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Original Article

115 kDa protein from *Xenopus laevis* embryos recognized by antibodies directed against the *Xenopus* homeoprotein XlHbox 1

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ABSTRACT Using antibodies against homeoprotein XlHbox 1 from *Xenopus laevis*, we have detected a new embryonic protein with a much larger molecular weight, 115 kDa. Antibodies fractionated according to their affinity for 3 different domains of the XlHbox 1 protein were used to show that this new protein is related to the C-terminal region of XlHbox 1 protein, downstream from the homeodomain. By immunohistochemistry, the protein was shown to be localized in nuclei of embryonic cells. On SDS-polyacrylamide gels, the 115 kDa protein appears as a set of closely spaced bands whose pattern varies with the stage of development and with the parental origin of the embryos. The protein could be extracted from embryos in a multiprotein complex of approximately 600 kDa. In contrast, the 18 and 27 kDa proteins predicted from the sequence of cloned cDNA to be transcribed and translated from the *XlHbox 1* gene could not be detected, suggesting that they are rare or unstable in embryos. These data suggest that the new protein is involved in the development of *Xenopus* embryos, with a function possibly related to that of the homeoprotein XlHbox 1.

KEY WORDS: *Xenopus embryogenesis, homeodomain protein, homeobox, immunoblot, immunohistochemistry*

Introduction

Analysis of the molecular mechanisms involved in the control of embryonic development has always been very difficult due to the small amount of material available for biochemical studies. Genetics has therefore been the most fruitful approach to these problems, particularly in *Drosophila*, and has led to the isolation of many genes controlling development, of which the homeobox-containing genes are perhaps the most fascinating (Gehring, 1992). Study of the products of these genes, again due to technical difficulties, has most often been carried out using fusion proteins produced in *E. coli* from expression vectors. The relatively large quantities of protein produced in these systems has permitted studies of protein structure and of protein/DNA interaction (Gehring *et al.*, 1990; Andrew and Scott, 1992; Treisman *et al.*, 1992). Direct study of these proteins in embryos is still necessary, however, to verify that the embryonic proteins possess the same characteristics as those expressed artificially, and to detect other proteins or macromolecules with which the embryonic proteins interact *in vivo* during development.

We set out to analyze the product of the homeobox gene *XlHbox 1* of *Xenopus* embryos, the first homeobox-containing gene found in vertebrates. XlHbox 1 was discovered through its homology with the gene *Antennapedia* of *Drosophila*, with which it shares 55 of the 60 amino acids in the homeodomain (Carrasco *et al.*, 1984). It has also been designated as *AC1* (Carrasco *et al.*, 1984), *Xeb1* (Carrasco and Malacinski, 1987), and *Hoxc-6* (Scott, 1992). *XlHbox 1* has been shown to produce 2 overlapping transcripts during early development through the use of 2 promoters separated in the genome by 9 kb. The corresponding proteins, whose predicted sizes are 17.6 kDa and 26.7 kDa, share the same homeodomain and C-terminus and differ only by an 82 amino acid extension at the N-terminus (Cho *et al.*, 1988). Transcripts of the *XlHbox 1* gene are limited to several specific embryonic regions (Carrasco and Malacinski, 1987), and the 2 proteins show a differential antero-posterior localization (Oliver *et al.*, 1988) which changes upon treatment of embryos with retinoic acid (López and Carrasco, 1992).

Here we have prepared rabbit antibodies to search for the products of the *XlHbox 1* gene in extracts from *Xenopus* embryos. Although by western blotting we could not detect either of the 2 expected proteins with the molecular weights predicted from the cDNA sequence (Cho *et al.*, 1988), the antibodies did recognize a new protein with a much higher molecular weight and allowed us to analyze some of the properties of this protein.

Abbreviations used in this paper: DTT, dithiothreitol; MBP, maltose-binding protein.

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Fig. 1. Maps of fusion proteins. *At the top is the map of the short protein product of Xenopus XlHbox 1 gene, as deduced from the nucleotide sequence of the shorter transcript of the gene (Cho et al., 1988). Its total length is 152 amino-acids, with the homeodomain composed of 60 amino-acids extending from residues 59 to 118. The ß-galactosidase fusion protein contains amino-acids 5 to 152 of XlHbox 1. The 4 fusion proteins with maltose binding protein contain residues 1-152 (T; total protein), 1-58 (N; amino-terminal part), 58-118 (H; homeodomain: the extra amino-acid at position 58 was included to preserve a cutting site for Factor Xa protease), and 119-152 (C; carboxy-terminal part).*

