The EMBO Journal vol.4 no.4 pp.957–964, 1985

Adenovirus VA RNA$_I$ mediates a translational stimulation which is not restricted to the viral mRNAs

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Communicated by L.Philipson

The effect of adenovirus VA RNA$_I$ on the translation of mRNAs expressing the bacterial chloramphenicol acetyltransferase (CAT) enzyme was studied by a transient expression assay in 293 cells. The CAT activity was determined in extracts prepared from cells transfected with mixtures of plasmids encoding CAT and VA RNA. The results showed that VA RNA$_I$ co-transfection resulted in a significant increase in CAT expression from a variety of constructs. Thus, expression of CAT from a SV40 mRNA, a β-globin mRNA and a chimeric mRNA containing the adenovirus-2 tripartite leader were all stimulated ~6-fold by VA RNA$_I$. Based on these results we conclude that the tripartite leader sequence is not required for the VA RNA-mediated stimulation of translation. Our results indicate instead that VA RNA$_I$ probably functions as a general enhancer of mRNA translation. A 2- to 3-fold stimulation of CAT expression was also obtained following transient expression of HeLa and CV-1 cells. The reduced efficiency was correlated with a 10- to 20-fold lower level of VA RNA expression in HeLa compared with 293 cells. Thus, it is likely that a product from region E1 indirectly enhances the translational efficiency by stimulating VA RNA transcription.

Key words: chloramphenicol acetyltransferase/translational regulation/tripartite leader/adenovirus VA RNA$_I$

Introduction

The adenovirus genome encodes two low mol. wt. RNAs designated virus-associated RNA (VA RNA) I and II (Reich *et al.*, 1966; Mathews, 1975; Söderlund *et al.*, 1976). The VA RNAs are transcribed from two adjacent DNA segments located around map co-ordinate 30 on the adenovirus type 2 (Ad2) genome (Mathews, 1975; Söderlund *et al.*, 1976). DNA and RNA sequence studies have shown that both RNAs are ~160 nucleotides long, exhibit scattered regions of sequence homology, and can be predicted to adopt similar secondary structures (Akusjärvi *et al.*, 1980). They are both transcribed by RNA polymerase III (Weinmann *et al.*, 1976) and accumulate in large amounts in the cytoplasm of lytically infected cells. VA RNA$_{II}$ is predominantly synthesized early, whereas VA RNA$_I$ continues to increase late after infection (Söderlund *et al.*, 1976). A fraction of the VA RNA population is found as ribonucleoprotein particles in association with the cellular lupus antigen, La, in infected cells (Lerner *et al.*, 1981; Habets *et al.*, 1983).

Although the VA RNAs were discovered almost two decades ago (Reich *et al.*, 1966), a specific function was not assigned to them until recently. Experiments using two adenovirus mutants, each of which fail to synthesize one of the VA RNA species, have shown that VA RNA$_I$ is required for an efficient translation of viral mRNAs at late times of infection (Thimmappaya *et al.*, 1982). The site of VA RNA action is most likely at the level of initiation of translation (Schneider *et al.*, 1984; Reichel *et al.*, 1985) although the exact mechanism has not yet been established.

These experiments, although pointing to an important role for VA RNA$_I$ in the control of protein synthesis, do not provide a complete explanation for how VA RNA functions. For example, the experiments do not answer the question whether VA RNA$_I$ is responsible for the discrimination between cellular and viral mRNAs that results in the selective translation of viral mRNAs at late times of infection (Bellow and Ginsberg, 1967; Andersson *et al.*, 1973; Beltz and Flint, 1979; Babich *et al.*, 1983), or if other, at the present time, unknown mechanisms control this selective shut-off of host protein synthesis. We have recently initiated experiments to specifically investigate this question. By a transient expression assay we have shown that VA RNA$_I$ functions as an enhancer of viral mRNA translation, increasing the efficiency of both early and late adenovirus mRNA translation (Svensson and Akusjärvi, 1984a). Here we extend these studies; we have determined whether VA RNA$_I$ requires viral sequences in order to function as a translational enhancer. For this purpose adenoviral as well as eukaryotic 5′- and 3′-non-coding regions were fused to the bacterial chloramphenicol acetyl transferase (CAT) gene (Gorman *et al.*, 1982). Following transfection of the various constructs into 293 cells, the effect of VA RNA$_I$ on CAT expression was measured. Our results show that the translational stimulation mediated by VA RNA$_I$ is not restricted to adenovirus mRNAs, but also includes mRNAs of non-viral origin. In conjunction with previously reported results we propose a mechanism by which VA RNA$_I$ mediates a control of translation in late adenovirus-infected cells.

Results

Experimental approach

We have used an immunoprecipitation assay to study the role of the adenovirus VA RNAs in the control of mRNA translation (Svensson and Akusjärvi, 1984a). In these experiments, 293 cells (Graham *et al.*, 1977) were co-transfected with plasmids encoding adenovirus structural genes and plasmids encoding the VA RNAs. The cells were then labeled with [^{35}S]methionine and a cell extract was prepared. The protein expression was subsequently monitored by immunoprecipitation, using monospecific antisera. To obtain a simpler and more sensitive assay we tested whether the bacterial CAT gene under the transcriptional control of various eukaryotic promoters (Gorman *et al.*, 1982) could be used in the co-transfection assay together with plasmids encoding the VA RNAs.

