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Genomic clones coding for some of the initial genes expressed during *Drosophila* development

(preblastoderm RNA synthesis/early embryo genome expression)

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ABSTRACT **Preblastoderm *Drosophila* embryos were made permeable and labeled *in vivo* with [^{32}P]phosphate-containing medium. Cytoplasmic polyadenylylated RNA was extracted from these embryos and used to screen a library of *Drosophila* genomic DNA sequences cloned in phage λ. Ten cloned sequences were selected for further study. These sequences were not complementary to mitochondrial DNA, nor did they contain the repeated nuclear genes coding for rRNA or histones. The cloned sequences each encode one or more unique genes expressed in preblastoderm embryos. RNA blot analysis indicated that some of these genes are also expressed at other times during embryogenesis. These results show that, in spite of the rapid nuclear divisions taking place during the preblastoderm stage, *Drosophila* nuclear genes are transcribed and that a subset of these genes show variable, stage-specific levels of expression during early embryogenesis.**

The newly fertilized *Drosophila* embryo contains a large number of maternally donated mRNAs whose presence is necessary for embryonic development (for example, see refs. 1–6). However, several studies have shown that zygotic genome expression is also required for correct early development (for example, see refs. 7–10). The exact timing of expression of these zygotic genes has not been determined, but it is known that substantial determination of embryo cell types has occurred by the end of the blastoderm stage (2.5–3.5 hr after fertilization) (1, 4, 11–13). Analysis of specific molecular events after fertilization will help to describe the first embryonic contributions to development and differentiation during *Drosophila* embryogenesis.

During the blastoderm stage, newly synthesized polyadenylylated transcripts rapidly associate with polysomes (14). When this observation is taken together with the relatively high rate of RNA synthesis per nucleus in the blastoderm, it appears that the initial surge of blastoderm transcription significantly contributes to the informational RNA at this time. Approximately 14% of the RNA in the embryo is synthesized between the onset and the end of the blastoderm stage (14). Analysis of the hybridization kinetics of this RNA population shows little change from the mature oocyte in complexity or coding capacity, reflecting the results of analysis of the embryo protein population (15, 16). Recent experiments, however, suggest that at least some of the sequences transcribed during the blastoderm stage may specify unique developmentally important gene products (17).

It has been argued that preblastoderm transcription is impossible due to the extremely rapid rate of DNA replication in cleavage nuclei. Probably the entire 3.4 min of interphase found during the 9.6-min division cycle of cleavage nuclei is devoted to DNA synthesis (18, 19). However, McKnight and Miller (20) observed active transcription on sister strands of replication forks in *Drosophila* cleavage chromatin. A small number of transcription units showed internal nascent fibril-less gaps that may have resulted from a brief interruption of RNA polymerase initiation by DNA replication. Similar electron microscopic observations of *Drosophila* preblastoderm chromatin revealed *Drosophila* nonnucleolar transcription units on 1% of the cleavage chromatin measured (21). Preblastoderm transcription units are densely packed with RNA polymerase molecules and contain small (< 2-μm) transcript fibrils. Transcription units of this class continue to be transcribed, at a much higher level, after blastoderm formation, suggesting that genes of this particular class are still expressed later in development.

We have also investigated preblastoderm nuclear transcription by labeling permeabilized early embryos with [^{32}P]phosphate-containing medium (unpublished data). A small fraction of the RNA synthesized in preblastoderm embryos represents transcription of nuclear genes. This RNA is small (7–12 S) and polyadenylylated and rapidly associates with polysomes. These results represent biochemical confirmation of the low level of nuclear transcription observed by McKnight and Miller (21). However, studies of heterogeneous RNA populations may obscure small and potentially developmentally important changes in embryonic gene expression. To form an accurate opinion of the influence of gene expression in early development, it is necessary to examine the expression of individual genes during embryogenesis. Therefore, preblastoderm ^{32}P-labeled RNA was used to select complementary *Drosophila* recombinant nuclear DNA sequences from a phage λ genomic library. These recombinant clones contain several unique genes that are expressed at low concentrations in preblastoderm embryos and in some cases are also expressed at other times during embryogenesis.

