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Nucleic Acids Research, 1994, Vol. 22, No. 23 4963–4968

Translation of 2′-modified mRNA *in vitro* and *in vivo*

Helle Aurup+, Angela Siebert, Fritz Benseler, David Williams§ and Fritz Eckstein*
Max-Planck-Institut für experimentelle Medizin, Hermann-Rein Strasse 3, D-37075 Göttingen, Germany

Received August 17, 1994; Revised and Accepted October 24, 1994

ABSTRACT

2′-Fluoro- and 2′-amino-2′-deoxynucleoside triphosphates have been used for *in vitro* transcription of 2′-modified luciferase mRNA. The 2′-modified deoxynucleoside-containing transcripts were tested for the expression of luciferase in *X.Laevis* oocytes as well as in rabbit reticulocyte lysate. Only 2′-fluoro-2′-deoxyadenosine-modified mRNA gave rise to luciferase as shown by SDS gel as well as by enzyme activity measurements *in vivo* as well as *in vitro*. 2′-Fluoro-2′-deoxy-pyrimidine nucleoside-modified mRNA did not give rise to luciferase activity. However, they directed incorporation of ^{35}S-labeled methionine into peptide fragments in rabbit reticulocyte lysate indicating premature termination of translation. No or only extremely little of such incorporation could be detected with 2′-amino modified transcripts.

INTRODUCTION

There is considerable interest in transcripts stabilized against degradation by nucleases as they would be useful for *in vitro* translational systems such as continous flow systems or for *in vivo* injections (1). This approach depends, first on the availability of adequately modified transcripts which in turn requires that the respective nucleoside triphosphates are substrates for a RNA polymerase. Secondly, it is essential that the modifications do not interfere with the translational machinery. We have recently shown that the introduction of 2′-fluoro or 2′-aminopyrimidine nucleosides into the hammerhead ribozyme enhances its stability against nucleases by several orders of magnitude without interfering with catalytic efficiency (2–4). Thus the introduction of such groups into transcripts might be a promising approach for the preparation of mRNAs with the desired properties provided these modifications do not compromise the translational machinery. The preparation of such modified transcripts by RNA polymerases has now become possible with the demonstration that 2′-fluoro and the 2′-aminonucleoside triphosphates are substrates for the T7 RNA polymerase (5).

We have chosen to examine the effect of 2′-modification on the translation of *in vitro* capped and polyadenylated transcripts coding for luciferase into active enzyme in two eukaryotic systems, *in vitro* in rabbit reticulocyte lysate and *in vivo* in *X.laevis* oocytes. These systems were chosen as the mRNAs are known to be stable and thus the interpretation of the results would not be complicated by instability of the message but directly indicate the effect of the 2′-modification on translation.

MATERIALS AND METHODS

Materials

Unmodified nucleoside triphosphates (special quality for molecular biology, 100 mM, pH 7) and P^1-5′-(7-Methyl)-guanosine-P^3-5′-guanosine-triphosphate were purchased from Boehringer Mannheim. [α-^{32}P] GTP (specific activity ca. 3000 Ci/mmol), [^{35}S]-methionine (specific activity 1200 Ci/mmol at 10 mCi/ml) and [^{14}C] methylated proteins (molecular weight standards) were obtained from Amersham Buchler (Braunschweig, Germany). The plasmids pGEM-luc and pGEM-4Z were from Promega Biotec. DNase I (10 units/μl, RNase-free) was from Boehringer Mannheim and restriction endonuclease *Nde*I from New England Biolabs. The 2′-modified nucleoside triphosphates were prepared as described (5) except for the 2′-azido-2′-deoxyadenosine triphosphate. The 2′-azido-2′-deoxyadenosine was synthesised as described (6) and phosphorylated according to Ludwig (7,8). It was subsequently reduced to the 2′-amino derivative according to Saneyoshi *et al.* (9) and analyzed by HPLC and ^{31}P NMR spectroscopy (10). T7 RNA polymerase was isolated from an overproducing *E.coli* strain as described Aurup *et al.* (5).