Results

Search for proteins encoded by the XlHbox 1 gene in Xenopus embryo extracts

Our initial objective was to detect in *Xenopus* embryo extracts the 17.6 kDa and 26.7 kDa proteins expected to be encoded by *XlHbox 1*. To produce antibodies directed against the XlHbox 1 protein we used an expression vector, kindly sent to us by Dr. Andrés Carrasco, which contained a fusion between the gene for *E. coli* ß-galactosidase and a portion of the *Xenopus XlHbox 1* gene (Fig. 1). This construct was expressed in *E. coli*, the fusion protein was purified by SDS-polyacrylamide gel electrophoresis, and was injected into rabbits to raise antibodies. Control western blotting experiments confirmed that immune sera from 2 different rabbits recognized the fusion protein as well as the ß-galactosidase control as expected (data not shown). These sera were then used in western blotting experiments with proteins extracted from *Xenopus* embryos (Fig. 2A). Although several bands were observed in the embryo extracts, no band reacting with the immune sera and not with the control preimmune or anti-ß-galactosidase sera was found in the lower region of the gel in the molecular weight range expected for XlHbox 1 proteins. Instead, a band with a molecular weight much higher than expected, approximately 115 kDa, was detected with the 2 immune sera and was absent from all controls.

Several different methods were employed for extracting proteins from embryos including homogenization of the embryos in buffers of different KCl concentrations, or directly incubating the embryos at 100°C for 10 min in 2% SDS and 0.1 M DTT. Again, no bands with the expected molecular weight were detected, whereas the 115 kDa band was present in each case (Fig. 2B). On other occasions, either micrococcal nuclease or DNAse I digestion was included in the protein extraction procedure, or much larger amounts of protein were loaded on the gels, or the time of electrophoretic transfer was varied between 20 min and 3 h. All such attempts also failed to reveal the expected proteins (data not shown).

To improve the specificity and sensitivity of the immunoblotting technique, antibodies were subsequently purified from the immune serum by immunoaffinity on a column of Sepharose coupled to the ß-galactosidase/XlHbox 1 fusion protein. As shown in Fig. 2C, the background was particularly low with these specific antibodies, and again no bands with the expected molecular weight were evident. Only the high molecular weight band was detected as before, this time appearing clearly as a doublet. Embryo extracts were also analyzed further by electrophoresis on a high resolution gel, as shown in Fig. 2D. Several bands around 115 kDa were observed with unfractionated immune sera and were not recognized by the preimmune serum nor by the control anti ß-galactosidase antibodies. Using our affinity-purified antibodies, however, only the 2 bands forming a doublet at 115 kDa were evident.

Analysis of the 115 kDa protein using antibodies directed against different domains of homeoprotein XlHbox 1

In order to further characterize the 115 kDa protein, we first wished to determine whether it contained a homeodomain. For that purpose, the XlHbox 1 protein was cleaved from its original vector and split into 3 contiguous parts: the N-terminal portion (N), the central homeodomain (H), and the C-terminal region (C) (Fig. 1).

