Adenovirus VA RNA₁: a Positive Regulator of mRNA Translation

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Received 3 November 1983/Accepted 19 January 1984

We have developed a sensitive transient expression assay in 293 cells to study the effect of VA RNAs on the translation of adenovirus mRNAs. Monolayers of 293 cells were transfected with mixtures of recombinant plasmids encoding adenovirus-specific transcription units and plasmids encoding VA RNAs. Transfected cells were labeled with [35S]methionine for ca. 15 h, and labeled cell extracts were prepared. Changes in the protein expression caused by VA RNA cotransfection were measured by immunoprecipitation, using monospecific antisera prepared against adenovirus-specific polypeptides. Using this experimental design, we demonstrate that VA RNA stimulates the translation of both early and late adenovirus mRNAs. Synthesis of the E3 19,000-dalton glycoprotein and the E2A 72,000-dalton DNA binding protein was stimulated between 10 and 20 times by VA RNA cotransfection. Synthesis of the late hexon polypeptide was also stimulated, although translation of hexon was from an aberrant mRNA lacking the second and third segments of the common tripartite leader attached to late adenovirus mRNAs. VA RNA₁, although very homologous to VA RNA₁, does not function as a translational enhancer.

Adenovirus gene expression is strictly regulated at multiple levels during the normal infectious cycle (reviewed in references 10 and 27). Many of the regulatory events occur at the level of RNA biogenesis, but evidence for the regulation of both early and late viral mRNA translation has been presented (6, 26, 33, 38).

After the onset of viral DNA replication, host protein synthesis is almost completely inhibited (5, 7). Although the deprivation of host cell mRNA sequences from the cytoplasm of late infected cells (8) may provide part of the explanation, recent experiments have indicated that a late adenovirus gene product causes a shut-off of host protein synthesis (6). In addition to a decline in cellular protein synthesis, recent experiments have shown that adenovirus VA RNA₁ in some way is required for an efficient translation of late adenovirus mRNA (38). In the present communication we have analyzed the specificity of this translational control.

VA RNAs are two small noncoding RNA species (VA RNA₁ and VA RNA₁₁) encoded by the adenovirus genome (25, 32, 35, 40). The genes for VA RNAs have been mapped as two tandem transcription units located around map coordinate 30 on the adenovirus type 2 (Ad2) genome (25, 29). Sequence studies of VA RNAs and their genes have shown that both RNAs are ca. 160 nucleotides in length, exhibit scattered regions of sequence homology, and can adopt secondary structures which resemble each other (2).

VA RNAs are unique among adenovirus genes in that they are transcribed by RNA polymerase III (31, 35, 40). In accordance with other polymerase III products, an intragenic transcriptional control region has been identified for VA RNA₁ transcription (12, 17). The two VA RNAs accumulate with different kinetics during a lytic infection. VA RNA₁ is predominantly synthesized early during the infectious cycle, whereas synthesis of VA RNA₁₁ increases late after infection, making it the most prominent RNA species in the infected cell (35).

Although VA RNAs were discovered in 1966 (32), a specific function was not assigned to them until recently. By constructing two Ad5 variants, each of which failed to synthesize one of the VA RNA species, Thimmappaya et al. (38) were able to demonstrate that VA RNA₁ in some way is required for an efficient translation of mRNA late during adenovirus infection. In the present communication, we present a transient expression assay in 293 cells which we have used to investigate the specificity of this translational control. We show that VA RNA₁ mediates a translational stimulation which is not restricted to late adenovirus mRNAs but also includes the mRNAs from at least two early adenovirus transcription units. VA RNA₁₁, although very homologous to VA RNA₁ (2), does not have the capacity to function as a translational enhancer.

MATERIALS AND METHODS

DNA, RNA, and enzymes. Cloning in plasmid vectors, including the addition of synthetic linker DNA, was by standard recombinant DNA techniques (23), using pBR322 as a vector. Isolation of cytoplasmic RNA from Ad2-infected HeLa cells in suspension and from 293 monolayer cells transfected with recombinant plasmids were as previously described (3). The synthetic HindIII linker d(CAAGCTT) and all restriction endonucleases used in this study were purchased from New England Biolabs. Nuclease S1 was obtained from Sigma Chemical Co.

Cell growth and transfection. Monolayer cultures of 293 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum and 50 μg of gentamicin per ml. Subconfluent cells were transfected as previously described (37) by using the calcium phosphate coprecipitation technique (41). Usually, a total of 15 μg of plasmid DNA was added per 5-cm petri dish. Plasmids encoding the VA RNA genes and plasmids encoding structural genes were added in amounts given in the figure legends. When necessary, pBR322 DNA was added as a carrier to bring the total amount of DNA to 15 μg per transfection.

