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RAPID COMMUNICATION

Stability and Nuclear Distribution of Mammalian Replication Protein A Heterotrimeric Complex

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Replication protein A (RPA), a stable complex of three polypeptides, is the single-stranded DNA-binding protein essential for DNA replication in eukaryotic cells. Previous studies of the subcellular distribution and stability of the RPA heterotrimer during the mammalian cell cycle have produced conflicting results. Here, we present evidence that these inconsistencies can be accounted for by the presence of an extractable pool of soluble RPA within the nucleus. Indirect immunofluorescence experiments in both CHO and HeLa cells showed that all three RPA subunits associated specifically with sites of ongoing DNA synthesis, similar to the replication fork protein proliferating cell nuclear antigen. Furthermore, we found no evidence for disassembly of the chromatin-bound heterotrimeric RPA complex *in vivo.* Our results are consistent with a role for RPA in the initiation and elongation steps of replication, as previously defined in the viral *in vitro* replication systems.

Key Words: **RPA complex; detergent extraction; replication foci; cell nucleus; cell cycle.**

INTRODUCTION

Replication protein A (RPA) is the single-stranded DNA-binding protein (SSB) required for replication of eukaryotic DNA. RPA is a complex of three polypeptides (designated RPA14, RPA32, and RPA70 according to their apparent molecular weights) conserved in all eukaryotes [1]. The genes encoding all three RPA subunits have been shown to be essential for viability in the yeast *Saccharomyces cerevisiae* [2]. Studies in viral and yeast replication systems have shown that the RPA complex is required for stabilization of unwound origin DNA [3–7] and assembly of a primosome at the initiation step [8]. RPA is also involved at the elongation stage of DNA replication [4, 9, 10]. The DNA-binding activity of the complex resides in the large subunit [7, 11], whereas the roles of the other two subunits are not clear [1, 11].

Studies of RPA in mammalian cells have posed several enigmas. One report [12] provided evidence that the three subunits of the extremely stable RPA complex [1] dissociated during mitosis and reassociated during early G1 phase to display a diffuse distribution throughout the rest of interphase in HeLa cell nuclei. Separation of the middle and large RPA subunits throughout the entire cell cycle was observed in myotubes that had been induced to reenter proliferative state by transformation with SV40 T antigen [13]. Surprisingly, only the large subunit of the RPA complex was found to colocalize with DNA replication sites in this report. Cell cycle-dependent disassembly of the RPA complex was also suggested by a recent *in vitro* study of biochemical properties of RPA [14]. Another puzzling aspect of RPA behavior is that a number of investigators found no evidence for significant changes in the nuclear distribution of RPA between replicative (S phase) and nonreplicative (G1 and G2 phases) stages of the cell cycle [12, 15, 16]. Clearly, these results are difficult to reconcile with the documented extreme stability of the RPA complex *in vitro,* as well as its role as a component of the replication machinery, which would be expected to dynamically associate with sites of ongoing DNA synthesis within S-phase nuclei. Resolving this confusion is critical to our understanding of the role of RPA in mammalian chromosome replication.

We have initiated a study of the events that prepare mammalian chromosomes for replication from the end of mitosis till the beginning of S phase [17–19]. In the course of these studies we examined the cell cycle-regulated changes in the nuclear distribution and chromatin association of hamster RPA in view of its potential role as a component of mammalian prereplication complexes. Here we show that RPA is present in the

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nucleus in a soluble form throughout the cell cycle in Chinese hamster ovary (CHO) cells and human HeLa cells. During S phase, a fraction of RPA associates tightly with nuclear components and is resistant to extraction with nonionic detergents. We have found no evidence for separation of the RPA subunits in the detergent-resistant RPA fraction *in vivo*. Contrary to some previous studies, our immunolocalization technique provides a positive and dynamic colocalization of all RPA subunits with sites of ongoing DNA synthesis in mammalian nuclei, consistent with its role as an essential replication protein.

MATERIALS AND METHODS

Cell culture and labeling of nascent DNA. CHOC 400 [20] and HeLa 229 cells (ATCC 2.1-CCL) were grown as monolayer cultures in Dulbecco's modified Eagle's medium (GIBCO) supplemented with nonessential amino acids and 5% fetal bovine serum (GIBCO) at 37°C in a 5% CO_2 atmosphere. Synchronization of cells in mitosis by nocodazole block and at the G1/S-phase border by aphidicolin block was done as described [18]. Cell cultures were pulse-labeled for 10 min by addition of 5-chloro-2′-deoxyuridine (CldU; Sigma) to the medium to a final concentration of 10 μM. At the end of the labeling period the CldU medium was removed, and the cells were transferred to ice, washed with cold PBS, and processed for indirect immunofluorescence labeling.

