# pEg7, a New *Xenopus* Protein Required for Mitotic Chromosome Condensation in Egg Extracts

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*Abstract.* We have isolated a cDNA, Eg7, corresponding to a *Xenopus* maternal mRNA, which is polyadenylated in mature oocytes and deadenylated in early embryos. This maternal mRNA encodes a protein, pEg7, whose expression is strongly increased during oocyte maturation. The tissue and cell expression pattern of pEg7 indicates that this protein is only readily detected in cultured cells and germ cells. Immunolocalization in *Xenopus* cultured cells indicates that pEg7 concentrates onto chromosomes during mitosis. A similar localization of pEg7 is observed when sperm chromatin is allowed to form mitotic chromosomes in cytostatic factor-arrested egg extracts. Incubating these extracts with antibodies directed against two distinct parts of pEg7 provokes a strong inhibition of the condensation and resolution of mitotic chromosomes. Biochemical experiments show that pEg7 associates with *Xenopus* chromosome-associated polypeptides C and E, two components of the 13S condensin.

Key words: chromatin condensation • mitotic chromosomes • *Xenopus laevis* • egg extracts • chromosome-associated proteins

I n most animal species, the early stages of embryonic development are characterized by a period of very rapid cell divisions called cleavages. In *Xenopus*, the first cleavage occurs 1.5 h after fertilization and is followed by 11 almost synchronous cell divisions that occur every 30 min (Newport and Kirschner, 1982). During this period, as well as during oocyte maturation, cell cycle progression is totally independent of transcription, but cannot proceed without protein synthesis. Qualitative analyses of the proteins synthesized in oocytes (Stage VI), unfertilized eggs, and embryos have shown that during oocyte maturation and after fertilization new proteins appear, whereas others are no longer synthesized (Bravo and Knowland, 1979; Meuler and Malacinski, 1984; Smith, 1986). This suggests that the synthesis of specific gene products, necessary for oocyte maturation, and then for the rapid division of blastomeres, is regulated at the translational level from the bulk of maternal mRNAs. Differential screening of a *Xenopus* egg cDNA library has been undertaken in order to isolate mRNAs encoding such proteins. 11 cDNAs corresponding to mRNAs that are either polyadenylated and loaded into polysomes (clones Cl1 and Cl2) or deadenylated and released from polysomes (clones Eg1–Eg9) after fertilization have been isolated by differential screening (Paris and Philippe, 1990). Three of the Eg proteins have already been characterized, all of these playing important roles in the control of cell cycle: Eg1/cdk2 controls the G1/S transition in higher eukaryotes (Paris et al., 1991), whereas Eg2 (Roghi et al., 1998) and Eg5 (Le Guellec et al., 1991; Sawin et al., 1992) are both required for mitotic spindle assembly. In the present report, we characterize another Eg protein, pEg7, which is localized on chromosomes during mitosis and is required for chromosome condensation.

During cell division, it is essential that each daughter cell receives a complete set of chromosomes. The proper segregation of sister chromatids, which occurs at anaphase, depends on the ability of chromosomes to be properly formed, and then aligned on the metaphase plate. This process requires the chromatin to be condensed, leading to the formation of resolved, fully compacted mitotic chromosomes (for review, see Hirano, 1995; Koshland and Strunnikov, 1996).

Chromosome condensation requires DNA topoisomerase IIα (Adachi et al., 1991; Uemura et al., 1987) and a

group of proteins called structural maintenance of chromosomes (SMCs).[1] A breakthrough in elucidating the mechanism of condensation was the discovery of the SMC proteins (for review, see Gasser, 1995; Hirano et al., 1995). These proteins, which are putative ATPases, are conserved from bacteria to human (Koshland and Strunnikov, 1996), and are involved in several processes such as chromosome condensation (Hirano and Mitchison, 1994; Saka et al., 1994; Strunnikov et al., 1995), sister chromatid cohesion and separation (Michaelis et al., 1997), gene dosage compensation (Lieb et al., 1998), and DNA repair (Jessberger et al., 1996).

The mechanisms by which the SMCs contribute to chromosome condensation are just starting to be elucidated. Two SMC proteins have been characterized in *Xenopus* by Hirano and Mitchison (1994) and given the names *Xenopus* chromosome-associated polypeptides C and E (XCAP-C and XCAP-E). Sequence analysis revealed that XCAP-C and XCAP-E are homologous to the budding yeast proteins SMC4 (Jessberger et al., 1998) and SMC2 (Strunnikov et al., 1995), and to the fission yeast cut3 and cut14 gene products, respectively (Saka et al., 1994). XCAP-C and XCAP-E were found to be associated with mitotic chromatids assembled from demembranated sperm nuclei incubated in egg mitotic extracts. The addition of anti–XCAP-C antibodies to extracts allowed a partial compaction that was blocked at a stage corresponding to long and extended chromosomes (Hirano and Mitchison, 1994). When added after chromosome condensation had been completed, these antibodies also destabilized the condensed chromosome structure, suggesting that XCAP-C activity is necessary for both the assembly and maintenance of condensed chromosomes (Hirano and Mitchison, 1994).

Recent data from *Xenopus* and *Schizosaccharomyces pombe* indicate that XCAP-C (cut3) and XCAP-E (cut14) are components of higher order complexes (Hirano et al., 1997; Sutani and Yanagida, 1997). In *Xenopus*, XCAP-C and XCAP-E are components of two complexes sedimenting at 8S and 13S. The 8S complex, which contains only XCAP-C and XCAP-E, is able to mediate a partial chromatin condensation in mitotic egg extracts. However, the 13S complex, which contains three additional proteins, is able to complete this process (Hirano et al., 1997).

In this paper, we investigated the role of pEg7. We showed that the addition of anti–pEg7 antibodies in egg extracts blocked chromosome condensation. Biochemical experiments indicate that pEg7 associates with XCAP-C and XCAP-E, but not with topoisomerase IIα. We propose that pEg7 is one component of the 13S condensin complex.

## Materials and Methods

### Cloning of cDNAs Encoding Xenopus pEg7

A partial Eg7 cDNA was isolated by differential screening of an unfertilized *Xenopus* egg λgt10 cDNA library as already described (Paris and Philippe, 1990). Four overlapping clones (Eg7.1–Eg7.4) were isolated

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1. *Abbreviations used in this paper:* CSF, cytostatic factor; SMC, structural maintenance of chromosomes; XCAP, *Xenopus* chromosome-associated polypeptide.

from the same library by using the partial cDNA as a probe. The NH2-terminal region of Eg7 cDNA was recovered with two nested PCR (see Fig. 1 A). The first nested PCR was performed on the same library using a vector forward primer and an Eg7 outer primer (5′CTTCGGTTTCAA-TATCCTCC3′, OP1). The PCR product was reamplified using the same vector forward primer and Eg7 inner primer (5′CCGAATTCTAA-CATGGCTCCC3′, IP1). This PCR product (Eg7.5) was digested by EcoRI and subcloned into the EcoRI site of pBluescript KS (Stratagene Inc.). The second PCR was performed with a *Xenopus* ovary Unizap cDNA library (Stratagene Inc.) using the vector reverse primer and an Eg7 outer primer (5′ACTGCATTCCTCATC3′, OP2). This PCR product was reamplified with the vector SK primer and an Eg7 inner primer (5′GGGGAATTCCTCCACCACAGACATG3′, IP2). The PCR product (Eg7.6) was digested with EcoRI and subcloned into the EcoRI site of pBluescript. Sequences were determined on both strands according to the method of Sanger et al. (1977). Searches in databases and sequence comparisons were performed with BLAST and FASTA programs (Pearson and Lipman, 1988).

