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In Vitro Protein-DNA Interactions at the Human Lamin B2 Replication Origin*

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The complexity of mammalian origins of DNA replication has prevented, so far, the *in vitro* studies of the modalities of initiator protein binding and origin selection. We approached this problem by utilizing the human lamin B2 origin, wherein the precise start sites of replication initiation have been identified and known to be bound *in vivo* by the origin recognition complex (ORC). In order to analyze the *in vitro* interactions occurring at this origin, we have compared the DNA binding requirements and patterns of the human recombinant Orc4 with those of preparations of HeLa nuclear proteins containing the ORC complex. Here we show that both HsOrc4 alone and HeLa nuclear proteins recognize multiple sites within a 241-bp DNA sequence encompassing the lamin B2 origin. The DNA binding activity of HeLa cells requires the presence of ORC and can be reproduced in the absence of all the other proteins known to be recruited to origins by ORC. Both HsOrc4 alone and HeLa nuclear proteins exhibit cooperative and ATP-independent binding. This binding covers nucleotides 3853–3953 and then spreads outward. Because this region contains the start sites of DNA synthesis as well as the area protected *in vivo* and preserves protein binding capacity *in vitro* after removal of a fraction of the protected region, we suggest that it could contain the primary binding site. Thus the *in vitro* approach points to the sequence requirements for ORC binding as a key element for origin recognition.

Regulation of DNA synthesis in eukaryotic cells, in analogy with the situation well described in prokaryotic replicons, occurs through the activation, at precise moments of the cell cycle, of precise sequences, defined as replication origins. Activation is promoted by a large protein complex whose assembly is initiated by the binding of the origin recognition complex (ORC)[1] to the sequences coinciding with actual origins (1, 2). In unicellular eukaryotes, the distribution of ORCs is directed by genetic elements analogous to bacterial replicators, whereas in mammals and in metazoa in general, the manner of the interaction of ORCs with DNA does not suggest comparable specificity (3, 4). The DNA binding requirements of mammalian ORCs have not been studied mainly because of the complexity and the small number of relatively well characterized mammalian origins of DNA replication (5). In order to extend the protein-DNA interaction studies to replication origins of mammals and to contribute to the establishment of an origin-dependent *in vitro* system, we have investigated the protein-DNA interactions at the human lamin B2 origin of DNA replication.

This origin is located in the short arm of human chromosome 19 and mapped with a high precision near the 3′ end of the lamin B2 gene (6–8). Studies conducted in human cells localized the origin area within ~250 bp. This area contains a more than 110-bp sequence involved in complex protein-DNA interactions during the cell cycle and accommodates both ORC-binding site(s) and the start sites of DNA synthesis (9–13). Because it apparently contains all the elements necessary for origin function, this area was chosen for DNA binding experiments. Prompted by recent results showing that in *Schizosaccharomyces pombe* it is the subunit 4 of ORC that provides the DNA binding entity to the whole complex (14–18), we have initially tested the DNA binding properties of the human recombinant Orc4 and then compared the DNA binding patterns of HsOrc4 with those of human nuclear proteins isolated from HeLa cells that contain all the ORC members. HeLa cells were chosen because they are known to activate the lamin B2 origin *in vivo* (19).

We report here the results of our investigations.

EXPERIMENTAL PROCEDURES

Cloning of HsORC4 Gene into Bacterial Vector, Expression and Purification of Recombinant Protein—The HsORC4 subunit was expressed in bacteria as recombinant fusion protein tagged with a His_6 epitope. The fragment that encodes the full-length human protein (1311 bp) was amplified by using the following pair of oligonucleotide primers: 5′-CGGGATCCATGAGCAGTCGTAAATCAAAGAGTAAC-3′ and 5′-TCCCCCCGGGTCATAACCAGCTTAGTGAGGATG-3′. The template was cDNA-generated by reverse transcriptase-PCR from total RNA isolated from HeLa cells. The PCR product was digested with *Bam*HI/*Sma*I and cloned into corresponding sites in the plasmid pQE-30 (Qiagen, Valencia, CA). Recombinant protein was expressed in *Escherichia coli* M15(pREP4) bacterial strain. Purification of recombinant HsORC4 protein was performed under native conditions, using TALON metal affinity resin, according to the instructions provided by the manufacturer (Clontech Laboratories Inc, Palo Alto, CA). Protein expression and purification were tested with a mouse monoclonal antibody against His_6 epitope, purchased from Clontech. Before use, the protein preparation was dialyzed against buffer (20 mM HEPES, pH 7.9, 30 mM NaCl, 0.1 mM EDTA, 6 mM $MgCl_2$, 0.2 mM $ZnCl_2$, 10% glycerol, 1 mM dithiothreitol, a mixture of protease inhibitors), supplemented with 0.1 mM ATP. Where necessary, an aliquot of the sample was additionally dialyzed in the same buffer but without ATP.

Preparation of Nuclear Extracts—Nuclear extracts were essentially prepared as described previously (20). Respective concentrations of NaCl and Nonidet P-40 in the extraction buffer were adjusted to 1 M and

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[1] The abbreviations used are: ORC, origin recognition complex; EMSA, electrophoretic mobility shift assay.

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1%, which significantly improved the yield in HsOrc2. Extracts were dialyzed against buffer containing 20 mM HEPES, pH 7.9, 30 mM NaCl, 0.1 mM EDTA, 6 mM $MgCl_2$, 0.2 mM $ZnCl_2$, 10% glycerol, 1 mM dithiothreitol, a mixture of protease inhibitors, with or without 0.1 mM ATP.

