Distribution of topoisomerase II-mediated cleavage sites and relation to structural and functional landmarks in 830 kb of Drosophila DNA

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ABSTRACT
The pattern of sites for cleavage mediated by topoisomerase II was determined in 830 kb of cloned DNA from the Drosophila X chromosome, with the objectives of comparing it with mapped structural and functional landmarks and examining if the correlations with such landmarks reported in individual loci can be generalized to a region ~100 times longer. The relative frequencies of topoisomerase II cleavage sites in 247 restriction fragments from 67 clones were quantified by hybridization with probes prepared from DNA fragments which abutted all cleavage sites in each clone, selected through the covalently bound topoisomerase II subunit; the specificity and quantitative nature of this method were demonstrated using a plasmid DNA model. The 12 restriction fragments with strong nuclear scaffold attachment (SAR) activity, of which seven possess autonomous replication (ARS) activity, show statistically strong coincidence or contiguity (P ≤ 0.11) with regions of high topoisomerase II cleavage site frequency. These regions show no correlation with repetitive sequence or A/T or C/G content and some extend over >10 kb; their sensitivity is therefore unlikely to be due to alternating purine–pyrimidine repeats or regions of Z conformation, which are preferred motifs. The hypothesis that they possess intrinsic curvature is consistent with the similarity of their length and spacing to regions of predicted curvature in the 315 kb DNA of Saccharomyces cerevisiae chromosome III and with the reported strong binding preference of topoisomerase II for curved DNA. The topoisomerase II cleavage pattern in this DNA further shows that its relationships to functional properties seen in individual loci, especially to MAR/SAR and ARS activity and to the restricted accessibility of DNA to topoisomerase II in vivo, can be generalized to much longer regions of the genome.

INTRODUCTION
The selection of sites for binding and reaction of topoisomerase II on DNA is determined by sequence and secondary structure motifs (1–11). Sequences of alternating purine–pyrimidine repeats provide preferred targets (1–4) and the enzyme binds strongly to regions with intrinsic curvature (5–7); its affinity for curved and Z conformations is two orders of magnitude greater than that for the B conformation (5–7). Topoisomerase II also binds to points of crossover of two duplex DNAs (8,9) and to regions in hairpin (10) and tetraplex (11) conformations. Binding sites can be revealed by inducing DNA cleavage by denaturation of the reaction intermediate, in which both strands are transiently interrupted and bound covalently to the enzyme (12). In genomic DNA, the cleavage sites which have been mapped in a number of loci show certain correlations with functional properties (13–24); for example in the Drosophila hsp70 (14,19) and histone (4,21) genes they flank the boundaries of coding regions, in the human and mouse c-fos gene they are clustered in promoters and enhancers (20) and they are highly reiterated in regions of DNA which bind to the nuclear matrix (MARs) and nuclear scaffold (SARs) (4,16,22,23,25).

This evidence that topoisomerase II recognizes functional and conformational motifs led us to examine its cleavage pattern in a region of genomic DNA two orders of magnitude longer than the individual loci previously studied, a cloned 830 kb continuum of Drosophila DNA covering this 830 kb region (26). Plasmid pRYG, a derivative of pUC19 (27), 11 of which are attached to functional properties.

MATERIALS AND METHODS
DNA and topoisomerase II-mediated cleavage
The λ clones of Drosophila DNA covering this 830 kb region have been described (26). Plasmid pRYG, a derivative of pUC19

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containing a 54 bp insert with strong topoisomerase II cleavage sites (2), was obtained from TopoGen (Columbus, OH). DNA was cleaved by topoisomerase II (bovine α-isofrom; TopoGen) by incubation in 20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 5% glycerol, 30 µg/ml BSA, 250 µg/ml ATP. The ratio of enzyme to DNA (by mass) was 1:10 and the topoisomerase II religation inhibitor VM-26 was added to 10 µg/ml. The reaction was terminated by adding SDS to 0.5% and EDTA to 10 mM; proteolytic enzymes were not used in order to conserve the DNA. After washing with the same buffer the bound DNA fragments were deproteinized and eluted in 1 ml 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% SDS, 500 µg/ml proteinase K for 16 h at 37°C and purified by extraction with phenol/chloroform.

