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Characterization of a Low-Molecular-Weight Virus-Associated (VA) RNA Encoded by Simian Adenovirus Type 7 Which Functionally Can Substitute for Adenovirus Type 5 VA RNA_I

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Human adenoviruses (Ads), like Ad type 2 (Ad2) and Ad5, encode a low-molecular-weight RNA (designated virus-associated [VA] RNA_I) which is required for the efficient translation of viral mRNAs late after infection. We cloned and characterized a VA RNA gene from simian adenovirus type 7 (SA7) which appears to have biological activity analogous to that of Ad2 VA RNA_I. Thus, SA7 VA RNA stimulates protein synthesis in a transient expression assay and can also functionally substitute for VA RNA_I during lytic growth of human Ad5. The SA7 genome encodes only one VA RNA species, in contrast to human Ad2, which encodes two distinct species. This RNA is transcribed by RNA polymerase III in the rightward direction from a gene located at about coordinate 30 on the viral genome, like its Ad2 counterparts. SA7 VA RNA shows only a limited primary sequence homology with the Ad2 VA RNAs (approximately 55%); the flanking sequences, in fact, are better conserved than the VA RNA gene itself. The predicted secondary structure of SA7 VA RNA is, however, very similar to that of Ad2 VA RNA_I, inferring that the double-stranded nature rather than the primary sequence of VA RNA is important for its biological activity.

The adenovirus (Ad) family comprises a large number of serotypes which infect a variety of animal species ranging from frogs to humans. The human Ads, which have been most thoroughly studied, can be subdivided into six subgroups based on their oncogenic, immunological, and biochemical characteristics (for a review, see reference 32). The nononcogenic subgroup C adenoviruses (Ad type 2 [Ad2], Ad type 5 [Ad5]) have been characterized in the most detail and may therefore be considered as the prototype members of the mammalian Ad genus.

The highly oncogenic simian Ad type 7 (SA7) is by far the best characterized nonhuman serotype (for a review, see reference 14). The overall sequence homology between SA7 and Ad2 is about 30% (47), although regions which have been subjected to nucleotide sequence analysis show patches of more highly conserved domains (15, 47). The organization of genes and transcription units appear to be identical, and regulatory functions seem also to be well conserved between the human Ads and SA7. For example, the SA7 E1A transcriptional activator protein can stimulate the expression of Ad5 early genes (15), and SA7 DNA-protein complexes replicate in extracts prepared from subgroup C Ad-infected cells (27). To this end we characterized the low-molecular-weight virus-associated (VA) RNAs encoded by the SA7 genome.

VA RNAs are small RNA polymerase III transcripts (50) which are produced in large amounts late during an Ad infection (34). The Ad2 and Ad5 genomes encode two VA RNAs, designated VA RNA_I and VA RNA_{II} (22, 40). These RNAs are transcribed from two closely spaced genes, located at about coordinate 30 on the viral genome. They are both approximately 160 nucleotides long, show scattered regions of primary sequence homology, and can be predicted to form extensively base-paired secondary structures which resemble each other (1).

Studies of viral mutants have shown that VA RNA_I is required for the efficient translation of viral mRNAs late after infection (45). The absence of VA RNA_I, and therefore late polypeptide synthesis, also causes severe defects in late mRNA production. Thus, a VA RNA_I negative mutant shows both aberrant splicing of late mRNAs and reduced accumulation of mRNA from the major late transcription unit (43a). The site of VA RNA action is at an early step of polypeptide chain initiation (35, 37). VA RNA_I rescues the translational capacity of infected cells by inhibiting the activity of a double-stranded RNA-dependent protein kinase, which phosphorylates the α-subunit of eucaryotic initiation factor 2 (36, 38). Both VA RNAs appear to serve a similar function, although VA RNA_I is much more efficient (6).

We show here that SA7 only encodes one low-molecular-weight VA RNA which shows a limited primary sequence homology with the Ad2 VA RNAs. SA7 VA RNA appears, however, to be a functional analog of VA RNA_I because it can functionally substitute for it during lytic growth of Ad5.

MATERIALS AND METHODS

Virus growth and DNA and RNA preparation. Ad5 mutant dl331 and SA7 were grown and titrated on HeLa and CV1 cells, respectively, as described elsewhere (45, 47). Viral DNA was prepared by the method of Pettersson and Sambrook (31). Cloning in pBR322 vectors, including the addition of synthetic *Eco*RI linker DNA (dGGAATTCC; New England Biolabs, Inc., Beverly, Mass.), was by standard recombinant DNA techniques (20). Total cytoplasmic RNA was isolated from monolayer cells as described previously (43, 44).

In vitro transcription. Virion and plasmid DNA were transcribed in a soluble HeLa whole-cell extract prepared as described by Manley (21). In a standard 10-μl reaction, 1 μg of DNA was incubated with 3 μl of cell extract (giving final concentrations of 12 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.9], 60 mM KCl, 7.5 mM $MgCl_2$,

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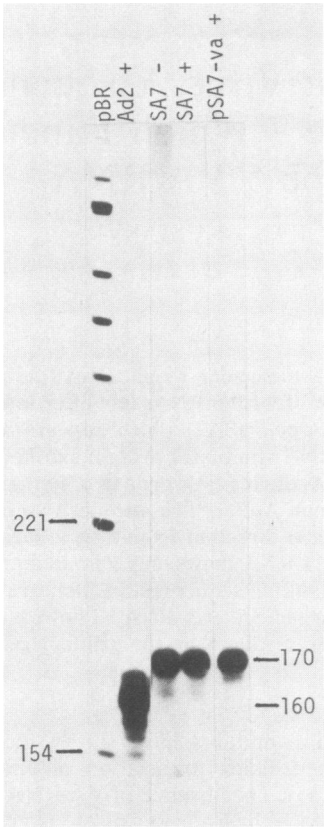

FIG. 1. RNA polymerase III transcription unit encoded by SA7. RNA synthesized in soluble HeLa whole-cell extracts (21), programmed with pSA7-va DNA (lane pSA7-va+), virion DNA purified from Ad2 (lane Ad2+), or SA7 particles (lane SA7− and SA7+), was separated by electrophoresis through a denaturating 8% polyacrylamide gel. The in vitro transcription reactions were performed either with (lanes Ad2+, SA7+, and pSA7-va+) or without (lane SA7−) 1 μg of α-amanitin per ml. Sizes (in nucleotides) of RNA transcripts (to the right) as well as a few pBR322 marker fragments (lane pBR; to the left) are indicated.

