Specific regulation of *Xenopus* chromosomal 5S rRNA gene transcription in vivo by histone H1

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The incorporation of histone H1 into chromatin during embryogenesis directs the specific repression of the *Xenopus* oocyte 5S rRNA genes. An increase in histone H1 content specifically restricts TFIIIA-activated transcription, and a decrease in histone H1 within chromatin facilitates the activation of the oocyte 5S rRNA genes by TFIIIA. Variation in the amount of histone H1 in chromatin does not significantly influence somatic 5S rRNA gene transcription. Thus, the regulated expression of histone H1 during *Xenopus* development has a specific and dominant role in mediating the differential expression of the oocyte and somatic 5S rRNA genes. This example demonstrates that histones can exert dominant repressive effects on the transcription of a gene in vivo in spite of an abundance of transcription factors for that gene.

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Impressive genetic evidence exists for the histone proteins having a supporting role in gene regulation (Grunstein 1990; Felsenfeld 1992; Herskowitz et al. 1992; Winston and Carlson 1992). Nucleosomes are recognized as potentially restricting the access of *trans*-acting factors to DNA (Adams and Workman 1993); this restriction can depend on the positioning of histones with respect to DNA sequence and on the modifications of the histones (Simpson 1991; Lee et al. 1993; Turner 1993). Histones and their modifications are also involved in establishing the structure and function of chromosomal domains (Braunstein et al. 1993; Renaud et al. 1993; Turner 1993). Nevertheless, a role for an individual histone in regulating the expression of a specific gene during a developmental process has not yet been established.

Linker histones such as H1 play a key role in the compaction of nucleosomes into the chromatin fiber (Thoma et al. 1979; Butler and Thomas 1980). They prefer to associate with DNA wrapped around an octamer of core histones rather than with naked DNA (Hayes and Wolffe 1993). Incorporation of a single molecule of linker histone into the nucleosome stabilizes the interaction of 166 bp of DNA with the histones (Simpson 1978; Allan et al. 1980). Linker histones may also influence the positioning of nucleosomes (Meersseman et al. 1991; Chipev and Wolffe 1992). Distinct variants of linker histones are expressed during the development of several organisms, including vertebrates (Poccia 1986). In the sea

urchin (*Strongylocentrotus*), there are distinct sperm, cleavage, and adult linker histones (Newrock et al. 1977; Levy et al. 1982; Knowles et al. 1987; Lai and Childs 1988). In *Xenopus laevis* during embryogenesis, there is a transition from a cleavage (B4) to an adult (H1), to a specialized linker histone found predominantly in terminally differentiated, nondividing cells (H1^o), (Perry et al. 1985; Smith et al. 1988; Dimitrov et al. 1993; Khochbin and Wolffe 1993). The structural and functional significance of these transitions has not been determined.

Linker histones have been proposed to act as general repressors of transcription (Weintraub 1984). In vitro reconstitution experiments using small DNA templates support this repressive role (Shimamura et al. 1989; Croston et al. 1991; Laybourn and Kadonaga 1991). In vivo, the overexpression of linker histones in somatic cells leads to cell cycle arrest and the reversible inhibition of DNA replication (Sun et al. 1989; Aubert et al. 1991). In contrast to these general inhibitory effects, evidence for a specific role for histone H1 in gene expression comes from experiments in *Xenopus*, where removal of histone H1 from the chromosomal chromatin of somatic cells selectively allows transcription factor access to the oocyte 5S rRNA genes (Schlissel and Brown 1984; Wolffe 1989a,b; Chipev and Wolffe 1992).

We have now examined the role of histone H1 in regulating gene expression during *Xenopus* embryogenesis. Surprisingly, the expression of many genes encoding small RNAs, including the somatic 5S rRNA genes, is

unaffected by overexpression of histone H1 or by deficiency of the protein. Nevertheless, we find that H1 has a dominant role in selectively repressing the oocyte 5S rRNA genes. Our results suggest that histones may exert their repressive effects on transcription even when transcription factors are in excess.

Results

Histone H1 inhibits the selective activation of chromosomal oocyte 5S rRNA gene expression by TFIIIA

In *X. laevis* 5S rRNA gene expression is developmentally regulated (Wakefield and Gurdon 1983; Wormington and Brown 1983; for review, see Wolffe and Brown 1988). The oocyte 5S rRNA genes (20,000 per haploid) are active in growing oocytes and transiently active at the mid-blastula transition (MBT) yet are repressed in somatic cells. In contrast, the somatic 5S rRNA genes (400 per haploid) are active in oocytes, at the MBT and in somatic cells. These genes share the same transcription factors TFIIIA, TFIIIB, and TFIIIC (Segall et al. 1980; Setzer and Brown 1985) yet assemble transcription complexes with differential stabilities (Wolffe and Brown 1987). The association of TFIIIA with the 5S rRNA gene is necessary for the subsequent formation of a transcription complex containing TFIIIB and TFIIIC (Setzer and Brown 1985). Transcription factors such as TFIIIA are abundant in growing oocytes (Pelham and Brown 1980; Shastry et al. 1984) yet are limiting for transcription in eggs and embryos (Engelke et al. 1980; Andrews and Brown 1987; Wolffe and Brown 1987). The product of transcription, 5S rRNA, has a role in titrating TFIIIA away from the oocyte 5S rRNA genes during embryogenesis (Rollins et al. 1993). This is because TFIIIA not only associates with the 5S rRNA gene but also with the transcription product, 5S rRNA (Pelham and Brown 1980). Raising the level of TFIIIA in developing *Xenopus* embryos will stimulate transcription of the oocyte 5S rRNA genes (Brown and Schlissel 1985; Andrews and Brown 1987).

