Cruciform DNA Binding Protein in HeLa Cell Extracts†

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ABSTRACT: We have analyzed by band-shift assays HeLa cell protein−DNA interactions on a stable cruciform DNA molecule. The stable cruciform was formed by heteroduplexing the *Hin*dIII-*Sph*I fragment of SV40 virus DNA that contains the origin of replication with a derivative mutant containing a heterologous substitution at the central inverted repeat. We have identified a novel binding activity in HeLa cell extracts with specificity for the cruciform-containing DNA and no apparent sequence specificity. The activity is protein-dependent, void of detectable nuclease activity, and distinct from that reported for HMG1. A cruciform binding protein (CBP) with an apparent molecular weight of 66 kDa was enriched from HeLa cell extracts. In addition to the CBP, we have detected sequence-specific binding activities to sites proximal to the cruciform. Binding to one such site is increased in the cruciform-containing heteroduplex DNA by comparison to its linear homoduplex counterpart, suggesting transmission of structural effects by the stem-loops to their local environment.

Inverted repeat (IR) DNA sequences are widely distributed in the chromosomal DNA of many eukaryotes (Wilson & Thomas, 1974; Schmid *et al.*, 1975; Klein & Welch, 1980). IRs that have dyad symmetry have the potential to form cruciform (stem-loop or hairpin) structures through intrastrand base pairing. It has long been suggested that such structures may form transiently *in vivo*, to serve as recognition signals for regulatory proteins of transcription, recombination, or replication (Gierer, 1966; Bell & Byers, 1979; Zannis-Hadjopoulos *et al.*, 1988; Bell *et al.*, 1991). The formation of cruciforms *in vivo* has been demonstrated for prokaryotic (Panayotatos & Fontaine, 1987; Zheng *et al.*, 1991; Dayn *et al.*, 1992), mammalian (Ward *et al.*, 1990, 1991), and eukaryotic virus SV40 DNA (Hsu, 1985), the latter at the viral origin of DNA replication.

The importance of IRs has been established for termination of transcription and attenuation in prokaryotes (Rosenberg & Court, 1979) and gene expression in prokaryotes (Horwitz, 1989) and eukaryotes (Shuster *et al.*, 1986; Greenberg *et al.*, 1987; McMurray *et al.*, 1991; Spiro *et al.*, 1993). In addition, transcriptional regulation by cruciforms has been demonstrated both *in vitro* (Horwitz & Loeb, 1988; Horwitz, 1989; Morales *et al.*, 1990; Waga *et al.*, 1990) and *in vivo* (Dayn *et al.*, 1992). This regulation can be controlled by cruciform-specific binding proteins (Tremethick & Molloy, 1986, 1988; Watt & Molloy, 1988; Singh & Dixon, 1990; Waga *et al.*, 1990).

IRs are commonly associated with replication origins of prokaryotic (Zyskind *et al.*, 1983; Hiasa *et al.*, 1990), viral (Muller & Fitch, 1982), eukaryotic (Tschumper & Carbon, 1982; Campbell, 1986), and mammalian (Hand, 1978; Zannis-Hadjopoulos *et al.*, 1984, 1985; Triboli *et al.*, 1987; Landry & Zannis-Hadjopoulos, 1991; Boulikas & Kong, 1993) organisms, as well as with amplified genes (Fried *et al.*, 1991). IRs, stem-loops, and cruciform structures have been shown to be functionally important for the initiation of DNA replication in plasmids (Masukata & Tomizawa, 1984; Noirot *et al.*, 1990), prokaryotes (Zyskind *et al.*, 1983; Hiasa *et al.*, 1990), and viruses of eukaryotes (Muller & Fitch, 1982; Deb *et al.*, 1986). The regulation of replication may be controlled by cruciform-specific binding proteins (Duguet *et al.*, 1977; Alexandrova *et al.*, 1984; Bonne-Andrea *et al.*, 1986; Alexandrova & Beltchev, 1988; Zannis-Hadjopoulos *et al.*, 1988; Hiasa *et al.*, 1990; Noirot *et al.*, 1990; Zechiedrich & Osheroff, 1990; Sun & Godson, 1993; Toth *et al.*, 1993). We have previously produced monoclonal antibodies (mAbs) with unique specificity to cruciform DNA structures (Frappier *et al.*, 1987, 1989). Using these mAbs we have shown that there is a dynamic distribution of cruciforms in mammalian nuclei, their numbers being at a maximum at the G1/S boundary (Ward *et al.*, 1990, 1991). Introduction of these antibodies into a permeablized cell system capable of carrying out DNA replication resulted in an enhancement of DNA replication (Zannis-Hadjopoulos *et al.*, 1988). These observations support the hypothesis that certain IR sequences may extrude into cruciforms and act as an attachment site or recognition structure for transcription, recombination, replication, or other protein factor(s).

In this study we have analyzed by band-shift assays HeLa cell protein−DNA interactions on a stable cruciform formed by heteroduplexing the *Hin*dIII-*Sph*I fragment of SV40 virus DNA, which contains the viral core origin of replication, with a derivative mutant containing a heterologous substitution at the central IR (Nobile & Martin, 1986). We have identified a novel binding activity from HeLa cell nuclear extracts with specificity to the cruciform-containing heteroduplex DNA. We have also detected sequence-specific binding of cellular factors to specific sites in the SV40 origin proximal to the IR. Binding to one such site is increased in the cruciform-containing heteroduplex DNA by comparison to its linear homoduplex counterpart.

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MATERIALS AND METHODS

DNA Substrates and Competitors. Plasmids pRGM21, pRGM29, pRGM67 (Nobile & Martin, 1986), pUC7 (Vieira & Messing, 1982), and pRGM128 (Frappier *et al.*, 1989) were used for construction of the heteroduplexes (Figures 1 and 11a). pRGM21 contains the *Hin*dIII-*Sph*I fragment (200 base pair (bp)) of the wild type SV40 origin of replication, cloned into pBR322. pRGM29 is identical to pRGM21 except that the wild type SV40 27 bp palindrome has been replaced by an unrelated 26 bp palindromic sequence. In pRGM67, the 13 bp inverted repeat of pRGM21 is substituted with an 8 bp *Sal*I linker containing a 4 bp palindrome.

Heteroduplexes were formed between the corresponding *Pvu*II fragments pUC7 (Vieira & Messing, 1982) and pRGM128 (Frappier *et al.*, 1989). In the pUC7 substitution plasmid (pRGM128), the 42 bp *Eco*RI segment from within the pUC7 48 bp palindrome has been replaced by a 56 bp inverted repeat composed of the *Eco*RI-*Cla*I fragment of pBR322 ligated head to head. When the *Pvu*II fragments of pUC7 and pRGM128 were heteroduplexed, stable cruciforms with unequal arms and unequal stem-loop structures formed (Figure 11a). In addition, the pUC7 × pRGM128 heteroduplexes contain the ability for limited branch migration (see Results and Figure 11a).

Prior to heteroduplexing, the *Hin*dIII-*Sph*I (pRGM21, pRGM29, or pRGM67) or the *Pvu*II (pUC7 and pRGM128) fragments were purified by electrophoresis on 4% polyacrylamide gels and eluted from the gel by isotachophoresis (Ofverstedt *et al.*, 1984). Heteroduplexing reactions were performed essentially as previously described (Nobile & Martin, 1986; Frappier *et al.*, 1987, 1989), by mixing equal amounts of the isolated double-stranded fragments, denaturing, and reannealing. Homo- and heteroduplex molecules were separated and purified from each other as described above, except where indicated otherwise. DNAs were dephosphorylated at the 5′ ends with calf intestinal alkaline phosphatase (New England Biolabs) and end-labeled with [γ-^{32}P]ATP (Amersham) using T4 kinase (BRL), as described (Maxam & Gilbert, 1980). Free nucleotides were removed by running the end-labeled products on Nick columns (Pharmacia).

Specific single-stranded competitors of pRGM21 and pRGM29 were isolated from each other by denaturation in DMSO and electrophoresis on native polyacrylamide, essentially as previously described (Maxam & Gilbert, 1980; Gaillard *et al.*, 1988), and eluted as above.