Immunofluorescence microscopy. Cells grown on coverslips were washed with cold cytoskeleton buffer (CSK; 10 mM Hepes/KOH, pH 7.4, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$) and either fixed directly or first extracted for 2 min on ice with 0.5% Triton X-100 (Triton; Sigma) in CSK buffer [supplemented with 1 mM PMSF (Boehringer Mannheim); 1 μg/ml each pepstatin, chymostatin, leupeptin, and aprotinin (Sigma); 50 mM sodium fluoride; and 0.1 mM sodium vanadate) and then fixed. Cells were fixed for 30 min at room temperature with 4% formaldehyde in PBS, washed with PBS, incubated for 5 min with 0.5% Nonidet-P40 (Boehringer Mannheim) in PBS, and stored in PBS at 4°C. The following primary antibodies were used for immunostaining: (i) rabbit polyclonal anti-RPA antibody [21], (ii) rabbit polyclonal anti-SSB/RP-A (a gift from Dr. M. Kenny, Albert Einstein College of Medicine, New York), (iii) mouse monoclonal anti-RPA70 (mAb-1; CalBiochem NA13), (iv) mouse monoclonal anti-RPA32 (mAb-2; CalBiochem NA18), (v) rabbit polyclonal anti-RPA14 (a gift from Dr. J. Borowiec, New York University), and (vi) mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (PC10 mAb; Santa Cruz Biotechnology, sc-56). From the various fixation protocols tested, the best PCNA-staining results were obtained by fixing the cells with ethanol:acetic acid (19:1) for 10 min at −20°C (a prior extraction with Triton was not necessary in this case). The secondary antibodies utilized were Texas red (TxRed)-conjugated donkey anti-rabbit IgG (Jackson Laboratories, 711-075-152), and FITC-conjugated donkey anti-mouse IgG (Jackson Laboratories, 715-095-151). Incubations with antibodies were carried out in a humidified chamber for 0.5–1 h at room temperature or for longer time periods at 4°C. All washes were done with 0.5% Tween 20 (Sigma) in PBS at room temperature.

In double-staining experiments in which RPA or PCNA was colocalized with sites of CldU incorporation, the cells were fixed and immunostained for the respective protein as described above. The cells were then fixed a second time, followed by incubations for 5 min in 0.5% NP-40 in PBS and for 30 min in 1.5 N HCl. The cells were washed with PBS/Tween and stained with rat anti-BrdU antibody (Harlan-Sera Laboratory, MAS250b), followed by FITC-conjugated donkey anti-rat IgG (Jackson Laboratories, 712-095-153). Coverslips were mounted in Vectashield (Vector Laboratories). Photographs were taken on Kodak Ektachrome P1600 films using a Nikon Labophot-2 microscope equipped with a Nikon PlanApo 100× 1.4 NA oil-immersion objective and a dual FITC/rhodamine (Merge images) or single FITC and TxRed fluorescence filters. Slides were scanned with Nikon Coolscan and Nikon LS-2000 devices and assembled in a Power Macintosh and Apple G3 computers using Adobe PhotoShop 5.0.2 and Claris Draw 1.0v4 software.

RESULTS AND DISCUSSION

RPA Exists in a Soluble and Chromatin-Bound Form in Mammalian Nuclei

Results of immunofluorescence studies can differ significantly depending on the fixation procedure applied [22, 23]. Therefore, we initiated our studies of RPA by comparing different fixation conditions. First, we found that the use of "precipitating" fixatives, such as methanol, ethanol, methanol:acetone (1:1), and ethanol:acetic acid (19:1), did not produce positive staining for RPA (not shown). Hence, they were not used further in this study.

