Int. J. Dev. Biol. 37: 509-517 (1993)

Original Article

Identification of an amphibian oocyte nuclear protein as a candidate for a role in embryonic DNA replication

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ABSTRACT **Monoclonal antibody $B_{24/3}$ recognizes a nuclear protein of 104 kD in germinal vesicles of newt oocytes. Immunohistostaining of oocytes at different stages of growth shows an accumulation of B_{24} protein throughout oogenesis. During development B_{24} protein is located inside embryonic cell nuclei from the onset of cleavage onwards. It gradually decreases from gastrulation and disappears at the tailbud stage. The NvB_{24} 17.1 clone was isolated from an ovary expression library of the newt *Notophthalmus viridescens* and then sequenced: the open reading frame is capable of encoding a polypeptide of 744 amino acids. Northern blot experiments have shown that the 17.1 clone recognizes a single transcript of about 3 Kb in the ovary. *In situ* hybridization experiments showed that B_{24} mRNA transcription starts from previtellogenic oocytes, and is followed by the appearance and gradual accumulation of B_{24} protein in germinal vesicles of medium and large size oocytes. Keeping in mind the sequence similarity shown by the B_{24} protein to the mouse P1 protein as well as to the budding yeast Mcm3 and fission yeast cdc21 proteins, B_{24} protein can be speculated to play a role in the events of DNA replication during early amphibian embryogenesis. As B_{24} antigen is located in the sphere organelles both inserted on the lampbrush chromosomes and free in the oocyte nucleoplasm, an additional possible role of B_{24} protein could be related to assembling and/or storing snRNPs during oogenesis.**

KEY WORDS: *oogenesis, embryogenesis, gene expression, immunofluorescence, in situ hybridization*

Introduction

The oocyte acts as a storehouse for chromosomal and nuclear proteins which are responsible, at least partially, for the genetic link existing between oogenesis and early development. It is well known that the oocyte transfers to the embryo common cellular components, such as polymerases, proteins involved in the constitution and assembly of nucleosomes, as well as factors which can play a role in the processes of determination and/or differentiation of embryonic cells (Davidson, 1986).

With that in mind, several authors have produced libraries of monoclonal antibodies against germinal vesicle antigens in order to identify and characterize possible maternal proteins which play a significant role in precocious events of embryogenesis (Dreyer *et al.*, 1981, 1982, 1985; Lacroix *et al.*, 1985; Dreyer, 1987; Roth and Gall, 1987).

The fate of antigens recognized by such antibodies was followed throughout oogenesis and development by immunostaining on isolated lampbrush chromosomes (Lacroix *et al.*, 1985; Roth and Gall, 1987; Ragghianti *et al.*, 1988) as well as on sections of oocytes and embryos (Krone and Franke, 1980; Dreyer and Hausen, 1983; Abbadie *et al.*, 1987; Boucher *et al.*, 1991).

In order to define the characteristics and the role of these maternal proteins during oogenesis and development, the sequences of some of them have been established from cDNA clones isolated by means of mAbs from expression libraries of ovary RNA (Miller *et al.*, 1989; Roth and Gall, 1989; Reddy *et al.*, 1991; Bellini *et al.*, 1993). Thus, Reddy *et al.* (1991) have described a clone from *Xenopus laevis* that encodes a protein, named xnf7, maternally expressed and preferentially located in the nuclei of both the central nervous system during late development and in the adult brain. Recently, Bellini *et al.* (1993) have isolated a clone from *Pleurodeles waltl* library that codifies a nuclear protein (A_{33}) localized on the loops of lampbrush chromosomes. These two proteins belong to a

Abbreviations used in this paper: $B_{24/3}$ mAb, $B_{24/3}$ monoclonal antibody; 17.1 clone, NvB_{21} 17.1 clone; aa, amino acid; ARS, autonomously replicating sequences; bp, base pairs; MW, molecular weight; ORF, open reading frame; snRNPs, small nuclear RNPs.

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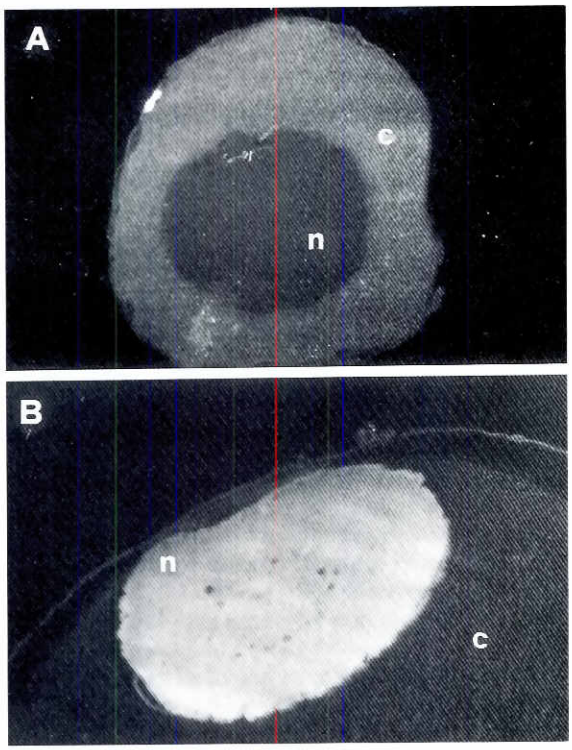

Fig. 1. Immunostaining of oocyte sections of *T. carnifex* with $B_{24/3}$ mAb. (A) *A previtellogenic oocyte: the nucleus (n) is unstained, while a weak fluorescence is visible in the cytoplasm (c).* **(B)** *A fully grown oocyte in which the germinal vesicle is heavily labeled and the cytoplasm is negative (x210).*

small family corresponding to a new class of zinc finger proteins (Bellini *et al.*, 1993).

