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RIP60, a Mammalian Origin-Binding Protein, Enhances DNA Bending near the Dihydrofolate Reductase Origin of Replication

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Replication of the Chinese hamster dihydrofolate (*dhfr*) gene initiates near a 281-bp *Hae*III fragment of stably bent DNA that binds RIP60, a 60-kDa origin-specific DNA-binding protein that has been purified from HeLa cell nuclear extract (L. Dailey, M. S. Caddle, N. Heintz, and N. H. Heintz, Mol. Cell. Biol. 10:6225–6235, 1990). Circular permutation assays showed that stable DNA bending in the *dhfr* origin region fragment was due to the presence of five oligo $(dA)_{3-4}$ tracts, designated bend elements B1 to B5, that are spaced 10 bp apart. DNA bending directed by elements B1 to B5, as assessed by anomolous migration of DNA fragments on polyacrylamide gels, was accentuated at 4°C. Bend element B5, which is in inverse orientation relative to elements B1 to B4, overlaps an ATT-rich motif that comprises the RIP60 protein-binding site. Gel mobility shift assays with circularly permuted bent DNA fragments and purified RIP60 showed that RIP60 markedly enhanced DNA bending of the *dhfr* origin region sequences. These results suggest that, as in many plasmids, bacteriophages, and eucaryotic viruses, mammalian DNA-binding proteins may enhance DNA bending near origins of replication during initiation of DNA synthesis.

Stably bent DNA has been implicated in nuclear organization, recombination, regulation of gene expression, and control of initiation of DNA synthesis (reviewed in references 37 and 38). First described for a restriction fragment derived from kinetoplast DNA of the trypanosome *Leishmania tarentolae* (27), bent DNA is characterized by abnormally slow migration on polyacrylamide gels (15, 27). Anomolous migration of bent DNA is most apparent when oligomeric A tracts, four to six residues in length, occur with a 10- to 11-base periodicity in phase with the twist of the DNA helix (23), and the bending center is located in the middle of the DNA fragment (43). Although several theoretical models for sequence-directed DNA bending have been proposed (4, 9, 39), as yet no single paradigm is able to account for the anomalous electrophoretic behavior of all naturally bent DNA sequences on polyacrylamide gels.

DNA bending, often enhanced by the binding of an initiator protein, has been implicated in the activation of a number of origins of replication. In plasmid pT181, the origin of replication contains a static DNA bend that is enhanced upon binding of the initiator protein RepC (22). Similarly, binding of the initiator protein to the origin of plasmid R6K induces DNA bending (28), as does binding of integration host factor to the origin sequences of plasmid pSC101 (35). In phage lambda, phased DNA bending is required for the functional interaction of the phage initiator O protein with reiterated origin sequences during initiation of DNA synthesis (44). Deletion mutagenesis and sequence substitution studies suggest that DNA bending in the simian virus 40 (SV40) A+T-rich domain is important for activation of the SV40 origin of replication (14). Although DNA bending in the A+T-rich domain of the SV40 origin is enhanced by a cellular protein called LOB, a role for LOB in SV40 DNA replication has not been established (5). Mutagenesis of the yeast origin of replication, the autonomously replicating sequence ARS1, suggests that DNA bending is an A+T-rich flanking sequence, domain B, may contribute to the efficiency of origin utilization (34, 42). Computer modeling of 11 origins of replication suggests that the relationship of bent and anti-bent DNA to initiator protein-binding sites may be a highly conserved feature of replication origins (12).

Bent DNA at origins of replication may serve multiple functions during initiation of chromatin replication. As a structural element of replication origins, bent DNA may promote directly helix disruption (31), foster the functional interaction of dispersed protein-binding sites (45), or serve to uniquely orient the topology of the DNA template for subsequent activation events (25). These functions need not be mutually exclusive.

To isolate sequences that function as origins of replication in mammalian chromosomes, we have previously investigated the replication of amplified dihydrofolate reductase (*dhfr*) gene domains in the methotrexate-resistant Chinese hamster cell line CHOC 400. Pulse-labeling studies in synchronized cells (18) or in nuclei isolated from synchronized cells (20) show that replication of the amplified domains commences within a series of restriction fragments, termed early-labeled fragments (ELFs), that map 3′ to the *dhfr* gene (19). Hybridization of replication intermediates formed during the onset of S phase to plasmids derived from the ELFs indicates that DNA synthesis initiates within a 4.3-kb *Xba*I fragment (6, 7). In-gel renaturation analysis of replication intermediates suggests that the *dhfr* gene origin is located within a 1.8-kb *Bam*HI-*Hin*dIII fragment contained entirely within the 4.3-kb *Xba*I fragment (26). This fragment also contains sequences homologous to those isolated in a origin-enriched DNA fraction (1).

