# PREPARATION OF COMPLEMENTARY DNA TO 5 S RIBOSOMAL RNA FROM *XENOPUS LAEVIS*

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## 1. Introduction

The 5 S rRNA genes of *Xenopus* provide a convenient system for study of the molecular mechanism of gene transcription and its control. The genes are abundant, each early oocyte containing nearly 100 000 copies in its 4C genome [1], and they can be isolated as a satellite from total DNA [2]. 5 S gene transcription is subject to control during normal oocyte development [3] and is carried out by a specific RNA polymerase [4], which retains its specificity in vitro [5]. The 5 S genes expressed in oocytes are different from those expressed in somatic cells [6,7].

Here we describe the preparation of radioactive DNA complementary to 5 S rRNA by reverse transcription and its use as a specific molecular probe for 5 S RNA sequences. We suggest potential uses for this cDNA in studies of 5 S RNA synthesis and accumulation during oogenesis in vivo and of 5 S gene transcription in vitro by RNA polymerase III. It may also be useful for studies with other animal species since it hybridizes to chicken 5 S RNA.

## 2. Materials and methods

### 2.1. *Materials*

*Xenopus laevis* were obtained from Xenopus Ltd., Redhill, Surrey, [2-$^3$H]adenosine 5′-triphosphate (16 Ci/mmol) and deoxy-[5-$^3$H]cytidine 5′-triphosphate (25.5 Ci/mmol) were obtained from the Radiochemical Centre, Amersham.

RNA:ATP adenyl transferase was prepared from 25 g *E. coli* B/r by the method in [8].

AMV reverse transcriptase was obtained from Dr J. W. Beard through Program Resources and Logistics of the US National Cancer Inst.

Oligo-(dT) cellulose (type T-3) was obtained from Collab. Res., and oligo($dT_{12-18}$) from Miles Biochem.

*E. coli* DNA, *E. coli* tRNA and deoxynucleoside-triphosphates were obtained from Sigma. Sephadex G-100 was obtained from Pharmacia.

### 2.2. *Isolation of 5 S RNA from* Xenopus laevis *ovary*

Adult *Xenopus laevis* ovary (100 g) was homogenized in 200 ml 10 mM $MgCl_2$–50 mM Tris–HCl (pH 7.6) using a Waring blender, followed by a Dounce hand-homogenizer. The homogenate was centrifuged at 10 000 × *g* for 10 min and the supernatant was centrifuged again at 100 000 × *g* for 2 h. The pellets from the second centrifugation were resuspended in 24 ml 0.5% SDS–130 mM NaCl–5 mM $MgCl_2$–20 mM Tris–HCl (pH 7.6) and extracted at room temperature with an equal volume of phenol equilibrated with 0.9% (w/v) NaCl. The phenol layer was re-extracted twice with 0.9% NaCl. The pooled aqueous layers were extracted twice with phenol and RNA was precipitated with 2.5 vol. ethanol at −20°C.

The RNA was redissolved in 50 mM NaCl and centrifuged at 10 000 × *g* for 10 min. The supernatant was adjusted to 1 M NaCl at 0°C, stirred for 30 min

*Abbreviations:* 5 S cDNA, complementary DNA to 5 S rRNA; globin cDNA, complementary DNA to rabbit globin mRNA; $R_0t$: [RNA conc. (μg/ml) × time (h)]/83; $R_0t_{½}$, value of $R_0t$ at half-maximal hybridization

and centrifuged again at 10 000 × *g* for 60 min to remove partially the 18 S and 28 S rRNA [9]. The pellets were re-extracted twice in the same way and the supernatants were pooled and precipitated with ethanol.

After Sephadex chromatography (fig.1), the purified 5 S RNA was dissolved in sterile, double glass-distilled water and its concentration determined accurately by absorbance measurements both before and after alkaline hydrolysis. Absorbances at 260 nm of a 1 mg/ml solution of RNA with a 1 cm pathlength were taken as 25 before and 32 after hydrolysis.

