VA RNAs from Avian and Human Adenoviruses: Dramatic Differences in Length, Sequence, and Gene Location

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Human adenoviruses encode low-molecular-weight RNAs, so-called VA RNAs, which are transcribed by RNA polymerase III. These RNAs are required for an efficient translation of viral mRNAs late after infection. The genes for the VA RNAs in the genome of CELO virus were mapped and characterized. The results showed a number of surprising differences between CELO virus and human adenovirus type 2 (Ad2). Thus, the CELO virus genome encoded only one VA RNA species, in contrast to human Ad2, which encoded two distinct species. The VA RNA from CELO virus was much shorter than the Ad2 VA RNAs (90 nucleotides compared with 160 nucleotides), and there existed no detectable primary sequence homology between them. The predicted secondary structure of CELO virus VA RNA was, however, similar to that of the Ad2 VA RNAs, implying that the folding rather than the primary sequence was the important feature for biological activity. CELO VA RNA also stimulated translation in a transient expression assay, as did the Ad2 counterparts, albeit with a much lower efficiency. The location of the gene for CELO VA RNA also differed from all previously characterized serotypes, suggesting that the genome organization of avian and human adenoviruses are different. Finally, termination of CELO VA RNA transcription occurred in a TTATT sequence which is unique as a stop signal for RNA polymerase III transcription.

Most of current knowledge about the structural and functional organization of the adenovirus genome stems from studies of the nononcogenic human subgroup C adenoviruses (Ad2, Ad5), which may therefore be considered as the prototype members of the mammalian adenovirus genus. The adenovirus family is, however, very large, containing serotypes with both a mammalian (mastadenovirus) and an avian host range (Aviadenvirus) (for a review, see reference 16). The avian serotypes are only distantly related to the human adenoviruses, with which they share a limited amount of DNA sequence homology (2). The classification of these viruses as adenoviruses rests on morphological and basic structural similarities. Thus, the genomes of both avian and human adenoviruses consist of a linear double-stranded DNA molecule with an inverted terminal repetition (3) and a protein, the terminal protein, attached to the 5' end of each DNA strand (19, 38). They also have similar mechanisms for DNA replication (4) and similar early and late phases of gene expression, and they share at least one nonstructural protein, the single-stranded DNA-binding protein (22). The main structural difference between them is the composition of the penton, the vertex capsomer, which in avian adenoviruses consists of two fiber molecules attached to the penton base (18, 30).

CELO virus (fowl adenovirus type 1) has been most thoroughly studied among the avian adenoviruses and can therefore be regarded as the prototype virus of the genus. Although the accumulated information about CELO virus is sparse and fragmentary compared with that of the human adenoviruses, it seems reasonable to suggest that regulatory mechanisms operating in the human adenovirus system will find a counterpart in CELO virus.

Transcription of the Ad2 and Ad5 genomes leads to the production of large amounts of two low-molecular-weight RNAs, designated the virus-associated RNAs, VA RNA1 and VA RNA2 (26, 34, 42). Both RNAs are around 160 nucleotides long and are transcribed by RNA polymerase III (50) from two closely spaced transcription units located near coordinate 30 on the viral genome (26, 28, 32). Nucleotide sequence studies have shown that they exhibit scattered regions of primary sequence homology and are capable of adopting similar secondary structures (1). The VA RNAs play an important role during the lytic growth of adenovirus by increasing the efficiency with which the viral mRNAs are translated late after infection (47). They function at an early step of polypeptide chain initiation (35, 40), probably by suppressing a protein kinase which phosphorylates the α-subunit of eIF-2 (39, 41). Both VA RNAs appear to serve a similar function, although VA RNA1 is much more efficient (7). The stimulatory effect of VA RNA on mRNA translation can also be reproduced in a transient expression assay (44); the synthesis of viral as well as nonviral proteins are markedly stimulated by cotransfection of plasmids encoding VA RNA1 (17, 44, 45).

To study the structure and function of VA RNAs from different adenoviruses, we characterized the low-molecular-weight RNAs expressed by the CELO virus genome. Our analysis shows a number of unexpected differences between the human and avian adenoviruses.

