MOLECULAR AND CELLULAR BIOLOGY, Apr. 1989, p. 1476–1487
0270-7306/89/041476-12$02.00/0

Vol. 9, No. 4

Sea Urchin Early and Late H4 Histone Genes Bind a Specific Transcription Factor in a Stable Preinitiation Complex

LIN TUNG, GILBERT F. MORRIS†, LAWRENCE N. YAGER,‡ AND ERIC S. WEINBERG*

Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6017

Received 15 August 1988/Accepted 21 December 1988

Early embryonic H4 (EH4) and H2B (EH2B) and late embryonic H4 (LH4) histone genes were transcribed in vitro in a nuclear extract from hatching blastula embryos of the sea urchin *Strongylocentrotus purpuratus*. The extract was prepared by slight modifications of the methods of Morris et al. (G. F. Morris, D. H. Price, and W. F. Marzluff, Proc. Natl. Acad. Sci. USA 83:3674–3678, 1986) that have been used to obtain a cell-free transcription system from embryos of the sea urchin *Lytechinus variegatus*. Achievement of maximum levels of transcription of the EH4 and LH4 genes required a 5- to 10-min preincubation of template with extract in the absence of ribonucleoside triphosphates. This preincubation allowed the formation of a stable complex which was preferentially transcribed compared with a second EH4 or LH4 template that was added 10 min later. Although the EH4 gene inhibited both EH4 and LH4 gene transcription in this assay and although the LH4 gene inhibited both EH4 and LH4 genes, neither of these genes inhibited transcription of the EH2B gene. Preincubation with the EH2B gene had no effect on the transcription of subsequently added EH4 or LH4 genes. Using this template commitment assay, we showed that the site of binding of at least one essential factor required for transcription of both EH4 and LH4 genes was located between positions −102 and −436 relative to the 5′ terminus of the EH4 mRNA. Moreover, deletion of this region resulted in a reduction in EH4 gene transcription in vitro. The sea urchin embryonic nuclear extract, therefore, should prove very helpful in the assay and purification of sea urchin gene-specific *trans*-acting factors, in the analysis of the *cis*-acting sequences with which they interact, and in biochemical studies on the formation of stable transcription complexes.

The histone genes of the sea urchin are among the best known examples of genes which are differentially regulated during early embryogenesis (for a review, see reference 41). During the cleavage and blastula stages of the sea urchin embryo, dramatic changes occur in the levels of mRNAs derived from the early embryonic (or α) genes and the late embryonic (or β, γ, and δ) genes. The early embryonic genes, which are reiterated several hundred-fold in a tandem array of a unit containing a gene for each of the five histones, are transcribed during oogenesis and between the 16-cell and midblastula stages (for reviews, see references 26 and 41). The level of early histone mRNAs per embryo increases about 10-fold during the cleavage and early blastula period and then rapidly decreases so that by the gastrula stage there is little remaining early mRNA (39, 43, 58). The late embryonic histone genes of *Strongylocentrotus purpuratus* and *Lytechinus pictus* are present in far fewer copies (2 to 12 members per genome, depending on the histone species) and are distributed in irregular clusters (9, 30, 31, 33, 34, 40). The late mRNAs are found in low levels in the egg, but in contrast to the early mRNA species, they do not increase to their maximum levels until the mid- to late blastula stage (7, 8, 20, 27, 30, 32, 34, 35, 40, 46) or even later in development (31).

The basis of these changes in early and late histone mRNA levels appears to be predominantly at the level of transcriptional control. In vivo measurements of instantaneous rates of RNA synthesis show that the rate of early gene transcription in *S. purpuratus* decreases over 10-fold in the early blastula stages (42, 58). In the case of late H2B mRNA synthesis, recent in vivo [^{3}H]uridine incorporation studies demonstrated an eightfold increase in the rate of RNA accumulation between the midblastula and late blastula stages (28a). Nuclear run-on assays, which are probably more informative than in vivo labeling experiments in indicating true transcription rates, also show a transcriptional turnoff of the early histone genes and an activation of the late genes. By this approach Knowles and Childs (32) have demonstrated that there is an 18-fold decrease in the transcription of early *L. pictus* H4 (EH4), H2A (EH2A), and H2B (EH2B) genes during blastulation and, over the same period, transcriptional increases of 5.5-fold for late H3 (LH3) and H4 (LH4) genes and 3.5-fold for late H2A (LH2A) and late H2B (LH2B) genes. These results have been repeated for the EH4 and LH4 genes of *S. purpuratus* (L. N. Yager and E. S. Weinberg, unpublished data).

Two different but complementary assay systems are now available for determining the control network that is involved in the transcriptional regulation of sea urchin genes. In one assay various DNAs are microinjected into unfertilized sea urchin eggs, and the timing of accumulation of the derived transcript during development is determined (11, 16–18, 28, 29, 35, 45, 54). This approach is beginning to be applied to the study of sequences which specify the correct temporal and spatial expression of sea urchin actin (29, 29) and H2B, H2A, and H1 histone genes (11, 35, 54) during embryonic development. A potential second assay for *cis*-regulatory sequences and *trans*-acting factors is provided by the ability to transcribe specific genes in nuclear extracts from sea urchin embryos. A nuclear extract derived from *Lytechinus variegatus* blastulae has been shown to be competent to transcribe sea urchin U1 small nuclear RNA genes (48). The combination of in vivo and in vitro assays provides a potentially powerful way to define gene sequences that are

* Corresponding author.
† Present address: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.
‡ Present address: Department of Biology, Temple University, Philadelphia, PA 19122.

important for attaining appropriate temporal and quantitative control of developmentally regulated genes.

In this report we present our initial results of studies on the in vitro transcription of EH4 and LH4 genes and an EH2B gene in which we used a nuclear extract derived from *S. purpuratus* embryos. We show that transcription of all three genes is initiated at or near the site expected from analysis of the 5′ terminus of the mature RNA. Achievement of maximum levels of transcription of the EH4 and LH4 genes requires a period of preincubation of template with extract in the absence of ribonucleoside triphosphates (NTPs). This period of preincubation with an EH4 or LH4 template allowed the formation of a transcription complex which was preferentially transcribed compared with a second EH4 or LH4 template that was added 10 min later. Moreover, the transcription of the second template was decreased when the concentration of the first template was increased. Although the EH4 gene inhibited both EH4 and LH4 gene transcription in this assay and although the LH4 gene inhibited both EH4 and LH4 genes, neither gene inhibited the transcription of the EH2B gene. We show that the site of binding of at least one essential factor that is required for the transcription of both EH4 and LH4 genes is located between positions −102 and −436 relative to the EH4 gene transcriptional start site. The transcription commitment assay, therefore, somewhat unexpectedly allowed the demonstration of a factor that is required for the maximal transcription of two different H4 genes that are expressed with quite different developmental programs. We have no evidence, however, for a factor which would act positively on the coordinately regulated EH4 and EH2B genes.

MATERIALS AND METHODS

Preparation of nuclear extracts. *S. purpuratus* eggs were fertilized and embryos were grown at 17°C until they reached the hatching blastula stage at 18 h of development. Each preparation was begun by filtering a volume of 20 ml of packed embryos on a 45-μm-pore-size nylon mesh (Nitex; Tetko, Inc.). Procedures that were previously used to prepare nuclei from *L. variegatus* embryos (47) were used with only minor modifications. All embryo washes and homogenizations were done on ice. Filtered embryos were washed twice with 40 ml of 0.55 M KCl; each wash was followed by centrifugation at 2,000 rpm in a Sorvall HB-4 rotor (DuPont Co., Wilmington, Del.). After a subsequent wash in 40 ml of 0.25 M sucrose–10 mM Tris (pH 8.0)–0.1 M EDTA, embryos were centrifuged at 3,500 rpm in the same rotor. The 20 ml of packed embryos was then suspended in an equal volume of homogenization buffer (0.32 M sucrose, 5 mM $MgCl_2$, 10 mM Tris [pH 8.0], 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 1 mM dithiothreitol, 1 mM spermidine, 1% Trasylol, and 0.1 mM phenylmethylsulfonyl fluoride) and disrupted with approximately 15 strokes of a tight-fitting B pestle in a Dounce homogenizer (until almost all cells were broken). The total volume was then adjusted to 180 ml (nine times the original volume of embryos) by adding 20 ml of homogenization buffer (without sucrose) and then 120 ml of 2 M sucrose (final sucrose concentration, 1.4 M). This suspension was layered over a 5-ml pad of the buffer containing 2 M sucrose and centrifuged at 19,000 rpm for 1 h at 2°C in a rotor (SW27). Pelleted nuclei were suspended by homogenization in glycerol storage buffer as described by Morris and Marzluff (47) and stored at approximately 10^9 nuclei per ml in liquid N_2.

Extracts were prepared from isolated nuclei by the procedure of Morris et al. (48), which involved the lysis and extraction of the nuclei in 0.4 M $(NH_4)_2SO_4$ followed by precipitation of protein by the addition of 0.25 g of $(NH_4)_2SO_4$ per ml of solution. The buffer which was added to the stored frozen nuclei was slightly different (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5], 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM spermidine, 0.1 mM phenylmethylsulfonyl fluoride), as was the buffer in which the final $NH_4(SO_4)_2$-precipitated protein was suspended (15% [vol/vol] glycerol, 25 mM HEPES [pH 7.5], 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride). The dissolved precipitate was dialyzed against 300 to 500 volumes of this buffer until the conductivity approximately equaled that of 100 mM KCl. Insoluble material was removed by centrifugation for 3 min in a microfuge, and the supernatant was stored in liquid N_2. Approximately 1 ml of extract containing 5 to 10 mg of protein per ml was obtained from 20 ml of packed hatching blastula embryos.

