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Interaction of a protein from rat liver nuclei with cruciform DNA

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We constructed a synthetic cruciform DNA which closely resembles Holliday junctions, a DNA structure formed during recombination or following the transition from interstrand to intrastrand base pairing in palindromic DNA sequences. We identified and partially purified a protein from rat liver that specifically binds to this cruciform DNA molecule and does not bind to single-stranded or double-stranded DNAs of the same sequence. This protein also binds to the cruciform structure formed by a 70 bp palindromic sequence cloned in plasmid pUC18. No detectable nucleolytic activity is associated with the rat liver cruciform-binding protein, in contrast to all cruciform-recognizing proteins known so far.

Key words: cruciform DNA/DNA-protein interactions/Holliday junctions/palindromes/rat liver

Introduction

Cruciform structures are postulated to occur in DNA as a consequence of (i) general and site-specific recombination, and (ii) the transition from interstrand base pairing to intrastrand base pairing in inverted repeated (palindromic) DNA sequences. These cruciform structures are commonly referred to as Holliday junctions, and molecules which contain them have been isolated repeatedly (Bell and Byers, 1979; Wolgemuth and Hsu, 1981; Mizuuchi *et al.*, 1982a). Essentially, Holliday junctions can be described as a four-way junction of two duplex molecules attached to each other by way of a reciprocal single-strand crossover.

Holliday junctions that arise during recombination must be eventually cleaved to restore the individuality of the interacting DNA molecules (Holliday, 1964). Several nucleases that recognize and cleave Holliday junctions have been identified: examples are phage T4 gene49 product (Mizuuchi *et al.*, 1982a), phage T7 gene3 product (de Massy *et al.*, 1984) and λ phage int protein (Hsu and Landy, 1984). A cruciform-cleaving nuclease from the lower eukaryote *Saccharomyces cerevisiae* has also recently been described (West and Koerner, 1985; Symington and Kolodner, 1985).

In addition to nucleases, however, other proteins may exist that recognize and bind to cruciform DNAs: they might do so in order to act as antagonists or modulators of the activity of the cruciform cleaving enzymes, or to facilitate or stabilize the extrusion of the potentially unstable cruciform structures generated by palindromic sequences. Inverted repeats have been associated with initiation of mammalian DNA replication (Frisque, 1983; Weller *et al.*, 1985; Zannis-Hadjopoulos *et al.*, 1984) and are rather common in the vicinity of structural genes, so that they might play a role in gene expression. The formation of cruciform structures from palindromic sequences has been demonstrated *in vitro* (Mizuuchi *et al.*, 1982b). It is not clear whether palindromes can give rise to Holliday junctions also in physiological conditions (Courey and Wang, 1983), but the inability of *Escherichia coli* to propagate large palindromes suggests that under some conditions cruciform structures can be extruded from such sequences (Lilley, 1981).

We have therefore set out to identify mammalian proteins that can interact specifically with cruciform DNAs, without selecting in favor of nucleolytic enzymes. We report here the construction of an artificial Holliday junction and the initial characterization of a protein from rat liver nuclei that binds to this molecule.

Results

Construction and properties of a synthetic cruciform DNA

Naturally occurring Holliday junctions are inherently unstable because of their internal sequence symmetry, which permits their resolution to double helices via spontaneous branchpoint migration. Kallenbach *et al.* (1983) have shown that non-symmetrical cruciform DNAs can be constructed from relatively short oligonucleotides, and that these are stable in solution. We have followed their strategy and constructed the non-symmetrical cruciform DNA represented in Figure 1. The sequences surrounding the four-way junction were so designed as to avoid alternative pairings through branch-migration: in addition, the four arms of the structure are all different in length, so that each could be recognized through its migration properties after cleavage by specific endonucleases. It should be noted that although the structure depicted in Figure 1 resembles a true Holliday junction in having four arms and a central four-way junction, unlike natural Holliday junctions its arms are all different in sequence, and no torsional stress is built into the structure. A further difference is that our artificial structure contains four double-stranded (ds) termini (which have a 5′ overhang for convenient labeling with DNA polymerase), while natural Holliday junctions occur in the middle of long DNA molecules without nearby termini. Cruciform structures extruded from palindromic sequence possess a single-stranded (ss) loop at the tip of two of their arms, and this feature is also lacking in our model molecule.

