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Original Article

Expression of mouse histone $H1^0$ promoter sequences following microinjection into *Xenopus* oocytes and developing embryos

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ABSTRACT Evidence from expression studies using transfected F9 teratocarcinoma stem cells indicates that the synthesis of the $H1^0$ histone is turned on very soon after the cells have been treated with retinoic acid, which causes them to differentiate into murine parietal endoderm. This increase in $H1^0$ at the time of commitment would allow a reorganization of the chromatin with a reprogramming of the gene activity of undifferentiated F9 cells to the differentiated state. The particular interest of the further development of this differentiation model is to answer the question whether this specific stimulation of expression can be induced only in homologous differentiation systems, or whether the identified $H1^0$ promoter sequences can also be specifically stimulated by heterologous factors. We therefore injected the mammalian $H1^0$ promoter sequences into *Xenopus* oocytes and fertilized eggs. The results of oocyte injection experiments indicate that $H1^0$ promoter sequence elements similar to those used in transfected F9 cells are specifically expressed in the oocyte. For analysis of $H1^0$ expression in *Xenopus* embryos we used promoter constructs ligated to ß-galactosidase sequences for microinjection. This procedure allows a particularly rapid and complete detection of expressed promoter clones within the differentiated tissues of the early *Xenopus* embryo.

KEY WORDS: *histone $H1^0$, Xenopus embryogenesis, injection, ß-galactosidase assay*

Introduction

Gene expression at the transcriptional level is controlled by cis- and trans-acting factors. Sequences located in cis-position relative to the transcribed gene are probably responsible for the accurate transcription of the gene and for the amount of RNA synthesized (Hatzopoulos *et al.*, 1988; La Thange and Rigby, 1988). A large proportion of transcribed genes are common in different cell types, e.g. the genes which control the synthesis of house-keeping proteins and/or structural proteins. Most of these genes are extremely well conserved during evolution, indicating the essential role of their function; several of these genes can be interchanged between species without disturbing the general function of the cell.

Analyses of gene function are extremely difficult. Only the actual reverse genetic or gene targeting by homologous recombination allows an analysis of the changes produced after a specific gene had been switched off (Frohman and Martin, 1989). These restricted possibilities are to some degree extended by two different methodological approaches which allow the analysis of genes when expressed in a foreign environment. One of these assays is the transfection of cloned sequences into different cell types (Pellicer *et al.*, 1980; Spandidos and Wilkie, 1984), the other is the injection of these sequences into oocytes or fertilized eggs of *Xenopus laevis* (Etkin *et al.*, 1986; Gurdon and Wakefield, 1986; Trendelenburg *et al.*, 1986). The employment of transfection allows a rapid analysis of the possible function of transfected sequences but does not

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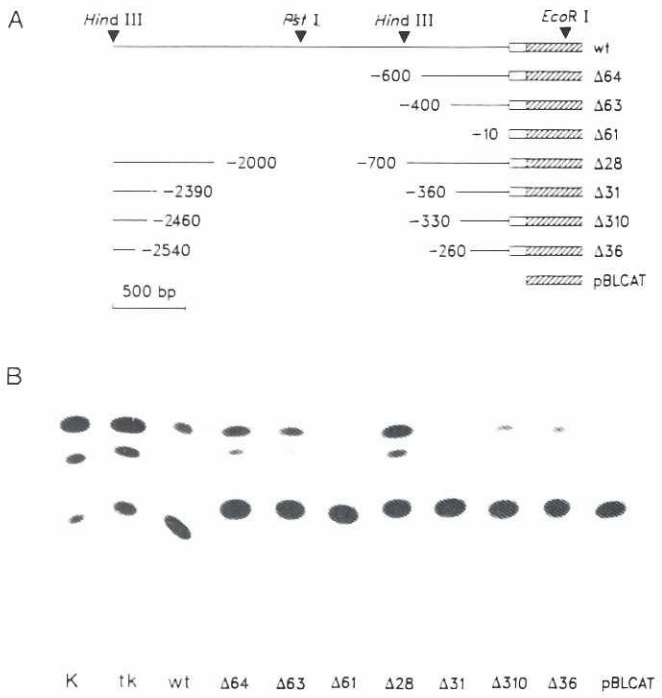