MATERIALS AND METHODS

DNA and RNA preparation. CELO virus was grown and purified as described by Laver et al. (18), and the viral DNA was prepared according to Pettersson and Sambrook (33). Cloning and preparation of plasmid DNA was by standard recombinant DNA techniques (23), with pBR322 as a vector. Total late RNA from CELO virus-infected chicken embryo kidney (CEK) cells was isolated 25 h postinfection by lysis in guanidinium isothiocyanate followed by centrifugation in CsCl (9). Isolation of total cytoplasmic RNA from monolayer cells transfected with recombinant plasmids was performed as previously described (44, 46).
In vitro transcription. Virion and plasmid DNAs were transcribed in a soluble HeLa whole-cell extract prepared as previously described (24, 25). A standard 10-μl reaction contained 1 μg of DNA, 3 μl of cell extract, 12 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.9), 60 mM KCl, 7.5 mM MgCl₂, 1.2 mM dithiothreitol, and 10.2% glycerol. Incubations were carried out for 1 h at 30°C with 5 μCi of α-32P-GTP 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 CELO VA RNA was analyzed by S1 nuclease cleavage (see Fig. 4), the template DNA was digested with RNase-free DNase I (Worthington Diagnostics, Freehold, N.J.) (20 μg/ml) for 5 min at 30°C before phenol extraction and ethanol precipitation.

DNA sequence analysis. The protocol of Maxam and Gilbert (29) was followed for end labeling of fragments and subsequent sequence analysis.

S1 endonuclease analysis. The protocol of Berk and Sharp (5) and Weaver and Weissman (49) was followed with minor modifications. RNA isolated from CELO virus-infected cells (5 μg), RNA (prepared 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 37°C (5' end analysis) or 45°C (3' end analysis) to the DNA fragments described in the legend to Fig. 4. S1 nuclease cleavage and electrophoretic separation were performed as previously described (46).

Hybridization. For Southern blot analysis, 0.2-μg samples of CELO virus DNA were cleaved with different restriction endonucleases and separated on a 1% agarose slab gel. The DNA was transferred to a nitrocellulose sheet according to the method of Southern (43) and hybridized with 32P-labeled DNA probes as described by Mathews and Pettersson (28).

For Northern blot analysis, 5-μg samples of total RNA isolated from CELO virus-infected cells were separated on an 8% polyacrylamide gel containing 8 M urea (29) and transferred electrophoretically to a Gene Screen plus membrane as described by the supplier (New England Nuclear Corp., Boston, Mass.). The filter was prehybridized for 6 h at 42°C in a buffer containing 50% formamide, 1% sodium dodecyl sulfate, and 1 M NaCl. For the hybridization, the same solution was used, containing in addition 100 μg of denatured salmon sperm DNA per ml. The different DNA probes were 32P-labeled by nick translation (36).

Transfections. Subconfluent monolayers of 293 cells were transfected by the calcium phosphate coprecipitation technique (14, 52) as previously described (44, 46).

CAT assay. Preparation of extracts and chloramphenicol acetyltransferase (CAT) assay conditions were as previously described (13, 45).

RESULTS

Identification of an RNA polymerase III transcript encoded by the CELO virus genome. RNA polymerase III genes from virtually all higher eukaryotes appear to be transcribed accurately in whole-cell extracts prepared from HeLa cells. Transcription both initiates and terminates correctly in the in vitro system, thus simplifying the identification of the RNA polymerase III products (24).

FIG. 1. RNA polymerase III transcription units encoded by the CELO virus genome. RNA synthesized in a soluble HeLa whole-cell extract (24), programmed either with plasmid pC-va DNA (lanes pC-va – and pC-va +) or with virion DNA purified from Ad2 (lane ad2 +) or CELO virus particles (lanes celo – and celo +) was separated by electrophoresis through a denaturing 8% polyacrylamide gel. The in vitro transcription reactions were performed either with (lanes ad2 +, celo +, and pC-va +) or without (lanes celo – and pC-va –) 1 μg of α-amanitin per ml. Sizes of RNA polymerase III transcripts (to the right) as well as a few pBR322 marker fragments (lane pBR; numbers at left) are indicated.

To determine whether the CELO virus genome encoded low-molecular-weight RNAs which are similar in structure to the human VA RNAs, we programmed an in vitro transcription system with purified CELO virus and Ad2 DNA (Fig. 1). As expected, Ad2 DNA directed the synthesis of the 160-nucleotide VA RNA₁ and VA RNA₂ species. CELO virus DNA, in contrast, directed the synthesis of two short RNA molecules with estimated lengths of 90 and 110 nucleotides, respectively. The synthesis of these RNAs were resistant to low concentrations of α-amanitin (1 μg/ml), suggesting that they were transcribed by RNA polymerase
III (51). As described below, the 90-nucleotide RNA species was also synthesized late during a CELO virus infection of CEK cells, and it will therefore be referred to as the CELO virus-associated RNA (CELO VA RNA). The 110-nucleotide species corresponded to a readthrough product of CELO VA RNA which used an alternative termination site of transcription and was therefore extended approximately 20 nucleotides at the 3' end (see below).