The formation of the four-stranded artificial Holliday junction was monitored in the following way (data not shown): oligonucleotide 1 was labeled with polynucleotide kinase and annealed to an equimolar amount of oligonucleotide 2; the half-junction thus formed migrated slower in a 15% polyacrylamide gel than oligonucleotide 1 alone. Sequential addition of oligonucleotides 3 and 4 produced molecules that migrated progressively slower. Having determined experi-

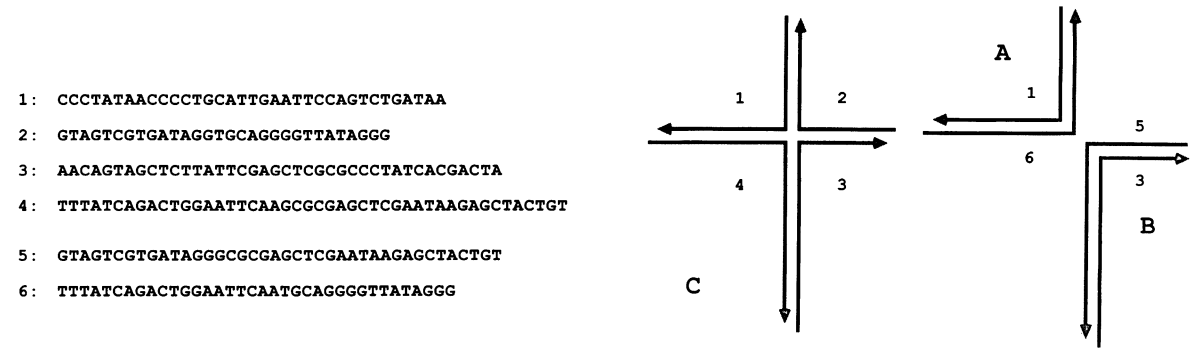

Fig. 1. Design of the synthetic cruciform DNA and of the control duplex DNAs. Oligonucleotides 1−4 are partially complementary to each other and, when annealed, assemble into the cruciform molecule indicated with **C**. This molecule, unlike natural cruciform DNAs, lacks any sequence symmetry about the centre, so that the central branchpoint is fixed and cannot migrate. Although the molecule is represented here on a plane, it probably is tetrahedral in solution and assumes a distorted tetrahedral configuration during gel electrophoresis. Oligonucleotides 5 and 6 are complementary to oligonucleotides 3 and 1, respectively. On annealing they form perfectly duplex molecules (indicated with **A** and **B**), whose sequences are the same as half of the cruciform DNA: to highlight this point, the duplex molecules are drawn containing an angle as the corresponding parts of molecule **C**, although they would presumably be perfectly straight both in solution and during gel migration.

mentally by titration the amounts of oligonucleotides to be mixed to obtain a 1:1:1:1 molarity, the cruciform DNA was constructed on all successive occasions by heating a mixture of the four oligonucleotides to 70°C and cooling it to 25°C over a 2 h period. The cruciform DNA migrates slower than any of the partially annealed products in a polyacrylamide gel, from which it can be extracted for further purification. The cruciform isolated from the gel is stable at 0°C in the presence of 100 mM NaCl and 10 mM $MgCl_2$ and can be rerun at room temperature in polyacrylamide gels yielding essentially a single band.

The identity of the putative cruciform DNA made in this fashion was verified by two criteria: its hydrodynamic properties and its oligonucleotide composition. The peculiar structure of Holliday junctions influences their electrophoretic behavior (Gough and Lilley, 1985). Our artificial cruciform DNA consists of only 151 nucleotides, but it behaves as a linear DNA of 210 bp in a 13% polyacrylamide gel and as a linear DNA of 115 bp in a 4% polyacrylamide gel (Figure 2a). Its hydrodynamic properties were similar also in exclusion chromatography, although this proved inferior in resolving power (data not shown). The most stringent test of its structure, however, was performed by reisolating from a gel the cruciform DNA formed by annealing four oligonucleotides labeled together in a single polynucleotide kinase reaction. The material eluted from the polyacrylamide slice was applied to a sequencing gel: the four oligonucleotides reappeared in equimolar ratio (Figure 2b). The intensity of the band corresponding to oligonucleotide 3 was in fact lower than the others, a result that can be attributed to the somewhat lower efficiency of labeling of this particular oligonucleotide.

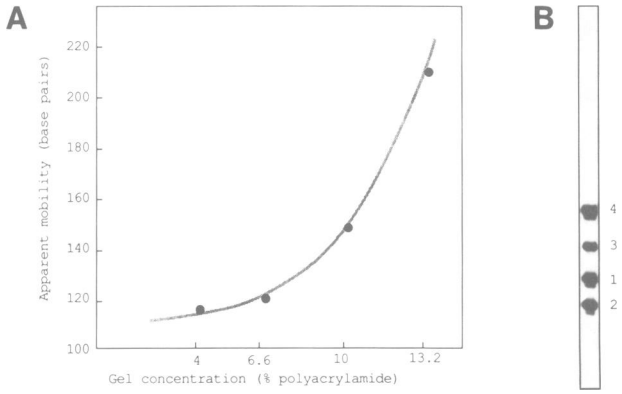

Fig. 2. Structural and hydrodynamic properties of the artificial cruciform DNA. **Part A.** Electrophoretic mobilities. Labeled cruciform DNA was subjected to electrophoresis in polyacrylamide gels of different concentration, and its apparent mol. wt (in bp) was determined by comparison with labeled DNA fragments from *Hin*fI-digested plasmid pUC19. **Part B.** Oligonucleotide composition. Ten pmol of oligonucleotides 1−4 were labeled with T4 polynucleotide kinase and 25 pmol of [γ-^{32}P]ATP. They were then mixed and annealed; the mixture was electrophoresed through a preparative 6.5% polyacrylamide gel. The slowest migrating band was cut out and incubated for 1 h at 80°C in 1 ml of distilled water. The eluted material was lyophilized and 1/20 of it was applied to a 10% polyacrylamide−urea sequencing gel alongside appropriate mol. wt markers.

