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PROSPECT

Inverted Repeats, Stem-Loops, and Cruciforms: Significance for Initiation of DNA Replication

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Abstract Inverted repeats occur nonrandomly in the DNA of most organisms. Stem-loops and cruciforms can form from inverted repeats. Such structures have been detected in pro- and eukaryotes. They may affect the supercoiling degree of the DNA, the positioning of nucleosomes, the formation of other secondary structures of DNA, or directly interact with proteins. Inverted repeats, stem-loops, and cruciforms are present at the replication origins of phage, plasmids, mitochondria, eukaryotic viruses, and mammalian cells. Experiments with anti-cruciform antibodies suggest that formation and stabilization of cruciforms at particular mammalian origins may be associated with initiation of DNA replication. Many proteins have been shown to interact with cruciforms, recognizing features like DNA crossovers, four-way junctions, and curved/bent DNA of specific angles. A human cruciform binding protein (CBP) displays a novel type of interaction with cruciforms and may be linked to initiation of DNA replication.

Key words: inverted repeats, cruciform DNA, secondary structure, DNA replication, cruciform binding proteins, structure-specific recognition, protein–DNA interactions

The double helical structure of DNA [Watson and Crick, 1953] now includes various conformational permutations of this molecule. "DNA structure" or "secondary structure of DNA" is a common term for many different—mostly double-stranded—DNA forms, of which the only common denominator is that they deviate from a canonical B-form DNA. These forms include intrinsic local variations, for example, changes in twist angle, a stably curved DNA, A-form DNA; inducible local and global secondary structures, e.g., melting, Z-form DNA, cruciforms, triplex DNA, superhelices, loops, the recently described slipped-stranded DNA (s-DNA) [Pearson and Sinden, 1996]; and covalent modifications of DNA, which also may affect its structure [for comprehensive reviews, see Cozzarelli and Wang, 1990; Sinden, 1994]. These variant structures can influence the interaction of DNA with proteins and consequently stimulate or repress processes that are governed by proteins such as transcription, repair, recombination or replication. One good example is supercoiling, which is known to regulate genetic expression in prokaryotes [Drlica, 1984; Pruss and Drlica, 1989] and eukaryotes [Weintraub and Groudine, 1976; Weintraub et al., 1986; Esposito and Sinden, 1988; Freeman and Garrard, 1992]. Therefore, DNA structure may possess regulatory potential on protein binding and, thus, on protein function.

Inverted repeat (IR) sequences are a common feature of prokaryotic and eukaryotic control regions, including replication origins. They have been shown to be functionally important for the initiation of DNA replication in plasmids, bacteria, eukaryotic viruses, and mammalian cells. IRs have the potential to form cruciform structures through intrastrand base pairing and under conditions of torsional strain on the DNA. Cruciform formation in vivo has been demonstrated in prokaryotes and in mammalian cells. Here, we present a discussion of IRs, their structural variations and possible biological roles of cruciform structures with an emphasis on initiation of mammalian DNA replication.

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STRUCTURAL CONSIDERATIONS

Occurrence and Significance of IRs

Inverted repeat, or palindromic sequences occur widely in the chromosomal DNA of many prokaryotes and eukaryotes such as plants [Bazetoux et al., 1978], yeast [Klein and Welch, 1980], *Neurospora* [Krumlauf and Marzluf, 1980], *Physarum* [Hardman and Jack, 1977], *Drosophila* [Wilson and Thomas, 1974; Schmid et al., 1975], mouse [Cech and Pardue, 1975; Cech and Hearst 1975], *Xenopus*, and human [Wilson and Thomas, 1974; Dott et al., 1976]. In the genome of many eukaryotes, IRs are distributed in a nonrandom fashion [Klein and Welch, 1980, and references therein]. IRs have been associated with regulation of gene expression in prokaryotes [Horwitz, 1989] and eukaryotes [Shuster et al., 1986; Greenberg et al., 1987; McMurray et al., 1991; Spiro et al., 1993], termination of transcription and attenuation in prokaryotes [Rosenberg and Court, 1979]. Additionally, IRs are commonly associated with replication origin sequences of prokaryotic [Zyskind et al., 1983; Hiasa et al., 1990], viral [Muller and Fitch, 1982], eukaryotic [Tschumper and Carbon, 1982; Campbell, 1986], and mammalian organisms [Hand, 1978; Zannis-Hadjopoulos et al., 1984; Zannis-Hadjopoulos et al., 1985; Landry and Zannis-Hadjopoulos, 1991; Boulikas, 1993], as well as amplified genes [Fried et al., 1991]. The occurrence of IRs at control regions, like promoters, terminators and replication origins, suggests a regulatory role. IRs have the potential to form cruciform (stem-loop or hairpin) structures [Platt, 1955]. However, not all palindromes function necessarily as cruciforms. Many remain in the linear state and may act as binding sites for protein dimers. Others exert their function only at the RNA level, for example, as RNA-hairpins at terminators or attenuators. However, there is accumulating evidence that, in several processes, palindromes may have functional significance also as cruciforms.

Conditions and Structural Effects of Cruciform Extrusion

A cruciform is an inducible four-branched DNA secondary structure that may form by *intra*-molecular base-pairing of the two complementary strands under appropriate conditions. Consider, for example, a circular covalently closed DNA molecule, into which torsional tension is introduced by breaking and unwinding the two strands, e.g., by the action of gyrase and ATP. In order to partially compensate for this stress, the molecule adopts a negative superhelical conformation, equivalent to a change in writhe. However, this negatively supercoiled DNA still has less helical turns than the same molecule would have if it were relaxed, i.e., the molecule is "untwisted" [Boles et al., 1990]. Deformation of either the writhe or the twist of a DNA molecule are energetically unfavorable. Therefore, a supercoiled DNA molecule has a higher free energy compared to its relaxed isomer. Beyond an energy threshold, the molecule collapses at a site, forming cruciforms at IRs by intrastrand base pairing [Hsieh and Wang, 1975; Lilley, 1980; Mizuuchi et al., 1982; reviewed in Lilley, 1989] or other specific local DNA secondary structures, depending on their nucleotide sequence [Sinden, 1994].

The local formation of these structures contributes a net twist change that brings about a further relaxation of the superhelical stress. Since cruciforms relax negative supercoiling, they effect a reduction of the free energy of the molecule and are, therefore, thermodynamically favorable in superhelical domains. Once formed, cruciforms are relatively stable (metastable), even in nicked, or linear, DNAs [Mizuuchi et al., 1982; Bell et al., 1991; Hyrien, 1989].

Mechanisms of Cruciform Formation

The formation of a cruciform is dependent on dyad symmetrical arrangement of the bases to permit stem formation. The stem extrudes as an intra-strand B-form helix [Sinden, 1994]. Two major mechanisms of transition from linear duplex to cruciform structure have been proposed that differ in salt dependent, effect of temperature and magnitude of activation energy [reviewed in Lilley, 1989; Murchie et al., 1992]. The S type (S refers to Salt-dependent), which has been observed in buffers approximating physiological ionic strength, is dependent upon supercoiling, temperature, ionic conditions, and the divalent cation [Singleton, 1983; Sinden and Pettijohn, 1984; Sullivan and Lilley, 1987]. Furthermore, the base composition at the center of the IR has a significant influence on the formation of a cruciform [Murchie and Lilley, 1987; Zheng and Sinden, 1988]. The S-type of transition commences with a small unwound state at the center of the inverted repeat, followed by *intra*-strand nucleation, giving rise to a protocru-

ciform, which proceeds by branch migration to a fully extruded cruciform.