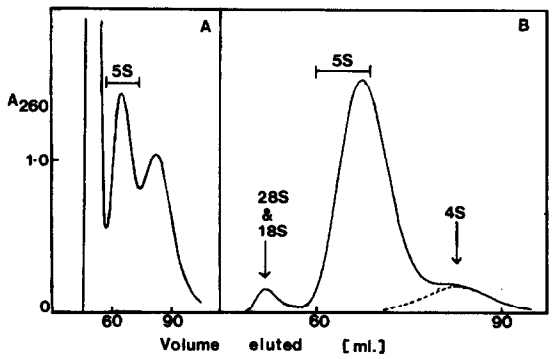


Fig.1. Isolation of 5 S RNA by Sephadex G-100 gel filtration. 5 S-enriched RNA (see section 2) was redissolved in 50 mM NaCl–5% sucrose and centrifuged at 2000 × *g* for 10 min. The supernatant, containing 6.8 mg RNA, was applied to a Sephadex G-100 column (80 × 1 cm) equilibrated at 4°C with 50 mM NaCl and eluted with 50 mM NaCl at 15 ml/h flowrate [9]. The 5 S fraction (A) was collected and precipitated with ethanol. The precipitate (1.44 mg) was redissolved in 50 mM NaCl and rechromatographed (B). The 5 S fraction indicated was collected, precipitated with ethanol and used in all subsequent studies.

2.3. *Synthesis of cDNA*

cDNA was prepared by the method in [10], as modified [11], and purified on alkaline sucrose gradients. Only the peak fractions shown in fig.2 were used and they were precipitated with ethanol in the presence of 60 μg of *E. coli* tRNA carrier and redissolved in sterile, double glass-distilled water. *E. coli* tRNA did not interfere with subsequent hybridization kinetics even at much higher final concentrations (100 μg/ml).

2.4. *Hybridization conditions*

Hybridizations were carried out at 70°C in 300–600 μl 0.24 M phosphate buffer (pH 6.8) as in [12]. Aliquots (25 μl) were removed at intervals and % hybrid was determined using S1 nuclease in the presence of 12.5 μg/ml *E. coli* DNA [12]. Each 25 μl aliquot contained 2000–5000 cpm c[$^3$H]DNA and S1 nuclease-treated zero-time backgrounds (<10% of input) were subtracted.

## 3. Results and discussion

3.1. *Preparation of 5 S rRNA and 5 S cDNA*

RNA was extracted with phenol/SDS (pH 7.6) from the cytoplasmic ribonucleoprotein pellet of adult *Xenopus* ovaries. This fraction includes a 42 S particle containing 5 S rRNA [3]. Large rRNAs were partially removed by 1 M NaCl precipitation and the remaining RNA was subjected to gel filtration on Sephadex G-100 [9]. The 5 S fraction (fig.1A) was precipitated with ethanol and rechromatographed (fig.1B). The heavy side of the 5 S peak (5SH) gave a single band on electrophoresis and was used in subsequent experiments. The light side of the 5 S peak (5SL) contained a faint additional 4 S band.

5S(H) RNA (100 μg) was polyadenylated at the 3′-end, using 50 units of freshly-prepared ATP–RNA adenyltransferase [8] for 5 min at 30°C in 1 ml final vol. These conditions were found to give an average poly(A) chain length of 29 nucleotides (table 1). The RNA was then extracted at pH 8.3 with chloroform/isoamyl alcohol in the presence of SDS, precipitated with ethanol and subjected to oligo(dT) cellulose chromatography. Over 50% of the 5 S RNA bound to oligo(dT) cellulose after this brief treatment and this could be increased to 75% by prolonging the enzyme treatment to 10 min (table 1).