A protein from rat liver nuclei binds to the synthetic cruciform DNA

We next investigated whether the synthetic cruciform DNA we had constructed could be used as reporter molecule to assay for the existence of mammalian cruciform DNA-binding proteins. A nuclear extract obtained from rat liver by the procedure of Dignam *et al.* (1983) was tested in a gel retardation assay for its ability to bind to our synthetic cruciform, which was labeled by kinasing oligonucleotide 1 prior to annealing. In a parallel series of experiments, the preformed cold cruciform DNA was labeled by incubation with the Klenow fragment of *E.coli* DNA polymerase I and labeled dCTP. The results obtained did not differ from those that we report here.

A preliminary experiment showed that the crude extract produced a smeared pattern of labeled molecules trailing behind the free cruciform DNA. The extract also contained a large amount of nucleases, which significantly reduced the intensity of the signal from the reporter molecule after several minutes of incubation at 37°C. Addition of increasing amounts of unspecific competitor DNA (i.e. sonicated ds calf thymus DNA) reduced the degradation of the labeled cruciform DNA and revealed the existence of one major retarded band (Figure 3a). The intensity of this retarded band was approximately proportional to the amount of extract add-

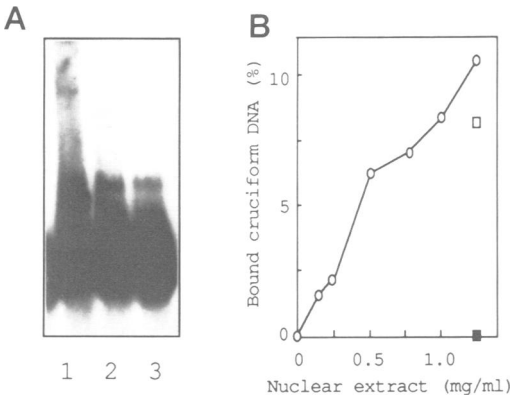

Fig. 3. The rat liver nuclear extract contains an activity that binds to cruciform DNA. **Part A.** One microliter (5 μg) of rat liver nuclear extract was added to 20 μl of binding mixture containing ~ 20 fmol of labeled synthetic cruciform DNA and 0.25 (**lane 1**), 1 (**lane 2**) or 4 μg (**lane 3**) of sonicated calf thymus DNA. After 5 min of incubation at 37°C, samples were withdrawn and subjected to electrophoresis as described in Materials and methods. **Part B**. The indicated amounts of rat liver nuclear extract (○——○), nuclear extract pretreated for 10 min at 37°C with 1 mg/ml of proteinase K (■), or nuclear extract pretreated for 10 min at 37°C with 1 mg/ml of pancreatic RNase (□) were added to 20 μl of binding mixture containing ~ 20 fmol of labeled synthetic cruciform DNA molecules and 2 μg of sonicated calf thymus DNA. After incubation for 10 min at 0°C, samples were withdrawn and subjected to electrophoresis as described in Materials and methods. The amounts of bound and free cruciform DNA were determined by densitometric scanning of the autoradiogram.

Fig. 4. The cruciform-binding protein binds to the synthetic cruciform DNA but not to control linear duplexes of the same sequence. Two microliters of fraction 5 of the cruciform-binding protein (**lanes** +) or its elution buffer (**lanes** −) were added to 20 μl of binding mixture containing ~20 fmol of the indicated gel-purified labeled DNA molecules and 0.6 μg of unlabeled calf thymus DNA. After incubation for 10 min at 0°C, samples were withdrawn and subjected to electrophoresis as described in Materials and methods. In order to achieve a good separation of the bound from the unbound cruciform molecules, and to avoid the loss from the gel of the faster-migrating linear control molecules, electrophoresis lasted the standard 4 h for the sample containing the cruciform DNA, but only 90 min for the samples containing the linear molecules.

ed (Figure 3b). The binding activity was resistant to treatment with RNase, but was completely eliminated upon incubation with proteinase K (Figure 3b).

The crude extract was then partially purified by batch adsorption to DEAE−Sepharose and chromatography on heparin−Sepharose, hydroxylapatite, MonoS and MonoQ. The pooled active fractions eluting from the MonoQ column still contained a mixture of polypeptide species, but were free of contaminating nucleases and could be characterized as shown below.

The cruciform-binding protein binds to the synthetic cruciform DNA but not to linear duplex molecules of identical sequence

To rule out the possibility that the cruciform DNA could in fact be bound by a protein recognizing a specific sequence present on the synthetic molecule, we synthesized two additional oligonucleotides (nos 5 and 6) which we utilized to produce two duplex molecules, each containing exactly the same sequences as one half of the cruciform DNA (see Figure 1).