1.2 mM dithiothreitol, 10.2% glycerol) for 1 h at 30°C with 0.5 mM each of ATP, CTP, and UTP and 50 μM GTP containing 5 μCi of [α-^{32}P]GTP (New England Nuclear Corp., Boston, Mass.) as a label. In some assays α-amanitin (Sigma Chemical Co., St. Louis, Mo.) was included at a concentration of 1 μg/ml. After transcription, the reactions were phenol extracted, ethanol precipitated, and suspended in a small volume of 60% formamide containing 0.5% sodium dodecyl sulfate. Electrophoretic separation was through 8% polyacrylamide gels containing 8 M urea. When the structure of the in vitro-transcribed SA7 VA RNA was analyzed by S1 nuclease cleavage (see Fig. 5), the template DNA was digested with RNase-free DNase I (20 μg/ml; Worthington Diagnostics, Freehold, N.J.) for 5 min at 30°C before phenol extraction and ethanol precipitation).

DNA sequence analysis. The protocol of Maxam and Gilbert (25) was followed for end-labeling of fragments and subsequent sequence analysis.

S1 endonuclease analysis. The protocol of Berk and Sharp (3) and Weaver and Weissman (49) was followed, with minor modifications. RNA isolated from SA7-infected (CV1) cells (5 μg), RNA from 293 cells transfected with recombinant plasmids (10 μg), or RNA made in vitro in a HeLa whole-cell extract (prepared from a 50 μl reaction mixture) was hybridized overnight at 50°C (5′-end analysis) or 56°C (3′-end analysis) to the DNA fragments described in the legend to Fig. 5. S1 nuclease cleavage and electrophoretic separation was carried out as described previously (44).

Southern blot hybridization. Fractions of SA7 DNA (0.2 μg) were cleaved with restriction endonucleases, and the fragment mixtures were separated on a 1.4% agarose slab gel. The DNA was transferred to a nitrocellulose sheet as described by Southern (41) and hybridized with ^{32}P-labeled SA7 VA RNA, prepared by in vitro transcription, and purified by gel electrophoresis as described by Mathews and Pettersson (24).

Transfections. Subconfluent monolayers of 293 cells were transfected by the calcium phosphate coprecipitation technique (11, 52), as described previously (42, 44).

Chloramphenicol acetyltransferase assay. Preparation of extracts and chloramphenicol acetyltransferase (CAT) assay conditions were as described previously (10, 43).

Complementation of the translational defect of mutant dl331. Subconfluent monolayers of 293 cells (6-cm-diameter petri dishes) were transfected with 10 μg of plasmid pHindB,

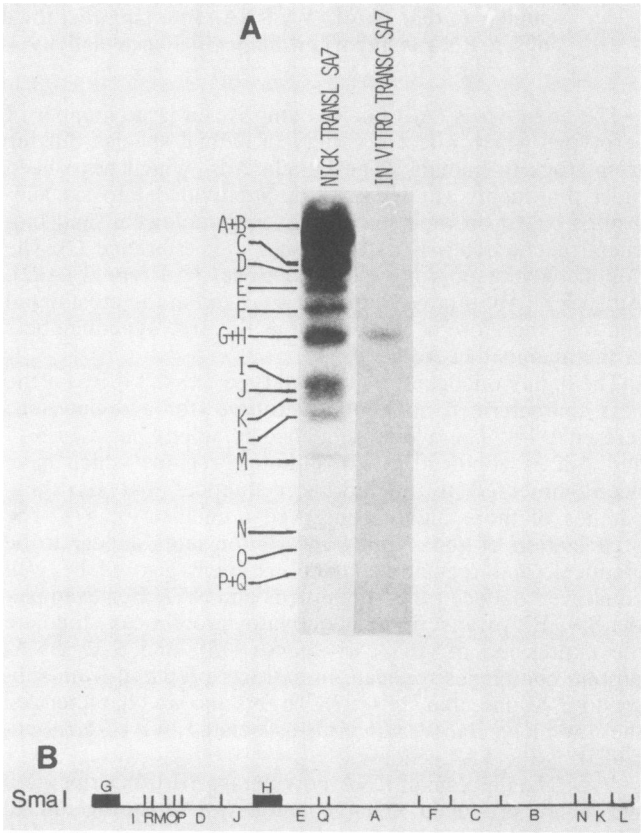

FIG. 2. (A) Hybridization between ^{32}P-labeled SA7 VA RNA and fragments of SA7 DNA transferred to a nitrocellulose filter by the method of Southern (41). ^{32}P-labeled SA7 VA RNA was prepared by in vitro transcription in a HeLa whole-cell extract progammed with SA7 DNA. The full-length RNA was eluted from a polyacrylamide gel and annealed to a nitrocellulose filter containing blotted *Sma*I fragments of SA7 DNA (lane marked in vitro transc [transcription] SA7). ^{32}P-labeled SA7 DNA was also hybridized to indicate the position of all *Sma*I fragments (lane marked nick-transl [translated] SA7). (B) Restriction endonuclease cleavage map of SA7 DNA for enzyme *Sma*I. ■, fragment mixture to which SA7 VA RNA hybridized.