We made use of the capacity to activate chromosomal oocyte 5S rRNA gene expression by raising the level of TFIIIA in *Xenopus* embryos to examine the influence of chromatin on transcription. TFIIIA protein is synthesized following the injection of TFIIIA mRNA into fertilized eggs. Without injection of TFIIIA mRNA, a slight excess of somatic 5S rRNA accumulates relative to oocyte 5S rRNA through gastrulation (Fig. 1, lane 7). Because there are 50 times more oocyte than somatic 5S rRNA genes, this represents at least a 50-fold preference for expression of the somatic genes. With injection of TFIIIA mRNA there is a large (>50-fold) increase in oocyte 5S rRNA accumulation and a much smaller increase (~2-fold) in somatic 5S rRNA accumulation based on densitometry of the autoradiographs (Fig. 1, cf. lanes 1 and 7 with lanes 2 and 8). The oocyte 5S rRNA genes have relatively weak terminators for RNA polymerase III, which leads to a diffuse "tail" of longer transcripts (Bogenhagen and Brown 1981). This increase in oocyte 5S

Figure 1. Selective repression of 5S oocyte RNA expression in gastrula embryos by injection of H1C mRNA. All embryos were injected with 0.1 μ Ci of [α - 32 P]GTP. Total nucleic acid from one control embryo (lanes 1,7) and mRNA-injected embryos (lanes 2–6,8–12) was separated on a 15% acrylamide, 4 M urea, 1 $\times$ TBE gel for 24 hr at 10 mA. Embryos were injected with 3 ng of TFIIIA mRNA alone (lanes 2,8), with 3 ng of H1C mRNA (lanes 5,11) or 8 ng of H1C mRNA (lanes 6,12), or injected with 3 ng of TFIIIA RNA and 3 ng of H1C mRNA (lanes 3,9) or 3 ng of TFIIIA RNA plus 8 ng of H1C RNA (lanes 4,10). Samples of 10 embryos were taken 9 hr (late blastula) or 14 hr (gastrula) after fertilization. The positions of U1, U2, somatic 5S, oocyte 5S RNA, and tRNAs are indicated.

rRNA reflects the maintenance of high levels of TFIIIA protein through gastrulation following injection of TFIIIA mRNA (Fig. 2A, 9 hr). This result also indicates that TFIIIA is limiting for oocyte 5S rRNA gene transcription and that TFIIIB and TFIIIC are present in excess. In vitro transcription experiments with chromatin isolated from control embryos and from embryos injected with TFIIIA mRNA confirm that this increase in 5S rRNA accumulation is directly attributable to a higher rate of transcription (data not shown; see Andrews and Brown 1987). RNase protection experiments (data not shown; see Fig. 7, below) confirmed that the increase in transcription was from the oocyte 5S rRNA genes.

Xenopus eggs sequester histone H1 mRNA but do not synthesize H1 protein until after fertilization (Woodland et al. 1979). Three variants of histone H1 accumulate: H1A, H1B, and H1C (Risley and Eckhardt 1981). H1A is

Figure 2. Translation of synthetic H1C and TFIIIA mRNA in developing embryos. Western blot of control and message-injected embryos. Proteins from one embryo equivalent were separated on an 18% polyacrylamide-SDS gel, transferred to nitrocellulose and probed with specific antibodies against H1 or B4, respectively (see Materials and methods). Control (–) and injected (+, +) embryos were taken at the indicated time. (A) Western blot of TFIIIA from control (–) and TFIIIA mRNA-injected (3 ng, +) embryos. Indicated amounts of purified TFIIIA were used as markers. (B) Western blot of H1 from control (–) and H1C mRNA-injected (3 ng, +) embryos. The same blot was incubated also with antibody against B4 protein as an additional control for the amount of loaded protein. (C) The overexpression of H1C protein does not change the expression of TFIIIA protein. The embryos were injected with TFIIIA mRNA alone or with both TFIIIA mRNA and H1C mRNA (3 ng, +, 8 ng, + +). The Western blot with immunopurified antibody against TFIIIA is shown.

the most abundant (~95% of total H1), H1B is next in abundance, and H1C is the least abundant in somatic cells (Risley and Eckhardt 1981). We chose to decrease H1A synthesis using ribozymes (see below) because of its abundance. We chose to overexpress H1C, because of the low levels normally present in embryonic chromatin and the capacity to easily monitor an increase in synthesis (see below). Similar data were obtained when histone H1 variants other than H1C were overexpressed (not shown). We have not systematically attempted to decrease synthesis of H1B or H1C because they make up <5% of total histone H1 at any time during *Xenopus* embryogenesis. Genes encoding all of these proteins have been cloned (Old et al. 1982; Perry et al. 1985).

In our first experiments we examined the consequences of increasing histone H1C synthesis for transcription in *Xenopus* embryos. Microinjection of in vitro-synthesized histone H1C mRNA into eggs directed a substantial increase in histone H1C synthesis through gastrulation (Fig. 2B). In this experiment the abundance

of histone H1 is compared with that of histone B4, because the amount of total B4 protein in the embryo remains constant through gastrulation (i.e., from fertilization to 14 hr postfertilization; Dworkin-Rastl et al. 1994). However, because of the rapid increase in the number of embryonic nuclei, the amount of B4 per nucleus decreases during development (Dimitrov et al. 1993). Thus, histone H1 accumulation progressively dilutes the number of nucleosomes containing histone B4 during *Xenopus* embryogenesis. The newly synthesized histone H1C is incorporated into embryonic nuclei (Fig. 3) without any obvious effect on the accumulation of small RNAs (Fig. 1, cf. U2, U1, and tRNA; lane 7 with lanes 11 and 12). However, overexpression of histone H1C does influence the transcription of the oocyte 5S rRNA genes (Fig. 1; see Fig. 7, below).

Figure 3. Overexpressed histone H1C is incorporated into chromatin. Two-dimensional electrophoresis of histones isolated from the chromatin of control and H1C mRNA-injected embryos. Embryos were injected with 3 ng of H1C mRNA, in 50 nl of ^3H -labeled lysine and ^3H -labeled arginine. Control embryos were injected with tritium-labeled amino acids only. After the indicated time of incubation, nuclei were prepared and histones were isolated by HCl treatment. Carrier histones from *Xenopus* erythrocytes were added, and the proteins separated by two-dimensional electrophoresis (Materials and methods). For identification of the tritium-labeled histones, the gels were stained with Coomassie blue to determine the position of unlabeled carrier histones that serve as markers, destained, treated with Amplify, dried, and autoradiographed. (Top panels) Autoradiograph of two-dimensional gel of histones isolated from control (left) and H1C mRNA-injected (right) embryos. Embryos were collected 6 hr after fertilization and histones extracted from them. (Bottom panels) Same as above but for embryos collected 9 hr after fertilization.