The other mechanism is the C-type, where C refers to ColE1, after the sequence in which this mechanism was first observed [Lilley, 1985; Murchie and Lilley, 1987]. This extrusion mechanism takes place in solutions lacking salt via a large unwound bubble in an AT-rich region distal to the IR. The bubble, initiated at this distal sequence, travels along the DNA strands, unwinds the first arm of the inverted repeat and, as the other arm also becomes unwound, intrastrand base pairing occurs leading to cruciform formation. AT-rich sequences distal to the IR are requisite to the C-type of transition. C-type transitions are independent of the sequence of the IR, but are completely dependent upon sequences located in *cis*.

Cruciform Structure: Geometry of Helices and Strands

Cruciforms have two major structural characteristics, the four-way DNA junction and the stems containing single-stranded loops. Structural analysis of four-way DNA junctions have been modeled on small stable synthetic oligonucleotides [reviewed by Lilley and Clegg, 1993]. The structure of the junction depends critically on the type and amount of counterion used in the solutions [Duckett et al., 1990]. Using stable junctions, it was found by several methods that, in the presence of Mg^{2+}: (1) four helices become pairwise colinear, adopting a compact, twofold symmetric, X-like shape, i.e., with angles subtended by the helix pairs of $\sim 120°$ and 60° [Duckett et al., 1988; Churchill et al., 1988]; (2) the two colinear helix pairs are in a right-handed conformation [Murchie et al., 1989]; (3) partner choice (isomerization) of helices for colinear arrangement depends critically on the DNA sequence, mainly at the junction, which thus determines the distribution of the stereoisomers [Chen et al., 1988; Duckett and Lilley, 1991]; (4) the most likely isomer is one in which the continuous strands display antiparallel polarity, with the consequence that the exchanging strands are in a noncrossed arrangement [Kimball et al., 1990; Murchie et al., 1991]; and (5) the strands at the junction fold in a way that allows all bases to be paired and stacked [Wemmer et al., 1985]. In the absence of Mg^{2+}, and at low-salt concentrations (<50 mM NaCl), the structure of a four-way junction is most likely in an extended, square–planar (or slightly pyramidal) [cf. von Kitzing et al., 1990], fourfold symmetric conformation, as opposed to a tetrahedron [Clegg et al., 1992, 1994; Cooper and Hagerman, 1987, 1989], displaying unstacking of base pairs at the junction [Duckett et al., 1990]. In the absence of Mg^{2+}, but at high concentrations of monovalent cations (>50 mM NaCl), the structure shares compactness with the Mg^{2+} species but lacks perfect twofold symmetry and still retains single-strand bases at the junction [Duckett et al., 1990]. Since the intracellular concentration of Mg^{2+} is rather high (mM amounts) [Duckett et al., 1990], the most probable (protein-free) physiological conformation of a cruciform may be the compact X-shaped structure, rather than an extended conformation [Lilley and Clegg, 1993; Clegg et al., 1992, 1994]. In another model, the given structure of a particular set of sequences does not have a stable steady-state structure [Eis and Millar, 1993], indicating that there is a certain amount of flexibility in four-way junctions [Petrillo et al., 1988].

Cruciform Structure: Stem-Loops

The tips of cruciform arms are sensitive to single-strand nucleases [Lilley, 1980; Panayotatos and Wells, 1981; Sheflin and Kowalski, 1985; Frappier et al., 1989], indicating that they are in a single-stranded state. For IRs with no intervening sequence between the repeats, in the cruciform conformation, the loops contain two to three unpaired bases [Scholten and Nordheim, 1986; Furlong and Lilley, 1986]. For other IRs, the loop size is dependent on the length of the intervening sequence. Hairpin loops probably involve base stacking [Blommers et al., 1989], nonWatson–Crick base pairing [Haasnoot et al., 1986; Hirao et al., 1994] and, under certain conditions, extraloop bases [Zhou and Vogel, 1993].

It has been shown that hairpin loop conformation and dynamics can be extremely sensitive to small base changes in the loop and adjacent stem sequences [Blommers et al., 1989; Williamson and Boxer, 1989]. Similarly, the presence of hairpin loops can affect the stem structure [Germann et al., 1990]. Hence, for cruciforms with short arms the presence of a hairpin loop may affect the overall conformation of the four-way junction [Petrillo et al., 1988]. The presence of loops could impart certain stiffness to the DNA structure, which may be important for protein recognition of the target DNA as a cruciform

[Crothers and Fried, 1982; Hogan and Austin, 1987; Travers, 1989], as opposed to similar structures, e.g., Holliday junctions, or crossovers, points where two double helices intersect by the process of supercoiling, looping or folding around a nucleosome (see below).

EVIDENCE FOR THE EXISTENCE OF CRUCIFORMS IN VIVO

Unrestrained Supercoiling In Vivo

As previously mentioned, a cruciform structure needs torsional tension, coming from unrestrained negative supercoils of sufficient density, in order to form from an IR. The required superhelical density σ, expressed as an average number of supercoils per helical turn, may vary from one IR to another [Zheng and Sinden, 1988]. Most DNAs in their natural state are negatively supercoiled, including chromosomal and plasmid DNAs of bacteria and the DNA contained in mammalian cells [Sinden, 1994]. In circular plasmids *isolated* from bacteria, a σ of -0.06 to -0.07 is generally observed [Zheng et al., 1991]. However, this relatively high negative superhelical density is greatly reduced in vivo due to the association of the bacterial genome with histone-like proteins [Pettijohn and Pfenninger, 1980]. Numerous methods have indicated that the in vivo superhelical density must lie between -0.025 and -0.05 [for discussion, see Zheng et al., 1991]. These values may be too low for a significant cruciform formation in vivo. However, many factors, including ongoing transcription [Liu and Wang, 1987; Wu et al., 1988; Tsao et al., 1989; Dayn et al., 1992; Bowater et al., 1994b]; growth conditions and stress [Haniford and Pulleyblank, 1985; McClellan et al., 1990; Dayn et al., 1991]; and topoisomerase I [McClellan et al., 1990; Zheng et al., 1991], may increase transiently the local superhelical density to a critical level sufficient for formation of a cruciform. After their formation, cruciforms efficiently resist reduction of torsional stress, since their reabsorption would require introduction of negative supercoils [Sinden et al., 1983].

Circular genomes, e.g., of prokaryotes, are topologically closed, a property that is essential for supercoiling. Eukaryotic chromosomes, on the other hand, are believed to exist as long linear molecules wrapped around nucleosomes. However, DNA in mammals is also thought to be attached to the nuclear matrix [Vogelstein et al., 1980], organized in closed topological domains. Because of the tight association of eukaryotic DNA with nucleosomes, however, virtually all supercoiling is restrained, so that, in essence, eukaryotic chromosomes should be tension free. Early attempts, which failed to detect unrestrained supercoiling in the bulk of DNA in eukaryotes, supported this general conclusion [Sinden et al., 1980]. However, a localized stress could be generated if nucleosomes were rearranged or removed from a topologically closed chromosomal domain [Leonard and Patient, 1991, and references cited therein]. In fact, recent evidence from yeast [Giaever and Wang, 1988; Brill and Sternglanz, 1988; Osborne and Guarente, 1988], insect [Jupe et al., 1993], frog oocytes [Leonard and Patient, 1991] and mammalian cells [Ljungman and Hanawalt, 1992] indicates that there are regions of localized unrestrained negative supercoiling in eukaryotic chromosomes. In some of these systems, supercoiling is apparently not merely a consequence of the twin model of ongoing transcription [Liu and Wang, 1987] but is independent of active transcription [Jupe et al., 1993] or preceeds the latter and is barely affected by blocking transcription elongation [Leonard and Patient, 1991]. These data indicate that, in spite of topoisomerase action, torsionally stressed topological domains do exist in eukaryotes and that localized supercoiling may be requisite for the progression of cellular processes.

