Rapid Purification of Biologically Active Individual Histone Messenger RNAs by Hybridization to Cloned DNA Linked to Cellulose†

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ABSTRACT: We describe a rapid and simple method for the purification of biologically active messenger RNAs. The method allows the isolation in a few hours of specific mRNAs from either whole cell or polysomal RNA even if the RNA represents less than 1% of the starting molecules. We used, as a model, cloned sea urchin (*Strongylocentrotus purpuratus*) histone gene fragments linked to cellulose by the method of B. E. Noyes & G. R. Stark ((1975) *Cell 5*, 301–310) as hybridization probes to isolate specific histone mRNAs from whole cell and polysomal RNA extracts. RNAs isolated in this manner maintain their biological activity, serving as templates for histone proteins in a wheat-germ, cell-free protein translation system. In addition, radiolabeled histone-specific RNA purified from cleavage stage sea urchin embryos, pulse labeled for short periods of time and analyzed on denaturing polyacrylamide gels, was the same size as mature histone mRNAs.

The quantitation and isolation of specific mRNAs is an essential prerequisite to a detailed understanding of the mechanisms which regulate gene activity. Most techniques used for the isolation of specific eukaryotic mRNA molecules have relied on an RNA's size and poly(A)[1]content. A more specific isolation procedure would depend on an RNA's unique nucleotide sequence. A hybridization method for preparative recovery of mRNA molecules as well as their quantitative measurement in solution requires an excess of homologous DNA. Until recently, recovery of RNA molecules by molecular hybridization has been restricted to circumstances in which large amounts of complementary DNA were available as in the case of viral genomes used to isolate viral mRNAs (Noyes & Stark, 1975; Shih & Khoury, 1976; Lewis et al., 1975) and situations in which very abundant poly(A)-containing mRNAs, such as globin mRNA, could be used as templates for the synthesis of large amounts of complementary DNA (Levy & Aviv, 1976; Wood & Lingrel, 1977). In some cases, DNA which is used to hybridize with the mRNAs has been covalently coupled to an insoluble support such as cellulose or Sepharose particles (Noyes & Stark, 1975; Shih & Khoury, 1976; Levy & Aviv, 1976; Wood & Lingrel, 1977). The purpose of this communication is to describe the advantages and utility of using cloned genes to isolate and examine specific mRNAs. We have utilized a procedure developed by Noyes & Stark (1975) to couple cloned sea urchin histone DNA to diazotized cellulose particles. The tandemly linked histone-coding DNA sequences of sea urchins appear to be of particular value for study of eukaryotic gene regulation. During the phase of rapid cell division found in early sea urchin embryos, histone proteins are synthesized in large quantities (Kedes & Gross, 1969). At this stage of development, histone RNAs are the most abundant mRNAs found on polysomes (Kedes & Gross, 1969; Nemer, 1975) as well as in newly made nuclear RNA (unpublished observations). Because they are so abundant, mRNAs coding for each of the five histone proteins have been resolved and purified by classical procedures using repeated gel electrophoresis under varying conditions (Levy et al., 1975; Grunstein et al., 1973; Gross et al., 1976a; Grunstein & Schedl, 1976). These purified RNAs can be radiolabled and sequenced (Grunstein et al., 1973; Grunstein & Schedl, 1976) and can act cell-free template for the synthesis of a single histone protein in cell-free protein-synthesizing systems (Levy et al., 1975; Grunstein et al., 1973; Gross et al., 1976a). Isolation of individual histone mRNAs by gel electrophoresis is time consuming and relies solely on the RNA's size and/or secondary structure. For example, because they are similar in size, it has been difficult to resolve H3, H2a, and H2b mRNAs from one another. We have found that use of individual histone DNA–cellulose probes allows a more rapid extraction procedure resulting in purer RNAs.

Hybridization of individual histone mRNAs to isolated, cloned segments of the histone gene repeat unit (Kedes et al., 1975a), electron microscopic analysis of heteroduplexes between histone mRNA and cloned histone DNA (Wu et al., 1976; Holmes et al., 1977), as well as direct DNA sequencing of several thousand nucleotide base pairs within these genes (Sures et al., 1976, 1978) have elucidated the basic organization of the *S. purpuratus* histone genes (Figure 1). The genes for the five major histone proteins are intermingled with one another and are separated by spacer sequences in a 6.54-kbp repeat unit. The order of the genes within this repeat unit and the transcription of all five mRNAs from only one DNA strand seem to be identical in all sea urchin species examined (Cohn et al., 1976; Gross et al., 1976b). Cloned DNA containing a single histone gene (H2a, H2b, or H4) as well as defined restriction endonuclease fragments corresponding to histone gene H1 or H3 (Figure 1) are readily isolated in large amounts and represent probes for detection and isolation of each individual histone mRNA. In this report, we describe the specificity of various histone DNA–cellulose probes used for selective purification of individual polysomal histone mRNAs and pulse labeled RNAs containing sequences complementary to histone genes. The technique is of general use for rapid purification of an intact, biologically active RNA

