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A Mechanism by Which Adenovirus Virus-Associated RNA_I Controls Translation in a Transient Expression Assay

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The mechanism by which adenovirus virus-associated RNA_I stimulates translational efficiency in a transient-expression assay in 293 cells was investigated. We showed that DNA transfection leads to activation of a protein kinase that phosphorylates the α subunit of eucaryotic initiation factor 2 and, as a consequence, inhibition of polypeptide chain initiation. Cotransfection of a plasmid encoding adenovirus type 2 virus-associated RNA_I recovered the translational capacity by preventing activation of the kinase.

The adenovirus genome encodes two approximately 160-nucleotide-long RNAs, the so-called virus-associated (VA) RNA_I and VA RNA_{II} (7, 13, 18). Both RNAs are transcribed by RNA polymerase III and accumulate to large amounts late after infection (18, 23).

Studies of viral mutants defective in VA RNA production have shown that VA RNA_I is required for efficient late protein synthesis (2, 22). The absence of VA RNA_I causes aberrant splicing and reduced accumulation of late mRNA (21). The translational defect, which has been characterized in great detail, occurs at the level of polypeptide chain initiation (14, 15). VA RNA_I appears to rescue translation in virus-infected cells by preventing activation of a protein kinase that phosphorylates the α subunit of eucaryotic initiation factor 2 (eIF-2) (16, 17). In its phosphorylated form, eIF-2 traps the guanine nucleotide exchange factor in an inactive complex and thereby inhibits protein synthesis. It is likely that VA RNA_I suppresses the activity of this kinase because of its partially double-stranded nature (1, 6, 8, 10, 16).

We have previously shown that VA RNA_I enhances the translational efficiency of viral, as well as nonviral, mRNAs in a transient-expression assay in 293 cells (19, 20). Considering the recently established mechanism for VA RNA_I action following a virus infection, it was not obvious how this regulation occurred during DNA transfection.

To analyze this, we determined whether VA RNA_I introduced by DNA transfection could complement the translational defect of the adenovirus type 5 (Ad5) mutant *dl*331 (22). This mutant is defective in VA RNA_I expression because of a small deletion in its intragenic promoter element (3). We infected 293 cells at a low multiplicity (21) with either Ad5 (wild type) or mutant *dl*331. Following a 23-h incubation, cells were pulse-labeled with [^{35}S]methionine (21) and fractionated into cytoplasm and nuclei by Nonidet P-40 extraction (19). Part of the cytoplasmic extract was used to measure eIF-2α kinase activity (17), and part was used to prepare a [^{35}S]methionine-labeled protein extract (19). Protein synthesis was reduced approximately 20-fold in *dl*331-infected cells compared with wild-type Ad5 infection (Fig. 1, lanes 1 and 2). Transfection of plasmid pHindB, encoding the Ad2 VA RNAs (19), 25 h before *dl*331 infection restored translational capacity almost to wild-type level (Fig. 1, lane 3). As expected (16, 17), extracts from *dl*331-infected cells showed enhanced eIF-2α kinase activity (Fig. 2A, lane 5). Cotransfection of plasmid pHindB resulted in almost complete inhibition of this kinase in mutant-infected cells (Fig. 2A, lane 6). Thus, VA RNA_I expressed from a transfected plasmid appears to regulate translation by the same mechanism as it does when expressed from the viral genome. Inhibition of eIF-2α phosphorylation was surprisingly efficient (between 80 and 90%; Fig. 2A, lane 6; data not shown), suggesting that most of the cells express VA RNA_I with high efficiency (transfected 293 cells contain between 10^5 and 10^6 copies of VA RNA per cell [19]). This is in contrast to protein-encoding genes in which we routinely obtained around 20% transfection efficiency (data not shown). We attribute this difference in efficiency to the fact that the VA RNAs are transcribed by a different RNA polymerase (23).

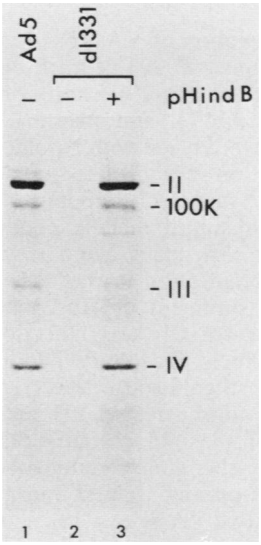

FIG. 1. Complementation of the translational defect of *dl*331. We infected 293 cells (6-cm-diameter plates), untransfected (−) or transfected by the calcium phosphate coprecipitation technique (24) with 10 μg of pHindB (+), at a low multiplicity (24 h posttransfection) with Ad5 (wild type) or *dl*331 (21). At 23 h postinfection, cells were pulse-labeled for 1 h with [^{35}S]methionine (21) followed by lysis in IsoB–Nonidet P-40 (19). A fraction of the lysate was subjected to electrophoresis in a 10% sodium dodecyl sulfate-polyacrylamide gel followed by fluorography.

