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Closely linked early and late histone H2B genes are differentially expressed after microinjection into sea urchin zygotes

(gene transfer/sea urchin development)

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ABSTRACT **An early and a late histone H2B gene from the sea urchin *Stronglyocentrotus purpuratus* were linked in a single plasmid and injected into the eggs of the sea urchin *Lytechinus pictus*. The levels of transcripts of injected early and late genes and of endogenous early genes were monitored during development by a ribonuclease protection assay. Transcripts of both the injected and endogenous early genes peaked during the blastula stage and decreased severalfold by the mesenchyme blastula stage. Transcripts of the injected late gene became detectable at the blastula stage and increased in amount subsequently, until at least the early gastrula stage, 28 hr after fertilization. Thus, the pattern of expression of the injected early and late *H2B* genes is similar to that of their endogenous counterparts. These results show that DNA sequences regulating the temporal pattern of early and late *H2B* gene expression must lie within the cloned DNA segments; i.e., within 600 base pairs of the early *H2B* gene and 3 kilobases of the late *H2B* gene.**

Understanding the processes that govern when genes are expressed during embryogenesis is a major goal of developmental biology. Despite considerable progress toward this goal, little is known about gene regulation during the earliest phases of development when important and possibly unique regulatory mechanisms operate. The histone genes in sea urchins provide an excellent model for the study of such mechanisms. The sea urchin synthesizes several different classes of histone proteins during early development. These proteins are the products of distinct sets of genes whose activity is modulated in a stage-specific manner (reviewed in ref. 1). Early histone genes, organized as tandem quintets and repeated several hundredfold in the genome, are expressed maximally at the early blastula stage (2–5). Late histone genes, arranged in irregular clusters and present in only 6–12 copies per genome, are maximally active in the late blastula and gastrula (6–10).

Our overall goal is to understand the roles of cis- and trans-acting elements in the regulation of early and late histone genes in the sea urchin embryo. As a first step in identifying the DNA sequences involved in the differential expression of these genes, we asked whether cloned early and late histone genes are expressed with appropriate developmental timing when injected into sea urchin eggs. To eliminate the possibility that observed differences between early and late *H2B* genes in the timing of their expression during development might be due to differences in their copy number arising after injection, we injected these two genes together on the same plasmid. We found that transcripts of the injected early *H2B* gene reach a sharp peak at the blastula stage, whereas those of the injected late *H2B* gene continue to accumulate at least through the early gastrula stage. This pattern of expression is very similar to that of the endogenous early and late *H2B* genes.

METHODS

Construction of a Plasmid Containing an Early and a Late Histone H2B Gene. The late *H2B* gene used in these studies is known as "L1" and is one of several variant late *H2B* genes so far characterized in *Strongylocentrotus purpuratus* (7, 10). A plasmid construct bearing both early and L1 late *H2B* genes was generated by replacing a segment of *S. purpuratus* DNA 3′ to the L1 late *H2B* gene with one copy of an early *H2B* gene (Fig. 1). The early *H2B* segment consisted of a 1.8-kilobase (kb) *Hha* I fragment containing ≈500 base pairs (bp) of 5′ gene flanking and 750 bp of 3′ gene flanking DNA. This segment was obtained from a cloned tandem repeat of early *S. purpuratus* histone genes [plasmid pCO2 (11)] and was inserted at a *Bgl* II site located 90 bp downstream from the late *H2B* transcription termination site. The late *H2B* segment, derived from the plasmid pSpl-1, was an *Eco*RI/*Bgl* II fragment 3 kb long. This fragment contains a late *H4* gene 1.6 kb upstream from the late *H2B* gene (7). The chimeric DNA segment bearing both early and late *H2B* genes was cloned in pBR322 and designated pSpEL-1.

Injection of Sea Urchin Eggs. *Lytechinus pictus* eggs were injected by the methods of Colin (12) and McMahon *et al.* (13). Briefly, dejellied eggs were attached to Petri dishes treated with 1% protamine sulfate and injected with ≈20 pl of plasmid DNA (50 μg/ml) in distilled water. This volume is ≈2% of the egg volume. The eggs were fertilized either immediately prior to injection or within 1 hr after injection and then cultured at 15°C. Mortality by the hatched blastula stage was <30%.