Previous studies have described another nuclear protein (B_{24}) occurring in germinal vesicles of several salamandrid species in association with spheres (Lacroix *et al.*, 1985; Ragghianti *et al.*, 1988). These oocyte organelles are present both free in the nucleoplasm and attached to the specific sphere organizers of lampbrush chromosomes. The spheres have been recently shown to contain snRNPs and to correspond to snurposomes C (Gall and Callan, 1989; Gall, 1992). MAb against B_{24} protein cross-reacts also with the spheres of germinal vesicles. According to Boucher *et al.* (1991), in *Pleurodeles*, the B_{24} protein accumulates in the nucleoplasm of growing oocytes and is retained in embryonic nuclei until the tailbud stage.

The present paper reports the pattern of expression of the B_{24} protein during oogenesis and embryogenesis in the newt species *Triturus carnifex*. Subsequently, 40 cDNA clones were isolated by the $B_{24/3}$ monoclonal antibody from an ovary expression library of the newt *Notophthalmus viridescens* (J.G. Gall, unpublished results). One of the clones, named NvB_{24} 17.1, was sequenced and used to study the pattern of the corresponding mRNA expression during oogenesis. The sequence analysis has shown that the B_{24} 17.1 cDNA clone encodes a putative protein which has a high similarity with a family of eukaryotic proteins involved in the initiation of DNA replication. The possible roles of that protein in oogenesis and development are discussed.

Results

Immunodetection of B_{24} antigen during Triturus oogenesis and embryogenesis

The distribution of B_{24} protein was studied by immunostaining of oocyte and embryo sections.

In previtellogenic oocytes (diameter smaller than 500 μm) the germinal vesicle is not stained, while a weak fluorescence is found in the cytoplasm, revealing that expression of B_{24} protein has already started (Fig. 1A). The intensity of fluorescence within nuclei progressively increases during oogenesis and becomes especially evident (intense and homogeneous) in larger vitellogenic oocytes (Fig. 1B). Follicular cells are negative.

During embryogenesis, nuclei are stained starting from early cleavage until gastrulation (Fig. 2A and B). At late gastrula nuclear staining is restricted to the endoderm cells, where it becomes progressively weaker until the tailbud stage. This observation appears clearly when the same immunostained sections (Fig. 2C and D) are also stained by DAPI (Fig. 2E and F). Antigen B_{24} was not detectable on sections of larvae after hatching (data not shown).

These results are in agreement with those previously obtained by immunoblotting of total protein extracts derived from germinal vesicles, staged embryos and adult tissues of both *Pleurodeles* and *Triturus* (Boucher *et al.*, 1991; Mancino, 1991). In those experiments $B_{24/3}$ mAb recognized a 104 kD polypeptide which is quite abundant in oocyte germinal vesicles and in embryos until the mid-gastrula stage. Afterwards it progressively decreases; the B_{24} antigen was not detectable in swimming larvae and in adult tissues, except the ovary.

Isolation and characterization of NvB_{24} 17.1 clone

Several clones have been obtained by screening an ovary cDNA expression library of *Notophthalmus viridescens* with $B_{24/3}$ mAb, and three of those clones having the largest inserts were selected (J.G. Gall, unpublished observations). A preliminary partial sequencing of the three clones showed that they represent different sized cDNAs from the same RNA (data not shown).

The largest clone, indicated as NvB_{24} 17.1, has been completely sequenced on both strands. The 17.1 clone is 2587 bp long and has an ORF of 2234 bp ending with a stop codon at position 2234. Two potential polyadenylation signals (AATAAA) occur in the untranslated region at 13 and 353 bp respectively, upstream of the end of the clone (Fig. 3). The ORF is capable of encoding a polypeptide of 744 aa with a calculated MW of 82 kD, a size which is 79% that of the full-sized B_{24} protein (104 kD), as determined by immunoblot of ovary and embryo extracts. These data, together with the finding of a deep internal location of the first *met* codon, indicate that the cDNA is truncated at its 5' end.

Although incomplete, the determination of the 17.1 clone sequence has provided some conceptual information concerning the protein it encodes. The derived amino acid sequence of the 17.1 cDNA shows a hydrophilic character generated by a content of charged amino acids of about 31%; moreover, it possesses a number of potential phosphorylation sites. In particular, a putative $p34^{cdc2}$ protein kinase phosphorylation site, TPRK (cf. Moreno and Nurse, 1990) encompasses amino acids 725-728 (Fig. 3). The sequence analysis did not reveal any motif commonly found in DNA binding proteins or protein-protein interactions.

