mRNA acetylated at 2'-OH-groups of ribose residues is functionally active in the cell-free translation system from wheat embryos

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Modification (acetylation) of 2'-OH groups of mRNA ribose residues does not result in a loss of their template activity in a cell-free translation system from wheat embryos and in some cases activates inactive mRNAs. Modification of 70–75% of 2'-OH groups makes mRNA stable to the effect of pancreatic ribonuclease.

Template ribonucleic acid; Chemical modification; Translation, in vitro

1. INTRODUCTION

Individual mRNAs have recently become widely accessible due to the development of methods of in vitro transcription of any cloned DNAs in transcription systems based on phage DNA-dependent RNA-polymerases and their cloned promoters [1,2]. Using these systems it is possible to obtain mRNAs encoding virtually any amino acid sequences in amounts sufficient to study and model in vitro different intracellular processes as well as to synthesize polypeptides in cell-free translation systems. In this connection the problem of mRNA protection against degradation by RNAses acquires particular importance. For example, under conditions of continuous translation in vitro [3] mRNA is effectively protected by ribosomes from nuclease attack; none the less, ribonuclease inhibitors are required at the initial stages of the process.

On the other hand, it has been shown before that treatment of tRNA in water solution with acetic anhydride results in acetylation of ribose 2'-oxy groups without affecting amino groups of nucleotide bases [4,5]. Such a modification is not accompanied by degradation of the polynucleotide chain, but even at a comparatively low level of modification (25–30%) tRNA loses its acceptor function as a result of complete breakdown of the secondary structure [5] and, what is especially important, the acetylated tRNA acquires a high stability against pancreatic ribonuclease. Such a modification could be used for stabilization of mRNA as the mRNA secondary structure is not essential for translation.

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The effect of acetylation of 2'-OH-groups of RNA ribose residues on its template activity is studied in a translation system from wheat embryos with the aim to increase the stability of mRNA to ribonuclease.

2. MATERIALS AND METHODS

Restrictase *Bam*HI, ribonuclease inhibitor from human placenta, DNA-dependent RNA-polymerase of phage SP-6, [^{3}H]leucine (56 Ci/mmol), and [^{3}H]phenylalanine (36 Ci/mmol) were from Amersham (UK). Creatine phosphate, creatine phosphokinase, ribonucleoside triphosphates, leupeptin, aprotinin, pepstatin, chymostatin were from Sigma (USA). Salts, dimethylformamide, acetic anhydride were from Merck (FRG). Plasmids containing SP-6 polymerase promoter with incorporated genes of calcitonin, atrially natriuretic factor and calcitonin precursor were kindly provided by Dr K.G. Skryabin (Institute of Molecular Biology, Academy of Sciences of the USSR).

2.1. *Preparation of mRNA*

Plasmids containing genes of calcitonin, atrially natriuretic factor and calcitonin precursor were linearized by restrictase *Bam*HI and transcribed using SP-6 phage polymerase according to [6]. RNA obtained after the reaction was separated from free ribonucleoside triphosphatases by precipitation in 2 M LiCl.

2.2. *RNA acetylation*

Acetylation of 2'-OH-groups of RNA ribose residues was carried out according to [7]. Five μl of dimethylformamide and 8 μl of acetic anhydride were added to 100 μl of 1 M sodium acetate, pH 7.0, containing 20–30 μg of RNA. The mixture was stirred and incubated for 2–3 h at room temperature. pH was maintained by adding 1 N NaOH solution to the reaction mixture. Upon completion of the reaction RNA was precipitated with 3 vols. of alcohol. The precipitate was collected by centrifugation, washed with 70% alcohol and dissolved in water to a concentration of 100–500 μg/ml.

2.3. *Determination of the degree of RNA acetylation*

The degree of acetylation of 2'-OH-groups of RNA ribose residues was determined using the hydroxam reaction [5]. One hundred μl of 0.4 M hydroxylamine sulfate and 50 μl of 1 N NaOH were added to 100 μl of the acetylated RNA solution. The solution was stirred, incubated for 10 min at room temperature and 50 μl of 0.1 N $Fe_2(SO_4)_3$

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MET LEU ARG ARG SER SER
mRNA ANF1 5′GAAUACAGGAAUUCU AUG UUG CGU AGA UCU AGU ...

MET SER LEU ARG ARG SER
mRNA ANF2 5′GAAUACAGGAAUUCU AUG UCU UUG CGU AGA UCU ...

Fig. 1. Differences in the encoding parts of ANF-1 and ANF-2 mRNA.

in 2 N H_2SO_4 were added. The optical density of the Fe^{3+}-hydroxomat complex was measured at 535 nm, assuming the molar extinction coefficient of acetylhydroxomat to be 509,000 [5].

2.4. *mRNA translation*

Translation of normal and modified RNAs was carried out in a cell-free translation system from wheat embryos prepared according to [8]. The amount of synthesized polypeptide was determined from the amount of TCA-precipitated [^{3}H]leucine. The analysis of the reaction products was performed using gel electrophoresis according to Laemmli [9].

