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Purification of cDNA complementary to sea urchin histone mRNA

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ABSTRACT

Complementary DNA (cDNA) was transcribed from a polyadenylated sea urchin histone mRNA preparation isolated by density gradient centrifugation. By hybridization, this cDNA was shown to be extensively contaminated (85% of hybridizable cDNA) with DNA complementary to RNA derived from the large ribosomal subunit. Purification of a mRNA specific cDNA fraction was achieved by hybridization of purified rRNA to cDNA followed by fractionation on hydroxylapatite. After further purification to remove nonhybridizable cDNA our purified cDNA showed only 8% hybridization to rRNA.

INTRODUCTION

The identification of newly synthesized histone mRNA as a majority species among the polysome associated mRNA templates in the cleaving sea urchin embryo (1-5), has made possible the isolation and fractionation of highly labelled individual histone mRNAs (6,7). Hybridization of these preparations to total and fractionated sea urchin DNA has led to an understanding as to the number (8,9) and structural organization (10,11) of histone genes. For the assay of histone mRNA however, a purified cDNA is required.

The preparation of a cDNA to histone mRNA is complicated by a number of factors. Histone mRNA, unlike other mRNA molecules, does not contain the 3' terminal poly(A) tail (12) required for reverse transcription. After *in vitro* enzymatic polyadenylation (13) however, cDNAs have been prepared to HeLa cell histone mRNA (14,15) and human reticulocyte ribosomal RNA (16). The absence of the poly(A) tail leads to another, and more serious, problem : the contamination of histone mRNA preparations from both HeLa cells (18) and sea urchin (17) with rRNA sequences. These rRNA contaminants become polyadenylated *in vitro* and transcribed with the mRNA molecules, thus resulting in cDNA preparations contaminated with rRNA complementary

sequences (18). The existence of these difficulties probably explains why the preparation of a purified cDNA to sea urchin histone mRNA has not been reported earlier.

In this paper we give evidence as to the extent of rRNA contamination of our histone mRNA preparation, and describe a method for the purification of a sea urchin histone mRNA-specific cDNA.

MATERIALS AND METHODS

AMV reverse transcriptase was generously supplied by Dr. J.W. Beard (Life Science Inc., Florida). All radioactive chemicals were obtained from the Radiochemical Centre, Amersham; S_1 nuclease (A. oryzae), M. lysodeikticus DNA and unlabelled deoxyribonucleoside triphosphates from Miles Laboratories; rat liver RNase inhibitor from Searle Biochemicals; oligo(dT) cellulose and oligo$(dT)_{12-18}$ from Collaborative Research, and actinomycin D from P.L. Biochemicals. All other chemicals were Analar (or equivalent) grade, and solutions used in the preparation of mRNA were rendered RNase free by treatment with diethylpyrocarbonate. Sea urchins, Parechinus angulosus, were collected on the Atlantic coast, 30 Km north of Cape Town.

Isolation and Characterization of Histone mRNA

Polysomes were isolated from early blastula embryos as described by Gross et al. (19). The polysomes were suspended in 2.5 mM Tris-HCl pH 7.4, 0.5% SDS and extracted with water saturated phenol. The polysomal RNA was precipitated with ethanol, dissolved in 10 mM Tris-HCl pH 7.4, 1% SDS, heated to 60°C for 10 min (20) and fractionated on 15-30% sucrose gradients (10 mM Tris-HCl pH 7.4, 1% SDS at 20°C, Beckman rotor SW 40Ti) at 281 000 x g for the times indicated in legend to Fig. 1. Rat liver ribosomal RNA and yeast tRNA were used in RNA markers to standardize the sucrose gradients. The 9S RNA was pooled, precipitated with ethanol and redissolved in distilled water. The 9S mRNA was translated in an ascites cell free system as described by Jacobs-Lorena et al. (21) using ^{3}H-lysine (10 µCi/ml) as radioactive precursor. After addition of 20 µg of sea urchin sperm histones (isolated by acid extraction) as carrier, the reaction mixtures were extracted with 0.2 M H_2SO_4, and the acid soluble fraction precipitated with acetone (-20°C overnight). Precipitates were dissolved in 8 M urea 0.5 M mercaptoethanol and analysed on 15% acid-urea polyacrylamide gels as described by Panyim and Chalkley (22). After staining in

amido black, gels were scanned (Vitatron TLD 100), sliced (1.5 mm slices) and the radioactivity per slice determined after oxidation (Packard Sample Oxidizer).