### Recombinant Proteins and Antibodies

The PstI(1492)–PstI(2150) fragment of Eg7.4 was subcloned into the PstI site of pQE30 (QIAGEN Inc.), giving the pQE-Eg7 P plasmid. The EcoRV(vector)–PstI(2903) fragment of Eg7.2 was subcloned into the SmaI-PstI sites of pQE30, giving the pQE-Eg7 G plasmid.

After induction at 37°C by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), TG1 bacteria transformed with the pQE-Eg7 P and pQE-Eg7 G plasmids produced polypeptides of ∼32 (pEg7 P1) and ∼31 (pEg7 G) kD, respectively. These proteins, containing six histidines in their NH2 terminus, were purified under denaturing conditions on a nickel-agarose column following the manufacturer's instructions (QIAGEN Inc.). Antisera were obtained by immunizing rabbits with purified His fusion proteins (Harlow and Lane, 1988). Antibodies were purified on affinity columns made by coupling purified His-tagged truncated proteins G and P1 to a CnBr-activated sepharose 4B (Pharmacia LKB Biotechnology Inc.). For this purpose, His-tagged polypeptides were first purified on Ni-Agarose columns and eluted in a buffer containing 8 M urea and 0.1 M NaH2PO4, pH 4.5. Samples were subsequently adjusted to pH 8.3 and the coupling reaction was performed as indicated by the manufacturer. The antibodies were purified against this affinity column as described in Harlow and Lane (1988). The elution fractions containing antibodies were pooled, dialyzed overnight against TNE (20 mM Tris-HCl, pH 8, 20 mM NaCl, 1 mM EDTA), and then concentrated with a centricon 30 (Amicon Corp.) following the manufacturer's instructions. The final concentration of purified antibodies was ∼3 mg/ml. Control IgGs were purified from nonimmune rabbit sera on protein A Sepharose columns (Pharmacia LKB Biotechnology Inc.) as described in Harlow and Lane (1988). Eluates were dialyzed against TNE and concentrated to a final concentration of 3 mg/ml.

Antitopoisomerase IIα antibodies were provided by D.F. Bogenhagen (State University of New York, Stony Brook, NY) (Luke and Bogenhagen, 1989). Anti–XCAP-C and –XCAP-E antibodies were provided by T. Hirano (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (Hirano and Mitchison, 1994).

### Preparation of Mitotic Egg Extracts

Mitotic egg extracts were prepared as previously reported (Hirano and Mitchison, 1991; Murray and Kirschner, 1989; Newport and Spann, 1987) with minor modifications.

Mitotic crude extracts were prepared by crushing unfertilized eggs in XB buffer (Murray, 1991) containing 10 mM EGTA (cytostatic factor [CSF]–XB). Mitotic extracts were further fractionated by ultracentrifugation at 50,000 rpm for 2 h at 4°C (TLS-55 rotor; Beckman Instruments, Inc.). The soluble fraction (high speed supernatant) was carefully removed and further clarified at 50,000 rpm for 30 min at 4°C. 50-μl aliquots were frozen in liquid nitrogen and stored at −80°C.

### In Vitro Chromosome Assembly and Blocking Assays

For chromosome assembly assays, high speed supernatants (HSS) were diluted twofold with XBE6 buffer (Hirano and Mitchison, 1994) containing twofold concentrated energy regenerating system (Hutchison et al., 1988). For blocking experiments, purified antibodies or Igs were added to a final concentration of 150 μg/ml and incubated at 4°C for 15 min before adding sperm heads. Demembraned sperm heads were prepared according to Hutchison et al. (1987), added at a final concentration of $10^3$/μl of HSS

and incubated at 21°C. For cytological studies, 5-μl aliquots were removed at different time points, processed, and fixed onto coverslips as described by Hutchison et al. (1988). The effects of antibodies were quantified by scoring the DNA masses containing condensed chromosomes. This was done by a blind test in which coverslips corresponding to each condition were randomly observed. For each field, the observer was asked for the presence of individual condensed chromosomes. The results were expressed as (positive pictures)/(total pictures) ratio for each condition.

## Cytological Analysis

For indirect immunofluorescence, coverslips were blocked in TBS containing 1% newborn calf serum (NBCS) for 1 h at room temperature. Anti–pEg7 sera were diluted 100-fold in TBS containing 1% NBCS and incubated for 15 h at 4°C. Coverslips were rinsed three times for 10 min in TBS at room temperature. The secondary antibody (fluorescein-coupled anti–rabbit Igs; Interchim) was diluted 100-fold in TBS containing 1% NBCS and incubated at room temperature for 3 h in a dark box. After rinsing three times in TBS, the coverslips were covered for 5 min by 200 μl of a 1-μg/ml solution of ethidium bromide made in TBS. The excess of ethidium bromide solution was removed and coverslips were mounted in antifade-containing 50% glycerol solution made in PBS. Slides were observed with a confocal microscope (Personal Confocal Microscope; Olympus Corp.).

## Protein Extraction

*From Oocytes and Eggs.* Fifteen oocytes, eggs, or embryos were homogenized in 150 μl of a buffer containing 75 mM Tris-HCl, pH 8, 2 mM dithiothreitol, 3% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. Samples were centrifuged at 3,000 g for 5 min at 4°C. Supernatants were then extracted with 1 vol of 1,1,2-trichlorotrifluoroethane and centrifuged at 12,000 g for 15 min at 4°C.

*From Adult Tissues.* Muscle, skin, intestine, stomach, liver, brain, and testis were homogenized in 3 ml/g of a buffer containing 50 mM Tris-HCl, pH 8, 10% glycerol, 5 mM magnesium acetate, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin. Protein extracts were centrifuged for 30 min at 15,000 g at 4°C, and supernatants were recentrifuged at 100,000 g for 1 h at 4°C.

*From Cultured Cells.* Cells were washed three times in PBS and lysed in Laemmli sample buffer. Samples corresponding to $1.6 \times 10^5$ cells were used for SDS-PAGE.

## Immunoprecipitation

Frozen aliquots of mitotic extracts (50 μl) were thawed and diluted 10× in CSF-XB. After centrifugation at 13,000 g at 4°C for 15 min, the supernatants were used for immunoprecipitations. Crude sera (1 μl/100 μl of diluted extract) were added to the clarified extracts and incubated at 4°C for 3 h. Immunoprecipitates were then recovered by adding 1/25 vol:vol Protein A Sepharose beads (Pharmacia LKB Biotechnology Inc.) and incubating for 1 h at room temperature with constant agitation. After centrifugation, beads were washed three times with 1 ml of CSF-XB buffer containing 1% NP-40. Beads were resuspended in 50 μl of Laemmli sample buffer and heat denaturated by incubation at 90°C for 10 min. 5 μl of each sample were analyzed by SDS-PAGE and Western blot.

