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Binding of the adenovirus VAI RNA to the interferon-induced 68-kDa protein kinase correlates with function

(virus-associated RNA/translational control/RNA secondary structure)

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ABSTRACT **In human cells infected with adenovirus, the virus-associated RNA VAI blocks the activation of the interferon-induced double-stranded-RNA-dependent 68-kDa protein kinase (p68) and maintains normal levels of protein synthesis at late times after infection. VAI antagonizes the kinase activity by binding to p68. The structure of VAI consists of two long, base-paired stems connected by a complex short stem–loop structure. Previous work using a series of adenovirus mutants showed that the structural determinants of the VAI RNA that are essential for function reside in the central complex short stem–loop structure and adjacent base-paired regions (functional domain); the long duplex regions were found to be dispensable for function. To determine whether binding of VAI to p68 correlates with function and whether the structural determinants that are essential for function are also essential for binding, we studied the interaction of wild-type and several mutant VAI RNAs with p68 in whole cells. The p68–VAI complexes from mutant- and wild-type-infected cells were immunoprecipitated by an anti-p68 monoclonal antibody. The mutant RNAs that functioned efficiently in the cells bound to p68 efficiently in the cells, whereas functionally impaired mutants failed to bind to p68, indicating that the binding of the VAI RNA to p68 correlates well with function. *In vitro* binding assays with immunopurified p68 confirmed these observations. Secondary-structure analysis of several mutant VAI RNAs suggests that the binding does not depend on the long duplex regions but requires all the elements of the functional domain. We propose that the functional domain and the p68-binding domain of the VAI RNA are identical.**

The virus-associated (VA) RNAs of adenovirus (Ad) are two small, RNA polymerase III-transcribed gene products of about 160 nucleotides, designated VAI and VAII, that accumulate to high levels at late stages of the virus growth cycle (1–4). VAI constitutes the major portion of the viral RNAs at late times after infection. Both RNAs are highly structured (5–8). During virus infection, the VAI RNA is required for efficient initiation of translation (9–11) and serves as a viral defense against the cellular antiviral response (12–14). It interacts with an important component of the cellular antiviral defense, the interferon-induced double-stranded RNA (dsRNA)-activated 68-kDa protein kinase (p68; also referred to as DAI and P1/eIF-2α kinase, refs. 15–20), which phosphorylates and thereby inactivates the vital translation initiation factor eIF-2 (21–24). The phosphorylated eIF-2 traps the second initiation factor, eIF-2B (guanine nucleotide exchange factor, GEF), leading to a translational arrest (21–24). In Ad infections the VAI RNA blocks the activation of p68 produced by the cell, thereby enabling protein synthesis to proceed (12–14). VAI can also function in transient assays, presumably by interacting with p68 (25–27).

The role of VAI RNA in p68-mediated translational control is direct, since the purified VAI can block p68 activation *in vitro* (refs. 12, 13, and 20; G.D.G. and B.T., unpublished results), bind to p68 in intact cells (28) and *in vitro* (28–30), and inhibit binding of dsRNA to p68 *in vitro* (20). The activity of p68 can be stimulated by low concentrations of both natural and synthetic dsRNAs, but high concentrations of duplex RNAs inhibit its activity (15–20, 29, 31–33). Analysis of VAI secondary structure shows that the RNA exists in solution as a highly base-paired molecule with two long, imperfectly base-paired stems of 23–25 base pairs (bp) joined at the center by a short stem–loop structure (7, 8). Because VAI RNA can block the activation of p68, its extended base-paired structure has been considered important for its function (12, 13). The long imperfectly base-paired duplex regions were thought to mimic dsRNA and prevent binding of dsRNA to p68; the VAI RNA would not mimic the dsRNA in activating the p68, because of several mismatches in its two long stems (7, 8, 12, 13).

Contrary to expectations, it has been shown (7, 8) by mutational analysis that the function of VAI is not dependent on the extended base-paired regions. Rather, function seems to correlate well with the integrity of the elements present in the central part of the molecule. This part of the VAI, designated the functional domain, consists of a complex short stem–loop structure and several base-paired nucleotides adjacent to it (7, 8). This observation is not consistent with the role proposed for the duplex regions of the molecule in the mechanism of VAI RNA function (12, 13). It seems that VAI may inhibit p68 by binding to the kinase at sites other than the dsRNA-binding site.