Fig. 1. Transcription properties of H1⁰ deletion mutants injected into *X. laevis* oocytes. (A) *A 3kb Nru I-Eco RI-fragment of the mouse histone H1⁰ gene containing the 5' flanking region (solid line), the transcription start site and 5' non-translated sequences (open bar) was cloned into the plasmid pBL3 carrying the CAT reporter gene (shaded bar) and designated as wild type (wt) construct. Construction of the Bal 31 deletion clones was done as described by Breuer* et al. *(1989).* **(B)** *CAT activity in oocytes injected with the H1⁰ CAT constructs: oocyte samples were injected with mouse H1⁰ CAT constructs as shown in Fig. 1A. 24 hours after injection, protein extracts of injected oocytes (1 oocyte equivalent, see Materials and Methods) were used to measure CAT activity. As a control, a CAT construct containing the HSV-thymidine kinase promoter in upstream position to the CAT gene was used (tk). (K) and (pBL CAT) indicate the result of CAT assays performed without extract (K) or using the pBL CAT probe without adding CAT enzyme (pBL CAT).*

allow quantitative determination of expression for the transfected sequences when the whole organism is considered. On the other hand, questions of this kind can be approached by using the *Xenopus* system, with which fully differentiated larvae may be easily obtained from injected eggs. Expression studies can also be conducted at different times during the early phase of embryonic development (Steinbeisser *et al.*, 1988).

The histones are very well conserved proteins, responsible for the organization of the basic chromatin structure (Weintraub, 1984). Histone H1 is known to be responsible for the formation of supranucleosomal structures, probably by intercalating between two nucleosomes containing the core histones. One of these histones, histone H1⁰ – a variant – is known to be abundant in terminally differentiated cells and in cells in which induced commitment has taken place (Gjerset *et al.*, 1982; Alonso *et al.*, 1988). We have isolated the promoter region of the mouse histone H1⁰ gene and demonstrated that this region is able to control specifically the synthesis of a reporter gene ligated to at least 600bp of the 5' upstream region (Breuer *et al.*, 1989). We were then interested to know whether this region is also recognized in a heterologous system and what the expression pattern of this kind of constructs is when injected into fertilized eggs. As control experiments we performed the same kind of experiments using two other promoter constructs which are known to be transcribed in all cell types. We were able to demonstrate that in fertilized *Xenopus* eggs and developing embryos, the expression of the reporter gene depends on the type of promoter region used and on the injection site.

Results

Transcription properties of H1⁰ deletion mutants injected into Xenopus laevis *oocytes*

To analyse the basic transcription characteristics of mouse histone H1⁰ promoter clones, batches of *Xenopus* oocytes were microinjected using a 3kb Nru I-Eco RI fragment of the mouse histone H1⁰ gene (Breuer *et al.*, 1989) which had been fused to the CAT gene (wt in Fig. 1A). In addition, a series of 7 different H1⁰ promoter deletion clones as well as the CAT sequence without H1⁰ promoter elements were injected into *Xenopus* oocyte nuclei (Fig.1A).

Microinjected oocytes were incubated for 24 hours and CAT activity was determined by thin layer chromatography. To allow direct comparison of transcription efficiencies, protein extracts equivalent to the protein content of one oocyte (250 µg; Gurdon and Wakefield, 1986) were used. As shown in Fig. 1B, H1⁰ constructs containing the following promoter sequences -700 (Δ28), -600 (Δ64), -400 (Δ63), as well as the wild type construct (wt) containing the complete sequence, were found to be very efficiently transcribed. To quantitate this expression efficiency, we had included a construct containing the thymidine kinase promoter (tk) of herpes simplex virus (HSV), a probe with a very high known transcription rate in injected *Xenopus* oocytes

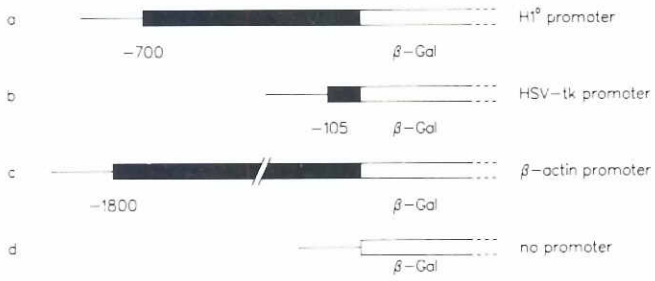

Fig. 2. Promoter ß-galactosidase constructs used for injection into fertilized *Xenopus* eggs. *Three different promoters were placed in front of the bacterial ß-galactosidase gene which had been ligated to the -700bp 5' flanking region of the mouse histone H1⁰ gene* **(a)**, *-105bp of the HSV thymidine kinase gene* **(b)** *-1800bp of the rat ß-actin gene* **(c)** *or no promoter* **(d).** *Thin lines: vector DNA; solid black bars: promoter fragments; white bars: lac Z gene.*

