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Virus-Associated RNAs of Naturally Occurring Strains and Variants of Group C Adenoviruses

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We compared the sequences of the virus-associated (VA) RNAs of group C adenoviruses, serotypes 1, 2, 5, and 6, and of three variants of adenovirus type 2 (Ad2) selected for loss of the *Bam*HI restriction site in the VA RNA_I gene. In the naturally occurring strains, VA RNA_I exists in two forms which differ by two nucleotides: one form is found in Ad2 and Ad6, and the other is found in Ad1 and Ad5. There are three sites of variation in VA RNA_{II}, the Ad1, Ad2, and Ad5 forms each differing from Ad6 VA RNA_{II} at one of the positions. One of the selected variants has a four-base duplication within the *Bam*HI cleavage site, whereas the two others have acquired a VA RNA_I sequence indistinguishable from that of Ad5. The findings are interpreted in terms of the secondary structures of the VA RNAs and the interrelationships among the viruses.

In addition to mRNA, transcription of adenovirus DNA leads to the production of large amounts of two small noncoding RNA species, virus-associated (VA) RNAs, which present several unusual features (22, 28, 36). The RNAs are found in cells infected with adenoviruses of all types examined, chiefly in the cytoplasmic fraction and overwhelmingly at late times in the infectious cycle, although their presence can be detected also in the nucleus and in the early phase of infection (9, 36, 40). Their synthesis, unlike that of mRNA, which is mediated by RNA polymerase II, is accomplished by RNA polymerase III (35, 40, 44-46). The two species, VA RNA_I and VA RNA_{II} (also known as 5.5S and 5.2S RNA), occur in a major form about 160 nucleotides in length and in an array of minor forms differing at both their 5' and 3' ends (5, 6, 16, 24, 43). Again in contrast to mRNA, the VA RNAs seem to be free of internal and terminal modifications such as methylation and capping, apart from a 5'-terminal polyphosphate vestige of RNA chain initiation (6, 25, 34, 36, 43). The genes that code for the VA RNAs are closely spaced, lying at or near the left (5') end of the main block of late structural genes, and are also transcribed in a rightward direction (22, 24, 32). The nucleotide sequences of both genes, their flanking regions, and the RNAs have been established by a combination of DNA and RNA sequence analyses (1, 5, 29, 31). Computer-assisted model building (1, 47) shows that the VA RNAs can adopt similar and rather stable hairpin-like conformations which presumably are important in their biological role. Their ability to bind mRNA has led to the suggestion that they may function in the splicing reaction (23), and hypothetical base-pairing schemes have been devised showing how VA RNA_I might facilitate the process by bridging across a splice point (27, 47).

Most of the available information on VA RNAs comes from studies of human adenovirus type 2 (Ad2), but existing data indicate that the properties of the VA RNAs of other adenoviruses are similar. Fingerprint analysis (2) shows that the primary sequence of the main species, VA RNA_I, is similar but not identical in the closely related Ad5, another group C strain, but is substantially different in the more distantly related human serotypes 7 and 12 (30, 42), belonging to groups B and A, respectively. To assess the range of variability which exists within a single family of viruses, we examined the structure of the VA RNAs of the group C adenoviruses, serotypes 1, 2, 5, and 6. In addition, we selected viable viruses mutant in the VA RNA_I gene to explore the permissible range of variability. The implications of the variations observed for the relationships of the group C viruses as well as for the secondary structures of the VA RNAs are considered.

MATERIALS AND METHODS

Cells and viruses. The human 293 (12) and HeLa cell lines were obtained from J. F. Williams. CV_1, an established line of African green monkey kidney cells, was obtained from J. Mertz. CV_1 and 293 cells were cultivated in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum and 100 μg of

streptomycin and of penicillin per ml. HeLa cells were cultivated in the same medium, with 10% calf serum replacing the fetal bovine serum. Suspension cultures of HeLa cells were grown in Eagle F13 medium with 5% calf serum.

Ad2 and Ad5 were originally obtained from U. Pettersson and J. F. Williams, respectively. Ad1 and Ad6 were obtained from the Centers for Disease Control, Atlanta, Ga. *1×51i* is an Ad2-Ad5 recombinant virus derived from a cross of the Ad5 mutant *ts*1 with the Ad2$^+$ND1- mutant *ts*51 (39). Viruses were propagated in suspension cultures or monolayers of HeLa cells. Plaque titrations were performed as previously described (15) except that $MgCl_2$ was omitted from the overlay medium for 293 cells.

Isolation of variants of *1×51i*. The virus *1×51i* was serially passaged undiluted in HeLa cells for 10 passages in order to accumulate mutant or recombinant viruses. The final stock was used to infect 1 liter of HeLa cells growing in suspension. Viral DNA was extracted from purified virions (33) and digested to completion with endonuclease *Bam*HI (New England Biolabs). A portion of the *Bam*HI-cleaved DNA was ligated with T4 DNA ligase (Miles Laboratories, Inc.). Ligated DNA or the *Bam*HI-restricted preparations of DNA were used to transfect 293 cells (1 μg of viral DNA per 60-mm plate) essentially as described by Graham and Van der Eb (13). The cells were treated with glycerol at 4 h postinfection to boost transfection efficiencies (11). Two hours after the boost, the medium was removed, an agar overlay was added, and the plates were incubated at 37°C for 7 to 10 days. Plaques were picked from the plates with a sterile pipette, the virus was suspended in phosphate-buffered saline, and the solution was sonicated. Stocks of virus were grown in HeLa cells and titrated by plaque assay on HeLa and 293 cells.

Extraction and analysis of viral DNA. A modification of the Hirt procedure (17) was used for separating viral DNA from large chromosomal DNA. Confluent monolayers of CV_1 or HeLa cells in 60-mm dishes were infected with virus at a multiplicity of about 10 PFU/cell. When a cytopathic effect on the cells was visible at about 24 h postinfection, the medium was removed, 2 ml of buffer (10 mM Tris, pH 7.0, 1 mM EDTA, and 0.5% sodium dodecyl sulfate) with pronase (0.5 mg/ml) was added, and the plates were incubated for 2 h at 37°C. After addition of 0.5 ml of 5 M NaCl, the contents of each plate were poured into a Beckman SW56 tube, held at 0°C for 4 to 16 h, and then centrifuged for 30 min at 35,000 rpm. The supernatant solutions were decanted and extracted twice with phenol (saturated with 50 mM Tris, pH 7.8) and once with chloroform-isoamyl alcohol (24:1). Nucleic acids were precipitated and reprecipitated with ethanol. One 60-mm dish yielded up to 25 μg of viral DNA which was sensitive to cleavage by restriction endonuclease. Digestions were carried out according to recommendations of the manufacturers of the enzymes (Bethesda Research Laboratories or New England Biolabs), and the fragments were resolved by electrophoresis through agarose gels (24) after degradation of RNA by incubation with pancreatic RNase (20 μg/ml).

Examination of VA RNAs. ^{32}P-labeled VA RNAs were isolated and analyzed essentially as previously described (24). Briefly, HeLa cells in suspension culture were infected with adenovirus at a multiplicity of 10 to 100 PFU/cell, washed in phosphate-free medium, resuspended, and labeled with [^{32}P]phosphate from 2 to 18 h postinfection. Cytoplasmic RNA was prepared by phenol and chloroform extraction, precipitated, and subjected to electrophoresis through polyacrylamide gels run at low temperature in the presence of Sarkosyl and glycerol. After autoradiography, the VA RNA bands were excised from this first gel, soaked in an 8 M urea solution, and applied directly to a second gel, which contained 7 M urea. The VA RNAs were eluted from the second gel, purified by chromatography, and analyzed by standard methods (2). After limit digestion with pancreatic or T_1 RNase, oligonucleotides were resolved by two-dimensional fingerprinting. Electrophoresis in the first dimension was carried out on cellulose acetate strips at pH 3.5 in 7 M urea; the second dimension was on DEAE-paper in 7% formic acid or, for pancreatic digests of VA RNA_I, in pH 1.9 buffer. The resultant oligonucleotides were located by autoradiography, quantified, and subjected to further digestions to elucidate their structures.

