Chromatin structure of the developmentally regulated early histone genes of the sea urchin *Strongylocentrotus purpuratus*

Jan Fronk⁺, Graeme A. Tank and John P. Langmore^{*}

Biophysics Research Division and Department of Biological Sciences, University of Michigan, Ann Arbor, MI 48109–2099, USA

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ABSTRACT

Chromatin organization of the early histone gene repeat was studied at the early embryonic stages of the sea urchin *S. purpuratus*. Micrococcal nuclease digestion showed a highly irregular packaging of the whole repeat at the period of transcriptional activity, which was progressively replaced by more regular nucleosomal arrays upon developmentally programmed inactivation. No evidence for unique positioning of the nucleosomes was found. Regions upstream of each of the genes were hypersensitive to DNAase I digestion in the active state. These regions contained one (H2A and H2B), or two (H3 and H4) well-defined DNAase I cutting sites, or two poorly-defined sites (H1). They mapped within DNA sequences shown previously to be required for proper expression of the genes. Hypersensitivity continued in the hatching blastula, which have a conventional nucleosomal structure and a much reduced transcriptional activity. Hypersensitivity of these regions during morula and early blastula was not dependent on the torsional strain in chromatin, as it was not influenced by extensive γ ray-induced nicking of the DNA in nuclei. By late blastula no hypersensitive regions were present.

INTRODUCTION

The knowledge of how chromatin composition, organization, and transcription influence each other is critical for understanding the mechanisms regulating the genetic activity of the eukaryotic cell. It is known that interactions of transcription factors with their recognition sequences may be modulated by the presence of nucleosomes (1, 2) and possibly by the higher-order chromatin structure (3). Abundant data indicate that transcribed chromatin regions are structurally and compositionally different from the silent ones (4–8). They are usually more susceptible to nuclease digestion, form less compact supranucleosomal structure, exhibit less well-defined nucleosomal organization, and contain short

regions hypersensitive to nucleases. The composition of histone sequence and modification variants, and the contents of HMG and other non-histone proteins are also found to differ significantly between active and inactive chromatin fractions. Some of these characteristics extend into large chromatin domains external to the genes themselves. The extent of these features varies strongly among different genes, showing no simple dependence on the intensity of their transcription. Most of these features are not limited to the period of actual transcription. They are found in potentially active chromatin regions and persist after the transcription is terminated. Therefore, the causal relationships between transcription and these modulations of chromatin structure remain uncertain.

The majority of the studies of transcriptionally active chromatin of higher Eukaryotes concerned genes expressed in highly specialized cell types (e.g., globin genes in erythroid cells (9), the ovalbumin gene in oviduct cells (10)), or genes responding to external stimuli (e.g., heat shock protein genes (11)). It is therefore of interest to extend such studies to developmentally regulated genes expressed in non-specialized cells. An ideal target for this investigation is the sea urchin early histone gene repeat (SUEHGR) of *Strongylocentrotus purpuratus*. The repeat is comprised of five genes coding for histones H1, H2A, H2B, H3, and H4 interspersed with nontranscribed spacers. This ~6.5 kbp long unit is tandemly reiterated ~300 times, constituting ~0.2% of the genome. The genes are transcribed only for several cell generations during the early development of the embryo between the early cleavage and mid-blastula stages, with maximum activity at the 64- to 128-cell morula stage (see 12 & 13 for reviews on the SUEHGR). The high content of the early histone genes in the *S. purpuratus* genome renders them particularly suitable for isolation as chromatin with good yield and high purity using nucleoprotein hybridization (14, 15, and Vincenz et al., submitted). Such isolated gene-specific chromatin is amenable to direct biochemical and physical studies.

Using indirect methods we wanted to study chromatin structure of the SUEHGR as a function of transcription, extending previous

* To whom correspondence should be addressed

⁺ Present address: Institute of Biochemistry, Warsaw University, 02089 Warsaw, Poland

studies (16,17,18) to completely characterize all five genes, and to include the early blastula stage, immediately after inactivation of the genes.

The results indicate that during active transcription the whole repeat does not have a periodic nucleosomal structure and harbors five prominent nuclease hypersensitive regions. These sites persist in the hatching blastula, despite the return of nucleosomal periodicity in both the coding and non-coding regions. Extensive γ ray-induced nicking of the DNA does not influence these hypersensitive sites. Partial restoration of nucleosomal packaging occurs immediately after transcription stops. Two cell generations later the DNAase I hypersensitive sites disappear and the nucleosomal packaging is completed.