To understand how this small RNA can inhibit the autophosphorylation of p68, we must identify the domain in the RNA with which p68 interacts and the structural features of the RNA essential for p68 binding and determine whether binding of VAI to p68 correlates with its function. To address these questions, we examined the interactions between p68 and various mutant VAI RNAs in living cells and *in vitro*. Our results show that the mutant RNAs that function efficiently in cells bind p68 efficiently in cells and *in vitro*, whereas functionally impaired mutants fail to bind p68 in both assays, indicating that the binding of VAI to p68 correlates well with function. Secondary-structure analysis of several mutant VAI RNAs suggests that binding does not depend on the two long duplex regions but requires all the elements of the functional domain located in the central part of the molecule. We propose that the structural features of the VAI RNA required for function and p68 binding are identical.

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Abbreviations: Ad, adenovirus; dsRNA, double-stranded RNA; mAb, monoclonal antibody; WT, wild type.

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MATERIALS AND METHODS

Mutant *dl*704 is a phenotypically wild-type (WT) variant of Ad type 5 (Ad5) and lacks the gene for the minor VA RNA species, VAII (34). Ad mutants with linker-scanning, deletion, and insertion mutations in the VAI gene and the plasmid that is capable of transcribing the VAI gene from the phage T7 RNA polymerase promoter (pA2WT/T7) have been described (8, 35). All Ad mutants used in this study lack the gene for the minor, VAII species (8, 35). p68–VAI RNA complexes formed in virus-infected cells (cell line 293, an Ad5-transformed human embryo cell line) and *in vitro* were analyzed exactly as described (28). The secondary structures of mutant VAI RNAs were analyzed as reported (8).

RESULTS

Analysis of p68–VAI Complexes Formed in Virus-Infected Cells. The experimentally derived secondary structure of VAI RNA consists of two extended base-paired regions, stems I and III, which are connected by a short base-paired region, stem II (Fig. 1*A*; refs. 7 and 8). Stems II and III are connected at the 3′ side by a complex short stem–loop (stem IV and loop C). In addition, in three regions RNA sequences loop out (loops A, B, and D). To determine the sequences and domains critical for function within this RNA structure, a series of Ad5 mutants in which the VAI gene was mutated by linker-scanning substitutions, deletions, and insertions were constructed and characterized (8, 35). Disruption of the base-paired regions in the distal parts of long 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 severe loss of function. These results led to the conclusion that the functional domain of the VAI RNA resides in the central part of the molecule. We have used several of these Ad5 mutants in this study to determine whether binding of VAI correlates with function and whether the functional domain of VAI is also the domain with which p68 interacts to modulate its activity. The locations of the mutations of the nondefective and defective mutant VAI genes are shown in Fig. 1 *B* and *C*, respectively. Five of these mutants are nondefective. Of these, substitution mutants *sub*706 (+1 to +11), *sub*707 (+18 to +27), and *sub*749 (+140 to +150) are linker-scanning mutants that presumably perturb base-paired regions of stem I (numbers in parentheses refer to location of mutations). Mutant *in*708 is an insertion mutant in which eight bases are inserted at +27. Mutant *dl*713 is a deletion mutant lacking six nucleotides (+72 to +79) near loop B. Substitution mutants *sub*719 (+26 to +45), *sub*709 (+43 to +53), *sub*741 (+76 to +90), *sub*743 (+90 to +105), *sub*745 (+105 to +117), *sub*746 (+116 to +126), *sub*747 (+122 to +134), and *sub*748 (+134 to +144) are linker-scanning mutants that disrupt the functional domain of the VAI RNA (ref. 8 and see below). Ad variants that carry these mutant genes are defective for polypeptide synthesis, growth yield, and p68 kinase activity (8).