MATERIALS AND METHODS

Isolation of ^{32}P-Labeled Preblastoderm RNA. The permeabilization and labeling of preblastoderm embryos will be described in detail elsewhere. Briefly, *Drosophila melanogaster* (Oregon R strain 2) embryos were collected for 45 min from large population cages after 1-hr precollections. Such precollection helps to alleviate contamination with older embryos held *in utero*. Light microscopic observation of 1,000 embryos collected in this manner showed that no more than 0.2% contamination of the preblastoderm embryos with older embryos had occurred. The dechorionated embryos were made permeable with octane and labeled with 200–250 μCi of monosodium [^{32}P]phosphate (500 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) according to Anderson and Lengyel (22). [^{32}P]Phosphate-labeled RNA from these

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Abbreviations: kb, kilobase pair(s); nt, nucleotide(s).

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embryos was isolated by extraction of embryo cytoplasm with phenol. The specific activity of this RNA was approximately 1,000 cpm/μg. However, because this RNA may represent transcription of genes whose RNA products are already present in the embryo in unknown amounts, the specific activity of the newly synthesized RNA alone cannot be determined.

Screening and Isolation of *Drosophila* Recombinant DNA Sequences from a Phage λ Genomic Library. The *Drosophila* genomic library contained in phage λ was obtained from T. Maniatis. This library was screened for recombinant phage-carrying *Drosophila* sequences complementary to preblastoderm ^{32}P-labeled RNA by using the methods of Maniatis *et al.* (23) and Benton and Davis (24). Phage DNA was isolated by a modification of the liquid culture method of Blattner *et al.* (25) as described by Mullins *et al.* (26).

Electron Microscopic Analysis of RNA Complementary to Cloned DNA Sequences. Polyadenylylated RNA extracted from 2-hr embryo cytoplasm was used to form R-loops with cloned DNA fragments exactly as described by Kaback *et al.* (27). R-loop reaction mixtures in 50% (vol/vol) formamide were prepared for electron microscopy by spreading over a 16.7% formamide hypophase (28). pBR322 plasmid DNA was added to R-loop preparations as a size standard. The concentration of clone sequence-specific transcripts was calculated in reference to rRNA hybridized to an excess of cDm103 plasmid DNA (29).

Isolation of *Drosophila* Nuclear, Plasmid, and Mitochondrial DNA. Nuclear DNA was isolated from dechorionated embryos by homogenizing them in 50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/250 mM sucrose with a Dounce homogenizer. A crude nuclear pellet was collected by centrifugation at 4,000 rpm in a Sorvall SS34 rotor for 15 min. The pellet was resuspended in homogenization buffer containing 0.1% Triton X-100 layered over 1.8 M sucrose and centrifuged 2 hr at 25,000 rpm in a Beckman SW 27 rotor. The nuclear pellet was resuspended in 10 mM Tris·HCl, pH 7.5/10 mM EDTA. Sarkosyl (10%) was added to bring the suspension to 1%, then 1.4 g of CsCl per ml was dissolved in the suspension to bring its density to 1.70 g/ml. CsCl solutions were centrifuged for 24 hr at 48,000 rpm in a Beckman SW 50 rotor. DNA-containing fractions from the center of the gradient were pooled and dialyzed extensively against 10 mM Tris·HCl, pH 7.5/1 mM EDTA and concentrated by ethanol precipitation.

The plasmids cDm103 [containing rRNA coding sequences (30)] and cDm500 [containing histone gene coding sequences (31)] were isolated exactly according to Anderson and Lengyel (32). Mitochondrial DNA was isolated by the procedure of Buttmann and Laird (33).

