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Simple, efficient *in vitro* synthesis of capped RNA useful for direct expression of cloned eukaryotic genes

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ABSTRACT

A simple and efficient method for direct in vitro synthesis of capped transcripts of cloned eukaryotic genes is described. As an example capped transcripts were made from a plasmid containing the human fibroblast interferon gene cloned under the control of a prokaryotic promoter. These transcripts were translated in vivo in Xenopus laevis oocytes and in vitro in reticulocyte and in wheat germ cell-free protein synthesizing systems.

INTRODUCTION

It is well documented that efficient eukaryotic protein synthesis requires a message with a capped 5'-end (1) although a few exceptions are known (2). Translation of certain prokaryotic mRNAs in a wheat germ cell-free system also improves dramatically by providing a 5' cap structure (3). In vitro addition of a cap structure has been obtained by making use of the enzymatic capping activity from vaccinia virions (3,4). This procedure is cumbersome as it requires the purification of the capping enzymes and a separate capping reaction, and is furthermore not very efficient (40-60%). The preparation of capped transcripts of cloned eukaryotic genes, however, is a very desirable objective both for screening and for detailed characterization of cloned genes by direct expression. Translation of authentic, non-manipulated, eukaryotic genes in prokaryotic systems is rather improbable because of the very different mechanisms for initiation of protein synthesis and the relevant control signals involved (1, 5). Eukaryotic translation systems, however, such as those of Xenopus laevis oocytes, wheat germ extracts or reticulocyte lysates are known to translate a very wide variety of messages provided they are capped properly (6).

In this paper we describe a one-step reaction in which it is possible to synthesize µg quantities of capped cDNA transcripts by priming the RNA polymerase with pre-synthesized, commercially available cap structures. The transcription is under the control of the strong prokaryotic promoter PL from bacteriophage λ, enabling commercially available Escherichia coli

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RNA-polymerase to be used. Such capped messages from the human fibroblast interferon gene (7) produce biologically active human interferon after injection into Xenopus oocytes at a rate 50-fold higher than for non-capped messages.

MATERIALS AND METHODS

Enzymes and radioactive products

Escherichia coli RNA polymerase (E.C. 2.7.7.6) was obtained from Boehringer or New England Nuclear, T1 ribonuclease (E.C. 3.1.4.8) and U2 ribonuclease (E.C. 3.1.4.-) from Sankyo; pancreatic ribonuclease (E.C. 3.1.4.22) and bacterial alkaline phosphatase (E.C. 3.1.3.1) from Worthington. Tobacco acid pyrophosphatase was purchased from BRL.

Radioactive α-^{32}P-NTPs and ^{32}P-orthophosphate were purchased from Amersham International Ltd.

Transcription and analysis of the transcripts

Transcription of a poly(dA-dT).poly(dA-dT) template was carried out as follows. The transcription mixture (50 1) contained 20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 10 mM 2-mercaptoethanol, 10 μM ATP, 1 μM [α-^{32}P]UTP (± 400 Ci/mmol), 500 μM GpppA or ^{7m}GpppA or ATP (also at 500 μM), 2 μg poly(dA-dT).poly(dA-dT) and 0.6 U RNA polymerase. The kinetics of the transcription reactions were followed as described by Smith et al. (8). After phenol extraction, the α-^{32}P-XTPs were removed by passage through a Sephadex G50 column (0.6 x 20 cm). The RNA was precipitated and analysed by digestion with T1 and/or pancreatic ribonuclease as described earlier (9). The resulting digests were fractionated by electrophoresis on cellulose acetate at pH 3.5 in the first dimension and chromatography on polyethylene imine plates (Macherey & Nagel, Düren, GFR) with 3.5 M formic acid-pyridine at pH 3.4 or homomixture β (10). 5'-terminal structures were further analysed by digestion with U2-ribonuclease, tobacco acid pyrophosphatase and bacterial alkaline phosphatase. Some reactions for primer dependent transcription contained 20% glycerol, but for transcription of β-interferon cDNA containing plasmids, glycerol was omitted because direct injection of glycerol-containing transcription mixtures caused an increased deterioration (lysis) of oocytes. The presence or absence of glycerol did not influence the efficiency of the capping reaction.

In vitro protein synthesis

Before addition to the wheat germ (11, 12) or reticulocyte lysate (Amersham International Ltd.) translation system, the RNA was repeatedly precipitated with ethanol to remove non-incorporated cap structures. Translation of the transcripts in Xenopus laevis oocytes was obtained by injection of 50 nl quantities of transcription mixture or concentrated

transcription products into the cytoplasm as reported previously (13), and subsequent incubation for 70 hrs at 20°C. The incubation medium was centrifuged for 3 minutes in an Eppendorf centrifuge and the supernatant assayed for interferon activity.