Recently, we used the strand specificity of Okazaki fragment synthesis in the *dhfr* gene origin region to map the transition zone between leading- and lagging-strand DNA synthesis that demarcates origins of bidirectional DNA replication (OBR) (7a). The *dhfr* OBR of CHO and CHOC 400 cells is located within the 4.3-kb *Xba*I fragment immediately 5′ to a 450-bp region that contains a novel, highly conserved repetitive element (ORR-1) and a fragment of

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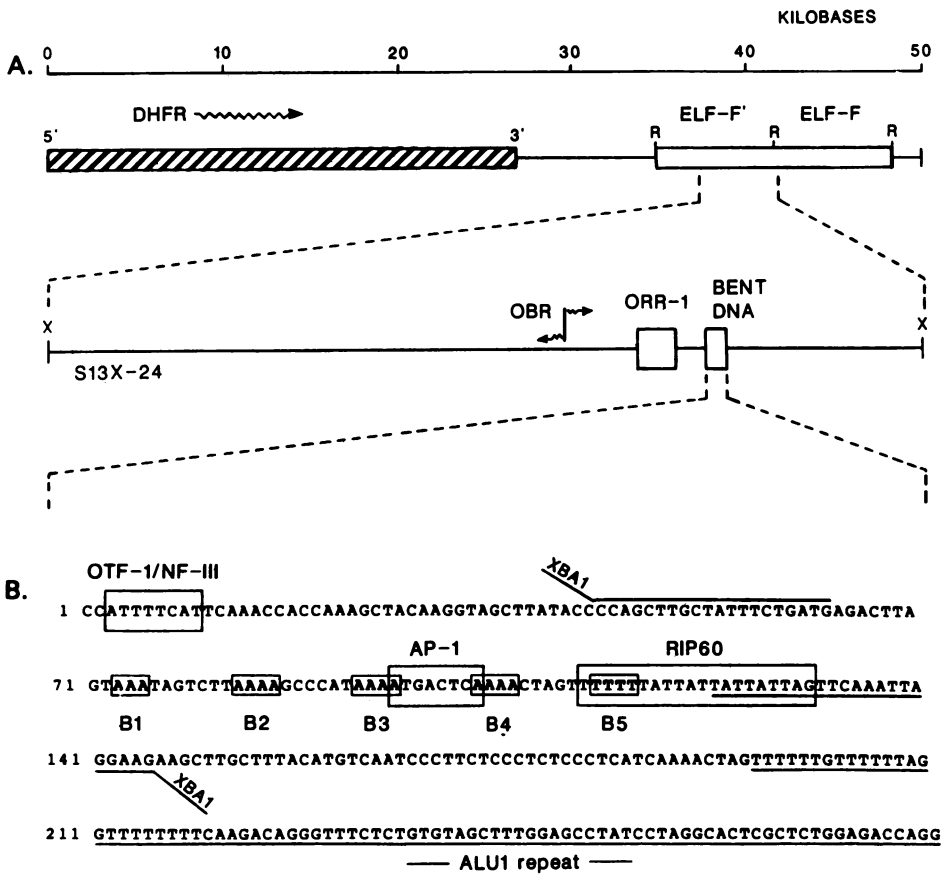

FIG. 1. Organization of the *dhfr* origin region. (A) The early-labeled *Eco*RI fragments ELF-F and ELF-F′ are shown relative to the transcribed sequences of the Chinese hamster *dhfr* gene (▨). Locations of the OBR defined by Okazaki fragment mapping studies, the highly conserved origin-related repeat (ORR-1), and the 281-bp *Hae*III bent DNA fragment within the 4.3-kb *Xba*I fragment that overlaps ELF-F and ELF-F′ are shown. (B) DNA sequence of the bent 281-bp *Hae*III fragment. The oligo(A) tracts B1 to B5 implicated in DNA bending are denoted by small open boxes. The binding sites for OTF1/NFIII and RIP60 and the consensus binding site for AP1 are denoted by large open boxes; see text for details concerning protein-DNA interactions at these sequences. The PCR primers used for subcloning a 102-bp segment of the *Hae*III fragment into the *Xba*I site of pBEND2 are indicated by solid lines with *Xba*I ends. The terminal 3′ 86 bp of the *Hae*III fragment are derived from an *Alu*I repeat as indicated.

stably bent DNA (7a, 8). The Okazaki fragment mapping experiments are in excellent agreement with all previous studies of initiation of DNA replication in the *dhfr* gene region (1, 6, 7, 16, 18–20, 26) with the exception of a study by Vaughn et al. (41a), who have interpreted two-dimensional (2D) gel replication mapping results as evidence that replication of the *dhfr* gene begins at random over a minimum of 28 kb of DNA.

The origin of replication associated with amplified *dhfr* genes of CHOC 400 cells is also active in the absence of gene amplification. Nucleosome condensation studies in CHO cells support the localization of the CHO *dhfr* gene replication origin to the ELF-F/ELF-F′ region (16), and stable transfection assays show that the ELF-F/ELF-F′ fragments contain all sequences necessary for the transfer of *dhfr* origin activity to new regions of the genome (7a, 16). Thus, the *dhfr* origin is a suitable model system for the study of initiation of DNA synthesis in mammalian cells.