Isolation of large and small ribosomal subunit RNA

Polysomes were isolated from 18 hour sea urchin embryos (early gastrula) as described by Gross et al. (19), dissociated by suspension in 50 mM Tris-HCl pH 7.6, 10 mM EDTA, 10 µg/ml polyvinylsulphate, and the ribosomal subunits fractionated on 15-30% sucrose gradients at 281 000 x g (SW 40Ti rotor) for 7 hours. The subunits were precipitated with ethanol, dissolved in NETS buffer (0.1 M NaCl, 10 mM Tris-HCl ph 7.4, 1 mM EDTA, 0.2% SDS) and the RNA extracted with phenol:chloroform (1:1).

Polyadenylation of histone mRNA

ATP-polynucleotidyltransferase was isolated from maize seedlings essentially as described by Mans and Huff (13), except that their final purification step on glycerol gradients was omitted. Histone mRNA (100 µg/ml) was incubated with ATP-polynucleotidyltransferase (900 µg enzyme protein/ml) at 30°C for 1 h, in the presence of 70 mM Tris-HCl pH 8.8, 1 mM $MnCl_2$, 10 mM dithiothreitol, 10 mM [^{3}H]-ATP (10 µCi/mM) and 100 units/ml of rat liver RNase inhibitor. The reaction was stopped by addition of an equal volume of NETS buffer and the RNA extracted with an equal volume of phenol:chloroform (1:1). The RNA was precipitated with ethanol, dissolved in a small volume of NETS buffer and fractionated on a 15-30% sucrose gradient (in NETS) at 281 000 x g (SW 40Ti rotor) for 26 h. The RNA sedimenting at 9S was pooled, precipitated with ethanol and dissolved in sterile distilled water.

Preparation of cDNA

cDNA was synthesized using essentially the method of Harrison et al. (23) with slight modifications. Polyadenylated mRNA (10 µg) was incubated at 37°C for 90 min in a final volume of 100-200 µl containing 400 µM each of dATP, dTTP and dGTP, 0.1 mCi/ml [^{3}H]-dCTP (23 Ci/mmole), 5 units/ml oligo$(dT)_{12-18}$, 50 µg/ml actinomycin D, 100 units/ml rat liver RNase inhibitor, 1000 units/ml reverse transcriptase, 50 mM Tris-HCl pH 8.2, 50 mM KCl, 10 mM dithiothreitol and 5 mM magnesium acetate. The reaction was stopped by addition of EDTA to a final concentration of 10 mM and 100 µg E.coli RNA was added as carrier. Unpolymerised nucleotides were removed by chromatography on Sephadex G-50, the RNA hydrolysed with 0.3 M NaOH for 16 h and the cDNA isolated by precipitation with ethanol followed by

chromatography on Sephadex G-50. The Sephadex (0.5 x 25 cm) was packed on a 1 cm pad of Dowex chelating resin (Sigma).

RNA:cDNA hybridization

RNA:cDNA hybridizations (in 2-20 µl), and analysis of hybrids by S_1 nuclease digestion were performed as described by Getz et al. (24). All reactions were carried out in duplicate.

Hydroxylapatite chromatography

Hydroxylapatite (Biogel HTP) columns (volume 1-2 ml) were prepared and fractionations carried out at 60°C (25). Prior to fractionation 100-200 µg of native M.lysodeikticus DNA was applied and the column washed with sodium phosphate buffers pH 6.8 (0.03 M, 0.12 M and 0.4 M). Hybridization reactions were stopped by rapid cooling to -20°C and diluted 20 x with 0.15 M NaCl. The sample was applied to the hydroxylapatite column and three fractions were eluted with 0.03 M, 0.12 M and 0.4 M sodium phosphate buffers, pH 6.8, respectively. The required fraction was desalted on a Sephadex G-25 column, the RNA hydrolysed, and the cDNA reisolated as described above.