RESULTS

Selection of variants in the VA RNA gene region. We used a slight modification of the procedure of Jones and Shenk (18) to select variants which lack the *Bam*HI restriction endonuclease site located near the middle of the gene for Ad2 VA RNA_I at position 29.0 on the viral genome. Viral DNA, digested with the *Bam*HI restriction enzyme, was religated with T4 DNA ligase and used to transfect human 293 cells. Molecules which escaped digestion or were reconstituted by ligation gave rise to plaques. The virus contained in each plaque was grown and screened for loss of *Bam*HI recognition sites. To increase the chances of isolating variants of the desired kind, we started with an adenovirus that is a recombinant between Ad5 and the nondefective adenovirus-simian virus 40 hybrid Ad2$^+$ND1, designated *1×51i* (39). The DNA of this recombinant from the left end of the genome to beyond the VA RNA genes is derived from Ad2, whereas the remainder derives from Ad5 with the exception of a short Ad2-specific stretch surrounding position 70. Consequently, this virus contains only two *Bam*HI sites, at positions 29 and 59.5, rather than the three found in Ad2. Loss of the site at position 29 leads to the generation of two fragments, 59.5 and 40.5 units in size, after *Bam*HI digestion instead of three fragments of 40.5, 30.5, and 29.5 units. Of 69 plaques analyzed, 2 virus stocks, variants 1 and 2, were isolated which had lost the *Bam*HI site at position 29 (Fig. 1). The remaining isolates gave the parental restriction pattern. One further stock, variant 3, was produced by a similar procedure

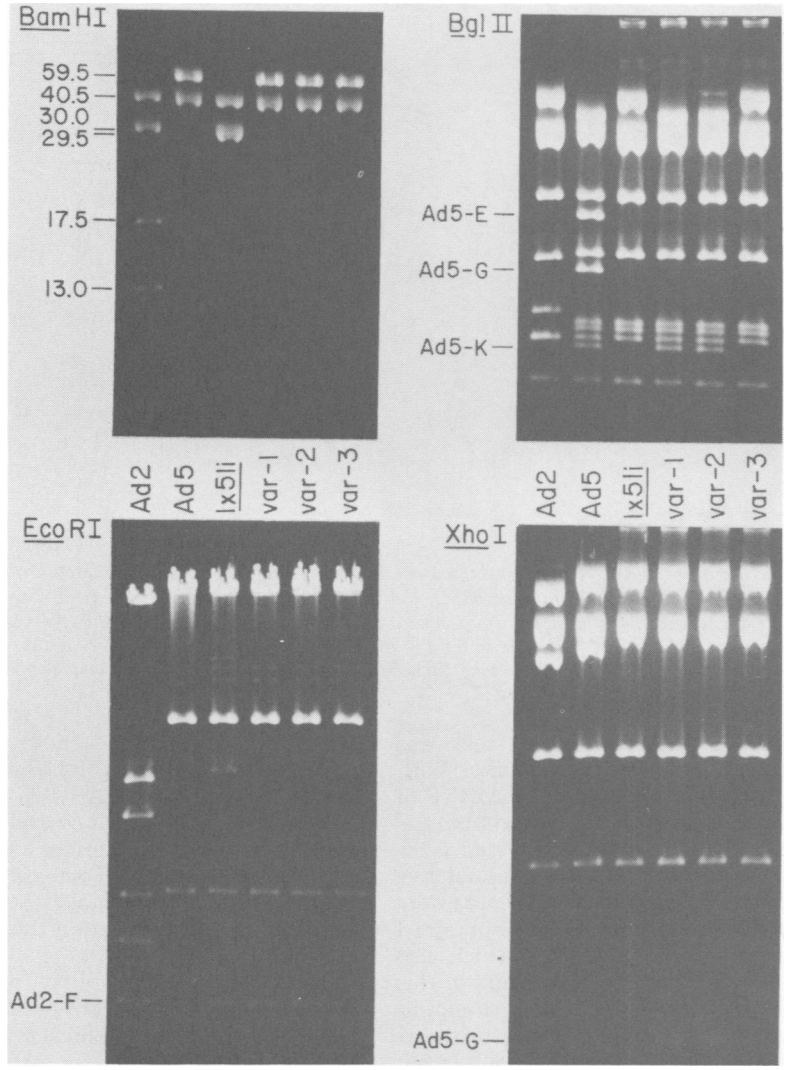

FIG. 1. *Restriction analysis of DNA of Ad2, Ad5, 1×51i, and variants 1, 2, and 3. Approximately 1 μg of DNA, isolated by the modified Hirt procedure, was digested with BamHI or EcoRI and analyzed in 0.7% agarose gels or with BglII or XhoI and analyzed in 1.2% agarose gels. Gels were photographed in UV light after soaking in ethidium bromide. The lengths of the BamHI fragments, in map units, are marked. Certain individual fragments of particular significance (see text) are identified.*

with the exception that the ligation step was omitted. Eleven sibling isolates from the same experiment had the parental distribution of *Bam*HI sites. No virus lacking the site at position 59.5 was obtained.

Properties of variants. The variants were all viable and did not differ from their parent in any way that could be detected by examination of (i) growth curves (Fig. 2), (ii) kinetics of VA RNA synthesis (data not shown), or (iii) nature and kinetics of synthesis of viral proteins (data not shown). It is particularly significant that all variants produced the 52,55-kilodalton (52,55K) protein, which maps between coordinates 29 and 34 (26). The 5′ end of the body of the mRNA encoding this product lies in or near the VA RNA genes (1, 26). Judging by its electrophoretic mobility, the 52,55K protein was of the Ad2, rather than the Ad5, variety in all cases.

As a result of the loss of the *Bam*HI site at position 29, the pattern generated by digestion of the variant DNAs with this endonuclease was identical to that derived from Ad5 DNA (Fig. 1). To distinguish between the DNA of the variants

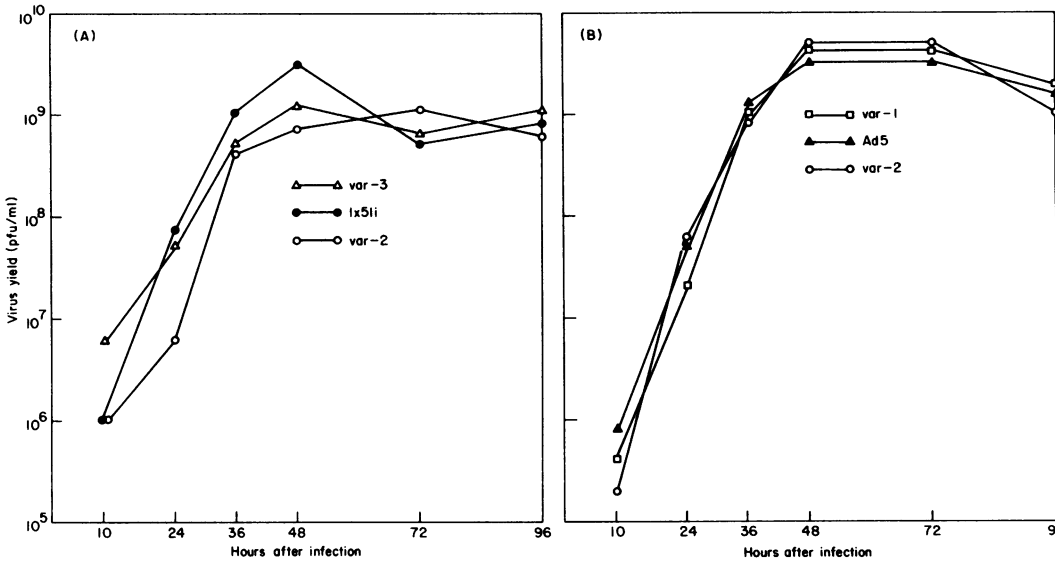

FIG. 2. *Growth curves of Ad5, 1×51i, and variants 1, 2, and 3. HeLa cells were infected at a multiplicity of infection of 20 PFU/cell. After 75 min at 38°C, the inoculum was removed, the plates were washed with phosphate-buffered saline, medium was added, and incubation was continued. At the times indicated, one 60-mm plate containing 2 × 10⁶ infected cells in 5 ml of medium was harvested, the virus was released by freeze-thawing and sonication, and the progeny was titrated by plaque assay on HeLa cells. Symbols: ●, 1×51i; ▲, Ad5; □, variant 1; ○, variant 2; △, variant 3.*

