Activation of an internal initiation site for protein synthesis during in vitro translation

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ABSTRACT

The major mRNA for adenovirus 2 polypeptide pVIII sediments at 18S as as-
sayed by in vitro translation in the messenger-dependent rabbit reticulocyte
lysate system. However, a small amount of messenger activity for pVIII sedi-
ments at about 27S, coincident with the mRNA for 100 K. Isolation and frac-
tionation of poly(A) containing RNA following in vitro translation of 27S 100 K
mRNA demonstrated the appearance of an 18S messenger activity for pVIII, which
is approximately the size of the authentic mRNA for this protein. Partial de-
gradation of 27S 100 K mRNA with alkali or ribonuclease T_1 also results in ac-
tivation of an 18S messenger activity for pVIII suggesting that in vitro mes-
senger activity for pVIII associated with 27S RNA is due to degradation of
100 K mRNA during translation in the cell-free system.

INTRODUCTION

The translation of specific RNAs in eukaryotic cell-free systems is a val-
uable tool for assigning messenger activities for particular proteins to spe-
cific RNA species. A tacit assumption in the use of these systems is that the
input RNA remains unchanged during translation in vitro. Many viral mRNAs are
expressed as families of 3'-co-terminal RNA molecules. Each member of a family
is thought to be active in directing the synthesis only of the protein coded at
the 5'-end of the molecule. A protein coded internally in such a mRNA is nor-
mally translated from a second mRNA in which its initiation site is at the 5'-
end. Examples of viral RNAs which have such arrangements are tobacco mosaic
virus (1), the late mRNAs of polyoma and SV40 (2,3,4), the late mRNAs of adeno-
virus 2 (5,6,7,8), and the mRNAs of RSV (9). We have observed that a number of
purified viral RNAs containing potential internal coding sequences have mes-
senger activity when translated in rabbit reticulocyte lysates not only for
the protein encoded in the 5'-region of the molecule but also a small but
easily detectable level of messenger activity for the protein coded internal-
ly. The mechanism by which such internal sequences are translated is of in-
terest because internal initiation is thought not to occur on eukaryotic

mRNAs (10). In the present study we have investigated the in vitro translation of adenovirus 2 100 K mRNA. When this 27S RNA is translated in a lysate from rabbit reticulocytes, messenger activity for the internally coded viral protein pVIII is also observed. We demonstrate that following translation in vitro the messenger activity for pVIII sediments at about 18S suggesting that its translation depends upon "processing" of the input RNA to generate a free 5'-terminal near the initiation site for pVIII. We also demonstrate that 18S messenger activity for pVIII can be generated from 27S 100 K mRNA by cleavage with alkali or ribonuclease T_1 indicating that the "processing" is likely to be non-specific.

MATERIALS AND METHODS

Cells : HeLa S3 cells were grown in suspension in Joklik-modified Eagle minimal essential medium supplemented with 5% calf serum.

Viruses : Stocks of Ad2 were obtained form Dr. Gernot Walter. Cells were infected at a multiplicity of 20 p.f.u./cell. Virus was adsorbed for 30' at room temperature and at a concentration of 4×10^7 cells/ml. Cells were then diluted to a concentration of 4×10^5/ml with fresh medium and infection was allowed to continue for 24 hours

Labeling of cells : Cells were labeled at 24 hrs after infection with [^{32}P]-orthophosphate (ICN, carrier free). 100 ml of infected cells were collected by centrifugation at 1000 rpm and washed once with Eagle minimal essential medium without phosphate (MEM-P). Cells were resuspended in 50 ml MEM-P supplemented with 2% dialyzed calf serum. 25 mCi [^{32}P]-orthophosphate was added and the cells were incubated for 3 hrs at 37°C.