Streptavidin Pull-down Assay—For pull-down experiments performed with HsOrc4, poly(dA-dT)-poly(dA-dT) was tailed, using terminal transferase with dATP substrate and attached to streptavidin-coated magnetic beads via biotinylated oligo(dT). Bacterial proteins purified over metal affinity resin were incubated with the attached DNA probe, washed, and stripped with SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE, and Western blotting was carried out using 5000-fold diluted, albumin-free, monoclonal antibody against His_6 (Clontech). Secondary antibody was goat anti-mouse horseradish peroxidase conjugate (Pierce), diluted 100,000-fold. The blot was developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

For pull-down experiments with streptavidin magnetic beads, a 241-bp fragment of origin DNA was amplified from human genomic DNA using CAAAAACGGAGCTGGGCTCAGCTG and GACATCCGCCGCTTCATTAGGGCAGAGGCC primer pair. The PCR fragment was either reamplified using one 5′-biotinylated primer (the other primer was not modified) or tailed, using terminal transferase with dATP substrate. Unilaterally biotinylated DNA fragments were directly bound to streptavidin-coated paramagnetic beads. The approximate efficiency of binding was 10 pmol of beads per pmol of DNA. Tailed fragments were bound to the beads via biotinylated oligo(dT) with approximate efficiency of 3–5 pmol of beads/oligo(dT) per pmol of DNA. Prior to binding to DNA, which was attached to the beads, protein samples were incubated with different competitors given in 50–100-fold molar or weight excess over attached DNA. Incubation with beads was continued for 2 h at 4 °C, with constant rotation. Typical binding mixtures contained 3 mg of proteins per 20 pmol of unilaterally biotinylated attached DNA or 600 μg of proteins per 5 pmol of attached tailed DNA. At the end of the incubation, samples were pulled down with a magnet and were washed several times with binding buffer. They were stripped from the beads using either SDS-PAGE sample buffer for 30 min at 37 °C or by DNase digestion using 1 unit of DNase per sample for 30 min at room temperature. Immunoblotting analyses were performed after separation of proteins in SDS-PAGE using the following antibodies: rabbit polyclonal antibody against HsCdc6 (Santa Cruz Biotechnology), monoclonal antibody against HsMcm3 (Medical and Biological Laboratories Co., Ltd.), monoclonal antibody against HsOrc2 (StressGen), and rabbit polyclonal antibody against HOXC13.

Agarose and Polyacrylamide Gel Mobility Shifts and Footprints—DNA binding reactions were performed with the 241-bp origin fragment, which was amplified from human genomic DNA using the CAAAAACGGAGCTGGGCTCAGCTG and GACATCCGCCGCTTCATTAGGGCAGAGGCC primer pair. A typical reaction was prepared in a buffer containing 20 mM HEPES, pH 7.9, 30 mM NaCl, 1 mM EDTA, 6 mM $MgCl_2$, 2 mM $ZnCl_2$, 2 mM dithiothreitol, 10% glycerol, with or without 1 mM ATP, with 10–500 ng of HsOrc4 or 1–30 μg of HeLa nuclear proteins and with 2–6 fmol of the DNA probe. The reaction mixtures contained also poly(dI-dC)-poly(dI-dC) in 2000-fold weight excess over labeled probe and, where indicated, cold origin or control DNA in 100-fold molar excess over labeled probe. Control DNA was a 166-bp fragment located 5 kb downstream of the origin and amplified from human genomic DNA using the CCTCAGAACCCAGCTGTGGA and GCCAGCTGGGTGGTGATAGA primer pair. Alternatively, poly(dI-dC)-poly(dI-dC) was replaced by other synthetic copolymers in the same amounts or combined with other competitors in the amounts indicated in the text. The reaction mixtures were subjected to EMSA in either 5% polyacrylamide or 1% agarose gels or subjected to DNase I protection assays. Polyacrylamide gels were 10 or 25 cm long and agarose gels were 25 cm long.

RESULTS

Human Orc4, tagged at the N terminus with 6 histidines, was cloned, expressed, and purified from *E. coli*; DNA and protein sequencing data confirmed the protein identity. The purification procedure resulted in a single band, as detected by Coomassie Blue staining and by immunoblots, using antibody against histidine tag (Fig. 1*A*). The purified protein was assayed for its ability to retard the electrophoretic mobility of a 241-bp DNA fragment containing the origin. This fragment encompasses the start site of leading strand synthesis, the area protected by proteins in *vivo* and the greatest part of the region

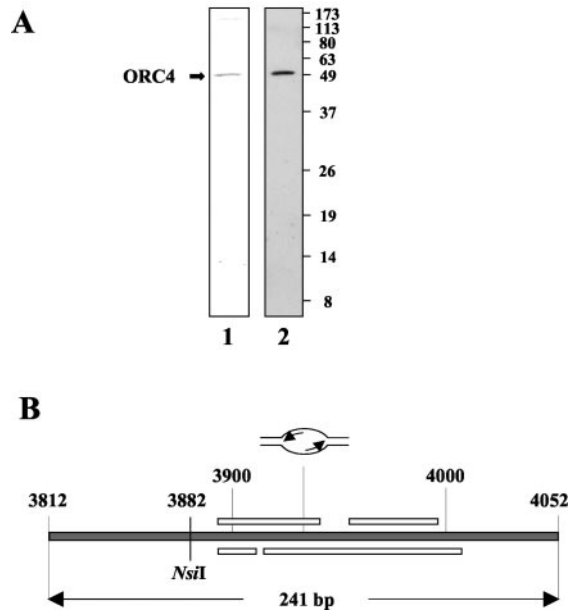

FIG. 1. **Purification of recombinant HsOrc4.** *A*, SDS-PAGE (*lane 1*) and immunoblot (*lane 2*) of the purified HsOrc4. *B*, schematic representation of the lamin B2 origin fragment. The start site of bidirectional DNA synthesis, the area protected by proteins *in vivo* in G_1, and the *Nsi*I restriction site are indicated. Fragment 3812–4052 was utilized either alone or bound to magnetic beads via poly(dT)-poly(dA) linkers, as indicated in the text.

that contains the binding sites for ORC (9–13) (Fig. 1*B*; the numbering corresponds to that of the humlambbb file of GenBank™). In the presence of HsOrc4, two retarded bands of origin DNA were observed in EMSA, of which the most retarded was less abundant. These bands were partly shifted toward still higher molecular weights when an antibody recognizing the recombinant HsOrc4 was present (Fig. 2*A*), whereas the same amount of control antibody had no effect (not shown).