We incubated the labeled cruciform DNA and the two duplex controls with an aliquot of cruciform-binding protein and a large amount of nonspecific competitor. Figure 4 shows that >50% of the cruciform DNA migrated as a single retarded band when incubated with cruciform-binding protein, while the control duplex DNAs were not bound.

As a further demonstration that the activity that binds to the synthetic cruciform DNA is structure-specific rather than sequence-specific, Figure 5 shows that the binding activity can be competed by cold cruciform DNA, but not by a mixture of the two duplex molecules which are equivalent to the linearized arms of the synthetic cruciform. The labeled cruciform DNA was incubated in the presence of the binding activity and a constant total amount of competitor DNA. When the sonicated calf thymus DNA (central lane) was progressively replaced by unlabeled cruciform DNA, the intensity of the retarded band gradually decreased; on the contrary, on replacing calf thymus DNA with unlabeled duplex arms, the intensity of the retarded band actually increased by ~50%, as revealed by densitometric scanning of the film. We observed that the decrease in intensity of the retarded band is not directly proportional to the ratio of unlabeled to labeled cruciform DNA present in the reaction mixture, which suggests that the large excess of calf thymus DNA can bind a substantial proportion of the cruciform-binding activity, in addition to all the contaminating duplex DNA binding proteins present in the partially purified extract. This may be due to the presence of a small amount of cruciform or branched DNA molecules in sonicated calf thymus DNA. In fact, linear duplex DNA from phage fd has approximately as low a competing ability as the duplex control molecules A and B, while fd circular ss DNA (which contains extensive secondary structures) has a moderate competing ability (Table I).

It has been shown that both synthetic and natural cruciform DNAs do not contain any unpaired bases (Wemmer *et al.*,

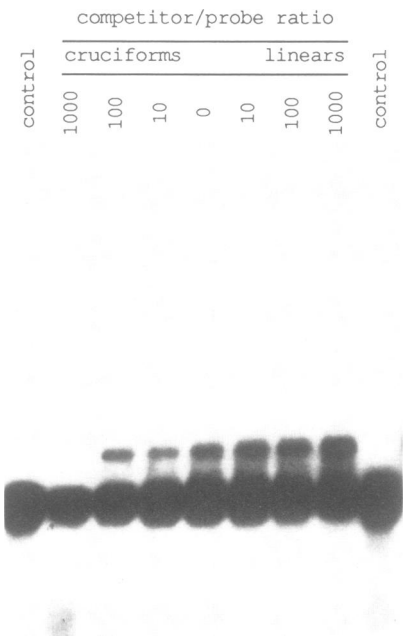

Fig. 5. Competition for binding between cruciform DNA and linear control duplexes. Nine reaction mixtures were prepared, each containing (in 20 μl) 10 fmol of labeled cruciform DNA and the indicated amount of unlabeled cruciform DNA or both unlabeled control linear duplexes (from 0.1 to 10 pmol). The total concentration of DNA in each reaction mixture was made equal to 55 μg/ml by adding the appropriate amount of calf thymus DNA (from a minimum of 0.6 μg in the reactions with the highest amount of specific competitor DNAs to a maximum of 1.1 μg in the reaction without specific competitor DNAs). After addition of 2 μl of fraction 5 of the cruciform-binding protein (or its elution buffer in the controls without protein) and 10 min of incubation on ice, the samples were electrophoresed as described in Materials and methods.

Table I. Competition to cruciform-binding by ds and ss phage fd DNA

Type of competitor DNA	Amount of cruciform DNA probe bound[a]		
	Concentration of competitor DNA (μg/ml)		
	0.5[b]	5.0	50.0
Linear ds fd DNA	0.94	0.83	0.53
Circular ss fd DNA	0.96	0.24	0.04
Synthetic cruciform DNA	0.66	0.03	<0.01

[a]The reaction mixture was as described in Materials and methods, except that calf thymus DNA was omitted. The amounts of bound and unbound cruciform DNA were estimated by densitometric scanning of the autoradiogram.
[b]At low total DNA concentrations, additional bands appeared that migrated slower than the cruciform DNA–cruciform-binding protein complex. These were probably due to contaminants present in the protein preparation, and were not taken into account in the calculation.

1985). However, in principle we could not rule out the possibility that, due to its small size or its actual base composition, our synthetic cruciform DNA could be incompletely base-paired: in this case, it could be a substrate for binding for a number of known mammalian proteins. We therefore tested whether the rat cruciform-binding protein could bind to ss DNA: each of the six oligonucleotides we had synthesized was labeled with kinase and incubated individually with an aliquot of the protein. In no case did the cruciform-

Fig. 6. Binding of the cruciform-binding protein to ss oligonucleotides. Two microliters of fraction 5 of the cruciform-binding protein (**lanes** +) or its elution buffer (**lanes** −) were added to reaction mixtures containing 5 fmol of the indicated kinase-labeled oligonucleotides and 0.6 μg of unlabeled calf thymus DNA. After incubation for 10 min at 0°C, the samples were subjected to electrophoresis as described in Materials and methods, except that a 12% polyacrylamide gel was used instead of a 6.5% gel.

binding protein retard the migration of any of the ss oligonucleotides (Figure 6), and we concluded that the cruciform-binding protein had no affinity for ss DNA with limited secondary structure.