Preparation of RNA : 4×10^7 HeLa cells, 24 hrs after infection with Ad2, were collected by centrifugation at 1000 rpm, washed once with cold saline and resuspended in 1.7 ml cold isotonic lysis buffer (0.15 M NH_4Cl, 10 mM Tris, pH 6.8, 2 mM $MgCl_2$, 50 µg/ml dextran sulfate). 0.2 ml of 10% NP 40 (Shell Chem. Co.) was added followed by brief vortexing. 0.1 ml 10% DOC was then added and the suspension vortexed for 30 sec. Nuclei were pelleted by centrifugation at 2000 rpm for 2 min. 90 µl of 0.5 M EDTA, pH 7.0, 80 µl of 4 M NaCl, 0.2 ml 10% SDS and 20 µl β-mercaptoethanol were added to supernatant. To make cytoplasmic RNA the supernatant was warmed to 30°C and extracted with 2 ml phenol. 2 ml of chloroform was added and the mixture reextracted. Phases were separated by centrifugation at 10,000 rpm for 20 min at 25°C. The aqueous phase was reextracted with 2 ml chloroform and the phases were separated as above. The aqueous phase was then collected and RNA was precipitated by the addition

of 2 volumes ethanol. Precipitated RNA was stored in aqueous ethanol at -20°C. Poly(A) containing cytoplasmic RNA was obtained by chromatography on oligo(dT)-cellulose (T-3, Collaborative Research).

Sucrose gradient centrifugation : RNA dissolved in a total volume of 0.5 ml of 10 mM Na Acetate, pH 5.2, 1 mM EDTA was layered on a 10.6 ml gradient of 10%-30% sucrose (wt./wt.) in 10 mM Na Acetate, pH 5.2, 1 mM EDTA, 0.1% SDS. Centrifugation was performed at 20°C for 15 hrs at 30,000 rpm in a Beckman SW41 rotor.

Cell-free translation : RNAs were translated in the messenger-dependent rabbit reticulocyte lysate system described by Pelham and Jackson (11) in the presence of [^{35}S] methionine (Amersham, 1000 Ci/mmole).

Polyacrylamide gel electrophoresis : Proteins synthesized in vitro were resolved by diluting reaction mixtures with 5 volumes of sample buffer and applying 5 μl to each lane of a 1 mm thick, 9.5 cm long slab polyacrylamide gel according to the system described by Laemmli and Maizel (12,13). Electrophoresis was for 4.5 hrs at a constant current of 15 mA.

In vitro activation of pVIII messenger activity : a) Incubation in rabbit reticulocyte lysate. Two μg of [^{32}P] labeled 27S poly(A) containing RNA isolated from Ad2 infected HeLa cells was incubated for 20 min at 30°C in 0.5 ml of a micrococcal nuclease treated rabbit reticulocyte lysate. Following incubation, 4.0 ml of 0.5 M LiCl, 10 mM Tris, pH 7.4, 1 mM EDTA and 2.0% SDS was added, the mixture was chilled on ice and poly(A) containing RNA was isolated by passing the mixture over a 0.2 ml column of oligo (dT) cellulose at 4°C. The column was washed with 20 volumes of 0.5 M LiCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 0.1% SDS (binding buffer). RNA was eluted with 2.0 ml of water at room temperature and precipitated by the addition of 20 μg yeast tRNA, one-tenth volume of 2M Na Acetate, pH 5.2 and 3 volumes ethanol. b) Alkaline hydrolsis. One μg of [^{32}P] 27S RNA in 50 μl H$_2$O was warmed to 50°C. 50 μl of 0.1 M Na$_2$CO$_3$ was added and incubation continued for 2 min at 50°C. The RNA was rapidly chilled and 1.0 ml of binding buffer at 4°C was added. Poly(A) containing RNA was then isolated as described above. c) RNase T$_1$ digestion. One μg of [^{32}P] 27S RNA was incubated in 1.0 ml of 0.15M NaCl, 50 mM Tris, pH 7.4, 1.0 mM EDTA containing 100 μg yeast tRNA and 0.1 units of RNase T1 (Calbiochem) for 3 min at room temp. 5 μl of 20% SDS was added and the reaction immediately extracted with 1.0 ml phenol and 1.0 ml choloroform. The mixture was centrifuged at 5000 rpm, 20°C for 10 min. The aqueous phase was removed and made 0.5 M LiCl. Poly(A) containing RNA was isolated as described above.

