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On the fidelity of mRNA translation in the nuclease-treated rabbit reticulocyte lysate system

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ABSTRACT

As a test of the fidelity of the rabbit reticulocyte lysate system, we have examined the products of translation of various different influenza virus mRNAs, produced by *in vitro* transcription. A common finding with all mRNA species was that the ratio of full-length translation product to incomplete products decreased with increasing mRNA concentration. These short products are a mixture of (i) polypeptides initiated at the authentic initiation site but terminated prematurely, and (ii) polypeptides initiated at internal sites and terminated at the correct site. Analysis of mRNA stability during the translation assay showed very little degradation, quite insufficient to be the principle cause of incomplete product synthesis. Investigation of the influence of various parameters on the ratio of full-length to incomplete products leads to the conclusion that a high fidelity of translation can be obtained provided certain precautions are followed: the use of capped, rather than uncapped, mRNAs at low concentrations, with KCl concentrations about 20 mM above the level that gives maximum incorporation.

INTRODUCTION

The nuclease-treated rabbit reticulocyte lysate system (1,2) has been widely used to assay mRNA and to identify the translation products of a given RNA species. For the most part it has proved to be efficient and accurate, arguably more so than any other system, and there appear to have been few cases where the wrong product has been synthesised in significant yield. However, in recent discussions of the scanning ribosome model Kozak has questioned the fidelity of this translation system (3), and has suggested that it may not be appropriate to use it to identify the products and initiation sites of a previously uncharacterised mRNA. Since these doubts are based on only a few specific examples, we wished to examine the question of the fidelity of the reticulocyte lysate translation system in more detail. Such studies require the use of a single mRNA species, which has hitherto been possible only with viral RNAs, and even these are not ideal as most virion RNA preparations are partially degraded. The advent of methods for *in vitro* synthesis of capped mRNA of defined sequence using bacteriophage T7 or SP6 RNA polymerases (4) now makes an analysis of fidelity more feasible. We show here that high fidelity of translation can be achieved under conditions of low concentrations of capped RNAs, with a KCl concentration slightly above that which gives maximum incorporation. Departure from these conditions leads to an increase in the yield of incorrect products, arising not only from initiation at internal sites but also from premature termination of translation.

MATERIALS AND METHODS

Materials

The plasmids used in this work were a generous gift of Dr. S.C. Inglis and Dr. V.C. Blok (Department of Pathology, University of Cambridge), and were derived by them from the influenza virus (strain A/PR/8/34) full-length cDNA clones described by Young *et al.* (5). Briefly, the PB1 cDNA of the clone pAPR206 of Young *et al.* was inserted into the Hind III site of pT7-2 (U.S. Biochemical Corp.); the PB2 cDNA of pAPR101 inserted into the BamH I site of pT7-2; and the NP cDNA of pAPR502 into the EcoR I site of pT7-1. Plasmid pJ'1, containing the influenza virus NS cDNA, was constructed in this laboratory by inserting the NS cDNA insert ultimately derived from pAPR801 (5) into the EcoR I site of pGEM-2 (Promega Biotec) to give pJ12. Plasmid pJ12 was digested with BamH I and Sal I, which removes 32 nucleotides of polylinker sequences (including an EcoR I site) and the whole 5'-untranslated region of the NS gene. The large fragment from this digestion was purified and ligated with synthetic oligonucleotides to give plasmid pJ'1, in which the 5'-untranslated segment of the NS gene is restored unchanged except for an extra C residue inserted to create a restriction site for BstE II.

Antisera raised against different segments of the influenza virus A/PR/8/34 PB2 protein, and against the A/PR/8/34 PA protein were kindly provided by Drs. S.C. Inglis and V.C. Blok, and are described in detail elsewhere (6).

m^7GpppG cap analogue was from New England Biolabs, RNasin and calf liver tRNA from Boehringer, nuclease-free bovine serum albumin from Bethesda Research Laboratories, and Protein A-Sepharose CL 6B from Pharmacia. Amplify, Hyperfilm β-max, [^{35}S]methionine (1200-1500 Ci/mmol) and [α-^{32}P]UTP (400 Ci/mmol) were from Amersham International. Bacteriophage T7 RNA polymerase was a gift from S. Mackie of this laboratory, and oligonucleotides were prepared in the Departments of Biochemistry and Pathology oligonucleotide production facility.

Plasmid Preparation and Transcription Reactions

Plasmids were propagated by standard methods (7), without chloramphenicol amplification, using *E. coli* TG1. Plasmid DNA for use in transcription reactions was purified by caesium chloride density gradient centrifugation in the presence of 0.3 mg/ml ethidium bromide. Ethidium bromide was subsequently removed from the plasmid DNA by extraction with butanol, followed by dialysis. The plasmid DNA was linearised by digestion with Sma I, except for pJ'1 (NS cDNA) which was linearised with EcoR I. A small sample was analysed by agarose gel electrophoresis to ensure that the digestion had gone to completion, and the linearised DNA was purified from the bulk of the reaction by extraction with phenol and subsequently with chloroform.