For these experiments, a hybrid plasmid containing a complete cDNA copy of the Ad2 tripartite leader fused upstream of the coding sequence for CAT (Figures 1A and 6) was constructed. The plasmid, which was designated pTripcat-2, has the transcriptional control region for the major late adenovirus promoter reconstructed upstream of the tripartite leader sequence. The

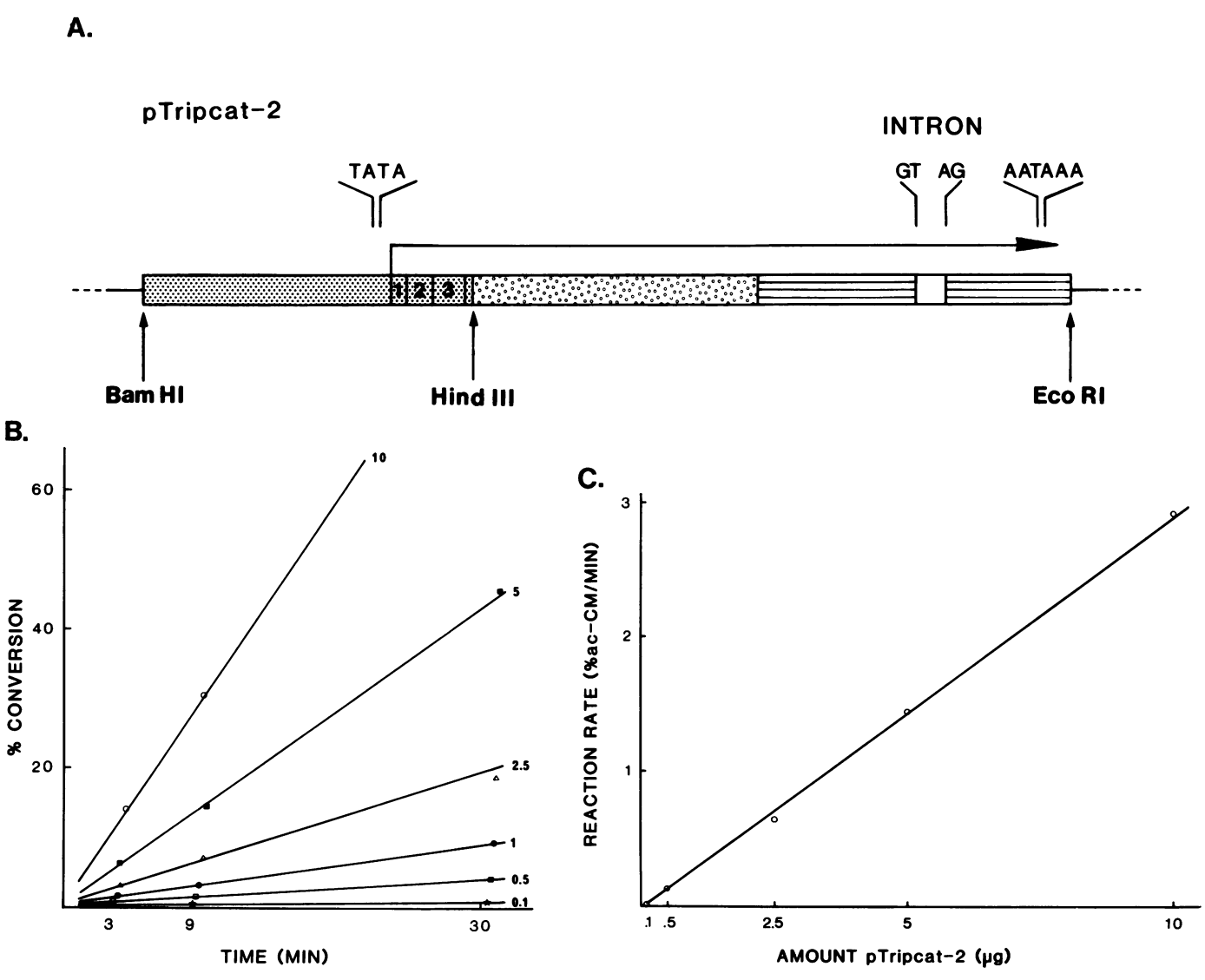

Fig. 1. CAT activity in 293 cells transfected with pTripcat-2. **(A)** Schematic drawing of the DNA insert in plasmid pTripcat-2. The TATA sequence designates the adenovirus major late transcriptional control region with an arrow showing the direction of transcription. The white box bordered by a GT/AG sequence indicates the location of an intron and the AATAAA sequence the position of the poly(A) addition site. Thin lines indicate pBR322 sequences, the stippled box Ad2 sequences, the dotted area CAT sequences and the hatched area SV40 sequences. The positions of selected restriction endonuclease cleavage sites are also indicated. **(B)** Kinetics of CAT expression. Variable amounts of pTripcat-2 DNA (0.1 – 10 μg) were transfected into 293 cells. The formation, in per cent of acetylated chloramphenicol, was plotted as a function of the incubation time. **(C)** To ensure that the comparisons in enzyme levels were done in the linear range of the assay the initial rate was determined for each assay in **panel B** and plotted as a function of μg pTripcat-2 added.

3'-flanking sequences, including RNA splicing signals and a poly(A) addition site, was derived from the SV40 early transcription unit.

To establish optimal conditions for CAT gene expression increasing amounts of pTripcat-2 DNA was mixed with carrier DNA (e.g., pBR322 DNA), to yield a total of 15 μg per tissue culture dish, and transfected into 293 cells. Following an incubation period of 50 h, cell extracts were prepared and assayed for CAT activity (Gorman *et al.*, 1982). Aliquots of the reaction mixture were withdrawn at different time points and the per cent conversion to acetylated chloramphenicol was measured (Figure 1B and C). As is evident from Figure 1C there was a linear relationship between the CAT activity and the amount of transfected plasmid. Thus, within the interval 0.1 – 10 μg the transcriptional and translational machinery in the transfected cells did not constitute a limiting factor in the assay system.

