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Transcription of similar sets of rare maternal RNAs and rare nuclear RNAs in sea urchin blastulae and adult coelomocytes

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SUMMARY

We studied the sequences transcribed in the rare class of hnRNA and the rare maternal RNA set in blastula embryos and a tissue of adult sea urchins, coelomocytes. About 26 % of labelled single-copy DNA formed hybrids which bound to hydroxyapatite after three cycles of hybridization with nuclear RNA from blastulae and coelomocytes. This corresponds to transcription of about 50 % of the single-copy genome by both cell populations. To compare the rare hnRNA sequences synthesized by blastulae and coelomocytes directly, labelled single-copy DNA was hybridized with blastula nuclear RNA to high RNA C_0t, fractionated into sequences complementary and non-complementary to blastula nuclear RNA by chromotography on hydroxyapatite, and then each fraction was rehybridized with nuclear RNA from blastulae and coelomocytes. About 62 % of the labelled DNA complementary to blastula nuclear RNA and about 1·5 % of the labelled DNA non-complementary to blastula nuclear RNA hybridized with nuclear RNA from both cell populations. Thus, coelomocytes and blastula embryos transcribe essentially the same single-copy sequences in the rare hnRNA class. A probe for the rare maternal RNA set was isolated by hybridizing single-copy DNA with total egg RNA to high RNA C_0t. 65–67 % of this probe hybridized with whole-cell RNA from eggs, blastulae, plutei and coelomocytes demonstrating that essentially all rare maternal RNAs are present, and presumably transcribed, in blastulae, plutei and coelomocytes.

INTRODUCTION

The sequence complexity and overlap of the rare nuclear and messenger RNA classes at various stages of sea urchin development have been measured by hybridizing a large excess of nuclear and cytoplasmic RNA with trace amounts of radiolabelled single-copy DNA and chromatography on hydroxyapatite (HAP).*

* *Abbreviations used*: C_0t, moles of nucleotides per litre × sec; C_0t 1/2, C_0t required for 50 % hybridization; CTAB, cetyltrimethylammonium bromide; HAP, hydroxyapatite; scDNA, single-copy DNA.

Key words: Rare hnRNAs, rare maternal RNA, sea urchins, RNA:DNA hybridization.

These studies have shown that the rare hnRNA class is transcribed from about 30% of the single-copy genome and contains essentially the same sequences in blastulae and plutei, although a small fraction of the sequences in the rare class in the intestine of adult sea urchins is not present in gastrulae (Hough, Smith, Britten & Davidson, 1975; Kleene & Humphreys, 1977; Wold *et al.* 1978; Rogers & Gross, 1978; Ernst, Britten & Davidson, 1979). By comparison, the rare maternal RNA set in the cytoplasm of the sea urchin egg is transcribed from about 6% of the single-copy genome, contains enough complexity to code for 15–20 000 different proteins, and about 80% of these sequences disappears from the polysomes between eggs and adult tissues (Galau *et al.* 1976; Hough-Evans *et al.* 1977; Duncan & Humphreys, 1981*a*). Since essentially all rare maternal RNAs are found in nuclear RNA of adult sea urchin cells (Wold *et al.* 1978), the comparatively large reduction in complexity of the rare mRNA class is believed to be controlled by post-transcriptional processes (Kleene & Humphreys, 1977; Wold *et al.* 1978; Davidson & Britten, 1979). We prefer to refer to the RNAs stored in sea urchin eggs as rare maternal RNAs, instead of mRNAs, because this population is a mixture of mRNAs and other RNAs which resemble nuclear RNA by being larger than typical mRNAs and containing oligo (A) and repetitive sequences (Costantini, Britten & Davidson, 1980; Lee *et al.* 1980; Duncan & Humphreys, 1981*b*).

Previous studies of the sequence overlap of rare hnRNAs in sea urchin development are incomplete because the fraction of embryonic rare hnRNAs which are present in adult tissues has not been measured directly. In the present study, we used single-copy DNA hybridization and HAP chromatography to study the sequence overlap of rare hnRNAs and rare maternal RNAs in hatched blasula embryos and coelomocytes in the Hawaiian sea urchin, *Tripneustes gratilla*. Coelomocytes are a tissue in the coelomic cavity of adult sea urchins consisting of about five cell types with functions in excretion, clotting and transport of nutrients (Endean, 1966) and have been reported to translate about fivefold fewer rare maternal RNA sequences than blastula embryos (Galau *et al.* 1976). We isolated specific probes for the rare hnRNA class in blastula embryos and the rare maternal RNAs in eggs and compared the rate and extent of hybridization of these probes with RNA from blastulae and coelomocytes. Our results demonstrate that essentially all rare maternal RNAs are present in blastulae and coelomocytes and that essentially the same single-copy sequences are transcribed in the rare hnRNA class in blastulae and coelomocytes.

MATERIALS AND METHODS

Isolation of nuclei from blastula embryos and coelomocytes

Eggs of *T. gratilla* were obtained, fertilized and grown to the hatched blastula and early pluteus stages as described previously (Kleene & Humphreys, 1977). Nuclear and cytoplasmic

fractions of blastula embryos were isolated as described previously (Kleene & Humphreys, 1977) except that pronase and EDTA were omitted from the dextrose washes.

Coelomocytes were obtained by draining coelomic fluid from adult sea urchins through an incision around the mouth into 1 ml of 1·7 M-acetic acid per 100 ml of coelomic fluid to inhibit clotting. The fluid was filtered through ten layers of cheese cloth, layered over 50 ml 1 M-dextrose, 0·02 M-Tris, pH 7·8 in a 250 ml centrifuge bottle and centrifuged for 10 min at 1000 r.p.m. at 4 °C in the Sorvall HS-4 rotor. The cells were lysed in TNM buffer (Kleene & Humphreys, 1977) containing 1 % Triton X-100, 0·5 % diethylpyrocarbonate, 20 μg/ml heparin, 30 μg/ml polyvinyl sulphate and 25 μg/ml spermine. The 'clot' which forms at this point can be rapidly digested with DNase, and therefore probably consists of DNA released by lysis of the exploding cells (Endean, 1966). The nuclei were collected by shearing the clot with five-strokes in a 40 ml Dounce homogenizer (Kontes, B pestle), the lysate was layered over 10 ml 0·5 M-sucrose, 5 mM-$MgCl_2$ in a 50 ml centrifuge tube, and centrifuged at 3500 *g* for 5 min at 4 °C. Using the inhibitors of RNase above and healthy adults, nuclear RNA which had been labelled by injecting 100 μCi [^{3}H]uridine into the coelomic cavity sedimented as a broad symmetrical peak at about 20S in SDS-sucrose gradients (Kleene & Humphreys, 1977). When the nuclei were isolated without these inhibitors of RNase, the labelled RNA sedimented slower than 4S.