Transcription reactions (usually 0.05 ml) for the production of capped transcripts were as follows: 40 mM Tris-HCl, pH 8.0; 15 mM $MgCl_2$: 1 mM ATP, CTP, UTP; 40 μCi/ml

[α-^{32}P] UTP; 0.1 mM GTP; 0.5 mM m^7GpppG; 5 mM dithiothreitol; 10 µg/ml nuclease-free bovine serum albumin; 2,000 units/ml RNasin; 150 µg/ml linearised template DNA; 50 µl/ml T7 RNA polymerase (activity > 40 units/µl). The reaction was incubated at 37° for 30 min, then made 1 mM in GTP, and incubation continued for a further 60 min. For the production of uncapped transcripts, the m^7GpppG cap analogue was omitted, and 1 mM GTP was present from the start of the reaction.

The extent of RNA synthesis was calculated from the percentage of [^{32}P]UTP incorporation determined by taking a 1 µl sample and diluting it into 200 µl water, then 50 µl of this was counted directly and 50 µl after cetyltrimethylammonium bromide precipitation to precipitate RNA (8). RNA was isolated from the rest of the reaction mix by extraction first with phenol, and then with chloroform. It was precipitated three times with ethanol in the presence of 2 M ammonium acetate to remove unincorporated nucleotides. The concentration of the final RNA solution was determined by counting Cerenkov radiation.

The efficiency of capping was determined by carrying out two parallel reactions, one to produce capped mRNA and the other uncapped, using pJ'1 linearised with BstE II so as to give a short, 45 nucleotide residues long, transcript. The labelled RNAs were run on a 6% acrylamide sequencing gel, and the resulting autoradiograph scanned. The presence of cap analogue in the transcription reaction was found to increase the size of the longest transcript by the equivalent of two additional nucleotide residues, thus allowing the ratio of capped to uncapped transcripts to be determined. It was found that about two-thirds of the transcripts produced under these conditions were capped.

For the analysis of labelled RNA by gel-electrophoresis, the method of McMaster and Carmichael (9) was used, with glyoxalated Hind III digested bacteriophage λ DNA or cucumber mosaic virus genomic RNAs as molecular weight standards.

Translation assays and analysis of translation products

Rabbit reticulocyte lysates were prepared and treated with micrococcal nuclease as described previously (2). The translation assays were based on previously described methods (2) with the following differences. Nuclease-treated lysate constituted 72% by vol. of the final assay mix, and the final concentration of the added components was: 90 mM KCl, 0.45 mM $MgCl_2$, 10 mM creatine phosphate, 100 mM each unlabelled amino acid (minus methionine), 45 µg/ml calf liver tRNA, 50 µg/ml creatine phosphokinase, 0.45 mCi/ml [^{35}S]methionine. L-cell postmitochondrial supernatants (L-cell S10) were prepared as described previously (10). For treatment with micrococcal nuclease the standard procedure was to incubate the L-cell S10 with 150 units/ml micrococcal nuclease (Worthington) and 0.3 mM $CaCl_2$ at 14°C for 90 min, followed by the addition of 2 mM ethyleneglycol-bis(2-aminoethylether)-N,N'-tetraacetate (EGTA) to stop the reaction.

Total incorporation of labelled methionine into acid-precipitable protein was determined as described previously (2). To analyse the products by gel electrophoresis, one volume of

translation assay was mixed with an equal volume of 100 μg/ml ribonuclease A, 10 mM EDTA (pH 7.2), incubated for 10 min at room temperature and then diluted with 18 vol. of the sample buffer described previously (11). Polyacrylamide slab gels were either 15% (w/v) acrylamide exactly as described before (11) or 20% (w/v) acrylamide, 0.066% (w/v) methylenebisacrylamide. The dried gels were exposed to Hyperfilm β-max, or were fluorographed (12) using Amplify and exposed to pre-flashed Fuji RX film. Where appropriate, the developed films were scanned using a Transidyne 2955 Scanning Densitometer. Track(s) loaded with radioactive marker proteins were included in each gel, and their positions are indicated on the figures, with sizes given as kDa.

For immunoprecipitation of the translation products, a 15 μl sample of the translation assay was diluted with 100 μl of lysis buffer: 10 mM Tris-HCl, pH 8.0, 0.14 M NaCl, 0.5% (v/v) nonidet P40. Antiserum (5 μl) was then added, and the mixture incubated for 30 min at room temperature with gentle agitation. An aliquot (50 μl) of a washed suspension of Protein A-Sepharose CL6B (approx. 0.1 mg dry resin suspended in 0.7 ml lysis buffer) was then added, and incubation continued for 30 min at 4°C, again with gentle agitation. The Protein A-Sepharose was pelletted in a microfuge and washed three times in 0.5 ml lysis buffer. Finally, the beads were resuspended in 30 μl of gel sample buffer, left at room temperature for 15 min and then boiled for 3 min to elute the bound proteins for analysis by gel electrophoresis.

RESULTS

Decrease in translation fidelity at high concentrations of mRNA

When capped transcripts of the various influenza virus cDNA clones were translated at different RNA concentrations, it was consistently noted that the higher the RNA level the lower was the ratio of full-length translation product to short polypeptide products, as illustrated in Fig. 1 for PB1 mRNA. The effect can be seen particularly clearly if equal amounts of labelled protein are loaded on each track (rather than equal volumes of each translation assay) as is also shown in Fig. 1. This phenomenon of decreasing 'signal to noise' ratio with increasing RNA concentration was noted with all the influenza mRNAs tested, with the qualification that it was more evident with some mRNA species (PB1, PB2 and NP) than others such as the NS mRNA (compare Fig. 1 and 2). It is possible that this is related to the shorter length of the NS mRNA coding sequence: 690 nucleotide residues, as opposed to 1494 for NP mRNA, 2277 for PB2 mRNA, and 2271 for PB1 mRNA (13).

