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The Origin Recognition Complex Marks a Replication Origin in the Human *TOP1* Gene Promoter*

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The locations of the origin recognition complex (ORC) in mammalian genomes have been elusive. We have therefore analyzed the DNA sequences associated with human ORC via *in vivo* cross-linking and chromatin immunoprecipitation. Antibodies specific for hOrc2 protein precipitate chromatin fragments that also contain other ORC proteins, suggesting that the proteins form multisubunit complexes on chromatin *in vivo*. A binding region for ORC was identified at the CpG island upstream of the human *TOP1* gene. Nascent strand abundance assays show that the ORC binding region coincides with an origin of bidirectional replication. The *TOP1* gene includes two well characterized matrix attachment regions. The matrix attachment region elements analyzed contain no ORC and constitute no sites for replication initiation. In initial attempts to use the chromatin immunoprecipitation technique for the identification of additional ORC sites in the human genome, we isolated a sequence close to another actively transcribed gene (*TOM1*) and an alphoid satellite sequence that underlies centromeric heterochromatin. Nascent strand abundance assays gave no indication that the heterochromatin sequence serves as a replication initiation site, suggesting that an ORC on this site may perform functions other than replication initiation.

Origins of DNA replication are the chromosomal regions where DNA replication forks for bidirectional duplication of replicons are established. The large and discontinuous genomes of eukaryotes require a large number of origins that are distributed throughout the genome to guarantee a complete replication within the limited time of the S phase in a cell division cycle (for reviews, see Refs. 1–9. The best characterized eukaryotic origins are those of the budding yeast *Saccharomyces cerevisiae* because they function as sites of replication initiation in extrachromosomal plasmid DNA and are, therefore, amenable to detailed molecular analyses (10, 11). Prototypic budding yeast origin (autonomously replicating sequences (ARSs)[1]) are composed of 100–200 base pairs and contain several essential sequence elements including a domain A with the AT-rich ARS consensus sequence and three short stimulatory elements, B1-B3, which are functionally important but divergent in sequence (12). The ARS consensus sequence and the adjacent B1 domain element constitute a binding site for proteins of the origin recognition complex (ORC), whereas the B3 domain element forms a binding site for the transcription factor Abf1 in some, but not all yeast origins (13).

ORC is a multimeric protein complex composed of six essential subunits (Orc1p-Orc6p) that associate in an ATP-dependent manner with ARSs (13–15). The major known function of ORC appears to be the recruitment of factors such as Cdc6, Mcm proteins, and others for the formation of functional prereplication complexes (16–19).

Origins of replication in multicellular organisms can usually not be investigated by ARS assays. Therefore, biochemical procedures such as two-dimensional gel electrophoresis (20, 21) or nascent strand length or nascent strand abundance assays (22, 23) were established to determine the sites where bidirectional genome replication initiates. Using these experimental strategies, a small number of mammalian origins have been identified. One major conclusion of these experiments is that, whereas the replication of genomes in differentiated mammalian and other metazoan cells begins at specific genomic loci that are quite stably inherited from one cell division cycle to the next, individual origins of a given organism differ greatly in size and sequence and are clearly less uniform and more complex in structure than budding yeast origins (3–5, 9, 10, 24). Many known mammalian origins are found to be located between transcribed regions and frequently in the vicinity of active transcriptional start sites (25–28). One reason for a preferred location of origins at transcriptional start sites might be the more loosely organized chromatin structure, allowing initiator replication proteins better access to their target DNA binding sites (for a recent review, see Ref. 29). It has been shown by footprinting analyses that the well studied lamin B2 origin is protected in a cell cycle-specific manner; however, it has not been directly demonstrated whether these or related sequence elements in differentiated metazoan cells are binding sites for ORC (30, 31).

A *Drosophila melanogaster* ORC localizes *in vivo* to the chorion gene amplification control element, which is active in ovarian follicle cells and determines the amplification of chorion gene clusters by repeatedly initiating DNA replication (32). Interestingly, amplification control element-bound ORC is in close contact with transcription factor E2F, which together with the Rb protein, regulates the initiation of replication (33). An ORC binding site has also been identified in the Epstein-Barr virus genome. The 165-kbp viral chromosome replicates as an episome in latently infected human cells in a regulated once-per-cell cycle manner dependent upon a functional bipartite viral origin. This origin binds the viral initiator protein, EBNA-1, in addition to proteins of the human ORC, which appear to be essential for viral genome replication (34, 35).

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[1] The abbreviations used are: ARS, autonomously replicating sequences; ORC, origin recognition complex; ChIP, chromatin immunoprecipitation; MAR, matrix attachment regions; SAF-A, scaffold attachment factor A.

Using a modified version of the chromatin immunoprecipitation (ChIP) protocol in combination with quantitative real-time PCR, we have recently identified an ORC binding site between two divergently transcribed human genes in a region that coincides with a start site for bidirectional DNA synthesis (36). We have now used the ChIP technique to investigate another transcription unit in the human genome, the *TOP1* gene, which occupies ~100 kbp of the chromosome 20 sequence (37). The *TOP1* gene promoter co-localizes with a CpG island and contains an A+T-rich element (38). In addition, the gene has two well characterized matrix attachment regions (MARs; Ref. 39). MARs are believed to connect chromatin loops to the non-chromatin ribonucleoprotein network known as the nuclear matrix (recently reviewed in Ref. 40). Several reports suggested that sites of DNA synthesis may be linked to the nuclear matrix (41, 42). Thus, *TOP1* offers an interesting opportunity to determine whether MARs are binding sites for ORC proteins and whether they function as replication origins. We have also used the ChIP assay to isolate and clone DNA sequences from immunoprecipitated ORC protein-bearing chromatin fragments and identified ORC binding regions in heterochromatic parts of the human genome.

MATERIALS AND METHODS

Cell Culture and Nucleoprotein Preparation—Asynchronous HeLa-S3 cells were cultivated on 145-mm dishes in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. Formaldehyde (Merck) was diluted to 1% in prewarmed medium (37 °C) and added to monolayers of 10^8 cells for 4 min if not otherwise indicated. After removal of the medium, cells were washed 3 times with cold phosphate-buffered saline (PBS), scraped off, washed twice in PBS and resuspended in hypotonic RSB buffer (10 mM Tris, pH 8.0, 3 mM $MgCl_2$, 10 mM sodium bisulfite, pH 8.0) for 10 min on ice. All centrifugation steps were carried out at 600 × *g* for 5 min at 4 °C. Cells were disrupted by Dounce homogenization (15 strikes). After centrifugation, nuclear material was washed twice in RSB buffer and once in high salt SNSB buffer (1 M NaCl, 10 mM Tris, pH 7.4, 0.1% Nonidet P-40, 1 mM EDTA, 10 mM sodium bisulfite, pH 8.0) and subsequently incubated on ice for 5 min. Finally, the nuclear material was resuspended at physiological salt concentration in NSB buffer (0.1 M NaCl, 10 mM Tris, pH 7.4, 0.1% Nonidet P-40, 1 mM EDTA, 10 mM sodium bisulfite, pH 8.0) and loaded onto gradients consisting of 1.3, 1.5, and 1.75 mg/ml CsCl diluted in gradient buffer (0.5% sarcosyl, 1 mM EDTA, 20 mM Tris, pH 8.0). Ultracentrifugation was carried out at 37,000 rpm for 24 h at 18 °C. The nucleoprotein fraction was collected from the gradients followed by overnight dialysis against Tris-EDTA (10 mM Tris, pH 7.4, 1 mM EDTA) supplemented with 10 mM sodium bisulfite, pH 8.

Nucleoprotein complexes were sonicated by a total number of 100 short pulses on ice. The concentration of nucleoproteins was determined (A_{260}) and adjusted to 2 μg/μl with Tris-EDTA buffer. Nucleoprotein fragments <1 kb were obtained by treatment with micrococcal nuclease (MBI Fermentas) at 10 units/mg of nucleoprotein in the presence of 3 mM $CaCl_2$ for 15 min at 37 °C. The reactions were stopped by adding 20 mM EDTA and analyzed on a 1% agarose gel.