and that of Ad5, DNA from each stock was digested with other restriction enzymes. With *Eco*RI, the fragment equivalent to *Eco*RI-F of Ad2 was released from *1×51i* DNA and from the DNA of the variants (Fig. 1). Since Ad5 lacks the *Eco*RI site at position 70.7 and does not give rise to the *Eco*RI-F fragment, the variants were distinct from Ad5. The same result was obtained with the enzymes *Bgl*II and *Xho*I, which also show differences between Ad5 and Ad2 in this region of the genome. The *Bgl*II site at coordinate 69 is present in Ad5, giving rise to the G and E fragments, but is absent from Ad2, *1×51i*, and variant 1, 2, and 3 DNAs (Fig. 1). Likewise, the *Xho*I site at 70 map units in Ad5 DNA is lacking in the other viruses. In *1×51i* and the variants, this leads to the appearance of a very large fragment (not resolved in Fig. 1) representing a fusion of the Ad5 A and D fragments: the resulting large fragment is dismembered in Ad2 DNA by cleavages at other positions (coordinates 53 and 66).

Digestion with endonucleases *Bgl*II and *Xho*I revealed additional differences between the DNAs of Ad2 and Ad5 a short distance to the left of the VA RNA_I gene. Because the VA RNA_{II} species of all of the variants were of the Ad2 type, as shown below, it was surprising to discover that variants 1 and 2 gave rise to fragments equivalent to the Ad5 *Bgl*II-K and *Xho*I-G fragments, which were absent from digests of *1×51i* DNA (Fig. 1). Thus, these variants resemble Ad5 at three closely linked sites in the immediate vicinity of the VA RNA_I gene: the *Xho*I, *Bgl*II, and *Bam*HI sites at coordinates 28.1, 28.3, and 29.0, respectively. Variant 3 lacks these distinctive *Bgl*II and *Xho*I sites and is indistinguishable from the parental *1×51i* by restriction digests at all locations except the *Bam*HI site at position 29.

Electrophoretic mobility of VA RNAs. ^{32}P-labeled VA RNAs were purified by consecutive electrophoresis in polyacrylamide gels under two different conditions (24). The first gel was run under nondenaturing conditions, which seems to accentuate the effects upon electrophoretic mobility of compositional differences and allow resolution of the VA RNAs of Ad2 and Ad5 (14). In this gel system, the VA RNA_I species of the three variants separated from the corresponding parental species, whereas the VA RNA_{II} species of the variants and parent comigrated (data not shown). The variants' VA RNA_I species all moved substantially faster than that of the parent, *1×51i*; in some gels, the variant 3 RNA trailed the VA RNA_I of variants 1 and 2 slightly.

The VA RNA_I species of Ad6 comigrated with that of Ad2, whereas the Ad5 and Ad1 species moved significantly faster. The VA RNA_{II} species of Ad2, Ad5, and Ad6 ran perceptibly ahead of the corresponding VA RNA_I species. The Ad5

VA RNA_{II} appeared to migrate the most rapidly, and the Ad6 species appeared to migrate the most slowly. Ad1 VA RNA_{II} was particularly difficult to purify because its slow electrophoretic mobility barely allowed it to resolve from the leading edge of the broad VA RNA_I band, which itself ran faster than the corresponding Ad2 species, and from the host cell U2 RNA species.

Fingerprint analysis of RNAs. Purified VA RNAs were analyzed by two-dimensional paper electrophoresis (fingerprinting) after digestion with T_1 or pancreatic RNase (Fig. 3 and 4). The yields of individual oligonucleotides were quantified by estimating their relative radioactivity, and their structures were determined or confirmed by standard procedures (2) (Tables 1–4).

(i) VA RNA_I of natural serotypes. For VA RNA_I of Ad2, the analyses were in good agreement with those of Ohe and Weissman (29) and closely matched predictions based on the DNA sequence (1). Indistinguishable results were obtained with Ad6 VA RNA_I, indicating that its structure is the same as that from Ad2.

Compared with their Ad2 counterpart, the Ad5 and Ad1 species exhibited several changes which permitted their sequence to be deduced (Fig. 3 and 4, Tables 1 and 2). The appearance of two new oligonucleotides, CCCCG (spot ITa) and UAUCCG (spot IT49′), and the increased yield of AG (spot IT3), with concomitant loss of the two neighboring Ad2 oligonucleotides, AACCCCG and AUCCG (spots IT17 and 47), are consistent with the substitution of the A and G residues, nucleotides 66 and 72 in the Ad2 sequence, by G and U, respectively (see Fig. 5). These alterations in the T_1 RNase products predicted changes in the pancreatic RNase digestion products that were in complete agreement with those observed. One copy of the oligonucleotide GAAC (spot IP21, nucleotides 64 through 67) was replaced by GAGC (spot IP27a), and one copy of the tetranucleotide GGAU (spot IP28, nucleotides 71 through 74) was replaced by two dinucleotides, AU and GU (spots IP4 and IP6). The only exception was that the increased molar yield of the dinucleotide GU was greater than expected; no explanation of this disparity is apparent at present. These two substitutions are consistent with the absence of a *Bam*HI cleavage site at coordinate 29 in Ad5 and Ad1 (M. Sleigh and J. Sambrook, unpublished data) and with the partial Ad5 DNA sequence (10, 41) which extends to nucleotide 114 in the VA RNA_I gene.

(ii) VA RNA_I of variants. As expected from restriction enzyme mapping of the viral DNA and from its electrophoretic mobility, the VA RNA_I of the parental virus *1×51i* was identical to that of Ad2 (Fig. 3 and 4, Tables 1 and 2).

The VA RNA_I species of variants 1 and 2 were identical to one another and to the corresponding species of Ad5 and Ad1, differing from the Ad2 form at two positions, nucleotides 66 and 72.

Variant 3 differed from its parent in that it contained an extra copy of the oligonucleotide GAU (spot IP12) and the novel oligonucleotide AUCG (spot ITb), which could be overlapped to give the sequence GAUCG or AUCGAU. Although the *Bam*HI restriction site at 29 map units was absent from this variant, none of the oligonucleotides from this (or any other) region of VA RNA_I was missing. This observation implies that the additional sequences are fitted into the *Bam*HI recognition sequence GGAUCC (nucleotides 71 through 76) in such a way as to destroy the endonuclease cleavage site without altering any of the RNase digestion products. This is achieved by a duplication of the four central bases of the site, GAUC (Fig. 5). The insertion would also alter the yields of the mononucleotides C and G by one residue (by one additional in the pancreatic and one less in the T_1 RNase digests, respectively), but these changes would be almost impossible to detect.