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[1] Abbreviations used: poly(A), poly(adenylic acid); EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; SSC, 0.15 M sodium chloride, 0.015 M sodium citrate; RNase, ribonuclease; kbp, kilobase pairs; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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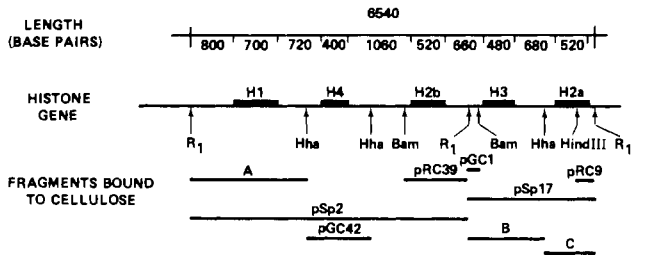

FIGURE 1: Map of the *S. purpuratus* histone genes. The organization of coding and spacer sequences of one tandem repeat of the five histone genes as determined by hybridization (Kedes et al., 1975a; Cohn et al., 1976) and electron microscopic analysis of heteroduplex structures (Wu et al., 1976; Holmes et al., 1977) is shown. The restriction endonuclease cleavage sites shown were used to generate specific histone DNA fragments subsequently coupled to cellulose particles. Those fragments which are identified as plasmids are separate clones, while fragments A, B, and C were derived by appropriate restriction endonuclease cleavage from longer cloned histone DNAs.

transcript from any cloned eukaryotic gene.

Materials and Methods

Culture of Sea Urchins and Isolation of Polysomal RNA. S. purpuratus were obtained from the University of California Bodega Bay Marine Station (Bodega Bay, Calif.). The embryos were grown at 15° C for 9 h to the early blastula stage of development in phosphate-depleted sea water containing 25 μCi/mL of [5-^{3}H]uridine (New England Nuclear, 27 Ci/mM). The RNA was isolated from polyribosomes (Kedes & Gross, 1969) and stored in 70% ethanol at −20 °C.

Isolation of Whole Cell RNA. S. purpuratus embryos (9 h) were pulse labeled in a 5% suspension (5 mL of packed eggs/100 mL of sea water) for 10 min with [5-^{3}H]uridine (100 μCi/mL). At the end of the pulse, the embryos were quickly chilled and washed twice in calcium–magnesium free sea water. The washed pellet of embryos was immediately disrupted and Dounce homogenized in 5 volumes of $NaDodSO_4$ buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 100 mM NaCl, 0.5% $NaDodSO_4$). This solution was then phenol extracted three times, chloroform extracted, ethanol precipitated, and used immediately or stored in H_2O at −20 °C.

Isolation of Plasmid DNA. E. coli (C600 rk$^-$ mk$^-$) strains containing plasmids pSp102, pSp117, pRC9, and pRC39 were grown in L broth, and covalently closed circular plasmid DNA was prepared (Cohen & Miller, 1970; Sures et al., 1976). The DNA was then digested with appropriate restriction enzymes (Kedes et al., 1975a; Sures et al., 1976; Cohn et al., 1976). Fragments of histone DNA were resolved by 0.8% agarose or 6% acrylamide gel electrophoresis (Kedes et al., 1975 a,b) and DNA was electrophoresed from excised bands.

Polyacrylamide Gel Electrophoresis of Histone mRNA. Electrophoresis in 6% acrylamide gels (1.5 × 100 mm) was carried out in E/10 buffer as previously described (Kedes et al., 1975a). Four percent formamide–polyacrylamide gels were polymerized as described by Pinder et al. (1974), using as running buffer 0.025 M diethylbarbituric acid, sodium diethylbarbiturate (Beckman-buffer B-2). Ethanol precipitates of RNA were collected by centrifugation, washed two times with 80% ethanol, and dried by lyophilization. Dry RNA pellets were dissolved in buffered formamide (Pinder et al., 1974), heated for several minutes at 65 °C, and electrophoresed at 25 mA for 5.5 h.

Diazotization of Aminocellulose and Nucleic Acid Coupling. m-Aminobenzyloxymethylcellulose (Miles Laboratories) was diazotized by the method of Noyes & Stark (1975) with the following modifications.

(1) Methyl sulfoxide (MCB Spectroquality) was used instead of distilled material. It was stored in aliquots at −20 °C.

(2) Just prior to addition of DNA to diazotized cellulose, the DNA was heated for 5 min at 70 °C and quickly chilled in 80% Me_2SO, 40 mM borate buffer, pH 8.0.

(3) When an entire closed circular plasmid chromosome was coupled to cellulose, it was first converted to linear form by digesting with a restriction endonuclease (purchased from New England Biolabs), phenol extracted, ethanol precipitated, and dissolved in 0.2 M borate buffer, pH 8.0. pSp102, pSp117, and pRC9 were digested with *Eco*RI, while pRC 39 was digested with *Bam*I.