In vitro transcription assays. The standard reaction consisted of the following components in a final reaction volume of 21 μl: 20 mM HEPES (pH 7.8), 70 mM KCl, 5 mM $MgCl_2$, 0.6 mM of each of the three unlabeled NTPs, 5.9 μCi of [α-^{32}P]UTP (800 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), 30 μM unlabeled UTP, 1 mM dithiothreitol, 3.5 U of RNasin (Promega), 5 μl of nuclear extract, and up to 7 μl of DNA template (to yield DNA concentrations up to 80 μg/ml). DNA templates were truncated by digestion with an appropriate restriction enzyme (New England BioLabs, Inc., Beverly, Mass.) (see Fig. 1 for the enzymes that were used). Reactions were performed at 16°C for 30 min and stopped by the addition of 125 μl of termination buffer (1% Sarkosyl [Sigma Chemical Co., St. Louis, Mo.], 100 mM NaCl, 100 mM Tris [pH 8.0], 10 mM EDTA). The samples were extracted with 100 μl of redistilled phenol saturated with TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). After separation and recovery of the aqueous phase, nucleic acids were precipitated by the addition of 400 μl of ethanol and NaCl to a final concentration of 0.25 M. The precipitated reaction products were dissolved in 5 μl of 80% formamide–20% 0.1× TBE (1× TBE is 50 mM Tris, 50 mM boric acid, 1 mM EDTA [pH 8.0]) and analyzed by electrophoresis on 8% polyacrylamide gels (acrylamide-methylene bisacrylamide; 30:1) containing 7 M urea and 50 mM Tris borate–1 mM EDTA buffer (47). The gel was stained in water with 1 μg of ethidium bromide per ml for at least 10 min and then destained in water. The stained gel was photographed under UV light, soaked in 5% acetic acid–5% isopropanol, and then dried and exposed to X-ray film (RX Fuji).

DNA templates. The embryonic LH4 gene construct used here (referred to as pLH4) was derived from the genomic clone λSpL22 (30) by subcloning a 1.6-kilobase-pair (kb) *Sal*I-*Eco*RI fragment into the *Sal*I and *Eco*RI sites of a pBS plasmid (Stratagene). The fragment contained 553 base pairs (bp) of sequence upstream of the mRNA cap site and 626 bp of sequence downstream of the 3′ mRNA terminus, in addition to the structural gene. The EH4 histone gene construct used here (pEH4) was derived from the 6.5-kb early embryonic histone gene repeating unit cloned in pCO2 (49). A 1.1-kb *Dra*I-*Ava*II fragment (extending 436 bp upstream from the cap site to 279 bp downstream from the 3′ mRNA terminus) was filled in with Klenow fragment and cloned into the *Hinc*II site of pUC118 (53). The EH2B gene used in these experiments was also derived from the 6.5-kb repeating unit that was previously cloned in pCO2. A 1.95-kb *Sal*I-*Xba*I fragment from this plasmid was subcloned into the *Sal*I and *Xba*I sites of a Bluescript vector (KS[−]; Strata-

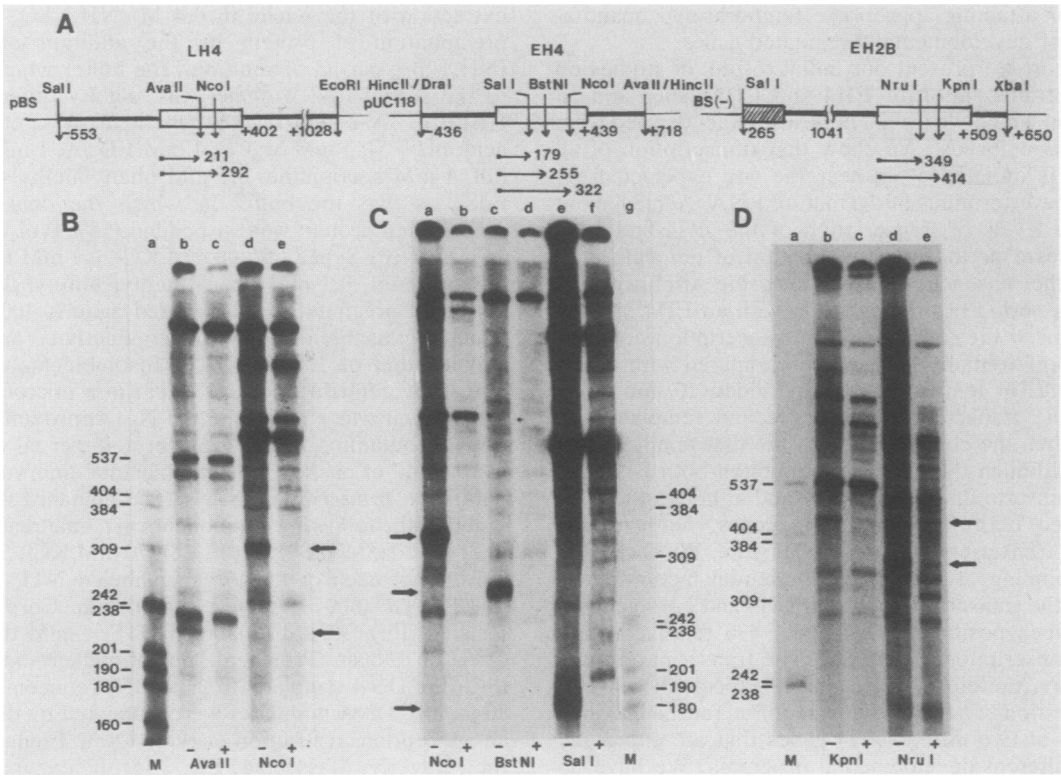

FIG. 1. Templates used for in vitro transcription reactions and their RNA products. (A) Maps of the three histone genes that were used as templates. Open boxes indicate the position of the mRNA sequences; thick lines represent intergenic spacer sequences, nontranscribed flanking sequences, or both; and thin lines represent plasmid vector DNA. In the case of the H2B template, the hatched box indicates the EH4 gene sequence, which is located upstream of the H2B gene. Numbers preceded by a plus or a minus sign refer to the distance (in nucleotides) from the 5'-terminus-encoded nucleotide of each mRNA (referred to as position +1 in each case). Below each map are indicated the sizes (in nucleotides) of the runoff transcripts expected from each truncated template if initiation occurred at position +1. (B) Transcripts derived from the LH4 gene templates. Lane a, Marker (M) DNA (*Hpa*II-digested pLH4 DNA labeled by filling in with the Klenow fragment and [α-^{32}P]dCTP; numbers are in nucleotides); lanes b and c, pLH4 DNA cut with *Ava*II used as the template; lanes d and e, pLH4 DNA cut with *Nco*I used as the template. In the reactions displayed in lanes c and e, α-amanitin (5 μg/ml; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added to the mixture prior to the addition of the DNA template. In all cases DNA templates were added at 40 μg/ml and were allowed to preincubate with extract for 10 min at 16°C prior to the addition of NTPs. The reaction was then allowed to proceed for 30 min at 16°C. (C) Transcripts derived from EH4 gene templates. The pEH4 DNA was cut with *Nco*I (lanes a and b), *Bst*NI (lanes c and d), or *Sal*I (lanes e and f) before it was added to the reaction mixture. The addition of α-amanitin (lanes b, d, and f), the DNA marker (M) (lane g), and reaction conditions were as described above for panel B. (D) Transcripts derived from EH2B gene templates. The pEH2B DNA was cut with *Kpn*I (lanes b and c) or *Nru*I (lanes d and e) before it was added to the reaction mixture. The addition of α-amanitin (lanes c and e), the DNA marker (M) (lane a), and reaction conditions were as described above for panels B and C.

gene); the resulting plasmid was designated pEH2B. The subcloned segment extended from within the H4 gene through 1,041 bp of the spacer between the H4 and H2B genes and the H2B structural gene to a position 141 bp downstream of the consensus 3' mRNA terminus sequence. Diagrams of all three inserts are shown in Fig. 1. EH4 gene upstream deletion templates (positions −102, −43, and −7) were derived from *Eco*RI-treated pEH4 by *Bal* 31 nuclease digestion. The digested DNA was filled in with Klenow fragment, cut with *Hin*dIII, and recloned into the *Hin*cII and *Hin*dIII sites of pUC118.

RESULTS

Histone transcripts are initiated in vitro at or near the proper mRNA cap site. We used a sea urchin embryo nuclear extract, which was prepared from 18-h hatching blastula embryos by the method of Morris et al. (48), to transcribe a variety of sea urchin histone genes in vitro. The transcription assay used in this study was based on the addition of a template that was truncated within the coding sequence. Proper initiation in vitro should result in the formation of an RNA with a predictable size. Subcloning of the EH4, LH4, and EH2B genes that were used as templates in this study was done as described above; maps of the three DNAs are presented in Fig. 1A.

Comparison of the 5' mRNA termini of the three RNAs as they exist in vivo with the site of initiation of transcripts in vitro indicated that the histone genes were appropriately transcribed in the nuclear extract. The 5' end of the LH4 mRNA specified by the SpL22 gene was determined by S1 nuclease mapping of total cytoplasmic RNA (data not shown) to be at the A or G residues 37 and 38 bases upstream of the AUG signal, respectively (we refer to the A residue 38 bp 5' of the ATG codon in the gene as position +1). The 3' end of the mRNA was assumed to be at the consensus position (26) just downstream from the highly conserved hyphenated inverted repeat that has been shown to be essential for the generation of correct histone mRNA 3' termini (2, 3). The mature RNA was therefore expected to be 402 bases long, which is consistent with the results of

Northern blots of cytoplasmic RNA (30). If proper initiation takes place in vitro, cutting of the pLH4 template with *Ava*II or *Nco*I before it is added to the reaction should result in the formation of RNAs of 211 and 292 bases in length, respectively. RNAs of these sizes could be seen (Fig. 1B, lanes b and d), indicating that initiation in the extract occurred at or near the site that was used in vivo. Transcription was catalyzed by polymerase II since the formation of RNA of predicted size in each case was sensitive to α-amanitin when it was present at 5 μg/ml (Fig. 1B, lanes c and e).

The EH4 histone gene template (pEH4) that was used is also shown in Fig. 1A. This gene is part of the 6.5-kb early embryonic histone gene repeating unit that has been cloned in pCO2 (49). The transcriptional start site was taken to be an A residue (which we designated as position +1) 67 bp upstream from the ATG codon signal in the gene, as determined by primer extension analysis of polysomal RNA and the coincidence of the 5′ RNA terminus with a consensus sequence that was present in each of the five early histone genes in the repeat unit (52). If pEH4 was cut with *Sal*I, *Bst*NI, or *Nco*I before it was added to the extract, correct transcriptional initiation should result in the formation of RNAs of 179, 255, and 322 bases, respectively. RNA products with these approximate sizes were seen (Fig. 1C, lanes a, c, and e, respectively). In adjacent lanes (Fig. 1C, lanes b, d, and f, respectively), synthesis of these species was shown to be sensitive to the addition of 5 μg of α-amanitin per ml.

