Chromatin Association of Replication Protein A*

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Replication protein A (RPA) is the major single strand-specific DNA-binding protein in eukaryotic cells. We have investigated the distribution of RPA in nuclei of proliferating HeLa cells and found that only one-third of the detectable RPA appeared to be bound to DNA in chromatin, whereas the remainder was free in the nucleosol. This distribution did not significantly change when cells were released from a double thymidine block into the S phase of the cell cycle. Single strand-specific endonucleases failed to mobilize RPA bound to chromatin in G1 phase and S phase HeLa cells. In contrast, brief treatments with pancreatic DNase I or with micrococcal nuclease sufficed to release RPA from its chromatin-binding sites. Sucrose gradient analysis of soluble miccoccal nuclease digests showed that the released RPA sedimented free of mono- or oligonucleosomal chromatin fragments, possibly indicating that most of the detectable RPA may be associated with chromatin sites, which are more open to nuclease attack than bulk chromatin. The surprising conclusion is that the majority of the detectable RPA is, either directly or indirectly, associated with double-stranded DNA regions in chromatin from HeLa cells in G1 phase and in S phase.

The major eukaryotic single strand-specific DNA-binding protein is known as replication protein A (RPA)‡ because it was originally discovered as a factor essential for simian virus 40 DNA replication in vitro. However, more recent evidence indicates that RPA is involved not only in DNA replication, but also in DNA repair and recombination as well as transcriptional regulation. These activities are dependent on the DNA binding properties of RPA, but also on its ability to interact with a variety of proteins such as DNA polymerases, DNA damage recognition proteins, and transcriptional activators (reviewed in Ref. 1).

RPA is composed of three subunits described as RPA70, RPA32, and RPA14 with reference to their approximate molecular masses. The major DNA-binding activity of RPA is associated with the RPA70 subunit, which contains a central region with two adjacent DNA-binding domains (2) and, possibly, a third and weak binding domain in the carboxyl-terminal region (3). An additional weak DNA-binding domain appears to reside on the RPA32 subunit, which may therefore contribute to the overall DNA-binding activity of RPA (3, 4). In addition, RPA32, together with RPA70, determines contacts between RPA and other proteins at sites of DNA replication or DNA repair (1).

The interactions with other proteins may be influenced by cell cycle- or DNA damage-dependent phosphorylation of the RPA32 subunit (5, 6). The function of the smallest subunit, RPA14, is less well known, but RPA14 appears to be involved in RPA complex assembly and stability (5).

RPA binds with high affinity and low cooperativity to single-stranded DNA with a preference for polypyrimidine tracts in vitro binding studies (7). Two binding modes have been identified: an intermediate and relatively weak binding mode, characterized by an occluded binding site of 8–10 nucleotides (8), and a more stable binding mode in which RPA covers ~30 nucleotides (7, 9, 10). Single-stranded DNA covered by RPA remains in an extended conformation under low ionic strength conditions, but more physiological salt concentrations induce a severe compaction of RPA-DNA complexes (11), which may reflect yet another DNA-binding mode possibly including ~70 nucleotides of bound DNA, as first suggested by work with yeast RPA (12). In general, the high affinity of RPA for single-stranded DNA is thought to contribute to the stabilization of single-stranded DNA intermediates and the removal of secondary structures from single-stranded regions. However, RPA also binds to double-stranded DNA, although with an affinity that is several orders of magnitude lower than its affinity for single-stranded DNA (13, 14). RPA prefers certain sequences or secondary structural elements in double-stranded DNA, with the interesting consequence that bound RPA is able to unwind double-stranded DNA regions up to several thousand base pairs at low ionic strengths in vitro (11).

RPA is an abundant cellular protein with $3 \times 10^4$ to $2 \times 10^5$ molecules/cell nucleus (13, 15, 16). Immunofluorescence studies indicate that RPA may change its localization in the nucleus in a cell-cycle dependent manner. This has been investigated in pseudonuclei that form in Xenopus egg extracts around added sperm chromatin or DNA. Immunofluorescence revealed that RPA accumulates as brightly stained dots in pre-replication centers before the initiation of DNA replication, but disperses throughout the nuclei when replication proceeds (17, 18). The intranuclear distribution pattern in this system appears to be accompanied by a change of RPA-binding sites. Before replication, RPA seems to be linked to double-stranded DNA in chromatin, probably via protein interactions, but is transferred to single-stranded DNA after the establishment of replication forks (19).

