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Original Article

Developmentally regulated chromatin acetylation and histone H1^0 accumulation

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ABSTRACT There exists a close relationship between core histone acetylation and the induced expression of the histone H1^0 gene. We took advantage of this fact to evaluate the influence of chromatin hyperacetylation on the developmentally regulated expression of this specific gene. In this study, the *in situ* immunodetection approach has been used to analyze both the acetylated histone H4 isoforms and histone H1^0 accumulation during early *Xenopus laevis* development. We have chosen two stages of development, gastrula stage, when H1^0 is not expressed and is not inducible by butyrate treatment, and stage 27 when H1^0 is not expressed but is inducible by butyrate. At stage 27 of development, the early induced accumulation of histone H1^0 under butyrate treatment, occurs mainly in tissues that express the protein normally during later development. These experiments suggest that histone acetylation may be part of a pathway which, in a specific set of cells, keeps H1^0 and probably a series of specific genes, competent for transcription, but cell-specific factors are involved in the induced expression of these genes.

KEY WORDS: *Xenopus laevis, deacetylase, transcription, enhancer, immunofluorescence*

Introduction

Histone H1^0, a member of the differentiation-specific subtype of linker histone family, starts to accumulate in cells upon cell commitment in many independent differentiation programs (for review see Khochbin and Wolffe, 1994; Zlatanova and Doenecke, 1994). It has been shown that the expression of this gene is induced by treatment of cells with sodium butyrate (Kress *et al.*, 1986), a known inhibitor of cellular deacetylases (Candido *et al.*, 1978; Sealy and Chalkley, 1978). Furthermore, by using cells which present different sensitivity to the highly specific deacetylase inhibitor, trichostatin A (Yoshida *et al.*, 1990), we were able to show the existence of a close correlation between the level of core histone acetylation and the induced expression of H1^0 (Girardot *et al.*, 1994). We have also shown that H1^0 gene expression can be induced during early *Xenopus* embryonic development by treating embryos with deacetylase inhibitors, butyrate (Khochbin and Wolffe, 1993) and trichostatin A (Almouzni *et al.*, 1994). Therefore, H1^0 gene may be considered as a model gene, the expression of which can be modulated by core histone acetylation. Thus this gene may be used as a marker enabling us to evaluate the probable role of a developmentally regulated histone acetylation in the regulation of gene expression.

The embryonic development of *Xenopus laevis* offers an opportunity to address this issue. Indeed, we show here that the first accumulation of H1^0 is highly tissue-specific and is observed in the nervous system, somites and cement gland. We took advantage of these observations to monitor the spatio-temporal pattern of the distribution of cell presenting hyperacetylated chromatin, and also, that of cells expressing H1^0. This will allow us to know whether the pattern of cell presenting hyperacetylated chromatin correlates with that of cells expressing H1^0.

In order to visualize acetylated histone H4, we used either an antibody recognizing acetylated isoforms of histone H4 in general (a kind gift of Dr. C.D. Allis), or an antibody recognizing preferentially hyperacetylated H4 isoforms (R 17/5 and 12, a kind gift of Dr. B.M. Turner). H1^0 was revealed with monoclonal antibody recognizing specifically this protein (Dousson *et al.*, 1989). Our data showed that acetylated histone H4 could be detected in every nucleus. The antibody recognizing hyperacetylated H4 did not show a preferential distribution of cells containing hyperacetylated chromatin. Interestingly, at stage 27, when H1^0 is not significantly expressed in normal embryos, treatment with butyrate triggers an important induction of H1^0 accumulation. This induced accumulation of H1^0 is highly tissue-specific and occurs in tissues where H1^0 accumulates in later stage of normal development. This treatment induces a hyperacetylation of chromatin in the majority of cells, but cells containing underacetylat-

Abbreviations used in this paper: CSA, C3H strain-specific antigen; NGS, normal goat sera; MHC, major histocompatibility complex; p.c., post coitum.

