

Identification of Nuclear Pre-replication Centers Poised for DNA Synthesis in *Xenopus* Egg Extracts: Immunolocalization Study of Replication Protein A

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Abstract. We demonstrate by immunofluorescence that a 70-kD protein (P70) purified from *Xenopus* egg extracts is associated with subnuclear foci (about 200) which we propose to be an assembly of DNA pre-replication centers (preRCs). A cDNA encoding this protein reveals that P70 is the *Xenopus* homologue of replication protein A (RPA also called RF-A). RPA is known to be a cellular, three-subunit single-stranded DNA binding protein, which assists T-antigen in the assembly of the pre-priming complex in the SV40 replication system. The punctated preRCs exist transiently; they form post-mitotically during the period of nuclear membrane breakdown and disappear during ongoing DNA replication. P70 is homogeneously as-

sociated with chromatin at the later stages of the S-phase and is displaced from chromatin post replication, so that P70 cannot be detected on mitotic chromosomes. Double-immunofluorescence studies using biotin-dUTP demonstrate that initiation of DNA synthesis is confined to preRCs, resulting in the punctated replication pattern observed previously by others. PreRCs form efficiently on decondensed chromatin in membrane-free egg extracts if ATP and divalent cations are present. Our results suggest that preRCs are composed of an assembly of a large number of pre-initiation replication complexes poised for initiation at discrete subnuclear regions prior to nuclear reconstruction and initiation of DNA synthesis.

CELL-free extracts derived from *Xenopus* eggs provide a suitable system to study many aspects of the cell cycle, including DNA replication. Egg extracts faithfully initiate and complete semiconservative DNA replication (Lohka and Masui, 1983; Blow and Laskey, 1986; Newport, 1987; Hutchison et al., 1987). Demembrated sperm nuclei incubated in low-speed supernatants (LSS)¹ made from crushed *Xenopus* eggs decondense, acquire a nuclear membrane-lamina structure and subsequently replicate their DNA (Lohka and Masui, 1983, 1984; Hutchison et al., 1987). These extracts can also replicate purified DNA in a semi-conservative manner after first assembling the DNA into chromatin and then forming so-called pseudo-nuclei (Blow and Laskey, 1986; Newport, 1987; Cox and Laskey, 1991).

A number of observations indicate that initiation of DNA replication is dependent on the formation of an intact nucleus. Removal of membrane vesicles from egg extracts by high-speed centrifugation (HSS extracts) prevents DNA replication and addition of a washed membrane fraction restores the replication capacity (Newport, 1987; Sheehan et al., 1988). Immuno-depletion of the nuclear lamins in the

complete LSS egg extracts prevents replication, despite formation of nuclear membranes around the input sperm nuclei (Newport et al., 1990; Meier et al., 1991). Thus, both the nuclear membrane and the lamina structure are required for DNA replication. Interestingly, Blow and Laskey (1988) have shown that the integrity of the nuclear membrane is necessary to limit DNA replication to one round within a single cell cycle.

DNA replication is known to occur at discrete foci (we will use the term replication centers, RCs), which can be visualized by in situ localization of bromodeoxyuridine or biotinylated-dUTP incorporated into pulse-labeled replicating nuclei (Nakamura et al., 1986; Nakayasu and Berezney, 1989; Cox et al., 1991). A discrete pattern of 100–300 RCs distributed throughout the nuclear lumen has been observed in nuclei replicating in the cell-free *Xenopus* system (Mills et al., 1989; Leno and Laskey, 1991), and each RC is estimated to consist of an assembly of 300–1000 replication forks (Mills et al., 1989). In tissue culture cells, the distribution of the RCs during S phase has been studied in some detail by pulse labeling of synchronized cell populations. In early S phase the replicating DNA foci are distributed throughout the nucleus, excluding heterochromatin and the nucleolus, while in late S phase they are localized to perinuclear and then intranuclear heterochromatin (Nakayasu and Berezney, 1989; Fox et al., 1991). Immunofluorescence studies of proliferating cell nuclear antigen, PCNA, (an aux-

1. *Abbreviations used in this paper:* ds, double stranded; HSS, high-speed supernatants; LSS, low-speed supernatants; PCNA, proliferating cell nuclear antigen; PVDF, polyvinylidene difluoride; RC, replication center; RPA, replication protein A; ss, single stranded.

iliary protein of DNA polymerase delta) have shown that the staining pattern of PCNA is akin to and follows that of the RCs through S phase. In some cases, RCs coincide with PCNA staining (Bravo and Macdonald-Bravo, 1987; Kill et al., 1991).

A central question concerning cell proliferation is to understand how initiation of DNA replication is regulated. In yeast, genetic evidence has identified well-defined transition points at the G1/S ("Start") and G2/M boundaries of the cell cycle. The *cdc2* kinase gene product has been implicated at both of these transition points; in *Xenopus* egg extracts, depletion of p33^{cdc2}, a kinase related to p34^{cdc2}, significantly decreases the ability to replicate added sperm nuclei (Blow and Nurse, 1990; Fang and Newport, 1991). One way to identify the targets downstream of the regulatory pathway of the G1/S transition is to examine at what stage the DNA replication machinery is assembled. That is, do pre-RCs which are poised for subsequent initiation exist in pre-S phase nuclei? This question could be addressed with the help of an antibody directed against a protein implicated in the initiation and priming events of DNA replication.

Biochemically, eukaryotic DNA replication is best understood in the viral SV-40 system. A number of cellular factors have been identified which, in conjunction with the SV-40 large T antigen, are essential for the replication of DNA containing an SV-40 origin (for reviews see Challberg and Kelly, 1989; Stillman, 1989). It is now possible to reconstitute this replication system from its purified components (Tsurimoto et al., 1990). Replication protein A (RPA or RF-A, the former term will be used) has been identified as a factor implicated in the early stages of SV-40 replication in vitro (Wold and Kelly, 1988; Fairman and Stillman, 1988). RPA is composed of three subunits of 70-, 34-, and 11-kD, and binds preferentially to single-stranded rather than double-stranded DNA. It is the only cellular factor required for the large T-antigen-mediated unwinding of DNA containing the SV-40 replication origin (Wold and Kelly, 1988). Thus, RPA is thought to be the eukaryotic counterpart of prokaryotic single-stranded DNA binding proteins (SSB; Alberts et al., 1968) and to play a role in the generation of a single-stranded region before the initiation of SV-40 DNA synthesis (Kenny et al., 1989; Fairman and Stillman, 1988; Wold et al., 1989).

In this report we use *Xenopus* egg extracts to characterize events that precede DNA replication, and show that a protein purified from these extracts is associated with transient subnuclear structures (~200 per nucleus), which we identify as preRCs. This protein, P70, purified on the basis of its resistance to elution by 2 M salt from DNA-Sepharose, is identified as the *Xenopus* homologue of the large subunit of RPA.

Materials and Methods

Preparation of *Xenopus* Egg Extracts

Xenopus laevis females were primed and ovulation was induced as described (Murray, 1991). Interphase extracts were prepared essentially according to Finlay and Forbes (1990). After extensive washing with extraction buffer, eggs were crushed by a 10-min centrifugation in a SW50.1 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 12,000 rpm (15,000 g). The cytoplasmic fractions were spun again (15,000 rpm, 10 min) to get rid of contaminating lipids and pigments, divided into 100 μ l aliquots, frozen in liquid nitrogen, and stored at -70°C (LSS). HSS were prepared by additional

centrifugation at 45,000 rpm (200,000 g) in the SW50.1 for 2 h at 2°C . The cleared cytoplasmic layer was recovered, divided into aliquots, frozen, and stored at -70°C . Mitotic extracts were prepared as described except that the high speed centrifugation was performed at 45,000 rpm in the SW50.1 for 2 h (Lohka and Maller, 1985; Newport and Spann, 1987; Adachi et al., 1991). Cycling extracts were prepared according to the procedure of Murray and Kirschner (1989) except that egg activation was effected with 0.2 $\mu\text{g}/\text{ml}$ of calcium ionophore A-23187 and Versilube F-50 oil was not used.

Fractionation of the Extract on DNA-Sepharose

Cyanogen-Bromide-activated CL-4B Sepharose was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). DNA-Sepharose was prepared according to the published protocol (Arndt-Jovin et al., 1975) using sonicated salmon sperm DNA. The concentration of DNA coupled to the Sepharose was estimated at 1.5 mg/ml of resin.

DNA-Sepharose was washed twice with HKM (5 mM Hepes, pH 7.5, 12.5 mM KCl, 3 mM MgCl₂, 0.3 mM EGTA) supplemented with 1 mM DTT, 0.2 \times protease inhibitor cocktail. Inhibitor cocktail was prepared as a 1,000 \times stock containing 10 mg/ml each of leupeptin, chymostatin, pepstatin A, and antipain in DMSO. The resin was equilibrated with an equal volume of HKM containing 2 mM ATP, 10 mM creatine phosphate, and 50 $\mu\text{g}/\text{ml}$ creatine kinase for 5 min at room temperature (22–23 $^{\circ}\text{C}$). HSS aliquots were thawed, supplemented with 2 mM ATP, 10 mM creatine phosphate, and 50 $\mu\text{g}/\text{ml}$ creatine kinase and then mixed with an equal volume of the equilibrated DNA-Sepharose. The mixture was incubated at room temperature for 2 h on a rotating wheel. The resin was pelleted by centrifugation and the supernatant was removed. The DNA-Sepharose was transferred to a column at 4°C and washed with 10 column volumes of HEK- β (5 mM Hepes, pH 7.5, 1 mM EDTA, 12.5 mM KCl, 10 mM β -glycerophosphate) supplemented with 1 mM DTT, 0.2 \times inhibitor cocktail. Proteins retained on the column were sequentially eluted with three column volumes of 0.5, 1, and 2 M KCl in HEK- β . The column was washed with HEK- β and residual proteins were eluted with 2 \times FSB (130 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 20% β -mercaptoethanol) to yield the SDS eluate.

Amino Acid Sequence Determination of P70

P70 was gel purified from the SDS eluates of DNA-Sepharose. 8 ml of mitotic extract or interphase HSS were processed as described above and 8 ml of the SDS eluates obtained were heated at 100°C for 3 min and loaded onto six 7.5–15% polyacrylamide gradient gels (1-mm thick, 12.5 \times 14.5 cm separating gel) with 13-cm-wide slots. The gels (including stacking gel) were made according to Laemmli (1970) the day before use and stored at 4°C . The electrophoresis buffer in the upper reservoir was supplemented with 0.1 mM sodium thioglycolate. After electrophoresis, gels were stained with 0.1% Coomassie blue R250 in 20% methanol for 10 min and destained for 10 min with 10% methanol. The P70 bands were excised, crushed between glass plates, and transferred to Eppendorf tubes (Brinkman Instruments Inc., Westbury, NY). About two crushed gel volumes of elution buffer (15 mM NH₄HCO₃, 0.025% SDS, 1 mM DTT, 0.1 mM PMSF) were added and the samples were incubated at room temperature on a rotating wheel overnight. The eluates were recovered by centrifugation, lyophilized in a speed-vac concentrator and stored at -70°C . Approximately 60 μg of P70 were recovered from 8 ml of extract.