Considering that SpOrc4 was shown to have affinity for AT-rich DNA (15, 18), we decided to check whether recombinant HsOrc4 shared this property. To this purpose, lysates of induced bacterial cells transfected with Orc4 expressing or empty vectors were purified over metal affinity resin, and the proteins bound to the resin were eluted and incubated with partially biotinylated poly(dA-dT)-poly(dA-dT) attached to streptavidin magnetic beads. After magnetic separation, the beads were washed, and the proteins were stripped and subjected to immunoblotting analysis using anti-histidine antibody. A single band corresponding to HsOrc4 was identified in the sample isolated from bacterial cells expressing HsOrc4 (Fig. 2*B*).

Recombinant Orc4 was also assayed for origin binding in the presence of different DNA competitors (Fig. 2*C*). One of these was a DNA fragment from a region located 5 kb away from the lamin B2 area, previously shown not to contain an active origin (19). The other competitor DNAs were three synthetic copolymers. As demonstrated in Fig. 2*C* the major retarded band was displaced by the cold origin DNA but was not affected by a 100-fold molar excess of the control DNA. Among the synthetic competitors tested, poly(dA-dT)-poly(dA-dT) was an efficient competitor (Fig. 2*C*, *lane 8*), whereas poly(dI-dC)-poly(dI-dC) and poly(dG-dC)-poly(dG-dC) were inefficient (*lanes 7* and *9*). It should be mentioned that the origin fragment has a 55% A-T content and shows stretches with much higher local AT content, characterized by frequent clusters of T residues in the upper strand. In contrast to this, the control DNA had uniformly distributed A and T residues, and an overall AT content of 36%.

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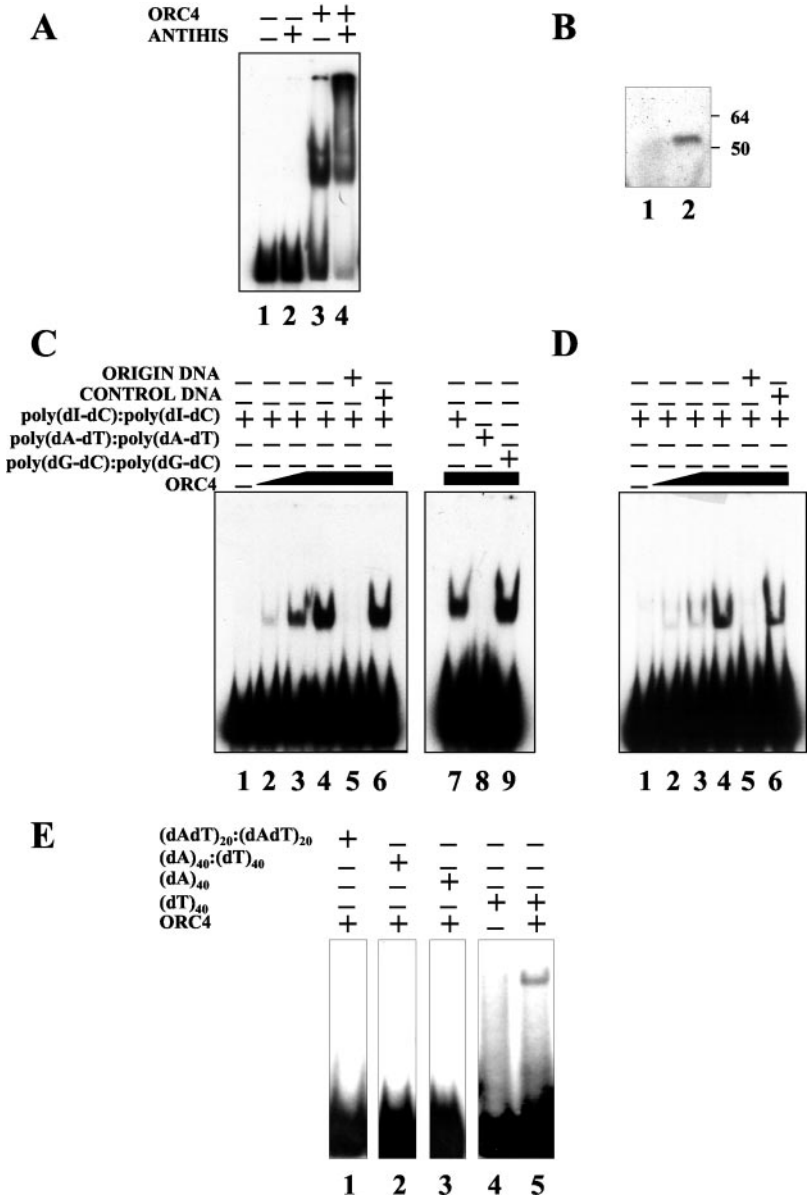