RNA Extraction and RNase Protection Assay. For each experiment, equal numbers of embryos (20–50) were washed in 0.5 M NaCl/50 mM EDTA/10 mM Tris·HCl, pH 7.4 (15,000 × *g* for 4 min) and dissolved in 200 μl of 0.1 M NaCl/1 mM EDTA/10 mM Tris·HCl, pH 7.4/0.5% $NaDodSO_4$/10 mM EGTA. Yeast tRNA (10 μg) was added as carrier. The RNA was extracted with 200 μl of phenol, 200 μl of chloroform, and then precipitated in 0.3 M NaOAc and 2.5 vol of ethanol. The precipitate was dissolved in sterile water and hybridized with [^{32}P]RNA probes generated by transcription *in vitro* according to Melton *et al.* (14). The early gene probe spanned nucleotides −2 to +142 of the early *H2B* gene and the late gene probe spanned nucleotides −43 to +62 of the L1 late *H2B* gene. Hybridization was for 20 hr at 47°C in 0.4 M NaCl/40 mM Pipes, pH 6.4/50 mM dithiothreitol/1 mM EDTA/70% (vol/vol) formamide. After hybridization, the RNA was digested with RNase A (40 μg/ml) and RNase T1 (7.5 units/ml) and the protected fragments were separated

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on a 6% acrylamide/7 M urea gel. The gels were exposed to preflashed x-ray film and an enhancing screen (Dupont Cronex). The autoradiographs were scanned in a densitometer (Hoefer, San Francisco).

RESULTS

As a first approach to identifying sequences involved in the differential regulation of sea urchin early and late histone genes, we asked whether cloned sea urchin early and late histone genes are expressed with appropriate developmental timing when reintroduced into sea urchin eggs by microinjection. To distinguish transcripts of the injected genes from those of endogenous genes, we microinjected *S. purpuratus* histone genes into eggs of a different species, *L. pictus*, whose histone sequences differ significantly from those of *S. purpuratus*. This difference enabled us to detect transcripts of the injected genes by using an RNase protection assay. Since early and late histone genes injected into eggs on separate plasmids might be replicated or degraded at different rates, resulting in misleading information about the timing of their expression, we decided to link early and late genes together on the same plasmid. Approximately 10^5 copies of this construct, shown schematically in Fig. 1, were microinjected into *L. pictus* eggs, and the expression of the linked early and late *H2B* genes was monitored in developing embryos by hybridization to radiolabeled RNA probes spanning the 5′ termini of the early or late H2B mRNAs.

In preliminary experiments, we found that when histone genes were injected as linear molecules, both early and late *H2B* genes were expressed at much higher levels than when they were injected in the circular form (data not shown). This is probably because linear DNA injected into sea urchin eggs forms concatenates and replicates during development while circular DNA does not (13). In subsequent experiments, we injected plasmids in the linear form. Preliminary experiments also indicated that $<10^5$ copies of the plasmid per egg resulted in fewer detectable transcripts, while $>10^5$ copies was toxic to the embryos (data not shown).

The expression of early *H2B* genes in host embryos is shown in Fig. 2. Total RNA was extracted from the embryos at the mid (11 hr) and the late (21 hr) blastula stage, hybridized to RNA probes complementary to early *H2B* gene transcripts, and digested with ribonuclease. RNA from both injected and uninjected embryos protected a probe fragment of ≈65 nucleotides, which represents cross-hybridization of the *S. purpuratus* early *H2B* probe with *L. pictus* early *H2B* transcripts. As expected, the endogenous early *H2B* transcripts were much more abundant in midblastulae than in late

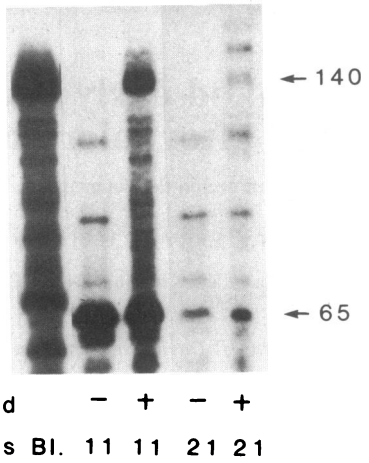

FIG. 2. Expression of endogenous and injected early histone *H2B* genes. RNA extracted from 40 uninjected *L. pictus* embryos or embryos injected with the plasmid containing both the early and late *H2B* genes was hybridized with a [^{32}P]RNA probe for *S. purpuratus* early *H2B* transcripts and digested with ribonuclease. The protected RNA fragments were electrophoresed on a urea/acrylamide gel and visualized by autoradiography. Only the relevant portion of the gel is shown. The first lane shows a control analysis of 0.05 μg of *S. purpuratus* 10-hr blastula RNA. The remaining lanes show the analyses of RNA from *L. pictus* embryos, uninjected or injected with the early/late gene construct, and collected at 11 and 21 hr after fertilization. The upper marked band is ≈140 nucleotides long and represents transcripts from the injected *S. purpuratus* early *H2B* gene. The lower marked band is ≈65 nucleotides long and represents cross-hybridization of the probe with endogenous *L. pictus* transcripts.