The cruciform-binding proteins binds with reduced affinity to imperfect cruciform molecules

To further define the specificity of the cruciform-binding protein, we also constructed incomplete cruciform molecules and tested them in the gel retardation assay. Oligonucleotide 1 was labeled at its 5′ end with T4 polynucleotide kinase and annealed with oligonucleotide 2, or with oligonucleotides 2 and 4, forming the molecules indicated by D and E in Figure 7, respectively. Molecule D is partially ss and partially ds, and is equivalent to the transition from paired to unpaired segments in ss DNA. Molecule E contains two segments of duplex DNA, which can rotate independently of each other about the center of the molecule, from which two segments of ss DNA also depart. The cruciform-binding protein does not bind at all to molecule D, while it has a low affinity for molecule E, which resembles more closely a complete cruciform DNA (Figure 7). By comparison, the complete cruciform was bound to >40% in the same experiment.

The rat cruciform-binding protein has no detectable nuclease activity

The only class of cruciform-recognizing proteins described so far are nucleases (Mizuuchi *et al.*, 1982a; Hsu and Landy, 1984; de Massy *et al.*, 1984; West and Koerner, 1985; Symington and Kolodner, 1985). We therefore tested for a nucleolytic activity of our protein. Prolonged incubations (2 h at 37°C) of the synthetic cruciform with the cruciform-binding protein did not yield any faster migrating DNA species (data not shown). The addition of such cofactors as ATP or NAD^+/NADH also had no effect.

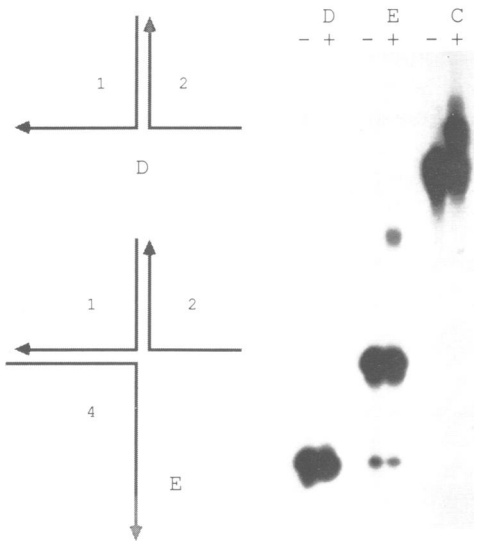

Fig. 7. Binding of the cruciform-binding protein to incomplete cruciform molecules. Incomplete cruciform molecules were generated by mixing labeled oligonucleotide 1 with unlabeled oligonucleotide 2 (molecule **D**), or with oligonucleotides 2 and 4 (molecule **E**). Complete cruciform molecules (**C**) were generated by mixing together oligonucleotides 1 − 4. The annealed molecules were then purified by gel electrophoresis. Incomplete and complete molecules (10 fmol) were then incubated under standard conditions with cruciform-binding protein (**lanes** +) or elution buffer (**lanes** −). Electrophoresis was performed on a 12% polyacrylamide gel.