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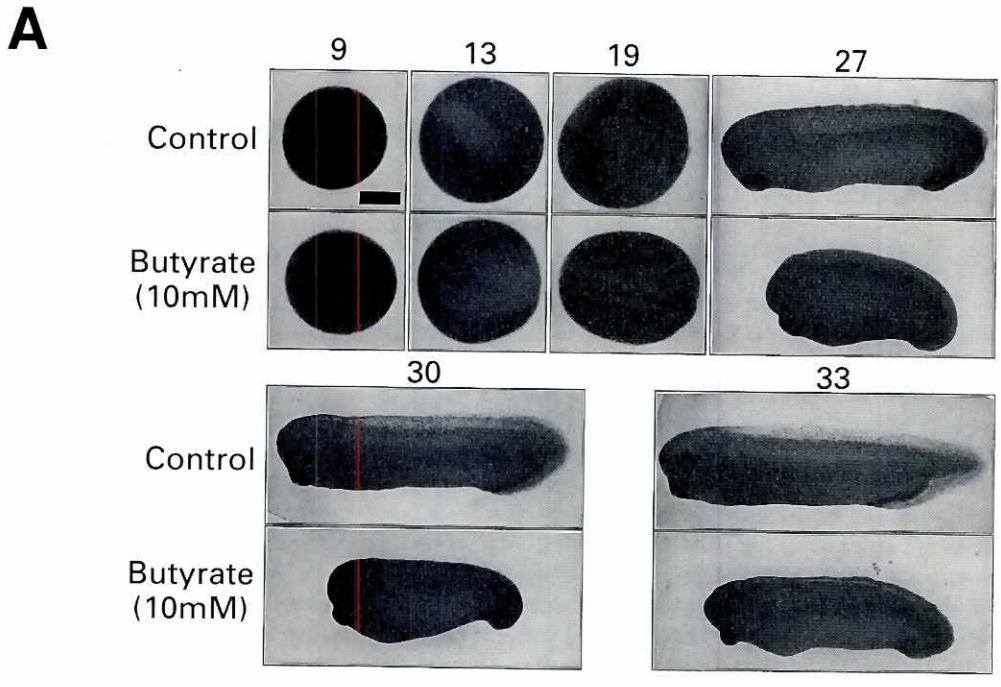

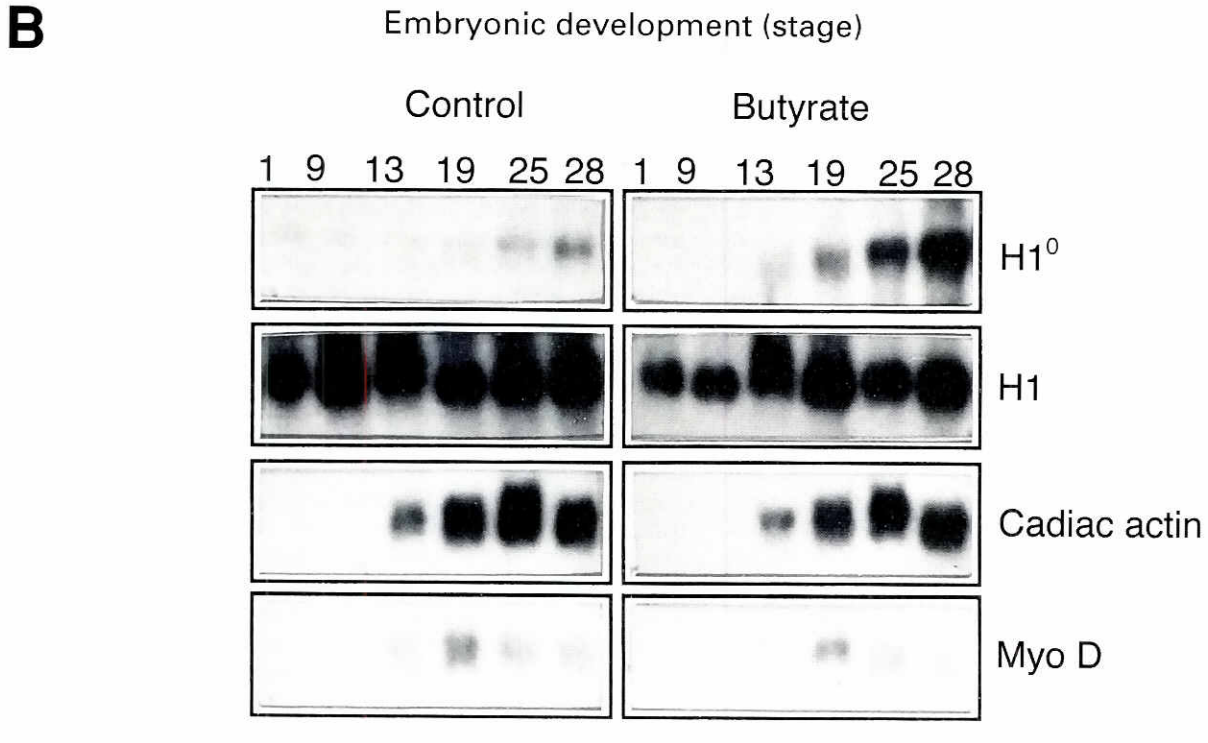

Fig. 1. Influence of sodium butyrate on embryonic development and specific gene expression. (A) *Fertilized eggs were either maintained in a normal medium (control) or in the presence of 10 mM sodium butyrate. Photographs were taken at the indicated developmental stage of the control embryos.* **(B)** *RNA was prepared from either control or butyrate treated embryos shown in* **(A).** *A Northern blot was obtained and hybridized with probes for H1⁰, H1, cardiac actin and Myo D.*