RNA purification

Our procedures for purifying total and nuclear RNA from blastulae, eggs, plutei and coelomocytes have been described previously (Smith, Hough, Chamberlin & Davidson, 1974; Hough, Smith, Britten & Davidson, 1975; Kleene & Humphreys, 1977). Briefly, eggs, embryos, coelomocytes and nuclei were lysed in urea-SDS buffer (Hough *et al.* 1975), extracted with phenol:chloroform, the interphase re-extracted twice with urea–SDS buffer and phenol: chloroform, the pooled aqueous phases were extracted with chloroform and precipitated with ethanol. The precipitated DNA and RNA were collected by centrifugation, resuspended and digested with RNase-free DNase (Smith *et al.* 1974), digested with proteinase K after addition of SDS to 0·5 %, extracted with phenol:chloroform and chloroform, chromatographed on Sephadex G-50 to remove partially digested DNA fragments, and precipitated with ethanol. The precipitated RNA was collected by centrifugation and digested with DNase and proteinase K and extracted with phenol: chloroform and chloroform and chromatographed on Sephadex again. The precipitated RNA was collected by centrifugation, and polysaccharides were removed with 2-methoxyethanol and cetyltrimethylammonium bromide (CTAB, Bellamy & Ralph, 1968) or by centrifugation through CsCl (Glison, Crkvenjakov & Byus, 1974). The use of CsCl or CTAB affects the rate of hybridization of some preparations of RNA depending on the cell type and cell fraction, so the method used for each preparation is noted in the Figure legend. Each RNA preparation was hydrolysed to a mode size of about 740 nucleotides in sodium phosphate buffer (pH 12·27) for 1·5 h at 0 °C, neutralized by addition of monosodium phosphate (Strauss & Sinsheimer, 1968), and then dialysed for 72 h against 1 mM-EDTA (pH 7·0) and distilled water.

Purification of RNA from coelomocytes was difficult due to large quantities of pigment, polysaccharide and other unidentified molecules. The maximum yield of coelomocyte nuclear RNA was about 50 μg/l of coelomic fluid. Even using the exhaustive purification procedures described above, about 20 % of our RNA preparations from coelomocytes were contaminated with pigment and were not used for hybridization. The absorbance ratios of RNA purified with CsCl were: 2·1–2·2 for both 260/280 and 260/230 nm; for RNA purified with CTAB; 260/230 nm, 2·3–2·4; 260/280 nm, 2·1–2·2.

DNA reassociation and RNA:DNA hybridization

Single-copy sequences in *T. gratilla* sperm DNA were purified and labelled to a specific activity of about 10 μCi/μg using *E. coli* DNA polymerase I (Boehringer – Mannheim), as described previously (Galau *et al.* 1976; Kleene & Humphreys, 1977). The mode size of the radioactive DNA was determined to be about 175 nucleotides long by sedimentation in alkaline sucrose gradients (Kleene & Humphreys, 1977). The zero-time binding (Britten, Graham & Neufeld,

1974) of the labelled single-copy DNA to HAP was about 0·45 %. The reactivity of the labelled DNA varied from 75–95 % depending on the levels of DNase contaminants in different batches of DNA polymerase.

DNA reassociation reactions were incubated at 60 °C in 0·12 and 0·41 M-PB (PB is an equimolar mixture of mono- and disodium phosphate). DNA C_0t values were corrected to equivalent C_0t by assuming that rates of reassociation in 0·41 M-PB are fivefold faster than in 0·12 M-PB (Britten *et al.* 1974; Van Ness & Hahn, 1982). The procedures used to measure DNA by chromatography on HAP have been described elsewhere (Britten *et al.* 1974; Kleene & Humphreys, 1977).

RNA: DNA hybridization reactions were incubated in 1–5 μl of 0·41 M-PB, 0·1 % SDS, 1 mM-EDTA. Recently Van Ness & Hahn (1982) reported that the cation acceleration for RNA:DNA hybridization in 0·41 M-PB compared with 0·12 M-PB should be 2·5 instead of 5. We have assumed an acceleration of 5 here to facilitate comparing our results with earlier studies of sea urchins.

RNA: DNA hybridization was analysed by minor modifications of the standard hydroxyapatite procedure of Galau, Britten & Davidson, (1974). Hybridization samples were expelled into 220 μl of 0·25 M-PB containing 10 μg/ml RNAse A and incubated for 30 min at 25 °C (Galau *et al.* 1976; Hough-Evans, Ernst, Britten & Davidson, 1977). To measure the fraction of labelled DNA in total duplexes (i.e., DNA duplexes and RNA: DNA hybrids), 100 μl of this high-salt RNAse digest was diluted to 2 ml with 0·12 M-PB and chromatographed on 0·4 g HAP after addition of 100 μg denatured 300-nucleotide-long fragment of calf thymus DNA. To measure the fraction of labelled DNA in duplexes, 0·1 ml of the high-salt digest was diluted to 0·05 M-PB, RNase A was added to 10 μg/ml, and the sample was incubated for 15–18 h at 37 °C, and then chromatographed as above. We used denatured DNA to suppress the background binding of single-stranded DNA to HAP because we find that 0·05 % SDS causes RNA: DNA hybrids and DNA duplexes to elute from HAP at lower phosphate buffer concentrations, and that SDS reduces the observed fraction of labelled DNA in RNA: DNA hybrids by 10–15 % (Kleene & Humphreys, unpublished). HAP column fractions were adjusted to 4 ml 0·12 M-PB and mixed with 16 ml scintillation cocktail (66 % toluene, 34 % Triton X-100, 0·4 % PPO). The fraction of labelled single-copy DNA and 'null-hnDNA' in RNA:DNA hybrids in each sample was calculated by subtracting the fraction of labelled DNA eluting in 0·48 M-PB after digestion with RNase in low salt (DNA duplexes) from the fraction of labelled DNA eluting from HAP in 0·48 M-PB after digestion in high salt (RNA: DNA hybrids and DNA duplexes).

Several precautions were taken to make sure that differences in hybridization were not caused by degradation of the DNA or RNA. Since the reactivity of highly radioactive DNA is known to decrease with time (Galau *et al.* 1976), hybridization reactions of various RNA preparations with each tracer were carried out within two weeks of each other. To minimize thermal degradation, incubations at 60 °C were restricted to less than 120 h. All of the hybridization reactions in this study and numerous others not reported here closely approximated pseudo-first-order kinetics over this interval. Fig. 1 shows a series of samples which were preincubated for 96 h at 60 °C, denatured by boiling, and then reincubated for up to 120 h. The data show that the preincubated samples hybridized at virtually the same rate and to the same extent as fresh samples. Finally, an aliquot of each mixture of RNA and tracer was incubated for 96–120 h at 60 °C and the size of the RNA measured by sedimentation in formaldehyde sucrose gradients (Kleene & Humphreys, 1977). In our hands, the amount of hybridization begins to decrease when the mode size of the RNA degrades to smaller than about 5·5S. The mode size of the RNA preparations used in this study was 9S before hybridization and at least 6S after incubation at 60 °C for 5 days.

Purification of hnDNA, null-hnDNA and egg-DNA

Sequences complementary to blastula nuclear RNA (hnDNA), not complementary to blastula nuclear RNA (null-hnDNA) and complementary to total egg RNA (egg-DNA) were isolated from labelled single-copy DNA by modifications of the first procedure of Galau *et al.* (1974). The basic procedure is described first, and then the specific conditions used in the isolation of the various probes. The appropriate amounts of RNA and labelled DNA were

incubated to the desired RNA C_0t in 0·41 M-PB, 1 mM-EDTA, 0·1 % SDS. The samples were diluted 50 times with 0·25 M-PB, digested with RNase A (10 μg/ml) for 30 min at 25 °C and chromatographed on 0·4 g HAP using 100 μg denatured 300-nucleotide-long fragments of *E. coli* DNA. Single-strand fragments partially depleted of sequences complementary to blastula nuclear RNA were eluted in 0·12 M-PB, the fine particles of HAP removed by centrifugation, the RNA hydrolysed with NaOH and then neutralized with acetic acid. The labelled DNA was purified by digestion with proteinase K (100 μg/ml, 2 h, 37 °C, 0·5 % SDS) and extracted once with phenol:chloroform, extracted twice with chloroform and passed over a bed of Chelex 100 (Bio-Rad). This procedure eliminates virtually all RNase from the labelled DNA judging from the rate of sedimentation of the RNA in formaldehyde sucrose gradients after incubation with labelled DNA for 5 days at 60 °C. The labelled DNA was concentrated by centrifugation for 24 h at 50 000 r.p.m. in the Beckman SW-65 rotor, the PB was withdrawn, and the DNA was dissolved in distilled water and lyophilized.