Chromatin Immunoprecipitations—Immunoprecipitations were performed with 1 mg of nucleoprotein in NET buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40). Affinity-purified antibodies were added at 15 μg (α-ORC1, IgG) and 10 μg (α-ORC2, α-SP1, α-p60/CAF-1) followed by 2-h incubation at 20 °C on a rolling platform. Immunocomplexes were collected by adding 50 μl of 50% protein A-Sepharose and further incubated for 2 h. Coupled protein A-Sepharose beads were washed 8× with radioimmune precipitation buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS), 3× in $LiCl_2$ washing buffer (10 mM Tris, pH 8.0, 250 mM $LiCl_2$, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA), and 5× in Tris-EDTA buffer. All buffers were supplemented with 10 mM sodium bisulfite, pH 8.0, as protease inhibitor. Beads were transferred to fresh tubes after each buffer change to reduce contamination of unspecific DNA sticking to the tube walls. The washed beads were divided for protein and DNA extraction, respectively.

Protein and DNA Extraction—For Western blotting experiments, proteins were eluted with 2% SDS, H_2O for 10 min at 37 °C. For a reversal of cross-links, nucleoproteins were incubated for 30 min at 65 °C and extracted with methanol/chloroform (43). Input and supernatant samples were treated accordingly. Proteins were separated by SDS page, transferred onto polyvinylidene difluoride membranes, and treated with specific antibodies.

Antibodies against human ORC1 and ORC2 have already been described (44). Monospecific antibodies against human ORC3-ORC6 were raised in rabbits using the N-terminal part of the ORC3 protein and the full-length ORC4 protein recombinant expressed in bacteria. Human ORC5 and ORC6 were expressed full-length in insect cells. Antibodies were characterized by immunoblotting using recombinant expressed proteins. To minimize the signal to noise ratio in the PCR, further extensive washing of the coupled protein A-Sepharose beads was crucial. Therefore, the whole washing procedure was repeated as described above. Finally, nucleoproteins were eluted with 1% SDS, Tris-EDTA at 37 °C for 10 min, and proteins were digested with 200 μg/ml proteinase K overnight at 37 °C. DNA was extracted by the standard phenol-chloroform procedure, ethanol-precipitated, and dissolved in 40 μl of Tris-EDTA.

Cloning of ORC DNA—Precipitated and extracted DNA was amplified by ligation-mediated-PCR (see Mueller and Wold (45)) using two overlapping linker oligonucleotides (5 pmol/μl/oligonucleotide) 5′-GCGGTGACCCGGGAGATCTGAATTC-3′ and 5′-GAATTCAGATC-3′, which were first annealed to double-stranded DNA by stepwise cooling from 90 °C. For blunt-end ligation, purified DNA fragments were first exposed to the exonuclease activity of the Klenow enzyme (2 units, 5 min, 37 °C). DNA synthesis reactions were started by adding a nucleotide mix (2.5 mmol each of dATP, dCTP, dGTP, dTTP) and further incubated for 30 min. The Klenow enzyme was inactivated at 70 °C for 20 min. DNA fragments were dephosphorylated with 1 unit of alkaline phosphatase at 37 °C for 1 h. Linker ligations were performed at 18 °C for 15 h using 1 unit of T4-DNA ligase and 2 μl of the double-stranded linker oligonucleotides. PCR reactions were performed in the presence of 10 mM each dideoxynucleotide, dATP, dCTP, dGTP, dTTP, 3 units of *Pfu* DNA polymerase, 6% glycerol, and 25 pmol of each oligonucleotide. The PCR was performed in a thermocycler at 30 cycles consisting of 1 min at 94 °C, 2 min at 63 °C, and 3 min at 72 °C.

Amplified DNA fragments were directly cloned in the pCR-BluntII-TOPO cloning system (Invitrogen) according to the manufacturer's manual. Plasmid DNA was extracted from bacteria, purified, and analyzed by sequencing and PCR methods.

Nascent Strand Abundance Assay—Approximately 1×10^8 HeLa S3 cells were trypsinized and washed twice in ice-cold phosphate-buffered saline and once in RBS (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$). Centrifugations were carried out at 600 × *g* for 10 min. Cells were resuspended in RBS on ice at about 5×10^6 cells/ml for 5 min. The same volume of 1% Nonidet P-40, RBS was added, and cells were further incubated on ice for 10 min. The nuclei were pelleted, washed twice in RBS, and resuspended at 5×10^7 nuclei/ml. The same volume of lysis buffer (20 mM Tris, pH 8.0, 20 mM EDTA, 2% SDS, 500 μg/ml proteinase K) was added and incubated overnight at 56 °C. Total genomic DNA was extracted with phenol/chloroform, precipitated with isopropanol, and dissolved in Tris-EDTA buffer at 2 μg/μl. DNA was denatured at 85 °C for 10 min followed by rapid cooling on ice and loaded on 5–30% (w/v) linear neutral sucrose gradients in Tris-EDTA buffer (plus 0.1 M NaCl). In a parallel tube, double-stranded size marker DNA (1-kb latter, MBI Fermentas) was loaded as a reference. Gradients were centrifuged at 20 °C in a Beckman SW28 rotor for 20 h at 26,000 rpm. Fractions of 1 ml were collected from top to bottom. The distribution of size markers in the gradient fractions was determined by agarose gel electrophoresis. DNA fractions corresponding to an average of 1-kb size (nascent DNA strands) and 2–10 kb were collected and precipitated with ethanol. The abundance of nascent DNA strands in the preparation was determined by quantitative real-time PCR.

Quantitative Real-time PCR—Real-time PCR was performed with the Light Cycler instrument (Roche Molecular Biochemicals) using a ready-to-use "hot start" reaction mix. The mix contains *Taq* DNA polymerase and a fluorescent dye, SYBR Green I, for real-time detection of double-stranded DNA. Reactions were set up in 10 μl including 0.5 mM each primer. PCR reactions were performed at 50 cycles routinely, using the standard settings recommended by Roche Molecular Biochemicals. Annealing temperatures of individual primers are indicated in Table I. Standard DNA samples (human genomic DNA) were serially diluted to 30 ng, 3 ng, 300 pg, 30 pg, and 3 pg. After PCR, the *x* axis crossing point of each standard sample was plotted against the logarithm of concentration to produce a standard curve. Genomic equivalents of DNA samples were determined by extrapolation from the standard curve (36).

