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DNA loop domains in a 1.4-Mb region around the human *hprt* gene mapped by cleavage mediated by nuclear matrix-associated topoisomerase II

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Abstract We have mapped the positions in a ~1.4-Mb region of genomic DNA around the human *hprt* gene which are accessible in vivo to cleavage by topoisomerase II associated with the nuclear matrix. These positions, which are interpreted as the boundaries of DNA loop domains, were mapped in K562 cells by examining the truncation of rare-cutter restriction fragments separated by pulsed field gel electrophoresis after topoisomerase II-mediated cleavage, using seven linked markers mapped in this region as probes for indirect end-labeling. Eleven cleavage positions were detected and were interpreted as defining ten loop domains of lengths between 70 and 210 kb (average ~135 kb); the *hprt* gene resides in a 150-kb loop domain. Loop domain boundaries coincided with three of the fifteen deletion breakpoints mapped in a 600-kb sector of this region in human lymphocytes, within the limits of resolution of pulsed field gel electrophoresis; this correlation was not statistically significant.

Key words DNA loop domains · Human *hprt* gene · Topoisomerase II · Nuclear matrix

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Introduction

A number of experimental approaches show that the genomic DNA of eukaryotic cells forms a series of topologically independent domains, and have led to a model in which the boundaries of these domains are attached to a matrix or scaffold in the nucleus (Blasquez et al. 1989; Laemmli et al. 1992; Roberge and Gasser 1992; Razin et al. 1994, 1995). Some observations suggest that loop domains are functional units of replication (Buongiorno-Nardelli et al. 1982; Marilley and Gassend-Bonnet 1989; Georgiev et al.1991), of transcription (Bode and Maass 1988; Phi-Van and Strätling 1988; Marilley and Gassend-Bonnet 1989; Levy-Wilson and Fortier 1990; Georgiev et al. 1991; Hanson and Ley 1992), and of recombination (Sperry et al. 1989). Although loop domains appear to be a ubiquitous feature of eukaryotic genomes, this level of DNA organisation is not considered in large-scale genome mapping studies because their detection is possible only where contiguous clones are available to map restriction fragments which bind specifically to the nuclear matrix (matrix attachment regions or MARs; Blasquez et al. 1989) or nuclear scaffold (scaffold attachment regions or SARs; Laemmli et al. 1992).

A new general approach to the mapping of loop domains exploits the ability of topoisomerase II associated with the nuclear matrix to cleave genomic DNA in MARs and SARs (Razin et al. 1994; Gromova et al. 1995b; Iarovaia et al. 1995, 1996); the enzyme mediates this cleavage upon denaturation after it has transiently interrupted and bound covalently to both strands of duplex DNA (Wang 1996). Topoisomerase II is a major component of the nuclear matrix (Berrios et al. 1985.) and can readily cleave DNA in MARs and SARs in vivo (Blasquez et al. 1989; Razin et al. 1991, 1994; Laemmli et al. 1992; Gromova et al. 1995b; Iarovaia et al. 1995, 1996). DNA cleavage also occurs at other sites which coincide in most cases with DNase I-hypersensitive or regulatory regions (Rowe et al. 1986; Udvardy et al.

1986), but this cleavage can be prevented by first extracting the topoisomerase II present in the nucleoplasmic compartment (Razin et al. 1994; Gromova et al. 1995b; Iarovaia et al. 1995, 1996). The prediction that DNA is cleaved only in MARs or SARs in these conditions has been confirmed in the cases of the c-*myc* (Gromova et al. 1995b) and ribosomal RNA (rRNA) genes (Razin et al. 1994; Iarovaia et al., unpublished), and the cleavage of the same sites by the single strand-specific nuclease Bal 31 in vivo (Iarovaia et al. 1995) supports their identification as MARs, which are characterised by ready unwinding (Bode et al. 1992). This correlation has been extended to a 500-kb cloned region of the X chromosome of *Drosophila melanogaster,* where ten cleavage positions coincide with SARs (Iarovaia et al. 1996). The further prediction that the DNA fragments which are excised by this cleavage reaction correspond to loop domains is supported by the similarity of their lengths to those estimated for domains (Benyajati and Worcel 1976; Igo-Kemenes and Zachau 1977; Buongiorno-Nardelli et al. 1982; Hancock and Hugues 1982; Hartwig 1982; Jackson et al. 1990) and more strongly by evidence suggesting that individual genes which have been examined each reside in an excised fragment of between 35 and 75 kb (Razin et al. 1994; Gromova et al. 1995a; Iarovaia et al. 1995, 1996).