(4) Diazotization was monitored by placing a small amount of the diazotized cellulose (after the final wash) in a saturated solution of β-naphthol in 0.2 M borate, pH 8.0. The cellulose particles should turn bright orange. *m*-Aminobenzyloxymethylcellulose stored for long periods of time lost its capacity to bind β-naphthol.

Binding was monitored by Cerenkov counting of trace amounts of ^{32}P "nick-translated" plasmid DNA (Maniatis et al., 1976). Binding of 40–60% of input DNA was usually obtained. Recently, at the suggestion of D. Kemp, we have obtained greater efficiency and better reproducibility of binding by carrying out the binding reaction of pH 6.5 in 25 mM potassium phosphate buffer.

DNA–RNA Hybridization. Hybridization was performed at 52 °C in 50% formamide, 10 mM Tris-HCl, pH 7.4, 0.6 M NaCl, 1 mM EDTA, 0.1% $NaDodSO_4$ containing 1 mg/mL yeast tRNA. RNA dissolved in hybridization buffer was mixed into a pellet of DNA–cellulose in 1.5-mL Eppendorf microcentrifuge tubes and incubated in a Haake water bath in racks which permitted the bottom of the tube to be gently agitated by the flow of the heating water. This allowed the cellulose to remain in a uniform suspension when the volumes were about 0.2 mL or less. Following the hybridization reaction, the cellulose was washed at 52 °C in hybridization buffer for 10 min and then three times more at room temperature followed by two washed with 1 mL of 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate). The hybridized RNA was eluted by resuspending the cellulose in 99% formamide and heating to 60 °C for 2 min. The heat eluting step was repeated and the pooled eluates were either ethanol precipitated for further analysis or Cl_3CCOOH precipitated. Following the elution step, the cellulose is regenerated for later use by one wash with formamide followed by another wash with hybridization buffer. After centrifugation the cellulose is again resuspended in hybridization buffer and stored at −20 °C. Some preparations have been stored for as long as 1 year without any apparent changes in hybridization properties. For unknown reasons, a few preparations have lost activity in much shorter times.

In some cases, RNase was used to further reduce nonspecific hybridization. Following the second 2 × SSC wash, cellulose was resuspended in 2 × SSC (1 mL) containing 10 μg/mL of RNase A (Sigma) and incubated at 24 °C for 30 min. Following centrifugation, the cellulose was suspended in 1 mL of 0.1 M sodium acetate, pH 5.5, 0.15 M iodoacetate (Bovre & Szybalski, 1971) at 57 °C for 40 min. The cellulose was then washed two times with 1 mL of 2 × SSC, and the RNA hybrids were recovered as previously described. We have not attempted to reuse iodoacetate-treated DNA–cellulose.

Cell-Free Protein Synthesis. Wheat germ S-30 extracts were prepared by the method of Roberts & Patterson (1973). Reaction mixtures contained in a final volume of 20 μL: 60 μg of preincubated wheat germ S-30 extract, 20 mM Hepes

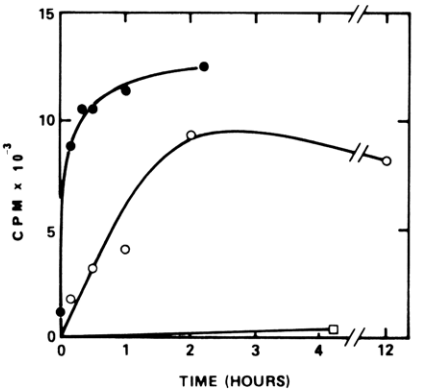

FIGURE 2: Kinetics of hybridization of polysomal RNA to histone DNA–cellulose. Identical amounts of [^{3}H]uridine-labeled polysomal RNA (65 000 cpm) from late blastula stage embryos were hybridized for various lengths of time to pSp2 DNA–cellulose (21 μg of eukaryotic mRNA coding strand DNA/mL) (●), pSp17 DNA–cellulose (2 μg of eukaryotic mRNA coding strand DNA/mL) (○), or *E. coli* DNA–cellulose (48 μg of total DNA/mL) (□). At the end of the hybridization, samples were chilled, and then processed as described in Materials and Methods. Eluted RNA was precipitated with cold 10% Cl_3CCOOH and radioactivity plotted.

(pH 7.6), 2 mM dithiothreitol, 1 mM ATP, 20 mM GTP, 8 mM creatine phosphate, 40 μg/mL of creatine phosphokinase, 30 μM each of the 19 unlabeled amino acids, 30 μM [^{3}H]lysine (38 Ci/mM), 90 mM KCl, 3 mM magnesium acetate, and varying amounts of mRNA (20–100 ng). Reactions were incubated at 21 °C for 90 min. Incorporation of [^{3}H]lysine into total protein was determined by precipitation of 2-μL aliquots in hot 20% trichloroacetic acid. The precipitates were heated to 90 °C for 10 min, chilled on ice, and filtered onto Whatman GF/C glass fiber filters. Radioactivity was measured by liquid scintillation spectrophotometry with 10 mL of toluene and Liquifluor (New England Nuclear).