The intranuclear distribution of RPA changes in a cell-cycle dependent manner also in cultured mammalian cells. Experiments with cellular systems as diverse as mouse myotubes, lymphoma cells, and human HeLa cancer cells have consistently shown that RPA is distributed throughout nuclei of cells in G1 phase, but appears in a punctate or dot-like pattern in most S phase nuclei (20–22). Myotube cells, induced to proliferate by expression of the SV40 transformation protein (T antigen), may be a special case though, as only RPA70, but not RPA32, could be detected at replication foci (20).

Since relatively little is known about the chromatin-binding mode of RPA in mammalian cells, we prepared chromatin from...
HeLa cells and determined that most of the nuclear RPA is free in the nucleosol, whereas only a minor fraction of nuclear RPA is associated with chromatin in proliferating cells. Interestingly, most of the chromatin-associated fraction appears to be bound to double-stranded DNA. The nuclear distribution of RPA does not detectably change when cells traverse from the pre-replicative G1 phase to the replicative S phase of the cell cycle. Bound RPA is released from chromatin by micrococcal nuclease digestion. Released RPA sediments in sucrose gradients just like isolated free RPA.

EXPERIMENTAL PROCEDURES

**Immunological Procedures**—RPA was expressed in bacteria and purified as described (11, 23). Isolated RPA was used as an antigen to raise antibodies in rabbits. RPA-specific antibodies were affinity-purified against isolated RPA according to standard procedures (24). The antibodies were used in immunoblotting experiments (Western blotting) essentially as originally described by Towbin et al. (25) with minor modifications (26). Immunoblots were visualized by the enhanced chemiluminescence procedure (ECL, Amersham Pharmacia Biotech).

**Cell Culture and Cell Fractionation**—HeLa cells (line S3; American Type Culture Collection CCL 2.2) were grown on plastic dishes in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum. For cell cycle synchronization, cells were arrested at the G1-S phase transition by two subsequent thymidine blocks (2.5 mM thymidine) for 14 h, separated by a period of 10 h without thymidine (27). Progression through S phase was monitored by pulse labeling with [3H]thymidine (1 μCi) for 60 min. Incorporated radioactivity was determined in acid-precipitated material by scintillation counting. The number of cells in mitosis was determined according to Ohyashiki et al. (28).

Indirect immunofluorescence was performed as described (29) with monospecific RPA antibodies and secondary fluorescein-labeled anti-rabbit antibodies (Sigma). Parallel immunofluorescence experiments were performed using a primary monoclonal anti-RPA70 antibody (Di-anova, Hamburg, Germany) and an anti-mouse Texas Red conjugate as secondary antibody (Sigma).

For cell fractionation, cells were washed in phosphate-buffered saline and resuspended in buffer A (110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 0.5 mM EGTA, 2 mM diethiothreitol, and 20 mM Hepes, pH 7.3) with 50 μg/ml digitonin (30). Nuclei were removed by low speed centrifugation after 15 min on ice with gentle agitation. The nuclear pellet was resuspended in hypotonic buffer B (1 mM Hepes and 0.5 mM EDTA, pH 7.5) with or without 0.5% Nonidet P-40 as indicated below.

Chromatin was prepared, according to Hancock (31), from washed cells in buffer B with Nonidet P-40. As first shown by Hancock (31), incubation in buffer B for 15 min on ice disrupts the nuclear envelope, whereas chromatin remains in a compacted form. Chromatin was further purified by centrifugation through a 100 mM sucrose cushion and finally resuspended in 0.25 mM EDTA, pH 8. DNA concentrations were determined with Hoechst 33258 (Hoefer Scientific Instruments, San Francisco) by fluorometry.

**Micrococcal Nuclease Digestion**—HeLa cell nuclei were incubated with 1 unit of micrococcal nuclease/40 μg of DNA (Boehringer Mannheim) in 0.25 mM sucrose, 10 mM Hepes, 10 mM KCl, 0.5 mM MgCl2, 1 mM diethiothreitol, and 2 mM CaCl2 at 37 °C. The reaction was stopped by the addition of 8 mM EDTA. Insoluble material was removed by centrifugation. The supernatants were analyzed by zone centrifugation through a 40 to 10% sucrose gradient in a Beckman SW 40 rotor at 4 °C and 40,000 rpm. The gradient was fractionated from the bottom into 0.6-ml aliquots. Samples from each fraction were analyzed by Western blotting for RPA and, after deproteinization, by agarose gel electrophoresis for the determination of DNA lengths (32).