Using the technique of Anderson (2), we previously identified a 281-bp *Hae*III fragment from the *dhfr* origin region (8) that displays the anomalous electrophoretic mobility in polyacrylamide gels that is the hallmark of bent DNA (15, 27). The 281-bp *Hae*III fragments maps approximately 450 bp 3′ to the *dhfr* OBR (Fig. 1). Analysis of protein-DNA interactions with the 281-bp *Hae*III fragment led to the identification of a novel 60-kDa protein, which we refer to as RIP60, that has been purified 9,000-fold from HeLa cell nuclear extract; RIP60 cofractionates with an ATP-dependent DNA helicase activity (10). RIP60 binds to the yeast origin of replication, ARS1, within domain B, a functionally important accessory sequence that also contains bent DNA (34). Gel shift competition assays show, however, that DNA bending alone is insufficient for binding of RIP60 (10).

In this study, we used circular permutation assays to identify the DNA sequence elements responsible for directing DNA bending within the 281-bp *Hae*III origin fragment. We show that binding of purified RIP60 to a site overlapping the 3′ end of the bent sequences markedly enhances DNA bending of origin region sequences. These results suggest that, as in many bacteriophages, plasmids, and viruses, eucaryotic proteins may enhance DNA bending at origins of replication during initiation of DNA synthesis.

MATERIALS AND METHODS

2D gel electrophoresis. The *dhfr* origin region was screened for bent DNA sequences by using the 2D gel assay described

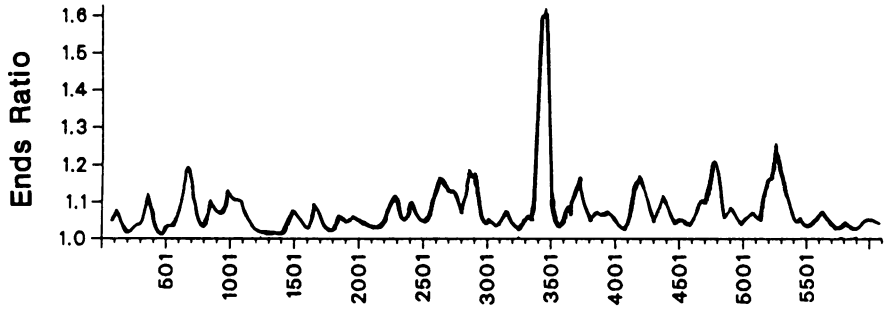

FIG. 2. Prediction by the ENDs ratio of DNA bending in the 281-bp *Hae*III fragment. The ENDs ratio (see text) was determined in 150-bp windows (with an 85% overlap between successive windows) throughout 6.2 kb of the origin region sequence reported by Caddle et al. (8). Pronounced DNA bending is predicted for the DNA sequences spanning nucleotides. 3301 to 3501. Bend elements B1 to B5 of the 281-bp *Hae*III fragment span nucleotides 3415 to 3459 of the origin region sequence.

by Anderson (2). A 281-bp *Hae*III fragment of plasmid S13X-24 (which contains the 4.3-kb *Xba*I origin fragment cloned into pUC13) that migrated 19% slower than expected in the second dimension was isolated, cloned, and sequenced as described by Caddle et al. (8). To examine the effect of temperature on migration of the 281-bp *Hae*III fragment, S13X-24 was digested with *Hae*III, and duplicate digests were resolved in the first dimension in a 2% agarose gel in TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA [pH 8.0]) electrophoresis buffer. Replicate gel slices were excised and reoriented by 90° across the top of duplicate 7% polyacrylamide gels. DNA fragments were resolved in the second dimension in one gel at 37°C and in the second at 4°C in TBE. Each gel was stained with ethidium bromide and photographed as described previously (8).

Circular permutation assays. To ascertain whether the putative bend elements B1 to B5 were responsible for bending of the 281-bp *Hae*III origin region fragment, a 102-bp fragment of DNA containing bend element B3 at its center and *Xba*I sites at the ends was generated by the polymerase chain reaction (PCR) (32) and cloned into the *Xba*I site of vector pBEND2 (47) to generate the derivative plasmid pMC-Bend. pMC-Bend was propagated in the *Escherichia coli* host strain DH5α; purified plasmid DNA was isolated by equilibrium sedimentation in cesium chloride and ethidium bromide as described previously (8). Plasmid DNA was digested with the indicated restriction endonucleases (Promega) and end labeled with [α-^{32}P]dATP and Klenow enzyme (13), and the resulting 228-bp circularly permuted fragments were resolved from the cloning vector on a 1% agarose gel. The labeled 228-bp fragments were isolated from the agarose gel by phenol-chloroform extraction and were then resolved along with end-labeled markers (Bethesda Research Laboratories) on a 10% polyacrylamide gel in TBE. Gel shift assays were performed with RIP60 purified from HeLa nuclear extract as previously described (10). Labeled DNA fragments and gel shift complexes were visualized by autoradiography.

Computer-aided sequence analysis of the *dhfr* origin region. DNA sequences were first analyzed for oligomeric A tracts with a 10-bp periodicity by scanning 6.2 kb of the *dhfr* origin region sequence (8) for the subsequence AAAAnnnnnn AAAA (where n is any nucleotide) with the software of Pustell and Kafatos (30). Determination of the ENDs ratio, which calculates the ratio of the contour length of a given axis of a DNA fragment to the shortest distance between its ends, by using the Olson wedge model was performed by M. Leffak and S. Kumar (Wright State University) as described previously (11).

