Specific binding of cruciform DNA structures by a protein from human extracts

Kieran M.Elborough and Stephen C.West*

Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts EN6 3LD, UK

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ABSTRACT

A gel electrophoresis binding assay has been used to probe extracts from cultured human lymphoblasts for proteins that bind cruciform structures in duplex DNA. Proteins have been detected that form complexes with synthetic X- and Y-junctions. Several lines of evidence suggest that binding is specific for DNA structure rather than sequence: (1) X- and Y-structures were bound whereas linear duplexes containing identical DNA sequences were not, (2) Binding occurred with equal efficiency to two X-junctions that were constructed from DNA strands of different sequence, (3) One X-junction successfully competed with another for binding whereas linear duplex DNA did not; and (4) protein-DNA complexes were observed at probe:non-specific competitor DNA ratios of 1:10,000.

INTRODUCTION

X-junctions in DNA are of considerable interest since they are present as intermediates, or have the potential to form, at sites of biological importance. During genetic recombination between homologous DNA molecules, Holliday junctions are formed as DNA strands are exchanged to form heteroduplex DNA (1-5), and subsequent enzymatic cleavage by a sequence-independent, structure-specific nuclease is necessary for their resolution (6). Similar crossovers occur transiently during site-specific recombination as the lambda or P1 bacteriophages integrate into their targets on the host chromosome (7-9). For other biological processes to occur, structural changes to the DNA may be necessary. For example, inverted repeat sequences which are able to extrude to form cruciform junctions *in vitro* (10-12), are found near replication origins in prokaryotes (15) and the viruses of eukaryotes (16-20). Cruciform extrusion might also serve as a structural signal for initiation of replication at yeast and mammalian origins (21, 22). In addition, inverted repeats have been implicated in transcription termination (23) and are present as intermediates during the replication of telomere sequences (24-26). Current models for the replication and maintenance of linear chromosomes involve

resolution of the telomere inverted repeats as a means of maintaining chromosome stability (27-29).

Several proteins are known that interact with X- and Y-junctions in DNA. Two bacteriophage enzymes (T4 endonuclease VII and T7 endonuclease I) cleave branched DNA structures *in vitro* to form linear duplex molecules containing ligatable nicks (30-32). Mutant T4 phage, which are defective in gene 49, produce highly branched multimeric DNA that results in abortive infections, apparently because the branched DNA cannot be packaged (33). The product of this gene, endonuclease VII, therefore appears to be required for the resolution of branches that are produced by the interlinked phage replication and recombination processes (34).

Enzymes capable of resolving cruciform junctions *in vitro* have also been isolated from *Saccharomyces cerevisiae* (35-37). Recent work from this laboratory has shown that resolution of model Holliday junctions by the yeast endonuclease is dependent upon homologous DNA sequences, and indicates a role for this enzyme in genetic recombination (6).

The biological significance of branched structures in DNA led us to the present study in which we have used a gel retardation assay to screen human cell-free extracts for proteins capable of specific interaction with X-and Y-junctions in DNA.

MATERIALS AND METHODS

Enzymes and Reagents

DNA polymerase I (Klenow fragment) was purchased from New England Biolabs or Bethesda Research Laboratories. Poly(dI-dC).poly(dI-dC), average length 3523 base pairs, was purchased from Pharmacia. Protein concentrations were determined using a modification of the Bradford assay (Bio-Rad) with ovalbumin as the standard.

Construction of DNA substrates

Oligonucleotides were prepared using an Applied Biosystems Model 380A DNA synthesizer. Equimolar amounts (10 μ g) of oligonucleotides A-K were annealed to form substrates 1-5 (Figure 1) by incubation at 65 °C for 2 h in 50 μ l 150 mM NaCl, 15 mM sodium citrate followed by gradual cooling to room temperature (38). Hybridized DNA was precipitated with ethanol and purified by electrophoresis through 12 % polyacrylamide. The DNA was labeled by filling in recessed 3'-termini using the Klenow fragment of DNA polymerase I and α -³²P-dCTP or α -³²P-dATP.