Cloning of the CELO VA RNA gene. To further characterize the CELO VA RNA, we cloned the fragment mixture generated by double digestion of CELO virus DNA with restriction endonucleases EcoRI and HindIII in plasmid pBR322. Small-scale plasmid preparations (15) were prepared from recombinants and assayed for their ability to direct the synthesis of CELO VA RNA in a soluble HeLa whole-cell extract. One positive clone, pC-va, which gave rise to the synthesis of both the 90- and 110-nucleotide species (Fig. 1), was selected for further characterization.

First a partial restriction endonuclease cleavage map was constructed for the 1,800-base-pair (bp) DNA insert in pC-va (Fig. 2B), and subsequently the location of the CELO VA RNA gene was mapped by Southern blot hybridization (43). DNA samples were digested with several different restriction endonucleases, the fragment mixtures were separated electrophoretically on agarose gels, and the DNA was transferred to a nitrocellulose sheet and hybridized with 32P-labeled CELO VA RNA prepared by in vitro transcription (data not shown). The results showed that both the 90- and the 110-nucleotide VA RNA transcripts (Fig. 1) were encoded within a 190-bp FokI-Tth1111I fragment located around position 1100 in clone pC-va (Fig. 2B).

Partial sequence analysis of plasmid pC-va. A 440-bp DNA sequence covering the FokI-Tth1111I fragment was determined (Fig. 2C). The established sequence (Fig. 3) showed several features expected for an RNA polymerase III transcription unit. For example, sequences homologous to the split promoter of RNA polymerase III genes could be identified (48). The Fig. 3, box A homology differed by two nucleotides from the established consensus sequence, whereas the Fig. 3, box B sequence showed a perfect identity. The sequence also contained two runs of T residues (positions 288 to 292 and 296 to 303), which could function as termination signals for RNA polymerase III transcription (8). Based on these considerations, we tentatively concluded that an RNA polymerase III transcription unit was located somewhere between positions 150 and 300 in the established sequence (Fig. 3).

Characterization of CELO VA RNA. To confirm this prediction and to map, at the nucleotide level, the position of the CELO VA RNA gene, the initiation and termination sites for transcription were determined by an S1 protection assay. Two DNA fragments were isolated for this analysis (Fig. 4A); to localize the 5' end, a 65-bp RsaI-AvaII fragment (position 175 to 241; Fig. 3), 5'-end-labeled at the AvaII cleavage site, was used. To position the 3' end, a 135-bp Ddel-FnuD2 fragment (position 205 to 339; Fig. 3), 3'-end-labeled at the Ddel cleavage site, was used. After S1 cleavage, the resistant material was separated through a
denaturing polyacrylamide gel in parallel with the corresponding DNA sequence ladder. RNA purified from a whole-cell extract programmed with plasmid pC-va protected an approximately 55-nucleotide DNA fragment (Fig. 4B, in vitro lane). This located the 5' end and transcriptional start site for CELO VA RNA close to position 185 as shown in Fig. 3. The exact initiation site was, however, ambiguous, since the S1 treatment gave rise to a slight heterogeneity at the end of the protected DNA fragment. The 3' end analysis (Fig. 4C) demonstrated the existence of two forms of CELO VA RNA in vitro; a major transcript having a 3' end located between positions 270 and 274 (Fig. 4C, 70 band) and a minor readthrough transcript (Fig. 4C, 85 band) terminating at the T cluster located between positions 288 and 293 (Fig. 3). The existence of alternative 3' ends explained the two transcripts observed during in vitro transcription (Fig. 1). We could predict from the S1 data that both species had the same 5' end. However, whereas the shorter, 90-nucleotide species had its 3' end at position 270 to 274, the 110-nucleotide species bypassed this transcription termination signal and extended for an additional 15 nucleotides to the T cluster located between positions 288 and 293, which functioned as a second stop signal.

We also examined whether the same low-molecular-weight RNAs were synthesized under in vivo conditions. The shorter, 90-nucleotide species was present in cytoplasmic RNA isolated from late CELO virus-infected CEK cells (Fig. 4B and C, in vivo lanes) as well as in RNA prepared from 293 cells transfected with plasmid pC-va (Fig. 4B and C, transfected lanes). However, the minor, 110-nucleotide readthrough product was hardly expressed under these conditions.