This strategy allows loop domains to be mapped in regions of the genome where detailed maps or clones are not available, by using linked probes to detect rare-cutter restriction fragments which have been truncated by topoisomerase II-mediated cleavage. Here we describe the application of this approach to a 1.4-Mb region of the human X chromosome surrounding the *hprt* gene, which has not been mapped in detail or cloned (Little et al. 1992; Lippert et al. 1995a, b), with the objective of examining loop domain organisation in a region that is much longer than those studied previously and investigating the relationship between loop domains and recombination breakpoints that map in this region (Morris and Thacker 1993; Lippert et al. 1995b; Rainville et al. 1995).

Materials and methods

Excision of DNA fragments mediated by nuclear matrix-associated topoisomerase II

K562 cells (ATCC CCL243) growing in DMEM medium with 10% fetal calf serum were washed, resuspended in phosphate-buffered saline (PBS), and $\sim 10^7$ cells in 50 µl of PBS were mixed rapidly with 50 µl of molten (42° C) 1.5% low-melting point agarose in PBS and transferred to a sample mold (Bio-Rad) at 4° C. The cleavage reaction by nuclear matrix-associated topoisomerase II was carried out as described previously (Gromova et al. 1995a; Iarovaia et al. 1996); nucleoplasmic topoisomerase II was removed by permeabilising and extracting the agarose blocks for 1 h at 4° C in 20 mM TRIS-HCl (pH 7.5), 2 M NaCl, 2 mM EDTA, 0.2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and after washing three times (30 min each) at 4° C in 2 ml of 20 mM TRIS-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, they were incubated for 1 h at 37° C in the same buffer with addition of ATP (1 mM) and the topoisomerase II religation inhibitor VM-26 (at the concentrations given in the Figure legends). DNA cleavage was induced by deproteinisation with proteinase K (500 µg/ml) in 0.5 ml of 0.9% Sarkosyl, 1% SDS, 400 mM EDTA, for 36–40 h at 55° C. The blocks were washed extensively with 100 mM EDTA. To map cleavage sites the blocks were further incubated with *Sfi*I, *Fsp*I, *Bss*HII, or *Sal*II (200–500 units/0.5 ml) using the supplier's recommended conditions and then placed in the wells of 1% agarose gels cast in 0.5 × TBE. DNA fragments were separated using a CHEF DR-II system (Bio-Rad) at 12–14° C for 20–22 h with a voltage gradient of 1.5 V/cm and switch time ramped linearly from 10 to 80 s or 20 to 90 s. Gels were stained with ethidium bromide, photographed, incubated in 0.25 M HCl (15 min), distilled water (10 min), and 0.4 M NaOH (30 min) at room temperature, and DNA was transferred to a Hybond N^+ membrane (Amersham) in 0.4 M NaOH for 6–12 h.

Probes and hybridisation

The probes employed were DXS53 (from B. Sylla), DXS86 (J-L. Mandel), DXS311 (J. J. A. Holden), 5′*hprt*, DXS1327, DXS1328, and DXS1329 (M. J. Lippert) and DXS10, DXS79, and DXS177 (ATCC) and were between 0.5 and 6 kb in length (Lippert et al. 1995a). Probes were labeled with [α-^{32}P]dCTP using the Multiprime or Rediprime systems (Amersham). Membranes were prehybridised for 3–6 h and hybridised for 16 h at 42° C in 25 mM TRIS-HCl (pH 7.5), 1 M NaCl, 50% formamide, 10% dextran sulphate, 1% SDS, 5 × Denhardt's reagent, with denatured sonicated salmon sperm DNA (200 µg/ ml), washed in 0.2 × SSC, 0.1% SDS for 20 min at room temperature and for 3 × 30 min at 65° C. Hybridisation signals were detected by exposure to Kodak X-omat AR film with an intensifying screen at −80° C. Fragment lengths were calculated by reference to λ DNA oligomers and a *Hin*dIII digest of λ DNA separated in adjacent lanes and detected by a λ probe after removing the first probe.