DNA binding assays were performed in the binding buffer of Elborough and West (1988), with a final concentration of 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, and 3% glycerol. Double-stranded poly(dI-dC) (Pharmacia) was also routinely added to a final concentration of 100 ng/μL. In a final volume of 20 μL and after a 15 min incubation on ice, the samples were placed in loading buffer (25% Ficoll, 25 mM EDTA, 0.2% bromophenol blue, 0.2% xylene cyanol) and electrophoresed on 4% polyacrylamide gels in 1× TBE at 12−13 V/cm for 1.5−2 h at room temperature. The gels were dried and exposed overnight for autoradiography with Kodak XOMAT-AR film and intensifying screens (DuPont) at −70 °C.

Extract Preparation. Experiments were performed with protein extracts from log phase HeLa S3 cells prepared essentially as described (Pearson *et al.*, 1991), with cytoplasmic extracts in hypotonic buffer (20 mM HEPES-KOH (pH 7.8), 5 mM potassium acetate, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT)) and nuclear extracts in hypotonic buffer + 500 mM potassium acetate. All experiments (except where indicated) were performed with nuclear extract. Extracts prepared by this method are capable of replicating the SV40 virus (Decker *et al.*, 1986), as well as plasmids containing mammalian (monkey CV-1 and human) DNA origin-enriched sequences (*ors*) in an *in vitro* replication system (Pearson *et al.*, 1991, 1994; Nielsen *et al.*, 1994).

Cruciform Binding Protein (CBP) Enrichment. To enrich for cruciform binding activity, crude extracts in buffer A (26 mM HEPES (pH 7.8), 82 mM potassium acetate, 5 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 1 mM PMSF, 10 mM $Na_2S_2O_4$, 4% glycerol) were loaded onto a DEAE-Sephadex A-50 (Pharmacia) column that was equilibrated with the same buffer. The column was washed with the equilibration buffer, and this flow-through was pooled (FT). Bound proteins were eluted off the column with a linear salt gradient of potassium acetate in buffer A (82−1000 mM). Fractions were collected and tested for CBP activity, by band-shift assay. The complete eluate was pooled into four separate pools: A, B, C, and D according to salt concentration (82−200, 200−450, 450−700, and 700−1000 mM potassium acetate in buffer A, respectively). CBP activity was contained in pool B, which was dialyzed against buffer B (0.01 M KH_2PO_4 (pH 7.4), 0.15 M NaCl, 2.5 mM EDTA, 1 mM PMSF, 2 μg/mL aprotininin, 1 × 10^{-7} M pepstatin A, 5% glycerol) and loaded onto an Affi-Gel Heparin Gel (BioRad) column. All of the CBP activity was recovered in the flow-through (FT_H), which was concentrated, dialyzed against buffer B, and subjected to sedimentation on a glycerol gradient (Malkas *et al.*, 1990). Briefly, an aliquot of FT_H was layered onto a preformed 10−40% glycerol gradient formed over a 0.5 mL cushion of 2 M sucrose and was centrifuged for 16 h at 150000*g* at 4 °C in a Beckman SW50.1 rotor. Parallel gradients were loaded with a set of standard protein markers (thyroglobulin, 669 kDa; aldolase, 158 kDa; hemoglobin, 64.5 kDa; chymotrypsinogen A, 25 kDa) and centrifuged simultaneously. Fractions were taken from the bottom and assayed for cruciform binding activity. In order to avoid the aggregation of HMG1 which occurs at pH 5.7 (Baker *et al.*, 1976), the pH was maintained at 7.4−7.8, at which HMG1 sediments as a monomer (Shooter *et al.*, 1974). Fractions with significant CBP activity were pooled, dialyzed, and used as pool H.

Western Blotting. Western blots were performed essentially as described (Burnette, 1981). Polyacrylamide gel electrophoresis was performed as previously described (Laemmli, 1970), using 20 μg of the various protein fractions obtained during the purification per lane. Proteins were electrotransferred to Immobilon-P (Millipore) and probed with anti-HMG1 antibody (a gift of Dr. M. Bustin). The blots were then developed by incubation with ^{125}I-conjugated protein A (Amersham). The membranes were exposed for autoradiography at −70 °C.

RESULTS

Construction and Properties of the Heteroduplex Cruciforms. A stable cruciform structure was generated by heteroduplexing the *Hin*dIII-*Sph*I fragments of plasmids

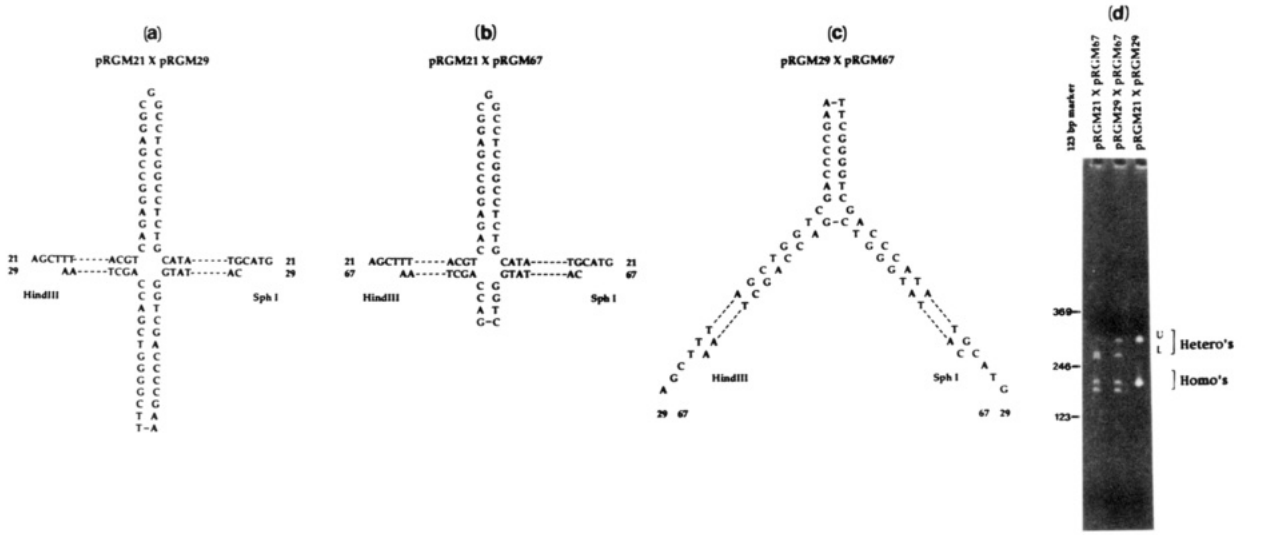

FIGURE 1: Heteroduplex DNA structures. Heteroduplex DNA structures between the *Hin*dIII-*Sph*I fragments of plasmids pRGM21 (200 bp), pRGM29 (199 bp), and pRGM67 (182 bp): (a) pRGM21 × pRGM29, (b) pRGM21 × pRGM67, and (c) pRGM29 × pRGM67. Only one of the two possible structures is shown in each case. Similar structures can also arise from the complementary strands (Nobile & Martin, 1986). (d) Electrophoretic pattern of the various heteroduplex structures on 4% polyacrylamide gel with reference to the 123 bp ladder marker (indicated by the numbers). The homoduplexes and the heteroduplexes are indicated. U and L indicate the upper and lower heteroduplexes arising from combinations of the different strands of pRGM29 and pRGM67.

pRGM21 × pRGM29 (Figure 1a, only one of the two possible heteroduplexes is shown) as previously described (Nobile & Martin, 1986; Frappier *et al.*, 1987, 1989). Briefly, the *Hin*dIII-*Sph*I fragment of pRGM21 (200 bp) is derived from SV40 DNA and comprises the viral core replication origin, while that of the substitution mutant pRGM29 (199 bp) is identical but for the replacement of the central inverted repeat with a heterologous 13 bp inverted repeat. Since the points of difference between the two strands are inverted repeats which are not complementary to each other, a fully base-paired duplex cannot form at this point, and upon hybridization of the mutant and wild type strands, two heteroduplexes, each containing a cruciform, are formed (Figure 1a, only one of the two possible heteroduplexes is shown). The cruciform structure is stable, as the symmetry is such that no alternative base pairing can occur through branch migration. This structure is stereochemically equivalent to a cruciform, except that it lacks some of the symmetry of a true cruciform in that the stems are not homologous. The two possible heteroduplexes (cruciform-containing molecules) between pRGM21 and pRGM29 migrate in polyacrylamide gel as a single band, slower than their respective homoduplexes (Figure 1d). We have previously shown that both heteroduplex molecules are susceptible at the loops to digestion by mung bean (MBN) and S1 nuclease and are recognized and restricted by T7 endonuclease I, which is specific for four-way DNA junctions (Nobile & Martin, 1986; Frappier *et al.*, 1989).

