Functional Dissection of Adenovirus VAI RNA

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During the course of adenovirus infection, the VAI RNA protects the translation apparatus of host cells by preventing the activation of host double-stranded RNA-activated protein kinase, which phosphorylates and thereby inactivates the protein synthesis initiation factor eIF-2. In the absence of VAI RNA, protein synthesis is drastically inhibited at late times in infected cells. The experimentally derived secondary structure of VAI RNA consists of two extended base-paired regions, stems I and III, which are joined by a short base-paired region, stem II, at the center. Stems I and II are joined by a small loop, A, and stem III contains a hairpin loop, B. At the center of the molecule and at the 3' side, stems II and III are connected by a short stem-loop (stem IV and hairpin loop C). A fourth, minor loop, D, exists between stems II and IV. To determine sequences and domains critical for function within this VAI RNA structure, we have constructed adenovirus mutants with linker-scan substitution mutations in defined regions of the molecule. Cells infected with these mutants were analyzed for polypeptide synthesis, virus yield, and eIF-2 α kinase activity. Our results showed that disruption of base-paired regions in the distal parts of the longest stems, I and III, did not affect function, whereas mutations causing structural perturbations in the central part of the molecule containing stem II, the proximal part of stem III, and the central short stem-loop led to loss of function. Surprisingly, one substitution mutant, *sub742*, although dramatically perturbing the integrity of the structure of this central portion, showed a wild-type phenotype, suggesting that an RNA with an alternate secondary structure is functional. On the basis of sensitivity to single-strand-specific RNases, we can derive a novel secondary structure for the mutant RNA in which a portion of the sequences may fold to form a structure that resembles the central part of the wild-type molecule, which suggests that only the short stem-loop located in the center of the molecule and the adjoining base-paired regions may define the functional domain. These results also imply that only a portion of the VAI RNA structure may be recognized by the host factor(s).

One way cells defend against virus infection is by blocking the protein synthesis machinery that the virus needs to reproduce. This inhibition of protein synthesis results from the induction or activation of a double-stranded RNA (dsRNA)-activated kinase, eIF-2 α kinase, which phosphorylates and thereby inactivates the vital protein synthesis initiation factor eIF-2 (44). Viruses have evolved various strategies to counteract this cellular defense. The best studied is that used by adenoviruses. They encode two small RNAs designated virus-associated (VA) RNAs I and II (VAI and VAI RNAs), transcribed by RNA polymerase III (28, 34, 38, 40, 50, 57; reviewed in reference 46 and references therein). Both RNAs are about 160 nucleotides (nt) long, and the VAI species constitutes the major portion of the viral RNAs at late times, reaching a concentration of 10^7 molecules per cell. During adenovirus infection, the VAI RNA blocks activation of the eIF-2 α kinase produced by the cell, thereby enabling protein synthesis to proceed (35, 41, 43, 48). In cells infected with a virus deficient in VAI RNA (*dl331*), initiation of translation is dramatically reduced late in infection (45, 55). During polypeptide chain initiation, eIF-2 forms a ternary complex with GTP and tRNA^{Met}. In the subsequent step of the initiation pathway, the GTP

moiety bound to eIF-2 is hydrolyzed to GDP, which is then exchanged for GTP before the eIF-2 can function in a new round of initiation. This exchange reaction is carried out by another factor, termed eIF-2B. The eIF-2 α kinase acts by phosphorylating eIF-2, which then forms a tight complex with the limiting amounts of eIF-2B, preventing the recycling of eIF-2 and thereby reinitiation of protein synthesis (42). A translation stimulation effect of VAI RNA is also demonstrated in simple transfection assays in which the VAI RNA blocks the activation of the kinase (2, 51, 52).

VAI RNA itself exists in solution as a highly base-paired molecule with nucleotides of the 5' half base paired with nucleotides of the 3' half in an extended base-paired stem-loop structure with a short, branched stem-loop in the central region (31, 33). This model is only slightly different from computer-generated secondary-structure models (1, 58). Although there is little homology at the level of DNA sequence between VAI RNAs of avian (23), simian (24), and other subgroups of human adenoviruses (11, 29, 47), VA RNAs of all of these viruses can fold to form highly base-paired stem-loop structures and in some cases can complement for the adenovirus type 2 (Ad2) VAI gene function in transfection studies (23, 24). The minor VAI species encoded by Ad5, though not homologous to VAI in primary sequence (1, 50), is also capable of forming a highly base-paired stem-loop structure and can partially complement for the VAI function in virus infection studies (7). Two small polymerase III products encoded by the Epstein-Barr virus that also diverge in sequence from Ad5 VAI can

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nonetheless partially complement the VAI function in adenovirus-infected human cells (6, 8), although contradictory results have been reported (16).

The involvement of VAI RNA in translation control is direct because (i) the purified VAI RNA can block the activation of latent eIF-2 α kinase in vitro (20, 35), (ii) a mutant RNA that fails to function in vivo also fails to block the activation in vitro (20), and (iii) the VAI RNA can bind to the cellular kinase in vivo and in vitro (18). Blocking the activation of the kinase is most likely the major function of VAI RNA in adenovirus infection. Although recent data suggest that splicing of late mRNAs is altered in cells infected with VAI⁻ mutants (53), this is probably an indirect effect reflecting the fact that some host or late viral proteins required for growth are limiting in mutant infections.

The activity of eIF-2 α kinase can be stimulated in vitro by low concentrations of both natural and synthetic dsRNAs, but high concentrations inhibit its activity (3, 12, 15, 17, 32). In virus-infected cells, the kinase is activated at least in part by the dsRNA generated by symmetrical transcription of the viral genome (27). Because the VAI RNA can block the dsRNA-dependent activation of the kinase (20, 34), its extended base-paired structure has been considered important for its function. The VAI RNA does not mimic the dsRNA in activating the eIF-2 α kinase, probably because of imperfections in its base-paired structure.