Results

The *hprt* gene region in K562 cells

Preliminary experiments showed that in K562 cells, the DNA fragments excised by topoisomerase II-mediated cleavage and separated by pulsed field gel electrophoresis (PFGE) showed the clearest pattern of hybridisation signals with *hprt* region probes, whereas

heterogeneous DNA fragments, probably released by endogenous DNases, caused variable background signals in other human cell lines examined and in cultured fresh lymphocytes (see Discussion). To provide a framework for mapping topoisomerase II cleavage sites, the organisation of the *hprt* gene region in K562 cells was compared with that reported for human T-lymphocytes (Lippert et al. 1995a) by hybridising *hprt*-linked probes to rare-cutter restriction fragments separated by PFGE. The length of the hybridising fragments coincided with those from lymphocytes within the limits of precision of PFGE measurements (estimated as ±10%: Cutting et al. 1988) and they could be placed contiguously by employing the marker positions reported for lymphocytes (Lippert et al. 1995a). Minor restriction fragments were detected by some probes (see Figs. 2, 3) and were ascribed to partial methylation of restriction sites. The positions of the *hprt*-linked markers in K562 cells did not appear to differ from those in lymphocytes (Lippert et al. 1995a).

Excision of DNA fragments mediated by nuclear matrix-associated topoisomerase II

The strategy for cleavage of genomic DNA in vivo by nuclear matrix-associated topoisomerase II has been extensively described previously (Razin et al. 1994; Gromova et al. 1995a, b; Iarovaia et al. 1995, 1996). Nucleoplasmic topoisomerase II was extracted from cells embedded in agarose and after incubation with the religation inhibitor VM-26 (Wang 1996) the accumulated enzyme molecules covalently bound to DNA were digested with proteinase K. Some DNA remained in the sample well and compression region of PFGE gels (Fig. 1) as noted in other studies, probably because topoisomerase II religation could not be completely inhibited by VM-26. DNA fragments ≥10 kb in length remain in the gel under these conditions (Iarovaia et al. 1996). The excised DNA fragments were 20–600 kb in length (Fig. 1) and showed a pattern similar to those seen in other cell types (Razin et al. 1991, 1994; Gromova et al. 1995a, b; Iarovaia et al. 1995, 1996).

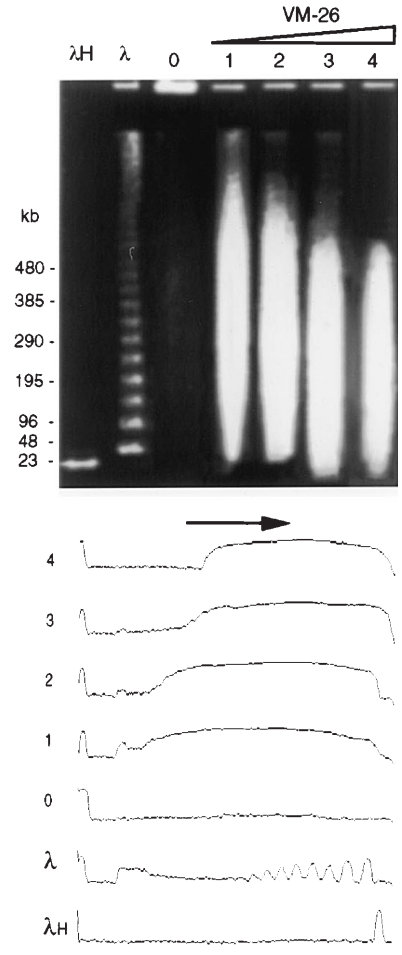

Fig. 1 Excision of fragments of genomic DNA mediated by nuclear matrix-associated topoisomerase II. Cells embedded in agarose were extracted to remove nucleoplasmic topoisomerase II, incubated with the topoisomerase II religation inhibitor VM-26 at a concentration of 10, 20, 30, or 45 μg/ml (lanes 1 to 4 respectively) for 1 h, and then treated with protease K to digest topoisomerase II molecules covalently bound to DNA (see Materials and methods). The excised DNA fragments were separated by PFGE and stained with ethidium bromide; the two upper bands in each lane represent DNA which remained in the sample well and the compression region. Markers were a *Hin*dIII digest (λH) and oligomers of λ DNA. Densitometer scans of each lane are shown below