The additional molecules with secondary structures of interest were heteroduplex molecules of pRGM21 with pRGM67 (Figure 1b, only one of the two possible heteroduplexes is shown) and pRGM29 with pRGM67 (Figure 1c, only one of the two possible heteroduplexes is shown) (Nobile & Martin, 1986; Frappier *et al.*, 1987). The pRGM21 × pRGM67 heteroduplex yields cruciform structures with stem-loops of unequal lengths, one of which is particularly short (Figure 1b, only one of the two possible heteroduplexes is shown); both heteroduplexes migrate slower than the homoduplexes, and although they migrate close to each other, they can be observed as distinct bands (Figure 1d). The pRGM29 × pRGM67 heteroduplex yields two possible Y- (or T-) structures (Figure 1c), both of which migrate slower than the homoduplexes, as distinct bands (Figure 1d). The migration of the heteroduplexes formed between pRGM21 × pRGM67 or pRGM29 × pRGM67 is as distinct bands and suggests that their conformation is different.

Cruciform Binding Activity in HeLa Cell Extracts. DNA binding activity was detected in HeLa cell nuclear extracts (Figure 2), in the presence of high concentrations (approximately 500-fold mole excess) of competitor poly(dI-dC). There are DNA binding activities to both pRGM21 and pRGM29 linear DNAs, as well as to the linear homoduplexes (mixture of pRGM21 and pRGM29) isolated after the heteroduplexing reaction. The band-shifted complexes with the homoduplexes are labeled A−C. There is no apparent difference in the pattern of shifted bands between the linear homoduplexes pRGM21 and pRGM29 or the mixture, suggesting that the binding occurs within regions of homology in these two fragments. A similar pattern of shifted bands is also observed with the heteroduplex (cruciform) molecules. In addition, however, there appears a series of fast migrating shifted bands that are unique to the cruciform molecules (Figure 2, complexes D). These shifted bands do not appear in any of the other linear DNA binding reactions, suggesting the presence of a new binding site(s) in the heteroduplex molecules that is absent in the homoduplex molecules. A parallel experiment run on 8% polyacrylamide gel reveals multiple shifted bands contained in the cruciform-specific shifted complex D (see below). Identical band-shift patterns have been obtained with uniquely labeled (5′ and 3′) pRGM21 heteroduplexed with cold pRGM29, and vice versa (data not shown), indicating that the binding activity giving rise to complexes D does not distinguish between the two possible cruciforms; this strongly suggests that the interaction is mainly structure-specific.

The above data suggested the presence of cruciform DNA-specific binding protein(s) in the HeLa nuclear extracts. To

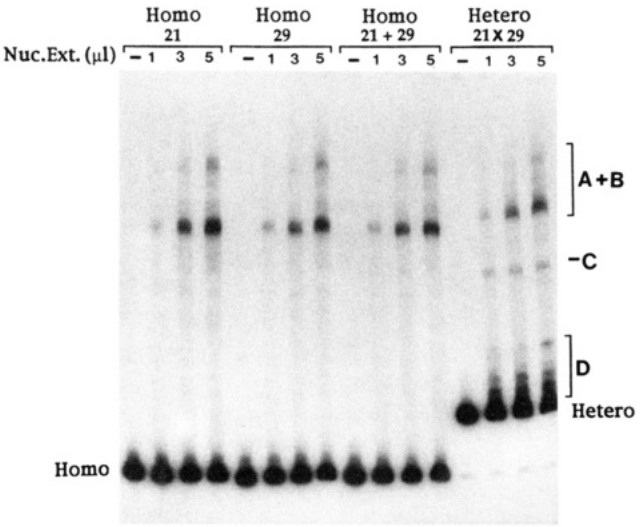

FIGURE 2: Binding to cruciform structure. Band-shift assays were performed using 2 ng/reaction of ^{32}P-labeled pRGM21, pRGM29, isolated pRGM21 + pRGM29 homoduplex DNAs, or isolated pRGM21 × pRGM29 cruciform DNA, as indicated. DNAs were incubated as described in Materials and Methods with the indicated volume of nuclear extract (165 ng of protein/μL). A−C denote band shifts common to both homoduplexes and cruciform molecules; D denotes band shifts unique to the cruciform. A small amount of homoduplex is evident in all reactions that use isolated heteroduplex; this is known to arise as a consequence of ethanol precipitation of the heteroduplex DNA (Svaren *et al.*, 1987).

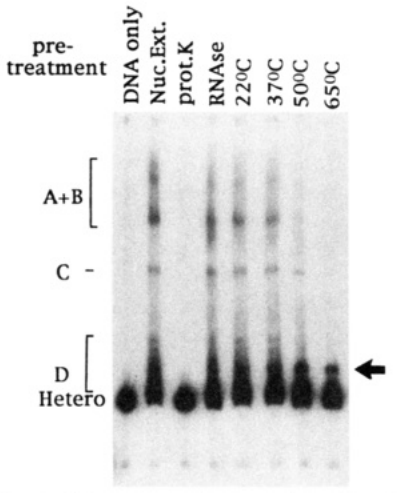

FIGURE 3: Band shifts are protein-dependent. Band-shift assays were performed on ice for 15 min as described in Materials and Methods, using 2 ng/reaction of ^{32}P-labeled cruciform (pRGM21 × pRGM29) and 5 μL of 165 ng of protein/μL nuclear extract that was pretreated for 30 min at 37 °C with either 1 mg/mL proteinase K or pancreatic *RNase* or pretreated for 30 min at 22, 37, 50, and 65 °C, as indicated. All pretreatments were performed immediately prior to binding reactions. A−D are as in Figure 2.

examine whether the retardation was due to protein binding, we analyzed the binding pattern after pretreating the extracts with proteinase K or *RNase* A (Figure 3). All binding activities are sensitive to proteinase K and resistant to *RNase*, indicating that the observed band shifts were due to protein factors present in the extracts. To further characterize the protein binding activity, we preincubated the extracts for 30 min prior to the binding assays at various temperatures between 22 and 65 °C (Figure 3). All protein−DNA complexes common to both homo- and heteroduplex DNAs (complexes A−C) were decreased by preincubation of the extracts above 37 °C and abolished by preincubation at 65

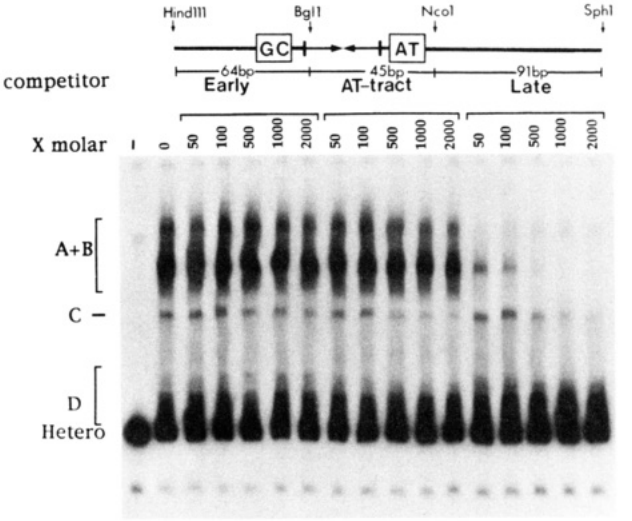

FIGURE 4: Sequence-specific competition assays. All DNA competition assays were performed in binding buffer, with 0.2 ng of isolated ^{32}P-labeled cruciform DNA, 100 μg/μL double-stranded poly(dI-dC), the indicated mole excess amount of competitor, and 3.5 μL of 165 ng of protein/μL nuclear extract in a final volume of 20 μL. The order of addition for all reactions was buffer, ^{32}P-labeled fragment, poly(dI-dC), competitor, nuclear extract. Reactions were allowed to incubate on ice for 30 min. (top) Map of the *Hin*dIII-*Sph*I fragment of wild type SV40 (pRGM21). The boxes indicate the early (GC), AT-tract (AT), and late elements. The central IR (→←) and the restriction sites of interest are indicated. Also shown are the sizes of the linear competitor fragments. (bottom) The early 64 bp (sites 5171−5235), the 45 bp (sites 5235−37) AT-tract, or the 91 bp (sites 37−128) late linear fragments of the wild type SV40 origin (*Hin*dIII-*Sph*I fragment of pRGM21 digested with *Bgl*I and *Nco*I). The mole excess used is indicated. A−D are as in previous figures.

