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Construction and Analysis of Additional Adenovirus Substitution Mutants Confirm the Complementation of VAI RNA Function by Two Small RNAs Encoded by Epstein-Barr Virus

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Adenovirus VAI RNA is essential for the efficient initiation of translation of viral mRNAs at late times after infection. Recently, by constructing an adenovirus type 5 substitution mutant, we showed that the Epstein-Barr virus encoded two small RNAs complemented for the VAI RNA function in the adenovirus type 5 lytic growth (Bhat and Thimmappaya, Proc. Natl. Acad. Sci. USA 80:4789–4793, 1983). This observation was based on our inability to propagate an adenovirus type 5 mutant lacking functional VAI and VAII genes. Subsequently, it was found that this mutant was viable and able to grow to a low titer. Therefore, we examined the complementation of the VAI RNA function by the Epstein-Barr virus-encoded RNAs by constructing additional adenovirus type 5 substitution mutants containing multiple copies of the Epstein-Barr virus-encoded RNA genes in nonessential early transcriptional region III. The new substitution mutants synthesized viral polypeptides at late times at levels comparable to those observed in wild type-infected cells. Our results convincingly demonstrated that the two Epstein-Barr virus-encoded RNAs can efficiently complement for the VAI RNA-mediated translational defect in adenovirus-infected cells.

Human cells infected by adenovirus synthesize large amounts of two low-molecular-weight RNAs designated virus-associated (VA) I and II RNAs (16, 22). The genes coding for these RNAs map around 29.0 map units (m.u.) on the adenoviral physical map, and they are transcribed by RNA polymerase III (22, 25). The VA RNAs are about 157 to 162 nucleotides long, and they can fold to form compact stem-loop structures (1). Portions of these RNAs are present in the cytoplasm as ribonucleoprotein particles complexed with a host-encoded 45,000-dalton phosphoprotein (La antigen) (9, 12, 13).

The major species, VAI RNA, has been shown to be required for the efficient initiation of translation of viral mRNAs at late times after infection (20, 24). The mechanism by which VAI RNA enhances translation appears to be by blocking a kinase activity which phosphorylates protein initiation factor eIF-2 (17, 19, 21). Because phosphorylated eIF-2 is unable to recycle, the block of eIF-2 phosphorylation is necessary for continued initiation of protein synthesis.

Cells harboring the genome of Epstein-Barr virus (EBV) sythesize large amounts of two low-molecular-weight RNAs designated EBV-encoded RNAs (EBER) I and II, which are also transcribed by RNA polymerase III (18). Although there is no striking nucleotide sequence homology between VAI RNA and the two EBERs, they are similar in gene organization (18).

To examine whether the EBERs could functionally substitute for VAI RNAs in adenovirus-infected cells, we previously constructed and characterized an adenovirus type 5 (Ad5) substitution mutant in which the two VA RNA genes were deleted and replaced by an EBV DNA segment coding for the two EBERs (*sub*731) (4). Because our previous attempts to construct a viable Ad5 mutant lacking VAI and VAII genes were unsuccessful and because Ad5 mutant *sub*731 containing single copies of the EBER genes was viable, we concluded that the two EBERs complemented the VA RNAs for growth. Subsequently, extensive experiments in our laboratory (3) and in the laboratory of T. Shenk showed that the Ad5 mutant lacking both of the VA RNA genes, although a poor grower, can reach a titer which is 60- to 80-fold less than that of a wild-type (WT) control, which is within 2-fold of the titer to which the EBER-substituted mutants can grow. These results led to some doubt concerning whether the two EBERs indeed complement for the VAI RNA function.

To clarify this point, we constructed two additional Ad5 substitution mutants in which multiple copies rather than a single copy of the EBER genes were inserted into the adenovirus genome and assayed the translation defect more directly by analyzing viral polypeptides. In this report, we show that these new substitution mutants synthesized late polypeptides at WT levels. Our results convincingly confirm and extend our previous observation that the EBERs are capable of functionally substituting for the VAI RNA function in adenovirus-infected cells.

