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# The effect of capping and polyadenylation on the stability, movement and translation of synthetic messenger RNAs in *Xenopus* oocytes

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ABSTRACT

Synthetic RNAs coding for chicken lysozyme, calf preprochymosin and Xenopus globin were transcribed in vitro using Sp6 RNA polymerase. The effects of capping and adding a poly(A) tail on the stability, movement and translation of these RNAs in Xenopus oocytes was examined.

Capping and polyadenylation increased stability of the transcripts, with at least 40% remaining intact 48h after injection into oocytes.

Capped poly(A)$^-$ transcripts moved more rapidly in oocytes than either capped poly(A)$^+$ transcripts or naturally occurring mRNAs.

The translational efficiency of most of the synthetic RNAs in oocytes increased with both capping and polyadenylation. The exception was one Xenopus globin transcript which had an unusual 3' end of 20As and 30Cs, where further polyadenylation decreased translational efficiency.

Polyadenylation was essential for detectable expression of the synthetic RNAs in cultured cells, but decreased translation of the synthetic RNAs in vitro.

## INTRODUCTION

It is now possible, using the RNA polymerase from Salmonella typhimurium phage SP6, to transcribe large quantities of discrete-sized RNAs in vitro from any DNA fragment introduced into an appropriate vector (1). Such transcripts have many uses: high specific activity synthetic RNAs have been used as probes for in situ hybridization (2), filter hybridization (1,3,4), mapping the 5' and 3' ends of transcripts (5) and mapping intron/exon boundaries in genomal DNA (1). Synthetic RNAs have also been used as mRNA analogues for translation in vitro (6,7,8) and for translation (6,9) or RNA processing studies (10,11,12) after microinjection into living cells. Finally injected anti-sense RNA transcripts have been used to interfere with the translation of complementary endogenous mRNAs during animal development (13,14).

In nearly all of these in vivo applications, Xenopus oocytes have been used. These cells have long provided a useful assay system for the expression of injected mRNAs and DNAs (15,16,17,18). In particular the microinjection of mRNAs has allowed studies on the stability (19,20,21,22,23,24), movement (24)

and translation of the mRNAs (15,16,18) as well as the subsequent processing of the protein products (16,17,18).

It is relatively simple to introduce specific structural modifications into RNA synthesised in vitro. We have exploited this to examine the effect of adding a cap structure or poly(A) tract on the behaviour of synthetic RNAs in oocytes.

Whilst capping increases the stability of mRNA in oocytes (20) the contribution of the poly(A) tail remains controversial. Some RNAs are reported to be destabilized by deadenylation (25,26,27,28) whilst others are unaffected (29,30,31). However, most of these studies use translation as a measure of RNA stability rather than a more direct method. We have re-investigated this question more directly using labelled RNA. We find that the stability of all the RNAs tested is enhanced by polyadenylation. Knowing the relative stability of injected poly(A)$^-$ and poly(A)$^+$ RNAs, we have measured the effect of polyadenylation on the translational efficiency of these RNAs. This is of interest since the degree of adenylation of an mRNA may regulate its translation during early development (32,33). We find that polyadenylation can result in much greater translational efficiency, although other aspects of the 3' terminal structure of an RNA can affect this efficiency. Polyadenylation also permits the translation of synthetic RNA after injection into cultured mammalian cells, thus introducing a further application for these RNAs.

Finally, we have followed the movement of the synthetic RNAs across the oocyte cytoplasm. Our previous study (24) using poly(A)$^+$ mRNAs found them to move very slowly, however recent work by Melton (13) suggests that synthetic, non adenylated RNAs can move rapidly. In this paper we confirm the faster movement of non adenylated synthetic RNAs, and find a slight retardation in rate of movement when a poly(A) tail is added.

## MATERIALS AND METHODS

### Chemicals

Chemicals were of 'Analar' grade purchased from BDH Chemicals (Poole, U.K.). Tris, Spermidine, Dithiothreitol and Nucleotide triphosphates were purchased from Sigma (Poole, U.K.).

### Antibodies

Rabbit anti-chicken egg white antibodies were prepared by standard methods after immunization of New Zealand White rabbits. Rabbit anti-prochymosin was a gift from P. Lowe (Celltech, U.K.). Monospecific rabbit anti-chick lysozyme

was a gift from Dr. A. Rees (Biophysics, Oxford, U.K.).

Radiochemicals

[$^{32}$P-α]rGTP (410 Ci/mmole), [$^{35}$S]methionine (>800 Ci/mmole) [$^{3}$H]leucine (120 Ci/mmole) and [$^{14}$C] protein molecular weight markers were obtained from Amersham International (Amersham, U.K.).

DNA Templates For in Vitro Transcription

pspLys$^{+}$. pspLys$^{+}$ is the chicken lysozyme cDNA, isolated as a Hind III fragment from pTK$_2$Lys$^{+}$ (34), inserted into the Hind III site of psp-64 (1), a vector which contains the Sp6 RNA polymerase promoter. For transcription pspLys$^{+}$ was linearised using EcoRI. The resulting 541 base Lys$^{+}$ transcript contains (in addition to the coding region); 14 out of 31 bases from the 5', and 17 out of 117 bases from the 3' untranslated regions of lysozyme mRNA (35,36).

psp 82$^{+}$. psp82$^{+}$ was prepared by inserting the Hind III fragment of pTK$_2$82$^{+}$ (34) (containing calf preprochymosin cDNA sequence) into the Hind III site of psp-64. psp82$^{+}$ was linearised with XbaI. The resulting Chym$^{+}$ transcript (1206 bases) contains in addition to the coding region, 14 out of 25 bases from the 5', and 6 out of 136 bases from the 3' untranslated region of calf preprochymosin mRNA (37).