**In Vivo Cross-linking**—The procedure used has been described by Göhring and Fackelmayer (33). Briefly, HeLa cells on plastic dishes were washed with phosphate-buffered saline to remove all traces of serum. Formaldehyde (1%) in warm Dulbecco’s modified Eagle’s medium without serum was then added for the times indicated below. Cells were lysed in 2% Sarkosyl. The lysate was first centrifuged through a CsCl step gradient to remove uncomplexed proteins. The nucleoprotein complex was recovered, sheared to pieces containing DNA fragments of 500–1000 base pairs, and recentrifuged in a CsCl equilibrium gradient. Cross-links can be broken by boiling in Laemmli sample buffer, allowing the analysis of proteins by standard denaturing polyacrylamide gel electrophoresis (34).

**RESULTS**

Characterization of RPA-specific Antibodies—Affinity-purified antibodies, raised in rabbits against recombinant human RPA, served as a major analytical tool to specifically determine RPA by immunoblotting in the presence of other nuclear proteins. To demonstrate the efficiency and specificity of the antibody preparation, we show in Fig. 1 that the antibodies specifically reacted with two polypeptides with apparent molecular masses of 70 and 32 kDa in an unfractionated extract of HeLa cells as well as in a protein extract of induced Escherichia coli cells harboring an expression plasmid with the three RPA sequences (5). Comparison with purified recombinant RPA showed that these polypeptides correspond to the large and middle subunits, RPA70 and RPA32, respectively, and that the antibodies do not recognize the smallest subunit, RPA14 (Fig. 1). Preimmune control antibodies gave negative results on Western blots (Fig. 1).

**Distribution of RPA in Nuclei**—Using RPA-specific antibodies, we show by immunofluorescence that all of the detectable RPA resides in HeLa cell nuclei and that RPA was evenly distributed in pre-replicative nuclei but partially appeared in a punctate form over a uniformly stained background in nuclei from S phase cells (Fig. 2A), as described in detail before by Murti et al. (22). The immunofluorescence experiment was repeated with a monoclonal RPA70-specific antibody with very similar results (data not shown).

To determine the fraction of free and structure-bound nu-
clear RPA proteins, proliferating HeLa cells were lysed in buffers containing digitonin at concentrations that are believed to disrupt the cytoplasmic membrane, but not the nuclear envelope (30, 35). The data show, however, that a considerable fraction of total cellular RPA appeared in the supernatants of digitonin-treated cells, suggesting that digitonin renders the nuclear envelope leaky for RPA (Fig. 2B, soluble fraction 1). Washing of the digitonin-treated nuclei in a hypotonic buffer and in a buffer with 0.5% Nonidet P-40 resulted in an additional release of free RPA (Fig. 2B, soluble fraction 2 and NP40 wash, respectively). The fraction of RPA remaining bound to structure in the final chromatin pellet was one-third of total nuclear RPA (Fig. 2B).

The cell fractionation procedure of Fig. 2 was performed using HeLa cells arrested by a double thymidine block at the G1/S phase transition as well as at different times after beginning of S phase. The results consistently showed that a fraction of ~20–30% RPA remained in a structure-bound form in pre-replicative nuclei as well as in nuclei from S phase HeLa cells (data not shown).

To further investigate the structure-bound fraction of RPA, we performed in vivo cross-linking experiments by treating proliferating HeLa cells on plastic dishes with formaldehyde for increasing lengths of times (33). Cross-linked nucleoprotein complexes were prepared from cell lysates by two consecutive centrifugations in CsCl gradients.

Analyses by denaturing polyacrylamide gel electrophoresis showed that histones were already cross-linked to DNA after a treatment with formaldehyde for 2 min and that the amount of cross-linked histones increased over time, until maximal values were reached at 30 min (Fig. 3A). Immunoblotting of polyacrylamide gels revealed that a significant fraction of RPA became cross-linked to DNA after 4 min of treatment with formaldehyde and that this fraction increased with longer cross-linking times (Fig. 3B). The results indicate that the RPA remaining in the nuclear pellet after cell fractionation is most likely associated with chromatin.