In another series of experiments, we followed a previously employed fixation procedure, which utilized formaldehyde as a cell-fixing agent [12, 15]. Exponentially growing CHO (CHOC 400) cells were fixed with formaldehyde and immunostained for RPA. Due to the low interspecies cross-reactivity of available anti-RPA antibodies, nearly all of which have been raised against the human RPA complex [1], only one antibody in our collection recognized hamster RPA (raised against the RPA trimeric complex) [21]. RPA was uniformly distributed throughout the nuclear interior (except in the nucleoli) in all interphase CHOC 400 cells, consistent with previous reports on human RPA [12, 15, 16]. A few cells (<5%) displayed several very bright foci superimposed on the strong homogeneous nuclear background. In mitotic cells, chromosomes stained negatively for RPA, and the protein complex was dispersed randomly in the cytosol (Fig. 1A, RPA). Notably, we were not able to observe punctate RPA staining that resembled patterns of DNA replication foci at any time during the cell cycle. Replication foci represent discrete sites of nuclear DNA synthesis that have been characterized in various cell types and can be visualized by immunostaining of nascent DNA which has been pulse-labeled with halogenated nucleotides [17, 23–26]. To directly examine the relative distribution of RPA and sites of DNA replication, CHOC 400 cells were pulse-labeled with CldU, fixed with formaldehyde, and double-stained with fluorescent antibodies specific for RPA and CldU. As shown in Fig. 1A (CHOC 400, Merge), the intensity of the overall RPA staining was so strong that the underlying CldU replication patterns were difficult to detect, demonstrating that RPA was also present at numerous sites outside of the