MATERIALS AND METHODS

Cells, viruses, and plasmids. Cell line 293 (an Ad5-transformed human embryo cell line) and HeLa cells (obtained from T. Shenk, Princeton University) were maintained in Dulbecco modified Eagle minimal essential medium containing 10% calf serum. Mutant *dl*704 is an Ad5 variant which has a 17-base pair (bp) intragenic deletion in the VAII RNA gene. As a result, the VAII RNA gene of this variant is not transcribed (4). Mutant *sub*731 is a variant in which a 230-bp DNA segment coding for the VAI RNA gene (29.0 to 30.0 m.u.) was replaced by a 1.1-kilobase (kb) DNA segment of the EBV genome coding for the two EBERs. Mutant *sub*730 is identical to mutant *sub*731 except that this variant contains a functional VAII RNA gene. Mutant *sub*722 has the DNA segment coding for the VAI RNA gene replaced

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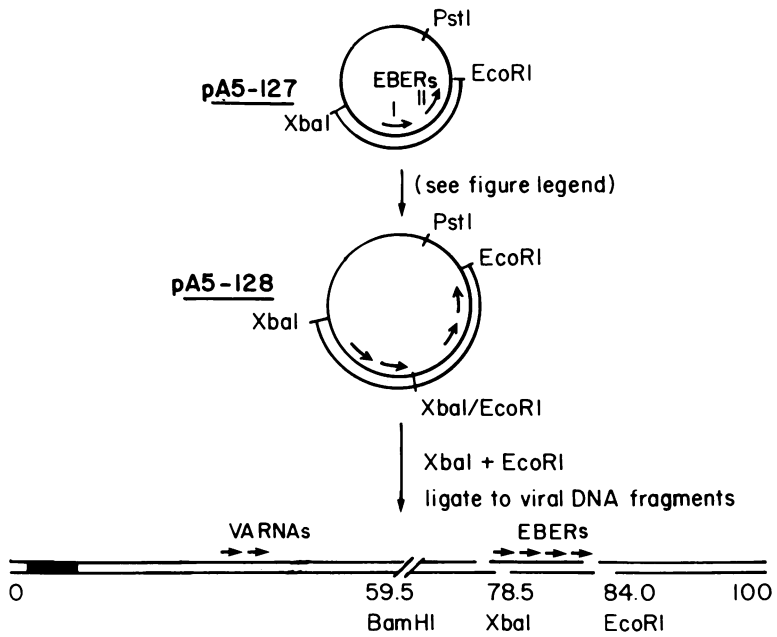

FIG. 1. Strategy used to construct Ad5 variant *dl-sub*750. A 1.1-kb DNA segment of the EBV genome coding for the two EBERs was cloned between the *Xba*I and *Eco*RI sites of a derivative of pBR322 (4). This 1.1-kb DNA segment was duplicated in tandem in pA5-128 as follows. Plasmid pA5-127 was cut with *Xba*I and *Eco*RI separately, and the cohesive ends were trimmed with nuclease S1. The plasmids were then cut with *Pst*I. Fragments from the *Pst*I site to the *Eco*RI site from one digest and from the *Pst*I site to the *Xba*I site from another digest were separated on an agarose gel, ligated, and amplified in *Escherichia coli* HB101 by using standard recombinant DNA procedures. The 2.2-kb DNA fragment containing a tandemly duplicated 1.1-kb EBV DNA segment coding for the EBER genes was ligated into the 0- to 78.5-m.u. (*Xba*I site) viral DNA fragment of *dl*321 and the 84.0-m.u. (*Eco*RI site) to 100.0-m.u. WT DNA fragment. The ligated sample was plaqued on cell line 293 cells by using a DNA plaque assay (11). The single solid lines in pA5-127 and pA5-128 are pBR322 portions. The solid box in viral DNA represents the deletion of the EIA region of *dl*312 (11).

by an Ad2 DNA segment from 88.3 to 89.7 m.u. (*Xba*I and *Eco*RI sites, respectively). As a result, this variant is VAI$^-$ VAII$^+$. The construction and characterization of the above mutants have been described previously (4). Double mutant *dl-sub*720 is VAI$^-$ VAII$^-$; it was derived from *sub*722. In addition to substitution of VAI RNA sequences by an unrelated sequence, *dl-sub*720 also has a 17-bp deletion in the coding sequences of the VAII RNA gene which is identical to the deletion of *dl*704. Additional details concerning this mutant have been described previously (3, 6). Mutant *dl*321 is similar to *dl*312 except that it retained the two *Xba*I sites located at 78.5 and 84.0 m.u. (11; B. Thimmappaya and T. Shenk, unpublished data).