A clear understanding of how the VAI RNA functions in vivo demands an understanding of the structural features and the sequence elements of the molecule that are important for its function. We have probed VAI RNA structure-function relationship by constructing 12 adenovirus mutants with linker-scan (LS) substitution mutations which span the entire length of the gene with the exception of the two intragenic promoter elements (6, 13, 14, 39). These mutants were then characterized with regard to polypeptide synthesis, growth yield, and eIF-2 α kinase activity. To determine the effect of mutations on the secondary structure of VAI RNA and to correlate features of secondary structure with ability to function in vivo, we analyzed the secondary structure of RNAs of several important mutants with RNases and evaluated the structural features of the VAI RNA molecule essential for function in virus-infected cells. Our studies show that the extended base-paired structures by themselves are not sufficient for function. Rather, it is the integrity of the central portion of the molecule, with its short stem-loop and the adjacent base-paired sequences, that is vital for function. This conclusion is supported by our finding that a mutant VAI RNA that is able to function efficiently despite a substantially altered secondary structure in the central portion of the molecule has a rearranged secondary structure that resembles this same central part of the native molecule.

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MATERIALS AND METHODS

Cells, viruses, and plasmids. Human 293 and monolayer HeLa cells were grown in Dulbecco modified minimal medium with 10% calf serum. Mutant *dl704* is a phenotypically wild-type (WT) variant of Ad5 and lacks a functional VAI RNA owing to a deletion within the intragenic promoter of VAI RNA gene. This mutant also contains an *EcoRI* site at 30.0 map units that is in the intergenic region of the two VA genes. Mutant *dl-sub720* is VAI⁻ VAI⁻, and *dl722* is VAI⁻

VAI⁺. Details of these variants have been published elsewhere (4, 7). Plasmid pA2-WT, described previously, contains the Ad2 VAI gene between *XbaI* (29.5 map units) and *EcoRI* (30.0 map units; formerly a *BalI* site) (6). Plasmid pA5-WT is identical to pA2-WT but contains the Ad5 VAI gene sequences. Plasmid m241-32 is an M13 clone containing Ad2 VAI sequences in which the T7 promoter was fused at the +1 position and the U-rich terminator sequence was extended into a stretch of 6 U residues followed by an *EcoRI* site (49). The VAI gene from this M13 plasmid was then transferred into the pA2-WT background by appropriate modifications (pA2-WT/T7). Mutations of *sub709*, *sub741*, and *sub742* (see below) were rebuilt into this plasmid by standard recombinant DNA methods (26).

Construction of adenovirus mutants with mutant VAI genes. Construction of adenovirus mutants *sub706*, *sub707*, *sub709*, *in708*, *in710*, *dl712*, *dl713*, *dl714*, *dl715*, and *dl717* has been described (4). Mutant *sub719* was constructed by filling a mutant in which sequences from +26 to +47 had been deleted with deletions terminating with a *HindIII* site and then inserting an 8-mer *BglII* linker. Mutants *sub741* and *sub742* were constructed as follows: 26-nucleotide-long oligonucleotide from +70 to +90 of the gene and its complementary sequence with mutations shown in *sub742* was synthesized and ligated to a pA2-WT derivative in which sequences between *BamHI* (+72) to *MboI* (+90) site were deleted. When the double-stranded oligonucleotide was cloned into this plasmid in two different orientations, mutations shown in *sub741* and *sub742* were generated. Mutations shown in *sub743*, *sub745*, *sub746*, *sub747*, *sub748*, and *sub749* were generated as LS mutations at the plasmid level as described previously (6) but with a *BamHI* site at the 5' side and an *EcoRI* site at the 3' side; these mutants were chosen for *Bal* 31 digestions. Appropriate matching deletion mutants with deletions terminating with *HindIII* sites were chosen to construct the LS mutants. The VAI mutants from the plasmid were then transferred into virus by a three-fragment ligation method as described elsewhere (7). Mutations in the LS mutants were confirmed by sequencing the DNA across the mutations.

Probing the structures of mutant VAI RNAs by structure-probing RNases. Human 293 cells were infected with adenovirus variants for 20 h, and total cytoplasmic RNAs were isolated. VAI RNAs were purified from these samples by subjecting the RNA to sucrose gradient centrifugation (15 to 30% sucrose, 41,000 rpm, 16 h in an SW41 rotor), followed by an 8% DNA-sequencing gel (54). About 0.5 μ g of the gel-purified VAI RNA was then dephosphorylated by incubation with calf intestine alkaline phosphatase for 30 min, and the dephosphorylated RNA was end labeled at the 5' end by T4 polynucleotide kinase and [γ -³²P]ATP (54). The labeled RNA was once again gel purified and subjected to controlled digestions with different RNases as described previously (31). End-labeled RNAs purified by native or denaturing polyacrylamide gels, followed by heating at 68°C and slow cooling, gave identical results in RNase cleavage experiments. The partial RNase digestion products were resolved on denaturing 14 or 8% DNA-sequencing gels, with HCO₃⁻-treated, end-labeled VAI RNAs as ladder markers. To analyze the 3'-end-labeled RNAs, plasmids containing the WT or mutant VAI genes were transcribed in vitro by T7 RNA polymerase, using a kit from Promega Biotec under conditions described by the supplier. The unlabeled RNAs were gel purified, and approximately 0.2 μ g of RNAs was 3' end labeled, using 8 U of T4 RNA ligase (Pharmacia, Inc.) and 70 pmol of 5'-[³²P]pCp (3,000 Ci/mmol; ICN Pharma-

FIG. 1. (A) Locations of the two VA genes on the Ad2 or Ad5 chromosome. The restriction sites are positioned on the Ad2 physical map. (B) Strategy used to construct Ad5 mutants with mutations in the VAI gene. ■, Deletion mutation in the coding region of VAI gene.

ceuticals Inc.). Reactions were carried out in 20- μ l volumes in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5)–0.05 mM $MgCl_2$ –3 mM dithiothreitol–0.2 mM ATP–10% dimethyl sulfoxide (36). Labeled RNAs were purified on 8% DNA-sequencing gels and subjected to RNase cleavage.