Methods

Plasmid construction. Cloning procedures were carried out essentially as described (11). Construction of the luc vector (pHAluc) is shown in Figure 1. A 1.8 kb DNA fragment containing the *Photinus pyralis* luciferase cDNA sequence was obtained as the *Hin*dIII/*Sac*I fragment from pGEM-luc and ligated to the 2.7 kb *Hin*dIII/*Sac*I fragment of pGEM-4Z. The resulting vector (pGEM-luc4Z) contained the sense luciferase sequence downstream of the T7 promotor. A 35-nucleotide poly(A) tail fragment was ligated to the *Nde*I/*Sac*I site of pGEM-luc4Z. This plasmid is called pGEM-luc4ZA+.

*To whom correspondence should be addressed

Present addresses: +Statens Seruminstitut, Artillerivej, DK-2300 Copenhagen, Denmark and §Medical Research Council, Laboratory for Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

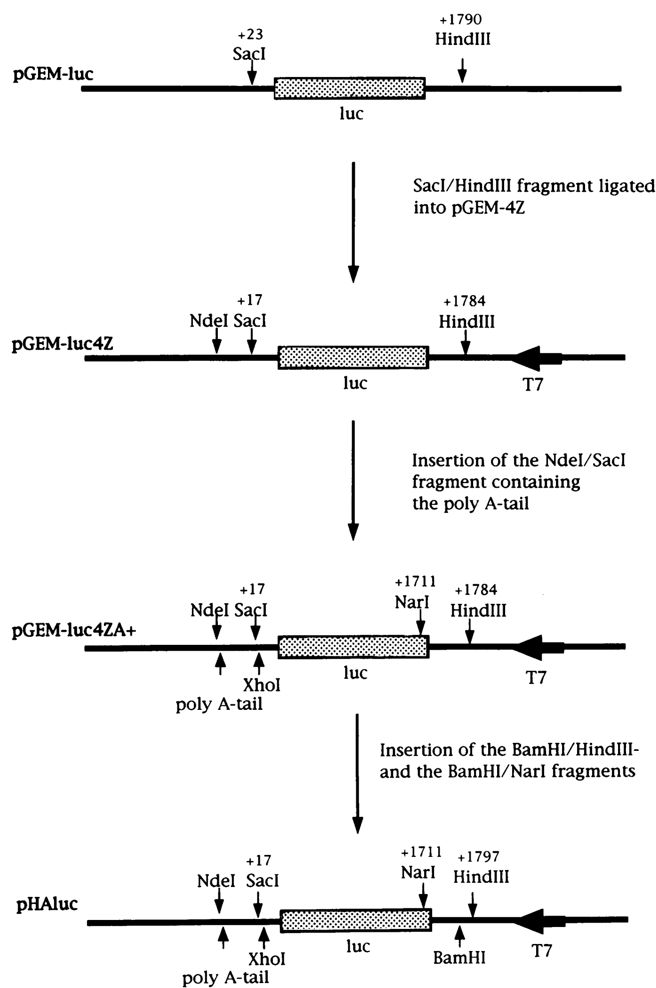

Figure 1. Construction of plasmid pHAluc. The *SacI/HindIII* luciferase cDNA fragment of pGEM-luc was ligated into the multiple cloning site of pGEM-4Z yielding pGEM-luc4Z. A poly(A)-tail was introduced by ligating the fragment; 5'TATG(T)$_{35}$CTGCAGGAGCT3' into the *NdeI/SacI* site of pGEM-luc4Z yielding the plasmid pGEM-luc4ZA$^+$. Finally two fragments, a *BamHI/HindIII* fragment: 5'-GATCCGGGCCCTCTAGATGCGGCCGCGAATTAATTCATT-CGA-3' and a *NarI/BamI* fragment: 5'-CGCCGGGCCTTTCTTTATGTTTTT-GGCGTCTTCCATTTTACCGTCGACG-3' were ligated into pGEM-luc4ZA$^+$ previously treated with *HindIII* and *NarI*. The resulting plasmid pHAluc contains the luciferase cDNA sequence under the control of the bacteriophage T7 promotor and optimized sequences for the 3'-downstream and 5'-upstream untranslated regions for the expression of luciferase.