To isolate the labelled DNA from RNA:DNA hybrids (i.e. hnDNA and egg-DNA), the fractions eluting from HAP in 0·48 M-PB containing total duplexes were dialysed against 0·05 M-PB for 24 h at 25 °C, digested with RNase A (10 μg/ml, 37 °C, 15 h) to release the labelled DNA in RNA: DNA hybrids as single-stranded DNA. To remove the DNA duplexes, the RNase digest was adjusted to 0·12 M-PB and fractionated on HAP using 100 μg denatured *E. coli* DNA. The 0·12 M-PB fractions containing the hnDNA and egg-DNA were extracted to eliminate RNase and concentrated as described above.

The conditions of hybridization used to purify the various tracers were as follows:

HnDNA (Fig. 3): 0·2 mg blastula nuclear RNA and 1×10^6 c.p.m. scDNA in 20 μl were incubated to RNA C_0t 45 000 M sec.

Null-hnDNA (Fig. 4): 0·6 mg blastula nuclear RNA and 2×10^6 c.p.m. labelled scDNA in 50 μl were incubated to RNA C_0t 77 000 M sec. Some of the labelled DNA which did not bind to HAP was used in Fig. 4A. The remainder was combined with an additional 0·3 mg blastula nuclear RNA in 25 μl and incubated to RNA C_0t 90 000 M sec, and chromatographed on HAP. The labelled DNA eluting in 0·12 M-PB was chromatographed on HAP again and the labelled DNA eluting in 0·12 M-PB constitutes the null-hnDNA used in Fig. 4B.

Egg-DNA (Fig. 5): 1 mg total egg RNA and 10^7 c.p.m. scDNA in 100 μl were incubated to RNA C_0t 40 000 M sec. The labelled DNA in RNA: DNA hybrids was purified by the sequential two HAP column procedure, combined with 0·5 mg egg RNA in 50 μl, incubated to RNA C_0t 40 000 M sec, and the labelled DNA in RNA: DNA hybrids again purified by the sequential, two HAP column procedure.

The fraction of unfractionated, labelled scDNA recovered as each of the various specific probes was as follows: hnDNA, *ca.* 10 %; null-hnDNA (Fig. 4A), 37 %; egg-DNA, 0·5 %. The amount of these probes used in each hybridization sample was: null-hnDNA (Fig. 4A), $0{\cdot}7\text{–}1{\cdot}0\times10^4$ c.p.m.; null-hnDNA (Fig. 4B), $3{\cdot}5\times10^3$ c.p.m.; hnDNA and egg-DNA, $0{\cdot}3\text{–}1{\cdot}0\times10^3$ c.p.m. The fraction of these probes binding to HAP after self-reassociation to DNA $C_0t\,10^{-4}$ M sec was: hnDNA, 1·0 %; null-hnDNA (Fig. 4A), 1·1 %; null-hnDNA (Fig. 4B); 1·2 %; egg-DNA, 3·5 %. There was no detectable self-reassociation of the hnDNA and egg-DNA after extended incubations with RNA as expected for probes complementary to assymetric RNA transcripts.

Data reduction

RNA:DNA hybridization and DNA reassociation data were subjected to least-squares analysis using a computer program described by Britten *et al.* (1974).

RESULTS

Fraction of single-copy DNA hybridized by blastula embryo and coelomocyte nuclear RNA

The fraction of the single-copy genome transcribed as nuclear RNA by blastula embryos and adult coelomocytes was determined by hybridizing nuclear RNA

with radioactive single-copy DNA (scDNA) and determining the fraction of labelled DNA whose binding to HAP is sensitive to digestion with RNase in low salt (see Materials and Methods). Fig. 1 shows the reassociation of a typical preparation of labelled scDNA with excess 300-nucleotide-long fragments of genomic DNA. There is no obvious reassociation of the labelled scDNA in this reaction at the rate of repetitive sequences. The second-order rate constant of the major component ($1{\cdot}2\times10^{-3}\,M^{-1}\,sec^{-1}$) is virtually the same as reported previously for single-copy sequences in the *T. gratilla* genome ($1{\cdot}25\times10^{-3}$

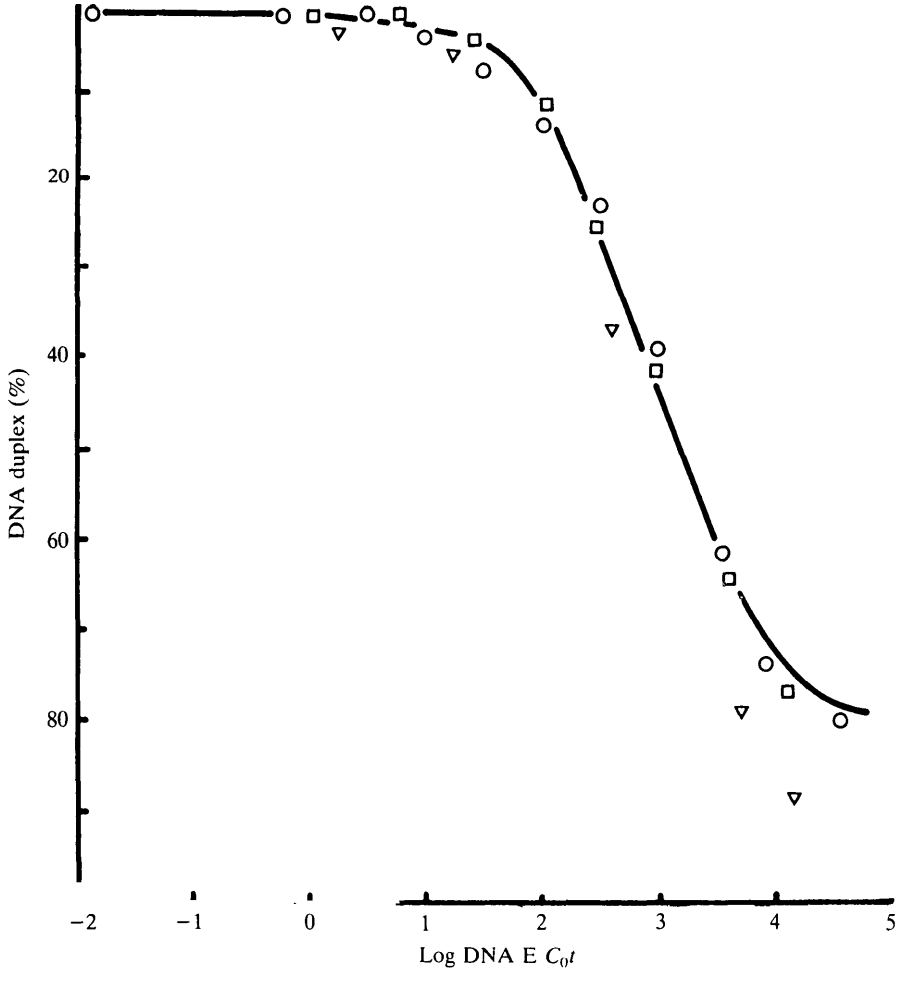

Fig. 1. Reassociation of radioactive single-copy DNA, hnDNA and egg-DNA with excess 300-nucleotide-long fragments of *T. gratilla* DNA. The total fraction of labelled DNA forming duplexes during the observed second order reaction, the total fraction of labelled DNA in duplexes at completion of the reaction, and the second-order-rate constant are respectively: single-copy DNA (□——□), 74·8%, 77·3%, $1{\cdot}2\times10^{-3}$ $M^{-1}\,sec^{-1}$; hnDNA (○), 75·6%, 79·6%, $9{\cdot}8\times10^{-4}\,M^{-1}\,sec^{-1}$; egg DNA (▽) 93·0%, 94·5%, $1{\cdot}2\times10^{-3}\,M^{-1}\,sec^{-1}$.