FIG. 2. **Binding of HsOrc4 to the lamin B2 origin and AT-rich DNA.** *A*, supershifts of the origin probe performed with recombinant HsOrc4 and with antibody against His_6 epitope: *lanes 1* and *2*, reactions without HsOrc4; *lanes 2* and *4*, reactions with anti-His_6 antibody; *lanes 3* and *4*, reactions with recombinant HsOrc4. *B*, immunoblots of proteins from bacterial cells transfected with empty vector (*lane 1*) or with vector expressing HsOrc4 (*lane 2*). The proteins were purified over metal affinity resin and bound to alternating AT copolymer attached to streptavidin paramagnetic beads. *C*, EMSA without (*lane 1*) or with 5 (*lane 2*), 10 (*lane 3*), and 50 ng (*lanes 4–9*) of recombinant HsOrc4 in the presence or absence of different competitors, as indicated. *D*, the same as *C* (*lanes 1–6*), but with 170-bp *Nsi*I origin fragment. *E*, binding of HsOrc4 to alternating (*lane 1*) and asymmetric (*lane 2*) double-stranded AT 40-mers and to single-stranded A (*lane 3*) and T 40-mers (*lanes 4* and *5*).

Given that the origin contains several tracts of asymmetric AT DNA, we have also included different alternating and asymmetric AT competitors. Under 1000-fold weight excess (~100-fold molar excess, assuming an average length of 2000 bp), asymmetric and alternating synthetic copolymers were equally efficient (as in Fig. 2*C*, *lane 8*) and under 100-fold weight excess (not shown) were equally inefficient competitors. (Alternating and asymmetric AT 40-mers were instead inefficient competitors at all concentrations tested.) In direct binding reactions HsOrc4 did not react either with double-stranded 40-mers or with single-stranded $(dA)_{40}$, but it exhibited low affinity for single-stranded $(dT)_{40}$ (Fig. 2*E*). Consequently, HsOrc4 seems to recognize essentially AT-rich DNA regardless of its sequence but with much lower affinity than for origin DNA and only if synthetic AT DNA is very long.

HsOrc4 was also demonstrated to form several protein DNA complexes of increasing size (Fig. 3*A*). The number of complexes depended on the excess of protein over the labeled probe; increasing amounts of Orc4 caused a shift of the major complex toward higher molecular masses, whereas several larger complexes appeared, indicating a sort of cooperative binding to multiple sites. At least one of these sites was preserved in the 170-bp fragment isolated after digestion of the 241-bp origin fragment with the restriction enzyme *Nsi*I (Fig. 2*D*). In addition, formation of the complexes was not dependent on the presence of ATP and was not affected by its non-hydrolyzable analogue (not shown).

The DNA sites recognized by the HsOrc4 protein were analyzed by DNase I protection on both strands of the DNA probe (Fig. 3*B*). Under the chosen protein:DNA ratio (as in Fig. 3*A*, *lane 4*), the clearest protection was observed between nucleotides at positions 3853–3953 on the upper strand and only within nucleotides 3893–3953 on the lower one (Fig. 4*B*). The protected regions have over 70% AT contents and several stretches of 3–10 A or T residues. The protection was more prominent on the T-rich strand of the DNA molecule. With still increasing protein concentrations, the boundaries of the protected regions spread beyond positions 3853 and 3953 (not shown).

In summary, these results show that human recombinant Orc4 exhibits DNA binding activity and recognizes the lamin B2 origin of DNA replication in ATP-independent manner. The competition binding experiments indicate a relatively low level of sequence specificity and preference for AT-rich DNA. At increasing protein:DNA ratios the complexes are shifted toward higher molecular masses, indicating that they contain

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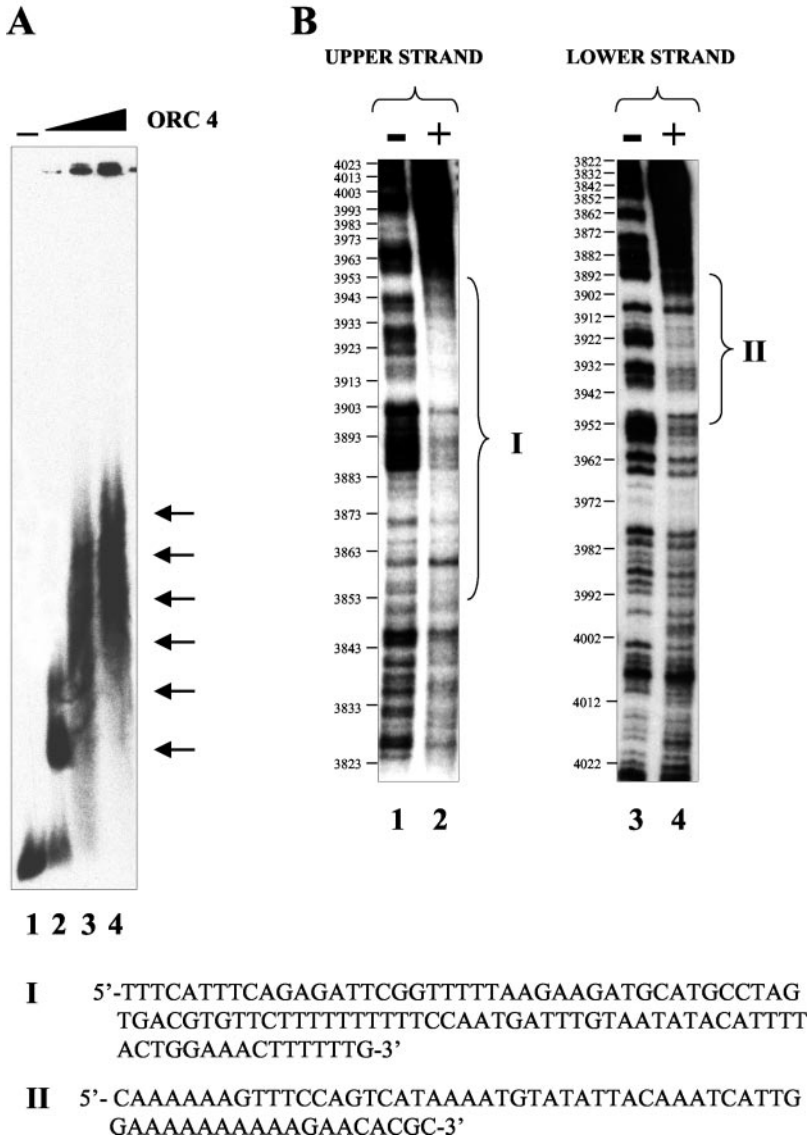