ed chromatin can also be observed. The absence of the induction of $H1^0$ accumulation in cells containing hyperacetylated chromatin but not programmed for $H1^0$ gene expression, i.e., cells in gastrula, shows that histone acetylation alone is not sufficient to induce $H1^0$ gene expression but probably acts synergistically with other specific factors to induce its expression.

Results

Histone $H1^0$ gene expression and cell differentiation under butyrate treatment

Fertilized eggs were split into two populations, one placed in a 10 mM sodium butyrate solution, and the other allowed to

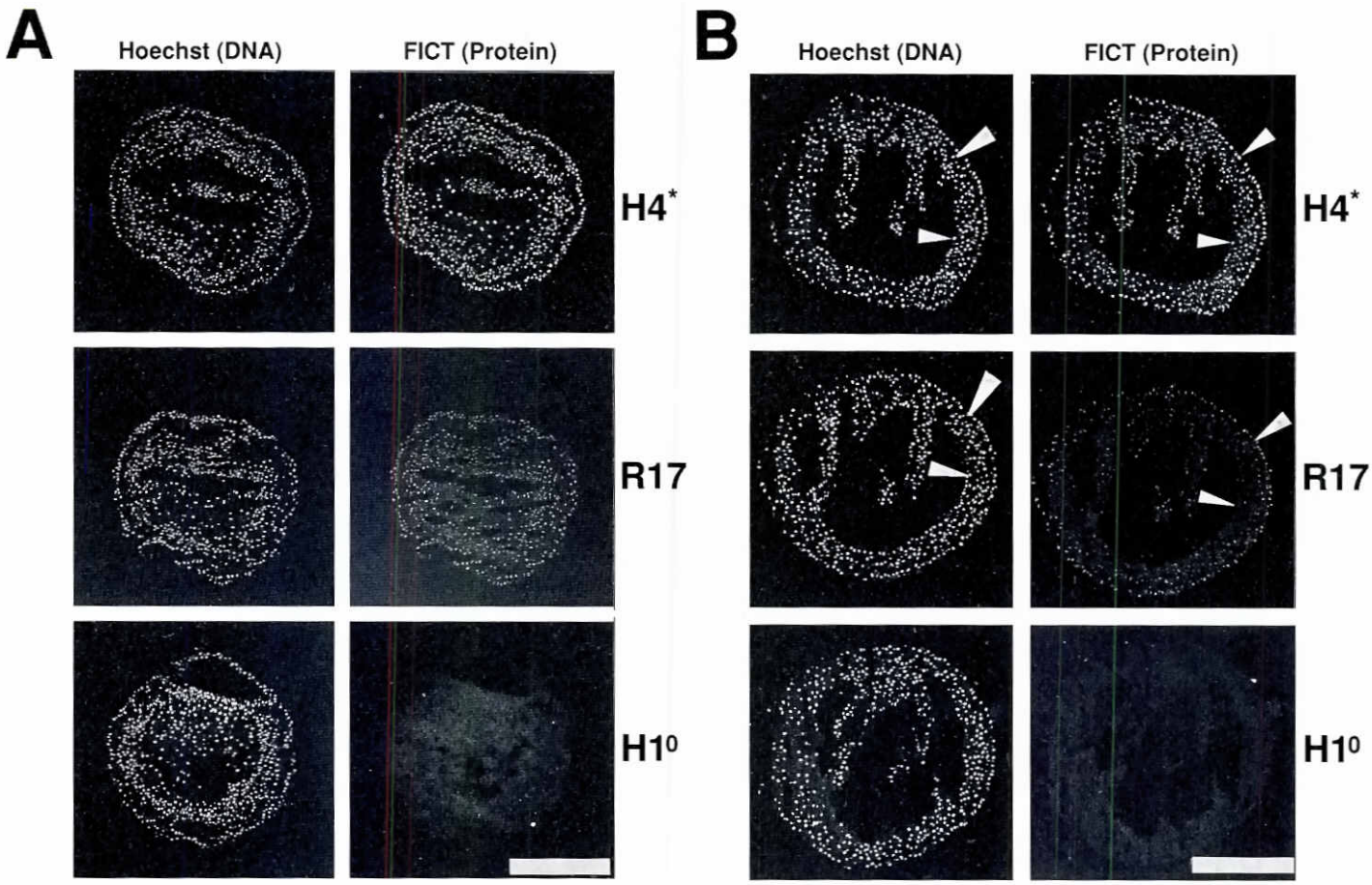

Fig. 2. Pattern of histone H4 acetylation and H1^0 accumulation in normal and butyrate treated gastrula. *Successive cryosections were obtained from control* **(A)** *and treated* **(B)** *embryos, taken at the gastrula stage of control embryos. These sections were used to immunodetect acetylated histone H4 by an antibody recognizing all acetylated isoforms of H4 (H4*) and an antibody recognizing preferentially hyperacetylated isoforms of histone H4 (R17) and finally H1^0 was detected by specific anti H1^0 antibody* **(right panel)**. *After immunodetection sections were counter-stained with Hoechst fluorochrome to visualize nuclei* **(left panel)**. *Arrows are used to underline the variation of immunofluorescence intensity in the indicated regions (compare Hoechst fluorescence. with immunofluorescence).*

develop normally. Figure 1A shows embryos representative of each population. A delay in the embryonic development is observable in treated embryos. The analysis of the exterior aspect of embryos shows that the normal development of the tail and eyes are repressed while an over-development of the cement gland can be observed. The effect of this treatment is better visible on cryosections presented in Fig. 4. Cryosections of treated embryos taken when normal embryos were at stage 27, showed that segmentation is not finished in the posterior part of the embryo, as unsegmented somitogenic mesoderm is still visible (Fig. 4, bracket). The organization of somites is also disturbed. These morphological modifications are accompanied with an early accumulation of H1^0 mRNA in cells (Fig. 1B). In order to monitor the normal commitment of cells in various differentiation pathways, we also analyzed the accumulation of Myo D and cardiac actin encoding mRNAs. The normal accumulation of these messengers in treated embryos showed that the commitment of cells takes place, at least in these differentiation programs. Therefore, it appeared that the treatment of embryos affects specifically H1^0 gene expression compared to other developmentally-regulated maker genes monitored in this study (Myo D and cardiac actin, as well as H1 gene). This observation prompted us to further analyze the relationship between histone acetylation and H1^0 gene expression.

Histone H4 acetylation in gastrula

Fertilized eggs were placed in a 10 mM sodium butyrate solution as above. The embryonic development was allowed to proceed until stage 12 (gastrula). Cryosections were obtained from normal and treated embryos. The presence of acetylated isoforms of histone H4 was monitored by indirect immunodetection using an antibody which recognizes all forms of acetylated H4 (Perry *et al.*, 1993). Acetylated H4 was observed in the nucleus of all cells (Fig. 2A compare DNA fluorescence and H4*-immunofluorescence). Using two dimensional gel electrophoresis, it has previously been shown that the diacetylated H4 isoform is the predominant form of histone H4 at this stage (Dimitrov *et al.*, 1993; Almouzni *et al.*, 1994). We then wished to know whether there was a specific set of cells presenting a nucleus containing hyperacetylated histone H4 in their chro-