## Western Blot Analysis

Electrophoresis on SDS-polyacrylamide gel and electrotransfer onto nitrocellulose membranes were performed as previously described (Laemmli, 1970; Harlow and Lane, 1988), respectively. Membranes were blocked in TBST (0.05% Tween in Tris-buffered saline) containing 5% skimmed milk for 2 h at 4°C. Membranes were incubated at 4°C for 1 h with antibodies diluted in TBST containing 2.5% skimmed milk. When purified anti–pEg7 P1 antibodies were used, they were diluted to a final concentration of 3 μg/ml. Crude sera were diluted 1,000-fold. Immunocomplexes were revealed with peroxidase-coupled anti–rabbit antibodies (Dako Corp.), using a chemiluminescence assay (Amersham Corp.) according to the manufacturer's instructions.

## Cell Cultures and Analysis

The *Xenopus laevis* Xl2 cells were grown at 25°C, without CO$_2$ in L15 Lei-

bovitz medium (GIBCO BRL) supplemented with 10% fetal calf serum (Bio Times) and antibiotic-antimycotic (GIBCO BRL).

For indirect immunofluorescence, the cells were washed with PBS (25°C), permeabilized by incubation in buffer containing 50 mM imidazole, pH 6.8, 50 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 5 mM MgCl$_2$, 0.1 mM β-mercaptoethanol, and 1% Triton X100 for 3 min at room temperature. After washing in the same buffer without Triton, cells were fixed by immersion in a mixture of 3% formaldehyde and 0.1% glutaraldehyde prepared in PBS for 30 min at room temperature. After washes in PBS, coverslips were treated by three successive baths of a 2-mg/ml solution of NaBH$_4$ in PBS. Cells were then rinsed six times in PBS, blocked in PBS containing 3% BSA for 30 min, and incubated with purified anti–pEg7 G antibodies at a final concentration of 30 μg/ml. The antibodies were revealed by FITC-conjugated goat anti–rabbit IgG (dilution 1/100; Interchim). All antibody reagents were diluted in PBS containing 1% BSA and incubations were performed at room temperature for 60 min. Cells were rinsed in PBS containing 1% BSA between each incubation. Coverslips were rinsed in PBS and mounted in Mowiol (Calbiochem Corp.). Samples were observed using a fluorescent microscope and photographed using a camera (AXIOLAB 35 and MC80DX; both from Carl Zeiss, Inc.).

## Nucleotide Sequence Accession Number

The sequence described here has been assigned GenBank/EMBL/DDBJ accession number AF072717.
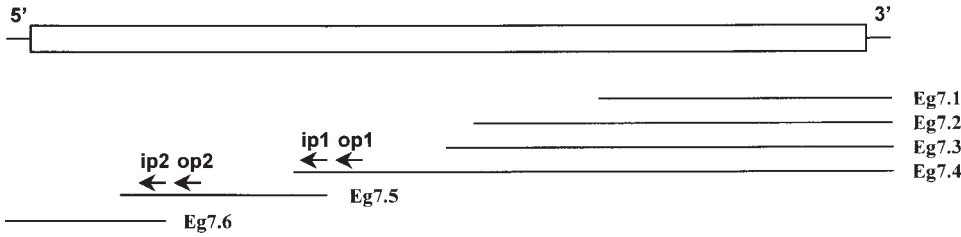
## Results

### Eg7 cDNA and Deduced Protein Sequence

A partial Eg7 cDNA clone was isolated from a *Xenopus* egg cDNA library by differential screening (Paris and Philippe, 1990). Several overlapping clones of different length were obtained by screening the same library with the Eg7 cDNA as a probe (Fig. 1 *A*). The full length open reading frame was obtained by two subsequent nested PCR as described in Materials and Methods (Fig. 1 *A*). The overlapping sequences obtained from three clones encoded a protein (pEg7) of 1,360 residues (predicted molecular weight: 150 kD; predicted isoelectric point: 5.8).

Comparison of the predicted amino acid sequence of pEg7 with sequence in the GenBank/EMBL/DDBJ was made. The best score (60% identity) was obtained with a human open reading frame of 1,401 amino acids (KIAA-0159 gene product) with unknown function (Fig. 1 *B*). This sequence comparison allowed confirmation of the position of the initiation codon. The closest sequence found in *Saccharomyces cerevisiae* (YLR272c) has only 22% identity with pEg7, the best conservation (45% identity) being confined between amino acids 975 and 1206. The amino acid sequence of pEg7 did not reveal any conserved motif that would allow classification of this protein in any previously described protein family.

### pEg7 is a 150-kD Protein Mostly Present in Gametes and Dividing Cells

We raised two different polyclonal anti–pEg7 antibodies (P1 and G) against two fusion proteins containing nonoverlapping regions of pEg7. The corresponding P (amino acids 489–710) and G (amino acids 720–960) regions are underlined in Fig. 1 *B*. Fig. 2 *a* shows that both serums recognized a band migrating as a 150-kD polypeptide in egg extracts. This is in agreement with the molecular weight calculated from the deduced amino acid sequence. This 150-kD band was not revealed by either preimmune serum. The detection of the 150-kD polypeptide was not af-

**A**

5'  [coding region box]  3'

————————— Eg7.1
——————————— Eg7.2
——————————————— Eg7.3
——————————————————— Eg7.4

ip1 op1
← ←

ip2 op2
← ←
——————— Eg7.5

——————— Eg7.6

**B**

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Xl-Eg7    MT---FHFHIPLAFRDLLKSGGIGHYVVQEVLPVRHVDAQFAAFQTSFRTEGPLCILQHF    57
Hs-ORF    MAPQMYEFHLPLSPEELLKSGGVNQYVVQEVLSIKHLPPQLRAFQAAFRAQGPLAMLQHF    60

Xl-Eg7    DTLYSILHHFRSLDIAIKEDVLEVMVKVASRHANELPAILEDLNMSVPQRAAHLNALKMN    117
Hs-ORF    DTIYSILHHFRSIDPGLKEDTLEFLIKVVSRHSQELPAILDDTTLSGSDRNAHLNALKMN    120

Xl-Eg7    CFILTQLIEAFEAETYKASLGSVEPSGKGKKAKSKPE-GFSWESERESILQALTHLLQPD    176
Hs-ORF    CYALIRLLESFETMASQTNLVDLDLGGKGKKARTKAAHGFDWEEERQPILQLLTQLLQLD    180

Xl-Eg7    IRRLWSMSVVEEEFVSMMTSCCYKMMGNPNIVMAKNKSTREALGHLLGVTVKRYNHMLSA    236
Hs-ORF    IRHLWNHSIIEEEFVSLVTGCCYRLLENPTINHQKNRPTREAITHLLGVALTRYNHMLSA    240