VA RNA stimulates expression of the bacterial CAT enzyme

We have shown that VA RNA_I stimulates the translational efficiency of both early and late adenovirus mRNAs (Svensson and Akusjärvi, 1984a). To extend these studies to include also non-viral genes, we determined the effect of VA RNA_I on the translation of a mRNA encoding the bacterial CAT enzyme. For this experiment monolayers of 293 cells were transfected with 1 μg of pTripcat-2 DNA and increasing amounts of plasmid pHindB which encodes both VA RNA_I and VA RNA_{II} (Akusjärvi *et al.*, 1980; Svensson and Akusjärvi, 1984a). The CAT activity was measured and plotted as a function of the amount of pHindB DNA added. As shown in Figure 2A, pHindB cotransfection resulted in a significant stimulation of the CAT activity. The most pronounced effect was obtained at very low quantities of pHindB. Above 5 μg of pHindB per dish we observed very little further stimulation of the translation efficiency. On

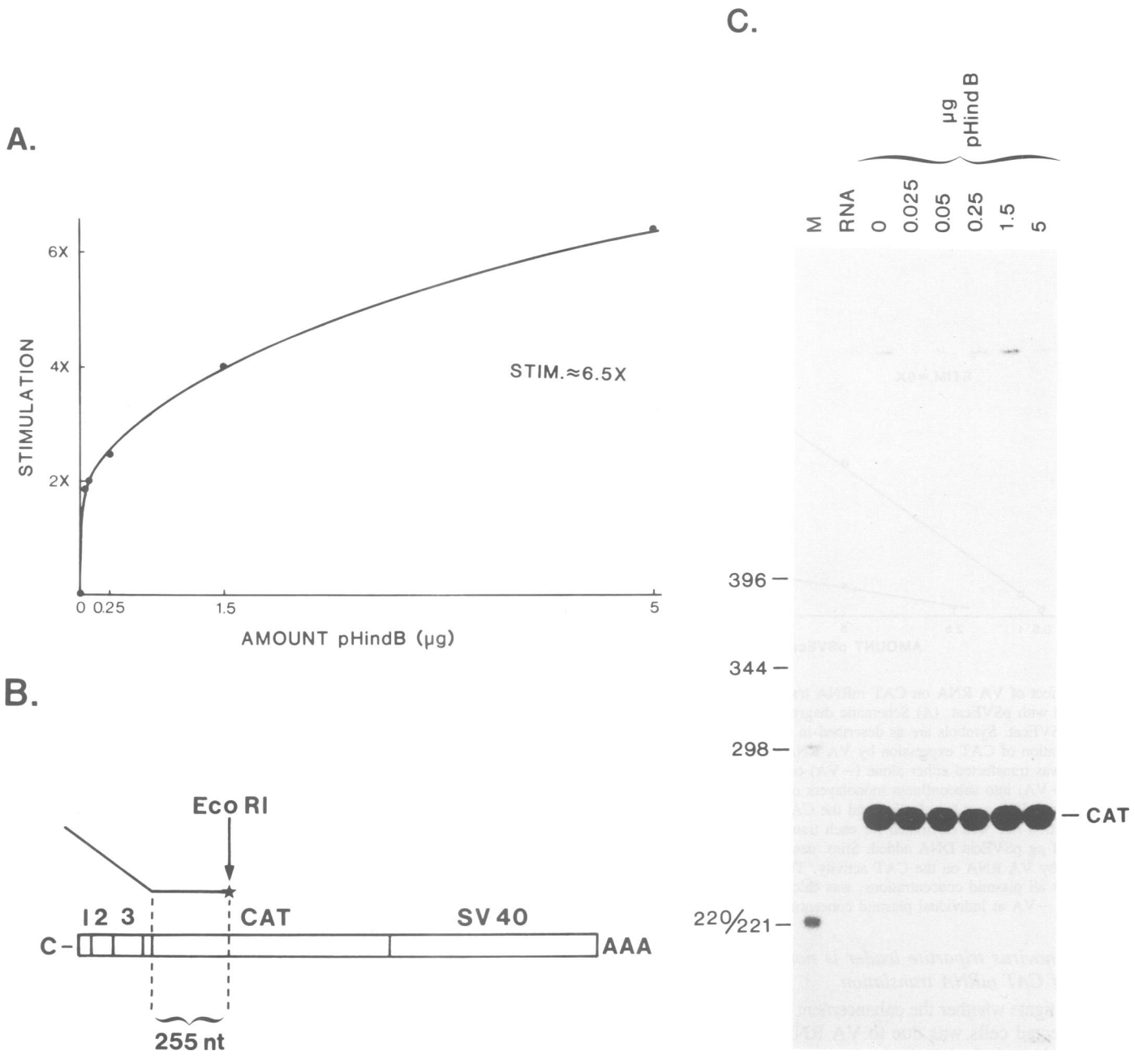

Fig. 2. Effect of VA RNA on CAT mRNA translation in cells transfected with pTripcat-2. **(A)** Increase in CAT activity caused by pHindB co-transfection. 1 μg of pTripcat-2 DNA was transfected together with increasing amounts of plasmid pHindB (0.1 – 5 μg) into subconfluent monolayers of 293 cells. A cell extract was prepared 50 h post-transfection and the CAT activity measured. The initial reaction rate was calculated for each transfection experiment and the stimulation plotted as a function of μg pHindB added. The stimulation was calculated as the ratio between the reaction rates obtained in assays where pHindB was co-transfected and the assay without any pHindB. **(B)** Strategy for the S1 nuclease analysis of CAT mRNA. An ~600 bp *SalI-EcoRI* fragment isolated from plasmid pβcat-2 was used as a DNA probe. The fragment was 5′ end-labeled at the *Eco*RI cleavage site located within the CAT coding sequence (position 450 in the sequence of Alton and Vapnek, 1979). **(C)** S1 endonuclease analysis of the CAT mRNA accumulating in 293 cells co-transfected with pTripcat-2 and pHindB **(panel A)**. Electrophoretic separation was through a 4% polyacrylamide gel containing 8 M urea.

average, CAT mRNA translation was stimulated ~6.5 times by VA RNA_I at the plateau level. To normalize the results obtained with different CAT constructs, all experiments described below were performed with a pHindB concentration of 5 μg per dish.

To exclude the possibility that the increase in CAT activity (Figure 2A) was due to a transcriptional stimulation of CAT mRNA synthesis, we analyzed the level of CAT mRNA in transfected cells by S1 endonuclease cleavage (Figure 2B and C). The results showed that the level of CAT mRNA was not affected by pHindB co-transfection, thus suggesting that the effect of VA RNA_I was at the level of mRNA translation.

The effect of the two VA RNAs on CAT mRNA translation was also tested. In separate experiments, plasmid pTripcat-2 was co-transfected with either plasmid pVAI or pVAII, which express VA RNA_I and VA RNA_{II}, respectively. The results confirmed our previous conclusion (Svensson and Akusjärvi, 1984a) that only VA RNA_I is capable of stimulating mRNA translation in our transient expression assay (data not shown).