Characterization of Nucleic Acids by Hybridization. Various DNA preparations were characterized by restriction endonuclease (Bethesda Research Laboratories) digestion followed by 0.8% agarose gel electrophoresis (34). Cloned and genomic DNAs were transferred to nitrocellulose paper by the procedure of Southern (35). Both RNA and DNA blots and λ replica screening filters were hybridized according to the methods described by Mullins *et al.* (34). DNA probes were radioactively labeled with [α-^{32}P]dCTP to an average specific activity of 10^8 cpm/μg of DNA by using a nick translation kit (New England Nuclear). Radioactive cDNA (10^8 cpm/μg) was synthesized from cytoplasmic polyadenylylated 2-hr embryo RNA according to the procedure detailed by Mullins *et al.* (34).

RESULTS AND DISCUSSION

Selection of Recombinant DNA Clones from a Genome Library. In order to examine the expression of the first genes transcribed during embryogenesis, preblastoderm RNA labeled with ^{32}P *in vivo* was used to select recombinant DNA clones from a library of *Drosophila melanogaster* genomic sequences cloned in phage λ. In the initial high-density screening procedure, recombinant phage, containing five genome equivalents of *Drosophila* DNA, were grown and their DNA was transferred to nitrocellulose filters. The filters were hybridized with preblastoderm RNA labeled with ^{32}P *in vivo*. Although approximately 500 plaques bound detectable radioactivity, only 50 were selected for subsequent screening. In the secondary screening procedure, the 50 positive plaques were individually plated at low density, replica transferred, and immobilized on filters, which were again hybridized with preblastoderm ^{32}P-labeled RNA. Forty-two plaques, which were derived from 18 of the original 50 plaques, were selected for secondary screening and found to bind detectable radioactivity, and these were subsequently plaque-purified.

In order to visualize hybridization with plaques more easily, an average of 2.5×10^7 phage of each purified stock were spotted on a bacterial lawn in a grid pattern and grown into colonies. The grid pattern of phage colonies was transferred and immo-

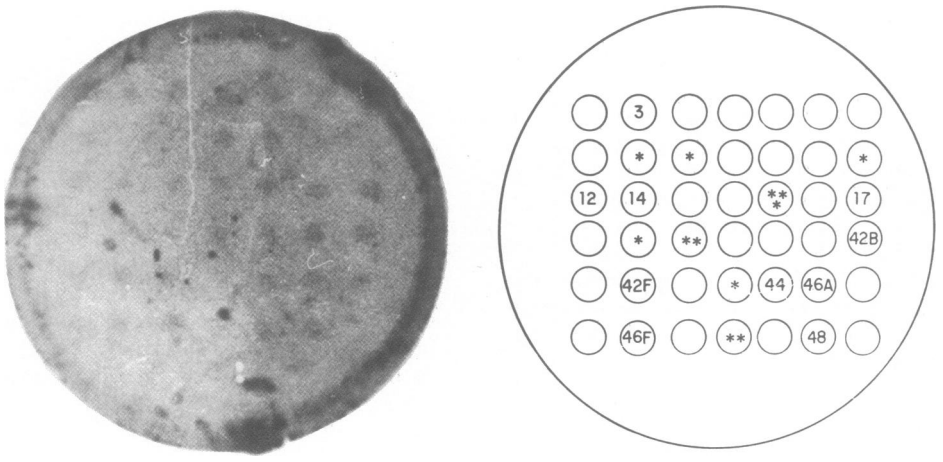

FIG. 1. Hybridization of selected recombinant phage with preblastoderm ^{32}P-labeled RNA. Phage selected by screening of the *Drosophila* genomic library were grown in a grid pattern, transferred to a nitrocellulose filter, and hybridized to ^{32}P-labeled preblastoderm RNA (approximately 10^7 cpm). The diagram of the autoradiograph indicates the grid pattern of phage (○), recombinant phage selected for further analysis (3–48), no growth of phage (*), phage growth but no ^{32}P-labeled RNA hybridization (**), and the one clone that cross-hybridized with the rRNA gene probe (cDm103) (***).

bilized on filters, which were hybridized with preblastoderm ^{32}P-labeled RNA. Most of the colonies showed hybridization with preblastoderm RNA. Identical filters were hybridized with radioactively labeled mitochondrial DNA as well as with histone and rRNA gene probes. The plasmid cDm500 was used as the source of histone gene sequences (31) and the plasmid cDm103 served as the source of rRNA gene sequences (30). The results of all these hybridizations are presented in Fig. 1.