°C. Only the most prominent, faster migrating protein−DNA complex which is unique to the cruciform DNA (Figure 3, arrow) is apparently heat resistant to 65 °C. Pretreatment of the extracts to 100 °C abolishes all binding (data not shown). These data indicate that the shifted bands represent protein−DNA complexes and that a binding activity specific for the cruciform structure is relatively heat resistant.

Sequence-Specific Binding. In order to distinguish between sequence- and structure-specific binding on the cruciform substrate (for complexes A−C, see above), we first examined which complexes are sensitive to competition with linear fragments. For this we used various linear duplex subfragments of pRGM21 as competitors (Figure 4) by digesting the 200 bp *Hin*dIII-*Sph*I fragment with *Bgl*I and *Nco*I (Figure 4, top). Using as much as a 2000-fold mole excess of the early fragment (64 bp) as competitor had no effect on any of the band-shifted complexes (Figure 4), indicating specificity in the protein−DNA interactions. Using the AT-tract (45 bp) did not affect complexes A, B, or D, but it affected complex C to a small extent (Figure 4). Complexes A + B were effectively competed out by 500-fold mole excess of the "late" fragment (91 bp) (Figure 4). Complex C was also mildly competed by the late fragment. Complex C was more effectively competed by the whole *Bgl*I-*Sph*I fragment (136 bp) than by either the 45 bp AT-tract or the 91 bp late fragment (data not shown). The band shifts that are unique to the cruciform (complexes D) were not affected by any of these linear fragments, consistent with the above data (see Figure 2). These results suggest that the protein(s) of complex C may bind near the *Nco*I site, while the protein(s) giving rise to complexes A and B

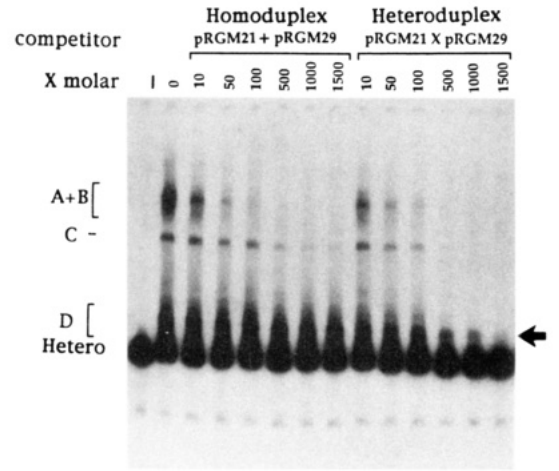

FIGURE 5: Structure-specific competition assays. Competitions were performed as in Figure 4. Isolated homoduplex (pRGM21 + pRGM29 mixture) or isolated cruciform (pRGM21 × pRGM29) competitor was included at the indicated mole excess amount. A−D are as in previous figures; the arrow marks the higher affinity band shift unique to the cruciform.

specifically interact with sequences on the "late" (91 bp) fragment. Thus, proteins generating complexes A−C interact in a sequence-specific manner with DNA regions present in both the homo- and heteroduplex molecules. The results moreover support the above data which indicate that the lowest shifted complexes (D) are specific to the cruciform-containing DNA. In addition, the fact that the band-shift patterns for either homoduplex pRGM21 or pRGM29 together or alone (Figure 2) do not contain complexes other than A−C, which are sequence-specific (Figure 4) and do not contain other complexes, such as the cruciform-specific complex D, suggests that there is no induction and/or stabilization of cruciforms from the linear homoduplexes. Furthermore, we have previously shown (Frappier *et al.*, 1989) by both S1 and *DNase*I digestions that there is no evidence for a cruciform structure in the linear homoduplexes pRGM21 or pRGM29.

Structure-Specific Binding. To demonstrate further that this activity binds specifically to only the cruciform-containing molecules, and recognizes its target DNA by structure rather than by sequence, we performed a series of competition binding assays using a variety of competitor DNAs. Using unlabeled isolated linear homoduplex (mixture of pRGM21 and pRGM29) or unlabeled isolated cruciforms (pRGM21 × pRGM29) as competitor DNAs, we were able to demonstrate unequivocally structure-specific binding to the cruciform molecules (Figure 5). The upper complexes (labeled A + B) are sensitive to both competitor DNAs and are absent at 500-fold mole excess of competitor. The intermediate complex (labeled C) is also absent at 500-fold mole excess of heteroduplex competitor and reduced at 1500-fold mole excess of homoduplex competitor. Surprisingly, complex C is more easily competed by the heteroduplex competitor than by the homoduplex competitor DNAs (Figure 5). Complex C is common to both the homo- and heteroduplex band shifts and is equally present when band shifts are done with either labeled homoduplex, heteroduplex, or single molecules of pRGM21 or pRGM29 (see Figure 2). Thus, it appears that binding to these common sites, although undoubtedly depending on sequence determinants (as demonstrated above), is enhanced (i.e., is of a greater stability and/or affinity) when the DNA molecule contains a cruciform or a cruciform−protein complex. The complexes that are unique to the cruciform-containing molecules (labeled D) are unaffected by as much as 1500-fold mole excess of either nonspecific or homoduplex competitor DNAs (Figure 5), while they start decreasing at 500-fold mole excess of heteroduplex competitor and are nearly abolished at 1500-fold mole excess of heteroduplex competitor (Figure 5). These experiments indicate that the binding that is unique to the cruciform DNA (D) is structure-specific. Furthermore, it appears that at least one protein−DNA interaction (C), which is common to both the linear and cruciform DNAs, may have increased stability due to the presence of a cruciform or a cruciform−protein in the DNA substrate.

We further tested the specificity of the binding activities to the cruciform DNA in a series of competition assays using a variety of DNA structures as competitors. Using an excess (>1500-fold mole excess) of either double- or single-stranded linear heterologous DNAs (pBR322) as competitors had no effect on any of the band-shifted complexes (data not shown). In contrast, using isolated single-strands of either pRGM21 or pRGM29 as competitors, we observed competition (Figure 6a). The four different single strands had no effect upon the band shifts unique to the cruciform (complex D; Figure 6a). The 3′−5′ strand of the *Hin*dIII-*Sph*I fragment of pRGM21 (SV40 late coding strand) was most efficient at competing for complexes A and B at 1000-fold mole excess. Both single strands of pRGM29 are as inefficient as the 5′−3′ strand of pRGM21 at competing for complexes A and B. Complex C is equally affected by all single strands at 1000-fold mole excess. Apparently, complexes A−C can be competed by the wild type (pRGM21), and not the substituted (pRGM29), late coding strand. The results indicate that the majority of the shifted material that is unique to the cruciform (D) is not due to single-stranded DNAs.

We also used a variety of Y-structures, made by heteroduplexing pRGM29 and pRGM67 DNAs, as competitors (Figure 6b). The two resulting heteroduplex molecules were isolated from each other by polyacrylamide gel electrophoresis (Figure 1d, L, lower, and U, upper) and used separately as competitors (Figure 6b). Both the lower (L) and upper (U) Y-structures (pRGM29 × pRGM67 heteroduplexes) competed similarly for complexes A−C, as well as for the cruciform-specific complex D. When a cruciform-like structure with stems of unequal length (asymmetric cruciform), produced by heteroduplexing pRGM21 with pRGM67 DNAs (Figure 1b), was used as a competitor, the results were similar to those obtained above with the Y-structures (Figure 6b). However, the competition of complex D by either the pRGM29 × pRGM67 (L or U) or the pRGM21 × pRGM67 heteroduplexes was not as efficient as competition by the symmetrical cruciform (pRGM21 × pRGM29) (Figure 5), that is, while the latter competed complex D fully at 1500-fold mole excess, neither the pRGM29 × pRGM67 nor the pRGM21 × pRGM67 heteroduplexes competed fully, even at 2000-fold mole excess. These results, taken together, indicate that the shifted material in D requires an intact cruciform DNA structure to interact specifically and stably (see also below).