The pspLys$^{+}$ and psp82$^{+}$ transcripts also have psp-64 and Hind III linker sequences at their 5' and 3' ends (see (1) and (34)).

psp64-Xβm. psp64-Xβm (a gift from P. Krieg, Harvard, USA) contains the entire cDNA sequence (including untranslated regions) of Xenopus β Globin mRNA inserted in psp64 (6). When linearised with Hinf I and transcribed the resulting Globin-Hinf transcript contains the entire 5' untranslated, coding, and 28 bases of the 3' untranslated region of Xenopus β globin mRNA. Linearising with Pst I gives a transcript (Globin-Pst) which contains the entire mRNA sequence plus a 'tail' of 20As and 30Cs (6).

In vitro Transcription

Uncapped Transcripts. Transcriptions were carried out as described by Melton et al. (1). Briefly a reaction containing 40 mM Tris pH 7.5, 6 mM $MgCl_2$, 2 mM Spermidine, 10 mM dithiothreitol, 2 units/μl Rnasin (Promega, Madison, USA), 100 μg/ml BSA (nuclease free, BRL), 500 μM rUTP, rCTP and rATP, 50 μM rGTP, 200 μCi/ml [$^{32}$P] rGTP, 50 μg/ml linearised DNA template and 300 units/ml Sp6 RNA polymerase (NEN, Southampton, U.K.) was incubated at 40°C for 1 h.

Capped Transcripts. To produce capped transcripts the reaction mix (above) was made 500 μM m$^{7}$G(5')ppp(5')G (monomethyl cap) or 500 μM m$^{7}$G(5')ppp(5')Gm (dimethyl cap) (PL-Pharmacia, Milton Keynes, U.K.) (8,38). Specific activity

of these transcripts was about $10^6$ dpm/$\mu$g.

Processing of Transcripts

After transcription the DNA template was digested by adding 20 $\mu$g/ml DNase I (Worthington, Irvine, UK) previously treated to remove residual RNAase activity (39), and incubating at 37°C for 10 min. Yeast tRNA (Boehringer, Lewes, U.K.) was added as carrier to 200 $\mu$g/ml and the reaction mix made 10 mM EDTA. The reaction mix was then extracted once with phenol:chloroform: isoamylalcohol (25:24:1) and twice with chloroform:isoamylalcohol (24:1) before adding ammonium acetate to 0.7 M, 2.5 volumes of EtOH and precipitating at -20°C. The precipitation was then repeated and the transcripts resuspended at 100 ng/$\mu$l in $H_2O$ for oocyte injection.

Polyadenylation of Transcripts

Transcripts were polyadenylated using poly(A) polymerase from E. coli (40). The purified transcript (up to 10 $\mu$g/ml) was polyadenylated in a reaction of 50 mM Tris pH 8.0, 100 $\mu$M rATP, 10 mM $MgCl_2$, 50 $\mu$g/ml BSA (nuclease-free, BRL, Paisley, UK), 250 mM NaCl, 1 mM $MnCl_2$, 35 u/ml polyA polymerase (BRL, Paisley, UK) at 37°C for periods up to 2 h (see fig. 2). The reaction was stopped by adding EDTA to 20 mM then phenol:chloroform extraction and ethanol precipitation as above.

The carrier tRNA used in the preparation of transcripts for polyadenylation (as well as the corresponding non-polyadenylated control) was first treated with periodate (41) to prevent the tRNA acting as substrate for the polyadenylation reaction.

Gel analysis of Polyadenylated Transcripts

The transcripts were dissolved in 80% (v/v) deionised formamide, 0.3% (w/v) Xylene cyanol FF, 0.3% (w/v) Bromophenol blue, 20 mM EDTA (pH 7.5) and analysed by electrophoresis on a 6% Acrylamide Urea sequencing-type gel (42).

Preparation of Natural mRNAs

Hen oviduct poly $A^+$ mRNA and $^{32}P$-reovirus mRNAs ($10^4$ dpm/$\mu$g) were prepared as described previously (24).

Microinjection of Oocytes

The preparation, microinjection and culture of oocytes was as described by Colman (18).

Analysis of Injected RNA Extracted From Oocytes

Gel Electrophoresis. RNA was extracted from whole oocytes (for stability studies) or from the animal and vegetal halves of oocytes (for movement studies), using at least 15 oocytes for each timepoint, and the total RNA recovered from 2.5 whole oocytes or 5 halves was separated on denaturing

formaldehyde agarose gels as described previously (24).

Autoradiography of Oocyte Sections. Oocytes were fixed in Bouin's liquid, embedded in paraffin wax (Paraplast. BDH, Poole, U.K.), sectioned and mounted on slides (43). After rehydration the sections were immersed in 5% TCA for 15 min at 4°C, rinsed in $H_2O$, air dried, dipped in NTB-2 emulsion (diluted 1:1 with $H_2O$) (Kodak, Rochester, New York) and exposed at 4°C in lightproof boxes. The autoradiographs were developed in Kodak D19 developer.

In vitro Translation

Message dependent nuclease-treated reticulocyte lysate (a gift from Tim Hunt, Cambridge University, U.K.) was used as described (44). Wheatgerm translation system (Amersham International, Amersham) was used according to the manufacturer's instructions. The final concentration of $K^+$ in this translation assay was adjusted to 140 mM. Before analysis on polyacrylamide gels (below), 5µl aliquots containing [$^{35}$S]-methionine-labelled polypeptides were incubated with pancreatic RNAase A (Sigma) at 100µg/ml for 30min at 25°C to degrade $^{32}$P-labelled transcripts.

Analysis of Translation Products

Incubation media or oocytes were prepared for electrophoresis on 15% SDS-polyactylamide gels with or without prior immunoprecipitation as described previously (45). Each track contains 0.25 processed oocyte and homogenate or processed media surrounding 2 oocytes. The gels were fixed, fluorographed (46) and the amount of each polypeptide quantified by band excision and scintillation counting.

Microinjection of Cells

Microinjection of RNAs at 100 ng/µl in distilled water into cultured CV-1 monkey cells was performed as described by Graessmann et al. (47). Injected cells were cultured at 37°C for 8 h before fixing in 3% (w/v) paraformaldehyde, permeabilized with 0.2% Triton X-100 and stained for indirect immunofluorescence by the method of Ash et al. (48) using FITC-conjugated goat anti-rabbit antisera (Miles-Yeda) as second antibody. Stained cells were examined with a Zeiss microscope using epifluorescence.

