6$)	Day 2 ($\times 10^8$)	Day 3 ($\times 10^8$)	Day 4 ($\times 10^8$)	Day 5 ($\times 10^8$)
*dl*704	2.1	1.49	3.8	3.8	3.6
*sub*706	1.85	1.1	1.67	1.4	1.57
*dl-sub*707	2.62	1.45	2.5	2.2	2.62
*in*708	3.1	1.6	3.0	3.19	3.49
*in*710	1.84	1.68	1.8	2.0	2.35
*dl*712	2.85	1.07	3.65	3.72	4.52
*dl*713	3.05	1.52	3.85	3.72	4.52
*dl*715[b]	4.5	2.0	1.9	1.8	2.0
(*dl*704)[b]	(3.5)	(1.6)	(2.5)	(3.5)	(3.2)

[a] See the legend to Fig. 5 for experimental details.

[b] The growth kinetics experiment for *dl*715 was performed independently of the other experiments; therefore, the yield of *dl*704 in this experiment is expressed separately (in parentheses).

the growth of this mutant. To clarify this point, we compared the growth properties of *dl*711 with another mutant which produced abundant quantities of VAII RNA. This mutant, *dl*726, has a 7-bp deletion in the B-block sequence of the intragenic promoter (5a). Because of this deletion, the mutant is $VAI^-/VAII^+$ and synthesized a ca. 16-fold-higher level of VAII than did *dl*703. Even with a compensatory increase in the quantities of VAII RNA, *dl*726 yielded a titer which was 1/10 of that of *dl*703 (Fig. 5D). Under these conditions, *dl*711 yielded a sixfold-higher titer than *dl*726 (Fig. 5D). *dl*711 synthesized only a small quantity of VAII RNA (Fig. 3B). Therefore, normal levels of VAII RNA probably contributed only to a very small extent in *dl*711 growth. From these results, we conclude that mutant VAI gene of *dl*711 is biologically functional.

Comparison of growth of partially defective mutants with a $VAI^-/VAII^-$ virus. By using a recombinant simian virus 40 containing a VAI gene we have recently attempted to isolate an Ad5 mutant lacking functional VAI and VAII genes (double mutant). Although earlier experiments showed that