Association of RPA with Chromatin during Cell Cycle Progression—As mentioned in the Introduction, RPA seems to be redistributed in mammalian cell nuclei at the beginning of S phase. The cell fractionation procedure of Fig. 2 was performed using HeLa cells arrested by a double thymidine block at the G1/S phase transition as well as at different times after beginning of S phase. The results consistently showed that a fraction of ~20–30% RPA remained in a structure-bound form in pre-replicative nuclei as well as in nuclei from S phase HeLa cells (data not shown).
phase, when a substantial fraction of immunologically detectable RPA concentrates in distinct dots (22). It was of interest to determine whether this process was accompanied by changes in the amounts of chromatin-associated RPA. To investigate this possibility, proliferating HeLa cells were arrested at the G2/S phase boundary using the double thymidine procedure. Excess thymidine was then removed to release the cells into S phase. As shown in Fig. 4A, maximal DNA synthesis was observed after 6 h, and most cells had started mitosis after 10 h.

Chromatin was prepared before and at 1-h intervals after release from the thymidine block. The protocol for chromatin preparation has originally been described by Hancock (31) and involves treatment of cells in buffers with low ionic strengths in the presence of the non-ionic detergent Nonidet P-40. This procedure is fast and simple and yields spherical chromatin bodies that are larger in volume and more homogeneous than chromatin in intact nuclei (31). Chromatin from mitotic cells does not, of course, have the spherical structure of chromatin bodies from interphase cells, but can nevertheless be pelleted by low speed centrifugation.

As shown by Western blotting with monospecific (Fig. 4B) and monoclonal (Fig. 4C) RPA antibodies, the amounts of chromatin-bound RPA did not significantly change in the first 5 h after cells had entered S phase. Even though cell synchrony was partially lost at the end of S phase, the data warrant the conclusion that RPA remains on chromatin during most of S phase, but dissociates at least partially from chromatin after DNA replication (Fig. 4). It is not possible to say whether the loss of RPA occurred already during the G2 phase or, more likely, at mitosis as suggested by Murti et al. (22).

**RPA on Isolated Chromatin—** Guided by the report on the DNA binding of RPA in replicating pseudonuclei of *Xenopus* egg extracts (19), we considered the possibility that RPA may also be bound to single-stranded DNA in S phase HeLa cell nuclei. To investigate this possibility, we prepared chromatin from cells before and 2, 4, and 6 h after release from the thymidine block (Fig. 4).

Chromatin preparations were incubated with single strand-specific mung bean nuclease under conditions known to be optimal for enzymatic activity. Insoluble chromatin was removed after incubation by centrifugation, and the amounts of RPA were determined in pellets and supernatants. Neither a decrease in chromatin-bound RPA nor an increase in free supernatant RPA could be detected (data not shown). Similar results were obtained using the single strand-specific S1 nuclease. Thus, single strand-specific nucleases failed to mobilize chromatin-bound RPA. To exclude the possibility that components in chromatin preparations were inhibitory to exogenous nucleases, we added radioactively labeled single-stranded M13 DNA to a chromatin preparation and treated the mixture with either mung bean nuclease or S1 nuclease. Both nucleases efficiently degraded the single-stranded DNA substrate (data not shown), demonstrating that their activities did not suffer in the presence of HeLa cell chromatin.

Our conclusion is that the majority of the chromatin-bound RPA is probably not associated with single-stranded DNA in S phase cells. To independently support this conclusion, the following experiment was performed. Recombinant RPA was bound to single-stranded phage M13 DNA and, in a parallel experiment, to double-stranded M13 DNA at increasing NaCl concentrations. RPA-DNA complexes were removed by ultracentrifugation, and free RPA was determined in the supernatants. The results showed that 0.4 M NaCl and higher was necessary to dissociate RPA from single-stranded DNA, whereas 0.1 M NaCl was sufficient to dissociate most of the RPA from double-stranded DNA (Fig. 5A).

Next we determined the salt sensitivity of RPA binding in isolated chromatin. For this purpose, chromatin from pre-replicative and S phase cells was treated with increasing salt concentrations of NaCl, and RPA was determined in pellets and supernatants. As shown in Fig. 5B, RPA was bound to chromatin at 0.1 M NaCl and remained associated with chromatin bound at concentrations of NaCl up to 1.0 M.

**Fig. 3.** *In vivo* cross-linking of RPA to DNA. HeLa cells, growing on plastic dishes, were treated with 1% formaldehyde for the times indicated. Cell lysates were first layered on top of a CsCl step gradient and centrifuged to remove free proteins. The nucleoprotein complex was collected, mechanically sheared to give fragments with 500–1000 base pairs of DNA, and further purified by CsCl equilibrium centrifugation. Nucleoprotein complexes with buoyant densities corresponding to their DNA/RNA ratios were adjusted to equal DNA concentrations, boiled in Laemmli sample buffer, and investigated by denaturing polyacrylamide gel electrophoresis. A, silver-stained polypeptides; B, Western blotting with RPA-specific antibodies. Only the RPA70 band is shown.