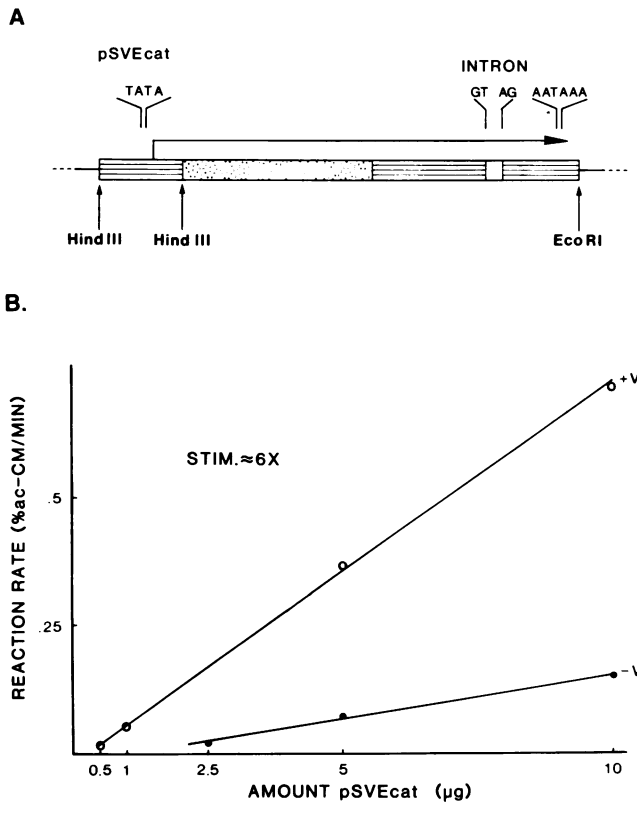

Fig. 3. Effect of VA RNA on CAT mRNA translation in 293 cells transfected with pSVEcat. **(A)** Schematic diagram of the DNA insert in plasmid pSVEcat. Symbols are as described in the legend to Figure 1A. **(B)** Stimulation of CAT expression by VA RNA. Between 0.5 and 10 μg of pSVEcat was transfected either alone (−VA) or together with 5 μg of pHindB (+VA) into subconfluent monolayers of 293 cells. A cell extract was prepared 50 h post-transfection and the CAT activity measured. The initial reaction rate was calculated for each transfection and plotted as a function of μg pSVEcat DNA added. Stim. denotes the stimulation conferred by VA RNA on the CAT activity. The stimulation, which was identical at all plasmid concentrations, was calculated as the ratio between +VA and −VA at individual plasmid concentrations.

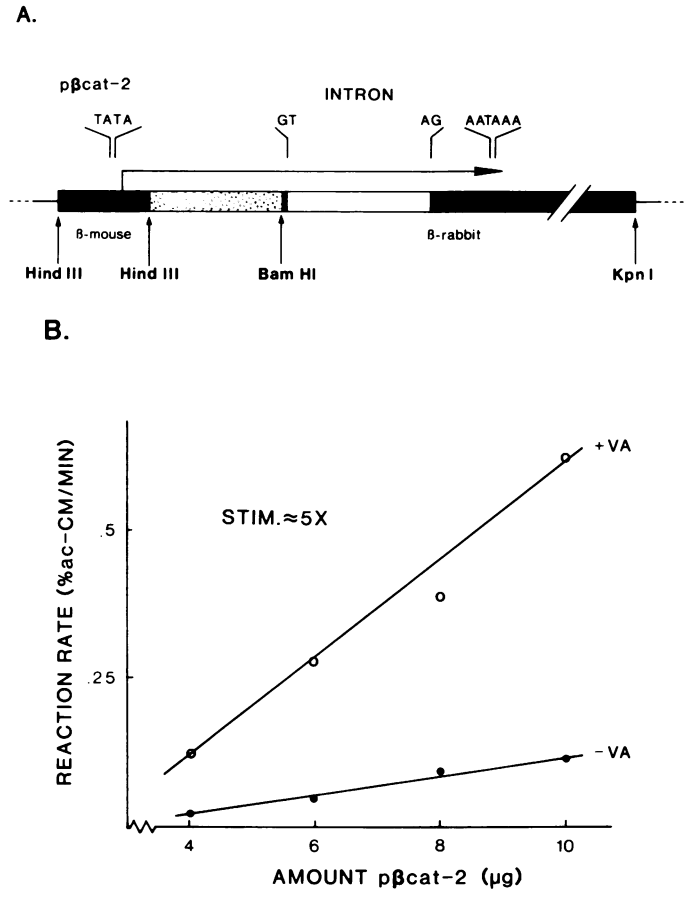

Fig. 4. Effect of VA RNA on CAT mRNA translation in cells transfected with plasmid pβcat-2. **(A)** Schematic drawing of the DNA insert in plasmid pβcat-2. The solid area represents sequences originating from the mouse and rabbit β-globin genes. The other symbols are as described in the legend to Figure 1A. **(B)** Stimulation of CAT expression by pHindB co-transfection. The transfections and calculations were as described in the legend to Figure 3B.

The adenovirus tripartite leader is not required for the stimulation of CAT mRNA translation

To investigate whether the enhancement of translation in pTripcat-2 transfected cells was due to VA RNA_I specifically recognizing the adenoviral tripartite leader sequence, we studied plasmid pSVEcat in our co-transfection assay. Recombinant pSVEcat (a derivative of pSV2cat) differs from pTripcat-2 in that the adenoviral major late promoter/tripartite leader fusion has been substituted by a fragment encoding the promoter and 5′-non-coding region from the SV40 early transcription unit (Figures 3A and 6).