$M^{-1}sec^{-1}$; Kleene & Humphreys, 1977). The reassociation of this preparation of labelled scDNA terminated when 77% of the labelled DNA was in DNA duplexes; other preparations of labelled scDNA used in isolating probes for rare hnRNAs and rare maternal RNAs in later sections of this study reacted to 90–95%. The cause of this difference in reactivity is explained in the Materials and Methods.

The hybridization reactions of blastula and coelomocyte nuclear RNA with scDNA are shown in Fig. 2. Parameters describing the rate and extent of these and all other hybridization reactions in this study were determined by computer least-squares analysis and are presented in Table 1. Both reactions are fitted reasonably well by a single pseudo-first-order component in which the rare hnRNA class hybridizes with 16% of scDNA with an RNA C_0t 1/2 of $1{\cdot}6\times10^4$ (blastula) or $3{\cdot}8\times10^4$ M sec (coelomocyte). These data and the data reduction are similar to previous studies of nuclear RNA in *T. gratilla* and other species of sea urchin (Hough *et al.* 1975; Kleene & Humphreys, 1977; Rogers & Gross, 1978; Wold *et al.* 1978; Hough-Evans *et al.* 1979; Ernst, Hough-Evans, Britten & Davidson, 1980). Nevertheless, we wish to point out several problems involving the kinetics of these reactions. First, the reactions of blastula and coelomocyte nuclear RNA are both clearly broader than an ideal pseudo-first-order transition. The broadness of this reaction can be at least partially accounted for by the major reaction of the rare hnRNA class and a minor reaction of abundant hnRNAs with $\leq 1\%$ of scDNA with an RNA C_0t 1/2 of about 1000 (Kleene & Humphreys, unpublished). Second, the reactions in Fig. 2 were incubated to high enough RNA C_0t to reach the end of the pseudo-first-order transition of the rare hnRNA class, but not to high enough RNA C_0t to demonstrate a plateau for kinetic termination due to limitations on the RNA C_0t attainable. We assume that these reactions have terminated because reactions of pluteus and intestine nuclear RNA terminate after the major reaction of the rare hnRNA class (Kleene & Humphreys, 1977; unpublished; Wold *et al.* 1978). Third, the cellular levels of rare hnRNAs have been calculated from their rate of hybridization relative to the rate of reassociation of the *E. coli* genome (Hough *et al.* 1975; Kleene & Humphreys, 1977; Wold *et al.* 1978; Ernst *et al.* 1979). Even though we routinely find that different RNA preparations purified by the same procedure and hybridized under the same conditions react at the same rate (e.g., Fig. 2), seemingly minor changes in RNA purification and hybridization conditions resulted in differences in rates of hybridization which introduce uncertainties into calculations of the levels of rare hnRNAs. The following examples give an idea of the variability and unpredictability of rates of hybridization we have observed using exhaustively purified RNAs. We found that the rare hnRNA class reacted at the same rate when blastula nuclear RNA was purified with CTAB and CsCl (an RNA C_0t 1/2 of approximately $1{\cdot}6\times10^4$ M sec). Surprisingly, the rare hnRNA class hybridized sevenfold faster in blastula whole-cell RNA which had been purified with CsCl instead of CTAB (RNA C_0t 1/2, $2{\cdot}2\times10^4$ *vs.* $1{\cdot}4\times10^5$ M sec, data not shown).

Since our nuclear RNA preparations contain about 7–10 % of total cellular RNA and greater than 95 % of pulse-labelled nuclear RNA (Brandhorst & Humphreys, 1972; Kleene & Humphreys, 1977; Griffith & Humphreys, unpublished), we expected that the rare hnRNA class in nuclear RNA would react 10- to 14-fold faster than in whole-cell RNA from blastulae. This prediction was contradicted by

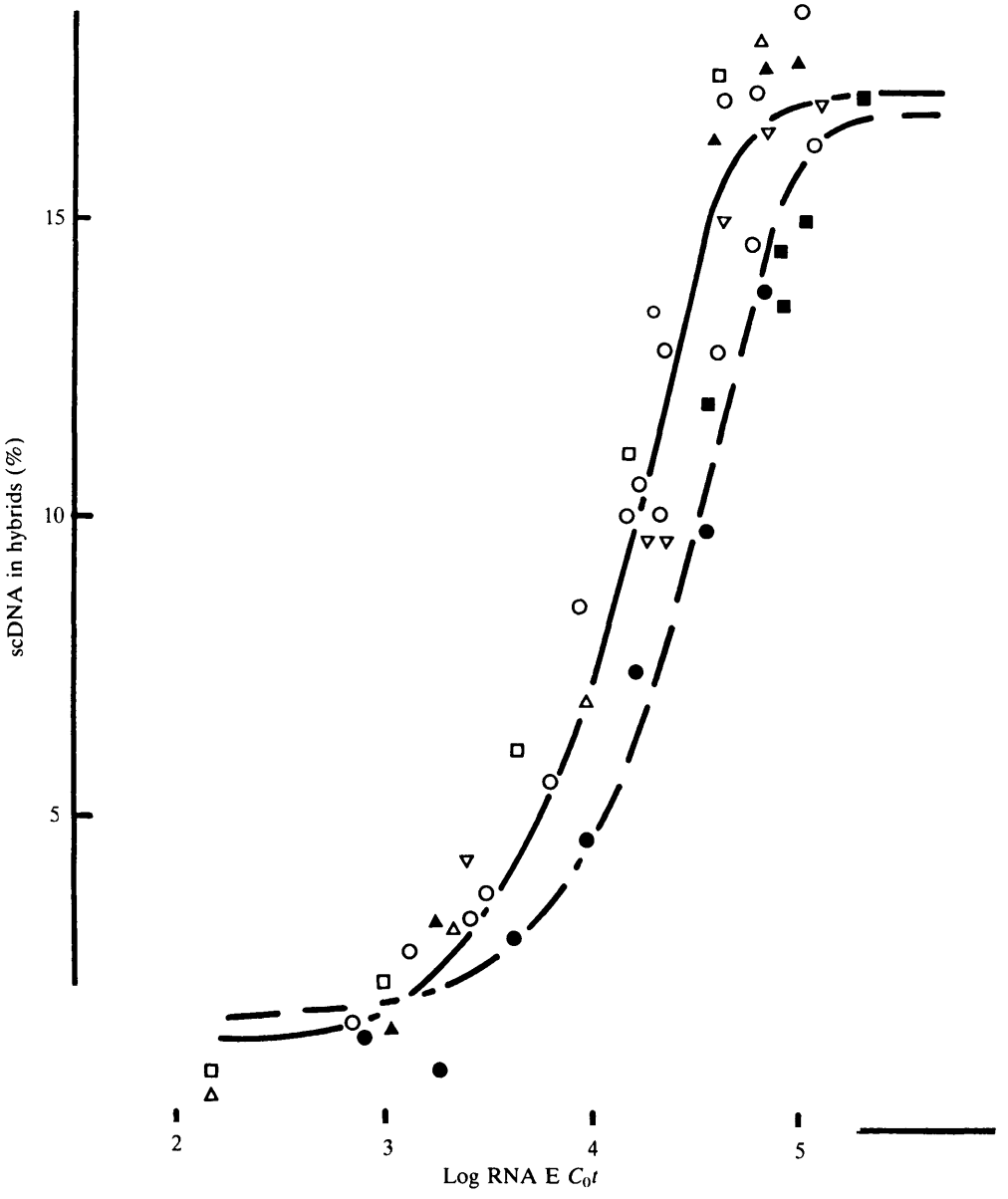

Fig. 2.