**Fig. 4.** Association of RPA with replicating chromatin. HeLa cells, arrested by the double thymidine method, were released into S phase by removing excess thymidine. A, cell cycle. Incorporated [3H]thymidine is expressed as a percentage of the maximal value (~10,000 counts/min). Cells in mitosis were determined after fixation and staining of cells on coverslips (28). B, RPA on chromatin. Chromatin samples with equal amounts of DNA (2 μg) were analyzed by Western blotting using monospecific RPA antibodies (see Fig. 1). C, same as B, except that a monoclonal anti-RPA70 antibody was used in the Western blotting experiment. D, loading control; histone bands as visualized by Coomassie Blue.
concentrations. The results showed that RPA already dissociated from chromatin at a salt concentration of 0.1 M NaCl (Fig. 5A), just like RPA bound to double-stranded M13 DNA. The results shown in Fig. 5B were obtained using chromatin from HeLa cells at 6 h after release from the double thymidine block, but the data with chromatin from other cell cycle stages were similar (data not shown). We conclude that most of the RPA in proliferating HeLa cells is not bound to single-stranded DNA, but could be bound to double-stranded DNA. We note, however, that RPA70 subunits appeared to be more sensitive to salt treatment than RPA32 subunits (Fig. 5B). The reason for this is presently not clear.

Chromatin-bound RPA Is Released by Micrococcal Nuclease—Since RPA could not be released from chromatin with single-strand-specific nucleases, we used pancreatic DNase I and micrococcal nuclease to determine whether RPA is directly or indirectly associated with double-stranded DNA in chromatin. We could indeed show that pancreatic DNase I (data not shown) and micrococcal nuclease (Fig. 6A) efficiently mobilized RPA from isolated chromatin. In fact, micrococcal nuclease already released a substantial fraction of RPA from S phase chromatin at short incubation times when the degradation of chromatin to soluble fragments became just detectable. Continued nuclease treatment mobilized approximately two-thirds of all of the chromatin-bound RPA under conditions when <20% chromatin was fragmented and appeared in the supernatants (Fig. 6B). The fast release of RPA could indicate that chromatin sites with bound RPA are more accessible to micrococcal nuclease than is bulk chromatin, but it has yet to be investigated whether this may be due to a more open chromatin conformation in the vicinity of bound RPA. We note that one-third of the chromatin-bound RPA was not released under standard conditions of incubation with micrococcal nuclease (Fig. 6). This fraction of RPA remained in a compact chromatin structure and could be pelleted by centrifugation. Higher nuclease concentrations or prolonged incubation times failed to decrease the fraction of nuclease-resistant RPA. The results may suggest that two types of RPA-binding sites exist in HeLa cell chromatin, only one of which may be accessible to nuclease attack under standard incubation conditions.

To determine whether solubilized RPA was associated with chromatin fragments, we performed sucrose gradient centrifugations of the soluble micrococcal nuclease digests. The results showed that almost all of the RPA sedimented free of mono- or oligonucleosomes (Fig. 7A). In fact, the sedimentation rate of mobilized RPA was ~5 S and was similar to that of isolated or recombinant human RPA (data not shown), indicating that very little DNA remained bound to RPA released from chromatin by nuclease treatment. To support this conclusion, we determined the sedimentation properties of RPA in chromatin digests after longer nuclease treatment. If RPA in the experiment of Fig. 7A carried DNA pieces of substantial lengths, we expected to find a reduction of its sedimentation rate upon further nuclease treatment. This was not observed even though continued nuclease digestion converted most oligonucleosomal chromatin fragments into nucleosomal core particles (Fig. 7B).

We note some overlap between the peak of RPA and that of mononucleosomes. But a direct association of RPA with other proteins appears to be unlikely because immunoprecipitations...
with anti-RPA antibodies precipitated all of the RPA, but no additional proteins (histones) or mononucleosomal DNA (data not shown). Furthermore, non-denaturing polyacrylamide gel electrophoresis (36) effectively separated mononucleosomes and RPA (data not shown).

The experiment of Fig. 7 was performed with chromatin prepared from cells at the G1/S phase transition as well as from cells in S phase. The results were similar and indicate that the chromatin binding properties of the detectable fraction of RPA do not significantly change in S phase.