## RESULTS

### Stability of transcripts

Capped transcripts of chicken lysozyme cDNA were synthesised by adding a "capping dinucleotide" (either $m^7$G(5')ppp(5')G or $m^7$G(5')ppp(5')Gm) to the transcription reaction. In 10 fold molar excess to GTP these dinucleotides are efficiently incorporated (8,38) at the 5' terminal position of transcripts in vitro, forming what we will refer to as monomethyl cap ($m^7$G(5')ppp(5')G) or

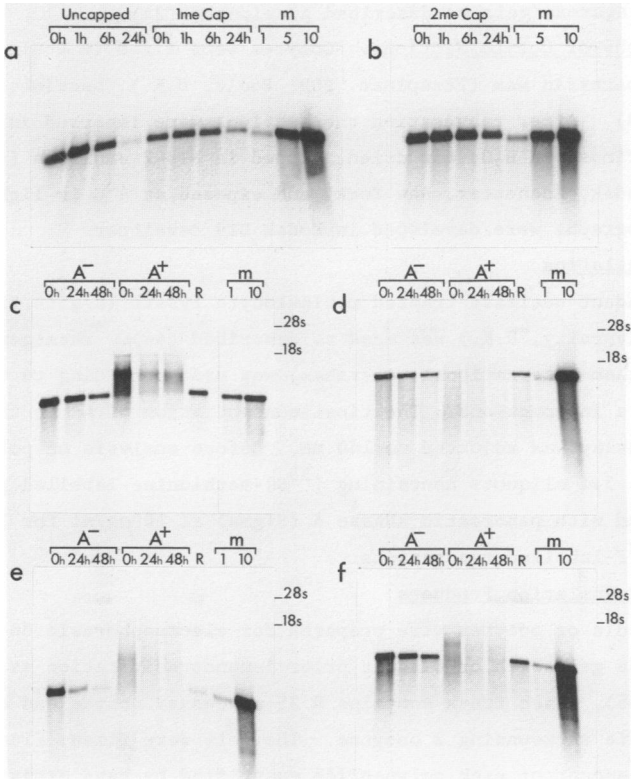


**Fig. 1. Stability of *in vitro* transcripts in oocytes.**
**At the times indicated RNA was extracted from batches of 15 intact oocytes previously injected with 5 ng per oocyte of the following $^{32}$P labelled *in vitro* transcripts. (a) Lys$^+$ uncapped, Lys$^+$ monomethyl cap (1me cap); (b) Lys$^+$ dimethyl cap (2me cap); (c) Lys$^+$ monomethyl cap poly(A)$^-$ and poly(A)$^+$; (d) Chym$^+$ monomethyl cap poly(A)$^-$ and poly(A)$^+$; (e) Globin-Hinf monomethyl cap poly(A)$^-$ and poly(A)$^+$; (f) Globin-Pst monomethyl cap poly(A)$^-$ and poly(A)$^+$. The extracted RNA was run on denaturing agarose formaldehyde gels which were dried down and autoradiographed. (A)$^-$ nonpolyadenylated, (A)$^+$, polyadenylated transcripts. The marker tracks (m) contain uninjected (poly(A$^-$)) transcript (1, 1ng; 5, 5ng; 10, 10ng). The track R is a repeat of the (A)$^-$, 48h track. 28S and 18S are the oocyte 28S and 18S ribosomal RNA bands. These bands become prominent due to reincorporation of labelled nucleotides from degraded transcript.**

**dimethyl cap ($m^7$G(5')ppp(5')Gm) transcripts. To compare their relative stabilities; uncapped, monomethyl capped or dimethyl capped/lysozyme (Lys$^+$) transcripts were injected into Xenopus oocytes (about 5 ng/oocyte). At various times after injection, RNA was extracted from the oocytes, separated on denaturing gels, autoradiographed (Fig. 1a and b) and the RNA bands excised**

**Stability of Capped RNA**

| Transcript | Amount of RNA | | |
|---|---|---|---|
| | 0h | 6h | 24h |
| Lys | 100 | 61 | 10 |
| Lys 1me Cap | 100 | 90 | 49 |
| Lys 2me Cap | 100 | 82 | 45 |

Table 1. Stability of Capped in vitro transcripts in oocytes.
The experiment in Fig. 1a and b was quantified by cutting the appropriate bands from the gels and counting in a scintillation counter. The amount of RNA at each time point is expressed as a percentage of the 0h value. Abbreviations as in Figure 1.

from the gel and quantified by scintillation counting (Table 1). Only 10% of the uncapped RNA remained intact after 24 h. In contrast 45% and 49% of the monomethyl cap and dimethyl cap transcripts respectively, remained intact after the same period.

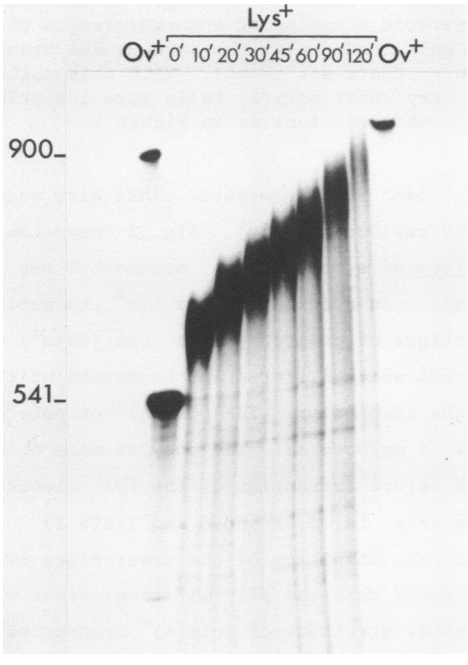


Fig. 2. Polyadenylation of Lys+ monomethyl cap transcripts.
Lys+ monomethyl cap transcript (541 nucleotides) was polyadenylated for 0, 10, 20, 30, 45, 60, 90 and 120 minutes (see Materials and Methods). Samples of the polyadenylated transcripts were then run on a 6% Acrylamide, urea sequencing type gel. The marker tracks (M) contain a 900b SP6 transcript.