(iii) VA RNA_{II} of natural serotypes. As in the case of VA RNA_I, the VA RNA_{II} species of the group C adenoviruses gave very similar fingerprints whether analyzed with T_1 RNase (Fig. 3 and Table 3) or pancreatic RNase (Fig. 6 and Table 4). The analyses of Ad2 VA RNA_{II} were in good agreement with expectations (1).

Compared with the Ad2 species, Ad6 VA RNA_{II} lacked the overlapping oligonucleotides CAG and AGGAC (spots IIT5a and IIP8), suggesting an alteration in or near the common dinucleotide AG (nucleotides 48 and 49). The increased yield of CG (spot IIT2) in the T_1 RNase digest and the appearance in the pancreatic RNase fingerprint of a new spot (IIPa) in the position expected for one of the isomers of GGGAC are consistent with an A → G substitution at nucleotide 48 (Fig. 6).

The same set of changes was apparent in Ad5 VA RNA_{II}. This species also lacked one copy of the trinucleotide CCG and the overlapping oligonucleotide GGGC (spots IIT3 and IIP6, nucleotides 69 through 74), suggesting a change at or near the common C residue (nucleotide 72). Concomitantly, there appeared an extra copy of the oligonucleotide $(G_2,A)C$ (spot IIP9) and a new oligonucleotide, $(A,C_2)G$ (spot IITa), consistent with the replacement of the C residue by an A at position 71 in the VA RNA_{II} sequence (Fig. 6).

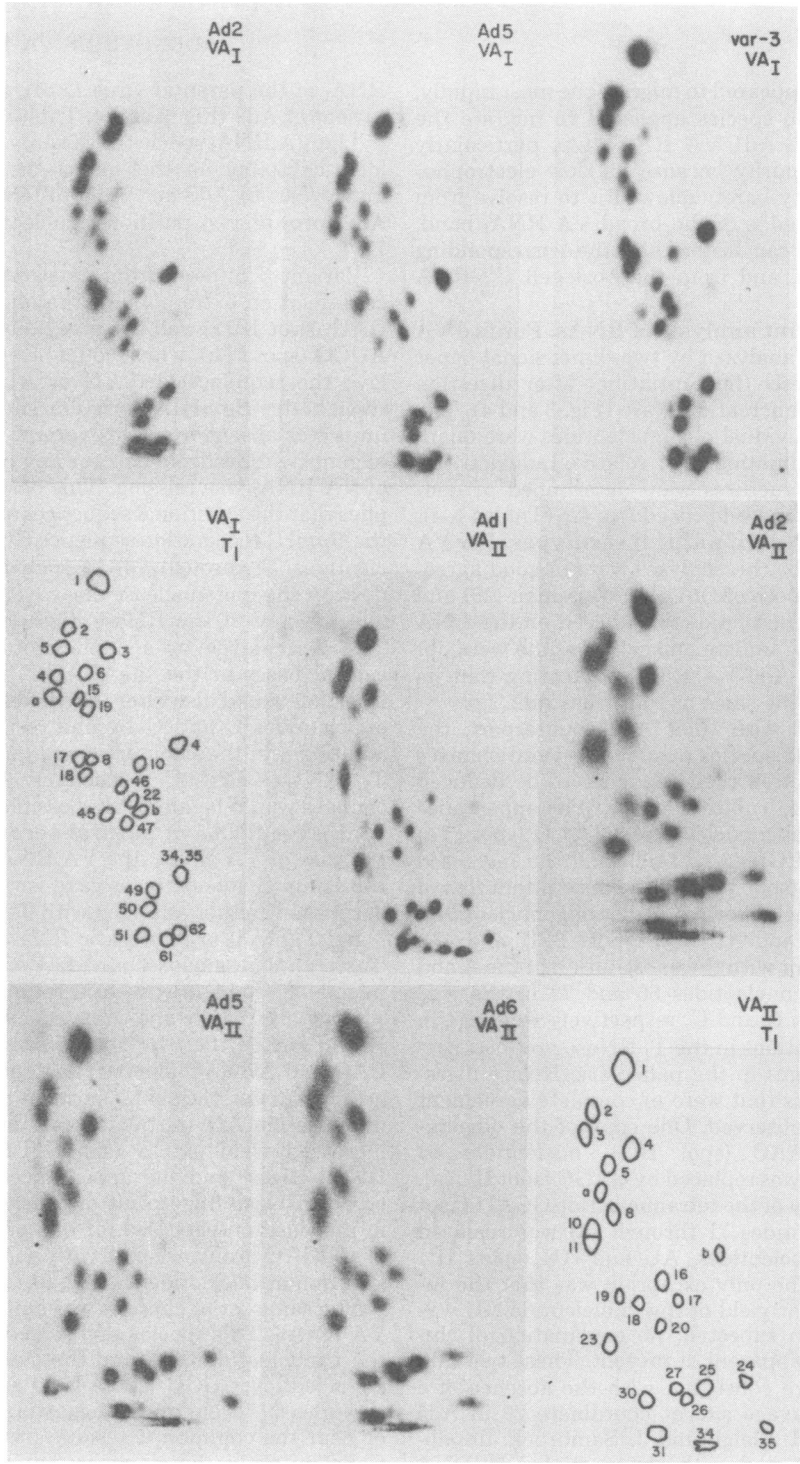

FIG. 3. *Fingerprints of VA RNA species digested with T_1 RNase. Electrophoresis was from left to right in the first dimension and from bottom to top in the second. The schematic diagrams give the nomenclature of the spots. Oligonucleotides from Ad2 are numbered; those found only in other serotypes are designated by letters. Spots present in low yield, including those from 5′ and 3′ termini, are not labeled. The following species yielded indistinguishable fingerprints: Ad2, Ad6, and 1×51i VA RNA_I; Ad5, Ad1, variant 1, and variant 2 VA RNA_I; Ad2, 1×51i, variant 1, variant 2, and variant 3 VA RNA_{II}. Oligonucleotide IIP19 was run off the second dimension of the Ad5 VA RNA_{II} fingerprint.*