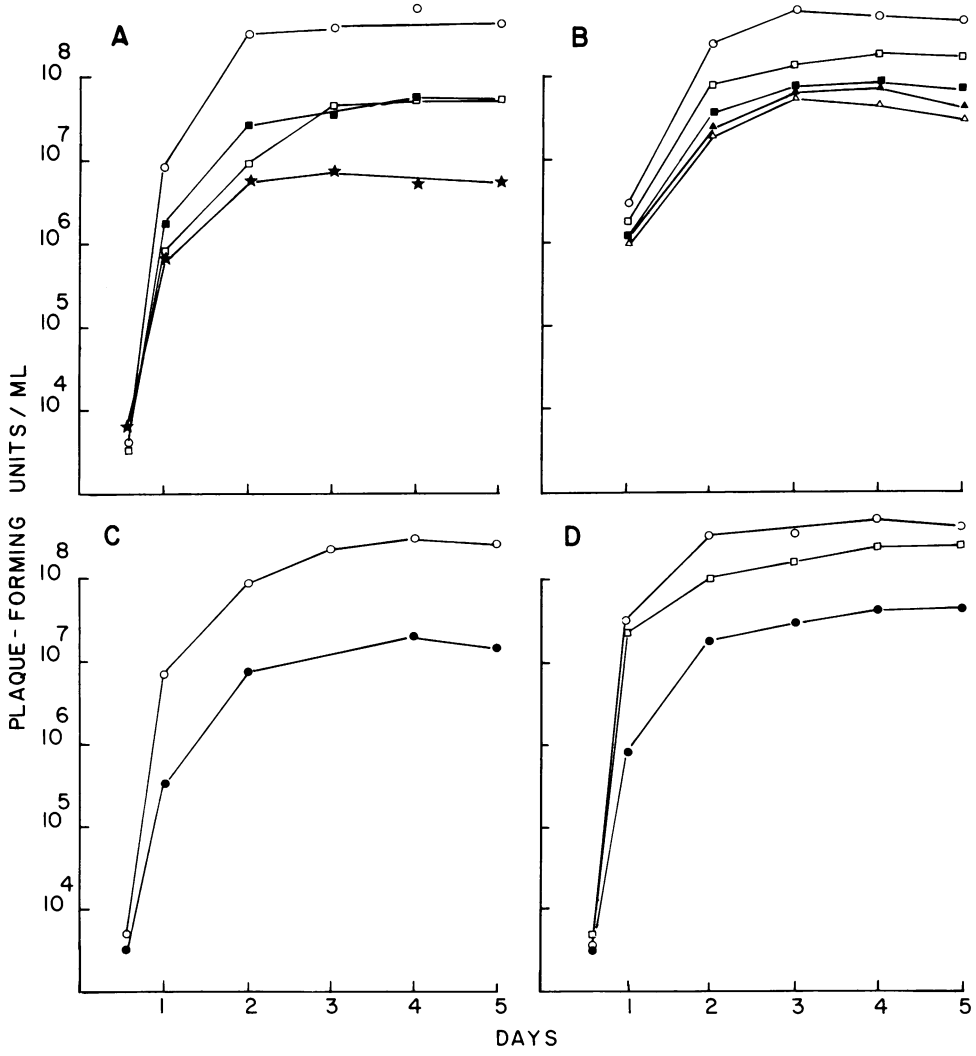

FIG. 5. Growth kinetics of mutant viruses. To monitor growth kinetics, HeLa cells were infected with various mutants at a multiplicity of 3 PFU per cell. After a 1-h incubation, the dishes were washed three times with Tris-saline and refed with DME containing 5% calf serum. At the indicated times, the virus yield was measured by plaque assay on HeLa cells. Symbols: (A) ○, *dl*704; □, *dl-sub*709; ■, *dl-sub*717; ★, double mutant; (B) ○, *dl*704; □, *dl*714; △, *dl*716; ■, *dl-sub*717; △, *dl*718; (C) ○, *dl*704; ●, *dl*721; (D) ○, *dl*703; □, *dl*711; ●, *dl*726.

the double mutant is nonviable (5), extensive experiments in the laboratory of T. Shenk and in our laboratory show that the double mutant is viable but grows very poorly, with a titer 1/60 of that of the WT control (see below). The construction of this mutant was described above. This mutant, *dl-sub*720, has the DNA sequences coding for VAI RNA of *dl*704 replaced by an Ad2 DNA segment from 88.3 to 89.7 m.u. This mutant is $VAI^-/VAII^-$ and grew very poorly, yielding a titer which was 1/60 to 1/80 of that of *dl*704. Because the double mutant was found to be weakly viable, it was necessary to determine whether the mutations in the VAI genes of the partially defective Ad5 mutants abolished their functions. We compared the growth kinetics of *dl-sub*709 and *dl-sub*717 with the growth properties of the double mutant (Fig. 5A). As can be seen, *dl-sub*709 and *dl-sub*717 yielded a ca. ninefold-higher titer when compared with that of the double mutant. These results suggest that these two mutants retain a portion of their activity. Our growth kinetics experiments, taken together, suggest that none of the mutations introduced into the gene abolish biological function. Mutations in two regions of the molecule (+43 to +53 and +107 to the 3′ end) resulted in partial loss of activity, whereas the mutations in the remaining parts of the molecule led to little or no loss in biological function.

The growth rate of the double mutant is somewhat anomalous for a virus which yields a titer which was 1/60 (day 3) to 1/80 (day 5) (Fig. 5A) of that of *dl*704, our WT control. Instead of a gradual increase in the titer value, at the 24-h point, *dl-sub*720 showed a titer only 1/15 that of *dl*704. The virus reached a maximum titer at day 3. We repeated this experiment twice with independently grown virus stocks and obtained identical results. We do not know the reasons for this observation at present.