Nuclease Activity. Most proteins that have been identified as recognizing four-way junctions are nucleases [reviewed in Duckett *et al.* (1992)], with a few reported exceptions (Bianchi *et al.*, 1989; Paillard & Strauss, 1991; Krylov *et*

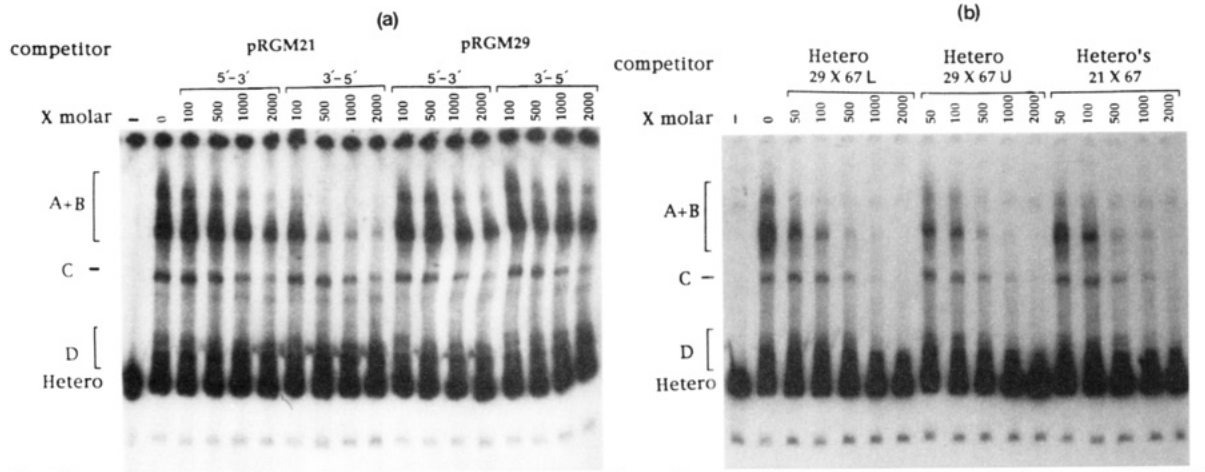

FIGURE 6: Other competitors. Competition reactions were performed exactly as in Figure 4. (a) Isolated single-stranded *Hin*dIII-*Sph*I fragments of either pRGM21 or pRGM29 are shown. (b) Isolated heteroduplex Y-structures formed between pRGM29 × pRGM67 L and U (see text and Figure 1) or isolated heteroduplex structures formed between pRGM21 × pRGM67 (see text and Figure 1) were included at the indicated mole excess amount. In both panels A−D are as in previous figures.

al., 1993; Varga-Weisz *et al.*, 1993). To investigate whether the binding activity that we are monitoring also contained nuclease activity, we incubated the binding reactions for 2 h at 37 °C, in the presence or absence of $MgCl_2$, ATP, and NAD^+/NADH (alone and in combination). Reactions were then treated with proteinase K and analyzed on either native or denaturing polyacrylamide gels. No decrease in the amount of labeled DNA was observed, nor was there any evidence of site-specific single-stranded nuclease activity (data not shown). We have demonstrated previously (Nobile & Martin, 1986; Frappier *et al.*, 1989) that the cruciform structure used herein is susceptible to digestion by S1 and MBN, which are specific for single-stranded regions (Sheflin & Kowalski, 1985), as well as the phage T7 endonuclease I, which is specific for DNA four-way junctions [reviewed in Duckett *et al.* (1992)].

Novel Cruciform Binding Activities. The ubiquitous and abundant high-mobility group 1 (HMG1) protein has been reported to bind specifically to Holliday-like junctions (Hamada & Bustin, 1985; Elborough & West, 1988; Bianchi, 1988; Bianchi *et al.*, 1989) as well as to sharply bent DNAs (Bianchi *et al.*, 1992). CBP(s), like HMG1 (Elborough & West, 1988; Bianchi, 1988) and cruciform-specific antibodies (Frappier *et al.*, 1987, 1989), also result in slightly shifted protein−DNA complexes. In order to test whether the observed complexes were due to binding of HMG1 or to a novel protein(s), band shifts were performed with calf thymus HMG1 (kindly provided by Dr. M. Bustin) and compared to those obtained with the HeLa cell extracts (Figure 7). HMG1 bound to the cruciform molecules but did not bind to the isolated homoduplex linear DNA fragments (Figure 7) nor to pRGM21 or pRGM29, separately (data not shown). Preliminary experiments using HMG1 reveals a single complex deriving from the cruciform (pRGM21 × pRGM29) (data not shown). Subsequently, when we compared the complexes obtained with CBP and HMG1, two complexes were detected for HMG1 (Figure 7). The upper most complex likely resulted from the complete HMG1 protein and the lower complex, probably, from a degradation product of HMG1, such as HMG3 (Goodwin *et al.*, 1978), which has been demonstrated to also have binding affinity for DNA (Bustin & Soares, 1985; Bianchi *et al.*, 1989). However, the possibility that the upper complex may represent multiple

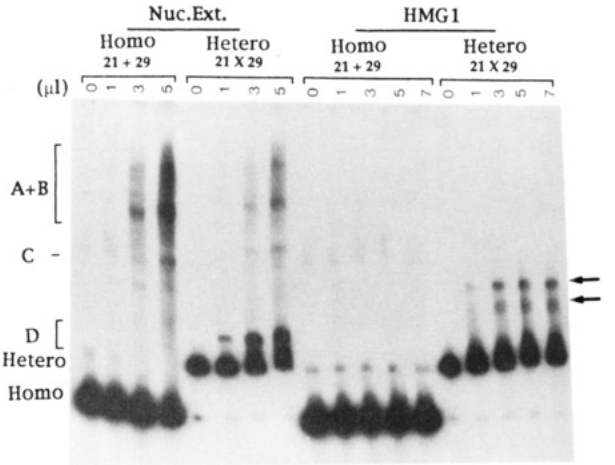

FIGURE 7: Unique cruciform DNA binding is a novel protein(s). Using ^{32}P-labeled isolated homoduplex (pRGM21 + pRGM29) and ^{32}P-labeled isolated cruciform (pRGM21 × pRGM29) as indicated, binding reactions were incubated on ice for 15 min with the indicated volumes of HeLa cell nuclear extract (165 ng of protein/μL) or with the indicated volumes of calf thymus HMG1 (43 ng/μL). A−D are as in previous figures. The arrows indicate the HMG1−cruciform complexes.

molecules of HMG1 on one cruciform molecule (Bianchi *et al.*, 1989) cannot be excluded. Both of the HMG1-specific complexes had a different mobility from the nuclear extract-shifted bands that are specific to the cruciform DNA (D).

CBP Sediments in Glycerol as a 66 kDa Protein. To further purify the cruciform-specific binding activity, crude HeLa cell extracts were enriched by chromatography (see Materials and Methods) and fractions were assessed by band-shift assays. Those fractions which were enriched for cruciform-specific binding activity were pooled. The binding activities from crude and purified fractions are shown in Figure 8a. The cruciform binding activity of the nuclear extract or a combination of the nuclear and cytoplasmic extracts yields similar cruciform-specific complexes (Figure 8a, complex D, arrow). Cruciform-specific binding activity was not bound to the Affi-Gel Heparin column and was recovered in the flow-through (FT_H; Figure 8a). A higher resolution gel (8% polyacrylamide) of the cruciform-specific binding complexes was also performed (Figure 8b); band-shifted complexes A and B did not enter into this gel. In