RESULTS

Organization of the *dhfr* origin region. A restriction map of the early-replicating region located 3′ to the Chinese hamster *dhfr* gene is presented in Fig. 1A. The early-labeled *Eco*RI fragments ELF-F/ELF-F′ that span the *dhfr* gene origin of replication are denoted; the 4.3-kb *Xba*I fragment of plasmid S13X-24 overlaps ELF-F/ELF-F′ as shown. The 281-bp *Hae*III fragment of S13X-24 previously identified as containing bent DNA by the 2D gel technique of Anderson (2) is denoted relative to the origin region repeat (ORR-1) (8) and the OBR as defined by strand-specific hybridization studies with Okazaki fragments (7a). The 281-bp *Hae*III fragment contains exact matches to the consensus binding sites for the transcription factors AP1 (3) and OTF1/NFIII (29) (Fig. 1B). As indicated, the terminal 86 nucleotides of the *Hae*III fragment are derived from an *Alu*I repeat.

Computer-aided analysis of the *dhfr* origin region sequence. To identify sequences within the *dhfr* origin region that may be involved in DNA bending, the origin region sequence was scanned with the sequence AAAAnnnnnnAAAA to identify oligomeric A tracts with a 10-bp periodicity. A single portion of the 6.2-kb DNA sequence contained four such tracts with precisely a 10-bp periodicity; these tracts are located within the 281-bp *Hae*III fragment and are referred to as bend elements B1 to B4 (Fig. 1B). An additional 7-bp tract of A residues, referred to as bend element B5, is located 10 bp 3′ to these sequences on the opposite strand.

Computer modeling using the ENDs ratio also predicted that DNA bending in the *dhfr* origin region is most pronounced in the 281-bp *Hae*III fragment that contains the oligo(dA)$_{3-4}$ tracts B1 to B5 (Fig. 2). Both 2D gel analysis (8) and computer analysis with the ENDs ratio (Fig. 2) failed to reveal additional sites of pronounced DNA bending in the immediate *dhfr* origin region. Hence, the 2D gel assay and the ENDs ratio analysis suggest that the immediate *dhfr* origin region contains a single region of bent DNA located within the 281-bp *Hae*III fragment of S13X-24.

Anomalous migration of the 280-bp *Hae*III fragment is enhanced at low temperature. An additional hallmark of stably bent DNA is that anomalous migration of bent DNA fragments in polyacrylamide gels is accentuated when the electrophoresis is performed at 4°C (2). Hence, we examined the effect of temperature on migration of the 281-bp *Hae*III fragment in the 2D agarose-polyacrylamide gel assay. In this technique, DNA digested with various restriction enzymes is resolved in a first-dimension agarose gel on the basis of size. The sample lanes are then excised from the agarose gel, reoriented 90° across the top of a polyacrylamide gel, and

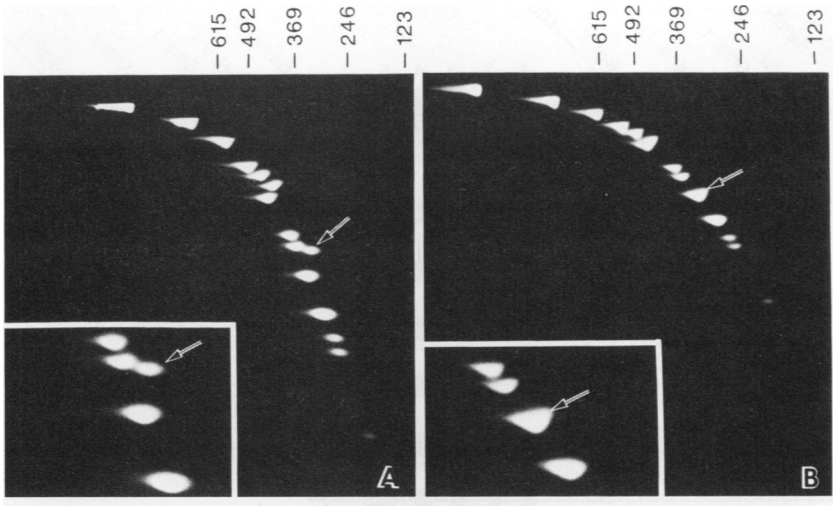

FIG. 3. Effect of temperature on anomalous migration of the 281-bp *Hae*III fragment in 2D agarose-polyacrylamide gels. Duplicate *Hae*III digests of plasmid S13X-24 were resolved in the first dimension in 2% agarose gels; the gel lanes were excised, rotated 90°, and subjected to electrophoresis in the second dimension at either 4°C (A) or 37°C (B) in 7% neutral polyacrylamide gels. The 2D electrophoretic patterns were visualized by staining with ethidium bromide. The position of the 281-bp *Hae*III fragment is denoted by arrows; regions of each gel that contain the 281-bp *Hae*III fragment are enlarged in the insets. The positions of marker DNA fragments resolved in the first-dimension agarose gel are indicated in base pairs.

resolved in the second dimension on the basis of size and shape. DNA fragments of random composition form a smooth arc in the second dimension, whereas those with unusual DNA structure migrate to positions discordant with their sizes. Thus, *Hae*III digests of S13X-24 were resolved in the first dimension in a 2% agarose gel and then in the second dimension in 7% polyacrylamide gels at either 4 or 37°C. At 4°C, the 281-bp *Hae*III fragment migrated with an apparent size of 330 bp, or approximately 19% slower than expected (Fig. 3A). In contrast, anomalous migration of the 281-bp fragment was less evident at 37°C (Fig. 3B). Because the 281-bp *Hae*III fragment migrated atop a second fragment of nearly equal size, the degree of retardation in the second dimension at 37°C could not be accurately calculated. On the basis of these results and those of the computer-aided analysis of the DNA sequence, we sought to determine whether bend elements B1 to B5 were indeed responsible for the anomalous migration of the 281-bp *Hae*III fragment.