the observation that the rare hnRNA class in CTAB and CsCl purified nuclear RNA hybridizes at approximately the same rate as in CsCl purified whole-cell RNA (RNA C_0t 1/2, $1{\cdot}6\times10^4$ *vs.* $2{\cdot}2\times10^4$ M sec).

The results in Fig. 2 which we wish to emphasize are that the reactions of blastula and coelomocyte nuclear RNA with scDNA are virtually identical in slope and extent. Therefore, the fraction of the single-copy genome transcribed as rare hnRNAs must be similar in both populations. This evidence for the similarity of the sequence complexity of rare hnRNAs in coelomocytes and blastulae is supported by results using fractionated scDNA below.

Similar sets of rare hnRNAs are transcribed by blastula and coelomocyte cells

To compare the rare hnRNA sequences transcribed by blastula embryos and adult coelomocytes directly, labelled scDNA was hybridized preparatively with blastula nuclear RNA to RNA C_0t $4{\cdot}5$–$7{\cdot}7\times10^4$ M sec and fractionated into sequences complementary (hnDNA) and non-complementary to blastula nuclear RNA (null-hnDNA) by chromatography on HAP as described in the Materials and Methods.

We found it surprisingly difficult to purify the hnDNA and null-hnDNA. The fraction of hnDNA which rehybridized with blastula nuclear RNA was unexpectedly small (62 % in Fig. 3 and Table 1; 50–55 % in other preparations) and the fraction of null-hnDNA which rehybridized with blastula nuclear RNA was unexpectedly great (7·5 % in Fig. 4A and Table 1; 9 % in other preparations). The hnDNA appears to have been maximally purified by a single cycle of hybridization because its reactivity with blastula nuclear RNA was not increased by a second cycle of purification by hybridization to blastula nuclear RNA (data not shown). It was, however, possible to remove practically all of the sequences

Fig. 2. Hybridization of blastula and coelomocyte nuclear RNA with radioactive scDNA. The percentage of scDNA hybridizing with three different preparations of nuclear RNA (○□▽) and two different preparations of coelomocyte nuclear RNA (●■) was measured after incubation to various RNA C_0t. The RNAs from both sources were purified with CTAB. As a control for degradation, several blastula nuclear RNA samples were preincubated at 60 °C for 96 h to RNA C_0t 90 000, boiled, and then reincubated to the indicated RNA C_0t (△). Another series of points represent the fraction of scDNA in RNA:DNA hybrids in the preparative reactions used in purifying labelled scDNA complementary to blastula nuclear RNA at high and low RNA C_0t (▲). The fraction of labelled DNA in RNA:DNA hybrids in the preparative reactions was calculated as the fraction of labelled DNA eluting from HAP in 0·48 M-PB from the first HAP column (total duplexes) multiplied by the fraction of labelled DNA eluting in 0·12 M-PB from the second column after digestion with RNase in low salt (single-strand DNA released from RNA:DNA hybrids). In calculating the fraction of labelled DNA in RNA:DNA hybrids in the preparative reaction, the zero-time binding of the scDNA (0·45–0·9 % depending on the preparation of labelled scDNA) was subtracted from the duplex fraction and added to the single-strand fraction. The data were fitted by computer-assisted least squares solution assuming a single pseudo-first-order component. The curves are drawn according to the parameters determined by least-squares solutions which are presented in Table 1.

Table 1. *Parameters for least-squares solutions to hybridization reactions of various types of RNA with various fractions of single-copy DNA*

Source of RNA	Tracer	Data shown	Total extent of hybridization (%)	Observed extent of hybridization (%)	Pseudo-first-order rate constant ($M^{-1}sec^{-1}$)	RNA C_0t 1/2 (M sec)
blastula, nuclear	scDNA	Fig. 2	17·3	16·1	$4·4\times10^{-5}$	$1·6\times10^{4}$
coelomocyte,nuclear	scDNA	Fig. 2	17·0	16·3	$1·8\times10^{-5}$	$3·8\times10^{4}$
blastula, nuclear	hnDNA	Fig. 3	61·5	50·4	$4·4\times10^{-5}$	$1·6\times10^{4}$
coelomocyte, nuclear	hnDNA	Fig. 3	63·5	44·5	$3·0\times10^{-5}$	$2·3\times10^{4}$
blastula, nuclear	null-hnDNA	Fig. 4A	7·5	7·2	$4·4\times10^{-5}$*	$1·6\times10^{4}$
coelomocyte, nuclear	null-hnDNA	Fig. 4A	7·5	7·2	$1·8\times10^{-5}$*	$3·8\times10^{4}$
blastula, nuclear	null-hnDNA	Fig. 4B	1·5†	–	–	–
coelomocyte, nuclear	null-hnDNA	Fig. 4B	1·5†	–	–	–
egg, total	egg-DNA	Fig. 5	65·5	58·6	$1·3\times10^{-4}$	$5·3\times10^{3}$
blastula, total	egg-DNA	Fig. 5	66·0	56	$1·5\times10^{-4}$	$4·6\times10^{3}$
pluteus, total	egg-DNA	Fig. 5	66·8	59·4	$8·2\times10^{-5}$	$8·4\times10^{3}$
coelomocyte, total	egg-DNA	Fig. 5	67·1	60·3	9×10^{-5}	$7·7\times10^{3}$

In general, the data were analysed with the least-squares computer program described by Britten *et al.* (1974). The data were fitted assuming a single, pseudo-first-order component and without placing restrictions on the rate or fraction of radioactive DNA hybridized. The exceptions to these generalizations are described in footnotes.

* The data were fitted assuming that the rates of hybridization of coelomocyte and blastula nuclear RNA with blastula null-hnDNA were the same as with single-copy DNA.

† The fraction of null-hnDNA hybridized is the average percentage of null-hnDNA hybridized by blastula and coelomocyte nuclear RNA at each RNA C_0t value.

complementary to blastula nuclear RNA by hybridizing the null-hnDNA a second time with blastula nuclear RNA to RNA C_0t 90 000 and two cycles of chromatography on HAP (Fig. 4B).