Pulse-chase experiments showed that none of the full-length translation products of any of the influenza virus mRNAs tested was unstable with time (data not shown) and thus the short products cannot be explained as merely degradation products of the full-length protein. In addition, the mRNAs were rather stable over the incubation period (Fig. 3), such that it is most unlikely that the synthesis of short products can be entirely explained as the result of translation of mRNA fragments. For incubation periods up to 30 min there was no loss of full-length mRNA, whilst between 30 and 60 min the yield of full-length RNA decreased slightly but

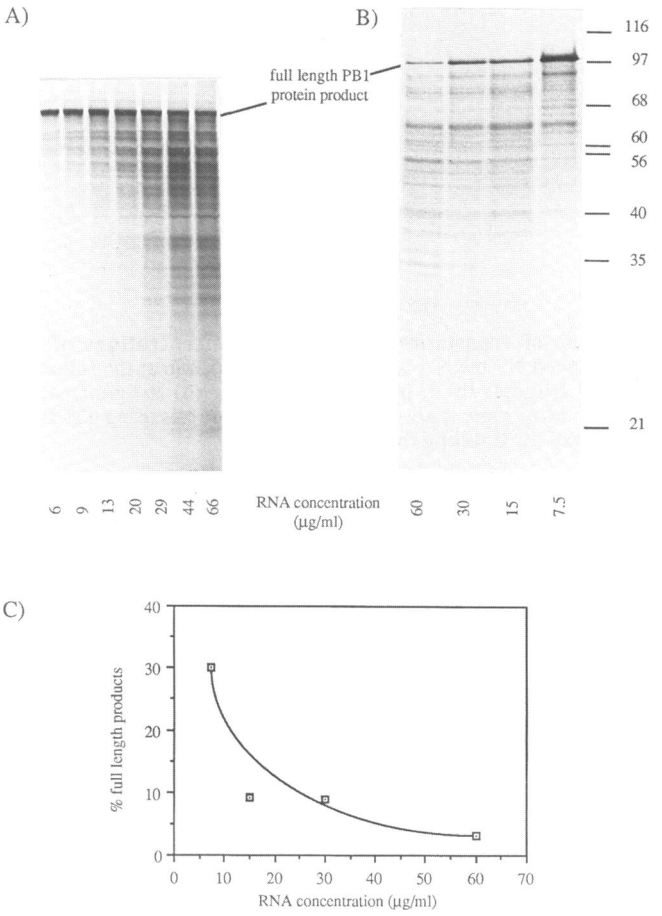

Figure 1. Products of translation of increasing concentrations of influenza virus PB1 mRNA. Capped PB1 mRNA was translated in two separate experiments for 45 min at the different mRNA concentrations specified. In (A) equal volumes of each assay were analysed by gel electrophoresis using a 15% polyacrylamide gel, and the gel fluorographed. In experiment (B) the ^{35}S-labelled protein in each assay was first determined, then appropriate volumes of each were taken to analyse equal amounts of labelled protein by gel electrophoresis, and the resulting fluorogram scanned to determine the percentage of full-length product (panel C) defined as the density of the upper band relative to the sum of the densities of all bands in that track.

without any obvious accumulation of shorter RNA species, yet the short translation products were synthesised as much in the early period as later (Fig. 4). The stability of the NP mRNA was not significantly affected by the presence of the initiation inhibitor edeine (Fig 3), which implies that protection of the mRNA by translating ribosomes is not a major factor determining the mRNA stability.

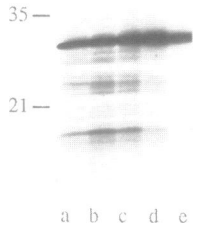

Figure 2. Products of translation of different concentrations of influenza virus NS mRNA. Capped NS mRNA was translated for 45 min at the following final RNA concentrations: (a) 126 μg/ml, (b) 63 μg/ml, (c) 32 μg/ml, (d) 16 μg/ml, and (e) 8 μg/ml. Equal volumes of each assay were analysed by gel electrophoresis using a 20% polyacrylamide gel. The lower portion of the resulting fluorogram is shown.

Origin of the short products

Inspection of the nucleotide sequences of these mRNAs shows that all the short products visible on these gels (i.e. larger than 15 kDa) must be encoded by the same reading frame as the full-length product. As the short products do not arise by proteolytic processing of the full-length product, they must arise either by premature termination of translation initiated at the correct site, or by initiation at incorrect internal sites. If the first of these possibilities is correct the short products should share common N-termini, whereas the alternative explanation predicts common C-termini.

One approach to distinguish between these alternatives was to linearise the DNA

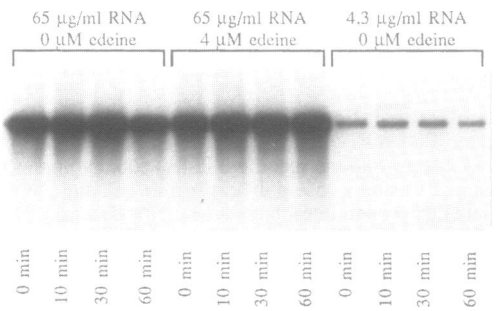

Figure 3. Stability of the influenza virus NP mRNA in the reticulocyte lysate. Capped ^{32}P-labelled NP mRNA was added at a final concentration of either 65 μg/ml or 4.3 μg/ml to standard translation assay reactions lacking [^{35}S]methionine but containing 4 μM edeine where indicated. At the times shown, 3 μl samples were removed for RNA isolation by phenol extraction. The RNA samples were glyoxalated before analysis by gel electrophoresis, and the gel autoradiographed.

