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Expression of a histone H1-like protein is restricted to early *Xenopus* development

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Genes whose expression is restricted to oogenesis and early development may have important functions in these processes. Northern analysis showed that *Xenopus* B4 mRNA is expressed in oogenesis and embryogenesis through to the neurula stage. Immunocytochemistry with anti-B4 antibodies showed that B4 protein is only detectable in preneurula stages; it is localized to nuclei and is associated with metaphase chromosomes. Immunoblotting revealed approximately constant levels of B4 protein per embryo for the first 2 days of development. Thus, as the number of nuclei increases during early development, the amount of B4 protein per nucleus is diluted out. Sequencing of two B4 cDNA clones revealed that the predicted B4 translation product is a 29-kD protein with 29% identity with histone H1, distributed over the entire length of its sequence. The B4 protein also has certain other H1 protein characteristics—a tripartite structure consisting of a mainly hydrophobic central domain flanked by an amino-terminal segment and a long hydrophilic carboxy-terminal tail containing a tandemly repeated amino acid motif. However, in contrast to histone H1 mRNA, B4 mRNA has a classic polyadenylation signal, is polyadenylated, and lacks the histone H1 3′ noncoding consensus sequence involved in RNA processing.

[*Key Words: Xenopus laevis*; development; maternal RNA; histone H1; nuclear protein]

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Zygotic transcription is minimal in the frog *Xenopus laevis* until approximately 8 hr after fertilization, at the midblastula transition (Bachvarova and Davidson 1966; Newport and Kirschner 1982; Kimelman et al. 1987). During the transcriptionally quiescent period prior to the midblastula transition, major events in pattern formation are known to take place. For example, cytoplasmic movements responsible for the establishment of the dorsal–ventral axis of the future embryo occur during the first cell cycle (Vincent et al. 1986; Vincent and Gerhart 1987), and inductive interactions necessary for mesoderm differentiation begin at the 32- to 64-cell stage (Jones and Woodland 1987). Prior to the midblastula transition, the embryo utilizes mRNAs and proteins synthesized during oogenesis and inherited through the egg.

Genes whose expression is restricted to oogenesis and early development may function in the regulation of the meiotic cell cycle in oocytes, in the rapid cleavage divisions of embryonic cells, or in pattern formation in the early embryo. A number of cDNA clones derived from mRNAs that are expressed at high levels in the *Xenopus* oocyte, but have decreased in titer significantly by the gastrula stage and do not accumulate further, have been isolated previously (Dworkin et al. 1985). The strategy followed for the study of the proteins encoded by these RNAs has been to sequence the cDNAs initially so as to identify the open reading frame, and then to compare the putative protein sequences with known proteins in the data banks. Then, using bacterially expressed β-galactosidase fusion proteins, antibodies raised against the proteins are used to examine both the spatial and temporal expression of the proteins in the oocyte and embryo. Here we describe the characterization of a maternal *Xenopus* mRNA, B4, whose expression is restricted to oocytes and early embryos. The B4 protein is shown to be nuclear and associated with chromatin in eggs and early embryos and to possess a number of features characteristic of the histone H1 family of proteins.

Results

Developmental expression of B4 mRNA

B4 mRNA is a moderately abundant maternal RNA that is restricted to oogenesis and early development of *X. laevis*. Northern analysis (Fig. 1) showed that the 1.2-kb B4 transcript is present in stage VI oocytes and remains at a constant level through the blastula stage. By the gastrula stage the level of B4 message has started to decline, and by the neurula stage it is barely detectable. The RNA is absent from later-stage embryos (stages 24–26 and stages 37–41) and from the adult tissues tested (muscle and liver). The slight increase in size of the RNA in the cleavage and blastula stages is most likely due to an increase in the length of the existing poly(A) tail.

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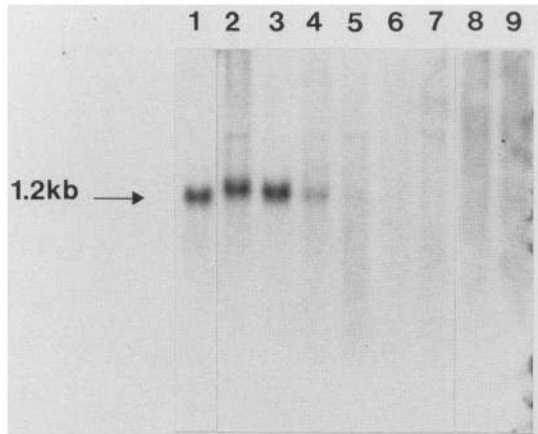

Figure 1. Northern blot analysis of *Xenopus* RNA probed with nick-translated clone B4.0 (Lanes *1–7*) 10 μg/lane of total RNA. (Lane *1*) Stage VI oocyte; (lane *2*) cleavage (stages 6.5–7); (lane *3*) blastula (stage 9); (lane *4*) gastrula (stages 10.5–12); (lane *5*) neurula (stages 17–20); (lane *6*) early tailbud (stages 24–26); (lane *7*) 3-day-old tadpole (stages 37–41); (lane *8*) 0.8 μg of liver poly(A)$^+$ RNA; (lane *9*) 0.4 μg of muscle poly(A)$^+$ RNA.

Characterization and sequencing of two B4 cDNA clones

The first B4 clone (B4.0; originally B4) was isolated from a *Xenopus* total ovary cDNA library (Dworkin et al. 1985) and has an insert of 793 bp. Since the B4 mRNA is approximately 1.2 kb in length, the clone B4.0 is incomplete. Screening of a *Xenopus* egg cDNA plasmid library for B4 sequences resulted in the isolation of a further clone, B4.1. This cDNA contains 894 bp of B4 sequence plus a poly(A) tail. The relationship between the two clones (based on their sequence, see below) is shown in Figure 2A. B4.1 has 387 bp of additional sequence at the 3′ end compared with B4.0, but it lacks 286 bp of sequence at the 5′ end that is present on B4.0. Together the two clones cover 1180 bp of sequence, excluding the poly(A) tail. Thus, virtually all of the B4 mRNA sequences should be contained within these two clones. Both B4.0 and B4.1 have been fully sequenced (Fig. 2B). Within the 507-nucleotide overlap between the two clones, five nucleotide differences were found (Fig. 2); four are base substitutions at positions 295, 337, 574, and 614, and one is an extra nucleotide at position 673 in B4.1 which was absent from B4.0. The two clones could be derived from different alleles of a single B4 gene, or represent two different B4 genes. The partial duplication of the *X. laevis* genome during its evolutionary history has resulted in increased genetic variability (Thiébaud and Fischberg 1977). Furthermore, many genes in *X. laevis* are polymorphic (Okada et al. 1985).

