Formation of Noncanonical DNA Structures Mediated by Human ORC4, a Protein Component of the Origin Recognition Complex[†]

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ABSTRACT: Many genomic sequences, DNA replication origins included, contain specific structural motifs prone to alternative base pairing. Structural rearrangements of DNA require specific environmental conditions and could be favored by chemical agents or proteins. To improve our understanding of alternative conformations of origins and the manner in which they form, we have investigated the effect of DNA-binding, AAA+ protein human ORC4 on single-stranded origin DNA or various oligonucleotides. Here we demonstrate that human ORC4 stimulated formation of inter- and intramolecular $T\cdot A\cdot T$ triplexes and created novel structures, such as homoadenine duplexes. Adenine-based structures were held together by Hoogsteen hydrogen bonds, as demonstrated on 7-deaza-dAMP- or dAMP-containing substrates, and characterized by increased thermal stability. Adenine pairing occurred only in the presence of human ORC4, in a neutral buffer supplemented with ATP and Mg^{2+} ions. The protein mutant that could not bind ATP was inactive in this reaction. Since the action of human ORC4 could be biologically important, its potential impact on DNA replication is discussed.

Almost each step of DNA metabolism requires local or global remodeling of cellular DNA induced by specific proteins. At initiation, this is achieved by initiators, members of the specific subgroup of the superfamily of ATPases associated with diverse cellular activities (AAA+ superfamily) (*1–4*). AAA+ proteins usually function as oligomeric, nucleotide-dependent molecular chaperones that couple the energy of ATP binding and hydrolysis to a change of various cellular substrates (*5*). The versatility of AAA+ initiators is reflected in their AAA+ domains which participate in a variety of events, from protein oligomerization to DNA binding and remodeling (*1–4*). With the aim of further exploring the manners in which these proteins affect origin DNA, we have chosen human ORC4, a typical AAA+ protein essentially involved in the assembly and maintenance of the human origin recognition complex (ORC)[1] (*6–8*).

Human ORC4 exhibits sequence-unspecific DNA binding activity (*9*). The protein prefers A+T rich origin or synthetic DNA sequences and binds to them in a cooperative manner. In addition, the protein prefers long alternating or homogeneous AT copolymers rather than double-stranded oligonucleotides of the same composition, and some origin fragments rather than the others, despite similarities in AT content and size. Given the fact that human ORC4 interacts with origin sequences prone to structural alterations, we were interested in testing whether it could function as a DNA restructuring agent. Our initial experiments were stimulated by the example of HMGB1, which was reported to promote noncanonical renaturation of certain tandemly repeated DNA sequences (*10, 11*). In a manner similar to that with HMGB1, we have followed human ORC4-mediated renaturation of the chosen origin fragments. We have also tested different combinations of complementary single-stranded oligonucleotides and registered noncanonical structures forming in the presence of human ORC4. Here we report that human ORC4 promotes origin restructuring and stimulates formation of noncanonical oligonucleotide structures. We also describe novel DNA structures which exclusively form in the presence of human ORC4 and discuss the potential importance of these structures in DNA replication.

EXPERIMENTAL PROCEDURES

Expression and Purification of Wild-Type and Mutant Human ORC4. Recombinant human ORC4 was expressed in *Escherichia coli* and purified over metal affinity resin as essentially described previously (*9*). To improve the yield of the active protein, treatment of bacterial lysates with DNase I was also included in the purification procedure. To remove insoluble aggregates, the protein was repurified by glycerol gradient centrifugation. Centrifugation was carried out in 10 to 30% glycerol gradients prepared in buffer A [20 mM HEPES (pH 7.9), 30 mM NaCl, 2 mM $ZnCl_2$, 6 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF]. Gradients were centrifuged in an SW 41 Beckman rotor at 38000 rpm, for 20 h at 8 °C. Gradient fractions were collected from the bottom and analyzed using an Agilent 2100 Bioanalyzer and a Protein 200 Plus LabChip kit, in combination with Protein 200 Plus assay software. Glycerol gradient fractions containing human ORC4 were

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[1] Abbreviations: ORC, origin recognition complex; HMGB1, high-mobility group protein B1; SSB, single-stranded DNA binding protein; TBM, Tris, boric acid, and magnesium-containing buffer.

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pooled and kept at −80 °C. The same protocol was used for purification of the wild type and the mutant protein. Site-directed mutagenesis of lysine to alanine in the GKT motif of human ORC4 was carried out by PCR amplification of the pQE-30 plasmid (Qiagen, Valencia, CA), containing a fragment encoding full-length human protein (1311 bp), with mutagenic primer CCCCGAGGATCAGGAGCAACTATGTTAATAAG and using the QuikChange multi site-directed mutagenesis kit (Stratagene, La Jolla, CA). PCR products were treated with *Dpn*I to digest the parental DNA template and used to transform M15 (pREP4)-competent cells.

DNA Binding Assays. Glycerol gradient pools were tested for DNA binding activity using a streptavidin pull-down assay. Double-stranded copolymers poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC) were tailed, using terminal transferase and dCTP substrate, and attached to streptavidin-coated paramagnetic beads via unilaterally biotinylated dG_{30}. Protein binding and washing were performed as described previously, and the bound protein was stripped from the beads by DNase I digestion (*9*). Stripped samples were separated by SDS−PAGE and visualized by silver staining. Binding reactions with triple-stranded oligonucleotides were performed in 25 μL of buffer A1 (the same as buffer A, but with 20 mM $MgCl_2$), with 8−80 fmol of the end-labeled oligonucleotide and with 250 ng to 1 μg of human ORC4. An EMSA of multistranded oligonucleotides was performed in TBM−PAGE (5% polyacrylamide gels prepared and run in 89 mM Tris base, 89 mM boric acid, and 2 mM $MgCl_2$) gels.