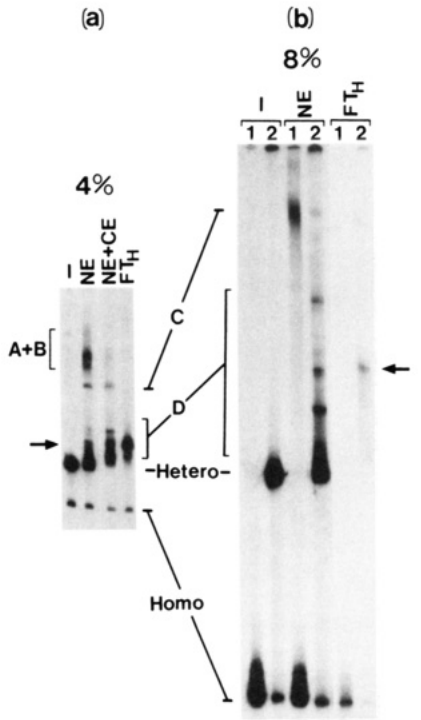

FIGURE 8: Enrichment of cruciform-specific binding. Crude extracts were enriched for cruciform DNA binding, as described in the text. Binding activity was assayed as in Figure 7, with either 3.5 μL of HeLa cell nuclear extract (NE; 165 ng of protein/μL), 3.5 μL of nuclear extract plus cytoplasmic extract (NE + CE; 1 μg/μL) used at a ratio of 8:15 (v:v), or 3.5 μL of enriched fraction (FF_H; 1 μg/μL). Reactions were run on (a) 4% polyacrylimide gels, using ^{32}P-labeled isolated cruciform (pRGM21 × pRGM29), or (b) long 8% polyacrylimide gels, using either ^{32}P-labeled isolated homoduplex (pRGM21 + pRGM29) DNA (lanes 1) or ^{32}P-labeled isolated cruciform (pRGM21 × pRGM29) DNA (lanes 2). Lanes containing the protein-free DNA are indicated by a slash. The panels are shown to scale. In both panels, A−D are as in previous figures. Complexes A and B did not enter into the 8% gel.

the crude nuclear extract (NE) lane there are several bands contained in the cruciform-specific band shift (D). The activities responsible for these different cruciform band-shift complexes eluted in different chromatographic fractions (unpublished results), suggesting that they may be different proteins. These results suggest that in addition to HMG1 and the CBP described here there may be other proteins that recognize and bind specifically to DNA cruciforms. The unbound fraction (FT_H) from the Heparin column is enriched for one of the cruciform-specific band shifts in complex D (Figure 8b) and does not bind to the linear homoduplex (homo).

The unbound fraction from the Heparin column (FT_H), enriched for cruciform-specific binding activity, was sedimented on a glycerol gradient (Malkas *et al.*, 1990; see Materials and Methods), and fractions were assayed for binding activity (Figure 9). Again, binding is specific for the cruciform-containing heteroduplex molecule (hetero); no binding is observed to the linear homoduplex DNA (homo). The cruciform-specific binding activity sedimented just above the marker hemoglobin (64 kDa), indicative of a molecular weight for CBP of approximately 66 kDa. It is important to note that we (Frappier *et al.*, 1987, 1989) as well as others (Bianchi, 1988) have found that the extent of retardation of a particular protein−cruciform DNA complex does not necessarily reflect the molecular weight of the binding protein. These results are in agreement with the above experiments that show distinct complexes generated by HMG1 (Figure 7). Further evidence that CBP is distinct from HMG1 was obtained by a Western blot analysis (see Materials and Methods) (Figure 10). The crude HeLa cell extracts (Figure 10, NE + CE), which were loaded onto a DEAE-Sephadex column (see Materials and Methods), the pools of fractions that were eluted from the DEAE-Sephadex column (Figure 10, FT and A−D), and the CBP-enriched fraction from the glycerol gradient (Figure 10, H) were subjected to SDS−polyacrylamide gel electrophoresis (Laemmli, 1970), blotted, and probed with anti-HMG1 antibodies (a gift from Dr. M. Bustin). Pool B, containing the CBP activity, did not contain an immunoreactive band at the expected molecular weight of HMG1 (28 kDa; Bustin *et al.*, 1990). Such a band was detected in both the crude cell extracts and in pool C (Figure 10, lanes NE + CE and C, see arrow), indicating that this is the only pool containing HMG1. [The series of cross-reactive bands present in all lanes (Figure 10) has also been previously observed in Western blots using anti-HMG antibody preparations (Pil & Lippard, 1993).] The above results (Figures 7 and 10) indicate that CBP is distinct from HMG1.

CBP Binds in a Structure-Specific Manner. To determine whether CBP can bind to cruciforms regardless of DNA sequence, we assayed the binding of the CBP-enriched glycerol gradient pool (H) to cruciform-containing heteroduplexes (pUC7 × pRGM128; Figure 11a, only one of the two possible heteroduplex structures is shown) with a sequence that is unrelated to that of pRGM21 × pRGM29. Heteroduplexes formed between pUC7 × pRGM128 (Frappier *et al.*, 1989) (Figure 11a) contain cruciforms formed by the 48 bp inverted repeat of pUC7 (Vieira & Messing, 1982) and the 56 bp inverted repeat of pRGM128 (Figure 11a). Both possible heteroduplex structures contain six bases that permit alternative base pairing at the junction point, thus allowing limited branch migration (Figure 11a, bold lines). This can lead to cruciforms that have been 18 and 24 bp in one stem-loop and between 22 and 28 bp in the other. The two resulting heteroduplexes migrate as a single distinct band, slower than either of the input homoduplexes in 4% polyacrylamide (Figure 11b). When isolated linear homoduplex or cruciform-containing heteroduplex pUC7 × pRGM128 molecules were used in band-shift assays with the enriched CBP fraction from the glycerol gradient, only the cruciform molecules were bound (Figure 11b). No binding of CBP to the linear homoduplexes could be detected, whereas the heteroduplexes were bound giving rise to a shifted band (Figure 11b, large arrow). Using unlabeled isolated linear homoduplexes (mixture of pUC7 + pRGM128) or unlabeled isolated cruciforms (pUC7 × pRGM128) as competitor DNAs, we observed that the CBP−cruciform complex could not be competed by increasing amounts of the linear pUC7 + pRGM128 homoduplex mixture but was competed when as low as 25-fold mole excess of the cruciform molecules was used, and binding was abolished with 100-fold mole excess of the cruciform competitor. These results indicate that CBP recognizes and binds to the pUC7 × pRGM128 cruciforms in a structure-specific manner. Furthermore, the complex of CBP bound to the pUC7 × pRGM128 cruciform could not be competed by increasing amounts of the linear pRGM21 + pRGM29 homoduplexes but was competed when as low as 25-fold mole excess of

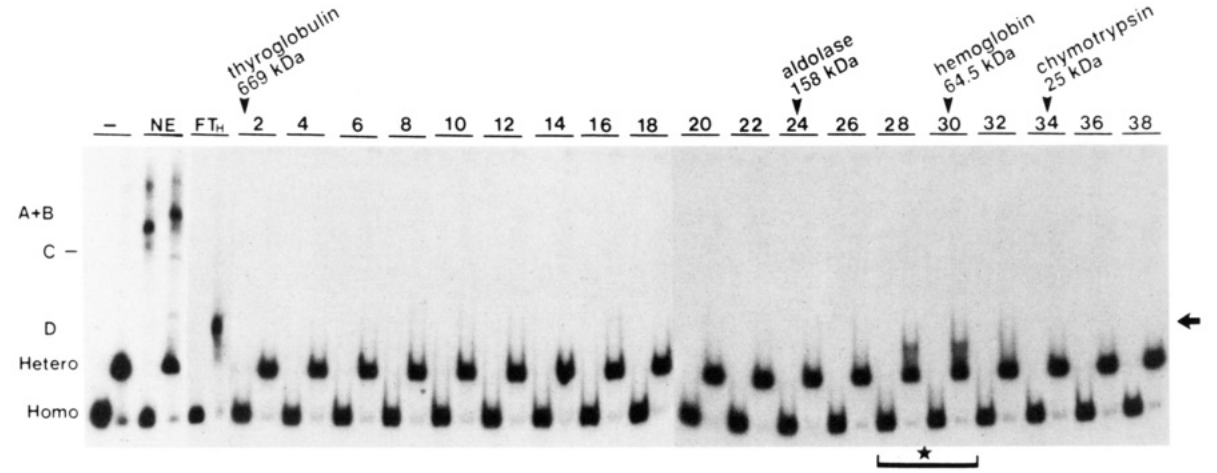

FIGURE 9: Glycerol gradient sedimentation. The fraction enriched for DNA cruciform-specific binding was sedimented on a 10–40% glycerol gradient, essentially as described (Malkas *et al.*, 1990; see Materials and Methods), 39 fractions of 100 μL each were taken from the bottom, and 15 μL of each fraction was used in band-shift assays with either ^{32}P-labeled isolated homoduplex (pRGM21 + pRGM29) or ^{32}P-labeled isolated cruciform (pRGM21 × pRGM29), as indicated. Also shown are the control DNAs alone and DNA with 3.5 μL of HeLa cell nuclear extract (NE; 165 ng/μL) or with 3.5 μL of enriched fraction (FT_H; 1 μg/μL). Arrow heads indicate the fractions in which the marker proteins sedimented in parallel gradients. An arrow indicates the cruciform-specific band-shifted complex. A bold bracket encompasses the fractions in which the CBP sediments. A star denotes the fraction containing the peak binding activity.