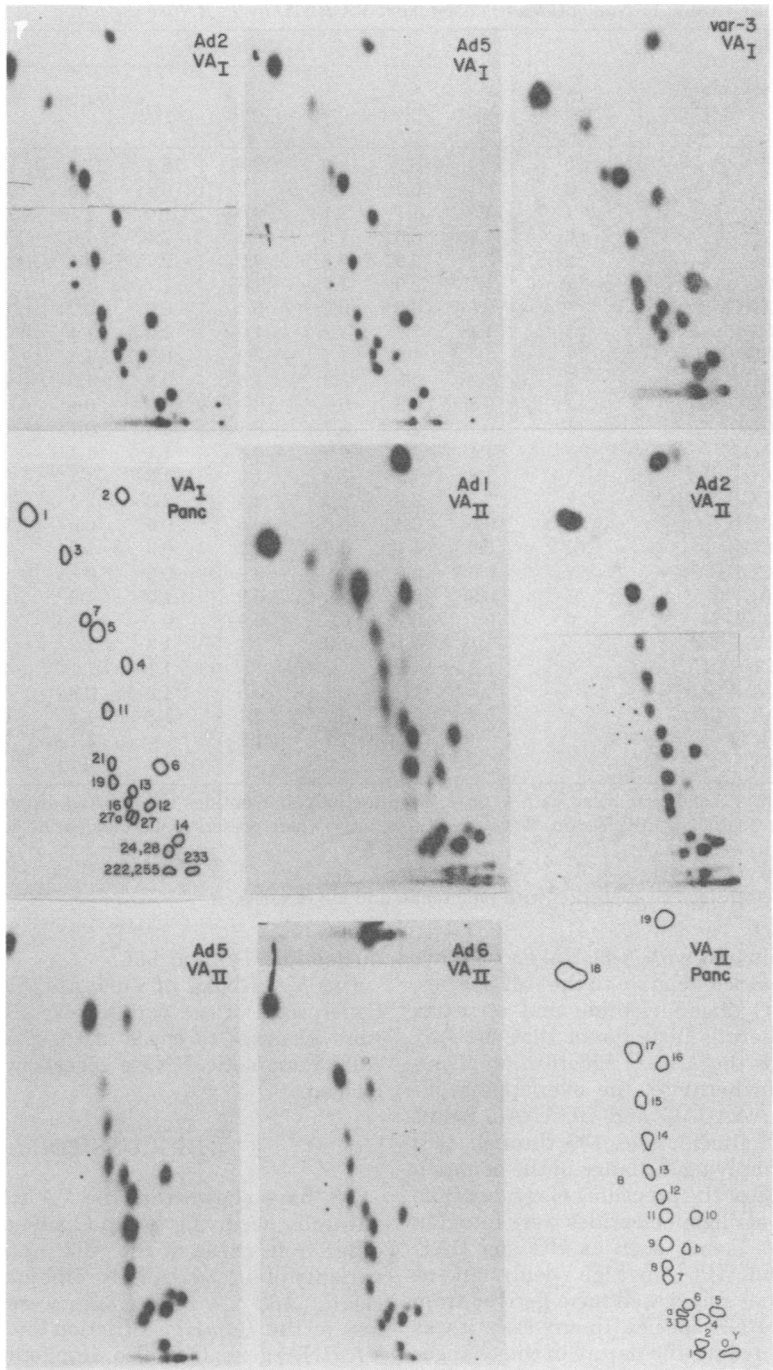

FIG. 4. *Fingerprints of VA RNA species digested with pancreatic RNase. Details are given in the legend to Fig. 3.*

The analysis of Ad1 VA RNA_{II} is less secure because of the difficulties in its purification; although the absence of particular oligonucleotides is reliable, the molar yields and presence of novel oligonucleotides must be regarded as suspect. The VA RNA_{II} of Ad1 shared with its counterparts from Ad5 and Ad6 the loss of one CAG oligonucleotide and the AGGAC oligonu-

TABLE 1. *Nucleotides released from VA RNA$_I$ by T$_1$ RNase digestion*

Spot no.	Structure[b]	Molar yield[a]								
		Ad2 predicted[b]	Ad2	Ad6	Ad5	Ad1	*1×51i*	Variant 1	Variant 2	Variant 3
IT1	G	20	20.4	18.9	18.0	19.6	18.1	17.9	17.2	17.7
IT2	CG	5	6.1	6.7	4.9	7.7	6.4	4.9	5.9	6.7
IT3	AG	1	1.3	1.1	2.1+	2.0+	1.0	2.1+	1.9+	1.2
IT4	UG	6	6.9	6.5	7.5	6.2	7.1	7.0	6.6	6.5
IT5	CCG	2	3.2	2.9	2.8	3.2	2.7	3.1	2.6	3.1
IT6	ACG	2	1.7	2.0	1.7	1.6	1.7	1.9	1.9	1.8
IT8	ACAACG	1	0.6	0.6	0.5	0.5	0.6	0.7	0.9	0.5
IT10	UCG	1	1.1	1.2	1.2	1.0	1.0	1.1	1.0	1.1
IT14	CCCG	1	1.1	1.2	1.1	0.9	0.9	1.1	1.2	1.1
IT15	ACCG	1	0.9	0.9	0.9	1.0	0.9	1.0	0.8	0.9
IT17	AACCCCG	1	0.8	0.9	0–	0–	0.9	0–	0–	0.8
IT18	AACCCAG	1	0.7	0.8	0.7	0.5	0.8	0.7	0.7	0.8
IT19	CAAG	1	1.1	1.0	0.9	0.8	1.1	1.1	1.3	1.0
IT22	UCAG	1	1.2	1.2	1.3	1.0	1.3	1.3	1.3	1.2
IT34	UCUG + UUCG	2	2.3	2.2	2.2	2.4	2.2	2.4	2.3	2.1
IT45	CCCUU	0–1	0.6	0.8	0.8	0.5	0.5	0.5	0.5	0.5
IT46	UCCG	1	0.9	1.1	0.9	1.2	0.8	1.0	0.8	0.9
IT47	AUCCG	1	0.9	0.9	0–	0–	0.9	0–	0–	0.9
IT49	UUACCG	1	0.9	0.9	0.9	0.9	1.0	1.2	0–	0.9
IT49′	UAUCCG[c]	0	0	0	0.8+	0.9+	0	1.1+	1.0+	0
IT50	AUCCAUG	1	1.1	1.1	1.0	1.1	1.3	1.2	1.1	1.1
IT51	CACUCUUCCG	1	1.2	1.1	1.1	1.0	1.1	1.1	1.1	1.0
IT61	AUAAAUUCG	1	1.0	1.0	1.1	0.8	1.0	0.9	1.1	0.9
IT62	UAUCAUG	1	0.7	0.7	0.7	1.1	1.3	1.1	0.9	0.9
ITa	CCCCG				1.1+	1.2+		1.1+	1.0+	
ITb	AUCG[c]									0.9+

[a] Deviations from Ad2 are marked with + or −. Terminal oligonucleotides are omitted. Spots ITa and ITb, absent from the prototype Ad2 strain, were quantified only when present. Oligonucleotide identification is shown in Fig. 3.

[b] From Akusjärvi et al. (1).

[c] Structures determined by digestion with pancreatic and U2 RNases.

cleotide as compared with Ad2. Taken together with the appearance, relative to the Ad2 species, of the GGGAC oligonucleotide and an extra copy of CG, there is little doubt that the Ad1 species contains the A → G substitution at nucleotide 48. Furthermore, the overlapping oligonucleotides AAACAG and GGAAAC (spots IIT19 and IIP7, nucleotides 139 through 146) were missing, implying a change in the common sequence AAAC or the preceding G residue (Fig. 6). Several novel oligonucleotides were found in the fingerprints (some, such as UG and GAU [spots IIT6 and IIP6], in high yield) but, as noted above, they might well have derived from contaminating RNA species. In any case, it was not possible to deduce the nature of the changes in this region of the Ad1 VA RNA$_{II}$ by examining the structure of the novel oligonucleotides. Because no oligonucleotide was observed to give rise to AAAC (or to AAAN or AAN) after digestion with both T$_1$ and pancreatic RNases, it is probably that one or more alterations have taken place in the run of A residues between nucleotides 141 and 143.

(iv) VA RNA$_{II}$ of variants. The T$_1$ RNase fingerprints of the variants' VA RNA$_{II}$ species were identical to those of their parent and of Ad2. Pancreatic RNase digests were not performed.

DISCUSSION

We have shown that the VA RNAs of some naturally occurring group C adenovirus strains differ from those of the Ad2 prototype. When variants of an Ad2-Ad5 recombinant virus that carries Ad2 VA RNA genes were selected for loss of the *Bam*HI restriction site lying in the VA RNA$_I$ gene, they also were found to produce altered VA RNAs.