The 5'-untranslated sequence upstream of the *luc* coding region was optimised for expression by excising the *HindIII/NarI* fragment of pGEM-luc4Z$^+$ containing two upstream start-codons and a non-optimal Kozak context in position −3 (12). An oligodeoxynucleotide of 50 base pairs, containing an internal *SalI* site and the original luciferase gene sequence AAA for position −1 to −3 (13), and a second oligodeoxynucleotide of 37 base pairs, with an internal *XmnI* site without upstream AUG sites were ligated into the *HindIII/NarI* site of pGEM-luc4ZA$^+$. This new plasmid is named pHAluc and includes in the 5'-untranslated region, 6 bases of luciferase cDNA sequences preceding the start codon as well as 47 bases of luciferase cDNA sequences downstream of the termination codon as part of the 3'-untranslated region (Fig. 2). pHAluc was used for *in vitro*

5'-m^{7}GpppGGAGACAAGCUUGAAUUAAUUCGGGCCGCAUCUAG AGGGCCCGGAUCCGUCGAC**GGUAAAAUG-{luciferase coding sequence}UAAAAUGUAACUGUAUUCAGCCGAUGACGAAA UUCUUAGCUAUUGUAAUCCUCCGAGG**CCUCGAGGUCGACGA AUUCGAGCUCCUGCAG(A)$_{35}$CAUA-3'

Figure 2. Primary sequences. Nucleotide sequences of the 5'- and 3'-untranslated region of the *in vitro* synthesized capped and polyadenylated *luc* mRNA transcripts. Bold letters indicate sequences which are identical to the original sequences from *Photinus pyralis* luciferase mRNA (13). The start and stop codons are underlined.

transcription of luciferase mRNA as described below. Templates used for transcription were prepared by linearizing pHAluc with *NdeI* by standard procedures (11).

The plasmid was isolated according to the Qiagen plasmid maxi preparation methods provided by Diagen (Düsseldorf, Germany), who also provided Centricon 100 filtration units (molecular weight cut off 10 kDa). The concentration of plasmid was determined by using 1 A_{260} unit equal to 50 μg of double-stranded DNA and the concentration of mRNA transcripts by using 1 A_{260} unit equal to 40 μg of mRNA (11).

In vitro transcription of capped and uncapped RNA. Capped mRNAs were produced by T7 *in vitro* transcription of linearized plasmid essentially as described to give rise to run-off (14). Transcription of *NdeI*-digested pHAluc DNA produces transcripts of 1838 nucleotides in total, with a 3'-noncoding sequence of 125 nucleotides including a poly(A) tail and a 5'-noncoding sequence of 61 nucleotides. The transcription reaction mixtures (total volume 50 μl) contained 40 mM Tris−HCl, pH 8.1, 3 μg linearized pHAluc, 1 mM spermidine, 8 mM $MgCl_2$, 10 mM DTT, 500 μM each of ATP, CTP and UTP, 100 μM GTP and 500 μM m^{7}G(5')ppp(5')G. For transcription reactions using analogues, UTP was substituted by either 3.5 mM 2'-F-UTP or 800 μM 2'-NH_2-UTP, CTP with either 3.5 mM 2'-F-CTP or 800 μM 2'-NH_2-CTP and ATP with either 3.5 mM 2'-F-ATP or 800 μM 2'-NH_2-ATP. To each sample was added 1000 units of T7 RNA polymerase. The samples were incubated at 37°C for 2 h. Reaction mixtures were then treated with 1 μl of DNase I for 15 min at 37°C, the samples were precipitated with ethanol/NaOAc, dried and dissolved in H_2O. A phenol−chloroform extraction of the samples was avoided as extensive loss to the phenol-phase of especially 2'-amino-modified transcripts has been observed. The samples were further purified by centricon 100 filtration at 1000×g for 30 min at RT. After washing three times with 2 ml of water, the yield was calculated by A_{260} measurements and the sample frozen immediately in liquid nitrogen and stored at −20°C until use. ^{32}P-labelled transcripts were prepared by adding 2 μl of [α-^{32}P]-GTP (final specific activity 1 Ci/mmol) to the transcription mixture. Quantitation of RNA yield was done spectrophotometrically. ^{32}P-labelled transcripts were analyzed by formaldehyde−agarose gel electrophoresis (11).