30 μg of P70 were dissolved in 1 \times FSB and loaded onto a SDS-polyacrylamide gel (5 μg per 5-mm slot). After electrophoresis, the protein was transferred onto an Immobilon polyvinylidene difluoride membrane (PVDF; Millipore Continental Water Systems, Bedford, MA). Transfer was done at 10 V/cm for 2 h in 25 mM Tris-192 mM glycine buffer with extensive cooling by water circulation (TE50 transfer apparatus; Hoefer Scientific Instruments, San Francisco, CA). The filter was stained with 0.1% amido black in 45% methanol, 7% acetic acid for 30 s, followed by washing with distilled water and the P70 bands were excised. In situ tryptic digest was performed essentially according to Aebersold et al. (1987) and the peptides were fractionated by reverse-phase HPLC using a C18 column (2.1 \times 250 mm; Vydac, Separations Group, Hesperia, CA). The manually collected peptide fractions were subjected to amino acid sequence analysis using the gas phase sequencer 470A (Applied Biosystems Inc., Foster City, CA).

PCR and Cloning of P70

The amino acid sequences of peptides LFSIEMVDESSEIR (peptide 3) and AWFDESGQVVE (peptide 5) were used to design PCR primers. Two de-

generate oligo-nucleotides corresponding to sense and anti-sense strands were made for each peptide. For peptide 3, these were 5'-CGGATCCAT-(TCA)GA(AG)ATGGT(TCAG)GA(TC)GA-3' (3A) and 5'-GGAATTCTC-(AG)TC(GATC)ACCAT(TC)TC(AGT)AT-3' (3B). For peptide 5 they were 5'-CGGATCCGA(AG)GG(TCAG)CA(AG)GT(TCAG)GT(TCAG)GA-3' (5A) and 5'-GGAATTTCGC(AGT)AT(TCAG)GG(TCAG)AC(TCAG)AC-3' (5B). Bases in parentheses represent mixtures. The primers contained BamHI or EcoRI sites at their 5' ends to facilitate cloning of the PCR products. Primer 3A was used in combination with 5B, and 5A with 3B. DNA purified from a *Xenopus* oocyte cDNA library in lambda gt10 (Rebagliati et al., 1985) was used as a template. 100 µl of reaction mixture contained 100 ng of the template and 100 pmol of each primer in 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 100 µg/ml gelatin. Amplification conditions were 30 cycles at 95°C for 1 min, 45°C for 2 min, and 65°C for 3 min. The last cycle was followed by incubation at 72°C for 10 min. The combination of primers 3A and 5B produced a major band of 0.6 kb, which was purified by phenol/chloroform extraction and ethanol precipitation. After digestion with BamHI and EcoRI, the 0.6-kb fragment was gel purified, cloned into the plasmid Bluescript SK⁻ (Stratagene, La Jolla, CA) and sequenced.

At the same time, 29-mer oligonucleotides, 5'-ATTTCICCGA(TC)-TCATCIACCATTTCIAT-3' and 5'-ATTTCICCACT(TC)TCATCIACCATTTCIAT-3' (I, inosine), were synthesized based on the amino acid sequence of peptide 3 and used to screen the lambda gt10 cDNA library. Plaques were transferred to nylon membranes (Hybond N+; Amersham Corp., Arlington Heights, IL) and hybridized at 50°C for 16 h with ³²P-labeled 29 mers in 6× SSC, 5× Denhardt's, 1% SDS, and 20 µg/ml sonicated salmon sperm DNA. The filters were washed three times at room temperature and once at 50°C for 10 min in 6× SSC. From 400,000 plaques screened, 22 positive clones were isolated and 14 clones out of the 22 were found to hybridize strongly with the 0.6-kb PCR product under stringent conditions.

Preparation of Anti-P70 Antibodies and Western Blotting

Immunization of hens and preparation of IgY from egg yolks were performed as described (Gassmann et al., 1990). 10 µg of gel-purified P70 were resuspended in 0.75 ml of PBS, emulsified with an equal volume of complete Freund's adjuvant, and then injected. Primary injection (day 0) was followed by two boost injections on days 12 and 20. Eggs were collected from 27 d and IgY was prepared from them. Generation of anti-P70 antibodies was checked by western blotting. One hen produced anti-P70 antibodies from day 35. Western blotting was performed as described (Towbin et al., 1979), using a 1:100 dilution of anti-P70 antibodies. Alkaline phosphatase-conjugated anti-chicken IgG was obtained from Sigma Chemical GmbH (Munich, Germany) and used as a secondary antibody.

P70 Immunofluorescence and Fluorescence Labeling of Replicating DNA In Vitro

For immunofluorescence staining, anti-P70 antibodies were affinity purified with a strip of nitrocellulose on which 2–5 µg of P70 had been immobilized (Smith and Fisher, 1984; Adachi and Yanagida, 1989). The antibodies eluted from the strips were used directly for immunofluorescence staining. The affinity-purified anti-P70 antibodies reacted only with P70 in western blotting of total egg extract (not shown). Antibodies affinity-purified with the carboxy-terminal of P70 expressed in *Escherichia coli* (Studier et al., 1990) showed the same immunofluorescence staining pattern with that of the antibodies affinity-purified with P70 obtained from the egg extracts (Adachi, Y., unpublished result). The *Xenopus* kidney cell line was grown in RPMI plus 5% FCS and processed for staining as described (Harlow and Lane 1988).

Demembrated *Xenopus* sperm nuclei were prepared according to Lohka and Masui (1983). In the cycling experiment, 2,250 demembrated sperm nuclei were added to a 15 µl aliquot of the cycling extract (Murray and Kirshner, 1989) and incubated at room temperature. The samples were fixed at 20 min intervals and double stained for P70 and DNA according to the procedure of Nagakawa et al. (1989). The first antibody was affinity-purified anti-P70 and the second was TRITC-labeled anti-chicken IgG (Sigma Chemical GmbH; 1:100 dilution). Experiments with interphase extracts were performed according to Mills et al. (1989). HSS and LSS aliquots were thawed and supplemented with 2 mM ATP, 10 mM creatine phosphate, 50 µg/ml creatine kinase, and 20 µg/ml cycloheximide. 30,000 sperm nuclei were incubated at room temperature for 1 h in 10 µl HSS; 30 µl of LSS were then added. DNA replication was monitored by pulse labeling with 20 µM biotin-dUTP (Bio-Rad Laboratories, Cambridge, MA) for 30 min before fixation. Samples were processed as above except that

Fluorescein-Streptavidin (Amersham Corp.; 1:100 dilution) was included in the secondary antibody solution.

Fluorescence-labeled samples were observed under a conventional fluorescence microscope (ICM405; Carl Zeiss, Oberkochen, Germany) or a confocal fluorescence microscope (MRC600; Bio-Rad Laboratories). To examine co-localization of P70 and replication foci, the settings of MRC600 were chosen to minimize the leakage between the two channels. The leakage of the rhodamine signal into the FITC channel was under these conditions ~3% and was not corrected. Conversely, ~17% of the FITC signal leaked into the rhodamine channel. This overlap was corrected for micrographs in which the FITC signal was dominant using a modified command file, bleed.cmd, which is supplied with the instrument.

Other Methods

Manipulation of DNA was done following the standard protocol (Sambrook et al., 1989). DNA sequence was determined by Sanger's method (Sanger et al., 1977) using T7 DNA polymerase (Pharmacia; Uppsala, Sweden). These sequence data are available from EMBL/GenBank/DDJB under accession number X67240.

Results

Isolation of P70 from *Xenopus* Egg Extracts as a High Salt-Resistant DNA-binding Protein

In attempts to identify proteins involved in chromosome structure, we fractionated mitotic *Xenopus* egg extracts by DNA-Sepharose chromatography. We hoped that fractionation of the extract with DNA-Sepharose might identify tightly binding scaffold proteins. We have focused our attention on DNA-binding proteins that resist elution by 2 M KCl and require denaturation with SDS for elution from double-stranded (ds) DNA-Sepharose. The protein composition of such an SDS eluate derived from egg extracts showed a fairly simple pattern by SDS-PAGE analysis (Fig. 1 a, lane δ). A protein of an apparent molecular weight of 70 kD (P70) was reproducibly observed. We roughly estimate that one microliter of the extract (=1.6 eggs) contained 10 ng of P70. We raised antiserum against a gel-purified fraction of P70. Immunoblot analysis of the various DNA-Sepharose fractions demonstrated that P70 was quantitatively bound to the DNA-Sepharose and eluted only with SDS (Fig. 1 b); the flowthrough fraction and fractions eluting at 0.5, 1 and 2 M KCl (Fig. 1 b, lanes 2 to 5) contained no detectable P70 as opposed to the input extract (lane I) and the SDS eluate (lane δ). Very similar results were obtained when the membrane-free, interphase HSS was used as a starting material (data not shown).

Although the DNA-Sepharose used for isolation of P70 was prepared with ds DNA, this column must also contain sufficient portions of single-stranded (ss) DNA since, as described below, P70 has strong preference for binding to ss DNA.