Injection of H1C mRNA into the egg at the same time as TFI_{IIA} mRNA leads to the selective reduction of oocyte 5S rRNA accumulation compared with the activated state in the presence of elevated TFI_{IIA} (Fig. 1, cf. lane 8 with lanes 9 and 10). This decrease in oocyte 5S rRNA gene transcription is at the transcriptional level (data not shown; see Fig. 7, below), indicating that histone H1C abundance could influence the assembly of transcription complexes on the oocyte 5S rRNA genes in spite of elevated levels of the TFI_{IIA} protein. The failure to activate the oocyte 5S rRNA genes is not attributable to any interference by elevated histone H1 mRNA or H1 protein on the synthesis of TFI_{IIA} protein (Fig. 2C). This is an important control because it excludes any limitation of translational capacity in the developing embryo (after H1 mRNA injection) leading to a decrease in TFI_{IIA} synthesis. The constant level of endogenous tRNA gene transcription in the presence of elevated histone H1C protein (see Fig. 7) indicates that histone H1C accumulation does not influence the activity of the other transcription factors (TFI_{II}C and TFI_{II}B) required both for tRNA and 5S rRNA gene transcription.

We conclude that a competition exists between the association of TFI_{IIA}, together with the other transcription factors with the oocyte 5S rRNA gene (Segall et al. 1980; Setzer and Brown 1985) and the association of histone H1 with these genes in the chromosome. Therefore, the presence of increased amounts of histone H1 in chromatin can have a dominant and specific repressive effect on oocyte 5S rRNA gene expression. This effect is dominant because it occurs in the presence of an excess of the transcription factor TFI_{IIA}. Furthermore, because TFI_{IIA} is the limiting transcription factor in the embryo at this time (Brown and Schlissel 1985; Andrews and Brown 1987), histone H1-directed repression of oocyte 5S rRNA gene transcription occurs when TFI_{II}C and TFI_{II}B are also in excess.

Elevation of histone H1 levels in vivo does not alter nucleosome structure

Addition of histone H1 to in vitro chromatin assembly systems can alter the spacing of the nucleosomal ladder following micrococcal nuclease digestion (Rodriguez-Campos et al. 1989; Kamakaka et al. 1993). This change in spacing may reflect the incorporation of more than one linker histone molecule per histone octamer into each individual nucleosomal unit (Weintraub 1978). Although the exact structure of such a nucleosomal unit has not been determined, we wished to examine whether unusual nucleosomal structures might be assembled within the *Xenopus* embryo in response to the presence of additional histone H1C protein.

We initially repeated the H1C-directed repression of oocyte 5S rRNA gene expression in the presence of excess TFI_{IIA} to confirm the activity of histone H1C by this functional assay (not shown). We then examined the incorporation of newly synthesized histone H1C into embryonic chromatin at 12 hr postfertilization (Fig. 4A). Microinjection of H1C mRNA leads to a more than

threefold increase (based on densitometry of the two-dimensional gel) in the accumulation of total histone H1 protein within embryonic chromatin, this increase is accounted for by increased accumulation of histone H1C protein. Increased levels of histone H1C are recovered in fractions containing mononucleosomal particles following injection of histone H1C mRNA into embryos (not shown). This additional histone H1 protein will conse-

Figure 4. Incorporation of histone H1C into *Xenopus* embryonic chromatin does not alter the nucleosomal spacing or the chromosome organization. (A) Two-dimensional electrophoresis of histones isolated from the chromatin of control and H1C mRNA-injected embryos. Embryos were injected with 8 ng of H1C mRNA and 3 ng of TFI_{IIA} mRNA, together with 50 nM of ³H-labeled lysine and ³H-labeled arginine. Control embryos were injected with tritium-labeled amino acids only. After 12 hr of incubation, nuclei were prepared and histones were isolated by HCl treatment. Histones were identified as described for Fig. 3. Autoradiographs of histone gels isolated from H1C mRNA-injected (left) and control (right) embryos are shown. H1C is indicated. (B) Micrococcal nuclease digestion patterns of chromatin isolated from control and H1C mRNA-injected embryos (+ H1C). As in A except 0.1 μ Ci of [α -³²P]dCTP replaces radiolabeled amino acids. After 12 hr of development, nuclei were isolated from both sets of embryos and their chromatin digested with increasing amounts of micrococcal nuclease (10, 20, and 50 units from right to left, open wedges). DNA fragments were deproteinized and resolved on a 10% nondenaturing polyacrylamide gel. An autoradiograph of labeled DNA fragments is shown. A 154-bp marker DNA fragment is indicated as are chromosome (Chr.) and core particle (Core) length DNA fragments. (C) As in B, except 0.5, 1, 2, 5, and 10 units of micrococcal nuclease are used, respectively, from left to right (open wedges) to digest chromatin from nuclei isolated from control and H1C mRNA-injected embryos. The DNA fragments were deproteinized and resolved on a 1.5% agarose gel. An autoradiograph of labeled DNA fragments is shown. A 517-bp marker DNA fragment is indicated.

quently dilute the number of nucleosomes containing the linker histone B4 (Dimitrov et al. 1993). It is important to note that all of the histone H1C protein may not be incorporated into individual nucleosomal particles, rather like transcription factors may interact with DNA both specifically or nonspecifically (Lin and Riggs 1975). Although histone H1 protein binds preferentially to DNA wrapped around the core histones rather than to naked DNA (Hayes and Wolffe 1993), under physiological ionic conditions histone H1 is readily exchanged between DNA that is either naked or wrapped around the core histones within a nucleosome (Thomas and Rees 1983; van Holde 1989). Nevertheless, increase in total H1 protein concentration will influence the equilibrium binding of histone H1 protein within the nucleosome (Hayes and Wolffe 1993). Under our experimental conditions in which the histone H1 protein content of chromatin is elevated in vivo, no increase in nucleosomal repeat is observed following micrococcal nuclease digestion (Fig. 4B,C). Moreover, the proportional protection of chromatosome (~166 bp) length compared with core particle (~146 bp) length DNA fragments appears unchanged in the presence or absence of additional histone H1 during extended micrococcal nuclease digestion (Fig. 4B). We conclude that elevated histone H1 is not perturbing the normal chromatin structure within the *Xenopus* embryo.