FIG. 3. **HsOrc4 recognizes multiple sites within the 241-bp origin fragment.** *A*, agarose gel mobility shift without (*lane 1*) and with 80, 150, and 250 ng of HsOrc4 (*lanes 2–4*, respectively). *Arrows* indicate different distinguishable retarded bands. *B*, DNase I protection assay: *lanes 1* and *2*, reactions on the upper strand without and with HsOrc4, respectively; *lanes 3* and *4*, reactions on the lower strand without and with HsOrc4, respectively; *I* and *II* are the sequences of the protected regions.

more than one HsOrc4 molecule per molecule of DNA. This effect can be explained either by protein multimerization or by cooperative recognition of multiple DNA sites. The observation that at high protein:DNA ratios the footprints reveal extended protection points to the later interpretation. In addition, it suggests preferential binding of HsOrc4 to the T-rich strand within AT-rich asymmetric sequences.

In order to determine how the binding pattern of HsOrc4 correlates with the events occurring in the presence of all the human proteins involved in origin activation, HeLa nuclear proteins were isolated and tested with the same DNA probes and using basically the same methodology used for HsOrc4. The nuclei were isolated by mechanical disruption of the cells, and the proteins were extracted with a buffer containing 1 M NaCl and 1% Nonidet P-40. The nuclear proteins were first tested for their ability to retard the electrophoretic mobility of the origin probe in 1% agarose gels (Fig. 4*A*). As expected, the complexes formed with HeLa nuclear proteins and the origin probe exhibited much larger molecular masses than with HsOrc4 alone. As observed with the recombinant molecule, the complexes were shifted toward higher molecular masses at increasing protein:DNA ratios and exhibited the same response as HsOrc4 to origin and non-origin DNA competitors. In addition, formation of the complexes was independent of ATP and not affected by its non-hydrolyzable analogue.

To investigate which portions of the origin sequence were covered by the nuclear proteins, we also performed DNase I footprinting analysis using the same protein:DNA ratio as in Fig. 4*A*, *lane 2*. As shown in Fig. 4*B*, extended protection consistent with binding to multiple sites within the origin DNA was detected on both strands of the probe. In contrast to that, nuclear proteins did not bind non-origin control when tested under identical conditions (not shown).

As with HsOrc4, the 5′ and 3′ limits of the protected region depended on the protein concentration (not shown), indicating that with both types of interaction, initial binding occurs within nucleotide positions 3853–3953 and then spreads outward. Protection of the lower strand was much more prominent with HeLa proteins than with HsOrc4 alone, which prompted us to probe into the identity of HeLa proteins interacting with origin DNA.

The nature of the proteins interacting with origin DNA was investigated by immunoblotting with antibodies against several candidate proteins. These were HsOrc2, HsCdc6, and HsMcm3, proteins that are assembled at origins in an ordered manner and belong to the pre-replicative complex involved in initiation of DNA replication in all eukaryotes (1–4). HsOrc2 was chosen as the representative of ORC complex, which, in extracts of HeLa cells, contains subunits 1–5 and is quantitatively precipitated by antibody against HsOrc2 (21), whereas

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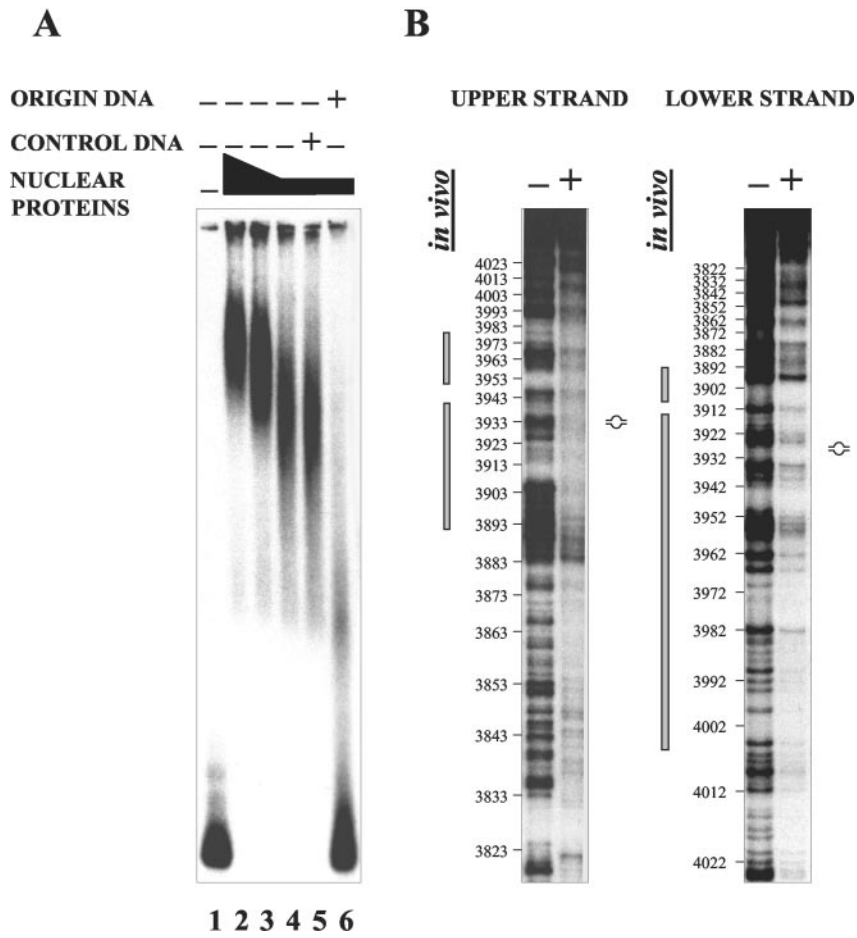