P70 Is the *Xenopus* Homolog of the Largest Subunit of Replication Protein A: cDNA Cloning

To obtain information about the primary structure of P70, we cloned the cDNA encoding P70. Tryptic peptides of P70 were used to obtain internal amino acid sequences (Aebersold et al., 1987). Oligonucleotides were designed according to the amino acid sequence information and used as probes to screen the cDNA library or as primers for PCR (see Materials and Methods). From 400,000 plaques of a lambda gt10 *Xenopus* oocyte cDNA library (Rebagliati et al., 1985),

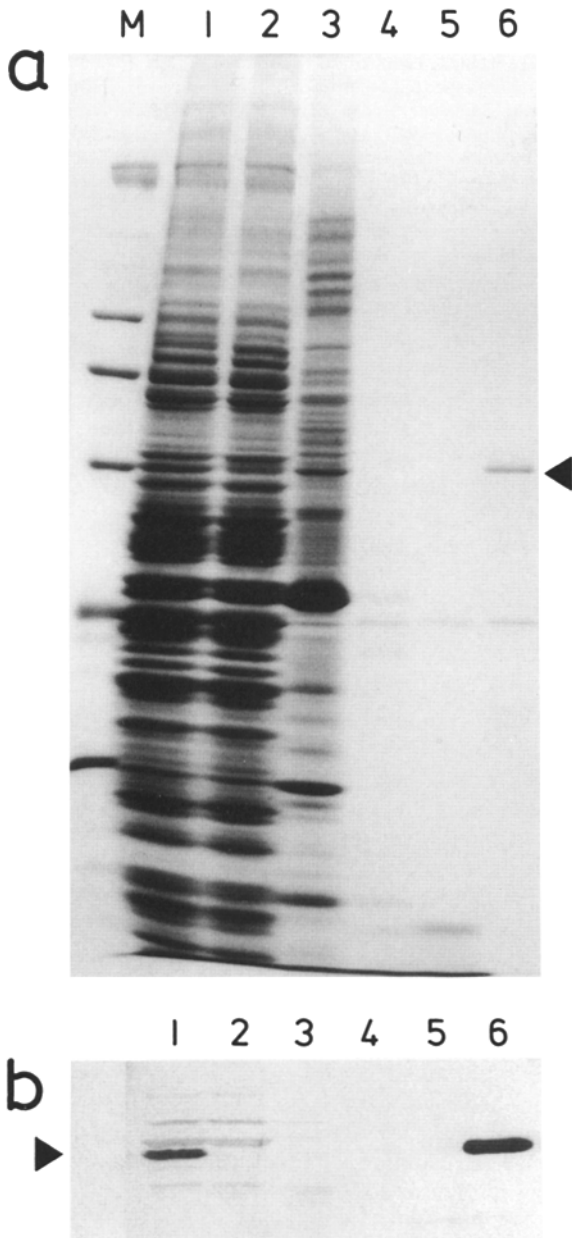


Figure 1. Fractionation of egg extract with DNA-Sepharose; P70 is a major protein resistant to 2 M KCl elution. A *Xenopus* egg extract was fractionated on DNA-Sepharose and the different eluates were subjected to SDS-PAGE and stained with Coomassie blue (A) or transferred to nitrocellulose and probed with anti-P70 antibodies (B). Lane 1, input extract; lane 2; flowthrough fraction; lanes 3–5 correspond to the 0.5, 1, and 2 M KCl eluates, respectively. Lane 6, SDS eluate. P70 is indicated by arrow heads. Lane M, molecular weight markers, from top to bottom, 205, 116, 97, 68, 45, and 29 kD.

22 positive plaques were isolated by oligonucleotide screening. PCR amplification yielded one major fragment 0.6-kb long which hybridized strongly to 14 out of the 22 lambda clones. The protein-encoding region of the cDNA was sequenced. Fig. 2 shows the determined DNA sequence and its predicted amino acid sequence. The open reading frame encodes a polypeptide of 609 amino acids with predicted molecular weight of 67.1 kD. All the amino acid sequences

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AAT TCC GGC GTC GTC GGT CCC AAA GTT CCT GTG CGT GGG ATT TGG CGG CAG GTT GAG
GTT GAG TGG AGG CGC GCG CGG GTA ATA CGC ACA GAA GGA GTC GGT AGT GTG AGT TGG

M A L P Q L S E
CGG CGG TCT TGT CCT GGG AAA ACA ACA AGC AGT ATG GCA CTT CCA CAA CTG AGC GAG
G A I S A M L G G D S S C K P T L Q V
GGC GCT ATC TCG GCT ATG TTG GGT GGC GAT TCT TCT TGC AAG CCA ACT CTG CAA GTG
I N I R P I N T G N G P P R Y R L L M
ATT AAT ATC CGC CCA ATC AAC ACT GGA AAT GGC CCT CCA CGT TAC CGT CTC TTA ATG
S D G L N T L S S F M L A T Q L N S L
AGC GAT GGC CTG AAT ACC CTT TCA TCT TTT ATG TTG GCA ACC CAG CTG AAC TCC CTT
V D N N L L A T N C I C Q V S R F I V
GTG GAT AAC AAT CTT CTT GCC ACA AAC TGT ATT TGT CAG GTG TCA CGG TTT ATT GTA
N N L K D G R R V I I V M E L D V L K
AAT AAT CTG AAA GAT GGA AGG AGA GTC ATC ATT GTA ATG GAG TTG GAC GTT CTG AAA
S A D L V M G K I G N P Q P Y N D G Q
TCT GCT GAT TTG GTT ATG GGA AAG ATT GGC AAT CCA CAG CCT TAC AAT GAT GGG CAA
P Q P A A P A P A S A P A P A P A P S K A L
CCG CAG CCT GCA GCA CCT GCA CCA GCA TCT GCT CCT GCT CCA GCA CCT AGC AAA CTA
Q N N S A P P P S M N R G T S K L F G
CAG AAC AAT TCA GCT CCT CCT CCT TCA ATG AAC AGA GGA ACG TCT AAA TTG TTT GGA
G G S L L N T P G G S Q S K V V P I A
GGG GGA AGT TTA TTG AAC ACA CCT GGG GGA TCT CAG TCC AAG GTA GTC TCC ATT GAG ATG
S L N P Y O S K W T V R A R V T N K G
AGT CTC AAT CCT TAC CAG TCC AAG TGG ACG GTT CGT GCT AGA GTT ACC AAT AAA GGT
Q I R T W S N S R G E G K L F S I E M
CAG ATC CGC ACG TGG ACG AAC TCC CGA GGG GAA GGG AAG CTA TTT TCA ATT GAG ATG
V D E S G E I R A T A F N E Q A D K E
GTG GAT GAG AGT GGT GAG ATC CGA GCT ACA GCT TTC AAT GAG CAA GCT GAC AAG TTC
F S I I E V N K V Y Y F S K G T L K A I
TTT TCC ATT ATT GAA GTC AAC AAG GTG TAT TAC TCC AAA GGC ACA TCC TTT GAA ATT
A N K Q Y T S V K N D Y E M T F N S E
GCC AAC AAG CAG TAC ACC TCT GTG AAG AAC GAC TAT GAG ATG ACT TTC AAC AGC GAG
T S V I P C D D S A D V P M V Q F E F
ACG TCT GTT ATT CCC TGT GAC GAC TCT GCT GAT GTC CCA ATG GTG CAG TTT GAA TTT
V S I G E L E S K N K D T V L D I I G
GTA TCT ATT GGC GAA TTA GAA AGC AAG AAT AAA GAT ACC GTT TTA GAT ATC ATA GGC
V C K N V E E V T K V T I K S N N R E
GTT TGT AAG AAT GTT GAA GAG GTA ACC AAG GTC ACA ATA AAA TCC AAC AAC AGA GAA
V S K R S I H L M D S S G K V V S T A T
GTT TCT AAG AGG AGC ATC CAT CTA ATG GAT TCA TCT GGC AAA GTG CTC TCA ACA TCT
L W G E D A D K F D G S R Q P V V A I
CTG TGG GGT GAA GAT GCC GAT AAA TTT GAT GGC TCC CGA CAG CCT GTT GTT GCA ATC
K G A R L S D F G G R S L S V L S S S
AAA GGA GCA AGG CTT TCA GAT TTC GGA GGG CGA TCA CTT TCA GTC TTG TCG TCT AGC
T V M I N P D I P E A F K L R A W F D
ACC GTA ATG ATC AAT CCT GAT ATT CCT GAA GCA TTC AAA CTC CGT GCA TGG TTT GAC
S E G O V V E G T S I S E S R R G G G T
TCG GAG GGG CAA GTG GTA GAA GGA ACA TCA ATC TCT GAA TCT CGC GGT GGT GGA ACG
G G G N T N W K S L L E V K N E N L T G
GGA GGT GGA AAC ACT AAT TGG AAG TCT TTG TTA GAA GTG AAA AAT GAA AAC CTT GGC
H G E K A D Y F T S V A T I V Y L R K
CAT GGT GAA AAG GCA GAT TAT TTC ACC AGC GTT GCT ACA ATC GTG TAT CTT CGG AAG
E N C L Y Q A C P S Q D C N K K V I D
GAA AAC TGT CTC TAT CAG GCC TGC CCC TCA CAA GAC TGT AAC AAA AAG GTG ATA GAT
Q Q N G L F R C E K C N K E F P N F K
CAA CAG AAT GGC CTT TTC CGC TGT GAA AAA TGC AAC AAA GAA TTT CCA AAC TTC AAA
Y R L I L S A N I A D F G E N Q W I T
TAT CGT CTC ATC CTT TCA GGT AAC ATT GCT GAC TTT GGG GAG AAT CAG TGG ATT ACC
C F Q E S A E S I L G Q N A T Y L G E
TGC TTC CAA GAA TCT GCT GAG AGT ATT CTG GGC CAG AAT GCT ACT TAT CTT GGA GAA
L K E K N E Q A Y D E V F Q N A N F R
CTT AAG GAA AAA AAC GAA CAA GCC TAT GAT GAG GTG TTT CAG AAT GCC AAC TTC CGT
S Y T F R A R V K L E T Y N D E S R I
TCT TAC ACG TTC AGG GCT CGA GTC AAG CTG GAG ACA TAC AAT GAT GAA TCT CGT ATA
K A T A V D V K P V D H K E Y S R R L
AAG GCC ACG GCC GTG GAT GTG AAA CCT GTT GAC CAC AAA GAA TAC TCT AGG AGG CTA
I M N I R K M A T Q G V
ATT ATG AAC ATT AGG AAA ATG GCA ACT CAA GGT GTC TGA TTT GCC ATA AAG ACC CTT
TGA TGA ATC ACT TAA AAG ATG CAG GAG CAA AAA ATA AAA TGT CCC AAT ACT AAA GCA
CTG AAG CTT

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Figure 2. Nucleotide and predicted amino acid sequences of the cDNA encoding P70. The predicted amino acid sequence is shown in single-letter code. Underlined amino acids correspond to sequences obtained from direct amino acid sequencing of tryptic peptides.