The EH2B gene used in these experiments was also derived from the 6.5-kb repeating unit that was previously cloned as pCO2. The 5′ mRNA terminus was regarded here as the A residue at a position 78 bp upstream of the ATG signal in the gene (termed position +1), as indicated by Sures et al. (52), on the basis of a consensus sequence (although primer extension on polysomal RNA gave sequence only to a point 7 bases downstream from this site). *Nru*I and *Kpn*I cut within the gene at positions 349 and 414 bp downstream, respectively, from the expected cap site position. RNAs with these approximate sizes were produced in vitro on these templates (Fig. 1D, lanes b and d, respectively), and of all the labeled bands on the gel, these species were the only ones that were specifically inhibited if 5 μg of α-amanitin per ml was added to the transcription reaction (Fig. 1D, lanes c and e, respectively).

These results indicate that the LH4, EH4, and EH2B genes are all transcribed faithfully in the nuclear extract. The exact initiation point could not be determined in these experiments, but the site was at or within a few bases of the position that is believed to be used in vivo. As has been shown previously with the sea urchin nuclear extract (48), and as has been found to be typical of many cell-free template runoff transcription assays (13–15, 21, 22, 25, 37, 56, 57), a number of α-amanitin-insensitive transcripts were also synthesized in vitro. We did not study the origin of these labeled RNAs; some may have been polymerase III transcripts, but others coincided on the gel with the positions of DNA fragments and probably represented end-labeled DNA molecules or end-to-end transcripts. The whole gel is displayed in Fig. 1 to show how the specific transcription products appeared in relation to these other background bands. In Fig. 2 to 10, we show only the regions of the gel containing the products of interest, although the background bands are present elsewhere in the lanes.

Although the EH4, LH4, and EH2B genes were transcribed in the nuclear extract, this was not the case for all the gene templates that we tested. The EH1 gene from the pCO2 repeat unit did not yield transcription products, and a LH3 template derived from the same λSpL22 clone which contained the LH4 gene described above was only weakly transcribed. In these cases the inability to transcribe the genes or the low level of transcription might have been caused by a lack of a *cis*-acting sequence of the particular template that was used, an absence in the extract of factors that were required for transcription of these genes, or a failure to optimize reaction conditions for these particular templates.

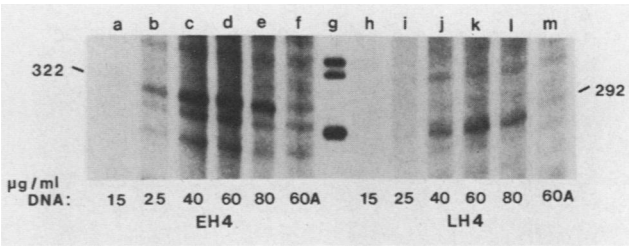

FIG. 2. Optimization of DNA concentration for transcription. Either pEH4 DNA digested with *Nco*I (lanes a to f) or pLH4 DNA digested with the same enzyme (lanes h to m) was added to a reaction mixture to achieve the final concentrations indicated below each lane (in micrograms per milliliter). In the case of the reactions containing 60 μg of DNA per ml, controls with 5 μg of α-amanitin per ml were also transcribed (lanes f and m). The approximate sizes of the α-amanitin-sensitive runoff transcripts (in nucleotides) are indicated to the left and right of the gel. Labeled marker DNA (lane g) was described in the legend to Fig. 1. NTPs were added to the reaction mixture directly after the DNA was added without a preincubation period.

Optimization of the transcription reaction. The amount of specific transcript obtained in a reaction was highly dependent on the concentration of DNA that was added to the extract. A threshold level of DNA must be added for transcription to occur. Figure 2 illustrates results of an experiment in which different amounts of either the EH4 (Fig. 2, lanes a to f) or the LH4 (Fig. 2, lanes h to m) template were added to an extract. A DNA concentration in excess of 15 μg/ml was required to produce more than a trace of specific transcript. If the input DNA concentration was raised to 25 μg/ml (for the EH4 gene) or 40 μg/ml (for the LH4 gene), the specific transcripts (and background bands) were increased dramatically. This effect was also obtained by adding 20 to 40 μg of nontranscribed carrier DNA (e.g., pUC118) per ml to the extract before adding the template DNA (data not shown). In such cases it was possible to obtain specific transcripts from as little as 5 μg of added pLH4 or pEH4 template per ml. This effect was therefore attributed to the titration, by any added DNA, of nonspecific inhibitors of transcription. The threshold amount of template or nonspecific DNA that was needed to allow transcription varied from extract to extract and had to be empirically determined for each nuclear extract preparation. Although the EH4 template appeared to be more effectively transcribed than the LH4 template (Fig. 2), this was not always the case. In many extracts, equal amounts of the two templates gave equal transcription levels. Figure 2 also shows that the addition of high amounts of template inhibited the level of transcription. The addition of 80 μg of either pEH4 or pLH4 per ml resulted in a decrease in transcription product compared with the amount produced in reactions in which 60 μg of template per ml was used. The exact concentration at which DNA started to inhibit the reaction also varied from extract to extract.

The transcription efficiency also depended on the amount

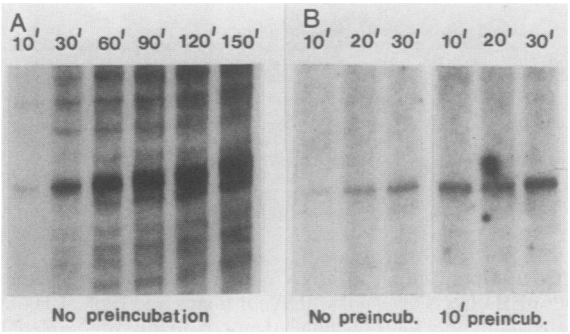

FIG. 3. Reaction kinetics with and without preincubation of template DNA. pLH4 DNA cut with *Nco*I was added to a series of tubes containing the reaction mixture. The DNA was added to attain a final concentration of 25 μg/ml; the final volume of each reaction was 21 μl. (A) NTPs were added directly after the addition of DNA template; and at the indicated times after the addition of NTPs, the reactions were stopped and RNAs were processed as described in the text. (B) Reactions were set up with the same DNA templates, concentrations, and volume as described above for panel A; however, the extract used was from a different preparation. The left three lanes of panel B were reactions in which NTPs were added directly after the DNA template (as in panel A); in the reactions displayed in the right three lanes of panel B, the DNA template was preincubated for 10 min at 16°C before the addition of the NTPs. At 10, 20, or 30 min in each case, the reactions were stopped and RNAs were processed as described in the text.

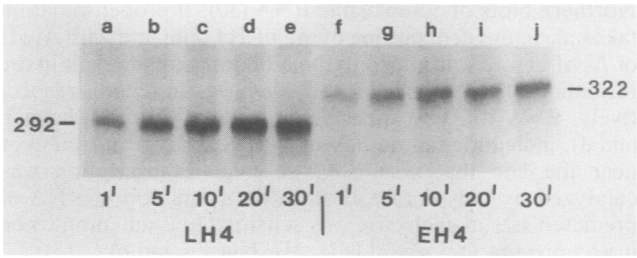

FIG. 4. Requirements for preincubation for maximal transcription of H4 genes. *Nco*I-cut pLH4 DNA (lanes a to e) or *Nco*I-cut pEH4 DNA (lanes f to j) (to attain a final concentration of 25 μg/ml) was added to a reaction mixture that lacked only the NTPs. The DNA was allowed to incubate at 16°C with the extract for the times indicated below each lane. At these times, NTPs were added to each reaction mixture and the reaction was allowed to proceed for an additional 30 min. The samples were placed on ice after stop buffer was added, and they were processed together for the isolation of RNA as described in the text. The sizes of the runoff RNAs produced from these templates are indicated (in nucleotides) to the left and right of the gel.

of extract that was used in the reaction. An optimum of 15 μl of extract per 40-μl reaction volume was often found (for extracts of 10 mg of protein per ml), and with some extracts transcription was suppressed when the extract input was increased to a higher concentration (data not shown). The level of transcription was also dependent on the concentration of KCl, with an optimum at 70 mM. All experiments described below were performed at this KCl concentration and with 5 μl of extract in a reaction volume of 21 μl (to conserve extract). The DNA concentrations were varied as described for each experiment.

Reaction kinetics suggest a requirement for stable complex formation. The production of specific transcripts proceeded in vitro for at least 90 min. LH4 transcription was followed over a 150-mm period (Fig. 3A). There appeared to be a lag in transcription since the amount of product in the 10-min reaction was very low (only one-seventh of the amount made in 30 min, as determined by densitometric analysis of the gel in Fig. 3A). To determine whether the lag could be eliminated, we decided to try a preincubation step, in which DNA template was added to the extract and allowed to incubate for 10 min in the absence of NTPs. Transcription of the LH4 gene over a 30-min period was followed both with (Fig. 3B, left three lanes) and without (Fig. 3B, right three lanes) the 10-min preincubation period (a different extract from that of Fig. 3A was used). The lag was not evident when the template was preincubated, indicating that a period of several minutes may be required for the assembly on the naked DNA of factors that are required for high levels of transcription. Densitometric analysis of the gel in Fig. 3B indicated that there was a linear accumulation over the 30-min period when the template was preincubated for 10 min, whereas when the template was not preincubated, the amount of product made during the first 10 min of the reaction was only one-quarter of that made in the first 20 min. The amount of product that was formed after 10 min of reaction after the preincubation step was even greater than the amount of product that was formed in a 30-min reaction period when the template was not preincubated. This suggests that the addition of substrate or the act of transcription itself inhibits the formation of the DNA-factor complex that is necessary for optimal transcription.