Xl-Eg7    SVKVIQLLQHFEHLASVLVHTVSLWATEYGMKPVIGEIMREIGQKCSQDLSRESSGFKAF    296
Hs-ORF    TVKIIQMLQHFEHLAPVLVAAVSLWATDYGMKSIVGEIVREIGQKCPQELSRDPSGTKGF    300

Xl-Eg7    ATFLTELAERIPAIMMPSISVLLDYLDGENYMMRNAVLTVMGEMVVRVLSGDQLEEAEKS    356
Hs-ORF    AAFLTELAERVPAILMSSMCILLDHLDGENYMMRNAVLAAMAEMVLQVLSGDQLEAAARD    360

Xl-Eg7    TRDQFLDTLQEHLHDVNTYVRSCVIQIYSRIVQEKALPLSRFQSVVTLVVGRLFDKSVNV    416
Hs-ORF    TRDQFLDTLQAHGHDVNSFVRSRVLQLFTRIVQQKALPLTRFQAVVALAVGRLADKSVLV    420

Xl-Eg7    CKNAIQLLASFLANNPFTCKLSSVDLKVPLEKETKKLKEMREKYQGPKPVVVISPEEEWE    476
Hs-ORF    CKNAIQLLASFLANNPFSCKLSDADLAGPLQKETQKLQEMRAQRRTAAASAVLDPEEEWE    480
                                                      ← P
Xl-Eg7    AMLPEVLEAFKILQQESKEEDIETE-EEIESSQHLARRILILLRKTSYKNSIRLTQKGI    535
Hs-ORF    AMLPELKSTLQQLLQLPQGEEEIPEQIANTETTEDVKGRIYQLLAKASYKKAIILTREAT    540

Xl-Eg7    ERFQEDPLFSE---GDSEAKSVLGILEKIFTEKKADLEQSTAKD-----------QDDA    580
Hs-ORF    GHFQESEPFSHIDPEESEETRLLNILGLIFKGPAASTQEKNPRESTGNMVTGQTVCKNKP    600

Xl-Eg7    QVNPPSQEVQKSDMDKQEMLVQYLSDAHHFALKIEEAIDVISKMMYETAVSVVQEVIEFF    640
Hs-ORF    NMSDPEESRGNDELVKQEMLVQYLQDAYSFSRKITEAIGIISKMMYENTTTVVQEVIEFF    660

Xl-Eg7    VTVSQFGVSQALLGVRRMLPLVWSKEPGVREAVLSAYRRLYLSSNGESERVKAQALVRSL    700
Hs-ORF    VMVFQFGVPQALFGVRRMLPLIWSKEPGVREAVLNAYRQLYLNPKGDSARAKAQALIQNL    720
              P→                        ← G
Xl-Eg7    SLLMVDSSAGILQCLEEIVSEFVQKGDIHPSVIQLLWEKFTQKSPCSALERRAAVMLLGM    760
Hs-ORF    SLLLVDASVGTIQCLEEILCEFVQKDELKPAVTQLLWERATEKVACCPLERCSSVMLLGM    780

Xl-Eg7    MTRGQPEIVMSNLDTLVSVGLGEQVQKDYQLAWEVCNCILKITDSQKQTLGKSTEPFRLP    820
Hs-ORF    MARGKPEIVGSNLDTLVSIGLDEKFPQDYRLAQQVCHAIANISDRRKPSLGKRHPPFRLP    840

Xl-Eg7    KDHSLFVCLTEAVAGGIGLSGLHWLPFKETAVRLVYELGEEPEEICSEILLRCSQNVLD-    879
Hs-ORF    QEHRLFERLRETVTKGFVHPDPLWIPFKEVAVTLIYQLAEGPEVICAQILQGCAKQALEK    900

Xl-Eg7    ------GHQTQDEVPNV-PAFLLTHLLSLAGDVALQQVVHLERAVSAELRRRRVLKEEQE    932
Hs-ORF    LEEKRTSQEDPKESPAMLPTFLLMNLLSLAGDVALQQLVHLEQAVSGELCRRRVLREEQE    960
                     G→
Xl-Eg7    AEKVGKQRKSKANESTMEEELGLVGASADDIEAELIRKICDTELLGGQQYLSAFLPLILR    992
Hs-ORF    HKTKDPKEKNTSSETTMEEELGLVGATADDTEAELIRGICEMELLDGKQTLAAFVPLLLK    1020

Xl-Eg7    ICNNPGRYSDPDLCTVATLALAKYMMISSDFCDTHLRLLFTLLEKSPLPSVRSNIMIALG    1052
Hs-ORF    VCNNPGLYSNPDLSAAASLALGKFCMISATFCDSQLRLLFTILEKSPLPIVRSNLMVATG    1080

Xl-Eg7    DLSIRFPNLIEPWTPNLYARLRDPSREVRKTAGMVMTHLILKDMVKVKGQVSEMAVLLIE    1112
Hs-ORF    DLAIRFPNLVDPWTPHLYARLRDPAQQVRKTAGLVMTHLILKDMVKVKGQVSEMAVLLID    1140

Xl-Eg7    SDQEISALARNFFNELSNKGNAVYNLLPDIISRLSDPDCGVEEEAFRTIMKQLLSYITKD    1172
Hs-ORF    PEPQIAALAKNFFNELSHKGNAIYNLLPDIISRLSDPELGVEEEPFHTIMKQLLSYITKD    1200

Xl-Eg7    KQTESLVEKMCHRFRTARTERQWRDLAHCLSLLPFSEKGLRKMQDCFDCYGDKLSDEAVY    1232
Hs-ORF    KQTESLVEKLCQRFRTSLTERQQRDLAYCVSQLPLTERGLRKMLDNFDCFGDKLSDESIF    1260

Xl-Eg7    NSFLTTVAKMRRGAKPELKALIDEFEQKLSRCHNKGLENMDVPEEPSAESDAQAPKAKRP    1292
Hs-ORF    SAFLSVVGKLRRGAKPEGKAIIDEFEQKLRACHTRGLDGIKELEIGQAGSQ-RAPSAKKP    1319

Xl-Eg7    PLASV--NVKKGKSEGDFQTPKPPAS---------RKSRRKVAVNFSSDE--ESDLEAEL    1339
Hs-ORF    STGSRYQPLASTASDNDFVTPEPRRTTRRHPNTQQRASKKKPKVVFSSDESSEEDLSAEM    1379