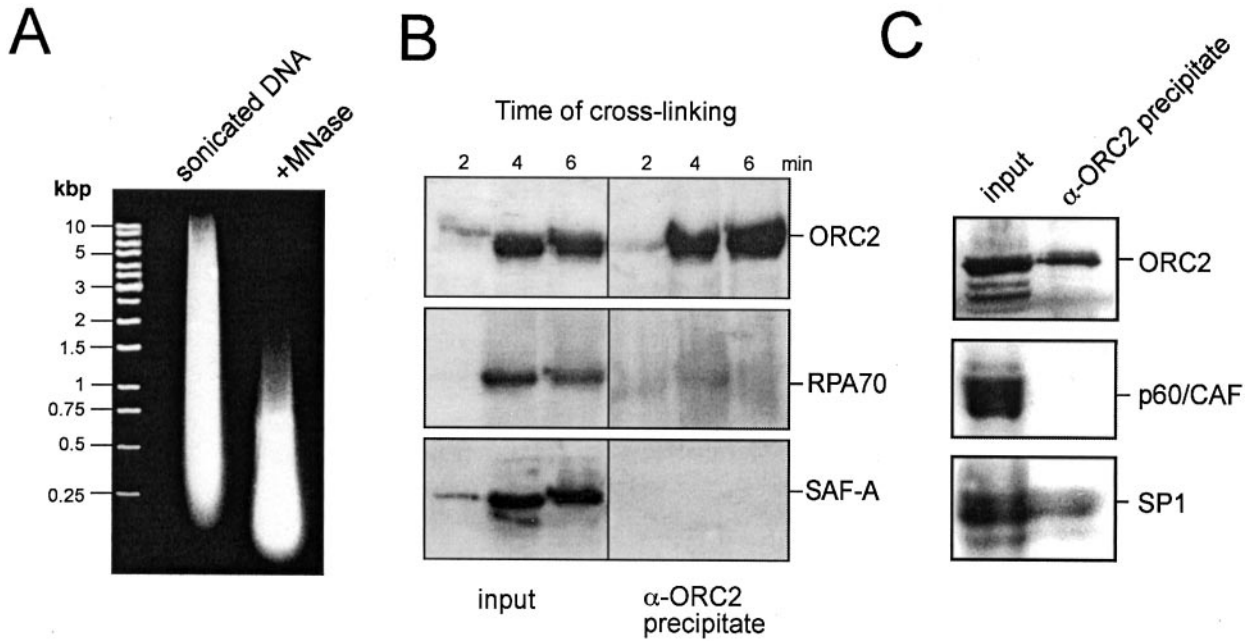

FIG. 1. **Efficiency of cross-linking and ChIP.** *A*, preparation of chromatin fragments. Cross-linked chromatin was first sonicated and then further trimmed by micrococcal nuclease (*MNase*). The extracted DNA was analyzed by agarose gel electrophoresis and ethidium bromide staining. *B*, time of cross-linking and specificity of ChIP. HeLa cells were treated with formaldehyde for 2, 4, and 6 min as indicated. Chromatin (100 μg) was prepared, trimmed (see *A*), and directly processed for Western blot analysis (*input*) using the antibodies indicated at the *right*. Another part of the same preparation was treated with Orc2-specific antibodies, immunoprecipitated, and then investigated by Western blotting (*α-ORC2 precipitate*). *C*, co-immunoprecipitations. Cross-linking (4 min) and immunoprecipitations were performed as in *B*. *Input*, Western blotting before immunoprecipitations.*α-ORC2 precipitate*, Western blots of immunoprecipitated chromatin using the antibodies indicated on the right.

RESULTS

Human Orc Proteins Are Recovered on Cross-linked Chromatin—Covalent cross-linking of chromatin proteins to DNA *in vivo*, and the isolation of cross-linked chromatin was performed as described (46–48). Isolated chromatin was sonicated and further trimmed by micrococcal nuclease to produce fragments with DNA of 0.2–1-kbp lengths (Fig. 1*A*). Chromatin fragments were either directly prepared for polyacrylamide gel electrophoresis and immunoblotting (*input*, Fig. 1) or first immunoprecipitated with specific antibodies (*precipitate*, Fig. 1) and then analyzed by immunoblotting.

Immunoblots showed that a cross-linking time of 4 min was sufficient to covalently link hOrc2p to DNA (44). This was also an optimal cross-linking time for other proteins such as the chromatin-associated fraction of the single strand-specific DNA-binding protein RPA and the scaffold attachment factor scaffold attachment factor A (SAF-A), an abundant nuclear protein that is bound to MAR elements *in vivo* (Ref. 49; Fig. 1*B*, *input*).

Orc2-specific antibodies efficiently precipitated chromatin fragments with covalently bound hOrc2p, but these precipitates contained little if any RPA and no detectable SAF-A (Fig. 1*B*, *precipitate*), indicating that hOrc2p and SAF-A were not cross-linked to the same chromatin fragments and, therefore, do most probably not reside at closely adjacent chromatin sites *in vivo*.

Fig. 1*C* shows the cross-linking to DNA of other nuclear proteins such as the p60 subunit of the chromatin assembly factor CAF1 (50) and transcription factor Sp1 (51). Immunoprecipitations with Orc2-specific antibodies indicate that the p60 subunit was not present in these immunoprecipitates (Fig. 1*C*). Interestingly, a fraction of transcription factor Sp1 always co-precipitated with hOrc2p-bearing chromatin (Fig. 1*C*), probably indicating that Sp1 and hOrc2p were occasionally cross-linked to the same chromatin fragments (see below).

Vashee *et al.* (53) and Dhar *et al.* (52) show that human

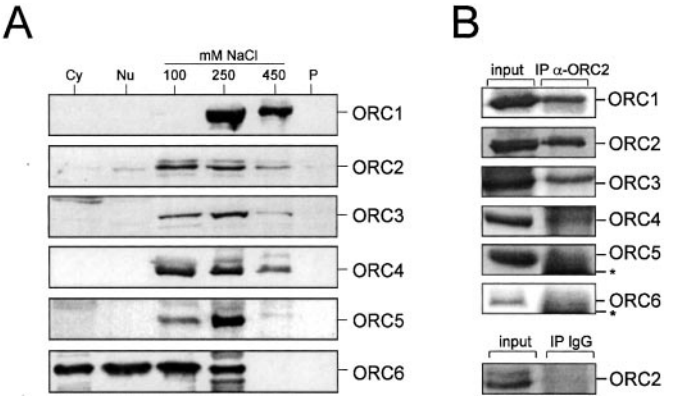

FIG. 2. **Intracellular distribution and cross-linking of human Orc proteins.** *A*, cell fractionation. Cytosolic proteins (*Cy*), soluble nuclear proteins (*Nu*), chromatin proteins in salt eluates (100, 250, and 450 mM NaCl), and insoluble nuclear pellet (*P*) are shown. Orc proteins were identified by polyacrylamide gel electrophoresis and Western blotting using the antibodies indicated on the *right*. *B*, cross-linking. *Input*, cross-linked chromatin before immunoprecipitation (*IP*). *IP α-ORC2*, immunoprecipitated chromatin (with Orc2-specific antibodies). *IP IgG*, immunoprecipitation with unspecific control antibodies. Immunoblotting was performed as in *A*. The *star* indicates the heavy or light chains of the respective antibody.

ORCs including subunits Orc1p-Orc5p can be extracted at high salt from HeLa cell chromatin and that human proteins Orc2p-Orc5p form a core complex to which proteins hOrc1p and hOrc6p are more loosely bound. It was, therefore, of interest to determine whether the other ORC proteins could be immunoprecipitated together with hOrc2p by Orc2-specific antibodies.

We first determined whether the six ORC proteins in asynchronously proliferating human cells could be identified by the available antibodies. For that purpose, HeLa cells were fractionated to yield cytosol (*Cy*), soluble nuclear proteins (*Nu*), and chromatin, which was treated with increasing salt (Fig. 2*A*).