We used an immunoprecipitation approach described by Katze *et al.* (28) to analyze the p68–VAI complexes formed in cells infected with the nondefective and defective Ad5 variants shown in Fig. 1 *B* and *C*. Previous work (8) showed that the mutant VAI genes of these viruses were transcribed efficiently; thus, RNA levels were not limiting for function or for interaction with p68. Cells (line 293) were infected with WT and mutant viruses shown in Fig. 1 *B* and *C* at 25 plaque-forming units per cell for 20 hr; [^{32}P]orthophosphate was present during the last 4 hr of infection. Cells were lysed and the p68 was immunoprecipitated from labeled cell lysates corresponding to equal amounts of trichloroacetic acid-precipitable radioactivity by incubation with an anti-p68 monoclonal antibody (mAb) (28). The labeled RNA that coimmunoprecipitated with p68 was recovered and analyzed by electrophoresis in a denaturing 8% polyacrylamide gel. To

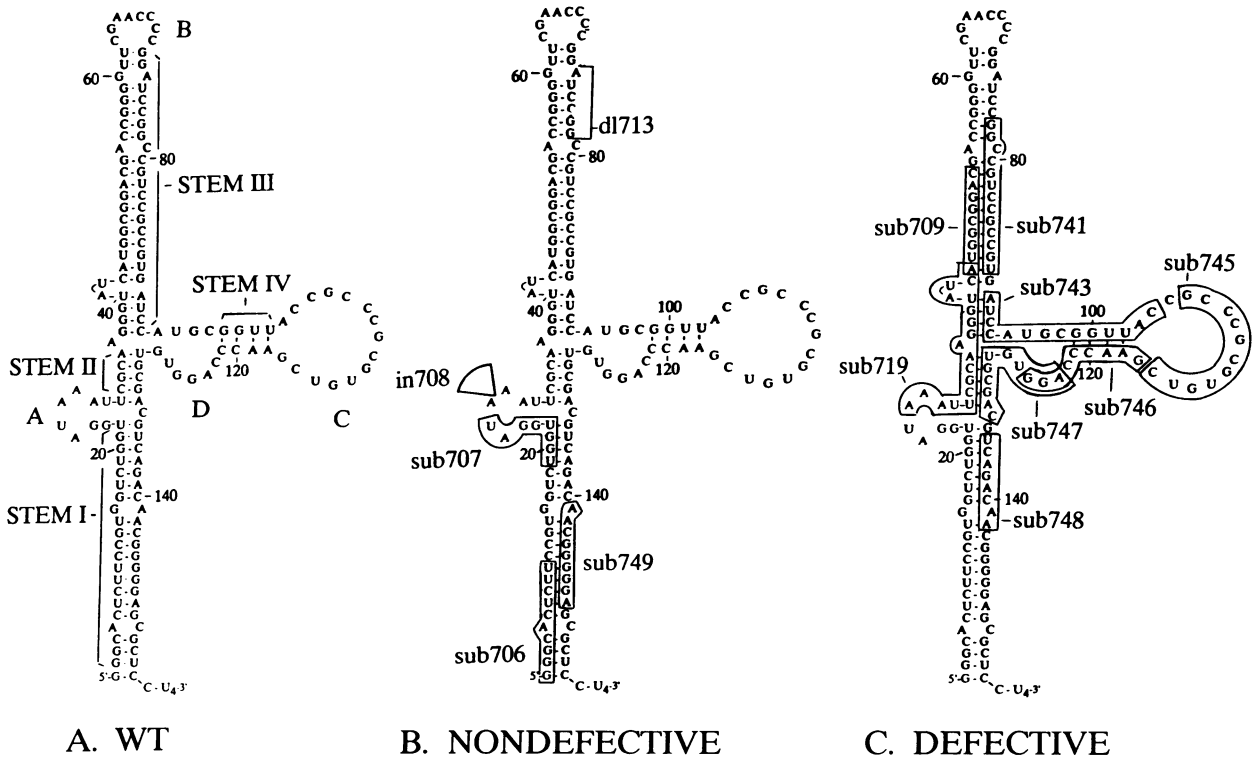

FIG. 1. Secondary-structure model of the Ad2 VAI RNA based on ribonuclease cleavage patterns (*A*) and location of the VAI mutations on the secondary structure of the WT VAI RNA (*B* and *C*). This model has features common to that proposed by others (5–7). A, B, C, and D are looped regions. The mutants are grouped into nondefective (*B*) and defective (*C*) based on their phenotype (8). Linker-scanning mutations are shown by boxes. Deletion mutation is shown by a bracket. Insertion mutation is shown by a large triangle.

ensure that the cells were infected efficiently, the expression of the VAI gene in these infections was monitored by extracting the VAI RNAs from a portion of the cell lysates and analyzing them in denaturing polyacrylamide gels (8, 35).