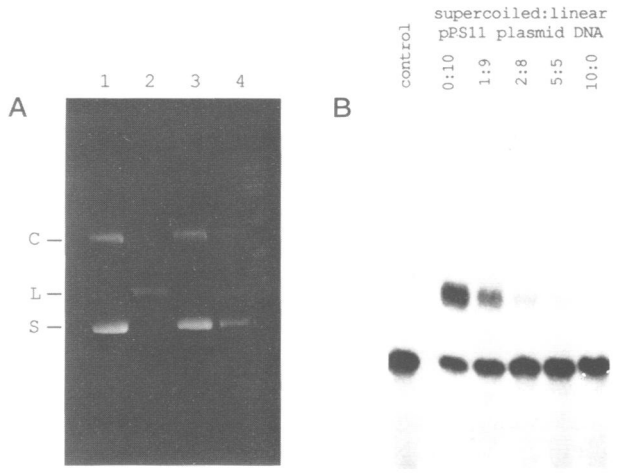

Fig. 8. Interaction of the cruciform-binding protein with pPS11 DNA. **Part A.** No detectable nucleolytic activity is associated with the cruciform-binding protein. A reaction mixture was set up containing 12.5 μg/ml of heat-treated supercoiled pPS11 plasmid, 50 mM NaCl, 10 mM Tris−HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT and 50 μg/ml BSA. Forty microliters of this mixture were incubated at 37°C for 1 h with 10 μl of buffer D + 200 mM NaCl (reaction 1), 10 μl of the same buffer containing 6 U of restriction endonuclease *Xba*I (reaction 2), 10 μl of fraction 5 of cruciform-binding protein (reaction 3), or 10 μl of buffer D + 200 mM NaCl containing 100 U of phage T4 endonuclease VII (reaction 4). The DNAs in the mixtures were then extracted with phenol and chloroform, precipitated with ethanol, resuspended in 5 μl and loaded on a 0.8% agarose gel. S indicates the position of supercoiled pPS11 DNA, L the position of linear DNA and C that of nicked circular DNA. **Part B.** The cruciform-binding protein binds to the cruciform structure formed by the palindrome of pPS11. Heat-treated linear and supercoiled pPS11 DNAs were mixed in five separate tubes in the following proportions: 0 supercoiled + 10 linear, 1 supercoiled + 9 linear, 2 supercoiled + 8 linear, 5 supercoiled + 5 linear, 10 supercoiled + 0 linear. These DNA mixtures were used to set up a series of reaction mixtures, each containing (in 31 μl) 50 μg/ml of combined pPS11 DNA, 50 mM NaCl, 10 mM Tris−HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 50 μg/ml BSA and 4% Ficoll. After the addition of 1 μl of cruciform-binding protein (fraction 5 diluted 5-fold in buffer D + 200 mM NaCl, see Materials and methods) or 1 μl of buffer D + 200 mM NaCl in the control without protein, the reaction mixtures were vortexed, supplemented with 1 μl of labeled synthetic cruciform DNA (8 fmol), and vortexed again. A sample was immediately applied to the gel and electrophoresed as described in Materials and methods.

The yeast cruciform nuclease is also incapable of cleaving synthetic cruciforms, although it can bind to them (Evans and Kolodner, 1987). This suggests that the presence of torsional stress in the DNA molecule which contains the cruciform structure may be a requisite for the activity of eukaryotic cruciform nucleases. We then assayed the nuclease activity of the cruciform-binding protein towards supercoiled ColE1 plasmid, which contains a cruciform which is readily cleavable both by phage T4 endonuclease VII (Lilley and Kemper, 1984) and the yeast cruciform nuclease (West and Koerner, 1985). Incubation of the plasmid for 2 h with the rat cruciform-binding protein, in the presence or in the absence of ATP or NAD^+/NADH, did not reveal any nuclease activity (data not shown). In contrast, endonuclease VII produced nearly complete cleavage of ColE1 plasmid after 20 min of incubation under the same conditions.

We also assayed the nucleolytic activity of the rat liver cruciform-binding protein towards plasmid pPS11, a derivative of plasmid pUC18 which contains a 70 bp palindrome cloned into the *Hin*dIII site of the polylinker (Scholten and Nordheim, 1986). Heating at 65°C causes the transition to a stable cruciform conformation in about 40−60% of the molecules, which become resistant to restriction endonuclease *Hin*dIII. A population of heat-treated pPS11 molecules were incubated for 1 h with a large excess of rat liver cruciform-binding protein or T4 endonuclease VII (Figure 8a). About 40% of the input plasmid molecules were cleaved by the phage endonuclease, whereas none were affected by the rat liver protein.

To rule out the possibility that the rat liver cruciform-binding protein might not recognize the specific cruciform structures present on ColE1 and pPS11 plasmids, the following experiment was performed. An aliquot of pPS11 was linearized with restriction endonuclease *Xba*I, and both the linear and supercoiled forms of the plasmid were incubated at 65°C. This treatment caused the transition of ~40% of the supercoiled molecules to the cruciform conformation and destabilized any cruciform structures that might still be present on the linear molecules. The heat-treated linear and supercoiled molecules were then mixed in various proportions, to a final concentration of 50 μg/ml. In this way the concentration of cruciform DNA structures was adjusted in the various mixtures to about 0, 1, 2, 5 and 10 nM, respectively, while the total amount of pPS11 DNA remained constant. These plasmid populations were incubated briefly with a subsaturating amount of cruciform-binding protein, which was then challenged with 0.25 nM labeled synthetic cruciform DNA. The amount of retarded synthetic cruciform should then be proportional to the amount of cruciform-binding protein not adsorbed to pPS11 DNA. Figure 8b clearly shows that the intensity of the retarded band is inversely pro-

portional to the concentration of cruciform-containing pPS11 molecules, which implies that the cruciform-binding protein can indeed bind to the cruciform structure formed by the palindrome contained in pPS11.

Discussion

In this communication we describe the construction of a synthetic Holliday junction and its use for the identification in a mammalian extract of an activity which binds to cruciform DNA. Both types of natural cruciform structures, those arising in genetic recombination and those extruded from palindromic sequences in superhelical DNAs, have distinct disadvantages that preclude their use as useful reagents in a wide variety of experimental systems. Both are embedded in large segments of duplex DNA, so that gel electrophoretic assays of the type we employed in this study are impossible. Cleavage of the cruciform structures in supercoiled plasmids can be expeditiously assayed, as is the case for the specific cleavages produced by restriction enzymes. However, this assay is complicated by the presence in crude extracts of non-specific nucleases and topoisomerases (which relieve the superhelicity required to maintain the palindromic DNA in cruciform configuration). More critically, a cleavage assay will not detect proteins that bind to cruciform DNAs without cleaving them.