A computer search analysis using the EMBL FASTP program (release no. 34) has revealed that 17.1 gene product is 71.5%

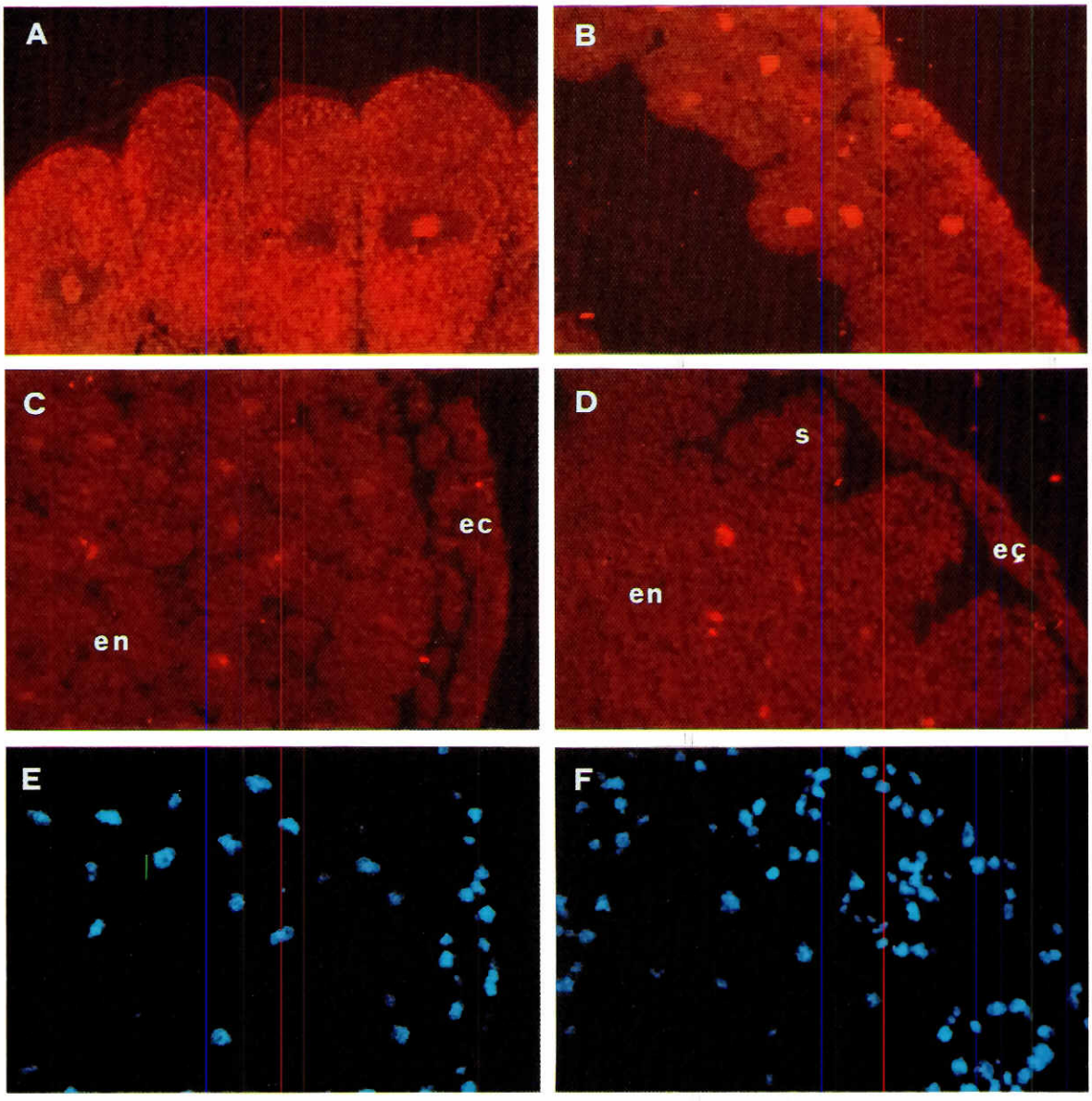

Fig. 2. Sections of *T. carnifex* embryos immunostained with $B_{24/3}$ mAb. *The nuclei are stained at cleavage* **(A)**, *blastula* **(B)**, *gastrula* **(C)** *and tailbud* **(D)** *stages with decreasing intensity and, starting from late gastrula, the staining is restricted to endodermal nuclei* **(C, D); (E** and **F)** *indicate the same sections as* **(C** and **D)**, *stained by DAPI. ec: ectoderm, en: endoderm, s: somite (x150).*

identical to the mouse P_1 protein (Thömmes *et al.*, 1992) in 715 aa overlap, 53.2% identical to *Saccharomyces cerevisiae* Mcm3 protein (Gibson *et al.*, 1990) in 513 aa overlap and 48.1% identical to *Schizosaccharomyces pombe* cdc21 protein (Coxon *et al.*, 1992) in 239 aa overlap (Fig. 4). In particular, a central region of about 200 aa is well conserved in all these proteins, suggesting an important functional role for this domain (Fig. 4).

Immunodetection of the bacterially expressed 17.1 fusion protein by $B_{24/3}$ mAb

In order to test whether 17.1 clone encodes the nuclear protein recognized by $B_{24/3}$ mAb, we expressed the 17.1 clone in the *E. coli* strain DH5. A band of M_r= 86 kD was recognized when translation products were analyzed on immunoblots with $B_{24/3}$ mAb (Fig. 5). This was expected because the calculated MW of 17.1 fusion protein is 86 kD. In fact 17.1 is cloned at the Eco RI site in Bluescript SK(-), in which 4 kD of the amino terminus of *E. coli* ß-galactosidase is in frame with the 744 aa of the 17.1 ORF.