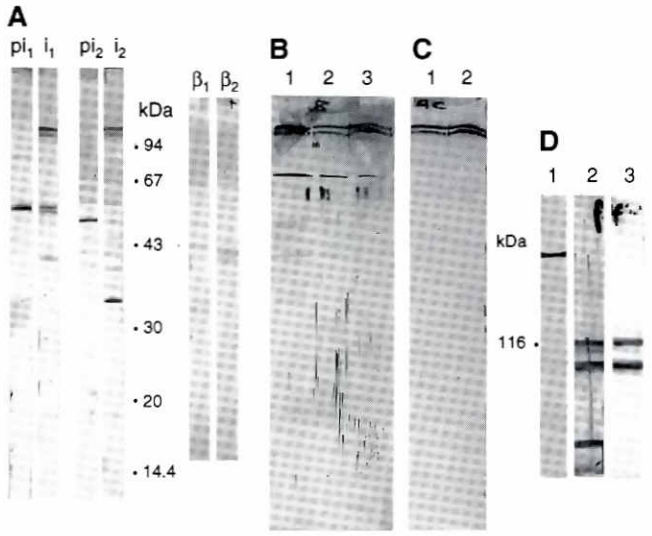

Fig. 2. Immunoblotting analysis of *Xenopus* embryos extracts with antibodies against XlHbox 1 protein. (A) *Extracts from Xenopus embryos were fractionated by electrophoresis on 18% polyacrylamide SDS gels and analyzed by western blotting using different sera. pi_1 and pi_2: preimmune sera from rabbits 1 and 2; i_1 and i_2: immune sera from rabbits 1 and 2; $ß_1$ and $ß_2$: two different sera directed against E. coli ß-galactosidase.* **(B)** *Three different extracts from Xenopus embryos were analyzed by western blotting with serum from rabbit 2. Lane 1, extraction of proteins with a buffer containing 50 mM KCl; lane 2, extraction with a buffer containing 250 mM KCl; lane 3, extraction by boiling embryos in 2% SDS, 0.1 M DTT. Composition of extraction buffers are given in Materials and Methods.* **(C)** *Immunoblotting analysis of Xenopus embryos extracts using affinity-purified antibodies against ß-gal-XlHbox 1 fusion protein. SDS extracts (lane 1) and 50 mM KCl extracts (lane 2) from Xenopus embryos were analyzed as in* **A** *and* **B**, *except that affinity-purified antibodies were used in place of total rabbit serum.* **(D)** *Immunoblotting analysis of extracts from Xenopus embryos on high resolution gels. The experiment is identical to the previous ones with 50 mM KCl extracts, except that electrophoresis was on a high resolution 6% polyacrylamide SDS gel to better analyze the doublet at 115 kDa. Lane 1, control with anti ß-galactosidase serum; lane 2, total rabbit serum; lane 3, affinity-purified antibodies. Molecular weight markers were LMW from Pharmacia, and E. coli ß-galactosidase (116 kDa) from Sigma.*