(McKnight *et al.*, 1981). As shown in Fig. 1B, the above mouse histone H1⁰ promoter deletions were all transcribed at a similarly high rate.

By contrast, injected pBL3-CAT constructs with deletions downstream of -400bp (Δ31, Δ310, Δ36) were found to be expressed at a much more reduced rate. The two extreme deletions tested (Δ61, pBL CAT) which contained either H1⁰ sequences downstream +10 bp or none, were found to be transcriptionally inactive in oocytes.

In summary, these results indicate that a minimum requirement for efficient transcription of the CAT reporter gene is the presence of upstream H1⁰ promoter sequences containing -360 to -600bp.

Expression of H1⁰ promoter constructs in Xenopus *embryos*

In order to analyze the expression of H1⁰ promoter constructs in injected *Xenopus* embryos, an experimental strategy different from the oocyte injection experiments was employed. Since we did not want to investigate H1° promoter expression during early *Xenopus* embryogenesis as an overall process, we used constructs fused to the bacterial ß-galactosidase gene (lacZ; Fig. 2) for identification of expressed promoters within the differentiated tissues of the early embryo (Sanes *et al.*, 1986). As internal controls for the histone, the H1⁰ constructs, a lacZ-coupled Herpes simplex virus (HSV) tk gene promoter sequence (Luckow and Schütz, 1987) and a rat cytoplasmic ß-actin promoter (Melloul *et al.*, 1984) were used (Fig. 2). The rat ß-actin promoter fragment consists of 1800 bp DNA and had been shown to be constitutively expressed in rat myogenic cells (Melloul *et al.*, 1984). Previous experience from our laboratory (unpublished observations) for the HSV-tk-promoter showed that a promoter sequence region downstream -105 bp is sufficient to obtain correctly initiated transcripts in injected *Xenopus* embryos at the

Using these two types of internal control genes, we wondered whether the regulatory elements located on the mouse histone H1⁰ promoter are recognized at all during *Xenopus* embryogenesis, and whether an expression would be restricted to defined cell types of the differentiated tissues in the early *Xenopus* embryo.

Injection of 200 pg plasmid DNA into the animal half of fertilized *Xenopus* eggs was started after *in vitro* fertilization and completed shortly before the onset of the first cleavage. Injected embryos were then kept at 23°C up to N.F. stage 41 (swimming tadpole; Nieuwkoop and Faber, 1967). The persistence and configuration of the injected DNA samples was analyzed at six different characteristic stages of early embryogenesis (9 h, early gastrula N.F. stage 10; 13 h, late gastrula, N.F. 12; 24 h, late neurula, N.F. 22; 36 h, early tailbud, N.F. 30; 48 h, heart-beat stage embryo, N.F. 34; cf. Fig. 3). As shown by Southern blot hybridizations, injected plasmid DNA could mainly be detected in the form of episomal circular DNA up to stage 34 (heart-beat embryo, 48 h). In some cases, significant amounts of high molecular weight DNA were identified in early embryos (13 h, late gastrula, mouse histone H1⁰ probe; 19 h, neurula, rat cytoplasmic ß-actin probe, Fig. 3). These bands are likely to represent concatemeric forms of linearized plasmids, which replicate during early cleavage but become degraded after completion of gastrulation (Etkin *et al.*, 1986; Trendelenburg *et al.*, 1986).