Ribozyme-mediated reduction in histone H1 gene expression during Xenopus embryogenesis: consequences for 5S rRNA accumulation

Ribozymes provide an attractive approach for the elimination of the expression of particular genes in vivo (Cotten and Birnstiel 1989). We have made use of a ribozyme approach to examine the consequences of a severe reduction in the accumulation of the major variant of histone H1 (H1A) for RNA accumulation in general and for the 5S rRNA genes in particular. A hammerhead ribozyme (Haseloff and Gerlach 1988) flanked by sequences homologous to H1A mRNA was introduced into a *X. laevis* tyrosine tRNA intron (Stutz et al. 1989). Following injection into a fertilized *Xenopus* egg, this ribozyme effectively restricts the accumulation of H1A protein in the *Xenopus* embryo (Kandolf 1992; see Materials and methods). Injection of the H1A ribozyme into the *Xenopus* egg leads to a four- to fivefold reduction in the accumulation of total histone H1 within the embryo by the beginning of gastrulation (Fig. 5A, 9 hr, measured by densitometric analysis of the autoradiograph). H1A is normally the predominant variant (~95% of total H1 protein) at this embryonic stage (Fig. 3). Moreover, because histone H1A mRNA normally comprises ~95% of total H1 mRNA at this stage (i.e., H1A + H1B + H1C; P. Bouvet, unpubl.), this four- to fivefold reduction of H1 protein synthesis is more severe than the two- to threefold reduction in H1A mRNA levels (Fig. 5B). Thus, it is possible that the H1A ribozyme both cleaves H1A mRNA and restricts synthesis of the protein through the formation of RNA duplexes that arrest the translation process

Figure 5. Injection of ribozyme in *Xenopus* embryos selectively inhibits the synthesis of histone H1. (A) Western blot of control and H1A ribozyme-injected embryos. HCl-extracted proteins from one equivalent embryo were separated on 18% polyacrylamide-SDS gel, transferred to nitrocellulose, and reacted with immunopurified antibody to *Xenopus* H1. Control (-) and H1A ribozyme-injected (10 ng, +) embryos were taken at the indicated time. Linker histones purified from *Xenopus* erythrocytes are used as a control. (B) Northern analysis of control and ribozyme-injected embryos. Total RNAs from one equivalent embryo were subjected to Northern blot hybridization with a ³²P-labeled H1A-specific gene probe (Perry et al. 1985). Control (-) and H1A ribozyme-injected (+) embryos were taken at the indicated times. (C) Western blot of TFIIIA protein levels in control and ribozyme injected embryos at 9 hr of development. Embryos were either injected with TFIIIA mRNA (3 ng) alone, with TFIIIA mRNA plus the H1 A ribozyme (10 ng), with ribozyme alone, or not injected. At 9 hr postfertilization, proteins from one equivalent embryo were separated on a 10% polyacrylamide-SDS gel, transferred to nitrocellulose, and reacted with immunopurified antibody to *Xenopus* TFIIIA. TFIIIA purified from *Xenopus* oocytes was used as a control, M (Smith et al. 1984).

(hybrid arrest of translation). It is also possible that the fraction of mRNA accessible to the ribozyme is limited through association of the H1 mRNA with the translationally repressive masking proteins in the embryo (Tafari and Wolffe 1993). An additional control determined that expression of the ribozyme had no influence on the abundance of TFIIIA protein in the embryo in the

presence or absence of microinjected TFIIIA mRNA (Fig. 5C). Furthermore, the ribozyme does not influence the apparent activity of the other class III gene transcription factors TFIIIB and TFIIIC, because endogenous tRNA gene transcription is unaffected when the ribozyme is injected into the developing embryo (see Fig. 7, below). Cloned class III genes requiring TFIIIB and TFIIIC are also transcribed equivalently in extracts prepared from control and ribozyme-treated embryos (not shown).

We examined the influence of reducing histone H1A synthesis during early embryogenesis on oocyte and somatic 5S rRNA gene expression (Fig. 6A, cf. lanes 1 and 2). This reduction in histone H1A synthesis has no major effect on the relative accumulation of oocyte and somatic 5S rRNA through early gastrulation (Fig. 6, cf. lanes 1 and 2). The approximately equivalent accumulation of somatic 5S rRNA and oocyte 5S rRNA reflects a 50:1 preference for expression of the somatic 5S rRNA gene at 12 hr postfertilization, even when histone H1A synthesis is reduced. A 50:1 transcriptional preference is observed when naked DNA templates encoding oocyte and somatic 5S rRNA genes are transcribed in egg extracts (Wolffe and Brown 1987). Thus, observing this differential expression probably reflects the major contribution transcription factor limitation makes to differential 5S rRNA gene regulation during the early stages of *Xenopus* embryogenesis (see Discussion). However, more sensitive RNase protection assays reveal changes in the transcription of the oocyte 5S rRNA genes by 15 hr in development when histone H1A synthesis is impaired (see Fig. 7, below). Thus, the influence of reduced histone H1 synthesis may predominate later in gastrulation, it is at this time that H1 normally replaces B4 almost completely within embryonic chromatin (Dimitrov et al. 1993).