FIG. 4. **Agarose gel mobility shifts and footprints with HeLa nuclear proteins.** *A*, reactions without (*lane 1*) and with 30 (*lane 2*), 20 (*lane 3*), and 10 (*lanes 4–6*) μg of nuclear proteins; in *lanes 5* and *6* a 100-fold molar excess of cold control or origin DNA, respectively, is also present. *B*, DNase I protection of the upper and lower strands in reactions without (−) and with (+) proteins. Positions of the start site and of the area protected in G_1 *in vivo* are indicated.

HsMcm3 was chosen as the representative of the MCM complex. In addition, HOXC13, a homeotic protein of yet undetermined function, but identified for binding to the lamin B2 origin by the yeast one-hybrid system, was also tested (22).

As shown by the results reported in Fig. 5*A*, the HeLa nuclear proteins contained significant amounts of HsOrc2, HsCdc6, HsMcm3, and HOXC13. To check whether these proteins interacted with origin DNA *in vitro*, we developed an assay based on the immunological detection of proteins interacting with unilaterally biotinylated origin probe attached to streptavidin paramagnetic beads and isolated by magnetic separation.

The nuclear extracts were divided in three equal portions and incubated with different combinations of DNA competitors, as indicated in Fig. 5*B*. They were pulled down with origin DNA attached to streptavidin, stripped, and subjected to immunoblotting. The experiments corresponding to *lane 1* were performed incubating the biotinylated fragment with the nuclear proteins in the presence of a 50–100-fold weight excess of poly(dI-dC)-poly(dI-dC) followed by stripping of the proteins and immunoblot analysis; in the experiments reported in *lane 2* the nuclear proteins were preincubated with a 100-fold molar excess of cold origin DNA and then challenged with the same incubation conditions as in *lane 1*; *lane 3* reports the results of experiments where the nuclear proteins were preincubated with a 100-fold molar excess of non-origin DNA and then challenged and analyzed again as in *lane 1*. Immunoblotting experiments were performed with antibodies to HsOrc2, HsCdc6, HsMcm3, and HOXC13. As the data show, HsMcm3 did not discriminate between different competitors used, but was sensitive to the overall amount of DNA. Differently from HsMcm3, HsOrc2, HsCdc6, and HOXC13 were selectively displaced only with origin DNA. 0

In order to check whether the amount of HsOrc2 correlates with DNA binding activity, we have also performed different assays with protein extracts that were partly or completely depleted of HsOrc2. For that purpose, protein extracts were incubated with increasing concentrations of origin DNA bound to paramagnetic beads, and the remaining proteins were tested for binding to the labeled origin. During these experiments we have observed that a large excess of unilaterally biotinylated DNA is needed in order to achieve quantitative binding of HsOrc2 and that the efficiency could be significantly improved by addition of poly(A) tails to origin DNA. Origin fragments were tailed with terminal transferase and dATP substrate and then bound to streptavidin beads via biotinylated oligo(dT). After incubation with DNA and magnetic separation, bound proteins were removed from DNA by either SDS-PAGE sample buffer or by complete DNase digestion of DNA attached to streptavidin magnetic beads. By using tailed DNA, we have repeated the competition binding experiments, as one presented in Fig. 5*B*, and the results were the same as with unilaterally biotinylated DNA. This was consistent with competition binding experiments done with HsOrc4, which have shown that a only very large excess of AT-rich DNA can affect protein binding to origin DNA. We cannot, however, exclude the possibility that, once nucleated by origin, protein binding spreads to the regions of tails, thus making tailed origin DNA more efficient than the unilaterally biotinylated one. Alternatively, an intervening spacer increases the distance from the beads and in that way facilitates binding of the big protein complexes.

Fig. 5*C* shows the results obtained with increasing concentrations of tailed DNA attached to paramagnetic beads. As demonstrated by the amounts of proteins present in the bound and free fractions, respectively, HsOrc2 and HsCdc6 could be

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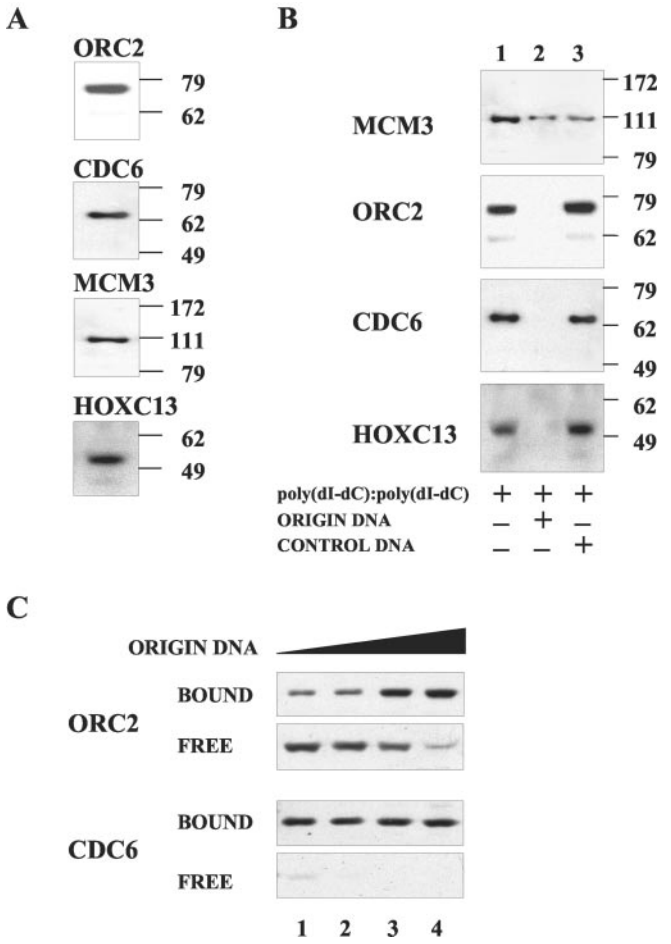