Topoisomerase II cleavage positions in the *hprt* gene region

All the *hprt*-linked probes described by Lippert et al (1995a) detected restriction fragments which had been truncated by topoisomerase II-mediated cleavage, but most were too distant from the ends of their cognate fragment to map the cleavage position unambiguously. Seven probes were employed which were situated close to the extremity of their corresponding restriction fragment, 10-15 kb away for 5′*hprt*, DXS1327, 1328, and 10, ~25 kb for DXS79 and 86, and ~50-kb for DXS1329 (Figs. 2, 3). Although these probes did not provide precise end-labeling conditions, this factor had relatively minor effects on the interpretation of the data, which are considered in the Discussion.

The pattern of truncation of restriction fragments after cleavage mediated by nuclear matrix-associated topoisomerase II is shown in Figs. 2 and 3. In some cases only partial truncation was seen; in the fragments detected by 5′*hprt* and DXS1327 (Fig. 2) and at two positions in fragments detected by DXS1329 and the *Bss*HII fragment detected by DXS86 (Fig. 3), this could be a consequence of different loop domain organisation of the two *hprt* alleles (see Discussion). DXS79 did not detect the cleavage position at −80 kb seen in the overlapping *Bss*HII fragment (Fig. 2) and similarly DXS10 did not detect that seen at 840 kb in the overlapping *Sac*II fragment (Fig. 3); this is likely to be due to the

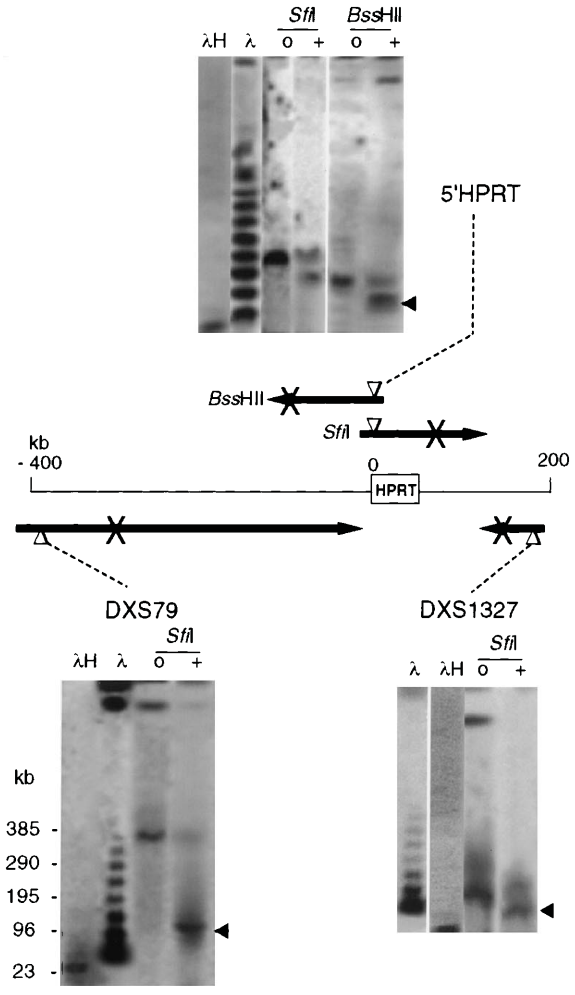

Fig. 2 Truncation of restriction fragments from the region centromeric to and including the *hprt* gene (−400 to +200-kb) after cleavage of DNA by nuclear matrix-associated topoisomerase II. Cells were incubated without (o) or with (+) VM-26 (45 μg/ml), topoisomerase II cleavage was induced as described in Fig. 1, and the agarose blocks were incubated with the restriction enzyme *Sfi*I or *Bss*HII. The resulting DNA fragments were separated by PFGE and hybridised with the indicated probes; the two upper bands in each lane represent signal from DNA remaining in the sample well and the compression region of the gel (Fig. 1). DNA fragments less than 10-kb in length remain in the gel in these conditions (Iarovaia et al. 1996). Truncated fragments are indicated by the *filled arrowheads*; their lengths were calculated from the positions of λ DNA oligomers and *Hin*dIII-digested λ DNA (λH) detected by rehybridisation with a λ probe. The map shows the positions of the restriction fragments corresponding to each panel and of the *hprt* gene; probe positions are shown by the *open arrowheads* and positions of topoisomerase II-mediated cleavage by X