Construction of new Ad5 substitution mutants. Because of technical reasons, the new Ad5 substitution mutants with multiple copies of the EBER genes were constructed in two steps. First, the 1.1-kb EBV DNA segment coding for the two EBER genes (pA5-127 [4]) was duplicated in another plasmid (pA5-128) by using standard recombinant DNA procedures (Fig. 1). The 2.2-kb DNA segment containing two copies each of the EBERI and EBERII genes was introduced into early transcriptional region III (EIII) of *dl*321 by a three-fragment ligation step; the 0- to 78.5-m.u. fragment from *dl*321 and the 84.0- to 100.0-m.u. fragment from WT Ad5 were ligated to the *Xba*I-to-*Eco*RI fragment of pA5-128. The ligated DNA sample was plaqued on cell line 293 cells to generate viral mutant *dl-sub*750 (Fig. 1). All of the Ad5 mutants described above and *dl-sub*750 contained unique *Bam*HI sites at 59.5 m.u. Mutants *sub*732 and *sub*733 were constructed from *sub*731 and *sub*722, respectively, by ligating the 0- to 59.5-m.u. fragments from these variants into the 59.5- to 100.0-m.u. fragment from *dl-sub*750. Therefore, mutant *sub*732 had one copy of the EBER genes between 29.0 and 30.0 m.u. and two copies of the EBER genes in EIII. Mutant *sub*733 had no VAI gene but contained a functional VAII gene and two copies of the EBER genes in EIII. The structures of these variants are summarized in Fig. 2.

RNA and protein analysis. ^{32}P-labeled total cytoplasmic RNAs were isolated from the mutant-infected cells and the RNA samples were analyzed on denaturing polyacrylamide gels as described previously (3).

To analyze the viral polypeptides, cell line 293 cells were infected at a multiplicity of 10 PFU per cell with the mutants and after 15 or 23 h were labeled with [^{35}S]methionine (50 μCi/ml; specific activity, >800 Ci/mmol; Amersham Corp.) for 1 h. The cells were then washed twice with phosphate-buffered saline (pH 7.4) and lysed in cold RIPA buffer (0.15 M NaCl, 0.1% sodium dodecyl sulfate, 1.0% sodium deoxycholate, 1.0% Triton X-100, 1 mM EDTA, 20 mM Tris, pH 7.4) at a concentration of 2.0×10^6 cells per ml as described by Cepco and Sharp (7). Cell extracts derived from equal numbers of cells were loaded directly onto a 20% sodium dodecyl sulfate–polyacrylamide gel to analyze late polypeptides.

RESULTS

Construction of mutant viruses. The protocol used to construct the Ad5 substitution mutants with multiple copies of the EBER genes is described in Materials and Methods (Fig. 1). Figure 2 shows the Ad5 mutants used in the experiments described below.

Mutant *dl*704 is a VAI$^+$ VAII$^-$ variant which grows to WT levels and was used as a WT control. Mutant *dl-sub*720 is a