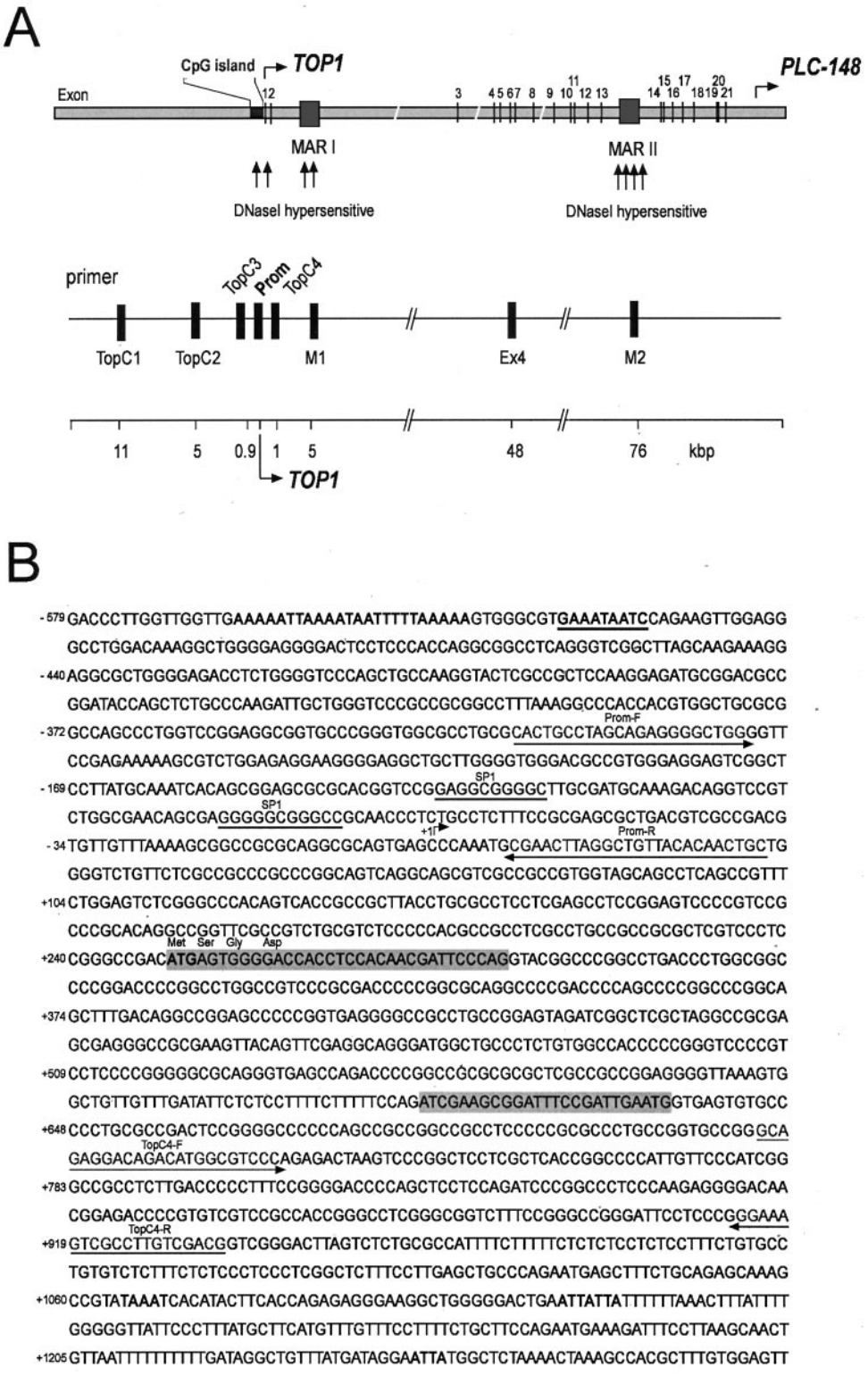

FIG. 3. **The *TOP1* gene.** *A*, overview. *Upper line*, exons (*vertical strokes* with *arabic numbers*), CpG island, and MARs as indicated. *Bent arrows*, transcription initiation of *TOP1* and the adjacent gene *PLC-148.* The DNase-I-hypersensitive sites have been mapped by Kunze *et al.* (38). *Middle line*, genomic sequences complementary to the PCR primers used (Table I). *Bottom line*, distances (in kbp) in both directions from the *TOP1* transcriptional start site. *B*, sequence around the major transcriptional start site (*bent arrow*). An upstream A+T-rich element is shown in *bold letters*, with a region homologous to the yeast ARS consensus sequence *underlined*. The Sp1 binding sites are indicated as are the coding part of exon 1 and the (small) exon 2 (*gray boxes*). The *arrows* indicated the positions corresponding to the forward and reverse primer sets *Prom* and *TopC4* (see *A* and Table I).

The six ORC proteins were detected on chromatin and could be mobilized with 0.1–0.25 M NaCl. However, a fraction of hOrc1p appeared to be more stably bound to chromatin since higher salt concentrations were required for an efficient elution. We also detected significant amounts of hOrc6p in soluble protein fractions (Fig. 2*A*).

Next we investigated whether the ORC subunits could be cross-linked to DNA. On isolated chromatin we detected signif-

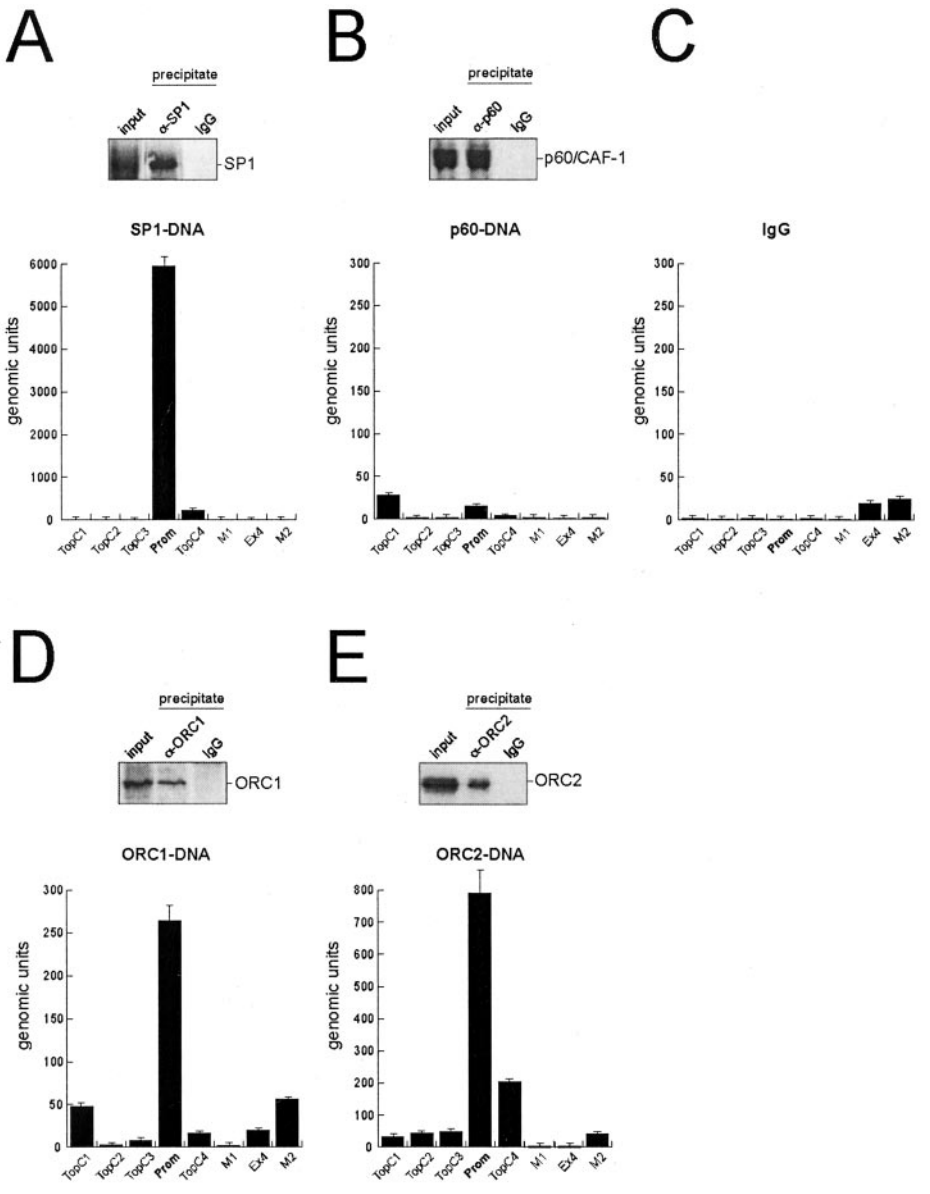

FIG. 4. **DNA sequences in immunoprecipitated chromatin.** Cross-linked chromatin was immunoprecipitated as described. *Insets*, to control the reaction, specific proteins were determined before (*input*) and after immunoprecipitation (*precipitate*) with specific antibodies or unspecific control antibodies (IgG). *Columns*, DNA was extracted from the immunoprecipitates and investigated by quantitative real-time PCR. For each primer pair, a standard calibration curve with serially diluted genomic DNA was constructed. The data obtained are expressed as genomic units, where one genomic unit corresponds to one amplifiable DNA copy in the sample examined. The antibodies used for the immunoprecipitations were either unspecific control antibodies (*C*) or specific for transcription factor Sp1 (*A*), chromatin assembly factor p60/CAF1 (*B*), hOrc1p (*D*), or hOrc2p (*E*).

icant amounts of subunits hOrc1p (Fig. 1) as well as hOrc2p–hOrc5p but reduced amounts of hOrc6p (Fig. 2*B*, *input*), which is in agreement with published data, suggesting that hOrc6p may not be a regular component of human ORC (52, 53).

Cross-linked chromatin was then immunoprecipitated with Orc2p-specific antibodies. The precipitates clearly contained hOrc1 as well as hOrc2p–hOrc4p (Fig. 2*B*) but very little, if any, hOrc6p (Fig. 2*B*). The Orc5p band overlapped to a significant extent with the IgG heavy chain band in the experiment. However, in independent experiment we clearly detected an Orc5p band in the immunoprecipitate. Comparing the input and supernatant, we estimate that between 30 and 50% of the cross-linked input sample could be immunoprecipitated. No ORC proteins were precipitated with unspecific IgGs (Fig. 2*B*) or with p60-specific antibodies (not shown). We have also tested antibodies against Orc3p, Orc4p, and Orc5p and found that the antibodies did not efficiently precipitate cross-linked proteins (not shown). Therefore, we used the Orc1p- and Orc2p-specific antibodies for the experiments reported below. Thus, ORC proteins could be covalently linked to chromatin and most likely occurred at the same chromatin sites either as one large complex or, alternatively, as subcomplexes.