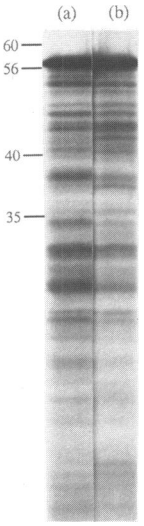

Figure 4. A comparison of the products translated from NP mRNA at early and late times. Capped NP mRNA was translated for 90 min under standard conditions except that the [^{35}S]methionine was added after either (a) 0 min, or (b) 30 min incubation, and 300 μM unlabelled methionine was added at (a) 5 min, or (b) 35 min. Samples of each assay were analysed by gel electrophoresis using a 15% polyacrylamide gel.

template at sites nearer the promoter, and thus generate mRNAs missing various lengths of the 3'-proximal sequences. If all the short products arise from premature termination of translation initiated at the same site, we would expect the same set of short products from translation of the truncated transcripts as from full-length. The results of such an experiment shown in Fig. 5 (left hand three tracks of panel B) indicate that the majority of the different short products translated from PB2 mRNA result from premature termination as most bands correspond closely between the truncated and full-length RNAs. However, a few of the short products of full-length RNA translation have no counterpart amongst the products translated from the truncated transcripts (and *vice-versa*), and these must be presumed to arise from initiation at internal sites. A very similar result was obtained when full-length and truncated forms of PB1 mRNA were translated (data not shown).

Another approach to this problem of identifying the origin of the short products was the use of antibodies raised against different segments of the PB2 polypeptide chain (6) to immunoprecipitate the products of translation of full-length or truncated PB2 mRNA (Fig. 5). It can be seen that there is a series of incomplete products larger than 35 kDa which are immunoprecipitated by the antibody against the extreme N-terminal segment, as well as by antibodies against the central region of the PB2 polypeptide (A2 and A3), but not by antibodies against C-terminal determinants. These products therefore share common N-terminal sequences with the full-length product, and most probably arise by premature termination of translation. On the other hand there are some incomplete products (highlighted by arrows in Fig. 5, panel B) that are immunoprecipitated by antibodies against C-terminal segments, but not by those raised against N-terminal or central segments. These products share common C-terminal