Expression and spatial distribution of B_{24} mRNA

Using the 17.1 clone as a probe, total RNA and poly(A^+) mRNA derived from ovary, liver and embryos of *T. carnifex* were analyzed by Northern blotting. The results indicate that only in the ovary does the 17.1 clone recognize a transcript of about 3 Kb, which is large enough to encompass the predicted ORF coding for a 104 kD B_{24} protein (Fig. 6).

Fig. 3. Nucleotide sequence of 17.1 clone and the deduced amino acid sequence. *Asterisks (*) mark the first stop codon downstream the open reading frame. The poly(A)-signals are indicated with (▼). A potential p34^{cdc2} protein kinase phosphorylation site is boxed. Since the amino terminus has not yet been determined, the amino acid encoded by the 5' most codon is arbitrarily designed as residue nr. 1.*

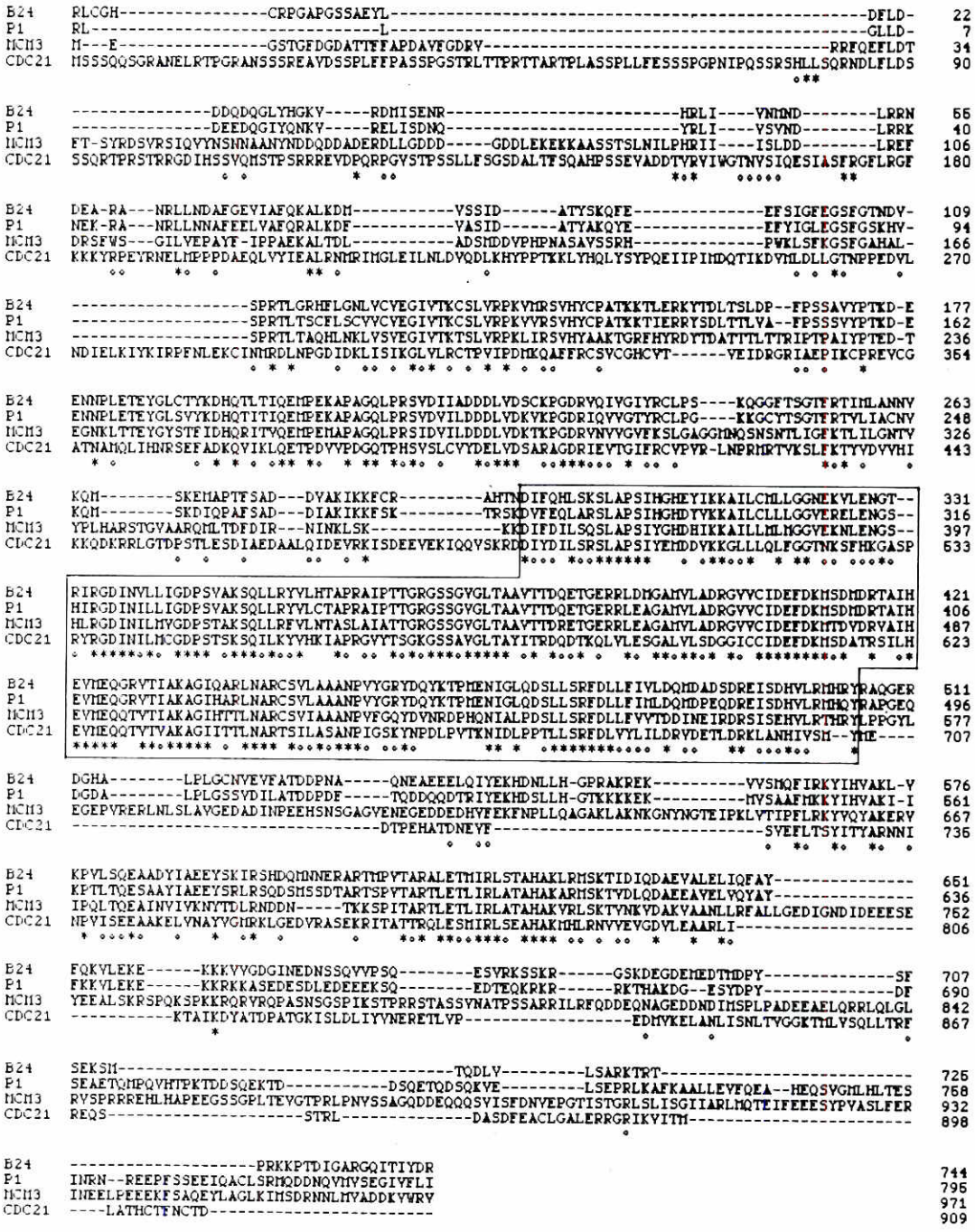

Fig. 4. Comparison of B_{24} sequence with related proteins. *The B_{24} protein sequence (top) is shown and compared to (order top to bottom) mouse P_1, Saccharomyces cerevisiae Mcm_3 and Schizosaccharomyces pombe cdc21. The alignment of the four sequences was generated using CLUSTAL V (fixed gap penalty= 10; floating gap penalty= 10; protein weight matrix= pam 250). Positions where all residues are identical are marked with a star (*) and positions where all residues are similar are marked with a dot (o). (—) indicates padding characters inserted to optimize the alignment. A central region of about 200 aa with the highest degree of similarity is boxed.*

In these experiments we could not detect any transcript either in liver or in embryos.

A possible explanation for this result is that either the B_{24} mRNA is not expressed in these tissues or is expressed at a level below detection by Northern blot. Thus further investigations are necessary to assess the expression of B_{24} mRNA either in somatic tissues or in embryos.