**Probes and hybridization**

Two different probes were hybridized successively to restriction fragments from cloned Drosophila DNA inserts, separated by electrophoresis in individual lanes of a 0.8% agarose gel and transferred to a Hybond N⁺ membrane (Amersham). The first (total DNA) probe served to quantify the relative amount of DNA in each fragment and was prepared from DNA of the same clones restricted with EcoRI, HindIII and BamHI. The second (topo II site) probe served to identify fragments which contained topoisomerase II cleavage sites and to quantify these sites and was prepared by further topoisomerase II cleavage and selection of DNA fragments which abutted cleavage sites, as described above. Probes were labelled by random priming with [³²P]dCTP using the Multiprime system (Amersham). Hybridization was at high stringency conditions (30°C) in 4× SET (20× SET is 0.6 M Tris–HCl, pH 8.0, 3 M NaCl, 40 mM EDTA), 2× Denhardt’s solution, 0.2% SDS, 100 µg/ml sheared denatured herring sperm DNA at 65°C for 18 h. Membranes were washed at room temperature with 1× SSC, 0.1% SDS, for 10 min, twice at 65°C with 3× SET, 0.2% SDS for 30 min and once at 65°C with 1× SET, 0.2% SDS for 30 min. The autoradiographic signals from the total DNA probe were quantified by scanning with a Vernon PH15 densitometer under conditions of linear response and the probe was removed by incubating the membrane in 0.5 M NaOH, 1.5 M NaCl for 30 min at 45°C and then in 1.5 M Tris–HCl, pH 8.0, 1.5 M NaCl for 30 min at 45°C. The efficiency of this stripping procedure is demonstrated in Figures 1 and 2. The membrane was rehybridized with the topo II site probe under the same high stringency conditions and this autoradiograph was scanned after adjusting the signal from a vector DNA fragment (Fig. 2) to the value given by the first probe. The cleavage site frequency was expressed as the ratio of the integrated signals (topo II site probe/total DNA probe) for each restriction fragment.

**Statistical analyses**

The use and interpretation of statistical tests to examine correlations between different properties of restriction fragments from this region of DNA have been described previously (26–31). Initial comparisons of the topoisomerase II cleavage site frequency in each fragment with a second property were made using the MacMul+GraphMu factorial correspondence analysis.
Figure 2. Detection of topoisomerase II cleavage sites in restriction fragments of cloned *Drosophila* DNA (26). The topo II site probes (lanes a) hybridized only to fragments which contained topoisomerase II cleavage sites and produced signals which were proportional to their frequency, while the total DNA probe (lanes b) detected all the fragments. Cloned DNA was restricted with *Eco*RI (R), *Sal*I (S), *Xho*I (Xo), *Xba*I (Xa) or *Hin*Ⅲ (H), electrophoresed in a 0.8% agarose gel, transferred to a membrane and hybridized successively with a total DNA probe and a topo II site probe as described in Materials and Methods. The signal from a 1.9 kb *Eco*RI–*Sst*I vector fragment, shown below the lanes, served to normalize the signal intensities in different lanes and on different membranes. Hybridization patterns for 24 of the 67 clones employed are shown. Markers were *Hin*Ⅲ/EcoRI-digested λ DNA (right, length in kb).

RESULTS

Comparison of topoisomerase II cleavage site frequency in restriction fragments

Rather than detecting topoisomerase II cleavage sites in each of the 247 fragments resulting from restriction of this region of DNA with *Eco*RI, *Hin*Ⅲ and *Bam*HI (26) by direct or indirect end-labelling (4,13–20), a more rapid procedure was devised to compare the relative frequency of cleavage sites in restriction fragments from each clone by hybridization with a probe which contained sequences abutting all topoisomerase II cleavage sites in the clone. This probe, termed a topo II site probe, was prepared from DNA fragments which bore a terminal covalently bound enzyme subunit, selected specifically by a filter binding procedure (33) after cleavage of restricted DNA from the clone by topoisomerase II; it therefore contained sequences complementary to those at all cleavage sites at a relative abundance which reflected the frequency of cleavage, so that the hybridization signal on each target restriction fragment was proportional to the number of cleavage sites which the fragment contained and to the frequency with which they were cleaved. This strategy greatly reduced the number of experiments required and is analogous to using a probe containing a mixture of DNA sequences of different abundance, reverse transcribed from a heterogeneous population of mRNAs, to compare the relative frequency of transcribed sequences in different restriction fragments (see for example 26).