The polyadenylated 5 S RNA eluted from the oligo(dT) cellulose was used directly for cDNA synthesis by the method in [10], using reverse transcriptase with oligo(dT) as primer. A similar method has been used to prepare cDNA to 18 S and 28 S ribosomal RNAs [13]. cDNA to rabbit globin mRNA was prepared at the same time. The rates of incorporation of d[$^3$H]CTP/μg RNA were similar for the two substrates.

Table 2 shows that 5S(H) RNA acts as a template for reverse transcriptase only after polyadenylation,

Table 1
Analysis of polyadenylation products by oligo(dT) cellulose chromatography

| Incubation conditions | Average poly(A) chain length (nucleotides) | Detection method | % Bound to oligo(dT) cellulose |
|---|---|---|---|
| 5 min at 30°C | 29 | $A_{260}$ | 53 |
| 10 min at 30°C | 53 | $A_{260}$ | 74 |
| | | Radioactivity | 79 |

Purified 5 S RNA (100 μg/ml) was incubated with freshly-prepared RNA–ATP adenyl transferase (50 units/ml) in 50 μl under the conditions in [8]. The average chain length was determined from the radioactivity incorporated from [$^3$H]ATP of known specific activity, assuming mol. wt 40 000 for 5 S RNA [3]. RNA eluted from an oligo(dT) cellulose column was detected either by $A_{260}$ or by radioactivity incorporated into the newly-synthesized poly(A) sequence

ruling out the possibility that we might be preparing cDNA to polyadenylated contaminants of 5 S RNA. On the other hand, both 5S(L) RNA and 4 S RNA do appear to contain traces of contaminants which act as templates for the enzyme. Only 5S(H) RNA was used in subsequent experiments.

After alkaline hydrolysis of the RNA, the cDNA was analysed on 5–20% alkaline sucrose gradients [14] using 4 S tRNA and 5 S ribosomal RNA from *Xenopus* as markers on parallel gradients. Globin cDNA ran at ~6–6.5 S, corresponding to a size of ~450 nucleotides, while 5 S cDNA ran at ~3–3.5 S (fig.2). This corresponds to ~80–140 nucleotides or between half- and full-length copy of the 120-nucleotide RNA, allowing for an additional 12–18 nucleotide oligo(dT) sequence. Specific activities were of the order of $10^7$ cpm/μg.

Table 2
Complementary DNA synthesis by reverse transcriptase

| Template | cpm incorporated per μg RNA (× $10^{-3}$) |
|---|---|
| 5S(H) RNA; poly ($A^+$) | 2964 |
| 5S(H) RNA; poly ($A^-$) | 0 |
| 5S(L) RNA; poly ($A^-$) | 80 |
| 4 S RNA; poly ($A^-$) | 200 |

Various RNA templates were incubated for 30 min at 37°C with 5 units of reverse transcriptase and 5 μCi of [$^3$H]CTP in 20 μl final vol. under the conditions in [11]. Reactions were terminated by the addition of 2 ml ice-cold 2% trichloroacetic acid and acid-insoluble radioactivity was determined by liquid scintillation. Background incorporation (no added template) was subtracted