A gel electrophoretic assay enabled us to detect an activity from rat liver nuclei which binds to cruciform DNA. This activity is a protein, as defined by its sensitivity to proteinase K and its chromatographic properties. The binding of this protein causes a small but reproducible reduction of the electrophoretic mobility of the synthetic cruciform DNA (~15%). Such a limited mobility shift, however, does not necessarily imply that the cruciform-binding protein has a low mol. wt: we have shown that a specific monoclonal antibody that binds to cruciform DNA (Frappier *et al.*, 1987) retards the migration of our synthetic molecule by a similar extent (Beltrame and Bianchi, unpublished results).

The rat liver cruciform-binding activity is distinctly specific for DNA structure, rather than for DNA sequence, as is demonstrated by the fact that it will readily bind to the synthetic cruciform DNA, but does not bind to control linear ss or duplex DNAs of the same sequence. This protein also interacts with pPS11 DNA when the 70 bp palindrome contained in the plasmid is extruded in cruciform conformation, but does not interact with linear pPS11 DNA, which contains no cruciform structures. These results also rule out the possibility that the putative cruciform-binding protein might in fact be recognizing features unique to synthetic cruciform DNAs, such as DNA termini or non-symmetrical arms. The well characterized cruciform of pPS11 (Scholten and Nordheim, 1986) is completely different in sequence from the synthetic cruciform DNA, contains no termini and is symmetrical.

The selectivity of the cruciform-binding protein towards cruciform DNA structures is quite high, since it does not bind to structures resembling stem-and-loops of ss DNA (molecule D, Figure 7), and it binds inefficiently to imperfect cruciform molecules consisting of only three strands (molecule E, Figure 7). We did not rule out the possibility that the putative cruciform-binding protein is in fact a protein that recognizes sharp bends in the DNA (the synthetic cruciform probably has a tetrahedral configuration in solution, and its four arms therefore stand at an angle of ~120° to each other). On the other hand, the ability to recognize such sharp bends could well be the mechanistic property that enables a cruciform-binding protein to recognize a Holliday junction, so that binding to bent DNA and cruciform DNA need not be mutually exclusive properties.

In contrast to the only other eukaryotic cruciform-recognizing protein known so far, that of *S.cerevisiae* (West and Korner, 1985; Symington and Kolodner, 1985), the cruciform-binding activity we describe appears to be devoid of nucleolytic activity. The yeast endonuclease does not cleave a synthetic cruciform similar to the one described in this communication, but it readily cleaves a number of supercoiled plasmids containing a palindromic sequence (Evans and Kolodner, 1986). The inability of the rat liver activity to cleave the same plasmids easily digested by both T4 endonuclease VII and the yeast endonuclease may have several explanations: (i) the activity requires a specific cofactor, (ii) the cruciform-binding activity is a proteolytic fragment or a subunit of a larger protein which has nucleolytic activity, or (iii) the intrinsic property of this activity is just the recognition of cruciform DNA. The first two explanations are possible, but in our opinion not very likely. The cleavage reaction is thermodynamically favored and both T4 endonuclease VII and the yeast cruciform endonuclease require no cofactor. If the rat liver cruciform-binding protein is a part of a nucleolytic enzyme, it must nevertheless contain the site required for binding to DNA. One would expect a protomer or proteolytic fragment of this type to retain at least part of the enzymatic activity, especially if the reaction requires no cofactor. On the other hand, it is impossible to rule out that the correct conformation of the active site depends critically on the presence of a particular segment of the polypeptide chain, which might be subject to degradation during purification.

We favor the hypothesis that the function of the rat liver cruciform-binding protein is actually the recognition and the stabilization/protection of cruciform DNA. Many proteins are known that bind to specific sequences of DNA, and their role is to provide spatial information to other proteins that perform vital functions in nucleic acid metabolism, such as gene expression or DNA replication. A protein that binds to specific structures interspersed in bulk DNA, rather than to specific sequences, might play a similar role. In this respect, it is quite interesting that palindromic, potentially cruciform DNA segments are found at the origins of replication of prokaryotes (Zyskind *et al.*, 1983), of several mammalian viruses (including SV40 and herpes simplex) (Frisque, 1983; Weller *et al.*, 1985), and in fractions of mammalian DNA enriched in replication origins (Zannis-Hadjopoulos *et al.*, 1984). Finally, it is quite possible that the actual function of the cruciform-binding protein is to shield cruciform DNA from specific endonucleases, so as to protect palindromes from nucleolytic attack or modulate the half-life of Holliday junctions during recombination.

Materials and methods

Reagents and enzymes

T4 polynucleotide kinase and restriction enzymes were purchased from New England Biolabs, *E.coli* polymerase I Klenow fragment from Boehringer Mannheim. T4 endonuclease VII was a gift from Dr B.Kemper (Univ. Köln).