3. RESULTS AND DISCUSSION

To study the effect of modification of 2′-OH-groups of ribose residues on the template activity of RNA we used a synthetic poly(U) polyribonucleotide 80 nucleotide bases long, two mRNAs encoding the atrially natriuretic factor (ANF-1 and ANF-2 mRNAs), one of which has an additional codon in the coding part (Fig. 1), human calcitonin mRNA (HCT mRNA) and calcitonin precursor mRNA (AluHCT mRNA). All the template RNAs obtained in the transcription system using the DNA-dependent phage SP-6 RNA-polymerase did not contain at the 5′-end the 'cap' structure which is usually present in eukaryotic mRNAs, and had a sequence of 9 nucleotide bases obtained at transcription of the SP-6 promoter.

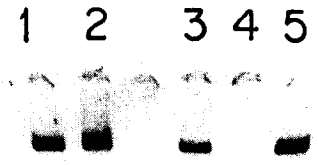

Fig. 2. Electrophoresis of modified and unmodified mRNA after pancreatic ribonuclease treatment. 1, AluHCT mRNA; 2, acetylated AluHCT mRNA; 3, AluHCT mRNA treated with 0.01 μg of pancreatic ribonuclease for 60 min at 25°C; 4, AluHCT mRNA + 1 μg of pancreatic ribonuclease; 5, acetylated AluHCT mRNA + 1 μg of ribonuclease.

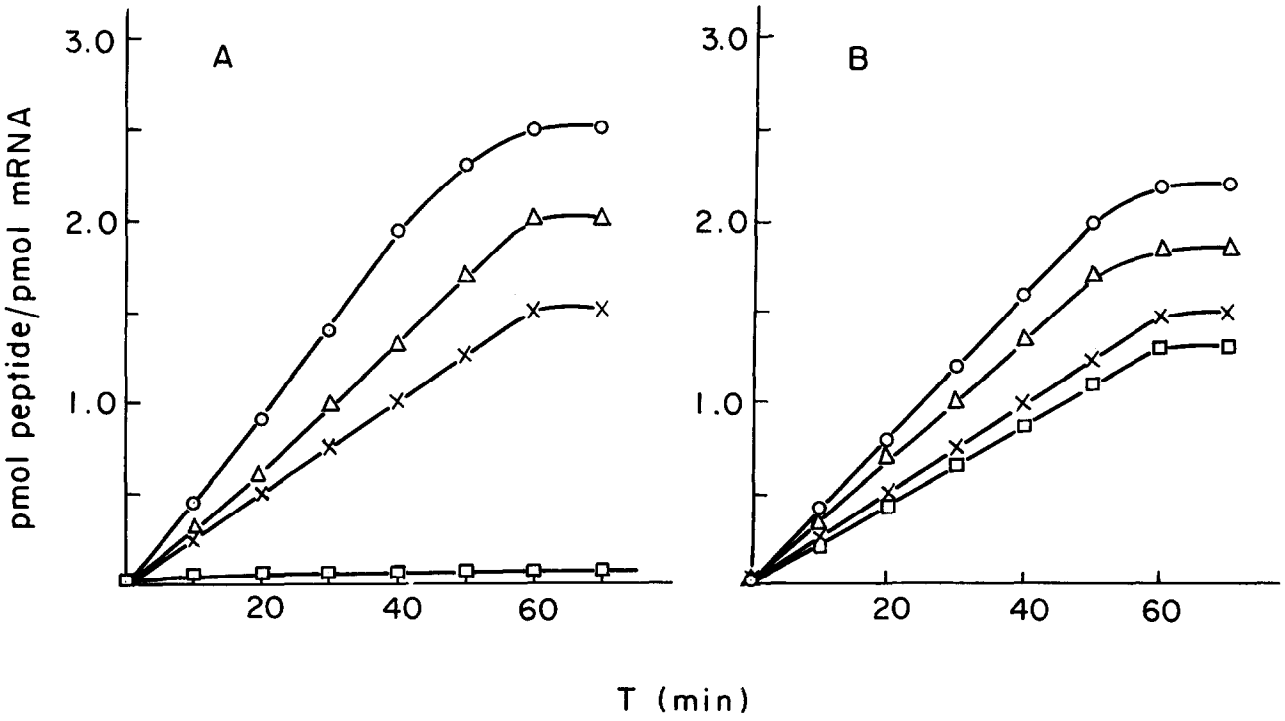

Fig. 3. Kinetics of synthesis of different polypeptides in a cell-free translation system from wheat embryos. A, unmodified mRNAs; B, acetylated mRNAs. (○), AluHCT mRNA; (△), HCT mRNA; (x), ANF-1 mRNA; (□), ANF-2 mRNA.