Measurement of VAI RNAs found in the cytoplasm of infected cells. In our transcription analysis experiments, we have observed reduced quantities of VAI RNAs in the cytoplasm of the HeLa cells infected with some of the mutants (Fig. 3). In the WT-infected cells, large quantities of VAI RNA are present in the cytoplasm, suggesting a possible relationship between the quantity of this RNA and its function. It was therefore possible that the slow growth observed for some of the mutants was due not to the inability of the mutant VAI RNAs to function in the cytoplasm during translation but to interference in some steps before cytoplasmic accumulation, leading to reduced quantities of VAI RNAs in the cytoplasm. These steps, for example, may be transcription termination, processing, or transport from nucleus to cytoplasm.

Therefore, the VAI RNA present in the cytoplasm of the mutant-infected cells at 15 h postinfection was quantitated as described above, and it was compared with the yield of the virus obtained during the growth kinetics experiment. The relative yield of the virus particles for some of the mutants and the relative amounts of the cytoplasmic VAI RNAs produced by the infected cells are shown in Table 2. There is no apparent correlation between the growth yields of the various mutants and ability to accumulate cytoplasmic VAI RNAs. For example, *dl-sub*709 grows 10-fold more slowly than WT virus, yet the VAI RNA produced by this mutant was present at 60% of the WT level. A similar situation was observed for *dl*716 and *dl-sub*717. A 40% decrease in cytoplasmic VAI RNA cannot explain low growth yield because *dl*715 and *sub*706 show less cytoplasmic VAI RNA, yet grow nearly as well as WT virus. Thus, it seems that nucleotide sequences between +43 and +53 and between +107 and the 3′ end play a more important role in VAI function than the nucleotides present in the remaining part of the molecule. The situation in the case of *dl*718 and *dl*721 is less clear. VAI RNA of these mutants was present in much-reduced quantities in the cytoplasm at 15 h after infection (Table 2). Perhaps, in these two cases, the reduced growth rate was due to a combination of the functional inability of the mutant VAI RNAs during translation and to the much-reduced quantities of these RNAs present in the cytoplasm.

Secondary structures of mutant VAI RNAs. VA RNAs show extensive base-paired stem-loop structures with nucleotide sequences at the 5′ end base-pairing with the nucleotide sequences at the 3′ end (1). Experimental evidence for the occurrence of such a hairpin structure in solution has been presented (18), suggesting that secondary structure may play an important role in biological function. It was therefore important for us to know whether the mutations that we introduced into the VAI RNA also disrupted their secondary structures. Computer-generated secondary structures of mutant VAI RNAs of *dl*711, *dl*716, *dl-sub*717, *dl*718, and *dl*721 are presented in Fig. 6. As can be seen, mutant VAI RNAs of all of these mutants can fold to give rise to stem-loop structures resembling the native VAI RNA. Only in *dl*718 is the stem structure disrupted with a bulge near the 3′-end uracil residues. In general, these results suggest that the partial loss of function is not related to loss of secondary structure.

DISCUSSION

We have constructed a series of adenovirus mutants with VAI-specific mutations which cover the entire VAI RNA gene, and we have shown that the majority of these mutants retained complete or nearly complete biological activity. The growth of mutants *dl-sub*709, *dl*716, and *dl-sub*717 was reduced, although they did grow significantly better than the double mutant. Figure 7 summarizes the mutation locations and relative growth of the mutants compared with that of *dl*704, our WT control. The importance of VAI RNA to the adenovirus growth cycle is demonstrated by the double mutant whose titer is only 1/60 of that of WT.

Our results can be interpreted in two ways. First, because activity may depend only on hairpin structure, mutants may show activity since most of these mutants can fold to form a hairpin structure. This reason is not likely because, although VAII RNA and Epstein-Barr virus RNAs also show a hairpin structure similar to that of VAI RNA (1, 5), Ad5

TABLE 2. Relative yield of the virus particles and the relative amounts of the cytoplasmic VAI RNAs produced by some of the mutants in infected cells[a]