occurrence of "strong" and "weak" topoisomerase cleavage sites (Udvardy et al. 1986; Wang 1996) which cause cleavage at a site distant from an indirect end-labeling probe to be undetectable if cleavage occurs more rapidly at a nearer site. The possibility that topoisomerase II cleavage between the probe and the near extremity of its cognate fragment escaped detection could be excluded in most cases by examining overlapping fragments. Cleavage close to DXS10 was detected by DXS86 in the overlapping *Bss*HII fragment, but none was seen close to DXS1328 (Fig. 3). In the regions of DXS79, 1329 and 86, overlapping fragments were not available, and undetected cleavage between these probes and the near extremity of their cognate fragments would cause errors of up to 50 kb in the other cleavage positions which they detected (see Discussion). Four of the cleavage positions were mapped independently within less than 20 kb by the truncation of different restriction fragments (at 360, 630, 840, and 970-kb) (Fig. 3) and were interpreted as single positions. The eleven positions of cleavage mediated by nuclear matrix-associated topoisomerase II were spaced at intervals of 70–210 kb (see Fig. 5).

An excised DNA fragment containing the *hprt* gene

The ~45-kb *hprt* gene appeared to be framed by topoisomerase II cleavage positions spaced by 150-kb (Fig. 2). To confirm this finding we determined the length of the DNA fragment excised by topoisomerase II cleavage which contained the *hprt* gene, as described earlier for other single-copy genes (Razin et al. 1994), by hybridising the 5′*hprt* probe to excised fragments separated by PFGE. A fragment of about 150 kb was detected (Fig. 4), in agreement with the length predicted from the cleavage map (Fig. 2).

Discussion

MARs and SARs could not be studied by conventional methods in this 1.4-Mb region due to the absence of detailed restriction maps and contiguous clones, but the boundaries of ten loop domains could be defined by the eleven positions of cleavage by nuclear matrix-associated topoisomerase II (Fig. 5). DNA cleavage under these experimental conditions is mediated by endogenous topoisomerase II; it depends on preincubation of cells with VM-26 (Fig. 1), a specific inhibitor of the enzyme's religation reaction (Wang 1996), and other studies show that religation inhibitors of disparate chemical classes cause cleavage at the same positions (Gromova et al. 1995a) and that the excised DNA fragments bear at their 5′ ends a covalently bound polypeptide whose size corresponds to that of a topoisomerase II subunit (Ralph and Hancock 1985; Razin et al. 1991). DNA cleavage was restricted to regions associated with the nuclear matrix by first extracting topoisomerase II from the nucleoplasmic compartment, which could cleave DNA at other sites; about 20% of both topoisomerase II isoforms remains in the agarose-embedded cells after this extraction with 2 M NaCl (Svetlova and Hancock, unpublished), conditions which operationally define the nuclear matrix and in which DNA loop domains are associated with it (Berrios et al. 1985; Phi-Van and Strätling 1988; Blasquez et al. 1989; Sperry et al. 1989; Razin et al. 1995). In unextracted cells the major

