Mutational Analysis of the Central Domain of Adenovirus Virus-Associated RNA Mandates a Revision of the Proposed Secondary Structure

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Protein synthesis in adenovirus-infected cells is regulated during the late phase of infection. The rate of initiation is maintained by a small viral RNA, virus-associated (VA) RNA_I, which prevents the phosphorylation of eukaryotic initiation factor eIF-2 by a double-stranded RNA-activated protein kinase, DAI. On the basis of nuclease sensitivity analysis, a secondary-structure model was proposed for VA RNA. The model predicts a complex stem-loop structure in the central part of the molecule, the central domain, joining two duplexed stems. The central domain is required for the inhibition of DAI activation and participates in the binding of VA RNA to DAI. To assess the significance of the postulated stem-loop structure in the central domain, we generated compensating, deletion, and substitution mutations. A substitution mutation which disrupts the structure in the central domain abolishes VA RNA function in vitro and in vivo. Base-compensating mutations failed to restore the function or structure of the mutant, implying that the stem-loop structure may not exist. To confirm this observation, we tested mutants with alterations in the hypothetical loop and short stem that constitute the main features of the wild-type model structure. The upper part of the hypothetical loop could be deleted without abolishing the ability of the RNA to block DAI activation in vitro, whereas other loop mutations were deleterious for function and caused major rearrangements in the molecule. Base-compensating mutations in the stem did not restore the expected base pairing, even though the mutant RNAs were still functional in vitro. Surprisingly, a mutant with a noncompensating substitution mutation in the stem was more effective than wild-type VA RNA_I in DAI inhibition assays but was ineffective in vivo. The structural and functional consequences of these mutations do not support the proposed model structure for the central domain, and we therefore suggest an alternative structure in which tertiary interactions may play a significant role in shaping the specificity of VA RNA function in the infected cell. Discrepancies between the functionality of mutant forms of VA RNA in vivo and in vitro are consistent with the existence of additional roles for VA RNA in the cell.

The virus-associated (VA) RNA_I of adenovirus type 2 (Ad2) is a key viral product which confers resistance to an interferon-induced cellular antiviral defense mechanism (for a review, see reference 23). VA RNA_I is a highly structured RNA of 160 nucleotides (nt), which is synthesized by RNA polymerase III (pol III) and accumulates to high concentration in the cytoplasm during the late phase of infection. It competes with double-stranded RNA (dsRNA) for binding to the protein kinase DAI, the dsRNA-activated inhibitor of protein synthesis. In the absence of VA RNA, activated DAI phosphorylates eukaryotic initiation factor 2, thereby indirectly impeding translational initiation (for a review, see reference 25). VA RNA can also stimulate the expression of genes introduced into uninfected cells by transfection (16, 36). No viral sequences are required in the reporter gene construct for this effect to be manifested. More recently, VA RNA has also been reported to increase the accumulation of mRNA from cotransfected plasmids: in one case VA RNA preferentially enhanced the accumulation of longer mRNAs (37), and in another it increased the stability of the plasmid-generated mRNA (35).

On the basis of nuclease sensitivity analysis (27), a sec-

ondary-structure model was proposed for VA RNA consisting of three main regions: the apical stem-loop, the central domain, and the terminal stem (Fig. 1A). In this model, the central domain, which resides between the apical stem and the terminal stem, contains a number of features typical of RNA secondary structure. The most prominent feature in the central domain is stem-loop 6, and there is also a bifurcation loop and an interior loop. Several aspects of this model structure are supported by mutational analyses in which foreign sequences or deletions were introduced into the VA RNA molecule. Examination of the secondary structure, biological function, and binding to DAI of these diverse mutants indicated that the central domain is required for the inhibition of DAI activation by dsRNA and implicated the apical stem in the binding of the VA RNA to DAI (6, 26, 27, 29). Subsequent studies suggested that the central domain also participates in the binding of VA RNA to DAI, but probably to a lesser extent than the apical stem (1, 7).