An experiment was designed where monolayers of 293 cells were transfected with increasing amounts of pSVEcat, either alone or together with 5 μg of plasmid pHindB. The reaction rate for each transfection experiment was calculated and plotted as a function of the quantity of pSVEcat added. As is shown in Figure 3B, the CAT activity was directly proportional to the amount of pSVEcat added, within the interval 0.5 − 10 μg. Furthermore, at all plasmid concentrations, pHindB co-transfection increased the CAT signal approximately six times. Since the enhancement of the CAT activity was identical at all plasmid concentrations, we conclude that the experiment was carried out under experimental conditions where the translational capacity of the transfected cells was not a limiting factor. Thus, the observed stimulation should reflect the contribution made by VA RNA_I to the efficiency of CAT mRNA translation. S1 endonuclease analysis of the CAT mRNA synthesis in pSVEcat transfected cells showed that pHindB co-transfection had no effect on transcription (data not shown), thus corroborating our conclusion that VA RNA_I acts at the level of mRNA translation.

Effect of the 3′-non-coding region on the VA RNA-mediated stimulation of translation

Since the origin of the 5′-flanking sequences appears to be of minor importance for the VA RNA-mediated stimulation of translation, we examined whether the 3′-non-coding region was of regulatory importance. For this experiment plasmid pβcat-2 was constructed (Figure 4A). Both pTripcat-2 and pSVEcat, used in the previous experiments, have identical 3′-non-coding sequences, originating from the SV40 early transcription unit (Figures 1A and 3A). Plasmid pβcat-2, in contrast, contains the promoter, cap site and first 26 nucleotides of the 5′-non-coding region from the mouse β-major globin gene (Konkel *et al.*, 1978) (Figure 6) and the 3′-flanking sequences including a poly(A) addition site and splice signals from the rabbit β-globin gene (Maniatis *et al.*, 1978). A hybrid between the mouse and the rabbit β-globin genes was constructed because of the presence of convenient restriction endonuclease cleavage sites in the respective genomic clones.

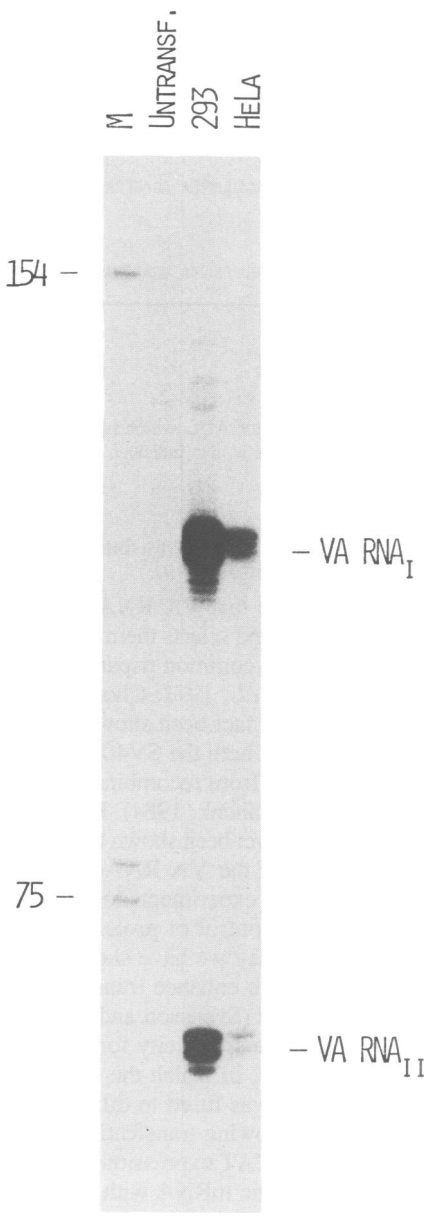

Fig. 5. Accumulation of VA RNA in pHindB transfected HeLa and 293 cells. Total cytoplasmid RNA was hybridized to a mixture of two 5′ end-labeled DNA probes specific for VA RNA$_I$ and VA RNA$_{II}$, respectively (Svensson and Akusjärvi, 1984a). After S1 cleavage, resistant material was separated through a 8% polyacrylamide gel containing 8 M urea. Untransf., RNA isolated from untransfected 293 cells. M, pBR322 marker fragments.

The effect of VA RNA$_I$ on CAT expression from the chimeric β-globin/CAT mRNA was tested. Subconfluent monolayers of 293 cells were transfected with increasing amounts of pβcat-2, either alone or together with 5 μg of pHindB. As shown in Figure 4B, co-transfection of plasmid pHindB resulted in an ~5-fold stimulation of the CAT activity. The stimulation was the same at all plasmid concentrations, demonstrating that the translational capacity of the transfected cells was not a limiting factor. Thus, we conclude that the 3′-flanking sequences do not significantly influence the enhancement conferred by VA RNA$_I$ on CAT mRNA translation.

As a by-product of the cloning procedure, plasmid pβcat-3 was obtained (Figure 7E). pβcat-3 is identical to pTripcat-2 and pSVEcat with the exception that the 5′-non-coding sequences and the transcriptional control region is from the mouse β-major globin gene. Co-transfection of pβcat-3 with pHindB resulted also in an ~5-fold stimulation of CAT mRNA translation (data not shown).

Role of region E1 in VA RNA-mediated stimulation of translation

Since a functional E1A region has been shown to enhance significantly the transient expression of both viral and cellular genes (Green *et al.*, 1983; Svensson and Akusjärvi, 1984b; Gaynor *et al.*, 1984) we have consistently used transfection into 293 cells (Graham *et al.*, 1977) as a way of ascertaining a high level of mRNA expression. The 293 cell line which is a human embryonic kidney cell line transformed by fragmented Ad5 DNA express constitutively both the mRNAs and proteins from region E1A and E1B (Aiello *et al.*, 1979). To analyze whether products from region E1 participate in the VA RNA-mediated stimulation of translation, we repeated our co-transfection assay in HeLa and CV-1 cells. We used plasmid pSVEcat (Figure 3A) for these experiments. Plasmid pSVEcat contains the SV40 enhancer element upstream of the transcriptional start site (reviewed by Khoury and Gruss, 1983) and is therefore efficiently expressed also in the absence of the adenovirus E1A function.