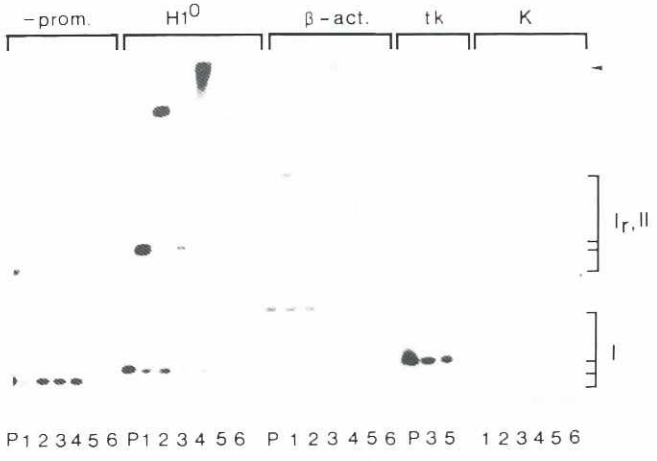

Fig. 3. Southern blot analyses of *Xenopus* embryo injected promoter constructs. *ß-galactosidase constructs without promoter (-prom) or containing the mouse histone H1⁰ promoter (H1⁰), the rat ß-actin promoter (ß-act.) or the HSV thymidine kinase promoter (tk) were injected into fertilized* Xenopus *eggs as circular DNA probes. The DNA was extracted from 9 h(1), 13 h (2), 24 h (3), 36 h (4), 48 h (5), 72 h (6) old embryos, analyzed on horizontal agarose gels, blotted on nitrocellulose filters and hybridized against ^{32}P "oligo primed" labeled pUC8. K: DNA from noninjected embryos. P: Plasmid controls: 1ng plasmid DNA of the different constructs. I: supercoiled plasmid DNA. Ir: relaxed circular DNA. II: nicked circular DNA, arrowhead indicates concatemers of H1⁰-ß-galactosidase*

TABLE 1

DISTRIBUTION OF ß-GALACTOSIDASE POSITIVE CELLS AND ORGANS IN 48 h *XENOPUS* EMBRYOS

promoter	H_1^0							tk			ß-actin		
Embryo no.	1	2	3	32	33	34	41	38	39	42	10	11	12
epidermis	●	●	●		●							●	
nerve													
neural tube		●	●		●	●	●	●			●	●	●
brain													
muscle	●	●	●	●	●	●	●	●	●		●	●	●
endoderm				●	●								

Embryos were injected with mouse histone $H1^0$, rat ß-actin and HSV thymidin kinase promoters ligated to ß-galactosidase constructs as shown in Fig 2.

We also tested supernatants containing the soluble proteins of homogenized, injected embryos for ß-galactosidase activity. No activity could be detected in uninjected embryos and embryos injected with the promoterless ß-galactosidase construct. In contrast, activity was detectable after midblastula transition (MTB) in embryos injected with ß-actin, $H1^0$- and tk-promoter probes (data not shown).

In addition to the above results from homogenized embryos, the lacZ gene assay allows a clear detection of expressed promoter sequences at the level of embryonic tissues. To get an overall idea of the promoter expression, embryos at the neurula stage (data not shown) and heart-beat stage embryos (48 h) were fixed and processed for histochemistry. Stained embryos were first analyzed as whole-mounts and then carefully dissected under the stereomicroscope in order to evaluate the internal staining of tissues. In addition, serial sections (7µm) of a few embryos embedded in paraffin were analyzed.

For the present study, 30 injected embryos were evaluated histochemically. The results can be grouped into the following categories: (i) non-injected control embryos were fixed and processed exactly as injected embryos and did not show any lacZ positive cells. (ii) As a further control, samples of fertilized eggs were injected with a lacZ gene construct lacking a 5' promoter sequence (Fig. 2). Again, no lacZ-positive areas could be detected in those embryos. (iii) After injection with one of the promoter-containing constructs, lacZ positive cells were seen in 90% of the embryos.

A representative diagram of the distribution of lacZ-positive areas in embryos injected with histone $H1^0$, HSV-tk and ß-actin promoter constructs is given in Table 1. For each of the embryos analyzed, the major lacZ-positive areas are listed. These results were obtained from lacZ histochemistry in 48 h heart-beat stage embryos (N.F. stage 34, as shown in Fig. 4). The distribution of positive areas in these embryos as well as in late neurulae (data not shown) indicates that expression of injected promoter constructs can be seen in derivatives of all three embryonic germ layers, in the ectoderm, mesoderm, and endoderm, and irrespective of the type of promoter construct used for injection. However, the distribution of positive areas within the tissues and organs derived from the three germ-layers showed that 55% of positive areas were located in ectodermal derivatives, 36.2% in those of mesodermal origin and 8.7% in derivatives of the endoderm. As shown in Table 1, the predominant type of lacZ-distribution was found in embryos exhibiting positive ectodermal *and* mesodermal areas. Significantly less frequent were embryos with positive areas in all three germ layers (21% of all embryos analyzed).