One μg of unprocessed 27S [^{32}P] RNA and the poly(A) containing RNAs iso-
lated following incubation of [^{32}P] 27S RNA in reticulocyte lysate, alkaline
hydrolysis or digestion with RNase T_1 were dissolved in 0.5 ml of 10 mM Na
Acetate, pH 5.2, 1.0 mM EDTA and fractionated on sucrose gradients as described
above. 18S RNA from these gradients was isolated and resedimented on a second
gradient. 18S RNA from the second gradient was isolated and dissolved in 10
μl H_2O. A small amount of this RNA was translated in 10 μl of the messenger-
dependent lysate. By measuring the radioactivity in isolated 18S [^{32}P] RNAs
the amount of RNA translated was normalized such that 18S RNA isolated from
unprocessed 27S RNA and 27S RNA incubated in reticulocyte lysate was from
the same amount of input 27S RNA and such that the same amount of 18S RNA gen-
erated by incubation in reticulocyte lysate, alkaline hydrolysis or hydrolysis
with RNase T_1 was translated.

RESULTS

Adenovirus 2 cytoplasmic RNA was fractionated on a sucrose gradient, RNA
from each fraction was translated in vitro in the messenger-dependent rabbit
reticulocyte lysate in the presence of [^{35}S]-methionine and the labeled pro-
ducts were analyzed on a polyacrylamide gel. The major species of messenger
activity for pVIII sediments at about 18S as determined previously by Anderson
et al. (14). Messenger activity for pVIII is also observed in fraction 9 (27S)
which contains the peak messenger activity for 100 K.

In order to determine if the 27S messenger activity for pVIII was the re-
sult of aggregation (27S) of 18S mRNA, messenger activity for 100 K was
partially purified by three cycles of sedimentation through sucrose gra-
dients. The peak of 100 K messenger activity from each gradient as assayed
by translation in vitro was collected by ethanol precipitation, dissolved in
buffer and denatured by heating at 100°C for 60 sec. prior to resedimentation.
The yield from 500 μg of poly(A) containing cytoplasmic RNA was about 5 μg
of 27S RNA. A small amount of messenger activity for pVIII continued to
sediment with 100 K messenger activity even after a fourth cycle of sedimen-
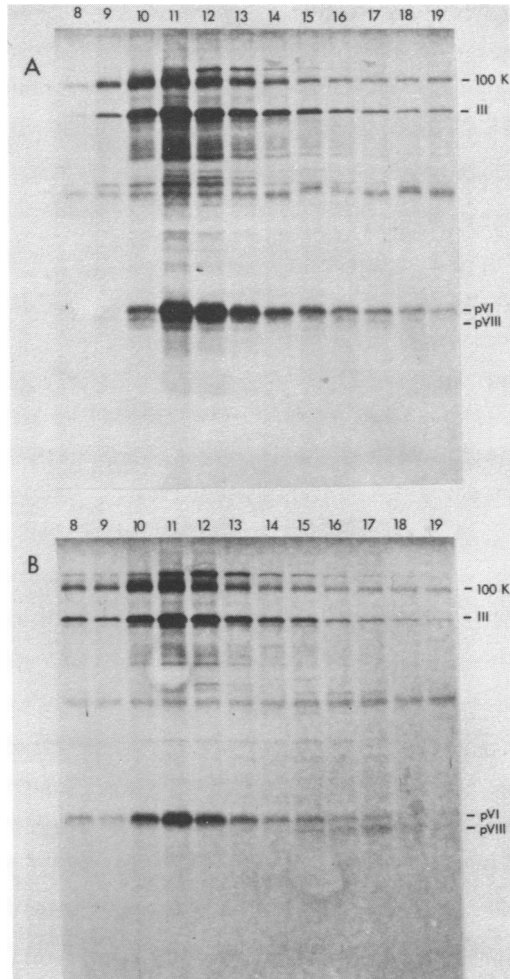tation and heat denaturation, (figure 2A).