We transfected 5 μg of pSVEcat DNA into monolayers of HeLa, CV-1 and 293 cells, either alone or together with 5 μg of plasmid pHindB. In the absence of VA RNA, the measured CAT activity was comparable in all three cell types (Table I). However, VA RNA co-transfection resulted in a 2- to 3-fold stimulation of CAT mRNA translation in HeLa and CV-1 cells as compared with ~6-fold stimulation in 293 cells. To investigate whether the difference in stimulatory potential was due to a direct effect of region E1 on translation, or caused by inefficiencies in VA RNA expression, we quantitated the amount of VA RNA synthesized in HeLa and 293 cells. As shown in Figure 5, the level of VA RNA expression was 10- to 20-fold higher in 293 cells compared with HeLa cells. Because of limitations in the DNA transfection assay (Graham and van der Eb, 1973; Wigler *et al.*, 1978) sufficient pHindB DNA cannot be added to the HeLa cells to reach a level of VA RNA expression equivalent to that obtained in 293 cells (Figure 5). Decreasing the amount of VA RNA expression in 293 cells to a level comparable with HeLa cells leads also to a 2.5-fold stimulation of CAT mRNA translation (Figure 2A). Based on these results we suggest that the lower stimulatory potential in HeLa cells probably is due to an inefficiency of VA RNA expression in this cell line.

Discussion

During an adenovirus infection expression of viral as well as cellular genes is subjected to control at both the transcriptional and the translational level. At late times of an infection an almost exclusive synthesis of viral polypeptides occurs in the infected cell (Bello and Ginsberg, 1967; Andersson *et al.*, 1973). Synthesis of cellular hnRNA is normal but the transport of host-cell specific mRNAs, from the nucleus to the cytoplasm, appears to be inhibited (Beltz and Flint, 1979; Flint *et al.*, 1983; Babich *et al.*, 1983). However, due to a long cytoplasmic half-life the concentration of some of the cellular mRNAs remains almost constant throughout the infection (Babich *et al.*, 1983; Khalili and Weinmann, 1984). Nevertheless, translation of the host-cell mRNAs is severely depressed at late times of an infection, suggesting that a viral gene product(s) causes a direct inhibition of host gene expression.

Recent experiments, using deletion mutant dl331, which is unable to synthesize VA RNA$_I$, have shown that VA RNA$_I$ is

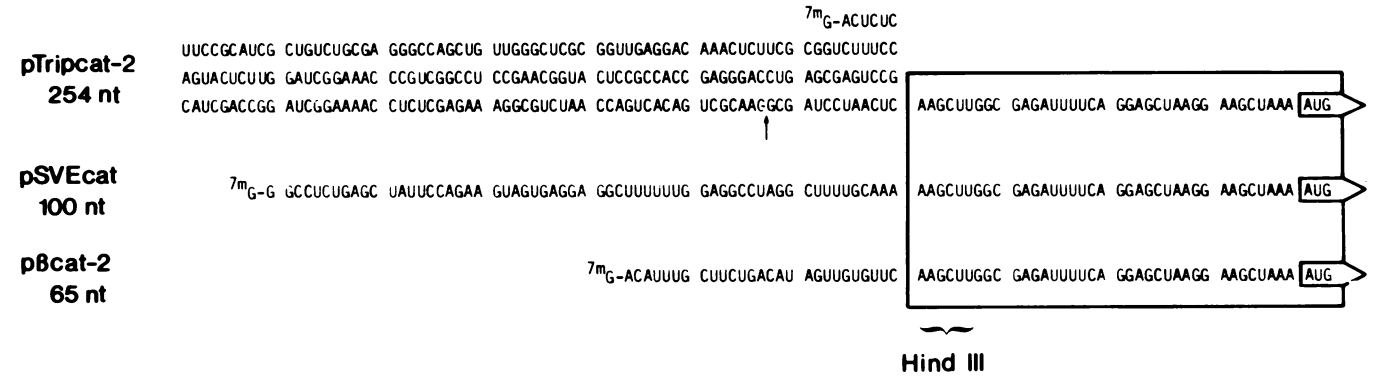

Fig. 6. Sequence of the 5′-non-coding region attached to the CAT mRNAs. The sequence up to −37 from the initiator AUG which is common to all three CAT constructs is represented by a box. The position of the *Hind*III site used in the construction of the recombinants is also indicated. The arrow points to the end of the adenovirus tripartite leader sequence (Akusjärvi and Pettersson, 1979, Zain *et al.*, 1979).

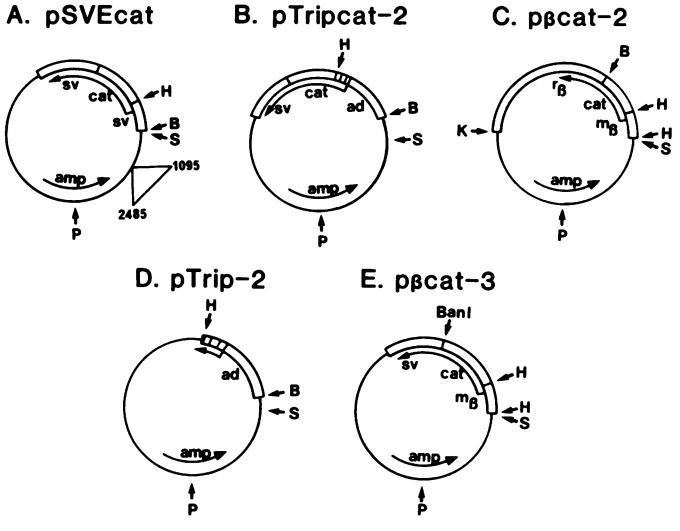

Fig. 7. Structure of plasmids. Thin lines represent pBR sequences and the boxed area the foreign DNA insert. B, *Bam*HI; H, *Hind*III; K, *Kpn*I; P, *Pvu*I; S, *Sal*I; mβ, mouse β-globin; rβ, rabbit β-globin.