## Stability of PolyA$^+$ RNA

| Transcript | | Length of poly A | | Amount of RNA | | |
|---|---|---|---|---|---|---|
| | | Median | Maximum | 0h | 24h | 48h |
| Lys 1me Cap | A$^-$ | — | — | 100 | 62 | 33 |
| | A$^+$ | 250 | 1400 | 100 | 47 | 54 |
| Lys 2me Cap | A$^-$ | — | — | 100 | 65 | 14 |
| | A$^+$ | 250 | 1400 | 100 | 79 | 54 |
| Chym 1me Cap | A$^-$ | — | — | 100 | 70 | 38 |
| | A$^+$ | 410 | 2100 | 100 | 90 | 71 |
| Globin Hinf 1me Cap | A$^-$ | — | — | 100 | 30 | 13 |
| | A$^+$ | 280 | 2900 | 100 | 58 | 42 |
| Globin Pst 1me Cap | A$^-$ | — | — | 100 | 48 | 33 |
| | A$^+$ | 250 | 2700 | 100 | 53 | 52 |

Table 2. Stability of Polyadenylated transcripts in oocytes.
The experiment in Fig. 1c, d, e and f plus a similar experiment using Lys$^+$ dimethyl cap RNA (gel not shown) was quantified by cutting the appropriate bands or regions (polyadenylated transcripts) from the gel and scintillation counting. The amount of RNA at each time point is expressed as a percentage of the 0h value. The median and maximum length of the poly(A) tail was estimated by densitometeric scanning of autoradiographs of denaturing agarose/formaldehyde gels loaded with the various RNA transcripts and suitable $^{32}$P labelled DNA markers (data not shown). With this method of sizing, transcripts that had very short poly(A) tails were indistinguishable from poly(A)$^-$ transcripts. Abbreviations as in Figure 1.

In addition to a "cap" most eukaryotic mRNAs also acquire poly(A) tails of between 150 and 200 residues (49,50). Fig. 2 demonstrates the polyadenylation *in vitro* of synthetic Lys$^+$ monomethyl cap transcripts using *E. coli* poly(A) polymerase. In addition to the Lys$^+$ transcript we also polyadenylated transcripts of preprochymosin cDNA (Chym$^+$) and two transcripts of Xenopus $\beta$ globin cDNA which differed in the extent of the 3' untranslated regions included in the transcript. The poly(A)$^+$ or poly(A)$^-$ transcripts were injected into oocytes (5 ng/oocyte). The oocytes were then incubated for periods of up to 48 h before extraction of the RNA, electrophoresis and quantitation as above (Fig. 1c, d, e and f and Table 2). Polyadenylation had only a small effect on the stability of the transcripts over the first 24 h. However after 48h we found that the poly(A)$^+$ transcripts were consistently more stable. The greater stability of poly(A)$^+$ transcripts was also indicated by the less pronounced appearance of radioactive 28S and 18S ribosomal rRNA in oocytes injected with poly(A)$^+$ transcripts compared with oocytes injected with poly(A)$^-$ transcripts; the radioactivity in these rRNAs arises from the reincorporation of nucleotides from degraded *in vitro* transcripts.

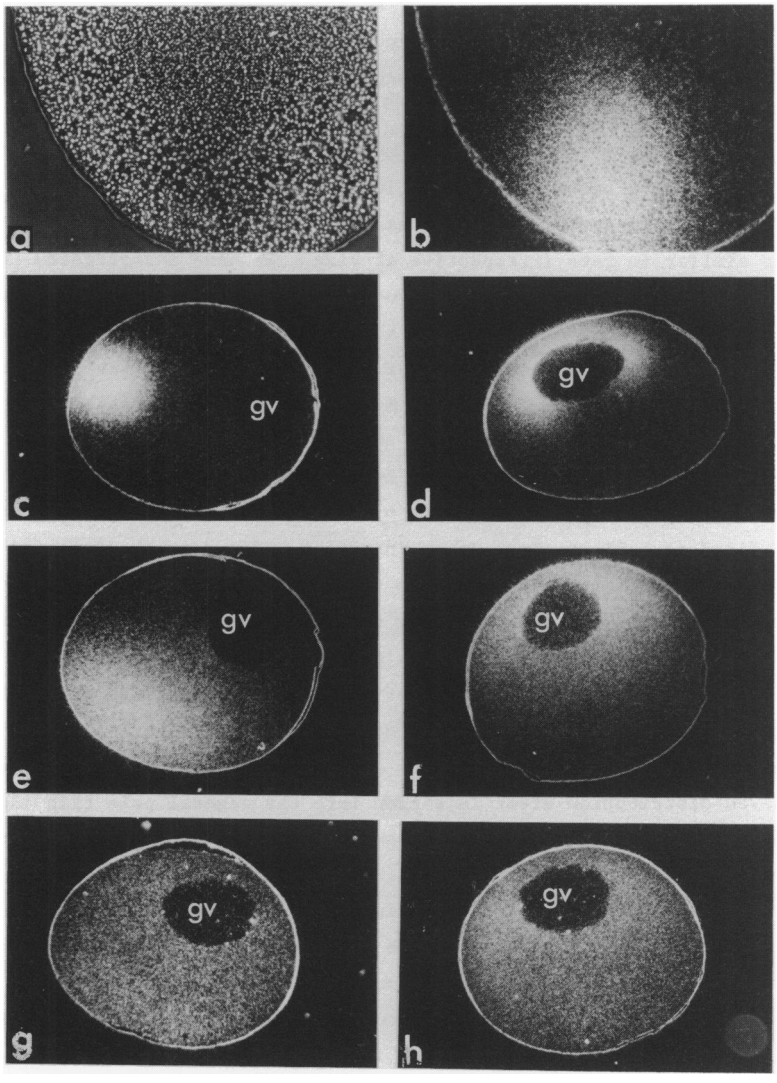


Fig. 3. Autoradiograph of injected oocytes.
$^{32}$P-Lys$^{+}$ dimethyl cap RNA was injected into oocytes at the vegetal (a,b,c,e and g) or the animal pole (d,f and h) and the oocytes fixed within 10 min (a,b,c,d), 6h (e,f) or 24h (g,h) and sectioned. After mounting on slides the oocyte sections were autoradiographed. The autoradiographed sections were visualized by phase-contrast (a) or dark-field (b-h) illumination. Panels a and b show the same oocyte section. The bright rim around the oocytes in panels b-h is due to the light scattering by dense pigment granules (not silver grains) distributed around the periphery of the oocytes. The nucleus of the oocyte is indicated (germinal vesicle, gv).