Detection of Cruciforms In Vivo

Early studies of cruciforms in mammalian cells led researchers to believe that these structures either did not form in vivo or that they formed but were not stable [Cech and Pardue, 1976]. These studies, however, were at the resolution level of electron microscopy and would not have detected small cruciforms or cruciforms stabilized by bound proteins. A further scepticism concerning the existence of cruciforms in vivo, came from results showing that there may be significant *kinetic* barriers to the formation of cruciforms under physiological conditions [Courey and Wang, 1983] and that extrusion was slow [Gellert et al., 1983]. Studies involving several IRs, however, revealed that different sequences have different extrusion rates [Sinden and Pettijohn, 1984; Lilley, 1985]. In vitro, cruciforms may be traced by virtue of their particular structure with several methods, including altered migration in native gels [Gough and Lilley, 1985]; susceptibility of the single-stranded tips to S1 nuclease [Lilley, 1980; Pan-

ayotatos and Wells, 1981]; resistance to DNase I digestion [Murchie et al., 1990]; failure of restriction endonucleases to cut their cognate site, when it was placed into the loop region [Mizuuchi et al., 1982]; discontinuous migration of topoisomers resolved in 2D gels [Mizuuchi et al., 1982]; and electron microscopy studies [Mizuuchi et al., 1982; Hsu, 1985]. These approaches cannot be, or were not used for the detection of cruciforms in vivo. In the following, we present the evidence, indirect and direct, for the occurrence of cruciforms in vivo, as detected by suitable techniques. Indirect evidence is mainly of genetic nature, i.e., the observed genetic changes are consistent with cruciform formation in vivo. Direct evidence, on the other hand, relies on direct (chemical immunochemical, enzymatic) probing of cruciform structures in the cell or in vitro. (For a critical discussions of in vivo methods, see Palecek [1991] and Sinden [1994].)

The first evidence suggesting that cruciforms or DNA hairpins may exist in vivo was the observation that cloned IRs > 150 bp are genetically unstable in plasmids in *E. coli* [Collins, 1981; Lilley, 1980; Mizuuchi et al., 1982; Leach and Stahl, 1983; Shurvinton et al., 1987; Williams and Muller, 1987; Muller et al., 1988; Sinden et al., 1991]. Those IRs may be eliminated by recombinational mechanisms, or by deletions due to slipped, mismatched alignment during DNA replication [Trinh and Sinden, 1991; Sinden, 1994; Leach, 1994]. The instability of short and long IRs does not hold true for eukaryotic organisms; both long and short IRs can be stably cloned in yeast [Klein and Welch, 1980; Weller et al., 1985; Henderson and Petes, 1993; Ruskin and Fink, 1993; Hayashi et al., 1993], insects [Schmid et al., 1975], or mammals [Wilson and Thomas, 1974]. Genetic evidence for stable stem-loop structures in eukaryotic cells comes from several reports aimed at the study of repair and recombination in yeast [Nag et al., 1989; Nag and Petes, 1991] and mammals [Bollag et al., 1992], indicating that in these organisms these structures are neither repaired by nucleolytic enzymes nor destroyed. Second, based on the observation that the magnitude of supercoiling in *E. coli* is stringently maintained on a constant level, Haniford and Pulleyblank [1985] provided an elegant demonstration of in vivo formation of cruciforms, by analysing the topoisomer pattern of a plasmid capable of cruciform extrusion. Two, rather than one gaussian distribution, were apparent when a subpopulation of the plasmid topoisomers carried a cruciform; this was due to an intracellularly increased superhelical density in these topoisomers, consistent with a compensation for the relaxation brought about by the cruciform extrusion. Third, Horwitz and Loeb [1988] concluded the formation of a cruciform in vivo by showing that transcription was inhibited under conditions of (high) intracellular negative supercoiling in *E. coli,* when an IR was cloned in the promoter of a tetracycline gene. Fourth, mutations at IRs which disrupt a putative cruciform structure but are functionally compensated for by a second mutation within the IR reconstituting the integrity of the cruciform stems were also interpreted as indicative for formation of cruciforms in vivo at the CD8α enhancer in human T cells [Hanke et al., 1995]. Fifth, formation of the diagnostic loop structure was demonstrated by treatment of whole cells with single-strand selective chemical probes, e.g., OsO_4 [McClellan et al., 1990; Bowater et al., 1994a] and haloacetaldehydes [Noirot et al., 1990; Dayn et al., 1991, 1992], indicating formation of cruciforms in vivo. The loop structure was also probed in situ by single-strand-specific nucleases, which function at physiological pH, e.g., P1 nuclease [Leonard and Patient, 1991; Hanke et al., 1995]. Sixth, psoralen has been another useful agent for proving the existence of cruciforms in prokaryotic [Zheng et al., 1991] and eukaryotic cells [Jupe et al., 1993], by locking the cruciform structure in vivo through interstrand crosslinks [Sinden and Ussery, 1992]. Seventh, formation of a natural cruciform on a prokaryotic plasmid in vivo was convincingly demonstrated by specific cleavage of this cruciform at its junction with intracellularly induced recombinant endonuclease I encoded by gene 3 of bacteriophage T7 [Panayotatos and Fontain, 1987]. Concurrently, the entire *E. coli* chromosome was cleaved at numerous sites, indicating native cruciforms or cruciform-like structures throughout the genomic DNA in vivo. Eighth, another approach involved the production of monoclonal antibodies directed to cruciform structures [Frappier et al., 1987, 1989]. These antibodies recognize conformational determinants specific to DNA cruciforms and do not bind linear double stranded DNA, linear single-stranded DNA, single-stranded DNA containing a stem-loop structure or tRNA. Moreover, they do not promote and/or induce the formation of cruciforms at IRs on linear DNA [Frappier et al., 1987,

1989]. The binding site of these antibodies has been mapped to the four-way (elbow-like) junction at the base of the cruciform [Frappier et al., 1989; Steinmetzer et al., 1995]. Using these antibodies, it was shown that cruciforms do exist within living mammalian (monkey and human) cells, with an estimated frequency of 0.6×10^5 to 3×10^5 cruciforms per cell [Ward et al., 1990] and are localized in discrete regions within the nucleus at the onset of S phase (see below). The reactivity of the anti-cruciform antibodies with distinct subnuclear sites confined to a certain time of the cell cycle phase indicates convincingly that cruciforms are not artificially induced by antibody binding.

The extrusion of cruciforms in vivo is likely to be primarily via the S-type (see above) [Zheng et al., 1991]. By contrast, standard C-type formation of cruciforms is not likely to occur in the cell, due to the rather high physiological ion concentration, which would strongly suppress the required large-scale opening of IR-flanking regions even in highly supercoiled DNA [Bowater et al., 1994a]. The extrusion of an IR progressing in vitro by C-type mechanism at low salt most probably changes in vivo to the S-type pathway that does not depend on opening of the flanking DNA [Sullivan and Lilley, 1988]. On the other hand, DNA with the propensity to undergo easy denaturation like, for example, DUEs (DNA unwinding elements) at origins of replications, also may be opened and/or stabilized in the single-strand state under intracellular conditions with the aid of proteins; this event may then elicit the formation of a cruciform in a nearby IR by a C-type mechanism.