Sequencing revealed one major open reading frame (ORF) in the B4 sequence (Fig. 2B) on the DNA strand that was confirmed to be the sense strand by hybridization of single-strand probes to B4 mRNA in Northern blots of oocyte poly(A)$^+$ RNA (data not shown). There are 277 nucleotides of 5′ noncoding region before the ATG codon. This ATG lies within a reasonable sequence context for translation initiation (Kozak 1987), and the presence of an upstream in-frame stop codon indicates this to be the beginning of the coding region. The B4.1-type sequence has the coding capacity for a protein of 273 amino acids, followed by 84 bp of 3′ noncoding sequence, with the polyadenylation signal AATAAA 24 nucleotides upstream of the poly(A) tail.

The four base substitutions between B4.0 and B4.1 are all in the coding region of the B4 protein and are silent changes. However, the missing nucleotide in B4.0 at position 673 causes a frameshift that results in a stop codon two amino acids later. This ORF would produce a protein of 134 amino acids sharing 132 amino acids with the B4.1 predicted protein but missing the very basic carboxyl half of the B4.1 protein (see below). The proportion of B4 mRNA in oocytes that contains the extra nucleotide at position 673 was determined by Northern analysis using 19-mer oligodeoxynucleotides covering the region around position 673 as probes. One of these oligomers was of B4.1 type (containing a nucleotide at position 673) and one of B4.0 type (lacking a nucleotide at position 673). Conditions under which labeled 19-mer B4 anti-sense oligomers (1014, 1015; Materials and methods; Fig. 2B) only hybridized to the perfectly matching template were established using as templates SP6 RNA transcripts that contained the B4 sequence around nucleotide 673 of the B4.1 or B4.0 type (Material and methods). Under these conditions oocyte poly(A)$^+$ RNA hybridized predominantly to the B4.1-type oligomer (1015) and very little, if at all, to the B4.0-specific oligomer (1014) (Fig. 3, lane O, 1.2-kb transcript arrowed). This demonstrates that the majority of B4 mRNA molecules contain a nucleotide at position 673 and that the larger ORF represents the major B4 translation product. B4 molecules lacking a nucleotide at position 673 must either be a minor B4 mRNA species, or

Figure 2. (*See following page.*) (*A*) Relationship between B4.0 and B4.1 cDNA clones. Below the horizontal line representing the clones are the nucleotide positions of the beginning and end of the separate cDNAs. The nucleotide differences between the two clones are shown with their respective nucleotide positions. (*) Absence of a nucleotide. (*B*) Nucleotide sequence of B4 cDNA and predicted amino acid sequence of B4 protein. The sequence is a composite of the B4.0 and B4.1 cDNA insert sequences. Numbering of nucleotides starts at the first nucleotide of the B4.0 clone. At five nucleotide positions there was a disparity in sequence between the two clones; the sequence of the B4.1 clone is given with the B4.0 nucleotide written above. (*) Nucleotide position that is absent from the B4.0 clone. The predicted amino acid sequence (three-letter code) is shown underneath the major open reading frame. Numbering of amino acids starts from the first amino acid of the B4 protein. The change in open reading frame caused by the absence of a nucleotide (*) in the B4.0 insert is shown beneath. (***) Stop codon of the B4.0 reading frame. The three direct amino acid repeats toward the carboxyl end are indicated by dashed arrows. The polyadenylation signal AATAAA is dot-underlined. The nucleotide sequence underlined at position 663–682 is that contained within the oligonucleotides used as hybridization probes (the first nucleotide was only present in the B4.0 type oligonucleotide; Materials and methods).

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A.

```
                    295 337     574 614 673

B4.0   5´ __________G_A_______C_C__*_____ 3´
                                       /
          1                          793


B4.1                5´ _A_T_______G_A__T____________________ poly(A) 3´

                      287                                1180
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B.

```
ggaggacagaagatctttggttaattctatcacttcctgcaaatctttctttagtttcagtaaatcgtcattctcgccattggccgt   87
taatgctgattcaacctgttgcaattgggctttgtaacccgccagctgcttagctagatcttctgccatcgcgccgctctctcgtct  174
cacagacttagttcccgacaacctctgcggcgtctcgcgagacttctccgcgcttttcgagtcgccccgccctcaacgctgacggtt  261

                                      g
ctctgttgattctcaaatg gct cct aag aag gca gtt gct gca cct gag gga ggc aac aag gaa aat gca  331
                met ala pro lys lys ala val ala ala pro glu gly gly asn lys glu asn ala   18

      a
gca gtt aaa gga tcc agt aaa gtt aag gtt aaa aga aaa tct atc aaa cta gtc aag acc caa tca  397
ala val lys gly ser ser lys val lys val lys arg lys ser ile lys leu val lys thr gln ser   40

cat ccc cca acc ctg tcg atg gtg gtg gag gtc ctg aaa aag aac acg gag cgg aaa ggg acc tct  463
his pro pro thr leu ser met val val glu val leu lys lys asn thr glu arg lys gly thr ser   62

gtg cag gcc att cgg acc cgg att ctg tct gca cat ccc aca gtg gat cca ctg agg ctg aag ttt  529
val gln ala ile arg thr arg ile leu ser ala his pro thr val asp pro leu arg leu lys phe   84

                                                      c
ttg cta cgg acg gcc ctg aac aaa ggg cta gag aag ggg att ctg atc aga cct cta aac tct agt  595
leu leu arg thr ala leu asn lys gly leu glu lys gly ile leu ile arg pro leu asn ser ser  106

                        c
gca aca gga gct aca gga aga ttc aaa ctt gcc aaa cca gta aaa act aca aag gct ggg aaa gaa  661
ala thr gly ala thr gly arg phe lys leu ala lys pro val lys thr thr lys ala gly lys glu  128