Xl-Eg7    SEAETPKNPTPI-RRTARSRAK                                        1360
Hs-ORF    TEDETPKKTTPILRASARRHRS                                        1401
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*Figure 1.* Cloning of Eg7 cDNA. (*A*) The open box represents the coding region (1,360 amino acids). Eg7.1–Eg7.4 correspond to partial cDNAs obtained by screening a *Xenopus* egg library. Eg7.5 and Eg7.6 were obtained by PCR from an oocyte library. The positions of primers used for nested PCRs are indicated by arrows. (*B*) Comparison of the deduced amino-acid sequences of *Xenopus* pEg7 (Xl-Eg7) and the human gene product KIAA0159 (Hs-ORF). The P (amino acids 489–710) and G (amino acids 720–960) regions used to construct the His-tagged recombinant polypeptides are delimited by arrows above the Xl–Eg7 sequence.
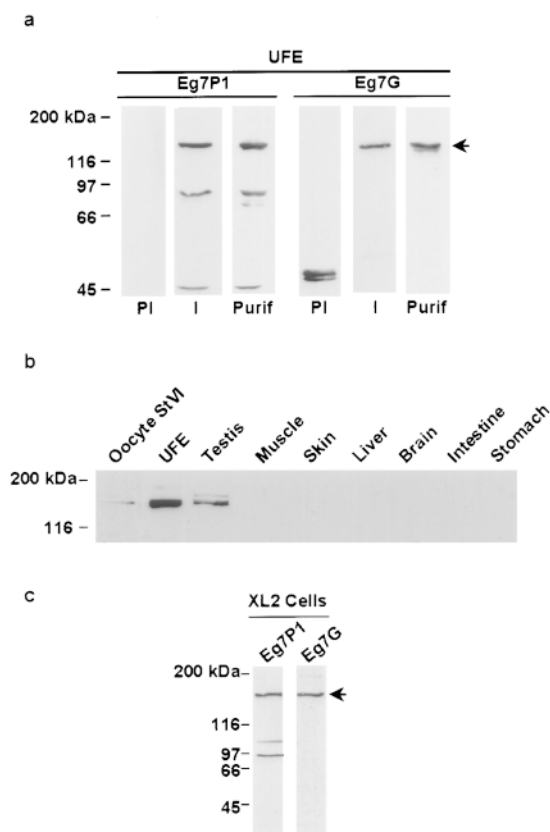
*Figure 2.* Expression of pEg7. (*a*) Pattern of the proteins revealed by anti–pEg7 P1 and anti–pEg7 G antibodies. Protein samples corresponding to one unfertilized egg were separated by SDS-PAGE and transferred onto nitrocellulose. The blots were probed with preimmune serums, immune serums, or purified antibodies as indicated. Positions of the molecular weight markers are indicated at the left. The position of pEg7 is indicated by an arrow. (*b*) Tissue expression of pEg7. Protein samples (40 μg) from each different tissue were separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with purified anti–pEG7 P1 antibodies. (*c*) Expression of pEg7 in Xl2 culture cells. Protein samples corresponding to 1.6 × 10⁵ cells were processed as described above and probed with purified anti–pEg7 P1 or anti–pEg7 G antibodies as indicated.

fected when the G and P1 antibodies were affinity purified against the corresponding fusion proteins.

The P1 antibody was used to study the expression pattern of pEg7 (Fig. 2 *b*). The expression of pEg7 strongly increases in unfertilized eggs as compared with stage VI oocytes. This is consistent with the polyadenylation of Eg maternal mRNAs that occurs during oocyte maturation (A. Couturier, unpublished data). The level of the protein progressively decreased after fertilization but remained detectable until the midblastula transition (data not shown). Besides its expression in eggs, pEg7 is readily detectable in testis but not in the other tissues examined. The samples were probed with an antitubulin antibody to confirm that equivalent amounts of proteins were loaded (data not shown).

## pEg7 Localizes to Condensing Chromatin in Mitotic Cells

The abundance of pEg7 in both eggs and testis suggests that the expression of this protein is correlated with the mitotic activity of cells. Therefore, the subcellular distribution of pEg7 in Xl2 *Xenopus* cells was examined at different stages of the cell cycle by indirect immunofluorescence microscopy. The specificity of antibodies was first tested by Western blot, using Xl2 cell extracts (Fig. 2 *c*). Purified P1 and G antibodies both detected the 150-kD polypeptide. Since the purified G antibody detected no additional bands, this was used for immunolocalization experiments (Fig. 3).

Cell cycle stages were identified by phase contrast and DNA staining with DAPI. Immunostaining of interphasic cells with G antibody gave a diffuse nuclear signal (Fig. 3 *C*). As cells entered into prophase and prometaphase, the immunofluorescence signal progressively localized on condensed DNA (Fig. 3, *F* and *I*). pEg7 was found to be distributed along the axis of the chromosomes over their entire length. The most intense chromosomal staining was observed on highly condensed chromosomes present at metaphase and anaphase (Fig. 3, *L* and *O*). During telophase (Fig. 3, *R* and *U*), the staining of chromatin progressively decreased to return at a level close to that observed in interphasic nuclei. The staining of condensed, mitotic chromatin was also observed with the P1 antibody (data not shown), confirming that this staining is specific for pEg7.

Hence, pEg7 is associated with chromatin throughout the process of chromosome condensation and segregation.

## pEg7 Is Required for Chromosome Condensation In Vitro

Chromosome condensation has been analyzed using an in vitro assay involving mitotic egg extracts. To assess the behavior of these extracts, demembranated sperm nuclei were added and observed for chromosome condensation by staining with Ethidium bromide (Fig. 4, *a, c, e, g, i,* and *k*). At the beginning of the incubation, sperm chromatin exhibited a highly compact, snake-like shape (Fig. 4 *a*). Upon incubation, this compact chromatin rapidly swelled and partly decondensed (Fig. 4 *c*). Further incubation led to local chromatin condensation (Fig. 4 *e*) and to the formation of entangled, prophase-like chromatin fibers (Fig. 4 *g*) that finally resolved into highly condensed individual chromosomes (Fig. 4, *i* and *k*). These dynamic changes in chromatin structure are in agreement with previous observations (Hirano and Mitchison, 1993).

As detected by anti–pEg7 G antibodies, pEg7 appeared to be associated with the DNA as soon as chromatin swelled (Fig. 4 *d*) and this localization persisted throughout the condensation process (Fig. 4, *f, h,* and *j*). The pattern of pEg7 staining followed the DNA images, detecting initially tangled fine fibers that resolved, by 2 h, into thick, condensed individual chromosomes. No signal was observed with preimmune serum (Fig. 4 *l*). Very similar observations were made with anti–pEg7 P1 antibodies (data not shown).