MATERIALS AND METHODS

Embryo cultures

Sea urchins (*S. purpuratus*) were obtained in their spawning season from Alacritty Marine (Redondo Beach, CA) and processed immediately upon arrival, or kept in an artificial sea water tank for not longer than 3 months. Spawning, fertilization, and rearing of the embryos in stirred suspension cultures at 15°C were as described (15). Hardening of the fertilization membranes was prevented by addition of 1 mM 3-aminotriazole. Only the egg batches showing perfect morphology and greater than 95% fertilization efficiency were used. For all embryo batches good synchrony, perfect viability, and correct tempo of development were found.

Preparation of nuclei

Nuclei were prepared as in ref. 15 with minor modifications. All operations were performed on ice, with protease inhibitors (0.1 mM PMSF and 5 mM iodoacetate) in all the solutions except when noted otherwise. Embryos harvested during morula and hatching blastula were gently squeezed through 52 μ m nylon mesh to remove fertilization membranes. Embryos harvested during late mesenchyme blastula were processed directly. After washing the embryos two times in 0.3 M sucrose in buffer A (60 mM KCl/15 mM NaCl/0.15 mM spermine/0.5 mM spermidine/15 mM Tris-HCl, pH 7.4) (19) they were homogenized in a glass-teflon Potter homogenizer in the same buffer and adjusted to 1.65 M sucrose. Homogenates were layered on 2.3 M sucrose cushions and centrifuged for 40 min at 30,000 rpm in a Beckman SW-40 rotor. Opaque nuclear bands were collected and washed three times by centrifuging and resuspending in progressively lower sucrose concentrations. For immediate processing, nuclei were suspended at 1 OD₂₆₀/ml in buffer A with 15mM β -mercaptoethanol, but without PMSF and iodoacetate. For storage, nuclei were suspended at 20 OD₂₆₀/ml in the above buffer containing 50% (w/v) glycerol. Small aliquots of the glycerol suspensions were quick-frozen in a dry ice-methanol bath and stored at -70°C. No changes in nuclei stored for periods exceeding one year could be observed by phase contrast microscopy, electrophoretic analysis of protein and DNA, and nuclease digestion patterns.

Nuclease digestion of nuclei

Most of the experiments described here were performed with nuclei immediately after their isolation. Identical results, however, were obtained for stored nuclei. Nuclei were suspended at 1 OD₂₆₀/ml in buffer A supplemented with 1 mM CaCl₂ for micrococcal nuclease (Worthington) digestion, or 1 mM MgCl₂

for DNAase I (Sigma, type II) digestion. After a 5 min equilibration at 37°C, digestions were started by adding the appropriate nuclease to final concentrations given in the legends to the figures. Reactions were terminated by adding one-fourth volume of 5% SDS/25 mM EDTA. After Proteinase K (50 μ g/ml) digestion for 3h at 45°C, solid NaCl was added to 2.5 M, and DNA was purified by two chloroform/isoamyl alcohol (24:1) extractions. RNAase digestion of the samples (50 μ g/ml, 1h, 37°C) did not lead to any changes in the results and hence was routinely omitted. DNA was precipitated with 2 volumes of ethanol, washed with 70% ethanol to remove salt, and dissolved at 0.3–1 mg/ml in 10 mM Tris-HCl/0.1 mM EDTA, pH 7.8.

Indirect end-labeling

For indirect end-labeling (20, 21) the purified DNA was cut to completion with restriction nucleases (2–3 units/ μ g, 2h at 37°C in manufacturers' buffer) and analyzed by electrophoresis, Southern transfer, and hybridization with the appropriate probe.

Several precautions are necessary to avoid errors in the indirect end-labeling experiments. The method measures positions of hypersensitive sites from a reference restriction nuclease cut, therefore it is imperative to distinguish DNA fragments beginning at this site from those generated by two cuts at different nuclease hypersensitive regions. It is also necessary to ensure that the physical map of the genomic DNA is unique (no sequence heterogeneity) and identical to that of the cloned sequence used as a reference. It should be noted that the SUEHGR has been shown to exhibit a significant degree of length and sequence heterogeneity (22). Finally, lane-to-lane variation in DNA migration may lead to erroneous size estimations.