We conclude that the major factor affecting the stability of the synthetic RNAs is the presence or absence of a cap structure, although long term stability of capped RNAs is enhanced by the presence of a poly(A) tail.

Movement of RNA in the oocyte

We have recently investigated the movement of natural poly(A)$^+$ mRNAs (chick lysozyme, chick ovalbumin, rabbit globin) and poly(A)$^-$ mRNAs (reovirus 18S and 12S) after their injection into oocytes (24). Movement of all the mRNAs was slow and similar in rate irrespective of size. We have investigated the movement of some synthetic RNAs for two reasons: first, the availability of labelled synthetic RNAs allows a more accurate quantitation of data than was previously possible and secondly recent work by Melton (13) has suggested that synthetic, non-adenylated transcripts may move rapidly within Xenopus oocytes. However the results shown in Figure 3 show that this is not the case. Oocytes were injected with radioactive capped Lys$^+$ RNA and the movement of the injected transcripts followed *in situ* by autoradiography of serial sections of oocytes fixed at various times after injection. Immediately after injection, the injected RNA remained localized to the region of injection (Fig. 3c and d); surprisingly there does not appear to have been any long-lasting (>10 min) disruption or displacement of the yolk granules at the site of injection (Fig. 3a and b). Movement of transcripts was evident after 6h (Fig. 3e and f), although they were still localised round the injection site. After 24h radioactivity was present in all parts of the oocyte (Fig. 3g and h), with an almost even distribution after injection into the vegetal pole (Fig. 3g), while ol parts of the oocyte (Fig. 3g and h), with an almost even distribution after injection into the vegetal pole (Fig. 3g), while ol parts of the oocyte (Fig. 3g and h), with an almost even distribution after injection into the vegetal pole (Fig. 3g), while oannot be attributed to degraded RNA alone. This conclusion is supported by the biochemical evidence below.

Radioactive RNAs were injected into the polar regions of oocytes and the oocytes, after freezing, were bisected into their animal and vegetal halves. RNA was then extracted from groups of separated halves and analysed by electrophoresis and autoradiography before quantification as described in Methods. In Fig. 4a the movement of radioactive Lys$^+$ monomethyl cap RNA is compared with that of coinjected radioactive reovirus mRNAs. Clearly the Lys$^+$ transcripts moved to the opposite oocyte half more rapidly than the 12S(S) or 18S(M) reovirus RNAs, and were almost equally distributed 24h after injection in the vegetal pole. We do not believe the faster movement of the Lys$^+$ transcript to be a consequence of its smaller size since the movement of Chym$^+$

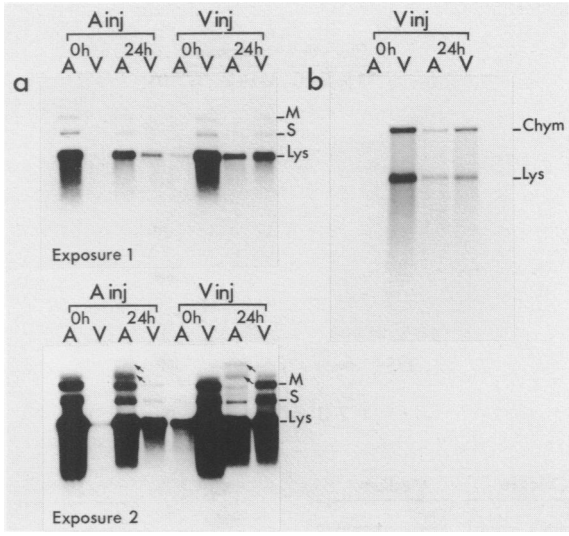


Fig. 4. Movement of RNA in oocytes.
Oocytes were injected (panel a) at the animal pole (A inj) or vegetal pole (V inj) with 60ng/oocyte $^{32}$P labelled Reovirus mRNA and 5ng/oocyte $^{32}$P labelled Lys$^+$ monomethyl cap RNA, or (panel B) in the vegetal pole with 2.5ng/oocyte $^{32}$P labelled Lys$^+$ monomethyl cap RNA and 2.5ng/oocyte $^{32}$P labelled Chym$^+$ monomethyl cap RNA.
RNA was extracted from the pooled animal (A) or vegetal (V) halves of the oocytes and run on denaturing agarose/formaldehyde gels. The gels were dried down and autoradiographed directly. Panel (a) contains two different exposures of the same gel.
Abbreviations. M, medium (18S) and S, small (12S) size class of the Reo mRNA; Lys, Lys$^+$ monomethyl cap RNA; Chym, Chym$^+$ monomethyl cap RNA. The arrows in exposure 2 of panel (a) mark the position of the 28S and 18S ribosomal bands which become prominent in the animal half 24h after injection due to reincorporation of radioactive nucleotides.

transcripts (1206 bases) and Lys$^+$ transcripts (541 bases) was similar (Fig. 4b) despite the fact that the Chym$^+$ transcripts are as long as the 12S reovirus mRNAs (1200, 1198, 1329 and 1418 bases (51,52,53)).