The distribution of B_{24} mRNA within oocytes at different growing stages was analyzed by *in situ* hybridization of ovary sections, using synthetic sense and antisense RNA probes.

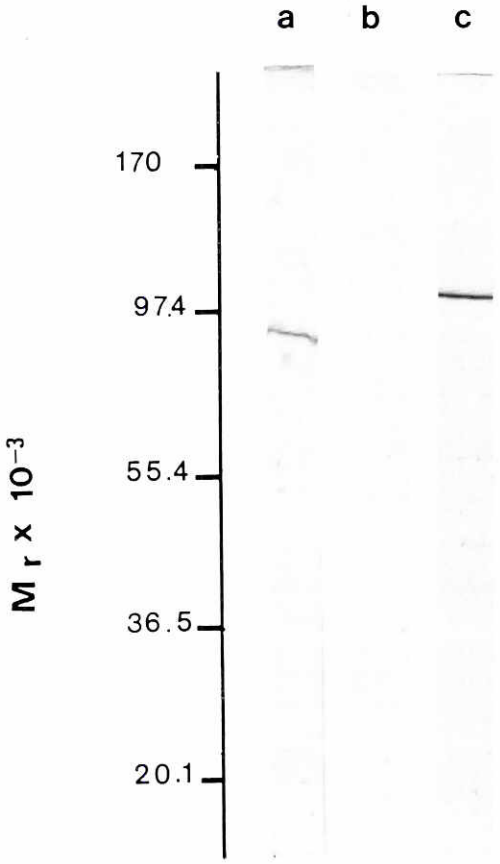

Fig. 5. Immunodetection of the 17.1 fusion protein by the $B_{24/3}$ mAb. *The B_{24} mAb recognizes a 17.1 fusion product of about 86 kD* **(lane a)**. *No product was detected with the $B_{24/3}$ mAb in protein extracted from bacteria transformed with Bluescript SK phagemid lacking any inserts* **(lane b)**. *The B_{24} protein of 104 kD is recognized by the $B_{24/3}$ mAb in total proteins of Triturus embryos at cleavage* **(lane c)**. *Molecular weight standards indicated on the left: trypsin inhibitor (20.1×10^3), lactate dehydrogenase (36.5×10^3), glutamate dehydrogenase (55.4×10^3), phosphorylase b (97.4×10^3), reduced α_2 macroglobulin (170×10^3), from bottom to top.*

The cytoplasm of previtellogenetic oocytes (with diameter smaller than 500 μm) is intensely labeled by using antisense RNA probe, while the germinal vesicles appear unlabeled, as do the surrounding follicular cells (Fig. 7A). In later stages, the vitellogenic oocytes show a lower grain density probably due to a dilution effect caused by the increase of cellular volume (Fig. 7A). Control oocyte sections, hybridized with sense RNA, show no labeling after comparable exposure times (Fig. 7B).

In situ hybridization experiments have been carried out on isolated lampbrush chromosomes of *T. carnifex*, with the aim of localizing the active sites of transcription of B_{24} gene. By using the antisense ^{3}H RNA probe to the nascent transcripts, a loop of normal matrix and size subterminally located on the long arm of chromosome I appears constantly labeled (Fig. 8). No labeling was observed using sense ^{3}H RNA probe (data not shown). This result suggests that transcription of B_{24} gene can occur also in vitellogenic growing oocytes at diplotene.

Discussion

Several germinal vesicle proteins of amphibian oocytes have been identified using monoclonal antibodies (Dreyer *et al.*, 1981, 1982; Dreyer and Hausen, 1983; Lacroix *et al.*, 1985; Abbadie *et al.*, 1987). In the present report, $B_{24/3}$ mAb was shown to identify a nuclear protein synthesized in the oocyte which behaves as a typical maternal protein since it does not disappear at the end of oogenesis and maturation. It survives inside the developing egg for some time. These results parallel those previously obtained on immunoblots where $B_{24/3}$ mAb recognizes a protein of MW= 104 kD which is detectable in oocytes and embryos until the tailbud stage (Ragghianti *et al.*, 1988; Mancino, 1991).

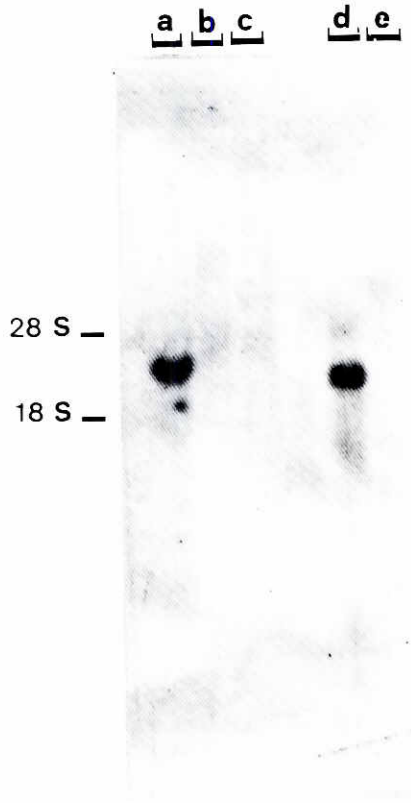

Fig. 6. Northern blot analysis of B_{24} mRNA expression. *Total RNA or poly(A)$^+$ RNA extracted from ovary, adult liver, and embryos were electrophoresed, blotted into Hybond membrane and hybridized with 17.1 cDNA probe. The samples contain: 80 μg of ovary total RNA* **(lane a)**; *40 μg of total RNA from both cleavage* **(lane b)** *and tailbud* **(lane c)** *embryos; 3 μg of ovary and liver poly(A)$^+$ RNA* **(lanes d and e)**. *A band at about 3 Kb is present in total and poly(A)$^+$ RNA from ovary* **(lanes a and d)**.