Other procedures. Low-molecular-weight ^{32}P -labeled RNAs synthesized in mutant-infected cells were analyzed as

described previously (7). Polypeptide analysis and growth yield assays of the mutant viruses were performed as described elsewhere (8). eIF-2 α kinase activity levels were determined as described by O'Malley et al. (34), using a >95% pure eIF-2 preparation (42). All recombinant DNA procedures were carried out as described previously (26).

RESULTS

Strategy for analyzing mutant VAI RNAs for function. The LS substitution mutants were first introduced into the VAI RNA gene at the plasmid level (30) and then reintroduced into an adenovirus vector that fails to synthesize the minor VAI RNA species (Fig. 1) (4, 7). Although the function of the VAI RNA has not been definitively established, our previous results show that it probably functions in the same way as does the VAI RNA (7; M. R. Furtado et al., unpublished results), and its deletion from the virus vector used allowed us to specifically evaluate the effects of mutations on VAI RNA function. Each adenovirus mutant was plaque purified twice, and the mutations in the VAI RNAs were confirmed by subjecting the gel-purified labeled VAI RNA isolated from mutant-infected cells to two-dimensional T_1 oligonucleotide mapping. Each mutant showed a characteristic T_1 map that was different from that of the WT molecule (4). Whenever mutant VAI RNAs failed to generate an altered T_1 pattern, the viral DNAs were sequenced directly to confirm the mutations. Figure 2 shows the nucleotides substituted in the 12 LS mutants for the WT VAI RNA sequence. In addition, two insertion mutants with short nucleotide sequences inserted in the 5' half and five deletion mutants with deletions in the 3' half of the VAI RNA are shown diagrammatically in Fig. 3. These mutants have previously been partially characterized for growth defects (4).

FIG. 2. Nucleotide sequence of LS substitution mutations. Mutated nucleotides are shown in reverse type. Nucleotides shown in parentheses at positions 66 and 72 differ from those found in the Ad5 sequence. *sub*706, -707, -719, -709, -741, -742, -743, and -746 contain the Ad2 VAI gene; *sub*745, -747, -748, and -749 contain the Ad5 VAI gene. *sub*707 and *sub*709 contain single-base deletions (shown by $\emptyset$). *sub*746 contains a 2-nt insertion at the mutated site. *Hind*III linkers with 10 or 12 bases were used to mutagenize the gene. Five U residues are shown at the termination site, although the number of U residues can be less than five in VAI RNA (9).

		RELATIVE YIELD	
	+ 40 + 80 + 120	293 HELA	
DL 704	→	1.0	1.0
SUB 706	+1 +11	0.75	0.37
SUB 707	+18 +27	0.96	0.58
SUB 719	+26 +45	0.02	0.1
SUB 709	+43 +53	0.03	0.1
SUB 741	+76 +90	0.04	0.06
SUB 742	+81 +90	0.6	1.8
SUB 743	+90 +105	0.12	0.34
SUB 745	+105 +117	0.16	0.34
SUB 746	+116 +126	0.14	0.34
SUB 747	+122 +134	0.14	0.38
SUB 748	+134 +144	0.07	0.09
SUB 749	+140 +150	0.48	0.93
DL 712	+69 +77	0.67	0.97
DL 713	+72 +79	0.83	0.97
DL 714	+90 +113	0.03	0.36
DL 715	+92 +108	0.06	0.54
DL 716	+107 +125	0.03	0.14
DL 717	+126 +152	0.03	0.11
IN 708	+27	0.88	0.84
IN 710	+53	0.36	0.53
DL-SUB720	---	(0.007-0.016)	(0.02-0.03)
SUB 722	---	(0.02-0.04)	(0.06-0.1)

FIG. 3. Schematic representation of the Ad5 VAI mutants discussed in the text. Virus yields for the mutant and WT viruses at day 4 were assayed in a growth yield experiment (4); the ratios of the yield of mutant virus to that of *dl704* are shown. Symbols: →, VAI RNAs; |, substitutions; ●, single-base deletions; ▽, insertions; □, deletions. The major initiation site at G is taken as nt 1 (54). Broken lines for *dl722* and *dl-sub720* indicate that the VAI gene is not transcribed.

To localize our substitution mutations on the secondary structure of VAI RNA, we needed an experimentally derived secondary structure. We investigated the secondary structure of the WT VAI RNA with single-strand-specific T_1 , U_2 , and *Bacillus cereus* RNases. Their cleavage patterns on the WT RNA were consistent with recently proposed secondary structures (31, 33) except for a minor modification in the small loop in the lower part of the molecule (see below). This structure has two extended base-paired regions, stems I and III, which are joined by a short base-paired region, stem II, at the center. Stems II and III are connected at the 3' side by a short stem-loop (stem IV and hairpin loop C). There is a loop of 7 nt between stems II and IV (loop D) (Fig. 4). Stem I consists of a 22-base-pair duplex structure in which nt 1 to 22 are base paired to nt 134 to 155. Stem II is a short duplex region in the center of the molecule which connects stems I and III. This part has nt 31 to 35 base paired with nt 128 to 132. Stems I and II are joined by a loop of 8 nt at the 5' side (nt 23 to 30; loop A). Stem III consists of a 26-base-pair duplex structure in which nt 37 to 62 are bonded to nt 71 to 94. A hairpin loop of 8 nt exists between nt 63 and 71 (loop B). The two long stems are not perfect Watson-Crick base-paired structures; they contain several G · U pairs and are interrupted by single-base mismatches. The short stem-loop in the center contains a duplex of 4 base pairs (nt 99 to 102 base paired to nt 118 to 121; stem IV).