Analysis of Cell-Free Translation Products. Fifteen percent polyacrylamide–$NaDodSO_4$ gels were prepared according to Laemmli (1970). Samples were mixed with 10× sample buffer, heated to 90 °C for 2 min, chilled, and electrophoresed on slab gels (1.5 mm × 10 cm × 0.13 cm) at 25 mA (constant current) for 4 h. The gel was removed and processed for detection of tritium by the fluorographic procedure of Bonner & Laskey (1974).

Results

Specificity of Histone DNA–Cellulose. The detailed map of the *S. purpuratus* histone genes (Cohn et al., 1976) has enabled us to select restriction endonuclease fragments of subcloned DNA fragments of defined gene structure for hybridization studies (Figure 1). In order to examine the properties of the various cellulose preparations, we initially measured the kinetics of their hybridization to ^{3}H-labeled polysomal RNA from early blastula stage sea urchin embryos (Figure 2). Such RNA is essentially a mixture of tRNAs, rRNAs, and heterogeneous mRNAs but only the mRNAs are extensively radiolabeled. The hybridization reactions were carried out at relatively low temperatures and in 50% formamide under relatively stringent conditions (see Materials and Methods and legend to Figure 2). As seen in Figure 2, the reaction is essentially completed by 2 h. Increasing the amount of cellulose-coupled DNA did not change the extent of the reaction. It is clear then that the reactions were in DNA excess. The rate of hybridization was several fold faster than a similar reaction using DNA on filters (Noyes & Stark, 1975; Levy & Aviv, 1976) and was proportional to the DNA concentration.

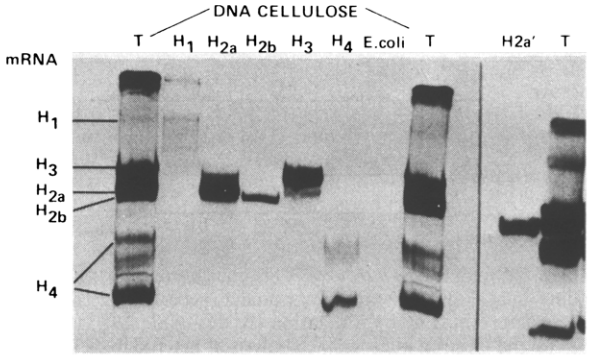

FIGURE 3: Autofluorogram of individual histone mRNAs purified by DNA–cellulose hybridization. Separate cellulose preparations with histone DNA complementary to only a single histone gene (see Figure 1) were hybridized in a volume of 0.2 mL to total [^{3}H]uridine-labeled blastula polysomal RNA for 1.5 h at 52 °C. After processing the hybrids (see Materials and Methods), eluted RNA was electrophoresed on a 6% polyacrylamide gel in E/10 buffer. The gel was then prepared for fluorography. Samples are: T, total unfractionated blastula polysomal RNA; H1, RNA hybridized to restriction endonuclease fragment (*Hha*I–2kbp–RI) from pSp2, containing only the H1 gene (19 μg of DNA/mL); H2A, RNA hybridized to a restriction endonuclease fragment (*Hha*I–0.8kbp–RI) of pSp17 DNA which contains only the H2a gene (5 μg of DNA/mL); H2b, RNA hybridized to pRC39 DNA–cellulose (5 μg of DNA/mL); H3, RNA hybridized to a restriction endonuclease fragment (*Hha*I–1.2kbp–RI) of pSp17 DNA containing only the H3 gene (5 μg of DNA/mL); H4, RNA hybridized to a restriction endonuclease fragment (*Hha*I–1kbp–RI) of pSp2, containing only the H4 gene (10 μg of DNA/mL); this fragment has been subcloned as pGC42; *E. coli*, RNA hybridized to total *E. coli* DNA bound to cellulose (50 μg of DNA/mL); H2A′, RNA hybridized to pRC9 DNA–cellulose (3 μg of DNA/mL).

The kinetic reaction of the RNA with the DNA–cellulose appears to be three- to sixfold slower than would be expected for an ideal second-order reaction for DNA–DNA renaturation in solution. To arrive at this estimate, we used the data of Figure 2 and calculated $C_0t_{1/2}$ for each of the reactions correcting for the fraction of the DNA complementary to the histone mRNAs (Cohn et al., 1976), the salt conditions of the reaction (Britten et al., 1974), and the slower rate of renaturation of RNA–DNA hybrids compared with DNA–DNA hybrids (Galau et al., 1977). Accordingly, we calculate a corrected $C_0t_{1/2}$ of 1.3×10^{-2} and 1.1×10^{-2}, respectively, for the pSp17 and pSp2 reactions in Figure 2. These compare with expected $C_0t_{1/2}$ values of 2×10^{-3} and 3×10^{-3}. If these values are correct, it may be that a combination of nonideal solution conditions and unavailability of some of the linked DNA limits the reaction rate.