Typical examples of heart-beat stage embryos (48 h; N.F. stage 34) positive for ß-galactosidase are shown in Fig. 4. Non-injected controls (4A) and embryos injected with the promoterless construct (4B) did not contain ß-galctosidase-positive cells. In contrast, embryos which had been injected with the HSV-tk promoter construct (4c) showed positive cells at the neural tube and adjacent myotomes (embryo No. 38, Table 1). The example shown for a ß-actin-injected embryo (Fig. 4D) exhibits positive cells throughout the entire epidermis including the ear placodes, brain, eyes and muscle (embryo No. 11, Table 1). With regard to histone $H1^0$-injected embryos, four typical examples are shown (Fig. 4E-H). A scattered distribution of small areas of positively stained cells is seen in embryos in Fig. 4E and G (4E: epidermis, neural tube, muscle, endoderm, embryo No. 33, Table 1; Fig. 4G: nerves, ear placodes, gill arches, muscle, embryo No. 41, Table 1). In Fig. 4F and H, embryos with much larger areas of positive cells are shown. In the embryo shown in Fig. 4F, only the left body site contains positive cells (epidermis, nerves, neural tube, muscle, mesenchyme, blood vessels, embryo No. 2, Table 1). In the embryo shown in Fig. 4H, ß-galactosidase-positive cells were found to be distributed more evenly over the entire epidermis, essentially similar to the ß-actin-injected embryo shown in Fig. 4D (Fig. 4H: epidermis, muscle, mesenchyme, blood vessels, embryo No. 1, Table 1).

As is also evident from the morphology of the embryos shown in Fig. 4, the distribution of lacZ-positive cells and/or the presence of clear embryonic deficiences in some cases enabled us to speculate on the putative site of DNA injection into the uncleaved egg. Such examples are: Fig. 4C, the embryo shown lacks the eye cups (arrowhead); Fig. 4E, severe malformation in the mouth region (arrowhead); Fig. 4F, only the left half of the embryo contains positive cells; Fig. 3g, craniolateral distribution of positive cells; and Fig. 4H, exclusive cranioventral area of positive cells.