The VAI RNAs synthesized in cells infected with nondefective mutants *sub*706, *sub*707, *in*708, *dl*713, and *sub*749, and the labeled RNAs recovered after immunoprecipitation of the same lysates, are shown in Fig. 2 *A* and *B*, respectively. The levels of VAI RNAs synthesized by these variants were comparable to that of WT, indicating that cells were infected with mutants efficiently. The VAI RNAs recovered from the p68–VAI complexes formed in cells infected with these mutants were also comparable (Fig. 2*B*), indicating that the VAI RNAs of these mutants were able to form stable complexes with p68 efficiently. The nature of the faster-moving bands in lanes corresponding to mutants *sub*719 (Fig. 2*A*) and *in*708, *dl*713, and *dl*704 (Fig. 2*B*) is not known; these may be degraded products or VAI RNAs with altered conformations that we see occasionally when heat is generated in gels during electrophoresis.

We then tested the ability of substitution mutants 709, 719, 741, 743, 745, 746, 747, and 748 to interact with p68. All these mutants are severely defective, and their mutations are located in the functional domain of the molecule (ref. 8 and Fig. 1*C*). Abundant quantities of VAI were detected in cells infected with these mutants, indicating that infection proceeded normally and the RNAs were intact (Fig. 2*A*). However, contrary to the results with WT or the nondefective mutants, negligible amounts of VAI were recovered from the immunoprecipitated p68 for these mutants (Fig. 2*B*). These results were reproduced in several independent experiments. Thus, we conclude that in living cells, the mutants that are able to function at WT levels can bind to p68 efficiently, whereas the VAI mutants that fail to function also fail to interact with p68.

Analysis of the p68–VAI Complexes Formed *in Vitro*. To determine whether the results with whole cells could be reproduced in cell-free assays, we examined the *in vitro* association of several mutant VAI RNAs with p68. The WT and the substitution mutants 709, 741, 743, 745, 746, 747, 748, and 749 were transcribed *in vitro* from the T7 promoter after cloning into a T7 vector (8), and the RNAs were purified in nondenaturing polyacrylamide gels (Fig. 3*A*). Interestingly, the mutant RNAs for *sub*709 and *sub*741 do not migrate with anomalous mobilities in nondenaturing gels, whereas they do in denaturing gels (Fig. 2*A* and ref. 8). p68 from virus-infected 293 cells was purified with mAb-Sepharose (28) and incubated with equal quantities of gel-purified, uniformly ^{32}P-labeled VAI RNAs obtained by *in vitro* transcription (Fig. 3*B*, + lanes). In parallel, labeled RNAs were also incubated with mAb-Sepharose that did not contain p68 (Fig. 3*B*, − lanes). After 20 min of incubation at 30°C, the p68–VAI complexes were washed, and bound RNAs were recovered and analyzed in denaturing polyacrylamide gels (Fig. 3*B*). In control experiments (WT and the nondefective mutant *sub*749), the VAI RNAs bound to p68 efficiently, whereas defective mutant VAI RNAs bound to p68 at negligible levels. Thus we conclude that the mutant VAI RNAs that are defective for function in virus-infected cells do not bind to p68 (as assayed by coimmunoadsorption) either in whole cells or *in vitro*. We cannot, however, rule out the possibility that these defective mutant RNAs may interact with p68 in a less stable association.