To test this idea further, we incubated pLH4 and pEH4 templates for various times before we added NTPs to start the transcription reaction, and the reaction was allowed to proceed for 30 min. The results presented in Fig. 4 show that a preincubation period of 5 to 10 min is required to obtain optimal transcription levels in the extract. Densitometric scans of the gel in Fig. 4 yielded transcript ratios for LH4 of 1.0:3.0:4.7:4.6:4.2 and for EH4 of 1.0:2.3:3.1:3.8:4.3 for preincubation times of 1, 5, 10, 20, and 30 min, respectively (each series was individually normalized to the amount of transcript made with 1 min of preincubation in each case). The data indicate that the addition of NTP substrates (or the act of transcription itself) inhibits further complex formation since transcription was very low with only a 1-min preincubation period, even though the template continued to be immersed in the extract over the 30-min reaction period. We would not have expected such a great difference between the use of templates whose total incubation period in extract was 31 min on one hand (1-min preincubation) and 36 min on the other (5-min preincubation). The complex appeared to be stable once it was formed, since the conditions established during the period of preincubation manifested their effects during the subsequent 30-min transcription period. This idea was tested further by performing commitment assays by using two templates which yielded distinguishable transcription products.

EH4 and LH4 genes form a stable complex, with at least one factor not being required for transcription of the EH2B gene. A property of stable transcription complexes is that once factors bind to the template, they are not available for transcription of a second template added to the reaction at a later time (1, 5, 10, 13, 15, 23, 25, 36, 38, 51, 55). We performed an experiment that was designed to test whether the binding of transcription factors to the histone genes commits a template to transcription even if a second template is added after a period of preincubation. Such a commitment assay could also be used to determine whether factors are shared by different genes. Results of an experi-

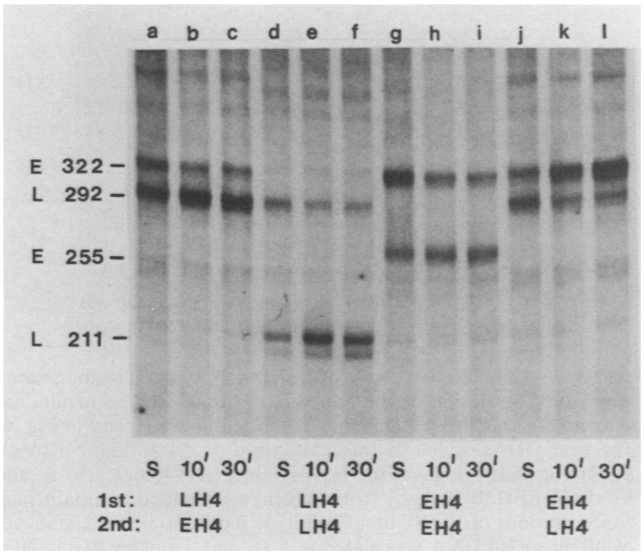

FIG. 5. Commitment assay demonstrates formation of a stable transcription complex. Two genes either were added simultaneously to the reaction mixture (lanes a, d, g, and j) or a second gene was added to the mixture 10 min (lanes b, e, h, and k) or 30 min (lanes c, f, i, and l) after the addition of the first gene. The reaction mixture was complete except for NTPs at the time of DNA addition. In all cases, an additional 10 min of preincubation preceded the addition of the NTPs, and the reaction was then allowed to proceed for 30 min. All operations after the addition of the first template were carried out at 16°C. The two DNAs added were as follows: *Nco*I-cut pLH4 as the first template and *Nco*I-cut pEH4 as the second template (lanes a to c); *Ava*II-cut pLH4 as the first template and *Nco*I-cut pLH4 as the second template (lanes d to f); *Bst*NI-cut pEH4 as the first template and *Nco*I-cut pEH4 as the second template (lanes g to i); *Nco*I-cut pEH4 as the first template and *Nco*I-cut pLH4 as the second template (lanes j to l). All templates were added to attain a final concentration of 40 μg/ml. Sizes of the runoff transcripts (in nucleotides) are indicated to the left of the gel.

ment of this design are presented in Fig. 5. Two genes were either simultaneously added to the extract or a second gene was added 10 or 30 min after the first gene was added. In all cases an additional 10 min of preincubation preceded the addition of NTPs, and the reaction was then allowed to proceed for 30 min. Results of an experiment in which two pLH4 templates were used are given in Fig. 5 (lanes d to f); the first template was cut with *Ava*II and the second was cut with *Nco*I to give transcripts of 211 and 292 bases, respectively (Fig. 1). The simultaneous addition of the two templates resulted in equal transcription levels from the two templates (Fig. 5, lane d). When the *Ava*II-cut template was added for 10 or 30 min before the *Nco*I-cut template was added, a higher level of transcription was seen for the first template and transcription of the second template was markedly lower (Fig. 5, lanes e and f). These results are consistent with those presented in Fig. 4, in which it is indicated that a 10-min preincubation period is sufficient to achieve maximal transcription. The results presented in Fig. 5, however, give the additional information that the complex was stable and that the factor(s), once bound to the DNA, transferred very slowly, or not at all, to a second template.

A similar experiment was done with two pEH4 templates. The results of an experiment in which an EH4 gene cut with *Bst*NI was used as the first template and the same gene cut with *Nco*I was used as the second template (to yield 255 and 322 base transcripts, respectively) are shown in lanes g to i of Fig. 5. In this case the simultaneous addition of templates (Fig. 5, lane g) resulted in preferential transcription of the *Nco*I-cut pEH4 template (for as yet unknown reasons, *Bst*NI-cut pEH4 templates were often not as effectively transcribed as *Nco*I-cut pEH4 templates; see also Fig. 1). As in the case of the two pLH4 templates, a 10-min preincubation with the first template resulted in an inhibition of transcription of the second template (Fig. 5, lane h). In this combination, the first template (*Bst*NI cut) was transcribed more efficiently after the 10-min preincubation, but the enhancement was not nearly as dramatic as that with the *Ava*II-cut pLH4 (Fig. 5) or with *Nco*I-cut pLH4 or pEH4 (Fig. 4 and 5). The result indicates, however, that at least one factor which is required for EH4 transcription is sequestered in a stable complex with the first pEH4 template when this DNA is allowed to preincubate with the extract. Results of similarly designed experiments are presented in Fig. 5, but in these cases the first and second templates were different genes (Fig. 5, lanes a to c and j to l). The LH4 gene sequestered a factor that is needed for maximal transcription of the EH4 gene (compare the 322-base transcript in lane a with that of lanes b and c in Fig. 5), and the EH4 gene bound a factor that is needed for transcription of the LH4 gene (compare the 292-base transcript in lane j with that in lanes k and l in Fig. 5). In all cases a 10-min preincubation was as effective as a 30-min period in enhancing transcription of the first template and in inhibiting transcription of the second template. These results indicate that there is at least one common factor that is required for transcription of both the LH4 and EH4 genes and that this factor can form a stable transcription complex with either gene template in vitro.

We wanted to know whether the stable complex involves a general factor that is required for transcription of any gene in the extract or whether it is a more specific transcription factor that is needed by only some genes. We therefore performed similar commitment assays in which we used the EH2B gene to determine whether the limiting factor is required for transcription of this gene as well. The protocol involved a 10-min preincubation with one of the three genes EH4, LH4, or EH2B as a first template; the addition of a second template, which could be any one of these three genes; a further 10-min preincubation period before the addition of NTPs; and a 30-min reaction period before the transcription was stopped. In these experiments we used three concentrations of the first template (10, 20, and 40 μg/ml), whereas the concentration of the second template was always 25 μg/ml (note that the experiment for which the results are shown in Fig. 5 was done with 40 μg of each template per ml).

The results of an experiment in which the second template was *Nco*I-cut pLH4 (which yielded a runoff RNA of 292 bases) are shown in Fig. 6. The first templates in this experiment were *Nco*I-cut pEH4 (Fig. 6, lanes d to f), *Ava*II-cut pLH4 (Fig. 6, lanes g to i), *Kpn*I-cut pEH2B (Fig. 6, lanes j to l), and *Nru*I-cut pEH2B (Fig. 6, lanes m to o), which yielded transcripts of 322, 211, 414, and 349 bases, respectively. As a control, pUC118 was added in identical concentrations in lieu of a first template (Fig. 6, lanes a to c). The addition of sufficient amounts of the nonspecific DNA prior to the addition of the pLH4 template had a stimulatory effect on the transcription of the subsequently added gene (compare lanes a and b in Fig. 6). We attributed this to the threshold DNA concentration effect described earlier (Fig. 2). The addition of 40 μg of the nonspecific DNA per ml (Fig. 6, lane c) resulted in no further enhancement of transcription over that of 20 μg/ml and, more importantly as a control for

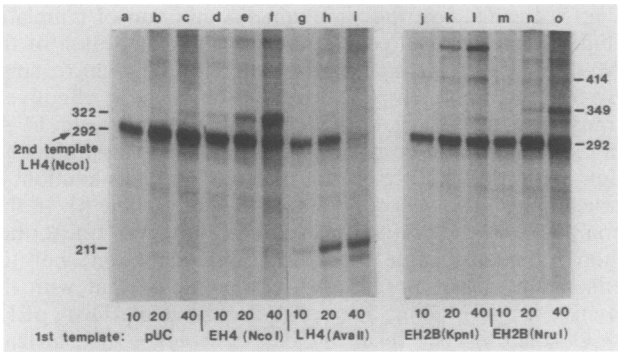

FIG. 6. Commitment assay with LH4 DNA added as the second template. pUC118 (lanes a to c), *Nco*I-cut pEH4 (lanes d to f), *Ava*II-cut pLH4 (lanes g to i), *Kpn*I-cut pEH2B (lanes j to l), or *Nru*I-cut pEH2B (lanes m to o) was added at 10, 20, or 40 μg/ml (as indicated below each lane) to a complete reaction mixture that lacked only NTPs. After a period of 10 min, *Nco*I-cut pLH4 was added to each reaction to attain a final concentration of 25 μg/ml, and after an additional 10 min NTPs were added to start the reaction, which was allowed to proceed for 30 min. All operations after the addition of the first template were carried out at 16°C. The RNA samples were run on two different gels, as indicated by the two panels. Sizes of the runoff RNA products (in nucleotides) are shown to the left and right of the gels.