              *
aat gta gcg tct gaa aac gta gac cca aat gca gag cag gaa acc caa aag aag gcc cca aag aaa  727
asn val ala ser glu asn val asp pro asn ala glu gln glu thr gln lys lys ala pro lys lys  150
                 lys thr ***

gaa aag aaa gcg aag act gag aaa gaa ccc aaa ggt gag aaa acc aaa gct gta gct aaa aag gcc  793
glu lys lys ala lys thr glu lys glu pro lys gly glu lys thr lys ala val ala lys lys ala  172

aag gaa gat tct gat gaa aaa ccc aaa gtt gcc aaa tct aag aaa gat aaa gag gca aaa gaa gtt  859
lys glu asp ser asp glu lys pro lys val ala lys ser lys lys asp lys glu ala lys glu val  194
                                                          <------------repeat 1

gac aag gct aat aaa gag gca aaa gaa gtt gac aag gct aat aaa gag gca aaa gaa gtt gac aag  925
asp lys ala asn lys glu ala lys glu val asp lys ala asn lys glu ala lys glu val asp lys  216
--------------> <--------------repeat 2--------------> <------------repeat 3----------

gct ccg gca aag aaa cca aaa gcc aaa aca gag gct gcg aaa gct gag ggg ggt ggc aag gca aag  991
ala pro ala lys lys pro lys ala lys thr glu ala ala lys ala glu gly gly gly lys ala lys  238
-->

aag gag ccc cca aag gcc aaa gcc aag gac gtg aaa gca cag aag gac tct aca gat gaa ggt gct 1057
lys glu pro pro lys ala lys ala lys asp val lys ala gln lys asp ser thr asp glu gly ala  260

cca gtt aag gct ggc aag aaa gga aag aaa gtg aca aac taggctgatatacttttaactctgtaaatctggtt 1131
pro val lys ala gly lys lys gly lys lys val thr asn                                      273

tttaatgtttaattctataaataaagtaagtaattgttttttttttctt poly (A)                              1180
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Figure 2. (*See previous page for legend.*)

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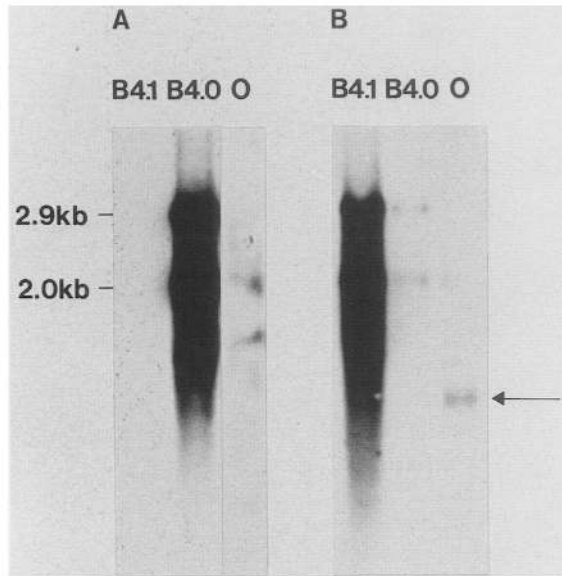

Figure 3. Differential hybridization of oligodeoxynucleotides to B4 SP6 transcripts and oocyte poly(A)+ RNA. Hybridization under differential hybridization conditions to SP6 transcripts (~150 ng/lane) of the B4.1 type (B4.1) or B4.0 type (B4.0) and to oocyte (O) poly(A)+ RNA (3 μg/lane) using as probes oligonucleotide 1014 (B4.0 type) (*A*) and oligonucleotide 1015 (B4.1 type) (*B*) (Materials and methods). (Arrow) Position of the 1.2-kb B4 mRNA. Both hybridizing bands in the SP6 transcript lanes are in vitro transcription products that contain the B4 sequence. The two left lanes in *A* are 6-hr autoradiographic exposures; all remaining lanes are 18-hr exposures.

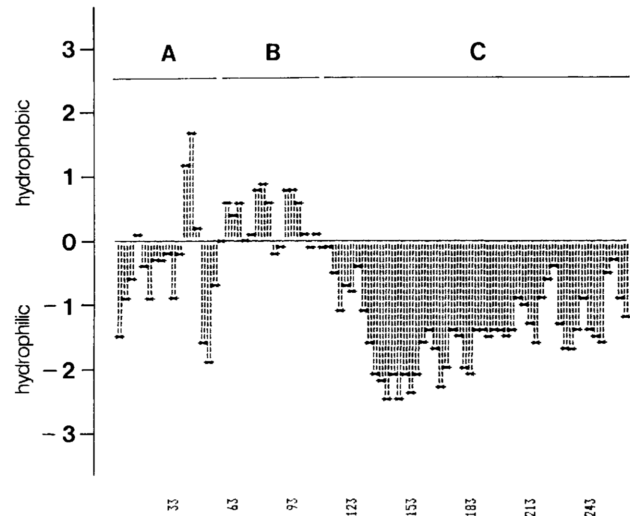

Figure 4. Hydropathy plot of the B4 protein. Numbers along the abscissa represent the amino acid positions of the B4 protein, with numbering starting at the amino terminus. The ordinant shows the hydropathic index. The segment length for analysis was 9 (Material and methods). A, B, and C are the three major domains of the B4 protein (Results).

the absence of this nucleotide in the B4.0 clone is artifactual. The designation 'B4' will be used to refer to the sequence containing the longer ORF.

The B4 sequence has the coding capacity for a protein of 29,290 kD. The amino acid content of the B4 protein is unusual in that it is 25% lysine, with most residues in the carboxy-terminal portion of the protein; there are no cysteine, tyrosine, or tryptophan residues. Overall the B4 protein is very basic, with a calculated pI of 10.62. A hydropathy plot of the B4 protein is shown in Figure 4. The B4 protein consists of three major domains: a central hydrophobic domain (region B) flanked by an amino-terminal domain (region A) and a carboxy-terminal domain which is strikingly hydrophilic (region C). In the carboxy-terminal domain of the protein are three tandemly repeated amino acid motifs, which are also exact repeats at the nucleotide level, and consist of two direct repeats of 10 amino acids followed by an incomplete repeat of 9 amino acids (Fig. 2B).

Searches of a protein data base with the B4 protein sequence revealed low but significant similarities with histone H1 proteins from a wide range of organisms, including plants, birds, mammals, fish, invertebrates, as well as amphibians (Table 1). The homology is throughout the coding region. Of the three histone H1 subtypes in *Xenopus* embryos, histone H1B shows the best alignment (29% amino acid identity) and is illustrated in Figure 5. A string of five identical amino acids is the longest consecutive match. B4 also shows comparable similarities to the two other *Xenopus* histone H1 subtypes: H1C (28% in 181 amino acid overlap) and H1A (25% in 206 amino acid overlap). These *Xenopus* H1 subtypes show 75–80% identity with each other (Turner et al. 1983; Perry et al. 1985).

Isolation of B4-specific antibodies and immunocytochemistry

Antibodies to the B4 protein were raised via production of an *E. coli* B4-β-galactosidase fusion protein. An expression vector was constructed that contained the *lacZ* gene fused to the B4.0 sequence in frame to yield upon translation amino acids 23–132 of the B4 protein fused to the carboxy-terminal end of β-galactosidase. The fusion protein was used to immunize rabbits, and antibodies against the B4 part of the fusion protein were isolated by affinity chromatography (Materials and methods).