When similar comparisons were made between poly(A)$^+$ or poly(A)$^-$ Lys$^+$ transcripts we found that the addition of a poly(A) tract decreased the rate of movement, the exact decrease varying over 24h from 25%-44% according to experiment (data not shown). However we cannot say whether the retardation caused by poly(A) addition, is sufficient to fully explain the differences in movement between synthetic and natural mRNAs (24, Fig. 4a).

Effect of capping on the translation of Lys$^+$RNAs in oocytes

In Fig. 5a we show the results of a translation experiment where uncapped,

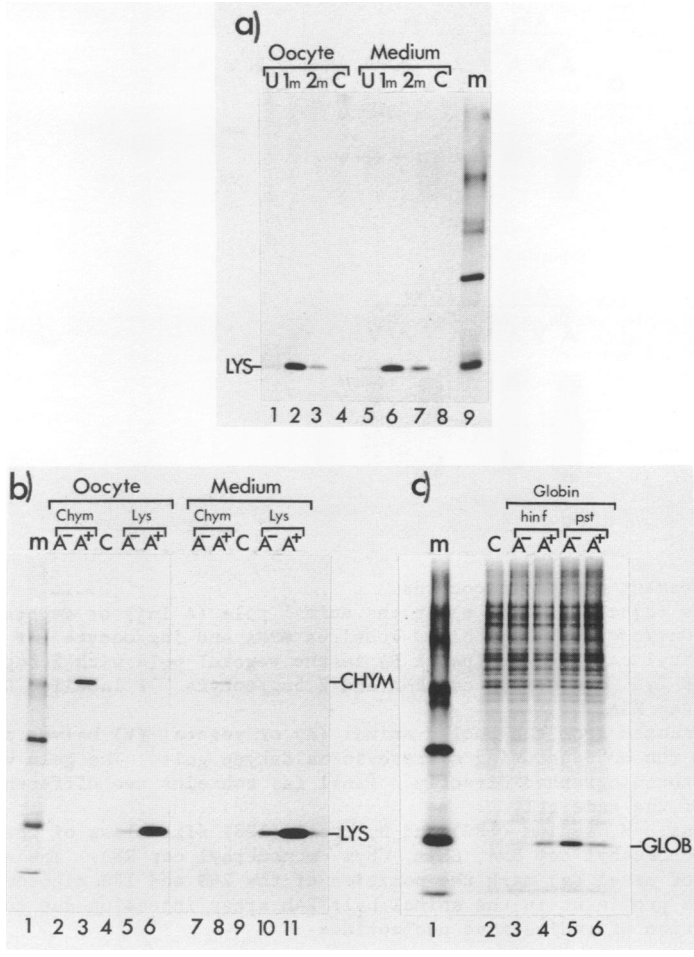


**Fig. 5. Translation of synthetic transcripts in oocytes.**
**Oocytes were injected with 5ng RNA/oocyte and labelled for 6h (panel a) or 24h (panels b and c) with $^{35}$S methionine (panels a and b) or $^{3}$H leucine (panel c). Oocytes (panel c) as well as their media (panels a and b) were then analysed on 15% SDS polyacrylamide gels before (panel c) or after (panels a and b) immunoprecipitation with the appropriate antibodies (see methods). In panel a the RNAs injected were all Lys$^{+}$ transcripts and were uncapped (U), monomethyl capped (1m) or dimethyl capped (2m). In panels b and c the RNAs, all monomethyl capped, are identified at the top of the tracks by their type (Chym, preprochymosin; Lys, lysozyme; Globin, hinf or Globin, pst, globin transcripts with different length 3' flanking sequences - see methods) and whether they possess (A$^{+}$) or do not possess (A$^{-}$) added poly(A) tracks. Other abbreviations: CHYM, LYS, GLOB - the positions of prochymosin, lysozyme and globin polypeptides. C, control, non-injected. m, marker track containing $^{14}$C protein molecular weight markers.**

monomethyl capped, and dimethyl capped Lys$^+$ transcripts were injected into oocytes and the oocytes cultured in $^{35}$S methionine for 6h. At the end of this period 61%, 90% and 82% of the uncapped, monomethyl capped, and dimethyl capped RNAs respectively remained intact (see Table 1). More lysozyme was obtained from the capped transcripts although some product was detected from the uncapped transcript. Clearly the monomethyl capped transcript was more efficiently used than the dimethyl capped transcript, a result which was consistently obtained in several batches of oocytes with several transcript preparations. From quantitation of the amount of lysozyme present in the gel (see Methods) and allowing for the different transcript stabilities (see Table 1) we estimate the translational efficiencies of the uncapped and dimethyl capped transcripts relative to that of the monomethyl capped transcripts (=1) as 0.04 and 0.20 respectively.

Effect of polyadenylation on translation of synthetic RNAs in oocytes

We have shown that polyadenylation of synthetic RNAs enhances their long term stability (Fig. 1). Since we knew the relative stabilities of poly(A)$^-$ and poly(A)$^+$ RNAs in oocytes over the first 24h (Table 2), we investigated whether the translation of these RNAs was proportional to their stability over this period. It is clear from Fig. 5b that the polyadenylated Lys$^+$ monomethyl capped transcripts (median length of tail 250 A residues) were translated more efficiently (20-fold in fact) than the unmodified transcripts, and that polyadenylation of Chym$^+$ monomethyl cap transcripts (median length of tail 410 A residues) also increased their translation (10 fold). The same stimulation was found using Lys$^+$ transcripts with shorter tails (median length 120 A residue) (data not shown). Although the extent of stimulation varied between batches of oocytes it was never less than 5 fold. Polyadenylation of the Lys$^+$ dimethyl cap transcript resulted in an increase in translation similar to that from polyadenylation of the monomethyl cap transcript, although the absolute amount of lysozyme made was lower (data not shown).