Because the mRNA for 100 K contains sequences coding for pVIII in its 3'-
terminal region (5), it is likely that the small amount of pVIII messenger ac-
tivity associated with 27S RNA was due to translation of these internal sequen-
ces. These sequences could either be translated by internal initiation of pro-
tein synthesis on intact 100 K mRNA or more likely that the RNA was processed
or degraded during translation in vitro producing a free 5'-end near the pVIII
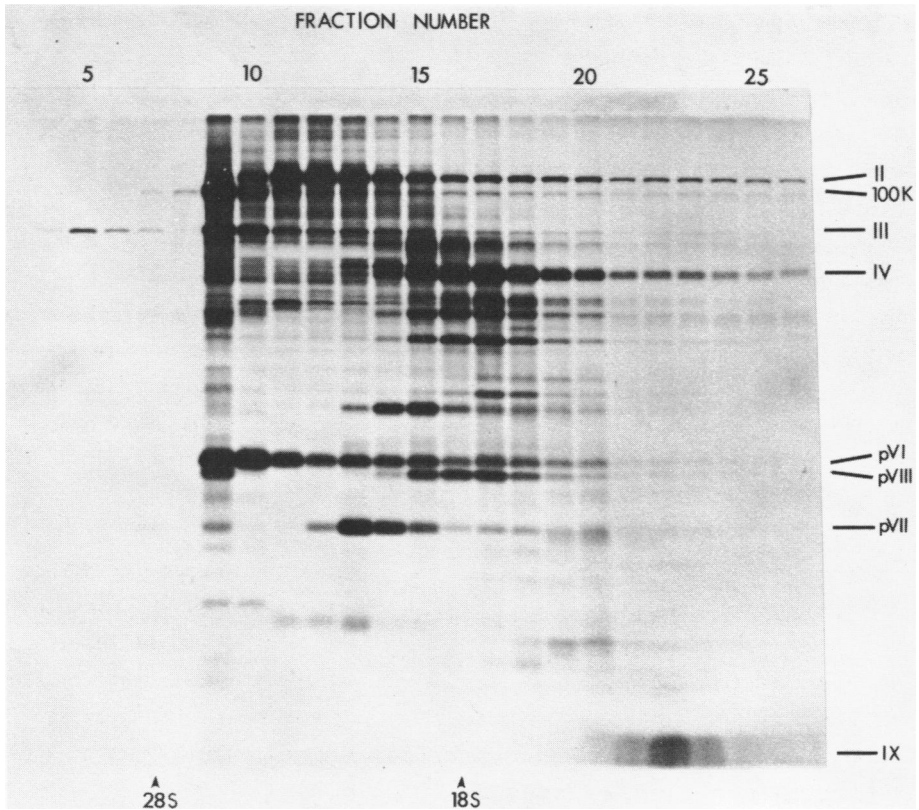
Figure 1. Translation of size fractionated adenovirus 2 mRNAs. 10 µg of poly(A) containing RNA isolated from the cytoplasm of HeLa cells infected for 24 hours with Ad2 was fractionated on sucrose gradients as described in Materials and Methods. The gradient was divided into 0.45 ml fractions and RNA in each fraction was collected by ethanol precipitation. RNA in each fraction was dissolved in 50 µl H$_2$O and 1 µl of each was translated in a 10 µl reaction mixture as described in Materials and Methods. One tenth of each reaction was analyzed on a 12.5% polyacrylamide gel and [^{35}S]labeled proteins visualized by fluorography.

initiation site on which translation could initiate. If the later possibility is correct, a RNA molecule with messenger activity for pVIII should be produced during the _in vitro_ translation reaction sedimenting at about 18S, the size of the authentic mRNA for pVIII.

This was tested by isolating poly(A) containing RNA following translation of 27S 100 K mRNA in the messenger-dependent rabbit reticulocyte lysate. The poly(A) containing RNA was fractionated on a sucrose gradient, RNA in each

Figure 2. Sedimentation of 27S Ad2 mRNAs before and after incubation in rabbit reticulocyte lysates. 27S Ad2 mRNA was purified by three cycles of heat denaturation and sedimentation in sucrose gradients. A) One μg of 27S RNA was fractionated on a sucrose gradients as described in Materials and Methods. RNA in each fraction was collected by precipitation in ethanol, dissolved in 20 μl H_2O and 1 μl of each was translated in vitro in the presence of [^{35}S] methionine. One-tenth of each reaction was analyzed on a 12.5% polyacrylamide gel and radioactive proteins visualized by fluorography. B) Two μg of 27S RNA was incubated for 20 minutes in 1.0 ml of rabbit reticulocyte lysate at 30°C. Following incubation the lysate was diluted with 10 ml of cold 0.5 M LiCl, 1 mM EDTA, 10 mM Tris, pH 7.4 and 10% SDS. This was passed over a 0.5 ml column of oligo(dT)-cellulose at 4°C. The column was washed extensively with 10 mM Tris, pH 7.4, 0.5 M LiCl, 1 mM EDTA, 0.1% SDS and bound RNA was eluted with H_2O and collected by ethanol precipitation, this RNA was subsequently analyzed on a sucrose gradient as in panel A. Sedimentation is from right to left and 28S and 18S rRNAs sedimented in fractions 10 and 17 respectively.