Treatment of lbo I and 7-Deaza-dAMP lbo I with OsO_4-Pyridine or DEPC. DNA fragment lbo I was amplified from the human genomic DNA using the CAAAAACGGAGCTGGGCTGCAGCTG and GACATCCGCTTCATTAGGGCAGAGGCC primer pair. 7-Deaza-dAMP lbo I was synthesized in the same manner, but using 7-deaza-dATP (Jena Bioscience) instead of dATP. In each reaction, only one primer was 5′-end-labeled. Lower or upper labeled strand-containing fragments were electrophoretically purified and precipitated. The purity and integrity of isolated DNA were checked by denaturing PAGE and their sequences by Maxam−Gilbert sequencing. Chemical reactions were performed as essentially described previously (*12, 13*); 100−125 fmol of each labeled fragment was dissolved in buffer A which was supplemented with 10% glycerol and 10 μg of poly(dI-dC)·poly(dI-dC). For lower strand modification, treatment was performed for 15 min at room temperature, in a total volume of 110 μL with 10 μL of DEPC (diethyl pyrocarbonate) added to reaction mixtures. For upper strand modifications, binding mixtures (24 μL) were supplied with pyridine (2 μL per reaction) and treated with 30 μL of a 2% solution of OsO_4, for 25 min on ice. After incubation reaction mixtures had been precipitated, dry pellets were resuspended in piperidine (150 μL) and incubated for 30 min at 90 °C. DNA fragments were extracted with *n*-butanol and reprecipitated. Reaction products were analyzed with 6% denaturing gels.

Protein-Mediated Renaturation of Origin DNA and Protein-Mediated Conversion of Single-Stranded Oligonucleotides into Multistranded Structures. End-labeled origin DNA was dissolved in water (1 fmol/μL) and incubated for 10 min at 95 °C. Three to four microliters of the DNA solution was quickly diluted into 50 μL of buffer A2 (the same as buffer A, but with 24 mM $MgCl_2$) which was preincubated and kept at 37 °C, with or without 1−500 ng of human ORC4. Where indicated, human ORC4 was replaced with either BSA (Sigma) or SSB (USB). Incubations were carried out for different periods of time, using a thermomixer at 37 °C and 600 rpm. At the end of the incubation, reaction mixtures were quickly cooled on ice, adjusted to 1 M NaCl and 1% SDS, and deproteinized with chloroform and isoamyl alcohol. Alternatively, the protein was digested with 1−5 μg of proteinase K dissolved in 0.1−0.5% SDS. Protein digestion was carried out for 15 min at 37 °C. Reaction products were analyzed in 5 or 8% TBM gels. TBM−PAGE was performed in the cold room. For protein-mediated conversion of single-stranded oligonucleotides into multistranded structures, end-labeled single-stranded oligonucleotides (1−4 fmol) were dissolved in buffer A1 and incubated for 15 min at 37 °C and 600 rpm, with or without 1−500 ng of human ORC4. The typical reaction volume was 25 μL. Where indicated, single-stranded oligonucleotides were incubated in the presence of excessive amounts of corresponding complementary strands. Deproteinized samples were analyzed by TBM−PAGE, followed by autoradiography. Chosen autoradiographic results were quantified using a Cyclone Storage Phosphor System (Perkin-Elmer) in combination with OptiQuant.

7-Deaza-dAMP lbo I Tailing. Gel-purified 7-deaza-dAMP lbo I was tailed using either dATP or 7-deaza-dATP. The tailing reaction was performed with terminal deoxynucleotidyl transferase (Fementas Life Sciences), as essentially described by the manufacturer.

RESULTS

Human ORC4 Stimulates Origin Restructuring. Human recombinant ORC4 was expressed in *E. coli* and purified as described previously (*9*). The protein was repurified by glycerol gradient centrifugation (Figure S1 of the Supporting Information), and its DNA binding properties were checked by streptavidin pull-down assays. This analysis revealed a single DNA-binding entity contained in human ORC4 (Figure S2 of the Supporting Information). Consistent with our previous results, human ORC4 predominantly recognized A+T rich DNA. Glycerol gradient fractions containing the purified protein were further tested for origin restructuring. In restructuring reactions, the substrates were A+T rich DNA fragments lbo I and *Sph*I−*Eco*RV, functionally essential and mutually interchangeable elements of respective lamin B2 and DHFR origins of replication (*14–17*). By several biochemical methods, both fragments were previously shown to harbor unorthodox structural elements, existing at neutral pH and in a buffer containing Mg^{2+} ions (ref *18* and unpublished work of J. Kusic). The same buffer was used for protein-mediated renaturation, and the products were analyzed by TBM−PAGE. End-labeled lbo I was first heat denatured and then quickly diluted into a buffer which was preincubated and kept at 37 °C. Upon addition of denatured DNA, reaction mixtures were further incubated at 37 °C, then quickly cooled on ice, and analyzed by TBM−PAGE performed at 4 °C. As presented in Figure 1A (lanes 2−4), only a very tiny fraction of lbo I renatured after being incubated for 30 min at 37 °C. In a parallel experiment, heat-denatured lbo I was transferred to reaction mixtures containing human ORC4, preincubated, and kept at 37 °C. In the