SIGNIFICANCE OF STEM-LOOPS AND CRUCIFORMS FOR INITIATION OF DNA REPLICATION

The extent of supercoiling has been shown to affect the regulation of DNA replication in *E. coli* [von Freiesleben and Rasmussen, 1992]. Since cruciform extrusion causes an effective relaxation in DNA [White and Bauer, 1987], cruciforms may indirectly influence the onset and regulation of replication by affecting the level of superhelicity, and thus the binding of specific protein factors [Horwitz, 1989]. In fact, supercoiling is known to affect specific binding of regulatory proteins for transcription, recombination, and replication [Wang and Liu, 1990; Cozzarelli and Wang, 1990].

Another instance, in which cruciforms may influence chromatin architecture and, in consequence, regulation of many processes on eukaryotic DNA, is in their interaction with nucleosomes. Nucleosomes interfere with the binding of initiation factors to promoters [Workman et al., 1991] and origins of replication [Cheng and Kelly, 1989; Simpson, 1990]. A yeast ARS placed within the nucleosome has severely reduced function, compared to its normal location in the linker region [Simpson, 1990]. In accordance, the SV40 origin of replication is nucleosome free [Jakobovits et al., 1980; Saragosti et al., 1980], a condition that favors initiation of replication [Cheng and Kelly, 1989]. Histones and/or nucleosomes bind poorly to inverted repeats [Weintraub, 1983], stem-loop [Nickol and Martin, 1983] or cruciform DNA structures [Nobile et al., 1986; Battistoni et al., 1988; Kotani and Kmiec, 1994; van Holde and Zlatanova, 1994], and it is likely that cruciform structures exist in either the spacer region between nucleosomes or orthogonal to the nucleosome surface [Nickol and Martin, 1983]. Thus, cruciforms at origins may exert a biological effect on replication either directly, by exposing nucleosome-free DNA and making it accessible to DNA binding proteins, or indirectly, by phasing nucleosomes in a replication-permissive array.

Although it has been unclear whether the replication of complex genomes initiates at unique and specific sites (origins), over the past few years the existence of specific origins has been well documented [DePamphilis, 1993]. However, according to the "Jesuit model" of replication initiation [DePamphilis, 1993], replication can start at any site that is easily unwound. Reopening of chromatin over a broad DNA region may induce a strong torsional tension in this domain due to unrestrained supercoiling (cf. above). This might lead to melting of several sites of low helical stability, provoking initiation at many "origins of bidirectional replication" (OBR). In fact, some complex origins may display an initiation zone extending over several kilobases, in addition to a predominant site of initiation. Thus, extrusion of cruciforms near origins of replication may be a further mechanism of cruciform action, by which absorption of excess torsional stress will suppress opening of "false" DNA sites and restrict or favor firing at the "right" OBR.

Finally, cruciforms may also participate directly in processes like transcription and replica-

tion by being themselves components of a nucleoprotein complex [Gierer, 1966; Bollum, 1975; Zannis-Hadjopoulos et al., 1988]. In the following sections, we summarize the evidence for cruciforms as active elements in DNA replication.

IMPLICATION OF STEM-LOOPS AND CRUCIFORMS IN INITIATION OF DNA REPLICATION

Prokaryotes

ss phage ϕX174. Primosome assembly on ϕX174 ssDNA requires stem-loop structures. This requirement can be satisfied by many different inverted repeat sequences regardless of the nucleotide sequence [Abarzua et al., 1984; Greenbaum and Marians, 1984; Soeller et al., 1984; Masai et al., 1990]. The *E. coli* replication factor PriA (formerly known as factor Y or protein n) is one of several proteins that constitute a multienzyme complex called the primosome, which functions to prime complementary strand synthesis during ϕX174 DNA replication [reviewed in Marians, 1992]. The first step in primosome assembly is the structure-specific recognition of a stem-loop by PriA [Soeller et al., 1984]. Similar primosome assembly mechanisms exist for many broad-host range plasmids [Miao et al., 1993].

ss phage G4. The origins of phage G4 and the closely related α3, St-1, and ϕK phages each contain a region of approximately 139 bases with three inverted repeats, which are required for priming [Lambert et al., 1986]. Insertional and substitutional mutations that preserve the secondary structures of the stem-loops also preserve the replication activity, indicating that structure, rather than sequence, is important for priming [Hiasa et al., 1989]. In fact, both the secondary structure and spacing of stem-loops of the bacteriophage G4 ori have been shown to be essential for the initiation of replication of the plasmid [Hiasa et al., 1990]. Following infection of the phage, the dnaG primase protein binds to the stem-loop of one of the IRs; this step is requisite for the synthesis of an RNA primer for the initiation of replication.

ds plasmid R1162. Initiation of replication of the broad host range plasmid R1162 occurs at two points on the double stranded molecule that are on opposite strands, each being at the outside edge of a 40 bp IR with a 40-bp intervening sequence [Lin and Meyer, 1987]. DNA synthesis is convergent and directed into the IR. Initiation occurs in a highly conserved 10-bp sequence present, in opposite orientation, at the outer edge of the IR. Varying the composition or the size of the intervening sequence by insertion or deletion had no effect on replication activity. However, reorienting the IRs to be direct repeats prevented initiation. Deletion of either of the repeats also abolished initiation, suggesting that initiation at one site is dependent on initiation at the other. A probable mechanism for the cooperative interaction of the two initiation sites might be the formation of cruciforms; the cruciform structure would be recognized by the replication machinery, and bidirectional (continuous only or semidiscontinuous) replication occurs [Lin and Meyer, 1987]. The single-stranded plasmid RSF1010 (related or identical to R1162) [Lin and Meyer, 1987] also requires an IR at its origin of replication for activity. It was recently demonstrated that both intrastrand base pairing and the base sequence were major determinants of replication activity [Miao et al., 1993].

ds plasmid pT181. The replication origin of the *Staphylococcus aureus* pT181 plasmid contains three short IRs [Wang et al., 1992, 1993]. It has been demonstrated that the initiation of DNA replication of the pT181 plasmid involves extrusion of one of the IRs from supercoiled double-stranded DNA as a cruciform in vivo [Noirot et al., 1990]. The plasmid-encoded initiator protein, RepC, binds to the origin region of either single- or double-stranded DNA and apparently facilitates and stabilizes the formation of the cruciform. After binding, the RepC protein introduces a nick in the cruciform DNA at the center of the IR, and the 3′-hydroxyl group at the nick serves as a primer for the initiation of dsDNA replication [Gennaro et al., 1989].

Organelles: Mitochondrial Origins of Replication

The initiation of replication on the supercoiled circular double-stranded genome of animal cell mitochondria involves stem-loop DNA structures [reviewed in Clayton, 1982, 1992]. There are two separately located origins of DNA replication, the H-strand (O_H) and the L-strand (O_L) origins. O_H initiates before O_L. The O_H origin is within the D-loop region. The D-loop is a region that consists of a three-stranded structure. The third strand of the D-loop is a nascent H strand (initiation sequence) with its 5′ end located at O_H [Chang et al., 1985]; its synthesis is likely directed by RNA priming and the initiation sequences vary from 520 to 690 nucleotides [Bogenhagen and Clayton, 1978]. These three-

stranded D-loops are unstable and have a high turnover rate (70 min) [Bogenhagen and Clayton, 1978]. The 3′ end of the D-loop strand serves as the primer for leading H-strand synthesis, which is continuous.