We do not believe that these problems in purifying hnDNA and null-hnDNA are peculiar to our methods because the purity of our hnDNA and null-hnDNA is greater than reported elsewhere (Hough-Evans *et al.* 1979; Ernst *et al.* 1979). Furthermore, the fraction of scDNA hybridized by blastula nuclear RNA in five preparative samples was the same as in analytical samples (Fig. 2) and we successfully purified probes for abundant hnRNAs and rare maternal RNAs which should be much more difficult because these classes of RNA are complementary to

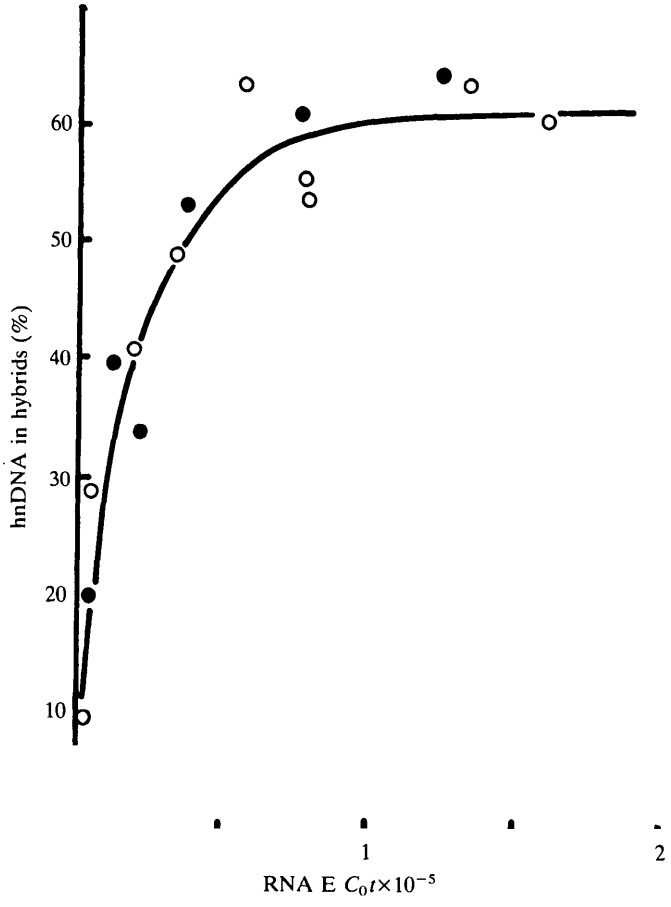

Fig. 3. Hybridization of blastula hnDNA with blastula (○) and coelomocyte (●) nuclear RNA. The blastula nuclear RNA was purified with CTAB and the coelomocyte nuclear RNA was purified with CsCl. The hnDNA was purified by hybridizing single-copy DNA with blastula nuclear RNA to RNA C_0t 45 000 as described in the Materials and Methods. The curves represent a least-squares solution of the data assuming a single pseudo-first-order component. The parameters for the least squares solutions are presented in Table 1.

much smaller fractions of scDNA than the rare hnRNA class. The low reactivity of the hnDNA and the high reactivity of the null-hnDNA with the homologous nuclear RNA could be explained either by the failure of HAP to bind all RNA: DNA hybrids or the failure to hybridize all complementary DNA during the preparative hybridization reaction (Van Ness & Hahn, 1982). We believe that these problems are primarily caused by the failure of HAP to bind all RNA: DNA hybrids because about 6% of the supposedly single-stranded DNA eluting from HAP in 0·12 M-PB after the first preparative hybridization binds to HAP when rechromatographed on a new HAP column. Only 0·8% of this labelled DNA bound to HAP after digestion with RNase in low salt demonstrating that RNA: DNA hybrids failed to bind to HAP. While the failure of HAP to bind all hybrids greatly increases the problems in purifying hnDNA and null-hnDNA and may be expected to produce underestimates of the fraction of the single-copy genome that is transcribed, it should have little effect on the interpretation of the experiments below comparing the hybridization of coelomocyte and blastula nuclear RNA with hnDNA and null-hnDNA.

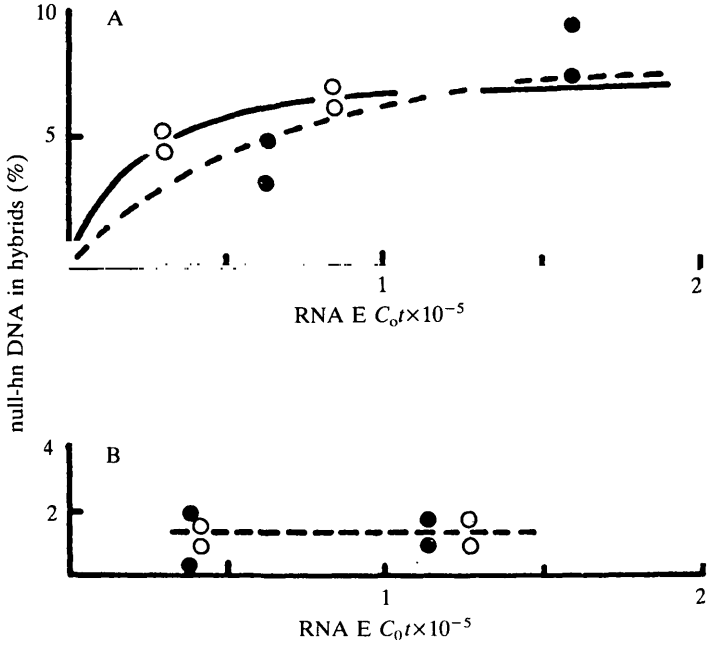

Fig. 4. Hybridization of null-hnDNA with blastula nuclear RNA (○) and coelomocyte nuclear RNA (●). Both types of RNA were purified with CTAB. (A) The null-hnDNA was purified by one cycle of hybridization of single-copy DNA with blastula nuclear RNA to RNA C_0t 77 000 and isolation of the unhybridized, single-strand DNA by chromatography on HAP. (B) the null-hnDNA used in Fig. 4A was purified by a second cycle of hybridization with blastula nuclear RNA to RNA C_0t 90 000 and isolation of the unhybridized DNA. The dashed line is the average percentage of null-hnDNA hybridized by blastula and coelomocyte nuclear RNA--1·5% with each type of RNA at both RNA C_0t values.

To determine whether coelomocytes transcribe the same sequences as blastulae, the hnDNA was hybridized with blastula and coelomocyte nuclear RNA. Fig. 3 and Table 1 show that nuclear RNA from both sources hybridizes essentially the same fraction of hnDNA (62–64 %). When the hnDNA was reassociated with excess sheared genomic DNA, at least 95 % of the hnDNA reacted at the same rate as single-copy sequences (Fig. 1). In combination, these results demonstrate that coelomocytes transcribe essentially all of the single-copy sequences transcribed by blastulae as rare hnRNAs.

To determine whether there are sequences present in coelomocyte nuclear RNA which are not present in blastula nuclear RNA, the null-hnDNA was hybridized with coelomocyte and blastula nuclear RNA. This experimental approach is very sensitive for rare transcripts which are present only in coelomocytes, because the background of hybridization to sequences which are present in both cell populations has been virtually eliminated. Fig. 4B and Table 1 show that about 1·5 % of the null-hnDNA hybridizes with nuclear RNA from both sources. In order to interpret this low level of hybridization, it is necessary to show that the null-hnDNA and RNA were not degraded. The null-hnDNA did not appear to be degraded because it reassociated as well with excess DNA as the unfractionated scDNA from which it was isolated (73 % duplexes at DNA C_0t 10 000 M sec). The absence of RNase activity in the null-hnDNA was shown by measuring the mode size of the RNA (6S) after incubation with null-hnDNA for 5 days at 60 °C in formaldehyde sucrose gradients (see Materials and Methods). These controls demonstrate that the failure of nuclear RNA to hybridize with the null-hnDNA is not an artifact of degradation of the DNA or RNA. The finding that essentially all of the sequences complementary to coelomocyte nuclear RNA were removed by hybridizing the scDNA with blastula nuclear RNA shows that coelomocytes transcribe few sequences in the rare hnRNA class in addition to those transcribed by blastulae.