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RFAXENO      MALPQLSEGAISAMLG-GDSSCKPT---LQVINIRPINTGNGPPRYRLLMSDGLNTLSSF
RFAHS       M-VGQLSEGAIAAIMQKGDNIKPI---LQVINIRPITGNSPPRYRLLMSDGLNTLSSF
RFASC       MSSVQLSRGDFHSIFTNKQRYDNPTGGVYQVYNTRKSDGANSNRKNLIMISDGIYHMKA-
            *   * * *   . . .   . *   * * *   * . . . . . * * * . . .
RFAXENO      MLATQLNSLVDNLLATNCICQVSRFIVNNLKDGRVIVME-LDVLKSADLVMGKIGNP
RFAHS       MLATQLNPLVEEEQLSSNCVCQIHRFIVNTLKDGRVVILME-LEVLSAEAVGVKIGNP
RFASC       LLRNQAASKFQSMELQRGDIIRVIAEPAIVRERKKYVLLVDDFELVQSRADMVNQTSTF
            . *   *   . .   *   . . .   . . . . . . . . . . *   . . .
RFAXENO      QP--YNDGQPQPAAPAPASAPAPAPSKLQNSAPPPSMNRGTSKLFG-----GGSL
RFAHS       VP--YNEGLQPQVAPPAPAASPAASSRPQPQNGSSGMGSTVSKAYGASKTFGKAAGPSL
RFASC       LDNYFSEHPNETLKDEDITDSGNVANQTNASNAGVPMHLNSNLSNANERKRFANENPNSQ
            . . .   .   . . .   . . . .   . *   *   . .   *
RFAXENO      LNTPGGSQSKVVP IASLNPYQSKWTVRARVNTKQIRTWSNSRGEKGLFSIEMVDESGEI
RFAHS       SHTSGGTQSKVVP IASLTPYQSKWTICARVTNKSQIRTWSNSRAEGKGLFSLELVDESGEI
RFASC       KTRP-----IFAIEQLSPYQNVWTIKARVSYKGEIKTWHNQRGDGKLFNVNFDLTSGEI
            .   . *   * * * * * * * * * * * * * * * * * * * * * * * * * * *
RFAXENO      RATAFNEQADKFFSIEIVNKVYVYFSKGLKIANKQYTSVKNDYEMTFNSETSVIPCDDSA
RFAHS       RATAFNEQVDKFFPLIEVNVKYYVFSKGLKIANKQFTAVKNDYEMTFNSETSVMPCEDDH
RFASC       RATAFNDFATKFNELQEGKVYVSKAKLQPAKPQFTNLTHPYELNLDLDRDVIIEECFDES
            * * * * * * * * * * * * * * * * * * * * * * * * * * *
RFAXENO      DVPMVQFEFVSIGELESKNKDTVLDIIGVCKNVEEVTKVTIKSNREVSKRSHLMDSSG
RFAHS       HLPVTVQFDFTGIDDLNENKSKDSLVDIIGICKSYEDATKITVRSNNREVAKRNIYLMDSG
RFASC       NVPKTHFNFIKLDIAIQNQEVNSVDVGLGIQTINPHFELTSRAGKK-FDRRDITIVDDSG
            . *   . * *   . . . . .   . . * * * . . .   . *   . . .   * *   . * *
RFAXENO      KVVSTTLWGEDADKFDGSRQPVVAIKGARLSDFGGRSLSVLSSSTVMINPDIPEAFKLR
RFAHS       KVVSTATLWGEDADKFDGSRQPVLAIKGARVSDFGGRSLSVLSSSTIIANPDIPEAYKLRG
RFASC       FISISVGLWNQALDFNLPEGSVAAIKGVVTVDFGGKSLSMGFSSTLIPNPEIPEAYALKG
            . .   * *   . *   *   . .   * * * * * * * * * * * * * * * * *
RFAXENO      WFDSEGQVVEGTSISESRGGGTGGGNT-----NWKSLEVKENLGHGEKADYFTSVAT
RFAHS       WFDAEQALDGVSISDLKSGGVGGGNT-----NWKTLYEVKSENLGQGDKPDYFSSVAT
RFASC       WYDSKGRNANFITLKQEPGMGGQSAASLTKFIAQRITIAQAENLGRSEKGDFFSVKAA
            * * *   . . . . .   *   . . .   . . . . .   * * * * * * * * * *
RFAXENO      IVYLRKENCLYQACPSQDCNKKVIDQONGLFRCEKCNKEFPNFYRRLISANIADFGENQ
RFAHS       VVYLRKENCMYQACPTQDCNKKVIDQONGLYRCEKCDTEFPNFYRMLISVNIADFQENQ
RFASC       ISFLKVDNFAYPACSNENCNKKVLEQPDGTWRCEKCDTNNARPWNRYILTISIIDETNQL
            . . *   . *   * * * * * * * * * * * * * * * * * * * * * *
RFAXENO      WITCFQESAESILGQATYLGELKEKNEQAYDEVFQANFRSYTFRARVKLETYNDESRI
RFAHS       WVTCFQESAEAAILGQNAAYLGELKDKNEQAFEEVFQANFRSIFRVRVKVETYNDDESRI
RFASC       WLTFLDQAKQLLGV DANTLMSLKEEDPNEFTKI TQSIQMNEYDFRIRAREDTYNDQSRI
            * * *   . .   * * *   * * *   . . . . .   . . * * *   . * * * * *
RFAXENO      KATAVDVKPVDHKEYSRRLIMNIRKMATQGV
RFAHS       KATVMDVKPVDYREYGRRLVMSIRRSAL--M
RFASC       RYTVANLHSLNYRAEADYLADELSKALLA--
            . *   . . . . .   . *   . .

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Figure 3. *Xenopus* P70 is homologous to the largest subunit of RPA. The amino acid sequences of *Xenopus* P70 (RFAXENO) and RPAs from human (RFAHS; Erdile et al., 1991) and *S. cerevisiae* (RFASC; Heyer et al., 1990; Brill and Stillman, 1991) were aligned using the Clustal V program (Higgins et al., 1991). Asterisks indicate the positions where all three sequences are identical. Dots indicate the positions where the amino acids of the three sequences are conservatively substituted.

of the tryptic peptides are found in this open reading frame (Fig. 2, peptides underlined).

We have found that the open reading frame is highly homologous to the largest subunit of human replication protein A (RPA or also called RF-A) which is an essential component for SV-40 DNA replication in vitro and the sequence of which has been recently published (Erdile et al., 1991). Fig. 3 shows a comparison of the amino acid sequences of P70 from *Xenopus*, *Saccharomyces cerevisiae* and human RPA

(Heyer et al., 1990; Brill and Stillman, 1991; Erdile et al., 1991). The homology between human and *Xenopus* P70 RPA is high; 72% of the amino acids are identical and 18% are conservative substitutions. The homology between *S. cerevisiae* and *Xenopus* RPA is lower; 30% for identical and 33% for conservative amino acid changes which are comparable to those between human and *S. cerevisiae* RPA (Erdile et al., 1991).

Perfectly in line with the molecular identification of P70,

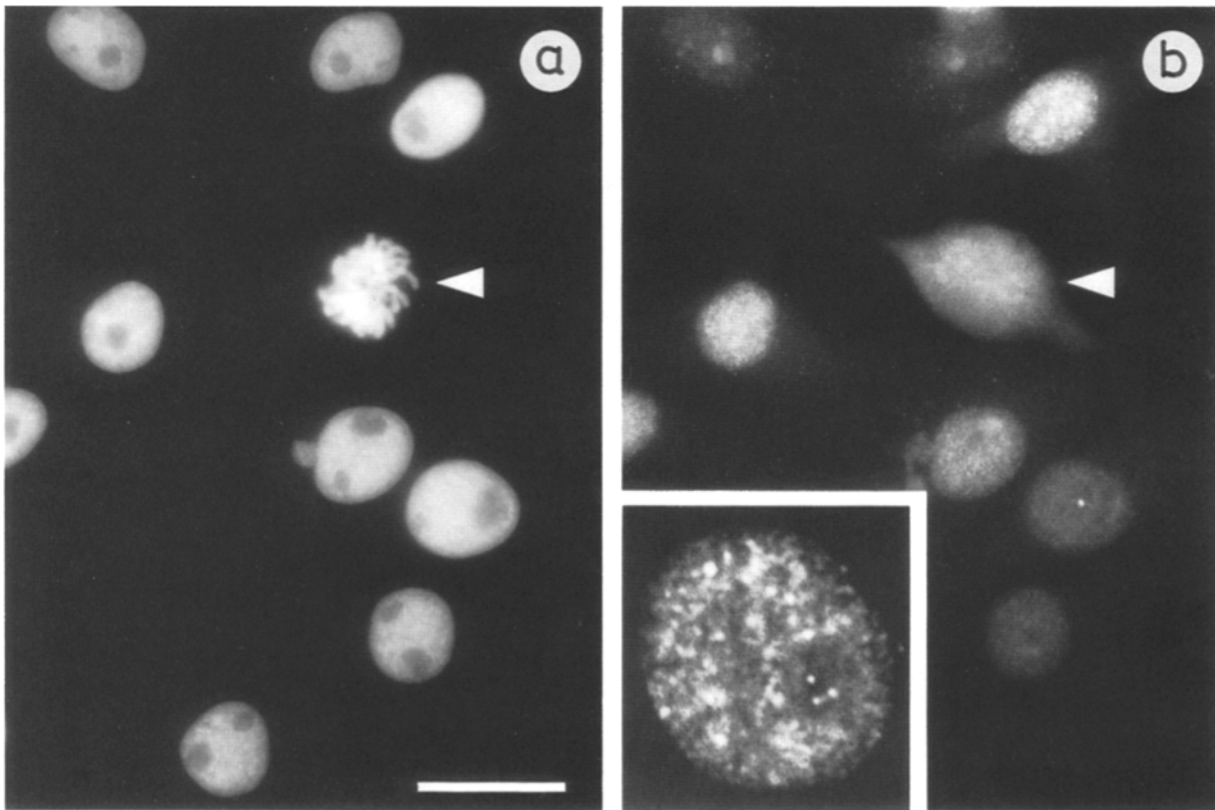


Figure 4. In cells, P70 is a nuclear protein which dissociates from chromosomes at mitosis. *Xenopus* kidney cells were grown on cover slips and processed for immunofluorescence staining with affinity-purified anti-P70 antibodies. *a* shows a DAPI staining and *b* displays the anti-P70 staining pattern. A mitotic cell is indicated with an arrow head: P70 is distributed throughout the cytoplasm in such cells and mitotic chromosomes stain negatively for P70. The inset of *b* shows a magnified image of a nucleus with a punctated pattern of P70 staining. Bar: (*a* and *b*) 25 μ m; (*inset*) 11 μ m.

we have confirmed by filter binding/competition studies that purified P70 had a strong preference for ss DNA over ds DNA. In addition, purified P70 rebinds our ds DNA-Sepharose and remains resistant to elution by 2 M KCl. We conclude that the tight binding is due to ss DNA regions on the column (Adachi, Y., data to be published).

Cell Cycle-dependent Distribution of P70

RPA is a single-stranded DNA-binding protein complex composed of 70-, 34- and 11-kD subunits (Wobbe et al., 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988). In the SV-40 replication system RPA cooperates with T antigen and topoisomerase I or II, before the onset of DNA synthesis, in the assembly of the prepriming initiation complex. Presumably, RPA binds the ori regions unwound by the DNA helicase activity of large T antigen and facilitates the formation of a single-stranded region prior to the initiation of SV-40 DNA synthesis (Wold et al., 1987).

A number of studies have demonstrated that DNA replication is restricted in the nucleus to discrete replication centers (RCs, see Introduction); in *Xenopus* sperm pronuclei incubated in egg extracts the RCs are thought to be composed of an assembly of 300–1,000 replication forks (Mills et al., 1989). We propose (see Discussion) that RCs may be derived from pre-replication centers (preRC) which would be composed of an assembly of prepriming initiation complexes. In view of the essential role played by RPA in the assembly of

the prepriming initiation complex, one would expect P70 to colocalize with the putative cellular preRC. Given a suitable antibody against the 70-kD subunit of RPA in the *Xenopus* egg extract system which can mimic many aspects of the cell cycle, we considered it would be worthwhile to pursue immunolocalization studies.