FIG. 4. Secondary-structure model of VAI RNA, based on RNase cleavage patterns. The model has features common to that proposed by others (31, 33). A to D are looped regions.

Nucleotides 103 to 117 exist as a large loop (loop C), and nt 122 to 127 between stems II and IV exist as another loop (loop D). In this report, we describe our mutants in the context of this secondary structure. Figure 5 shows the locations of the 12 substitution, 2 insertion, and 5 deletion mutations in the secondary structure of the VAI RNA. Mutants were classified as defective or nondefective on the basis of their phenotypes in 293 cells; mutants that showed more than fivefold-reduced polypeptide synthesis and growth yield were considered defective.

Electrophoretic analysis of the mutant VAI RNAs. To determine whether the substitution mutants synthesized VAI RNAs at levels adequate for function, human 293 cells were infected with adenovirus variants and immediately labeled with ^{32}P . Cells were harvested at 20 h postinfection, and total cytoplasmic RNAs were isolated and analyzed on denaturing polyacrylamide gels as described previously (Fig. 6) (7). All mutants synthesized RNAs at levels comparable to that of the WT, and in no case was the difference more than twofold, indicating that RNA was not limiting for function in mutant infections. A striking observation was the mobility of *sub709* and *sub741* RNAs, which have mutations disrupting the proximal base-paired region of stem III. These RNAs migrated on the gel as though they carried large deletions, migrating much faster than the WT VAI RNA. Similarly, *sub719* VAI RNA also migrated slightly faster than the WT RNA. The other substitution mutant RNAs migrated with a

FIG. 5. Locations of the VAI mutations on the secondary structure of the VAI RNA. The mutants are grouped as defective or nondefective according to phenotype. LS mutants are boxed; deletion mutants are bracketed; insertion mutants are shown by triangles.

mobility similar to that of WT. Under these same electrophoretic conditions, deletion and substitution mutants migrated according to size. For example, the mutant RNA of an insertion mutant in which 10 nt was inserted at nt 27

FIG. 6. Electrophoretic analysis of VAI RNAs synthesized by the Ad5 VAI mutants. Conditions for RNA analysis are described in Materials and Methods. Arrowheads show the positions of VAI RNAs. The top 4 cm and bottom 10 cm of the autoradiograms are not shown. Mutant *sub745* was analyzed in a separate experiment.

(*in708*) and a deletion mutant in which 15 nt was deleted in the 3' half (*dl714*) migrated slower and faster, respectively (Fig. 6) (data for the rest of the mutants are not shown). Mutant *sub742*, however, was an exception. Although mutations in this molecule disrupted the same base-paired residues as in *sub709* but at the 3' side of loop B and overlapped with the *sub741* mutation (Fig. 5), the mobility change was much less. That the dramatic mobility change observed for mutants *sub709* and *sub741* and a considerable mobility change noted for mutants *sub719* and *sub742* were due to the perturbation in structure of the VAI RNA is supported by results of the nuclease sensitivity analysis of these molecules (see below). Mobility is most likely dependent on the integrity of the central region of the molecule, since mutant RNAs with mutations that do not disrupt this region migrated according to their size.

Phenotypes of the adenovirus VAI mutants. To determine the effects of mutations on the function of VA RNA, we assayed the mutants for viral polypeptide synthesis at late times, growth yield, and in vitro phosphorylation of the exogenously added eIF2.

Polypeptide synthesis. Human 293 cells were infected with the adenovirus variants for 19 h and then labeled with [³⁵S]methionine for 1 h. Labeled polypeptides were extracted and then analyzed on sodium dodecyl sulfate-polyacrylamide gels as described in Materials and Methods. A WT control (*dl704*), a VAI⁻ VAI⁺ mutant (*sub722*), and a mutant that did not synthesize VAI or VAI⁺ RNA (*dl-sub720*; double mutant) (7) were included for comparison.

TABLE 1. Relative hexon polypeptide synthesis by VAI mutants in 293 cells^a

Mutant	Hexon synthesis
Nondefective	
<i>dl704</i> (WT).....	1.0
<i>sub706</i>	0.96
<i>sub707</i>	1.1
<i>in708</i>	1.0
<i>in710</i>	1.1
<i>dl712</i>	1.0
<i>dl713</i>	1.0
<i>sub742</i>	0.9
<i>sub749</i>	0.5
Defective	
<i>sub719</i>	0.09
<i>sub709</i>	0.08
<i>sub741</i>	0.1
<i>sub743</i>	0.14
<i>sub745</i>	0.15
<i>sub746</i>	0.15
<i>sub747</i>	0.13
<i>sub748</i>	0.08
<i>dl714</i>	0.09
<i>dl715</i>	0.14
<i>dl716</i>	0.09
<i>dl717</i>	0.09
<i>dl-sub720</i> (VAI /VAII)	0.08
<i>sub722</i> (VAI /VAII ⁺)	0.12

^a The hexon bands in the protein gels were quantitated by scanning the autoradiograms with a laser densitometer. WT values were arbitrarily taken as 1.0. The values shown are for a typical experiment. The relative values were highly reproducible and did not change more than 5% from experiment to experiment.

Mutant *sub722* is similar to *dl331*, described earlier (55), and synthesized viral polypeptides at much reduced levels (eight-fold; quantitation based on densitometer scanning of the autoradiograms is given in Table 1) in comparison with the WT control (Fig. 7), whereas the translation defect in

dl-sub720 was even more severe and hexon polypeptide synthesis was reduced by about 12-fold. Mutants *sub706* and *sub749*, which disrupt the secondary structure of the distal portion of stem I, two deletion mutants, *dl712* and *dl713*, which disrupt the distal portion of the stem III, and another substitution mutant, *sub707*, which disrupts part of the stem I and loop A (Fig. 5), synthesized late polypeptides at WT levels, indicating that perturbations in these regions of the molecule are tolerated. Similarly, two insertion mutants, *in708* and *in710*, in which mutations were located in loop A (at nt 27) and the distal part of stem III (at nt 54), respectively, were not defective for translation. Paradoxically, mutant *sub742*, which disrupts the proximal part of stem III, was not defective for translation. This is a unique mutant and is discussed in detail below.