An ORC Binding Site in the Human TOP1 Gene Is Located at an Upstream Promoter Site but Not in Matrix Attachment Regions—Next we addressed the question of whether ORC binds to particular genomic regions. The well characterized *TOP1* gene locus appeared to be a interesting region because it contains several features that have frequently been found in known origins such as A+T-rich elements, MARs, nuclease hypersensitive sites, and a higher than average G+C content in the gene promoter.

The *TOP1* gene is composed of 21 exons (37) and contains MAR I, located at an intronic site immediately after exon 2, and MAR II, which occurs further downstream, between exons 13 and 14 (Ref. 39; Fig. 3). MAR I and MAR II specifically attach to components of the nuclear matrix and exhibit specific binding sites for the SAF-A both *in vitro* and *in vivo* (39, 49).

The *TOP1* promoter is composed of several elements that function as binding sites for transcription factors including the ubiquitous Sp1 protein (54). The promoter has a G+C content of 67% and co-localizes with a CpG island. This is of interest because it is assumed that origins of DNA replications are predominantly located in the vicinity of CpG islands (55–57). The upstream promoter region also contains an A+T-rich track

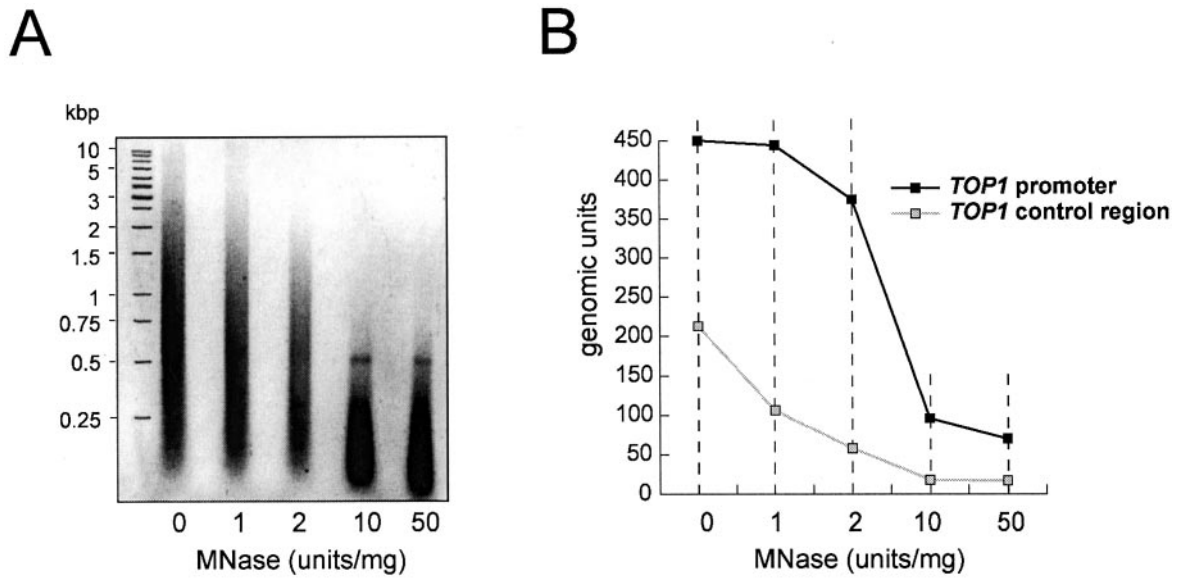

FIG. 5. **Protection of *TOP1* promoter proximal elements against shearing and nuclease attack.** *A*, DNA fragmentation. Sheared cross-linked chromatin was treated with increasing amounts of micrococcal nuclease (*MNase*). The DNA was extracted and visualized by ethidium bromide after agarose gel electrophoresis. *B*, differential protection. DNA fragments in *A* were analyzed by quantitative PCR using primers for the *TOP1* promoter region (*Prom*; Fig. 3) and a control region (*TopC1*; Fig. 3).

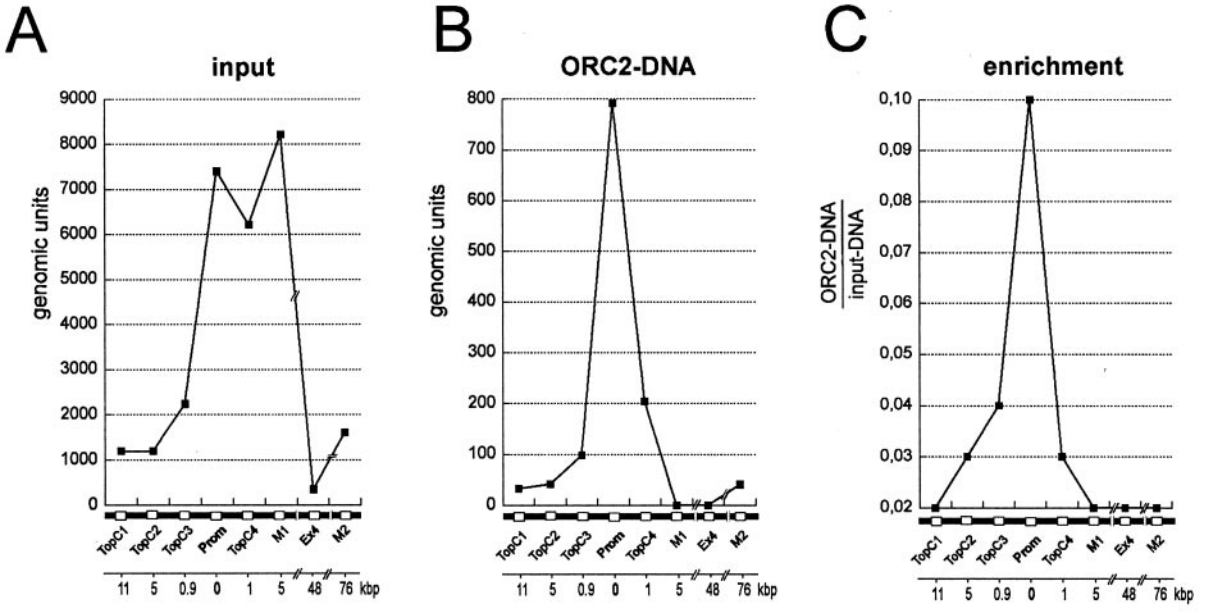

FIG. 6. **Enrichment of promoter-proximal sequences by immunoprecipitation.** *A*, before immunoprecipitation. DNA was extracted from cross-linked chromatin and amplified by quantitative PCR using the primer sets indicated (see Fig. 3). The results are expressed in genomic units relative to serially diluted genomic control DNA. *B*, after immunoprecipitation with hOrc2p-specific antibodies. The PCR analyses were performed as in *A*. *C*, enrichment. Ratios of precipitated over input amplifiable DNA are plotted against primer sites on the *TOP1* map.

as found in the vicinity of replication origins (2). For the identification of ORC binding sites, chromatin immunoprecipitations were performed with Orc2p-specific antibodies as described above.

Nucleoproteins were extracted from the immunoprecipitates for Western blotting, and DNA was extracted for an analysis by a quantitative real-time PCR procedure that allows for the detection of sequences that were specifically precipitated (see "Materials and Methods" (36)). For PCR analyses we used eight primer sets corresponding to different parts of the genomic section investigated (Fig. 3). The primers gave approximately equal amplification results with unsheared genomic DNA (fragment size greater than 20 kbp) as template under the conditions of quantitative PCR (not shown; see Fig. 7).

As a positive control, cross-linked chromatin was immunoprecipitated with antibodies against the transcription factor Sp1. Because the *TOP1* promoter contains two G+C boxes serving as Sp1 binding sites (54), we expected an enrichment of promoter sequences in the immunoprecipitate. Indeed, quantitative PCR revealed the presence of almost 6000 copies of the promoter sequence but only very few copies of other sequences in and around the gene (Fig. 4*A*).