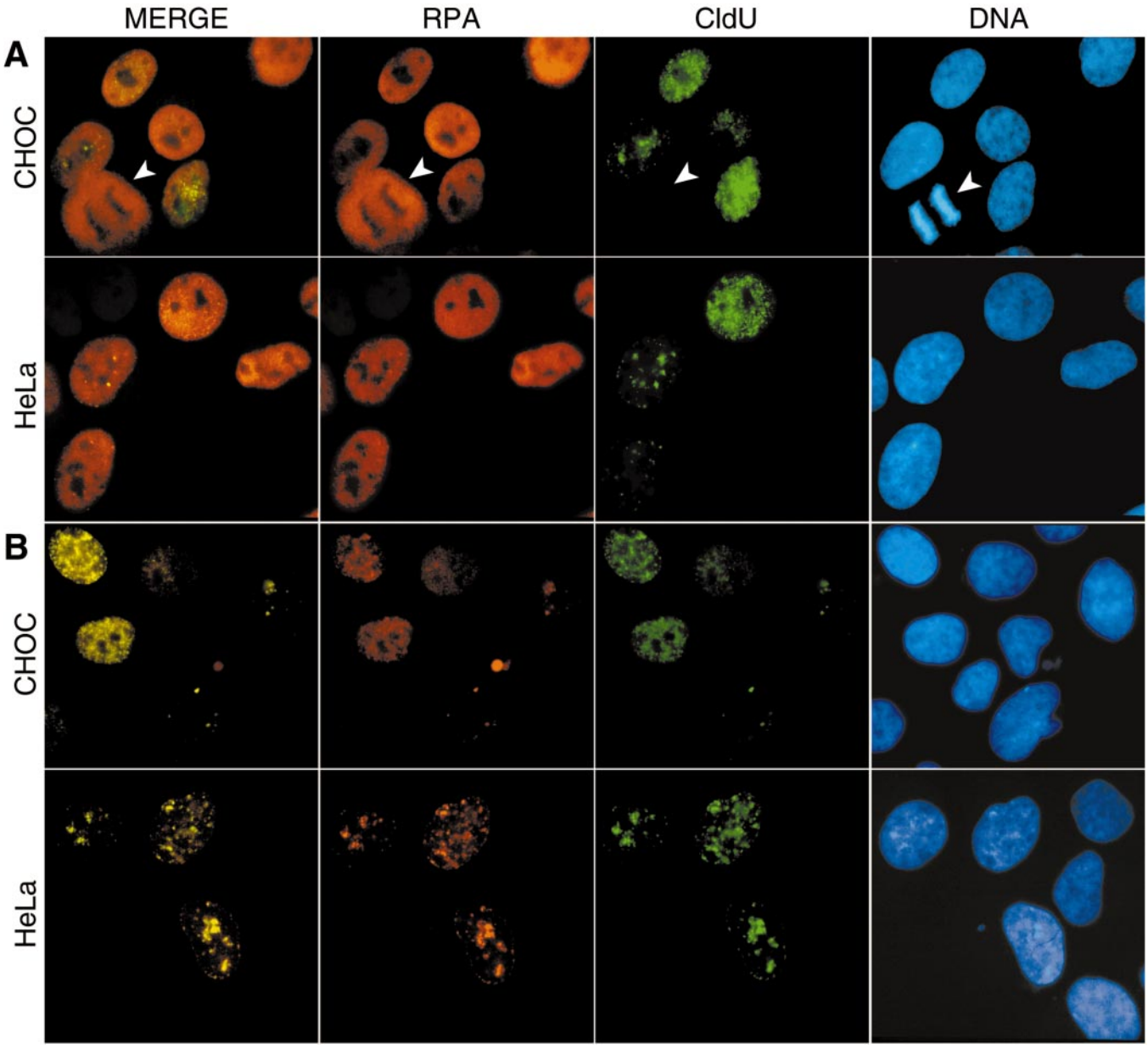


FIG. 1. Anti-RPA antibodies stain homogeneously the nuclei of nonextracted mammalian cells but specifically illuminate DNA replication sites in Triton-extracted cells. Asynchronous cultures of CHOC 400 or HeLa cells were pulse-labeled with CldU as described under Materials and Methods. Indirect immunofluorescent staining of RPA (red) and CldU (green) sites was performed with aliquots of these cells that were either untreated (A) or Triton-extracted (B) before fixation with formaldehyde. DNA was stained with 0.2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). The arrowhead points to an anaphase cell.

CldU-labeled replication foci. One potential caveat of our study was that we were using an antibody against the human RPA complex to immunostain the hamster homologue of RPA. To eliminate the formal possibility that interspecies differences could produce artifactual results, we performed immunolocalization experiments with HeLa cells (Fig. 1A, HeLa). Human RPA exhibited nuclear distribution identical to its hamster homologue. Thus, the use of an anti-human RPA antibody cannot account for the results in CHOC 400 cells.

One plausible explanation for the lack of colocalization of RPA with sites of ongoing replication is that the conditions for fixation prior to immunolocalization had stabilized a population of excess soluble RPA molecules within the nucleus. A similar soluble pool of protein has been identified for another replication factor—

PCNA [23]. Bravo and MacDonald-Bravo demonstrated the existence of two populations of PCNA in mouse cells: (i) a population detectable only during S phase, which interacts with nuclear structures and is resistant to extraction with nonionic detergents, and (ii) a second population, which is present throughout the entire cell cycle and is easily removable from nuclei by brief extraction with Triton X-100. To test whether analogous subpopulations of RPA exist in mammalian nuclei, both CHOC 400 and HeLa cells were pulse-labeled with CldU, briefly extracted with Triton X-100, and fixed with formaldehyde. When these cells were stained with anti-CldU and anti-RPA antibodies (Fig. 1B) less than half of the nuclei reacted with the antibodies. All positive nuclei were stained with both antibodies, indicating that they were in S phase. The fact that only S-phase Triton-extracted nuclei stained positively for RPA argues that RPA is not tightly bound to chromatin during nonreplicative (G1 and G2) phases of the cell cycle. We confirmed this observation using synchronized populations of CHOC 400 [18] and HeLa cells (not shown). In S-phase nuclei, RPA-positive sites colocalized with CldU-labeled replication foci (Fig. 1B, Merge). Thus, treatment of cells with Triton prior to fixation removed a soluble pool of excess RPA within the nucleosol and uncovered the punctate RPA patterns typical for replicating nuclei. Parallel control experiments (not shown) in which aliquots of the same cells were stained with anti-PCNA and anti-CldU antibodies produced analogous staining results, consistent with previous reports on the nuclear distribution of PCNA [23].

The RPA Subunits Form a Stable Complex in the Chromatin-Bound, Detergent-Resistant RPA Fraction

Next, we considered the possibility that our results might be incomplete due to the use of an antibody raised against the entire RPA complex, particularly in light of data suggesting the separation of the RPA subunits during the cell cycle. In one report, RPA70 colocalized with DNA replication sites, while RPA32 appeared to be scattered throughout the nucleus during interphase and absent from DNA replication sites [13]. These findings contrast with another study in which some, but not all, of the nuclear RPA32 and RPA70 sites colocalized with replication foci [27]. Furthermore, *in vitro* biochemical analysis of chromatin-bound RPA presented evidence for potential dissociation of the heterotrimeric complex [14]. RPA14 has been shown to uniquely associate with nucleoli [12]. Preferential localization of RPA32, but not RPA14 or RPA70, to mitotic chromosomes has been reported by some investigators [12, 13], while other labs did not detect RPA on mitotic chromosomes [15].