Radioactive labeling of cells and preparation of protein extract. Approximately 32 h posttransfection, cells were washed with phosphate-buffered saline and labeled with 20 μCi of [35S]methionine (1.000 Ci/mmol; New England Nucle-
FIG. 1. Experimental scheme to study the effect of VA RNAs on viral mRNA translation. Subconfluent monolayers of 293 cells were transfected (37) with a mixture of recombinant plasmids encoding VA RNA and adenovirus-specific transcription units. After an incubation period of 6 h, the transfected cells were treated for 2 min with 15% glycerol. At ca. 35 h posttransfection cells were refed with medium containing 10 μCi of [35S]methionine ([35S-MET]) per ml. At ca. 50 h posttransfection, cells were lysed, and RNA and protein extracts were prepared for subsequent quantitations of gene expression by S1 endonuclease cleavage and immunoprecipitation. NP40, Nonidet P-40.

ar Corp.) per ml in Eagle medium containing 2.5% of the normal concentration of methionine and 10% fetal calf serum (11). After a labeling period of 15 h, cells were washed three times in phosphate-buffered saline and then fractionated into cytoplasm and nuclei by IsoB–Nonidet P-40 extraction (3). Half of the cytoplasmic extract was used for the isolation of total RNA by phenol extraction (3). Nuclei were washed with RIPA buffer (28), and half of the material was combined with the remaining cytoplasmic fraction, thus giving a [35S]methionine-labeled protein extract.

Immunoprecipitation and in vitro translation. The procedure described by Persson et al. (26) was essentially followed. Briefly, identical amounts (usually 5 × 10⁶ to 10 × 10⁶ cpm) of the [35S]methionine-labeled protein extracts were incubated together with antisera raised against the purified Ad2 hexon polypeptide (30), the E2A 72,000-dalton (72K) polypeptide (22), or the E3 19,000-dalton (19K) polypeptide (28). The antigen-antibody complexes were isolated by binding to protein A-Sepharose (Pharmacia Fine Chemicals). The complexes were washed thoroughly in phosphate-buffered saline–1% Triton–1 mM phenylmethylsulfonfluoride and analyzed (after boiling in sample buffer)

FIG. 2. Effect of VA RNA on the synthesis of the E3 19K glycoprotein. (A) Schematic representation of recombinant plasmid pAL E2/E3 used in the experiment. The stippled box indicates the 11-kilobase Ad2 DNA insert (map coordinates, 58.5 to 89.5) with arrows showing the direction of transcription for early regions 2A and 3. Thin lines denote pBR322 sequences. Only the EcoRI sites bordering the DNA insert are shown. This figure has not been drawn to scale. (B) Immunoprecipitation of [35S]methionine-labeled 293 cell extracts. Monolayers of 293 cells were transfected with 10 μg of plasmid pAL E2/E3 and increasing amounts of plasmid pHindB encoding the VA RNAs. Transfected cells were labeled with [35S]methionine for 15 h, and a cell extract was prepared and immunoprecipitated with an antiserum directed against the E3 19K glycoprotein (28). The radioactivity at the top of the gel is caused by background precipitation of cellular proteins since a normal rabbit antiserum gives an identical result (data not shown). Lanes: UNTRANS-, [35S]methionine-labeled extracts prepared from untransfected 293 cells; 1:0, no plasmid pHindB added; 1:0.1, 1 μg of pHindB added; 1:0.25, 2.5 μg of pHindB added; and 1:0.5, 5 μg of pHindB added. (C) Schematic diagram showing the position of the 5'-end-labeled TaqI fragment (base pairs 9 to 567 in the sequence of Hérisse et al. [18]) used for the S1 endonuclease analysis of E3 mRNAs. (D) S1 endonuclease analysis of E3 RNAs accumulated in 293 cells cotransfected with pAL E2/E3 and pHindB. Electrophoretic separation of the S1-resistant material was through a 5% polyacrylamide gel containing 8 M urea. The 330-nucleotide-long S1-resistant DNA fragment corresponds to the distance from the E3 mRNA cap site to the TaqI cleavage site at position 567 (18) (Fig. 2C). Transfections and symbols are the same as those described for Fig. B. Early RNA, total cytoplasmic RNA isolated from cells maintained in the presence of 1-β-d-arabinofuranosylcytosine (25 μg/ml) from 1 to 7 h postinfection.
by electrophoresis in 10 or 13% sodium dodecyl sulfate-polyacrylamide gels, followed by fluorography. Changes in protein expression were measured by densitometer scanning of autoradiographs. Total cytoplasmic RNA was translated in a mRNA-dependent rabbit reticulocyte cell-free system (Amersham Corp.), and the total translation mixture was immunoprecipitated as described above.