As negative controls, immunoprecipitations with antibodies against the p60/CAF1 protein (see Fig. 1) as well as with unspecific control antibodies were performed. The p60/CAF1-specific antibodies efficiently precipitated the p60 protein (*precipitate*, Fig. 4*B*), but the associated DNA could not be amplified to a significant extent with the primer sets used (Fig. 4*B*).

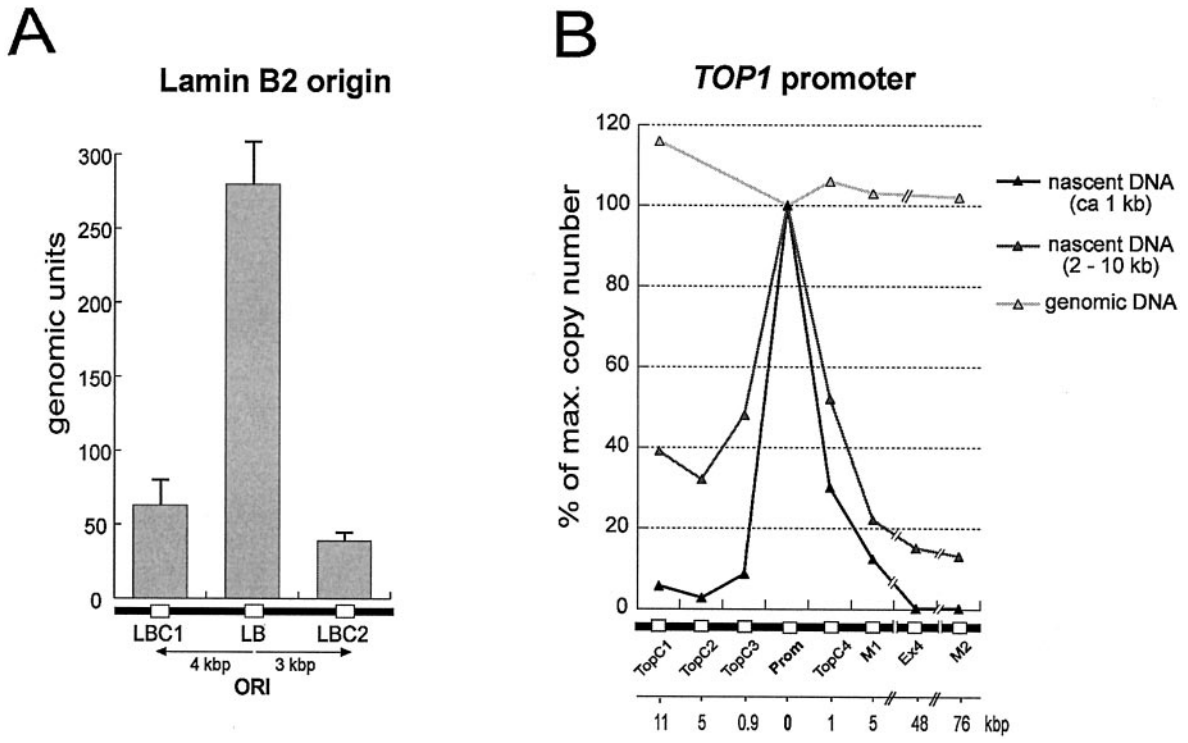

FIG. 7. **Start of bidirectional replication as determined by a nascent strand abundance assay.** *A*, control. Sequences corresponding to the well established human lamin B2 origin are more abundant than more distal sequences in the 1-kb-size fraction of denatured DNA. *B*, *TOP1* sequences. PCR analyses were performed with denatured DNA of the 1-kb-size class and the 2–10-kb-size class. The data are expressed as the percent of maximal copy number in a given size class because the two size classes contain different amounts of DNA (maximally ~250 copies in the 1-kp class and maximally ~3000 copies in the 2–10-kb class). The data with genomic DNA (fragments larger than 20 kb) are included to demonstrate that all primers function with essentially equal efficiencies in the PCR assays.

Similarly, control antibodies failed to precipitate any chromatin fragments with specific DNA sequences (Fig. 4*C*; values of <50 copies in real-time PCR reactions are considered to be non-significant).

In contrast, chromatin immunoprecipitated with Orc1 or with Orc2 antibodies contained DNA sequences corresponding to the upstream promoter region of the *TOP1* gene (Fig. 4, *D* and *E*). More precisely, we estimated about 800 copies of amplifiable promoter sequences in the Orc2 precipitate and about 260 copies in the Orc1 precipitate. The difference in copy numbers between precipitates with Orc1 and Orc2 antibodies was observed in all experiments of this kind and has been noted before in studies on ORC binding sites around another human gene (36). However, the elements MAR I and MAR II appear to be much reduced in immunoprecipitated cross-linked chromatin relative to promoter sequences (Fig. 4). This seems to indicate that ORC is bound to a promoter site in the *TOP1* gene but not to the MAR elements.

It is possible though that promoter sequences were densely covered by cross-linked proteins and, therefore, more protected against shearing and nuclease digestion than MAR elements. This would result in an increase of the copy numbers of promoter sequences in input cross-linked chromatin and, consequently, also in the immunoprecipitates.

To investigate this possibility, isolated cross-linked chromatin was sheared to DNA fragment sizes between <0.25 and 2 kbp and further digested by micrococcal nuclease (Fig. 5*A*). DNA was extracted from fragmented chromatin before and after nuclease digestion and analyzed by quantitative PCR assays. The data indicate a 2-fold higher abundance of promoter fragments compared with control DNA in sheared chromatin. In addition, promoter DNA was more resistant than control DNA to nuclease digestion (Fig. 5*B*).

We note, however, that similar experiments with other genes (36) showed less protection of promoter elements and conclude that the degree of protection by cross-linked proteins varies among ORC binding sites (not shown). Thus, the digestion step is crucial in these experiments, and it was therefore of interest to confirm the results of Fig. 4 comparing the number of sequence copies before and after immunoprecipitation to estimate the enrichment achieved. This was done in the experiment of Fig. 6, where DNA from cross-linked chromatin fragments (0.3–1 kbp) was analyzed by quantitative PCR. The input sample (before immunoprecipitation) contained several thousand copies of amplifiable promoter and promoter-proximal DNA sequences but fewer copies of distal sequences (about 1000 copies or less; Fig. 6*A*). Orc2-specific antibodies immunoprecipitated ~800 copies of the promoter sequence, 200 copies of the closely adjacent TopC4 sequence, and clearly less than 100 copies of more distal sequences (Fig. 6*B*). Control antibodies immunoprecipitated no significant amounts of promoter or adjacent sequences (see Fig. 4*C*). The values of Fig. 6*B* were used to determine the ratios of precipitated over input DNA and showed that up to 10% of the promoter sequences but less than 2% of more distal sequences could be recovered from the immunoprecipitates (Fig. 6*C*).

Thus, in this particular experiment, immunoprecipitated *TOP1* gene promoter sequences were at least 5-fold enriched over more distal sequences. Although these numbers are lower than the enrichment achieved in the immunoprecipitates of ORC-bearing Epstein-Barr virus fragments (35) and also lower than the enrichment for an ORC binding region in the *MCM4* gene promoter (36), it clearly confirms that an ORC binding site is located in the promoter region of the *TOP1* gene and excludes ORC binding sites in MARs.