Because the genomic library was constructed from nuclear DNA, as expected, no colonies showed hybridization to the mitochondrial DNA. On the other hand, histone and rRNA genes are middle repetitive sequences in the *Drosophila* genome (220 copies and 260 copies per haploid genome, respectively) and represent a large portion of the total embryo RNA [0.1% and 90%, respectively (22, 32)]. Because we were interested in identifying potential developmentally specific, unique genes transcribed in preblastoderm embryos, we wished to exclude the histone and rRNA genes from further selection. DNA from one selected phage colony hybridized with the rRNA gene probe. This colony was not used for further study. No hybridization of the phage grid colonies to the histone gene probe was detected. Ten of these recombinant phage displaying positive hybridization to newly synthesized preblastoderm RNA were selected for further characterization. These are indicated in Fig. 1.

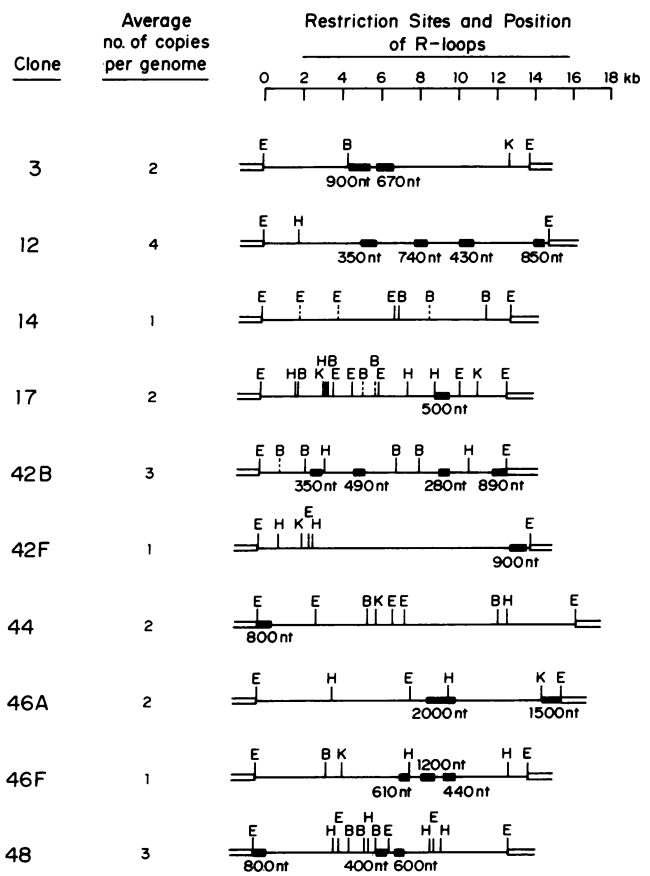

FIG. 2. Restriction maps of cloned sequences and location of regions transcribed in preblastoderm embryos. Cloned DNA was digested with the restriction endonucleases *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), and *Kpn* I (K) and electrophoresed on agarose gels. A mixture of phage λ *Hin*dIII and phage φX174 *Hae* III fragments was electrophoresed in an adjacent lane and used as molecular weight markers. The location of the gene(s) expressed in preblastoderm embryos was determined by the measurement of cloned sequences containing R-loops viewed by electron microscopy. The positions of the R-loops are shown by bars on the restriction maps. Clone 14 gave no observable R-loops in 250 molecules visualized. The estimated gene copy numbers of the cloned sequences listed are derived from the minimum number of bands that hybridized to digested genomic DNA in excess of the number of bands expected from restriction enzyme analysis of each cloned sequence. kb, Kilobases; nt, nucleotides.