We initially examined a *Xenopus* kidney cell line using affinity-purified anti-P70 antibodies; these antibodies stain nuclei brightly (Fig. 4) indicating that P70 is a nuclear protein not restricted to eggs. The intensities and staining pattern vary between cells; some cells display a punctated and others a more homogeneous nuclear staining pattern (Fig. 4 *b*, for punctated staining see its inset). P70 belongs to the large class of nuclear proteins which dissociate from mitotic chromosomes into the cytoplasm. In M phase, P70 is not localized on mitotic chromosome plates but is distributed homogeneously throughout the cytoplasm (Fig. 4, *a* and *b*, arrow head). This is in contrast to the staining behavior of anti-*Xenopus* topoisomerase II antibodies used as a control; nuclei and metaphase chromosomes stain brightly for topoisomerase II (not shown; Earnshaw et al., 1985; Gasser et al., 1986).

To examine more precisely the distribution of P70 throughout the cell cycle, we have used *Xenopus* egg extracts which proceed through several rounds of cell cycles (Lohka and Masui, 1983; Hutchison et al., 1987; Murray and Kirschner, 1989). The staining pattern for P70 is strongly cell cycle dependent; the most conspicuous pattern observed

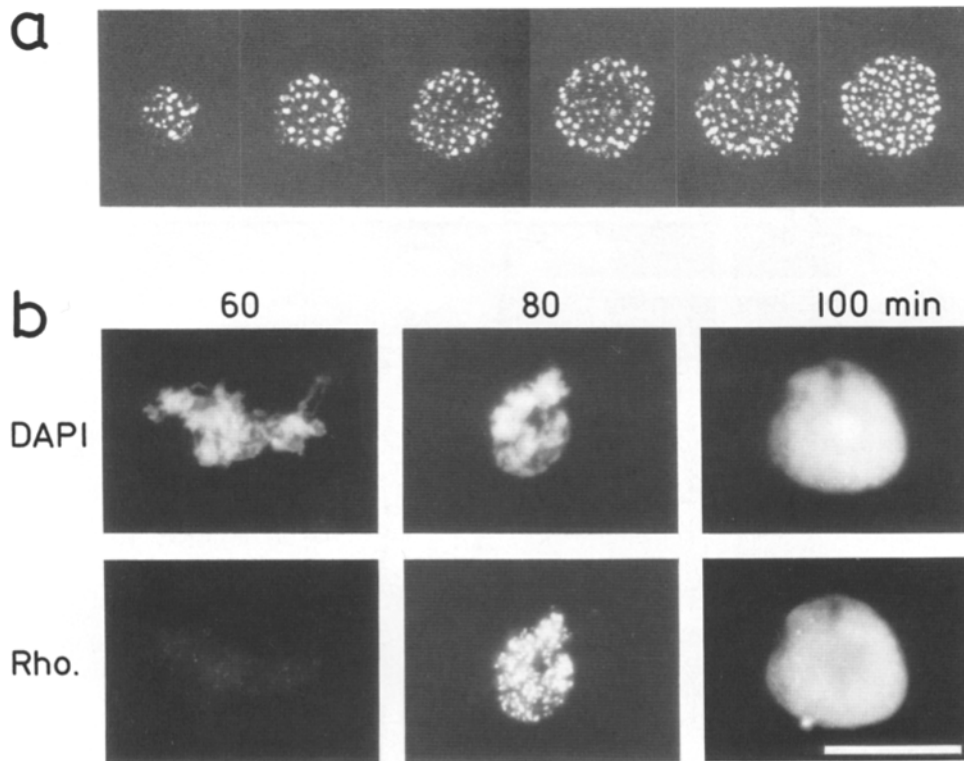


Figure 5. P70 distribution in cycling extracts: P70 foci formation occurs after mitosis. The distribution of P70 was examined by immunofluorescence using affinity-purified anti-P70 antibody staining on sperm chromatin incubated in cycling extracts (Murray and Kirschner, 1989). In this particular experiment, mitotic chromosomes were observed at 60 (60 min, *DAPI*) and again at 140 min. *a* shows optical sections at 1- μ m intervals through a nucleus with P70 foci after 80 min in cycling extract. These images were recorded with a confocal microscope. *b* show the DNA staining (*DAPI*) and P70 staining (*Rho*) pattern of samples taken at 60, 80, and 100 min. These photographs were taken through a conventional fluorescence microscope. Bar: (a) 17 μ m; (b) 25 μ m.

postmitotically (see below) is composed of a large number of bright foci. We have examined P70 foci of such nuclei by confocal optical sectioning; consecutive sections are shown in Fig. 5 *a*. The P70-foci are distributed throughout the interior nuclear lumen and they do not line up along the inner nuclear lamina. From optically sectioned nuclei, we have determined that, on the average, nuclei contain 200 P70 foci, with a range of 80 to 400 per nucleus.

The cell cycle behavior of P70 is shown in Fig. 5 *b*. Samples were taken from the cycling extract every 20 min and immuno-stained for P70 with the help of a rhodamine-tagged secondary antibody and stained for DNA with DAPI. In this particular experiment, chromosome condensation (M phase) was observed at around 60 and again at 140 min. The mitotic-like chromosomes observed at 60 min were essentially negative for P70 staining in line with the observation made in tissue culture cells. But, following mitosis, during decondensation of chromosomes and prior to nuclear reconstruction, a striking P70 foci pattern was observed (Fig. 5 *b*, 80 min). The P70 foci seemed to form just after mitosis as chromosomes started to decondense, since some P70 foci formation was already noticeable on the partially decondensed chromosomal regions in the 60 min samples (data not shown). At later stages (100 min), the P70 staining pattern of nuclei changed; the population of nuclei with P70 foci decreased and was replaced by those with homogeneous P70 staining (Fig. 5 *b*, 100 min).

P70 Localizes to Foci on Xenopus Sperm Nuclei Incubated in Interphase HSS: Dependence on ATP, Divalent Cations, and Temperature

Since P70 foci formation was observed to start just after mitosis in cycling extracts and presumably before the onset of DNA replication, we examined this question in more detail

by analyzing foci formation on *Xenopus* sperm nuclei in membrane-free interphase HSS extracts which are defective for DNA replication (Newport, 1987; Sheehan et al., 1988). In the interphase HSS, condensed demembranated sperm nuclei undergo rapid decondensation within 10 min (Philpott et al., 1991). After incubation at room temperature (22–23°C) for a period of 20 min in HSS, a fraction of nuclei started to acquire a P70 foci pattern. The number of foci increased during further incubation and at 60 min, an average of 200 P70 foci was observed in all of the input nuclei as shown in Fig. 6 *b*. Note that P70 foci are also apparent in the phase image of Fig. 6 *b*, presumably due to the heavy complexing of these centers by antibodies. Input sperm nuclei showed only background levels of staining for P70 (Fig. 6 *a*).

The tight interaction of P70 on the DNA-Sepharose column is a spontaneous process requiring no ATP, divalent cations or incubation at ambient temperature, probably due to the presence of ss DNA regions on the column. In contrast, the assembly of the P70 foci on demembranated sperm chromatin in HSS is not a spontaneous process and is blocked by any of the following treatments (Fig. 6): incubation on ice (*c*), after chelation of divalent cations with EDTA (*d*) or in the absence of ATP (*e*). To deplete the extract of endogenous ATP, we either dialyzed the extract or incubated it with hexokinase and 20 mM glucose (Newmeyer et al., 1986). Addition of an ATP regenerating system to the dialyzed extract restores the ability of the extract to form P70 foci on sperm nuclei (Fig. 6 *f*).

Either of these treatments also reduced the extent of decondensation of the input sperm chromatin (compare Fig. 6, *b* and *c*, *d* or *e*). To rule out the possibility that incomplete decondensation might prevent P70 foci formation the following experiment was performed: the supernatant of heated extracts (80°C, 5 min) is known to decondense sperm nuclei

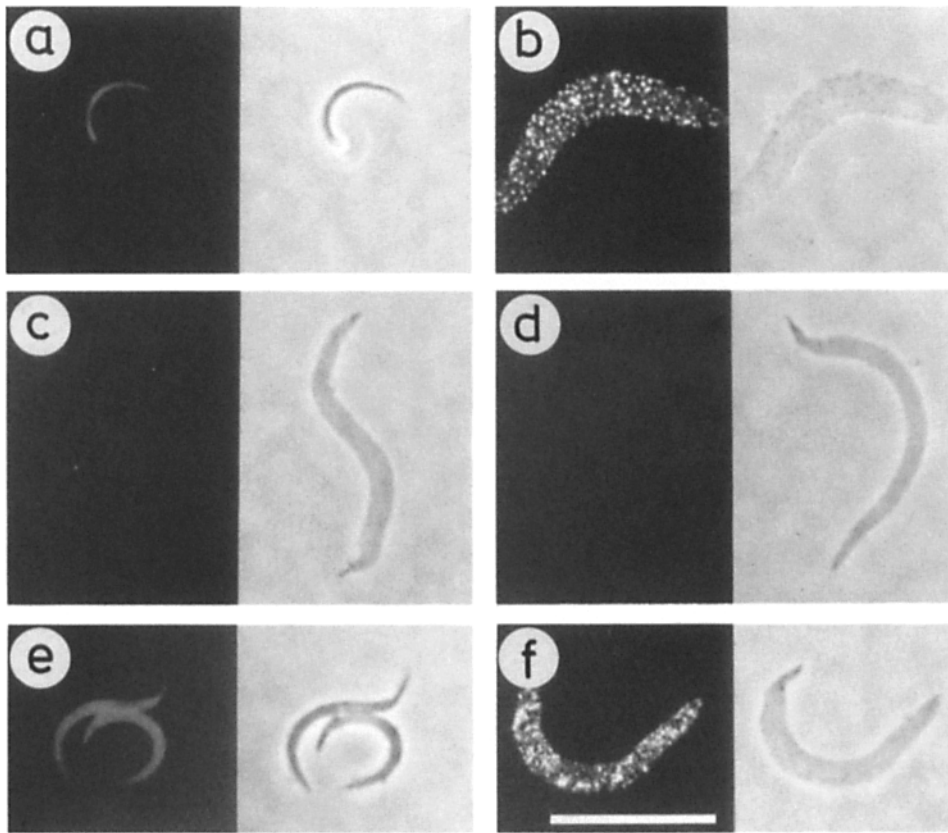


Figure 6. P70 foci form on decondensed sperm chromatin in the interphase HSS. Demembrated sperm nuclei were incubated in an interphase HSS supplemented with an ATP regenerating system for 0 (a) and 60 min (b). Incubation was performed for 60 min in the presence of EDTA (c), on ice (d), in dialyzed HSS without ATP addition (e). f is the same as e but supplemented with an ATP regenerating system. Samples were fixed and processed for immunofluorescence staining with affinity-purified anti-P70 antibodies. Micrographs are shown as pairs, on the left side the P70 staining pattern and the right side the corresponding phase contrast micrographs. Bar, 25 μ m.

efficiently (Pfaller et al., 1991; Philpott et al., 1991). Such nuclei acquire a normal P70 complement of foci, but only if mixed and incubated with complete interphase HSS. Again, we observed that P70 foci formation required ATP, divalent cations, and incubation at ambient temperature despite the fact that the input nuclei were already decondensed. (not shown.)