It is well established that secondary structural elements in RNA, such as duplexes, hairpins, internal loops, bulged bases, and pseudoknots, are intimately connected with biological functions (30, 31). Despite its critical role in VA RNA function, the complex stem-loop structure of the central domain has not been subjected to a detailed examination. To define the features of this stem-loop structure, we introduced foreign sequences into a 20-nt region within the central domain and made deletions in the proposed loop structure. The structural and functional consequences of these muta-

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FIG. 1. Secondary structure models for Ad2 VA RNA_I. (A) Schematic diagram based on reference 27 showing the principal regions of the molecule. (B) As in panel A, with the cleavage sites marked according to the following convention: large arrowheads with shaft, very strong cuts; large arrowheads, strong cuts; small arrowheads, weaker cuts; solid symbols, single-strand-specific sites; open symbols, nuclease V₁ sites. (C) Alternative model with cleavage sites marked as in panel B.

tions were investigated. Secondary-structure analysis showed that in all cases the structural changes were confined to the central domain, including loop 2a, stem 3, and stem-loop 6 (Fig. 1A), and did not affect the apical stem-loop or the terminal stem, implying that the central domain is a relatively autonomous structural element with considerable intrinsic tertiary interactions. Surprisingly, the stem-loop, which is the principal feature of the central domain in the original model, is not supported by structural analysis or by study of the function of the mutant VA RNAs *in vivo* and *in vitro*. These findings suggest that an alternative conformation of the central domain is important for VA RNA function.

MATERIALS AND METHODS

Construction of mutants. All deletion and substitution mutants constructed here were generated within the VA RNA gene of plasmid pT7VA (28) by oligonucleotide-directed mutagenesis (18, 41) with *Escherichia coli* CJ 236. For experiments conducted *in vivo*, the *Dra*I restriction endonuclease site (TTTAAA) was modified to TTTTGG by using oligonucleotide-directed mutagenesis to allow termination of VA RNA transcription by pol III *in vivo*. The fidelity of termination was verified in pol III transcription experiments *in vitro* (data not shown). Two RNA bands were generated,

one the size of wild-type VA RNA_I and the other a few nucleotides shorter, possibly because of 5'-end changes (4, 40). The efficiency of site-specific mutation was approximately 50%. Oligonucleotides were synthesized by using a 380-A DNA synthesizer (Applied Biosystems), and mutants were analyzed by dideoxy sequencing (33) with a kit (United States Biochemical Corporation).

Kinase assay. Wild-type and mutant VA RNAs were purified by sequential electrophoresis through denaturing and nondenaturing 8% polyacrylamide–7 M urea gels, followed by cellulose CF-11 chromatography (28). The VA RNA was then incubated with DAI (purified to the hydroxylapatite stage [41]) and [γ -³²P]ATP (ICN Biochemicals, Inc.) in either the presence or absence of reovirus dsRNA as described previously (28).

RNA secondary structure. The secondary structures of wild-type and mutant VA RNAs were determined by the RNase sensitivity technique (27). Each mutant RNA was labeled at its 5' or 3' end and subjected to partial digestion under conditions such that most RNA molecules were not digested and the remainder were cleaved only once. We employed the single-strand-specific RNase T₁, *Bacillus cereus* RNase, RNase U₂, and RNase T₂ (specific for G residues, pyrimidines, A residues, and any exposed base, respectively), as well as nuclease V₁ from cobra venom (specific for helical and stacked nucleotides). Digestion

TABLE 1. Sequence and function of central-domain mutants

VA RNA	Sequence ^a			DAI inhibition (5 µg/ml)	Reticulocyte assay	CAT stimulation
	Stem 6	Loop 6	Stem 6			
	100	110	120			
Wild type	GCGGUU	ACCGCCGCGUGUCG	AACCCA	++	++	+++
<i>ls3</i>	-----CA	GAUCUGx-----	-----	-	-	-
<i>ls3a</i>	-----	GAUCUGx-----	-----	-	-	-
<i>ls3b</i>	-----CA	GAUCUGx-----	UG-----	-	±	-
<i>del103-109</i>	-----	xxxxxxx-----	-----	++	-	-
<i>del110-117</i>	-----	-----xxxxxxxx	-----	+	-	-
<i>sub110-117</i>	-----	-----UACACAUC	-----	+	-	-
<i>sub101,102</i>	-----CA	-----	-----	+++	+++	-
<i>sub118,119</i>	-----	-----	UG-----	-	-	-
<i>sub102,118,119</i>	-----A	-----	UG-----	-	-	-
<i>sub101,102,118,119</i>	-----CA	-----	UG-----	+	-	-

^a -, same as wild type; x, deleted nucleotide. The sequence shown includes two nucleotides flanking stem 6 at both the 5' and 3' ends.

products were analyzed in denaturing gels run for various lengths of time to resolve all regions of the RNA molecules, and the positions and intensities of nuclease cleavages were noted. RNA secondary-structure models were derived by manual folding and the use of a computer program which incorporated both empirical data and estimates of energies inherent in base pairing (15, 42).