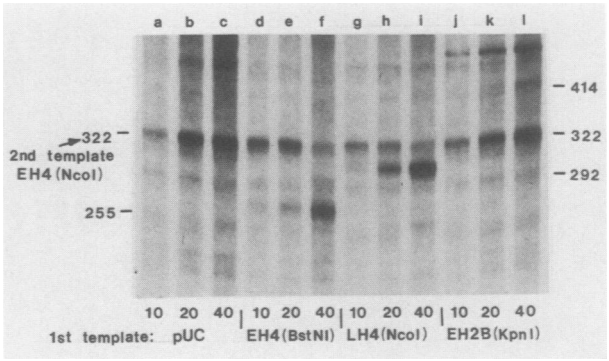

FIG. 7. Commitment assay with EH4 DNA added as the second template. The design of the experiment for which the results are shown here was the same as that described in the legend to Fig. 6. The first DNAs added in this case were pUC118 (lanes a to c), *Bst*NI-cut pEH4 (lanes d to f), *Nco*I-cut pLH4 (lanes g to i), and *Kpn*I-cut pEH2B (lanes j to l), which were added to attain final concentrations of 10, 20, or 40 μg/ml, as indicated below each lane. *Nco*I-cut pEH4 DNA was added as a second template 10 min later at a final concentration of 25 μg/ml. After an additional 10 min of preincubation, NTPs were added to start the reaction.

this experiment, did not inhibit transcription. In the case of the LH4, EH4, and EH2B genes that were used as the first DNA template, an increase in the amounts of the first template from 10 to 20 and 40 μg/ml allowed a more efficient use of the first DNA template for transcription, again illustrating the threshold effect. When the *Ava*II-cut pLH4 was added as the first template (Fig. 6, lanes g to i), transcription of the *Nco*I-cut pLH4 second template was inhibited; this was most evident when the highest concentration of first template was used (Fig. 6, lane i). Although the amount of transcript derived from the second LH4 template did not decrease when the concentration of first template was raised from 10 to 20 μg/ml, it is probable that even 20 μg of LH4 first template per ml was inhibitory, since the increase in the second template transcript observed when 20 μg (instead of 10 μg) of pUC118 DNA per ml was added first (compare lanes a and b in Fig. 6) was not seen when these amounts of pLH4 DNA were added as first template (compare lanes g and h in Fig. 6). Preincubation with 40 μg of pLH4 per ml had a dramatic effect on the inhibition of transcription of the second pLH4 template (Fig. 6, lane i). When 40 μg of *Nco*I-cut pEH4 DNA was used as the first template (Fig. 6, lane f), some inhibition of the second pLH4 template was seen when compared with that of the 40-μg/ml pUC control (the 292-base transcript in lane f was 40% of that in lane c in Fig. 6, as determined by densitometry). Furthermore, a 1.6-fold decrease in the 292-base transcript was seen when 40 μg of pEH4 DNA per ml was used as the first template compared with that when 20 μg/ml was used (compare lanes e and f in Fig. 6). Similar experiments done with the same pEH4 first template (Fig. 5, lanes h and i; see also Fig. 10, lane l) showed an even more impressive inhibition of LH4 transcripts. On the other hand, when the truncated EH2B template was used for preincubation, no decrease in the second LH4 gene transcription was detected when the amount of first template was increased. The results that were obtained when *Kpn*I-cut pEH2B DNA and *Nru*I-cut pEH2B DNA were used are shown in Fig. 5 (lanes j to l and m to o, respectively). As noted above, the *Nru*I-cut DNA (yielding a 349-base RNA) is a more effective template than the *Kpn*I-cut DNA (yielding a 414-base transcript). The threshold level for transcription was reached as the amount of either template was increased. However, as in the case when pUC118 DNA was added first, but unlike the situation when pLH4 or pEH4 DNA was added as the first template, preincubation with pEH2B DNA at the highest DNA input (Fig. 6, lanes l and o) had no inhibitory effect on transcription of the second pLH4 template, compared with lower concentrations (Fig. 6, lanes j and k, and lanes m and n). (The right and left panels of Fig. 6 were derived from two different gels, so the intensities of the pEH2B-containing lanes could not be compared directly with those of the pUC-containing lanes.)

Results of a similar experiment in which the EH4 gene (cut with *Nco*I to yield a 322-base RNA in the runoff transcription assay) was used as the second template is presented in Fig. 7. As in the experiment in which pLH4 DNA was the second template, sufficient concentrations of pEH4 (Fig. 7, lane f) and pLH4 (Fig. 7, lane i), when they were added as the first template, inhibited the utilization of the second pEH4 templates. Even at the highest DNA concentrations used, neither pUC118 DNA (Fig. 7, lane c) nor pEH2B DNA (Fig. 7, lane l) inhibited transcription of the second template. When pUC118 or pEH2B DNA was added first, an increase in the DNA concentration from 10 to 20 μg/ml enhanced transcription of the second template. In other words, as was the case for the experiment for which the results are illustrated in Fig. 6, at least 40 μg of total DNA per ml was necessary for efficient transcription in the extract. Since this enhancement was not seen when first-template pEH4 or pLH4 DNA was boosted from 10 to 20 μg/ml (compare the 322-base band in lanes d versus e and that in lanes g versus h), it is probable that inhibition of second-template transcription was obtained with even 20 μg of first-template pEH4 or pLH4 DNA per ml. We conclude that the EH2B gene does not form a stable complex with a limiting factor that is used by the EH4 gene.

A question that remains to be answered is whether pEH2B DNA can be transcribed when the extract is depleted of the factor(s) which binds to the EH4 and LH4 genes. Figure 8 presents data which indicate that EH2B gene transcription is not dependent on these factors. When *Nru*I-cut pEH2B

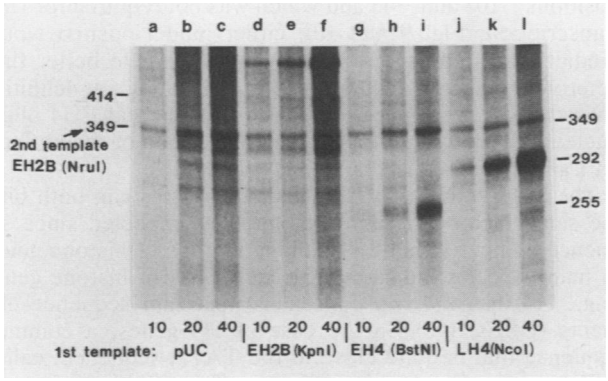

FIG. 8. Commitment assay with EH2B DNA added as the second template. The design of the experiment for which the results are shown here the same as that described in the legend to Fig. 6 and 7. The first DNAs added in this case were pUC118 (lanes a to c), *Kpn*I-cut pEH2B (lanes d to f), *Bst*NI-cut pEH4 (lanes g to i), and *Nco*I-cut pLH4 (lanes j to l), which were added to attain final concentrations of 10, 20, or 40 μg/ml, as indicated below each lane. *Nru*I-cut pEH2B DNA was added as a second template 10 min later at a final concentration of 25 μg/ml. After an additional 10 min of preincubation, NTPs were added to start the reaction.

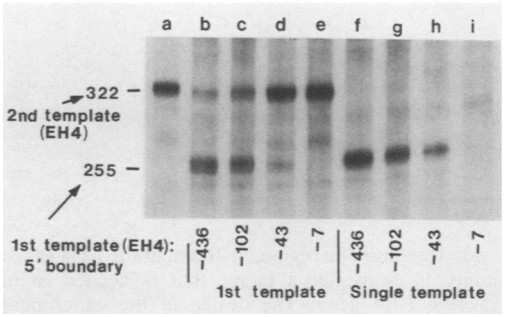

FIG. 9. EH4 upstream deletions indicate sequence requirements for maximal transcription and stable complex formation. *Bal* 31-generated deletions (−102, −43, and −7 refer to the 5′ limit of the resulting deletion) were compared with the undeleted pEH4 construct (−436) for their ability to transcribe a single template (lanes f to i) and for their ability to compete for a factor that was needed to transcribe a second EH4 DNA template (lanes b to e). The four DNAs, which were cut with *Bst*NI, were added to the mixture to attain a final concentration of 40 μg/ml, and 10 min later *Nco*I-cut pEH4 was added to the same final concentration (lanes b to e). As a control, the *Nco*I-cut pEH4 template was also tested alone at 40 μg/ml (lane a). As in the previously described experiments (Fig. 6 to 8), the reaction mixture was preincubated for an additional 10 min before the addition of NTPs to start the reaction, and all preincubations were done at 16°C. Sizes of the runoff transcripts are shown to the left of the gel: 322 bases for the RNA derived from the *Nco*I-cut template; 255 bases for the RNA derived from the *Bst*NI-cut templates.

(yielding a 349-base RNA) was used as the second template, none of the DNAs used as a first template, including the *Kpn*I-cut EH2B gene, was inhibitory. There was no decrease in the 349-base band even at the highest concentration used for each competitor DNA. The 414-base RNA band produced by the *Kpn*I-cut pEH2B was present, although it was difficult to see because of the high background (Fig. 8, lane f). The three bands with mobilities of greater than that of the 349-base band (Fig. 8, lanes a to f) and the higher-molecular-weight band above the 414-base band (Fig. 8, lanes d to f) were not α-amanitin sensitive (Fig. 1) and are not relevant to this discussion. It is unclear why one of the three-lower-molecular weight bands is intensified in lane f of Fig. 8.

The overall conclusion drawn from the experiments for which the results are illustrated in Fig. 6 to 8 is that at least one factor forms stable interactions with, and is required for transcription of, both LH4 and EH4 genes, but this factor is not required for transcription of the EH2B gene. The lack of competition by the EH2B gene (when used as a first template) on transcription of any of the three genes cannot be regarded as conclusive evidence against the formation of a stable complex involving other specific transcription factors with EH2B DNA, or even as evidence against the use of some additional common factor by all three genes. Such factors may exist in the extract at a sufficiently high concentration so that they were not depleted by the EH2B gene when it was present at a concentration of 40 μg/ml. We did not use higher concentrations of pEH2B as a first template because at 80 μg of total DNA per ml, transcription of all templates begins to be inhibited in the reaction (Fig. 2).