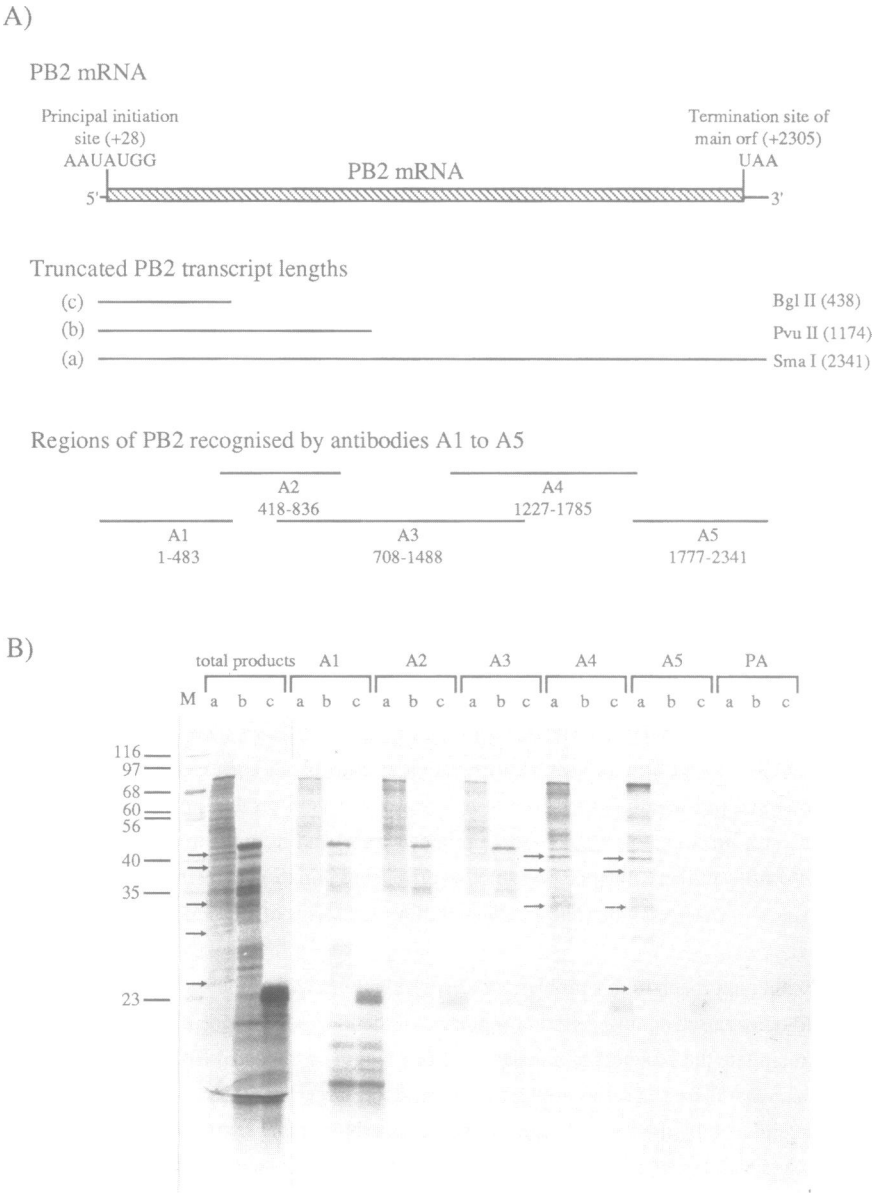

Figure 5. Immunoprecipitation of the products of translation of PB2 mRNA. Capped PB2 mRNA was prepared in (a) full-length form (2341 nt.) using plasmid DNA linearised by digestion with Sma I, or in two different truncated forms by linearising with either (b) Pvu II (giving a 1174 nt. RNA) or (c) Bgl II (438 nt. RNA) prior to transcription, as illustrated in panel (A). These mRNAs were translated under standard conditions for 30 min at final RNA concentrations of (a) 69 μg/ml. or (b) and (c) 46 μg/ml. Equal volumes of each

sequences with the full-length protein, and must have arisen by initiation at internal sites. Therefore this experiment again suggests that the majority of the incomplete products are the result of premature termination of translation initiated at the correct site, but some originate from initiation at internal sites. In a similar experiment Brierley *et al.* noted that the majority of incomplete products were precipitated with antibodies against C-terminal determinants rather than by those against N-terminal segments (6), which indicates that initiation at internal sites was the main cause of short product synthesis in their hands. Their experiments used precisely the same batch of reticulocyte lysate as was used for most of the present work, and very similar assay conditions, except for a slightly different mRNA sequence, and, possibly the most significant, a lower mRNA concentration (6).

Parameters affecting the relative synthesis of short products

We investigated the effect of various parameters on the ratio of synthesis of short peptides to full-length product at high RNA concentration, with a view to maximising expression of the complete coding sequence. When the concentration of added KCl was varied, maximum overall incorporation occurred at 90-110 mM added KCl for all mRNAs tested, but the relative synthesis of full-length products (the signal to noise ratio) was better at higher KCl concentrations in the range 120-140 mM (Fig. 6). This suppression of short product synthesis by supra-optimal salt was more evident with KCl than with KOAc (data not shown).

The translation of capped and uncapped transcripts was compared, together with the influence of cap analogue, m^7GTP, on the translation of capped mRNAs. With the two mRNAs examined, PB1 and NP mRNAs, translation of the capped transcript in the presence of cap analogue yielded a product pattern very similar to that obtained using the uncapped form of the mRNA (Fig. 7). The absence of a 5'-cap, or the presence of m^7GTP, reduced the yield of labelled products by about 4-fold in the case of NP mRNA, and even more so with other mRNAs such as NS (data not shown). However, not all the products were equally reduced in yield. Synthesis of the full-length product and some of the short products was strongly inhibited, whilst the yield of other short products was reduced much less or not at all. It is tempting to speculate that the former class of highly cap-dependent short products are principally those that arise by premature termination of translation initiated at the correct site, whilst the other class of less cap-dependent or entirely cap-independent products are those

translation assay were analysed by gel electrophoresis using a 20% polyacrylamide gel, as were the products of immunoprecipitation of each of the three assays by each of the five antisera preparations (A1 - A5) directed against different regions of the PB2 protein, and a non-immune serum (PA) directed against the unrelated influenza PA protein. The fluorograph of this gel is shown in (B), where the designations of the tracks corresponds to translations of the three types of mRNA preparation (a), (b) and (c) depicted in panel (A). Panel (A) also shows the segments of the PB2 mRNA encoding the antigens used to prepare the antisera A1 - A5 (6). Arrows indicate a representative set of products that (i) were precipitated by A5 (and the larger of them also by A4), but not by any other antibodies, and also (ii) were found amongst the translation products of full-length mRNA but not the truncated transcripts. These products are considered to arise by initiation at internal sites.

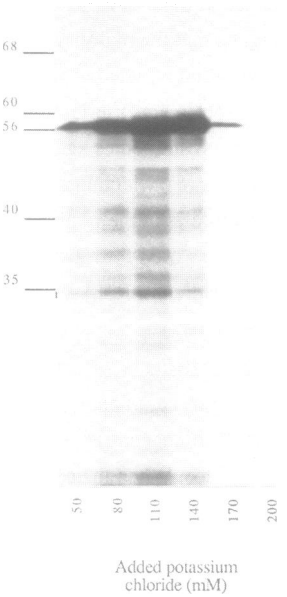

Figure 6. Influence of KCl concentration on the products of translation of influenza virus NP mRNA. Capped NP mRNA was translated at a final concentration of 40 μg/ml RNA for 45 min with added KCl at the final concentrations shown. Equal volumes of each assay were analysed using a 15% polyacrylamide gel, which was autoradiographed.

originating from internal initiation. Certainly, the translation of PB1 mRNA yields two prominent products in the range 60-65 kDa, whose synthesis is entirely cap-independent (Fig. 7), and has all the hallmarks of internally initiated proteins.