Enhancement of CAT expression. Human 293 cells (8) were cotransfected with 3 µg of β-globin-CAT12 (37) and 10 µg of the appropriate VA RNA-encoding plasmids as described previously (12). Transfections were performed in duplicate. The cells were ~60% confluent in 6-cm dishes, and the total quantity of DNA was adjusted to 15 µg with pUC119 DNA. Following a glycerol shock at 6 h posttransfection, the cells were harvested at 48 h for chloramphenicol acetyltransferase (CAT) assay (12). Negative controls contained the vector pUC119 or pEM-7, which carries the T7 RNA polymerase promoter and human immunodeficiency virus type 1 TAR sequence (11) in place of the VA RNA sequence. Dot blot hybridization showed that comparable amounts of wild-type and mutant VA RNA had accumulated in the cytoplasm by 48 h posttransfection.

Cell-free translation. DAI activation was assayed by translation in vitro in two stages. First, rabbit reticulocyte lysate was preincubated for 15 min at 30°C with reovirus dsRNA and purified wild-type or mutant VA RNA (28). Then the reaction mixture was completed by addition of globin mRNA (4 µg/ml; Bethesda Research Laboratories), [³⁵S]methionine (5 mCi/ml; ICN Biomedicals), 72 mM potassium acetate, 0.4 mM magnesium acetate, 0.4 mM GTP, and other components specified previously (3). After incubation for a further 30 min at 30°C, the translation products were resolved in a 15% polyacrylamide-sodium dodecyl sulfate (SDS) gel and visualized by fluorography. The globin band was quantified by using a Fuji Bio-image Analyzer.

RESULTS

The current model for the secondary structure of VA RNA (Fig. 1A) contains two imperfectly duplexed stems joined by a complex stem-loop structure, the central domain. This model was derived as a fit for the empirical data obtained by RNase sensitivity analysis, placing nucleotides that are cleaved by single-strand (ss)-specific nucleases in unpaired regions and assigning nucleotides that are cleaved by nucleases V₁ in regions of secondary structure (Fig. 1B). However,

the data can be accommodated equally well by other models, such as that shown in Fig. 1C, with alternative structures in the central domain. The apical stem is at least partially responsible for the efficient binding of VA RNA to DAI (26, 29), while the central domain is implicated in the inhibition of DAI activation (6, 26, 27). In this study we wanted to explore the role of stem-loop 6 in the central domain in determining the inhibition function of VA RNA. A previous study (26) had shown that large deletions or substitutions in the central domain abolished function and caused major rearrangements in the structure of the VA RNA molecule. Here we have introduced small deletions and substitutions by site-directed mutagenesis to test the important features of stem-loop 6 and to challenge the existence of this predicted structure in the central domain.

The mutations are listed in Table 1, and their sequences are displayed schematically in Fig. 2 superimposed on the model structure of Fig. 1A. All the constructs were introduced into a vector which allows efficient production of VA RNA in vitro by using T7 RNA polymerase (28), and the secondary structures of the mutant RNAs were studied by the nuclease sensitivity technique (27). This analysis indicated that the structures are perturbed primarily in the central domain, while the rest of the structure remains the same as in wild-type VA RNA. To assess the functional consequences of the mutations, we investigated their ability to inhibit the activation of DAI in vitro, as measured by autophosphorylation. None of the RNAs synthesized in vitro activated DAI when tested over a wide range of concentrations (5 ng/ml to 50 µg/ml [data not shown]), verifying that they were free of dsRNA contaminants (28) and confirming that the interaction between VA RNA and DAI is fundamentally different from that between dsRNA and DAI.

Attempts to restore the stem-loop structure in the central domain. Base-compensating mutations are often designed to lend support to a predicted secondary structure. We focused initially on the mutant *ls3*, in which part of the short stem and the top of the presumed loop of stem-loop 6 were substituted by the linker-derived sequence CAGAUCUG (Fig. 2). This substitution should not allow the pairing necessary for the formation of stem-loop 6. Mutant *ls3* is nonfunctional both in vivo and in DAI autophosphorylation inhibition assays in vitro (26, 27, 29). To determine whether function is restored by reestablishing the ability to form stem-loop 6, we first made the base-compensating mutants

FIG. 2. Schematic of mutant sequences. The sequences are superimposed on the original secondary-structure model (27).

ls3a and *ls3b* (Fig. 2). Substitution *ls3a* was obtained by replacing nt 101 and 102 in *ls3* (CA) with the wild-type sequence UU to restore the proposed pairing with the wild-type sequence AA (nt 118 and 119). Conversely, substitution *ls3b* was made by replacing the sequence AA (nt 118 and 119) in *ls3* with the sequence UG to allow the pairing of nt 118 to 102 and 119 to 101, thereby recreating a presumptive short stem in stem-loop 6 but with a different sequence (Fig. 2).