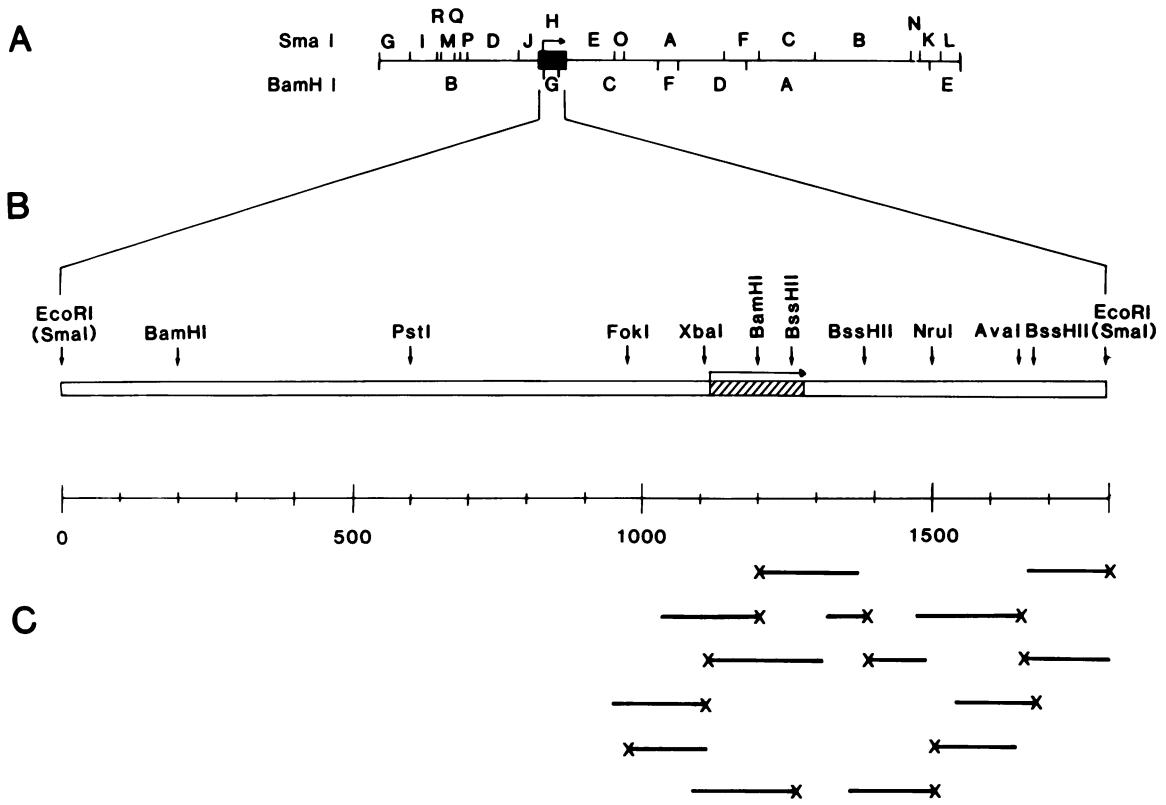

FIG. 3. Organization of the SA7 genome. (A) Restriction endonuclease cleavage map of SA7 DNA for enzyme *Sma*I and *Bam*HI (46). Symbols: ▬ position of the DNA insert in plasmid pSA7-va; arrow, direction of SA7 VA RNA transcription. (B) Enlargement of the 1,800-bp *Sma*I fragment insert in pSA7-va. Symbols: ▨, position of the SA7 VA RNA gene; arrow, direction of SA7 VA RNA transcription. The *Sma*I H fragment was cloned in plasmid pBR322 after the addition of *Eco*RI linkers. (C) Sequencing strategy. Symbols: ×, sites of end-labeling and extent and direction of the sequences that were determined. Numbers are in nucleotides.

pSA7-va, or pBR322. At 30 h posttransfection cells were infected for 45 min at 37°C with 10 fluorescent-forming units of mutant dl331 per cell (33). After removal of unadsorbed virus, cells were incubated for an additional 20 h at 37°C. At this time cells expressing late Ad polypeptides were visualized by indirect immunofluorescence, as described by Linné et al. (19), with an antibody directed against the Ad2 hexon polypeptide (30).

RESULTS

Identification of an RNA polymerase III transcript encoded by SA7. To determine if the SA7 genome encodes low-molecular-weight RNAs similar to the human VA RNAs, virion DNA from Ad2 and SA7 was used to program RNA synthesis in a HeLa whole-cell extract (21). As expected, Ad2 DNA directed the synthesis of a heterogeneous class of RNAs with a size of about 160 nucleotides corresponding to the VA RNA_I and VA RNA_{II} species (Fig. 1). SA7 DNA, on the other hand, directed the synthesis of a discrete transcript with a length of 170 nucleotides (Fig. 1). This RNA was also made in reactions containing 1 μg of α-amanitin per ml, suggesting that it most likely is transcribed by RNA polymerase III (51). As shown below, the 170-nucleotide species accumulated late during a lytic SA7 infection and therefore is referred to as the SA7 VA RNA.

Cloning of the SA7 VA RNA gene. To be able to characterize SA7 VA RNA in detail we constructed a recombinant plasmid encoding the SA7 VA RNA gene. A fragment suitable for cloning was identified by hybridization of ^{32}P-labeled SA7 VA RNA to Southern blots of SA7 DNA cleaved with restriction endonucleases. SA7 VA RNA hybridized to a doublet consisting of *Sma*I fragments G and H (Fig. 2). These fragments were isolated by gel electrophoresis and cloned into plasmid pBR322 after conversion of the *Sma*I terminus to *Eco*RI sites by the addition of synthetic linker DNA. To identify a recombinant plasmid encoding SA7 VA RNA small-scale plasmid preparations (13) were assayed for their ability to direct the synthesis of SA7 VA RNA in a soluble HeLa whole-cell extract. One positive clone, designated pSA7-va (Fig. 1), was selected for further characterization.

A partial restriction endonuclease cleavage map was constructed for the 1,800-base-pair (bp) DNA insert in plasmid pSA7-va (Fig. 3B), and the position of the SA7 VA RNA gene was mapped by Southern blot analysis, with ^{32}P-labeled SA7 VA RNA used as a hybridization probe (data not shown). The results demonstrate that SA7 VA RNA is encoded within a 400-bp DNA fragment that is located between the unique *Xba*I and *Nru*I cleavage sites in clone pSA7-va (positions 1100 and 1500; Fig. 3B) and, furthermore, that it, like Ad2 VA RNA_I (22), traverses a *Bam*HI restriction endonuclease cleavage site (Fig. 3B).