In vitro luciferase synthesis and gel analysis of protein production. The mRNAs were translated in a nuclease-treated rabbit reticulocyte lysate according to the recommendations of the supplier (Promega Biotec). The reaction conditions described below are from the supplier, optimized for the expression of luciferase from unmodified mRNA. The standard reaction

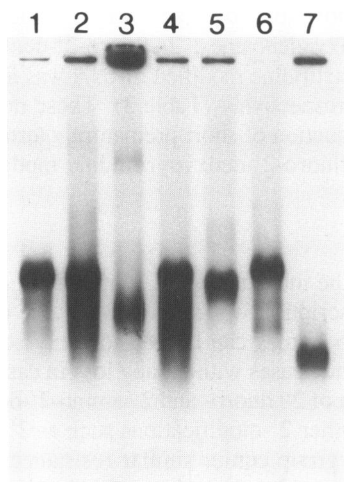

Figure 3. *In vitro* transcription of luciferase mRNA. Autoradiography of a dried agarose gel displaying [^{32}P]GMP-labelled uncapped transcripts using *Nde*I-linealized pHAluc as template. Lane 1, unmodified transcript; lane 2, 2′-F-AMP-modified transcript; lane 3, 2′-NH_2-AMP-modified transcripts; lane 4, 2′-F-CMP-modified transcript; lane 5, 2′-NH_2-CMP-modified transcript; lane 6, 2′-F-UMP-modified transcript; lane, 7, 2′-NH_2-UMP-modified transcript. Details are described in Materials and Methods.

mixture was incubated at 30°C for 2 h and contained in 50 μl: 10 mM creatine phosphate, 50 μg/ml creatine phosphokinase, 2 mM DTT, 50 μg/ml calf liver tRNA, 79 mM potassium acetate, 0.5 mM magnesium acetate, 0.02 mM hemin, 35 μl rabbit reticulocyte lysate and 1 μg mRNA. The transcripts were translated in the presence of 4 μl [^{35}S]methionine, and the translation products were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Transcripts were analysed for luciferase activity by measuring light emission as described below and for the incorporation of [^{35}S]methionine by trichloroacetic acid precipitation as described in the protocol provided by the supplier of the rabbit reticulocyte lysate (Promega Biotec).

Luciferase assay. Adult *Xenopus laevis* frogs were maintained and oocytes of stage 5/6 isolated as described (15,16). After cytoplasmic injection with 40–50 nl/oocyte of transcript solution (2 ng/50 nl) they were incubated in modified Barth medium at 18.5°C (18). Oocytes were homogenized in cell culture lysis buffer (Promega Biotec) and the extent of translation was determined by assaying aliquots for luciferase activity 48 h after injection of transcript, by addition of 100 μl of substrate solution (Promega Biotec)} to the homogenate. Luciferase activity was detected with a standard scintillation counter equipped with a chemiluminescence measurement program (LS6000LL Beckman). Samples were introduced immediately into the counting chamber and counted for 1 min. When luminescence intensity was over 3×10^6 c.p.m. samples were diluted in 1×cell culture lysis reagent and recounted. At each time point luciferase assays were performed in duplicate for each oocyte extract. They varied ± 15%. Data is expressed as c.p.m./oocyte.

RESULTS

The vector pHAluc, linearized with *Nde*I, directs the synthesis of luciferase mRNA of 1838 nucleotides in length. Transcripts were obtained in the presence of the 5′-triphosphates of 2′-fluoro-2′-deoxy- and 2′-amino-2′-deoxyuridine, -cytidine and -adenosine. Formaldehyde agarose gel electrophoretic analysis (Fig. 3) reveals that the transcripts obtained with the three 2′-fluoro-2′-deoxynucleoside triphosphates have the same mobility as that obtained with the natural triphosphate indicating that a full length transcript was obtained (lanes 2, 4, 6). However, the mobility of the transcripts obtained with the 2′-amino modified triphosphates is somewhat faster than that of the unmodified transcript (lanes 3, 5, 7). Interestingly, the mobilities are not identical for all three 2′-amino modified transcripts which one would expect if the amino group was responsible for this higher mobility. Thus, one has to conclude that these products are indeed shorter and, therefore, represent prematurely terminated material. However, it can not be excluded at present that the 2-amino groups interfere with the mobility in a sequence dependent manner. The yield of the modified transcripts was determined by UV absorption after purification of the products (Table 1). Except for the low yield of transcript obtained with the 2′-aminoadenosine triphosphate, the yields are comparable to that obtained with the normal triphosphates.