Genomic Representation of Cloned Sequences. To determine the number of copies of the cloned preblastoderm transcribed sequences in the *Drosophila* genome, nuclear DNA from 0- to 2-hr embryos was digested with several restriction endonucleases, electrophoresed, and blotted according to the method of Southern (35). Genomic blots were hybridized with radioactively labeled cloned sequences and subsequently autoradiographed (data not shown). On the basis of these results and the detailed restriction maps of the 10 selected cloned sequences (shown in Fig. 2), it was determined that the *Drosophila* genome contains an average of one to four copies of each cloned sequence. The average copy number is also listed in Fig. 2.

Identification of Coding Sequences on the Cloned DNA. Several methods were used to localize the coding sequence(s) expressed in preblastoderm embryos within the selected recombinant phage DNA. Initially, preblastoderm ^{32}P-labeled RNA was hybridized to blots of restriction fragments of cloned sequences. High nonspecific binding, the low level of clone-specific sequences within the ^{32}P-labeled RNA population, and possibly competing nonradioactively labeled maternal sequences contributed to ambiguous results using this method. Therefore, the coding region(s) in the selected clones was identified by electron microscopic observation of R-loops formed with total preblastoderm RNA. The position of the R-loops is presented in Fig. 2. Representative examples of these R-loops are shown in Fig. 3.

Level of Expression of the Cloned Sequences. In order to evaluate the amount of RNA transcribed from the cloned sequences during the preblastoderm stage, cloned DNA immobilized on nitrocellulose filters was hybridized to preblastoderm ^{32}P-labeled RNA. DNA from each of the 10 clones hybridized between 0.01% and 0.16% of the total preblastoderm ^{32}P-la-

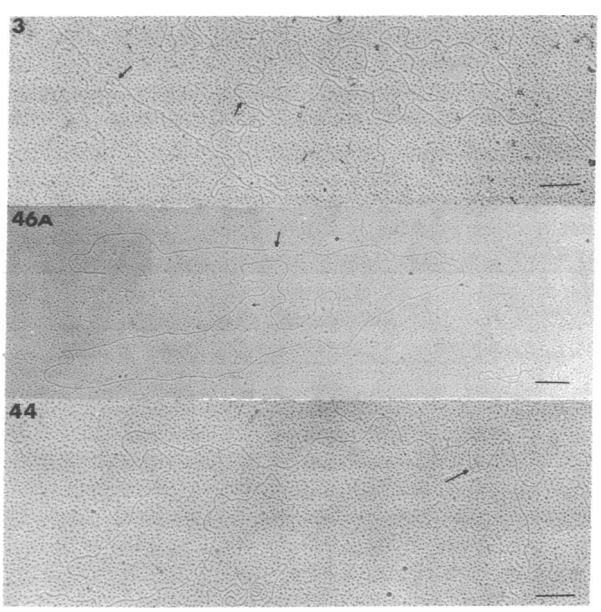

FIG. 3. R-loops of preblastoderm RNA and cloned sequences. Polyadenylylated RNA from preblastoderm embryo cytoplasm was used to form R-loops with each of the cloned DNAs. Typical structures observed in the electron microscope are shown here. The arrows point to R-loops. The bars indicate the length of 1 kb of double-stranded DNA.

beled RNA in three separate experiments, as compared to no DNA or prokaryotic DNA filter controls.

The concentration of total preblastoderm RNA complementary to the cloned sequences was obtained from R-loop data. The fraction of cloned DNA molecules containing R-loops can be compared to that obtained in a standard reaction, that is, a known amount of rRNA is hybridized under identical conditions to an excess of a cloned DNA fragment carrying the 18S and 28S rRNA genes [cDm103 (30)]. The ratio of R-loops observed is a measure of the concentrations of the RNA present in the reaction mixture containing the unknown (29). The concentrations of total preblastoderm polyadenylylated transcripts complementary to cloned sequences determined in this way range up to 0.28% as shown in Table 1. Except for clone 14, for which no R-loops were seen among 250 molecules, the low level of total preblastoderm RNA that hybridized to each clone was similar to the amount of complementary newly synthesized RNA found for these sequences.