The ORC Binding Region Coincides with an Origin of Replication—We next addressed the question of whether the identified ORC binding site in the *TOP1* gene promoter region

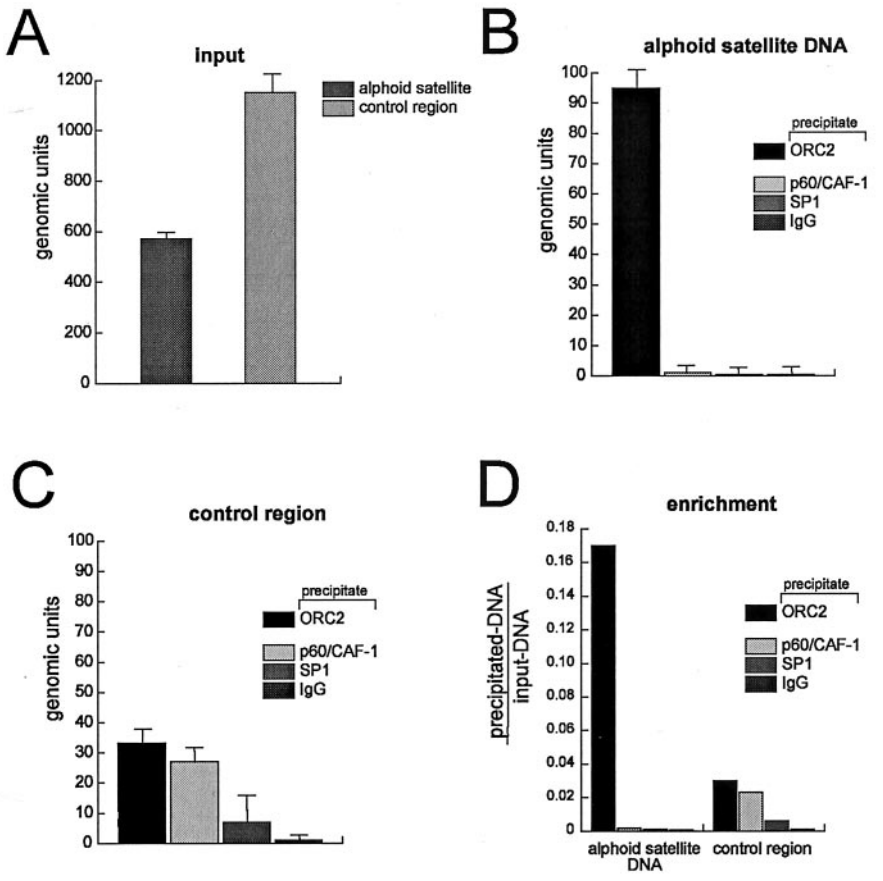

FIG. 8. **Chromatin immunoprecipitations of an alphoid satellite DNA region.** *A*, input. Sheared and digested cross-linked chromatin was deproteinized and investigated by quantitative real-time PCR to determine the abundance of alphoid satellite (primers ASD) and control DNA sequences (primers TopC1) before immunoprecipitation. *B*, alphoid satellite DNA in immunoprecipitates. The chromatin preparation in *A* was immunoprecipitated with specific antibodies as indicated. The abundance of alphoid satellite DNA in the precipitates was determined by quantitative PCR. *C*, control DNA in immunoprecipitates. Same experiment as in *B* but with the TopC1 primers. *D*, enrichments. Ratios of the amounts of precipitated over input DNA are shown.

coincides with an active replication origin. For this purpose, the nascent strand abundance assay of Giacca *et al.* (22) was used. This assay determines the abundance of DNA strands of about 1 kbp in length extracted from denatured genomic DNA. These strands exclusively occur in the vicinity of replication start sites because leading strands at more advanced replication forks are much longer, and lagging strands consist of smaller Okazaki fragments of 0.1–0.2-kbp lengths.

Denatured genomic DNA from proliferating HeLa cells was centrifuged through sucrose gradients, and fractions containing DNA of fragments sizes of 1–2 kbp and of 2–10 kbp were collected. To verify that the 1-kbp fraction of DNA strands was enriched for nascent DNA, primer pairs corresponding to the well characterized lamin B2 origin and, as controls, primer pairs for more distal regions on both sides of the lamin B2 origin were used (58). Quantitative PCR confirmed a higher abundance of origin over flanking sequences (Fig. 7*A*). Therefore, the preparation of denatured DNA fragments was suitable for a determination of nascent DNA strands.

Accordingly, DNA strands in the 1–2- and 2–10-kbp sucrose gradient fractions were used as templates for quantitative PCR with the eight *TOP1* gene primer sets described above (Fig. 3). The results clearly showed a higher abundance of promoter-proximal over more distal sequences (Fig. 7*B*), suggesting that a replication start site is located in the upstream promoter region of the *TOP1* gene. As expected for nascent DNA strands, the enrichment of promoter-proximal DNA sequences relative to distal sequences was higher in the 1–2-kbp size fraction than in the 2–10-kbp size fraction (Fig. 7*B*) because larger nascent DNA strands include not only sequences from the replicative start but from adjacent regions as well. We thus conclude that an origin of DNA replication is located in the promoter region of the human *TOP1* gene, most likely in close association with the ORC binding site.

ORC Binding Sites Occur in Genomic Regions with Repetitive DNA—So far we have demonstrated that the ChIP protocol is useful in detecting ORC binding sites in the vicinity of active genes of known nucleotide sequences. Next, we wished to determine whether the method is suitable for the identification of ORC binding sites elsewhere in the genome. For that purpose, DNA was extracted from immunoprecipitated chromatin (with Orc2-specific antibodies) and cloned in plasmid vectors.

To confirm that a cloned sequence serves as an ORC binding site *in vivo*, independent immunoprecipitations were performed and investigated by quantitative PCR using primers complementary to the sequence in question. A given sequence was considered to be specifically associated with cross-linked ORC if it occurred in enrichments of more than 5-fold greater than control sequences.

Twenty different ChIP clones were thus isolated and partially sequenced (average insert length, 1250 bp). Two of the sequences were detected at high copy numbers in independent immunoprecipitates of cross-linked chromatin.

One of the sequences corresponded to a region close to the gene *TOM1* on human chromosome 22q13.1 (59). The cloned sequence revealed a higher than average G+C content (51%), with consecutive CpG dinucleotides and is, therefore, related to mapped ORC binding regions at active genes. However, we have not yet determined whether the *TOM1* ORC binding site coincides with a replication origin.

The second sequence is homologous to the human alphoid repetitive satellite DNA, which occurs in the centromeric regions of human chromosomes (for a review, see Ref. 60). Several independent ChIP and quantitative PCR assays were per-

TABLE I
Oligonucleotides used for quantitative real-time PCR assays

TOP1 locus

Primer	Sequence (5′ - 3′)	Amplicon position (*)	Amplicon size (bp)	Annealing temperature (°C)
TopC1-F	GACTGCAGAATCTGATGAGCAAC			
TopC1-R	CTAAGAGGTGACAAGCTGCTTCC	1574 - 1873	299	60
TopC2-F	CTAGCTGAGACTGCAGGCACAC			
TopC2-R	GTATGTAGATTAGGGAGCTGCTC	7439 - 7729	290	60
TopC3-F	GCACATTGTATTAATCCAAGCAG			
TopC3-R	CTCTGTGAGGTCGCCTGTATTAC	11603 - 11873	270	60
Prom-F	CACTGCTACCAGAGCGGCTGGGC			
Prom-R	GCAGTTGTGTAACAGCCTAAGTTCG	12461 - 12749	288	68
TopC4-F	GCAGAGGACAGACATGGCCTCCC			
TopC4-R	CGACCGTCGACAAGGCGACTTTCC	13434 - 13660	226	60
M1-F	CATGTCTTAAAGTTTATATTCC			
M1-R	CAGAATCATTTTCCATACCATTAG	17370 - 17550	180	60
Ex4-F	GTCCGATGTAGCCAAGACCC			
Ex4-R	CTACTAGAAAACTCTGTACC	59917 - 60217	354	60
M2-F	GCAGTCATTAAAGGCCTGGAAAAG			
M2-R	GTTTCACTAAAAATAAAACCCC	88619 - 88859	240	60

(*) Accesion No.: AL 035652 (5′ - end of *TOP1* gene) und AL022394 (3′ end)

LaminB2 locus

Primer	Sequence (5′ - 3′)	Amplicon position (#)	Amplicon size (bp)	Annealing temperature (°C)
LBC1-F	GTTAACAGTCAGGGGCATGGGCC			
LBC1-R	CCATCAGGGTCACCTCTGGTTCC	1 - 240	240	66
LB-F	GGCTGGCATCGACTTTCATTTCAG			
LB-R	GTGGAGGGATCTTTCTTAGACATC	3839 - 4070	232	68
LBC2-F	CACAGCATGCGGCTGCTGATCTG			
LBC2-R	CCTCGTCCCTCCCATCTGCCTGC	6648 - 6932	285	68

alphoid satellite DNA / *TOM 1* locus

ASD-F	GGGTTTCAGACCTGCTCTGTCAAGAGG			
ASD-R	CCATTCAACTCACAGAGTTGACCCTTGC	1806-2199	394	60
TOM1-F	CCACAGCACCTTGCTGTACCCC			
TOM1-R	CTTGCTGTCTCATCCAGGTAGTC	-	196	60

(#) Accession No.: M64363 (lamin B2 locus); M28031 (alphoid satellite DNA); AC005290 (analog to h*TOM1* gene)

formed to investigate the association of hOrc2p with the alphoid satellite DNA sequence (Fig. 8*C*). For this purpose, we used primers corresponding the cloned satellite DNA and to a genomic control region (primer set TopC1; see Table I). Sheared and digested cross-linked chromatin contained about twice as much control DNA than alphoid satellite DNA (Fig. 8*A*, *input*), but hOrc2p-specific antibodies clearly precipitated more chromatin-containing satellite DNA than control DNA, whereas antibodies against p60/CAF-1 and SP1 did not (compare Fig. *8*, *B* and *C*). We found that satellite DNA was about 8-fold enriched in the hOrc2p precipitate but not in the control precipitates (Fig. 8*D*). Thus, these data suggest that at least hOrc2p is associated with alphoid satellite DNA *in vivo*.