S1 endonuclease analysis. The protocol described by Svensson et al. (37) was followed with a few minor modifications. Usually 10 \( \mu \)g of cytoplasmic RNA (0.1 \( \mu \)g for the analysis of VA RNAs) was hybridized to the 5'-end-labeled DNA fragments described in the figure legends. Hybridizations were carried out overnight at 56°C for the hexon, E2, and E3 probes and at 50°C for the VA RNA\(_1\) and VA RNA\(_{II}\) probes. S1 endonuclease cleavage and electrophoretic separation were performed as previously described (37).

RESULTS

Experimental design. We developed a simple cotransfection assay in 293 cells to study the effect of adenovirus VA RNAs on translation of mRNAs (Fig. 1). Since an efficient viral transcription requires a functional E1A region, we used transfection into 293 cells (15) to ascertain a high level of viral mRNA expression. We have shown (C. Svensson and G. Akusjärvi, submitted for publication) that the 293 cell line, which constitutively expresses the E1A functions (1), stimulates specific adenovirus gene expression ca. 1,000-fold after transfection. Recombinant plasmids encoding adenovirus-specific transcription units were constructed. These plasmids were transfected (16) together with plasmids encoding the VA RNAs into monolayers of 293 cells (Fig. 1). Transfected cells were maintained for ca. 32 h in Eagle medium before a 15-h labeling with \([\text{35S}]\)methionine. At ca. 50 h posttransfection the cells were harvested, and a cell extract was prepared. One half of the cells was used to prepare cytoplasmic RNA, whereas the other half was used to prepare a \([\text{35S}]\)methionine-labeled protein extract. The mRNA expression in the transfected cells was quantitated by S1 endonuclease cleavage (39) and correlated to the protein expression as determined by immunoprecipitation, using monospecific antisera prepared against purified adenovirus polypeptides. The experimental approach was proven functional by demonstrating that synthesis of the late hexon
polypeptide was drastically enhanced by VA RNA cotransfection (see below).

Stimulation of early protein synthesis by VA RNA. To study the effect of VA RNAs on the translation of early viral mRNAs, we constructed recombinant plasmid pAL E2/E3 which includes the complete Ad2 E2A and E3 transcription units (Fig. 2A). The clone, which contains an 11-kilobase DNA insert (map coordinates, 58.5 to 89.5), was constructed by inserting a partial EcoRI digest of Ad2 DNA into the unique EcoRI cleavage site of pBR322.

Plasmid pHindB (map coordinates, 17.1 to 31.6) (36) encoding both VA RNAI and VA RNAII (see Fig. 4A) (2, 4, 14) was cotransfected with pAL E2/E3 into 293 cells. Immunoprecipitation with a rabbit antiserum directed against the E3 19K glycoprotein (28) of a [35S]methionine-labeled cell extract showed that the synthesis of the E3 19K polypeptide is enhanced ca. 10 times by pHindB cotransfection (Fig. 2B). To show that the increased synthesis of the E3 19K polypeptide was not the result of an increased E3 mRNA expression in cotransfected cells, we analyzed the RNA concentration by 5'-end-labeled S1 endonuclease cleavage (Fig. 2C) (39). The amount of E3 mRNA was not affected by cotransfection with plasmid pHindB (Fig. 2D).

Since pAL E2/E3 encodes both the E2A and E3 transcription units (Fig. 2A), the effect of VA RNA on E2A expression was also analyzed. Figure 3A shows the result of an immunoprecipitation, using an antiserum directed against the E2A 72K DNA binding protein (22); the synthesis of the
72K DNA binding protein was stimulated ca. 20-fold by plasmid pHindB cotransfection.