We then asked whether the reduction of histone H1 in early embryonic chromatin would have consequences for the activation of oocyte 5S rRNA gene expression in the presence of excess TFIIIA. Injection of TFIIIA mRNA leading to an elevation of TFIIIA concentration of approximately four- to fivefold (not shown) leads to a more substantial activation of oocyte 5S rRNA gene transcription (twofold) from histone H1-deficient chromatin (Fig. 6B), than from chromatin containing the normal mixture of histone H1 and B4 (Fig. 6, cf. lanes 3 and 4, and 7 and 8). Thus, embryonic chromatin deficient in histone H1 is more accessible to transcription factors.

These results suggest that the abundance of histone H1 in early embryonic chromatin will only influence the differential expression of the oocyte and somatic 5S rRNA genes beyond the level of differential expression (i.e., the 50-fold somatic preference) established through the differential interaction of transcription factors with the genes as naked DNA (Wolffe and Brown 1987).

The influence of increase or reduction in histone H1C accumulation during normal Xenopus embryogenesis on 5S rRNA gene transcription

Our experiments so far have established that an increase

Figure 6. Effects of histone H1A depletion on oocyte 5S rRNA gene expression. (A) Control embryos were injected with 10 ng of tyrosine tRNA alone (lanes 2,6) or together with 3 ng of TFIIIA mRNA (lanes 4,8). Other embryos were injected with 10 ng of H1A ribozyme alone (lanes 1,5) or together with 3 ng of TFIIIA mRNA (lanes 3,7). All embryos were coinjected with 0.1 μ Ci of [α - 32 P]. After the indicated time, total RNAs were extracted and the RNA of one equivalent embryo was separated on a semidenaturing gel for 24 hr at 10 mA. (Bottom panel) A shorter exposure of top panel. The positions of U2, U1, somatic 5S, oocyte 5S and tRNAs are indicated. (B) Quantitation by scanning densitometry of the 15 hr results shown in A (lanes 5–8). Absolute values (arbitrary units) are shown for U1, somatic 5S, and oocyte 5S rRNA. The oocyte 5S rRNA values in the absence of microinjected TFIIIA mRNA were not significantly above background on the autoradiograph used for this densitometric analysis.

in histone H1C protein early in *Xenopus* embryogenesis will repress oocyte 5S rRNA gene expression under con-

Figure 7. Regulation of oocyte 5S rRNA transcription by histone H1 during normal development. (A) Overexpression of histone H1C by 9 hr postfertilization selectively represses oocyte 5S rRNA gene transcription. Embryos were either not injected (–) or injected (+) with H1C mRNA (8 ng). After 9 hr postfertilization, nuclei were fractionated from both sets of embryos and run-on transcription allowed in the presence of radioactive precursors (Materials and methods). RNAs synthesized under these conditions are shown resolved on a 10% denaturing polyacrylamide gel (Run on). In a parallel reaction, radiolabeled RNAs synthesized by run-on RNAs were hybridized to an excess of antisense RNA corresponding to oocyte or somatic 5S rRNA, followed by digestion with RNase (Materials and methods). Protected RNAs corresponding to oocyte or somatic 5S rRNA are shown. Finally, histone H1 protein present in the control and injected embryonic nuclei is shown by immunoblotting (Immunoblot). (B) Inhibition of histone H1A synthesis through 15 hr postfertilization selectively facilitates oocyte 5S rRNA gene expression. As in A, except embryos were either not injected (–) or injected with the H1A ribozyme (10 ng). After 15 hr postfertilization, the abundance of histone H1 in nuclei (Immunoblot), the transcriptional properties of the nuclei (Run on), and the relative transcription of oocyte, and somatic 5S rRNA (RNase Prot.) were assayed.

ditions of TFIIIA excess (Fig. 1). We have also determined that normal histone H1A expression is not essential for a substantial part of differential oocyte and somatic 5S rRNA gene expression (Fig. 6). Thus, in principle, both TFIIIA limitation and histone H1 accumulation may have a role in 5S rRNA gene regulation during development. Nevertheless, we have not established that altering histone H1 levels will influence differential 5S rRNA gene expression during development in the absence of excess TFIIIA. These experiments require a more sensitive combination of assays because specific oocyte 5S rRNA and somatic 5S rRNA gene transcription must be examined under conditions where 5S rRNA gene transcription is already substantially reduced attributable to TFIIIA limitation (Figs. 1, 2, and 6).

First, we overexpressed histone H1C through injection of H1C mRNA without injection of TFIIIA mRNA. Synthesis of H1C by early gastrulation (9 hr postfertilization) is substantially elevated (Fig. 7A, Immunoblot). Run-on transcription from chromatin isolated at this time (Fig. 7A, Run on) shows that total 5S rRNA gene transcription is reduced under conditions where H1C protein is elevated. RNase protection experiments (Fig.

7A, RNase Prot) show that the reduction in total 5S rRNA gene transcription can be specifically attributed to a reduction in transcription of the oocyte 5S rRNA genes. Somatic 5S rRNA gene transcription is unaffected. Control RNase protection assays also indicated that tRNA transcription was unaffected by overexpression of H1C. We conclude that overexpression of histone H1C protein during normal development will specifically inhibit oocyte 5S rRNA gene transcription.

Second, we reduced histone H1A expression through injection of the ribozyme without injection of TFIIIA mRNA. Synthesis of H1A through gastrulation (15 hr postfertilization) remains substantially reduced (Fig. 7B, Immunoblot). Run-on transcription from chromatin isolated at this time (Fig. 7B, Run on) shows that total 5S rRNA gene transcription is increased under conditions where H1A protein is reduced. RNase protection experiments (Fig. 7B, RNase Prot.) show that the elevation of total 5S rRNA gene transcription can be specifically attributed to an increase in transcription of the oocyte 5S rRNA genes. We conclude that reduction of histone H1A protein will specifically facilitate oocyte 5S rRNA gene transcription. Thus, alterations in histone H1 levels will

influence differential 5S rRNA gene expression in the presence of normal levels of TFIIIA protein within the embryo.