These potentially base-compensating mutants did not fully restore wild-type function. As illustrated in Fig. 3 and shown in Table 2, only at the highest concentration tested (50 $\mu\text{g/ml}$) did mutant *ls3a* inhibit DAI autophosphorylation as efficiently as wild-type VA RNA did. It was less efficient at the lower concentration of 20 $\mu\text{g/ml}$ and completely lost its inhibitory function at 5 $\mu\text{g/ml}$. Mutant *ls3b*, on the other hand, completely failed to restore the lost inhibitory function of mutant *ls3* (29) and was inactive even at the high concentration of 50 $\mu\text{g/ml}$ (Fig. 3, Table 2).

To seek an explanation for these results, we examined the changes in the secondary structure of each of the compensating mutants. The nuclease sensitivity pattern of *ls3a* RNA in stem-loop 6 (Fig. 4A) is more similar to that of wild-type VA RNA than to that of the nonfunctional mutant *ls3* (Fig. 5). However, the three A bases in loop 2a which are very susceptible to attack by ss-specific nucleases in wild-type VA RNA are resistant in mutant *ls3a*. Moreover, mild cleavages in stem 3b suggest melting of the stem and new nuclease V_1 cuts that appear in the substituted sequence indicate that this sequence may be involved in a secondary or tertiary structure. These changes are diagrammed in Fig. 5, in which the cleavage patterns are superimposed on the wild-type central domain structure. Thus, the structure and partial function of *ls3a* RNA are compatible with pairing in stem-loop 6, and alterations elsewhere in the central domain

FIG. 3. Inhibition of DAI activation by VA RNA mutants. Kinase assays were conducted with or without reovirus dsRNA and the indicated RNAs at the concentrations indicated. Shown are sections of autoradiograms illustrating DAI autophosphorylation.

could explain its lack of function at 5 $\mu\text{g/ml}$. However, structural analysis of mutant *ls3b* suggests that the substituted bases CA (nt 101 and 102) and UG (nt 118 and 119) do not pair since there is an ss cut after the C at nt 101 and there are no nuclease V_1 cuts after nt 99 (Fig. 5). One interpretation of these results is that nt 101, 102, 118, and 119 are important for function but do not form the stem structure depicted in Fig. 1A.

Functional and structural analysis of mutants with changes in loop 6. The reduced function of mutant *ls3a* could be

TABLE 2. Inhibition of DAI activation in vitro by central-domain mutants

VA RNA	Inhibition when present at ^a :		
	50 $\mu\text{g/ml}$	20 $\mu\text{g/ml}$	5 $\mu\text{g/ml}$
Wild-type VA RNA ₁	+++	+++	++
Loop mutants			
del103-109	+++	+++	++
del110-117	++	++	+
sub110-117	+++	++	+
ls3a	+++	++	—
Stem mutants			
sub118,119	+++	++	—
sub101,102	+++	+++	+++
sub102,118,119	+++	+++	—
sub101,102,118,119	+++	+++	+
Stem-loop mutants			
ls3	+	—	—
ls3b	—	—	—

^a +++ , complete inhibition; ++ , partial inhibition; + , slight inhibition; — , no inhibition.

FIG. 4. Nuclease sensitivity analysis. Wild-type VA RNA₁ and *ls3a* RNA were labeled with ³²P at their 5' ends and then partially digested with the enzymes marked. (The specificities of the enzymes are given in parentheses.) Shown are autoradiograms of polyacrylamide gels run for short (A) and long (B) times. The regions of the molecule and nucleotide numbers are marked. Abbreviations: T1, RNase T₁; BC, *B. cereus* RNase; U2, RNase U₂; T2, RNase T₂; V1, nuclease V₁ from cobra venom.

attributed to the presence of the mutant sequence GAUCUG in the top part of the proposed loop. This sequence could interfere with the formation of wild-type structure (as indicated by the *ls3a* RNA digestion pattern shown in Fig. 5), or it could be that the wild-type primary sequence is essential per se for function. To test these possibilities, we made mutant *del103–109*, corresponding to *ls3a*, by deleting the loop sequence between nt 103 and 109. In addition, to determine whether the lower part of the potential loop is important for function, we introduced two mutations into this region. Nucleotides 110 to 117 were deleted to produce mutant *del110–117*, and the same bases were substituted by the sequence CUACACAU to produce mutant *sub110–117* (Table 1; Fig. 2).