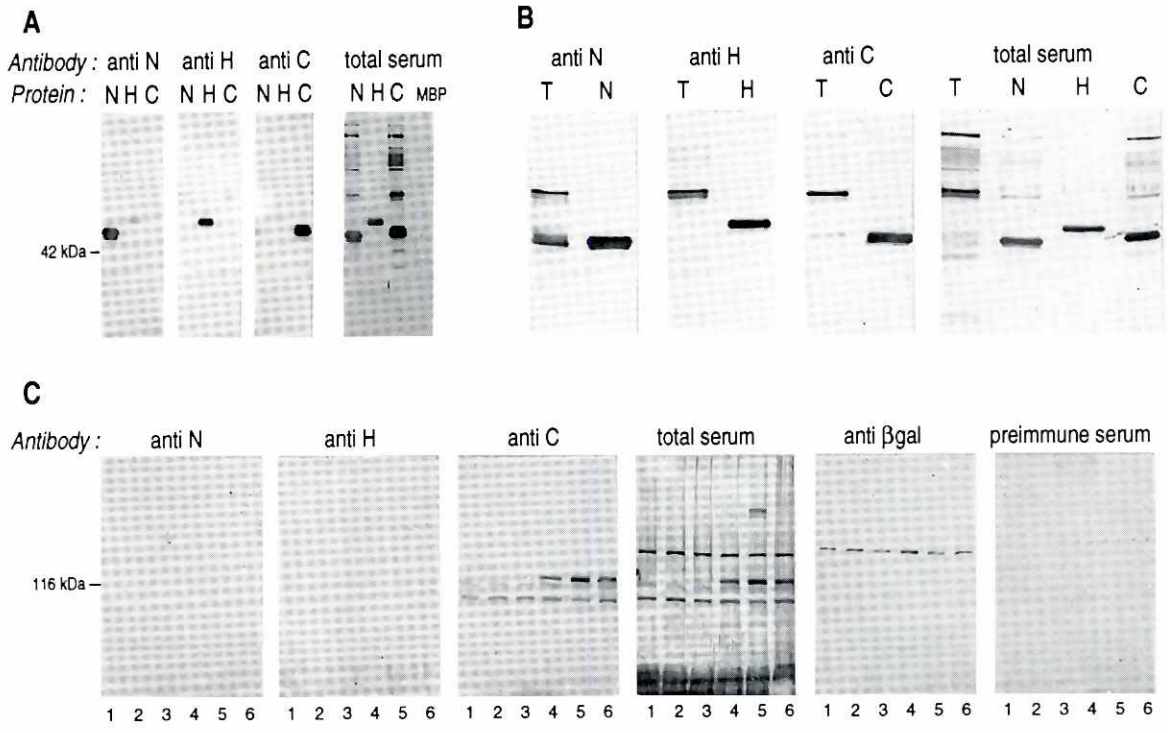

Fig. 3. Analysis of *Xenopus* embryo extracts with antibodies specific to different domains of XIHbox 1 protein. *Antibodies specific for 3 different domains of the XIHbox 1 protein were purified from total rabbit serum using 3 different affinity columns prepared with fusion proteins containing the amino-terminal region, the central homeodomain, or the carboxy-terminal region of the total protein.* **(A)** *Affinity-purified antibodies to one domain do not bind to the other 2 domains. Three identical SDS-polyacrylamide gels were loaded with fusion proteins N, H and C, and analyzed by western blotting using affinity-purified antibodies anti-N, anti-H, and anti-C, as indicated. A similar gel containing in addition pure maltose binding protein (Mr 42,000) was analyzed with total rabbit serum; in this case, bands in the upper part of the gel are due to ß-galactosidase.* **(B)** *Control of the affinities of the purified antibodies. Three western blots containing fusion protein T (with an extra band at ~48 kDa due to premature termination of translation in the homeodomain, see Materials and Methods; this band only appears with anti-N) and equal amounts of fusion proteins N, H, and C were probed with affinity-purified antibodies against domains N, H, and C, respectively. A fourth blot containing all 4 proteins was probed with total rabbit serum. On this latter blot, as in panel A, the bands of high molecular weight are due to ß-galactosidase.* **(C)** *Analysis of Xenopus embryo extracts with the affinity-purified antibodies. Six identical blots prepared with proteins extracted from Xenopus embryos at different stages of development were probed with the following antibodies: affinity purified anti-N, anti-H, anti-C, total immune serum, anti ß-galactosidase, and preimmune serum. Stages of development: 4, 8-9, 10-12, 20-21, 22, 23-31, in lanes 1-6, respectively.*

The 3 gene fragments and the uncleaved *XIHbox 1* gene were linked to the C-terminal end of maltose binding protein of the expression vector pMal-c2 (Fig. 1). These constructs were then expressed in *E. coli* and the fusion proteins obtained. They were used to prepare affinity columns which were, in turn, used to fractionate the immune serum according to its specificities for the 3 parts of the XIHbox 1 protein. These purified antibodies showed no cross-reactivity, since antibodies against any one part of the protein did not bind either of the 2 other parts (Fig. 3A). It is also interesting to note that the unfractionated serum recognizes all 3 parts of the protein with roughly the same intensity, and does not bind to maltose binding protein. Figure 3B shows that the 3 fractionated antibodies give signals of comparable intensities when comparable amounts of the corresponding proteins are loaded on the gels. It also confirms that the unfractionated serum recognizes similarly all 3 parts of the protein.

An analysis of the *Xenopus* extracts with these 3 purified antibodies is shown in Fig. 3C. Surprisingly, no band is evident with the antibodies specific for the N-terminal or for the homeodomain of XIHbox 1, whereas the antibodies specific for the C-terminal region detect the same 115 kDa protein as before. Therefore it appears that the antibodies which bind the 115 kDa protein correspond to epitopes of protein XIHbox 1 localized in its C-terminal region, downstream of the homeodomain.

Localization of the 115 kDa protein in Xenopus embryos

The 3 affinity-purified antibodies were used in immunohistochemistry experiments to study the localization of the new protein in embryos. Experiments performed with whole-mount embryos (Fig. 4A) or with sections (Fig. 4B) gave the same result: no significant signal was visible with anti-N and anti-H antibodies, whereas a strong signal was observed with anti-C antibodies, in perfect agreement with the results of the immunoblotting experiments. In these experiments, the 115 kDa protein was found to be localized in nuclei of embryonic cells.

Band pattern changes with embryos originating from different parents

Western blotting analysis of extracts from embryos from 7 different parental couples at a given stage of development is illustrated in Fig. 5. The band pattern in the 115 kDa region of the gel varies markedly from one egg batch to another, with the