To determine whether this particular ORC site could serve as an origin of replication, nascent strand abundance assays were performed (see Fig. 7), but an enrichment of clone alphoid DNA sequences in the 1-kbp fraction of nascent DNA was not detected (not shown).

We can, therefore, not decide whether hOrc2p on alphoid repetitive DNA marks a replication origin. It might instead serve to establish the special chromatin conformation at the centromeres of chromosomes just as Orc proteins are involved in the organization of chromatin in yeast and *Drosophila* genomes (for a review, see Ref. 61). Clearly, this point deserves further investigation.

The number of clones investigated is certainly too low for a statistical evaluation on the abundance of ORC-bearing fragments in immunoprecipitated chromatin. However, the results show that cloning of DNA fragments in immunoprecipitated chromatin could be an interesting method of identifying ORC binding sites in the mammalian genome.

DISCUSSION

We have searched for an ORC binding site in a 100-kbp human genome section that includes a typical housekeeping gene, *TOP1*, encoding a type I DNA topoisomerase (37). We provide evidence by ChIP and quantitative PCR that an ORC binding site is located at the upstream promoter region of the *TOP1* gene.

The antibodies used for chromatin immunoprecipitation are directed against hOrc2p and, therefore, precipitated chromatin fragments with covalently linked hOrc2p. The same chromatin fragments also carried other Orc proteins such as hOrc1p, hOrc3p, hOrc4p, and most probably, hOrc5p, suggesting that these proteins form an ORC when bound to chromatin. Interestingly, hOrc6p was barely detectable in these complexes, consistent with results obtained with biochemically isolated human ORC that is composed of a core of proteins hOrc2p–hOrc5p, to which hOrc6p is bound only loosely (52, 53).

Although hOrc1- and hOrc2-specific antibodies precipitate chromatin fragments with the same DNA sequence, the copy numbers in the Orc1-specific precipitates were always one-half or less of the copy numbers in the hOrc2p-specific precipitates (Fig. 4). This could be explained by the observation that hOrc2p remains on chromatin during all phases of the HeLa cell cycle, whereas hOrc1p is present in reduced amounts on chromatin in S-phase cells as previously described and discussed in detail (36, 62).

ORC in Promoter Regions—Although the resolution of the ChIP method is limited and the ORC binding site cannot be located precisely, our data are consistent with the possibility that ORC is located close to the binding sites of the transcription factor Sp1. We conclude this because Orc2-specific antibodies precipitated chromatin fragments that carried not only hOrc2p and other Orc proteins but also significant amounts of the transcription factor Sp1 (Fig. 1). Moreover, antibodies against transcription factor Sp1 precipitated the same DNA sequences that were precipitated by Orc antibodies, suggesting that ORC and Sp1 most probably reside at closely adjacent sites within the *TOP1* promoter. It cannot be excluded though that Sp1 forms physical contacts with ORC.

We note, however, that Sp1 precipitates usually contained 7–8 times more promoter copies than Orc2-precipitates (Fig. 4). This difference could be due to properties of the respective antibodies or to different accessibilities of the cross-linked proteins. Furthermore, the PCR-amplified sequence contains several potential Sp1 binding sites but probably only one ORC site. However, another and potentially more interesting possibility is that, whereas most or all *TOP1* promoters contain transcription factor Sp1, only a fraction of the promoters may contain ORC, implying that the *TOP1* origin is not regularly established in HeLa cells. If this could be substantiated by more direct experiments, it may indicate that ORC binding sites are rather flexible genetic elements in mammalian genomes (29).

The ORC site in the *TOP1* gene coincides with an origin of replication as indicated by the results of the nascent strand abundance assay. This conclusion agrees well with earlier reports showing that many origin sequences have been mapped in the vicinity of genes and frequently in promoter/enhancer regions (for review, see Ref. 55). Furthermore, many sequences that replicate early after the entry of cells into S phase are rich in CpG dinucleotides that are frequently found in the upstream promoter regions of housekeeping genes (57). Indeed, it is well established that genomic sections with housekeeping genes always replicate early in S phase as do regulated genes in cells expressing these genes, whereas regulated genes replicate late in the S phase of differentiated cells that do not express these genes (63). Thus, given the close correlation of early DNA replication with active transcription, it is quite likely that other housekeeping genes also carry ORCs in their upstream regions.

It is conceivable that the more open chromatin structures

that characterize active promoters may facilitate an access of ORC to DNA. Indeed, the acidic transcriptional activation domain of BRCA 1 alters the local chromatin structure and thereby stimulates chromosomal DNA replication (64). In addition the chromatin accessibility complex, CHRAC, clears the simian virus 40 origin of nucleosomes and facilitates an interaction of the viral replication initiator, T antigen, with the origin (65).

Furthermore, the transcription factor Sp1 appears to stimulate manyfold the function of the viral initiator, T antigen (66–68). Similar results have been described for other viral systems (69, 70). In yeast, transcription factor Abf1 on the B3 element of ARSs (13) and the recruitment of an RNA polymerase II transcription complex (71) activate replication. The localization of ORC to the *Drosophila* chorion gene amplification unit depends on direct interactions with the E2F transcription complex (33). Future experiments may show that ORC and transcription factors such as Sp1 not only bind to adjacent DNA sites but also interact functionally to promote replication initiation.

Wyrick *et al.* (72) recently used a ChIP procedure to determine the genome-wide distribution of ORC binding sites in the budding yeast genome with its densely packed genes (72). Most yeast ORCs are located at ARS origins within intergenic sequences, in particular in long terminal repeats of transposable elements which contain transcription and termination signals that may establish chromatin domains suitable for replication initiation, a conclusion that is consistent with the interpretation discussed above.

MAR and Replication—We have chosen the *TOP1* gene for the determination of ORC binding sites because it contains two well mapped MAR elements. The nuclear matrix has frequently been found to be associated with nascent DNA, and it has been proposed that MAR elements are sites where DNA replication is initiated (for recent reviews, see Refs. 7, 24, and 73).

We have now shown that SAF-A, a major MAR binding protein, was not cross-linked to the same chromatin fragments that carry Orc proteins. In addition, the two MAR elements in the *TOP1* gene could not be specifically precipitated with ORC-specific antibodies and are, therefore, not associated with cross-linked ORC, and these MAR elements do not function as replication origins, as suggested by the results of the nascent strand abundance assay. However, MARs may be important to spatially structure replicons through their association with the nucleoskeleton and to form links between sites of DNA replication and the chromosomal architecture (74).

ORC in Late-replicating Heterochromatin—The dense packing of heterochromatin excludes the possibility that ORCs are guided to DNA via promoter DNA sequences and existing open chromatin conformations. However, replication of early chromatin regions may change the structure of chromatin such that previously inaccessible chromatin opens up and can then engage replication complexes.

We have now detected hOrc2p at centromeric alphoid satellite DNA that is probably organized as heterochromatin *in vivo*, but we were unable to demonstrate by the nascent strand abundance assay that this region functions as the replication origin. Thus, hOrc2p on human satellite DNA sequences could have functions in heterochromatin assembly just like Orc proteins in *Drosophila* (75) and yeast (76). But it remains an important task to use ChIP or other techniques to locate Orc proteins in late-replicating human heterochromatin and to determine the exact role for human ORC in these regions.

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