Most of the other parameters tested had at most a marginal influence on the signal to noise ratio. The addition of extra calf liver tRNA, over and above the 45 μg/ml that is standard in all our assays, did not increase the relative yield of full-length products. High concentrations (0.5 mg/ml) of *E. coli* rRNA, a mixture of 23S and 16S rRNA purified by sucrose density gradient centrifugation to remove contaminants that are inhibitory to protein synthesis, slightly decreased the signal to noise ratio. Perhaps not surprisingly, the addition of a translatable RNA, for example capped β-globin mRNA produced by *in vitro* transcription, decreased the yield of full-length influenza virus products and increased the yield of incomplete products. As the *in vitro* synthesised mRNAs used in these experiments would be contaminated with the plasmid DNA template, we tested whether the addition of linearised plasmid DNA affected the signal to noise ratio, but found no influence up to 100 μg/ml final concentration.

Addition of L-cell extract improves the fidelity of translation

The findings reported above suggest that high concentrations of mRNA out-titre the capacity of some endogenous reticulocyte factors that are critical for the fidelity of translation.

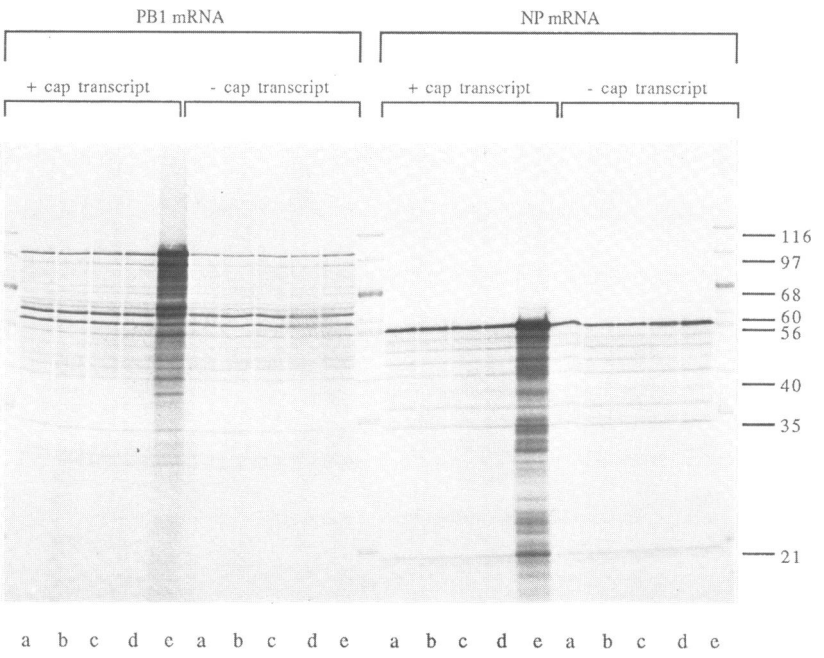

Figure 7. Cap-dependence of synthesis of incomplete products of translation of PB1 and NP mRNAs. Capped or uncapped PB1 mRNA (left panel) or NP mRNA (right panel) were translated for 80 min at a final RNA concentration of 85 μg/ml in each case, and with m^7GTP present at the following final concentrations: (a) 1.0 mM, (b) 0.75 mM, (c) 0.5 mM, (d) 0.25 mM and (e) none. Equal volumes of each assay were analysed using a 15% polyacrylamide gel, which was fluorographed.

This raises the question of whether such factors might be more abundant or more active in cell-free extracts from other sources. To investigate this we examined the effect of supplementing the reticulocyte lysate with postmitochondrial supernatant (S10) from L-cells, which we have found to be a more active system than the HeLa cell S10 (10). When L-cell S10 was added to the reticulocyte lysate, overall [^{35}S]methionine incorporation was depressed. This, however, can be entirely explained by the increase in the pool of unlabelled methionine contributed by the L-cell extract: in the mixed system the methionine pool is approximately four times higher than in the reticulocyte lysate alone (R.M. Lane and R.J.J., unpublished observations). Apart from this decrease in incorporation, the presence of the L-cell extract caused a small but reproducible improvement in the signal to noise ratio at high RNA concentrations (Fig. 8). Densitometry of the fluorogram shown in Fig. 8 revealed that with mRNA at 12 μg/ml the full-length product represented just under 50% of the total incorporation, irrespective of the presence or absence of L-cell extract. However, when the RNA concentration was raised to 60 μg/ml , the full-length product was only 22% of total incorporation in the unsupplemented reticulocyte lysate, but 36% in the mixed system. The five-fold increase in mRNA concentration elicited only a 2.1-fold