FIG. 5. **Replicative proteins present in HeLa nuclear extracts and the interaction with the lamin B2 origin.** *A*, immunological detection of HsOrc2, HsCdc6, HsMcm3, and HOXC13 present in nuclear extracts from HeLa cells. *B*, interaction of HsOrc2, HsCdc6, H6Mcm3, and HOXC13 with the lamin B2 origin; immunoblots of proteins bound to unilaterally biotinylated origin DNA attached to streptavidin paramagnetic beads, in the presence of competitors, as indicated in the figure. *C*, binding of HsOrc2 and HsCdc6 to increasing amounts of tailed origin DNA attached to streptavidin beads. The fractions of proteins bound to the DNA probe or remaining free in solution are indicated in *lanes 1–4*.

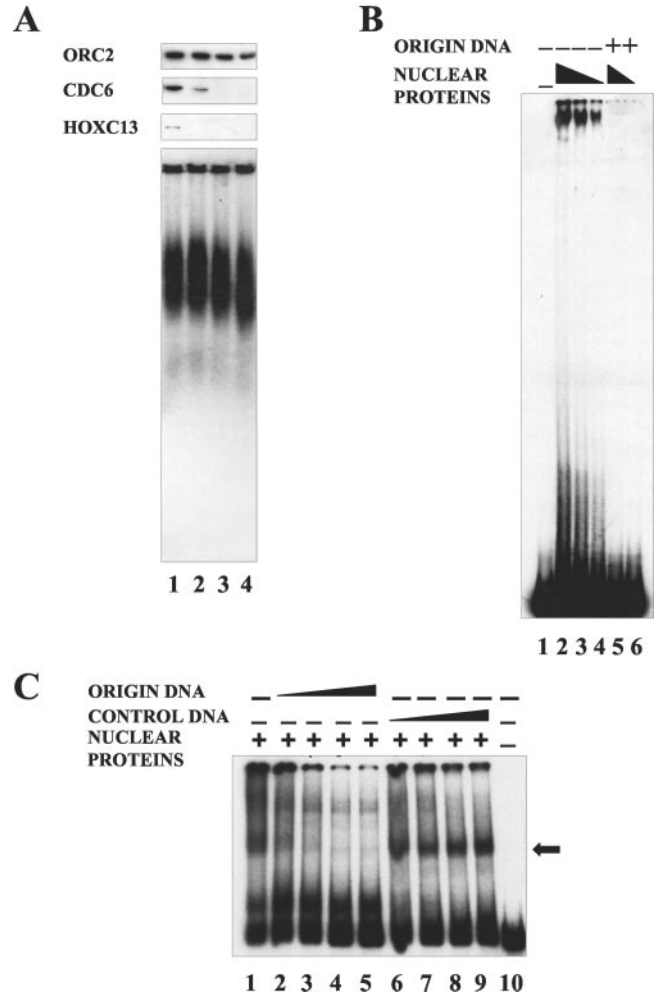

FIG. 6. **Agarose and polyacrylamide gel mobility shifts of HeLa nuclear proteins devoid of HsCdc6 and HOXC13.** *A*, *lower panel*, EMSA (agarose gels) binding reactions with protein extracts containing HsOrc2, HsCdc6, and HOXC13 (*lane 1*), devoid of HOXC13 (*lanes 2–4*), and devoid of both HOXC13 and HsCdc6 (*lanes 3* and *4*). *Upper panel*, actual amounts of HsOrc2, HsCdc6, and HOXC13 in the samples that were assayed for gel mobility shifts (*lanes 1–4*), as determined by immunoblotting analysis. *B*, EMSA (PAGE) of 241-bp origin DNA fragment with protein preparations depleted of HsCdc6 and HOXC13; *lane 1*, reaction mixture without proteins; *lanes 2–4*, with decreasing amounts of proteins; *lanes 5* and *6*, the same as *lanes 2* and *3* but in the presence of 50-fold molar excess of cold origin DNA. *C*, EMSA (PAGE) of the 170-bp *Nsi*I origin DNA fragment with the same proteins as in *B*; *lanes 2–5* and *lanes 6–9*, respective reactions with increasing amounts (10-, 25-, 50-, and 100-fold molar excess over DNA probe) of either origin or control DNA competitors. *Arrow* indicates position of the complex specific for origin DNA.

quantitatively bound to tailed origin DNA. Interestingly, the amount of DNA necessary for quantitative protein binding was 10-fold higher for HsOrc2 than for HsCdc6, which indicated differences in their overall amounts in favor of HsOrc2, which binds to DNA even in the absence of Cdc6. ATP was present in all reactions, but binding of proteins was independent of ATP and not affected by the presence of its non-hydrolyzable analogue.