The immunolocalization of pEg7 on condensed chromatin suggests that the protein could be involved in the pro-
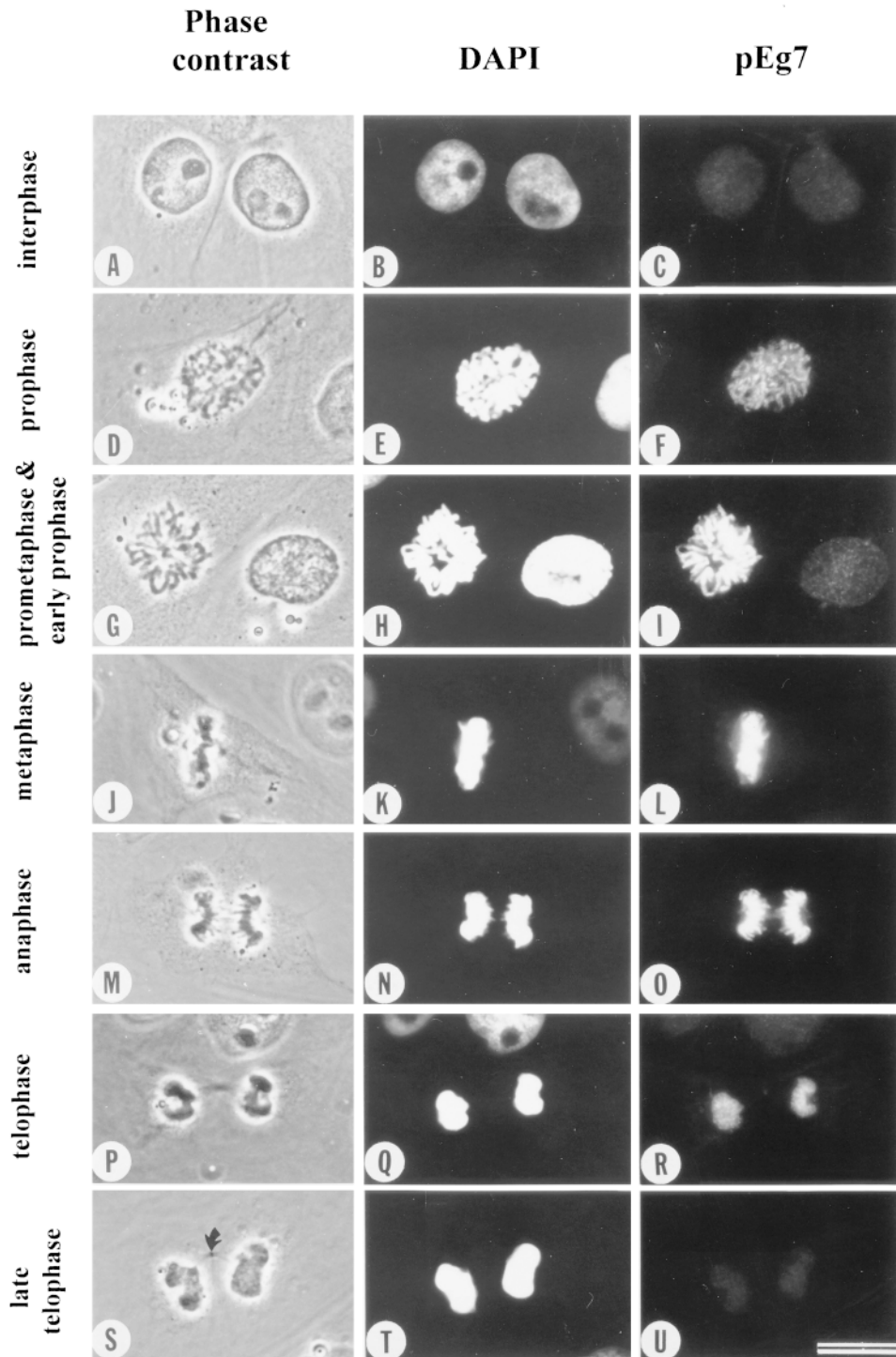
Phase contrast | DAPI | pEg7

interphase (A, B, C)
prophase (D, E, F)
prometaphase & early prophase (G, H, I)
metaphase (J, K, L)
anaphase (M, N, O)
telophase (P, Q, R)
late telophase (S, T, U)

*Figure 3.* Immunolocalization of pEg7 in *Xenopus* Xl2 culture cells. The cells were observed in phase contrast (*A, D, G, J, M, P, S*), and DNA was stained with DAPI (*B, E, H, K, N, Q, T*). pEg7 was detected by indirect immunofluorescence using purified anti–pEg7 G antibodies (*C, F, I, L, O, R, U*). (*S*) Arrow indicates the midbody. Bar, 10 μm.

cess of chromosome condensation. This issue was addressed by immunoblocking experiments using purified anti–pEg7 antibodies. We first verified the pattern of the proteins revealed by these antibodies in high-speed mitotic extracts. The Fig. 5 (*left*) shows that the purified G and P1 antibodies recognized essentially one 150-kD polypeptide in these extracts. This 150-kD protein was not detected by nonimmune immunoglobulins.

Mitotic extracts were then incubated with either rabbit nonimmune immunoglobulins or purified anti–pEg7 anti-

bodies, and the chromatin behavior was observed after addition of sperm heads. Adding nonspecific antibodies to the extracts had no effect on the dynamics of chromatin condensation (Fig. 5, *a–c*). In particular, chromosomes were fully condensed and resolved after 2 h of incubation (Fig. 5 *c*), and numerous individual chromosomes were observed (one example is given in Fig. 5 *c, inset*). When the extracts were preincubated with anti–pEg7 G antibodies, the swelling step occurred apparently normally (compare Figs. 5 *d* and 4 *c*). However, the chromatin fibers remained
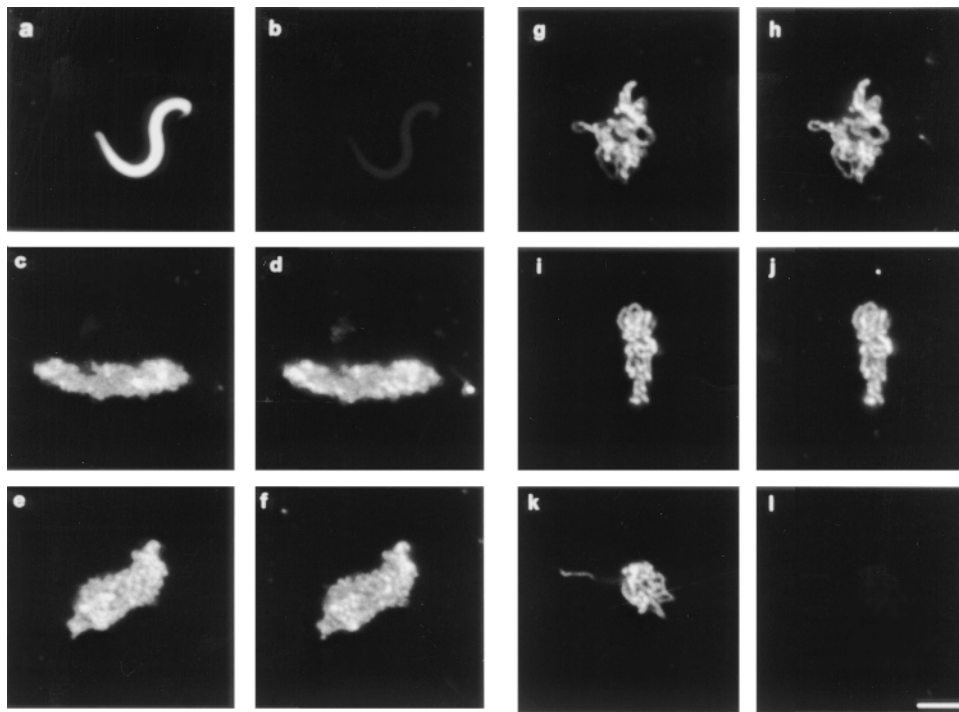
*Figure 4.* Immunolocalization of pEg7 on mitotic chromosomes assembled in vitro. Chromosomes were assembled under standard conditions (see Materials and Methods) and aliquots were taken to analyze DNA at different time points. Samples were fixed and DNA was stained with ethidium bromide (*a, c, e, g, i,* and *k*) and pEg7 was detected by indirect immunofluorescence using anti–pEg7 G immune serum (*b, d, f, h,* and *j*) or preimmune serum as a control (*l*). (*a* and *b*) 0 min, (*c* and *d*) 15 min, (*e* and *f*) 30 min, (*g* and *h*) 60 min, (*i–l*) 120 min. Bar, 5 μm.

tangled at 1 h incubation (Fig. 5 *e*) and, although partial chromosome condensation occurred, the resolution was not complete. Chromosomes remained in a mass where they appeared thicker and slightly less condensed than in control. In addition, individual chromosomes were never observed after 2 h (compare Fig. 5, *f* and *c*) and even after 3 h of incubation (not shown).