Virus	Relative yield of virus mutant per *dl*704	Relative amounts of VAI RNA mutant per *dl*704
*dl*704	1.0	1.0
*sub*706	0.42 ± 0.04	0.59 ± 0.05
*dl-sub*709	0.1 ± 0.03	0.59 ± 0.16
*in*710	0.61 ± 0.1	0.64 ± 0.16
*dl*711	0.52 ± 0.03	0.64 ± 0.08
*dl*714	0.41 ± 0.08	0.71 ± 0.23
*dl*715	0.64 ± 0.11	0.39 ± 0.05
*dl*716	0.11 ± 0.03	0.56 ± 0.16
*dl-sub*717	0.14 ± 0.03	0.45 ± 0.14
*dl*718	0.08 ± 0.02	0.26 ± 0.04
*dl*721	0.06 ± 0.02	0.18 ± 0.01

[a] The experiments were repeated three times, and average values and error bars are presented. VAI RNA was quantitated as described in the text.

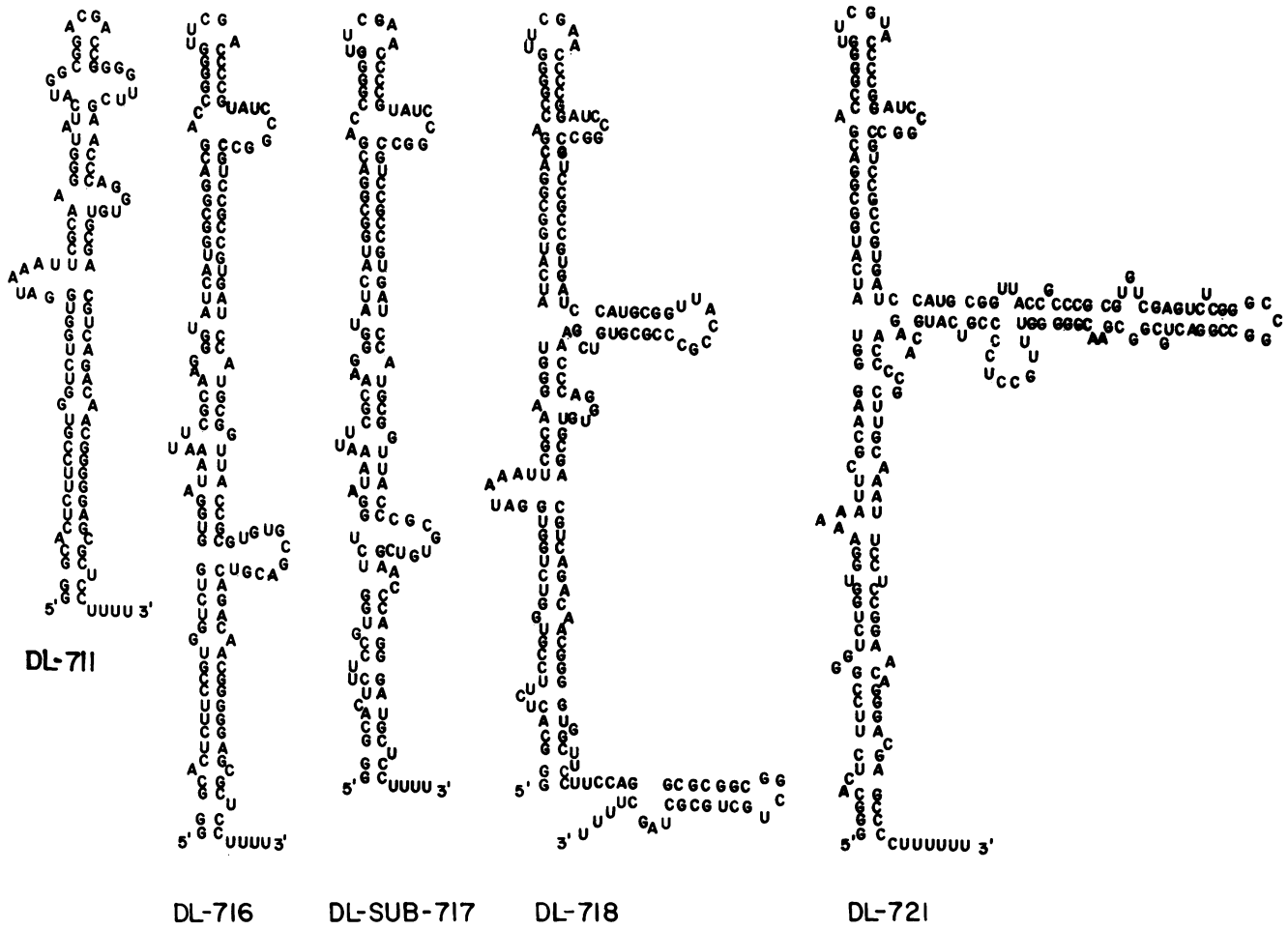

FIG. 6. Secondary structures of mutant VAI RNAs. The structures of VAI RNAs were derived by using computer analysis, with a program described by Zuker and Stiegler (32). The secondary structure of VAI RNA of *dl-sub*709 (not shown) is very similar to that of WT VAI RNA.

mutants which synthesize abundant quantities of these RNAs do not grow to WT levels (5, 5a, 26).

An alternative and more attractive interpretation is that the VAI RNA has multiple functional sequences which may be short nucleotides of 4 to 6 bases. We suggest that these are quite short nucleotide sequences, because all long nucleotide sequences can be interrupted by deletion, substitution, and insertion mutations without a major loss of function. If these multiple sites were distributed throughout the entire molecule, then deletion of some of them would not lead to a loss of function because other active sequences would remain.

The specific function of VAI RNA is not yet entirely worked out. It is thought that the VAI RNA serves as a link between the translation initiation complex and viral mRNAs, because in the VAI$^-$ mutant-infected cells, conversion of 43S preinitiation complex to 48S preinitiation complex is blocked (21). In addition, short