Position of CELO VA RNA on the viral genome. To map the position of the CELO VA RNA gene on the viral chromosome, we hybridized 32P-labeled pC-va DNA to different sets of restriction endonuclease cleavage fragments of CELO virus DNA (Fig. 5A). The results (Fig. 5B) demonstrated that clone pC-va (located at 1,800-bp HindIII-EcoRI fragment located between coordinates 88 and 92 on the CELO virus genome. Since the position and orientation of the VA RNA gene was known in plasmid pC-va (Fig. 2B), we concluded that it was transcribed in the leftward direction from a gene located at coordinate 90 on the CELO virus genome (Fig. 2A). This is in contrast to the human VA RNAs, which are transcribed in the rightward direction from genes located around coordinate 30 (11, 26, 32).

Low-molecular-weight RNAs expressed during CELO virus infection. To determine whether CELO virus encoded additional VA RNAs, which for some reason had escaped detection under our in vitro transcription conditions, we studied the virus-encoded low-molecular-weight RNAs expressed during a lytic CELO virus infection. Total RNA isolated from late infected CEK cells (25 h postinfection) was separated in a denaturing polyacrylamide gel, transferred electrophotographically to a nylon sheet, and hybridized with different 32P-labeled DNA probes. Hybridization with CELO virus DNA, purified from virions, disclosed the existence of only one virus-encoded low-molecular-weight RNA species (Fig. 6). This RNA corresponded in size and genomic location, as shown by hybridization with subfragments of pC-va (Fig. 6), to the 90-nucleotide transcript characterized above.

Biological activity of CELO VA RNA. Ad2 VA RNA1 is required for efficient translation of viral mRNAs late after infection (47). The effect of VA RNA1 can also be reproduced in a simple transient expression assay in 293 cells (44). Cells transfected with plasmids expressing the Ad2 VA RNAs complement the growth of the Ad5 mutant d1331 (47), which is defective in late mRNA translation due to the lack of the VA RNA1 species (C. Svensson and G. Akusjarvi, manuscript in preparation). Also, the synthesis of viral as well as nonviral proteins can be significantly enhanced by cotransfection of plasmids encoding the Ad2 VA RNA1 species (44, 45).

CELO VA RNA was correctly synthesized in 293 cells
RsAl  Ddel  Avall  FnuD2

3'-probe *

* 5'-probe

A

B

C
transfected with plasmid pC-va (Fig. 4). To determine whether it could also function as a positive effector in mRNA translation, we tested whether CELO VA RNA enhanced protein synthesis in a cotransfection assay.

For this experiment, a chimeric plasmid, pTripcat-2 (45), expressing the CAT enzyme under the transcriptional control of the major late adenovirus promoter, was used. In separate experiments, plasmid pTripcat-2 DNA was cotrans-
FIG. 6. Northern blot analysis of low-molecular-weight RNAs expressed during a CELO virus infection. (A) Total RNA isolated from CELO virus-infected CEK cells was separated on a denaturing polyacrylamide gel and transferred electrophoretically to a Gene Screen plus membrane. Hybridization was with the following 32P-labeled DNA probes: CELO DNA purified from virus particles (lane CELO), plasmid pC-va (lane pC-Va), and subfragment of plasmid pC-va depicted in panel B (lanes 1 through 4). Arrow, position of CELO VA RNA; pBR322 marker fragments (lane pBR) are included, with sizes (in bp) on the left. (B) Schematic diagram showing the position of subfragments of plasmid pC-va used for the Northern blot hybridization. 1-2, Position of CELO VA RNA; small arrow, direction of transcription. E, EcoRI; F, FokI; H, HindIII; K, KpnI; T, Tth111I; X, XbaI; pBR, pBR322.

TABLE 1. Stimulation of CAT expression in 293 cells

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<th>Plasmid</th>
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<tr>
<td>pBR322</td>
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<td>pC-va</td>
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<td>pHindB</td>
<td>6.3</td>
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* Cells were transfected with 3 μg of pTripcat-2 DNA and 5 μg of either pBR322, pC-va, or pHindB DNA.

mRNA expression (data not shown), suggesting that the observed effects of both were at the level of mRNA translation.