DNAs

Oligonucleotides were synthesized by the phosphotriester method and purified by HPLC. Calf thymus DNA was sonicated before use to an average length of 400 bp. Plasmid pPS11 was a gift from Dr A.Nordheim (Zentrum für Molekulare Biologie, Heidelberg). Supercoiled and *Xba*I-cut linear pPS11 DNAs, both at a concentration of 200 μg/ml, were heated for 2 h at 65°C in 50 mM NaCl, 10 mM Tris−HCl, pH 8.0, 10 mM $MgCl_2$, 1mM dithiothreitol (DTT), and then immediately cooled on ice. Complete and incomplete cruciform DNAs and control linear duplexes were made by mixing the appropriate amounts of oligonucleotides in 10 mM Tris−HCl pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT, heating them at 70°C for 3 min and allowing them to cool to room temperature (0°C for the incomplete cruciform DNAs) over a 2 h period. DNA molecules were labeled by filling-in after annealing or with T4 polynucleotide kinase before annealing. The crude labeled preparations of cruciform or duplex molecules were then loaded on a 6.5% gel in TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) and electrophoresed at 20 V/cm. The band of pure material was identified by autoradiography, cut out and placed in an Eppendorf tube with a hole in the bottom. The punctured tube was then placed inside a second intact tube and spun at 10 000 *g* for 1 min at 0°C. The fragmented polyacrylamide gel in the bottom tube was resuspended in 10 mM Tris−HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 10 mM $MgCl_2$ and shaken at 0°C for 4−16 h on a rotary wheel. The gel fragments were removed by passing the resulting slurry through a glass fiber filter: the filtered material was kept at 0°C until used.

Assay for DNA binding

Standard reaction mixtures contained 8% Ficoll, 10 mM $MgCl_2$, 10 mM Hepes, pH 7.9, 5 mM KCl, 1 mM EDTA, 1 mM spermidine, 0.5 mM DTT, 200 μg/ml BSA, the indicated amounts of sonicated calf thymus DNA as aspecific competitor and variable amounts of reporter and competitor DNA molecules. Incubations were performed routinely on ice for 10 min; however, after sufficient purification the fractions could be assayed at 37°C with identical results. Samples (6 μl) were then applied to a 1 mm thick 6.5% polyacrylamide gel in 0.5 × TBE that had been extensively prerun. Electrophoresis was performed at room temperature for 4 h at 10 V/cm; however, electrophoresis at 0°C gave identical results. The gel was then transferred onto a sheet of DE81 paper (Whatman), dried and autoradiographed with Kodak XAR film at −80°C with intensifying screens.

Purification of the cruciform-binding protein

Frozen rat livers were stored at −80°C. Several livers (30 g) were reduced to small pieces with a hammer and thawed in 75 ml of buffer A [10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 10% v/v glycerol, 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 0.5 mM DTT]. All subsequent operations were performed at 0°C. The tissue was homogenized mechanically with two strokes in a Dounce homogenizer; the homogenate was checked microscopically for cell lysis and filtered through Kimwipe paper tissues to remove the macroscopic debris. It was then transferred to several 50 ml Falcon tubes and centrifuged for 10 min at 2000 r.p.m. in a swinging bucket Beckman TJ-6 centrifuge. The pellet was resuspended in the original volume of buffer A and centrifuged once more in the same way. The pellet was then resuspended in 50 ml of buffer A; this suspension was layered over the same volume of buffer A + 0.5 M sucrose in three 50 ml Falcon tubes and centrifuged as before. The supernatant was discarded and the nuclear pellet was resuspended in 25 ml of buffer D (20 mM Hepes, pH 7.9, 0.2 mM EDTA, 10% v/v glycerol, 0.5 mM PMSF and 0.5 mM DTT) + 0.42 M NaCl with a glass Dounce homogenizer. The suspension was stirred slowly with a magnetic stirrer for 30 minutes and then centrifuged for 15 min at 12 000 r.p.m. in a SS-34 rotor. The resultant clear supernatant was dialyzed against several changes of buffer D + 0.1 M KCl, and centrifuged again for 15 minutes at 12 000 r.p.m. in a SS-34 rotor. The supernatant nuclear extract was loaded on a 150 ml column of DEAE−Sepharose; the column was extensively washed with buffer D + 0.1 M KCl and eluted with buffer D + 0.3 M KCl. The eluate (fraction 1) was diluted with 0.5 volumes of buffer D and applied to a heparin−Sepharose column, which was eluted with a linear gradient of KCl in buffer D. The pooled active fractions (fraction 2) were applied to an hydroxylapatite column and eluted with a linear gradient of K phosphate (pH 7.0) in buffer D. The pooled active fractions (fraction 3) were concentrated by centrifugation through an Amicon cartridge, diluted with buffer D + 0.2 M KCl, applied to a FPLC MonoS column and eluted with a linear gradient of KCl in buffer D. The pooled active fractions (fraction 4) were diluted with 4 vol of buffer D, applied to a FPLC MonoQ column and eluted with a linear gradient of KCl in buffer D. The pooled active fractions from the MonoQ column (fraction 5) were used for the experiments described in the text. On a SDS−polyacrylamide gel, fraction 5 still contained several bands, from which the active species could not be identified with certainty.

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