Immunocytochemistry of paraffin-embedded *Xenopus* embryo sections using the affinity-purified anti-B4 antibodies and a fluoresceinated secondary antibody revealed staining of the nuclei of early embryos (Fig. 6A,B). The same pattern of staining was seen at stage 7 using three different fixatives, including precipitant and cross-linking types (data not shown). All nuclei appeared to stain equally. Nuclear labeling was bright in eight-cell embryos (the earliest stage examined; data not shown), as well as at later cleavage and blastula stages. By the gastrula stage labeling was in general fainter, and in neurulae and later stages (stages 24–27, stages 40–41) labeling was no longer detectable (Fig. 6B and data not shown). Labeling was absent from a range of adult

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Table 1. *Sequence similarity of B4 protein to the histone H1 family of proteins*

Protein	Code or reference	Sequence identity (%)	Amino acid overlap
Frog histone H1B	HSXL1B	29	219
Frog histone H1A	HSXL1A	25	206
Frog histone H1C	a	28	181
Rainbow trout histone H1	HSTR1R	29	181
Gonadal sea urchin histone H1	HSUR1P	28	178
Rat testis-specific histone H1	b	23	201
Trout histone H1	HSTR1	25	166
Goose histone H5	HSGS5	27	108
Pea histone H1	c	31	107
Human histone H1	A24850	35	116

The code for a particular protein is its entry name in the PIR sequence data bank (Release 10.0). For proteins not listed in the PIR, the reference containing the sequence is: (a) Turner et al. (1983); (b) Cole et al. (1986); (c) Gantt and Key (1987).

tissues tested (liver, kidney, leg muscle, heart, brain; data not shown). Cleavage-stage sections have a high proportion of nuclei at the metaphase stage of mitosis, and these nuclei showed a similar staining pattern with the DNA stain DAPI as with the anti-B4 antibodies (Fig. 6B,a), indicating that the B4 antigen is associated with the chromosomes. In the unfertilized egg the one set of metaphase chromosomes stained brightly (data not shown). In the stage VI oocyte the germinal vesicle was slightly brighter than background (data not shown) but since the chromatin is only partially condensed it was difficult to be certain whether the chromosomes themselves were stained. Identical results were obtained with anti-B4 antibodies purified from serum of two separate rabbits. Anti-β-galactosidase antibodies at the same concentration as the B4-specific antibodies gave a negative reaction to all sections tested (data not shown).

Because the B4 protein and *Xenopus* histone H1 exhibit amino acid similarity, the possibility existed that the observed staining of nuclei was due to cross-reaction of the anti-B4 antibodies to endogenous histone H1. To test this, rabbit antiserum raised against *Xenopus* histone H1 (a gift of Dr. A. Moorman) was reacted to *Xenopus* sections. This antiserum labeled nuclei at all stages tested (egg, blastula, gastrula, neurula, tailbud, and tadpole), with the later stages showing the brightest labeling (Fig. 6C and data not shown). This result proved that the B4 staining pattern was not due to cross-reaction to endogenous histone H1 and demonstrated that the nuclei of post-gastrula stages were fully accessible to antibody staining.

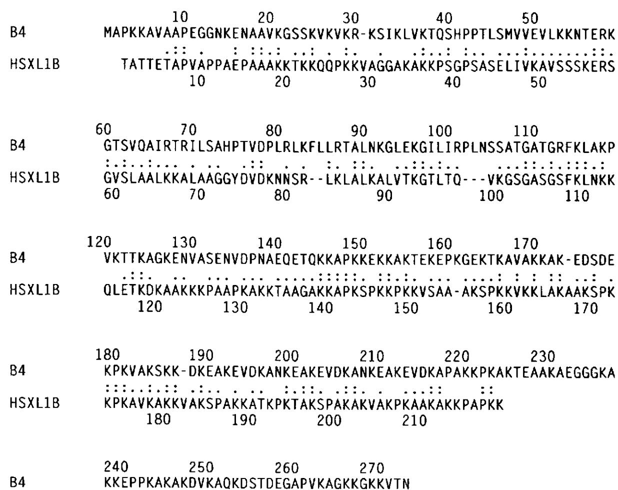

Figure 5. Comparison of B4 protein with *Xenopus* histone H1B (HSXL1B). Double dots indicate identity and single dots indicate conservative replacements.

Immunoblotting

Anti-B4 antibodies, even at low dilutions (~15 μg/ml), did not label any protein in Western blots of total *Xenopus* embryonic lysates. Therefore, to visualize B4 protein on Western blots it was necessary to prepare samples enriched in B4 protein. Immunoblotting of nuclear extracts from cleavage embryos showed staining of one major band at 36 kD and two minor bands at 34 kD and 40 kD (Fig. 7A). Staining with anti-B4 antibodies isolated from a different rabbit (rabbit 2) stained the major 36-kD band as well as the 40-kD minor band but not the 34-kD band (Fig. 7B and data not shown). Anti-β-galactosidase antibodies at the same concentration did not label these proteins (Fig. 7A). The 36-kD band probably represents the B4 protein, as it is the major band recognized by both sera. The minor band(s) could be due to post-translational modification of the protein affecting its mobility, or cross-reaction of the antibodies to B4-related proteins. The higher molecular weight than predicted from sequencing (29 kD) is not surprising since many histone and nuclear proteins are retarded on gels due to their unusual amino acid composition (Bürglin et al. 1987). The very basic carboxyl terminus of B4 (and histone H1) (Fig. 4) suggested that advantage could also be taken of solubility in 5% perchloric acid as a method of enrichment of B4 (Materials and methods). Immunoblots of perchloric acid extracts from total embryos (Fig. 7B) showed reaction (with antibodies from rabbit 2) to a 36-kD protein and a 40-kD protein in all

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stages tested (from oocyte to early tailbud embryo), although the relative intensities of these two proteins changed through development. Anti-histone H1 antiserum labeled a protein migrating at ~31 kD in these perchloric acid extracts (Fig. 7C). The presence of B4 protein in the oocyte is consistent with the partial polysomal distribution of B4 RNA at this stage (Dworkin et al. 1985). Since the level of B4 mRNA declines rapidly between the oocyte and neurula, whereas the B4 protein level remains approximately constant, the protein itself appears to be relatively stable. However, although the total amount of B4 protein per embryo remains unchanged during early development, the amount of B4 protein per nucleus decreases substantially as cell division proceeds.