In analogy with the human subgroup C Ads the *Sma*I H fragment (coordinates 27.5 to 32.2; Fig. 3A) would be expected to encode the SA7 VA RNA gene. This prediction was confirmed by mapping the chromosomal position of the DNA insert in plasmid pSA7-va by Southern blot hybridization (data not shown). In summary, these results demonstrate that SA7 VA RNA is transcribed in the rightward

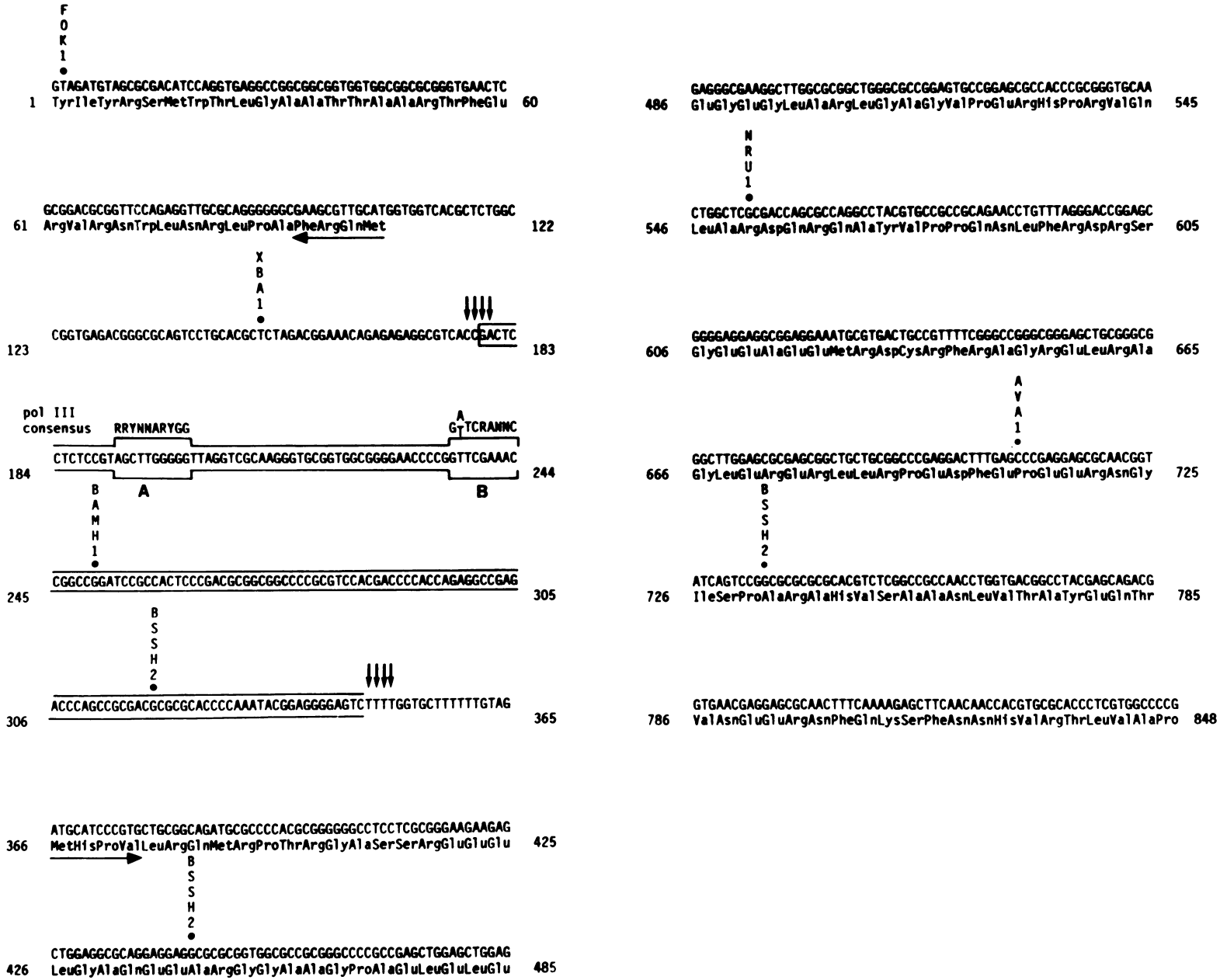

FIG. 4. Nucleotide sequence of the SA7 VA RNA gene and its flanking sequences. The DNA sequence of the sense strand of SA7 VA RNA is shown. The position of the SA7 VA RNA gene, as determined by S1 nuclease analysis, is shown (□). Nucleotides shown within the expanded boxes (A and B) correspond to the SA7 VA RNA sequences homologous to the control sequence for RNA polymerase (pol) III promoters. Vertical arrows indicate the position of 5′ and 3′ ends of SA7 VA RNA, as determined by S1 nuclease analysis. The position of two ORFs encoded by the viral l-strand (←) and r-strand (→) are also indicated. Symbols and abbreviations ●, position of cleavage sites for selected restriction endonucleases; R, pyrine; Y, pyrimidine; N, any base.