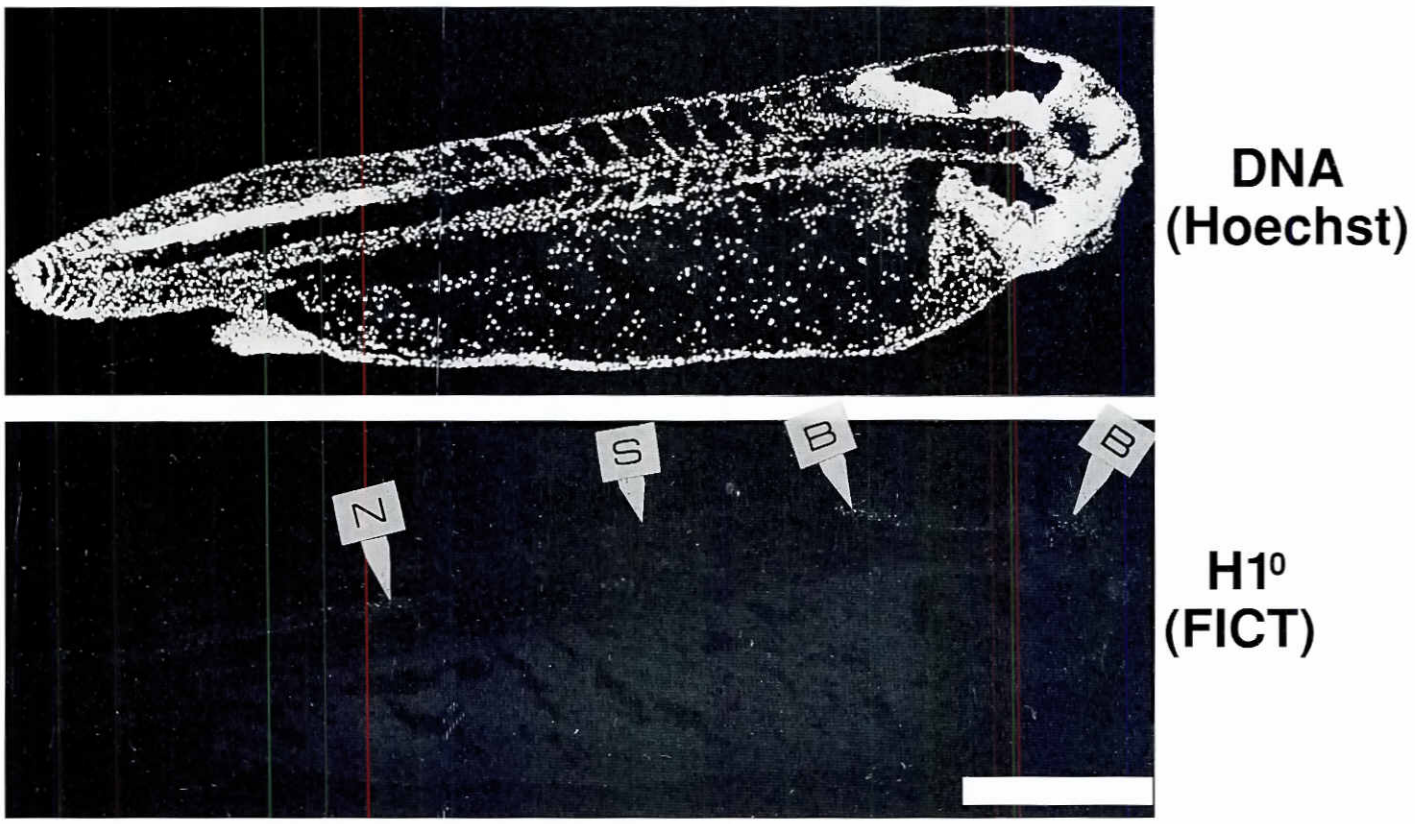

Fig. 3. Early accumulation of H1⁰ during normal development. *Cryosections obtained from embryo at stage 36 of normal development were used for H1⁰ immunodetection. Sections were counter-stained with the DNA-specific fluorochrome Hoechst, which allows for the detection of all nuclei. S, somites; N, neural tube; B, brain.*

matin. To this end we used an antibody specifically recognizing histone H4 acetylated on lysine 5 and 12. This antibody recognizes preferentially hyperacetylated isoforms of histone H4 (Jeppesen and Turner, 1993). The immunostaining procedure did not allow us to show any difference in immunofluorescence intensity of labeled nuclei within an embryo. It has previously been shown that the diacetylated H4 isoform is the predominant form of histone H4 present at this stage (Dimitrov *et al.*, 1993). Therefore, we interpreted the observed uniform labeling of nuclei to be due to the lack of hyperacetylated chromatin in specific sets of cells. Dimitrov *et al.* (1993) observed that hyperacetylated histone H4 appeared first at the gastrula stage of development upon treatment of embryos with butyrate. We used the antibody described above to know whether cells containing hyperacetylated chromatin display a preferential tissue-specificity. Figure 2B shows that the outer layer cells of the embryo is more intensely labeled (Fig. 2B, compare DNA fluorescence and the R17 mediated immunofluorescence). This pattern of labeling is confirmed when we used anti-acetylated H4 antibody recognizing all isoforms, which labeled the outer layer cells of the gastrula more intensely (Fig. 2B, compare DNA fluorescence and H4* immunofluorescence. Note that the exposure conditions were chosen to show the difference of labeling intensity between the outer layer and the rest of cells).