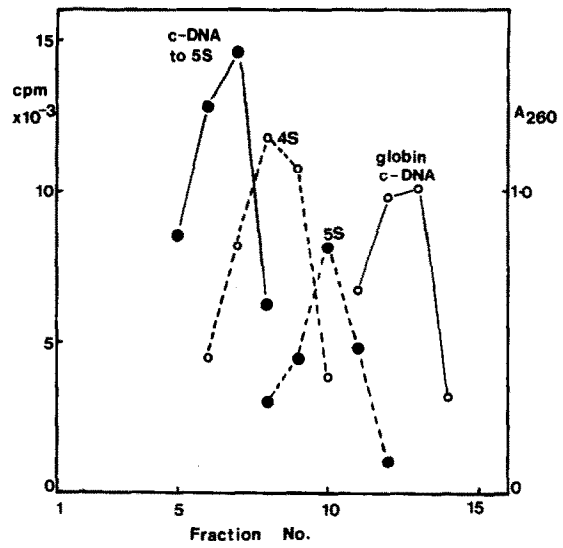


Fig.2. Isolation and approximate size determination of cDNAs on alkaline sucrose gradients. Samples were applied to 5–20% (w/v) linear sucrose gradients (5 ml) in 100 mM NaOH–1 mM EDTA–0.9 M NaCl [14] and centrifuged at 40 000 rev./min for 20 h in the Spinco SW 50.1 rotor at 10°C. *Xenopus* ovary 4 S and 5 S RNA markers were run on parallel gradients in 1 mM EDTA–0.9 M NaCl–50 mM Tris–HCl (pH 7.4). Fractions (0.3 ml) were collected and neutralized with acetic acid, where necessary. Samples (10 μl) from cDNA gradient fractions were precipitated with 5% trichloroacetic acid, filtered and counted by liquid scintillation (5 S cDNA (• - •); globin cDNA (○ - ○)). 4 S and 5 S peaks on RNA gradients were located by absorbance measurements: 4 S (○ — ○); 5 S (• — •). Approximate *S* values and molecular sizes of the cDNA were estimated using the relationship in [22], and a size of 470–530 nucleotides for 6.5 S DNA [11,23].

3.2. *Hybridization kinetics*

Kinetics of hybridization of both 5 S and globin cDNA to their respective RNAs are shown in fig.3. The $R_0t_{1/2}$ for the globin mRNA/DNA reaction was determined as $6.1 \times 10^{-4} \pm 0.3 \times 10^{-4}$ [3], which is similar to that obtained by others under these conditions [12]. The expected $R_0t_{1/2}$ for the 5 S RNA/cDNA reaction can be calculated from the following formula, assuming that the reaction rate is proportional to the square root of cDNA size [15,16]:

$$\text{Expected } R_0t_{1/2}\,(5\text{ S}) = R_0t_{1/2}\,(\text{globin mRNA}) \times 0.5 \times \frac{\text{mol. wt 5 S RNA}}{\text{mol. wt globin mRNA}} \times \sqrt{\frac{\text{mol. wt globin cDNA}}{\text{mol. wt 5 S cDNA}}}$$

The factor of 0.5 allows for rabbit globin mRNA consisting of two heterologous chains coding for α- and β-globin. Inserting known values for RNA sizes and 450 nucleotides for globin cDNA, we obtained $R_0t_{1/2}$ (5 S) values of $1.47 \times 10^{-4}$ and $1.56 \times 10^{-4}$ for 5 S cDNA sizes of 90 and 80 nucleotides, respectively. The observed value of $1.53 \times 10^{-4} \pm 0.03 \times 10^{-4}$ [4] falls within this range and is consistent with an average 5 S cDNA size of 84 nucleotides.

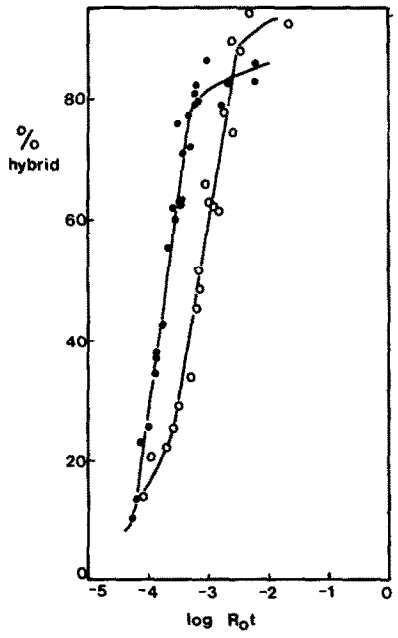


Fig.3. Hybridization kinetics of excess RNAs to their respective cDNA. The 5 S RNA–5 S cDNA curve (• - •) contains the combined points of 3 separate expt and the globin mRNA–globin cDNA curve (○ - ○), of 2 expt. Maximum hybridization levels for estimation of $R_0t_{1/2}$ were taken as 83% for 5 S cDNA and 92% for globin cDNA.