Discussion

B4 mRNA is a moderately abundant *Xenopus* RNA that is expressed during oogenesis and the first day of embryonic development. Sequencing of two independently isolated B4 cDNA clones has revealed the putative amino acid sequence of the B4 protein. The B4 protein shows a weak sequence similarity to histone H1, an identity of about 25–30% over the entire length of the protein. Although histone H1 proteins are the least conserved of the histone family, nevertheless histone H1 subfamily members are highly homologous. In *Xenopus* embryos the three known histone H1 subtypes (H1A, H1B, H1C) are 75–80% identical at the amino acid level (Turner et al. 1983; Perry et al. 1985). The central globular domain of the histone H1 molecule is the most conserved region of the histone H1 protein (70% identity in all animal histone H1 proteins, with 90–100% conservation around a phenylalanine residue within the globular domain; Coles et al. 1987; Gantt and Key 1987), but B4 has no higher similarity to this part of the H1 molecule than to any other. However, the B4 protein does possess the tripartite structure characteristic of histone H1; a central globular hydrophobic core surrounded by an amino–terminal segment and a carboxy-terminal hydrophilic tail. The larger size of the B4 protein (29 kD) compared to the characterized animal histone H1s (20–25 kD) is due mainly to longer carboxy- and amino-terminal domains. The presence of three carboxy-terminal direct amino acid repeats in B4 is also a feature of histone H1 proteins (Von Holt et al. 1979; Mezquita et al. 1985); however, the sequence of the B4 repeat is not similar to any known repeat in histone H1.

Despite similarities between B4 and histone H1 at the protein level, B4 mRNA does not show histone-like features. The B4 mRNA does not contain the 3′ dyad symmetry sequence that is thought to be involved in RNA processing; neither does it contain the second further downstream purine-rich 3′ sequence element conserved in histone H1 mRNAs (Krieg and Melton 1984; Coles et al. 1987). The B4 mRNA contains a classic polyadenylation signal in the 3′ noncoding sequence and the message is known to be polyadenylated. This is in contrast to histone mRNAs, which generally do not contain a polyadenylation signal and lack a poly(A) tail. As an exception to this generality, the majority of *Xenopus* histone mRNA is polyadenylated in the oocyte (Ruderman et al. 1979), but these polyadenylated histone mRNAs lack a polyadenylation signal (Ballantine and Woodland 1985). A further difference between B4 and histone H1s is the gene frequency in the genome. Southern analysis of the B4 gene shows a pattern typical of low-copy-number genes (E. Dworkin-Rastl and M. Dworkin, unpubl.), in contrast to each of the *Xenopus* histone H1 genes which are reiterated in the genome 10–25 times (Perry et al. 1985).

In situ immunofluorescence experiments using antibodies raised against a bacterial B4 fusion protein showed that the B4 protein is localized in the nuclei of early embryos until the gastrula stage, after which it is no longer detectable. The B4 protein was clearly chromatin-associated during mitotic metaphase in cleavage embryos. Immunoblotting showed that the B4 protein is inherited maternally and persists at approximately constant levels from the oocyte to the early tailbud stage. Thus, during early cleavage stages, before the amount of

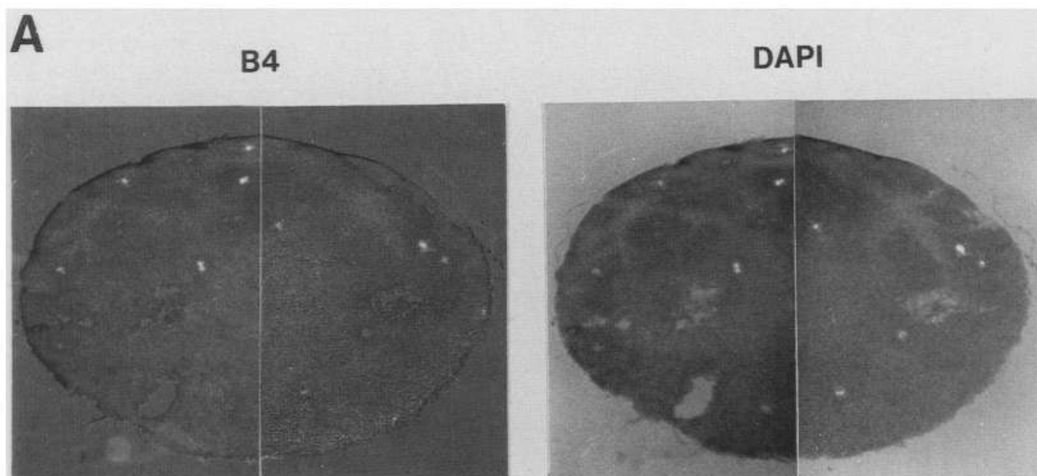

Figure 6. (*Continued on following page.*)

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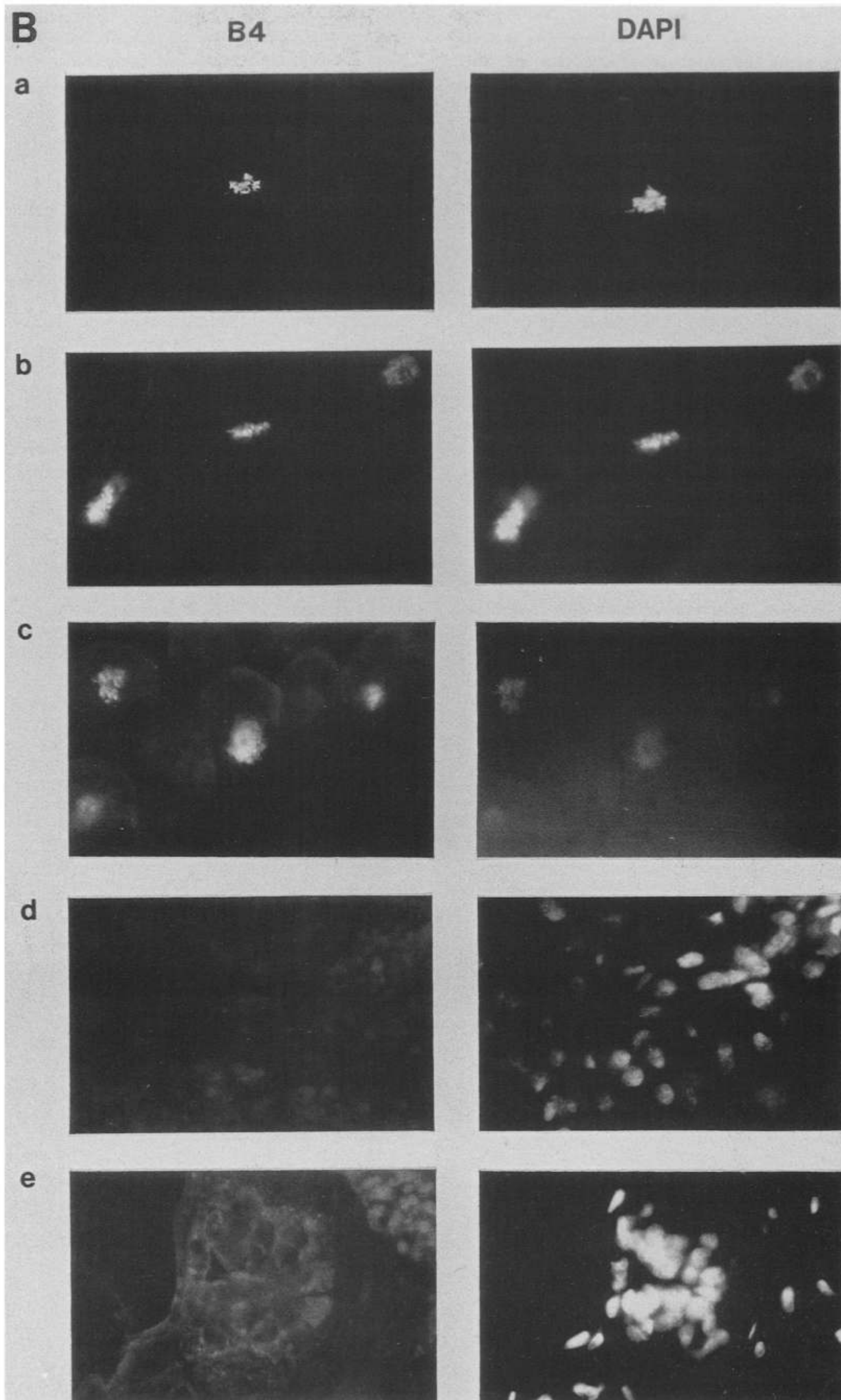