We performed numerous control experiments to ensure that our assignments of hypersensitive sites were correct. Size markers were run in the same lane as the DNA being analyzed. Physical maps were constructed for all the genomic DNA preparations. Only those preparations showing a unique map identical to that of the cloned SUEHGR (pCO2A) (22) were used. Hybridization patterns obtained for DNA after appropriate restriction nuclease cutting were compared with those obtained for non-restricted DNA. Only the bands absent in the latter were used to measure the positions of hypersensitive sites. The extent of restriction nuclease cutting of the genomic DNA was checked by probing Southern gel blots with the whole cloned SUEHGR. It was found that some restriction nucleases were inhibited completely or partially by a contaminant (possibly a polysaccharide) present in DNA preparations from late mesenchyme blastula nuclei. These enzymes were not used for the analysis. The results of these control experiments will not be shown.

DNA probes and markers

All DNA fragments used as radioactive probes for hybridization were derived from the complete SUEHGR clone, pCO2A (22). Plasmid DNA was cut to completion with restriction endonucleases and electrophoresed in preparative agarose gels (0.8–2%); appropriate bands were cut out, electroeluted in Elutrap (Schleicher and Schuell), and purified by ion-exchange chromatography (Elutip, Schleicher and Schuell). To avoid contamination when necessary, subclones of the SUEHGR were used as starting material, or a larger fragment containing the sequence of interest was first isolated and subsequently re-cut with the appropriate restriction nuclease, followed by a second round of preparative electrophoresis. Probes were labeled with

^{32}P using a random-primer kit (Boehringer Mannheim) to $\sim 1-5 \times 10^8$ dpm/ μg .

A HindIII digest of λ DNA and a HaeIII digest of ϕX174 DNA were used as size markers for ethidium bromide staining. To precisely measure molecular weights in the autoradiograms, hybridizable size markers were used. For the indirect end-labeling experiments they were obtained by partial digestion of pCO2A with MspI or HhaI and complete digestion with the restriction nuclease used for the particular experiment. For nucleosome size measurements a complete MspI digest of *S. purpuratus* sperm DNA was used.

DNA analysis

DNA (3 to 6 $\mu\text{g}/\text{lane}$) was electrophoresed in 14 cm long agarose gels (1.6% for indirect end-labeling and 2% for nucleosomal pattern analysis) in TBE buffer (89 mM Tris-HCl/3mM EDTA/89mM boric acid, pH 8.3) at 2–4 V/cm. Gels were stained in 3 $\mu\text{g}/\text{ml}$ ethidium bromide, photographed under 302 nm illumination, and electrotransferred in $0.5 \times \text{TBE}$ onto Zeta-Probe (BioRad) membranes. After transfer the DNA was denatured and fixed by a 15 min treatment with 0.4 M NaOH. Filters were prehybridized for at least 6h at 68°C in 0.5% Carnation non-fat dry milk/1% SDS/0.5 mg/ml denatured salmon sperm DNA/1.5 $\times$ SSPE and then hybridized for 12–48h in the same mixture containing ^{32}P -labeled probe. After hybridization filters were washed at room temperature in $2 \times \text{SSC}/0.1\%$ SDS, $0.5 \times \text{SSC}/0.1\%$ SDS, and $0.1 \times \text{SSC}/0.1\%$ SDS for 15 min each, and finally in $0.1 \times \text{SSC}/0.1\%$ SDS at 50°C for 30 min. Wet filters were exposed without screens at room temperature against Kodak XAR film, or with one screen (DuPont Lightning Plus) at –70°C against preflashed film. When necessary, filters were stripped by a 30 min wash in 0.4 M NaOH at 37°C, and the effectiveness of the stripping was verified by autoradiography with a screen for at least 48h. Filters were rehybridized as above. Gel photographs and autoradiograms were densitized using a Photometrics Star 1 CCD camera coupled with a Northgate 386 computer.

γ -ray irradiation

Nuclei at 1 OD₂₆₀/ml in buffer A were placed in small ice-containing plastic beakers at a distance of 0 to 140 cm from a ^{60}Co source and exposed for periods from 1 to 45 min so as to receive a combined dose from 1 krad to 1 Mrad. Immediately after irradiation nuclei were digested with DNAase I as above.

Nucleotide sequences

Sequences of the fragments of the *S. purpuratus* SUEHGR were from GenBank. Histone gene transcription start sites were assigned after ref. 23. Conservative sequence motifs in 5' regions of histone genes were from ref. 24.

RESULTS

To correlate chromatin organization of the SUEHGR with its transcriptional activity we compared three periods during early development:

1. the period of maximal SUEHGR transcriptional activity — the 64–128 cell stage (morula), corresponding to 8–10h of development under our conditions;
2. the period just after the SUEHGR transcription is shut off — the hatching blastula stage (18h); and
3. the period several cell divisions after the transcription of the

SUEHGR ceases — the late mesenchyme blastula/early gastrula stage (36–39h).