At saturation, 40% of the histone RNA hybridized to pSp17 cellulose and 60% to pSp2 cellulose. Since pSp17 DNA contains coding regions for two histone mRNAs while pSp2 contains the remaining three histone mRNA coding regions, this was the result expected if the probes measure only the mRNAs complementary to the genes bound to the cellulose, and if the radiolabel is distributed equally among the five mRNAs. Similar hybridization kinetics were found using all the other probes described in Figure 1.

In order to test the specificity of our cellulose preparations, individual DNA fragments corresponding to a single histone gene (Figure 1) were independently linked to cellulose. The resulting matrices were hybridized with blastula stage ^{3}H-labeled polysomal RNA and washed, and the hybridized RNA was eluted and collected. We analyzed this RNA by polyacrylamide gel electrophoresis (Figure 3). Since we know the identity of each individual histone mRNA in this gel system (Levy et al., 1975; Cohn et al., 1976), we could easily

ascertain both the purity of each eluted RNA and its identity. The autofluorogram shown in Figure 3 demonstrates the specificity and sensitivity of hybridization in DNA excess. Wells labeled H2b, H4, and H2a′ contain RNA eluted after hybridization with DNA celluloses prepared from DNA clones containing sequences homologous only to a single mRNA. In each case only one RNA type was obtained. The multiple H4 RNAs are isocoding variants of these mRNAs (see Grunstein & Schedl, 1976). Wells labeled H2a, H3, and H1 contain RNA eluted after hybridization with DNA cellulose prepared from purified restriction endonuclease fragments. Unlike the subcloned DNA cellulose probes, these seemingly pure restriction endonuclease fragments contain, in addition to the excised fragment, small quantities of DNA corresponding to other histone genes contained on the same plasmid. Therefore, H2a cellulose hybridizes, in addition to H2a mRNA, small amounts of H3 mRNA, and H3 cellulose hybridizes, in addition to H3 mRNA, small amounts of H2a mRNA. These two genes are contained in the pSp 17 plasmid. The H1 cellulose hybridizes, in addition to H1 mRNA, small amounts of H2b mRNA. The H1 restriction fragment is contained in plasmid pSp 2, which also contains the H2b and H4 genes. Hybridization in DNA excess therefore reveals specific cross-contamination of small amounts of other DNA fragments contained in the same cloned plasmid. To ensure a single mRNA population, an attempt to use subcloned pieces of DNA should be made. This point is best demonstrated by comparison of wells H2a and H2a′ (Figure 3). Hybridization of radiolabeled blastula polysomal RNA to *E. coli* DNA cellulose was negligible.

The H2a and H2b mRNAs are of similar size and do not separate well in this gel system, especially when the gel is overloaded with mRNA. The H2a mRNA contaminant is seen as a faint band in the H3 well. This H2a mRNA is separated from the pure H2b mRNA. The H2a well, overloaded to demonstrate cross-contamination with H3 mRNA, does not separate the H2a from the adjacent H2b well (Figure 3).

Translation of Purified mRNA in a Wheat-Germ, Cell-Free, Protein-Synthesizing System. Sequence selectivity fulfills only partially the requirements for a generally applicable method for mRNA purification. It is equally important to maintain both the integrity and biological activity of each purified mRNA. Each mRNA preparation shown in Figure 3 was translated in a wheat-germ, cell-free, protein-synthesizing system, and the products were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4). Purified mRNAs which have been subjected to hybridization reactions translate at least as efficiently as unfractionated mRNA provided that no carrier RNA (which inhibits cell-free translation) is used to precipitate eluted mRNA fractions. As predicted from the analysis of radiolabeled mRNA (Figure 3) *E. coli* cellulose enriched no translatable mRNA species and H1 translation product contained detectable H2b contamination. The translation products obtained using H3 mRNA as template contain, in addition to authentic H3 protein, what is most likely a prematurely terminated H3 peptide which comigrated with H4 protein. On longer polyacrylamide gels (not shown) this peptide migrates slightly ahead of the H4 product. We were surprised to find two polypeptides stimulated by both H2a and H2b mRNAs since these seemed to be the purest radiolabeled mRNA preparations (Figure 3). In both cases, the lower molecular weight protein synthesized by these templates comigrates only with its authentic H2a and H2b counterpart. The higher molecular

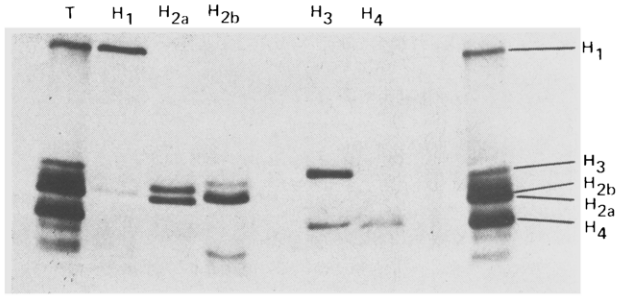

FIGURE 4: Autofluorogram of proteins synthesized in vitro using as templates RNAs eluted from DNA–cellulose. RNAs eluted from the DNA–cellulose described in Figure 3 (approximately 100 ng) were translated in a wheat-germ, cell-free protein-synthesizing system as described in Materials and Methods. A portion of each reaction was electrophoresed on 15% polyacrylamide gels in the presence of $NaDodSO_4$. The gels were then fluorographed. The positions of unlabeled *S. purpuratus* histones which were stained with amido black are indicated.