Two substitution mutants in the 5' half (*sub709* and *sub719*) and five substitution mutants in the 3' half (*sub741*, -743, -745, -746, -747, and -748) synthesized viral polypeptides at 7- to 12-fold-lower levels than did *dl704*. *sub719*, -709, -741, and -748 were nearly as defective as the mutant that synthesized neither VAI nor VAII RNAs (*dl-sub720*; Fig. 7). All of these mutants have disruptions in the central portions of the molecule. Mutations in *sub709* and *sub741* disrupt a duplex region in the proximal part of stem III, whereas the *sub748* mutation disrupts the proximal part of stem I. The mutation in *sub719* partly overlaps loop A, stem II, and the 5' side of the proximal portion of stem III. Mutations in *sub743* and *sub747* disrupt the 3' side of stem II, the proximal part of stem of III, and the short stem-loop. On closer examination, there was a difference in the levels of polypeptide synthesis among these mutants. *sub743*, -745, -746, and -747 synthesized polypeptides at two- to three-fold-higher levels than did the double mutant (Table 1). This difference was noted in numerous experiments and therefore we believe it to be real. The difference was also reflected in growth yields and eIF-2 α kinase activities (see below).

We tested six deletion mutants lacking 6 to 23 nt for phenotype. Two of these mutants, *dl712* and *dl713*, with deletions located in the distal part of stem III (Fig. 5), showed the WT phenotype. The other four deletion mutants,

FIG. 7. Polypeptide synthesis of adenovirus mutants with mutation in the VAI RNA gene. Human 293 cells were infected with the mutants at 5 PFU and labeled 20 h postinfection with [³⁵S]methionine for 1 h. Cells were lysed and polypeptides are analyzed on 20% sodium dodecyl sulfate-polyacrylamide gels as described previously (10). A and B are results of two separate experiments. Positions of hexon and 100-kilodalton polypeptides are shown.

FIG. 8. Analysis of eIF-2 α kinase activity in human 293 cells infected with *dl704* (WT control) and Ad5 VAI mutants. Cells were infected with selected Ad5 mutants for 20 h, cell lysates were prepared, and the eIF-2 α kinase activity in the cell lysates was determined by the agarose-poly(I-C) binding method as described by O'Malley et al. (33). HCl, Heme-controlled inhibitor; α , position of the phosphorylated alpha subunit of eIF2.

which overlap in a region between nt 90 and 152, were all as defective as the double mutant. Interestingly, mutant *dl715*, in which 17 nt between nt 90 and 108 (Fig. 5) are deleted, was not as defective; it synthesized polypeptides at slightly higher levels (Fig. 7 and Table 1) and yielded slightly more virus particles (Fig. 3; see below).

Growth yields of VAI mutants. All mutants were assayed for growth yield in human 293 cells, in which the VAI⁻ mutant shows the most dramatic phenotype (21, 55; unpublished observations). They were also assayed on a monolayer HeLa cell line in which a VAI⁻ mutant showed a moderate phenotype, probably because of a delayed activation of the eIF-2 α kinase (21) (Fig. 3). Substitution, deletion, and insertion mutants that synthesized normal levels of viral polypeptides grew to WT levels. In 293 cells, *sub719*, -709, -741, and -748, with disruptions in the central region of the molecule, grew to a titer 1/20 to 1/50 that of the WT. Mutants overlapping the short stem-loop (*sub743*, -745, -746, and -747) grew to titers that were one-seventh to one-eighth that of the WT but still three- to fourfold higher than that of mutants in which the adjacent base-paired regions were mutated (*sub719*, -709, and -741). Reduction in mutant virus yield was more dramatic when assayed on monolayer HeLa cells. Viruses mutated in stem II and the proximal part of stem III grew more slowly and yielded titers 1/10 that of the WT, whereas mutants with substitution (*sub743*, *sub744*, *sub745*, *sub746*, and *sub747*) and deletion (*dl715*) of sequences in the short stem-loop grew to near-normal levels. In both assays, alterations in the duplex regions that hold the short stem-loop in a particular configuration were more deleterious than those in the short stem-loop itself.

Determinations of eIF-2 α kinase activity in mutant infections. Figure 8 shows the extent of eIF-2 phosphorylation in vitro by cell extracts prepared from human 293 cells infected with some of the VAI mutant viruses. Human 293 cells were infected with 20 PFU per cell for 20 h, and the cell extracts were prepared and assayed for the ability to phosphorylate exogenously added eIF-2 as described by O'Malley et al. (33). Kinase activity in WT-infected cells was comparable to that of mock-infected cells, whereas the mutant lacking both VAI RNA genes (*dl-sub720*) and a mutant lacking only the VAI gene showed fourfold-higher levels of activity. Levels

of eIF-2 phosphorylation for substitution mutants were in agreement with polypeptide synthesis and growth yields in 293 cells; only the defective mutants exhibited considerably higher levels of phosphorylation.