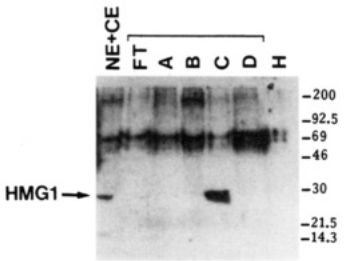

FIGURE 10: Distribution of HMG1. A Western blot was performed with anti-HMG1 (see Materials and Methods) using crude nuclear and cytoplasmic extracts (NE + CE), pooled fractions from the DEAE-Sephadex column flow-through (FT), 82–200 mM salt fraction (A), 200–450 mM salt fraction (B), 450–700 mM salt fraction (C), 700–1000 mM salt fraction (D), and the enriched fraction from the glycerol gradient (H). Molecular weight marker sizes are indicated in kilo Daltons. The HMG1 band, present in the crude extracts, NE + CE, and fraction pool C, is indicated by an arrow.

the cruciform (pRGM21 × pRGM29) molecules was used, and binding was abolished with 100-fold mole excess of the cruciform competitor (Figure 11b). Both the pUC7 × pRGM128 and the pRGM21 × pRGM29 cruciform molecules competed with similar efficiency, indicating that CBP recognizes and binds to them with similar affinity. Identical results were obtained when unlabeled linear homoduplex or cruciform pUC7 × pRGM128 molecules were used as competitors against complexes formed by CBP binding to labeled pRGM21 × pRGM29 cruciforms (Figure 11c). When isolated linear homoduplex or cruciform-containing heteroduplex pRGM21 × pRGM29 molecules were used in band-shift assays with the enriched CBP fraction from the glycerol gradient, only the cruciform molecules were bound (Figure 11c). No binding of CBP to the linear homoduplexes could be detected, whereas the heteroduplexes, again, were bound giving rise to a shifted band (Figure 11c, large arrow). These results are in agreement with those shown in Figure 9. Using unlabeled isolated linear homoduplexes (mixture of pUC7 + pRGM128) or unlabeled isolated cruciforms (pUC7 × pRGM128) as competitor DNAs, we observed that the CBP–cruciform complex could not be competed by increasing amounts of the linear pUC7 + pRGM128 homoduplex mixture but was competed when as low as 25-fold mole excess of the cruciform molecules was used, and binding was abolished with 100-fold mole excess of the cruciform competitor. The unlabeled pUC7 × pRGM128 cruciform competed against the complex of CBP bound to labeled pRGM21 × pRGM29 cruciform (Figure 11c) with similar efficiency as the unlabeled pRGM21 × pRGM29 cruciform competed against the complex of CBP bound to labeled pUC7 × pRGM128 cruciform (Figure 11b). These results demonstrate that CBP recognizes DNA cruciforms in a structure-specific manner that is independent of DNA sequence.

DISCUSSION

Cruciform extrusion from an inverted repeat requires negative supercoiled tension (Mizuuchi *et al.*, 1982). Supercoiling of the DNA can regulate many biochemical processes, including transcription (Pruss & Drlica, 1989), replication (von Freiesleben & Rasmussen, 1992), and recombination (Cozzarelli & Wang, 1990). Cruciform extrusion will absorb/relax as many negative supercoils as the number of turns that exist in the stems of the cruciform (White & Bauer, 1987). In this manner cruciform extrusion or melting may regulate the supercoiled state of a particular region of DNA, which, in turn, may affect protein recognition/binding to distal sequences. Cruciforms may form transiently *in vivo*, to act as special regulatory signals on the DNA for transcription, recombination, or replication and serve as the attachment site for protein factors. Cruciform-specific proteins can affect the regulation of these processes (Zannis-Hadjopoulos *et al.*, 1988; Hiasa *et al.*, 1990; Noirot *et al.*, 1990; Waga *et al.*, 1990; Zechiedrich & Osheroff, 1990; Sun & Godson, 1993; Toth *et al.*, 1993).

Using monoclonal antibodies with unique specificity to cruciform DNA structures (Frappier *et al.*, 1987, 1989), we have shown that there is a dynamic distribution of cruciforms in mammalian nuclei, their numbers being at a maximum at the G1/S boundary, suggesting a possible role at the onset of DNA replication (Ward *et al.*, 1990, 1991). In support of this hypothesis, introduction of the anti-cruciform mAbs

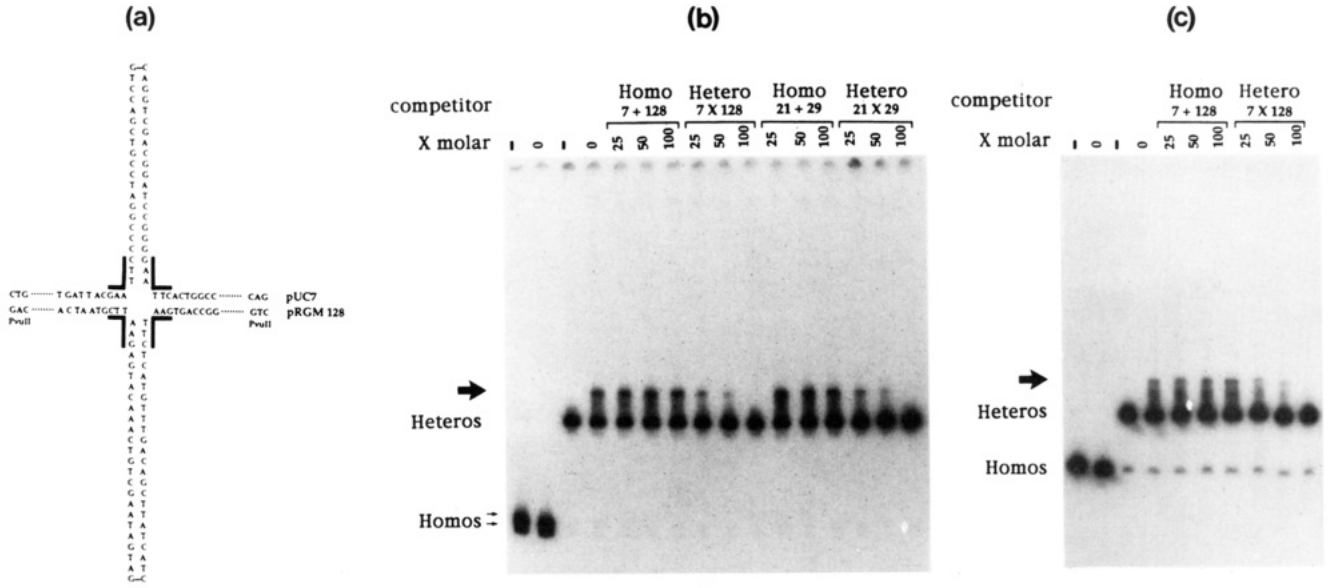

FIGURE 11: CBP is cruciform-specific. (a) Cruciform-containing heteroduplex composed of the *Pvu*II fragments of pUC7 and pRGM128 is shown. Only one of the two possible pUC7 × pRGM128 heteroduplex molecules is shown. The bases which allow limited branch migration are indicated by bold lines. (b) ^{32}P-labeled isolated *Pvu*II homoduplexes (pUC7 + pRGM128 mixture) or isolated cruciform (pUC7 × pRGM128) molecules were incubated with 4 μL of the CBP-enriched fraction H (0.45 μg/μL) from the glycerol gradient. Note that both the pUC7 × pRGM128 cruciforms migrate as a single distinct band of slower mobility than either of the linear homoduplexes (small arrows). The large arrow indicates a cruciform-specific band-shifted complex. (c) ^{32}P-labeled isolated *Hin*dIII-*Sph*I homoduplexes (pRGM21 + pRGM29 mixture) or isolated cruciform (pRGM21 × pRGM29) molecules were incubated with 4 μL of the CBP-enriched fraction H (0.45 μg/μL) from the glycerol gradient. The large arrow indicates a cruciform-specific band-shifted complex. Binding and competition assays were performed as in Figure 4, except that poly(dI-dC) was omitted. The various competitors and the mole excess amounts are indicated.

to a permeabilized cell system yielded up to a 6-fold enhanced nucleotide incorporation into replicating DNA and an enhanced copy number of early replicating DNAs such as *ors* 8, *DHFR*, and *c-myc* (Zannis-Hadjopoulos *et al.*, 1988). *ors* 8, an origin-enriched sequence activated at the onset of S phase (Zannis-Hadjopoulos *et al.*, 1985), contains an IR that extrudes into a cruciform (Bell *et al.*, 1991).