O_L is only activated after H-strand synthesis has proceeded beyond it, thus leaving it exposed as a single-stranded template. O_L has relatively small sequence requirements for activity, but it is thought to be structurally functional [Hixson et al., 1986]. O_L consists of a short, highly conserved, 30-bp IR with a T-rich loop; this IR has the potential to form a stem-loop structure [Crews et al., 1979; Gillum and Clayton, 1979]. Development of a mtDNA in vitro replication assay demonstrated that mitochondrial primase, a ribonucleoprotein, was able to structurally recognize the stem-loop of O_L, initiate RNA priming and subsequent DNA synthesis [Wong and Clayton, 1985a,b]. RNA priming occurs within the loop, and DNA synthesis begins at the base of the stem. It is likely that replication of the mitochondrial genome of yeasts are accomplished by similar mechanisms [de Zamaroczy et al., 1981].

Eukaryotic Viruses

IRs have been shown to be important for the initiation of DNA replication in viruses of eukaryotes [Frisque et al., 1983; Deb et al., 1986b; Prives et al., 1987; Stow and McMonagle, 1983; Weller et al., 1985; Reisman et al., 1985; Lochson and Galloway, 1986], some of which have been used as models for eukaryotic DNA replication.

Simian virus 40 (SV40) contains two palindromes in its origin. Both are required for the initiation reaction, and one of them, the "early palindrome," melts upon formation of the preinitiation complex [Borowiec and Hurwitz, 1988]. Previous EM studies suggested the presence of a cruciform structure in intracellular SV40 DNA [Hsu, 1985], but more recent studies indicated that neither SV40 palindrome is extruded into a cruciform [Kim and Kang, 1989].

The origin regions in the herpes simplex virus (HSV) genome, ori_{L1}, ori_{L2}, and ori_S, all contain AT-rich palindromes with the capacity to form cruciforms [Weller et al., 1985; Lochson and Galloway, 1986]. However, the ori_S palindrome seems to be required in the linear, rather than cruciform conformation, in the initiation of HSV replication [Lochson and Galloway, 1988; Deb and Doelberg, 1988].

Epstein-Barr virus (EBV) has been an attractive model for cellular DNA replication, because it replicates at the same rate as cellular chromosomes, is restricted to S-phase and maintains a low number of genome copies per cell through the action of the plasmid maintenance replication origin, *ori*P [Yates and Guan, 1991]. Recent evidence indicates that both the family of repeats (FR) and the dyad symmetry (DS) element of *ori*P, are sensitive to single-strand nucleases [Williams and Kowalski, 1993]. In duplex DNA, the structure of the DS element is a large single-stranded bubble containing a stem-loop formed by the 65-bp dyad, while the FR element is in the cruciform conformation. These investigators concluded that the intrinsic ability of the *ori*P elements to form alternative structures may be important in the initiation process, specifically to facilitate the access of the replication machinery to the parental DNA strands. The unwound single-strand bubble containing a base-paired hairpin is reminiscent of the *ssi* signals of plasmids and phages [reviewed in Marians, 1992]. It is tempting to speculate that the unwound *ori*P is recognized by replication factors in a similar fashion as the *ssi* signals.

Eukaryotes

An important question in cellular and molecular biology is how the cell limits DNA synthesis to one round per cell cycle. The mechanism that inhibits re-initiation is not known, although chromatin conformation [Hewish, 1976; Solomon and Varshavsky, 1987; Dhar et al., 1989; Forrester et al., 1990], DNA methylation [Wilson, 1987; Leonhardt et al., 1992], chromatid pairing [Roberts and Weintraub, 1986, 1988], and nuclear membrane permeability [Laskey et al., 1989; Leno et al., 1992] may be involved in the process.

Replication origins, or replicators, the specific sequences that control the initiation of DNA replication, are also poorly defined in mammalian cells [DePamphilis, 1993; Stillman, 1993]. Unlike the simple genomes of prokaryotes and viruses that possess a single origin of replication, the multi-chromosome mammalian genome is replicated from multiple origins. With this increased genomic complexity one might also expect more complex or more numerous modes of regulation at the level of initiation [reviewed by Huberman, 1995].

Structures associated with eukaryotic replication origins. Although the existence of specific consensus sequences in mammalian cells is an evolving area of study, certain types of sequences and/or (potential) structures, e.g., curved DNA [reviewed in Hagerman, 1990, 1992], DNA unwinding elements (DUEs) [Natale et al., 1992, 1993], matrix attachment regions (MARs) [Boulikas, 1992], and inverted repeats (IRs) [Zannis-Hadjopoulos et al., 1984, 1985, 1988; Landry and Zannis-Hadjopoulos, 1991; Bell et al., 1991; Nielsen et al., 1994] are common to many replication origins of prokaryotic, lower eukaryotic, and mammalian organisms [Muller and Fitch, 1982; Campbell, 1986; DePamphilis, 1993]. Some or all of them may constitute an origin and/or mediate the proper origin function and regulation in concert with the cell cycle.

Mammalian early replicating origin enriched sequences (*ORS*) and IRs. Early replicating sequences enriched for replication origins that are activated at the onset of S phase were isolated by extrusion of nascent strands [Zannis-Hadjopoulos et al., 1981] of replicating monkey (CV-1) cells synchronized at the G1/S-phase border [Kaufmann et al., 1985]. The nascent DNA, ranging in size from several hundred bp to approximately 2 kb, were cloned into pBR322 generating a library of cloned early replicating sequences. These sequences, by the nature of their isolation, should contain replication origins at or near their center [Zannis-Hadjopoulos et al., 1983] and thus have been called origin enriched sequences (*ors*).

The *ors* clones were found to be enriched for snap-back sequences, implying that some mammalian origins of DNA replication contain palindromic (IR) sequences [Zannis-Hadjopoulos et al., 1984, 1985]. This finding was later confirmed by nucleotide sequence analysis of the *ors* fragments [Rao et al., 1990; Landry and Zannis-Hadjopoulos, 1991]. Such analyses revealed that common to all the *ors* and other known replication origins (prokaryotic and viral) is the presence of IRs, both perfect and imperfect, as well as AT-rich sequences [Zannis-Hadjopoulos et al., 1984; Rao et al., 1990; Landry and Zannis-Hadjopoulos, 1991]. The IRs are generally flanked by AT-rich regions [Rao et al., 1990] that could facilitate C-type cruciform formation (see above). In addition, the *ors* sequences were statistically enriched for perfect and near perfect matches of the yeast ARS consensus sequence [Palzkill and Newlon, 1988], scaffold attachment regions (SAR) of *Drosophila* [Gasser and Laemmli, 1986], and the CACCC transcriptional control region consensus [Dierks et al., 1983]. Other laboratories have subsequently obtained by similar techniques, libraries of early-replicated DNAs from mammalian cells (human and avian) containing putative replication origins. Those studies also revealed an enrichment of sequences containing IRs, AT-rich regions, SARs and transcriptional regulatory elements [Razin et al., 1986; Triboli et al., 1987; Dimitrova et al., 1993].

The *ors* clones were examined for their ability to replicate autonomously upon transfection into CV-1, COS or HeLa cells [Frappier and Zannis-Hadjopoulos, 1987; Landry and Zannis-Hadjopoulos, 1991]. At least 17 of 35 *ors* clones tested were able to support autonomous replication, as assayed by the *Dpn*I assay [Peden et al., 1980] or by semiconservative bromodeoxyuridine incorporation. Four of the 17 autonomously replicating *ors* were also tested and found capable of initiating replication in a HeLa cell-free in vitro DNA replication system [Pearson et al., 1991]. In both the in vivo [Frappier and Zannis-Hadjopoulos, 1987] and in vitro [Pearson et al., 1991] assays, replication was dependent on the presence of an *ors* insert, initiation was within the ors sequence, and synthesis was semiconservative and bidirectional [Frappier and Hadjopoulos, 1987; Pearson et al., 1991]. Both in vivo [Frappier and Zannis-Hadjopoulos, 1987] and in vitro [Pearson et al., 1994a] initiation within the *ors* insert has been mapped by electron microscopy.