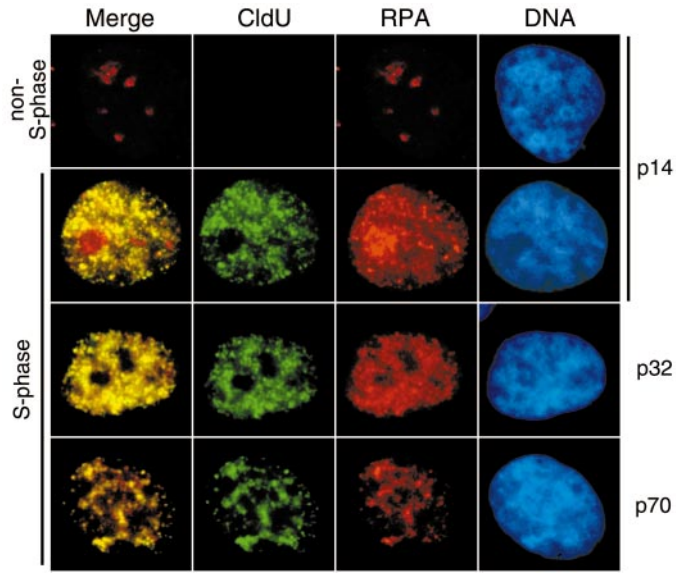
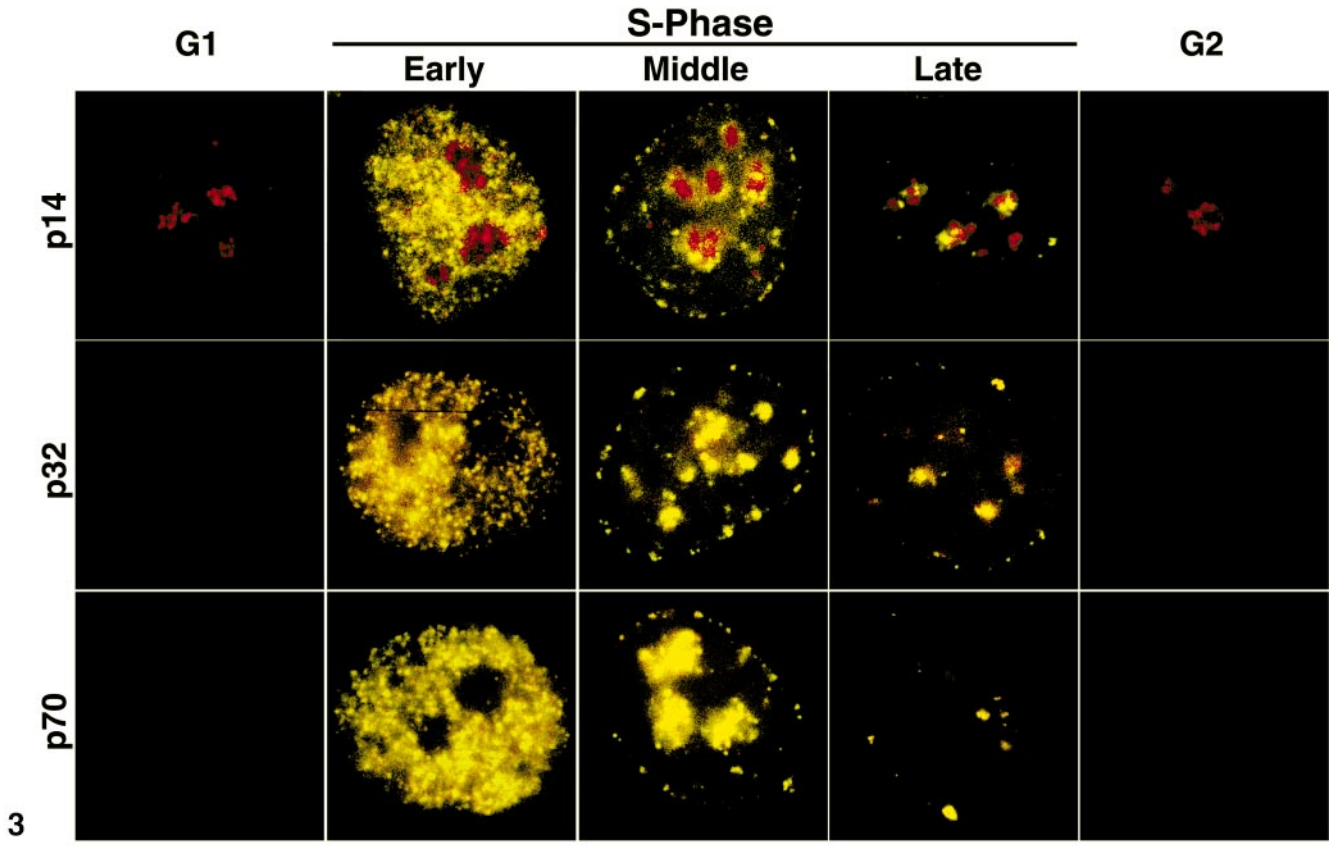