Presence of rare maternal RNAs in blastulae, plutei and coelomocytes

The rare maternal RNA set in eggs of *T. gratilla* hybridizes with about 2·7 % of single-copy DNA (Duncan & Humphreys, 1981*a*). 60 % and 15 %, respectively, of the rare maternal RNA set are on the polysomes of plutei and coelomocytes of *S. purpuratus* (Galau *et al.* 1976; Wold *et al.* 1978). Since we were unable to detect differences between the rare hnRNA sequences of blastulae, plutei and coelomocytes (Kleene & Humphreys, 1977), we wondered how much of the rare maternal RNA set is transcribed in plutei and coelomocytes. We purified a probe for the rare maternal RNA set in the egg by two cycles of hybridization of total egg RNA with labelled scDNA to RNA C_0t 40 000 M sec, and hybridized this probe, referred to as egg-DNA, with whole cell RNA from eggs, blastulae, plutei and coelomocytes.

Fig. 5 and Table 1 show that total egg RNA hybridizes 65 % of the egg-DNA probe demonstrating that the 2·7 % of unfractionated scDNA which is

complementary to the rare maternal RNA set had been enriched by 23-fold. This reaction follows pseudo-first-order kinetics which means that the various maternal RNA sequences reacting with the egg-DNA are at similar concentrations in total egg RNA. The RNA C_0t 1/2 of this reaction ($5{\cdot}5\times10^3$ M sec) was similar to that reported previously ($2{\cdot}4\times10^3$ M sec; Duncan & Humphreys, 1981*a*). When the egg-DNA was reassociated with excess *T. gratilla* DNA, essentially all of the observed reaction occurred at the rate of single-copy sequences (Fig. 1).

The reactions of egg-DNA with RNA from unfractionated (whole-cell) blastulae, plutei and coelomocytes also closely approximated ideal pseudo-first-order kinetics (Fig. 5). The RNA C_0t 1/2 of all four of these reactions differed by

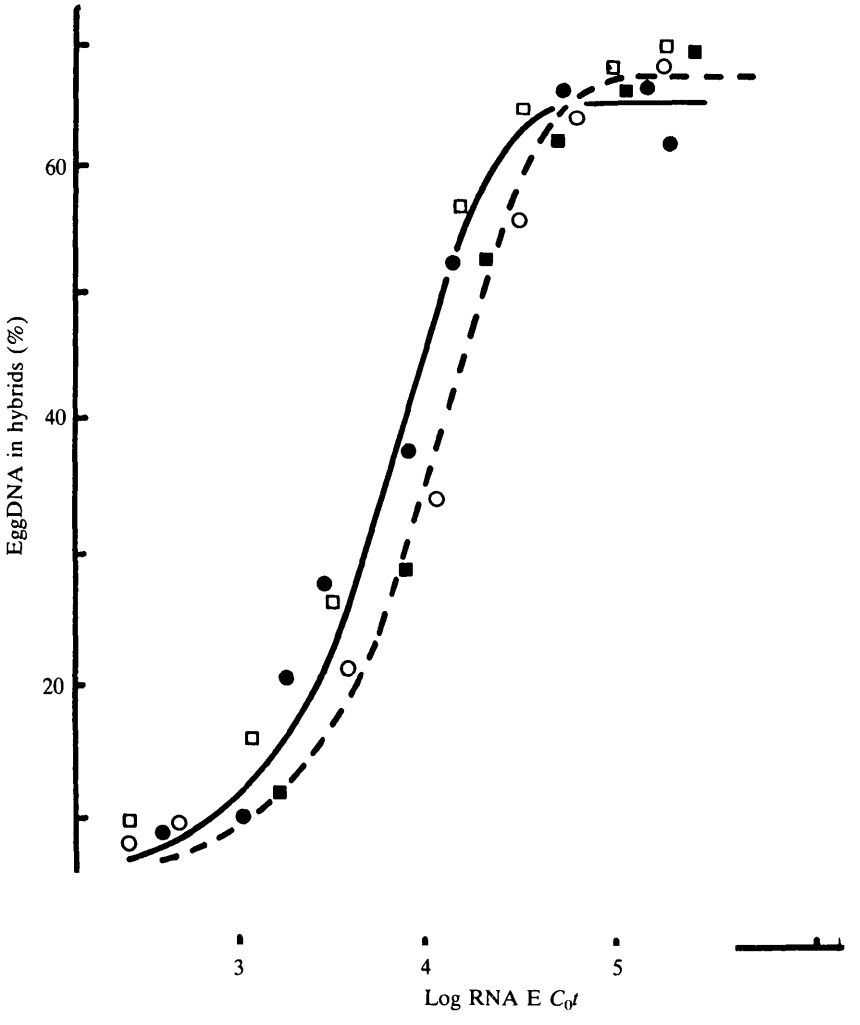

Fig. 5. Hybridization of egg-DNA with whole-cell RNA from eggs (●——●), blastulae (□), plutei (○--○) and coelomocytes (■). All four types of RNA were purified with CsCl. The data were fitted assuming a single pseudo-first-order component. The parameters for the least-squares solution are presented in Table 1.

less than a factor of 2 ($8{\cdot}4\times10^3$ *vs.* $4{\cdot}6\times10^3$ M sec, Table 1). The RNA from all four cell populations hybridized practically identical fractions of egg-DNA at termination (65·5 % to 67·1 %, Table 1). Thus, essentially the entire rare maternal RNA set is present in blastulae, plutei and coelomocytes.

Unfortunately, we encountered a variety of unexpected problems in attempting to measure the fraction of the rare maternal RNA set which is associated with the polysomes of coelomocytes and blastulae. Isolation and characterization of intact, highly purified polysomal mRNA from coelomocytes is difficult due to high levels of RNase, polysaccharide, pigment and lysis of the nuclei of exploding cells (Endean, 1966). At the same time, our preparations of total polysomal and EDTA-released polysomal mRNA from blastula embryos were contaminated with sufficient levels of abundant and rare hnRNAs to preclude measurement of the fraction of the rare maternal RNA set associated with the polysomes. In our hands, puromycin release of mRNA from polysomes (Galau *et al.* 1974, 1976) was an unsatisfactory procedure for eliminating nuclear RNA contaminants from polysomal RNA because equal amounts of labelled RNA were released from the polysomes by incubation at 37 °C in the absence and presence of puromycin (Griffith & Humphreys, unpublished).

DISCUSSION

We consistently find that about 17 % of labelled scDNA hybridizes with nuclear RNA from blastulae, plutei and coelomocytes forming RNA:DNA hybrids which bind to HAP after one round of hybridization (Kleene & Humphreys, 1977). The fraction of the single-copy genome transcribed is usually calculated from the fraction of labelled scDNA hybridized by correcting for the reactivity of the labelled DNA with RNA as determined from the reassociation of the labelled scDNA with excess genomic DNA (77 %, Fig. 1), the fraction of labelled DNA purified from RNA:DNA hybrids that reassociates at the same rate as single-copy sequences in the genome ($\geq$ 95 %, Fig. 1), and assuming transcription of one strand of DNA (Hough *et al.* 1975). We calculate accordingly that 40 % of the single-copy genome is transcribed (i.e., $17\,\%\times2\times95\,\%/77\,\%$).