Upstream regions are essential for effective transcription of the EH4 gene. We used the in vitro transcription assay to begin to define the *cis*-acting regions that are essential for the template activity of the EH4 gene. *Bal* 31 deletions were created starting from the *Dra*I site 436 bp upstream of the mRNA cap site in the EH4 gene. Three of the deletions were tested for transcription and compared with the construct containing the 436 bp of 5′-flanking sequence (Fig. 9, lanes f to i). Removal of the region between positions −436 and −102 resulted in an approximately twofold decrease in activity. Additional removal of the sequence between positions −102 and −43 led to a further threefold decrease in transcription. A further deletion to position −7 (which included the ATA box) resulted in a complete loss of transcription. These results (and additional unpublished data) suggest that the upstream region consists of a series of positive regulatory signals, each of which is necessary for maximal transcription in vitro. A more detailed analysis of these data will be published elsewhere.

Stable complex formation with the EH4 gene is dependent on upstream sequence. A two-step commitment assay similar to those used earlier (Fig. 6 to 8) was performed with the 5′ deletion mutants of the EH4 gene (cut with *Bst*NI to give a 255-base transcript) as a first template. Figure 9 (lanes b to e) shows the results of an experiment in which an *Nco*I-cut EH4 gene (which gave a 322-base RNA) containing the 5′ sequence up to position −436 was used as the second template. There was considerable competition when the DNA at position −436 was used as the first template (Fig. 9, lane b; these results are similar to those presented in Fig. 5, lanes h and j, and Fig. 7, lane f). Removal of the region between positions −436 and −102 from the first template still allowed the inhibition of transcription of the second template, but to a lesser extent (Fig. 9, lane c). Removal of the additional sequence between positions −102 and −43 or −7 resulted in the complete loss of ability to compete (Fig. 9, lanes d and e). We interpret these results to mean that there are positively acting factors which bind with sequences between positions −102 and −43 and between positions −102 and −436. The stable complex formation must involve one or more of these factors. The result also shows that the region downstream from position −43 by itself cannot form a stable transcription complex with the limiting factor(s), although it contains essential *cis*-acting sequences.

We then studied the effect of preincubation of the three EH4 deletion mutants on transcription of an LH4 gene when

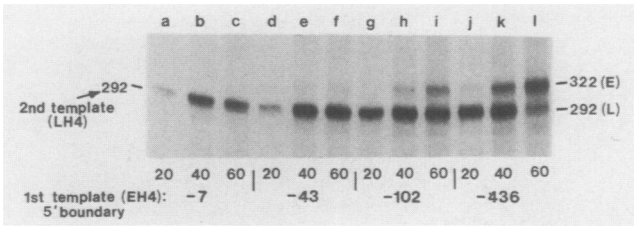

FIG. 10. Deletion of a region upstream of the EH4 gene results in the inability to sequester a factor that is needed to maximally transcribe the LH4 gene. The design of the experiment was as described in the legend to Fig. 9, except that the four EH4 first templates were cut with *Nco*I and added to attain final concentrations of 20, 40, or 60 μg/ml, as indicated below each lane; and the second template in this case was *Nco*I-cut pLH4 DNA, which was added at a final concentration of 25 μg/ml.

it was added as a second template. The LH4 DNA contained a 5′-flanking sequence to position −553 as in all previous experiments. Figure 10 shows that the result differs from the case in which the EH4 gene was used as the second template (Fig. 9). Figure 10 (lanes j to l) shows the results that were obtained when the EH4 gene containing 436 bases of upstream sequence was used as a first template. As in Fig. 6 (lanes d to f), we observed a decrease in transcription of the LH4 second template, although inhibition was not seen unless 60 μg of EH4 DNA per ml was added in this case. If the first template extended only to position −102, inhibition was not observed (Fig. 10, lanes g to i). Further deletions to position −43 (Fig. 10, lanes d to f) and to position −7 (Fig. 10, lanes a to e) gave a similar lack of inhibition. These results indicate that the shared limiting factor that is involved in the formation of the stable transcription complexes of the LH4 and EH4 genes must bind to a region upstream of position −102 of the EH4 gene. The region of the EH4 gene downstream from this position, although it contains sequences that are important for recognition of other factors (see above), does not form a stable complex with a factor that is essential for maximal LH4 gene expression.

DISCUSSION

A common factor may be essential for maximal LH4 and EH4 activity. We have presented evidence demonstrating that maximal transcription of the EH4 and LH4 genes in vitro requires binding of at least one factor that is not needed for transcription of the EH2B gene. The factor is therefore not required for transcription of all genes, but whether it is strictly H4 specific or is required for transcription of a wider spectrum of genes can be determined only when additional templates are tested in the commitment assay. Although the data indicate that a common factor can bind to both LH4 and EH4 genes, the results are consistent with two models. In the simplest case, there is a single limiting factor which is required for maximal transcription of both the LH4 and EH4 genes. In the second case, there are two similar factors, each with the potential to bind to either H4 gene template, but one binds with a greater affinity to the LH4 gene and the other binds with a greater affinity to the EH4 gene. Theoretically, the template commitment assay should allow us to distinguish between these possibilities, but this experiment would only be meaningful if a common binding factor(s) was the only component of the actively transcribed complex. Since gene-specific factors also seem to be required for maximal transcription (e.g., the EH4 factor which bound between positions −102 and −43 and which was not required for LH4 transcription; Fig. 9 and 10), either model posited would predict that homologous genes would compete better than heterologous genes in the commitment assay. A definitive answer must await the use of specific LH4 and EH4 oligonucleotides as competitors and a comparison of their effects on transcription of LH4 and EH4 templates.

The observation that LH4 and EH4 genes can both bind the same factor(s) is not completely unexpected since sequence comparisons of 5′-flanking regions of histone genes of many species indicates that each class of histone genes (e.g., H4) has its own consensus upstream sequence elements (USEs) (50). In the case of H4 genes, a common sequence that is quite close to the TATA sequence, called USEI, is present in histone genes from *Xenopus laevis*, humans, chickens, and sea urchins; but no other upstream sequence was found to be common to histone genes from these four species (50). This sequence has been found in both LH4 and EH4 genes from *S. purpuratus* (30) and was the only common upstream motif detected in that comparison. A reexamination of the *S. purpuratus* upstream LH4 and EH4 sequences, however, revealed a second homologous sequence, AGGNGGCNCACTC, which was located between positions −120 and −108 of the EH4 gene and between positions −82 and −70 of the LH4 gene (sea urchin H4 USEII). The site in the EH4 gene was within the region between positions −436 and −102, which are implicated in the binding of the limiting factor that is required for stable complex formation of both LH4 and EH4 genes. Recently, we have shown by DNase I footprinting and competition experiments that the sequences between positions −133 and −102 of the EH4 gene and positions −94 and −66 of the LH4 gene bind a common factor (I. J. Lee, L. Tung, and E. S. Weinberg, manuscript in preparation). Preincubation of the nuclear extract with a 33-bp synthetic oligonucleotide which contained a sequence that was identical to the footprinted region of the LH4 gene resulted in a strong inhibition of the subsequently added LH4 and EH4 genes, but not of the EH2B gene. It is likely that these regions of the EH4 and LH4 genes are involved in the stable complex formation demonstrated in this study (see above).

From sequence comparisons of all early and late sea urchin histone genes, there was no obvious candidate for a *cis*-acting region which would modulate the coordinate regulation of the five early genes or particular groups of late genes. Moreover, the most striking homologies were those between genes of the same class with different regulatory patterns. Is it possible that a common binding site on the EH4 and LH4 genes has any bearing on the regulation of these genes? This system is reminiscent of the differentially regulated oocyte and somatic 5S genes of *X. laevis* and the binding of common factors to these sequences in a stable preinitiation complex (for a review, see reference 61). The differential transcription of these genes is now thought not to be the result of a difference in binding affinity of the 5S specific factor, TFIIIA, but, possibly, the result of differential stability of the stable complex (44, 60), perhaps because of the ability of TFIIIC to bind to the two different TFIIIA-5S DNA complexes (59). Similar events may occur on the two different histone genes, but further speculation must await the results of additional experiments.

Stable complex formation with H4 genes. Formation of a stable preinitiation complex between *trans*-acting factors and promoter sequences is a property of genes that are transcribed by RNA polymerases III (1, 5, 36), I (10, 55; for a review, see reference 51), and II (13, 15, 23, 25, 38); and

the formation of such complexes has been considered as a mechanism to maintain the differential expression of particular genes during development (5, 6). In the case of genes that are transcribed by RNA polymerase II, stable complexes have been demonstrated with TFIIA and TFIID by the sequential addition of two templates to extracts or to reconstituted fractions that are competent for transcriptional initiation (13, 15), and such stable complexes can undergo some reinitiation in a partially purified nuclear extract (23). Surprisingly, there is little evidence for stable complexes involving gene-specific factors which interact with polymerase II-transcribed templates. One such case is, in fact, the demonstration by Heintz and Roeder (25) of an interaction of a factor in a HeLa cell S-phase nuclear extract with the cell cycle-responsive human histone H4 gene discussed above. In a two-step incubation protocol similar to that used in our experiments described above, Heintz and Roeder (25) showed that preincubation of the H4 gene with nuclear extract results in a marked decrease in the transcription of a second H4 gene template. However, neither in that study nor in a subsequent study in which the S-phase-dependent promoter sequences of this gene were determined (21) were the sequence requirements for stable complex formation delineated.

In the case of the sea urchin H4 genes, two lines of evidence strongly suggest that the factors involved are not general transcription factors such as TFIID. First, under conditions in which the LH4 and EH4 genes sequester a factor that is required for transcription of a subsequently added LH4 or EH4 template, transcription of a subsequently added EH2B gene is not affected. Second, the ability to sequester required factors is lost when regions upstream of the TATA box are deleted from the EH4 gene. An EH4 template lacking sequences upstream of position −43 no longer competes for transcription of EH4 or LH4 genes in the template commitment assay (Fig. 9 and 10). Interestingly, an EH4 template containing the region between positions −43 and −102, but lacking sequences upstream of position −102, can compete for transcription of EH4 but not of LH4 genes in the commitment assay. The region upstream of position −102 binds a factor(s) which is required for maximal transcription of both EH4 and LH4 genes. Another factor, which is needed for maximal transcription of only the EH4 gene, apparently interacts with the region between positions −102 and −43.