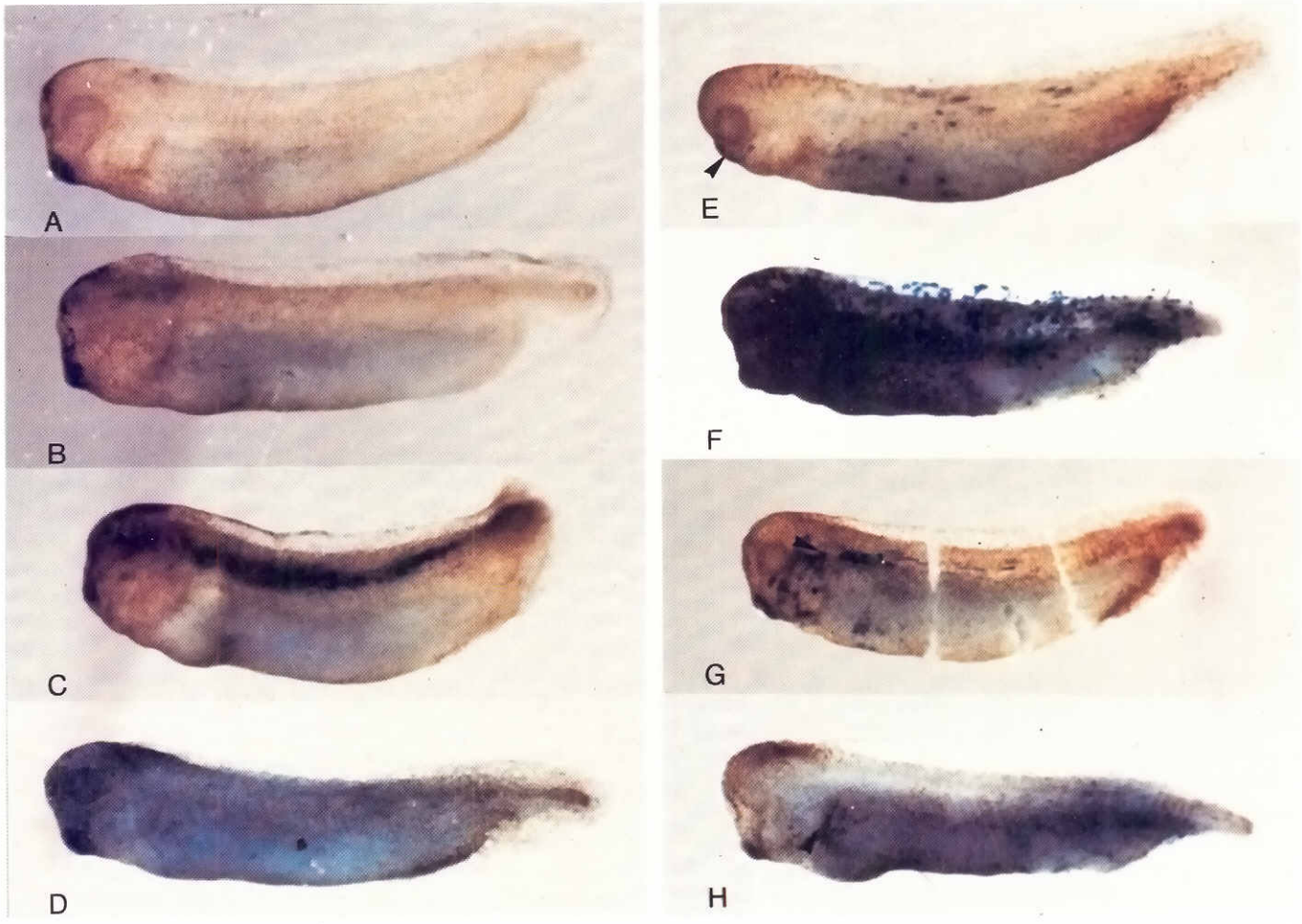

Fig. 4. Histochemical ß-galactosidase assay on *Xenopus* embryo whole-mounts. *48 h old* Xenopus *embryos, which were non-injected* **(A)**, *injected with tk* **(C)**, *ß-actin* **(D)**, *H1⁰ promoter* **(E-H)** *or the promoterless ß-gal. constructs* **(B)** *were fixed and assayed for ß-galactosidase activity (see Materials and Methods).*

Discussion

Histone H1 genes as differentiation markers

Differentiation is accompanied by programmed changes in gene expression, with the synthesis of some new gene products and the shut-off of others. Such a reprogrammed gene expression is probably correlated with changes in chromatin structure, which would allow the demasking of some genes with a subsequent transcription. It has been shown that the amount of histone H1⁰ is greatly increased in differentiating tissues, thus correlating with the postulated reorganization of chromatin structure.

The mammalian histone H1⁰, which is closely related to the H5 of birds, is expressed in terminally differentiated mammalian cells (Doenecke and Tönjes, 1986) and in murine teratocarcinoma cells committed to parietal endoderm (Alonso *et al.*, 1988). H1⁰ is not regulated in a cell cycle-dependent way and not coupled to DNA synthesis. Replacing main type H1 with H1⁰ alters the higher order chromatin structure leading to reprogramming of the cells and to changes in their transcriptional activity (Doenecke *et al.*, 1988). Expression of the mammalian histone H1⁰ can be induced when murine teratocarcinoma stem cells are differentiated to parietal endoderm. It was found that the increased synthesis takes place very early during differentiation, thus suggesting that the increase in steady-state histone H1⁰ mRNA is directly related to the commitment process (Alonso *et al.*, 1988). Regulatory elements of the mouse H1⁰ promoter were identified by transfection experiments (Breuer *et al.*, 1989).