*Ors*8 (483 bp in length; <5 copies per haploid CV-1 genome) [Zannis-Hadjopoulos et al., 1985; Rao et al., 1990] (GenBank Accession No. M26221) is replicated in the early part of S phase [Zannis-Hadjopoulos et al., 1988]. When present on plasmids, it replicates autonomously upon transfection into mammalian cells [Frappier and Zannis-Hadjopoulos, 1987] and in vitro [Pearson et al., 1991]. The various landmarks of *ors*8 include, among other characteristics, an internal 44-bp imperfect IR sequence and an AT-rich sequence domain [Rao et al., 1990]. Recently we demonstrated by deletion mutagenesis that an internal portion (186 bp) of *ors*8 containing the IR is essential for replication activity in vivo and in vitro [Todd et al., 1995]. Certain deletions flanking the IR appear to enhance the replication activity both in vitro and

in vivo, suggesting that there may be negative acting sequences inhibiting replication. Similar results have also been obtained with *ors*12 [Pelletier et al., in preparation]. Finally, *ors*12, which associates with the nuclear matrix in a cell cycle dependent manner [Mah et al., 1993], has also been demonstrated to act as a bona fide replication origin at its native chromosomal locus [Pelletier et al., submitted].

Mammalian cells: effect of anti-cruciform-DNA antibodies. Monoclonal antibodies (mAbs) have been produced with unique specificity to cruciform DNA structures [Frappier et al., 1987, 1989] (cf. above). Introduction of the anti-cruciform DNA mAbs into a permeabilized cell system capable of carrying out DNA replication, resulted in a 2- to 11-fold enhancement of DNA synthesis and, particularly, an enhanced replication of known early replicating sequences such as *ors*8, DHFR and c-*myc* [Zannis-Hadjopoulos et al., 1988]. This observation is consistent with stabilization by antibody of cruciforms near the origins of replication and facilitation of multiple initiations at these sites.

Using the anti-cruciform DNA mAbs in the same system, Ward et al. [1990, 1991] were able to quantify the number of cruciform structures in living cells by fluorescent flow cytometry. A bimodal distribution of cruciforms was observed throughout the S phase, with their numbers at a maximum (estimated 3×10^5 cruciforms per cell) at the G_1/S boundary, and a second wave occurring at approximately 4 h into S phase, but at a lower level (estimated 0.6×10^5 cruciforms per cell) than that observed at the G_1/S boundary [Ward et al., 1990]. The timing of these waves correlates with both the rates of maximal DNA synthesis [McAlear et al., 1989] and the relative enhancement of DNA synthesis by the mAb [Zannis-Hadjopoulos et al., 1988]. There are no detectable cruciforms in G_2/M nuclei [Ward et al., 1991]. These data indicate that formation of cruciforms appears to be cell-cycle regulated. Consistent with this, Collins et al. [1977, 1982] had found that although there were no S1 nuclease sensitive sites in nonproliferating cells, the number of such sites increased when the cells were stimulated to go from G_0 to G_1 and peaked at the onset of S phase. As previously mentioned, cruciforms are sensitive to single-strand-specific nucleases such as S1 [Lilley, 1980; Panayotatos and Wells, 1981] and mung bean nuclease [Frappier et al., 1989] by virtue of their single-strandedness at the tip of the stem-loops. In addition to these studies, there are several reports of different non-B DNA structures that appear to be dynamically regulated throughout the eukaryotic cell cycle, including triplex DNA [Burkholder et al., 1988], Z-DNA [Staiano-Coico et al., 1985], and single-stranded DNA [Klein et al., 1967; Tan and Lerner, 1972; Collins et al., 1977; Conrad and Newlon, 1983].

The above observations support the hypothesis that certain IRs may represent potential initiation sites for DNA replication, as previously suggested [Bollum, 1975; Edenberg and Huberman, 1975; Hobom et al., 1979; Meijer et al., 1979], serving as the attachment site for initiator or other protein factors [Gierer, 1966; Hand, 1978; Muller and Fitch, 1982; Mizuuchi et al., 1982; Tschumper and Carbon, 1982; Hsu, 1985; Bianchi, 1988; Elborough and West, 1988]. Bell et al. [1991] took advantage of the specificity of the anti-cruciform DNA antibody to affinity-purify and clone DNA fragments from CV-1 genomic DNA. Fragments isolated in this fashion were enriched in palindromic sequences. In addition, up to 50% of the clones from the cruciform-DNA affinity-purified library were able to replicate autonomously upon transfection into mammalian cells. *ors*8, an early replicating CV-1 DNA sequence with autonomous replicating activity [Frappier and Zannis-Hadjopoulos, 1987; Todd et al., 1995] (see above) was also recovered in this library. Other libraries were also prepared from human genital fibroblasts, embryo lung fibroblasts (WI-38) and colorectal cancer cells (SW48), by affinity purification with anti-cruciform antibody. These libraries were also shown to be enriched for autonomous replication activity in assays both in vivo and in vitro [Nielsen et al., 1994].

PROTEINS INTERACTING WITH CRUCIFORMS AND CRUCIFORM-LIKE STRUCTURES

In order to critically evaluate the interaction of proteins with cruciform substrates, one must be conscious of the fact that cruciforms possess several structural elements, which may be shared also by other structures in DNA. These elements include (1) single-strand DNA at the cruciform tips; (2) possible mismatches and/or bulges at the stems due to imperfect intramolecular complementarity of the IR; (3) intercrossing helices (crossovers); and (4) angles between the helices or helix-*pairs* of apparently defined degree, depending on the ionic strength (cf. above). For these reasons, cruciforms may interact with

proteins involved in the recognition of single-strand and/or perturbed DNA, intersecting helices at nucleosome entry-exit sites, interwound supercoils, recombination intermediates (Holliday junctions), and intrinsically curved or bent DNA. Therefore, interaction of several proteins with cruciforms studied in vitro, although valuable for basic principles of molecular recognition, may have limited significance for the protein's exact cellular function.

DNA Crossovers

DNA crossovers (crossing helices without exchanging strands) occur in several instances, for example, at the site where DNA enters and exits a nucleosome, or at the intersection of two helices in a plectonemic (interwound) supercoiled molecule. DNA crossovers, like cruciforms, apparently obey the same basic principles of DNA self-fitting [Timsit and Moras, 1991, 1994], thus giving rise to similar structures with similar crossing angles and groove faces [von Kitzing et al., 1990; Lilley, 1992]. Proteins, specifically binding to DNA crossovers, may therefore be able to interact with cruciforms or four-way junctions. For example, histones H1 and H5, which are implicated in tight packaging of chromatin by interacting with the nucleosome linker regions, interact preferentially with DNA crossovers [Krylov et al., 1993]. It was found that H1 could also bind specifically to a stable four-way DNA junction [Varga-Weisz et al., 1993].

Another example are the eukaryotic topoisomerases I and II. These enzymes have been shown to interact preferentially with helix intersections of superhelical molecules [Zechiedrich and Osheroff, 1990], a feature anticipated from their molecular action, which is relaxing positive and negative supercoils. In accordance, extruded cruciforms may be also recognized by topoisomerase II [Pognan and Paoletti, 1992]. By contrast, neither *prokaryotic* topoisomerase I (ω protein), which acts by recognizing single-stranded DNA [Kirkegaard and Wang, 1985], nor topoisomerase II (gyrase), which must introduce negative supercoils, was found to associate preferentially with DNA crossovers [Zechiedrich and Osheroff, 1990, and refs. therein].