For brevity, embryos at these stages of development will be referred to as 10h, 18h, and 36h.

Using extensive digestion with micrococcal nuclease and Southern blotting, we assayed the nucleosomal structure of the SUEHGR chromatin at these stages. The nuclease hypersensitive sites were mapped after mild digestion with DNAase I, using the indirect end-labeling approach.

Nucleosomal organization of the SUEHGR

Nuclei from 10h, 18h, and 36h embryos were digested to various extents with micrococcal nuclease, and the purified DNA was electrophoresed in an agarose gel. The ethidium bromide-stained patterns, corresponding to the bulk of the DNA, were similar for all stages, with susceptibility to digestion decreasing with the age of the embryos (Fig. 1 A). The average nucleosome repeat was 218 bp for the 10h embryos, 220 bp for the 18h embryos, and 227 bp for the 36h embryos. When a Southern blot of this gel was probed with the whole cloned SUEHGR, a dramatically different picture emerged (Fig. 1 B). In the 10h digest the SUEHGR sequences were present in a broad smear, with faint bands of ~ 140 and ~ 280 bp. This suggests that nucleosomes were largely absent from the SUEHGR or substantially altered during morula. In the 18h digests well-pronounced nucleosomal patterns were present, with a rather high background. In the 36h digests the nucleosomal pattern was very sharp, with no background. The repeat lengths were not significantly different from those of the bulk chromatin.

To see if the differences in the nucleosomal organization revealed above are exhibited by transcribed and nontranscribed regions of the SUEHGR alike, we rehybridized the same Southern blot with short probes derived from different sites of the repeat: the 347 bp PvuII-HhaI fragment contained entirely within the H3 coding sequence (Fig. 1 C), the 318 bp XbaI-EcoRI fragment contained entirely within the H2B-H3 spacer (Fig. 1 D), and the 365 bp XhoI-SalI fragment abutting the 5' end of the H4 gene (not shown). In all three cases the hybridization patterns were very similar to those obtained using the whole SUEHGR as a probe. This indicates that at particular developmental stages functionally different regions of the SUEHGR have indistinguishable structures.

DNAase I hypersensitive sites

To identify DNAase I hypersensitive regions in the SUEHGR we first performed low resolution mapping of the whole repeat. Mild digestion of 10h, 18h, and 36h nuclei and deproteinized DNA with DNAase I, followed by digestion of the purified DNA with XhoI and indirect end-labeling using the 365 bp SalI-XhoI probe highlighted four prominent bands specific to 10h and 18h nuclei (Fig. 2). Their sizes indicated the presence of four DNAase I hypersensitive sites located at the 5' ends of the H1, H2A, H3, and H2B genes. The 5' flank of the H4 gene could not be mapped using this probe. Under the conditions employed the endogenous nuclease activity was very low (data not shown), so the cutting was due mostly to the action of the added DNAase I.

To map the positions of these hypersensitive sites with greater accuracy we used the strategies shown in Fig 3. Reference restriction nuclease cutting sites were chosen to lie within 500–1600 bp from the expected position of the closest DNAase I hypersensitive site, i.e. in the range of good resolution of agarose gels. Mapping was done for 10h, 18h, and 36h nuclei

Fig. 1. Nucleosomal organization of the SUEHGR at different developmental stages. Nuclei from morula (10h), hatching blastula (18h), and late mesenchyme blastula (36h) were digested for 5 min with micrococcal nuclease. For each developmental stage three concentrations of nuclease were used: 1 U/ml, 5 U/ml, and 20 U/ml (left to right). DNA was purified, electrophoresed in an agarose gel, and transferred to a nylon membrane for hybridization. **A.** Ethidium bromide-stained pattern of the bulk DNA. **B.** Southern blot of the gel shown in A, probed with the whole cloned SUEHGR. **C.** Same blot rehybridized with the gene-specific 347 bp PvuII-HhaI fragment. **D.** Same blot rehybridized with the spacer-specific 318 bp XbaI-EcoRI fragment. M—size markers

and also for deproteinized DNA. Figs. 4 and 5 show the results of such mapping experiments employing probes ending at PvuII sites (Fig. 4) and at the SalI site (Fig. 5). In all four cases prominent bands that are unique to the 10h and 18h nuclei are seen. Their sizes allow precise localization of the DNAase I hypersensitive sites: a single site 100 bp upstream of the H2B transcription start site (Fig. 4 A), a single site 115 bp upstream of the H2A transcription start (Fig. 4 B), two sites 175 and 95 bp upstream of the H4 transcription start site (Fig. 4 C), and a broad region at the 5' end of the H1 gene, extending from ~70 bp upstream to ~30 bp downstream of the transcription start