The translational stimulation was not detected when the total cytoplasmic RNA isolated from cotransfected cell was translated in vitro in a mRNA-dependent rabbit reticulocyte lysate (cf. Fig. 3A and B). The apparent difference in E2A 72K polypeptide synthesis in Fig. 3B is caused by a slight variation in the transfection efficiency. Based on these results, we conclude that the enhanced expression on both the E2A 72K DNA binding protein and the E3 19K glycoprotein is due to a translational stimulation mediated by VA RNA. In agreement with earlier reports (6, 38), we found that the translational regulation is not reproduced in an in vitro translation system, suggesting a complex interaction between VA RNA and the translational machinery of the cell. Due to the enormous efficiency of VA RNA transcription in transfected 293 cells (see below), we found that a very small amount of plasmid pHindB is required to obtain the maximal translational stimulation (Fig. 2B and 3A).

Construction of plasmids encoding single VA RNA genes. To determine whether both VA RNA transscripts contribute to the observed stimulation in our cotransfection assay, we constructed two recombinant plasmids, pVAI and pVAlI, which separately encode the two VA RNAs (Fig. 4A). Clones pVAI (map coordinates, 28 to 29.6) and pVAlI (map coordinates, 29.6 to 31.6) were constructed by converting the BalI cleavage sites present in plasmid pHindB to HindIII cleavage sites by the use of synthetic linkers. The two DNA fragments encoding the VA RNA genes were isolated and inserted into the unique HindIII cleavage site of plasmid pBR322 (Fig. 4A).

Plasmids pVAI and pVAlI gave rise to an efficient synthesis of VA RNA1 and VA RNA2, respectively, when transfected into 293 cells (Fig. 4B). As expected, the parental pHindB plasmid directs the synthesis of both VA RNAs. VA RNAs are transcribed with an enormous efficiency in pHindB-transfected cells. We estimate that the transfected 293 cells contain between 10^5 and 10^6 copies of VA RNA per cell.

Translational stimulation is mediated by VA RNA1. The effect of the two VA RNAs on late adenovirus gene expression was also tested. Clone pKGO-hexon encoding the gene for the hexon polypeptide was constructed by fusing the 8.5-kilobase-long HindIII fragment A of Ad2 (map coordinates, 50.2 to 72.2), encoding the hexon gene and its regulatory signals, to the adenovirus major late promoter (Fig. 5A). The hexon mRNA which is synthesized in pKGO-hexon-transfected 293 cells has the first leader segment spliced to the body of the hexon mRNA sequences (data not shown). Thus, the hexon mRNA synthesized in our transfection experiments differs from mRNA transcribed in late adenovirus-infected cells by lacking the second and third leader segments of the common tripartite leader attached to late mRNAs (reviewed in reference 27).

Monolayers of 293 cells were cotransfected with pKGO-hexon and either plasmid pVAI or pVAlI. Immunoprecipitation of [35S]methionine-labeled cell extracts with a rabbit antiserum directed against the Ad2 hexon polypeptide (30) showed that only pVAI cotransfection resulted in an en-

FIG. 5. Effect of VA RNA1 and VA RNA2 on the synthesis of the late hexon polypeptide. (A) Schematic drawing illustrating the structure of the recombinant plasmid pKGO-hexon. Fragment HindIII-A (map coordinates, 50.2 to 72.2) encoding the hexon gene was fused to a 871-base-pair BamHI-HindIII fragment (map coordinates, 14.7 to 17.1) (22) in plasmid pBalE (24), which encodes the major late adenovirus promoter and the first segment of the common tripartite leader. The hexon mRNA thus generated has the first leader segment spliced to the acceptor site for the hexon gene (3). (B) Immunoprecipitation of [35S]methionine-labeled 293 cell extracts with an antiserum directed against the hexon polypeptide. Monolayers of 293 cells were transfected with 10 μg of plasmid pKGO-hexon and 3 μg of either plasmid pBR322, pVAI, or pVAlI. [35S]methionine labeling and immunoprecipitation were as described in the text. Electrophoretic separation was on a 10% sodium dodecyl sulfate-polyacrylamide gel. UNTRANSF., [35S]methionine-labeled extracts prepared from untransfected 293 cells; Ad2, [35S]methionine-labeled marker virus.
enhanced hexon polypeptide synthesis (Fig. 5B). pVAlII-co-transfected cells showed no significant stimulation. The failure of plasmid pVAlII to stimulate translation of the hexon mRNA was not caused by an inefficient VA RNA function since increasing the amount of plasmid pVAlII in the transfection mixture did not result in an enhancement of hexon polypeptide synthesis. Furthermore, cotransfection of pVAl and pVAlII with pAL E2/E3 shows that VA RNA also is responsible for the translational stimulation of the E3 19K and E2A 72K polypeptides described in the previous section (data not shown).