The analysis of an extract prepared from the whole embryo by Western blot showed that H1⁰ cannot be detected at this stage (not shown), and moreover, its expression is not inducible by butyrate treatment (Khochbin and Wolffe, 1993). It seemed interesting to know whether an accumulation of H1⁰ could be detected in nuclei presenting hyperacetylated chromatin. The *in situ* immunodetection of the protein showed that, as expected, the protein is not present in nuclei of a normal gastrula and moreover, it could not be detected either in nuclei presenting hyperacetylated chromatin after butyrate treatment (Fig. 2A,B).

Histone H4 acetylation at stage 27

Fertilized eggs were allowed to develop in the presence of butyrate as above, until control embryos reached stage 27 of development. Cryosections were prepared from these treated embryos as well as from control embryos taken at stages 27 and 36. The analysis of the normal development showed that the first significant immunodetection of H1⁰ was observed at stage 36 in a specific set of tissues, i.e., nervous system, somites and cement gland (Fig. 3). When treated embryos were examined (taken when normal embryos were at stage 27), an important accumulation of H1⁰ was observed (Fig. 4). The induced accumulation of H1⁰ also appeared to be tissue-specific. The protein accumulated in cells forming the nervous system and somites (Fig. 4). It is interesting to note that H1⁰ accumulation is not visible in the unsegmented somitogenic mesoderm compared to somites (Fig. 4). Tissues, in which accumulation of H1⁰ can be induced, are those that first accumulate H1⁰ nor-