nucleotide sequences complementary to VAI RNA are found in tripartite leader segments of the mRNAs encoded by the major late transcription unit and also in the 5′ untranslated regions of mRNAs coding for some of the early viral mRNAs (our unpublished data). Tripartite leader segments have been shown to enhance translation (3, 15, 27): VA RNAs have been shown to bind to viral mRNAs (16). Therefore, it is possible that multiple RNA-RNA or RNA-protein interactions or both among VAI RNA, the 5′ untranslated segments of the viral messages, and the translation apparatus of the host cell result in efficient translation. These multiple interactions could be mediated by short nucleotide sequences of VAI RNA. We have also shown that the VAI mutations between +43 and +53 or between +107 and the 3′ end of the gene lead to considerable loss of activity. Perhaps these sequences in particular may play a more important role in the VAI interaction with cellular or viral components or both.

Nucleotide sequences from the two control elements (+10 to +18 and +53 to +69) and nucleotides +26 to +44 are unchanged in the group of mutants described in this report. However, an Ad5 mutant with only the 5′ half of the gene did not grow significantly better than the double mutant, suggesting that these sequences alone are not sufficient for the function (our unpublished data).

Our observation that the deletion mutations in the 3′ half of the VAI RNA (*dl*715, *dl*716, *dl-sub*717 and *dl*718) led to a reduced accumulation of mutant RNAs in the cytoplasm is intriguing. Some or all of the VA RNAs have been shown to be present as ribonucleoprotein particles complexed with the La antigen (9, 13, 14), and this protein binds to terminal uracil residues (24) and probably to several nucleotides near the termini of VAI RNA (9). If La antigen binds to VAI RNA