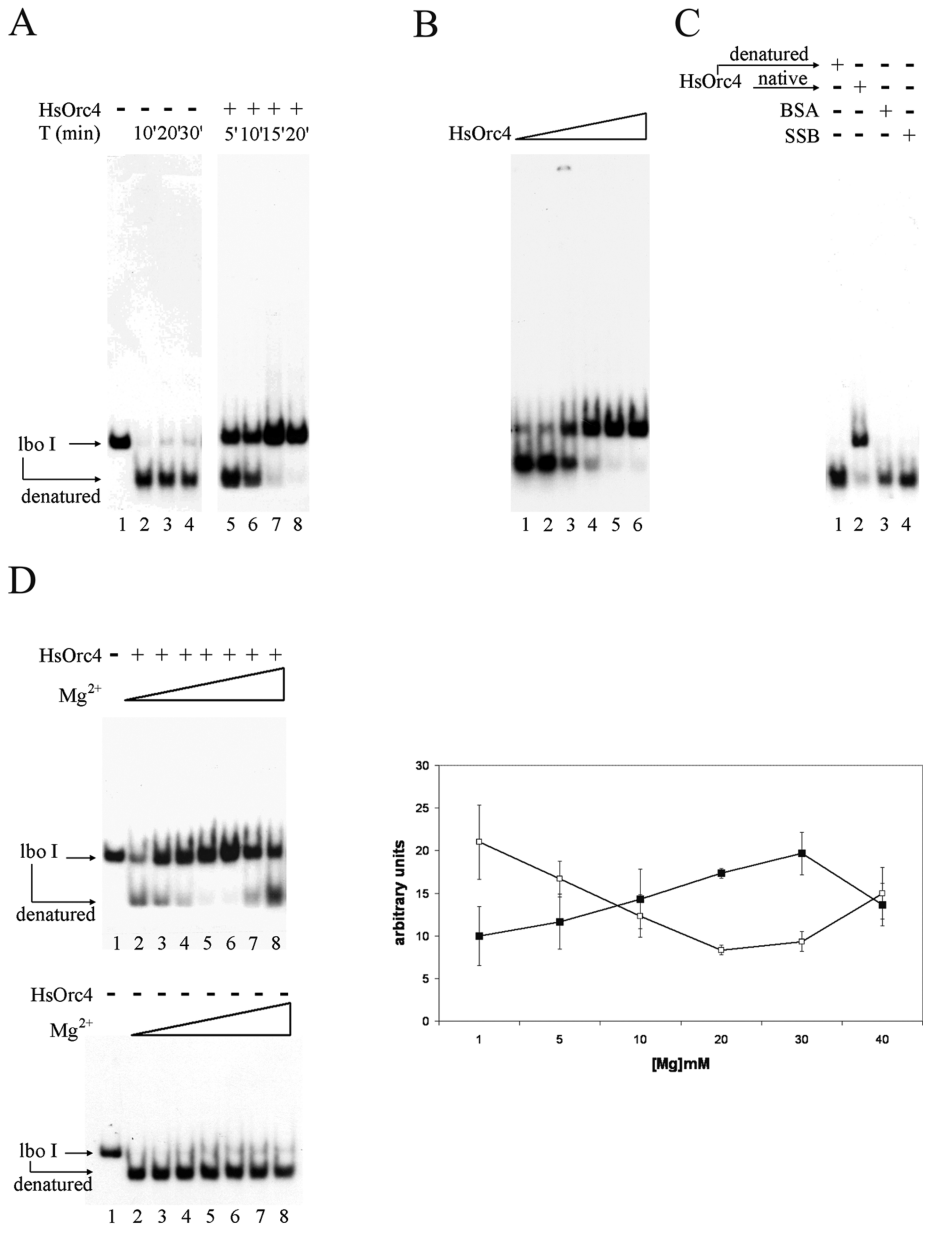

FIGURE 1: Human ORC4 stimulates reassociation of the complementary origin strands. (A) End-labeled lbo I (lane 1) was heat denatured (lanes 2−8) and incubated at 37 °C without protein (lanes 2−4) or with 150 ng of human ORC4 (lanes 5−8). Incubation periods are indicated in the figure. (B) Denatured lbo I (lanes 1−6) was incubated for 15 min at 37 °C with 25 (lane 1), 50 (lane 2), 100 (lane 3), 150 (lane 4), 250 (lane 5), and 500 ng (lane 6) of human ORC4. (C) Denatured lbo I (lanes 1−4) was incubated with identical amounts (200 ng) of heat-inactivated human ORC4 (lane 1), native human ORC4 (lane 2), BSA (lane 3), and SSB (lane 4). (D) In the top left panel, lbo I (lane 1) was heat denatured (lanes 2−8) and incubated with 150 ng of human ORC4 (lanes 2−8) in the presence of increasing concentrations of Mg^{2+} (lanes 2−8). The bottom left panel was the same as the top, but without human ORC4. In the right panel, human ORC4-mediated origin renaturation was quantified and plotted as a function of Mg^{2+} concentration. Double-stranded (■) and single-stranded (□) DNA from three different experiments.

same manner as without protein, reaction mixtures were incubated for different periods of time at 37 °C and then cooled on ice. Reaction products were deproteinized and precipitated (Figure 1A, lanes 5−8). Alternatively, complementary strands were first incubated with human ORC4 for 15 min at 37 °C, and the protein was then destroyed via addition of proteinase K and incubation of reaction mixtures for an additional 15 min (Figure 1B−D). Reaction products were cooled on ice and analyzed as described before.