The association of P70 with sperm nuclei was examined by Western blotting of the nuclei collected by centrifugation. P70 was only detected in the nuclear pellets from the samples in which the P70 foci were formed. No detectable P70 was associated with nuclei which did not assemble P70 foci (data not shown).

In summary, efficient P70 foci formation occurs in membrane-free HSS interphase extracts which are defective for DNA replication. Nuclear membranes are not required for P70 foci formation. This is in line with the observation made in cycling extracts in which foci formation immediately follows the decondensation of mitotic chromosomes. P70 foci formation in HSS is a non-spontaneous process requiring ATP, divalent cations and incubation at ambient temperature, in contrast to the tight binding of P70 to DNA-Sepharose.

P70 Foci Disassemble during DNA Replication

As described above, P70 foci formation occurs in the membrane-free HSS (Fig. 6 b) which is defective in DNA replication of input sperm nuclei (Newport, 1987; Sheehan et al., 1988). Do P70 foci represent poised pre-initiation complexes? The punctated staining pattern of P70 is actually quite similar to that of the replicating DNA foci which can

be observed on sperm pronuclei incubated in egg extracts (Mills et al., 1989, see also Introduction) and the number of P70 foci and RCs are about the same. If P70 is also implicated in early stages of replication in chromatin, as it is in the SV-40 system, then colocalization of P70 foci and replicating DNA foci would be expected. On the other hand, the cycling experiment showed that P70 foci formed just after mitosis disappear and that the protein is redistributed homogeneously in the nucleus (or on chromatin) during progression into the following cell cycle (Fig. 5 b, 100 min). This change might be related to the ongoing DNA replication.

It is possible to initiate synchronized DNA replication on demembrated sperm nuclei pre-incubated in the HSS by the addition of LSS (Mills et al., 1989). We studied the rela-

Table I. Distribution of P70 and Biotin-dUTP Labeling at Various Times after LSS Addition

Time of fixation (min)	P70 foci	dUTP foci	P70 homogeneous (%)	dUTP homogeneous
30	94	14	6	6*
60	42	26	58	58
90	6	6	94	48
120	2	2	94	26

Samples were pulse labeled with biotin-dUTP for 30 min before fixation and processed as described in the legend to Figure 7. The percentage of nuclei with the indicated staining pattern for P70 or for biotin-dUTP incorporation is shown.

* Note that these nuclei at 30 min have much weaker, homogeneous biotin-dUTP signals than those at later times.

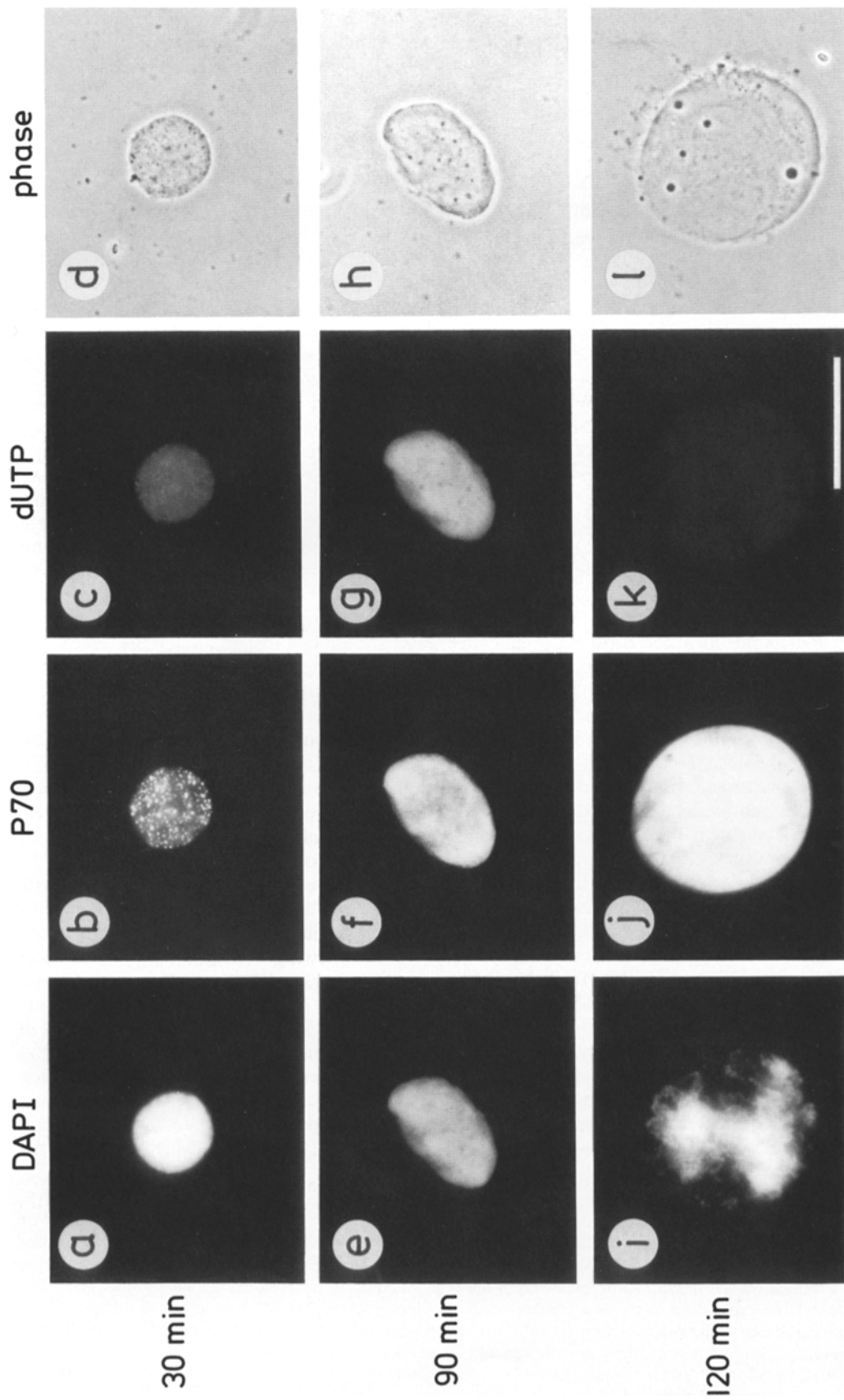


Figure 7. Redistribution of P70 after the onset of DNA replication. DNA replication of decondensed sperm nuclei in HSS was induced by the addition of 3 volumes of LSS. DNA replication was monitored by a 30-min pulse-label with biotin-dUTP before fixation. At 30 min (*a* to *d*), 90 min (*e* to *h*), and 120 min (*i* to *l*) samples were fixed and processed for triple staining. *a*, *e*, and *i* are stained with DAPI. *b*, *f*, and *j* show the distribution of P70. *c*, *g*, and *k* show the incorporation of biotin-dUTP detected with fluorescein-streptavidin. *d*, *h*, and *l* are phase contrast images. Photographs were taken using a conventional microscope. Bar, 25 μ m.

tionship of the P70 foci and replication centers by exposure of sperm nuclei to HSS extracts, which allows decondensation and the formation of P70 foci, and subsequent addition of LSS extract to permit nuclear reconstruction and initiation of DNA replication. Cycloheximide was added to the LSS extract to limit the extract to a single round of replication and to prevent entry into mitosis. Samples were pulse labeled (for 30 min) with biotin-dUTP at different times to reveal the replication foci and the slides were then triple stained with DAPI for DNA, with fluorescein-streptavidin for replicating DNA and with anti-P70 antibodies.

Table I summarizes the quantitative aspects of the temporal changes in P70 staining and biotin-dUTP incorporation patterns. All the demembrated nuclei exposed to the HSS have well-developed P70 foci and, upon addition of the LSS, ~94% of the input nuclei retain the foci pattern during the first labeling period (30 min, see Table I, Fig. 7 *b*). Subsequently, the foci pattern is progressively replaced by a homogeneous P70 pattern (Fig. 7 *f*); at 60 min only 42% of the nuclei retain P70 foci, at 90 and 120 min, this number is down to 6 and 2%, respectively (Table I). This time course confirms the P70 redistribution phenomenon observed in the cycling extracts described above (Fig. 5 *b*). Note that the homogeneous P70 pattern observed coincides with that of the DNA distribution (DAPI) at 60 (not shown) and 90 min (Fig. 7, *e* and *f*). At late post-replicative times (120 min), however, the chromatin condenses and P70 is not localized on the chromatin but is redistributed homogeneously throughout the entire nuclear lumen (Fig. 7, *i* and *j*, see also Fig. 8 *b*). Above, we demonstrated that P70 is not chromosome-bound at mitosis. The dissociation of P70 from chromatin observed here at the post-replicative, late G2-like stage (entry into mitosis is inhibited by cycloheximide) is consistent with observations made in tissue culture cells and in the cycling extracts.

In line with observations made by others (Mills et al., 1989), DNA replication commences in discrete foci (RCs). During the first 30-min-labeling period, most of the nuclei (80%) show little incorporation of biotin-dUTP, but ~14% of the nuclei display a punctated replication pattern. Nuclei with bright homogeneous staining are not observed (Table I). The punctated replication pattern is best observed with the confocal microscope (discussed below). Nuclei with punctated biotin-dUTP staining (26%) are also observed during the second pulse labeling period (30 to 60 min), the fraction of these nuclei then decreases to 6 and 2% at 90 and 120 min, respectively. The nuclei with punctated replication foci are thought to be precursors to those with the bright homo-

neous staining (see also Mills et al., 1989). The fraction of replicated nuclei with homogeneous bright staining is 0% at 30 min, highest (58%) at 60 min, but decreases to 26% at later stages (120 min) indicating that the S phase is nearly complete.