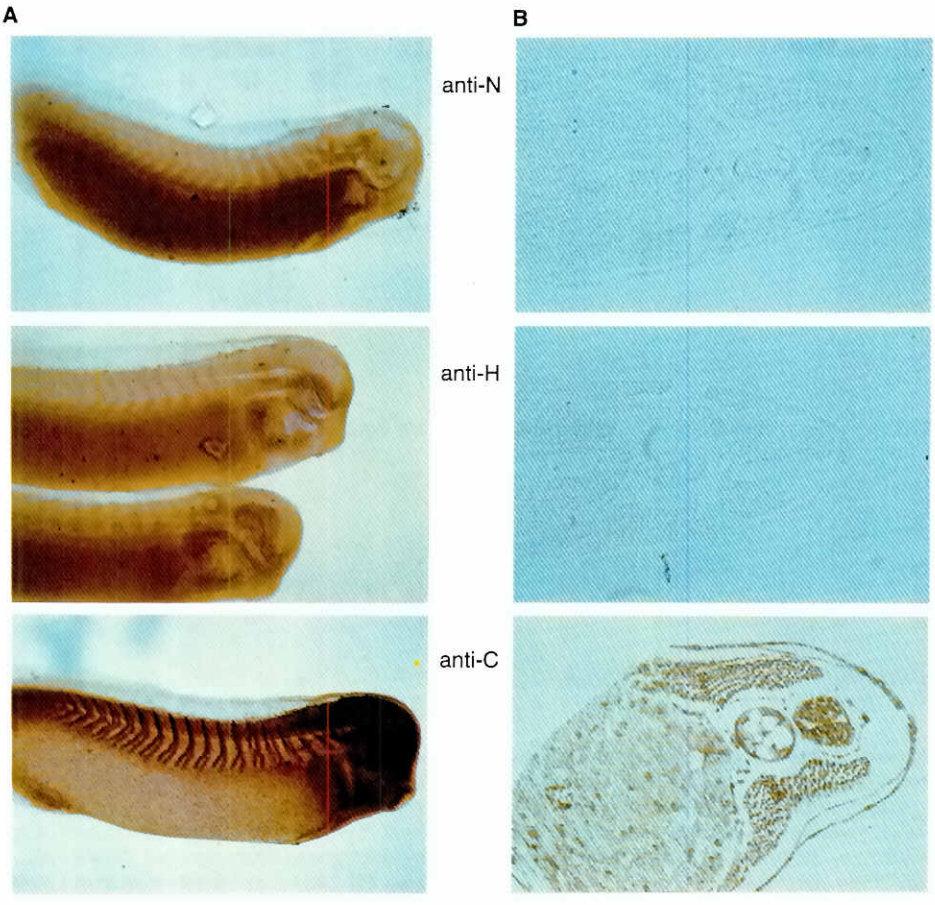

Fig. 4. Immunolocalization of the 115 kDa protein in *Xenopus* embryos. *The 3 affinity-purified antibodies anti-N, anti-H, and anti-C were used in immunohistochemistry experiments to localize the protein in whole-mount embryos at stage 25 of development* **(A)** *or in serial sections of embryos. A transversal section at the trunk level of a stage 25 embryo is shown* **(B)**, *other sections gave similar results.*

apparent molecular weights of the proteins differing by almost 10 kDa between the 2 extremes. This finding is likely to be the result of differences at the gene level, as the animals used in these experiments were not genetically homogeneous, as discussed later.

The band pattern varies in the course of development

In Fig. 6, embryos originating from 2 different couples were analyzed at different stages of development. An evolution of the pattern is observed with the stages of development, with new bands appearing before stage 20 while the bands that were initially present remain visible with the same intensity. The same result can also be partially seen in Fig. 3. The difference in apparent molecular weight of the bands is at least 3 kDa. This phenomenon is probably the result of post-translational modifications of a protein, presumably phosphorylations, although other hypotheses are also possible as discussed later.

The 115 kDa protein can be extracted from embryos in the form of a complex of 600 kDa apparent molecular weight

In an effort to purify the 115 kDa protein by HPLC, an extract from *Xenopus* embryos was analyzed by gel filtration on a Superose 6 column. The fractions were assayed by western blotting for the presence of the 115 kDa protein (Fig. 7). Except for a small percentage which eluted with the excluded volume (fraction 16), most of the 115 kDa protein eluted with an apparent molecular weight of approximately 600 kDa (fractions 25-26). This result suggests either that the protein is present in embryos in multimeric form, or that it is associated with other macromolecules in a complex which is preserved, at least partially, during the extraction process.

Discussion

We have used rabbit antibodies directed against different regions of the *Xenopus* homeodomain protein XlHbox 1 to study proteins from *Xenopus laevis* embryos. The transcripts of this gene previously characterized by Cho *et al.* (1988) are expected to give rise to 2 proteins of 17.6 and 26.7 kDa. However, we could not detect a protein with this molecular weight. All the signals in this molecular weight range, which we observed by western blotting using total serum, were extremely weak and corresponded in fact to non-specific interactions, as they all disappeared when we used purified antibodies. We tried several different methods for extracting proteins, such as boiling in SDS or nuclease digestion of embryo extracts, and we also tried to increase as much as possible the amount of protein loaded on the gels but the results were always negative. This is in agreement with the work of Oliver *et al.* (1988) on the same protein, who also mention that they did not detect it by western blotting. The most likely explanation is that the protein is very rare in embryos, either because it is expressed in very small amounts, or because it is very unstable, as noted by Harvey *et al.* (1986) for the Xhox-1 protein synthesized in embryos after injection of synthetic mRNA. There is little doubt, however,