In all reaction mixtures containing human ORC4, lbo I renatured in minutes (Figure 1A, lanes 5−8, and Figure 1B, lanes 1−6). The amount of DNA product increased when the length of incubation with the protein (Figure 1A, lanes 5−8) or its amount in renaturing reactions (Figure 1B) was increased. The structural integrity of human ORC4 was crucial for its activity which disappeared after heat denaturation of the protein (Figure 1C, lanes 1 and 2). Other tested proteins did not affect renaturation of lbo I, as exemplified by BSA and SSB, used as controls (Figure 1C, lanes 3 and 4). Similar results were also obtained with DHFR fragment *Sph*I−*Eco*RV (not shown). In reaction mixtures containing human ORC4, the amount of DNA product was also dependent on Mg^{2+} ions (Figure 1D, top left panel, lanes 2−8) and showed a broad maximum at Mg^{2+} concentrations between 20 and 30 mM (Figure 1D, right panel). In reaction mixtures with no protein added, variations in the concentration of Mg^{2+} ions did not have a significant effect (Figure 1D, bottom left panel, lanes 2−8).

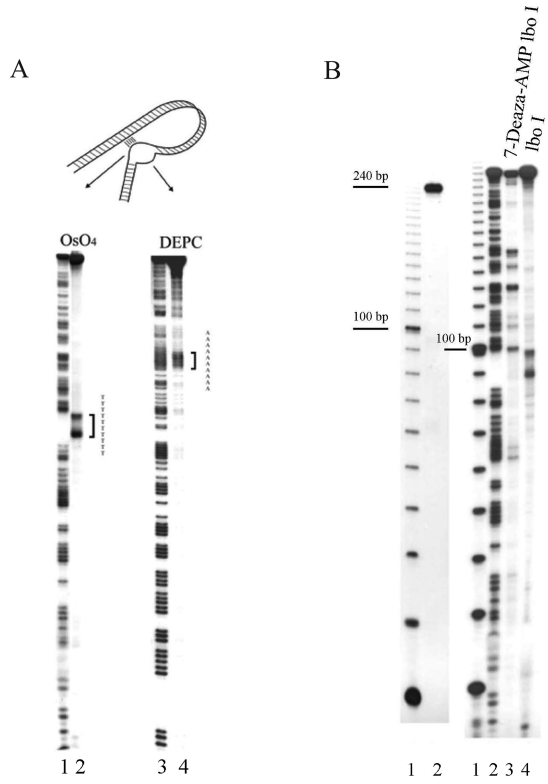

FIGURE 2: Intramolecular *T*·A·T triplex, the product of human ORC4-mediated renaturation of lbo I. (A) Schematic representation of the *T*·A·T triplex detected in lbo I (top) and Maxam and Gilbert G+A sequencing reactions of respective upper and lower strands of lbo I (lanes 1 and 3) and OsO_4-pyridine cleavage pattern of the upper (lane 2) and DEPC cleavage pattern of the lower strand (lane 4) of lbo I (bottom). Complementary strands of the bubble are denoted with brackets. (B) Ten-base pair ladder (lane 1) and 7-deaza-dAMP lbo I analyzed intact (lane 2) in the left panel or after OsO_4-pyridine cleavage in the right panel (lane 3). Lane 1 in the right panel represents the 10 bp ladder, whereas lanes 2 and 4 represent respective G+A sequencing and OsO_4-pyridine cleavage reactions, respectively, of the control lbo I.

Human ORC4 Stimulates Formation of Intra- and Intermolecular Triplexes. Origin fragment lbo I was previously shown to assume a noncanonical form, consistent with intramolecular, partly triple-stranded structure. As initially detected by a combination of chemical agents specific for either single- or double-stranded DNA and with antibody that interacted with triple-stranded DNA, lbo I triplex was characterized by the bubblelike AT element (*18*) (Figure 2A, schematic representation). Since the bubble donated its thymine strand for intramolecular interaction with complementary double-stranded sequence, a characteristic cleavage pattern with single-strand-specific agents OsO_4-pyridine and DEPC reflected its predominantly single-stranded nature and partial protection of its thymines by a *T*·A·T structure. To probe the structure of the product of human ORC4-mediated renaturation, the corresponding band was isolated from the gel and treated with OsO_4-pyridine and DEPC (*1*). The cleavage pattern of lbo I, which was renatured in the presence of human ORC4, confirmed that the protein helped create previously identified noncanonical structure (Figure 2A).

Triple-helical nucleic acids always have a central purine strand simultaneously engaged in Watson−Crick and Hoogsteen hydrogen bonding. Interactions that hold the duplex together involve Watson−Crick hydrogen bonding surfaces, whereas Hoogsteen bonds attach a third strand to a preexisting duplex. In lbo I, Hoogsteen bonds are expected to prevent reassociation of thymine and adenine strands of the bubble and to keep it open. This could be tested by substituting 7-deaza-dATP for dATP and by probing lbo I structure with OsO_4-pyridine. Since 7-deaza-dATP can form Watson−Crick but not Hoogsteen bonds, substitution is expected to change the sensitivity of the bubble thymines to OsO_4-pyridine.