fraction was translated in vitro in the presence of [^{35}S] methionine and the labeled products were analyzed by polyacrylamide gel electrophoresis. Figure 2B shows a 18S peak of messenger activity for pVIII (fractions 15-18) associated with RNA isolated following translation in vitro. This peak of activity is not present in the 27S preparation of 100 K mRNA prior to in vitro translation (figure 2A). As discussed above, this result suggests that the translation of pVIII sequences in 27S mRNA is dependent upon cleavage of 100 K mRNA near the initiation site for pVIII.

An interesting question is whether this apparent activation of internal initiation sites for protein synthesis is performed by endonucleases which cleave 100 K mRNA at specific sites (possibly RNA processing enzymes) or whether activation can be caused by random degradation of 100 K mRNA at positions near the initiation site for pVIII. This was tested by comparing the activation of 18S messenger activity for pVIII by incubation in rabbit reticulocyte lysates to activation caused by degradation induced by alkaline hydrolysis or hydrolysis with ribonuclease T$_1$. Purified [^{32}P] labeled 27S 100 K mRNA was either incubated in rabbit reticulocyte lysate, subjected to degradation by alkali or incubated with RNase T$_1$. Poly(A) containing RNA was isolated and then fractionated on sucrose gradients. The 18S region from each gradient was pooled and rerun on a second gradient. RNA in the 18S region of the second gradient was pooled, precipitated and translated in vitro in the presence of [^{35}S]-methionine. The amount of RNA translated was normalized such that the 18S RNA in the control and reticulocyte lysate activated preparations was from the same amount of [^{32}P]-labeled 27S input RNA, and such that the same amount of 18S [^{32}P] RNA generated by incubation in reticulocyte lysate, alkali or RNase T$_1$ was translated. Figure 3 shows that incubation in the reticulocyte lysate results in a significant increase in 18S messenger activity for pVIII. Alkaline hydrolysis and digestion with RNase T$_1$ also activates 18S messenger activity for pVIII (figure 3, lanes 4 and 5) which is about as active as 18S RNA generated in the reticulocyte lysate. Thus, two methods for randomly degrading 100 K mRNA result in activation of pVIII 18S messenger activity suggesting that the pVIII messenger activity associated with 27S RNA is due simply to degradation of the input mRNA during translation.

To confirm that the protein synthesized from 18S RNA generated during translation in vitro is pVIII, the 26K proteins synthesized in response to 18S RNA generated by incubation in the reticulocyte lysate (figure 3, lane 3) and authentic pVIII mRNA (figure 3, lane 6) were compared by peptide analysis by the method of Cleveland et al. (15).

Figure 3. In vitro production of 18S messenger activity for pVIII from 2/S RNA by incubation in rabbit reticulocyte lysate, alkali or RNase T_1. [^{32}P]-labeled 27S Ad2 RNA was either incubated in rabbit reticulocyte lysate or hydro-hydrolyzed with mild alkali or ribonuclease T_1 as described in Materials and Methods. 18S polyadenylated RNA was isolated and translated in vitro or in the presence of [^{35}S] methionine and analyzed on a 12.5% polyacrylamide gel. Lane 1: no RNA added; lane 2: 18S RNA isolated from untreated 27S mRNA; lane 3: 18S RNA isolated following incubation of 27S RNA in rabbit reticulocyte lysate; lane 4: 18S RNA isolated following incubation of 27S RNA in mild alkali; lane 5: 18S RNA isolated following incubation of 27S RNA with ribonuclease T_1; lane 6: translation of Ad2 poly(A) containing 18S RNA.