Table I. Stimulation of CAT expression in 293, HeLa and CV-1 cells[a]

	% Acetylated CM		
	293	HeLa	CV-1
−pHindB	2.9	5.1	3.4
+pHindB	18.6	12.5	9.2
Stimulation	6.4	2.4	2.7

[a]Cells were transfected with 5 μg of pSVEcat and 5 μg of pBR322 (−pHindB) or 5 μg of pHindB. CAT activity was measured and quantitated as described in Materials and methods.

required to obtain an efficient translation of the virus specific mRNAs late after infection (Thimmappaya *et al.*, 1982). Since host protein synthesis is blocked also in dl331-infected cells, VA RNA_I cannot be responsible for the turn-off of host protein synthesis. Taken together, these results indicate that the preferential translation of adenovirus polypeptides in late infected cells is regulated at least at two levels. Firstly, a viral function, which is unrelated to VA RNA_I, alters the translational machinery of the cell in such a way that neither host- nor adenovirus-specific mRNAs can be efficiently translated. Secondly, VA RNA_I confers to the translational machinery a capacity which seemingly enables the ribosomes to selectively translate the late adenovirus mRNAs.

It is tempting to speculate that VA RNA_I indeed recognizes the late adenovirus mRNAs and selects them for translation, since the majority of them carry a common tripartite leader sequence at their 5′ termini (Berget *et al.*, 1977; Chow *et al.*, 1977). The intact tripartite leader has in fact been shown to play an important role in the translation of both the SV40 large T antigen and the adenovirus E1A proteins from recombinant genomes (Thummel *et al.*, 1983; Logan and Shenk, 1984). However, as pointed out by the authors, it has not yet been shown that this phenomenon is related to the function of the VA RNAs.

We have recently initiated experiments to study the specificity of the VA RNA-mediated control of protein synthesis. By use of a transient expression assay we have shown that VA RNA_I, but not VA RNA_{II}, is able to enhance translation of both early and late adenovirus mRNAs (Svensson and Akusjärvi, 1984a). To study, in more detail, the specificity for VA RNA_I we constructed a series of plasmids, in which the coding sequence for the bacterial CAT enzyme was fused to different 5′- as well as 3′-non-coding regions. Following transfection into 293 cells the effect of the VA RNAs on CAT expression was measured. The results showed that a chimeric mRNA with the CAT coding sequences fused to the adenovirus tripartite leader (pTripcat-2; Figure 2) is not more efficiently translated than a comparable mRNA having the 5′-non-coding sequences derived from the SV40 early transcription unit (pSVEcat; Figure 3). In both cases the translational efficiency was increased approximately six times by VA RNA. Furthermore, we observed a similar stimulation of CAT expression from a chimeric mRNA where the 5′-non-coding sequences were derived from the mouse β-major globin gene. Thus we conclude that the adenovirus tripartite leader does not, by itself, confer a translational advantage to a mRNA such that it automatically becomes more susceptible to stimulation by VA RNA_I.

In conclusion our experiments show that stimulation of CAT mRNA translation is more or less independent of the origin of the 5′- as well as the 3′-flanking sequences. Since the length of the 5′-non-coding region varies from 65 nucleotides in pβcat-2 to 254 nucleotides in pTripcat-2 (Figure 6), we can also conclude that the length of the 5′-flanking sequences does not significantly influence the degree of the translational stimulation. The stimulation of CAT was, in fact, only slightly less than that we previously observed for the adenovirus E3 19 K glycoprotein (Svensson and Akusjärvi, 1984a). Based on these results,

we conclude that VA RNA_I probably possesses a general capacity to stimulate translation and does not function by preferentially selecting the adenovirus mRNAs for translation.

We have found that the VA RNA stimulates translation ~2- to 3-fold better in 293 cells compared with HeLa and CV-1 cells (Figure 5). This difference was correlated with an ~20-fold higher efficiency of VA RNA expression in 293 cells. From these results we conclude that VA RNA functions as a positive regulator of translation also in the absence of other adenovirus gene products, although a product from region E1 enhances the translational efficiency, probably indirectly, by allowing for a more efficient VA RNA transcription. It is well documented that the 289 amino acid protein from region E1A has a capacity to activate transcription of RNA polymerase II genes (Jones and Shenk, 1979; Berk *et al.*, 1979; Nevins, 1981; Ricciardi *et al.*, 1981; Montell *et al.*, 1982). Our results suggest the intriguing possibility that a product from region E1A also functions as an enhancer of RNA polymerase III transcription. We are currently experimentally testing this hypothesis.

It is not known, at the present time, how VA RNA_I enhances the efficiency of translation. It appears, however, likely that VA RNA_I functions at an early stage of the initiation process of translation (Schneider *et al.*, 1984; Reichel *et al.*, 1985), possibly by positioning the ribosome to the initiator codon. In cells infected with mutant dl331 (VA RNA_I negative), the efficiency of translation initiation is drastically reduced and the large majority of the ribosomes accumulate as non-functional 80S monosomes not associated with mRNA (Schneider *et al.*, 1984). Since host cell gene expression also is inhibited in late infected cells (Bello and Ginsberg, 1967; Andersson *et al.*, 1973; Beltz and Flint, 1979; Babich *et al.*, 1983), VA RNA_I may be required in order to compensate for loss of an essential component of the normal translational machinery.

To explain both the preferential translation of viral mRNAs late after infection (Bello and Ginsberg, 1967; Andersson *et al.*, 1973), and the general stimulatory effect of VA RNA as observed in our transfection assays, we propose that VA RNA_I acts by specifically stimulating the translation of newly synthesized mRNA. For example, VA RNA_I perhaps becomes associated with newly synthesized mRNA in the nucleus, and functions as a tag that enables the ribosome to select a specific mRNA for translation. It is likely that the cellular and viral hnRNAs compete with each other in binding VA RNA in the cell nucleus. However, since only the viral mRNAs are transported to the cytoplasm (Beltz and Flint, 1979; Babich *et al.*, 1983) the hypothesis provides an explanation for the preferential synthesis of virus specific polypeptides seen late during an adenovirus infection (Bello and Ginsberg, 1967; Andersson *et al.*, 1973). The hypothesis predicts, furthermore, that the cellular mRNAs still present in the cytoplasm of late infected cells (Babich *et al.*, 1983; Khalili and Weinmann, 1984) will not be efficiently used for translation. This is due to the fact that these mRNAs have been synthesized before infection or during the early phase of the infectious cycle, when the VA RNAs are made in very small amounts (Söderlund *et al.*, 1976), and as a consequence are not associated with VA RNA_I.