Figure 6. (*See facing page for legend.*)

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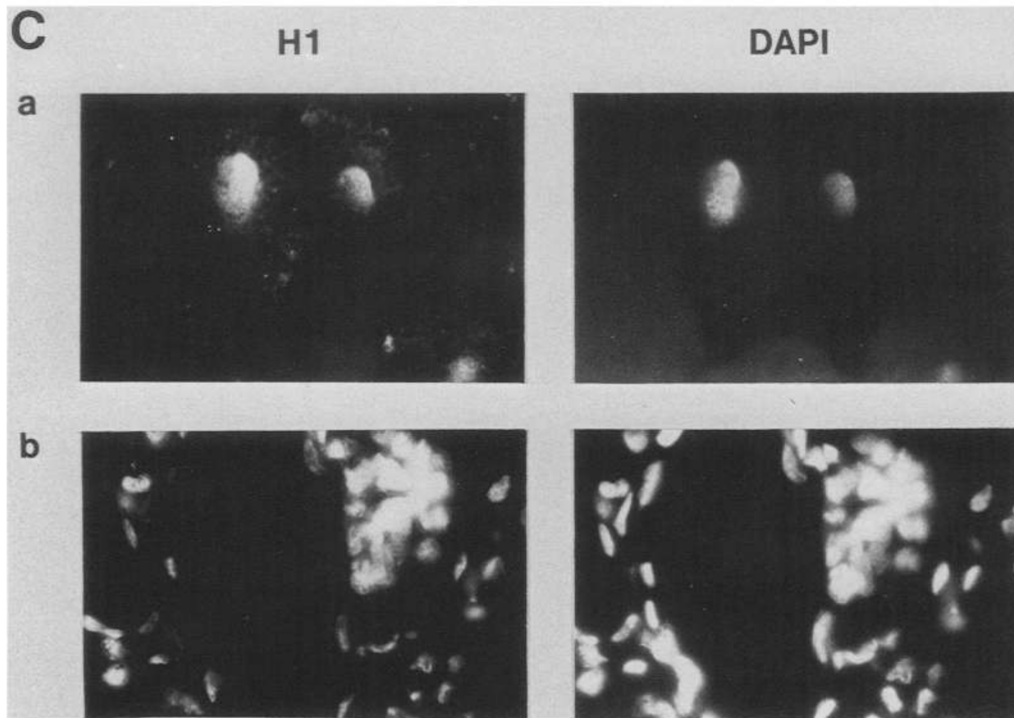

Figure 6. Immunocytochemistry of *Xenopus* sections. Paraffin-embedded *Xenopus* tissue sections were stained with either B4-specific antibodies (*A* and *B*, ~80 μg/ml), or *Xenopus* anti-histone H1 antiserum (*C*, 1 : 100 dilution) and visualized with a fluoresceinated secondary antibody. Left-hand-side panels show antibody staining; right-hand-side panels show counterstaining of the same section with DAPI. The length of photographic exposure in seconds for each of the antibody : DAPI pairs, respectively, is given in parentheses (exp.x,x) below. (*A*) Low-power view of a cross-section of a blastula embryo (stage 8) with animal hemisphere at the top of the picture (exp. 60,8). The embryo is approximately 1.2 mm in diameter. (*B*) High-power view (6.3× higher magnification than in *A*) of: (*a*) cleavage (stage 6.5), centering on a set of metaphase chromosomes (exp. 60,8); (*b*) blastula (stage 8) (exp. 60,8); (*c*) gastrula (stages 11–11.5) (exp. 60,15); (*d*) neurula (stage 19) (exp. 60,8); (*e*) 3-day-old tadpole (stages 40–41), centering on neural tube (exp. 240,4). (*C*) High-power view of: (*a*) blastula (stage 8) (exp. 60,8); (*b*) 3-day-old tadpole (stages 40–41) (exp. 30,8).

chromatin increases sufficiently to bind all available B4 protein, there must be B4 protein in the embryo not associated with chromatin. Such extrachromosomal stores, however, were not detected immunocytochemically, and therefore are probably not localized. The failure to detect B4 protein in nuclei from later stages is probably a result of the decrease in amount of B4 protein per nucleus due to its distribution into increasing numbers of nuclei as development proceeds.

There is little evidence for developmentally specific histone H1 variants in *Xenopus*, except for the description by Koster et al. (1979) of a cleavage-specific lysine-rich protein (Destrée et al. 1973; Flynn and Woodland 1980; Risley and Eckhardt 1981; Perry et al. 1986). However, histone H1 variants specific to early development have been described in the sea urchin (Levy et al. 1982) and mud-snail (Flenniken and Newrock 1987), and specific to the testis in rat (Cole et al. 1986). The best-characterized sea urchin variants conform to standard histone family characteristics and show high sequence similarity to the other sea urchin histone H1 proteins (Levy et al. 1982). The divergence of B4 protein and mRNA from the histone H1 family suggests that B4 should be considered either a distant member of the histone H1 family or a novel protein with histone H1 characteristics.