RESULTS AND DISCUSSION

Isolation and characterization of histone mRNA

The method used for the phenol extraction of polysomal RNA (low pH, low ionic strength) was chosen in order to preferentially isolate poly(A)$^-$ RNA (26). After centrifugation of the polysomal RNA on sucrose gradients a peak of 9S material was present (Fig. 1a) which was pooled and purified on a second sucrose gradient (Fig. 1b). When analysed on a standardized sucrose gradient the pooled RNA sedimented as a uniform peak of 8.8 - 9S, and was shown, by chromatography on oligo(dT) cellulose (27), to contain less than 3% poly(A)$^+$ RNA in agreement with the results of Fromson and Duchastel (28). This 9S RNA preparation supported the synthesis of major sea urchin histones (Fig. 2) when incubated in as ascites cell free system and is therefore referred to as 9S histone mRNA. The apparent absence of histone H2A synthesis can be explained by the slower migration of embryo H2A compared with sperm H2A (unpublished observations). We have not excluded the possibility of low levels of contamination with other non-polyadenylated mRNAs in this histone mRNA preparation, but it has been shown that sea urchin 9S polysomal RNA does not support the *in vitro* synthesis of any proteins except histones (19,29).

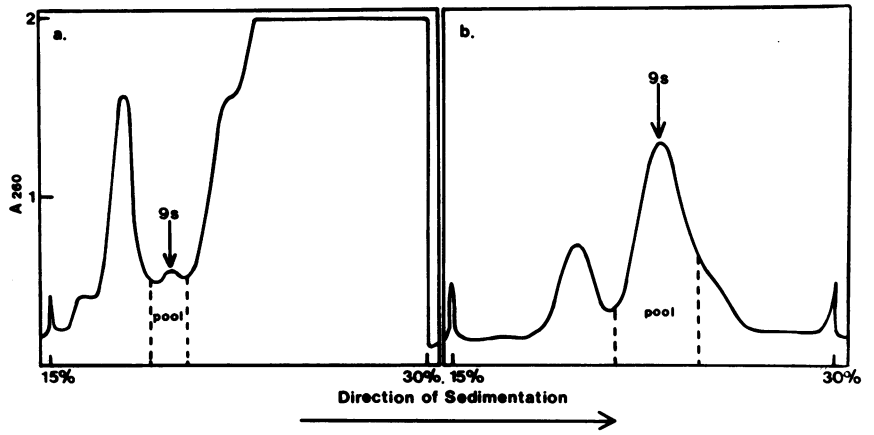

Fig. 1 : Isolation of 9S histone mRNA by sucrose density gradient centrifugation. a) Total sea urchin polysomal RNA fractionated on 15-30% gradients by centrifugation for 17 h. b) Pooled 9S RNA fraction from (a) refractionated on a 15-30% gradient for 22 h.

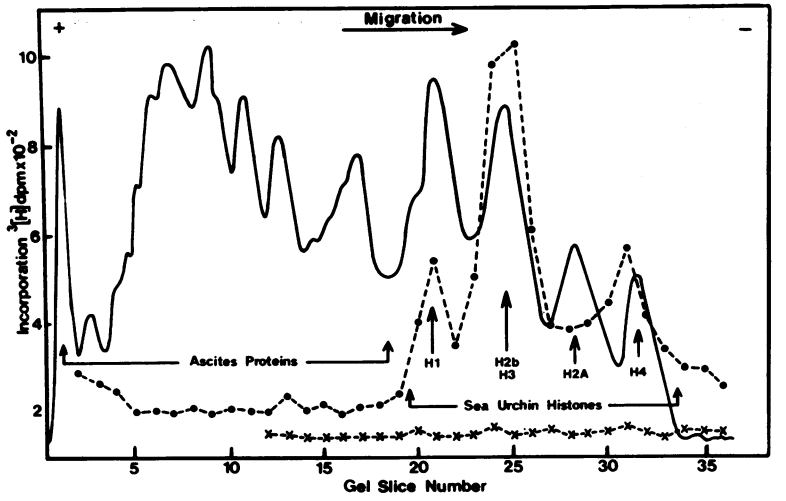

Fig. 2 : Analysis of products of cell free translation of 9S mRNA on 15% acid-urea polyacrylamide gels. Sea urchin sperm histones were added as carrier. —— optical density; ●----● radioactivity of [^{3}H]-labelled proteins synthesised in the presence of 9S mRNA; x——x incorporation in the absence of added mRNA.