Bend elements B1 to B5 direct DNA bending in new sequence contexts. To determine the role of elements B1 to B5 in sequence-directed DNA bending in the 281-bp *Hae*III fragment, we used circular permutation assays to examine the ability of these sequences to promote DNA bending in new sequence contexts. Previous work by others has demonstrated that the effect of DNA bending on anomalous electrophoretic migration is most pronounced when the bend is located in the center, rather than at the end, of a DNA fragment (44). Position-specific effects of the bend on anomalous migration can be tested by generating permuted DNA fragments of uniform length that have the bend located at various distances from the ends of the molecule. Hence, a 102-bp fragment of DNA encompassing bend elements B1 to B5 was generated by PCR and cloned into vector pBEND2. pBEND2 contains two repeats of a polylinker containing 17 restriction endonuclease sites, thereby allowing the facile generation of restriction fragments of uniform length that contain putative bend elements at different positions relative to the ends of a DNA fragment. The PCR primers were selected such that the center of the 102-bp fragment, after cloning, is located in the middle of bend element B3 (Fig. 1B). The organization of the derivative plasmid, pMC-Bend, was confirmed by DNA sequencing (not shown) and is depicted in Fig. 4.

The 228-bp restriction fragments generated by the digestion of pMC-Bend with six different restriction enzymes (Fig. 4) were purified from 1% agarose gels, labeled, and subjected to polyacrylamide gel electrophoresis. All six 228-bp fragments migrated in identical fashion as 230 ± 15-bp fragments in agarose gels (not shown). When resolved on a 10% polyacrylamide gel at 4°C, all of the fragments migrated slower than expected (Fig. 5A). Retarded migration was most pronounced with the *Xho*I fragment (relative mobility, or actual migration over expected, equal to 1.51), indicating that the center of the DNA bend maps approximately 114 bp from the end of the *Xho*I fragment, or near bend element B3. The similarity of the migration patterns of the *Bam*HI and *Mlu*I fragments (Fig. 5A) also supports the localization of the bend center near element B3. In the *Bam*HI fragment the B3 oligo(A) tract is located 68 bp from the end of the fragment, while in the *Mlu*I fragment this sequence is located 66 bp from the end. These results show that the DNA sequences that encompass bend elements B1 to B5 are able to induce stable DNA bending in new sequence contexts and therefore confirm that bending of the 281-bp *Hae*III fragment is due to these sequences. These experiments also establish that the center of DNA bending in linear DNA containing these sequences is located near bend element B3.

RIP60 enhances DNA bending in the *dhfr* origin region. The 281-bp *Hae*III fragment from the *dhfr* origin region contains consensus binding sites for several cellular factors, including a novel 60-kDa protein, RIP60, that protects a portion of bend element B5 and the adjacent ATT repeats in DNase I footprinting reactions (Fig. 1B; 10). RIP60 binds to domain B of the yeast origin of replication, ARS1, and copurifies with an ATP-dependent DNA helicase activity (10). To determine whether RIP60 affects DNA bending of the origin region sequences, the 228-bp fragments used in the circular permutation assay were incubated with purified RIP60 under gel shift conditions, and the resulting protein-DNA complexes