site, with positions -70 and -30 showing enhanced sensitivity. Similar indirect end-labeling experiments were conducted using KpnI-cut DNA and the 227 bp KpnI-XbaI probe to map the H3 5' region. Two sites at 130 and 60 bp upstream of the transcription start site were hypersensitive to DNAase I (not shown). All these sites appear to be slightly more sensitive in the 10h nuclei than in the 18h ones (note that twice as much nuclease was used in the latter case), but traces of an endogenous nuclease activity found in 10h nuclei, which was absent from the 18h nuclei, may also be responsible for this difference. In the 36h nuclei no prominent hypersensitive regions can be

Fig. 2. Low resolution mapping of the DNAase I hypersensitive sites in the SUEHGR. Nuclei from morula (10h), hatching blastula (18h), and late mesenchyme blastula embryos (36h), and deproteinized sperm DNA (D) were digested for 30 sec with DNAase I at 10 U/ml, 20 U/ml, 100 U/ml, and 4 U/ml, respectively. The DNA was purified, cut to completion with XhoI and analyzed by indirect end-labeling using the 365 bp XhoI-SalI probe. Migration is from left to right. Open boxes represent protein-coding regions, small flags show transcription start sites and direction, and the filled box depicts the DNA fragment used as the radioactive probe. Note that the SUEHGR map is in a logarithmic scale, with the H4 gene region and the 365 bp probe not drawn to scale.

Fig. 3. Indirect end-labeling strategy used to map the positions of the DNAase I hypersensitive sites. The upper diagram shows the map of the SUEHGR (clone pCO2A). The lower diagram shows the probes used for indirect end-labeling. Symbols are as in Fig. 2.

discerned, regardless of the extent of DNAase I digestion. Their digestion pattern closely resembles that of the naked DNA.

An additional apparent hypersensitive site unique to the 10h nuclei mapping to the middle of the H2A gene can also be observed (Fig. 4 B). Its presence, however, varied between different 10h nuclear preparations (e.g., Fig. 5), and therefore we are not sure of its significance.

Fig. 6 summarizes the results of the hypersensitive site mapping for the whole SUEHGR. The nucleotide sequences encompassing these sites and their significance will be evaluated in the Discussion.

Nucleosome positioning

We attempted to map the positions of nucleosomes along the SUEHGR using micrococcal nuclease digestion followed by indirect end-labeling, using the same strategy as for the DNAase I hypersensitive site mapping. The digestion patterns obtained for nuclei are qualitatively identical to the pattern for naked DNA (see Fig. 7). The cutting sites are concentrated in the spacer regions, probably as a consequence of their higher AT content. Several regions exhibit enhanced micrococcal nuclease sensitivity; these coincide closely with the DNAase I hypersensitive regions, but no site that is readily cut in naked DNA is protected in nuclei. A possible exception is a region in the H4-H2B spacer, where a site sensitive in naked DNA, marked by an asterisk, is protected in nuclei. In fact, this region could accommodate three phased nucleosomes, as the spacing of four micrococcal nuclease cutting sites in naked DNA is close to the nucleosome repeat length. With the above qualification, the results do not indicate the presence of uniquely positioned nucleosomes at any of the

Fig. 4. High resolution mapping of the DNAase I hypersensitive sites in the SUEHGR from the PvuII sites. Nuclei from morula (10h), hatching blastula (18h), and late mesenchyme blastula (36h), and deproteinized DNA from sperm (D) were digested for 30 sec with DNAase I at 10 U/ml, 20 U/ml, 100 U/ml and 4 U/ml, respectively. DNA was deproteinized, cut to completion with PvuII and analyzed by indirect end-labeling. A. The 326 bp PvuII-EcoRI probe was used to map the 5' end of the H2B gene. The star marks the position of the intact ~2.5 kbp PvuII-PvuII fragment showing strong cross-hybridization with the 326 bp probe. B. The 347 bp PvuII-HhaI probe was used to map the 5' region of the H2A gene. C. The 197 bp PvuII-PstI probe was used to map the 5' region of the H4 gene. Symbols are as in Fig. 2.