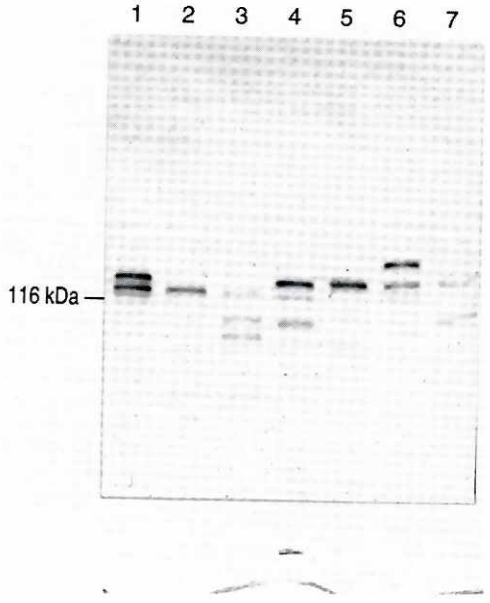

Fig. 5. Variation of the band pattern in embryos originating from different parents. *Extracts from Xenopus embryos at stage 22 of development but originating from 7 different parent couples* **(lanes 1-7)** *were analyzed by immunoblotting using affinity-purified antibodies against total XlHbox 1 protein.*

that this protein is synthesized in embryos at some stages of development, and it could probably be detected with improved immunoblotting or extraction techniques: despite technical difficulties, some homeodomain proteins have already been detected by western blotting with protein extracts prepared from embryos (Krause *et al.*, 1988; Gavis and Hogness, 1991; Wall *et al.*, 1992). A major technical problem in such experiments should arise from the high degree of conservation of the homeodomain between different homeoproteins, which implies that an antibody raised against a specific homeodomain should also recognize many other homeodomain proteins. However, studies of XlHbox 1 by immunolocalization in embryos (Oliver *et al.*, 1988; López and Carrasco, 1992; this work) have always given very weak signals, pointing again to a lack of stability of the homeoproteins during development.

Instead of the expected signal, we observe a protein with an apparent molecular weight which is much higher: 115 kDa. In our western blots, this signal is always present in a clearly visible manner, is obtained with the unfractionated serum as well as with purified antibodies, and corresponds to a protein which is extracted by homogenization of embryos in a buffer containing 50 mM KCl. This protein appears as a set of close bands with a pattern that is different when embryos originate from different parents, and which shows a variation in the course of development. The changes with the parents most probably result from allelic difference between animals, and a genetic study in progress should clarify this point, if it is not too complicated by tetraploidy and gene duplications (Fritz *et al.*, 1989). The variations of pattern during the stages of development can be explained in different ways. The simplest possibility is that they are due to post-translational modifications, for example phosphorylations, occurring at a given stage of development, as is the case for the Ultrabithorax protein of *Drosophila* (Gavis and Hogness, 1991). Alternatively, they could be the result of differential expression of different alleles in the course of development. Again, the genetic study of the band pattern may clarify this point.

As shown by using affinity-fractionated antibodies in immunoblotting and immunohistochemistry experiments, the 115 kDa protein is recognized by antibodies directed against the C-terminal region of XlHbox 1 protein downstream of the homeodomain, but it is not recognized by antibodies directed to the homeodomain itself, nor by antibodies to the N-terminal region of XlHbox 1 protein. This C-terminal domain, which is only 35 amino-acids long, contains 8 acidic residues which are clustered within the last 20 amino-acids, but shows no significant similarity with protein motifs of known function. The question is what is the exact relationship between this new 115 kDa protein and the homeoprotein XlHbox 1. The fact that the 115 kDa protein is strongly recognized by the antibodies against the C-terminal region of XlHbox 1 suggests that in this region their amino-acid sequences are very similar or even partly identical. As the evidence is only immunological, however, other possibilities cannot be excluded. For example, the amino-acid sequences might be different but share common epitopes. This seems unlikely, however, since the signal on western blots is very strong, which would imply that the number of epitopes in common should be large. We can therefore hypothesize that there is some amino-acid sequence homology between the 115 kDa protein and the C-terminal region of XlHbox 1. If this is indeed the case, 2 possibilities exist. It could either be a different gene coding for a protein with a partly similar sequence, or it could be the same gene, and the 115 kDa protein would be the product of post-transcriptional events. It might, for example, be the result of alternative splicing. In this respect, it is worth noting that in their analysis of the transcripts of this gene, Carrasco *et al.* (1984) have noted the existence of a large RNA, possibly a pre-mRNA, which has not been characterized further. The protein could also be the result of an unknown post-translational mechanism. From the data presented here and considering our preliminary attempts, the purification of the 115 kDa protein from *Xenopus* embryos may not be out of reach, and should allow an unambiguous characterization of this new protein and its relationship with the XlHbox 1 homeodomain protein.