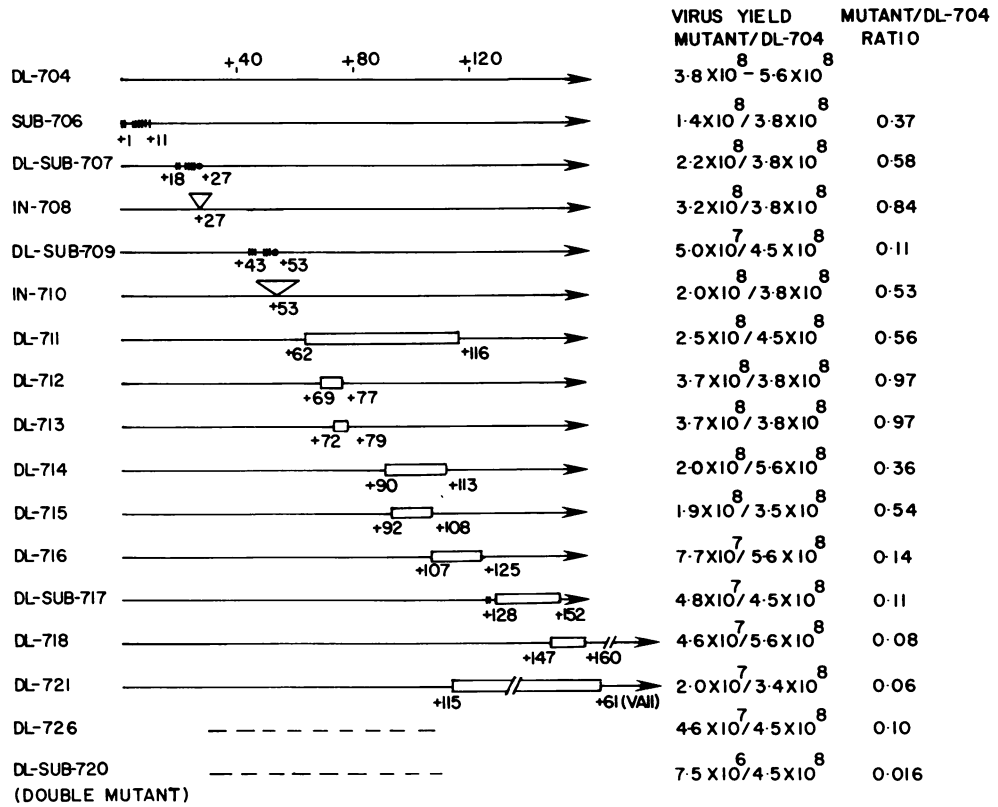

FIG. 7. Schematic representation of the mutant VAI RNAs of the various Ad5 mutants described in the text, yield of the mutant virus particles of day 4 of the growth kinetics experiment, and the ratio of the yield of mutant virus to that of *dl*704. All the mutant viruses were not assayed at the same time, so the range of the titer of *dl*704 is given. The yield of *dl*711 was compared with that of *dl*703. For the double mutant, the titer on day 3 was found to be maximum. This value was compared with the titer of *dl*704 on day 5. Symbols: horizontal lines with arrows, VAI RNAs; vertical bars, substitutions; solid circles, single base deletions; triangles, insertions; open boxes, deletions; broken lines for *dl*726 and the double mutant, the VAI gene was not transcribed.

in a sequence-specific manner and if this protein has any role in transcription or transport of VA RNAs from the nucleus to cytoplasm, inefficient binding of this protein to mutant RNAs would explain the reduced accumulation of VA RNAs in the cytoplasm.

The mutant *sub*706 has nucleotides from +1 to +10 substituted. Transcription in this case is initiated with two consecutive adenine residues at +1 and +2, although entire 5′-flanking sequences of this mutant gene are intact. Although the preference for purine as the initiating base is conserved, we have not detected initiation from the upstream guanosine residues (our unpublished data). Quantitatively, VAI RNA of *sub*706 is severalfold more abundant than the WT VAI-A species. Mutation in *sub*706 also overlaps with an 8-nucleotide-long sequence (+6 to +13) of the VAI RNA which was shown to be complementary to the 3′ end of the 18S eucaryotic rRNA (18). Since *sub*706 grows at about WT levels, these sequences may not be critical to VAI function.

Mutant *dl*718 synthesizes a ca. 188-nucleotide-long transcript as result of a 12-bp deletion overlapping the normal transcription termination signal located between +157 and +160. Four or more adenine residues in the template strand form a part of the polymerase III transcription termination signal (6). Transcription of VAI gene of *dl*718 terminates very likely between +195 and +201, because of six consecutive adenine residues on the template strand in this region. A 200-nucleotide-long transcript of the WT VAI gene has been observed both in vivo and in vitro (28, 30), presumably owing to a readthrough of the normal termination signal. It seems that *dl*718 is able to utilize the second terminator efficiently. It is possible that, in the WT situation, the second terminator serves as a safety mechanism.