Preincubation of template with extract for at least 10 min in the absence of NTPs was required for maximal transcription (Fig. 4). This observation demonstrates that there is a slow limiting step involving an interaction between template and a component of the extract. Results of the template commitment experiments (Fig. 5 to 10) indicate that this limiting step involves the assembly of stable preinitiation complexes. What is puzzling is that the results presented in Fig. 4 imply that the assembly process is inhibited once the NTPs are added to the reaction mixture. Consider that the only difference between lanes a and b (and similarly for lanes f and g) in Fig. 4 is that the template was incubated for either 1 or 5 min in the presence of extract before the NTPs were added. Since the total subsequent incubation with NTPs was 30 min in each case, there was only a very minor difference in the total length of time that the templates were in contact with the extract (31 versus 35 min). The striking difference in the amount of transcript in the two reactions, therefore, indicates that the assembly process cannot continue once NTPs are added, once transcription begins, or both. Results of preliminary experiments (L. Tung and E. S. Weinberg) indicate that the addition of ATP to the extract during preincubation with template dramatically inhibits transcription of LH4 and EH4 genes and that this inhibition cannot be overcome by the addition of ATP along with the other three NTPs after the preincubation period. These observations strongly suggest that the inhibitory effect of ATP is not operative once factors have assembled into the stable preinitiation complex on the DNA template. This is highly reminiscent of the effect of 0.015% Sarkosyl on the adenovirus major late promoter studied in reconstituted and intact HeLa cell nuclear extracts (22, 23); but whether the ATP effect operates on the conversion of a committed complex to a rapid start complex, as does Sarkosyl at this low concentration, or inhibits the formation of the committed stable complex is not yet known.

The experiments described above do not address the issue of reinitiation of transcription on the committed templates. It is of interest that the efficiency of template utilization in the sea urchin nuclear extract is high in comparison with that in other in vitro transcription systems. For example, 1.57×10^4 dpm was recovered from the LH4 runoff transcript band of lane k in Fig. 10. This corresponded to 12.7 fmol of transcript produced from 186 fmol (0.525 μg) of template in the 30-min reaction, or a template utilization efficiency of 6.87%. Considering that the reaction was linear for at least 90 min, template efficiencies of at least 20% could be obtained in these extracts. Furthermore, these reactions have not been optimized for a maximal transcription. Higher transcriptional levels per template molecule could probably be obtained by increasing the amount of extract in the reaction mixture, by removing nonspecific transcription inhibitors, and optimizing UTP and DNA concentrations. It may, in fact, be possible to obtain efficiencies greater than one transcript per template in this system. Even at the levels observed here, however, there is a reasonable possibility that reinitiation is taking place on the committed complexes since it is probable that most of the template was not used at all for transcription in these reactions.

Developmental regulation and transcriptional controls studied with the in vitro system. Ideally, in vitro transcription systems can be used to study the physiological and developmental regulation of genes. One of the first examples of such an approach was the demonstration that a particular human histone H4 gene was transcribed more efficiently in nuclear extracts from synchronized HeLa cells in the S phase than in extracts from non-S-phase cells (25). Subsequently, the sequences responsive to a positively acting factor in the S-phase extract were localized to a region between −70 and −100 bp from the transcriptional start site (21), and a protein was found to bind to this region by electrophoretic band retardation assays and DNase I footprinting (12). Several genes, including the mouse albumin gene (19) and the human growth hormone gene (4), have been shown to be transcribed far more efficiently in nuclear extracts derived from appropriate differentiated tissues, and the responsive *cis*-acting sequences have been identified. More recently, the distal promoter of the *Drosophila melanogaster* alcohol dehydrogenase (Adh) gene was shown to be transcribed in nuclear extracts that were prepared from different embryonic stages with the same relative efficiency as that in vivo at these times in development (24).

Results of these studies indicate that a similar approach might be successful if it is applied to developmentally regulated sea urchin genes. Unfortunately, thus far we have not observed preferential transcription of the appropriate gene with extracts from different embryonic stages. By using

extracts from nuclei of 36-h gastrula embryos, a stage at which only the embryonic LH4 gene is transcribed in vivo (32; L. N. Yager and E. S. Weinberg, unpublished data), both EH4 and LH4 genes are effectively transcribed in vitro. The transcriptional turnoff of the EH4 gene that was seen during embryogenesis was therefore not reproduced in the extracts. We have not done extensive experiments with extracts from cleavage or morula embryos, but preliminary data with 10-h morula nuclear extracts shows that both EH4 and LH4 genes are transcribed in this case as well. The transcription of the LH4 gene should have been severalfold lower than that of the EH4 gene in this extract if the developmental controls were preserved. The reasons for the lack of retention of the differential transcription of these two genes seen in vivo may have been because of limitations inherent in our experimental plan or they may reflect particular mechanistic requirements for control. Purely technical considerations might be (i) the loss of negatively acting factors during preparation of the extract, (ii) the absence of the responding negative *cis*-acting sequences on the templates that were used, and (iii) failure to optimize for the conditions that are needed for interaction of the putative negatively acting factor with the DNA templates. Mechanistic limitations might include (i) a requirement for assembly of the gene into chromatin for appropriate factor-template assembly, (ii) a need for the use of supercoiled templates rather than linearized templates for the appropriate interactions with factors, or (iii) the inability of the in vitro system to allow appropriate interactions between factors if their binding sites are too far apart on the DNA template.

Some of these possibilities are reasonable. It is probable that some factors are lost from the extract as it is prepared. The EH1 gene, for example, is not transcribed by the extract at all, indicating that some essential factor for transcription of this gene is not present. It is also possible that particular *cis*-acting sequences might not be present on the templates that we used. We have performed all of our experiments thus far with LH4 and EH4 templates containing only 553 and 436 bp of upstream sequence, respectively. Furthermore, if there were one master enhancer or silencer of transcription on the early gene repeat unit, it could be located in or near any of the other four early histone genes. Similarly, such a sequence, if it were present in the late gene cluster, could be located downstream of the LH4 gene or even in, or downstream of, the linked LH3 gene. These possibilities as well as the other potential rationales for the lack of a developmental response in vitro are subject to further studies.

Although the results described above are not illuminating from the standpoint of the temporal control of the H4 genes, they do provide information on regions of the EH4 and LH4 genes that are essential for maximal activity. Furthermore, the data demonstrate that the in vitro transcription system derived from embryonic nuclear extracts is a useful tool in the identification and purification of transcription factors and in characterizing the nature of the complexes that are involved in the initiation of RNA synthesis. The competition protocols can be used to determine the range of templates which require a particular factor and are useful in identifying the site of interaction of the factor with a particular template. Work is currently in progress to identify the exact sequences which bind the essential H4 transcription factor.

ACKNOWLEDGMENTS

We thank W. F. Marzluff for helpful advice and for providing guidance and laboratory facilities to G.F.M. and L.N.Y. in the preliminary stage of this study. We also thank N. Heintz and D. Reinberg for advice and stimulating discussions.

This work was supported by Public Health Service grant GM27322 from the National Institutes of Health (to E.S.W.).

LITERATURE CITED

1. **Bieker, J. J., P. L. Martin, and R. G. Roeder.** 1985. Formation of a rate-limiting intermediate in 5S RNA gene transcription. Cell **40**:119–127.
2. **Birchmeier, C., W. Folk, and M. L. Birnstiel.** 1983. The terminal RNA stem-loop structure and 80 bp of spacer DNA are required for the formation of 3′ termini of sea urchin H2A mRNA. Cell **35**:433–440.
3. **Birchmeier, C., R. Grosschedl, and M. L. Birnstiel.** 1982. Generation of authentic 3′ termini of an H2A mRNA in vivo is dependent on a short inverted DNA repeat and on spacer sequences. Cell **28**:739–745.
4. **Bodner, M., and M. Karin.** 1987. A pituitary-specific *trans*-acting factor can stimulate transcription from the growth hormone promoter in extracts of nonexpressing cells. Cell **50**: 267–275.
5. **Bogenhagen, D. F., W. M. Wormington, and D. D. Brown.** 1982. Stable transcription complexes of Xenopus 5S RNA genes: a means to maintain the differentiated state. Cell **28**:413–421.
6. **Brown, D. D.** 1984. The role of stable complexes that repress and activate eucaryotic genes. Cell **37**:359–365.
7. **Busslinger, M., and A. Barberis.** 1985. Synthesis of sperm and late histone cDNAs of the sea urchin with a primer complementary to the conserved 3′ terminal palindrome: evidence for tissue specific and more general histone gene variants. Proc. Natl. Acad. Sci. USA **82**:5676–5680.
8. **Childs, G., R. Maxson, and L. H. Kedes.** 1979. Histone gene expression during sea urchin embryogenesis: isolation and characterization of early and late messenger RNAs of *Strongylocentrotus purpuratus* by gene-specific hybridization and template activity. Dev. Biol. **73**:153–173.
9. **Childs, G. C., C. Nocente-McGrath, T. Lieber, C. Holt, and J. Knowles.** 1982. Sea urchin (Lytechinus pictus) late stage H3 and H4 genes: characterization and mapping of a clustered but non-tandemly linked multigene family. Cell **31**:383–393.
10. **Cizewski, V., and B. Sollner-Webb.** 1983. A stable transcription complex directs mouse ribosomal RNA synthesis by RNA polymerase I. Nucleic Acids Res. **11**:7043–7056.
11. **Colin, A. M., T. L. Catlin, E. H. Davidson, and R. Maxson.** 1988. Closely linked early and late H2B histone genes are differentially expressed after injection into sea urchin zygotes. Proc. Natl. Acad. Sci. USA **85**:507–510.
12. **Dailey, L., S. M. Hanly, R. G. Roeder, and N. Heintz.** 1985. Distinct transcription factors bind specifically to two regions of the human histone H4 promoter. Proc. Natl. Acad. Sci. USA **83**:7241–7245.
13. **Davison, B. L., J.-M. Egly, E. R. Mulvihill, and P. Chambon.** 1983. Formation of stable preinitiation complexes between eukaryotic class B transcription factors and promoter sequences. Nature (London) **301**:680–686.
14. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
15. **Fire, A., M. Samuels, and P. A. Sharp.** 1984. Interactions between RNA polymerase II, factors, and template leading to accurate transcription. J. Biol. Chem. **259**:2506–2516.
16. **Flytzanis, C. N., R. J. Britten, and E. H. Davidson.** 1987. Ontogenic activation of a fusion gene introduced into sea urchin eggs. Proc. Natl. Acad. Sci. USA **84**:151–155.
17. **Flytzanis, C. N., A. P. McMahon, B. R. Hough-Evans, K. S. Katula, R. J. Britten, and E. H. Davidson.** 1985. Persistence and integration of cloned DNA in post embryonic sea urchins. Dev. Biol. **108**:431–442.
18. **Franks, R. R., B. R. Hough-Evans, R. J. Britten, and E. H. Davidson.** 1988. Spatially deranged though temporally correct expression of a *Strongylocentrotus purpuratus* actin gene fusion in transgenic embryos of a different sea urchin family. Gene Dev. **2**:1–12.
19. **Gorski, K., M. Carneiro, and U. Schibler.** 1986. Tissue-specific