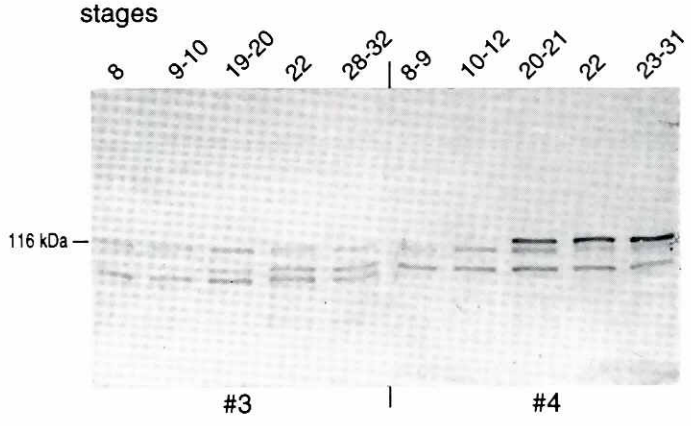

Fig. 6. Variation of the band pattern during the course of development. *Embryos originating from 2 different couples (couples number 3 and 4 in Fig. 5) were collected at the indicated stages of development and protein extracts were analyzed by immunoblotting using affinity-purified antibodies against total XlHbox 1 protein.*

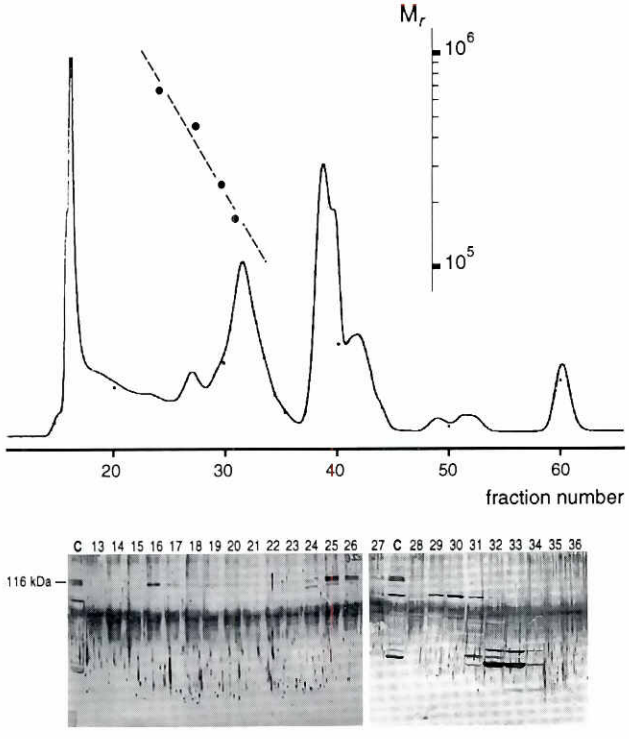

Fig. 7. Analysis of extracts from *Xenopus* embryos by gel filtration. *A protein extract from embryos was fractionated on a Superose 6 column. The top of the Figure shows the optical density of the fractions at 280 nm, and a calibration curve of the column obtained with proteins of known molecular weights. Fractions were analyzed by immunoblotting using total immune serum, as shown by the western blots at the bottom of the Figure. Lanes labeled C are controls loaded with the starting protein extract. The 115 kDa protein is present in fraction 16 (excluded volume) and predominantly in fractions 25-26, the bands of lower molecular weights in fractions 29-34 do not stain with affinity-purified antibodies.*

Materials and Methods

Fusion proteins

Schematic maps of the constructs used are shown in Fig. 1. All cloning experiments were performed according to standard procedures (Sambrook *et al.*, 1989). The protein used for rabbit immunization was a fusion protein between *E. coli* ß-galactosidase and part of the XlHbox 1 protein. It was produced by the vector pUR290 containing the PstI fragment of the *XlHbox 1* gene inserted at the *PstI* site in the polylinker downstream of the *lacZ* gene. The fusion proteins used for fractionating the antibodies according to their affinities to different domains of the XlHbox 1 protein were made by linking the appropriate regions of the gene downstream of the sequence coding for maltose binding protein in the expression vector pMal-c2 (New England Biolabs) at the *XmnI* site. This would allow us to recover the inserted polypeptide by site-specific proteolytic cleavage with Factor Xa protease, a property which was, however, not used in the present work.