The oligonucleotide composition of X-junction DNA was confirmed by annealing 4 oligonucleotides that had been prelabeled with polynucleotide kinase and ^{32}P - γ -ATP. The X-junction was run on a 12 % polyacrylamide gel and the band cut out and eluted. The purified DNA was denatured using 90 % formamide and applied to a 20 % polyacrylamide gel. Comparison with marker oligonucleotides indicated the presence of all four oligonucleotides in approximately equimolar ratio.

The electrophoretic mobility of X-junction DNA was found to be anomalous in comparison with duplex DNA fragments of the same length and was dependent upon acrylamide gel concentration (38). X-junctions were resistant to digestion by S1 nuclease as determined by electrophoresis on a sequencing gel following denaturation.

Electrophoretic Binding Assay

In the standard binding assay, cell-free extract was incubated for 10 min at 25 °C with 1 μg poly(dI-dC).poly(dI-dC) in 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA and 3 % glycerol. 0.3 ng ^{32}P -labeled substrate DNA was then added (final volume 10 μl) and incubation continued for 20 min. Samples were loaded onto a 4 % polyacrylamide gel (30:1 acrylamide: bisacrylamide) and electrophoresed using 6.7 mM Tris-HCl, pH 7.5, 3.3 mM sodium acetate, 2 mM EDTA as the buffer system. Electrophoresis was at 500 V for 1 min followed by 160 V for 90 min for a 16 x 16 cm gel. Following electrophoresis, gels were dried and the DNA located by autoradiography.

Extract Preparation

Human cell lines were obtained from the Human Genetic Mutant Cell Repository, Camden, New Jersey, and were grown in RPMI 1640 medium supplemented with 15 % fetal calf serum. Extracts were prepared by a modification of published methods (39). 1 litre cultures (approx 1×10^9 cells) were grown, harvested by centrifugation, washed in iced phosphate-buffered saline and resuspended in 6 ml of iced hypotonic buffer (10 mM Tris-HCl, pH 7.9, 1 mM EDTA and 1 mM dithiothreitol). The cells were broken by 16 strokes using a 5 ml Dounce homogenizer, and an equal volume of 50 mM Tris-HCl, pH 7.9, 10 mM MgCl_2 , 2 mM dithiothreitol, 25 % sucrose and 50 % glycerol was added dropwise. Saturated ammonium sulphate (1.5 ml) was slowly added and the solution stirred gently for 30 min. Cell debris was removed by centrifugation at 45,000 rpm for 3 h in a Beckman SW 50.1 rotor. To the supernatant, solid ammonium sulphate was added at 0.33 g per ml and the suspension stirred gently for 30 min. The resulting precipitate was collected by centrifugation, resuspended in 1 ml dialysis buffer and dialyzed for 5 h against 25 mM Hepes-KOH, pH 7.9, 100 mM KCl, 12 mM MgCl_2 ,

0.5 mM EDTA, 2 mM dithiothreitol and 17 % glycerol. Cell-free extracts (typically 10 mg/ml protein) were rapidly frozen and stored as 25 μ l aliquots. Under these conditions, extracts were found to be stable for a period of 12 weeks. Storage for longer periods resulted in decreased junction-binding activity.

Partial Purification of DNA-Binding Activity

Whole cell extract (225 mg) prepared from GM3403 lymphoblasts was dialyzed against phosphate buffer (20 mM sodium phosphate, pH 6.8, 1 mM dithiothreitol, 1 mM EDTA, 0.1 mM phenylmethyl sulphonyl fluoride (PMSF) and 10 % glycerol), and applied to a 1.6 ml single-stranded DNA cellulose column equilibrated with the same buffer. The column was washed and eluted stepwise with phosphate buffer containing 50 mM, 200 mM and 400 mM NaCl. Fractions were collected and 4 μ l samples assayed for the presence of X-junction binding activity. The bulk of the activity was observed in the 400 mM eluate (5 ml, 0.7 mg/ml protein).