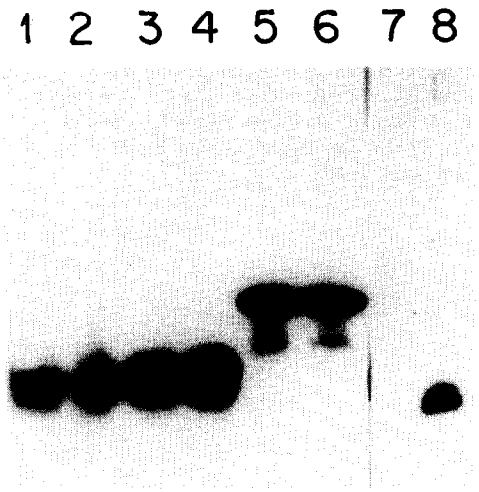

Fig. 4. Electrophoresis of translation products of different normal and modified mRNAs: 1, ANF-1; 2, acetylated ANF-1; 3, HCT; 4, acetylated HCT; 5, AluHCT; 6, acetylated AluHCT; 7, ANF-2; 8, acetylated ANF-2.

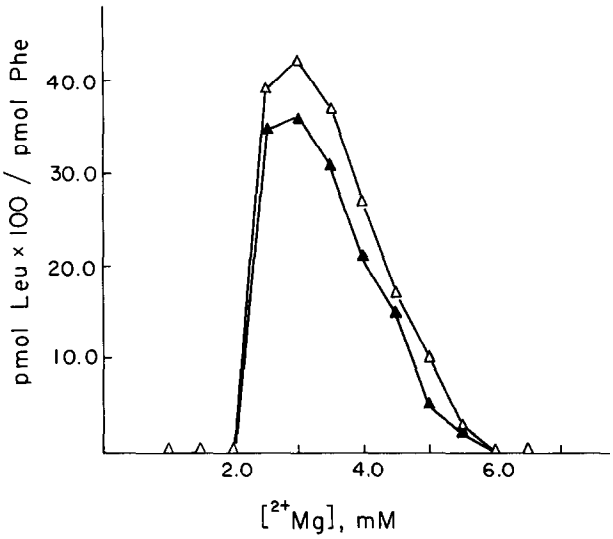

Fig. 5. Percentage of leucine incorporation upon translation of the normal (▲) and acetylated (△) poly(U).

2′-OH-groups were modified by treating mRNAs with acetic anhydride. Acetylation was carried out at pH 7 in 5% aqueous dimethylformamide to prevent side reactions of amino groups, thus ensuring homogeneity of the reaction mixture and a good reproducibility of the results of modification. Judging by the hydroxam reaction, 70–75% of all 2′-OH-groups of ribose residues in mRNA were acetylated; as a result, RNA acquired a high stability to pancreatic ribonuclease (Fig. 2) which requires free 2′-OH-groups for its activity.

The effect of acetylation on the functional activity of mRNAs was estimated from synthesis of encoded polypeptides in a cell-free system from wheat embryos. Translation was done at an optimum of potassium ion concentration of 75 mM which is consistent with the optima described earlier for uncapped mRNAs [10].

Fig. 3 represents the kinetics of synthesis of different polypeptides on normal and acetylated mRNAs. All the modified mRNAs studied in the system of wheat embryos are translated with an efficiency equal to, or close to that of normal mRNAs. An interesting effect of activation of template activity after modification of 2′-oxy groups of ribose residues has been observed for ANF-2 mRNA. As mentioned above, ANF-2 mRNA differs from ANF-1 mRNA by an additional codon (coding for Ser) which seems to promote the formation of the secondary structure, thus inactivating it in translation. However, as has been shown earlier for tRNA [9], acetylation of even 25–30% of RNA 2′-OH-groups leads to a complete breakdown of the secondary structure; for ANF-2 mRNA this results in its inactivation.

Analysis of the translation products by gel electrophoresis has shown (Fig. 4) that mRNAs always yield products whose molecular mass is in conformithy with that of the encoded polypeptides.

Knorre et al. studied the products of poly(A) and poly(U) acetylation [11] and noticed different capabilities of modified polyribonucleotides to stimulate binding of aminoacyl-tRNA with ribosomes. Completely acetylated poly(A) is as efficient in binding Lys-tRNA as untreated polymers. At the same time, 88% acetylated poly(U) shows a considerable decrease of activity relative to Phe-tRNA. It could be assumed from these data that the degree of miscoding increases at translation of 2′-AcO-mRNA.

However, the experiments on leucine incorporation at translation of modified and unmodified poly(U) (Fig. 5) have shown that the degree of miscoding for acetylated poly(U) changes by about 5% whereas the range of magnesium ion concentrations stimulating this process remains the same.

Thus, it is shown that mRNA does not lose (in some cases even acquires) template activity at acetylation of 70–75% of 2′-OH-groups of ribose residues and can be effectively translated in the eukaryotic cell-free system from wheat embryos.

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