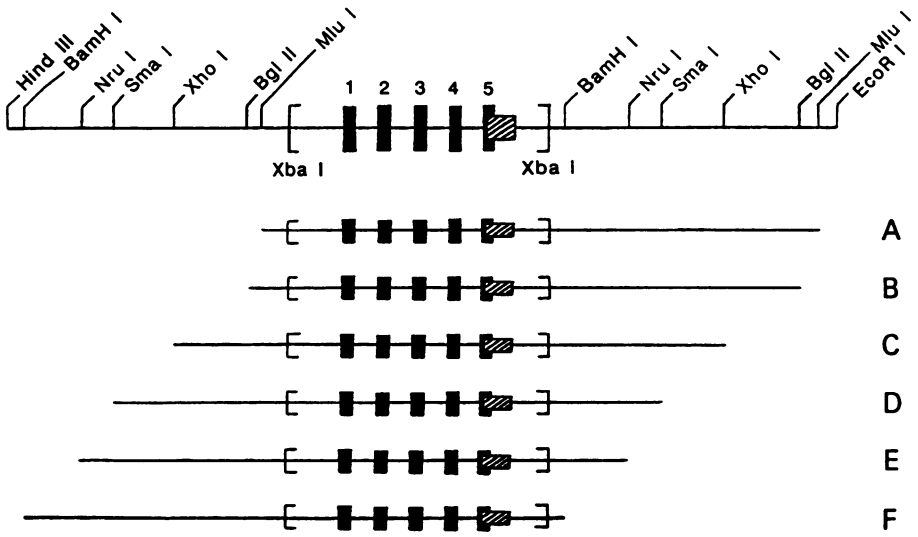

FIG. 4. Organization of plasmid pMC-Bend. A 102-bp segment of the 281-bp *Hae*III fragment was amplified by PCR (Fig. 1) and cloned into the *Xba*I site of pBEND2 to generate pMC-Bend. The orientation of the 102-bp insert within pMC-Bend relative to the indicated reiterated restriction endonuclease cleavage sites was determined by DNA sequencing. The positions of bend elements B1 to B5 (■) and the RIP60-binding site defined by DNase I footprinting (▨) are indicated. The 228-bp DNA fragments A to F were generated by digestion of pMC-Bend with *Mlu*I (A), *Bgl*II (B), *Xho*I (C), *Sma*I (D), *Nru*I (E), and *Bam*HI (F).

were resolved on neutral 4% polyacrylamide gels (Fig. 5B). The principle of this experiment, as described by Mukherjee et al. (28), is that the electrophoretic mobility of a fragment of double-stranded DNA is dependent on the root mean square of the end-to-end distance of the DNA molecule. If a protein bends DNA upon binding to its specific sequence, the effect of bending on mobility of the protein-DNA complexes will be most pronounced when the bend is located in the center of the fragment. As the binding site is located closer and closer to the end of test fragments, the electrophoretic mobility of the corresponding protein-DNA complexes will increase as the DNA fragment more closely approximates a flexible, linear rod. Others have also shown that this assay is a sensitive measure of protein-induced DNA bending in a variety of regulatory sequences (33, 47).

The position of the RIP60-binding site relative to the end of the 228-bp cyclic permuted DNA fragments dramatically affected the electrophoretic mobility of RIP60-bent DNA complexes (Fig. 5B). When the RIP60-binding site was at or near the center of the 228-bp fragment, as for the *Mlu*I, *Bgl*II, and *Xho*I fragments, little difference in the mobility of the RIP60-DNA complexes was observed in the gel shift assay (Fig. 5B, lanes 3 to 5). In contrast, when the center of the RIP60-binding site was located 36 bp from the 3′ end of the 228-bp fragment, as in the *Bam*HI fragment, the RIP60-bent DNA complexes migrated 40% more rapidly than did the *Mlu*I, *Bgl*II, and *Xho*I DNA fragment-RIP60 complexes (Fig. 5B; compare lanes 3 to 5 with lane 8). More rapid migration of the gel shift complexes was first observed for the *Nru*I fragment, which contains the center of the RIP60 binding site 62 bp from the end of the probe (lane 7). Control gel shift reactions with oligonucleotide competitors (e.g., lane 2) confirmed that this effect was specific for the binding of RIP60. Note that the migration of the free probe in the gel shift experiments was retarded relative to that of the marker DNA and reiterated the pattern of anomalous migration observed in the absence of protein (Fig. 5B).

To compare the effect of RIP60 on the migration of protein-DNA complexes with that observed with free probe, the relative migration of the DNA fragments and the gel shift complexes was measured and plotted versus the position of bend element B3 in each of the six test fragments (Fig. 6). Comparison of the curves generated from each experiment showed a marked discordance in the migration of the gel shift complexes for the *Mlu*I and *Bam*HI fragments. While the *Mlu*I and *Bam*HI fragments displayed similar migration patterns in the absence of protein (relative mobility of 1.40 versus 1.37), as is expected if the bending center is near bend element B3, in the presence of RIP60 the *Bam*HI fragment migrated almost 40% faster than the *Mlu*I-RIP60 complex. In light of the work of others (33, 46), these results suggest that RIP60 not only markedly enhances DNA bending in the origin region sequences but also displaces the center of bending 25 to 30 bp 3′ to bend element B3.

DISCUSSION

The sequences that regulate initiation of DNA synthesis in mammalian cells have remained remarkably elusive. While considerable evidence suggests that DNA replication begins at preferred sites in the mammalian genome (reviewed in reference 41), no definitive assay for mammalian origin activity has yet been devised. The lack of a reliable assay for origin function has hindered the identification of sequences involved in initiation of DNA synthesis and has thwarted the isolation of proteins that participate in this process. There is considerable experimental evidence to suggest that an origin of DNA replication is located approximately 17 kb 3′ of the Chinese hamster *dhfr* gene. As in SV40 (17), phage lambda (40), and polyomavirus (21), the *dhfr* OBR has been biochemically defined by mapping the zone in which leading- and lagging-strand DNA synthesis switches template strands (7a). To complement the Okazaki fragment mapping studies, the DNA sequence of the *dhfr* origin region was determined, and these sequences were examined for DNA structures common to other well-characterized replication origins (8). These studies identified a highly conserved repetitive element (ORR-1) and a fragment of stably bent DNA that are located immediately 3′ to the OBR.