The specificity of this approach has been demonstrated in studies of the chicken α-globin locus, where a single restriction fragment known to contain cleavage sites was identified among six contiguous fragments (35). Its quantitative nature was assessed using a plasmid containing a 54 bp insert with strong topoisomerase II cleavage sites, pRYG (2), which was restricted to produce three fragments of which one contained the insert (Fig. 1A). After further cleavage by topoisomerase II the fragments abutting the cleavage site were selected to prepare a topo II site probe; this probe recognized only the restriction fragment which contained...
Figure 3. The relative frequency of topoisomerase II cleavage sites in 247 restriction fragments covering the 830 kb region of Drosophila DNA. The clones and their restriction maps have been described previously (26). The cleavage site frequency is shown by the height of the column above each fragment; for clarity, the baseline was set at the mean value for the entire continuum and regions with lower cleavage site frequencies are not shown. Restriction fragments with strong SAR activity are shown by filled rectangles and ellipses; the latter fragments and those shown by open ellipses show ARS activity (27,29). Positions of cleavage by nuclear matrix-associated topoisomerase II in vivo between 300 and 800 kb (28) are shown by xxxx.

topoisomerase II cleavage sites (Fig. 1A). To simulate differences in the frequency of cleavage sites in a particular fragment, varying amounts of pRYG were mixed with pUC19, which lacks the insert but is otherwise identical (2), restricted and subjected to electrophoresis; the hybridization signal from the topo II site probe relative to that from the total DNA probe was proportional to the amount of the pRYG fragment containing topoisomerase II cleavage sites (Fig. 1B). This procedure thus specifically detects restriction fragments which contain topoisomerase II cleavage sites and allows the relative frequency of these sites to be quantified. To obtain topo II site probes for all 67 clones of Drosophila DNA employed, the amount of topoisomerase II required was prohibitively high and we therefore examined whether the religation inhibitor VM-26 (32) could be used to increase the yield of cleaved DNA to prepare these probes. Probes prepared using VM-26 produced higher hybridization signals than those prepared without VM-26 but using a 10-fold greater quantity of enzyme (Fig. 1C). The signals on 21 restriction fragments from five representative clones were higher by a factor of 4.0; the small standard deviation of ±0.9 shows that the relative cleavage site frequencies determined by this low resolution approach using fragments of average length 3.4 kb were not significantly distorted by the use of probes prepared using VM-26 (see Discussion).

Topoisomerase II cleavage in restriction fragments of Drosophila DNA

Inserts of Drosophila DNA covering the 830 kb region excised from λ clones (26) were restricted and processed as described for plasmid pRYG. The number of hybridizations required was reduced by separating fragments from eight clones in individual lanes of a single gel and hybridizing them with probes prepared from pooled DNA of the same clones. The hybridization patterns shown in Figure 2 illustrate the different signals produced by the topo II site probe (lanes a) and the total DNA probe (lanes b) on individual restriction fragments, reflecting their different frequency of topoisomerase II cleavage sites. As examples, the two smallest fragments from clone λ535.2 (Fig. 2, upper left lane) showed a lower signal from the topo II site probe than from the total DNA probe, whereas for the smallest fragment from λ53 (Fig. 2, lower left lane) the signal from the topo II site probe was higher. The ratio of the signals (topo II site probe/total DNA probe) was calculated for each restriction fragment after normalizing the integrated signals with respect to those from a vector DNA fragment in the same lane to correct for differences in transfer efficiency, probe specific activity and autoradiographic conditions between different experiments.

The assembled data are summarized in Figure 3, where only those regions where the topoisomerase II cleavage site frequency exceeded the mean value for the entire continuum are shown for clarity. The distribution of these regions was compared with those of other landmarks by applying and interpreting statistical tests as described previously (26–30).

Correlations of topoisomerase II cleavage site frequency with functional and structural properties

Only strong SAR activity and ARS activity showed a correlation with cleavage site frequency by factorial correspondence analysis (34) and none was detected with repetitive sequence content, A/T
Figure 4. Correlations between the frequency of topoisomerase II cleavage sites and other properties of the restriction fragments. The fragments were ranked in classes according to their cleavage site frequency and in each class the number which showed or lacked a second property was displayed as a histogram. The left panels show direct comparisons, while the panels marked displacement 1, 2 and 3 show tests of proximity relationships performed after attributing to each fragment the cleavage site frequency of the first, second or third adjacent fragment leaving the other parameters unchanged; a displacement by one average fragment length corresponds to 3.4 kb. The probability that the observed distributions could have resulted from a random distribution, calculated by the homogeneity and Wilcoxon tests, is shown by the values of $p_1$ and $p_2$ respectively; low $P$ values shown in bold type indicate a statistically strong correlation.

or C/G richness, weak SAR activity or transcriptional activity (not shown). These correlations were examined quantitatively by the homogeneity and Wilcoxon tests, in which the number of fragments in each cleavage frequency class which possessed or lacked a second property and the probability ($P$) that these numbers could arise from a random distribution were calculated.