Table I. Yields of *in vitro* transcription of luciferase mRNA

Transcript	Transcription yields total (μg)
unmodified	10
2′-F-AMP-modified	7.5
2′-NH_2-AMP-modified	1.8
2′-F-CMP-modified	12
2′-NH_2-CMP-modified	12
2′-F-UMP-modified	12
2′-NH_2-UMP-modified	11

Amount of uncapped transcript determined by A_{260} absorption as described in Materials and Methods.

Table II. *In vivo* (*Xenopus laevis* oocytes) and *in vitro* (rabbit reticulocyte lysate) translation of unmodified- and 2′-F-AMP-modified capped mRNA

Transcripts	Luciferase activity[a] *in vivo*	*in vitro*
unmodified transcript	11156	128
2′-F-AMP-modified transcript	983	13

Yields of luciferase obtained *in vivo* at 48 h after injection of luciferase mRNA into *X.laevis* oocytes or obtained in a *in vitro* translation system after 2 h of incubation. The activity was measured as described in Materials and Methods. Background activity determined with 100 μl of substrate solution (between 10 000–30 000 c.p.m.) was subtracted from all sample values.
[a] $\times 10^6$ c.p.m./oocyte or $\times 10^6$ c.p.m./μl rabbit reticulocyte lysate.

The ability of 2′-modified mRNA to act as messenger for the expression of luciferase was tested in *X.laevis* oocytes and in an eukaryotic cell-free system from rabbit reticulocyte lysate. 2′-Fluoro-2′-deoxy-adenosine modified mRNA was successfully used as messengers for the expression of luciferase in both systems. After 48 h of incubation in *X.laevis oocytes* and after 2 h of incubation in rabbit reticulocyte lysate the activities of luciferase in both cases were ~10-fold lower than when the unmodified mRNA was used as messenger (Table 2). No detectable translation of enzymatically active luciferase was obtained when either of the two 2′-fluoro-2′-deoxypyrimidine-nucleotide modified mRNAs or the three 2′-amino-2′-deoxy-modified RNA were used as messengers *in vivo* or *in vitro*.

In order to check the integrity of the protein synthesised, samples from the *in vitro* translation were subjected to SDS–denaturing gel electrophoretic analysis (Fig. 4). The luciferase protein of 61 kDa is clearly visible when unmodified- and 2′-fluoro-2′-deoxy-adenosine modified mRNA are used as messengers. The luciferase protein is accompanied by a smaller number of premature terminated peptides in both cases.

When the 2′-fluoro-2′-deoxypyrimidine modified mRNAs or the 2′-amino-2′-deoxynucleotide modified RNAs are used as messengers no detectable premature terminated polypeptides could be detected within the range of 30–60 kDa (Fig. 4). In order to check whether any translation had occured, samples from the *in vitro* translation experiments were analysed for the incorporation of [^{35}S]-methionine into smaller peptide fragments by acid precipitation. In addition to the incorporation of [^{35}S]-methionine when the unmodified- and the 2′-fluoro-2′-deoxyadenosine modified mRNA are used as messenger, the 2′-fluoro-2′-deoxypyrimidine modified mRNAs stimulate the incorporation of [^{35}S]-methionine. No incorporation was detected when 2′-amino-2′-deoxynucleoside modified mRNAs were applied as messengers (Table 3). The relative incorporation of radioactive label when using 2′-fluoro-2′-deoxyadenosine-, 2′-fluoro-2′-deoxycytidine- and 2′-fluoro-2′-deoxyuridine modified mRNA was found to be 0.20, 0.10 and 0.12, respectively (Table 3). These numbers strongly indicate the production of short prematurely terminated peptides when using 2′-fluoro-2′-deoxypyrimidine modified mRNAs.