FIG. 2. All three subunits of the heterotrimeric RPA complex colocalize with DNA replication foci. Exponentially growing HeLa cells were briefly pulse-labeled with CldU, extracted with Triton, and fixed with formaldehyde as described under Materials and Methods. Subunit-specific antibodies were used to examine the relative distribution of the small (p14, red), middle (p32, red), or large (p70, red) RPA subunits and DNA replication sites (CldU, green). DNA was stained with DAPI as in Fig. 1.

We reinvestigated this controversial issue using antibodies specific for the individual RPA subunits in combination with antibodies raised against the trimeric RPA complex. This analysis was performed only with HeLa cells, since our subunit-specific antibodies did not cross-react with hamster RPA. First, exponentially growing HeLa cells, prepared according to the optimal fixation conditions described in the previous section, were double-stained for CldU and RPA14, RPA32, or RPA70 (Fig. 2). Results revealed that each of the three subunit-specific antibodies stained exclusively S-phase nuclei and that RPA sites specifically colocalized with CldU replication sites. In addition, unlike RPA32 and RPA70, the anti-RPA14 antibody decorated nucleoli in all interphase cells (Fig. 2, p14), consistent with the results of Murti *et al.* [12]. We confirmed these results using synchronized populations of HeLa cells. Again, all three RPA subunits were found to colocalize with DNA replication sites at all stages of S phase (Fig. 3, S phase). G1- and G2-phase nuclei did not bind the p32- and p70-specific antibodies. Punctate intranucleolar staining with the anti-RPA14 antibody during both nonreplicative (Fig. 3, G1 and G2) and replicative (Fig. 3, S phase) phases of the cell cycle was observed [the nucleolar localization of p14 was confirmed by double-staining with anti-RPA14 and nucleolar-specific antibodies (data not shown)]. Extranucleolar RPA14 foci appeared yellow (due to