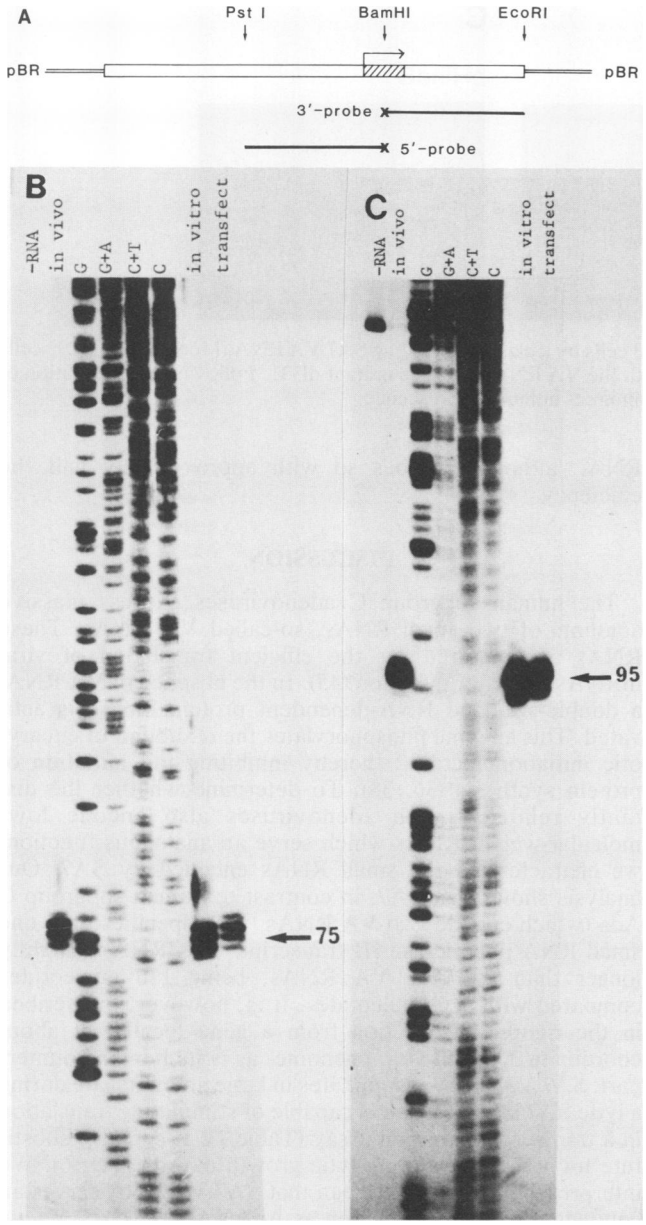

FIG. 5. S1 endonuclease analysis of the structure of SA7 VA RNA. (A) Strategy for the S1 endonuclease analysis. Total RNA was hybridized to the indicated 5'- and 3'-end-labeled DNA probes (asterisks show the position of the ^{32}P label) and treated with S1 nuclease. Resistant material was separated in parallel with the corresponding sequence ladder (lanes G, G+A, C+T, and C in panels B and C) through an 8% denaturing polyacrylamide gel. Symbols and abbreviations ▨, position of the SA7 VA RNA gene; arrow, direction of transcription; pBR, pBR322 vector DNA. (B) Mapping of the initiation site for SA7 VA RNA transcription. (C) Mapping of the position of the 3' end for SA7 VA RNA. Lanes for panels B and C: in vivo, RNA isolated from SA7 infected CV-1 cells; in vitro, RNA prepared from a whole-cell extract programmed with pSA7-va; transfect, RNA isolated from 293 cells transfected with plasmid pSA7-va. Sizes (in nucleotides) of S1 nuclease-protected fragments are indicated to the right.

direction from a gene located close to coordinate 30 on the SA7 genome.

Partial sequence analysis of plasmid pSA7-va. An 848-bp DNA sequence (Fig. 4) covering the *Xba*I-*Nru*I fragment was determined (Fig. 3C). Examination of the established SA7 DNA sequence revealed the likely position of the SA7 VA RNA gene (Fig. 4). Sequences homologous to the split promoter of RNA polymerase III genes (48), as well as two runs of thymidine residues (positions 347 to 350 and 356 to 361) which could function as termination signals (7) for SA7 VA RNA transcription, were identified in the established sequence. Furthermore, the SA7 VA RNA gene crosses a *Bam*HI restriction endonuclease cleavage site, as predicted from the hybridization data (Fig. 3B). Based on these considerations we tentatively conclude that an RNA polymerase III transcription unit is located somewhere between positions 175 and 360 in the established DNA sequence (Fig. 4).

Characterization of SA7 VA RNA. To confirm this prediction and to map, at the nucleotid level, the position of the SA7 VA RNA gene, the initiation and termination sites for transcription were determined by the S1 nuclease protection assay.

Two end-labeled DNA fragments were used for this analysis (Fig. 5A). To map the transcription start site a 600-bp *Pst*I-*Bam*HI DNA fragment (positions 600 to 1200; Fig. 3B), which was 5' end labeled at the *Bam*HI cleavage site, was used; and to position the 3' end a 600-bp *Bam*HI-*Eco*RI DNA fragment (positions 1200 to 1800; Fig. 3B), which was 3' end labeled at the *Bam*HI cleavage site, was used. After S1 nuclease cleavage the resistant material was separated on a denaturing polyacrylamide gel, together with the corresponding DNA sequence ladder.

Three sources of RNA were analyzed in parallel: (i) total cytoplasmic RNA isolated from late (25 h postinfection) SA7-infected CV1 cells (Fig. 5B and C, lane in vivo), (ii) RNA purified from whole-cell extracts programmed with plasmid pSA7-va (lane in vitro, Fig. 5B and C), and (iii) cytoplasmic RNA prepared from 293 cells transfected with plasmid pSA7-va DNA (lane transfect, Fig. 5B and C). The same protected DNA fragments were observed in all three RNA preparations (Fig. 5B and C). The 5'-end analysis (Fig. 5B) positioned the major transcriptional start site for SA7 VA RNA close to nucleotide 180 (Fig. 4). The exact initiation site is, however, ambigous because the S1 nuclease treatment left a slight heterogeneity at the end of the protected DNA fragment. Longer S1 nuclease-protected fragments, which most likely correspond to minor upstream transcription initiation sites, were also visible in the autoradiogram. The 3'-end analysis (Fig. 5C) positioned the termination site for SA7 VA RNA transcription to the first run of thymidine residues located between nucleotides 347 and 350 (Fig. 4). The thymidine cluster that serves as a termination signal is bordered by GC base pairs and is therefore expected to function as a very efficient transcription stop signal (7).

Northern blot analysis of RNA prepared late after a lytic SA7 infection failed to demonstrate the existence of additional virus-encoded low-molecular-weight RNAs (data not shown). Therefore, we conclude that SA7 encodes only one VA RNA: a low-molecular-weight RNA which resembles the Ad2 VA RNAs both in its basic structural features and genomic organization (see below).