RESULTS AND DISCUSSION

Transcription of synthetic poly(dA-dT).poly(dA-dT) template and of PL or PL/R promoter containing plasmids

In a transcription experiment, using poly(dA-dT).poly(dA-dT) as a template and E. coli RNA polymerase in the presence of high concentrations of GpppA or ^{7m}GpppA (500 μM) and low concentrations of ATP (<20 μM) and α-^{32}P-UTP (1 μM), it was found that the 5'-terminal structure pppApUp almost disappeared while it was replaced by a faster moving spot in the second dimension (see Fig. 1a, b and c). The newly labelled structures were further characterized by digestion with bacterial alkaline phosphatase, T1 ribonuclease, U2 ribonuclease, U2 ribonuclease + bacterial alkaline phosphatase and tobacco acid pyrophosphatase + bacterial alkaline phosphatase. All digestion products indicated that these structures were GpppApUp and ^{7m}GpppApUp respectively (results not shown). It was therefore concluded that the RNA was initiated with a cap structure instead of pppA. Reactions that occur at low concentrations of ATP and with high concentrations of cap-structure proceeded at about 80% with GpppA as primer and at between 20 and 50% with ^{7m}GpppA as primer when measured relative to the rate at a high ATP concentration. When GpppAm, Gppp^{m}Am or ^{7m}GpppAm were added to the transcription mixture, the rate of RNA synthesis was reduced but the RNA contained predominantly a pppApUp 5'-terminus. These results suggest that methylation at the 2' position results in a 2-3-fold reduction in the rate of RNA synthesis and made the cap-structure unsuitable as a primer. When ^{7m}GpppU was added, pppA was again preferred for initiation. Preliminary experiments with poly(dG-dC).poly(dG-dC) suggest that GpppG, ^{7m}GpppG and ^{7m}GpppC are accepted by the RNA polymerase for initiation of RNA synthesis.

Next, we wished to determine whether cap-primer dependent transcription could also be obtained if the RNA was synthesized under the control of a prokaryotic promoter. For this purpose, a plasmid containing the PL promoter of phage λ and the termination region of phage fd (Fig. 2) was transcribed in various conditions. It is evident that the transcription patterns did not change when high concentrations of ATP were substituted for GpppA or ^{7m}GpppA (Fig. 2). Once again, the presence of ^{7m}GpppA led to a reduction in the rate of RNA synthesis. The predominant PL specific transcript was analyzed by double digestion with pancreatic ribonuclease and T1 ribonuclease and fingerprinted in two