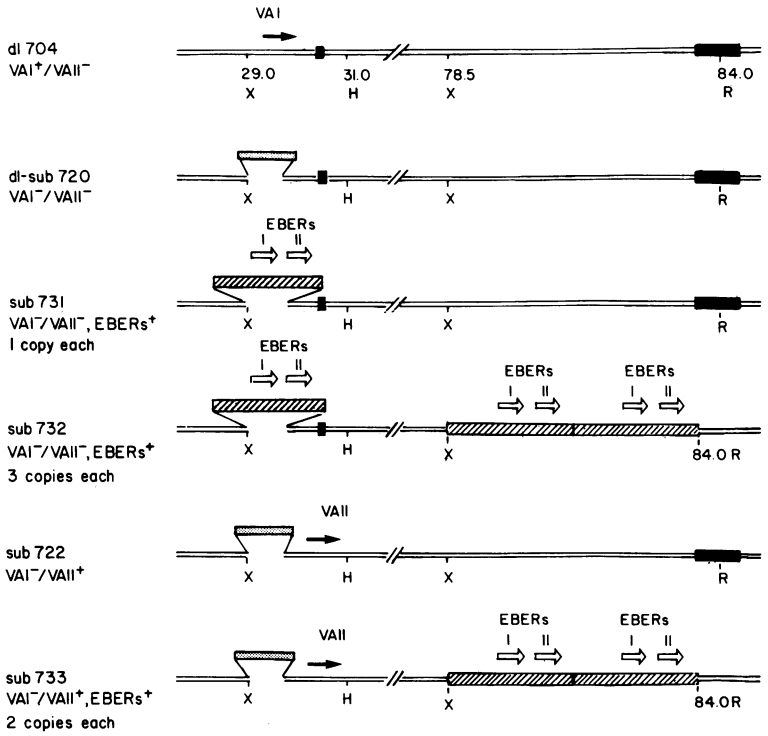

FIG. 2. Adenovirus substitution mutants. The solid boxes represent deletions. The stipplied regions in *dl-sub*720, *sub*722, and *sub*733 represent a 430-bp Ad2 DNA segment (88.3 to 89.7 m.u.) inserted between *Xba*I and *Eco*RI sites (29.0 and 30.0 m.u., respectively). The cross-hatched areas in *sub*731, *sub*732, and *sub*733 represent the EBV DNA segment coding for the EBER genes. The solid box between 30.0 and 31.0 m.u. is a 17-bp deletion within the putative intragenic promoter of the VAII RNA gene. The solid box at 84.0 m.u. represents a deletion of approximately 1% of the Ad5 genome found in the parental strains used to construct the substitution mutants (11). Restriction endonuclease site abbreviations: X, *Xba*I; H, *Hin*dIII; R, *Eco*RI. For all of these variants, only the portions of the genomes containing the VA RNA genes (between 29.0 and 31.0 m.u.) and the EIII regions are shown.

VAI^- $VAII^-$ double mutant, and *sub*731 is a substitution mutant which is VAI^- $VAII^-$ and contains a single copy of the EBER genes. Mutant *sub*722 is VAI^- $VAII^+$ and thus is similar to *dl*331 with respect to the VAI RNA-specific mutation and phenotype (24) (see Materials and Methods).

The two new Ad5 substitution mutants which contained multiple copies of the EBER genes were designated *sub*732 and *sub*733. Mutant *sub*732 had three copies of the EBER genes, with one copy between 29.0 and 30.0 m.u. and the two other copies in the nonessential EIII region. This mutant contained neither the VAI RNA gene nor the VAII RNA gene. *sub*733 was a VAI^- $VAII^+$ mutant and contained two copies of the EBER genes in the EIII region. Restriction endonuclease digestion of the DNAs of these variants (data not shown) and an analysis of the low-molecular-weight RNAs which they synthesized (see below) confirmed the mutational alterations with respect to either VA RNA or EBER genes. The genomes of mutants *sub*732 and *sub*733 were found to be stable after five passages.

RNA analysis of mutant-infected cells. To study transcription of the VAI or EBER genes of the mutants, HeLa cells were infected with the mutants at a multiplicity of 10 PFU per cell and labeled with $^{32}P_i$, and total cytoplasmic RNAs were analyzed on a 8% denaturing polyacrylamide gel containing 8 M urea (3). Figure 3 shows the an autoradiogram of such a gel. As expected, the lane corresponding to the mutant with a single copy of the EBER genes (*sub*731) contained neither VAI RNA nor VAII RNA but did contain two new RNA bands corresponding to EBERI and EBERII. The identities of these RNAs at these positions were confirmed previously (4). The lane corresponding to the mutant containing three copies of the EBER genes (*sub*732) was similar to the *sub*731 lane, except that the EBERI and EBERII bands were more intense, as expected. Mutant *sub*730 was identical to mutant *sub*731, except that it had a functional VAII RNA gene. This mutant synthesized increased quantities of VAII RNA (4) and provided a marker for Ad5 VAII RNA, as well as the EBERs. Mutant *sub*733, containing two copies of the EBER genes and a functional VAII RNA gene, sythesized increased quantities of the two EBERs and slightly increased quantities of the VAII RNA. Mutant *sub*722, the parent of *sub*733, synthesized large quantites of VAII RNA but no VAI RNA or EBER. The lane containing RNA from the mutant lacking both of the VA RNA genes (*dl-sub*720) did not contain either of the VA RNAs.