weight polypeptides synthesized in vitro by H2a and H2b mRNAs are only seen when the RNA is extracted from embryos at those specific developmental stages at which we are able to detect developmentally regulated histone protein variants (G. Childs & R. Maxson, unpublished experiments). The preparations of H2a and H3 mRNAs (prepared from restriction fragments) used as templates in the experiment shown in Figure 4 were slightly cross-contaminated. Longer exposures of the films of translation products of these two mRNA preparations did, in fact, show trace amounts of contaminating H3 and H2a protein.

Purification of Newly Made Histone RNA Sequences. Whole cell RNA from cleavage stage embryos radioactively labeled for a 10-min pulse with [^{3}H]uridine was hybridized to a histone DNA–cellulose probe containing the entire histone gene repeat unit (pSp2 plus pSp17 DNA) and the hybridized RNA was eluted. Half of this RNA was subjected to a second round of hybridization and elution and all the hybridized and nonhybridizable material was analyzed on 4% formamide–polyacrylamide denaturing gels (Figure 5). Radiolabeled RNA eluted from histone DNA–cellulose after the first cycle of hybridization contained an RNA with a mobility of 28S in addition to mature 9S histone mRNAs (Figure 5C). Our initial hypothesis was that this large RNA contained histone mRNA sequences and might be a polycistronic nuclear transcript. Further analysis of this potential precursor RNA clearly showed that, although it survived a second round of hybridization, it was not histone specific (Figure 5E). Under these conditions of labeling and hybridization to histone gene probes we have been unable to detect in whole cell RNA RNAs larger than mature polysomal messengers. Shorter labeling times and analysis either by sucrose gradient sedimentation or denaturing polyacrylamide gels have not revealed higher molecular weight histone RNAs (unpublished results).

Nonspecific cellulose binding can be significant when purifying an RNA species less abundant than blastula stage polyribosomal histone mRNA (the 28S RNA, Figure 5C, is an example). Two cycles of hybridization were sufficient to purify newly made pulse labeled histone RNA to apparent radiolabeled purity. In analytical quantitative experiments we found that background can be lowered significantly further and sensitivity therefore increased by treating DNA–cellulose with ribonuclease. Usually only a small portion of the gene is necessary to recover an entire, intact mRNA (e.g., our H2a probes); however, if RNase is used in preparative experiments, the cellulose-linked DNA should be long enough to protect the entire messenger. RNA which comigrates with untreated

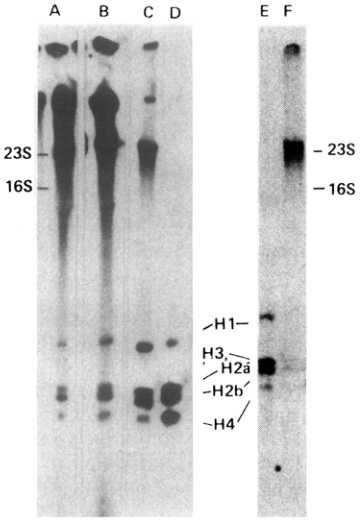

FIGURE 5: Autofluorogram of pulse-labeled whole embryo RNA. Whole cell RNA (200 000 cpm) which had been pulse labeled for 10 min with [^{3}H]uridine was hybridized for 2 h to pSp2 + pSp17 cellulose (containing all five histone genes). One-half of the hybridized material was subjected to a second round of hybridization. The hybridized RNAs and unhybridized supernatants were all subjected to electrophoresis on 4% polyacrylamide gels containing 98% formamide. (A) RNA remaining in the supernatant after hybridization (30 000 cpm); (B) total cell pulse labeled RNA (30 000 cpm); (C) RNA which hybridized to histone DNA–cellulose (4000 cpm); (D) total polysomal RNA (10 000 cpm); (E) RNA identical with sample C (4000 cpm) which hybridized a second time to histone DNA–cellulose; (F) RNA remaining in the supernatant after the second round of hybridization. Wells E and F were run on a separate gel.

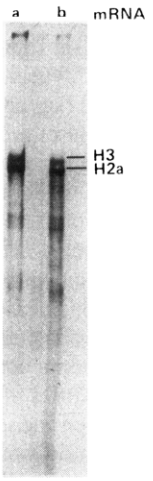

FIGURE 6: Effect of RNase on the ability to recover intact histone mRNA from DNA–cellulose. Polysomal RNA radiolabeled with [^{3}H]uridine was hybridized to pSp17 DNA–cellulose. One-half the cellulose was processed without RNase treatment, while the other half was treated with RNase as described in the text. The eluted RNAs were then analyzed on 6% polyacrylamide gel in E/10 buffer. (a) No RNase treated; (b) RNase treatment.