Mutant *del103–109*, in which the 15-base loop was shortened to 8 nt, inhibited DAI autophosphorylation nearly as efficiently as wild-type VA RNA did (Fig. 3; Table 2), indicating that the sequence between nt 103 and 109 is not essential for function in vitro and implying that nt 103 to 109 in *ls3* are detrimental, perhaps because they interact with other sequences in the VA RNA molecule, thereby disturbing its structure. However, deleting nt 110 to 117 impaired VA RNA function to some extent at all concentrations tested (Table 2). Even at the high concentration of 50 µg/ml, this mutant RNA did not completely inhibit activation of DAI by dsRNA. The loop substitution mutant *sub110–117* at concentrations of 20 and 5 µg/ml was less efficient than wild-type VA RNA (Fig. 3; Table 2). These results suggest that, unlike the upper part of stem-loop 6, the wild-type VA RNA

sequence in the lower part of the loop may be important for function.

The digestion pattern of the functional deletion mutant, *del103–109*, is compatible with that of wild-type VA RNA, except that nuclease V₁ cuts appear in stem-loop 6 and stem 3a (Fig. 5). These cuts are compatible with the model structure suggested for wild-type VA RNA (Fig. 1A) and with alternative structures such as that illustrated in Fig. 1C. In contrast, the deletion of nt 110 to 117 caused major rearrangements in the central domain, as can be deduced from the structure analysis (Fig. 5). Strong ss-specific cleavages appeared in the sequence immediately adjacent to the deletion, and weaker cuts appeared in stem 3b. New nuclease V₁ cuts in stem 3a and between nt 95 and 99 suggest that these sequences are involved in base pairing. The cleavage pattern of *sub110–117* depicted in Fig. 4B shows new strong ss cuts between nt 118 and 122 and nt 129 and 136, while nt 123 to 127, which are extensively cut by ss-specific nucleases in wild-type VA RNA, are resistant in the mutant and new nuclease V₁ cuts appear after nt 126 and 128 (Fig. 5). These changes may correlate with the reduced function of the mutant.

Mutants with substitutions in the short stem of stem-loop 6: functional and structural analysis. An essential feature of the proposed central domain structure is the formation of a stem containing a minimum of 3 bp. Analysis of the structure of the base-compensating mutant, *ls3b*, suggests that this pairing does not exist, possibly because of the new sequence introduced between nt 103 and 109. Strong nuclease V₁ cuts

FIG. 5. Cleavage sites in the central domain. The results of analyses such as in Fig. 4 are summarized by using the convention defined in Fig. 1. Wt, wild type.

in the substituted bases in the loop may indicate that they are involved in a secondary structure, perhaps involving other parts of the molecule, that can interfere with the pairing at the base of the loop (Fig. 5).

To examine this possibility, we generated four "stem

mutants" based on the model of Fig. 1B, by substituting two or more nucleotides in the proposed stem of stem-loop 6 (Fig. 2). Mutant *sub101,102,118,119* is a base-compensating mutant expected to pair in the base of the central-domain loop. Unlike mutant *ls3b*, in which the substituted bases in

FIG. 6. Activity of mutant VA RNAs in vivo. Plasmids encoding the RNAs indicated were cotransfected with a CAT expression plasmid, and the units of CAT resulting were measured. (A) CAT activity is expressed relative to a negative control transfected with pUC119 instead of a VA RNA plasmid. The control marked (–) was transfected with pEM-7 instead of a VA RNA plasmid. (B) CAT activity is expressed relative to a pEM-7 control. WT, wild type.

the loop may create spatial disturbances, *sub101,102,118,119* has wild-type sequences in the loop. Indeed, *sub101,102,118,119* is effective in blocking DAI activation (although it is less effective at 5 $\mu\text{g/ml}$ [Table 2]), but nuclease sensitivity analysis suggests that the expected pairing of C at nt 101 with G at nt 119 does not occur. The nuclease digestion pattern (Fig. 5) shows a single-stranded cut after C at nt 101, and new nuclease V_1 cleavages appear in stem 3a and between nt 95 and 99. Also notable is a strong V_1 cut after C at nt 116 (missing in wild-type VA RNA) and weak V_1 cleavages at the base of loop 2a. These minor changes in the digestion pattern, taken together, lead to the conclusion that in this mutant the central domain is composed of a different structure from the one suggested for wild-type VA RNA. An attractive possibility is that nt 96 to 99 (UGCG) pair with nt 35 to 32 (ACGC), as indicated in the alternative model (Fig. 1C).