The level at which the mutants *dl*718 and *dl*721 can function in the infected cells is somewhat difficult to determine. These mutants grow to titers 1/10 and 1/17 that of *dl*704 and have reduced levels of cytoplasmic VAI RNA. If cytoplasmic VAI RNA is limiting, these mutant VAI RNAs may not be as defective as the growth titer indicates. However, at present, such an estimation is not possible because we do not know whether the VAI RNA is overproduced, nor can we yet calculate the optimum quantity of VAI RNA required for efficient translation.

The present group of Ad5 mutants provides us with an opportunity to dissect the function of VAI RNA at the nucleotide sequence level. It will be interesting to know whether there is a quantitative difference in the synthesis of the various viral polypeptides in cells infected with these mutants and whether these mutant RNAs interact differently with the translation apparatus of the host cell. We are pursuing these aspects at present.

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LITERATURE CITED

1. **Akusjarvi, G., M. B. Mathews, P. Andersson, B. Vennstrom, and U. Pettersson.** 1980. Structure of genes for virus-associated RNA_I and RNA_{II} of adenovirus type 2. Proc. Natl. Acad. Sci. U.S.A. **77**:2424–2428.
2. **Barrell, B. G.** 1971. Fractionation and sequence analysis of radioative nucleotides, p. 751–779. *In* G. L. Cantoni and D. R. Daries (ed.), Procedures in nucleic acid research, vol. 2. Harper & Row, Publishers, New York.
3. **Berkner, K. L., and P. A. Sharp.** 1984. Expression of dihydrofolate reductase, and the adjacent E1b region, in an Ad5-dihydrofolate reductase recombinant virus. Nucleic Acids Res. **12**:1925–1941.
4. **Bhat, R. A., B. Metz, and B. Thimmappaya.** 1983. Organization of the noncontiguous promoter components of adenovirus VAI RNA gene is strikingly similar to that of eucaryotic tRNA genes. Mol. Cell. Biol. **3**:1996–2005.
5. **Bhat, R. A., and B. Thimmappaya.** 1983. Two small RNAs encoded by Epstein-Barr virus can functionally substitute for the virus-associated RNAs in the lytic growth of adenovirus 5. Proc. Natl. Acad. Sci. U.S.A. **80**:4789–4793.

5a. **Bhat, R. A., and B. Thimmappaya.** 1984. Adenovirus mutants with DNA sequence perturbations in the intragenic promoter of VAI RNA gene allow the enhanced transcription of VAII RNA gene in HeLa cells. Nucleic Acids Res. **12**:7377–7388.