Discussion

The regulation of the chromosomal oocyte 5S rRNA genes during *Xenopus* embryogenesis depends on the abundance of TFIIIA and on the incorporation of histone H1 into chromatin (Figs. 1 and 7). TFIIIA specifically activates and histone H1 specifically represses oocyte 5S rRNA gene transcription (Fig. 8). We suggest that the developmentally regulated expression of linker histones, in which the cleavage stage linker histone B4 is diluted within chromatin by histone H1 during *Xenopus* embryogenesis (Dimitrov et al. 1993), will have specific consequences for how the transcriptional machinery utilizes a chromatin template. Moreover, our results demonstrate that histones can exert dominant repressive effects on transcription both in the presence of an excess of transcription factors (Fig. 1) and during normal development (Fig. 7).

Histone H1 and the selective repression of the oocyte 5S rRNA genes

The roles of individual histones and their modifications in vitro in mediating general transcriptional repression have been defined using the *Xenopus* 5S rRNA genes (Shimamura et al. 1988, 1989; Tremethick et al. 1990; Almouzni et al. 1990, 1991; Clark and Wolffe 1991; Hayes and Wolffe 1992; Lee et al. 1993). Histones and transcription factors were found to compete for association with the 5S rRNA gene, yet the first histones to associate with the 5S rRNA genes during chromatin assembly were less repressive toward transcription. Chromatin assembly in

vivo and in vitro is optimally coupled to replication (Worcel et al. 1978; Almouzni et al. 1991; Gruss et al. 1990) such that an acetylated histone tetramer (H3/H4)₂ is sequestered before H2A/H2B and, finally, H1. This creates a window of opportunity for the programming of the 5S rRNA genes with transcription factors, because the (H3/H4)₂ tetramer is less repressive than the complete (H2A/H2B, H3/H4)₂ octamer, whereas chromatin containing histone H1 is essentially inert and inaccessible to the class III gene transcriptional machinery. If the transcription factors bind stably to DNA before nucleosome assembly is complete a gene is potentially active. However, if they do not they are subject to displacement attributable to competing histone–DNA interactions (for review, see Wolffe 1992). Thus, the somatic 5S rRNA genes that rapidly form a stable complex with transcription factors TFIIIA and TFIIIC (Lassar et al. 1983; Wolffe 1988; Keller et al. 1992; Siedel and Peck 1992) might resist competing histone–DNA interactions, whereas the oocyte 5S rRNA genes that do not rapidly form a stable complex with these same transcription factors will be open to repression.

Consistent with this hypothesis, increasing the level of transcription factor TFIIIA in egg extracts will selectively activate the oocyte 5S rRNA genes (Wolffe and Brown 1987; Wolffe 1989a). Likewise elevating TFIIIA levels in developing embryos will selectively activate oocyte 5S rRNA gene expression (Figs. 1, 6, and 7; Brown and Schlissel 1985; Andrews and Brown 1987). Thus, limitation of transcription factors plays a key role in the inactivation of the oocyte 5S rRNA genes. Importantly, the oocyte 5S rRNA genes are inactive but are not repressed under these conditions, because addition of transcription factors activates their transcription.

Evidence for a competing histone–DNA interaction influencing the association of transcription factors with the oocyte 5S rRNA genes came initially from in vitro experiments. The key protein believed to be important in repressing the oocyte 5S rRNA genes is histone H1 (Schlissel and Brown 1984). Removal of histone H1 from somatic cell chromatin allows transcription factors to gain access to the oocyte type 5S rRNA genes. The oocyte 5S rRNA genes in somatic cells in vivo are known to be packaged into nucleosomes (Gottesfeld and Bloomer 1980; Young and Carroll 1983) and the positioning of histone–DNA contacts appears dependent on histone H1 (Chipev and Wolffe 1992). Sliding of nucleosomes following H1 removal may allow transcription factors access to the oocyte 5S rRNA genes. The addition of histone H1 to chromatin deficient in the protein in vitro can cause the selective repression of oocyte 5S rRNA genes and the displacement of transcription factors (Wolffe 1989b; Chipev and Wolffe 1992).

We have now determined that histone H1 will direct the selective repression of the oocyte 5S rRNA genes in vivo (Figs. 1 and 7). This establishes for the first time in a metazoan that the regulated expression of a specific histone can influence the expression of a specific gene. Importantly, histone H1C association with chromatin (Figs. 3 and 4) has a dominant-repressive effect on the

Figure 8. Model for differential oocyte and somatic 5S rRNA gene expression. The somatic 5S rRNA gene (solid arrow) and oocyte 5S rRNA gene (open arrow) are shown associated with transcription factors TFIIIA, TFIIIB, and TFIIIC. The somatic transcription complex is stable as indicated by the ellipsoid around it; the oocyte transcription complex is unstable indicated by the arrows. Additional TFIIIA promotes oocyte transcription complex retention; additional histone H1 promotes oocyte transcription complex displacement through nucleosome formation.

transcription of the oocyte 5S rRNA genes in spite of a continued excess of transcription factor TFIIIA (Fig. 2C).

Histone H1 prefers the prior assembly of histone octamers onto DNA for efficient incorporation into chromatin (Hayes and Wolffe 1993) and will direct the formation of nucleosomal arrays on the oocyte 5S rRNA genes (Chipev and Wolffe 1992). Association of histone octamers with the oocyte 5S rRNA genes are necessary for the selective repression of oocyte 5S rRNA genes within genomic DNA in vitro (Wolffe 1989b). In our assays accumulation of an excess of histone H1 does not alter the physiological features of chromatin attributed to the presence of linker histones, including the presence of a normal chromatosome stop (Simpson 1978), and nucleosome spacing as revealed by digestion with micrococcal nuclease (Fig. 6). This suggests that the increase in histone H1 is not leading to the general formation of different nucleosomal structures (Weintraub 1978) but is, instead, leading to a more stable equilibrium association of histone H1 with nucleosomal DNA and to a more efficient replacement of histone B4 by histone H1 in the nucleosomes of embryonic chromatin. We suggest that either H1 association with the octamer or the folding of the nucleosomal array dependent on histone H1 may direct the dissociation of TFIIIA and other weakly associated transcription factors from the oocyte 5S rRNA genes. These same transcription factors are stably associated with the somatic 5S rRNA gene and, hence, cannot be displaced by the sequestration of histone H1 (Fig. 7). Another contributory factor in the selective repression of oocyte 5S rRNA genes by histone H1 might be the preference of H1 for AT-rich DNA (Renz and Day 1976; Jerzmanowski and Cole 1990). The oocyte 5S rRNA genes all have AT-rich spacers while those of the somatic genes are GC rich, which may lead H1 to associate preferentially with the oocyte genes (Schlissel and Brown 1984). Maintenance of the final repressed state is likely to be a local effect of histone-DNA interactions because the developmental inactivity of the oocyte 5S rRNA genes persists when the chromosomes are cleaved by restriction enzymes between the genes (Gurdon et al. 1982).