DISCUSSION

The human subgroup C adenoviruses express massive amounts of two low-molecular-weight RNAs, the VA RNAs. These RNAs are required for an efficient initiation of polypeptide chain synthesis during lytic growth of human adenoviruses (35, 39–41, 47). To determine whether the distantly related avian adenoviruses coded for RNA(s) which serves an analogous role, the low-molecular-weight RNAs encoded by CELO virus were characterized. We found that the CELO virus genome encoded one small RNA which was transcribed by RNA polymerase III. This RNA was expressed in large amounts late during a lytic infection and therefore fulfills the basic requirement for a VA RNA (34). However, as shown here, the CELO VA RNA differed drastically from its Ad2 counterparts in several respects.

In contrast to the human subgroup C adenoviruses, which all encode two low-molecular-weight RNAs (27), CELO...
virus specified only one VA RNA species (Fig. 6). This RNA was considerably shorter than the human VA RNAs (90 nucleotides long, compared with 160 nucleotides). The gene and its flanking sequences also exhibited only a limited primary sequence homology with the human VA RNA genes (data not shown); it is doubtful that the observed similarities were statistically significant.

CELO VA RNA also differed in its chromosomal localization compared with previously characterized serotypes. Thus, CELO VA RNA appeared to be transcribed in the leftward direction from a gene located close to coordinate 90 on the viral genome (Fig. 2). This is in contrast to VA RNAs from the human adenoviruses (12, 27) and also with Simian adenovirus type 7 (S. Larsson, C. Svenson, and G. Akusjärvi, manuscript in preparation), which all are transcribed in the rightward direction from genes located around coordinate 30. The orientation of the CELO virus genome is uncertain since few genes have so far been mapped on the viral genome. However, with the orientation shown in Fig. 2, the positions of the genes coding for the T antigens (30), as well as the DNA sequence required for encapsidation, are oriented toward the left end of the genome (20) as is the case with the human adenoviruses. With the opposite orientation, the CELO VA RNA gene would be placed on the left side, transcribed in the rightward direction but still displaced about 20 map units relative to the genes for the human VA RNAs. Furthermore, since CELO DNA is approximately 30% longer than human adenovirus genomes (53), the CELO VA RNA gene is located around 4,000 bp from the end of the genome, compared with 10,700 bp in the case of Ad2 (37). The differences in arrangement of VA RNA genes hints at large differences in gene organization between avian and human adenoviruses.

The Ad2 VA RNA gene has been extensively mutated in a search for nucleotide sequences which are of importance in its function (6). These data have indicated that many short regions, or more likely the secondary structure of the RNA, is of importance. A characteristic of the human VA RNAs is
that computer-generated secondary structures show extensively base-paired stem loops where the sequences at the 5' and 3' ends of the molecule are annealed to each other (Fig. 7) (1). Experimental evidence for the existence of such hairpin structures free in solution has also been presented (31), suggesting that the folding of the RNA chain may be a key factor in biological function. In this respect it is noteworthy that CELO VA RNA, which has no significant primary sequence homology with the Ad2 VA RNAs, can be predicted to fold into a highly base-paired secondary structure which shows features resembling the Ad2 VA RNA (Fig. 7).

CELO VA RNA functioned, with a low efficiency, as a translational enhancer in a transient expression assay (Table 1). However, whether CELO VA RNA also serves this function during viral growth in chicken cells is not known. The lower efficiency by which CELO VA RNA functioned in our assay system may have been due to its poor expression in human cells (Fig. 1) or alternatively may have been caused by cell type specificity. Theoretically, CELO VA RNA may still be an efficient translational enhancer in the natural host of the virus. It is also possible that CELO VA virus, which has a lytic growth cycle that is considerably longer than the human subgroup C adenoviruses (4, 22), is less dependent on a VA RNA. In fact, the speed by which the infectious cycle advances may be correlated to the efficiency by which VA RNAs rescue the translational capacity of virus-infected cells.

Much to our surprise, the termination signal for CELO VA RNA transcription was found to be different from all previously characterized RNA polymerase III genes. Previous studies have shown that the 3' end of RNA polymerase III products is generated by transcription termination rather than processing of a longer precursor transcript (10). A stretch of T residues (four Ts or more), preferably bordered by GC base pairs, has been shown to be sufficient to cause termination (8). Unlike other RNA polymerase III genes, CELO VA RNA terminates at a TTATT sequence (position 270 to 274; Fig. 3). This sequence is also efficiently recognized by the transcription machinery in HeLa and 293 cells (Fig. 4B), excluding the possibility that the control sequences for transcription termination differ between avian and human cells. Instead it appears that RNA polymerase III can recognize a TTATT sequence as a stop signal under certain conditions. Since this has not been described before, it seems likely that additional features, in addition to a run of T residues, determine transcription termination in eucaryotic cells. These are currently under investigation.

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