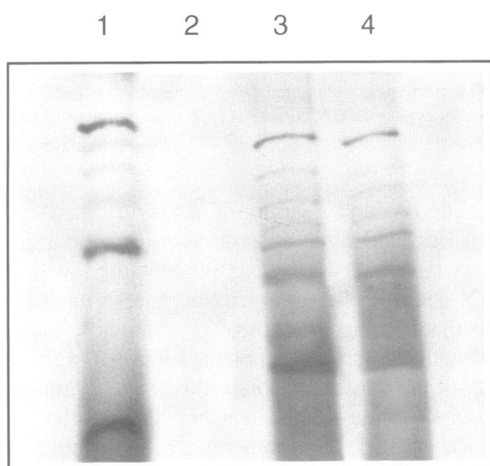

Figure 4. *In vitro* translation of unmodified and 2′-modified luciferase mRNA. Autoradiography of a dried 12.5% SDS–polyacrylamide gel displaying the synthesis of [^{35}S]methionine-labelled proteins and polypeptide fragments after incubation of unmodified- and 2′-modified mRNAs in a cell free system from rabbit reticulocyte lysate. Incubation with: Lane 1, Molecular weight markers, 69 kDa, 50 kDa, 30 kDa; lane 2, 2′-NH_2-AMP-modified RNA; lane 3, 2′-F-AMP-modified mRNA; lane 4, unmodified mRNA. Details as described in Materials and Methods.

DISCUSSION

Our interest in the introduction of 2′-modifications into mRNA by *in vitro* transcription was stimulated by the observation that hammerhead ribozymes can be considerably stabilized against degradation by nucleases without any loss in catalytic efficiency by incorporation of 2′-fluoro- and 2′-amino-2′-deoxynucleotides (2–4,19,20). Other 2′-modifications such as 2′-O-alkyl groups or the 2′-deoxy group confer similar resistance (21,22). It was thus tempting to explore whether mRNAs could be modified similarly in order to stabilize them against degradation, and whether these modifications might affect the efficiency of translation. One pre-condition for such an approach is that the modified nucleoside triphosphates must be substrates for a template-dependent RNA polymerase. Indeed, 2′-fluoro and 2′-aminonucleoside triphosphates are substrates for T7 RNA polymerase and can be used for the preparation of modified transcripts (5). 2′-Deoxynucleoside triphosphates can not fully substitute for the ribonucleoside triphosphates in reactions catalyzed by this enzyme except possibly to a small extent (Aurup and Eckstein, unpublished). No report has been published in which the substrate properties of 2′-O-alkyl nucleoside triphosphates with a RNA polymerase has been described.

2′-Modified polynucleotides have been tested before as templates in translation (23,24). However, these templates were all prepared by polymerisation of the nucleoside diphosphates with polynucleotide phosphorylase, an enzyme which does not require a template. As a result these polynucleotides did not code for a particular protein but only specified the polymerisation of certain amino acids. This precluded any analysis of the production of a full length and active protein.

We have used 2′-fluoro- and 2′-aminonucleoside-modified transcripts of the luciferase gene to assay the effects of the modifications on the translational system in two eukaryotic systems, *X.Laevis* oocytes and a rabbit reticulocyte lysate. There is ample evidence that capped and polyadenylated mRNAs and transcripts are very stable in both these systems (25,26). This allows to correlate the expression of luciferase directly with the efficiency of translation of the modified transcripts without interference from differences in stability. The luciferase gene was

Table III. Incorporation of ^{35}S-methionine using unmodified- and 2′-modified capped transcripts as messengers in the *in vitro* translation system from rabbit reticulocyte lysate

Messenger RNA	Incorporation of ^{35}S-methionine (c.p.m./μl lysate)	Relative incorporation
unmodifed mRNA	22500	1
2′-F-AMP-modified mRNA	4500	0.2
2′-NH_2-AMP-modified mRNA	bg	bg
2′-F-CMP-modifed mRNA	2200	0.1
2′-NH_2-CMP-modified mRNA	800	0.04
2′-F-UMP-modified mRNA	2700	0.12
2′-NH_2-UMP-modified	bg	bg

Background translation of 1500 c.p.m./μl lysate was subtracted from all values. bg, only background values. The incorporation of ^{35}S-methionine was determined as described in Materials and Methods.

chosen for translation as the enzyme can easily be assayed and therefore facilitates the quantitation of the translational efficiency.