Biological activity of SA7 VA RNA. Ad2 VA RNA_I is required for the efficient translation of viral mRNA late after infection (45). To determine whether SA7 VA RNA is also capable of serving as a positive effector in mRNA translation, we determined its biological activity by two experimental approaches. First, we tested whether SA7 VA RNA could enhance protein synthesis in a cotransfection assay. We have shown previously (42, 43) that the effect of VA

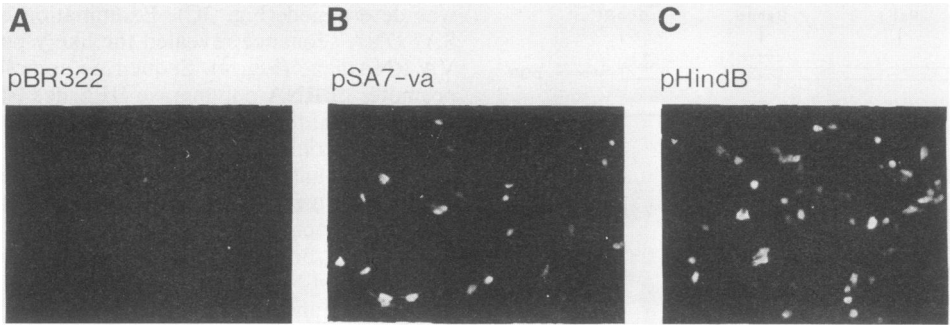

FIG. 6. Complementation of the translation defect in dl331-infected 293 cells by a plasmid encoding SA7 VA RNA. Monolayers of 293 cells transfected with plasmid pHindB, pSA7-va, or pBR322 were infected with the VA RNA_I-negative mutant dl331. Following an incubation of 20 h cells were assayed for the production of the hexon polypeptide by indirect immunofluorescence.

RNA on translation can be reproduced in a transient expression assay in 293 cells. The synthesis of viral as well as nonviral proteins can be significantly enhanced by cotransfection of plasmids that encode Ad2 VA RNA_I. Second, we determined whether SA7 VA RNA could complement the translational defect of Ad5 mutant dl331. This mutant is defective in late mRNA translation because of a small deletion in the VA RNA_I gene which prevents its expression (45).

For the first approach, a chimeric plasmid, pTripcat-2 (43), which expresses the bacterial CAT enzyme under the transcriptional control of the major late Ad promoter, was used. In separate experiments, monolayers of 293 cells were transfected with pTripcat-2 DNA, either together with plasmid pHindB, which encodes the Ad2 VA RNAs (42), or together with plasmid pSA7-va. Following an incubation period of 50 h, cell extracts were prepared and assayed for CAT activity (10). SA7 VA RNA was almost as effective as Ad2 VA RNA_I in stimulating CAT expression (Table 1). pHindB cotransfection resulted in an approximately eightfold stimulation of CAT compared with an approximately fourfold stimulation by pSA7-va. The cotransfections did not affect the level of CAT mRNA in the transfected cells (data not shown), suggesting that the effect is due to increased translational efficiency in VA RNA-producing cells.

For the second approach, monolayers of 293 cells were transfected with plasmid pHindB or pSA7-va. Approximately 30 h posttransfection the cells were superinfected with the VA RNA_I-negative mutant dl331 (45). Following an additional incubation period of 20 h, cells were fixed and assayed for the production of the hexon polypeptide by indirect immunofluorescence. pSA7-va cotransfection yielded approximately half the amount of brightly fluorescent cells as compared with pHindB cotransfection (Fig. 6). Results of these experiments indicate that SA7 VA RNA serves an analogous biological function as the Ad2 VA RNA_I, although it does so with approximately half the efficiency.

TABLE 1. Stimulation of CAT expression in 293 cells[a]

Plasmid	CAT activity (fold)[b] for the following expt:			Average
	1	2	3	
pBR322	1.0	1.0	1.0	1.0
pSA7-va	4.3	4.7	3.3	4.1
pHindB	9.8	7.7	7.8	8.4

[a] Cells were transfected with 2 μg of pTripcat-2 DNA and 10 μg of pBR322, pSA7-VA, or pHindB DNA.

[b] CAT activity was measured as described previously (10). The degree of acetylation obtained with pBR322 was arbitrarily set as 1.

DISCUSSION

The human subgroup C adenoviruses express massive amounts of two small RNAs, so-called VA RNAs. These RNAs are required for the efficient translation of viral mRNAs late after infection (45). In the absence of VA RNA_I a double-stranded RNA-dependent protein kinase is activated. This enzyme phosphorylates the α-subunit of eucaryotic initiation factor 2 thereby inhibiting the initiation of protein synthesis (36, 38). To determine whether the distantly related simian adenoviruses also encode low-moleular-weight RNAs which serve an analogous function, we characterized the small RNAs encoded by SA7. Our analysis shows that SA7, in contrast to human subgroup C Ads (which encode two VA RNAs [23]), specifies only one small RNA polymerase III transcript. This RNA is slightly longer than the Ad2 VA RNAs, being 170 nucleotides compared with 160 nucleotides. It is, however, transcribed in the rightward direction from a gene located at about coordinate 30 on the viral genome, as is its human counterpart. SA7 VA RNA accumulates in large amounts late during a lytic SA7 infection and is capable of stimulating translation in a transient expression assay (Table 1). It can also substitute for VA RNA_I during lytic growth of Ad5 (Fig. 6). We interpret these results to mean that SA7 VA RNA serves an equivalent biological function as Ad2 VA RNA_I