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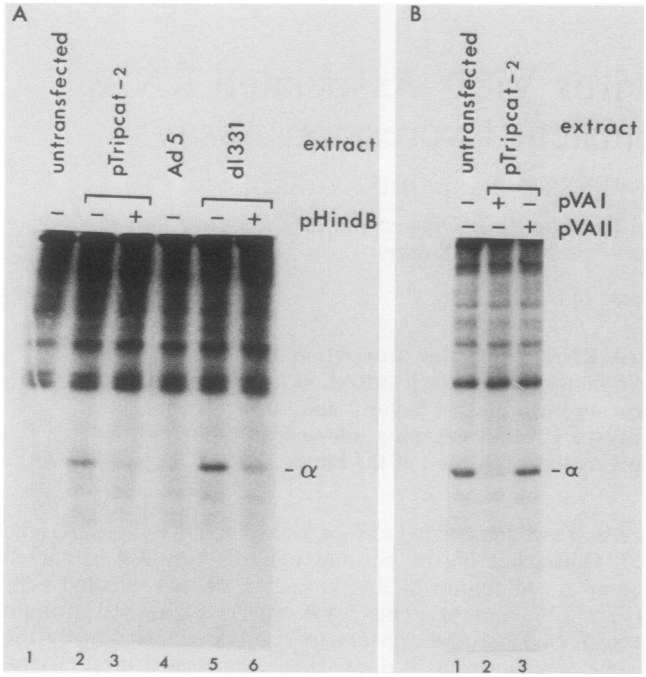

FIG. 2. Activity of eIF-2α kinase in transfected and infected 293 cells. Kinase levels were measured as described by Siekierka et al. (17). The assays were supplemented with 2 μg of partially purified rat liver eIF-2 (11). (A) Lanes 1 to 3, kinase levels in cell extracts prepared in the DNA transfection experiment shown in Fig. 3. Lanes 4 to 6, kinase levels in cell extracts prepared in the virus complementation experiment shown in Fig. 1. (B) Kinase levels in cell extracts prepared from 293 cells transfected (24) with 3 μg of pTripcat-2 DNA and 10 μg of pVAI, pVAII (+) or pBR322(−). Plasmid pVAI contains a 600-base-pair DNA insert which encodes the Ad2 VA RNA_I gene (19). Plasmid pVAII contains a 750-base-pair fragment encoding the Ad2 VA RNA_{II} gene (19).

To study the function of VA RNA further, we measured eIF-2α kinase activity in 293 cells cotransfected with plasmids pTripcat-2 (Fig. 3C; 20), expressing chloramphenicol acetyltransferase (CAT), and pHindB. Following a 50-h incubation, cells were lysed with Nonidet P-40 (19), and the cytoplasmic fraction was isolated and used to measure protein kinase (17) and CAT activities (4). pHindB cotransfection resulted in approximately eightfold stimulation of CAT activity (Fig. 3A, lanes 2 and 3) without affecting the CAT mRNA concentration in the cells (19, 20) (Fig. 3B). Surprisingly, measurement of eIF-2α kinase activity indicated that pTripcat-2 DNA transfection had activated a protein kinase which phosphorylated the eIF-2α subunit (Fig. 2A, lane 2). Significantly lower levels of this kinase were observed in untransfected 293 cells (Fig. 2A, lane 1). Cotransfection of plasmid pHindB resulted in complete disappearance of the kinase activity (Fig. 2A, lane 3). Cotransfection of plasmids pVAI and pVAII, which separately encode the two VA RNAs (19), showed that VA RNA_I was efficient in preventing activation of the eIF-2α kinase (Fig. 2B, lane 2), whereas VA RNA_{II}, which was without significant effect on translation in our transient expression system (19), showed only a marginal effect on the kinase activity (Fig. 2B, lane 3).