Mutant *sub102,118,119*, which is another base-compensating mutant with a C $\rightarrow$ U transversion compared with *sub101,102,118,119* (Fig. 2), lends support to the conclusion that the central-domain structure in these mutants is incompatible with the original model structure for the central domain. The digestion pattern of *sub102,118,119* RNA is similar to that of *sub101,102,118,119*, except that there is a ss cut after G-100, again indicating that the short stem is not formed (Fig. 5). *sub102,118,119* RNA is effective in blocking DAI activation at the high concentration of 50 $\mu\text{g/ml}$ and at 20 $\mu\text{g/ml}$ but does not inhibit activation at 5 $\mu\text{g/ml}$ (Table 2). This argues strongly in favor of the view that the stem-loop structure per se is not important for function in vitro but that changing the sequence either of nt 101 and 102 or of nt 118 and 119 is deleterious.

Substitution mutants *sub101,102* and *sub118,119* distinguish between these two possibilities. The AA-to-GU change in *sub118,119* impaired the inhibition function at 20 and 5 $\mu\text{g/ml}$ but did not cause significant changes in the nuclease digestion pattern (Fig. 5). This result may suggest that the bases AA in positions 118 and 119 are important for function and that replacing them affects the efficiency of the RNA in blocking DAI activation. Mutant *sub101,102* was

designed to test simultaneously the significance of the AA dinucleotide for function and the validity of stem-loop 6 structure in the central domain by replacing UU with CA in the presumed short stem (Fig. 2). When *sub101,102* RNA was tested in in vitro kinase assays, it was found to block DAI activation efficiently even at the low concentration of 5 $\mu\text{g/ml}$ (Fig. 3; Table 2). Moreover, when the activity of *sub101,102* RNA was compared with wild-type VA RNA by making serial dilution of the RNAs, it was estimated that *sub101,102* is four times more efficient than wild-type VA RNA in blocking DAI activation by dsRNA (data not shown). Hence the sequence UU in wild-type VA RNA is not essential for function in vitro and can be replaced by the sequence CA. This is consistent with the conclusion that a stem-loop structure in the central domain is not essential for function.

Mutant function in transient-expression and cell-free translation assays. In previous studies, the ability of VA RNA mutants to rescue protein synthesis in vivo was tested in cells infected with the VA RNA $_1$ -negative virus Ad5 *dl331* (27). To assess the functional consequences of some of the new central domain mutants, we examined mutants *ls3*, *ls3a*, and *ls3b* in this assay. For this purpose, the original 3'-end sequence was restored to allow pol III termination: the T7-based wild-type plasmid stimulated CAT expression as efficiently as did pMHVA (27), which contains the unmodified VA RNA gene (data not shown). On the basis of the kinase assays described above, *ls3* and *ls3b* RNAs were expected to be inactive while *ls3a* might exhibit partial activity compared with wild-type VA RNA. All three mutants proved to be inactive, however (data not shown). Because the range of the *dl331* rescue assay is small (<six-fold) and the activity of *ls3a* might therefore have been missed, we turned to an alternative assay in which VA RNA enhances the expression of a cotransfected CAT reporter gene (37). As seen in Fig. 6A, in this assay wild-type VA RNA $_1$ increased CAT expression by over 70-fold while the mutants *ls3*, *ls3a*, and *ls3b* were almost ineffective, giving increases of only 2- to 3-fold over the results obtained with the control plasmid. Thus, in the case of *ls3a* there is a

FIG. 7. Activity of mutant RNAs in cell-free translation. Reticulocyte lysate assays contained the RNAs indicated at 3 μ g/ml (left column) or 6 μ g/ml (right column) as well as 20 μ g of reovirus dsRNA per ml. Globin synthesis is presented as a percentage of that obtained in the absence of reovirus dsRNA.

discrepancy between the kinase assay results and those obtained *in vivo*. We considered the possibility that this discrepancy is related to the fact that *ls3a* RNA is completely defective *in vitro* at the low concentration of 5 μ g/ml, even though it is active in the kinase assay at high concentration. Therefore we tested the activity of *sub101,102*, a central-domain mutant that is fully functional at this concentration. This mutant was assayed for its ability to enhance CAT expression in parallel with three other functional and partially functional central-domain mutants, *sub118,119*, *del103–109*, and *sub101,102,118,119* (Table 1). Surprisingly, *sub101,102* and the three other mutants were also all ineffective in this assay (Fig. 6B). Thus, the discrepancy does not seem to be attributable to an effect of RNA concentration.