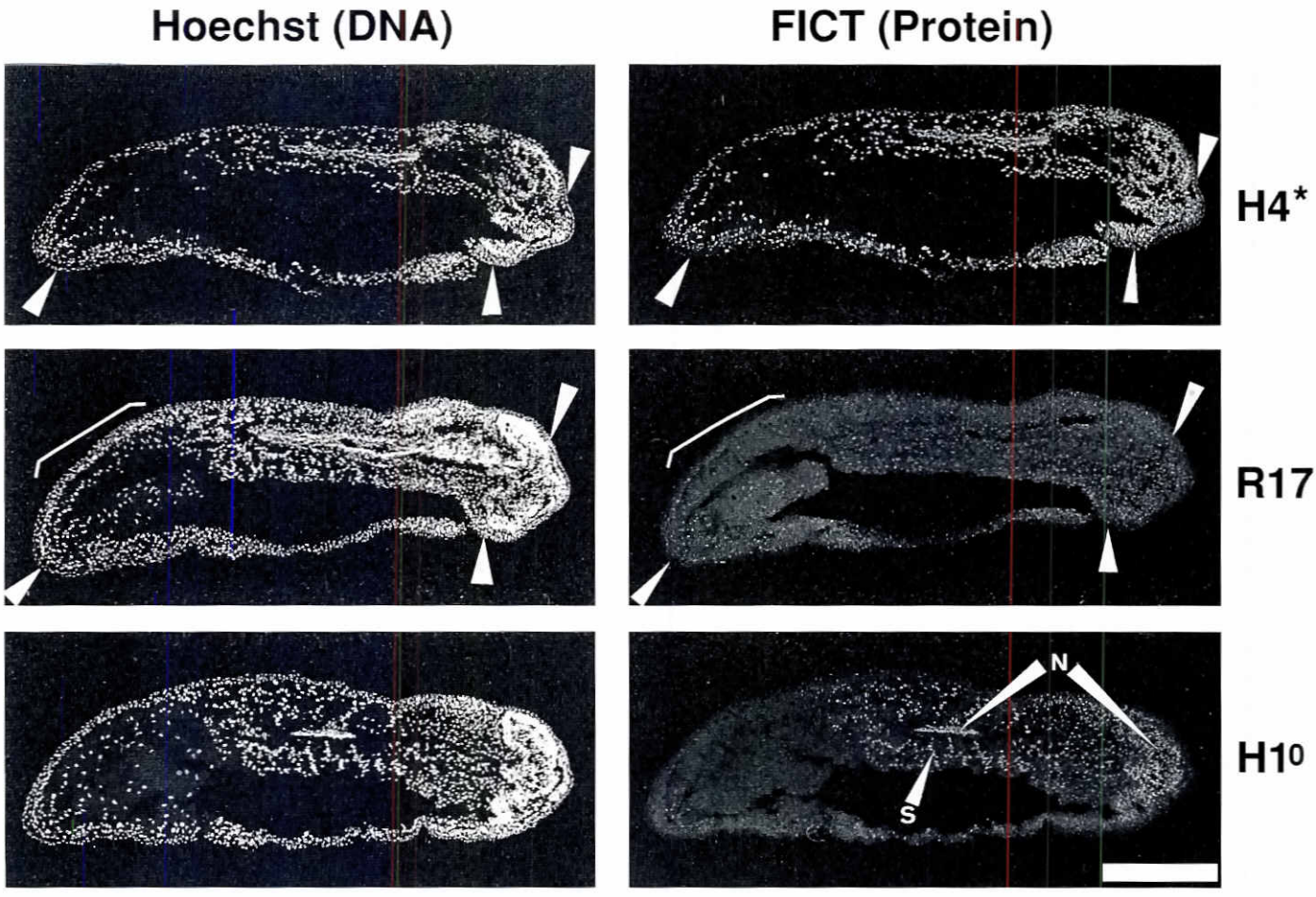

Fig. 4. Pattern of histone H4 acetylation and H1^0 accumulation in butyrate treated embryos taken at stage 27. *Successive cryosections were obtained from treated embryos taken at stage 27 of development and immunodetection of these sections was carried out as in Fig. 2. Here again H4* indicates acetylated H4, R17 shows nuclei containing hyperacetylated H4, and H1^0 indicates the presence of histone H1^0. As in Fig. 2, nuclei in each section are visualized by Hoechst fluorescence and shown in the left side of the immunofluorescence (FITC) panel. Arrows are used to show underacetylated regions. In H1^0 panel, N refers to the nervous system and S to the somites.*

mally at stage 36 (Fig. 3). This result shows that treatment of embryos with butyrate accelerates the developmentally regulated process of H1^0 expression, but does not violate its specificity: tissues expressing H1^0 in normal development are the first to overexpress this protein after butyrate treatment, but they do earlier.

We then wished to monitor the pattern of cells displaying hyperacetylated chromatin under butyrate treatment. Butyrate-treated embryos were taken when control embryos were at stage 27. They were used to immunodetect, both histone H4 acetylation and hyperacetylation. Figure 4 shows that cells containing hyperacetylated H4 (R 17/5 and 12 positive) are widely distributed throughout the whole embryo. However, nuclei in the anterior part of the embryo are preferentially labeled. A region with only a few labeled nuclei can be detected in the posterior part of the embryo, mainly in the unsegmented somitogenic mesoderm. The peripheral cells were underacetylated (weak immunodetection, even with the antibody recognizing all acetylated isoforms of H4), and therefore appear to be insensitive to butyrate treatment (Fig. 4).

Discussion

There are several lines of evidence showing that histone acetylation can be considered as a marker of chromatin domain containing genes that are competent for transcription (Hebbes *et al.*, 1994; for review see, Turner, 1993, 1991 and Csordas, 1990). The reversible acetylation of lysine in the conserved N-terminal tails of core histones appears to be a way to modulate the interaction of these tails with DNA (Hong *et al.*, 1993). These interactions may play an important role in the regulation of the structure and function of chromatin. Indeed, they can control the access of a transcription factor to a target sequence in a nucleosome (Lee *et al.