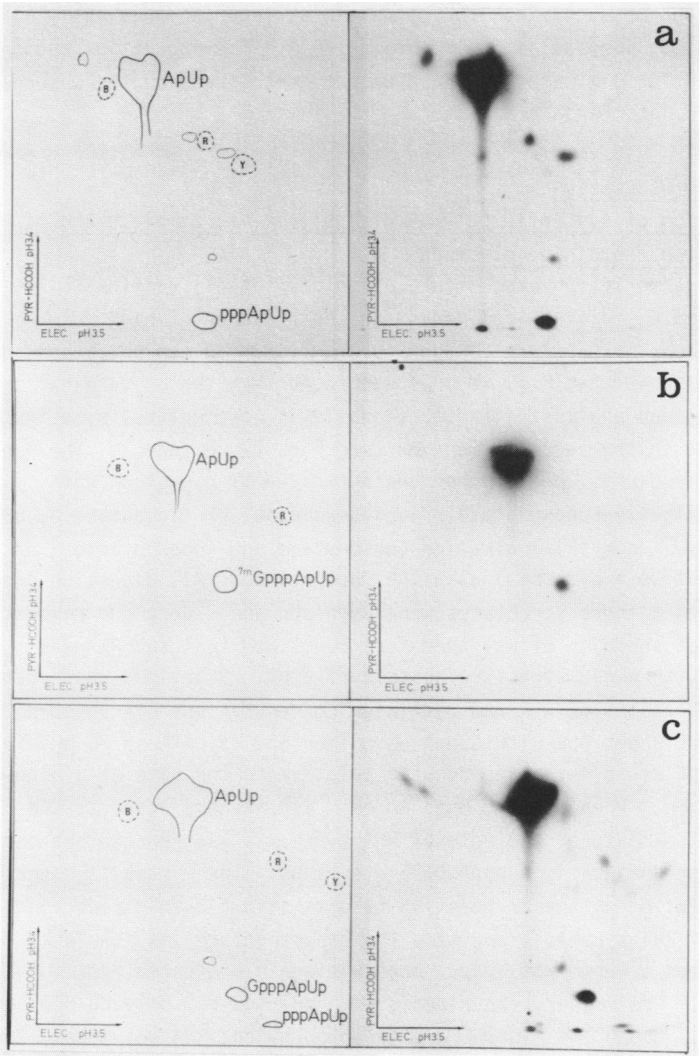

Fig. 1. Analysis of the 5'-terminal nucleotides released by pancreatic ribonuclease digestion of RNA synthesized by Escherichia coli RNA polymerase on poly(dA-dT).poly(dA-dT) in the presence of α-^{32}P-UTP and: a, ATP (500 μM); b, ATP (20 μM) + ^{7m}GpppA (500 μM); c, ATP (20 μM) + GpppA (500 μM). The first dimension was run by electrophoresis on cellulose acetate paper at pH 3.5 and the second dimension was ascending chromatography on polyethylene imine plates with 3 M formic acid-pyridine at pH 3.4. Spots encircled by dashes are dye references (10).

dimensions on polyethylene imine plates. As before the pppApUp spot was replaced by the GpppApUp or ^{7m}GpppApUp structures for 80% and about 70% respectively (Figure 3). Proof for the 5'-terminal position of the cap

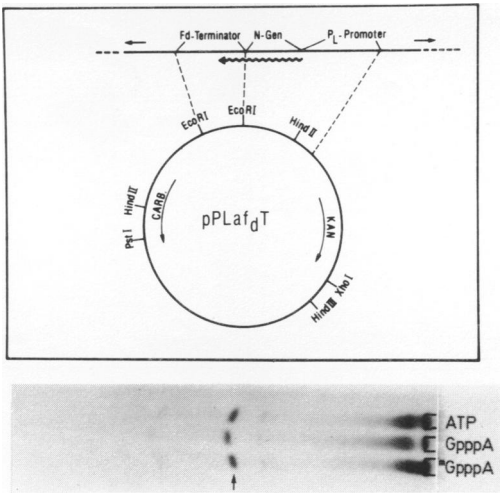

Fig. 2. Composition and polyacrylamide gel analysis of transcripts synthesized on plasmid pPLa fdT. This plasmid contains the PL promoter of phage λ in the anticlockwise orientation and the terminator of phage fd(16). PL promoter specific initiation produces an RNA product of 165 nucleotides which is terminated at the fd terminator. The product (arrow) has been characterized by RNA sequencing techniques (24).

structure was obtained by digestion of the PL specific transcript with T1 ribonuclease and analysis of the 5'-terminal oligonucleotide with pancreatic ribonuclease. In Fig. 4, it can be seen that the 5'-terminal oligonucleotide pppApUpCpApGp of the PL transcript disappears (spot 1) and that a new component is present which migrates together with oligonucleotide II. This new component contains the structure GpppApUp. The electrophoretic and chromatographic shift observed is in good agreement with the change in the charge of the oligonucleotide when a G residue is added to pppApUpCpApGp.

As it is the intention to use these capped transcripts for the expression of cloned eukaryotic genes it is very important to show that the cap structure present in the transcription mixture does not inhibit the synthesis of long RNA chains. Therefore, a transcription reaction was carried out on a recombinant plasmid containing the Hind A fragmemt of simian virus 40 cloned after the strong PL promoter of phage λ. The construction and transcription patterns of the pPLcSVt5-2 plasmid (16) in the presence and absence of GpppA and ^{m}GpppA structures is shown in Fig. 5. It is clear that there is very little change in the respective transcription patterns, although the longest transcripts contain 1270 nucleotides. 5'-end analysis of the two longer transcripts showed that they were capped for more than 90%. The experiments described here were