Paradoxical phenotype of *sub742*. Results with the LS mutants clearly showed that perturbations of the base-paired region in the proximal part of stem III of VAI RNA lead to severe loss of function. Mutants *sub709* and *sub741*, with altered bases in the 5' and 3' sides of this region, respectively, are expected to disrupt this duplex region and are defective. However, mutant *sub742*, which disrupts this same region, exhibited WT polypeptide synthesis (Fig. 7), growth yield (Fig. 3), and eIF-2 phosphorylation (Fig. 8). Mutants *sub709* and *sub742* carry mutations that affect the same base-paired residues (44-89, 45-88, 46-87, 49-84, 50-83, and 51-82; the numbers correspond to residues at the 5' side hydrogen bonded to residues to the 3' side of the duplex in WT VAI RNA) (Fig. 5). Also, nucleotides mutated in nondefective *sub742* are a subset of those mutated in defective *sub741* (Fig. 2). One explanation for this surprising result comes from RNA folding. Either mutant RNA of *sub742* but not *sub709* and *sub741* may retain this central structural feature or rearranged sequences may provide an alternate secondary structure close enough to that of the native molecule to allow efficient interaction with the kinase or other host factors. To test these possibilities, we carried out secondary-structure analysis of the WT and three mutant VAI RNAs.

Secondary-structure analysis of WT and mutant RNAs of *sub709*, *sub741*, and *sub742*. Unlabeled mutant VAI RNAs were isolated from virus-infected cells or by transcribing the mutant VAI genes from a plasmid containing a T7 promoter immediately upstream of the RNA start site and an *EcoRI* site next to the terminator sequences (49). The in vitro transcription product generated from this construct differs from that of virus-infected cells only in the addition of an extra U at the 3' end (49). The in vitro-synthesized RNAs were then labeled at the 3' end with [³²P]pCp by T4 RNA ligase or at the 5' end with [γ -³²P]ATP by T4 polynucleotide kinase. Labeled RNAs were then subjected to single-strand-specific T₁, U₂, and *B. cereus* RNases and a dsRNA-specific RNase (V₁) under conditions designed to cleave less than 5% of the molecules (31). An RNA sequence ladder generated by treatment of VAI RNA with HCO₃⁻ at 90°C for 3 min was used as a marker. The partial cleavage products were resolved on 14 or 8% denaturing polyacrylamide gels. The cleavage maps obtained by using 5'-end-labeled RNAs compared well with those obtained with 3'-end-labeled RNAs. Representative gel patterns obtained from the 3'-end-labeled RNAs for the WT and three mutant RNAs are shown Fig. 9. By varying the periods of time for which gels were run, we could resolve the cleavage products from all regions of the molecule.

The cleavage pattern obtained for the WT VAI RNA is shown in Fig. 10. Most of the cleavages except those in loop A are consistent with the structure proposed by Mellits and Mathews (31) (Fig. 4). In most of our experiments, we were unable to see detectable cleavages at residues 20, 21, and 22. We therefore present the structure in this region of the molecule according to that proposed by Monstein and Philipson (33). In our studies, we have observed three types of cleavages: bands that appear very dark in the gels due to pronounced cleavages, bands that appear with moderate intensity, and bands that appear weak but detectable in most experiments (Fig. 10). Cleavages were observed only in loops A, B, C, and D for the WT RNA.

FIG. 9. Nuclease sensitivity analysis of the VAI RNAs synthesized by the WT molecule and *sub709*, -741, and -742. In vitro-synthesized RNAs were labeled at the 3' end as described in Materials and Methods and digested with the indicated RNases. The products were resolved in 14% DNA-sequencing gels. NE, No RNase; M, ladders generated by partial digestion of the labeled RNA under alkaline conditions as described in Materials and Methods.

Cleavage patterns revealed that substitution of nt 44 to 46 and 49 to 51 in *sub709* RNA had a profound effect on the structure of the molecule (Fig. 10). Base-paired sequences between nt 31 and 52, 79 and 82, 90 and 94, and 128 and 133 were all cleaved by RNases in the mutant but not the WT. In loop B, only nt 61 to 64 were cleaved in the mutant, whereas all nucleotides of loop B were cleaved in the WT structure. In addition, residues from 94 to 102 in the short stem-loop, which were resistant to RNases in the WT structure, were all cleaved in this mutant. Thus, the central portion of the mutant VAI RNA of defective *sub709* is considerably more open than that of the WT, a rearrangement that almost certainly destroys the integrity of the short stem-loop and the adjacent base-paired regions. The structures of stem I and loop A remained unperturbed in all three substitution mutants, indicating that the effects of the mutations were restricted to the central and top portions of the molecule. Mutant *sub741* VAI RNA also showed a high degree of sensitivity to RNase digestion. Several base-paired residues

in stem III were cleaved by RNases at moderate to pronounced levels (Fig. 10). The only long stretches of sequences that were resistant to cleavage were from nt 53 to 60 and from nt 64 to 73, which likely exist as a base-paired structure. The cleavage pattern in the short stem-loop is so different from that of the WT molecule that the molecule cannot exist in its native configuration. Thus, the dramatic mobility change for these RNAs in polyacrylamide gels is reflected in their high degree of sensitivity to single-strand-specific RNases.

Except for stem I and loop A, the cleavage pattern of *sub742* VAI RNA (Fig. 10) deviated from those of both the WT RNA and defective mutants. Nucleotides 34 to 46, which overlap part of stem II and the proximal part of stem III, were cleaved by RNases, as were all substituted nucleotides on the 3' side (between nt 80 and 95) that form the proximal part of stem III. In the loop B region, only nt 61 to 64 were cleaved. In the short stem-loop (stem III and loop C), the cleavages appeared somewhat similar to those of WT

FIG. 10. RNase sensitivity of WT, *sub709*, *sub741*, and *sub742* VAI RNAs. Cleavage maps are shown on the WT RNA structure. Symbols: $\blacktriangle$, RNA cleavage; $\bullet$, pronounced cleavage; $\circ$, moderate cleavage; ∇ , weak but reproducible cleavage. The mutated nucleotides are shown in lowercase.