When considering these similarities in function, it is surprising that a nucleotide sequence comparison between Ad2 and SA7 VA RNA shows so little primary sequence homology, about 55% (Fig. 7). In fact, the flanking sequences were better conserved than the VA RNA genes themselves (exceeding 74%; Fig. 7). The Ad2 VA RNA_I and Ad2 VA RNA_{II} genes are about 60% homologous relative to each other (1). However, VA RNA_{II} shows only marginal biological activity compared with VA RNA_I (6, 42) and SA7 VA RNA (Table 1 and Fig. 6). Because SA7 VA RNA and Ad2 VA RNA_I appear to be functionally more related, we have aligned their nucleotide sequences against each other (Fig. 7). The 5′ half of SA7 VA RNA (nucleotides 1 to 70) shows a high degree of homology with VA RNA_I (approximately 68%). This region includes the two promoter elements (designated A and B, Fig. 7) that have been shown by mutational analysis to be required for Ad2 VA RNA_I transcription (5, 9, 12). The 3′ half (nucleotides 70 to 170; Fig. 7) shows a much lower primary sequence homology (approximately 45%). This region is, in fact, more homologous to Ad2 VA RNA_{II} (data

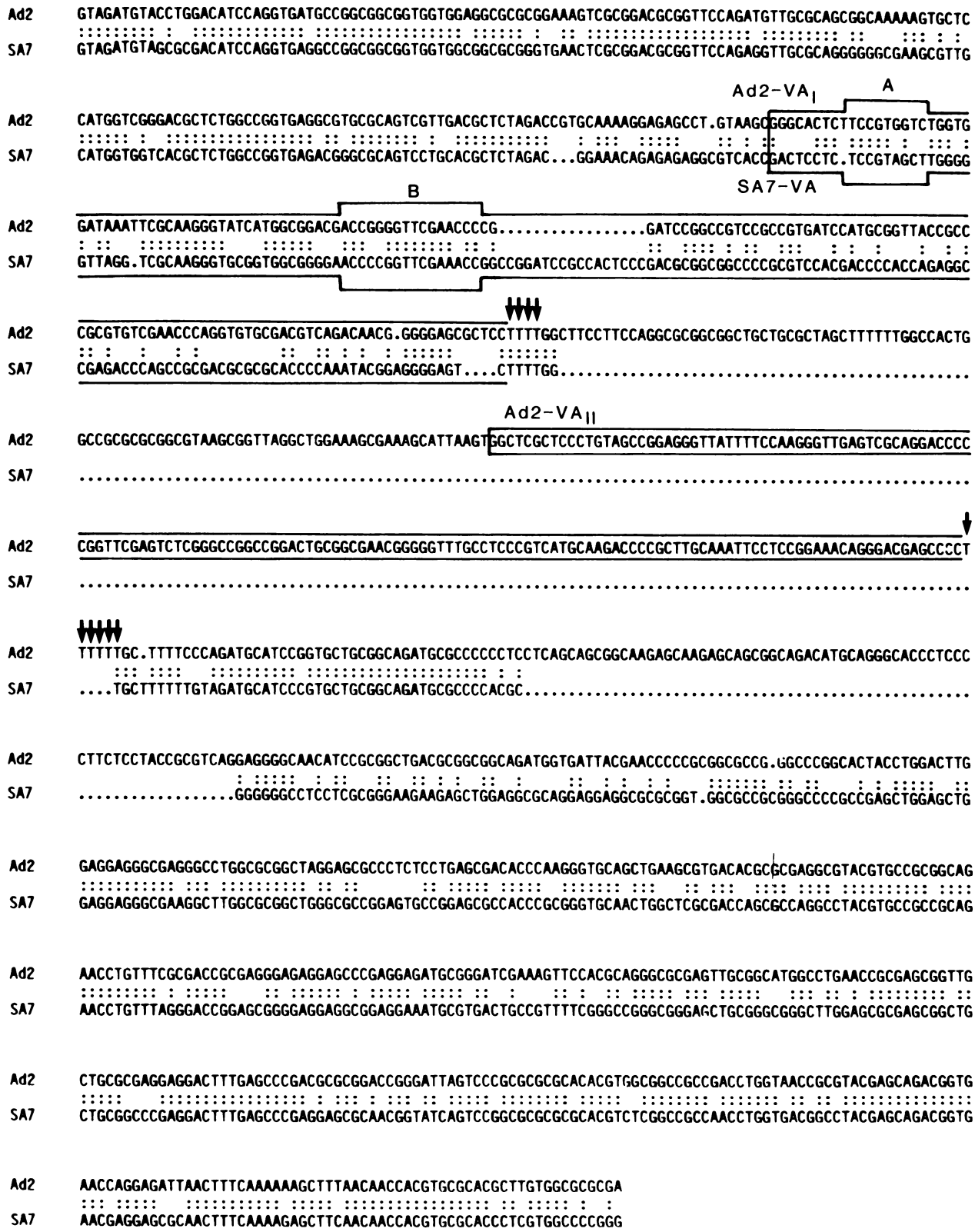

FIG. 7. DNA sequence comparison between the Ad2 and SA7 VA RNA gene regions. Nucleotides which are identical in the two genomes are indicated with a colon between the two sequences, and deletions are indicated (. . . .). The position of the Ad2 VA RNA_I, Ad2 VA RNA_{II}, and SA7 VA RNA genes are boxed in the sequence. Elements A and B correspond to the control sequences required for maximal VA RNA_I transcription (5). Arrows denote the position of the termination signals for VA RNA transcription. The homology was derived by computer analysis with the gap progarm developed by Devereux et al. (8).