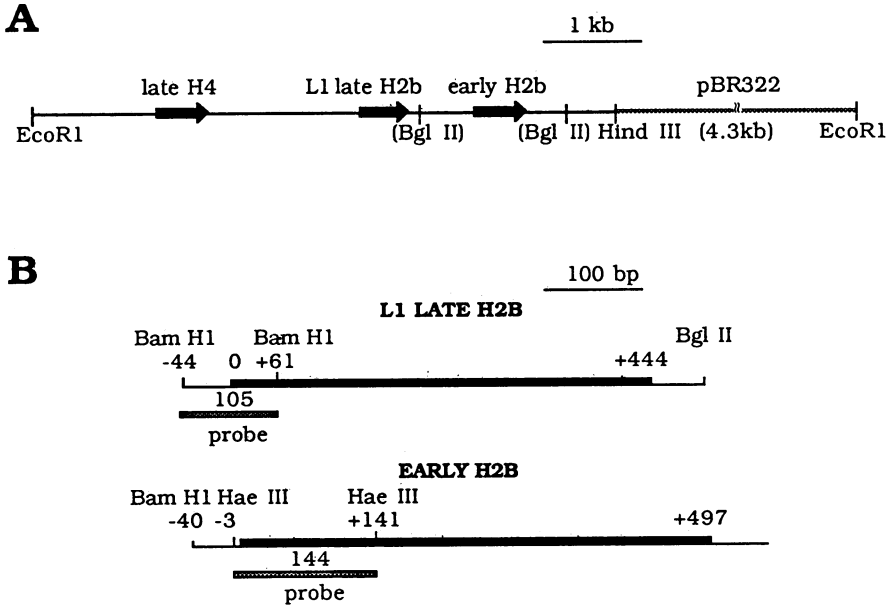

FIG. 1. Schematic map of plasmid pSpEL-1. This plasmid contains an early and a late histone H2B gene as well as a late histone H4 gene. The early *H2B* segment (1.8 kb) was derived from a cloned tandem repeat unit of *S. purpuratus* early histone genes. This segment was inserted into the plasmid pSpl-1 (7) at a *Bgl* II site 90 bp downstream from the L1 late *H2B* transcription termination site. (*A*) Low resolution map showing entire plasmid construct. (*B*) High resolution maps of early and late *H2B* genes showing segments used as probes (hatched lines). These segments were cloned into the RVIIΔ7 vector and radiolabeled RNA probes were generated using SP6 RNA polymerase (see *Methods* and Fig. 2).

blastulae. A probe fragment of ≈140 nucleotides, the expected size for transcripts from the injected *S. purpuratus* early *H2B* gene, was protected only by RNA from injected embryos. As with the endogenous early H2B mRNAs, transcripts of the injected early *H2B* genes were substantially more abundant in midblastulae than in late blastulae.

In contrast to the decrease in the expression of the injected early *H2B* gene in the late blastula stage, transcripts of the injected late *H2B* gene increased in amount during this time. Fig. 3 shows the ribonuclease protection products of embryonic RNA hybridized with the late *H2B* probe. A group of fragments migrating at 60–65 nucleotides, the expected size for *S. purpuratus* L1 late H2B mRNA RNase protection products (10, 15), was found in increasing amounts 11, 22, and 28 hr after fertilization. These fragments were not detected in RNA from uninjected embryos or injected embryos 7 hr after fertilization. We could not compare levels of injected late *H2B* transcripts with their endogenous counterparts as our late probe did not cross-hybridize detectably with *L. pictus* late H2B mRNAs. The observed increase in transcripts of injected late genes is consistent, however, with the expression profile of *L. pictus* late genes (see also Fig. 4), since it occurs when the level of *L. pictus* early *H2B* transcripts is decreasing (7).

The results of several injection experiments are summarized in Fig. 4. For each experiment, we plotted the relative levels of transcripts at the various time points. Fig. 4*A* summarizes the levels of endogenous early H2B transcripts at four time points for two experiments. The level of transcripts increased ≈10-fold between 6 and 12 hr after fertilization and then dropped by a factor of 10 by 21 hr. In Fig. 4*B*, the relative levels of injected *H2B* gene transcripts are shown. As with the endogenous early transcripts, those of the injected early gene increased ≈10-fold during cleavage and blastulation and then decreased by a factor of ≈10 by late blastula stage. However, the peak accumulation of these early *H2B* transcripts came somewhat later than that of the endogenous H2B mRNAs (see *Discussion*).