Based on the observation that we could selectively deplete nuclear samples of individual proteins (see Fig. 5*C*) by incubating the extracts with increasing amounts of tailed origin DNA attached to streptavidin beads, we prepared samples containing HsOrc2, HsCdc6, and HOXC13, devoid of HOXC13 and devoid of both HOXC13 and HsCdc6 (Fig. 6*A*, *upper panel*). These samples, containing proteins that remain in free fractions after incubation with increasing amounts of tailed origin DNA attached to streptavidin paramagnetic beads and magnetic separation, were assayed for agarose gel bandshifts of the labeled origin probe. As shown in Fig. 6*A*, gradual decrease and complete absence of Cdc6 and HOXC13 did not significantly affect the pattern of the shifted complexes. The average mass of the complexes was decreasing in correlation with the overall amount of HsOrc2, and no binding was detected in extracts devoid of HsOrc2 (hence presumably of the whole complex, not shown). Even in the absence of Cdc6 and HOXC13, the size variations of detected protein-DNA complexes were consistent with recognition and incomplete saturation of multiple DNA sites.

In order to get complexes of uniform size, protein samples were further diluted and analyzed by EMSA, under conditions of origin DNA excess. By using very small amounts of nuclear proteins (samples were 5-, 10-, and 15-fold diluted with respect to the amount presented in Fig. 6*A*, *lane 4*) and vast amounts of DNA competitors (see "Experimental Procedures"), a single band close to the top of a 25-cm long polyacrylamide gel was detected (Fig. 6*B*). With lower amounts of competitors, this band was rapidly converted into a form too large to enter the gel. To prevent this conversion into larger complexes and to isolate a potential single binding site, we used the *Nsi*I frag-

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ment, previously shown to bind HsOrc4 (see above, Fig 2*D*). This fragment was also bound to streptavidin paramagnetic beads and tested as described previously for binding to HsOrc2. Because it gave positive results (not shown), it was further tested for EMSA in short polyacrylamide gels (Fig. 6*C*). With this fragment, a single specific complex was detected.

DISCUSSION

Altogether, our results show that HsOrc4 and HeLa nuclear proteins recognize multiple sites within 241 bp of the lamin B2 origin DNA. The DNA binding activity of HeLa nuclear proteins is correlated with the presence of ORC and can be reproduced in the absence of HsCdc6 (and hence of all the other proteins which are recruited to origins by ORC) as well as in the absence of HOXC13, a protein that exhibits specific affinity for origin DNA *in vivo* and *in vitro*. Both HsOrc4 and HeLa nuclear proteins exhibit origin-specific, cooperative, and ATP-independent binding properties. In footprint reactions, both activities protect extended regions on both strands; protection of the upper strand is much more prominent, particularly with Orc4. With both types of protein preparations, the limits of the protected areas depend on the protein:DNA ratio, indicating that interaction is not random, initiates at nucleotide positions 3853–3953, and then spreads outward. The DNA fragment isolated after *Nsi*I digestion (3882–4052) contains a portion of this sequence, and we suggest that binding initiates in this region, an inference supported by the fact that the same region accommodates the start sites of DNA replication *in vivo*. The area protected *in vitro* corresponds to the one protected *in vivo*, but extends even further in both directions; this discrepancy may reflect restrictions imposed on DNA by features of the local organization of chromatin that constrain the interaction into a more limited zone.

From this study it appears that HsOrc4 alone produces the major part and dictates the pattern of the interactions occurring at the lamin B2 origin *in vitro*. The other components of ORC, or even of the whole pre-replicative complex, may have a modulator role. Essentially, the overall binding pattern at the lamin B2 origin of DNA replication resembles the ones previously obtained at amplification origins of insects. The results presented here should in fact be compared with the data previously obtained with purified or recombinant ORC at amplification origins of insects (23–26), or with SpORC and SpOrc4 (14–18). As in our case, the experiments performed with insect origins show that one origin region contains more than one binding site for ORC. Experiments with *Drosophila* ORC demonstrate a cooperative binding that results in formation of very big complexes with disperse boundaries, an observation very similar to what we obtained with both Orc4 and HeLa proteins. This pattern was interpreted in terms of two subpopulations of ORCs, one small and sequence-specific, and a nonspecific larger one. We propose that the phenomenon reflects a certain hierarchy in the specificity of DNA-binding sites rather than a distinction among ORC molecules.

Differently from insect ORCs and similarly to *S. pombe*, we do not detect ATP-dependent binding. In addition, our results are in many ways similar those obtained with *S. pombe* proteins; *S. pombe* ORC also shows preference for AT-rich DNA, more precisely for the T-rich strand in AT-rich asymmetric origin sequences and for consecutive runs of clustered T residues. DNA binding of the whole complex is promoted by its subunit 4 and occurs through an AT-hook domain situated within the N-terminal portion of the protein molecule. In HsOrc4 this domain is missing, and yet yeast and human proteins share a fairly similar DNA binding specificity. In *S. pombe* and in humans the common denominator of the interaction of ORC with DNA appears to be the recognition of multiple AT-rich sequences. Indeed, the lack of a metazoan origin sequence consensus and the example of *S. pombe* which shares with metazoa relatively similar origin structure, but interacts with DNA through the domain not present in metazoa, prompted many scientists to speculate that factors other than ORC determined the specificity of origin selection (3, 4). However, such factors have never been found. Previous work on *S. pombe* and the results presented here give an interesting example of how non-identical but functionally analogous structures could determine similar DNA binding properties of ORCs isolated from different species and how these properties are being conserved during evolution. We feel that the apparently low specificity of ORCs could be overcome by ordered binding to many low affinity sites and that the overall specificity of recognition could be dictated by the spatial arrangement of these sites. The non-random ordered binding that we detect in our experiments and the preference for the T-rich strand of the origin support this notion.

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