*, 1993), or they may influence the higher order structure of chromatin (Ridsdale *et al.*, 1990). Moreover, acetylation seems to affect the structure of the nucleosome itself (Ebralidse *et al.*, 1993; Bauer *et al.*, 1994). Previously, we gathered data showing that histone H1^0 is a gene whose expression can be influenced by inhibiting deacetylases during *Xenopus laevis* embryonic development (Khochbin and Wolffe, 1993; Almouzni *et al.*, 1994). Moreover, a striking correlation was

found between the level of H1^0 gene activity and the degree of core histone acetylation (Girardot *et al.*, 1994). These observations designate H1^0 as a model gene, and the study of its developmentally regulated expression may allow us to understand the influence of chromatin acetylation on gene expression. It has been previously shown that a developmentally regulated deacetylase activity can be evidenced during early *Xenopus* embryogenesis (Dimitrov *et al.*, 1993), which correlates well with the timing of H1^0 gene inducibility (Khochbin and Wolffe, 1993). In this study, using an *in situ* approach, we tried to show whether a developmentally regulated chromatin hyperacetylation under butyrate treatment correlates with the expression of our model gene, H1^0, which had a tissue-specific pattern of expression during the early development. Our data showed that, early during development, at gastrula stage, when for the first time hyperacetylated H4 has been observed upon butyrate treatment (Dimitrov *et al.*, 1993), hyperacetylated chromatin is present preferentially in the outer layer cells of the embryo. Interestingly, H1^0 accumulation could not be evidenced in these cells. We then examined embryos at stage 27 of development. This stage was chosen since H1^0 is not significantly expressed in cells, but the analysis of the whole embryo showed that the gene is inducible upon butyrate treatment. When butyrate-treated embryos taken at the time corresponding to the stage 27 of normal development were examined, a clear accumulation of H1^0 was observed in specific sets of tissues, which are essentially those that normally accumulate the protein later in development, at stage 36. Interestingly, hyperacetylation did not occur in tissues like the somatogenic mesoderm and the epidermis, which are precisely those where H1^0 accumulation can not be observed.