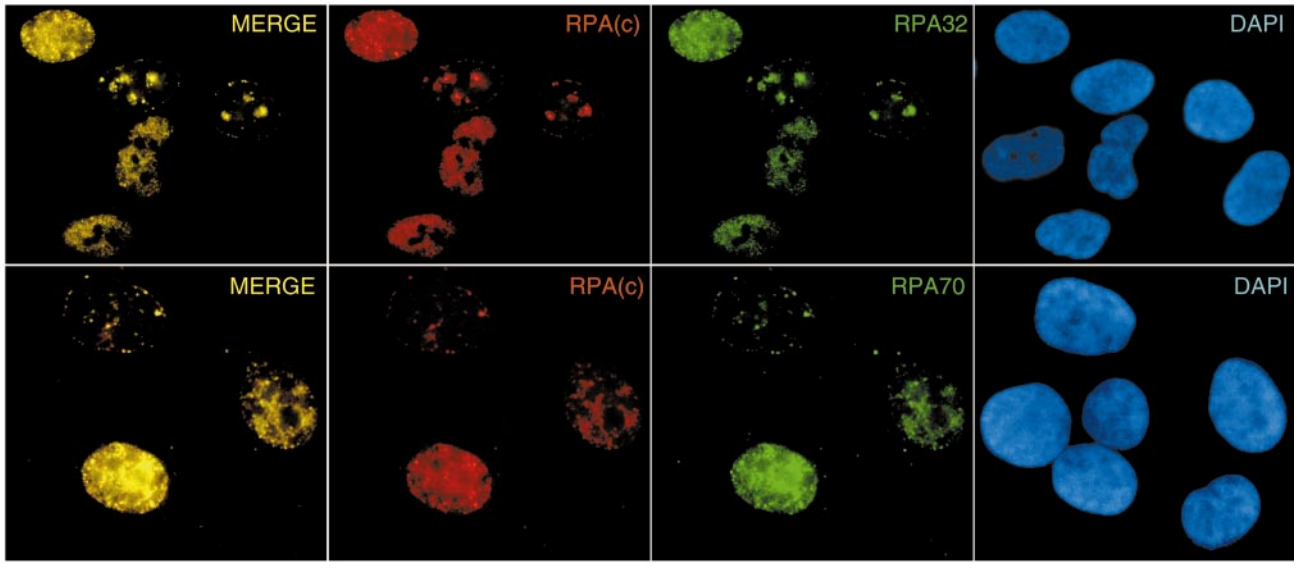


FIG. 3. Localization of the RPA subunits in synchronized human cells. HeLa cells were synchronized in mitosis following a previously described procedure [18]. Metaphase cells were released in the next cell cycle and aliquots of the cells were either collected 4 h later (G1 phase) or blocked at the G1/S-phase border with 10 μg/ml aphidicolin as described [18] and subsequently released in free medium for 2 h (early S), 6 h (middle S), 10 h (late S), or 14 h (G2 phase). The cells were pulse-labeled for 10 min with CldU, Triton-extracted, fixed, and stained for the respective RPA subunits (red) and CldU (green) as in Fig. 2. Shown are images directly photographed using a dual FITC/rhodamine filter. Colocalization of the RPA subunits with sites of DNA synthesis at all stages of S phase is visible as yellow coloration. G1- and G2-phase nuclei stain negatively for p32 and p70. Positive staining of nucleoli with the p14-specific antibody was observed in all interphase cells.

FIG. 4. The individual RPA subunits colocalize with the RPA heterotrimer in the Triton-resistant nuclear RPA population. Exponentially growing HeLa cells were extracted with Triton X-100, fixed with formaldehyde, and immunostained for the trimeric RPA complex (red) and either RPA32 (green) or RPA70 (green) subunits as described under Materials and Methods. DNA was stained with DAPI as in Fig. 1.

colocalization of red-labeled RPA14 with green-labeled CldU sites) in all S-phase nuclei. The RPA14 nucleolar foci were not labeled by the anti-CldU antibody during early S phase and thus appeared red. During mid/late S phase, when nucleolar DNA replicates, RPA14 and CldU foci colocalized within nucleoli.

Next, we investigated whether individual RPA subunits could be detected outside of the trimeric RPA complex. Triton-extracted HeLa cells were immunostained with rabbit polyclonal antibodies specific for the RPA complex (two different antibodies were utilized) and either RPA32-specific or RPA70-specific mouse monoclonal antibodies. Results showed that both RPA32 and RPA70 colocalized exclusively with the heterotrimeric complex (Fig. 4). The anti-RPA14 antibody could not be used in this analysis, since it is a rabbit polyclonal antibody, like the antibodies specific for the trimeric complex. Nonetheless, simultaneous staining with anti-RPA14 and either anti-RPA32 or anti-RPA70 antibodies revealed the complete colocalization *in vivo* (with the exception of the nucleolar signal unique to RPA14) between the small subunit and the other two subunits of the heterotrimeric RPA complex (not shown). We observed intranucleolar colocalization of the RPA subunits only during mid/late S phase, when nucleolar DNA replicates. Still, it remains formally possible that the p32 and p70 subunits are present in the nucleolus also during the rest of the cell cycle but their epitopes are inaccessible to our antibodies. Finally, immunolocalization experiments revealed that mitotic chromosomes were not stained by any of the anti-RPA antibodies in both nonextracted (Fig. 1A and data not shown) and Triton-extracted cells (not shown). In our hands, inactive anti-RPA antibodies that did not detect the respective RPA-specific protein bands by Western blot analysis occasionally produced diffuse nonspecific nuclear staining which could explain at least some of the previous observations on differential distribution of RPA subunits.

In conclusion, the results presented in this report clarify a body of inconsistent data in the literature concerning the nuclear distribution of RPA during the mammalian cell cycle. We demonstrate the existence of two populations of hamster and human RPA proteins: a soluble nucleosolic form present throughout the cell cycle and a detergent-resistant form immunodetectable only during S phase. The detergent-resistant RPA population dynamically colocalizes with sites of ongoing DNA synthesis, similar to the replication fork protein PCNA. We find no evidence for disassembly of the chromatin-bound RPA complex *in vivo.*

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