In the South African clawed frog, *X. laevis* 5 histone H1 variants (H1, A, B, C, D, E) were identified. Two of them (H1, D, E), were exclusively expressed in adult tissues, indicating a possible regulatory function of these histones in gene transcription (Risley and Eck-

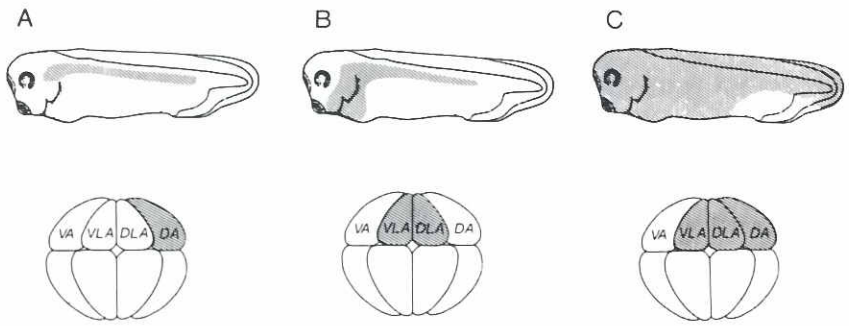

Fig. 5. Correlation between ß-galactosidase expression and the putative injection site. *Upper line shows schematic drawings of 48 h old* Xenopus *tailbud embryos with different patterns of ß-galactosidase positive cells (hatched areas). A, B, C correspond to embryos shown in Fig. 3.* **(A)***: Fig. 3c;* **(B)***: Fig. 3g;* **(C)***: Fig. 3f. Lower line shows corresponding hypothetic 16-cell stage Xenopus em bryos. The animal blastomeres DA (dorsal, animal), DLA (dorsal, lateral, animal), VLA (ventral, lateral, animal) and VA (ventral, animal) were designated according to Wetts and Fraser (1989). Hatched areas indicate those blastomeres which were thought to contain most of the injected DNA sample of an individual injection experiment for generating the expression pattern shown above.*

hardt, 1981). The oocyte injection experiments shown above demonstrate that murine H1^0 regulatory sequences are recognized in *Xenopus laevis* oocytes.

Characteristics of mouse histone H1^0 expression in microinjected Xenopus *oocytes*

We have found that the mammalian H1^0 variant upon injection into *Xenopus* oocytes is recognized and actively transcribed. This transcription is dependent on the presence of sequences located at the 5' upstream region of the promoter region, in a similar way as found for transfected murine cells (Breuer *et al.*, 1989) using identical mutant deletions. In the heterologous oocyte expression experiment, the H1^0 sequences required for efficient transcription are located in a region comprising -360 to -600 bp upstream of the gene. The construct Δ28, containing additional upstream sequences (Fig. 1), did not show a markedly different expression as compared to the wild type clone (wt) or the construct lacking the distal 5' flanking region (Δ64). These results strongly indicate that no potential inhibitor or enhancer sequences – as recognized in the oocyte experiment – are located in the region -700 to -3200 bp upstream of the 5' histone H1^0 promoter.

Identification of expressed H1^0 sequences in differentiating tissues of Xenopus *tadpoles with the ß-galactosidase assay*

The developing *Xenopus* embryo is a widely used model system for studying the differential expression of injected genes like the ß-globin gene, the vitellogenin gene, the SV40 early gene and the *Xenopus* α-actin gene (Steinbeisser *et al.*, 1988; for reviews see Etkin *et al.*, 1986; Gurdon and Wakefield, 1986; Trendelenburg *et al.*, 1986).

To identify the cells in which the H1^0 promoter is active during the very fast embryogenesis of *Xenopus* we placed the promoter upstream to the bacterial ß-galactosidase gene. In a histochemical assay it is possible to detect ß-galactosidase-containing areas of the embryo. The conformation and persistence of the injected plasmid DNA can be simultaneously assayed by Southern blot hybridization and the activation pattern of the injected promoter can be microscopically examined in whole-mounts and dissected embryos. The ß-galactosidase technique allowed us to follow the expression pattern of injected DNA at the cellular level. Using other strategies such as examination of dissected embryos or tissues from adult frogs, it was possible to rule out a mosaic pattern of H1^0 expression. For SV40-CAT constructs, any kind of pattern of expression with regard to tissue type or regional localization could be observed (Etkin and Pearman, 1987). This time-consuming procedure can be overcome by the histochemical ß-galactosidase assay which allows the analyses of expression of the injected genes in late blastula, gastrula and neurula stages as well as in early tadpoles. Our results indicate that the injected mouse histone H1^0 promoter as well as the other two promoters (HSV tk, rat ß-actin) showed no tissue-specific or germ layer-specific expression. Despite this fact, the individual expression pattern in the embryos was caused by an asymmetric DNA distribution in the fertilized eggs as had also been previously observed (Etkin and Pearman, 1987). It thus appears that this approach directed to analyze cell-lineage pattern, e.g. analysis of tissue grafting experiments (Epperlein *et al.*, 1988; Krotoski *et al.*, 1988), or injection of fluorescent tracers (Gimlich and Cooke, 1983; Cooke, 1985; Wetts and Frazer, 1989).