About 8–10 ng of histone H1A protein is inherited by the *Xenopus* embryo from the oocyte, sufficient theoretically to provide the newly synthesized chromatin in embryos with histone H1 until the early blastula stage (Van Dongen et al. 1983). The translation of stored histone H1 mRNA is not significant until late cleavage stages (Woodland et al. 1979; Flynn and Woodland 1980). Despite the presence of H1 protein in the early embryo it is not clear when it is actually incorporated into chromatin. Destrée et al. (1973), examining histones in chromatin from late blastula and gastrula embryos, showed that histone H1 was underrepresented relative to the other histones until the neurula stage, when it reached its expected proportion. Experiments on chromatin assembly in extracts from unfertilized eggs showed an absence of histone H1; nevertheless the chromatin of nuclei assembled in *Xenopus* egg extracts displayed the nucleosome digestion patterns typical of histone H1-containing chromatin (Dilworth et al. 1987). These data suggest that an unknown histone H1 variant may exist in *Xenopus* eggs and early embryos. It is intriguing to speculate that B4 is this protein.

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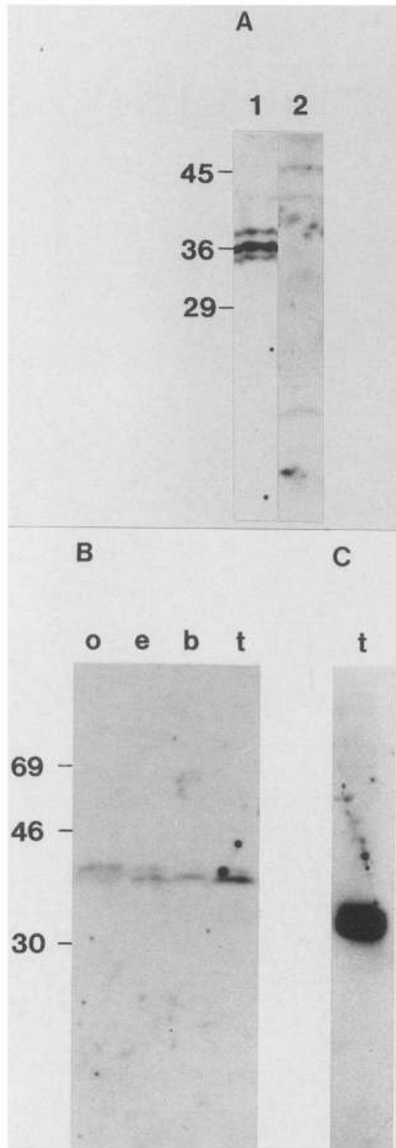

Figure 7. Immunoblotting of an embryonic nuclear extract and perchloric acid protein extracts. (*A*) A Western blot of a nuclear extract from preneurula embryos was probed with B4-specific antibodies (~15 mg/ml) (lane *1*) or β-galactosidase-specific antibodies (~15 mg/ml) (lane *2*). (*B,C*) Western blots of perchloric acid extracts from stage VI oocytes (lane *o*), unfertilized eggs (lane *e*), stage 9 blastulae (lane *b*), and stages 26–27 early tailbud embryos (lane *t*) were probed with anti-B4 antibodies (~15 ml, derived from a different rabbit from those in *A*) (*B*); or *Xenopus* anti-Hl antiserum (1 : 500 dilution) (*C*). (*B*) Five-day autoradiographic exposure; (C) 18-hr autoradiographic exposure. Antibodies were visualized with ^{125}I-labeled protein A. The numbers on the left refer to the molecular weight in kilodaltons.

Materials and methods

RNA extraction and Northern analysis

Oocytes were staged according to Dumont (1972) and embryos according to Nieuwkoop and Faber (1967). RNA extraction was performed as described previously (Dworkin and Dawid 1980). Poly(A)$^+$ RNA was isolated and Northern analysis with plasmid probes was carried out as described previously (Dworkin et al. 1985) with hybridization and washing conditions as detailed by Church and Gilbert (1984).

Construction and screening of an egg cDNA library

An egg cDNA plasmid library was prepared from poly(A)$^+$ RNA isolated from unfertilized eggs as described under RNA extraction, except that the RNA was not digested with DNase. Synthesized double-stranded cDNA (Gubler and Hoffman 1983) was size-fractionated by passage over a Sepharose 4B column and cDNA larger than ~500 bp was inserted into the *Pst*I site of pGEM4 (Promega) using oligo(dG:dC) tailing. Clones of the egg cDNA library were screened for B4 clones using as probe a nick-translated 549-bp *Pvu*II–*Acc*I fragment of B4.0 (nucleotides 133–681 in Fig. 2B). The final wash stringency of the filters was 0.2× SSC, 1% SDS at 65°C.

DNA sequencing and computer programs

The majority of DNA sequencing was performed using the method of Maxam and Gilbert (1980) with labeling of DNA fragments as described previously (Dworkin-Rastl et al. 1984). Some sequences were obtained by the chain-termination method with double-stranded DNA templates (Chen and Seeburg 1985) performed with a Sequenase sequencing kit (USB) and using an SP6 primer (for inserts cloned into pGEM vectors) or a primer oligodeoxynucleotide of B4 sequence.

Homology searches of the protein sequence database of the Protein Identification Resource data bank (Release 10.0) were performed using the FASTP program (Lipman and Pearson 1985). Construction of the hydropathy plot, based on the method of Kyte and Doolittle (1982), and calculation of the pI value were performed using a DNA/Protein Sequence Analysis software package (IBI).

Differential oligonucleotide hybridization

Oligodeoxynucleotides were synthesized with an Applied Biosystems DNA synthesizer, purified by reverse-phase HPLC following the protocol of Applied Biosystems, and dissolved in water.

SP6 RNA transcripts of 2.0 kb and 2.9 kb, produced by standard methods (Melton et al. 1984), contained 19 nucleotides of sense B4.0 or B4.1 sequence centered at the position of the extra nucleotide in clone B4.1 (position 673; Fig. 2B). They were used as template RNAs to determine differential hybridization conditions such that a 19-mer antisense B4.0 oligomer (5′ labeled with T4 polynucleotide kinase to a specific activity of 5000 Ci/mmole) only hybridized to the B4.0-type RNA and not to the B4.1-type, and vice versa (B4.0-specific probe, oligonucleotide 1014, 5′-TACGTTTTCGACGCTACAT-3′; B4.1-specific probe, 1015, 5′-TACGTTTTCAGACGCTACA-3′ Fig. 2B). Differential hybridization conditions were obtained by hybridization overnight at 45°C in 1% BSA, 7% SDS, 1 mM EDTA, 0.75 M $NaHPO_4$ (pH 7.2). Filters were washed for 15 min in solution A (0.9 M NaCl, 0.09 M Na-citrate, 5% SDS, 0.5% BSA) at room temperature (RT), 15 min in solution B (0.9 M NaCl, 0.09 M Na-citrate, 1% SDS) at RT, and then 5 min in 200 ml of solution B, which was initially heated to 65°C but cooled down as washing was carried out at RT. Northern analysis of oocyte poly(A)$^+$ RNA (3 μg/lane) was carried out under these conditions with labeled B4.1 and B4.0 oligomers as probes (10^6 cpm/lane).