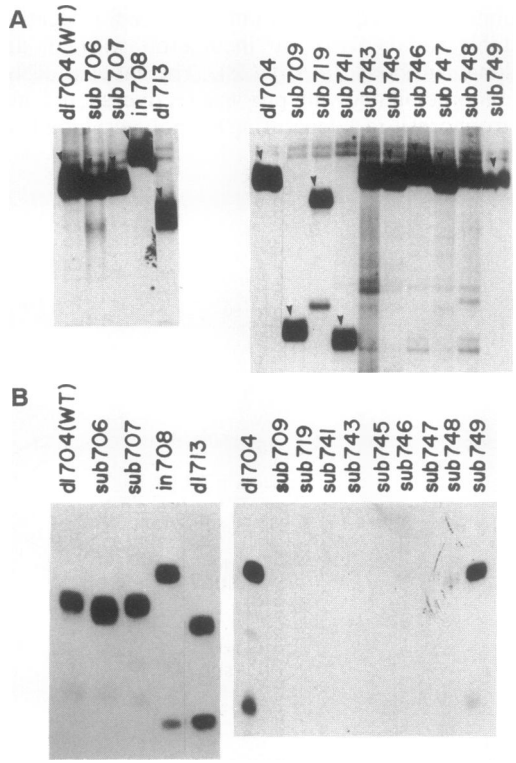

FIG. 2. Binding of p68 to WT, defective, and nondefective VAI RNAs in Ad-infected human cells. p68–VAI complexes were immunoprecipitated from virus-infected cells, by an anti-p68 monoclonal antibody (28), and the RNAs recovered from the complexes were analyzed in denaturing 8% polyacrylamide gels (35). (*A*) Electrophoretic analysis of VAI RNAs isolated from infected cell lysates used in immunoprecipitation analysis. Arrowheads show the positions of VAI RNAs. (*B*) VAI RNAs recovered from the p68–VAI complexes formed by Ad5 mutants in virus-infected cells. Only relevant portions of the autoradiograms are shown.

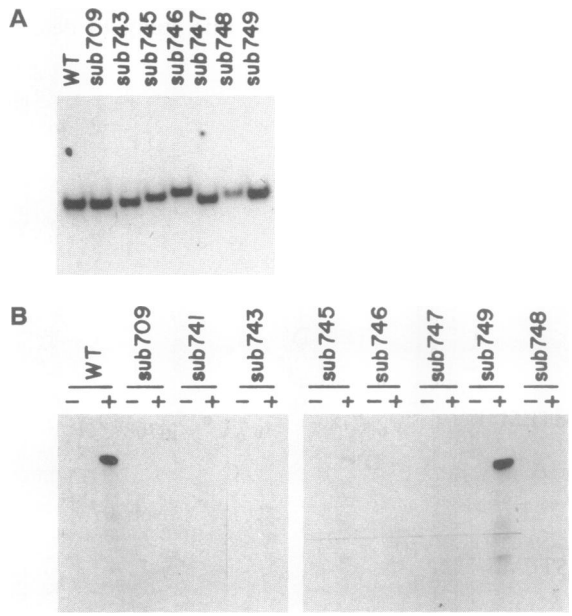

FIG. 3. Binding of p68 to WT and mutant VAI RNAs *in vitro*. (*A*) Electrophoretic analysis of WT and mutant VAI RNAs transcribed *in vitro*. The VAI genes were transcribed by T7 RNA polymerase *in vitro* and purified by electrophoresis in a nondenaturing 6% polyacrylamide gel. Data for *sub*741 RNA are not shown. (*B*) Electrophoretic analysis of RNAs bound to p68 *in vitro*. VAI RNAs were recovered from p68–VAI complexes (28) and analyzed in denaturing 8% polyacrylamide gel. Only relevant portions of the autoradiograms are shown. Lanes correspond to experiments in which RNA was added to mAb-Sepharose that was not previously reacted with interferon-treated cell extracts (−) or to experiments with interferon-treated cell extracts (+).

Secondary Structure of Mutant VAI RNAs. For correlation of the structural alterations in mutant VAI RNAs with their ability to function and to bind p68, we determined the secondary structure of four functionally defective mutants that also do not bind p68. Two of these, *sub*709 and *sub*741, contain mutations in the proximal part of stem III; the other two, *sub*745 and *sub*748, have mutations in the short stem–loop and the base-paired region extending to stem I, respectively (Fig. 1*C*). *In vitro* synthesized mutant and WT RNAs were labeled at either the 3′ or the 5′ ends and digested with single-strand-specific RNases and the partial cleavage products were resolved in denaturing polyacrylamide gels (ref. 8 and data not shown). The unpaired bases identified in the RNase cleavage maps were taken into consideration when the mutant RNA sequences were folded with a computer-assisted folding program (36, 37). The structures derived for the mutant RNAs with this combined approach are shown in Fig. 4. In all four mutants the integrity of the functional domain was destroyed. Substitution mutants *sub*709 and *sub*741, which contain mutations in the 5′ and the 3′ region, respectively, of the proximal part of stem III, had very open structures. In *sub*709, most of the sequence of stem III existed as a large single-stranded bulge, whereas in *sub*741, stem III was interrupted by four loops. In both mutants the structure of most of stem I was intact. Major perturbations in the secondary structures of *sub*745 and *sub*748 RNAs were also evident. Although mutations in *sub*745 RNA were located exclusively in loop C, the sequences of the short stem–loop in the functional domain were rearranged. Similarly, the structure of the functional domain was also dramatically altered in *sub*748. Interestingly, in both cases most of stem III, including the apical stem–loop structure, was intact. The structure of *sub*741 also contained an apical stem–loop with five G·C pairs. In spite of this, p68 failed to bind appreciably to these mutant VAI RNAs. We conclude that the extended base-paired duplex regions, including the apical stem–loop structure of stem III, are not sufficient for p68 binding; the binding of p68 to VAI requires the elements present in the functional domain.