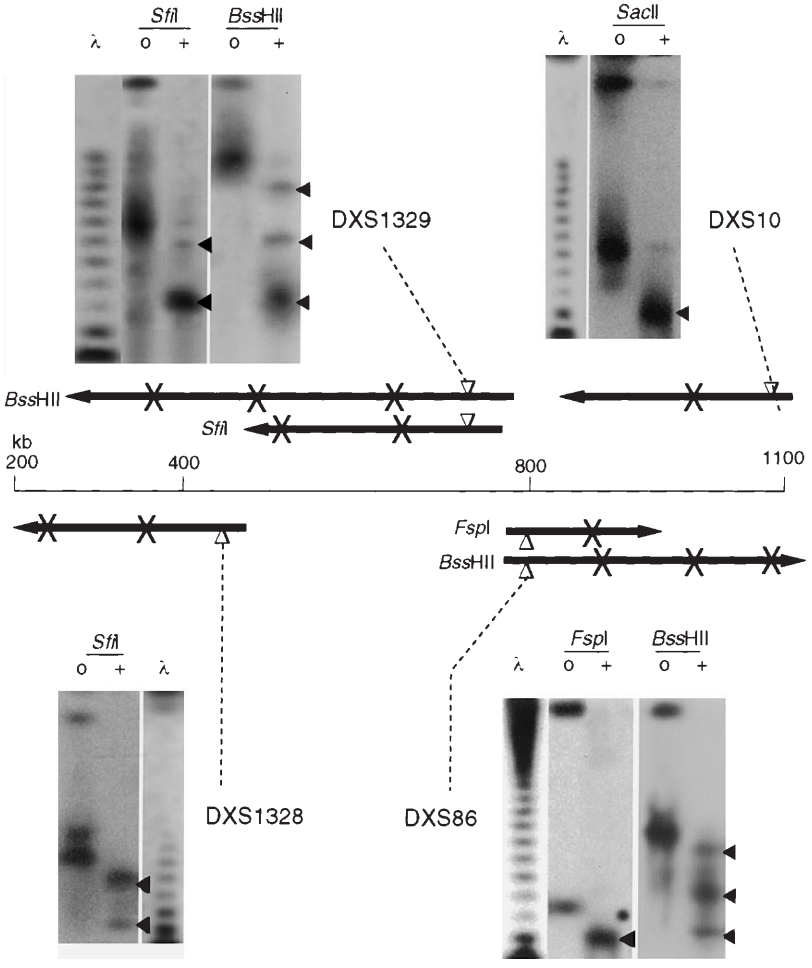

Fig. 3 Truncation of restriction fragments in the region telomeric to the *hprt* gene (200 to 1100 kb) by nuclear matrix-associated topoisomerase II. The experimental conditions and symbols are as described in Fig. 2; after topoisomerase II-mediated cleavage the agarose blocks were incubated with *Sac*II, *Sfi*I, *Fsp*I, or *Bss*HII. Incomplete restriction was attributed to partial methylation of restriction sites (see Discussion)

cleavage positions are the same, but a background of cleavage at other sites, believed to be mediated by nucleoplasmic topoisomerase II *within* loop domains, is eliminated by the extraction step (Gromova et al. 1995b). These considerations show that the observed cleavage positions exist in vivo and do not result from artefactual rearrangements of DNA during the extraction procedure (Razin et al. 1995). The existence of these two classes of cleavage site further suggests that topoisomerase II recognition sequences are necessary but not sufficient to define the boundaries of loop domains. Mapping of the cleavage positions at high resolution is not yet feasible since no genomic clones or sequence data have been reported for this region except for the *hprt* gene itself, but cleavage positions in other loci show reiterated cleavage sites clustered within a region several kb long (Razin et al. 1991, 1994).

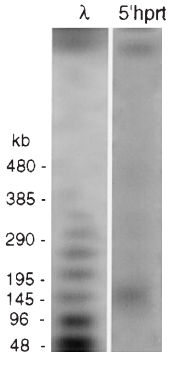

Fig. 4 Hybridisation of the 5'*hprt* probe to DNA fragments excised from cells processed as described in Fig. 1 (45 μg/ml VM-26)

The periodical spacing of cleavage positions for nuclear matrix-associated topoisomerase II around the *hprt* gene is characteristic of long genomic regions, including the rRNA genes (Razin et al. 1994; Iarovaia et al. 1995), a 500-kb region of the *Drosophila* X chromosome (Iarovaia et al. 1996), and the human dystrophin gene locus (Lagarkova et al., unpublished); it reflects the spacing of discrete regions where DNA is accessible to topoisomerase II in the nuclear matrix, because cleavage of genomic DNA by topoisomerase II in vitro yields only a continuous spectrum of fragments cleaved at random sites (Gromova et al. 1995a; Iarovaia et al. 1996). Further topoisomerase II cleavage positions were detected in the region of −1900-kb by probe DXS53, but