RNA, although there were differences. C94 was cleaved in *sub742* RNA but not in the WT molecule. Similarly, C109 was cleaved in WT VAI RNA but not in *sub742*. The intensity of cleavage of residues in loop C was also different in this RNA than in the WT molecule. A very pronounced cleavage was observed for A103 in *sub742* compared with that of the WT (Fig. 10). Undoubtedly, as in *sub709* and *sub741*, the structure in the central part of the RNA in *sub742* is perturbed by the mutation, but the RNA seems to have a more compact structure than that of *sub709* and *sub741*.

A novel secondary structure for *sub742* VAI RNA. On the basis of the sensitivity of this mutant molecule for the single-strand-specific RNases, we propose a secondary structure for this RNA (Fig. 11) that consists of five base-paired regions (stem I to V) and six loops (A to F). Stems I and II and loop A are equivalent to that of the WT. Stem III consists of four base-paired sequences in which nt 47 to 50 are paired with nt 108 to 111. Stem IV contains eight base-paired sequences in which nt 53 to 60 are base paired to nt 67 to 75. The last stem, V, consists of five base-paired sequences in which nt 78 to 82 are base paired to nt 97 to 101. Stems II and III are separated by two loops, B (nt 36 to 46) and F (nt 112 to 126), on the 5' and 3' sides, respectively. Loop C (nt 61 to 66) is the smallest of the loops, and the position of this loop is somewhat similar to that of the WT molecule. Loop D consists of 14 nt (83 to 96) and contains all of the substituted nucleotides. Six nucleotides between stems II and IV (nt 102 to 107) form loop E. This structure is compatible with almost all of the single-strand-specific RNase cleavages and several V_1 cleavages. On the basis of energy values given by Tinoco et al. (56), the molecule has a calculated free energy value of $\Delta G = -59.7$ kcal (ca. -249.8 J)/mol, which compares well with that of the WT molecule (31, 33). Furthermore, the existence of stem IV, loop C, stem V, and loop D structures is also supported in part by a computer-predicted structure based on programs of Zucker

and Steigler (59) (data not shown). Interestingly, part of the structure of this RNA, encompassing stems III, IV, and V and loops C, D, and E, very closely resembles the structure present in the central region of the WT molecule. Since our mutational analysis suggested that the central region of the

FIG. 11. Experimentally derived secondary structure for the VAI RNA of *sub742*. A to F are looped regions.

WT molecule was critical for function, these alternate stems and loops may be what allowed the drastically altered *sub742* to produce a WT phenotype.

DISCUSSION

We have constructed and characterized a group of adenovirus mutants that contain a clustered set of point mutations in defined regions of the VAI RNA. Since all of these mutants synthesized abundant quantities of VAI RNAs in virus-infected cells, we determined the effects of each mutation on RNA function during virus infections.

The only known function for the VAI RNA in virus-infected cells is to block the activation of the host eIF-2 α kinase. This kinase is a 68-kilodalton ribosome-associated polypeptide containing two distinct activities: an intramolecular autophosphorylation reaction in which the 68-kilodalton polypeptide is phosphorylated, and a kinase activity that phosphorylates an exogenous substrate, the 36-kilodalton subunit of the initiation factor eIF-2 (3, 19, 22; reviewed in reference 25). Low concentrations of both natural and synthetic dsRNAs are able to stimulate the activity of the kinase in vitro, but high concentrations inhibit its activity (3, 12, 15, 17, 32). The VAI RNA blocks the activation of the kinase at the autophosphorylation step (20, 35; G. Ghadge et al., unpublished results). It has been proposed that monovalent binding of the kinase with the VAI RNA molecule prevents two kinase molecules from contacting each other, a step that is probably necessary for autophosphorylation (20, 35). The extended base-paired structure of the VAI RNA has been considered important for its function, since such an RNA could mimic the dsRNA and yet presumably fail to activate the kinase because of its imperfectly base-paired structure.

Our mutational analysis results are not fully consistent with this explanation. First, the long base-paired region, stem I, cannot play a significant role in VAI RNA function. Nuclease sensitivity experiments show that the three substitution mutants *sub709*, -741, and -742 all contain an intact double-stranded stem I and yet only one, *sub742*, can inactivate the kinase (Fig. 8). Mutational effects in *sub706*, -707, and -749 would definitely disrupt stem I, yet these mutants show the WT phenotype. Second, most of the base-paired stem III in *sub742* is sensitive to RNases and may exist as loops in the middle of the molecule (Fig. 11; see below), yet the mutant is able to function as efficiently as the WT, indicating that the extended base-paired stem III is not critical for function. Several other observations support this notion. For example, one insertion and two deletion mutants in the distal portion of stem III (*in710*, *dl712*, and *dl713*; Fig. 5) show the WT phenotype. Mutations here probably do not disrupt the secondary structure of the central portion and do not affect function. Another report recently examined the structural features of the VAI RNA necessary for function in transfection assays by constructing deletion and LS mutants restricted to the 3' half the molecule (31). Two of the deletion mutants (*dl1* and *Ad2dl2*; the mutation in *Ad2dl2* is identical to that of *dl713* reported here) fall in this area of the molecule and do not disrupt either the structure of the central part of the molecule or the function of the molecule. Furthermore, naturally occurring adenovirus variants that contain insertion and substitution mutations at nt 72 grow normally (29). All of these results taken together suggest that the function of VAI RNA is not dependent on its extended base-paired structure. It is possible, therefore, that the VAI RNA inhibits by binding to the kinase at sites other than the dsRNA-binding site.