The topoisomerase II cleavage site frequency was not correlated with the known structural properties of repetitive sequence content (Fig. 4, upper row) or A/T or C/G richness (not shown) nor with the functional properties of weak SAR or transcriptional activity (not shown). Restriction fragments with strong SAR activity were clearly over-represented in classes with higher cleavage site frequencies (Fig. 4, second row, left panel). The correlation between these properties was statistically highly significant, as shown by the low $p_1$ and $p_2$ values ($P \leq 0.11$; this correlation was still seen after a relative displacement by one or two fragment lengths (one average fragment length is 3.4 kb). Fragments with ARS activity were also over-represented in the classes with a high cleavage site frequency and the correlation between these properties was statistically highly significant ($P \leq 0.06$; Fig. 4, bottom row).

**DISCUSSION**

**Comparison of topoisomerase II cleavage site frequency in restriction fragments**

The strategy used here to examine topoisomerase II-mediated cleavage in a region of DNA much longer than those studied previously is more rapid than direct or indirect end-labelling methods (4,13–24,36–41), which, further, are not suited to quantitative measurement or comparison of cleavage site frequencies because rapid cleavage at sites close to the labelled extremity or to the probe can mask cleavage occurring at more distant sites. This approach shows the relative frequency of cleavage sites in different fragments, but does not distinguish those with a few strong cleavage sites from those with more frequent but weaker sites. The use of probes prepared using the religation inhibitor VM-26 had little influence on the relative frequency of cleavage sites at this relatively low resolution using fragments 3.4 kb in average length (Fig. 1C), when the differential stimulation seen at nucleotide resolution (35–41) is likely to be masked, consistent with reports that cleavage patterns mapped by indirect end-labelling do not show significant differences in the presence of VM-26 (17,19,20). The strong preference of topoisomerase II for binding and cleaving in alternating purine–pyrimidine sequences is not influenced by VM-26 (42,43). Topoisomerase II of bovine origin was employed to cleave Drosophila DNA because of its ready availability, but the sequence preference of the enzyme does not show significant species differences (2,42) and a heterologous enzyme is therefore commonly used in binding and cleavage studies (2,16,17,20,21,37,43).

**Functional correlates of topoisomerase II cleavage site frequency**

Of the functional properties mapped previously in this 830 kb region of DNA, only strong SAR and ARS activities show a statistically strong correlation ($P \leq 0.11$; Fig. 4) with a higher frequency of topoisomerase II cleavage sites. A proximity relationship was seen by statistical analysis in cases where a SAR fragment did not itself show frequent cleavage sites, with one exception (Figs 3 and 4), consistent with the contiguity in the chicken lysozyme locus (44) of a MAR and a strongly curved region which would provide a preferred cleavage target (5–7). The present results thus extend the evidence that strong cleavage sites occur in a number of individual MARs and SARs (4,16,17,22,23,25) to the 12 strong SARs in this 830 kb region, considerably strengthening the hypothesis that this is a general
characteristic of MARs/SARs. The inverse relationship was not seen, however; a high frequency of cleavage sites is not predictive of SAR activity (Fig. 3). ARS activity also showed a strong correlation with higher cleavage site frequency (P ≤ 0.1, Fig. 4), possibly reflecting the implication in ARS function of cruciform fragments and are >10 kb in length (Fig. 3), suggesting that their higher than the mean extend over several contiguous restriction organized within the nucleus.


REFERENCES

22 Constitution of SARs, which lie in curved regions of S.cerevisiae (47) and they are coincident or contiguous with strong SARs, which show intrinsic curvature (49), and with ARSs, which lie in curved regions of S.cerevisiae chromosome III (46) (Fig. 4). As well as raising this hypothesis, which remains to be explored, this study of the topoisomerase II cleavage pattern in 830 kb of Drosophila DNA shows that its relationships to functional properties reported in individual loci, especially to MAR/SAR and ARS activity, and to the restricted accessibility of genomic DNA to topoisomerase II in vivo, can be generalized to much longer regions of the genome.

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