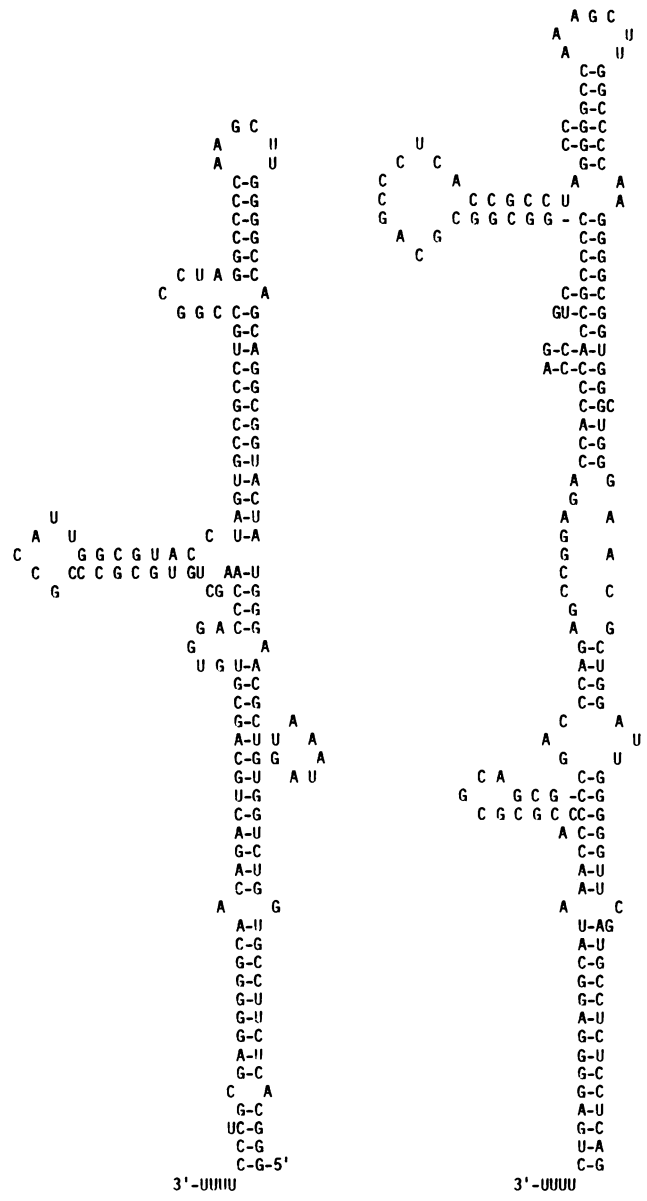

FIG. 8. Secondary structure of VA RNAs. The most stable structures for Ad2 VA RNA_I and SA7 VA RNA were derived by computer analysis with a program developed in our laboratory (1).

not shown). This result indicates that SA7 VA RNA consists of a hybrid between the VA RNA_I and VA RNA_{II} genes. This homology, however, is also weak and therefore we cannot conclusively trace the origin of the 3′ half of SA7 VA RNA.

Bhat et al., (4) have constructed a large number of mutant viruses in a search for sequences in the VA RNA_I gene which are of importance for its translational enhancement function. These data have shown that large pieces of VA RNA_I can be altered without any drastic impairment of the growth properties of the mutant viruses. However, sequences located between positions 43 and 53 and between position 107 and the 3′ end of the VA RNA_I gene appear to be of more critical importance for its biological activity. These sequences have been only partly conserved in SA7 VA RNA. The region from positions 43 to 53 (located immediately upstream of element B; Fig. 7) is better conserved (6 of 11 nucleotides) than the second functional domain located between position 107 and the 3′ end which shows only a fragmentary homology. Because of this low primary sequence conservation, it is unlikely that VA RNA mediates its translational enhancement function through the interaction of viral or cellular components with specific nucleotide sequences.

An attractive alternative is that the double-stranded nature of the VA RNAs is of functional importance. Computer-generated secondary structures predict that VA RNA_I folds into an extensively base-paired stem-loop structure in which the sequences at the 5′ and 3′ ends form a duplex region (1) (Fig. 8). Partial nuclease digestion data (28) have confirmed the basic principles of these predictions and have shown that VA RNA_I, free in a solution, contains a number of stable duplex regions. Direct support for the hypothesis that the secondary structure of VA RNAs is biologically significant comes from the study of O'Malley et. al. (29), who showed that VA RNA_I blocks activation of DAI (the double-stranded activated inhibitor of protein synthesis) during a lytic infection. This enzyme is activated by low concentrations and inactivated by high concentrations of double-stranded RNA (for a review, see reference 17). Furthermore, an avian adenovirus, chicken embryo lethal orphan virus, which is only distantly related to the human Ads, shows stronger conservation at the secondary structure level than does the primary sequence (16). Therefore, it can be predicted that SA7 VA RNA, which has only a limited primary sequence homology, assumes an extensively base-paired secondary structure that, in its key features, resembles Ad2 VA RNA_I (Fig. 8).

The sequences flanking the SA7 VA RNA gene are highly conserved and show homology with the sequences 5′ of the VA RNA_I gene and 3′ of the VA RNA_{II} gene (Fig. 7). Thus, the DNA segment equivalent to the intragenic spacer region and the Ad2 VA RNA_{II} gene are absent from the SA7 genome.

It is likely that the high level of conservation of the flanking sequences is due to their importance as protein coding sequences. Two open translational reading frames (ORFs), one on each side of the SA7 VA RNA gene, can be identified in the established DNA sequence (Fig. 4). These show high degrees of homology with sequences flanking the VA RNA genes (Fig. 7). The l-strand (the DNA strand transcribed in the leftward direction into mRNA) sequence upstream of SA7 VA RNA most likely encodes the amino terminus of the 87,000-molecular-weight terminal protein precursor (39). A potential initiator, methionine, followed by an ORF, is located 73 nucleotides upstream of the initiation site for SA7 VA RNA transcription (Fig. 4). The homology is high both at the DNA sequence (approximately 81%; Fig. 7) and amino acid sequence levels (data not shown). The r-strand (the DNA strand transcribed in the rightward direction into mRNA) sequence downstream of the SA7 VA RNA gene encodes an ORF which extends from position 366 to the right terminal end of the established sequence (Fig. 4). The amino terminus of this ORF shows an almost complete identity with the N terminus of the so-called 52,000, 55,000-molecular-weight polypeptide (31 of 32 nucleotides; Fig. 7) which is located immediately downstream of the Ad2 VA RNA_{II} gene (2, 18, 26). A patch homology to the 52,000, 55,000-molecular-weight gene continues after a gap of approximately 70 nucleotides in the SA7 sequence. The similarity in sequence (approximately 74%; Fig. 7) makes it very

likely that this ORF encodes the SA7 homolog of the Ad2 52,000, 55,000-molecular-weight polypeptide.

The stronger conservation of nucleotide sequences and ORFs on both sides of the SA7 VA RNA gene, compared with the VA RNA gene itself, lends further support for the hypothesis that the double-standed nature rather than the primary sequence of VA RNAs is the biologically significant feature.

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