A marked stimulation of its translation in oocytes was also found on polyadenylation of Globin-Hinf transcripts which, like Lys$^+$ and Chym$^+$, possess only a short 3' untranslated region (Fig. 5c, cf tracks 3 and 4). However much better translation was obtained with the Globin-Pst transcripts which possesses all the 3' untranslated region plus a section of 20 A residues (from the natural poly A tail) and 30 C residues (from cDNA cloning) (6). Surprisingly, *in vitro* polyadenylation of the Globin-Pst transcripts reduced their translational efficiency (Fig. 5c, cf tracks 5 and 6).

We conclude that the polyadenylation of a transcript lacking stretches of

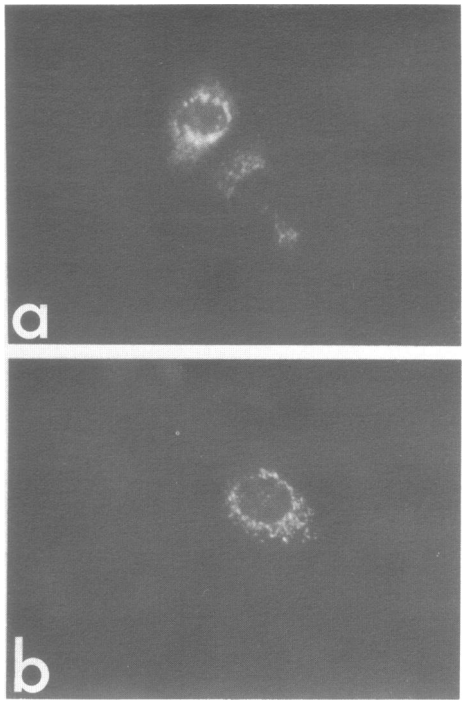


Fig. 6. Synthesis of lysozyme in cultured cells.
Subconfluent monkey CV-1 cells were injected with (a) oviduct mRNA (1mg/ml) or (b) Polyadenylated monomethyl cap $Lys^+$ RNA (100µg/ml) and cultured for 8h. Cells were fixed, stained with mono-specific rabbit anti-chick lysozyme followed by FITC-goat antirabbit antibody and viewed by epifluorescence illumination.

As can cause an increase in its translation in vivo which is disproportionately greater than the effect of poly(A) on transcript stability.

Translation of Synthetic RNAs in cultured cells

We have recently described the synthesis of lysozyme after microinjection of lysozyme mRNA into cultured mammalian cells (34). Monkey CV1 cells were injected with $Lys^+$ monomethyl cap $poly(A)^+$ transcripts, incubated for 8h and examined for lysozyme production using indirect immunofluorescence (Fig. 6b). The distribution of lysozyme was indistinguishable from that in cells injected with chick oviduct mRNA (Fig. 6a). However no detectable expression was obtained from $Lys^+$ monomethyl cap transcripts that were not polyadenylated (data not shown).

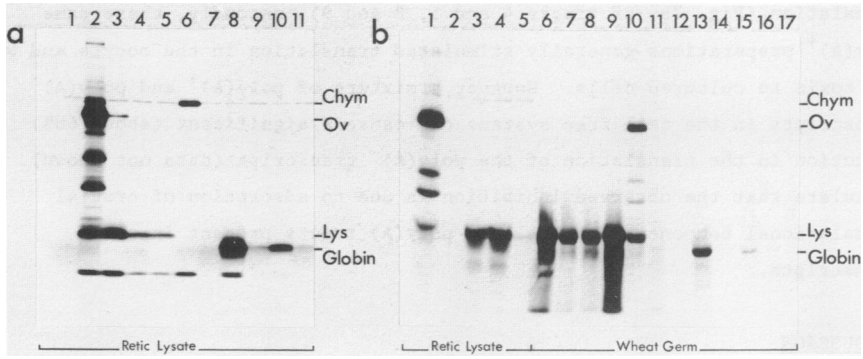


**Fig. 7. Translation of synthetic transcripts in vitro.**
**100ng aliquots of synthetic RNAs were translated in the reticulocyte lysate or wheat germ cell free translation systems as shown, and the protein products separated on 15% SDS polyacrylamide gels.**
**Panel (a): track 1, no RNA; track 2, oviduct mRNA; track 3, $Lys^+$ RNA; track 4, $Lys^+$ poly(A)$^+$ RNA; track 5, $Lys^+$ poly(A)$^+$ RNA, separate preparation; track 6, $Chym^+$ RNA; track 7, $Chym^+$ poly(A)$^+$ RNA; track 8, Globin-Hinf RNA; track 9, Globin-Hinf poly(A)$^+$ RNA; track 10, Globin-Pst RNA; track 11, Globin-Pst poly(A)$^+$ RNA.**
**Panel (b): track 1, oviduct mRNA; track 2, no RNA; track 3, $Lys^+$ RNA; track 4, $Lys^+$ RNA after incubation in the polyadenylation reaction at 4°C; track 5, $Lys^+$ poly(A)$^+$ RNA; track 6, $Lys^+$ RNA; tracks 7 and 8, $Lys^+$ poly(A)$^+$ RNA, different preparations; track 9, $Lys^+$ RNA after incubation in polyadenylation reaction at 4°C; track 10, oviduct mRNA; track 11, $Chym^+$ RNA; track 12, $Chym^+$ poly(A)$^+$ RNA; track 13, Globin-Hinf RNA; track 14, Globin-Hinf poly(A)$^+$ RNA; track 15, Globin-Pst RNA; track 16, Globin-Pst poly(A)$^+$ RNA; track 17, no RNA.**
**All transcripts contained an $m^7G(5')ppp(5')G$ (monomethyl) cap structure. Lys, Chym, Ov and Globin indicate the position of lysozyme, chymosin, ovalbumin and globin respectively.**

## Translation of Synthetic RNAs in vitro

All of the non-polyadenylated monomethyl transcripts ($Lys^+$, $Chym^+$, Globin-Hinf, Globin-Pst) were successfully translated (with similar relative efficiencies) in both wheatgerm and reticulocyte lysate cell free systems (Fig. 7). Surprisingly, in view of the result obtained with the same transcript preparations in oocytes, the Globin-Hinf transcripts were reproducibly more efficient than the Globin-Pst transcripts (Fig. 7a, cf tracks 8 and 10; Fig. 7b, cf tracks 13 and 15).