Polyadenylation and transcription

Low molecular weight degradation products produced during polyadenylation were removed by sucrose density gradient fractionation (Fig. 3). The inclusion of the rat liver RNase inhibitor into the polyadenylation

incubation mixture was shown to substantially increase the amount of 9S RNA remaining after polyadenylation and to have no effect on the activity of the ATP-polynucleotidyltransferase. The yield of 9S RNA was 30-40% of the input RNA.

The polyadenylated 9S histone mRNA was very effective as a template for reverse transcriptase; the yield of cDNA synthesized being 15-20% of the input polyadenylated 9S mRNA. The cDNA was analysed on a standardized 15-30% sucrose gradient and shown to sediment as a broad peak at 6.5S (Fig. 4). The specific activity of the cDNA was calculated to be

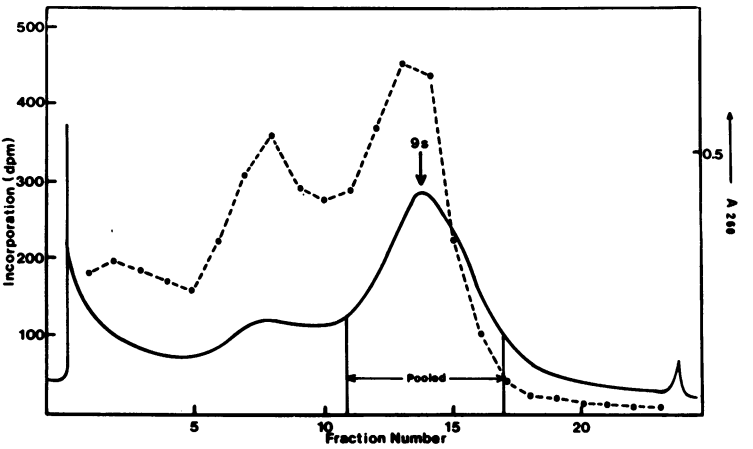

Fig. 3 : Fractionation of polyadenylated RNA on 15-30% sucrose gradients ——— A_{260}; ●----● [^{3}H]-AMP incorporation.

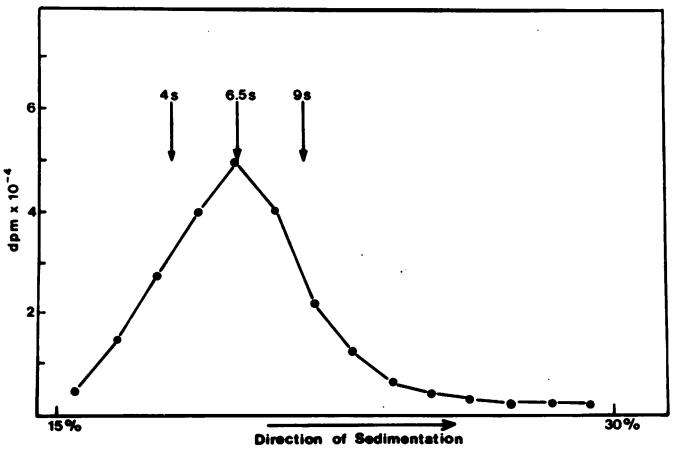

Fig. 4 : Analysis of complementary DNA to histone mRNA on standardized 15-30% sucrose gradients (in NETS buffer), ●——● dpm of [^{3}H]-labelled cDNA.

36.1 x 10^6 dmp/µg assuming that the cDNA consists of approximately 25% dCMP (nucleotide analysis of [^{32}P]-labelled 9S mRNA from several species of sea urchin shows a G+C content of 50-56% (30).