DISCUSSION

The purpose of this study was to determine the structural requirements of the VAI RNA for p68 binding and to determine whether the functional domain identified in mutagenesis studies is also the kinase-binding domain. By analyzing p68–VAI complexes formed in WT- and mutant-infected cells, we showed that the mutant VAI RNAs that are unable to function are also unable to bind the kinase. As shown earlier, these mutations cluster in the functional domain (8). Secondary-structure analysis showed that mutants *sub*745 and *sub*748 (and very likely mutants *sub*746 and *sub*747) retain stem III and the loop (B) associated with it (apical stem–loop; Figs. 1*A* and 4). An apical stem–loop structure with five G·C pairs is also present in *sub*741 (Fig. 4). Mutants *sub*709, *sub*741, and *sub*745 also retain most or all of stem I (Fig. 4). All these mutants synthesize abundant quantities of VAI RNAs upon infection and yet these RNAs cannot complex with p68. The *in vitro* results complement these observations extremely well. Negligible levels of binding were observed *in vitro* for functionally impaired mutant RNAs. Thus, p68 does not bind to the defective VAI RNAs, although they contain extended base-paired regions or apical stem–loop structures. Instead, binding seems to correlate well with the integrity of the central part of the molecule. Since only functionally impaired VAI mutants fail to complex with p68 both in cells and *in vitro*, it is reasonable to conclude that binding correlates with function and that the functional domain of the RNA is also the p68-binding domain. Exactly what features of the functional domain are recognized by p68 will require further mutational analysis of the RNA as well as