Holliday Junctions, Four-Way Junctions

A structure most resembling a cruciform is the so-called Holliday junction [Holliday, 1964], which displays intersecting helix-pairs and reciprocal strand exchange, but lacks the characteristic loops of a cruciform, a feature which may have implications for the structure and the interaction with enzymes (see above). A Holliday junction is an intermediate in homologous general recombination and, consequently, several recombination enzymes, implicated in generation, processing and resolution of a Holliday structure, may be also relevant for interaction with (and instability of) cruciform structures.

In *E. coli,* the main pathway of general recombination is initiated by exonuclease V, encoded by the *rec*BCD genes [reviewed by Kowalczykowski et al., 1994]. Earlier reports suggested a function for *rec*BCD not only in the generation of a recombination intermediate, but also in its resolution [Thaler and Stahl, 1988]. In fact, *rec*BCD is able to introduce a double-strand cut into a model four-way junction in vitro, provided that two ends are open [Taylor and Smith, 1992]. However, no cleavage (and no interaction) takes place, when the ends are blocked, as in the case of a cruciform. This suggests that *rec*BCD may be able to interact with and resolve four-way junctions in whose formation it participates, but precludes an involvement of *rec*BCD in direct recognition of preformed cruciforms. Moreover, resolution of Holliday intermediates in vivo seems to be accomplished by different enzymes (see below).

After the formation of the primary Holliday junction, strand transfer takes place, mediated by the products of the *ruv*A and *ruv*B genes [Iwasaki et al., 1992; Tsaneva et al., 1992; Parsons et al., 1992; for reviews of the late recombination steps, see West and Connoly, 1992; Kuzminow, 1993; West, 1994, 1995; Shinagawa and Iwasaki, 1995]. Apparently, RuvA is the protein specifically interacting with the preformed four-way junction [Iwasaki et al., 1992; Tsaneva et al., 1992; Parsons et al., 1992]. Accordingly, the RuvA–RuvB complex is able to recognize and promote reabsorption of a cruciform on a superhelical plasmid (at the expense of ATP) [Shiba et al., 1991; Shinagawa et al., 1991]. RecG is a RuvA–RuvB substitute protein, also able to recognize four-way junctions and promote strand transfer [Lloyd and Sharples, 1993a,b].

The resolution of Holliday junctions takes place through the action of resolvases. Although the interaction of the latter with cruciforms conceivably may not result in anything else other than cruciform deletion, these enzymes were the prototype of proteins binding to four-way

junctions, and their interaction with DNA has been investigated in great detail. Therefore, the results obtained with these systems may also be significant for protein–cruciform interactions.

Recognition and resolution of Holliday junctions generated by all *E. coli* pathways of general recombination is performed by the RuvC resolvase [Iwasaki et al., 1991], a thoroughly studied enzyme. RuvC has a greater specificity for "true" recombination intermediates than the T4 endonuclease VII [Benson and West, 1994] (see below) and, also in contrast to the latter, cleaves the *continuous* pair of crossing helices [Bennett and West, 1995]. However, it requires a region of homology at the junction point [Iwasaki et al., 1991; Benson and West, 1994]. Upon binding, RuvC brings about a profound structural distortion of two of the four strands of the four-way junction revealed by hydroxyl radical footprinting [Bennett et al., 1993] and unfolds it into a two-fold symmetric structure, different from those described for protein-free junctions [Bennett and West, 1995]. Surprisingly, no contacts to the DNA backbone are evident; this may be explained by interaction of the enzyme, mainly with base pairs, in accordance with the sequence specificity of the cleavage reaction [Bennett et al., 1993]. The DNA-free crystal structure of RuvC has been resolved recently [Ariyoshi et al., 1994]. Another *E. coli* protein, called Rus, shares with RuvC the ability to interact and endonucleolytically resolve four-way junctions [Sharples et al., 1994].

Many other resolvases interacting with four-way junctions have been isolated from different sources and characterized in varying detail. The most prominent members are the debranching enzymes endonuclease VII, encoded by gene 49 of bacteriophage T4 [Pottmeyer and Kemper, 1992, and refs. therein], and endonuclease I, encoded by gene 3 of bacteriophage T7 [Parsons and West, 1990, and refs. therein]. Hydroxyl radical footprinting of T4 endonuclease VII complexed with a four-way junction revealed contacts with the backbone of two out of the four strands at the base of the junction, but no obvious distortion of the DNA structure [Parsons et al., 1990]. By contrast, with the same method all four strands were found to be contacted by T7 endonuclease I, again without structural distortion [Parsons and West, 1990]. This suggests that interaction may be simultaneously with all four junction elbows, or, alternatively, with a different pair of strands of each of two equally abundant stacking stereoisomers (cf. above). The specificity of T4 endonuclease VII, besides cleavage of the *exchanging* strands of four-way junctions and cruciforms, was investigated and found to extend over a broad range of substrates with a clear dependence on local nucleotide sequence [Bhattacharyya et al., 1991; Pottmeyer and Kemper, 1992]. Similarly, T7 endonuclease I cleaves a multitude of structures, has an even 100-fold higher affinity for *single*-strand DNA and shows a sequence-influenced bias in cleavage [refs. in Parsons and West, 1990]. The relaxed specificity of these enzymes is in accordance with their high toxicity for the cell when induced from expression plasmids [Panayotatos and Fontaine, 1987; Kosak and Kemper, 1990] and admonishes to cautiously interpret results obtained with them as probes for cruciform formation (cf. above).

Four-way junction- and/or cruciform-recognizing proteins with the capacity to resolve their substrate by double-strand endonucleolytic cleavage were also isolated from eukaryotic sources. They include the CCE-1 (mgt-1) X-solvase from yeast mitochondria [Kleff et al., 1992; Ezekiel and Zassenhaus, 1993], several yeast proteins [Symington and Kolodner, 1985; Symington et al., 1985; Evans and Kolodner, 1987, 1988; West and Korner, 1985; West et al., 1987; Jensch et al., 1989], calf thymus enzymes [Elborough and West, 1990; Hyde et al., 1994], and resolvases from hamster cells [Hyde et al., 1994], mouse cells [Hyde et al., 1994; Solaro et al., 1995], HeLa cells [Waldman and Liskay, 1988], and human placenta [Jeyaseelan and Shanmugam, 1988]. Additionally, proteins interacting specifically with four- and three-way junctions and/or cruciforms, of which *no* endonuclease activity was reported, were found in *Ustilag maydis* [Kotani et al., 1993], and human lymphoblasts [Elborough and West, 1988]. Similarly, human nuclear poly(ADP-ribosyl)transferase is also likely to interact with cruciforms in vitro and in vivo [Sastri and Kun, 1990; Oei et al., 1994].

Curved/Bent DNA

Perhaps the most dominant feature of a cruciform for interaction with proteins, over a particular sequence, homology, interstrand complementarity, single strand regions, crossing helices and strand exchange, is the presence of defined angles. This became apparent by the elegant experiments of Bhattacharyya et al. [1991], who

demonstrated that several four-way junction-recognizing enzymes, such as T4 endonuclease VII, a yeast and a thymus resolvase (cf. above), are able to recognize (and cleave in the presence of Mg^{2+}), not only their classic substrate, but also three-way junctions, nonbranched bulged duplexes, and intrinsically curved DNA, provided that these substrates present an angle of $\sim 120°$. This suggests that, at least some four-way junction interacting proteins, actually measure angles. However, a curved DNA–RNA hybrid strand was not a substrate, indicating that the helical parameters of a B-form DNA are also requisite for interaction [Bhattacharyya et al., 1991].