Developmental Sequence of Expression of the Cloned DNAs. The size and pattern of expression of RNAs encoded by the 10 selected cloned sequences was further investigated by hybridization with blots of electrophoresed cytoplasmic RNA from different early embryo stages. Multiple RNA species were detected for several cloned sequences. The detected RNA species are generally small (less than 1,000 nt), reflecting the RNA size ranges found in R-loops and in the population of preblastoderm RNAs labeled with ^{32}P *in vivo* (Fig. 2; unpublished data).

Three types of temporal expression patterns were found for the selected cloned sequences. First, clones 12, 14, and 17 hybridized only to RNA species from 0- to 1.25-hr embryos (represented by clone 12 in Fig. 4). The multiple transcripts from clones 12 and 14 appear in approximately equal concentrations, suggesting that these genes may be coordinately regulated. Exclusive transcription of genes in preblastoderm embryos is surprising, and these sequences may specify early fate-determination products for the blastoderm embryo. During differential screening of a *Drosophila* cDNA library with pre- and postblastoderm RNA, Scherer *et al.* (17) also observed a low number of sequences found exclusively at the preblastoderm stage. They, however, attributed this phenomenon to a maternal contribution in accordance with Arthur *et al.* (36), who defined a similar transient maternal RNA population by hybridization kinetics.

Table 1. Concentration of cloned sequence transcripts in preblastoderm polyadenylylated RNA as determined by R-loops

Clone	Total no. of molecules observed	% of total RNA
3	252	0.06
12	245	0.23
14	250	<0.01
17	300	0.08
42B	257	0.28
42F	252	0.05
44	240	0.15
46A	256	0.14
46F	254	0.13
48	312	0.21

All R-loop reactions were carried out in the presence of excess DNA coding sequence. Percent of total RNA was calculated relative to an identical hybridization between rRNA and cDm103. An average molecular length of 750 nt was assumed for the polyadenylylated RNAs hybridized.

Transcripts that hybridized with clone 46A DNA (Fig. 4) also displayed a coordinated pattern of expression in early embryos. Two 46A RNA species present in low concentration at the preblastoderm stage appear to be maximally synthesized at the blastoderm stage, regress at gastrulation, and are no longer detectable in older embryos.

A second type of expression pattern is represented by clones 42F and 44, which hybridize with RNA species that remain at approximately the same low concentrations throughout the embryo stages analyzed. Clone 44 DNA hybridized to a surprisingly large species (4.5 kb) from total cytoplasmic RNA; this reaction might represent a cross-hybridization with contaminating mitochondrial DNA or a large precursor transcript. The continuing presence of similar amounts of these transcripts during early embryogenesis suggests that they might encode housekeeping functions.

The third type of expression pattern is seen in four of the selected cloned sequences that showed differentially variable expression patterns for the multiple-sized RNAs they encoded. These cloned sequences hybridized with several RNAs, each of which were present at different stages of early development. Similarly, genomic clones selected by Scherer *et al.* (17) carrying a gene transcribed specifically at the blastoderm stage also encoded genes expressed at other times in development. DNA from clones 3 and 48 (clone 3 is shown in Fig. 4) each hybridized to two RNA species barely detectable in preblastoderm and blastoderm embryos, but at gastrulation these transcripts and other additional transcripts were found to be present in much higher concentrations. One RNA species complementary to clone 46F DNA is found at low levels throughout early embryogenesis, but three other 46F transcripts are present only in blastoderm embryos.

Finally, a complex pattern of expression was also displayed by clone 42B transcripts. Two encoded RNA species are detected throughout early embryogenesis and four other complementary transcripts appear and disappear during other stages of development.