Hybridization of cDNA

The cDNA preparation was hybridized back to histone mRNA (unpolyadenylated) in RNA excess and 72% became hybridized. The remaining 28% is referred to as nonhybridizable cDNA. 5-10% of the cDNA was resistant to S_1 nuclease digestion even in the absence of RNA (background hybridization) which indicates the presence of double stranded regions of cDNA. Similar observations have been made with other systems (23).

The kinetics of the hybridization of cDNA to histone mRNA are shown in Fig. 5. The reaction proceeded with a $C_o t_{\frac{1}{2}}$ value of 2.7 x 10^{-2} mole sec/litre. Using the kinetic standard, globin mRNA to cDNA hybridization (31), the base sequence complexity was calculated (24) to be 8100 nucleotides which is about 3 x the expected complexity if five histone mRNAs are present. Stein et al. (33) have shown a complexity of twice the expected value for HeLa cell histone mRNA. Either the histone mRNA is contaminated with other RNA species (e.g. rRNA) or there may be many more than 5 histone

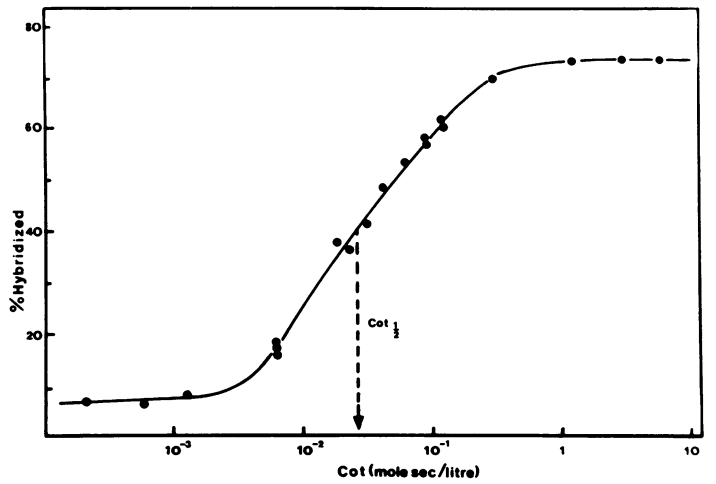

Fig. 5 : Kinetics of the [^{3}H]-cDNA:mRNA annealing reaction. Hybridizations were carried out using 0.2 ng of cDNA per 20 µl incubation. The mRNA concentration (0.05 - 50 µg/ml) and the time of incubation (1-20 h) were varied to obtain the range of $C_o t$ values used. Hybrids were analysed by S_1 nuclease digestion (24).

mRNAs present, both of which could contribute to the high complexity (amino acid sequence analysis of sea urchin histones shows that there are in fact more than 5 histones present in P. angulosus (33,34).

Titrations of cDNA to increasing amounts of ribosomal RNA, isolated from the large and small ribosomal subunits (Fig. 6), showed that the cDNA (and therefore the mRNA) was highly contaminated with DNA complementary to rRNA, and in particular to RNA sequences of the large ribosomal subunit. The RNAs isolated from the large and small ribosomal subunits showed, by polyacrylamide gel electrophoresis, a low level of cross contamination which could explain the high RNA:cDNA ratio required for saturation with the small subunit RNA preparation. This observation, together with the monophasic nature of the saturation hybridization reaction of cDNA:large ribosomal subunit RNA (Fig. 6) indicates that the level of 18S rRNA contamination in the histone mRNA was very low.

A comparison of the $C_o t$ curves of mRNA:cDNA and large subunit RNA:cDNA hybridizations (Fig. 7) shows that 85% of the hybridizable cDNA (61% of the total cDNA) was complementary to rRNA. Therefore only 15% of the hybridizable cDNA was complementary to mRNA indicating that only 15% of our 9S histone mRNA preparation was mRNA. Contamination of histone mRNA and therefore cDNA prepared from HeLa cells has been described by Boss et al.