The levels of injected late *H2B* gene transcripts at several time points in three separate experiments are shown in Fig. 4*B*. Like the endogenous late *H2B* transcripts (Fig. 4*A*), these mRNAs became detectable at the early blastula stage (≈10 hr after fertilization) and their levels increased ≈25-fold during the blastula stage (10–28 hr after fertilization). Levels of injected late *H2B* gene transcripts decreased later in development, since gastrula stage embryos (40 hr postfertilization) contained about half the number of injected late *H2B* gene transcripts as did late blastula stage embryos (data not shown).

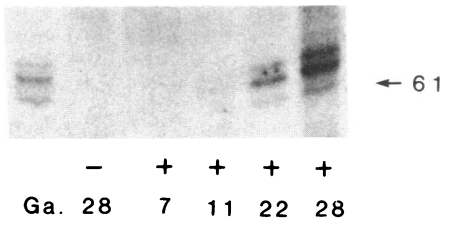

FIG. 3. Expression of injected late histone H2B genes. RNA extracted from 20 uninjected and plasmid-injected *L. pictus* embryos was analyzed using a radiolabeled RNA probe for late H2B mRNA as described in the legend to Fig. 2. Only the relevant portion of the gel is shown. The first lane shows the fragments protected by 0.5 μg of *S. purpuratus* gastrula RNA. The second lane shows the analysis of RNA from uninjected eggs collected 28 hr after fertilization. The remaining lanes show analyses of plasmid-injected eggs collected 7, 11, 22, and 28 hr after fertilization. Our late *H2B* probe did not detect *L. pictus* late *H2B* transcripts.

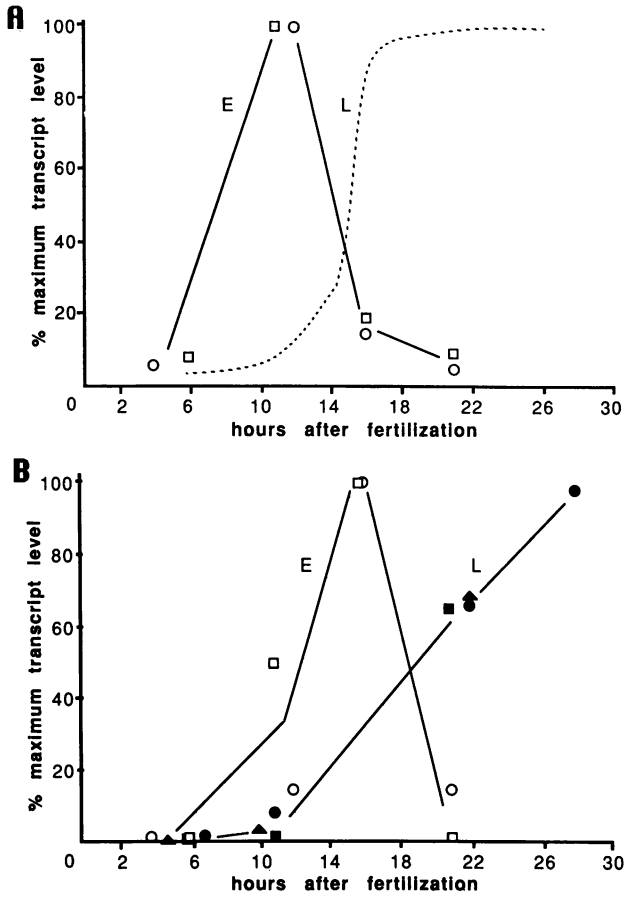

FIG. 4. Temporal pattern of endogenous and injected early and late *H2B* gene expression in *L. pictus* embryos. RNA from embryos injected with *S. purpuratus* early and late histone H2B genes was analyzed by ribonuclease protection. For each experiment, the relative amounts of protected fragments at the various time points were determined by densitometry and the maximum value was set at 100%. Nondetectable levels of hybridization are plotted on the *x* axis. (*A*) Relative amounts of endogenous early (labeled E) and late (labeled L) *H2B* gene transcripts. (*B*) Relative amounts of injected early (labeled E) and late (labeled L) *H2B* gene transcripts. Data from different experiments are indicated by different symbols. In *B*, solid symbols refer to late H2B mRNA measurements. Solid circles represent the same experiment shown in Fig. 3. Endogenous late histone mRNA curve in *A* is derived from the data of Knowles and Childs (7) on *L. pictus* late H3 and H4 mRNAs.