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Expression vector construction and fusion protein isolation

The 1.37-kb *Bam*HI–*Pst*I fragment from B4.0, which contained the sequence from position 342 of B4 sequence (Fig. 2B) to the end of the B4.0 insert plus 778 bp of pBR322 sequence, was cloned into the *Bam*HI–*Pst*I site in the polylinker of pUR291 (Rüther and Müller-Hill 1983). This resulted in the fusion of the *lacZ* gene to the B4.0 open reading frame yielding upon translation amino acids 23–134 of the B4.0 protein fused to the carboxy-terminal end of β-galactosidase. *E. coli* strain K12 71–18 (Messing et al. 1977) was transformed with this vector and transformants were maintained on minimal medium. Fusion protein was induced by adding isopropyl-β-D-thiogalactoside (Pharmacia) to a final concentration of 1.5 mM to log-phase bacteria (OD_{590} = 1.0) in L-broth and growth for approximately 2 hr until OD_{590} = 1.7–2.0. Induced cultures (2.4 liters) were harvested and the bacteria lysed by resuspension in 24 ml of 0.2 M Tris-HCl (pH 7.6), 10 mM Mg-acetate, 5% glycerol, 0.25 M NaCl, 10 mM 2-mercaptoethanol (ME), 0.5 mg/ml lyzozyme (Sigma), and repeated cycles of freezing and thawing. After centrifugation for 30 min at 18,000 rpm in a Sorvall SS34 rotor, over 90% of the fusion protein was associated with the pellet fraction. This material was solubilized in 10 ml of 8 M urea, 50 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 1 mM EDTA, 30 mM ME, 1 mM phenylmethylsulfonylfluoride (PMSF), and fractions (200 μl) were separated on SDS–polyacrylamide gels (Laemmli 1970), from which the fusion protein was cut out and electroeluted.

Immunization protocol and antibody purification

Rabbits were immunized with 200 μg of B4 fusion protein in 1 ml of PBS emulsified with 3 ml of complete Freund's adjuvant subcutaneously at multiple sites on the back. After 3–4 weeks rabbits were boosted with 100 μg of fusion protein in incomplete Freund's adjuvant. A second boost of 100 μg was given subcutaneously 2 weeks later. Two weeks after this, a boost of 100 μg was given in a combination of subcutaneous and intravenous injections. Further boosts were given at intervals of 4 weeks to maintain high antibody titer. Bleeds (1–3 ml, 25 ml, or total exsanguination) were taken 1–2 weeks after boosts.

Serum was applied twice to a β-galactosidase affinity column to remove anti-β-galactosidase antibodies. This column was prepared by linking β-galactosidase (Sigma; 8 mg/ml resin) to CNBr-activated Sepharose 4B (Sigma). Bound anti-β-galactosidase antibodies were eluted with 0.2 M glycine, 0.5 M NaCl (pH 2.3) and neutralized immediately with 1 M Tris-HCl (pH 9.5). The flowthrough fraction (containing B4-specific antibodies) was applied to a B4-β-galactosidase fusion protein affinity column. This column was prepared by linking purified B4-β-galactosidase fusion protein to CNBr-activated Sepharose 4B (3 mg/ml resin). B4-specific antibodies were eluted in a similar manner as described for the anti-β-galactosidase antibodies, precipitated, resuspended in PBS, and dialyzed extensively.

Immunocytochemistry

Oocytes, dejellied eggs, or embryos were fixed at 4°C overnight in 100% methanol and paraffin-embedded (Paraplast, Monoject) by standard procedures. Sections of 10 μm were cut and dried onto subbed slides (0.5% gelatin, 0.05% chromium potassium sulfate). Slides were deparaffinated and incubated with 3% BSA in PBS for 45 min at 4°C. The slides were then incubated with primary antibody (purified antibodies or diluted antiserum) at 4°C overnight. After two washes with PBS (1× 30 min, 1× 15 min) at RT, slides were incubated again in 3% BSA in PBS for 5–10 min at 4°C. Incubation with FITC-conjugated anti-rabbit IgG (Sigma) at 1 : 100 dilution in PBS was for 4–6 hr at 4°C in the dark. Slides were washed 2× 30 min with PBS at RT, counterstained by incubation with 0.5 μg/ml 4,6-diamidino-2-phenylindole (DAPI) for 5 min, and washed for 30 min with PBS. Slides were mounted with pH 8.5 buffered glycerol-polyvinylalcohol mounting medium containing 25 grams/liter NaI as an antibleach (Böck et al. 1985). Sections were examined using epifluorescence microscopy, with appropriate filters for viewing DAPI and fluorescein fluorescence.

Preparation of extracts and immunoblotting

Nuclear protein extracts from 150 mixed-stage preneurulae embryos (majority stages 9 and 10) were prepared according to the method of Risley and Eckhardt (1981), except that L-1-tosyllamide-2-phenylethylchloromethylketone was not added to the buffers.

To prepare extracts of acid-soluble proteins, oocytes, eggs, or embryos were homogenized in 20 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1 mM PMSF, 13 mM ME, 0.5 μg/μl of pepstatin A, 0.7 μg/μl of leupeptin, and precipitated on ice with 70% perchloric acid to give a final concentration of 5% perchloric acid. After brief sonication, the sample was left on ice for 30 min. The precipitate was collected by centrifugation (10,000 rpm, 30 min) and the supernatant precipitated by addition of 0.2 volume of 100% trichloroacetic acid. This precipitate was collected, washed once in acidified acetone, twice with acetone, and finally dissolved in sample buffer for gel analysis.

Protein extracts were separated by SDS–polyacrylamide gel electrophoresis and immunoblotting performed by standard procedures (Towbin et al. 1979; Burnette 1981).

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Note

Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries.

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Expression of a histone H1-like protein is restricted to early Xenopus development.

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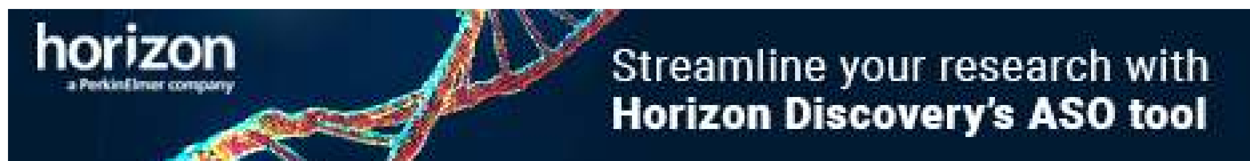

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