VA RNA gene structure. Both VA RNAs can adopt very stable secondary structures (Fig. 7). Computer-aided model building (1) has revealed a single preferred conformation for VA RNA$_I$ and two for VA RNA$_{II}$; of the latter, one is very similar to the VA RNA$_I$ structure in

TABLE 2. *Nucleotides released from VA RNA$_I$ by pancreatic RNase digestion*

Spot no.	Structure[b]	Molar yield[a]								
		Ad2 predicted[b]	Ad2	Ad6	Ad5	Ad1	*1×51i*	Variant 1	Variant 2	Variant 3
IP1	C	28	19.6	19.7	18.6	19.5	22.5	22.7	22.2	26.4
IP2	U	10	9.1	8.2	8.9	9.0	8.0	7.6	7.6	8.2
IP3	AC	2	2.4	2.3	2.2	2.2	2.5	2.6	2.1	2.4
IP4	AU	3	3.5	3.5	5.0+	5.1+	3.7	4.8+	4.7+	3.5
IP5	GC	7	7.9	8.0	7.7	7.9	7.7	8.	7.7	7.6
IP6	GU	7	8.3	8.2	10.1+	9.8+	7.4	9.8+	11.5+	7.2
IP7	AAC	1	1.0	1.0	0.8	1.3	1.3	1.1	0.8	1.1
IP11	GAC	2	2.1	2.3	2.3	2.5	1.7	2.2	2.1	2.2
IP12	GAU	1	1.1	1.2	1.3	1.3	1.2	1.2	1.1	2.0+
IP13	GGC	2	1.9	1.9	1.9	2.5	1.7	2.0	1.8	1.8
IP14	GGU	3	3.1	3.1	3.1	3.5	3.4	3.3	3.3	3.1
IP16	AAAU	1	1.3	1.2	1.3	1.2	1.3	1.3	1.4	1.1
IP19	AGAC	1	1.1	1.1	1.0	1.4	1.3	1.1	1.2	1.0
IP21	GAAC	2	2.0	2.3	1.1−	1.2−	1.9	1.0−	1.0−	2.0
IP24	AGGU[c]	1	1.0	1.0	1.0	1.2	1.1	1.1	1.1	1.0
IP27	GGAC[d]	1	1.0	1.0	1.0	1.3	1.0	1.0	1.1	1.1
IP27a	GAGC[d]				0.9+	1.2+		1.0+	1.0+	
IP28	GGAU[c]	2	2.0	2.0	1.0−	1.2−	2.3	1.1−	1.1−	2.0
IP253	GGGGU	1	0.5	1.1	0.6	0.9	0.8	0.9	0.7	0.7
IP222	AAGGGU	1	0.5	0.4	0.6	0.7	0.8	0.7	0.7	0.7
IP255	GGGGGAGC	1								

[a] Deviations from Ad2 are marked wih + or −. Terminal oligonucleotides are omitted. Spot IP27a, absent from the Ad2 prototype, was quantified only when present. Oligonucleotide identification is given in Fig. 4.

[b] From Akusjärvi et al. (1).

[c] Oligonucleotides not always separated; yields estimated after redigestion with T_1 RNase.

[d] Determined by redigestion with U2 and T_1 RNases.

overall shape and thermodynamic stability, whereas the other is slightly different in form and is favored energetically.

Two of the serotypes (Ad5 and Ad1) and two of the variants (1 and 2) exhibit two single-base changes in their VA RNA$_I$ sequences relative to the Ad2 serotype. The A → G change at position 66 does not affect the base-pairing scheme of the most stable theoretical structure for this molecule, since the residue in question is located in a loop (Fig. 7A). The G → U alteration at position 72, however, destroys a terminal G·C base pair in a stem containing six such pairs and would be expected to destabilize the structure somewhat. This effect could be mitigated by a slight rearrangement of the pairing scheme (inset, Fig. 7). The four-base insertion in the VA RNA$_I$ of variant 3 would also have little or no impact on the base-pairing scheme because it merely expands the preexisting loop between nucleotides 73 and 79.

When compared with the Ad2 form, the VA RNA$_{II}$ species of the other group C viruses exhibit changes in three regions. The A → G substitution at nucleotide 48, found in all of the other group members, replaces a U·A base pair with a U·G pair. The resultant destabilization in secondary structure is more severe for the conformation of lesser stability (Fig. 7B), in which the G·C pair (nucleotides 49 and 81) would become isolated from the nearby three-long run of G·C pairs. In the more stable VA RNA$_{II}$ structure (Fig. 7C), the U·A pair terminates a stem, and the change to a U·G pair would be expected to be less damaging. The G → A substitution at nucleotide 71 in Ad5 VA RNA$_{II}$ would have little or no effect on the more stable structural form, in which it would be located in a loop, and would enhance the stability of the less favored form by converting a G·U pair in an eight-long paired stem into an A·U pair. It is difficult to evaluate the impact of the third VA RNA$_{II}$ alteration in the vicinity of nucleotide 142, since it has not been fully defined. The five nucleotides which could be affected include a paired G at the end of a long paired stem, two unpaired A residues, and a paired A and C residue at the end of another long stem. None of these alterations should be very disruptive; in any event, the effect on the two forms would be the same, since the structures are indistinguishable in this region.

Functional considerations. Clearly, the alterations in VA RNA sequences that are specified here—the substitutions at nucleotides 66 and 72 in VA RNA$_I$ and at 48, 71, and approxi-

mately 142 in VA RNA_{II} and the tandem duplication of nucleotides 72 through 75 in VA RNA_I—do not affect the viability of the virus or the production, stability, or function of the VA RNAs. In the *Xenopus* 5S RNA gene, the most important signals governing the initiation of transcription are located within the coding sequence (5, 37). Studies with tRNA genes (8, 19) and, more recently, VA RNA genes (10; R. Guilfoyle and R. Weinmann, personal communication) suggest that this may be the norm for genes transcribed by RNA polymerase III. In the VA RNA_I gene, the intragenic control region certainly includes nucleotides 12 through 58 and may extend as far as nucleotides 11 through 68. A similar region probably also exists in the VA

TABLE 3. *Nucleotides released from VA RNA_{II} by T_1 RNase digestion*

Spot no.	Structure[b]	Molar yield[a]				
		Ad2 predicted[b]	Ad2	Ad6	Ad5	Ad1
IIT1	G	20	20.3	17.4	16.2	17.5
IIT2	CG	2	2.0	2.8+	3.0+	4.7+
IIT3	CCG	3	3.2	2.8	2.0−	2.4
IIT4	AG	4	4.4	3.7	3.6	3.7
IIT5a	CAG[c]	1	0.9	0−	0−	0−
IIT5b	ACG[c]	1	0.8	1.0	1.0	1.0
IIT8	CAAG, AACG	2	1.3	1.4	1.2	0.8
IIT10	ACCCCG	1	0.7	0.8	0.9	0.6
IIT11	ACC_4G	1	0.8	0.7	0.7	0.6
IIT16	UCG	1	1.2	1.1	1.0	1.5
IIT17	UAG	1	0.8	0.9	0.9	0.8
IIT18	CUCG	1	1.0	1.0	1.1	1.0
IIT19	AAACAG	1	0.5	0.7	0.7	0−
IIT20	ACUG	1	1.0	1.0	0.9	0.8
IIT23	$CCUC_4G$	1	0.9	1.0	1.0	0.9
IIT24	UUG	1	0.9	0.9	1.0	0.9
IIT25	UUCG, CUUG	2	1.9	1.9	2.0	1.8
IIT26	UCAUG	1	0.9	0.9	1.0	0.8
IIT27	UCUCG	1	1.1	1.0	0.8	0.8
IIT30	CUC_3UG	1	1.1	1.1	1.1	0.9
IIT31	$CA_3U_2C_2UC_2G$	1	1.0	0.9	1.1	0.6
IIT34	$U_2AU_4C_2A_2G$	1	0.4	0.7	0.8	0.7
IIT35	UUUG	1	1.1	0.9	1.0	0.9
IITa	$(A,C_2)G$				1.0	
IITb	UG					1.0

[a] Deviations from Ad2 are marked with + or −. Terminal oligonucleotides are omitted. Spots IITa and IITb, absent from the prototype Ad2 strain, were quantified only when present. Oligonucleotides were identified as shown in Fig. 3.

[b] From Akusjärvi et al. (1).

[c] Oligonucleotides not separated; yields estimated after redigestion with T_1 RNase.