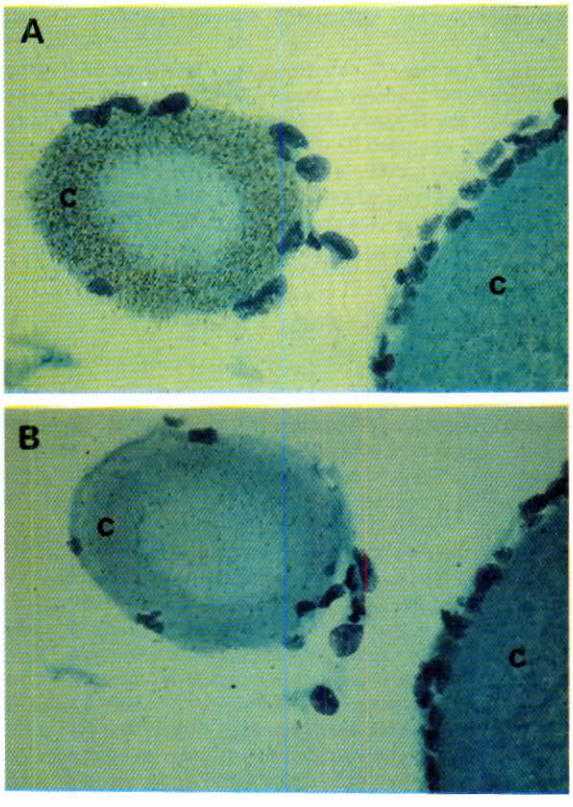

Fig. 7. *In situ* hybridization of *in vitro* synthesized 17.1 RNA to different sized oocytes. (A) *Oocyte sections hybridized with antisense ^{35}S RNA probe: cytoplasm (c) of the previtellogenic oocyte is heavily labeled; the autoradiographic signal is weaker in cytoplasm of the vitellogenic oocyte, probably due to a dilution effect of the B_{24} mRNA level.* **(B)** *Oocyte sections probed with sense 35S RNA: both nucleus and cytoplasm are not labeled (x210).*

By means of $B_{24/3}$ mAb, several cDNA clones have been isolated from an ovary expression library of *N. viridescens* and the clone containing the largest insert has been sequenced. Although 17.1 clone is truncated at the 5' end, the determination of its sequence has provided valuable information about the conceptual protein it encodes. A computer search analysis within EMBL data library revealed that the putative protein encoded by 17.1 clone is 71.5% identical to mouse P_1 protein (Thömmes *et al.*, 1992), 53.2% identical to *S. cerevisiae* Mcm3 protein (Gibson *et al.*, 1990; Yan *et al.*, 1991) and 48.1% identical to *Schizosaccharomyces pombe* cdc21 gene product (Coxon *et al.*, 1992). These similarities strongly suggest that 17.1 gene product may be an amphibian equivalent member of a recently discovered family of conserved nuclear proteins which have been implicated in early events of eukaryotic chromosome replication (Coxon *et al.*, 1992). In particular, mouse P_1 protein is associated with the polymerase-α primase complex and is believed to have a function in the replication of cellular DNA similar to the role played by T antigen in SV40 DNA replication. The Mcm3 protein of yeast has been shown to be involved in the early steps of DNA replication, probably activating the ARS elements. The cdc21 gene was originally identified in a screen for cdc mutants affecting S phase and nuclear division. Since the highest rate of identity is found between 17.1 gene product and the mouse P_1 protein, 17.1 gene may be considered the amphibian homologue of mammalian P_1 gene.

We suggest that the protein encoded by the 17.1 clone corresponds to the endogenous 104 kD protein recognized by the $B_{24/3}$ antibody. This assumption is supported by the consideration that the bacterially expressed protein from 17.1 clone and endogenous B_{24} protein share an epitope recognized by $B_{24/3}$ mAb. Moreover, in Northern blot experiments, the 17.1 clone reveals a single RNA species of about 3 Kb, which is the right size to encode a 104 kD protein. Further evidence for the claim that the protein encoded by 17.1 clone corresponds to the endogenous B_{24} protein is based on the observation that B_{24} mRNA expression occurs in the ovary starting from previtellogenic oocytes and precedes the appearance of B_{24} protein within the nucleus.

At present, we can only speculate on the possible functions of the B_{24} protein. Given the similarity of B_{24} to P_1, Mcm3 and cdc21 proteins, we can hypothesize an involvement of B_{24} protein in DNA replication initiation during embryogenesis. The way by which B_{24} protein could be involved in the replication processes may be discussed in light of two models concerning DNA replication in eukaryotes, elaborated by Callan (1973) and recently reviewed by Diffley and Stillman (1990). The first model postulates the existence of different classes of replication origins recognized by different initiator proteins in order to explain the differences in origin usage between somatic cells in culture and early embryonic cells. According to this model, all classes of initiator proteins are present in early embryos in which replication is fast, while only some of them are present in somatic cells in culture, where replication proceeds slowly. Although this model needs better experimental evidence, it should be recalled that Mcm3 mutants are unable to maintain plasmids containing only certain ARS elements, suggesting a specific interaction of this protein with specific ARS elements (Yan *et al.*, 1991). It is also likely that the cdc21 protein might be needed to activate only a subset of replication origins (Coxon *et al.*, 1992). In line with this first model, the B_{24} antigen, which is present in precocious embryonic stages, may be considered as a factor which is able to interact with replication origins specific to early development.