RESULTS

Gel Retardation of X-structure DNA

In order to assay human cell-free extracts for proteins capable of specific binding to branched structures in DNA, we prepared substrates 1 - 5 shown in Figure 1. The products of annealing various combinations of oligonucleotides are shown in Figure 2, and include X-junctions (lanes e and f), Y-junction DNA (lane g) and linear duplex DNA (lanes h and i). Annealed structures were purified by preparative gel electrophoresis and 32 P-labeled at recessed 3'-termini. In the first experiments, we used a cruciform or X-structure (substrate 1, Figure 1) which was constructed by annealing equimolar amounts of four single-stranded oligonucleotides (strands A-D, Figure 1). Linear duplex DNA was also prepared by annealing strand A and strand C with their complementary sequences (substrates 4 and 5, Figure 1). The two linear duplexes therefore contain all DNA sequences present in the X-structure, and serve as controls for sequence-specific DNA binding.

Since X-junctions contain four arms that flank a central junction or crossover point, they are analogous to a Holliday junction. However, in contrast to a true Holliday junction (which is inherently unstable due to branch migration) sequence symmetry constraints built into the X-structures make branch migration of the crossover point impossible, and therefore ensure their stability.

The assay used to detect proteins that bind to X-junction DNA was a modification of the gel electrophoresis DNA binding assay (40-42). The assay is based on the observation that protein-DNA complexes migrate through low ionic

Figure 2. Polyacrylamide gel electrophoresis of oligonucleotides and various annealed mixtures.

Lanes a-d contain oligonucleotides H, I, J and K respectively. Subsequent lanes contain the products of annealing oligonucleotides H I, J and K (lane e), A, B, C and D (lane f), A, D and G (lane g), A and E (lane h) and F and C (lane i). DNA was visualized by ethidium bromide staining of a 12 % polyacrylamide gel.

cultures (GM3403) were preincubated with an excess of competitor DNA [poly(dI-dC).poly(dI-dC)] prior to the addition of ^{32}P -labeled X-junction DNA probe. After a further incubation, reactions were loaded directly onto gels and electrophoresed. In the absence of extract, the X-junction DNA migrated as a discrete band (Figure 3, lane a). When a constant amount (0.3 ng) of X-junction was incubated with increasing concentrations of whole cell extract, one distinct band of lower mobility was observed (Figure 3, lanes b-f).

Treatment of binding reactions containing extract and X-junction DNA with 1 % SDS or 200 $\mu\text{g/ml}$ proteinase K for 15 min at 37 $^{\circ}\text{C}$, converted all the low mobility band to a form that comigrated with the unbound DNA. The binding was unaffected by treatment with 50 $\mu\text{g/ml}$ RNase, but was eliminated by heating the extract for 2 min at 100 $^{\circ}\text{C}$. These results strongly suggest that the low mobility band represents a discrete protein-DNA complex.

Specificity of Binding

To determine whether the complexes represented specific protein-DNA

Figure 3. Specific binding of X-structure DNA by whole cell extract from GM3403 lymphoblasts.

Varying concentrations of cell-free extract were assayed for specific binding as described in Materials and Methods. Following electrophoresis, gels were dried and the DNA located by autoradiography. Lanes a-f, X-structure DNA (substrate 1, Figure 1), lanes g-l, linear duplex DNA (substrate 4, Figure 1). Cell-free extract was present at the following concentrations: Lanes a and g, control without extract; lanes b and h, 2.5 μ g; lanes c and i, 5 μ g; lanes d and j, 7.5 μ g; lanes e and k, 10 μ g, lanes f and l, 12.5 μ g.

interactions, a series of control experiments were performed. We tested to see whether ^{32}P -labeled linear duplexes (substrates 4 and 5, Figure 1) that contained the DNA sequences present in the X-junction could be retarded by the protein extract. The results obtained with one of these duplexes is shown in Figure 3 (lanes g-l). Bands of lower mobility were not detected and the products of binding reactions yielded DNA species that comigrated with the linear duplex probe. Similar results were obtained with the other linear duplex. To eliminate the possibility that the X-junctions were incompletely base-paired and that the observed binding was due to an interaction with single-stranded DNA, extract was incubated with X-junction DNA in the presence of 1 μ g of each of the individual single-stranded oligonucleotides. Binding to the X-junction was not reduced (data not shown).