6. **Bogenhagen, D. F., and D. D. Brown.** 1981. Nucleotide sequences in xenopus 5S DNA required for transcription termination. Cell **24**:261–270.
7. **Celma, M. L., J. Pan, and S. M. Weissman.** 1977. Studies of low molecular weight RNA from cells infected with adenovirus 2. I. The sequence at the 3′ end of VA-RNAI. J. Biol. Chem. **252**:9032–9043.
8. **Fowlkes, D. M., and T. Shenk.** 1980. Transcriptional control regions of the adenovirus VAI RNA gene. Cell **22**:405–413.
9. **Francoeur, A. M., and M. B. Mathews.** 1982. Interaction between VA RNA and the lupus antigen La: formation of a ribonucleoprotein particle *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **79**:6772–6776.
10. **Graham, F. L., J. Smiley, W. C. Russel, and R. Narin.** 1977. Characteristic of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
11. **Green, C., and C. Tibbets.** 1980. Targeted deletions of sequences from closed circular DNA. Proc. Natl. Acad. Sci. U.S.A. **78**:3378–3382.
12. **Guilfoyle, R., and R. Weinmann.** 1981. Control region for adenovirus VA RNA transcription. Proc. Natl. Acad. Sci. U.S.A. **78**:3378–3382.
13. **Lerner, M. R., N. C. Andrews, G. Miller, and J. A. Steitz.** 1981. Two small RNAs encoded by Epstein-Barr virus and complexed with protein are precipitated by antibodies from patients with systemic lupus erythematosus. Proc. Natl. Acad. Sci. U.S.A. **78**:805–809.
14. **Lerner, M. R., J. A. Boyle, J. A. Hardin, and J. A. Steitz.** 1981. Two novel classes of small ribonucleoproteins detected by antibodies associated with lupus erythematosus. Science **211**:400–402.
15. **Logan, J., and T. Shenk.** 1984. Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection. Proc. Natl. Acad. Sci. U.S.A. **81**:3655–3659.
16. **Mathews, M. B.** 1980. Binding of adenovirus VA RNA to mRNA: a possible role in splicing. Nature (London) **285**:575–577.
17. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end labelled DNA with base specific chemical cleavages. Methods Enzymol. **65**:499–559.
18. **Monstein, H., and L. Philipson.** 1981. The conformation of adenovirus VAI RNA in solution. Nucleic Acids Res. **9**:4239–4250.
19. **Ohe, K., and S. M. Weissman.** 1971. The nucleotide sequences of a low molecular weight RNA from cells infected with adenovirus 2. J. Biol. Chem. **246**:6991–7009.
20. **Reich, P. R., B. Forget, J. Rose, and S. M. Weissman.** 1966. RNA of low molecular weight in KB cells infected with Ad2. J. Mol. Biol. **17**:428–438.
21. **Schneider, R. J., C. Weiberger, and T. Shenk.** 1984. Adenovirus VAI RNA facilitates the initiation of translation in virus-infected cells. Cell **37**:291–298.
22. **Shortle, D., D. Koshland, G. M. Weinstock, and D. Bostein.** 1980. Segment directed mutagenesis: construction *in vitro* of point mutations limited to a small predetermined region of a circular DNA molecule. Proc. Natl. Acad. Sci. U.S.A. **77**:5357–5379.
23. **Soderlund, H., U. Pettersson, B. Vennstrom, L. Philipson, and M. B. Mathews.** 1976. A new species of virus-coded low molecular weight RNA from cells infected with adenovirus type 2. Cell **7**:585–593.
24. **Stefano, J. E.** 1984. Purified lupus antigen La recognizes an oligouridylate stretch common to the 3′ terminii of RNA polymerase III transcripts. Cell **36**:145–154.
25. **Thimmappaya, B., N. Jones, and T. Shenk.** 1979. A mutation which alters initiation of transcription by RNA polymerase III on the Ad5 chromosome. Cell **18**:947–954.
26. **Thimmappaya, B., C. Weinberger, R. J. Schneider, and T. Shenk.** 1982. Adenovirus VAI RNA is required for efficient translation of viral mRNAs at late times after infection. Cell **31**:543–551.
27. **Thummel, C., R. Tjian, S. L. Hu, and T. Grodzicker.** 1983. Translational control of SV40 T antigen expressed from the adenovirus late promoter. Cell **33**:455–464.
28. **Weil, P. A., J. Segall, B. Harris, S. Y. Ng, and R. G. Roeder.** 1979. Faithful transcription of eukaryotic genes by RNA polymerase III in systems reconstituted with purified DNA templates. J. Biol. Chem. **254**:6163–6173.
29. **Weinberger, C., B. Thimmappaya, D. M. Fowlkes, and T. Shenk.** 1981. Regulation of adenovirus VA RNA gene expression. ICN-UCLA Symp. Mol. Cell. Biol. **23**:507–514.
30. **Weinmann, R., T. G. Brendler, H. J. Raskas, and R. G. Roeder.** 1976. Low molecular weight RNAs transcribed by RNA polymerase III during Ad2-infection. Cell **7**:557–566.
31. **Weinmann, R., H. J. Raskas, and R. G. Roeder.** 1974. Role of DNA dependent RNA polymerase II and III in transcription of the adenovirus genome late in productive infection. Proc. Natl. Acad. Sci. U.S.A. **71**:3426–3430.
32. **Zucker, M., and P. Stiegler.** 1981. Optimum computer folding of large RNA sequences using thermodynamics and auxiliary information. Nucleic Acids Res. **9**:133–148.