Fig. 5. High resolution mapping of the DNAase I hypersensitive sites in the SUEHGR from the SalI site. The 365 bp SalI-XhoI probe was used to map the 5' region of the H1 gene. Digestion conditions are as in Fig. 4.

developmental stages tested. However, several different positioning arrangements on different copies of the SUEHGR cannot be excluded by this analysis. Also the presence of very strong cutting sites in the naked DNA renders the results of this

Fig. 6. DNAase I hypersensitive sites in the SUEHGR — a summary. DNAase I hypersensitive sites and the hypersensitive region are marked, respectively, by arrows and a box below the SUEHGR map. See Discussion for details concerning nucleotide sequences at the hypersensitive sites.

Fig. 7. Micrococcal nuclease cutting sites in the SUEHGR. Nuclei from morula (10h), hatching blastula (18h), and late mesenchyme blastula (36h), and deproteinized DNA from sperm (D) were digested for 30 sec with micrococcal nuclease at 6 U/ml, 6 U/ml, 20 U/ml, and 2 U/ml, respectively. DNA was deproteinized, cut to completion with PvuII, and analyzed by indirect end-labeling exactly as in Fig. 4. Arrows are drawn only to align the autoradiograms with the map of the SUEHGR and are not intended to mark any special sites. Stars mark the position discussed in the text.

experiment inconclusive, as it is known that micrococcal nuclease preference for linker versus core DNA is not absolute (25).

Effect of DNA nicking on the hypersensitive sites

Maintenance of the torsional strain in chromatin domains has been shown to be required for maximal transcription (26) and increased overall nuclease sensitivity of active chromatin regions (27), and has been implicated in the generation of S1 hypersensitive sites in plasmids (28). To check whether the DNAase I hypersensitivity of the sites identified in the SUEHGR depends on topological constraints, we mapped the hypersensitive pattern after irradiating the isolated nuclei with γ rays, using doses of up to 1 Mrad. Based on inactivation of phiX-174 with ^{60}Co radiation, this dose

Fig. 8. Influence of γ irradiation on the presence of DNAase I hypersensitive sites. Nuclei from hatching blastula embryos were irradiated with a ^{60}Co source with doses (in rads) indicated in the figure. Immediately after irradiation nuclei were digested for 30 sec with DNAase I at 5 U/ml and 20 U/ml and probed from the XhoI site as in Fig. 2.

should result in an average of 2.8 nicks per SUEHGR (28a). Much smaller amounts of radiation are known to relax the torsional strain present in chromatin domains (29). Fig. 8 shows that even at the highest dose of irradiation the DNAase I hypersensitive pattern remains virtually unchanged. Quantitative densitometry of the Southern blot of alkaline agarose gels of restricted DNA was used to quantitate the integrity of the SUEHGR chromatin before and after irradiation. After a dose of 1 Mrad, the sample shown in Fig. 8 had experienced >69% cleavage of the single stranded SUEHGR monomers. Thus, <31% of the single strand SUEHGR monomers remained intact and <9.7% of the double stranded monomers of SUEHGR were left uncleaved by radiation. Assuming Poisson statistics, the probability of cleavage of each 6.5kb SUEHGR fragment was >2.3. This means that after 1 Mrad of radiation more than 90% of the SUEHGR monomers had one or more nicks and more than 40% of the monomers had three or more nicks. Because the hypersensitivity did not change as the result of this 1 Mrad dose, we conclude that covalent integrity of the SUEHGR is not necessary for the maintenance of nuclease hypersensitivity *in vitro*. Because it would be impossible to map the hypersensitivity at much higher doses, and because the extent of local constraint in supercoiling of SUEHGR is unknown, we cannot exclude the possibility that local torsion is required to maintain hypersensitivity.

DISCUSSION

Disruption of chromatin organization accompanies transcription of the SUEHGR

Transcription of the early histone genes of *S. purpuratus* is accompanied by a transient loss of the regular nucleosomal packaging of the whole repeat, as shown by micrococcal nuclease digestion of nuclei during embryonic development. Similar data were also reported for the SUEHGR of *P. lividus* (17). What

is the mechanism for this disruption? Any explanation offered must account for the fact that the sequences that are not transcribed, i.e. the intergene spacers, also undergo this transition. It is known that significant portions of the SUEHGR intergene spacers are not transcribed, even though the sites of termination are unknown (30,31). Consequently, if the non-transcribed and gene regions were organized in distinguishable structures the hybridization pattern obtained with the total repeat used as a probe should differ from the pattern obtained using a gene-only probe. The virtual identity of these patterns proves that the non-transcribed sequences also lose nucleosomal packaging when the genes are active. On the other hand, as soon as the transcription ceases, the regular packaging of the whole repeat is restored. The high background seen between nucleosomal bands in the 18h pattern may result from incomplete restoration of the regular organization of all the copies or from the superposition of two kinds of patterns, diffuse 10h-like and regular 36h-like, which would indicate heterogeneity among individual copies of the SUEHGR.