TABLE 4. *Nucleotides released from VA RNA_{II} by pancreatic RNase digestion*

Spot no.	Structure[b]	Molar yield[a]				
		Ad2 predicted[b]	Ad2	Ad6	Ad5	Ad1
IIP0	G_5U	1	0.5	0.9	1.2	0.3
IIP1	G_2AG_3U	1	nd[c]	nd	nd	1.0
IIP2	AAG_3U	1	0.7	0.6	0.6	0.3
IIP3	AG_3AC	1	0.7	0.6	0.8	0.4
IIP4	GAGU	2	2.3	2.2	2.5	2.0
IIP5	GGU	1	1.2	1.1	1.4	1.5
IIP6	GGGC	1	0.9	0.9	0−	0.8
IIP7	GGA_3C	1	0.9	0.8	1.0	0−
IIP8	AGGAC	1	0.9	0−	0−	0−
IIP9	$(G_2,A)C$	2	2.1	2.1	3.5+	1.9
IIP10	GU	2	2.7	2.7	3.0	2.8
IIP11	GGC	2	2.4	2.0	2.3	2.1
IIP12	AAAU	1	1.1	1.2	1.2	0.9
IIP13	AAGAC	1	1.0	0.9	1.0	0.6
IIP14	GAAC	1	1.2	1.1	1.0	0.8
IIP15	AGC	1	1.2	1.2	0.9	1.2
IIP16	AU	2	2.7	2.4	2.3	3.0
IIP17	GC	7	8.8	7.6	7.9	6.5
IIP18	C	33	19.4	21.0	12.6	19.0
IIP19	U	20	18.6	19.1	17.0	19.4
IIPa	$(A,G_3)C$			0.8	0.9	0.4
IIPb	GAU					1.3

[a, b] See Table 3.

[c] nd, Not determined.

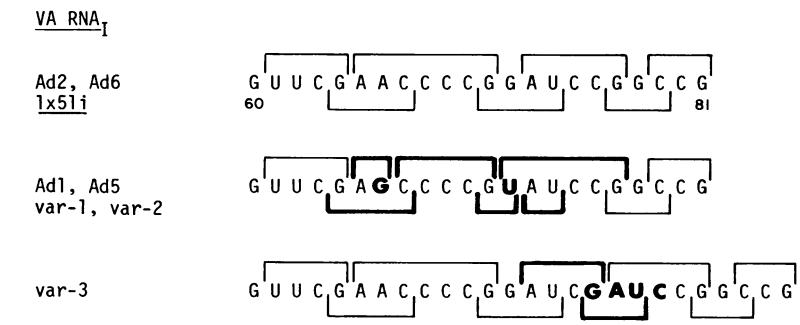

FIG. 5. *Sequence changes in VA RNA_I species. Nucleotides 60 through 81 of the Ad2 VA RNA_I sequence (1) are shown on the top line, with the oligonucleotides released by T_1 and pancreatic RNases bracketed above and below the line, respectively. Alternative sequences found in other serotypes and variants (var-1, etc.) are shown on the lower lines. Nucleotide and oligonucleotide differences from Ad2 are emphasized by darker lettering and brackets.*

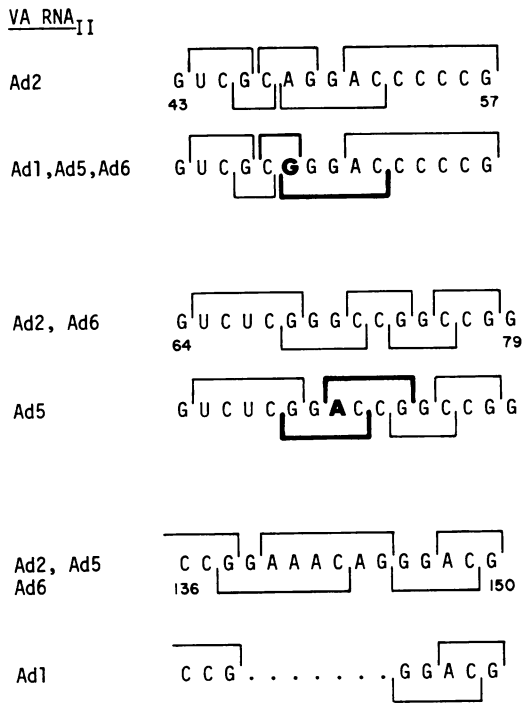

FIG. 6. *Sequence changes in VA RNAII species. Segments of the Ad2 VA RNAII sequence (1) are shown on lines 1, 3, and 5. Alternative sequences found in other serotypes are indicated on lines 2 and 4 as described in the legend to Fig. 5. The Ad1 sequence between nucleotides 139 and 145 has not been defined and is shown on line 6 as a series of dots.*

RNAII gene (10). Clearly, the changes specified here must lie outside the signal region or else be neutral in effect.

Circumstantial evidence (23) suggests that the VA RNA species may play a role in the splicing reaction involved in the production of late viral mRNA's, but the details of this process remain to be clarified. In earlier studies (38), a large number of viable recombinants between Ad2 and Ad5 were created and mapped; their existence indicates that a wide variety of, if not all, combinations of genes from these two serotypes can coexist in a single virus. Particularly relevant is the observation that, within the limits of precision inherent in the analyses, all regions of Ad5 DNA were found in combination with Ad2 VA RNAI and vice versa. The sequence differences between Ad2 and Ad5 VA RNAI do not affect their ability to function together with most, if not all, genes from either serotype. Furthermore, data presented here show that variants 1 and 2 have an Ad2-type VA RNAII but an Ad5-type VA RNAI. Thus, we can conclude that if the two VA RNAs act in concert, they can be of different serotype origin.

The alterations in the VA RNA genes could also result in protein coding changes if they are located in translated regions of mRNA's. All three reading frames are blocked somewhere in the VA RNA gene region as discussed previously (1), so the protein-coding potential of the region is limited. Most of the alterations described here lie in possible coding regions and would result in amino acid substitutions. The four-base duplication of variant 3, however, would change the reading frame and lead to a foreshortening of the protein. The same is true of the two-base deletion 25 bases upstream from the VA RNAI 5′ end in Ad5 DNA (41); the variant carrying this mutation fails to produce a VA RNAI subspecies that initiates with the A residue three bases to the left of the usual G start. Since this virus too is viable, the extended VA RNAI species cannot be essential.

Origin of the variants. The genetic structure of the *1×51i* recombinant was deduced from an analysis of restriction sites (39) and is reinforced by observations reported here: (i) the crossover to Ad2 DNA responsible for the rescue of the Ad5 *ts*1 mutation has led to the loss of Ad5-specific *Bgl*II and *Xho*I sites at 69 and 70 map units (Fig. 1), as well as the acquisition of the neighboring Ad2-specific *Eco*RI site at coordinate 70.7; (ii) the presence of Ad2 DNA sequences to the left of the *Bam*HI site at coordinate 29 is correlated with the absence of the neighboring Ad5-specific *Bgl*II and *Xho*I sites at 28.3 and 28.1 map units (Fig. 1); (iii) both VA RNAs are of the Ad2 variety; and (iv) the E1A (21) and 52,55K (G. P. Thomas and M. B. Mathews, unpublished data) polypeptides of Ad2 and Ad5 are electrophoretically distinct, as are several of the late proteins of these viruses (14). Cell-free translation of the *1×51i* mRNA's encoding these polypeptides reveals that they are of the Ad2 variety (data not shown), indicating that the recombinant also carries Ad2 markers between coordinates 1.5 and 4.4 and between 29 and 34 and supporting the view that it contains Ad2 sequences extending from the left end of the genome into, or beyond, the coding region for the 52,55K polypeptide (29 to 34 map units).