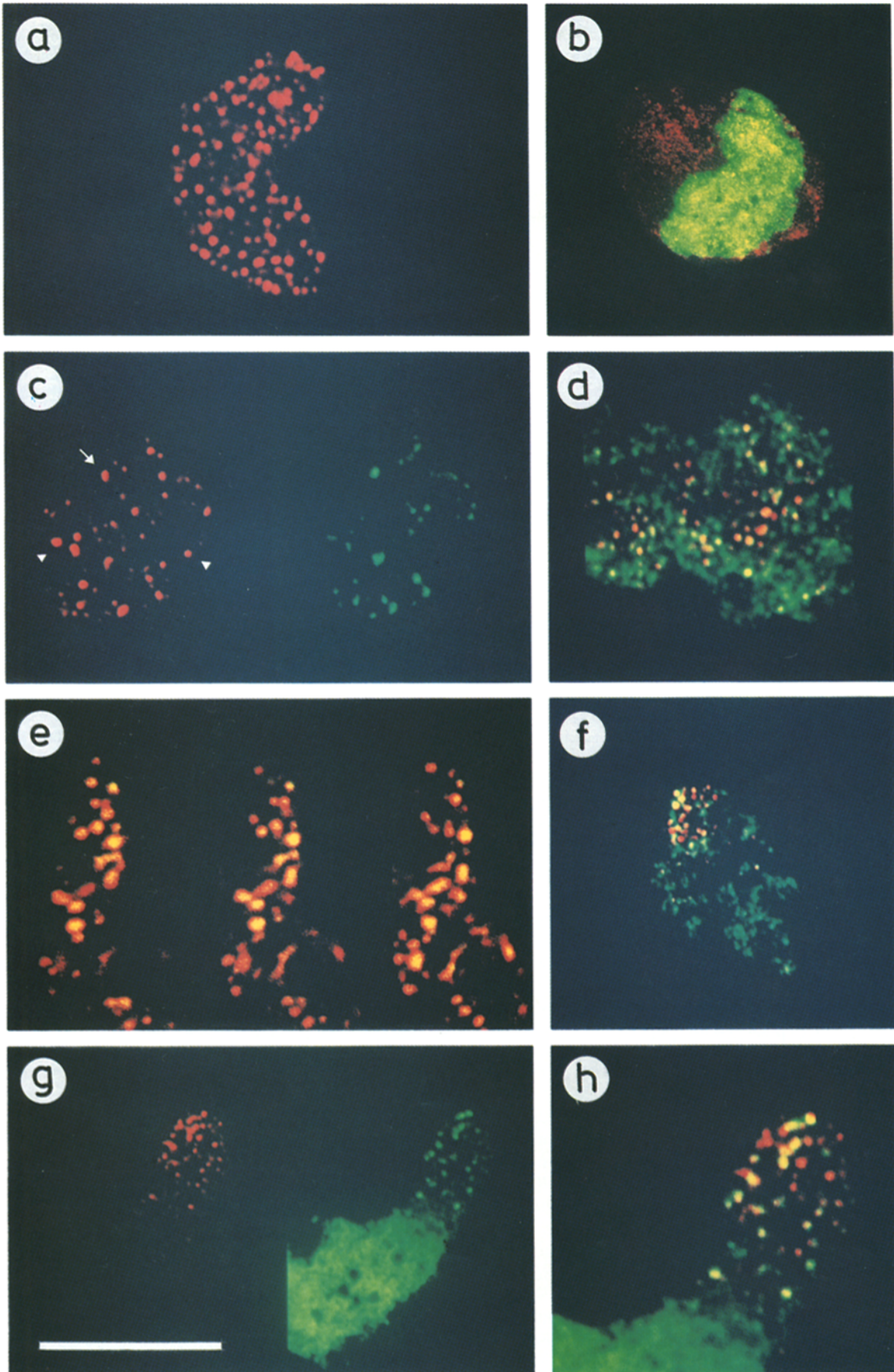
P70 Foci Colocalizes to DNA Replication Centers

The kinetic analysis discussed above, suggests a temporal link between the changing P70 pattern and DNA replication. We have used the confocal microscopy to study the spatial relationship between P70 foci and the replication centers (RCs) in samples derived from the time-course experiment discussed above. We observe that early (initiating) replication foci (RCs) co-localize to the P70 foci, hence called preRCs. Fig. 8 *a* shows a pre-replication nucleus with typical preRCs of P70 foci in a red color (a rhodamine-labeled secondary antibody was used to localize P70). A post-replicative, late G2-like nucleus is shown in Fig. 8 *b* in a merged picture. As mentioned above, P70 dissociates from the partially condensed chromatin at this stage. The red P70 signal can be seen to be distributed throughout the nuclear lumen surrounding the replicated DNA (in green; fluorescein-tagged streptavidin was used to localize the incorporated biotin-dUTP).

Fig. 8 *c* is representative of a nucleus at a very early replicative stage; the left half displays in red the typical pattern of P70 foci. In the right half, the replication initiation pattern of RCs is shown in green. This latter pattern is relatively simple, it consists of a few RCs which co-localize exactly with the P70 foci (compare the red and green patterns).

In some of the panels of Fig. 8 we have merged the red P70 signal and the green replication signal, resulting in a pattern with variable color shading depending on the ratio of the red to green signals (Fig. 8, *d*, *f*, and *h*). The red foci correspond to preRCs which are replication negative. Foci with a yellow appearance are due to co-localization of red P70 foci with the green RCs; they are observed in early S phase nuclei or nuclei which still contain "early" replication or initiation domains. The dominantly green staining pattern, which has a more streaked fibrous appearance, represents more advanced replication stages; in this case the green replication signal dominates the red P70 signal due to the high level of biotin-dUTP incorporation. In addition, P70 is being displaced by ongoing replication as shown above. Replication initiation within a nucleus is by no means synchronous; nuclei usually contain domains with an "early" initiation pattern (composed of red and yellow foci) which are often spa-

Figure 8. Colocalization of P70 foci and RCs. In all panels the P70 staining pattern (rhodamine-conjugated secondary antibody) is in red and replicating DNA, detected with the biotin-dUTP/fluorescein-streptavidin system, is in green. All images were recorded by the confocal microscope. *a* shows P70 preRCs formed on sperm chromatin incubated in the replication-defective HSS extract for 60 min. After this incubation LSS was added to induce DNA replication. *b* shows a post-replicative nucleus observed at 120 min after LSS addition. Note the P70 staining throughout the nuclear lumen. *c* shows a nucleus predominantly at an initiation stage of replication. Note the colocalization of many red P70 foci to the green replication foci (one example is indicated with an *arrow*). Several P70 foci are still replication negative (*arrow heads*); they have a very weak signal in the green channel due to leakage. *e* shows merged, optical 0.3- μ m sections through such an early replication stage nucleus demonstrating the three-dimensional colocalization of the P70 and the replication/initiation pattern. *d* and *f* show merged examples of nuclei at slightly later stages of replication. The yellow foci arise from replication initiation; the red P70 and the green replication signal colocalize. A rare nucleus which contains at the bottom a homogeneously green post-replication region and at the top, an early initiation domain is shown in *g*. The early replication domain of this nucleus is shown enlarged and merged in *h*. Bar: (*a*, *c*, *d*, and *e*) 12.5 μ m; (*b*) 25 μ m; (*f* and *g*) 20 μ m; (*h*) 10 μ m.



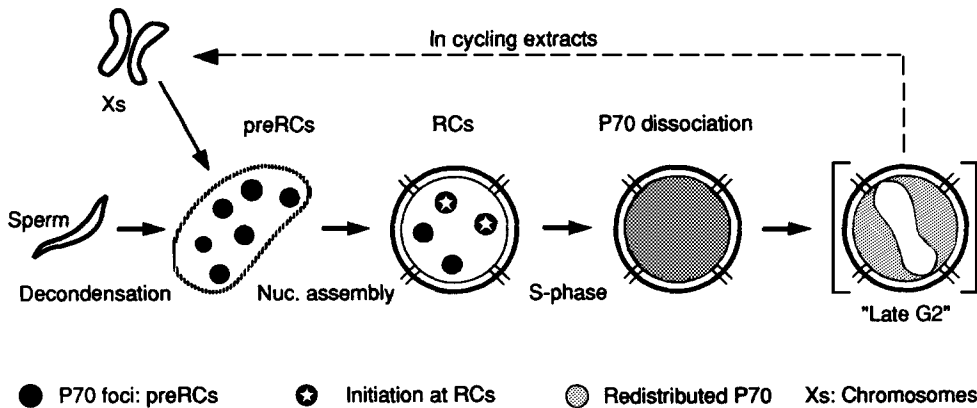


Figure 9. P70 localization through cell cycle in egg extracts. The distribution changes of P70 in egg extracts are summarized. P70 foci (preRCs) form on decondensing chromatin. DNA replication initiates from these preRCs after nuclear membrane-lamina assembly. Ongoing replication induces redistribution of P70. If the extract is blocked from entering mitosis by cycloheximide, then post-replicative nuclei with prophase-like condensed chromosomes (shown in brackets) accumulate; in these nuclei P70 dissociates from the condensing chromatin into the nuclear lumen. P70 is not chromosome bound at mitosis.

tially separated from more advanced, post-initiation areas (composed largely of green streaks).

Fig. 8, *g* and *h* show a nucleus which contains at the bottom a homogeneously green post-replication region and at the top, an early initiation domain (the latter is enlarged). The red and the green channel are presented separately for this nucleus as well as merged. Again, the early domain contains red, replication-negative foci, as well as yellow foci that are indicative of initiation of replication. The red foci observed in the unmerged panel *g* (see also Fig. 8 *h*) serve to illustrate an important control; the crossover leakage from the rhodamine into the fluorescein channel is negligible (~3%) at the instrument settings used. The leakage from the fluorescein into the rhodamine channel is greater (~17%) and was corrected by image processing. This correction, however, is not of crucial importance for the conclusions drawn here, since the green signal arising from replication initiation is weaker than the red P70 signal at the early stages during which co-localization is observed. Co-localization of P70 foci and RCs is not due to cross-contamination of the channels, as is evident by the presence of red and yellow foci in the same picture, that is, the ratio of the two signals changes. The channel overlap is more of a problem at advanced stages of replication when the green replication pattern dominates the red P70 signals.

To rule out the possibility that the yellow (initiation) foci of the merged pictures arises from a simple superposition of red and green signals at different levels in space, we have optically sectioned replicating nuclei. Fig. 8 *E* shows a nucleus thought to be at a very early initiation stage of replication. The channels were merged in this case. This panel demonstrates that initiation of replication occurs within the P70 foci, that is, the yellow signal is quite central to that of the red P70 foci even at different levels of focus.

In summary (Fig. 9), these experiments suggest that P70 foci represent pre-replication centers (hence, the term preRCs); replication initiation occurs predominantly (probably exclusively) within the confines of the preRCs. The preRCs become RCs and ongoing DNA replication induces re-distribution of P70, leading to a more homogeneous dis-

tribution over chromatin. At later post-replicative stages, P70 dissociates from the condensing chromatin into the nuclear lumen; this is clearly observed in cycloheximide-blocked extracts which do not enter mitosis. As observed in cycling extracts as well as in tissue culture cells, the dissociation of P70 from chromatin is complete at mitosis.