The circular permutation assays presented here confirm

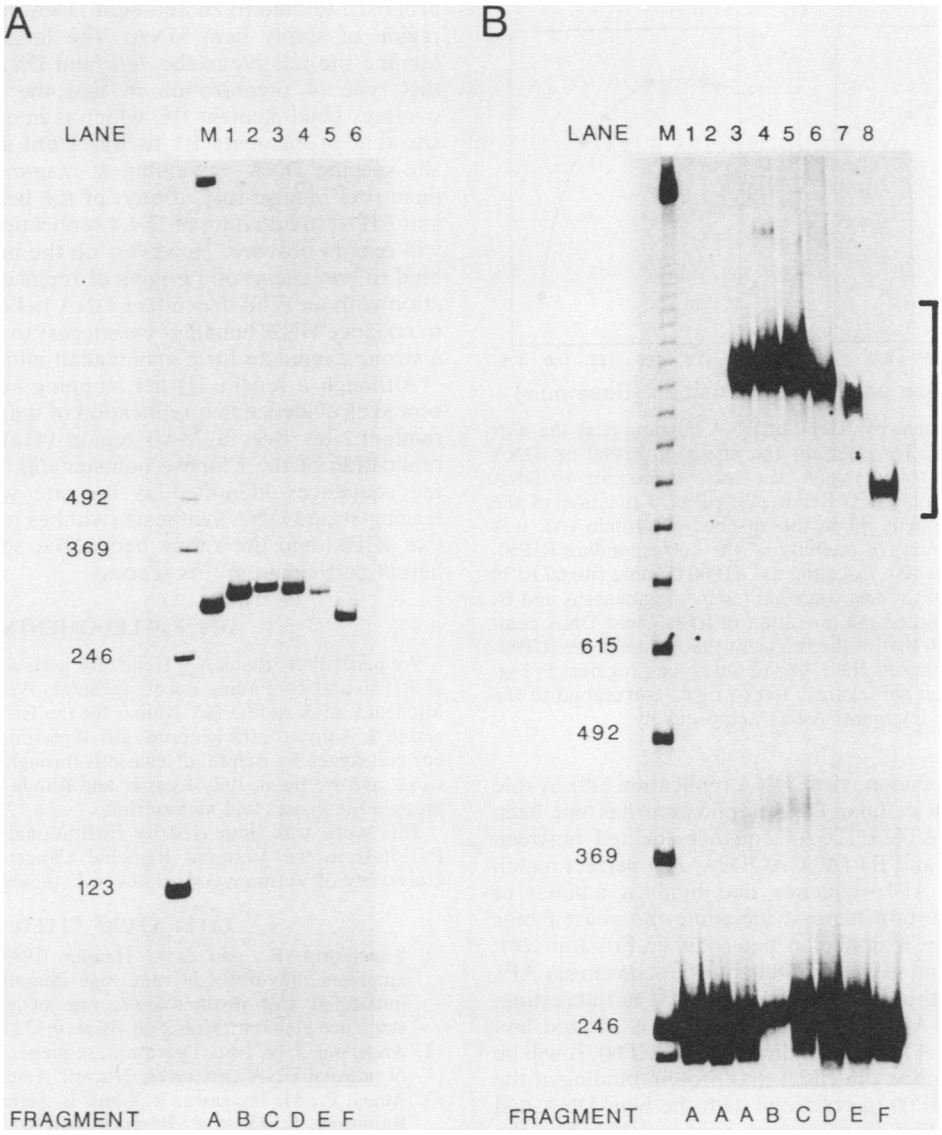

FIG. 5. Position-specific effects of RIP60 on the migration of the circularly permuted 228-bp DNA fragments of pMC-Bend. (A) Effect of the position of bend elements B1 to B5 on the anomalous migration of DNA fragments in polyacrylamide gels. Fragments A to F of pMC-Bend (Fig. 4) were purified from an agarose gel, end labeled, and resolved on a 10% polyacrylamide at 4°C along with end-labeled marker DNA fragments (indicated in base pairs). The gel was dried, and the labeled DNA fragments were visualized by autoradiography. The migration of each of the 228-bp test fragments A to F was retarded relative to that of the marker fragments. (B) Gel shift analysis of RIP60 protein-bent DNA complexes. The end-labeled fragments A to F from panel A were incubated with purified RIP60 as described elsewhere (10), and the resulting protein-DNA complexes were resolved on a 4% neutral polyacrylamide gel at 24°C. The gel was dried, and RIP60-bent DNA complexes were visualized by autoradiography. Although the unbound 228-bp DNA probes migrated slightly faster at 24 than at 4°C (compared with the 246-bp marker, for example), the position-specific effect of elements B1 to B5 on anomalous migration was also observed under gel shift conditions. The RIP60 protein-bent DNA complexes (bracket) are specific in that no band shift was observed in the absence of protein (lane 1) or in the presence of a 100-fold molar ratio of specific RIP60 competitor DNA (lane 2). The specific activity of fragment E in these experiments resulted in a weaker autoradiographic signal in both panels.

that the *dhfr* origin region contains phased oligo(dA) tracts that are able to direct DNA bending in new sequence contexts. The degree of DNA bending induced by these sequences is enhanced at low temperature, a property characteristic of bent DNA (2). Because stably bent DNA is a functional component of many procaryotic and eucaryotic replication origins, we expect that the bent DNA sequences from the *dhfr* origin region, perhaps acting at a distance in concert with other sequence elements, are involved in initiation of DNA synthesis at the nearby OBR defined by the Okazaki fragment mapping studies. This expectation is reinforced by the presence of multiple consensus binding sites for cellular transcription factors within the bent DNA sequences.