Gene expression in H1-deficient embryos

Substantial experimental evidence supports the hypothesis that the limitation of transcription factor activity in the early embryo is a major component of the differential expression of the oocyte and somatic 5S rRNA genes (Andrews and Brown 1987; Wolffe and Brown 1987; Rollins et al. 1993). This limitation leads to at least a 50-fold preference for transcription of a somatic over an oocyte 5S rRNA gene on naked DNA (Wolffe and Brown 1987). Because there are 50 times more oocyte 5S rRNA genes than somatic 5S rRNA genes, this would lead to equivalent accumulation of oocyte versus somatic 5S rRNA. Thus, it is not surprising that reduction in histone H1 content within chromatin does not influence the relative expression of the oocyte and somatic 5S rRNA genes substantially beyond this 50-fold ratio especially at early

times of embryogenesis when chromatin is still relatively deficient in histone H1 (Dimitrov et al. 1993; Fig. 6). However the histone H1-deficient chromatin is more easily programmed with transcription factors (Fig. 6). More importantly, sensitive RNase protection assays using transcripts generated directly from embryonic chromatin at 15 hr postfertilization (Fig. 7) reveals that oocyte 5S rRNA gene expression is facilitated at physiologic transcription factor levels by a reduction in the content of histone H1 protein. TFIIIA activation of transcription is therefore more vigorous within the histone H1-deficient chromatin later in development (15 hr postfertilization) when histone H1 would have normally substantially replaced histone B4 in chromatin (Dimitrov et al. 1993). The cleavage linker histone B4 is much more acidic in the carboxy-terminal tail than histone H1 (Smith et al. 1988; Dimitrov et al. 1993); hence, like phosphorylated histone H1, it might be expected to have a weaker interaction with DNA (Jerzmanowski and Cole 1990). Thus, B4 may not compete effectively with transcription factors for association with the oocyte 5S rRNA genes. This suggests that specific linker histone variants may exert selective effects on the transcription of the chromatin template with which they are associated.

At the MBT, during *Xenopus* embryogenesis, there is a general activation of transcription of many genes, including the oocyte 5S rRNA genes (Newport and Kirschner 1982a,b; Wakefield and Gurdon 1983; Wormington and Brown 1983; Andrews and Brown 1987; Rupp and Weintraub 1991). We find that normal levels of histone H1 in chromatin are not necessary for the activation of transcription at the MBT (Fig. 6). Thus, a reduction in histone H1 does not appear to favor or disfavor expression of U1, U2, somatic 5S, or the bulk of the tRNA genes. The only genes that we have so far detected whose transcription is influenced by H1 protein are the oocyte 5S rRNA genes. The oocyte 5S rRNA genes are normally activated at the MBT and are subsequently repressed as gastrulation proceeds (Andrews et al. 1991; Rupp and Weintraub 1991). This transition correlates with the accumulation of levels of histone H1 in the embryo that are stoichiometric with the core histones (Dimitrov et al. 1993). Because increasing levels of histone H1 in the MBT embryo will direct the specific repression of oocyte 5S rRNA gene transcription before gastrulation is complete (Figs. 1 and 7), we suggest that the transient nature of oocyte 5S rRNA gene activation following expression of TFIIIA in the cleavage-stage embryo is probably attributable to accumulation of endogenous histone H1 toward the end of gastrulation (Andrews and Brown 1987). It will be of interest to examine whether the transcription of other genes transiently activated at the MBT (Rupp and Weintraub 1991) will be repressed in a comparable H1-dependent way. Significant changes in the utilization of regulatory elements for transcription occur during early mammalian development (Martinez-Salas et al. 1989; Majumder et al. 1993). Our results indicate that these events may also be related to transitions in linker histone expression during

the first cleavage events following fertilization (Clark et al. 1992).

Materials and methods

Plasmid constructions

The coding region of H1C (Old et al. 1982) was cloned by PCR from *X. laevis* genomic DNA using the primers 5'-GAATTT-AAGCTTCAAAGATGACAG-3' and 5'-GGAAGTCTAGAGT-TACTTTTTAGC-3'. This fragment was then subcloned into the *Xba*I and *Hind*III sites of pSP64pA (Promega). The coding region of TFIIIA RNA was subcloned from pSP7F15 (Andrews and Brown 1987) into the *Xba*I and *Hind*III sites of pSP64pA. pSP5Soo was constructed by subcloning a 195-bp fragment excised by *Bam*HI from pX1o316 (Wormington et al. 1981), containing the *X. laevis* major oocyte 5S rRNA gene, into pSP72 (Promega).

The construction of H1A ribozyme was performed according to Kandolf (1992). A hammerhead ribozyme (Haseloff and Gerlach 1988) was introduced into the intron of the *Xenopus* tyrosine tRNA (Stutz et al. 1989). The tyrosine tRNA was cloned by PCR from genomic DNA using the primers 5'-GCC-CAAGCTTAACAATTGAAG-3' and 5'-GCGGGATCCTCA-TCTCCTTC-3'. The 188-bp fragment was subcloned into the *Bam*HI and *Hind*III sites of pSP72 (Promega). An *Apal* site was introduced into the tRNA intron (Cotten and Birnstiel 1989). The histone H1A complementary sequence (Perry et al. 1985) was then introduced in this *Apal* site using these oligonucleotides: 5'-CTTTTTAGCGgctgatgagtcgtaggacgaaaCTCTTG-GTGGCGGGCC-3' and the opposite strand 5'-CGCCACCAAGAGTtctctcagcagctcatcagcCGCTAAAAAGGGCC-3'. The ribozyme sequence is in lowercase letters; H1A sequences are in uppercase. All the constructions were confirmed by sequencing.