Purification of fusion proteins

For production of fusion proteins, cultures of *E. coli* were induced with IPTG, grown for 2 h at 37°C, and harvested by centrifugation at 3,500g for 15 min. Cells from 1 vol of culture were resuspended in 1/20 vol of 10 mM Na phosphate pH 7.2, 30 mM NaCl, 0.25% Tween 20, 10 mM 2-mercaptoethanol, 10 mM EDTA, 10 µg bestatin and chymostatin/ml, 25 µg leupeptin/ml, 2 µg pepstatin/ml (protease inhibitors from Boehringer). The cells were frozen and thawed once, sonicated on ice for 3 periods of 45 s, NaCl was added to 0.5 M, the extract was centrifuged 30 min at 10,000g, and the supernatant was recovered. The ß-galactosidase fusion protein used for rabbit immunization was purified by preparative electroelution from SDS-polyacrylamide gels. The MBP fusion proteins were initially intended to be purified by binding to an amylose affinity column. However, whereas MBP could be purified very efficiently by affinity chromatography on such columns, none of the 4 fusion proteins bound significantly to the amylose column. Therefore, fusion proteins N and C, which were produced in high yield, were purified by preparative electrophoresis and electroelution. In contrast, fusion protein H, which contains the homeodomain, was expressed with a very low yield. This was presumably due to the large number of AGG codons in the homeobox, an arginine codon which is a frequent codon in vertebrates but an extremely rare codon in *E. coli* (Wada *et al.*, 1991). Such low level of expression made it necessary to add a purification step, and this protein was purified by phosphocellulose chromatography as follows. An *E. coli* extract from 3 l of culture expressing the fusion protein was prepared by sonication in 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, plus the 4 protease inhibitors as before. It was centrifuged 30 min at 10,000g, the supernatant was diluted 10-fold with 20 mM K phosphate pH 7.5, 1 mM DTT, 1 mM EDTA, and loaded on a 35 ml phosphocellulose column previously equilibrated with the same buffer, at 4°C. After washing the column, proteins were eluted with a linear gradient of 20 mM to 1 M K phosphate containing 1 mM DTT and 1 mM EDTA. 4 ml fractions were collected and analyzed by western blotting. The fusion protein containing the homeodomain was found to elute much later than the bulk of the proteins, as a sharp peak around 0.4 M K phosphate. It is interesting to note that MBP did not bind to phosphocellulose under these conditions, implying that the homeodomain is responsible for this very tight binding of the fusion protein to phosphocellulose.

Preparation and purification of antibodies

Immunological experiments were performed as described by Harlow and Lane (1988). Two rabbits were injected intradermally at ~40 points in their backs with 100 µg of purified fusion protein, in 500 µl of buffer homogenized with an equal volume of complete Freund's adjuvant. Boosts at 3 and 6 weeks after the first injection were with the same amount of protein and incomplete Freund's adjuvant.

Antibodies were purified by passing the antisera through a column prepared by linking the antigen to CNBr-activated Sepharose following the conditions given by the manufacturer (Pharmacia). Elution of bound antibodies was with 0.15 M glycine pH 2.5, followed by immediate neutralization with 0.1 volume of 1 M Tris-base.

Extraction of proteins from Xenopus embryos

Embryos staged according to Nieuwkoop and Faber (1967) were dejellied, washed in Barth's solution, and stored at -70°C in the presence of 10% glycerol. For protein extraction, embryos were thawed and homogenized in 50 mM Tris-HCl pH 7.9, 50 mM KCl, 10 mM DTT, 10 mM EDTA, 10 µg bestatin and chymostatin/ml, 25 µg leupeptin/ml, 2 µg pepstatin/ml, at 0°C. The homogenate was centrifuged 10 min at 13,000g, and the supernatant was collected and centrifuged again for 1 h at 35,000g.

Immunoblotting

After electrophoresis in SDS-polyacrylamide gels, proteins were transferred electrically to nitrocellulose membranes (BA85 from Schleicher and Schuell) in 25 mM Tris, 190 mM glycine, 20% methanol. The non-specific sites on the membranes were blocked by a 1 h incubation in 5% non-fat dry milk in TBST (10 mM Tris-HCl pH 8, 150 mM NaCl, 0.05% Tween 20). The membrane was incubated with the appropriate dilution of antibody in TBST, usually 1:1000 for crude rabbit serum and 1:100 for purified antibodies. After 3 washes of 10 min in TBST, the bound antibodies were reacted with a secondary antibody directed to rabbit IgG and coupled to alkaline phosphatase, and alkaline phosphatase was detected by the appearance of a dark purple color in the presence of a chromogenous substrate (ProtoBlot from Promega).

Immunohistochemistry

Stage 25 embryos from albino *Xenopus laevis* were prepared and treated with specific antibodies as described by Harland (1991). The secondary antibody was a goat anti-rabbit IgG coupled to horseradish

peroxydase, which was visualized by the reaction with diaminobenzidine and hydrogen peroxide.

Gel filtration of extracts from Xenopus embryos

200 μl of embryo extract were loaded on an HPLC Superose 6 gel filtration column (Pharmacia) equilibrated in 50 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM DTT, 1 mM EDTA, at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and assayed by western blotting. The column was calibrated with proteins from the Pharmacia calibration kit: aldolase, 158 kDa; catalase, 232 kDa; ferritin, 440 kDa; thyroglobulin, 669 kDa.

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