Detection of the specific protein-DNA complex was dependent upon the inclusion of poly(dI-dC).poly(dI-dC) to compete for non-specific DNA-binding proteins. Figure 4 shows the results obtained with varying amounts of competitor DNA. In the absence of competitor, incubation of extract with either X-junction (Figure 4A, lane b), or linear duplex (Figure 4B, lane b), resulted in retardation of all substrate DNA. However, non-specific protein-DNA complexes were eliminated by the addition of 0.3 μ g or more competitor DNA (Figure 4, lanes c-i). Above this level of competitor, only specific protein-DNA complexes were observed when X-junctions were used. These were formed even in the presence of 3 μ g competitor, i.e at a probe:competitor ratio of 1:10000 (Figure 4A, lane i). In contrast, no protein-DNA complexes were detected at high competitor levels when

Figure 4. Comparison of specific and non-specific binding by crude extract.

The 32 P-labeled DNA fragment (0.3 ng) in A was an X-junction (substrate 1, Figure 1) and in B was a linear duplex (substrate 4, Figure 1). Reactions b-i contained 10 μ g protein extract. Non-specific competitor DNA [poly(dI-dC).poly(dI-dC)] was present in reactions c-i at specific fragment to competitor DNA ratios of 1:1 (c), 1:10 (d), 1:100 (e), 1:1000 (f), 1:3333 (g), 1:6666 (h) and 1:10000 (i). Reactions a and b contained no competitor DNA. Gel retardation experiments were performed as described in Materials and Methods.

linear duplex DNA was used as substrate in the assay (Figure 4B, lanes f-i).

Since the phage nucleases that cleave X-junctions are also able to cut Y-junctions (32, 44), we also assayed to see whether Y-junction DNA (substrate 3, Figure 1) could be retarded by the extract. The results presented in Figure 5, lanes c and d, show the Y-junction in the absence and presence of extract. Again the junction-containing DNA was bound by the cell-free extract to produce one distinct band of lower mobility (Figure 5, lane d). This band was of a similar mobility to that observed when X-junction DNA was used as substrate (Figure 5, lane b). In contrast, the two linear duplexes (substrates 4 and 5, Figure 1) which contained all the DNA sequences present in the Y-junction were not retarded in the same experiment (Figure 5, lanes f and h).

Figure 6, lane b shows binding of a whole cell extract prepared from GM3403 lymphoblasts to a second X-junction (substrate 2, Figure 1) constructed from DNA sequences unrelated to those used in substrate 1. The formation of a band with lowered mobility identical to that produced by the first X-junction confirms that

Figure 5. Specific binding of Y-structures DNA by crude extract.

Gel retardation experiments were performed as described in Materials and Methods. The ^{32}P -labeled fragments (0.3 ng) in the assays were: reactions a and b, X-structure DNA (substrate 1, Figure 1): reactions c and d, Y-structure DNA (substrate 3, Figure 1): reactions e and f, linear duplex DNA (substrate 4, Figure 1): reactions g and h, linear duplex DNA (substrate 5, Figure 1). Crude extract (10 μg) was present in reactions b, d, f and h.

Figure 6. DNA-cellulose elution of X-structure binding protein.

Cell-free extracts from GM3403 lymphoblasts were partially purified by chromatography on single-stranded DNA-cellulose as described in Materials and Methods. X-structure DNA (substrate 2, Figure 1) was used in each assay. Reaction a, no extract; reaction b, 5 μ g whole cell extract; reaction c, 4 μ l (0.4 μ g) 50 mM NaCl eluate; reaction d, 4 μ l (2 μ g) 200 mM NaCl eluate; reaction e, 4 μ l (2.8 μ g) 400 mM NaCl eluate. The assay and gel retardation experiment were performed as described in Materials and Methods except the competitor DNA concentration was 0.5 μ g.

the protein-DNA complexes are structure-specific and are not dependent on DNA sequence. Subsequent fractionation of this extract on single-stranded DNA cellulose as described in Materials and Methods resulted in greater than 50-fold purification, as the bulk of the activity eluted at 200 - 400 mM NaCl (Figure 6, lane e). Using this partially purified fraction, it was shown that binding to 32 P-labeled X-junction (substrate 1, Figure 1) was competed by addition of unlabeled X-junction (substrate 2, Figure 1) but not by the same concentration of linear duplex DNA (substrate 4, Fig. 1). The results of this experiment are shown in Figure 7, and support the view that binding is specific for DNA structure rather than sequence.