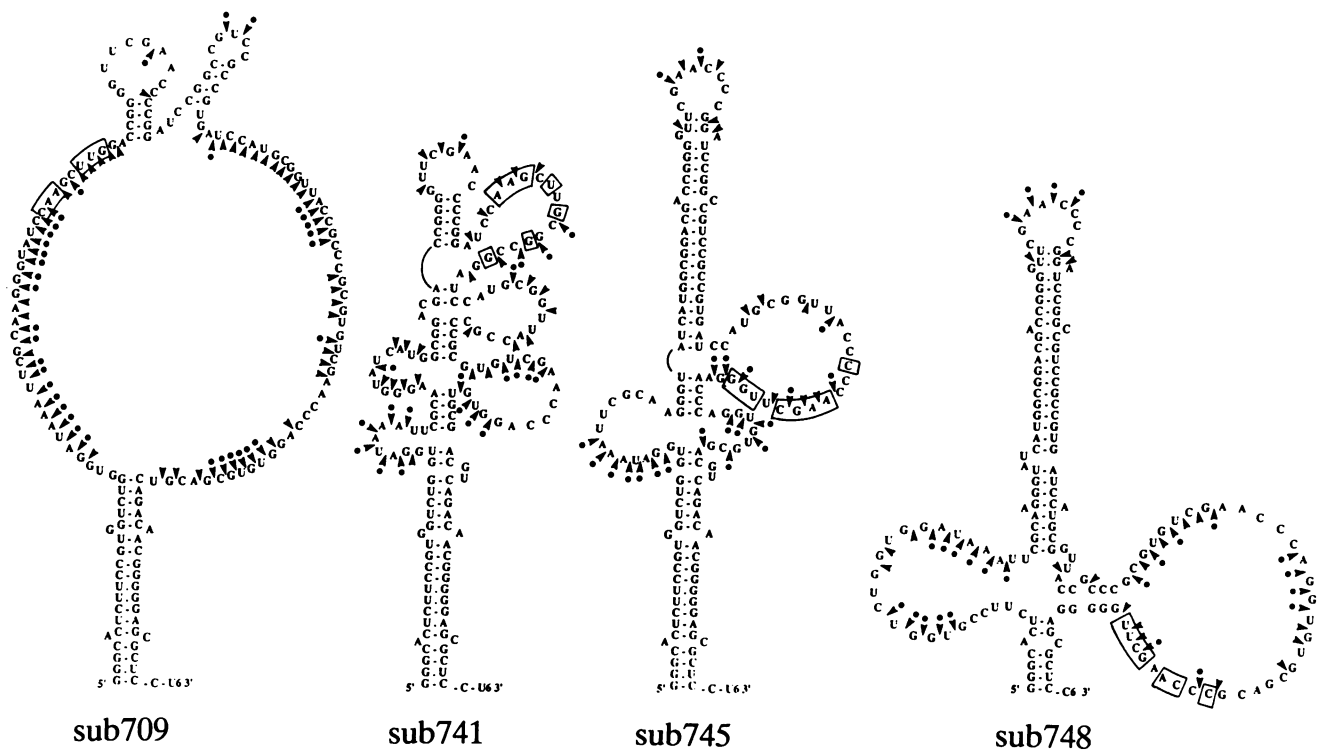

FIG. 4. Experimentally derived secondary structures for the VAI RNAs of *sub*709, *sub*741, *sub*745, and *sub*748 based on single-strand-specific RNase cleavage patterns (8). RNase cleavages are shown by arrowheads. Pronounced cleavages are denoted by dots. The mutated nucleotides are boxed. Nucleotide sequences of Ad2 and Ad5 VAI RNAs differ at two positions, and the Ad mutants used in this study contain VAI genes of both Ad2 and Ad5 origin (8, 35). The mutant VAI genes used for secondary structure analysis are of Ad2 origin. All VAI RNAs derived from T7 constructs contain six uridine residues rather than the four found *in vivo* (8).

knowledge of the three-dimensional structure of the RNA and its target, p68.

Mellits *et al.* (30) investigated interactions of several mutant VAI RNAs with a partially purified p68 preparation *in vitro* and found a lack of correlation between binding and function. Several severely defective mutants with mutations in the functional domain bound to p68 efficiently. Those authors concluded that all mutant molecules that can form an apical stem–loop structure can bind p68 efficiently. The short stem–loop in the functional domain was required for function but not for efficient binding, implicating the presence of two domains in the RNA, one for p68 binding and the second for inhibition of p68. Our results do not support this conclusion. Our results show that mutant VAI RNAs that contain the apical stem–loop identical to that of WT (*sub*745 and *sub*748, for example) or a newly formed apical stem–loop with five G·C pairs (*sub*741) do not bind to p68 in whole cells or *in vitro*. Binding requires all the elements present in the complex stem–loop structure and the adjoining duplex regions, providing evidence that the kinase-binding domain and the functional domain of VAI are indistinguishable. The discrepancies between our results and those of Mellits *et al.* (30) most likely can be explained based on the protocol differences. We used an immunoprecipitation assay whereas Mellits *et al.* (30) used filter binding and UV crosslinking followed by immunoprecipitation and recovery of RNA by antibodies on beads. The results of Mellits *et al.* are based solely on *in vitro* studies. Our results are based on the analysis of p68–VAI complexes formed in virus-infected cells and are supported by *in vitro* data. There is a very good agreement between the results of the two types of experiments. Therefore, we believe that these results most likely reflect the *in vivo* situation.

How does binding of VAI to p68 lead to the inhibition of p68 activity? Several important questions need to be addressed before a model can be proposed for the inactivation of p68 by VAI. Do the inhibitor (VAI RNA) and the activator (dsRNA) of p68 compete for the same site on p68? What elements in the functional domain are required for p68 binding? What is the significance of extremely large quantities of VAI in an infected cell at late times? Does VAI also interact with other host proteins (38), and if so, are they functionally significant?

The mechanism by which the VAI RNA blocks the activation of p68 is unknown. It is likely that VAI interferes with the binding of dsRNA to the kinase. Experimentally derived secondary structures show that the extensively base-paired stem III that is present in WT is also present in the defective mutants *sub*745 and *sub*748 (Fig. 4). And yet these mutants fail to bind p68. These and the p68 binding results of nondefective mutants such as *sub*706, *sub*749, and *dl*713, which presumably perturb the long duplex regions but retain the integrity of the functional domain, suggest that VAI may bind to the kinase at a site(s) other than that which is involved in dsRNA binding. It is possible that VAI binding may lead to a conformational change in the kinase molecule such that the activator cannot bind to it. A cDNA clone for p68 has been described (39). A structure–function map of this protein may provide clues to some of these questions.

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