Gel shift and DNase I footprinting experiments have shown that the ATTTTCAT sequence at the 5′ end of the 281-bp *Hae*III fragment binds purified OTF1/NFIII (N. H. Heintz, unpublished observations). OTF1/NFIII is an abundant cellular transcription factor involved in S-phase regulation of the histone H2B gene that is also required for

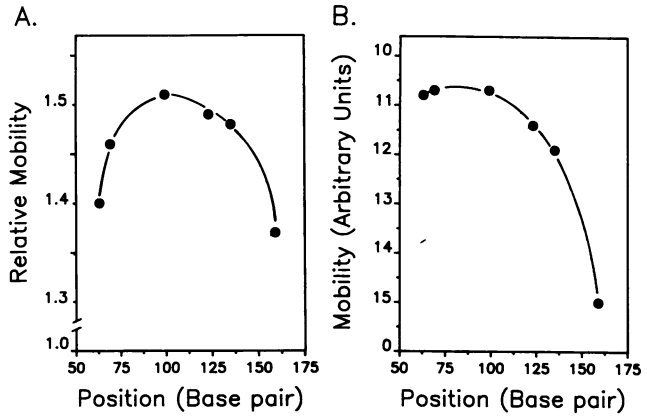

FIG. 6. Enhancement by RIP60 of DNA bending near the *dhfr* origin of replication. To ascertain the effect of RIP60 on DNA bending in the *dhfr* origin region, the relative mobility of DNA fragments A to F from pMC-Bend, plotted as a function of the position of bend element B3 in the absence of protein (A), was compared with the relative mobility of the corresponding RIP60-bent DNA complexes (B). Locating the RIP60 binding site 60 to 70 bp from the 5′ end of the test fragment (as for fragments A and B; Fig. 5) markedly retarded the migration of RIP60-bent DNA complexes compared with that of the fragment that contains the RIP60-binding site 60 to 80 bp from the 3′ end of the probe (fragment F; Fig. 5). The points in each curve, from left to right, correspond to the values calculated for fragments A to F, respectively.

efficient initiation of adenovirus DNA replication (29). A role for OTF1/NFIII in cellular DNA replication has not been established. The ATGACTCA sequence located between bend elements B3 and B4 (ATGACTCA) is a perfect match to the consensus AP1 sequence that binds a number of eucaryotic transcription factors, including the yeast factor GCN4 (36) and the mammalian heterodimer Fos-Jun (24). Binding of these and related proteins to the consensus AP1 site has not been thoroughly investigated. It is interesting, however, that the AP1 consensus sequence is located less that 20 bp 5′ to the ATT-rich binding site for RIP60. It will be important to determine the effect that protein binding at the AP1 site has on RIP60 interactions with the bent DNA and the effect, if any, that these factors have on recruiting the RIP100 helicase to the *dhfr* origin region.

As discussed by Mukherjee and colleagues (28) and Zwieb and co-workers (46), gel shift analysis with circularly permuted DNA fragments is a highly sensitive assay for protein-induced DNA bending. The gel shift experiments with circularly permuted DNA fragments from pMC-Bend presented here show that the position of the RIP60-binding site relative to the ends of the 228-bp permuted fragments markedly affects the migration of RIP60-DNA complexes. Hence, RIP60 enhances DNA bending of the *dhfr* origin region sequences. The gel shift analysis also suggests that RIP60 moves the center of DNA bending 20 to 30 bp 3′ to element B3. In several other replication systems, protein-induced DNA bending mediates the interaction of dispersed protein-binding sites during the formation of higher-order protein-DNA structures (22, 28, 35, 45). By analogy, RIP60 may foster the interaction of dispersed regulatory sequences that bind protein factors in the *dhfr* origin region.

Computer-aided sequence analysis of 11 origins of replication has suggested to Eckdahl and Anderson that the relationship of initiator-binding sites to bent and anti-bent DNA sequences at origins may be highly conserved throughout evolution (12). In this model, initiator proteins are proposed to bind to an anti-bent DNA domain adjacent to a region of stably bent DNA. The location of the RIP60-binding site relative to the *dhfr* bent DNA sequences shows this type of organization in that the RIP60-binding site overlaps bend element B5, which is in an orientation opposite that of elements B1 to B4. Until a reliable assay for site-specific DNA unwinding at mammalian origins is refined, the precise role, if any, of the bent DNA sequences and RIP60 in initiation of DNA replication at the *dhfr* origin will remain obscure. However, on the basis of its ability to bind to two eucaryotic origins of replication, its cofractionation with an ATP-dependent DNA helicase, and its ability to enhance DNA bending, we suggest that RIP60 represents a strong candidate for a mammalian initiation factor.

Although a recent 2D gel mapping study has been presented as evidence that replication of the *dhfr* gene occurs at random sites over a 28-kb region (41a), we propose that replication of the Chinese hamster *dhfr* gene initiates near the sequences identified as the site where leading- and lagging-strand DNA synthesis switches template strands and that RIP60 and the stably bent DNA sequences described herein participate in this process.

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