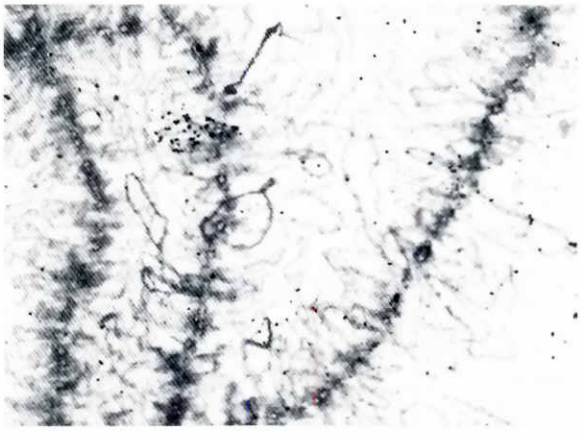

Fig. 8. *In situ* hybridization of the ^3H 17.1 antisense riboprobe to the nascent transcripts of lampbrush chromosomes of *T. carnifex*. *A single loop is labeled on chromosome I (x720).*

Callan's alternative model (1973) predicts that the number of origins used is regulated by the availability of key proteins: in this case, the high concentration of B_{24} protein in embryonic cell nuclei could be responsible for the greater number of origins used in DNA replication in early development.

B_{24} antigen represents the first protein detected in the spheres of lampbrush chromosomes (Lacroix *et al.*, 1985). More recently, these organelles have been studied by means of monoclonal antibodies and ^3H riboprobes (Gall and Callan, 1989; Boucher *et al.*, 1991; Wu *et al.*, 1991; Gall, 1992) and were shown to contain snRNPs (Gall and Callan, 1989). Boucher *et al.* (1991) believe that B_{24} antigen could not represent a constitutive protein of snRNPs according to its immunostaining pattern and MW, and suggest that B_{24} has a role in assembling and/or storing snRNPs during oogenesis. Thus, the spheres can be assumed to play a role in the assembly of snRNP complexes for the nucleus, comparable to the way that nucleoli assemble ribosomal RNP complexes for the cytoplasm (Gall and Callan, 1989). However, the parallel between spheres and nucleoli in amphibian oogenesis appears limited, because U snRNA genes do not undergo amplification as ribosomal genes do (Phillips *et al.*, 1992).

Therefore, the results here reported show that the B_{24} protein may have a different function in both oogenesis (a role in the growth and the organization of the snurposomes C) and development (a role in the precocious events of DNA replication). These two potential functions require further investigation.

Materials and Methods

Oocytes, embryos and larvae studied in the present work refer to the newt species *Triturus carnifex* (Laurenti, 1768) from Pisa. The stages of development of embryos were determined taking into account various normal tables, particularly those of Gallien and Durocher (1957).

The procedures for hybridoma production and ELISA tests have been described elsewhere (Lacroix *et al.*, 1985).

Immunohistology

Fragments of ovaries, whole embryos and larvae were fixed in 0.25 M NaCl, 2% formaldehyde, 5% acetic acid for 1 h and embedded in paraffin (fusion point: 56°C). The sections, 5-8 µm thick, were first deparaffinized with xylene and rehydrated. They were then incubated in inactivated rabbit serum (Dakopatts: X302), 1:20 diluted in PBS (130 mM NaCl, 15 mM Na_2HPO_4, 2 mM NaH_2PO_4, pH 7.8) for 10 min. Next, the sections were incubated with mAb $B_{24/3}$ diluted 1:250 in PBS for 1 h and washed 3 times in PBS for 10 min each. The antigen-antibody complexes were visualized using a TRITC-conjugated second antibody (Dakopatts: R270) diluted 1:50 in PBS with 0.4 µg/ml DAPI added (Sigma: D-1388). Incubation in the latter solution, in the dark, lasted for 30 min. After 3 rinses in PBS of 10 min each, the sections were mounted in glycerol and PBS (2:1) and observed under a Zeiss photomicroscope equipped for phase-contrast and epifluorescence observations.

DNA sequencing

MAb $B_{24/3}$ was used to select some clones from an expression library of *N. viridescens* ovary cDNA cloned in the vector lambda Zap (J.G. Gall, unpublished results). Three clones (designated as NvB_{24} 33.1, NvB_{24} 27.1, NvB_{24} 17.1), converted to the corresponding Bluescript phagemid, were selected for the present study. Only the longest clone (NvB_{24} 17.1) was completely sequenced on both strands using the Sanger chain-termination method (Sanger *et al.*, 1977). Unidirectional exonuclease III deletions of the insert were constructed according to Henikoff (1987). After size selection, the antisense strand of individual deleted clones was sequenced using the primer T_7 with the T_7 sequencing kit (Pharmacia). To complete the sequence, the sense strand was sequenced with synthetic oligonucleotides used as primers.