To confirm the importance of Hoogsteen bonds, the lbo I fragment was prepared with 7-deaza-dATP instead of dATP. The DNA fragment was isolated via 10% TBE−PAGE and, after its size and integrity had been checked by denaturing PAGE (Figure 2B, left panel), subjected to chemical cleavage. The control lbo I fragment was prepared in the same manner, but with dATP. As presented in Figure 2B (right panel), the cleavage pattern of 7-deaza-dAMP lbo I exhibited a dramatic decrease in OsO_4-pyridine sensitivity within the bubble area, thus confirming that Hoogsteen interactions keep the bubble open. Several new signals appearing in double-stranded portions of lbo I indicated that bubble interactions also stabilized the other parts of the molecule. To stimulate noncanonical renaturation of the separated origin strands, human ORC4 could interact with triplex-forming DNA elements and/or deform the overall structure of renaturing DNA strands in a manner favorable for triplex formation. To single out possible targets of human ORC4, its DNA binding and restructuring potential was also tested with simple intermolecular structures, like to the triple-stranded portion of lbo I.

Intermolecular *T*·A·T triplexes form upon incubation of single-stranded oligoadenine with excessive amounts of oligothymine, at neutral pH and in the presence of Mg^{2+} ions (*19*). To determine whether human ORC4 could also affect formation of this structure, end-labeled dA_{34} was incubated with increasing amounts of unlabeled dT_{34}, without or with human ORC4. Incubation was carried out for 15 min at 37 °C in a buffer containing 20 mM Mg^{2+}. At the end of the incubation, the protein was destroyed with proteinase K. Reaction products were analyzed via 8% TBM−PAGE.

As presented in Figure 3A, addition of increasing amounts of dT_{34} first resulted in formation of AT duplexes (Figure 3A, lane 2) and then intermolecular *T*·A·T triplexes (Figure 3A, lanes 3−7). Oligomers were converted into triplexes at lower dT_{34}:dA_{34} ratios with human ORC4 than without it (Figure 3A, top and bottom panels). To determine whether the protein stably interacted with triplexes, the same oligonucleotide substrates were incubated with human ORC4, but only the control samples were treated with proteinase K. After all the samples had been subjected to an EMSA in TBM gels, the smeared pattern of bound DNA was detected in non-deproteinated samples (Figure 3B). As judged by the three relatively discrete but weak protein−DNA complexes which were hidden in the smear, only a small fraction of DNA exhibited stable association with human ORC4, whereas the bulk of reaction products remained protein-free. In combination, experiments presented in Figure 3 demonstrated that human ORC4 stimulated formation of the intermolecular *T*·A·T triplex in a manner which did not require stable protein−DNA interaction.

Human ORC4 Mediates Self-Association of Homoadenine Oligonucleotides. To further investigate the action of human ORC4 in restructuring reactions, we have tested a variety of oligonucleotide substrates and we have observed that ho-