Our results show that the approach used in our experiments can be used to complement other biological approaches directed to analyze cell lineage patterns, either by analysis of tissue-grafting experiments (Epperlein *et al.*, 1988; Krotoski *et al.*, 1988) or by injecting fluorescent tracers (Gimlich and Cook, 1983; Cooke, 1985; Wetts and Frazer, 1989).

Taking together the results of these studies and the localization of ß-galactosidase-positive cells in injected embryos, the resulting pattern in distribution of expressed promoters can be explained by tracing back the presumptive injection site. Depending on the area of injection in the non-cleaved fertilized egg (which is, of course, not precisely known), a possible preferential

deposition of DNA might have occurred within the early blastomeres DA, VLA, DLA, or VA, which is likely to have led to the type of expression observed (Fig. 5). This view was strengthened by the analysis of the injection site, which can often be traced back indirectly from the observed partial differentiation asserts seen at the early tailbud embryo stage (Fig. 4C,E,G,H). In Fig. 5, three typical examples of embryos with ß-galactosidase-positive areas are shown, together with the presumptive DNA distribution in the corresponding early blastomeres. These observations imply that the DNA distribution in the injected embryos is not a random mosaic but can be interpreted as the result of an ordered DNA propagation together with the intrinsic distribution of the endogenous components. Thus, this approach may now open the possibility of investigating the tissue-specific regulation of expression in greater detail by using injection into defined areas of the uncleaved or cleaved egg.

Materials and Methods

DNA probes used for microinjection

The following DNA samples were used: mouse histone gene promoter fragments (Breuer *et al.*, 1989), a rat cytoplasmic ß-actin gene promoter sequence (Melloul *et al.*, 1984) and a Herpes-Simplex virus (HSV) tk gene promoter (Luckow and Schütz, 1987) which were ligated to the chloramphenicol-acetyl transferase or ß-galactosidase gene.

Injection into oocytes and fertilized eggs of Xenopus laevis

25nl DNA solution (100ng/µl) was injected into the nucleus of non-centrifuged stage 6 *Xenopus laevis* oocytes. Oocytes were then incubated in 1x Barth Medium for 24 hours at 19°C. Eggs of *X. laevis* were fertilized *in vitro* and the jelly coats removed by treatment with 0.2% Cystein-HCl. Eggs were then injected with 200pg DNA in a volume of 20nl before the first cell division, as described previously (Steinbeisser *et al.*, 1988).

Chloramphenicolacetyl transferase (CAT) assay

Oocytes were homogenized in 0.25M Tris pH7.8 (30-50µl per oocyte) and the insoluble fraction was removed by centrifugation (10 min 10.000xg). The CAT assay was performed as described by Gorman *et al.* (1982) using 30-50µl oocyte extract. The reaction mixture (90µl final volume) was incubated for 1 hour at 37°C.

DNA preparation from embryos

Nucleic acids from embryos were extracted by the proteinase K method (Hofmann *et al.*, 1985), analyzed on horizontal agarose gels, blotted on nitrocellulose filters and hybridized against ^{32}P oligo-primed labeled pUC8 (Maniatis *et al.*, 1982).

ß-Galactosidase histochemistry in whole-mounts

Precisely staged *Xenopus* embryos (Nieuwkoop and Faber, 1967) were fixed in 2% formaldehyde, 0.2% glutaraldehyde in PBS for 60-90 min and washed with PBS. Fixed embryos were placed in 500µl reaction mixture containing 1mg/ml 4-Cl-5-Br-3-indolyl-ß-galactosidase (X-Gal), 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 2mM $MgCl_2$ in PBS and were incubated at 30-35°C for 18-24 hours. The reaction was stopped by rinsing the embryos with 3% DMSO in PBS (Sanes *et al.*, 1986) and the whole-mounts or the sections of paraffin-embedded specimens were examined microscopically.

Acknowledgments

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