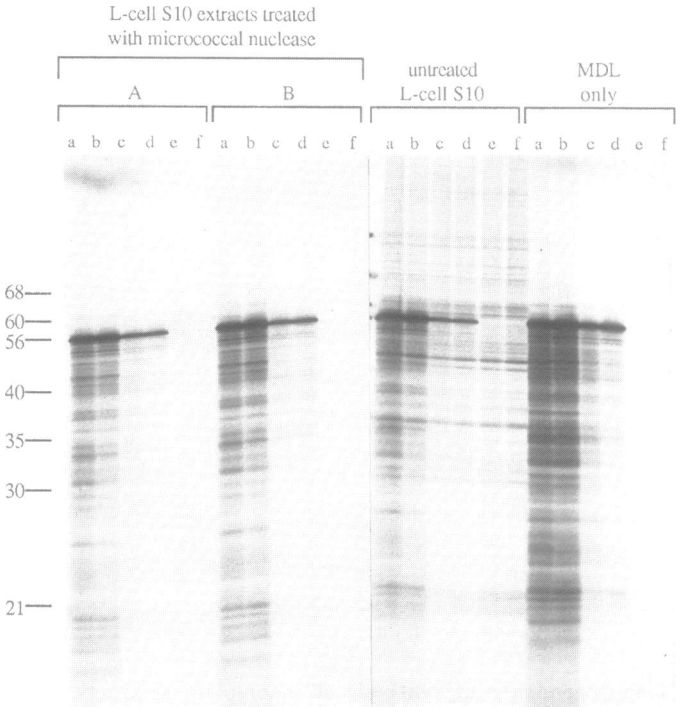

Figure 8. Addition of L-cell S10 increases the fidelity of translation of NP mRNA. Aliquots of L-cell S10 were treated with micrococcal nuclease (A) as described in Materials and Methods, or (B) with the modification of using 0.5 mM $CaCl_2$ and an incubation time of 60 min at 14°C. After treatment, these samples and the untreated L-cell S10 were each supplemented with 60 μg/ml calf-liver tRNA, and each was mixed with reticulocyte nuclease-treated message-dependent lysate (MDL) in a ratio of 1 vol. L-cell extract to 3 vol. MDL. Translation assays were carried out using these mixtures or unsupplemented MDL exactly as described in Materials and Methods, with either (a,b) 60 μg/ml capped NP mRNA, or (c,d) 12 μg/ml capped NP mRNA, or (e,f) no added mRNA. Samples were taken afer 45 min (a,c,e) or 90 min (b,d,f) incubation, and analysed using a 15% polyacrylamide gel.

increase in the yield of the full-length product in the absence of L-cell extract, but a 3.2-fold increase when it was present. The yield of all the short products seemed to be decreased to the same extent by the addition of L-cell extract (Fig. 8), which implies that the L-cell extract not only inhibits initiation at incorrect, internal sites, but also suppresses premature termination of translation.

DISCUSSION

The results presented here show that when a single species of RNA is translated in the reticulocyte lysate, increasing concentrations of RNA lead to a decrease in the signal to noise

ratio, where 'signal' refers to the yield of the full-length product and 'noise' to the yield of incomplete products. Low RNA concentrations give the expected product with very little noise. As the RNA level is increased there is a greater increase in the yield of short products than full-length product, and ultimately, at very high RNA concentrations, the absolute yield of full-length product falls. The short products fall into two classes: (i) proteins whose synthesis is initiated at the correct site but terminates prematurely, and (ii) proteins whose synthesis is initiated at incorrect (internal) sites.

Although it is commonly assumed that partial degradation of the mRNA by nucleases is the cause of incomplete product synthesis, our results show that there is very little mRNA degradation during the assay, certainly not enough to be the sole explanation for the high yield of short products seen at high RNA concentrations. It has long been known that the reticulocyte lysate contains significant RN'asin (ribonuclease inhibitor) activity (14), as we have confirmed for our lysate preparations in previous work on the translation of mRNAs produced by vaccinia virus core particles (15). Moreover, if degradation of the RNA during the assay were the cause of short product synthesis we would expect the signal to noise ratio to be worse at low RNA concentrations, when a higher proportion of the input RNA should be degraded. This is precisely the converse of what was actually observed. In addition we would expect that the addition of high concentrations of an inert RNA, such as *E. coli* rRNA, should improve the signal to noise ratio by providing an alternative substrate for the putative ribonuclease, yet our results were precisely the opposite. In the one instance when we have knowingly encountered a ribonuclease problem, as a contaminant of a particular preparation of papaya mosaic virus RNA, the signal to noise ratio, though atypically low at all RNA concentrations, actually increased with increasing RNA levels, as predicted, whilst uncontaminated preparations showed the decreasing signal to noise ratio recorded here (R.J.J., unpublished observations).

The decrease in fidelity of translation with increasing mRNA concentration was seen with all the mRNAs tested, although some species, such as the NS mRNA, seem less prone to this phenomenon than others. In addition, it is possible that the contributions of each of the two classes of incomplete products defined above may differ with different mRNAs. For example, the short products translated from NS mRNA seem to be mainly those which arise from internal initiation, since their abundance increases as the 'strength' of the authentic initiation site is decreased (manuscript in preparation), either by changing the AUG initiation codon, or by mutating the local nucleotide sequence context to a less favourable context as defined by Kozak (16,17).

A similar phenomenon of a decrease in the signal to noise ratio with increasing RNA concentration has been routinely observed in our standard calibrations of nuclease-treated lysate preparations using TMV RNA (2), as well as with other capped viral RNAs. Although virion RNA preparations, especially those from plant viruses, always contain some fragmented RNA (18), which could contribute to the synthesis of short products, this does not provide an

adequate explanation for the fact that the signal to noise ratio decreased with increasing RNA concentrations. A more extreme example is seen with poliovirus RNA translation where maximum yield of products initiated at the authentic site is seen at the very low concentration of 5-10 μg/ml; increasing the RNA concentration above this level leads to a drastic decrease in the yield of authentic products, and a sharp increase in incorrect products, most of which arise by initiation at internal sites located in the 3'-region of the RNA (19-21). In this case, too, the addition of HeLa cell or L-cell S10 markedly improves the signal to noise ratio (19-21). On the other hand, another picornavirus RNA, encephalomyocarditis virus RNA, is translated very efficiently and accurately in the reticulocyte lysate system (unsupplemented with L-cell S10), with few incorrect products even at RNA concentrations as high as 50 μg/ml (11,20). Our conclusion is that all RNA species may be prone to inaccurate translation at high RNA concentrations to a certain extent, but differences between various RNA species are seen in (a) the magnitude of the effect, (b) the critical RNA concentration at which incorrect product synthesis becomes significant, and (c) the relative contribution of the two classes of incorrect products. In addition, we find that different batches of lysate show differences in the signal to noise ratio when tested with the same RNA species.