Variant 3 is identical to its parent in all respects other than the absence of the *Bam*HI cleavage site at position 29, which results from a duplication of the four central nucleotides in the *Bam*HI recognition sequence. Bearing in mind that variant 3 was derived from *Bam*HI-restricted DNA that was not religated before transfection, it is possible that this structure

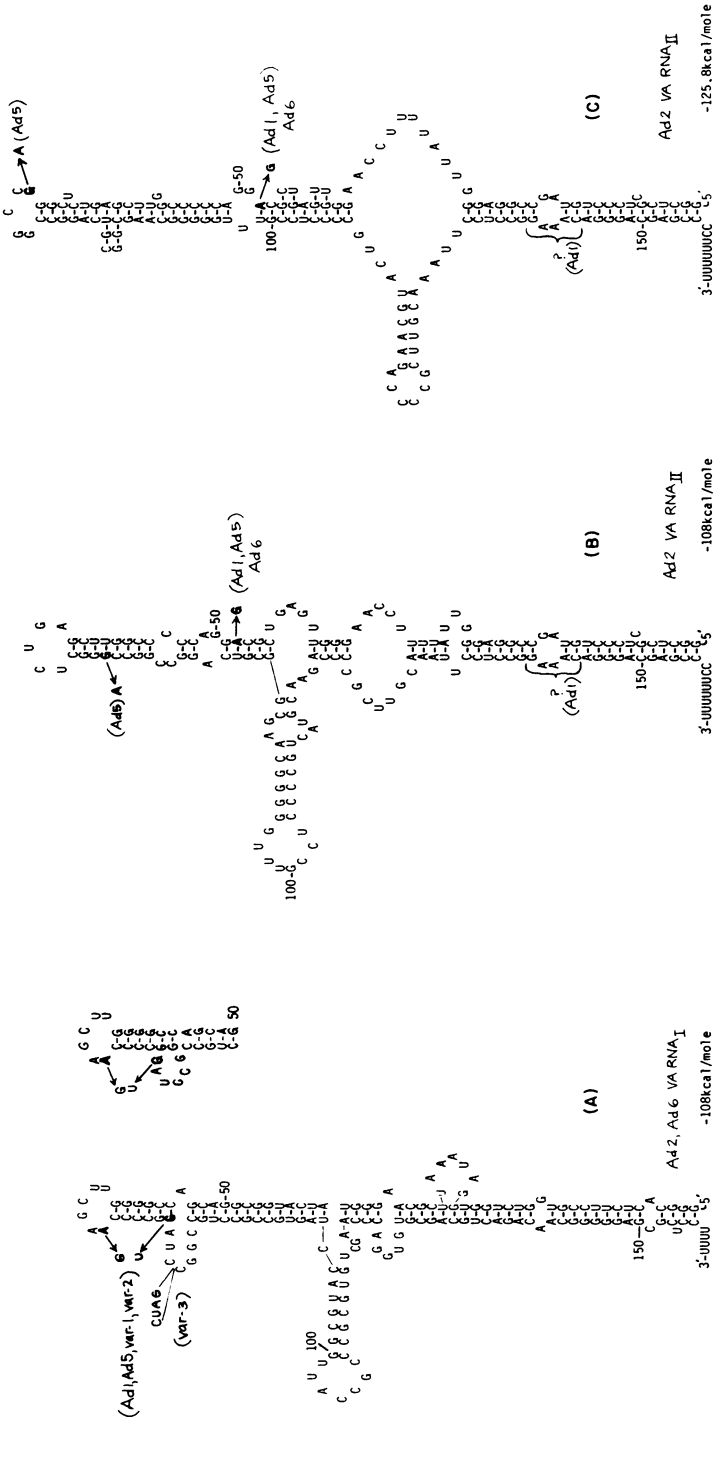

FIG. 7. *Secondary structure of VA RNAs. The most stable structures for Ad2 VA RNA$_I$ (A) and VA RNA$_{II}$ (B and C) are taken from reference 1. The positions and nature of the sequence alterations found in other serotypes and variants are indicated. An alternative structure for nucleotides 50 through 83 of Ad2 VA RNA$_I$ is shown in the inset to (A).*

arose by the blunt-ended sealing of the two arms of the site after filling in of the four-base protrusion at their 5′ termini. These reactions would be carried out by the recipient cell. Alternatively, it could be that the duplication was present in the original preparation of *1×51i* DNA, presumably as a result of an unequal crossover event during the high-multiplicity passage of the virus stock. Because of its resistance to cleavage with *Bam*HI nuclease at position 29, only one ligation (instead of two) would be required for reconstitution of the genome after transfection, and its growth in the recipient cells would be favored. Experiments to distinguish between these possibilities have not been performed, but it is relevant to point out that transfection with linear simian virus 40 DNA carrying cohesive ends has generally given rise to deletions rather than insertions (7, 20).

Variants 1 and 2 are identical to one another in all respects, and it cannot be excluded that they represent siblings rather than independent isolates. They differ from the parent, *1×51i*, in lacking the *Bam*HI site not only at position 29, by virtue of a G → U transversion at nucleotide 81 in the VA RNA_I sequence, but also at three other locations: nucleotide 66 in the VA RNA_I sequence has been altered by an A → G transition, and *Xho*I and *Bgl*II sites are present at coordinates 28.1 and 28.3. These four features are characteristic of Ad5. Other markers, such as the VA RNA_{II} and E1A and 52,55K polypeptides, are of the Ad2 variety, as in the parent, *1×51i*. The concurrent acquisition of this set of Ad5 markers spanning about 200 base pairs raises the possibility that they were accidentally incorporated en bloc by recombination with a fragment of Ad5 DNA contaminating one of the reagents. In this case, the right border of the crossover can be specified with considerable precision as lying between nucleotide 72 in the VA RNA_I gene and nucleotide 48 in the VA RNA_{II} gene, a span of 232 base pairs. An alternative is to suppose that these four alterations preexisted in the high-passage *1×51i* stock, presumably linked as a coordinate set. At present, however, we know of no reason why these changes should be correlated.

Relationships among group C adenoviruses. Group C adenoviruses comprise a homogeneous family in which the four canonical serotypes share a variety of biological and molecular properties which set them apart from members of the other adenovirus groups. Little or no recombination occurs between adenoviruses of different groups, which are therefore isolated from one another. Despite their probable common origin, the groups are presumably evolving separately from one another. However, the viruses within a group engage in promiscuous recombination in vitro and apparently also in nature (38). Thus, the group is presumably evolving as a unit.

The naturally occurring strains of group C adenoviruses exhibit two VA RNA_I sequences, one characteristic of Ad2 and Ad6 and the other characteristic of Ad1 and Ad5. On the other hand, each serotype has a unique VA RNA_{II} sequence: that of the Ad6 species differs at one of three separate positions from those of Ad1, Ad2, and Ad5. Thus, it would seem that the Ad6 sequences are closely related to those of Ad2 and more distantly related to those of Ad1 and Ad5.

The genomes of these viruses have also been compared over their full length by restriction endonuclease mapping (Sleigh and Sambrook, unpublished data; 4). The data confirm that the closest relationship is between Ad6 and Ad2, followed by the Ad6 and Ad5 pair, but Ad5 and Ad2 are not particularly akin. Ad1 is the furthest removed serotype, its closest affinity (with Ad6) being no more intimate than the weakest affinity (Ad2-Ad5) among the three other members of the group. Of the canonical serotypes, Ad6 most closely approaches a hypothetical prototype, but how closely it approximates an archetypal group C adenovirus is a moot question.

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ADDENDUM IN PROOF

The Ad2-specific *Bcl*I restriction site at coordinate 11.3 is missing from *1×51i* DNA (F. Asselbergs and C. Thummel, unpublished data), suggesting the existence of Ad5-derived sequences in this region of the genome.

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