The variable expression patterns of these cloned sequences

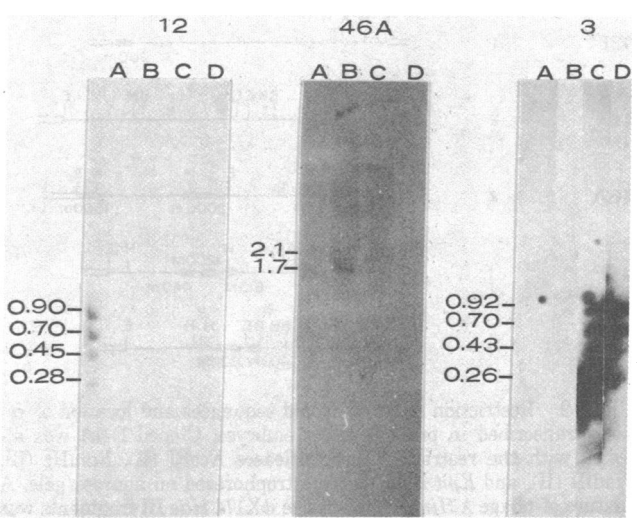

FIG. 4. Expression of cloned sequences during embryogenesis. Ten micrograms of polyadenylylated cytoplasmic RNA was isolated from 0- to 1.25-hr, 1.5- to 2.5-hr, 3- to 4-hr, and 4.25- to 5.25-hr embryos (lanes A–D, respectively), electrophoresed in denaturing agarose gels, and transferred to nitrocellulose filters. RNA sizes are indicated in kb. *Hae* III fragments of ϕX174 DNA were electrophoresed in an adjacent lane to serve as molecular weight markers. Blots were hybridized with nick-translated cloned sequences and ϕX174 DNA and subsequently autoradiographed.

indicate that simple spatial clustering of genes is not a sufficient qualification for coordinate regulation. Yet many of products of these genes seem to be required at specific stages of early embryogenesis, suggesting their possible influence on the phenotype of the embryo at that time.

Further Discussion. Both the RNA blots and the R-looping experiments confirm the low abundance and small size of the RNAs encoded on the preblastoderm RNA-selected clones. However, the RNA blots revealed that half of the cloned sequences hybridized to multiple preblastoderm stage polyadenylylated RNA species, while R-loop analysis in some cases detected fewer coding regions. Because multiple RNA species seen in the RNA blots appeared to be present in approximately equal amounts, the lack of their detection in R-loop structures due to a disproportionate concentration of each species seems unlikely. An alternative explanation of this result may be the occasional clustering of coding regions in a small portion of the cloned DNA. This phenomenon has been seen in many other coordinately regulated gene families in *Drosophila* [e.g., glue protein genes (37), chorion genes (38), cuticle protein genes (39), small heat shock protein genes (40), tRNA genes (41), and histone genes (31)]. Very tightly clustered genes would be difficult to detect by electron microscopic analysis of large recombinant molecules (here up to 45 kb) because the uncertainty of R-loop positions may be as great as 5% (28). These results may indeed indicate such a clustered sequence arrangement. Finally, it is known that overlapping but distinct RNA sequences can be transcribed from the same gene region (for example, see refs. 42 and 43). This would also give rise to multiple RNA species on a RNA blot but only one R-loop position observed in the electron microscope.

In summary, the fact that the selected cloned sequences hybridize to preblastoderm RNA labeled with ^{32}P *in vivo* indicates that the transcripts detected by this hybridization are synthesized by the embryo. These results complement those of McKnight and Miller (21), who observed a low level of preblastoderm RNA transcription by electron microscope analyses of chromatin. Such transcription in early embryos may possibly function to replenish maternal supplies of sequences, especially those required for housekeeping functions. However, because the majority of cloned sequences displayed stage-specific patterns of expression, it may be suggested that their products influence the determination of phenotype during embryogenesis.

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