Fusion protein

Because 17.1 is cloned into the phagemid Bluescript SK(-) at the Eco RI site, an inducible *lac* promoter upstream from the *lac* Z gene permits fusion expression with the ß-galactosidase gene product *in vivo*. The bacterial strain *E. coli* DH5 was used to express the fusion protein as described by Maniatis *et al.* (1982). Bacteria were transformed with the 17.1 clone and the *lac* promoter was induced with 5 mM IPTG. The bacterial protein extracts were prepared according to Maniatis *et al.* (1982), separated in a SDS 8%-15% polyacrylamide gradient, and blotted to a nitrocellulose filter. Translation products were analyzed on immunoblots with $B_{24/3}$ mAb (Ragghianti *et al.*, 1988). Controls consisted of proteins from bacteria transformed with Bluescript SK phagemid that lacked any insert.

Northern blotting

Total RNA was extracted from ovary, liver and embryos using a guanidinium isothiocyanate/hot phenol procedure (Kit Clontech, USA). All samples were treated with 1 unit of RQ1 DNase (Promega) per microgram of total nucleic acid, reextracted with phenol and chloroform/isoamyl alcohol (24:1 vol/vol), and precipitated with ethanol. RNA degradation was monitored by ethidium bromide staining of gel prior to blotting.

The poly(A^+) RNA was purified by chromatography on oligo(dT)-cellulose successively performed twice or by Dynabeads mRNA purification kit (Dynal).

Total RNA (80 µg from ovary and liver; 40 µg from embryos) and RNA poly(A^+) (1-3 µg) was denatured in 50% formamide, 15% formaldehyde and separated on 1% agarose gels containing 2.2 M formaldehyde. The RNA was transferred and UV-crosslinked to Hybond N membranes (Amersham-Buchler) or nitrocellulose membrane (Schleicher & Schuell BA85, 0.45 µm). The membrane-bound RNA was hybridized with ^{32}P-labeled 17.1 clone overnight at 42°C in 40% formamide. Filters were washed at high stringency conditions (a first wash in 0.5xSSC, 0.1% SDS for 30 min at 42°C, followed by a second wash in 0.1xSSC, 0.1% SDS for 15 min at 42°C), then processed for autoradiography by standard procedures (Maniatis, 1982). The cDNA probe was labeled to a specific radioactivity of $3x10^8$ c.p.m./µg DNA using a random-primed DNA labeling kit from Boehringer Mannheim.

In situ hybridization

Oocyte sections

Fragments of ovaries were fixed in 0.25 M NaCl, 2% formaldehyde, 5% acetic acid for 1 h and embedded in paraffin. 5-8 µm sections were deparaffinized and postfixed with 4% paraformaldehyde in PBS for 20 min and treated with proteinase K (3 µg/ml) for 15 min at 37°C. After incubation in 0.1 M triethanolamine containing acetic anhydride (0.25% vol/vol) for 10 min, slides were washed in PBS. RNA probes were made by *in vitro* transcription in the presence of ^{35}S-UTP (1000 Ci/mmol, Amersham) (Krieg and Melton, 1987). Sense and antisense RNAs were synthesized using as template the 17.1 clone linearized at the Cla I or Sma I sites, respectively, and transcribed by either T_3 or T_7 RNA polymerases; probes were hydrolyzed to an average of 150 bp. Hybridization was done at 42°C overnight with $3x10^6$ c.p.m. of RNA probe per slide (in 55% formamide, 0.34 M NaCl, 0.02 M Tris-HCl pH 7.4, 5.65 mM EDTA pH 8.0, 0.01 M NaH_2PO_4 pH 7.6, 11% dextransulphate, 1.1x Denhardts, 0.56 mg/ml tRNA *E. coli*). The hybridized slides were washed in 5xSSC, 50% formamide at 50°C for 30 min; the final wash after RNase digestion was made in 2xSSC at 37°C for 15 min. Slides were dipped into NTB2 emulsion (Kodak), diluted 1:1 with H_2O, and exposed at 4°C for 5-15 days. After development, slides were stained with Giemsa.

Lampbrush chromosomes

The preparations of lampbrush chromosomes were made according to Old *et al.* (1977). They were pretreated with proteinase K (0.1 µg/ml) for 30 min at 42°C, washed 3 times in 2xSSC and dehydrated. Sense and antisense RNA probes were made by *in vitro* transcription in the presence of ^3H-UTP (42 Ci/mmol, Amersham). Hybridization was done at 42°C overnight with $4x10^5$ c.p.m. of RNA probe per slide (in 40% formamide, 4xSSC, 0.1 M Tris-HCl pH 7.5). The hybridized slides were washed in 2xSSC and then in 1xSSC at 65°C for 1 h. Some preparations were washed in 0.5xSSC at 65°C for 1 h. The final wash after RNase digestion was made

in 2xSSC. Control experiments were carried out by doing some preparations with sense RNA as a probe, and by treating some other preparations with RNase before hybridizing them with antisense probe. The slides were dehydrated, dipped into NTB2 emulsion and exposed at 4°C for 1-2 months. After development, lampbrush chromosomes were stained with Coomassie blue R.

Acknowledgments

We are particularly indebted to Dr. J.G. Gall (Baltimore) for his generous gift of NvB_{24} clones and for providing encouragement to carry on this research. The skilled computer assistance of Dr. Paolo Deri is gratefully acknowledged. The work was supported by grants from C.N.R. and M.U.R.S.T. (Roma) to G.M.

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Accepted for publication: November 1993