Figure 4 indicates that the two proteins are identical and confirms that messenger activity generated in vitro from 27S RNA does indeed code for pVIII.

DISCUSSION

Other investigators have previously demonstrated the in vitro synthesis of proteins from initiation sites activated by degrading RNA. Purchio et al.
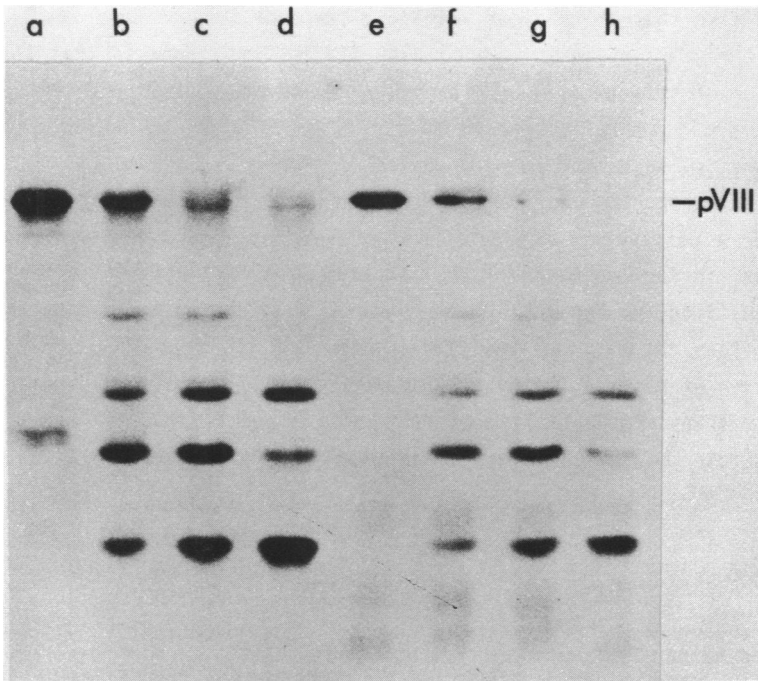
Figure 4. Peptide analysis of the 26 K protein synthesized in response to 18S RNA genereated from Ad2 27S RNA or from authentic Ad2 18S RNA. The 26 K protein (pVIII) synthesized in response to 18S RNA generated from 27S RNA by incubation in a rabbit reticulocyte lysate (lanes e-h) or synthesized in response to authentic Ad2 18S RNA (lanes a-d) were subjected to peptide analysis by the method of Cleveland et al. (15) on a 16% gel using Staphylococcus aureus V8 protease. Lanes a and e: no protease; lanes b and f: 0.1 μg protease; lanes c and g: 0.4 μg protease; lanes d and h: 1 μg protease.

(16) have employed the translation of degraded avian sacroma virus RNA to confirm that the src protein is coded within viral 35S RNA. Pelham (17) has recently demonstrated that protein synthesis is initiated at spurious sites on partially degraded cowpea mosaic virus RNA and tobacco mosaic virus RNA when translated in vitro in rabbit reticulocyte lysates. In these instances initiation of protein synthesis is presumably occuring at AUG codons which lie just downstream of free uncapped 5'-termini generated by degradation of the RNA.

The present work extends these observations by demonstrating that internal initiation sites for protein synthesis can actually be activated during in vitro translation and is likely to be due to random degradation during the reaction. The in vitro synthesis of pVIII directed by its authentic 18S mRNA is relative-

ly insensitive to the inhibitor m^7GTP (C. Lawrence, unpublished result), which inhibits the translation of capped mRNA (18). This suggests that the normal translation of this mRNA is not strongly dependent on the presence of a cap, and may explain the efficient synthesis of pVIII from RNA which is degraded during in vitro translation

The main importance of this result is that it indicates that messenger activity for a particular protein in vitro does not insure that a specific RNA species is, in fact, a mRNA for that protein in vivo. While in vitro translation of purified RNA species is a very useful tool in the assignment of messenger activities for particular proteins, some caution must be taken in the interpretation of the results of cell-free translations. On the other hand, the ability to translate partially degraded RNAs is quite useful in some instances to demonstrate the presence of particular coding sequences in certain RNA species.

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