**DISCUSSION**

The experiments described here were performed to learn more about the distribution of RPA in HeLa cells. A first result was that the majority of RPA is present in the soluble fraction even after gentle lysis of cells and that maximally one-third remains in the chromatin pellet. Since immunofluorescence gave no indication of a cytoplasmic localization of RPA, it can be concluded that the soluble RPA fraction originates from the nucleol. RPA in the pellet is at least partially bound to chromatin because RPA appeared in CsCl gradients of crosslinked nucleoprotein at the same buoyant density positions as histones. This result does not necessarily indicate that RPA is directly bound to DNA. It could be linked to DNA via another protein (37). This would explain why RPA appears during cross-linking some minutes later in nucleoprotein compared with histones. In any case, the fraction of chromatin-bound RPA did not significantly change when HeLa cells became actively engaged in DNA replication after their release from a double thymidine block. This may indicate that the fraction of chromatin-bound RPA is large enough to satisfy the needs for RPA in replication. It is possible though that the pool of free RPA serves as a reservoir for fresh and active RPA during replication. This would be required when chromatin-bound RPA changes its properties during replication, e.g. by phosphorylation (6, 38, 39), and when modified RPA is released from chromatin and regenerated in the nucleol. In this case, molecules from the pools of free and chromatin-bound RPA proteins should be in equilibrium, and the rates of exchange should increase when cells move from the G1 phase into the S phase of the cell cycle. This point has not yet been investigated. Another and possibly simpler explanation for the excess of free RPA would be that free RPA serves as a background system that protects single-stranded DNA during DNA repair or that RPA is required in high amounts during other stages of the cell cycle such as mitosis when RPA70 has been reported to accumulate at spindle poles and RPA32 at chromosomes (22).

With regard to the chromatin-bound form of RPA, we had expected to detect at least some RPA on single-stranded DNA during S phase. However, we were unable to mobilize detectable amounts of RPA with single strand-specific endonucleases. This does not, of course, exclude the presence of RPA on single-stranded regions at replication forks. In fact, numerous experiments have clearly demonstrated that the DNA-binding activity of RPA is indispensable for eukaryotic DNA replication (reviewed in Ref. 1), and biochemical experiments have located RPA directly in the vicinity of replication forks (40). Furthermore, single strand-specific nucleases have been shown to mobilize RPA from replicating sperm chromatin in *Xenopus* egg extracts (19). It is known though that the use of origins in *Xenopus* egg systems is more relaxed than in adult cells (41–44), with the consequence that many more replication forks are established, and consequently, much more RPA is engaged at replication forks than in adult cells, in which S phase is 8–10 times longer than in *Xenopus* egg extracts. Thus, our data could mean that only rather small and undetectable amounts of RPA are in direct contact with single strands at any given time in S phase HeLa cells.

Another reason for the inability of single strand-specific nucleases to release RPA from replicating mammalian chromatin could be that RPA is a component of a large multiprotein complex that protects single-stranded regions against nucleolytic attack. But again, this can be true only for a minor fraction of chromatin-bound RPA because RPA sites in chromatin are readily accessible to micrococcal nuclease, which can already mobilize most of the bound RPA after short incubation times. Our conclusion is that most of the RPA appears to be associated with double-stranded DNA. It is not known whether this association is direct or mediated by a protein tether (37). We note though that RPA molecules released by nuclease treatment sediment through sucrose gradients at the rate of free uncomplexed RPA proteins. Thus, if RPA binds indirectly to chromatin, either its protein partner must be too small to affect the sedimentation properties, or the protein partner dissociates from RPA during incubation with micrococcal nuclease. We
have excluded the possibility that the partial overlap between RPA and nucleosomal core particles in some of the sucrose gradient fractions was due to an interaction with a nucleosome.

Finally, only two-thirds of the bound RPA could be released by either DNase I or micrococcal nuclease. We do not know whether the remaining RPA is associated with an inaccessible part of isolated chromatin or bound to the nuclear matrix, which cannot be solubilized by nuclease treatment. In either case, the fraction of nucleosol-resistant RPA is similar in pre-replicative and replicating HeLa cells and therefore is not related to a cell cycle-dependent physiological event. In summary, we draw the surprising conclusion that RPA, the prototypic eukaryotic single strand-specific DNA-binding protein, either occurs free in the nucleosol or is associated to a considerable extent with double-stranded DNA in mammalian cell chromatin and, furthermore, that this distribution does not detectably change when HeLa cells proceed from the G1 phase to the S phase of the cell cycle.

Acknowledgments—We thank Marion Ritzi for help with the cross-linking experiments and Frank O. Fackelmayer for discussions.

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