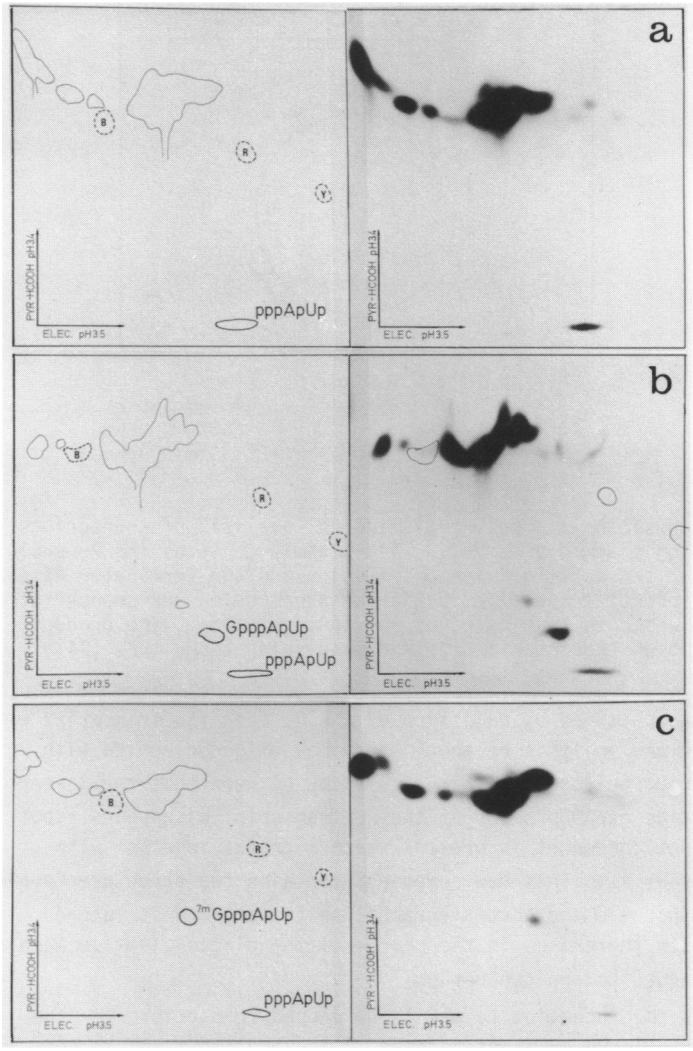

Fig. 3. Analysis of the 5'terminal nucleotides released by pancreatic and T1 ribonuclease digestion of the 165 nucleotide RNA synthesized on the pPLafdT plasmid in the presence (b, GpppA; c, ^{7m}GpppA) or absence (a) of cap structures. The digestion and fractionation of the products were carried out as described in the legend to Fig. 1.

designed to obtain transcripts starting with a cap structure and are similar in design to the procedure used for priming transcription by ribo-oligonucleotides (18-22). The structure of the cap primers, however, is more similar to a nucleoside triphosphate analogue.

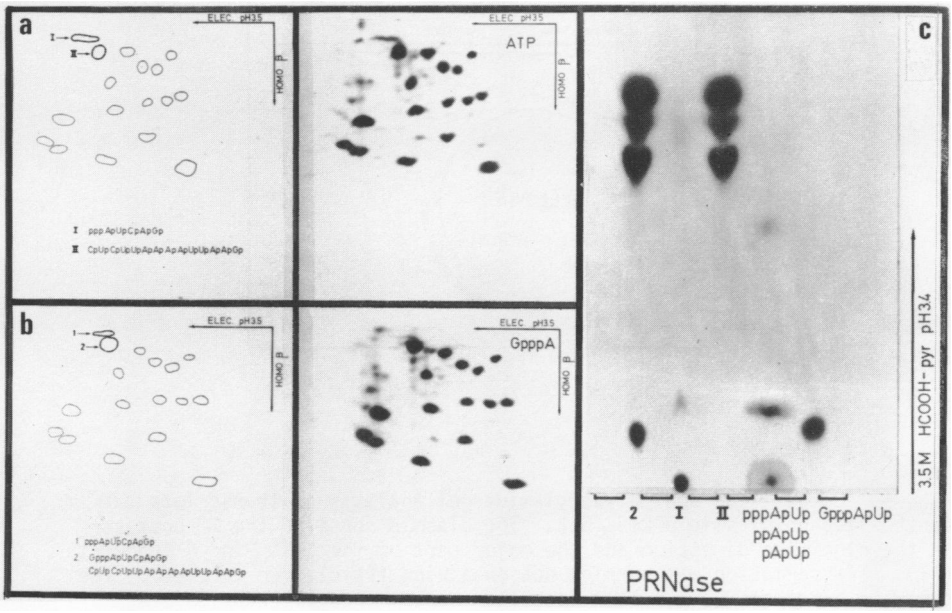

Fig. 4. Analysis of the 5'-terminal T1 ribonuclease oligonucleotide of the 165 nucleotide RNA synthesized on the pPLa fdT plasmid in the absence (a) or presence of GpppA (b). The right hand part of the figure shows a one-dimensional chromatogram of a pancreatic ribonuclease digest of the 5'-terminal oligonucleotide pppApUpCpApGp (I), of the individual spot CpUpCpUpUpApApApApUpUpApApGp (II), and of a mixture of GpppApUpCpApGp and CpUpCpUpUpApApApApUpUpApApGp (2). Marker nucleotides GpppApUp, pppApUp, ppApUp and pApUp were obtained from transcription experiments with poly(dA-dT).poly(dA-dT) as template (see Fig. 1). The relative intensities of the spots I and II are 1 to 5 because the RNA was labeled with α-^{32}P-UTP.