**DISCUSSION**

Previous work, using the Ad5 deletion mutants dl330 and dl331 (VA RNA\(\rightarrow\)VA RNA\(\rightarrow\)) has shown that production of viral polypeptides is reduced in late infected cells (38). This suggests that VA RNA\(\rightarrow\) in some way is required for an efficient translation of late viral mRNAs. When early viral polypeptide synthesis was analyzed, it was unexpectedly found that mutant dl330 produced enhanced levels of the E2A 72K DNA binding polypeptide at 5 to 7 h postinfection. This effect does not seem to be due to an inhibitory effect of VA RNA\(\rightarrow\) on early polypeptide synthesis since a derivative of dl330 with a functional VA RNA\(\rightarrow\) gene inserted in region E3 has an identical phenotype (38). Since adenovirus gene expression is subjected to a very complex regulation by mechanisms which still are largely unknown, we have developed a transient expression assay in 293 cells to analyze the effect of VA RNAs on mRNA translation. Although having some limitations, the transfection assay simplifies interpretation of the experimental results by minimizing the influence of other adenovirus genes on the process to be studied.

We demonstrate in this paper that adenovirus VA RNA\(\rightarrow\) functions by stimulating translation of a variety of mRNAs. The synthesis of the adenovirus E2A 72K DNA binding protein, involved in viral DNA replication, and the E3 19K glycoprotein, a membrane protein found associated with the major transplantation antigens in both humans and rodents (19, 34), are stimulated by VA RNA\(\rightarrow\). The synthesis of the late hexon polypeptide is also efficiently stimulated, although the hexon polypeptide in our experiments is translated from an aberrant mRNA lacking the second and third segments of the tripartite leader which is normally attached to late adenovirus mRNAs (reviewed in reference 27). It will be of interest to determine whether a mRNA carrying the intact tripartite leader functions more efficiently in enhancing late polypeptide synthesis. Whether VA RNA stimulates expression from both viral and cellular mRNAs, rather than selecting adenovirus mRNAs for translation, remains to be firmly established. Our preliminary experiments indicate that the translational stimulation by VA RNA may in fact lack specificity, since we find that the bacterial chloramphenicol acetyltransferase gene when under the control of the simian virus 40 early promoter is stimulated by the VA RNAs (unpublished data). We are currently analyzing nonviral genes in our cotransfection assay to clarify the specificity of VA RNA-mediated translational control.

How does VA RNA\(\rightarrow\) function in translation? Although our experimental design does not allow us to exclude the possibility that VA RNA\(\rightarrow\) functions by increasing the cytoplasmic stability of proteins, pulse-labeling experiments with VA RNA\(\rightarrow\)-negative mutants have shown that the reduced level of viral polypeptides in mutant infected cells is due to a reduction in the rate of protein synthesis. The rate of chain elongation is the same, but fewer ribosomes are attached to each mRNA in mutant infected cells (38), suggesting a role for VA RNA\(\rightarrow\) in the initiation of translation. VA RNAs exist in the infected cells as ribonucleoprotein particles in association with the cellular protein, La, which is recognized by the La class of lupus antisera (13, 21). The Epstein-Barr virus genome also encodes small RNAs which are associated with the same cellular antigen (20). These RNAs can, although very inefficiently, substitute for VA RNAs during adenovirus replication (9). The La antigen does not seem to be necessary for VA RNA transcription, at least not in vitro (13), but the association between VA RNAs and the La antigen may be of importance for the translational control mediated by VA RNA. Whether VA RNAs regulate protein synthesis by associating with ribosomes is at present unanswered. It should certainly be possible to test the hypothesis with the transfection assay described in the present communication.

We have previously shown that the two VA RNA\(\rightarrow\) genes, at the primary sequence level, are very closely related. Both RNAs are similar in length and can adopt secondary structures which resemble each other (2). Despite these similarities, only VA RNA\(\rightarrow\) functions as a stimulator of protein synthesis under our experimental conditions. Furthermore, infection of HeLa cells with the dl328 mutants results in wild-type yields of virus, suggesting that the VA RNA\(\rightarrow\) gene is also dispensable for virus growth in tissue culture cells (38). Thus, at this time the function of VA RNA\(\rightarrow\) remains a puzzle.

**ACKNOWLEDGMENTS**

We thank Marie Lager for excellent technical assistance and Marianne Gustafson for patient secretarial help. We also thank Ulf Pettersson for fruitful discussions and help in the preparation of this manuscript.

This work was supported by grants from the Swedish Cancer Society and the Swedish Natural Science Research Council.

**LITERATURE CITED**