The steady-state levels of the two EBERs were determined in cell line 293 cells infected with these mutants by excising the two bands together from the polyacrylamide gel and measuring the radioactivity. Cells infected with the mutant containing three copies of the EBER genes and with the mutant containing two copies of the EBER genes (*sub*732 and *sub*733, respectively) contained approximately twice as much of the EBERs as cells infected with the mutant containing a single copy of the EBER genes (*sub*731).

Polypeptide analysis. Previous results showed that in HeLa cells infected with a VAI^- Ad5 mutant (*dl*331), although normal amounts or properly spliced, polyadenylated, and capped messages were present, viral polypeptide synthesis at late times was reduced 8- to 10-fold compared with

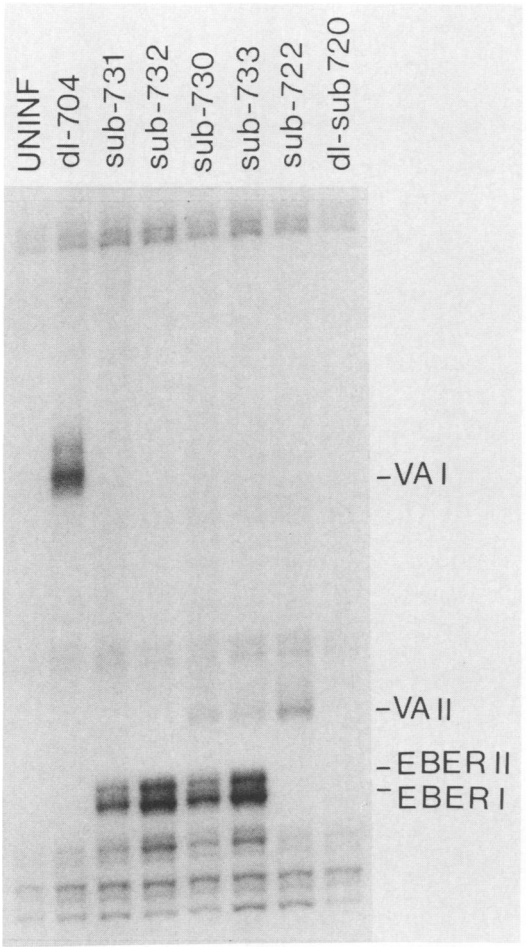

FIG. 3. Analysis of low-molecular-weight RNAs synthesized by the Ad5 mutants described in the text. ^{32}P-labeled total cytoplasmic RNAs isolated from mutant-infected cells were electrophoresed on an 8% polyacrylamide–8 M urea gel (thickness, 0.4 mm; length, 40.0 cm; 600 V in Tris-borate buffer) and autoradiographed. The top 8 cm and bottom 16 cm of the autoradiogram are not shown. UNINF, Uninfected.

WT-infected controls (24). The viral polypeptides synthesized by the Ad5 substitution mutant containing the EBER genes were examined by labeling the infected cells with [^{35}S]methionine and analyzing the labeled proteins on sodium dodecyl sulfate-polyacrylamide gels as described in Materials and Methods. (Fig. 4). At 16 h postinfection, the cells infected with *dl-sub*720, which lacked both the VAI and VAII RNA genes and the EBER genes, showed a drastic reduction in polypeptide levels compared with cells infected with *dl*704, which was used as a WT control (Fig. 4A). Slightly increased quantities of late polypeptides were observed in cells infected with *sub*731, which had one copy of the EBER genes, and with *sub*722, which was VAI$^-$ VAII$^+$. Mutant *sub*732, which contained multiple copies of the EBER genes, synthesized significantly higher quantities of late proteins than the double mutant. Mutant *sub*733, which contained two copies of the EBER genes, synthesized late proteins at levels near WT levels.