However, we believe that more than 40 % of the single-copy genome is transcribed because an additional 9 % of single-copy DNA in our 'null-hnDNA' was still hybridizable by nuclear RNA after a single cycle of preparative hybridization and removal of the hybridized scDNA by chromatography on HAP (Fig. 4, Table 1). Difficulties in purifying labelled DNA fractions not complementary to mRNA and hnRNA using HAP have been reported by others and have been attributed to the failure of HAP to bind all RNA:DNA hybrids (Williams & Penman, 1975; Hastie & Bishop, 1976; Ernst *et al.* 1979). We agree that these problems were caused primarily by the failure of HAP to bind all RNA:DNA hybrids and believe that a similar fraction of labelled DNA

complementary to hnRNA failed to be measured in our analytical reactions because the same fraction of labelled scDNA hybridized in our analytical and preparative reactions (Fig. 2). We also believe that using DNA reassociation to correct for the reactivity of the labelled scDNA with RNA results in underestimates of the fraction of the genome transcribed because short RNA:DNA hybrids bind less tightly to HAP than DNA duplexes (Martinson, 1973; Doktor, Brenner & Miller, 1974; Kleene & Humphreys, unpublished). Since the total amount of labelled scDNA hybridized after three cycles of hybridization was 26 % (Figs 2 & 4), about 50 % of one strand of the single-copy genome appears to be transcribed in *T. gratilla*. This is about 1·5-fold greater than previous estimates (Hough *et al.* 1975; Kleene & Humphreys, 1977; Wold *et al.* 1978; Hough-Evans *et al.* 1979; Ernst *et al.* 1979). The vast majority of these sequences is usually assumed to be present at similar levels in the rare hnRNA class (Hough *et al.* 1975; Kleene & Humphreys, 1977), and a much smaller fraction of sequences is presumably present as abundant hnRNAs (Kleene & Humphreys, 1977; Busby & Bakken, 1979). Unfortunately, we found that the levels of rare hnRNAs cannot be accurately calculated by comparing their rate of reaction to rates of reaction in model systems, because of large and unpredictable fluctuations in rates of reaction of rare hnRNAs related to the method of RNA purification and cell fraction. In addition, Van Ness & Hahn (1982) and Kovesdi & Smith (1982), have questioned the accuracy of some of the corrections previously used (Hough *et al.* 1975) in calculating levels of rare hnRNAs from rates of reaction. Previous estimates that there is an average of 0·07–1·0 copy of each rare hnRNA sequence in each embryonic sea urchin nucleus are probably reasonable first approximations (Hough *et al.* 1975; Kleene & Humphreys, 1977).

We did not detect a difference between the rare hnRNA sequences in blastula embryos and adult coelomocytes in this study (Figs 2, 3, 4) or in our previous study of blastula and pluteus embryos (Kleene & Humphreys, 1977). We admit that in one experiment with 'null-hnDNA' in our previous study (Fig. 4, Kleene & Humphreys, 1977), our data show a small but distinct difference between the rare hnRNA sequences of blastulae and plutei such as reported for gastrulae and adult intestine cells in *S. purpuratus* (Ernst *et al.* 1979). However, in our hands, the difference was much smaller (5 % *vs.* 20 % of the complexity of the rare hnRNA class), and was not observed in any other experiment. Although saturation hybridization is too imprecise a technique to rule out the existence of a small difference between the rare hnRNA sequences of blastulae, plutei and coelomocytes, our data indicate that the difference must be smaller than reported for gastrulae and intestine cells. Irrespective of whether there are small differences between the rare hnRNA populations of various sea urchin cell types, all of the available studies agree that a large majority of the same sequences are present in the rare hnRNA class of the immature oocyte, blastulae, gastrulae, plutei and two adult tissues (Kleene & Humphreys, 1977; Wold *et al.* 1978; Hough-Evans *et al.* 1979; Ernst *et al.* 1980). The sequences comprising the rare hnRNA class have not

been detected in micromeres of 16-cell embryos, apparently due to a low overall rate of RNA synthesis (Rogers & Gross, 1978; Ernst *et al.* 1980).

Our probe for the rare maternal RNA set in the egg hybridized to the same extent with whole-cell RNA from eggs, blastulae, plutei and coelomocytes (Fig. 5). These results confirm the report of Wold *et al.* (1978) that transcripts complementary to a similar probe for rare mRNAs in blastula embryos are present in nuclear RNA of adult intestine and coelomocytes. The finding that only about 20 % of the sequence in blastula rare mRNAs is present in cytoplasmic RNA of the intestine (Wold *et al.* 1978), while all rare maternal RNAs are present in nuclear RNA of adult tissues supports speculations that the accumulation of rare maternal RNAs is regulated post-transcriptionally (Kleene & Humphreys, 1977; Wold *et al.* 1978; Davidson & Britten, 1979; Lev *et al.* 1980).

We wish to emphasize that we have mixed feelings about the post-transcriptional regulation of rare maternal RNAs. On the one hand, we were unable to confirm an earlier report (Galau *et al.* 1976) that the fraction of the rare maternal RNA set which is translated in coelomocytes is fivefold lower than in blastulae due to high levels of RNase in coelomocytes and high levels of nuclear RNA contaminants in our preparations of blastula polysomal RNA. On the other hand, saturation hybridization experiments on eggs from three species of sea urchin concur that the rare maternal RNA set contains only about one-fifth the complexity of the rare hnRNA class (Hough *et al.* 1975; Galau *et al.* 1976; Hough-Evans *et al.* 1977; Rogers & Gross, 1978; Ernst *et al.* 1980; Duncan & Humphreys, 1981*b*; Kleene & Humphreys, unpublished). This observation gives credibility to the conclusion that the rare maternal RNA set in eggs is a discrete population of RNAs which resembles nuclear RNA by virtue of being about twofold longer than normal sea urchin mRNAs and containing oligo (A) and repetitive sequences (Costantini *et al.* 1980; Lee *et al.* 1980; Duncan & Humphreys, 1981*b*; Thomas, Britten & Davidson, 1982). The resemblance of maternal RNAs to nuclear RNAs implies that some of the very high sequence complexity of rare maternal RNAs in eggs and early embryos is due to sequences which do not code for protein, and leads us to suspect that some of the decreasing sequence complexity of rare maternal RNAs during sea urchin development reflects the disappearance of these non-coding sequences. It follows that the numbers and kinds of proteins translated from rare maternal RNAs during sea urchin development can only be elucidated by studies of individual RNAs.

Considering the very great developmental difference between the undifferentiated cells in blastula embryos and differentiated coelomocytes in the adult sea urchin, it is striking that essentially all rare hnRNA and rare maternal RNA sequences in blastula embryos are present in coelomocytes at or above the levels of the rare hnRNA class. The simple generalization that the rare hnRNA class generally represents a constituitive low level of transcription of all genes in eukaryotic cells appears to be ruled out by findings that there are marked differences between the rare hnRNAs in different tissues in rats (Chikaraishi,

Deeb & Sueoka, 1978), that stage-specific rare hnRNAs and mRNAs are transcribed during aggregation and culmination in slime molds (Blumberg & Lodish, 1980, 1981), that mRNAs for globin and ovalbumin cannot be detected in nuclear RNA of cells which do not make these proteins (Groudine & Weintraub, 1975; Harris, Rosen, Mean & O'Malley, 1978) and tissue specific rare hnRNAs are synthesized in the adult intestine of sea urchins (Ernst *et al.* 1980). At present, it is a possibility that all embryonic rare hnRNAs and rare mRNAs are transcribed in all transcriptionally active cells at all stages of sea urchin development.

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