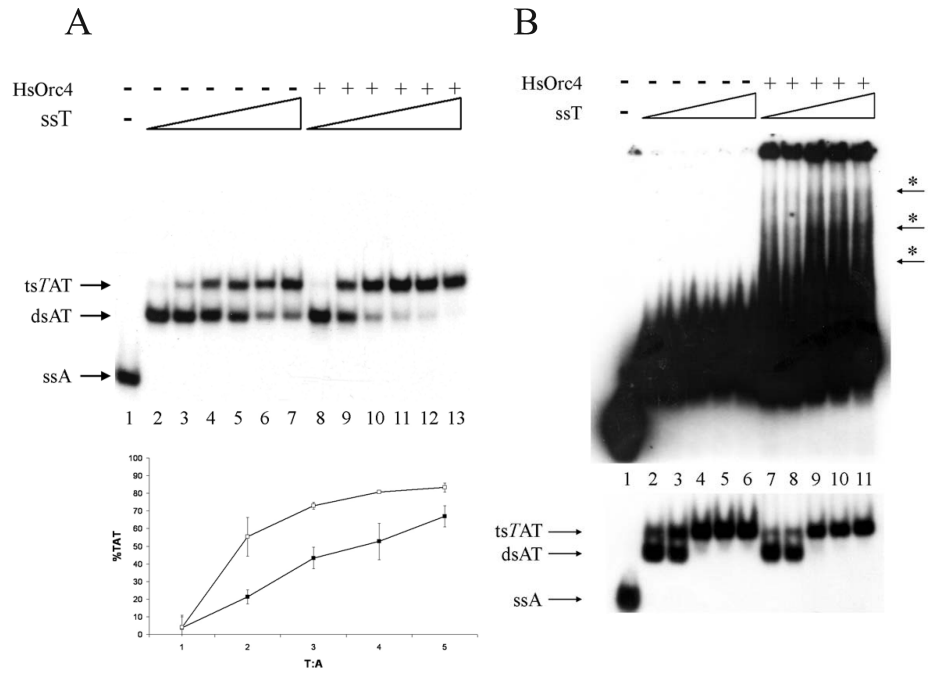

FIGURE 3: Formation of the intermolecular $T\cdot A\cdot T$ triplex is stimulated by human ORC4. (A) The top panel shows end-labeled dA_{34} (lanes 1−13) incubated with equal (lanes 2 and 8), doubled (lanes 3 and 9), tripled (lanes 4 and 10), quadrupled (lanes 5 and 11), quintupled (lanes 6 and 12), and sextupled (lanes 7 and 13) amounts of unlabeled dT_{34} (lanes 2−7 and 8−13), without (lanes 2−7) or with (lanes 8−13) 150 ng of human ORC4. The bottom panel shows the amounts of $T\cdot A\cdot T$ from three separate experiments performed without (■) or with (□) human ORC4 quantified and plotted as a function of T:A ratio. (B) End-labeled dA_{34} (lanes 1−11) was incubated with 2-fold (lanes 2 and 7), 3-fold (lanes 3 and 8), 6-fold (lanes 4 and 9), 12-fold (lanes 5 and 10), and 25-fold (lanes 6 and 11) excesses of unlabeled dT_{34} (lanes 2−6 and 7−11) in the presence of 500 ng of human ORC4. Control samples (lanes 2−6) were digested with proteinase K. Protein−DNA complexes present in undigested samples are denoted with asterisks (top panel). The structure of free DNA is made clear by shorter exposition of the same gel (bottom panel). Triple-, double-, and single-stranded DNAs are denoted with arrows.

moadenine oligonucleotides most clearly exemplified different aspects of human ORC4's action. Unlike the $T\cdot A\cdot T$ triplex-forming oligonucleotides that were stimulated by the protein to adopt a multistranded form but, under appropriate conditions, could undergo structural conversion even without it, homoadenine restructuring occurred only in the presence of human ORC4. Upon incubation with increasing amounts of the protein and after its destruction by proteinase K, dA_{34} or A_{40} was converted into larger products (Figure 4A). When separated by electrophoresis and compared with single-stranded (Figure 4A, lanes 1 and 4), double-stranded (Figure 4A, lane 2), and triple-stranded (Figure 4A, lane 3) molecules composed of adenines and thymines, these products corresponded by size to homoduplexes and possibly homoquadruplexes (Figure 4A, lanes 5−11). Protein-mediated structural conversion required Mg^{2+} ions and was inhibited with 20 mM EDTA added to reaction mixtures at the beginning of the incubation with the protein (Figure 4B,C). The concentration of Mg^{2+} necessary for maximum activity was 15−20 mM, whereas a further increase, from 20 to 50 mM, did not affect the amount of product formed. Homoadenine structures were also characterized by an unusual thermal stability. In reaction mixtures presented in Figure 4D, single-stranded homoadenine oligonucleotides were incubated with equal amounts of human ORC4 which was then destroyed by proteinase K. After protein destruction, reaction products were additionally incubated for 10 min at temperatures ranging from 37 to 95 °C and analyzed as described previously. The bulk of the products remained stable even at 95 °C. From a comparison of numerous experiments similar to those presented in panels A and C of Figure 4, it appeared that single-stranded oligoadenine first formed duplexes and then duplexes interacted to form the structures corresponding to quadruplexes and occasionally even larger. Upon prolonged incubation at high temperatures, these structures slowly disintegrated, first into smaller structures and then into single-stranded molecules. This indicated that duplex building blocks could form multistranded molecules.

In the next step, we have investigated the role of nucleotide binding in the reactions described above. Since human ORC4 belongs to the AAA+ family of adenosine triphosphatases, one could expect that ATP regulates its function. To test this possibility, we have mutated lysine in the GKT consensus sequence of the protein's Walker A motif to alanine. Mutant and wild-type protein were expressed and purified in the same manner and, after repurification by glycerol gradient centrifugation, tested with homoadenine oligonucleotide substrates. As presented in Figure 4E, mutation designed to block nucleotide binding by human ORC4 completely abolished the protein's activity in the restructuring reaction.

Self-Association of Homoadenine Oligonucleotides Requires Hoogsteen Bonding. Assuming that in the structures presented in Figure 4 single-stranded homoadenines interact through hydrogen bonds, we have also tested whether they use Watson−Crick or Hoogsteen hydrogen bonding surfaces. For that purpose, we have tailed 7-deaza-dAMP lbo I with terminal transferase using either dATP or 7-deaza-dATP as a substrate. Since 7-deaza-dAMP lbo I cannot form Hoogsteen bonds involving adenines and has Watson−Crick hydrogen bonding surfaces already engaged in a normal duplex, it can react through only single-stranded tails. If this reaction involves Hoogsteen bonding, 7-deaza-dAMP lbo I