When the *Nde*I-cut plasmid pHAluc is used as template for T7 RNA polymerase-catalysed transcription of luciferase RNA with 2′-fluoro-2′-deoxyadenosine- and 2′-fluoro-2′-deoxypyrimidine modified substrates, transcripts were obtained in yields comparable to that of the normal transcript. Only the yield of 2′-amino-2′-deoxyadenosine modified RNA was decreased 4-fold (Fig. 3). When the three 2′-amino-2′-deoxynucleoside triphosphates were used, transcription products were obtained which ran faster than the unmodified mRNA in the gel electrophoretic analysis. It is difficult to decide whether this represents shorter than full length transcript or a change in gel mobility caused by the modification. In general the 2′-amino modification decreases the stability of duplexes. 2′-Amino groups have been shown to decrease the thermal stabilities of polynucleotides (27,28) and of oligonucleotide duplexes (29−31). Decreased thermal stability between the RNA transcript and the DNA template has been identified as a factor for termination of transcription (32−37). It is therefore conceivable that the 2′-amino modification can cause premature termination by weakening of the template/RNA interaction. If it did, this pre-termination is sequence dependent as the length of the transcripts varies for the three analogues examined. However, an affect on gel mobility by this modification can not be excluded at this time.

The translational efficiency of the 2′-modified transcripts differed greatly. Only the transcript containing 2′-fluoroadenosine supported the synthesis of luciferase as monitored by an activity assay. In both systems, the *X.Laevis* oocytes and the rabbit reticulocyte lysate, the amount was 10% in comparison to that obtained with the unmodified transcript. The results seen with the activity assay were confirmed by the analysis of the protein in a SDS gel (Fig. 4). Again, only the 2′-fluoroadenosine transcript gave rise to the full length protein. Measurement of ^{35}S-incorporation in a precipitation assay indicated that the amount of protein synthesized with the 2′-fluorouridine and -cytidine transcripts was about half of that seen with the 2′-fluoroadenosine, i.e. 10% of that obtained with the unmodified transcript (Table 3). These proteins must be fragments which do not give rise to luciferase activity and therefore must be prematurely terminated.

These results indicate that the presence of 2′-fluoro and 2′-aminopyrimidine nucleosides in the transcript as well as that of 2′-aminoadenosine strongly interfere with translation whereas 2′-fluoroadenosine is reasonably well tolerated. Apparently the latter does not severely interfere with the function of the polyA tail. The translational efficiency we observe here is less than that seen by Fukui *et al.* (24) who followed the polymerisation of ^{14}C-lysine with poly(2′-fluoro-2′-deoxyadenylic acid) as template in a prokaryotic translational system. However, their template was not homogeneous in size and therefore full length protein could not be assayed for by gel analysis, nor could this protein be assayed for activity.

Why the other modified transcripts were not efficient templates beyond the production of short peptide fragments as seen in the ^{35}S precipitation assay, can not be answered with certainty at present. The decrease in thermal stability of duplexes by the 2′-amino modification as discussed above might also be the cause for the inability for such transcripts to serve as templates for translation. This destabilization might interfere with the interaction of mRNA and ribosomal RNA in the initiation stage or even with the tRNA anticodon. Conversely, the 2′-fluoro modification tends to enhance thermal stability of polynucleotide and oligonucleotide duplexes (31,37−40). This might facilitate secondary structures which interfere with the translocation of the mRNA on the ribosome or with the secondary structure which prevents the scanning process of initiation of translation in the 5′-untranslated region. Strong secondary structures in the 5′-untranslated region of *in vitro* transcribed RNA are known to abolish translation in *X.laevis* oocytes and in an *E.coli* cell-free translation system (41,42).

In summary, it is shown that 2′-fluoro-2′-deoxyadenosine modified RNA can function as messenger for the expression of luciferase *in vitro* as well as *in vivo*. No expression of luciferase was detected, however, when 2-amino-2′-deoxynucleoside modified mRNAs or 2′-fluoro-2′-deoxypyrimidine nucleoside modified mRNAs were used as messenger.

ACKNOWLEDGEMENTS

We thank S.Sigurdsson, J.Thomson and T.Tuschl for careful reading of the manuscript. Financial support by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie is acknowledged.

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