Our knowledge of the regulation of H1^0 gene transcription may provide an insight into the mechanism involved in the induction of gene expression. Previously we showed that the basal transcription of H1^0 gene is controlled essentially by three cis-acting elements, the proximal H4 and H1 boxes and a distal UCE element (Khochbin and Wolffe, 1993). Microinjection experiments using mutated H1^0 gene promoters demonstrate that there is a developmentally regulated order in the use of the regulatory elements discussed above. In oocytes we observed that transcription depended on the TATA box, which was independent from the integrity of the H4 box, H1 box and the UCE. During early development up to neurulation, basal transcription is dependent on the H4 box and H1 box but not on the UCE (Almouzni *et al.*, 1994). Finally in an established somatic adult cell line (A6 cells), the enhanced expression of the H1^0 promoter following transfection was dependent on the integrity of these elements (Khochbin and Wolffe, 1993). The UCE element therefore seems to act relatively late during development. We were able to show that the binding of factors to this element can influence the binding of factors to the proximal promoter region allowing the transcription of the gene under a repressive environment (Khochbin and Lawrence, 1994). Therefore, the early induction of H1^0 upon butyrate treatment, when probably the UCE element is not yet functional, may be due to hyperacetylation that mimics the action of UCE-binding factors and facilitates the binding of factors to the proximal promoter region. Moreover, upstream of the UCE, several binding sites for transcription factors have been described, i.e., thyroid hormone-responsive element and retinoic acid receptor- binding element (Bouterfa *et al.*, 1993). It has recently been shown that the latter binds retinoic acid receptor (Mader *et al.*, 1994). The acetylation-mediated modulation of the chromatin structure may also allow for the action of these factors earlier in development. Microinjection of the H1^0 promoter-containing transgene in *Xenopus* (Steinbeisser *et al.*, 1989) may provide a good assay to investigate this possibility.

Acetylation (at least that of histone H4), may be a necessary step for making a developmentally regulated gene competent for expression, but it is not sufficient to induce the expression of the gene. Cell-specific factors are certainly involved in the process.

Materials and Methods

In situ immunodetection of proteins

Embryos at different stages after fertilization were fixed on ice in 4% PFA, 0.6xPBS for 1 h, and impregnated with 10%, and then 20% sucrose in PBS (1 h each, on ice). After embedding in Tissue Tek II (Miles Scientific), embryos were frozen and cut with a cryostat in 10 µm thick sections. Sections were mounted onto gelatin-coated slides, treated with 4% PFA for 10 min at room temperature, and washed with 3 changes of PBS at 4°C (10 min each). Before immunolabeling, slides were treated with 0.25% Triton X100 in PBS for 5 to 10 min. Then, they were incubated for 1 h at 37°C with different antibodies (antibody raised against acetylated isoforms of H4 was a gift of Dr. Allis, and R17/5 and 12, an antibody recognizing preferentially hyperacetylated isoforms of histone H4, was a gift of Dr. Turner). Antibodies were diluted in PBS containing 0.1% Triton, 0,5% BSA, 5% lamb serum. For H1^0 immunodetection, pure hybridoma supernatant fraction containing anti-H1^0 antibody was used. After incubation with the anti-histone antibodies, slides were washed with PBS, BSA 0.5%, glucose 0.1% (PBS-G-BSA) at 4°C (3x10 min). Then they were incubated with a fluorescein isothiocyanate (FITC)-conjugated antibody. For H4 isoforms, we used an anti-rabbit antibody (Jackson, 1/200 dilution), and for H1^0, an anti-mouse antibody (Cappel, 1/800 dilution). Incubation was carried out in PBS, 0.1% Triton, BSA 0.5%, 5% lamb serum, for 30 min at 37°C. After 3 washes in PBS-G-BSA, slides were counterstained with the DNA-specific fluorochrome Hoechst 33258 (2 mg/ml).

RNA preparation and analysis

RNA was prepared from staged embryos and analyzed exactly as described previously (Khochbin and Wolffe, 1993).

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