DISCUSSION

We examined the timing of expression of cloned *S. purpuratus* early and late histone H2B genes after injection into *L. pictus* embryos. Since genes injected on separate plasmids might appear to be differentially expressed simply because of differences in replication or degradation rates, we injected early and late genes linked in a single plasmid. The cloned early *H2B* segment contained ≈600 bp of 5′ and 700 bp of 3′ flanking sequence; the late *H2B* segment contained 3 kb of 5′ and 100 bp of 3′ flanking sequence.

We found that the injected early and late *H2B* genes were transcribed accurately and with a nearly normal temporal pattern in the host embryos. Early gene transcripts reached peak accumulation in the midblastula and then decreased several times by the late blastula stage. Late gene transcripts continued to accumulate through the blastula stage. This pattern was identical to that of the endogenous *H2B* genes in the most critical respect: the decline in early *H2B* expression in late blastula embryos.

Our results thus strongly suggest that the sequences

involved in the decrease in early *H2B* gene expression and the increase in late gene expression in late-stage embryos reside in the cloned DNA. Davidson and coworkers (16, 17) have similarly shown that cloned 5′ leader and upstream sequences of a sea urchin early histone H2A gene and a cytoskeletal actin gene allow developmentally appropriate expression of a fused chloramphenicol acetyltransferase gene injected into sea urchin eggs.

The differential expression of closely linked early and late *H2B* genes during development provides arguments against two hypotheses concerning the regulation of early and late histone gene expression. First, since the early *H2B* gene used in these experiments was removed from the context of the early tandem repeat structure, this structure probably does not play a major role in the decline in early *H2B* gene expression in late embryogenesis. Second, differential timing of DNA replication during the cell cycle, which appears to be important for the differential expression of oocyte and somatic 5S ribosomal RNA genes in *Xenopus* (18), is probably not necessary for the developmental regulation of early and late histone genes since the two closely linked gene should replicate nearly simultaneously. In addition, our data suggest that if alterations in chromatin structure are responsible for changes in transcription rates, these alterations can be very localized.

Are the injected *H2B* genes subject to transcriptional regulation like their endogenous counterparts? In developing *S. purpuratus* embryos, early histone genes are regulated at the posttranscriptional as well as the transcriptional level (3, 5): the decrease in early H2B mRNA levels in late blastula embryos is caused by a decrease (2–3 times) in histone mRNA half-life together with a decline by a factor of 10 in histone mRNA transcription rate. Transcripts of early genes injected into *L. pictus* embryos drop in amount by a factor of 10–20 during the late blastula stage, far in excess of the decline expected if only the post-transcriptional (i.e., changing half-life) mechanism were operating. Thus, the injected early histone genes are probably transcriptionally regulated.

We cannot be certain, however, that injected late histone genes undergo appropriate transcriptional regulation similar to that of the endogenous genes. The transcription rate of late *H2B* genes in sea urchin embryos increases severalfold at the midblastula stage without a change in half-life of the mRNA (M. Ito, J. Bell, and R.M., unpublished observations). The accumulation of transcripts from the injected late genes is similar to that expected for endogenous genes (7). However, it is formally possible that the transcription rate of the injected genes has a different profile from that of the endogenous genes.

Although the level of transcripts derived from injected early genes declined as late gene transcripts accumulated, these early transcripts peaked several hours later than their endogenous counterparts. This difference in timing was probably not caused by the differential activity of the *L. pictus* transcriptional machinery on endogenous versus injected *H2B* genes since, in species hybrids formed between *L. pictus* and *S. purpuratus*, maternal and paternal forms of histone genes are expressed identically (ref. 19; R.M., unpublished observations). It is possible that the timing difference was the result of the L1 late *H2B* gene's proximity to the injected early *H2B* gene. Against this possibility is the observation of Davidson *et al.* (16) who found that an early H2A–chloramphenicol acetyltransferase fusion construct was maximally expressed 15 hr after fertilization, not at 10–12 hr as would be expected. However, the levels of chloramphenicol acetyltransferase activity were not directly compared with endogenous H2A mRNA levels in the injected embryos and thus may not have peaked later than the endogenous H2A mRNA. Finally, the peak of our injected early gene transcripts may have been delayed because the injected genes replicated at a different rate from the endogenous genes. Nevertheless, early and late histone H2B genes were expressed with essentially normal developmental timing after injection into sea urchin zygotes, demonstrating that the major control is exerted solely by sequences adjacent to the genes.

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