Discussion

Extracts prepared from human cells supplemented with purified SV-40 large tumor antigen (T-AG) are known to support replication of plasmids containing the SV-40 origin of replication (Li and Kelly, 1984; Stillman and Gluzman, 1985; Wobbe et al., 1985). Biochemical fractionation of the cell extract has led to the identification of a number of protein factors, one of which is RPA. RPA is a multi-subunit protein (70, 34, and 11 kD) which preferentially binds to ss DNA and is thought to be a eukaryotic SSB, which facilitates the generation of a single-stranded DNA region (Wold and Kelly, 1988; Fairman and Stillman, 1988). RPA is known to cooperate with T-AG, before the onset of DNA replication, in the formation of the prepriming complex consisting of the extensively unwound SV-40 origin of replication. RPA is also proposed to be involved in the elongation process of the replication fork (Kenny et al., 1989; Tsurimoto and Stillman, 1989).

The single-stranded DNA binding capacity of RPA is known to reside in the 70-kD subunit (Wold et al., 1989). The P70 protein studied in this report was identified, initially by tryptic peptide sequences and subsequently by sequencing of a cDNA clone, as the *Xenopus* homolog of the largest, 70-kD subunit of RPA (Fig. 3). P70 is observed following fractionation of *Xenopus* egg extracts on a DNA-Sepharose column. This protein is resistant to extraction by 2 M KCl and is the most prominent protein in the subsequent SDS-eluate (Fig. 1). Quite likely, our ds DNA-Sepharose contains a portion of ss DNA which is sufficient for P70 binding. Indeed, additional DNA binding studies using ds versus ss DNA have also revealed a strong preference of the *Xenopus* RPA for ss DNA (not shown). Because RPA is known to be

composed of three subunits, we re-examined our SDS-polyacrylamide gels and found that the smaller subunits of 34 and 11 kD were co-eluted with P70 from the DNA-Sepharose (data not shown).

The most significant observations reported here are the following: (a) The localization of P70 to subnuclear structures that form several hundred foci on decondensing chromosomes. These foci appear post-mitotically but prior to the assembly of the nuclear membrane-lamina complex and the onset of DNA synthesis; (b) The identification of these foci as preRCs which are poised for DNA replication. The importance of these observations lies in the foundation they provide for future analyses of the early events of DNA replication as well as of the relationship of nuclear structure to this metabolic event.

These notions, summarized in Fig. 9, stem from immunofluorescence studies of sperm nuclei in cycling *Xenopus* egg extracts. The P70 staining pattern changes dramatically through the cell cycle. Metaphase chromosomes at mitosis stain negatively for P70 in the egg extract as well as in *Xenopus* kidney cells (Figs. 4 and 5). In the latter case, P70 is distributed homogeneously throughout the cytoplasm, indicating displacement from chromosomes rather than degradation of this protein at mitosis (Fig. 4). This behavior is akin to that of the nuclear lamina.

In the cycling extracts, the preRCs become visible immediately upon decondensation of the mitotic chromosomes (Fig. 5 b, 80 min) and before incorporation of detectable biotin-dUTP, which is detected about 20 min after mitosis (data not shown). Between 70–400 discrete foci can be observed per nucleus, distributed throughout the nuclear lumen as established by optical sectioning (Fig. 5 a). Upon progression in the cell cycle (to an advanced or post S phase stage), homogeneous P70 staining is observed. Morphologically, preRC formation precedes the reconstruction of the nuclear membrane and initiation of replication.

The striking and efficient formation of preRCs in membrane-free HSS extract is in perfect agreement with the timing of P70 foci formation observed in cycling extracts. The HSS extracts decondense input demembrated *Xenopus* sperm nuclei efficiently but they are defective in the assembly of the membrane-pore-lamina complex as well as in DNA replication (Newport, 1987; Blow and Laskey, 1988; Sheehan et al., 1988; Philpott et al., 1991; Newport et al., 1990). Despite this deficiency, preRC formation is observed in demembrated *Xenopus* sperm nuclei. Thus, preRC formation does not require an active nuclear import system or an integral nuclear membrane. The assembly of P70 prior to nuclear reconstruction is in contrast with that of PCNA (an auxiliary protein of DNA polymerase delta) and DNA polymerase alpha since their accumulation in nuclei occurs only after the assembly of the nuclear membrane and lamina (Hutchison and Kill, 1989). This is consistent with the notion that the P70 foci represent preRCs. An intact nuclear lamina structure is also probably not involved in preRC formation, since lamin assembly has been shown to be dependent on the presence of the nuclear membrane (Newport et al., 1990). However an involvement of lamina proteins in preRC formation is not ruled out.

We do not know yet whether the P70 foci or preRCs formed in the HSS include ss regions of unwound DNA. The HSS alone cannot induce DNA replication of the decon-

densed input nuclei, although it contains primase and DNA polymerase alpha activities and can replicate ss naked DNA templates (Mechali and Harland, 1982). These facts suggest that in HSS, the formation of single stranded DNA regions or the synthesis of RNA primers are suppressed in the decondensed sperm nuclei on which P70 preRC foci are already formed. Clearly, replication initiation requires other activation steps (e.g., protein modification) and/or some structural support.

Most, if not all, of the initiation of DNA replication is confined to P70 foci. Experimentally, this has been studied by the addition of a low speed, membrane-containing egg extract (LSS) to a HSS extract containing pre-decondensed sperm nuclei. Synchronous DNA replication occurs after a 30-min lag during which the nuclear membrane-pore-lamina assembly and general nuclear reconstruction presumably occur (Mills et al., 1989; Newport et al., 1990). During this lag period no significant changes are observed in the preRC pattern of P70 foci. Co-localization of P70 foci to RCs is seen in the fraction of early-replicating nuclei (Fig. 8 c) but also in nuclei at later stages of S phase containing late as well as "early" initiation regions such as the nucleus shown in Fig. 8, g and h. Parenthetically, replication initiation, although usually occurring in confined areas of the nucleus, appears not to commence at a particular nuclear region, such as the periphery. This may be due to the use of sperm nuclei which appear to contain no heterochromatin.

Co-localization of the P70 preRC foci with RCs is best seen in merged color micrographs as yellow (or orange) spots. This yellow colour stems from the superposition of the red P70 signal with the green biotin-dUTP signal. Particularly convincing evidence for the spatial confinement of replication initiation to P70 foci was obtained by optical sectioning using the confocal microscope; the yellow spots are due to a true co-localization in space of the two signals and not to a simple superposition (Fig. 8 e). The global time-course and temporal link of the P70 staining and the biotin-dUTP labeling pattern through the cell cycle (Table I) together with the co-localization results strongly support the notion that the P70 foci represent preRCs. We consider the terminology prereplication centers, preRCs, for the subnuclear P70 foci justified. The known participation of RPA in the formation of the SV-40 prepriming complex is in perfect agreement with the results reported here. As an interesting possibility, preRCs may represent an assembly of pre-replication structures, possibly interacting with the (all elusive) cellular origins of replication.

Colocalization or enrichment of replication proteins such as PCNA and DNA polymerase alpha to nuclear replication centers has been observed previously (Bravo and MacDonald-Bravo, 1987; Kill et al., 1991). Interestingly, the herpes simplex protein, ICP8, a viral SSB protein, colocalizes to the replication foci generated by viral replication (Kops and Knipe, 1988; Ruyechan, 1983). ICP8 was shown to redistribute from a punctated pre-replication position to the replication centers in a DNA synthesis dependent manner (Quinlan et al., 1984).

P70 foci formation on decondensed sperm nuclei is not a spontaneous process. In the absence of ATP or of divalent cations, or following incubation on ice, preRCs are morphologically absent and, biochemically, P70 is not associated with sperm chromatin. PreRC formation may require

unwinding of the DNA targets on sperm nuclei, possibly explaining the requirement for ATP. This contrasts to the spontaneous binding of P70 to DNA-Sepharose; a tight interaction is observed under all of these conditions. Therefore it is unlikely that the P70 foci simply result from an interaction with preexisting ssDNA on sperm chromatin. In support of this, in HSS extracts, which are known to replicate ssDNA efficiently (Mechali and Harland, 1982), we have not observed any punctate biotin-dUTP incorporation on sperm chromatin (data are shown).

The link between replication initiation and the presence of nuclear structures such as the nuclear membrane and lamina is not understood mechanistically. These structures may be required to permit assembly of the nuclear matrix, an operationally defined nuclear substructure which has defied a satisfactory characterization, both biochemically and structurally, despite considerable efforts by many laboratories. It has repeatedly been suggested that eukaryotic DNA replication occurs in association with the nuclear matrix, which may serve as a scaffolding for the organization of replication forks (Berezney and Coffey, 1975; Jackson and Cook, 1986; Vaughn et al., 1990). The organization of replication forks into foci supports this view (Nakamura et al., 1986; Nakayasu and Berezney, 1989; Fox et al., 1991). The association of nascent DNA (including replication forks) observed following a variety of nuclear dissociation procedures, has led to models which suggest that the DNA strand may be spooled through fixed, attached replication sites (reviewed by Cook, 1991). Since preRCs assemble in HSS extracts on sperm chromatin prior to reconstruction of the nucleus, one would have to conclude that such pronuclei already possess an organizing matrix. This question needs to be addressed experimentally, but we favor an alternative view that avoids the difficulty of spooling several hundred strands through fixed, attached sites. We propose that preRCs and RCs may arise simply by dynamic self-aggregation of spatially neighboring replication forks or pre-initiation structures, possibly, to achieve molecular crowding. At least on the lagging strand, replication elongation is known to occur discontinuously in nucleosome-sized bursts (reviewed by Hand, 1978). If the replication machinery briefly dissociates from its "attached" stage temporarily during the elongation steps ("hop and go"), then spooling of DNA through fixed sites can be avoided.

DNA replication in *Xenopus* egg extracts containing cycloheximide is limited to a single round of semi-conservative replication (Blow and Laskey, 1988). An important question of DNA replication is to understand how cells suppress the re-replication of replicated DNA. Blow and Laskey (1988) have demonstrated that the integrity of the nuclear membrane is necessary to suppress re-replication, since permeabilization of the membrane leads to a second round of replication. They suggested the existence of a "licensing factor" implicated in initiation of replication that can interact with chromatin only during the period of nuclear membrane breakdown possibly due to the absence of nuclear localization sequence. The licensing factor is proposed to be inactivated following use. Our experiments do not identify P70 as the licensing factor, but this protein fulfills some of its proposed properties; P70 foci or preRCs assemble on the decondensing chromosomes during the period of nuclear membrane breakdown, prior to initiation of DNA replication and

the P70/RPA complex is likely to be involved in the initiation of replication. It will be of interest to see whether preRCs formation is prevented in nuclei containing an intact nuclear membrane.

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Note added in proof. We have recently demonstrated, by immunodepletion of *Xenopus* extracts (LSS) for RPA and subsequent complementation of the depleted extracts with the purified protein, that RPA is an essential factor for DNA replication of intact sperm nuclei (Adachi, Y., unpublished results).

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