This calculation is based on the assumption that *Xenopus* ovary 5 S RNA behaves as a single species, rather than a mixture of non-crossreacting molecules, like the two globin mRNAs. The contribution of somatic 5 S RNA, which differs in ~7 bases from oocyte-specific 5 S RNA [6,7,17], to total ovary 5 S RNA is known to be negligible [18]. However, the oocyte-specific 5 S RNA is itself known to be heterogeneous, although the differences are in only a few bases [7]. In order to satisfy ourselves that these differences would not prevent crossreaction under these hybridization conditions, the 5 S cDNA was hybridized to total chick RNA from myoblast cell cultures, the 5 S RNA of which differs from *Xenopus* oocyte 5 S RNA in 16 bases [19]. The cDNA hybridized to this material to the same extent as to *Xenopus* ovary 5 S RNA (>80%; results not shown). The effect of these changes on the rate of hybridization, however, was not studied. Heterogeneities in oocyte 5 S RNA might have the effect of slowing down the RNA–cDNA hybridization quite appreciably [11,20]. If this were so, our determination of the $R_0t_{1/2}$ for 5 S RNA–cDNA hybridization would be higher than the value for a single 5 S RNA sequence and the estimate we have made of the cDNA size from this $R_0t_{1/2}$ would be too low.

These considerations, however, should not affect our use of the cDNA to measure 5 S RNA levels in oocyte extracts, since in each case we are comparing the unknown with our purified 5 S standard. This assumes only that the sequence composition of 5 S RNA of the unknowns is the same as in the purified standard. Somatic 5 S RNA from the follicle cells is present in RNA extracted from the whole ovaries, but at <1% [18].

3.3. *Applications*

We have used radioactive cDNA to measure 5 S RNA levels in isolated oocytes of different developmental stages. The % 5 S of total RNA can be determined in an extract of known total RNA concentration or the absolute amount of 5 S RNA per oocyte can be determined using RNA from a known number

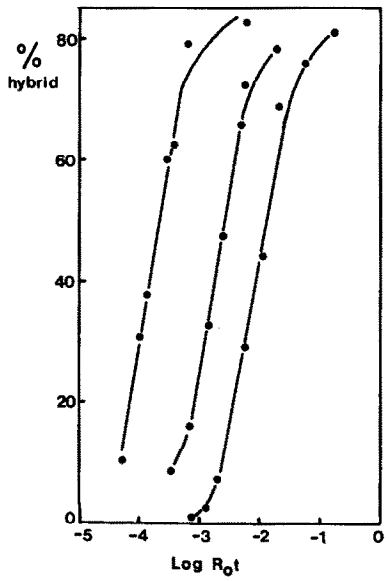


Fig.4. Hybridization of cDNA and 5 S RNA. Typical hybridization curves of 5 S cDNA with pure 5 S RNA (the first, most-rapidly hybridizing curve) and with 2 unknown RNA samples from isolated oocytes. The amount of 5 S RNA in these samples was determined, from the ratios of their $R_0t_{1/2}$ values to that of pure 5 S RNA [11], as 0.025 and 0.008 μg, respectively, per 300 μl hybridization.

of oocytes. The method can easily measure 0.01 μg, which is less than the content of a single early oocyte (fig.4).

Unlabelled 5 S cDNA could be used for the detection and isolation of radioactive newly-synthesized 5 S RNA or of in vitro RNA transcripts of any size containing 5 S sequences. Recently, *Xenopus* 5 S genes have been obtained by molecular cloning and their transcription has been studied by reinjection into nuclei of *Xenopus* oocytes [21]. In vitro transcription studies using 5 S cDNA as a probe will complement studies using directly-isolated, or cloned, 5 S DNA, which also contains 'spacer' DNA sequences.

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