On the basis of the hypothesis that there exist specific cellular recognition proteins for DNA cruciforms, we have identified and partially characterized a novel cruciform DNA binding activity, CBP, from HeLa cell extracts. CBP is specific for cruciform DNAs, regardless of sequence, may require length symmetry in the stems, is void of nuclease activity, and has an apparent molecular weight of 66 kDa.

A number of mammalian cellular proteins has been reported, which recognize Holliday-like four-way DNA junctions [for a review see Duckett *et al.* (1992), Zechiedrich and Osheroff (1990), Krylov *et al.* (1993), Varga-Weisz *et al.* (1993)] and other cruciform-like structures (Paillard & Strauss, 1991), among them HMG1 (Bianchi, 1988). HMG1 binds to single-stranded DNA (Hamada & Bustin, 1985), B−Z DNA junctions (Hamada & Bustin, 1985), and four-way junctions (Hamada & Bustin, 1985; Bianchi, 1988). Although HMG1 is an abundant nuclear protein (Bonne-Andrea *et al.*, 1986; Bustin *et al.*, 1990), we did not detect the HMG1-specific complex in band-shift assays with crude HeLa nuclear extracts. This suggests that the CBP described in this paper has a greater affinity for the cruciform DNA substrate used in this study over and above that of HMG1. One explanation might be the difference in the cruciform DNA substrates used; in contrast to other DNA binding studies (Bianchi, 1988; Elborough & West, 1988; Bianchi *et al.*, 1989), the cruciform structure used in the present study is composed of two and not four separate molecules (Figure 1). Stem-loop conformation and dynamics are sensitive to small changes in the loop and adjacent stem sequences (Blommers *et al.*, 1989; Williamson & Boxer, 1989; Germann *et al.*, 1990). Hence, the presence of loops on short stems may affect the overall structure of the four-way junction. The qualities of the two-molecule structure may affect the flexure, the writhe, and the intratorsional strain experienced by the molecule (Crothers & Fried, 1982; Travers, 1989; Petrillo *et al.*, 1988). These qualities could impact certain stiffness to the DNA structure which may be important for protein recognition (Crothers & Fried, 1982; Hogan & Austin, 1987; Travers, 1989) of the target as a cruciform, as opposed to a four-way junction or a DNA crossover (Timsit & Moras, 1991).

The CBP(s) observed in this study are novel and distinct from HMG1, by two independent criteria: molecular weight [HMG1 is 28 kDa (Bustin *et al.*, 1990)] and differing chromatographic behavior (see Results). Several proteins, such as topoisomerase I and II [100 and 170 kDa, respectively (Cozzarelli & Wang, 1990)] (Zechiedrich & Osheroff, 1990), HMG1 (Lilley, 1992), and the histone H1 [approximately 21 kDa (Cole, 1984)] (Krylov *et al.*, 1993), have been implicated in binding specifically to DNA crossovers which occur at points of DNA looping and folding and at nucleosome linkers (Timsit & Moras, 1991). Recently, it was reported that H1 could bind specifically to a stable four-way DNA junction (Varga-Weisz *et al.*, 1993), which is structurally similar to DNA crossovers that occur at the point where DNA strands enter and exit the nucleosome (Hartman *et al.*, 1980; Timsit & Moras, 1991). Based on its molecular weight, CBP is different from these proteins.

In addition to the CBP protein(s), sequence-specific binding activities were observed. These activities might be related to other cellular proteins binding the SV40 core origin

that have been reported by others (Baur & Knippers, 1988; Alliger *et al.*, 1988; Gaillard *et al.*, 1988; Jones *et al.*, 1988; Malkas & Baril, 1989; Galli *et al.*, 1992; Carmichael *et al.*, 1993). The competition experiments reported here indicate that the sequence-specific binding proteins interact with sites that are present in the late region of the origin. The AT-tract, directly adjacent to the central inverted repeat of the SV40 origin of replication, is known to be specifically recognized by the AT-tract binding protein (Malkas & Baril, 1989) that cofractionates with a multiprotein DNA polymerase α-primase complex of HeLa cells (Malkas *et al.*, 1990). Another factor, the LOB (late origin binding protein), binds specifically to the AT-tract and has been demonstrated to induce bends into the DNA (Baur & Knippers, 1988). The apparent molecular weight of the AT-tract binding protein is 62 kDa, while the LOB protein sediments in sucrose at 3.5 S, just below hemoglobin. Both of these proteins were reported to bind in a sequence-specific manner.

An effect of the cruciform upon sequence-specific binding in *cis* was observed. It appears that the presence of a cruciform stabilizes the binding of a protein(s) at a site proximal to it. Cruciforms are known to effect structural alterations in the flanking sequences (Gough & Lilley, 1985; Diekmann & Lilley, 1987; McLean & Wells, 1988; Klysik, 1992). This mode of transmission along the DNA molecule (Crothers & Fried, 1982) has been previously observed [Sullivan and Lilley (1986) and Klysik (1992) and references therein] and is referred to by Wells and co-workers as "telestability" (Burd *et al.*, 1975). This transmission has been demonstrated to occur over long distances and can affect protein DNA interactions such as the interaction of RNA polymerase with promoters (Klein & Wells, 1982) and nuclease specificities (Drew & Travers, 1985). However, the effect of a cruciform upon proximal protein binding has not been previously reported.

Histones bind poorly to inverted repeats (Weintraub, 1983) or DNA stem-loop structures (Nickol & Martin, 1983; Nobile *et al.*, 1986; Battistoni *et al.*, 1988); thus, it is likely that cruciform structures exist in the spacer region between nucleosomes. Cruciforms may play a role in nucleosome phasing, such that they expose nucleosome-free DNA sequences allowing them to be bound by specific DNA binding proteins for processes such as transcription, recombination, and replication (Nickol & Martin, 1983; Nobile *et al.*, 1986; Battistoni *et al.*, 1988). A yeast ARS placed within the nucleosome severely reduced function compared to its normal location in the linker region (Simpson, 1990). We (Frappier *et al.*, 1989) and others (Parsons *et al.*, 1990; Lu *et al.*, 1989; Murchie *et al.*, 1990) have observed that DNA four-way junctions are particularly resistant to digestion by *DNase*I. This is probably due to the inaccessibility of the DNA at the junction point. It is interesting that the observed inherent resistance of the cruciforms to digestion by *DNase*I is partially relieved by the presence of adjacent nucleosomes (Nobile *et al.*, 1986), that is, the stem-loop structures showed an increased relative accessibility to *DNase*I in nucleosomes when compared with naked heteroduplexes. This suggests an alteration in the structure of the DNA four-way junction by nucleosome formation.

It is conceivable that the extrusion of certain cruciforms may be facilitated and/or stabilized by nuclear factors, causing nucleosome phasing that renders the cruciform and the DNA proximal to it available for other transcription, recombination, or replication factors. Cruciform formation may also induce flexural alternations in proximal DNA flanking the junction, which would effect the binding of other factors, and may facilitate unwinding of the duplex for transcription or replication priming. As we have previously suggested (Zannis-Hadjopoulos *et al.*, 1984, 1988), transiently forming cruciforms would be ideally placed at replication origin sites, since such sites must be activated only once per cell cycle in a normal cell. Temporal regulation of cruciform formation at these sites could potentially be achieved by cruciform binding proteins, which might stabilize or facilitate the formation of cruciforms.

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