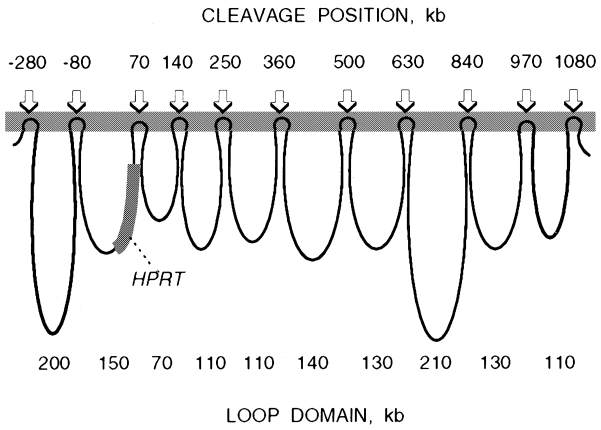

Fig. 5 The positions of DNA cleavage in the *hprt* gene region mediated by nuclear matrix-associated topoisomerase II (Figs. 2 and 3) interpreted as the boundaries of ten contiguous DNA loop domains. Four cleavage positions (at 360, 630, 840, and 970 kb: Fig. 3) were mapped independently within a region of less than 20-kb by the truncation of different restriction fragments and are interpreted as single positions

are not shown because this and the intervening region have not been mapped (Lippert et al. 1995a). The average spacing of cleavage positions is ~135 kb (Fig. 5), a value that coincides with lengths of DNA loop domains estimated by other methods (Benyajati and Worcel 1976; Igo-Kemenes and Zachau 1977; Buongiorno-Nardelli et al. 1982; Hancock and Hugues 1982; Hartwig 1982; Jackson et al. 1990). The ~45-kb *hprt* gene, the only transcription unit identified within this region, is framed by two cleavage positions separated by 150-kb (Figs. 2 and 4). This is consistent with the reported absence of SARs near the *hprt* gene (Beggs and Migeon 1989); the finding that restriction fragments from the gene itself show MAR activity (Sykes et al. 1988; Chong et al. 1995) can be understood if genomic DNA contains a repertoire of MARs from which only certain loop domain attachments are selected according to the cell's status, as suggested by the change of loop domain length during development in *Xenopus* (Micheli et al. 1993). Since K562 cells are derived from a female leukemia, the partial topoisomerase II cleavage in some fragments (Figs. 2, 3) could reflect different loop domain organisation of the two *hprt* alleles only one of which is transcribed (Morris and Thacker 1993; Lippert et al. 1995a, 1995b). Cleavage positions for nuclear matrix topoisomerase II also frame transcription units in the α-globin (Razin et al. 1991) and rRNA genes (Iarovaia et al. 1995) – like MARs and SARs, which in most but not all cases frame coding regions (Bode and Maass 1988; Phi-Van and Strätling 1988; Blasquez et al. 1989; Hanson and Ley 1992; Laemmli et al. 1992; Roberge and Gasser 1992; Iarovaia et al 1996).

It has been hypothesised that certain recombinational events may occur in loop domain attachment regions and be mediated by topoisomerase II (Sperry et al. 1989; Bodley et al. 1993; Felix et al. 1995; Broeker et al. 1996) and some breakpoint regions in the *hprt* gene contain consensus topoisomerase II recognition sequences (Morris and Thacker 1993; Rainville et al. 1995). Fifteen deletion breakpoints have been mapped between 0 and 600 kb in the *hprt* gene region in human lymphocytes (Lippert et al. 1995b) of which three, at 70, 140, and 500 kb, coincide with the loop domain boundaries mapped here within the limits of resolution of PFGE, but the statistical probability that this coincidence arises from random distributions is high (0.2, χ^2 test); the possibility that a subset of recombinational events may occur at domain boundaries remains open, however. A breakpoint region in cells of a secondary leukemia examined following chemotherapy with topoisomerase II religation inhibitors coincides with a SAR (Broeker et al. 1996), and it will be of interest to compare the positions of recombination induced by these agents in the *hprt* and other regions (Charron and Hancock 1991; Yu et al. 1994) with loop domain boundaries. The use of this strategy to relate loop domain organisation to physical and genetic maps could contribute to understanding the functional significance of this feature of genome organisation.

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