Undoubtedly the RNA polymerase accepts the cap-structure as an RNA primer because it is not very inhibitory during the first steps of the RNA synthesis elongation reaction.

In vitro translation of capped transcripts

In a first experiment on expression of a eukaryotic gene by means of such capped transcripts, a recombinant plasmid containing the PL promoter followed by the human fibroblast interferon gene (pHFIF-67-11 (23)) was transcribed in the presence or absence of the GpppA or ^{7m}GpppA cap structures. Injection of the different RNA preparations into the cytoplasm of Xenopus oocytes produced biologically active interferon proteins as assayed by a cytopathogenic effect inhibition assay. The oocytes injected with the ^{7m}GpppA capped RNA synthesized ten times more interferon than those injected with GpppA capped RNA. The non-capped RNA

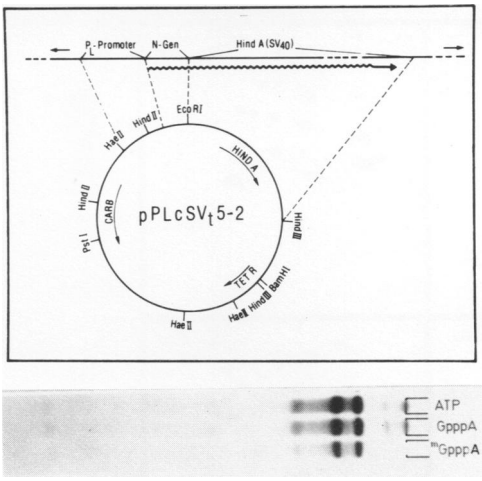

Fig. 5. Composition and polyacrylamide gel analysis of transcripts synthesized on plasmid pPLcSV$_t$5-2. The plasmid contains the PL promoter in the clockwise direction and the major part of the SV40 Hind A fragment (15). Transcription was carried out on a Hind III cleaved template. The two main bands are PL promoter specific (unpublished observation).

produced 50 times less interferon than the ^{7m}GpppA capped RNA. Since the pre-interferon initiation triplet in the pHFIF-67-11 plasmid is preceded by another AUG triplet which allows the synthesis of a polypeptide 47 amino acids long, a new plasmid derivative was made in which this first AUG signal was lacking. For this purpose, a promoter which was a hybrid between PL and PR was synthesized. It contained the left part of PL, up to the Hind II site, and the right part from PR. Injection of oocytes with these PL/R started interferon gene transcripts produced 10 to 30 times more interferon than the PL transcripts.

The capped transcripts have also been translated in a wheat germ extract (11) and in a reticulocyte lysate. The translation was cap-dependent since addition of ^{7m}GpppA inhibited the incorporation of ^{35}S-methionine by more than 90%. However, it was surprising to discover a major band with the molecular weight corresponding to the β-lactamase protein, and only a faint band with the molecular weight corresponding to the interferon protein. This is remarkable because the β-lactamase promoter was expected to be much weaker than the PL or PL/R promoters (e.g. the major transcripts in the gel of Fig. 5 are all PL promoted). The small (47 amino acid) polypeptide is well synthesized in the wheat germ extract, but apparently less well in the reticulocyte lysate. It may be necessary to optimize the structure of the RNA 5'-leader sequence for more efficient expression. Examples of expression of cloned eukaryotic genes from capped transcripts synthesized in vitro will be reported elsewhere.

In conclusion, we have described a simple method for the in vitro synthesis of capped transcripts which is useful for the direct expression of eukaryotic genes when cloned under control of a suitable promoter. This procedure is simple and may be used to screen a eukaryotic cDNA bank by direct expression, for example on the basis of a biological activity like interferon. Since the amount of interferon produced by the oocytes (100 I.U. ml^{-1}) is approximately 100 times above the minimal detection level, in principle it should be possible to detect a positive signal by transcribing a mixture of one hundred recombinant plasmids one of which contains an interferon gene. Obviously, a eukaryotic cDNA gene bank can also be screened on the basis of other criteria, such as immunological detection. In addition, this method is useful for characterization of cloned cDNA genes and for studies on structure-function relationships in the initiation of protein synthesis in eukaryotes.

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