At 24 h postinfection, mutants containing three copies of the EBER genes (*sub*732) and mutants containing two copies of the EBER genes (*sub*733) synthesized late polypeptides at WT levels and in quantities dramatically higher than those observed in double mutant- or *sub*731-infected cells (Fig. 4B). Mutant *sub*733, which had two copies of the EBER genes, synthesized much more protein than its parent, *sub*722, which lacked the EBERs. These results clearly demonstrated that the increase in the quantities of the EBERs increased the efficiency with which they compensated for the translational defect of either the adenovirus double mutant or a mutant that lacked only the VAI RNA gene.

Growth yields of the mutant viruses. The mutants described above were also assayed for growth yield on cell line 293 cells as described previously (3). Table 1 shows the yields of the virus particles for various mutants on days 4 and 5. The double mutant grew to a titer which was about 100-fold less than that of *dl*704, which is in agreement with our previous observation (3). The titer of *sub*731 increased only marginally compared with that of *dl-sub*720, whereas *sub*732, which contained three copies of the EBER genes, yielded a titer which was about sixfold higher than that of the double mutant. Similarly, *sub*733, which contained two copies of the EBER genes, yielded a titer which was sixfold higher than that of *sub*722, which contained no EBER genes. Although mutants *sub*732 and *sub*733 did not grow to WT levels, they were capable of synthesizing polypeptides at WT levels (Fig. 4A and B).

DISCUSSION

The molecular basis for the complementation of VAI RNA function by the EBERs is unclear at present. The two viruses that encode these RNA polymerase III genes are similar in that they replicate in animal cell nuclei and they both contain linear double-stranded DNAs as their genomes. When normal B lymphocytes are infected with EBV in vitro, the cells are transformed into continuously dividing permanant cell lines (15), and they synthesize abundant quantities of the EBERs (2). Infection of human cells by human adenoviruses always leads to a lytic response, and the cells transformed by adenoviruses do not synthesize VA RNAs (2)

The mechanism by which VAI RNA enhances translation is by suppressing the activity of a kinase which phosphorylates the α-subunit of protein synthesis initiation factor eIF-2 (19, 21). Phosphorylated eIF-2 is unable to recycle, resulting in the cessation of protein synthesis. One of the kinases that phosphorylates eIF-2 is the double-stranded RNA- activated kinase (8). This enzyme is activated by low concentrations of double-stranded RNAs but is inactivated by high concentrations (8, 10, 14). VAI RNA displays an extensive double-stranded structure, and it is conceivable that large quantities of this double-stranded RNA may block eIF-2 α-kinase activity. Although the EBERs do not display a compact hairpin structure similar to that of VAI RNA, potential secondary structures have been drawn for EBERs in which the 5′ terminus can base pair to its 3′ terminus (18). These structures also contain double-stranded regions in several places. Therefore, it is likely that these hairpin-like structures may be one of the structural requirements of the small RNAs for their translation enhancement function and that high concentrations of such RNAs may suppress the activity of the eIF-2 α-kinase.

Our mutational analysis of VAI RNA showed that, in addition to secondary structure, two regions of the molecule (positions +43 to +53 and position +107 to the 3′ end) also play an important role in its function (3). Nucleotide sequences homologous to these regions in identical locations in EBERI and EBERII are not detectable. Nucleotide se-