In both translation systems polyadenylation caused a large (>70%) decrease in the translation of all of the transcripts. We do not believe that this decrease is due to the presence of non-specific inhibitory factors acquired during the polyadenylation reaction for the following reasons: first passage of $Lys^+$ transcripts through a "mock" polyadenylation reaction (reaction conducted at 4°C) before re-extraction and assay did not affect

translation (Fig. 7b, cf tracks 4 and 5, 8 and 9), secondly, these same poly(A)$^+$ preparations generally stimulated translation in the oocyte and were not toxic to cultured cells. However a mixture of poly(A)$^+$ and poly(A)$^-$ transcripts in the cell free systems did cause a significant (about 60%) reduction in the translation of the poly(A)$^-$ transcript (data not shown). We speculate that the observed inhibition is due to adsorption of crucial translational components by the long poly(A) tracts present in our transcripts.

## DISCUSSION

RNA transcribed *in vitro* from cloned DNA is becoming widely used *in vivo* (mainly in Xenopus oocytes) to investigate RNA processing (10,11,12), translation (6,9), or to block translation with antisense transcripts (13,14). We have investigated the effect of adding a 5' cap or 3' poly(A) tail (common features of most eukaryotic mRNAs) on the stability, movement and translation of several transcripts following injection into *Xenopus* oocytes.

### Stability of Transcripts in Oocytes

*Effect of Capping* All eukaryotic and many viral mRNAs have a cap structure consisting of a 7 methylguanosine base joined by a triphosphate bridge to their 5' end. In addition the first (and sometimes the second) nucleotide of the RNA is methylated (56). The cap structure increases the stability of natural mRNAs (20,57) and *in vitro* transcripts (6,10) injected into *Xenopus* oocytes. We have compared the stability of an uncapped Lys$^+$ transcript with two forms of capped transcript. One had the 7 methyl guanosine base at the cap (called the monomethyl cap), while the other had, in addition, the first nucleotide of the transcript methylated (dimethyl cap). We found the uncapped Lys$^+$ transcript to be more stable (61% left after 6h) than has been previously reported for other *in vitro* transcripts (none detectable 15 min after injection (6)). However reports of absolute values for RNA stability in oocytes are notoriously varied (cf 20 and 22; also cf 23 and 24), probably reflecting differences in the batches of oocytes used. Nevertheless within the same batch of oocytes we found that both cap structures enhanced the stability of the transcripts by about 5 fold after 24h.

*Effect of Polyadenylation* The role of poly(A) in stabilizing mRNAs injected into oocytes remains controversial. Using cDNA hybridization to RNA recovered from injected oocytes, Marbaix *et al*. (27) have shown that rabbit globin mRNA is destablizied by deadenylation. However most of the other studies on the role of polyadenylation have used the relative translation of the mRNA at

various times after injection as a measure of the stability of the mRNA. Using this criterion for stability, polyadenylation stabilizes globin (26,27) and histone mRNA (28) but has no effect on the stability of interferon (29,30) or -2U globulin mRNAs (31). In this paper we have directly measured the stability of radioactive poly(A)$^+$ or poly(A)$^-$ synthetic transcripts injected into oocytes by the combined use of electrophoresis, autoradiography and scintillation counting of the excised bands. The stability of all transcripts tested increased upon polyadenylation.

Translation of Synthetic Transcripts

Effect of Capping on Translation in Oocytes In addition to its role in stabilizing eukaryotic mRNAs (20,57), a cap structure is required for efficient translation of mRNA (20,57,59,60). In agreement with these previous reports we find that uncapped synthetic Lys$^+$ RNAs are used (about twenty-five times) less efficiently than monomethyl capped RNAs. Also the monomethyl cap RNAs are used five times more efficiently than the dimethyl cap RNA. This is surprising as it has been reported that oocytes will methylate injected, capped (but unmethylated) RNAs to form both the monomethyl and dimethyl capped pattern of methylation (20), thus raising the question whether the oocyte can regulate translation by such a differential methylation mechanism. Although the monomethyl cap and dimethyl cap RNAs differ in their ability to stimulate protein synthesis, we find them to have similar stabilities. This result conflicts with those of Huez et al. (61) which indicated that deadenylated globin mRNA was destabilized by translation, but fits in with our findings (not shown) that capped sense and antisense transcripts were similarly stable.

Effect of Polyadenylation on Translation in vivo Two general hypotheses for the role of poly(A) tails in the cytoplasm have been advanced. In one, the role of poly(A) is to stabilize mRNAs (27,21,25); in the other polyadenylation is implicated in regulating the efficiency of translation (32,33,62,63). Both hypotheses are satisfied by the results reported in this paper. We have found that polyadenylation by synthetic RNAs both confers stability and leads to a sometimes dramatic (20 fold) increase in translation in oocytes (the increase in translation being greater than the increase in stability). A similar, though less striking, effect has been noted by Deshpande et al. (31). We have not ascertained the point in the translational mechanism which is stimulated by the polyadenylated RNAs. It is possible that the poly(A)$^+$ synthetic RNAs can compete more efficiently with the endogenous poly(A)$^+$ mRNA for translational components than the poly(A)$^-$ RNAs. That the poly(A) tract itself might be responsible for this is indicated by our observation that co-

injection of poly(A) (25 ng/oocyte) and the synthetic RNAs inhibits the translation of the poly(A)$^+$-, but not the poly(A)$^-$-synthetic RNAs (Drummond and Colman, unpublished data).

Polyadenylation of synthetic RNAs appears to be essential for their translation in cultured cells (Fig. 6). Huez et al. (54) could detect no translation product after the injection of deadenylated globin mRNA into HeLa cells and attributed this to mRNA instability. We have not determined if the effect of polyadenylation is due to increased stability, increased translational efficiency, or both as in the oocyte. However, this result further extends the applications of synthetic RNA by offering a new method for transient expression of cloned cDNAs in cultured cells.

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