The synthesis of prematurely terminated products is puzzling, but it is important to emphasise that limiting tRNA is unlikely to be the cause. We have previously shown that the reticulocyte lysate needs to be supplemented with tRNA from a heterologous and less specialised source, e.g. calf liver, in order to translate non-erythroid mRNAs efficiently (1,2). In the case of tobacco mosaic virus (TMV) RNA translation, omission of calf liver tRNA causes the signal to noise ratio to decrease very sharply with increasing TMV RNA concentration, and at high levels (50 μg/ml) no full-length product is made within 60 min incubation (T. Hunt and R.J.J., unpublished observations). Whilst this decreasing signal to noise ratio seen when increasing concentrations of TMV RNA are translated in the absence of calf liver tRNA is superficially like the observations noted in this paper, there are important differences. In the presence of calf liver tRNA, the lag time before the first appearance of the major full-length TMV product is quite short (typically 15-20 min) and hardly varies with increasing TMV RNA concentration, whereas in its absence this lag time increases dramatically as the TMV RNA concentration is raised. However, in the present work, the time before the first appearance of full-length PB1 or PB2 protein was identical irrespective of whether low or high RNA concentrations of mRNA were used. Moreover, all lysates used in this work were supplemented with 45 μg/ml calf liver tRNA, and additional tRNA was found to have no influence on the signal to noise ratio, nor on the lag time before the first appearance of full-length product. Thus mRNA concentration appears to have no influence on the rate of elongation by those ribosomes that complete translation of the open-reading frame, but does seem to affect the probability that a given ribosome will stop elongation prematurely.

As for the incomplete products that arise from initiation at internal sites, at least some of these initiation events appear to be cap-independent as they are not inhibited by m^7GTP, and

they are synthesised in the same yield irrespective of whether a capped or uncapped transcript is assayed. Initiation events on fragmented RNA molecules would, of course, fall into this category, but the yield of some of the products in this class, for example the two products of ca. 60-65 kDa translated from PB2 mRNA (Fig. 7), seems too high for partial mRNA degradation to be the sole explanation for their appearance. We would also stress that some of these incorrrectly initiated products may arise not from cap-independent initiation events, but by the scanning ribosome (16,17) by-passing the authentic initiation site and using downstream AUG codons. Whilst the studies reported here cannot easily identify this sub-class of internally initiated products, we have developed another system which is more suitable to reveal such 'leaky' scanning. This consists of an influenza NS mRNA derivative in which the initiation site is reiterated to provide two closely separated in-frame AUG codons: the frequency of initiation at the second (downstream) of these AUG codons is not only dependent on the local sequence context of the upstream AUG codon (as predicted by the scanning ribosome model), but also increases with increasing mRNA concentration (in preparation). It therefore appears that increasing mRNA levels may lead to increasingly leaky scanning.

The general decrease in fidelity of translation with increasing RNA concentrations suggests that high RNA levels out-titre the capacity of endogenous reticulocyte factors that are critical for the accuracy of initiation and for sustained elongation. Attempts to identify these factors are in progress, concentrating on the approach suggested by this work that such factors may be more abundant or more active in L-cell extracts than in reticulocyte lysates.

From the strictly operational viewpoint, our results show that the reticulocyte lysate can achieve sufficiently high fidelity to be used with confidence to identify the translation products and initiation sites of an unknown mRNA, provided certain precautions are taken: low concentrations of capped mRNAs should be used, and KCl concentrations optimised not for maximum total incorporation but for maximum signal to noise ratio. Although doubts have recently been voiced as to the fidelity of the reticulocyte lysate system (3), only two cases of apparent infidelity have been cited, one of which gives too little information to allow independent evaluation (22). The other, concerning p53 mRNA translation, is very interesting, because the capped p53 mRNA isolated from SV80 cells was actually translated in the reticulocyte lysate with high fidelity to give only the expected full-length product (23). Low fidelity with many short products occurred only when uncapped mRNAs generated by *in vitro* transcription were studied (23). In this particular case, therefore, the fault clearly lies not in the translation system itself, but in the use of inappropriate RNA preparations. In another case where cell-free translation of *in vitro* synthesised transcripts gave many short products thought to arise by initiation at internal sites (24), it is again striking that uncapped mRNAs were used at fairly high concentration (50 μg/ml) in the presence of potassium acetate rather than KCl, precisely the conditions which we would advise against. Although we found only a slight decrease in the signal to noise ratio when KOAc was used in place of KCl for the translation of the capped transcripts used in this work, we will show elsewhere that with naturally uncapped

mRNAs, such as cowpea mosaic virus RNA, KOAc is highly permissive, and KCl restrictive to the synthesis of incorrect products initiated at internal sites (R.J.J., in preparation).

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