In comparison with anti–pEg7 G antibodies, anti–pEg7 P1 antibodies had earlier effects. Once swelled (Fig. 5 *g*), sperm chromatin exhibited only partial and local condensation, appearing as a mass of entangled fibers that re-

mained stable throughout the incubation (Fig. 5, *h* and *i*). Not only were no individual chromosomes observed, but it was impossible to distinguish one chromosome from another, even after 3 h of incubation (not shown). The differences observed between these antibodies cannot be accounted for by differences in titer or in efficiency since both antibodies titrated >80% of pEg7 in the blocking conditions (data not shown).

The results of these experiments were quantified by scoring the DNA masses that harbored fully condensed individual chromosomes (see Materials and Methods). The
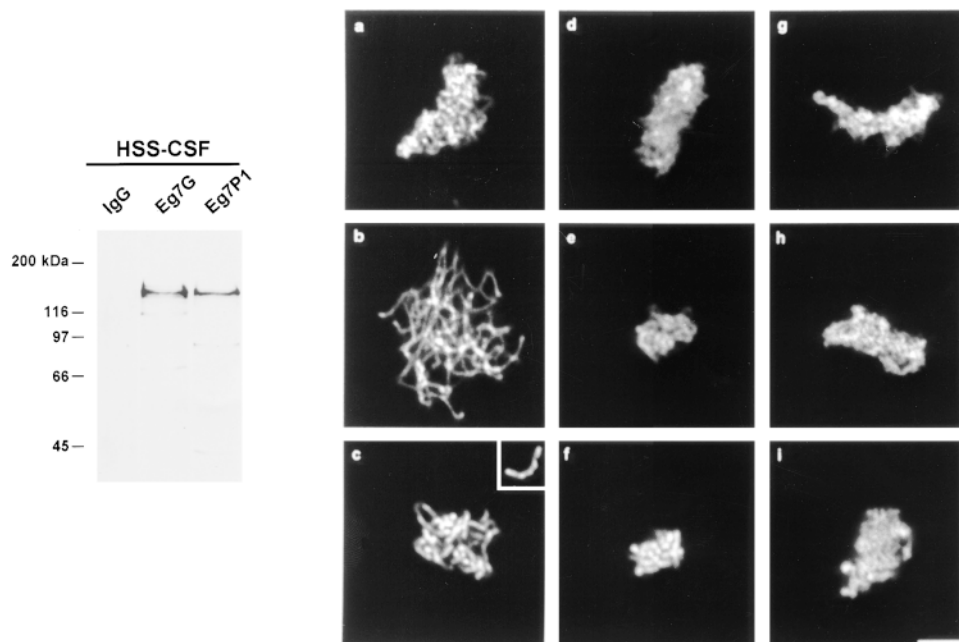


*Figure 5.* pEg7 is required for assembly of mitotic chromosomes in vitro. (*Left*) Samples from high-speed mitotic extracts (1 μl) were separated by SDS-PAGE, transferred to a nitrocellulose filter, and probed with nonimmune immunoglobulins, purified anti–pEg7 P1 or purified anti–pEg7 G antibodies as indicated. (*Right*) Chromosomes were assembled in the presence of IgG (*a–c*), purified anti–pEg7 G antibodies (*d–f*), or purified anti–pEg7 P1 antibodies (*g–i*). Samples were taken at different time points and fixed. DNA was stained with ethidium bromide: (*a, d,* and *g*) 30 min, (*b, e,* and *h*) 60 min, (*c, f,* and *i*) 120 min. Bar, 5 μm.
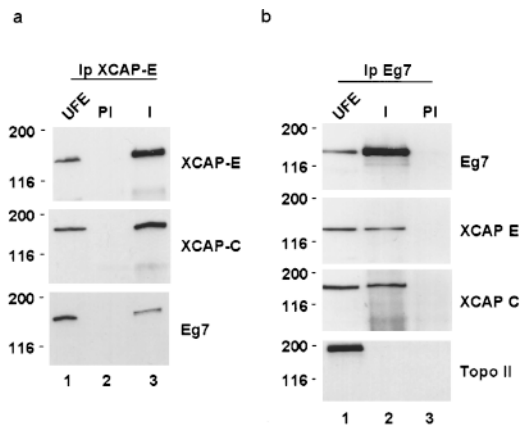
*Figure 6.* pEg7 is a component of the 13S condensin complex. (*a*) Immunoprecipitation of XCAP-E was performed using preimmune serum (lane *2*) or immune serum (lane *3*). Immunoprecipitates were analyzed on SDS-PAGE, transferred to a nitrocellulose filter, and probed with anti–XCAP-E (*top*), anti–XCAP-C (*middle*), or anti–pEg7 P1 (*bottom*). An unfertilized egg was used as an internal standard (lane *1*). (*b*) Immunoprecipitation of pEg7 was performed using anti–pEg7 P1 immune serum (lane *2*) or preimmune serum (lane *3*). Immunoprecipitates were analyzed on SDS-PAGE, transferred to a nitrocellulose filter, and probed with anti–pEg7 P1, anti–XCAP-E, anti–XCAP-C, and anti–topoisomerase IIα (*top* to *bottom*).

scores obtained were of 10/11, 9/10, 0/15, and 0/12 for the assays done in the presence of buffer, nonimmune immunoglobulins, G antibody, and P1 antibody, respectively. These results indicate that pEg7 function is required for both the compaction and resolution of mitotic chromosomes in egg extracts.

### pEg7 Interacts with the XCAP-C and XCAP-E Components of the Condensin Complex

Several proteins had been reported to be required for chromosome condensation, including topoisomerase IIα (Adachi et al., 1991) and the 13S condensin complex (Hirano et al., 1997). XCAP-C and XCAP-E, which form a heterodimer (Hirano and Mitchison, 1994), are also components of a higher order protein complex (13S condensin), which contains three other proteins.