RNA in polyacrylamide gels can be eluted from the probe after inactivating remaining RNase with iodoacetate (Figure 6). The H2a and H3 mRNAs purified in this manner appear to be undegraded; however, their biological activity was not tested.

Discussion

Others have commented on the advantages of using solid phase techniques where DNA is covalently associated with the support matrix (Noyes & Stark, 1975; Levy & Aviv, 1976; Wood & Lingrel, 1977). The major advantages that we have found over other solid phase bound DNA methods are: (1) Hybridized RNA may be eluted from DNA-cellulose intact, maintaining its biological activity. (2) DNA bound to cellulose can be reused many times resulting in the saving of time required for isolation of large amounts of valuable restriction endonuclease fragments. We have reused some preparations as many as a dozen times. Because DNA excess conditions are still maintained, we see no apparent decrease in the ability of the DNA cellulose to extract biologically active mRNA. (3) The kinetics of hybridization resemble those of liquid hybridization, and reactions are completed much more rapidly than by filter hybridization (Figure 2). (4) Due in part to short incubation periods, RNA is not degraded during the hybridization (Figure 5). (5) DNA is covalently bound and is minimally lost during the hybridization reaction. Measurement of the loss of DNA during hybridization reactions revealed that 0.5–2.0% of covalently bound DNA may be lost during the hybridization reaction (R. Cohn, personal communication). For certain purposes this may represent a significant amount of contamination, but, if necessary, the DNA can be selectively removed from the mRNA by DNase treatment or precipitation with 3 M sodium acetate.

This report describes a method for the rapid purification of histone RNA which is dependent not on the size of the RNA but on its complementarity to histone genes. Hybridization and elution from gene specific histone DNA–cellulose provide higher and purer yields of specific sequences than those techniques previously used in this laboratory (Levy et al., 1975; Grunstein et al., 1973; Grunstein & Schedl, 1976). We have primarily focused on preparative isolation of individual histone mRNAs and pulse labeled histone RNA. Quantitative analysis of gene specific RNAs using this technique has already been described (Noyes & Stark, 1975). We have, in addition, shown that histone DNA–cellulose can be used to quantitate histone specific RNA in both in vivo and in vitro transcripts from isolated nuclei (Levy et al., 1978). However, if isolation of large amounts of specific DNA represents no problems and reutilization of DNA becomes unnecessary, then filter hybridization remains a useful method to quantitate newly made RNA since one RNA solution may be used with multiple filters and washing can be done by batch methods.

The purity of the final RNA product depends, of course, on the purity of the DNA–cellulose probe. This point is best illustrated by examining the RNA products eluted from our original preparation of H2a DNA–cellulose (Figure 3). We did not suspect that the restriction endonuclease fragment used to construct the cellulose probe was contaminated with H3 DNA. Because the reactions take place in vast DNA excess, a minor DNA contaminant is capable of pulling out significant amounts of contaminating RNA. This problem can only be eliminated by subcloning of the gene. When we did this with the H2a gene (pRC9 DNA) the eluate was pure H2a mRNA with no trace of H3 messenger (Figure 3). This point emphasizes the sensitivity of this method and demonstrates the advantage of using this procedure with a cloned DNA fragment, if possible, rather than using a restriction fragment or preparing cDNA cellulose (Levy & Aviv, 1976; Wood & Lingrel, 1977). Similarly, cDNA probes are only as pure as the original mRNA preparation used to make the cDNA–cellulose itself. In DNA excess experiments, impurities can become significant.

The purity of the final product also depends on the initial concentration of the RNA species of interest. Pulse-labeled whole cell RNA which contains a 30-fold lower concentration of radiolabeled histone sequences than blastula polysomal RNA (2% vs. 60%, data not shown) requires two cycles of

hybridization and elution using a specific DNA–cellulose probe. Backgrounds can be lowered significantly further, and sensitivity, therefore, increased, by treating DNA–cellulose with RNase (Figure 6).

The construction of histone DNA–cellulose has provided a means for the rapid and selective purification of histone mRNAs and pulse-labeled histone RNA from a heterogeneous population of RNA molecules. The technique has been used in our laboratory to prepare large amounts (50–60 μg) of highly purified, unlabeled histone mRNA for use in RNA–DNA electron microscope analysis (R. Cohn, unpublished) and RNA sequence analysis (I. Sures, unpublished). We have also used this approach to search for histone mRNA precursors or evidence of spacer transcription. We have cloned a *BamI–EcoRI* restriction endonuclease fragment from the spacer region in front of the 5′ end of the H3 gene. This clone, pGC1 (Figure 1 and unpublished data), has also been used as a hybridization probe. Consistent with the results shown in Figure 5, we detect no significant hybridization of whole cell cleavage stage RNA pulse labeled in vivo for 5 min to this spacer DNA probe (Childs, unpublished observation).