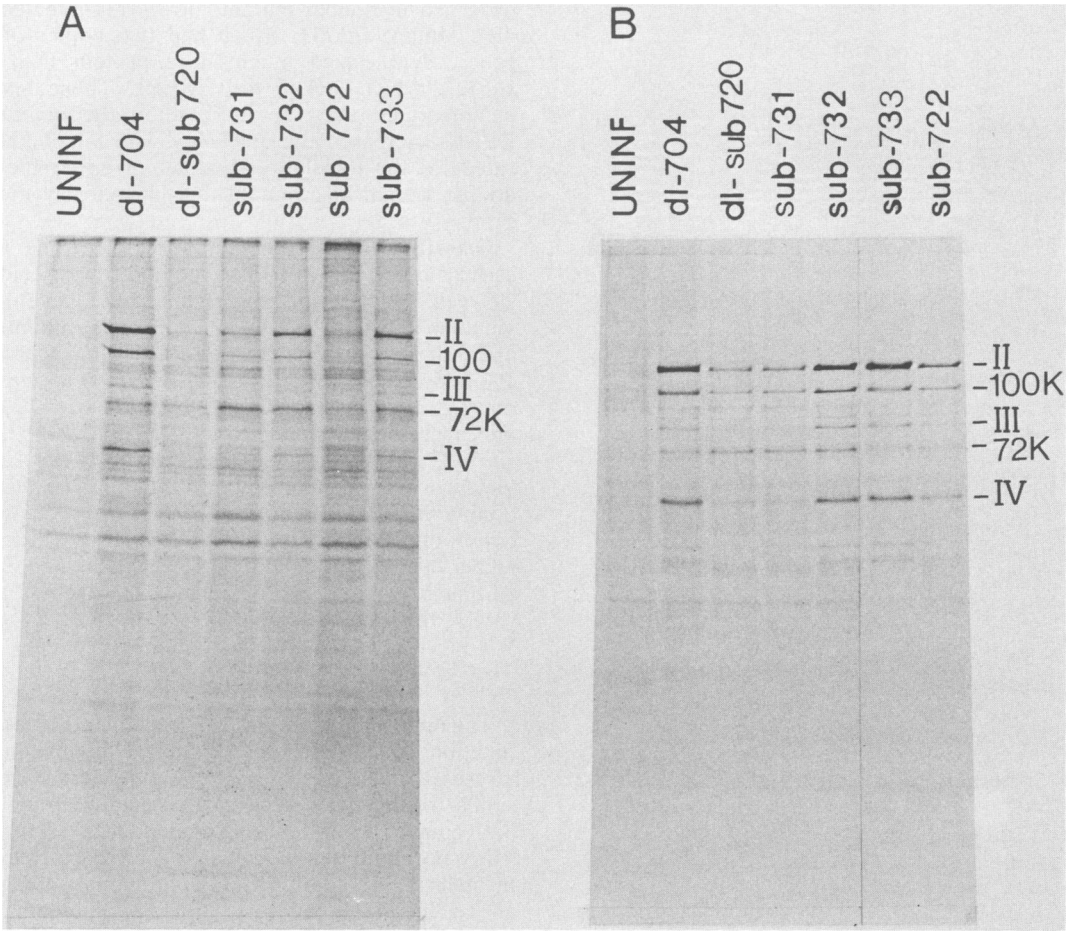

FIG. 4. Analysis of polypeptides synthesized in cell line 293 cells at late times after infection with *dl*704, *sub*722, a double mutant, and various Ad5 substitution mutants containing single or multiple copies of the EBER genes. Cells were infected at a multiplicity of 10 PFU per cell and labeled with [^{35}S]methionine (50 μCi/ml) for 1 h at 15 or 23 h postinfection. Cell extracts were prepared from equal numbers of cells as described in Materials and Methods and loaded onto a 20% sodium dodecyl sulfate–polyacrylamide gel. Electrophoresis was carried out at 15 mA for 18 h. Each lane contained extract derived from 3×10^4 cells. (A) Viral polypeptides synthesized at 16 h postinfection. (B) Viral polypeptides synthesized at 24 h postinfection. UNINF, Uninfected.

quences from position +52 to position +74 and from position +68 to position +93 of VAI RNA show considerable homology with nucleotide sequences from position +60 to position +82 and from position +110 to +135 of EBERI, respectively (Fig. 5). Similarly, nucleotide sequences from position +52 to position +72 and from position +130 to position +150 of VAI RNA show significant homology with nucleotide sequences from position +59 to position +79 and from position +64 to position +84 of EBERII, respectively. Of these, the nucleotide sequences from position +52 to position +72 of VAI RNA contain the B block promoter sequence (5). Whether these sequences play an important role in biological function and whether one of the domains (positions +43 to +53) identified as important for VAI RNA function is an extension of this sequence are difficult to determine, as mutations in this part of the gene abolish transcription (5). The nucleotide sequences from position +130 to position +150 of VAI RNA which show significant homology to the EBERII sequence from position +64 to position +84 belong to the second functionally important region of VAI RNA (3).