Microinjection of embryos and RNA analysis

In vitro transcription reactions were performed as described by Krieg and Melton (1984) using linearized plasmids. To synthesize a 5'-terminal capped mRNA, 0.5 mM M⁷G(5')ppp(5')Gm was introduced in the reaction. The in vitro-synthesized mRNA was extracted with 1:1 phenol/chloroform and then ethanol precipitated in the presence of 2.0 M ammonium acetate.

Xenopus eggs were fertilized in vitro and maintained in water. Fertilized eggs were dejellied with 2% cysteine (pH 8.0) and injected during the first cleavage. Embryos were maintained in 0.1×OR2/5% Ficoll for 2–3 hr before being transferred to water. About 30 nl of solution containing the various mRNAs (for details, see figure legends) and 0.1 μCi of [α-³²P]GTP were injected into each embryo. Embryos were allowed to develop at 22°C to the appropriate stage, and RNA was then extracted as described by Harland and Misher (1988). The radiolabeled RNAs were separated on a semidenaturing gel according to Wakefield and Gurdon (1983) to separate the 5S oocyte-type RNA from the somatic 5S RNA (15% acrylamide, 4 M urea, 1× TBE gels were run in 1× TBE for 24 hr at 10 mA. Gels were fixed for 30 min in 10% acetic acid, 10% methanol, before drying). Quantitation of labeled RNAs was by laser densitometric analysis of autoradiographs using a Molecular Dynamics densitometer.

Preparation of nuclei and isolation of proteins

Embryos were thawed in ice, and nuclei were isolated as described (Wolffe 1989b). Histones and the protein B4 were extracted from nuclei by overnight treatment with 0.25 N HCl, followed by precipitation with 80% TCA to a final concentra-

tion of 20% (Dimitrov et al. 1993). After centrifugation the pellets were washed twice with acetone containing 0.2% HCl, followed by one wash with acetone only, and dried.

Total proteins from embryos were prepared by homogenizing the embryos in 1.0 M Tris-HCl (pH 8.9), 2% SDS, and 1% dithiothreitol (Andrews and Brown 1987), followed by centrifugation for 15 min at 4°C in a top bench centrifuge. The supernatant was used for further analysis by SDS–polyacrylamide electrophoresis (see below).

Transcription extract and in vitro transcription of embryonic chromatin

Run-on transcription of *Xenopus* embryonic chromatin was as described (Wolffe 1989b). Briefly, 50 injected embryos at mid-blastula transition were washed twice in ice-cold extraction buffer (50 mM HEPES-KOH at pH 7.5; 50 mM KCl, 5 mM MgCl₂, 2 mM β-mercaptoethanol). Embryos were placed in 0.65-ml microtubes, and excess buffer removed and centrifuged at 4°C at 9000g for 10 min. The cytoplasmic layer that contains endogenous chromatin was removed carefully. Radiolabeled ribonucleoside triphosphate [α-³²P]GTP and 0.5 mM ATP, UTP, and CTP were added, and run-on transcription allowed.

Transcription reactions with oocyte nuclear extract and plasmid pXP10 (Wolffe et al. 1986) were as described by Wolffe and Brown (1987). Transcription reaction products were dissolved in 90% formamide and analyzed on a 6% acrylamide 8 M urea gel.

RNase protection assays

Plasmids containing either the *X. laevis* major oocyte 5S rRNA gene (pXP5Soo) or the somatic 5S rRNA gene (pSP64-X1s11; Guinta et al. 1986) were linearized with *Hind*III and *Bam*HI, respectively. Unlabeled antisense RNA was generated using either SP6 RNA polymerase (pXP64-X1s11) or T7 RNA polymerase (pSP5Soo).

RNase protection experiments were performed according to the protocol RPA11 (Ambion, Austin, TX). Briefly, 400 ng of unlabeled antisense RNA were mixed with one-fifth of the radiolabeled RNA synthesized in an in vitro run-on transcription of embryonic chromatin (see above). After an overnight hybridization in 80% formamide, 100 mM sodium citrate (pH 6.4), 300 mM sodium acetate (pH 6.4), and 1 mM EDTA, the samples were digested with a mixture of RNase T1 and RNase A (10 μg/ml) for 30 min at 37°C. The reaction was stopped by precipitation with ethanol. RNAs were resolved on a 6% polyacrylamide gel (see Guinta et al. 1986; Andrews et al. 1991).

Electrophoretic and immunochemical analyses of proteins

Polyacrylamide SDS–electrophoresis was performed as described by Laemmli (1970). Two-dimensional electrophoresis was carried out essentially as described by Russanova et al. (1980). Briefly, the proteins were first separated in a 15% polyacrylamide slab gel containing 7 M urea and 5% acetic acid. The strip with the separated proteins was then excised and placed on top of a second gel, which was made of a 3-cm 5% stacking gel and 15-cm separating gel, containing 0.4% Triton X-100 and 6 M urea. Usually, electrophoresis was run for 14–16 hr at 7 mA. For identification of the tritium-labeled histones, the gels were first stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad) to determine the positions of the unlabeled carrier histones, which served as markers, destained, treated for 15–30 min with Amplify (Amersham), dried, and autoradiographed.

Polyclonal antibodies against *X. laevis* H1, B4, and TFIIIA were prepared after injection of the proteins into rabbits (An-

drews and Brown 1987; Wolffe 1989b; Dimitrov et al. 1993). The antibodies were immunospecifically purified from IgG by affinity chromatography with antigen conjugated to CNBr-Sepharose (Harlow and Lane 1988). All Western blots were made by standard methods (Harlow and Lane 1988), and they were visualized using India ink before incubation with the antibody to ascertain transfer. Purified TFIIIA (Smith et al. 1984) and histone H1 (Wolffe 1989b) were used as standards.

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