The SUEHGR DNA in 10h nuclei is certainly still complexed with histones. However, the presence of bands corresponding to the nucleosome core and a compact dimer suggests that extensive sliding of the cores has taken place; the resulting randomization of nucleosome spacing would result in a smear after micrococcal nuclease digestion. It should be noted that transcriptionally active chromatin regions have been found to be depleted in H1 (32) and that removal of H1 facilitates sliding of nucleosome cores (33). The cause of the proposed core sliding could be the unwinding and overwinding of DNA due to the passage of the RNA polymerase complexes (34). This would explain why transcription also influences the adjacent, nontranscribed regions. A less likely explanation is that the majority of nucleosomes are temporarily dissociated during transcription of the SUEHGR. The overall extent of micrococcal nuclease-mediated fragmentation of the SUEHGR at 10h is comparable to that of the bulk chromatin (Fig. 1 A and B).

Studies of the nucleosomal organization of other polII-transcribed genes have produced conflicting results. In yeast, transcribed sequences have a typical nucleosomal organization, only marginally less regular than that of the bulk of chromatin (35). The hormonally regulated rat phosphoenolpyruvate carboxykinase gene displays nucleosomal packaging regardless of its transcriptional activity (36). Transcribed β - and α -globin genes in induced murine erythroleukemia cells have been reported to exhibit regular nucleosomal organization (9), but showed no evidence of nucleosomal organization in another study (37). Transcribed Ig κ genes exhibit no evidence of nucleosomal packaging (38), although weak core- and dinucleosome-sized bands have been observed (39). Upon transcriptional activation, *D. melanogaster* hsp70 genes (11), the *Chironomus tentans* secretory protein gene (40), and the 5' flanking region of the hen ovalbumin gene (10) lose nucleosomal packaging. The histone gene repeat of *D. melanogaster* has a dual organization: genes show no evidence of nucleosomal organization, while spacer sequences do (41). There is no simple dependence of the extent of the disruption of nucleosomal packaging on the intensity of transcription. An elegant study (42) of four developmentally regulated genes in *Dictyostellium discoideum*, showing similar intensities of expression, revealed the following picture: upon transcriptional activation one of the genes underwent a very pronounced disruption of, but not a complete loss of nucleosomal organization, while two others showed only a very moderate

blurring of the nucleosomal pattern. The fourth gene had an irregular structure both in the inactive as well as in the active states.

Inspection of the above data shows that all of the genes reported to have non-nucleosomal organization when active are vigorously transcribed. The SUEHGR stands out as an exception — it has a non-nucleosomal organization even though its rate of transcription is rather low, about 2–3 transcripts per gene per minute at the peak of activity (43). This apparent discrepancy can be explained if one assumes that it is the density of transcription averaged over the whole chromatin domain, rather than the absolute rate of transcription, that determines the stability of the nucleosomal organization. A typical eukaryotic gene constitutes less than 10% of the length of the chromatin domain, or loop, in which it resides. In contrast, in the SUEHGR the transcribed sequences constitute almost 50% of the whole repeat. Thus, a moderate level of transcription of the SUEHGR will lead to a density of transcription averaged over the chromatin domain comparable to that resulting from vigorous transcription of a single-copy gene. This may explain why low levels of SUEHGR transcription are sufficient to disrupt its nucleosomal organization.

DNAase I hypersensitive sites are transiently present at the 5' ends of the early histone genes

Nuclease hypersensitive sites have been described in numerous instances (4–6). While usually associated with potential or actual transcription of the chromatin region, the mechanism of their generation and maintenance remains largely unknown. Binding of sequence-specific transcription factors has been implicated, as well as torsional strain in the DNA (28). In most cases the sites remain hypersensitive long after transcription ceases.

In the case of the SUEHGR, the DNAase I hypersensitive sites map to the short regions in the 5' flanks of each of the genes. The positions of the nuclease hypersensitive sites described in this work are in good agreement with the low resolution study of the *P. miliaris* SUEHGR (44) and other studies of H3, H2A, and H2B in *S. purpuratus* (16,17,18). The sites of hypersensitivity fall within the DNA regions found to be necessary for proper expression of some of the sea urchin early histone genes.