The hypothesis also provides an explanation of our observation that synthesis of the bacterial CAT enzyme is efficiently stimulated by VA RNA_I. In cells transfected with plasmid DNA both the mRNA and the VA RNAs are concomitantly expressed without the restriction in host cell gene expression being operative. Therefore, we observe an enhancement of protein synthesis which is not restricted to the adenovirus mRNAs. The hypothesis predicts, furthermore, that translation of cellular mRNAs would be stimulated in the VA RNA co-transfected cells. We have not, however, been able to detect a general increase in protein synthesis after DNA transfection (data not shown). This is most likely due to the low transfection efficiency which is obtained by the calcium phosphate co-precipitation technique (Loyter *et al.*, 1982).

Finally, we would like to point out that our finding that VA RNA_I has a general ability to stimulate translation probably can be developed into a useful method for improving the yield from eukaryotic expression vehicles.

Materials and methods

Plasmid construction

pSVEcat, which is a pML2 (Lusky and Botchan, 1981) derivative of pSV2cat (Figure 7A), was kindly provided by Dr M.Yaniv (Institute Pasteur, Paris, France). pTripcat-2 (Figure 7B) was constructed as follows. pSVEcat was cleaved with restriction endonucleases *Hind*III and *Pvu*I. The 2200-bp DNA fragment specifying the CAT coding sequences and the SV40 3′-flanking sequences were isolated by gel electrophoresis and inserted between the unique *Hind*III and *Pvu*I sites in plasmid pTrip-2 (Figure 7D). Plasmid pTrip-2 has a complete cDNA copy of the adenovirus tripartite leader fused to a 670-bp DNA fragment (position 5370 – 6039, Aleström *et al.*, 1982; Gingeras *et al.*, 1982) encoding the transcriptional control region for the major late adenovirus promoter (Akusjärvi and Svensson, in preparation). pβcat-2 (Figure 7C) was constructed by a two-step cloning procedure. Step 1: construction of pβcat-3 (Figure 7E). The SV40 transcriptional control region was removed from plasmid pSVEcat by cleavage with *Hind*III and replaced by a *Hind*III-*Hinc*II (converted to *Hind*III by linker addition) fragment encoding the mouse β-major globin promoter (Konkel *et al.*, 1978). Step 2: construction of pβcat-2 (Figure 7C). The *Ban*I cleavage site located 20 bp downstream of the TAA sequence terminating CAT translation (position 903 in the sequence of Alton and Vapnek, 1979) in pβcat-3 was converted to a *Bam*HI site by linker addition. The *Sal*I-*Bam*HI fragment was subsequently isolated and inserted into plasmid pβ2x (Banerji *et al.*, 1981) similarly cleaved with *Sal*I *and Bam*HI. All cloning experiments connected with this study were carried out according to the guidelines of the Swedish Recombinant DNA Committee, using standard recombinant DNA techniques (Maniatis *et al.*, 1982).

Cell growth and transfection

Monolayer cultures of 293 cells were maintained in Dulbecco's modified Eagle medium containing 10% (v/v) fetal calf serum and 50 μg of gentamycin/ml. Subconfluent cells were transfected as previously described (Svensson *et al.*, 1983) using the calcium phosphate co-precipitation technique (Graham and van der Eb, 1973; Wigler *et al.*, 1978). A total of 15 μg of plasmid DNA was added per 6 cm Petri dish. Plasmids expressing CAT and VA RNA were added in the amounts given in the figure legends. In all transfections pBR322 DNA was used as a carrier to bring the total amount of DNA to 15 μg per transfection.

Assay for CAT activity

Following a 50 h incubation period, transfected cells were harvested and a cell-extract prepared by brief sonication. The CAT activity was measured essentially as described by Gorman *et al.* (1982). Briefly, 5 – 20 μl (of 100 μl total) of the cell extract was incubated at 37°C in a final volume of 180 μl together with 1 μCi of [^{14}C]chloramphenicol. Aliquots of the reaction mixture were withdrawn at different time points and the reaction terminated by extraction with 0.5 ml of cold ethyl acetate. ^{14}C-labeled chloramphenicol was separated from acetylated forms by ascending t.l.c. (Merck silica gel 60). Following autoradiography, radioactive spots were excised and counted. The rate of conversion was calculated by determining the percentage of acetylated chloramphenicol.

S1 endonuclease analysis

In experiments where RNA was isolated from transfected cells, half of the washed cell pellet (Gorman *et al.*, 1982) was fractionated into nuclei and cytoplasm by IsoB-Nonidet P-40 extraction (Akusjärvi and Pettersson, 1978). Total cytoplasmic RNA was subsequently isolated by phenol extraction. S1 endonuclease digestion was as previously described (Berk and Sharp, 1978; Weaver and Weissman, 1979; Svensson *et al.*, 1983). When CAT-specific mRNA was quantitated, 10 μg of total cytoplasmic RNA was hybridized overnight at 45°C to the 5′ end-labeled *Eco*RI-*Sal*I fragment indicated in the legend to Figure 2B. For analysis of VA RNA expression 1 μg of total cytoplasmic RNA was hybridized overnight at 50°C to the 5′ end-labeled probes described by Svensson and Akusjärvi (1984a). The conditions for S1 endonuclease cleavage and electrophoretic separation was as previously described (Svensson *et al.*, 1983).

Acknowledgements

We thank Marie Lager for excellent technical assistance and Jeanette Backman for patient secretarial help. We also wish to thank M.Yaniv and colleagues in his laboratory for providing us with plasmid pSVEcat. We are also indebted to Ulf Pettersson and Göran Magnusson for many fruitful discussions and help in the preparation of this manuscript. This work was supported by grants from the Swedish Natural Science Research Council and the Swedish Cancer Society.

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Received on 7 November 1984; revised on 24 January 1985