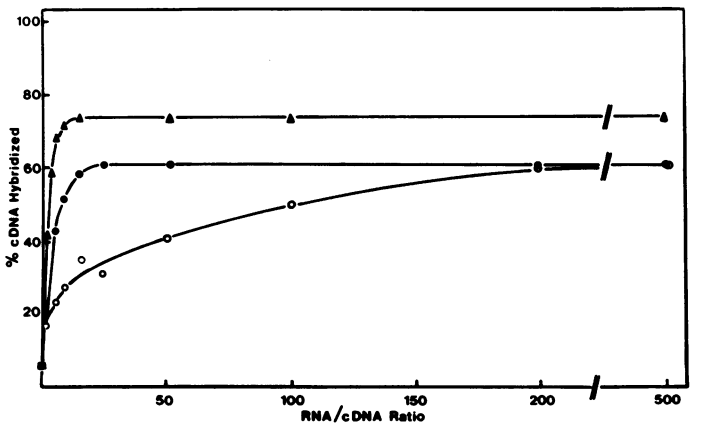

Fig. 6 : Titration of [^{3}H]-cDNA to mRNA (▲——▲), large ribosomal subunit RNA (●——●) and small ribosomal subunit RNA (o——o). Reaction mixtures containing 0.5 ng of [^{3}H]-cDNA were incubated to a $C_o t$ value of 0.3 mole sec/litre (> 100 h) with increasing amounts of RNA and the hybrids were analysed by S_1 nuclease digestion. Background hybridization values are shown (■).

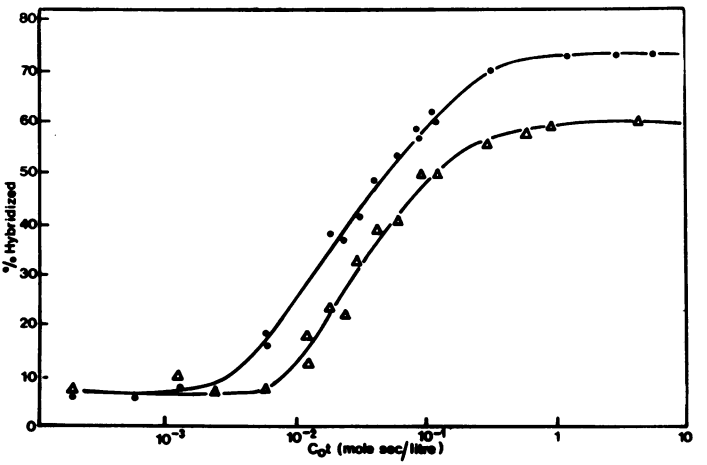

Fig. 7 : A comparison of the kinetics of [^{3}H]-cDNA:mRNA. (●——●) and [^{3}H]-cDNA : large ribosomal subunit RNA (▲——▲) annealing reactions carried out as described in the legend to Fig. 5.

(18). Stein and coworkers (32), also working with HeLa cells, have prepared a cDNA which shows no hybridization to rRNA indicating a rRNA free mRNA preparation. The contamination of our mRNA preparations with large ribosomal subunit RNA could be due to breakdown products of the 28S rRNA which would be released from the 28S fraction by the heat treatment to which polysomal RNA is subjected prior to sucrose gradient fractionation (60°C for 10 min). 28S rRNAs extracted from ribosomes of a number of tissues have been shown to contain intramolecular "nicks" (caused by nuclease activity during isolation) whereas these do not occur in the 18S rRNAs indicating that they are not as susceptible to nuclease activity (35). This would explain the absence of small subunit RNA contamination in our mRNA preparation.

The isolation of *in vivo* labelled histone mRNA from cleaving sea urchin embryos and its use in hybridization experiments are not complicated by rRNA contamination because of the absence of rRNA synthesis during the cleaving stages. The labelled mRNA preparations are contaminated with unlabelled rRNA (17) but this does not affect the hybridization experiments carried out with these labelled mRNA preparations.