The *in vivo* experiments indicated that all of the central-domain mutants tested were defective in stimulating CAT expression, whereas, as shown above, some changes were tolerated in the kinase assay. We therefore decided to test some of these mutants in a reticulocyte lysate translation system which is sensitive to the dsRNA-mediated activation of DAI (10). Figure 7 shows that wild-type VA RNA is able to protect globin synthesis from activation of DAI in this system while the defective mutants *ls3b* and *sub118,119*, as expected, exhibited little or no function. In accordance with the kinase assay results (Fig. 3), *sub101,102* was more active than wild-type VA RNA at low concentration but equivalent at high concentration. Thus, protein synthesis in the reticulocyte system responds to changes in the central domain of VA RNA in the same way as the direct kinase assay but differently from assays conducted *in vivo*.

DISCUSSION

The original model structure for Ad2 VA RNA (Fig. 1A) was based principally on nuclease sensitivity analysis (Fig. 1B) and supported by mutagenic studies (6, 26, 27, 29).

These studies defined the central-domain structure but did not test the structure or disclose its functionally important features. To examine the structural elements of the central domain that are important for function, we started with substitution mutant *ls3*, which replaces 9 bases of the wild-type sequence with 8 different bases and prevents the formation of stem-loop 6 (26). The *ls3* mutation leads to structural changes in the central domain, and *ls3* RNA function is impaired *in vivo* (26, 27) and *in vitro* (29). On the basis of results with mutant *ls3*, we made two base-compensating mutants, *ls3a* and *ls3b*, and seven additional deletion and substitution mutants to test specific features of the proposed structure of the central domain. The mutants were tested for their function *in vitro* and *in vivo*, and the results are summarized in Table 1.

Structural and functional implications. The two potentially base-compensating mutants *ls3a* and *ls3b* failed to restore the function of the central-domain mutant *ls3*; *ls3a* was only partly active in DAI inhibition assays while *ls3b* was completely inactive. Structural analysis of *ls3a* and *ls3b* RNAs showed perturbations in the central domain and suggested that neither mutant regained the proposed wild-type structure (Fig. 5). This observation led to two avenues of investigation. First, a set of mutants with changes located in the hypothetical loop (*del103–109*, *del110–117*, and *sub119–117*) was designed to test whether the wild-type sequence in loop 6 is essential for the formation of a functional structure or, alternatively, is important *per se* for function. A second set of mutants, with changes in the hypothetical stem (*sub102,118,119*; *sub101,102,118,119*; *sub101,102*; and *sub118,119*), was designed to challenge the existence of stem-loop 6 in the central domain (Fig. 1).

The “loop mutations” showed that the upper part of the loop can be deleted with little impairment of function (*del103–109*), while the deletion or substitution of the lower part of the loop (*del110–117*, *sub110–117*) was more deleterious. Since the nuclease digestion pattern of deletion mutant *del103–109* is very similar to that of the wild type, we conclude that bases 103 to 109 are not important for structure or function *in vitro* (although they are required *in vivo*, as discussed below).

Results obtained with the “stem mutants” extend the conclusions obtained with the potentially base-compensating mutants *ls3a* and *ls3b*. Although mutants *sub102,118,119* and *sub101,102,118,119* efficiently inhibited DAI activation *in vitro*, their nuclease digestion pattern in the central domain was not consistent with the proposed wild-type structure (Fig. 1A). Thus, it seems that even without possible interference from adjacent bases (as in *ls3a* or *ls3b*), bases 101 and 102 do not pair with bases 119 and 118 to form the proposed stem-loop structure of the central domain.