The initiator protein RepC recognizes IR-III [Koepsel et al., 1986] and stabilizes the cruciform at the pT181 origin by simultaneous interaction with one stem-loop extruded from the adjacent IR-II [Noirot et al., 1990; Wang et al., 1993]. The pT181 origin was shown to contain a DNA curvature, which is bent further by RepC binding to the linear origin [Koepsel and Khan, 1986], indicating that bent DNA is a major determinant for stable interaction. RepC, although clearly sequence specific, may therefore be another example of a cruciform binding protein, whose stable interaction relies mainly on fitting to the "elbow" formed by two cruciform helices.

A large eukaryotic, nonendonuclease protein family, known to interact with cruciforms, are the HMG-box proteins and their prokaryotic counterpart HU [Bianchi et al., 1992; Ner et al., 1994]. The abundant HMG1 and HMG2 prototypes ($\sim 3–5 \times 10^4$ per nucleus), have been shown to interact preferentially with many structured DNAs, including cruciforms, negative supercoils, crossovers, and *cis*-platinated DNA [Ner et al., 1994, and refs. therein]; for a division of the HMG proteins into three subtypes see Ferrari et al. [1992]. An approx. 80° bent, L-shaped HMG-box domain [Weir et al., 1993; Read et al., 1993; Jones et al., 1994; King and Weiss, 1993; Werner et al., 1995] either interacts with the minor grove of linear DNA bending it [Ferrari et al., 1992; Pil et al., 1993; King and Weiss, 1993; Haqq et al., 1994; Werner et al., 1995; Churchill et al., 1995], or favors binding to DNA angles [Pil and Lippard, 1992; Bruhn et al., 1992; Pil et al., 1993; Churchill et al., 1995]. The latter is probably the cause for the particular capability of HMG-box proteins to interact with cruciforms [Bianchi et al., 1989; Peters et al., 1995]. However, HMG1 has been reported to not be able to protect a four-way junction from T4 endonuclease VII digestion [Bhattacharyya et al., 1991]. This may be due to a different association of HMG1 with its substrate, than that of T4 endonuclease VII [Bhattacharyya et al., 1991]. No footprints of a HMG-box protein on a four-way junction have been reported yet, and the actual protein contacts on DNA are therefore unknown.

Human Cruciform Binding Protein

Based on the hypothesis that there exist specific cellular recognition proteins for DNA cruciforms, we identified and partially characterized a novel DNA-binding activity from HeLa cell nuclei [Pearson et al., 1994b]. The binding activity was enriched from HeLa cells and appears as a 66-kDa cruciform binding protein (CBP). It is specific for cruciform containing molecules, appears to require stem length symmetry and is void of nuclease activity. Cruciform-binding is apparently not biased by the particular sequence. CBP does not recognize linear dsDNA, or ssDNA [Pearson et al., 1994b]. It does, however, show some affinity to Y-shaped molecules. Moreover, by a series of criteria [Pearson et al., 1994b], it is different from the abundant HMG1 protein. Thus, CBP is a novel, structure-dependent DNA binding activity. Hydroxyl radical footprinting studies demonstrated that the protein binds at the base of four-way junctions, recognizing the (asymmetric) arrangement of the helices and contacting the DNA backbone of three strands at both groove surfaces; binding induces structural alterations in the DNA substrate [Pearson et al., 1995]. Protein-induced structural alteration in DNA is common among replication proteins such as dnaA of *E. coli* [Bramhill and Kornberg, 1988], bacteriophage lambda O protein [Schnos et al., 1988], UL9 of herpes simplex virus [Koff et al., 1991], and T antigen of SV40 virus [Borowiec and Hurwitz, 1988]. With respect to these structural aspects, CBP interacts with a four-way junction in a radically different manner from other proteins studied in comparable detail [Pearson et al., 1994b] (cf. above). Interestingly, the monoclonal anti-cruciform antibody can compete against CBP for binding to cruciform DNAs (unpublished data). Recently, using hydroxyl radical footprinting, the antibody binding site was mapped to the cruciform's four-way junction [Steinmetzer et al., 1995]. This indicates that the site(s) recognized by the antibody are similar to those recog-

nized by CBP. This may be biologically significant, as the antibody is known to enhance replication in the cell [Zannis-Hadjopoulos et al., 1988].

Recent studies indicate that CBP activity is regulated with the cell cycle and is maximal in late G_1 phase (in preparation). Cells in late G_1/early S phase are enriched for CBP activity, while G_0 and G_1 cells are not. This pattern of regulation is consistent with the pattern of cruciform formation [Ward et al., 1990] and is similar to that observed for many eukaryotic replication proteins, including c-*myc* protein [Studzinski, 1989] and others [reviewed in Huberman, 1990; and Norbury and Nurse, 1992]. It is interesting that CBP activity may be regulated with the cell cycle and that this activity is maximal at the same stage in the cell cycle (G_1/S) at which the number of observed cruciforms is maximal [Ward et al., 1990]. Thus, cruciform formation may be linked to the appearance of CBP activity.

CONCLUSIONS

Current models of eukaryotic replicon activation [Held and Heintz, 1992; Benbow et al., 1992; Fangman and Brewer, 1991; Hamlin, 1992; DePamphilis, 1993; Stillman, 1993] postulate that different classes of replication origins are successively activated throughout the S phase. Not every IR with dyad symmetry in mammalian cells is associated with an origin of replication. It is clear that formation of cruciforms is important for other functions such as gene expression in both prokaryotes [Horwitz and Loeb, 1988; Horwitz, 1989; Waga et al., 1990; Dayn et al., 1992] and eukaryotes [Greenberg et al., 1987; Martinez-Arias et al., 1984; Shuster et al., 1986]. Furthermore, not all mammalian origins may contain IRs or require cruciform formation for functionality. However, it is likely that the regulation of initiation of some replicating sequences does require an IR, and possibly cruciform formation, as both AT-rich tracts and IRs are present on all functional *ors* sequences, as well as other isolated early replicating mammalian DNAs [Triboli et al., 1987; Razin et al., 1986; Anachkova and Hamlin, 1989; Leu et al., 1989; Dimitrova et al., 1993].

The possibility that certain IRs may act as triggering signals for the initiation of replication is based on numerous previous studies [Zannis-Hadjopoulos et al., 1984, 1988; Ward et al., 1990, 1991; Bell et al., 1991; Nielsen et al., 1994; Todd et al., 1995]. As a result of those studies, we suggested that 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.

Cruciform-specific proteins may stabilize cruciform structures, or promote cruciform extrusion or their reabsorption by branch migration, as this process is slow [Johnson and Symington, 1993] and likely to require an energy source [Robinson and Seeman, 1987]. A protein that could promote branch migration of the four strands at a cruciform junction could either force cruciform reabsorption or catalyse the unwinding of the two strands. Unwinding would be accomplished following the intrastrand base pairing of the complete IR; as there exists no homology in the regions flanking the IR, further branch migration would render these regions single-stranded. The unwound regions would then be available for the entry of the replication/transcription/recombination factors. We propose that cruciform extrusion would cause nucleosome phasing, rendering itself and the DNA proximal to it nucleosome-free, and thus available for other replication initiation factors. In addition, we suggest that cruciformation may induce flexural alterations in proximal DNA flanking the junction. These alterations could affect the binding of other factors (or vice versa), and may facilitate unwinding of the duplex for transcription or replication priming. It may very well be that the site of initiation is not the cruciform; however, initiation would be relatively close. Suitable models, like the ors sequences, should enable the study of cruciform-facilitated initiation of eukaryotic DNA replication.

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