TABLE 1. Growth yields of adenovirus substitution mutants[a]

Mutant	Virus yield (titer) on:	
	Day 4	Day 5
*dl*704	2.1×10^9	2.0×10^9
*dl-sub*720	2.3×10^7	2.0×10^7
*sub*731	3.8×10^7	5.0×10^7
*sub*732	1.5×10^8	1.5×10^8
*sub*722	5.5×10^7	6.0×10^7
*sub*733	3.3×10^8	3.5×10^8

[a] To monitor growth kinetics, cell line 293 cells were infected with various mutants at a multiplicity of 3 PFU per cell. After 1 h of incubation, the dishes were washed three times with Tris-saline, and the cultures were fed with Dulbecco modified Eagle medium containing 5% calf serum. On days 4 and 5 the virus yields were measured by plaque assay on HeLa cells.

The relationship between the increase in the quantities of the EBERs synthesized by the mutants and the degree to which these mutants rescue the translational defect in virus-infected cells is somewhat difficult to quantitate. Only two-fold-increased quantities of the EBERs were found in cells infected with *sub*732, which contained three copies of the EBER genes, compared with cells infected with *sub*731,

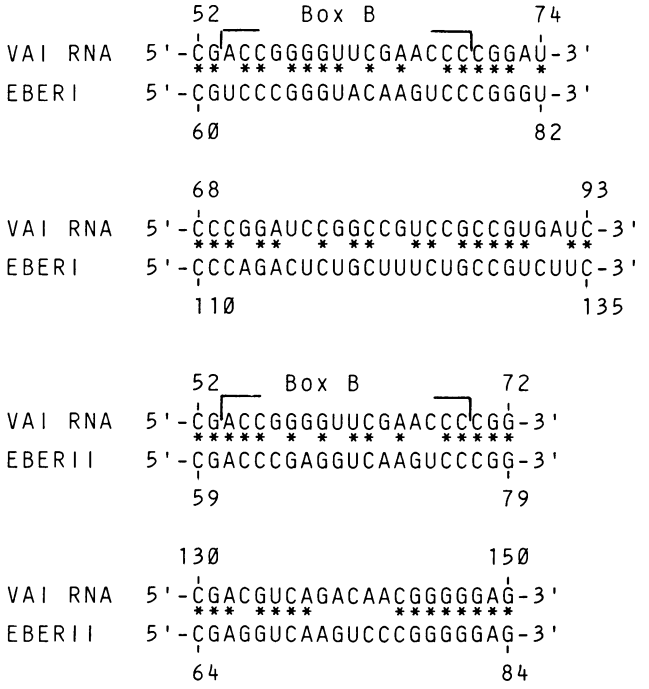

FIG. 5. Nucleotide homologies found between VAI RNA sequences and EBERI and EBERII sequences. Sequence homologies were identified by a computer analysis. The nucleotide sequences shown in box B belong to the box B sequences of the intragenic promoter of the VAI RNA gene (5). Homologous nucleotides are indicated by asterisks.

which contained a single copy of the EBER genes. Yet, at 24 h after infection, only *sub*732 synthesized near-normal levels of late polypeptides. It is possible that in *sub*731-infected cells the levels of one or both of the EBERs were limiting and thus not able to complement the VAI RNA function efficiently. If this hypothesis is true, it is similar to the situation in WT adenovirus-infected cells, in which VAI RNA is the most efficient effector of translation and VAII RNA can function only very weakly (6, 23).

The reasons for the reduced yields of infectious viruses from the mutants with multiple copies of EBER genes are not clear at present, but several explanations can be offered. The increased quantities of EBERs could interfere with virus encapsidation, or the DNA sequences coding for the EBERs could have some kind of *cis*-acting inhibitory effect on DNA packaging. Alternatively, the VAI RNA could have another as-yet-unidentified function required for viral maturation for which the EBERs do not complement. Finally, the mutants with multiple copies of the EBER genes may synthesize one or two minor polypeptides required for virus maturation in suboptimum quantities. Whatever the reasons for the reduced virus yield, the polypeptide analysis clearly shows that the EBERs can enhance translation of adenoviral messages in the absence of VAI RNA.

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