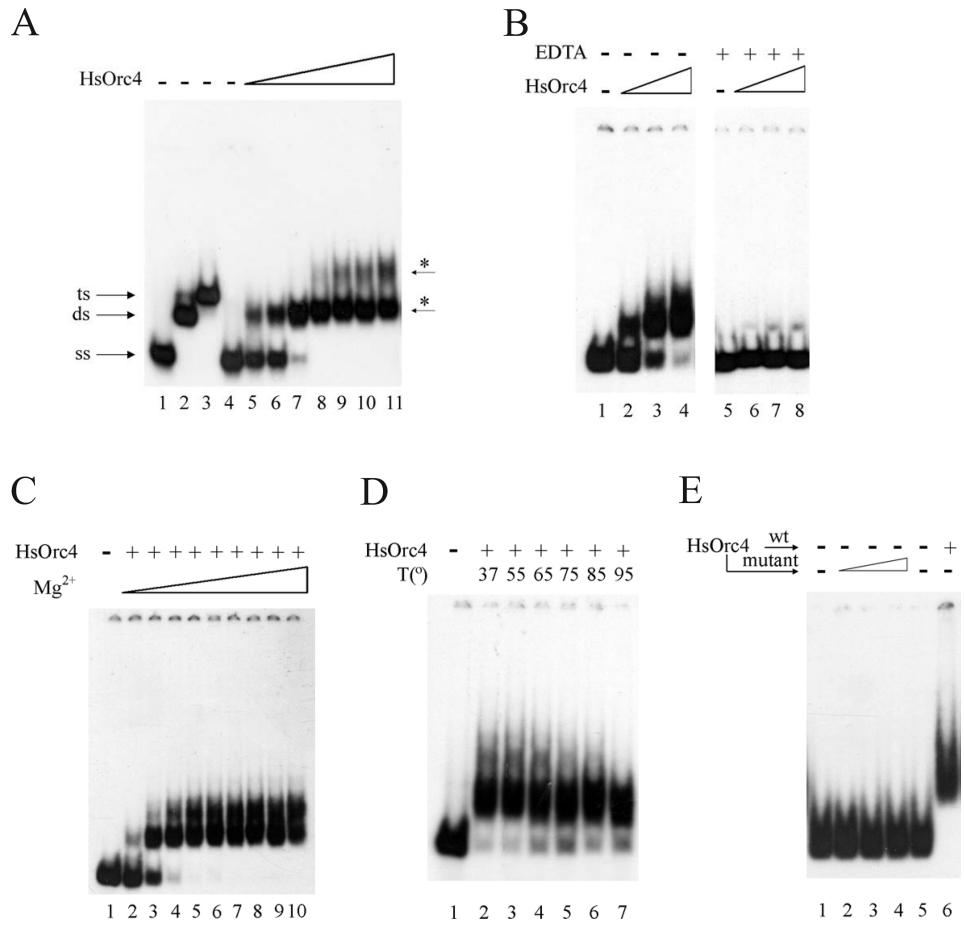

FIGURE 4: Human ORC4, but not its ATP-binding mutant, mediates A·A pairing. (A) End-labeled dA_{34} incubated without protein (lane 4) or with 25 (lane 5), 50 (lane 6), 100 (lane 7), 150 (lane 8), 200 (lane 9), 250 (lane 10), and 500 ng (lane 11) of human ORC4. Respective single-stranded (A), double-stranded (AT), and triple-stranded (*T*AT) 34-mers are presented in lanes 1−3, respectively, as size markers. Homoadenine structures are denoted with asterisks. (B) End-labeled dA_{34} (lanes 1−8) incubated with 50 (lanes 2 and 6), 100 (lanes 3 and 7), and 150 ng of human ORC4 (lanes 4 and 8), without (lanes 1−4) or with 20 mM EDTA (lanes 5−8). (C) End-labeled dA_{34} (lanes 1−10) incubated with 75 ng of human ORC4 (lanes 2−10) in the presence of 1, 5, 10, 15, 20, 25, 30, 40, and 50 mM Mg^{2+}, respectively. (D) End-labeled dA_{34} (lanes 1−7) was treated with 100 ng of human ORC4 (lanes 2−7), and the products were additionally incubated at 37 (lane 2), 55 (lane 3), 65 (lane 4), 75 (lane 5), 85 (lane 6), and 95 °C (lane 7). (E) End-labeled dA_{34} (lanes 1−6) incubated without protein (lanes 1 and 5), with 50 (lane 2), 100 (lane 3), and 150 ng (lane 4) of mutant human ORC4, or with 150 ng of wild-type human ORC4 (lane 6).

could react through tails composed of dATP, but not through 7-deaza-dATP tails.

For restructuring reactions, tailed substrates were isolated via 10% TBE−PAGE and the size of their tails was checked by denaturing PAGE. Isolated substrates were incubated with human ORC4 and analyzed as described previously. As presented in Figure 5, the substrate tailed with dATP formed larger structures, whereas the other, tailed with 7-deaza-dATP, remained unchanged. Nucleic acid substrates presented in Figure 5 had single-stranded tails containing approximately 50−90 nucleotides of dAMP or 90−140 nucleotides of 7-deaza-dAMP. In other experiments, dATP-tailed substrates having tails in the range from several dozens to one hundred or more nucleotides could also be converted into larger structures, whereas 7-deaza-dATP-tailed substrates could not form larger structures regardless of their length. As with single-stranded oligonucleotides, reaction with tailed substrates and wild-type protein required Mg^{2+} ions and was inhibited by EDTA. In addition, the GKT mutant protein was completely inactive in this restructuring reaction (Figure 5, lanes 5 and 10).

Although previously undetected in duplexes, A·A pairing occurs in noncanonical structures such as the *A*·A·T triplex. In this structure, the A·T duplex and homoadenine third strand interact in a reverse Hoogsteen hydrogen bonding scheme. As already described, under our experimental conditions, this structure did not form from complementary single-stranded oligonucleotides. To determine whether it could form from different constituents, we have first preformed unlabeled A·A duplexes and then added end-labeled single-stranded homothymine oligonucleotides to this reaction mixture. Incubation with a potential third-strand-forming sequence was carried out with or without human ORC4, but *A*·A·T triplexes were not detected. Therefore, although we cannot exclude the possibility that adenines forming A·A duplex interact in a reverse Hoogsteen hydrogen bonding scheme as in the *A*·A·T triplex, our current results do not support this conclusion.