A powerful argument for an alternative structure in the central domain comes from the surprising observation that mutant *sub101,102*, which introduces nucleotides that interfere with base pairing in the stem of stem-loop 6, is more efficient than the wild type in inhibiting DAI activation by dsRNA. On the basis of the nuclease digestion pattern of *sub101,102* RNA and the other “stem mutant” RNAs, a possible alternative model structure can be advanced (Fig. 1C). The pairing scheme in the alternative model structure is identical to the structural model originally proposed for VA RNA (Fig. 1B) except in the central domain. nt 96 to 99 pair with nt 35 to 32 to form a more open secondary structure in the central domain. The large loop that composes the central domain has sequences that are protected from ss cuts (nt 119 to 121 and nt 139 to 135), and this, together with the two

major nuclease V_1 cuts after nt 115 and 131, suggests that tertiary interactions are involved in shaping the central domain. More support for a tertiary structure comes from mutant *ls3a*, in which substitutions in nt 103 to 109 affect the digestion pattern in loop 2a on the opposite side of the model (Fig. 5). It should be emphasized, however, that other secondary structures are also compatible with the data available (20). Furthermore, Furtado et al. have suggested that a drastically altered VA RNA molecule can rearrange to form an active structure (6). Evidently the critical features of the central domain remain to be discovered.

Implications of the discrepancies between in vivo and in vitro experiments. Some of the new central-domain mutants behaved differently in the in vivo assays used in this study, the *dl331* rescue assay and the CAT cotransfection assay, than in the in vitro kinase and reticulocyte assays. While wild-type VA RNA increased CAT gene expression by over 70-fold, even central-domain mutants that were functional in vitro failed to show a similar effect. Mutant *sub101,102*, which does not allow the formation of stem-loop 6 and is ineffective in vivo, is fully functional in vitro and is even more active than the wild type at low concentrations. This observation supports our suggestion that VA RNA adopts an alternative structure in which base changes in the central domain can be functionally tolerated.

What is the structure of the central domain, and why is there a discrepancy between the in vivo and in vitro assays? Our data do not suggest any additional Watson-Crick base pairing in the central domain of the secondary structure proposed in Fig. 1C, forcing us to invoke other types of interaction that can contribute to the tertiary structure. Possibilities include base stacking and hydrogen bonds between bases and phosphates or sugar 2' OH groups. Such interactions are seen in yeast phenylalanine-tRNA (14) and in RNA hairpins that contain the GNRA loops found in the most frequently occurring RNA hairpins in 16S and 23S rRNA (13). Tertiary structures are also crucial for function in the tRNA-like structure at the 3' terminus of turnip yellow mosaic virus RNA and other plant viruses (2, 32). In these viral RNAs, the formation of a specific three-dimensional structure based on an anomalous cloverleaf secondary structure is the key to faithful recognition of the RNA terminus by aminoacyl-tRNA synthetases. It is altogether likely that a specific three-dimensional structure is necessary for the recognition of VA RNA by DAI.

Several central-domain mutants are active in vitro, as shown here, but no central-domain mutant has been produced so far which is functional in vivo (6, 27; see above). The discrepancy between the two kinds of assay could be explained by assuming that VA RNA exerts two different actions, one of which is observed only in vivo. In the reticulocyte lysate translation system, as in the in vitro kinase assay, VA RNA competes with dsRNA for binding to the RNA-binding site on DAI (9, 17). Previous data showed that the integrity of the apical stem is essential for the binding of VA RNA at low RNA concentrations (26, 29) and that the central domain may stabilize the binding (1, 7). This effect of VA RNA may be less dependent on an optimal structure of the central domain, and therefore some base changes can be accommodated. In the transient-expression assay, VA RNA increases the expression of a reporter gene at least in part by preferentially enhancing the accumulation of certain mRNAs, probably by regulating their stability on polysomes (35, 37). This second effect of VA RNA may be dependent on an optimal structure of the central domain, which can be achieved only with the wild-type primary

sequence. It is also possible that some changes in the central domain can be tolerated for this function as well but that no such changes were included in the central-domain mutants tested. Resolution of these issues must await further analysis of the interactions between RNA and DAI (9, 22), as well as refinement of the VA RNA structure by new methods (5) including three-dimensional modeling (21) and phylogenetic analysis (20). An alternative resolution of the discrepancy recognizes that assays conducted in vitro are influenced by the assay conditions selected. For instance, deviations in the ionic conditions can lead to changes in the RNA structure (1). Moreover, cells contain many potential ligands and other factors which may affect the interaction between VA RNA and DAI. Both of these entities are to some extent ribosome associated in the cell (17, 34), and a fraction of the VA RNA is also associated with the La antigen (19, 24); furthermore, the related Epstein-Barr virus RNAs (EBERs) are associated with a ribosomal protein (38, 39). Interactions of this sort may place restrictions on the structure of VA RNA such that only optimal central-domain structures can be tolerated in vivo. The significance of these additional interactions for VA RNA structure and function is not yet understood.

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