The preparation of a pure histone mRNA specific cDNA, for the analysis of histone mRNA, could be achieved in two ways : either by isolation of a mRNA preparation free of rRNA contamination, or by purifying the mRNA

specific sequences from a cDNA prepared from contaminated mRNA. Owing to the difficulties of separating poly(A)$^-$ histone mRNA from rRNA, we have approached the problem by the latter method, removing complementary rRNA contaminants by fractionation of rRNA:cDNA hybrids on hydroxylapatite and isolating the single stranded cDNA.

Fractionation of RNA:cDNA hybrids by hydroxylapatite chromatography

A comparison of results obtained from the analysis of hybrids by S_1 nuclease digestion and hydroxylapatite chromatography are shown in Table 1. In all cases, the percentage double stranded material apparent by hydroxylapatite chromatography is higher (11%) than that by S_1 nuclease digestion. This can be explained by the presence of single stranded "tails" of non-hybridized cDNA attached to double stranded regions. The recovery of cDNA after hydroxylapatite chromatography was in all cases greater than 90%. cDNA isolated from mRNA:cDNA hybrids, purified on hydroxylapatite, became 82% S_1 nuclease resistant (maximum possible nuclease resistance is 86%, see Table 1) when hybridized back to mRNA, showing that cDNA purified on hydroxylapatite and reisolated as described was not affected in its capacity to form stable hybrids.

TABLE 1 : Analysis of hybridization reactions by S_1 nuclease digestion and hydroxylapatite fractionation. The values given show average results of several experiments.

Hybridization reaction	% cDNA hybridized	
	S_1 nuclease digestion	Hydroxylapatite fractionation
cDNA:mRNA	72.0	83.5
cDNA:rRNA	61.4	72.8
cDNA:no RNA	6.8	17.0

Purification of histone mRNA specific cDNA

The ability to fractionate single stranded DNA from DNA:RNA hybrids by hydroxylapatite chromatography has made possible the purification, from our total cDNA preparation, of a histone mRNA specific cDNA. cDNA was incubated with sea urchin large ribosomal subunit RNA (RNA:cDNA ratio = 50; $C_o t$ 10 mole sec/litre) and the hybrids fractionated on hydroxylapatite. The single stranded material (20-25%), containing mRNA specific and non-hybridizable cDNA, was pooled, and the cDNA reisolated, and hybridized to histone mRNA

(RNA:cDNA ratio = 50; $C_o t$ 10 mole sec/litre). The resulting double stranded material (approximately 40%) was isolated by chromatography on hydroxylapatite. The cDNA isolated from this fraction is referred to as histone mRNA specific cDNA ($cDNA_{his}$). The possible existence of self complementary regions in the $cDNA_{his}$ could result in loss of $cDNA_{his}$ by isolation with the double stranded cDNA:rRNA hybrids on hydroxylapatite. However this seems unlikely in view of the results shown in Table 1 where 11% of the total cDNA preparation is shown to be histone mRNA specific when analysed by both hydroxylapatite and S_1 nuclease digestion.

The hybridization of $cDNA_{his}$ to different RNA preparations (RNA:cDNA ratio = 5000) is shown in Table 2. The low level of hybridization, 49%, when $cDNA_{his}$ was hybridized to mRNA could be due in part to the small average size of $cDNA_{his}$ which was determined as 4.5S (15-30% sucrose gradient in NETS buffer). The preparation showed only 8% hybridization to rRNA which corresponds to 16% of the hybridizable cDNA. In other words, the fraction of

TABLE 2 : Hybridization of total and purified cDNA to mRNA and rRNA determined by S_1 nuclease resistance.

Hybridization reaction	% cDNA Hybridized	% of Hybridizable cDNA	% Hybridizable cDNA specific for mRNA
cDNA:mRNA	72	100	
cDNA:rRNA	61	85	15
cDNA:no RNA	7	10	
$cDNA_{his}$:mRNA	49	100	
$cDNA_{his}$:rRNA	8	16	84
$cDNA_{his}$:no RNA	2	4	

hybridizable cDNA which is specific to histone mRNA has been increased from 15% to 84% (Table 2) making the $cDNA_{his}$ very much more suitable as a probe for the qualitative and quantitative analysis of histone mRNA in sea urchin eggs and embryos.

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