Based on these results, we propose a model in which VA RNA_I enhances protein synthesis in a transfection assay by securing the availability of an active form of eIF-2. Apparently, DNA transfection per se results in enhanced eIF-2α kinase activity, which probably reduces protein synthesis (9) in transfected cells. Hence, VA RNA_I cotransfection restores the translational capacity of these cells by suppressing the activity of this kinase. We do not know whether DNA transfection increases the eIF-2α kinase concentration or modulates the activity of a pre-existing enzyme complex.

Calcium phosphate precipitates as such did not enhance the kinase level. However, inclusion of a number of plasmids induced a similar increase in eIF-2α kinase activity in transfected cells (data not shown). In fact, transfection of pBR322 DNA alone induced a small increase in kinase level. It is believed that symmetrical transcription of the viral genome (12) produces the double-stranded RNA which activates the eIF-2α kinase in *dl*331-infected cells (16, 17); the activity of the kinase is regulated by double-stranded RNA (6, 10). Similarly, complementary transcription from natural and cryptic promoters within the plasmid sequences

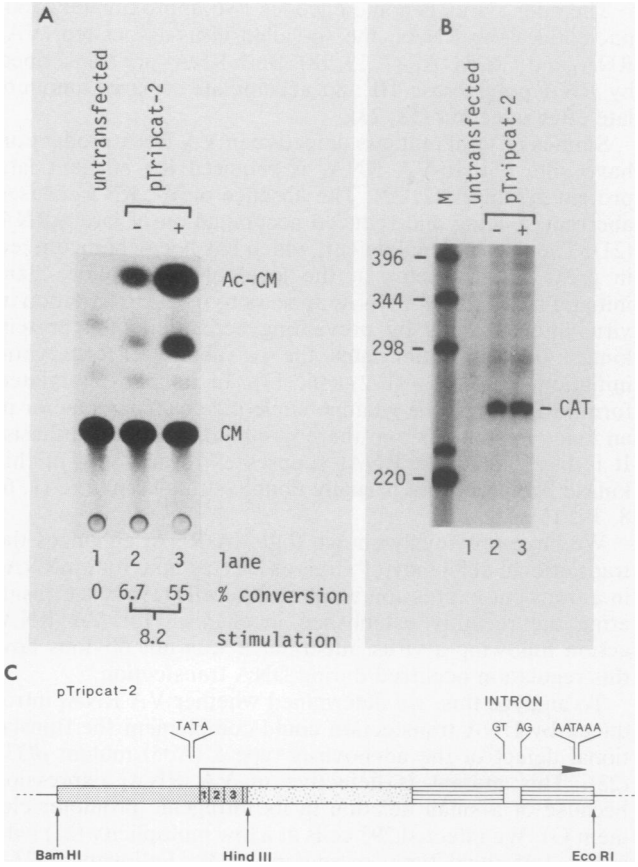

FIG. 3. Effect of VA RNA on CAT mRNA translation. (A) We transfected (24) 293 cells with 3 μg of pTripcat-2 DNA and 10 μg of pHindB (+) or pBR322 (−). A cell extract was prepared 50 h posttransfection by lysis in IsoB–Nonidet P-40 (19), and CAT activity was measured as described by Gorman et al. (4). (B) S1 endonuclease analysis of CAT mRNA accumulation in 293 cells transfected with pTripcat-2 DNA and pHindB (panel A). As a DNA probe, a 600-base-pair *Sal*I-*Eco*RI fragment previously described (20) was used. The fragment was 5′ end labeled at an *Eco*RI cleavage site which is located within the CAT coding sequences. (C) Schematic diagram of the DNA insert in pTripcat-2 (20). The stippled box indicates Ad2 sequences (the major late transcriptional control region plus a cDNA copy of the tripartite leader), the dotted area indicates CAT sequences, and the striped area indicates simian virus sequences.

may generate double-stranded RNA that affects eIF-2α kinase activity during DNA transfection.

The proposed mechanism provides an explanation for our failure to detect a nucleotide sequence requirement for VA RNA-mediated translational enhancement (20). We have, however, previously found a slight variation in VA RNA-mediated translational stimulation (19). It is not obvious how an mRNA-specific translation control could be exerted by the eIF-2α kinase activation mechanism alone. It is therefore possible that the situation is more complex and that, for example, VA RNA serves additional functions or that mRNA competition results in selective translation in a situation in which initiation factors are present in limiting amounts.

VA RNA-mediated translational stimulation is not limited to 293 cells (5, 20), and it should therefore be a useful tool to increase protein expression in cells in which DNA transfection leads to inhibition of translation because of eIF-2 phosphorylation.

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