For the H2A gene, we find a hypersensitive site at position –115 (numbering relative to the transcription start site); this differs slightly from the result of a previous study of the *S. purpuratus* SUEHGR (16), which reported a major site at –70. Interestingly, a region of the *P. miliaris* H2A promoter from –139 to –111 was identified as necessary for proper regulation of transcription *in vitro* (45). We do not know the nucleotide sequence of this region for *S. purpuratus*, but a high level of sequence conservation of the early histone gene flanking regions has been observed between these species (24).

For the H2B gene, the hypersensitive site is at –100; this region is somewhat broader than for the H2A, H3, and H4 genes, and it extends in the 3' direction to ~–40. Wu and Simpson (16) identified 2 preferred cutting sites at –30 and –70. The nucleotide sequence of the 5' flank of the H2B gene (5'... –110 ANCTGCGACG CCTAAGACCA ATGAAAGGAT CGAGACCGAG GCTCATTTGC ATACGGACCG ...) reveals the presence of two motifs (bold letters) close to position –100: a CCAAT box and a highly conserved H2B-specific motif, the tridecamer CTCATTTGCATAC (24), which encompasses a ubiquitous ATTTGCAT octamer (46). Several CCAAT-binding and octamer-binding proteins have been identified as transcription factors (47, 48). It is reasonable to speculate that interaction of

such factors with their recognition sequences results in an alteration of the DNA structure, recognized as a hypersensitive site.

For the H3 gene promoter, our results (two sites at -60 and -130, not shown) agree very closely with the previous study (16) (sites at -50 and -120). A recent detailed study (49) identified several factor-binding sites (bold) in this region: 5'... -140 **TTGCACATAC** **GGCATCGCCA** **AGCCCCCTTC** **CCGTCACGCG** **CTAAACAAAA** **GAGCAACCCG** **GTTGACCAAT** **CAAGAGAGCT** **TTACAAACGG**... The centers of the two two hypersensitive sites found in the present report (underlined) fall precisely at the borders of these factor-binding sequences.

In the H4 5' region, we found two hypersensitive sites at -175 and -95. The DNAase I hypersensitivity of this gene has not been investigated in *S. purpuratus* previously and has only been roughly estimated for *P. miliaris* (44). Regions between -102 and -436 and between -46 and -102 were found to bind transcription factors and to be required for transcription of the early H4 gene of *S. purpuratus in vitro* (50). In the human H4 gene promoter a transcription factor binds sequences between -80 and -110 (51). One of the hypersensitive sites that we identified, at -95 (underlined), maps to the sequence AGTCTCCGCA (bold) that is repeated almost perfectly four bp downstream: 5'... -110 CGAATGGGGA **GTCTCCGCAC** **TCCAGTCCCG** **CATACCGTAA**... While the meaning of this particular sequence arrangement is unknown, direct and inverted repeats of short oligonucleotides are often found in enhancers and promoters of numerous genes (52).

For the H1 gene, a broad region spanning positions -70 to +30 shows DNAase I hypersensitivity, with distinguishable sites at -70 and -30 (underlined in the sequence below). The previous study of the *S. purpuratus* SUEHGR (16) gave no data for the H1 gene, while for *P. miliaris* SUEHGR a picture similar to that reported here was found (44). A conserved sequence (GGGCGG) just upstream of -70 is a potential transcription factor Sp1-binding element and was shown to influence the expression of *S. purpuratus* H1 genes (53). Just downstream of the -30 hypersensitive site lies the TATA box: 5'... -80 **CATGGGGGCG** **GACGACCCGG** **GACTGTCTCC** **TCCCACGTAC** **GCAACAATGC** **CTTATATTGA**...

Two interesting features of the nuclease hypersensitive sites in the SUEHGR were observed. First, their presence is limited to the period of the actual transcription and a short time thereafter. Second, their enhanced nuclease sensitivity does not require the topological continuity of the DNA. γ ray-induced nicking, which should allow relaxation of any elastic strain in chromatin domains, does not influence the relative sensitivity of the sites. Another observation suggesting that hypersensitive sites retain their structure after relaxation of chromatin is the presence of fragments generated by two cuts at these sites by endogenous nuclease (53) or DNAase I (our unpublished results). These facts indicate that it is probably the interaction with specific factors and the local perturbation of the DNA structure that result in the generation of the DNAase I hypersensitivity. The disappearance of all the nuclease hypersensitivity some time after the cessation of the developmentally-programmed transcription may reflect the tight control of this regulation and the lack of transcription of these genes until the next generation of the sea urchin.

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