To determine if pEg7 is part of the condensin complex, we carried out immunoprecipitation experiments with anti-XCAP-E antibodies (Fig. 6 *a*). Immunoprecipitates were resolved by SDS-PAGE. After transfer onto nitrocellulose, the blot was probed with anti–XCAP-E, anti–XCAP-C, and anti–pEg7 P1 antibodies. In addition to XCAP-E and XCAP-C, pEg7 was detected in the immunoprecipitates. Relative to the levels found in total unfertilized eggs extracts, the levels of pEg7 in the immunoprecipitates were lower than those of XCAP-E and XCAP-C. This can be accounted for by the existence of an additional 8S complex that contains only XCAP-E and XCAP-C (Hirano et al., 1997). To confirm that pEg7 is physically bound to both XCAP-E and XCAP-C, an immunoprecipitation experiment was performed using the anti–pEg7 P1 antibody (Fig. 6 *b*). This produced an immunoprecipitate

that contained not only pEg7, but also XCAP-E and XCAP-C. Probing the immunoprecipitate with anti–topoisomerase IIα antibodies showed that this protein was absent from the complex. This is in agreement with the previous observation that topoisomerase IIα, although being physically associated with chromatin (Earnshaw et al., 1985), is not a component of the 13S condensin complex (Hirano et al., 1997). Similar results were observed when immunoprecipitations were made using the anti–pEg7 G antibodies (data not shown).

Since XCAP-E and XCAP-C exist in either a 8S heterodimeric or a 13S pentameric complex (Hirano et al., 1997), the above data indicate that pEg7 is a component of the 13S condensin complex.

## Discussion

We report the cloning and the characterization of a novel *Xenopus* protein, pEg7. pEg7 is hardly detectable in stage-VI oocytes and its expression strongly increases in matured oocytes. This expression pattern presumably reflects the polyadenylation of Eg7 maternal mRNA during oocyte maturation, as previously reported by Paris and Philippe (1990). This polyadenylation behavior is in agreement with the presence, in the 3′ untranslated region, of three cytoplasmic polyadenylation elements (for review, see Osborne and Richter, 1997). The coding part of Eg7 cDNA does not exhibit any conserved motifs that might have given clues about the function of the protein. However, pEg7 shows significant amino acid similarity (60%) to a human protein, suggesting that the protein is conserved at least among vertebrates, but no data exist about the function of the human protein.

Immunofluorescence staining of Xl2 cultured cells showed a weak diffuse nuclear distribution of pEg7 in interphase cells. This is in agreement with the presence of potential nuclear localization signals in the amino acid sequence (RRRR at position 922 and PASRKSR at position 1314). As cells enter mitosis, pEg7 begins to concentrate on condensing chromatin. This chromatin staining increases concomitantly with mitotic chromosome condensation, and complete colocalization of DNA and pEg7 is observed at metaphase. The chromosomal staining persists during anaphase but disappears at the end of telophase. pEg7 localization on chromosomes is very similar to that of XCAP-C in somatic cultured cells (Hirano and Mitchison, 1994). XCAP-C, which with XCAP-E belongs to the SMC family, was shown to be required in the chromosome condensation process (Hirano and Mitchison, 1994). Therefore, the localization of pEg7 suggests that this protein could also be involved in the chromosome condensation process. This proposal is supported by the inhibitory effect of anti–pEg7 antibodies on chromosome assembly in CSF-arrested egg extracts.

The assembly of mitotic condensed chromosomes involves at least two distinct processes. (*a*) The resolution of entangled chromatin into individual chromosomes by decatening DNA fibers. This process is mainly ensured by topoisomerase IIα. (*b*) The compaction of chromatin to give fully condensed rod-shaped chromosomes. This process requires XCAP-C and XCAP-E, which exist in two different complexes, the 8S and 13S condensins. The 8S con-

densin is a heterodimer containing only XCAP-C and XCAP-E, whereas the 13S condensin contains three additional subunits (XCAP-D2, XCAP-G, and XCAP-H) (Hirano et al., 1997).

It is formally conceivable that pEg7 acts on chromosome condensation alone or as a cofactor of either topoisomerase IIα or the condensin complex. Immunoprecipitation experiments show that pEg7 forms a complex with two components of the condensin complexes (XCAP-C and XCAP-E), but not with topoisomerase IIα. Since, among the two complexes that contain XCAP-E and XCAP-C, only the 13S complex contains three additional proteins, our data indicate that pEg7 is a component of the 13S condensin complex. Among the three additional subunits of the 13S condensin, the XCAP-D2 is the only component having an apparent molecular weight (150 kD) that is compatible with that of pEg7. pEg7 is therefore possibly identical to XCAP-D2. Whereas immunodepleting the 13S condensin abolishes chromosome condensation in egg extracts, the exact role of individual components in this process is poorly understood. Nevertheless, a direct requirement for XCAP-C was demonstrated by adding specific antibodies to the chromosome assembly assay (Hirano and Mitchison, 1994). By using the same strategy, we have shown here that anti–pEg7 antibodies disturb the assembly of mitotic chromosomes (Fig. 5). Once the condensation process was achieved, adding anti–pEg7 antibodies had no effect on the architecture of mitotic chromosomes (data not shown). This may indicate that, in contrast to the observation for XCAP-C (Hirano and Mitchison, 1994), pEg7 is not necessary for the maintenance of mitotic chromosomes. Alternatively, once pEg7 is associated with chromatin, these antibodies may be unable to displace or inactivate it.

The two antibodies used here are directed against two distinct parts of the protein and have different effects. The anti–pEg7 P1 antibodies almost completely abolish the condensation process, giving axis-like structures within masses of chromatin. This corresponds to a level of compaction obtained when only the 8S complex is present in the extract (Hirano et al., 1997). These observations suggest that anti–pEg7 P1 antibodies totally inactivate the 13S complex. In contrast to the results with anti–pEg7 P1 antibodies, the anti–pEg7 G antibodies only partially inhibit condensation, giving chromosomes that are distinguishable but thicker than in control extracts. In addition, individual chromosomes were never observed, suggesting that the anti–pEg7 G antibodies disturb the resolution process. It is possible that the partial compaction defect is due to the inhibition of resolution only. This would be in agreement with an emerging model in which compaction and resolution are coupled. That two distinct antibodies against pEg7 affect either compaction or resolution seems paradoxical. However, whereas the resolution process is thought to be driven essentially by topoisomerase IIα (Hirano, 1995), it is not precluded that the 13S condensin complex directly or indirectly participates in this process. In this context, it is interesting that one component of the 13S condensin, XCAP-H, is homologous to the *Drosophila* barren gene product that is an activator of topoisomerase II (Bhat et al., 1996). From these data, we propose that the anti–pEg7 G antibodies might disturb the interaction between either XCAP-H and the rest of the 13S complex or between XCAP-H and topoisomerase IIα. It may be that pEg7 is both a physical and a functional link between the compaction and resolution processes.

*Note Added in Proof.* The immunoblocking experiments described in Fig. 5 were repeated in cycled extracts: this gave similar defects in chromosome condensation. While this paper was under revision the sequence of XCAP-D2 was published (Kimura, K., M. Hirano, R. Kobayashi, and T. Hirano. 1998. *Science.* 282:487–490). The deduced amino acid sequence is 99% identical to pEg 7.

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