All of our evidence suggests that it is the central part of the molecule whose integrity is critical for function. Eight adenovirus LS mutants with mutations overlapping this region of the molecule (Fig. 5) are unable to function in virus-infected cells. Structural analyses of two of these mutants, *sub709* and *sub741*, which affect the proximal region of stem III, indicate that the mutations lead to a dramatic change in sensitivity to RNases. RNase cleavages are widespread in stems II and III and in the short stem-loop in both *sub709* and *sub741* RNAs, indicating that the original compact structure of the molecule is destroyed. Another defective LS mutation reported recent (*ls1*) also falls in the same region as that of *sub741*, and nucleotides mutated in *ls1* are a subset of those mutated in *sub741* (31). The effects of the *ls1* mutation on structure and function are similar to those of *sub741* reported here. Mutants *sub719*, *sub743*, and *sub746*, which affect the central base-paired regions in part and also stem III, and mutant *sub745*, which exclusively falls on loop C, are all defective for function. Our structural analysis studies of *sub709* and *sub741* RNAs indicate that mutations in these molecules almost certainly lead to disruption of structure in this region. As a result, we would argue, these mutants fail to function. We would also argue that *sub748* and other deletion mutants in the 3' half failed to function in vivo because the mutation interfered in the correct folding of the critical central part of the molecule. Thus, function seems to correlate well with the integrity of the elements present in the central region of the molecule, which contains the short stem-loop and the adjacent base-paired regions.

How rigid are the structural requirements in this central region of the molecule for function? What elements of this part of the molecule are recognized by host factors? Analysis of mutants *sub709*, *sub741*, and *sub742* provides some clues. Mutant *sub709*, which contains mutations at nt 44 to 46 and 49 to 51, and *sub742*, which mutates these same base pairs on the 3' side of this duplex region (nt 82 to 84 and 87 to 89), have opposing phenotypes. In addition, bases that are mutated in *sub742* are a subset of those mutated in *sub741* (nt 77 to 79, 81, 83, 86, 88, and 89) yet *sub742* has a WT phenotype whereas *sub741* is defective. Nuclease digestion analysis reveals that these two RNAs differ considerably in structure. Stem II and most of stem III and the short stem-loop of *sub709* RNA are sensitive to RNases whereas the WT is not, indicating that the mutant RNA has little secondary structure in this region. RNAs of mutants *sub741* and *sub742* also show a high degree of sensitivity to RNase digestion, but the digestion patterns of these two RNAs differ significantly. Large numbers of nucleotides on both the 5' and 3' sides of stems II and III as well as nucleotides in the short stem-loop are attacked by RNases in *sub741* RNA. Thus, this RNA also lacks secondary structure in the central part. The cleavage map of *sub742* RNA is dramatically different from that of WT RNA but not as extensive as that of *sub709* or *sub741*. On the basis of the RNase cleavage data, we propose a secondary structure for this RNA (Fig. 11) that reproduces on one stem the critical short stem-loop found in the center of the active molecules. Stems I and II and loop A of this structure are identical to those of the WT, but stem II is followed by two large loops, B and F, at the 5' and 3' sides of the stem. These loops consists of 11 and 16 nt, respectively, and are held by a short stem of 4 base pairs (stem III). The remaining sequences fold to form a hairpin loop (stem IV and loop C) and a short, branched stem-loop at the 3' side consisting of a short stem (V) of 5 base pairs and a loop (D) of 16 nt. Between stem II and IV, 6 nt do not base pair and exist as another loop (E). These results also show that even

a small difference, such as the type of bases substituted, can make profound difference in VAI RNA folding.

Our mutational analysis thus suggests that even minor damage to the structure in the central region (stems II, III [proximal part], and IV and loops C and D) of the WT molecule leads to loss of function. How then does *sub742* RNA function efficiently? We propose that the unexpected WT phenotype for *sub742* RNA is due to its unique structure in the top third of the molecule. This structure and the structure in the central part of the WT molecule have a number of similarities. For example, stem IV and loop C of *sub742* RNA resemble stem III and loop B of WT RNA. Similarly, stem V and loops D and E of *sub742* RNA can be compared with stem III and loops C and D of the WT molecule. If these similarities are more than a coincidence, the eIF-2 α kinase or other host factors may recognize these elements common to both WT and *sub742* RNA. If this is the basis for the WT phenotype of *sub742*, one can draw several conclusions. First, there is some flexibility with regard to minimal length of duplex regions in the stem regions and the number of nucleotides in loops C and D of the active molecule. For example, stem IV of *sub742*, which may be equivalent to the proximal part of stem III of the WT molecule, contains only 8 base pairs. The number of nucleotides in the branched stem-loop structure varies in these two molecules. Second, the specific bases contained in these structural elements also vary.

RNAs from several different DNA viruses with little overall similarity to VA RNA or to one another can complement adenovirus VAI function, which supports the above interpretations. Two small RNA polymerase III transcripts transcribed from Epstein-Barr virus substitute partially for VAI function (6, 8), and this complementation is based on the ability of these transcripts to inactivate the kinase *in vivo* (M. R. Furtado and B. Thimmappaya, manuscript in preparation). The VA RNAs of simian and avian adenoviruses can complement adenovirus VAI RNA function in transfection assays (23, 24). Although the structures of these RNAs have not been determined experimentally, all of the RNAs are capable of folding to form molecules with branched configurations that meet the minimal structural requirements described here for function.

Our mutational analysis studies show that sequences in the branched stem-loop are less sensitive to mutational effect than are those of the short duplex regions of stems II and III. Mutations in this region lead to synthesis of polypeptides at levels two- to threefold higher than in stems II and III. Growth yield and eIF-2 phosphorylation data also support this observation. This effect is more dramatic when we examine these mutations in HeLa cells. Mutations in the short stem-loop allow normal growth, whereas mutations in the adjacent duplex regions reduce the titer 10-fold. These effects have not been observed for similar mutations in less sensitive transfection assays (31). Although we do not have a good explanation for this effect at present, it is possible that the protein-binding domain is located in the short duplex region in the proximal part of the stem III. Sequences in the short stem-loop may help to stabilize the tertiary interactions within the molecule. Further mutational analysis of the VAI RNA, elucidation of its precise three-dimensional structure, and determination of bases in the VAI RNA that make contact with the eIF-2 α kinase should unveil the precise mechanism by which these abundant small RNAs are able to intercede in the autophosphorylation that activates the kinase.

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