in vitro transcription from the mouse albumin promoter. Cell **47**:767–776.
20. **Grunstein, M.** 1978. Hatching in the sea urchin *Lytechinus pictus* is accompanied by a shift in histone H4 activity. Proc. Natl. Acad. Sci. USA **75**:4135–4139.
21. **Hanly, S. M., G. C. Bleecker, and N. Heintz.** 1985. Identification of promoter elements necessary for transcription regulation of a human histone H4 gene in vitro. Mol. Cell. Biol. **5**:380–389.
22. **Hawley, D. K., and R. G. Roeder.** 1985. Separation and partial characterization of three functional steps in transcription initiation by human RNA polymerase II. J. Biol. Chem. **260**: 8163–8172.
23. **Hawley, D. K., and R. G. Roeder.** 1987. Functional steps in transcription initiation and reinitiation from the major late promoter in a HeLa nuclear extract. J. Biol. Chem. **262**: 3452–3461.
24. **Heberlein, U., and R. Tjian.** 1988. Temporal pattern of alcohol dehydrogenase gene transcription reproduced by *Drosophila* stage-specific extracts. Nature (London) **331**:410–415.
25. **Heintz, N., and R. G. Roeder.** 1984. Transcription of human histone genes in extracts from synchronized HeLa cells. Proc. Natl. Acad. Sci. USA **81**:2713–2717.
26. **Hentschel, C. C., and M. L. Birnstiel.** 1981. The organization and expression of histone gene families. Cell **25**:301–313.
27. **Hieter, P. A., M. B. Hendricks, K. Hemminki, and E. S. Weinberg.** 1979. Histone gene switch in the sea urchin embryo. Identification of late embryonic histone messenger ribonucleic acids and the control of their synthesis. Biochemistry **18**: 2707–2715.
28. **Hough-Evans, B. R., R. R. Franks, R. A. Cameron, R. J. Britten, and E. H. Davidson.** 1987. Correct cell-type-specific expression of a fusion gene injected into sea urchin eggs. Dev. Biol. **121**:576–579.
28a. **Ito, M., J. Bell, G. Lyons, and R. Maxson.** 1988. Synthesis and turnover of late H2B MRNA in developing embryos of the sea urchin, *Strongylocentrotus purpuratus*. Dev. Biol. **129**:147–158.
29. **Katula, K. S., B. Hough-Evans, R. J. Britten, and E. H. Davidson.** 1987. Ontogenic expression of a Cyl actin fusion gene injected into sea urchin eggs. Development **101**:437–447.
30. **Kaumeyer, J. F., and E. S. Weinberg.** 1986. Sequence, organization, and expression of late embryonic H3 and H4 histone genes from the sea urchin, *Strongylocentrotus purpuratus*. Nucleic Acids Res. **14**:4557–4576.
31. **Kemler, I., and M. Busslinger.** 1986. Characterization of two nonallelic pairs of late histone H2A and H2B genes of the sea urchin: differential regulation in the embryo and tissue-specific expression in the adult. Mol. Cell. Biol. **6**:3746–3754.
32. **Knowles, J. A., and G. Childs.** 1984. Temporal expression of late histone messenger RNA in the sea urchin *Lytechinus pictus*. Proc. Natl. Acad. Sci. USA **81**:2411–2415.
33. **Knowles, J. A., and G. Childs.** 1986. Comparison of the late H1 histone genes of the sea urchins *Lytechinus pictus* and *Srongylocentrotus purpuratus*. Nucleic Acids Res. **14**:8121–8133.
34. **Knowles, J. A., Z. Lai, and G. Childs.** 1987. Isolation, characterization, and expression of the gene encoding late histone subtype H1-γ of the sea urchin *Strongylocentrotus purpuratus*. Mol. Cell. Biol. **7**:478–485.
35. **Lai, Z.-C., R. Maxson, and G. Childs.** 1988. Both basal and ontogenic promoter elements affect the timing and level of expression of a sea urchin H1 gene during early embryogenesis. Gene Dev. **2**:173–183.
36. **Lasser, A. B., P. L. Martin, and R. G. Roeder.** 1983. Transcription of class III genes: formation of preincubation complexes. Science **222**:740–748.
37. **Manley, J. L., A. Fire, A. Cano, P. A. Sharp, and M. L. Gefter.** 1980. DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. Proc. Natl. Acad. Sci. USA **77**: 3855–3859.
38. **Mattaj, I. W., S. Lienhard, J. Jiricny, and E. M. De Robertis.** 1985. An enhancer-like sequence within the *Xenopus* U2 gene promoter facilitates the formation of stable transcription complexes. Nature (London) **316**:163–167.
39. **Mauron, A., L. Kedes, B. R. Hough-Evans, and E. H. Davidson.** 1982. Accumulation of individual histone mRNAs during embryogenesis of the sea urchin *Strongylocentrotus purpuratus*. Dev. Biol. **94**:425–434.
40. **Maxson, R. E., T. J. Mohun, G. Childs, and L. H. Kedes.** 1983. Distinct organizations and patterns of expression of early and late gene sets in the sea urchin. Nature (London) **301**:120–125.
41. **Maxson, R. E., T. J. Mohun, R. Cohn, and L. Kedes.** 1983. Expression and organization of histone genes. Annu. Rev. Genet. **17**:239–277.
42. **Maxson, R. E., and F. H. Wilt.** 1981. The rate of synthesis of histone mRNA during the development of sea urchin embryos (*Strongylocentrotus purpuratus*). Dev. Biol. **83**:380–386.
43. **Maxson, R. E., and F. H. Wilt.** 1982. Accumulation of the early histone messenger RNAs during the development of *Strongylocentrotus purpuratus*. Dev. Biol. **94**:435–440.
44. **McConkey, G. A., and D. F. Bogenhagen.** 1988. TFIIIA binds with equal affinity to somatic and major oocyte 5S RNA genes. Gene Dev. **2**:205–214.
45. **McMahon, A. P., C. N. Flytzanis, B. R. Hough-Evans, K. S. Katula, R. J. Britten, and E. H. Davidson.** 1985. Introduction of cloned DNA into sea urchin egg cytoplasm: replication and persistence during development. Dev. Biol. **108**:420–430.
46. **Mohun, T. J., R. Maxson, G. Gormezano, and L. Kedes.** 1985. Differential regulation of individual late histone genes during development of the sea urchin (*Strongylocentrotus purpuratus*). Dev. Biol. **108**:491–502.
47. **Morris, G. F., and W. F. Marzluff.** 1983. A factor in sea urchin eggs inhibits transcription in isolated nuclei by sea urchin RNA polymerase III. Biochemistry **22**:645–653.
48. **Morris, G. F., D. H. Price, and W. F. Marzluff.** 1986. Synthesis of U1 RNA in a DNA-dependent system from sea urchin embryos. Proc. Natl. Acad. Sci. USA **83**:3674–3678.
49. **Overton, G. C., and E. S. Weinberg.** 1978. Length and sequence heterogeneity of the histone gene repeat unit of the sea urchin, S. purpuratus. Cell **14**:247–257.
50. **Perry, M., G. H. Thomsen, and R. G. Roeder.** 1985. Genomic organization and nucleotide sequence of two distinct histone gene clusters from *Xenopus laevis*. Identification of novel conserved upstream sequence elements. J. Mol. Biol. **185**: 479–499.
51. **Sollner-Webb, B., and J. Tower.** 1986. Transcription of cloned eukaryotic ribosomal RNA genes. Annu. Rev. Biochem. **55**: 801–830.
52. **Sures, I. S Levy, and L. H. Kedes.** 1980. Leader sequences of *Strongylocentrotus purpuratus* histone mRNAs start at a unique heptanucleotide common to all five histone genes. Proc. Natl. Acad. Sci. USA **77**:1265–1269.
53. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153**:3–34.
54. **Vitelli, L., I. Kemler, B. Lauber, M. L. Birnstiel, and M. Busslinger.** 1988. Developmental regulation of micro-injected histone genes in sea urchin embryos. Dev. Biol. **127**:54–63.
55. **Wandelt, C., and I. Grummt.** 1983. Formation of stable preinitiation complexes is a prerequisite for ribosomal DNA transcription *in vitro*. Nucleic Acids Res. **11**:3795–3809.
56. **Wasylyk, B., C. Kedinger, J. Corden, O. Brison, and P. Chambon.** 1980. Specific *in vitro* initiation of transcription on conalbumin and ovalbumin genes and comparison with adenovirus-2 early and late genes. Nature (London) **285**:367–373.
57. **Weil, P. A., D. S. Luse, J. Segall, and R. G. Roeder.** 1979. Selective and accurate initiation of transcription at the Ad2 major late promoter in a soluble system dependent on purified RNA polymerase II and DNA. Cell **18**:469–484.
58. **Weinberg, E. S., M. B. Hendricks, K. Hemminki, P. E. Kuwabara, and L. A. Farrelly.** 1983. Timing and rates of synthesis of early histone mRNA in the embryo of *Strongylocentrotus purpuratus*. Dev. Biol. **98**:117–129.
59. **Wolffer, A. P.** 1988. Transcription fraction TFIIIC can regulate differential *Xenopus* 5S RNA gene transcription in vitro. EMBO J. **7**:1071–1079.
60. **Wolffe, A. P., and D. D. Brown.** 1987. Differential 5S RNA gene expression in vitro. Cell **51**:733–740.
61. **Wolffe, A. P., and D. D. Brown.** 1988. Developmental regulation of two 5S ribosomal RNA genes. Science **241**:1626–1632.