Several cultured human cell lines, both normal and those containing inheritable DNA repair defects, have been assayed for activity that binds X-junction DNA. These include Hela, GM1953 and GM0621 (from phenotypically normal individuals), GM2249 (Xeroderma pigmentosum complementation group C), and GM3403 (Bloom's syndrome). In all extracts, specific protein-DNA binding activities have been observed.

Figure 7. Competition for binding by two X-junctions but not by duplex DNA.

Gel retardation assays were performed as described in Materials and Methods using 0.3 ng labeled X-junction DNA (substrate 1, Figure 1), 2.1 μ g of the 400 mM NaCl eluate from single stranded DNA cellulose and 0.2 μ g poly(dI-dC).poly(dI-dC). Reaction a, without protein; reaction b, with protein; reaction c, as b but supplemented with 1 μ g unlabeled X-junction (substrate 2, Figure 1); reaction d, as b but supplemented with 1 μ g unlabeled duplex (substrate 4, Figure 1).

DISCUSSION

The results presented here provide the first demonstration of specific binding to branched DNA structures by a protein, or proteins, from human cell-free extracts. Several lines of evidence suggest that the binding is structure rather than sequence dependent: (1) Binding was specific for branched DNA since linear duplexes containing identical DNA sequences were not bound, (2) Binding occurred with equal efficiency to two X-junctions that were constructed from DNA strands of different sequence, (3) One X-junction successfully competed with another for binding whereas linear duplex DNA did not, and (4) protein-DNA complexes were observed even at probe:non-specific competitor DNA ratios of 1:10000.

The specific protein-DNA complexes formed by the interaction of the human cell-free extract with X-junction DNA may be similar to those formed between the yeast Holliday junction-resolving protein and X-junctions (45). In both cases, the X-junctions were bound and DNA-protein complexes were stable during gel electrophoresis. In experiments with the yeast nuclease, it was reported that the X-junctions used in the binding assay were not cleaved by the yeast nuclease whereas other DNA substrates containing cruciform junctions were cut efficiently. It was presumed that the X-junctions constructed by annealing short

oligonucleotides were either too small to be cleaved or that DNA sequences necessary for cleavage were not present (45). Recent work from this laboratory has shown that symmetrical cleavage across model Holliday junctions by the yeast endonuclease is dependent upon sequence homology between the arms that flank the junction (6). Experiments to detect specific cleavage of cruciform DNA by the human cell extracts have so far been unsuccessful due to the presence of non-specific nucleases.

Since junctions have been implicated in biologically important processes such as recombination, replication and transcription termination, the objective of our present work was to demonstrate the presence of a junction-binding protein in human cells. A number of normal and mutant human cell lines have been screened for specific binding to X-structures, and in all cases protein-DNA complex formation has been observed. These lines include HeLa, GM1953 and GM0621 (from phenotypically normal individuals), GM2249 (xeroderma pigmentosum complementation group C), and GM3403 (Bloom's syndrome).

Preliminary purification studies suggest that the X-junction binding protein may be partially purified by DEAE-biogel, ssDNA-cellulose and dsDNA-cellulose chromatography. The protein is relatively stable at 4 °C, the activity remaining for approximately 24 hours. However, at the present time we have little knowledge of the protein(s) responsible for specific binding, or of the role the protein may play in any particular aspect of nucleic acid metabolism. Efforts are underway to purify and characterize this novel protein.

While this manuscript was in preparation Dr M. E. Bianchi kindly communicated to us results, prior to publication, describing the interaction of a protein from rat liver nuclei that binds to cruciform junctions (46).

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*To whom correspondence should be addressed

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