DISCUSSION

Summarized, our experiments demonstrate that human ORC4 acts on single-stranded origin DNA or oligonucleotides and converts them into noncanonical structures. Structural alterations do not seem to be a consequence of the stable protein−DNA interactions but occur in the process

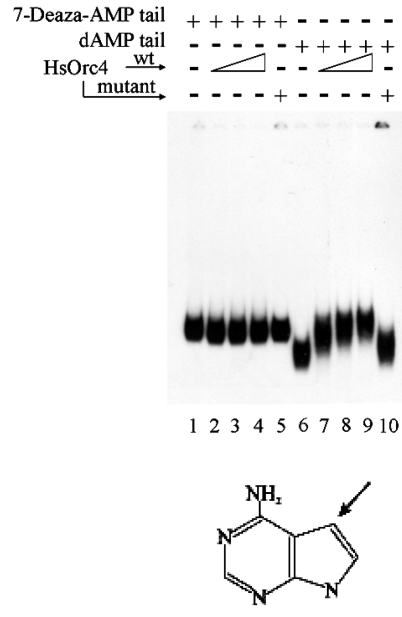

FIGURE 5: A•A pairing requires Hoogsteen hydrogen bonding surfaces. 7-Deaza-dAMP lbo I tailed with either 7-deaza-dATP (lanes 1−5) or dATP (lanes 6−10) incubated with 100 (lanes 2 and 7), 150 (lanes 3 and 8), and 200 ng (lanes 4 and 9) of human ORC4 or with 150 ng of mutant human ORC4 (lanes 5 and 10). In the bottom panel, an arrow indicates a 7-deaza-dATP surface that cannot hydrogen bond.

which requires human ORC4. This is a novel mode of DNA manipulation by a protein, currently best represented by human ORC4's action toward homoadenine oligonucleotides. Only in the presence of this protein do adenine nucleotides pair to form duplexes and even larger structures. Adenines pair through Hoogsteen hydrogen bonding surfaces, and the products of this reaction are characterized by increased thermal stability. Consistent with the canonical mode of action of AAA+ proteins, this reaction requires Mg^{2+} ions and is regulated by nucleotide binding to human ORC4.

In addition to the large body of evidence demonstrating that guanine rich nucleic acids can self-associate (*20* and the references cited therein), here we show that, under specific conditions, even adenines could share the same property. While potential G4-forming elements usually occur in telomeric and promoter regions, possible natural substrates of human ORC4 occur in DNA replication origins. Origin regions that could be involved in the protein-mediated restructuring comprise multiple pairs of short homothymine/homoadenine sequences relating to each other as either mirror or glide reflection images (*18*). Stimulated by human ORC4, such sequences could build *T*•A•T triplexes and thus displace stretches of single-stranded adenines prone to alternative pairing (or vice versa). The overall origin structure resulting from these rather simple interactions could be a complex composition of multistranded and looped elements. Given that human ORC4 could stimulate pairing rearrangements of origins only if it gets a chance to act on single-stranded DNA, its action would require unwinding and transient breaking of interacting origin strands promoted by negative supercoiling and by topoisomerase action. In that respect, it is interesting to note that DmORC and human ORC4 efficiently interact with negatively supercoiled DNA (ref *21* and unpublished work of J. Kusic and B. Tomic) and that mutations of the lbo I bubble area change the sensitivity of LMNB2 origin to Topo I (*22*).

If the complex origin structures could form in living cells, the structures are expected to be an instrument of ORC's action. Initiators from different domains of life, ORC included, share similar architecture and mechanistic properties at initiation but differ in details such as the specificity of origin recognition. Recently reported crystal structures of archaeal initiator−origin complexes emphasize the importance of DNA structural features in species in which origin recognition occurs in a sequence-unspecific manner (*3, 4*). The mechanism via which ORC recognizes DNA structural elements and shapes them to fit into it would complement the mechanism in which Cdc6 discriminates between origin and nonorigin DNA and, by means of its ATPase activity, disassembles complexes formed on wrong sequences (*23*). In this scenario, human ORC4 could, for instance, pair appropriate stretches of adenines to expose stretches of single-stranded thymines which activate MCM helicases on bubbled substrates (*24*). Additionally, complex origin architecture could provide correct positioning and orientation of replisomes. Since DNA synthesis occurs on fixed replisomes (*25*), initiator-induced duplex melting cannot be sufficient to accommodate opposing movements of the leading and lagging strand polymerases. This problem could be solved by the lagging strand looping, as proposed by the trombone and butterfly models of DNA replication (*26, 27*), and the loops could be created by DNA−DNA interactions catalyzed by the initiator. Alternatively, the ability of human ORC4 to mediate DNA−DNA interactions may be important for other ORC functions, such as cohesin-independent sister-chromatid cohesion (*28*). In a broader prospective, the example of human ORC4 is interesting, because it shows that proteins could expand the structural repertoire of cellular genomes by producing energetically unfavorable DNA structures.

SUPPORTING INFORMATION AVAILABLE

Gel-like image of purified human ORC4 analyzed with the Agilent 2100 Bioanalyzer (Protein 200 Plus assay) and results of streptavidin pull-down experiments performed with purified human ORC4 and poly(dA-dT)•poly(dA-dT) or poly(dC-dG)•poly(dC-dG) tailed and attached to streptavidin-coated magnetic beads. This material is available free of charge via the Internet at http://pubs.acs.org.

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