If higher molecular weight precursors to histone mRNAs exist in sea urchins only at late developmental stages, as has been suggested by Kunkel et al. (1978), then we should be able to isolate fragments of DNA complementary to those regions of such transcripts which are not part of cytoplasmic mRNAs. These fragments will provide a powerful tool for both isolation and quantitation of the dynamics of RNA synthesis and degradation. This approach should help answer a number of questions about mRNA precursors. For example, several genes coding for mRNAs for highly differentiated proteins, including the β-globin gene, the ovalbumin gene, and an immunoglobulin gene, contain inserts in the middle of their protein coding regions (Tilghman et al., 1977; Brethnach et al., 1977; Brack & Tonegawa, 1977). The histone genes of sea urchins which are active at early cleavage do not have such inserted sequences (Wu et al., 1976). It is likely that the inserted sequences are transcribed as mRNA precursors and that the initial transcripts represent the mRNA plus the insert (Tilghman et al., 1978). Subcloning of these inserted sequences, free of mRNA coding regions, and binding them to cellulose would then allow a quantitative and kinetic analysis of the sequential steps involved in processing of specific high molecular weight nuclear RNA.

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References

Bonner, W. N., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Bovre, K., & Szybalski (1971) *Methods Enzymol. 21*, 350–382.

Brack, C., & Tonegawa, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5652–5656.

Brethnach R., Mandel J. L., & Chambon, P. (1977) *Nature (London) 270*, 314–319.

Britten, R. J., Graham, D. E., & Neufeld, B. R. (1974) *Methods Enzymol. 29*, 363–418.

Cohen, S. N., & Miller, C. A. (1970) *J. Mol. Biol. 50*, 671–687.

Cohn, R. H., Lowry, J. C., & Kedes, L. H. (1976) *Cell 9*, 147–161.

Galau, G. A., Smith, M. J., Britten, R. J., & Davidson, E. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2306–2310.

Gross, K., Probst, E., Schaffner, W., & Birnstiel, M. (1976a) *Cell 8*, 455–470.

Gross, K., Schaffner, W., Telford, J., & Birnstiel, M. (1976b) *Cell 8*, 479–484.

Grunstein, M., & Schedl, P. (1976) *J. Mol. Biol. 104*, 323–349.

Grunstein, M., Levy, S., Schedl, P., & Kedes, L. (1973) *Cold Spring Harbor Symp. Quant. Biol. 38*, 717–724.

Holmes, D. S., Cohn, R. H., Kedes, L. H., & Davidson, N. (1977) *Biochemistry 16*, 1504–1512.

Kedes, L. H., & Gross, P. R. (1969) *Nature (London) 223*, 1335–1339.

Kedes, L. H., Cohn, R. H., Lowry, J. C., Chang, A. C. Y., & Cohen, S. N. (1975a) *Cell 6*, 359–369.

Kedes, L. H., Chang, A. C. Y., Housman, D., & Cohen, S. N. (1975b) *Nature (London) 255*, 533–538.

Kunkel, N., Hemminki, K, & Weinberg, E. (1978) *Biochemistry 17*, 2591–2598.

Laemmli, U. K. (1970) *Nature (London), New Biol. 227*, 680–685.

Levy, S., & Aviv, H. (1976) *Biochemistry 15*, 1844–1847.

Levy, S., Wood, P., Grunstein, M., & Kedes, L. (1975) *Cell 4*, 239–248.

Levy, S., Childs, G., & Kedes, L. H. (1978) *Cell 15*, 151–162.

Lewis, J. B., Atkins, J. F. Anderson, C. W., Baum, P. R., & Gesteland, R. F. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1344–1348.

Maniatis, T., Kee, S. G., Efstratiadis, A., & Kafatos, F. C. (1976) *Cell 8*, 163–182.

Nemer, M. (1975) *Cell 6*, 559–570.

Noyes, B. E., & Stark, G. R. (1975) *Cell 5*, 301–310.

Pinder, J. C. Staynov, D. Z., & Gratzer, W. B. (1974) *Biochemistry 13*, 5373–5378.

Roberts, B. E., & Patterson, B. M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2330–2334.

Shih, T. Y., & Khoury, G. (1976) *Biochemistry 15*, 487–493.

Sures, I., Maxam, A., Cohn, R., & Kedes, L. H. (1976) *Cell 9*, 495–502.

Sures, I., Lowry, J., & Kedes, L. H. (1978) *Cell 15*, 1033–1044.

Tilghman, S. M., Tiemeier, D. C., Polsky, F., Edgell, M. H., Siedman, J. G., Leder, A., Enquist, L. W., Norman, B., & Leder, P. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4406–4410.

Tilghman, S. M., Curtis, P. J., Tiemeier, D. C., Leder, P., & Weissmann, C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1309–1313.

Wood, T. G., & Lingrel, J. B. (1977) *J. Biol. Chem. 252*, 457–463.

Wu, M., Holmes, D. S., Davidson, N., Cohn, R., & Kedes, L. H. (1976) *Cell 9*, 163–169.