Sequence dependent modulating effect of camptothecin on the DNA-cleaving activity of the calf thymus type I topoisomerase

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ABSTRACT

High-resolution mapping of topol cleavages in the regions of human DNA including the oncogene c-Ha-ras and p53, has revealed three kinds of topol cleavage sites: cleavage sites not affected by camptothecin; cleavage sites reinforced only in the presence of camptothecin, and cleavage sites which weaken in the presence of camptothecin. Statistical analysis of sequences revealed certain nucleotide or dinucleotide preferences for three groups studied. The preferences in camptothecin-reduced sites predominate upstream from the cleavage point, whereas in camptothecin-induced sites the situation is reversed. The influence of camptothecin on cleavage sites induced by two molecular forms of topol has been also studied.

INTRODUCTION

One of the main problems confronting the study of enzymes involved in genetic processes is their polyfunctional behavior and interaction with a high-molecular-weight DNA or RNA substrate characterized by structure-dependent properties. Understanding the principles of DNA -protein interaction and the organization of functionally active DNA domains is indispensable in the elucidation of fine mechanisms governing genetic processes and their regulation.

Many scientists have recently become keenly interested in DNA topoisomerases, the enzymes known to change DNA conformation and to be an integral part of the genetic apparatus of the cell, because of the increasing amounts of information showing the involvement of these enzymes in the processes of replication, transcription and recombination [1,2]. A characteristic and unique property of these enzymes is the ability to form an intermediate covalent complex with one of the cleaved DNA end [3]. On this premise,a method has been devised allowing topoisomerase fixation on DNA without chemical crosslinking agents. The method essentially consists in the termination of the topoisomerase reaction with the aid of a detergent or other protein denaturing substances [4]. Analysis of the DNA-protein complexes reveals the nucleotide sequence preference for the DNA-topoisomerase interaction.

From the start of the studies on nucleotide sequence determination and nucleotide distribution at the site of topoI-induced DNA cleavage [5,6]; the non-random distribution of topoisomerase-mediated breaks in SV40 DNA was successfully shown and, consensus of 5 nucleotides was noticed to exist in the region of cleavage [6]. Subsequent work was devoted to mapping topoisomerase binding to Tetrahymena genes 16S and 23S that were found to contain a 16-nucleotide consensus [7]. The latter was tested using model systems and interaction with topoisomerase was proved [8]. However, insufficient amounts of the mapped genes significantly hindered the proper interpretation and discussion of experimental results. This shortage in turn was brought on by the absence of optimal conditions for entire mapping, i.e., localizing all sites of interaction with topoisomerase throughout the cloned gene. Cleavage, had to be performed with an high molar excess of topoisomerase compared to the substrate. To overcome this difficulty, camptothecin, a specific topoI inhibitor, was used [9]. The property of camptothecin which reinforces topoI- induced DNA cleavage, shifting the balance towards 'cleavable complexes', has opened a new stage in mapping individual genes in vivo and in vitro [10–12]. Intensive use of camptothecin for in vivo mapping has prompted a question as to whether this inhibitor could be affecting topoI site specificity. The data reported by Westergaard et al.[13,14] and Perez-Stable et al. [16] together with our previous experimental results [15] demonstrate a change brought about in the topo I-mediated DNA cleavage pattern by camptothecin. Analysis of cleavage sites indicated the existence of several types of sites: in one the degree of cleavage remained unchanged in the presence of the inhibitor, while in two others the latter caused either an increase and a decrease in the cleavage level, respectively. Some other studies on SV40 virus mapping [17,18] however did not reveal any appreciable difference between topoI -cleaved sequences in the presence or absence of camptothecin, except cleavage 'reinforcement' in the detected topoI sites. This work provides consecutive proofs that the modulating effect of camptothecin action is sequence-dependent and the nucleotide composition of flanking sequences believed to play an essential role in low-specific DNA-protein recognition is a determining factor in the ultimate development of each site [27].

MATERIALS AND METHODS

Camptothecin was a gift from the National Cancer Institute (lactone form, no.94600). Stock solutions of camptothecin, 10 mM in 100% dimethyl sulfoxide (DMSO), were stored in samples at −20°C. The DMSO was from Fluka. The Micrococcal nuclease, Proteinase K, Tris and agarose were obtained from

Sigma. A Klenow fragment of E.coli DNA polymerase I and a nick-translation kit were from Amersham; the Bio-Rex70 and SDS from Bio-Rad; the acrylamide and EDTA from Serva. Restriction endonucleases were purchased from the Institute of Applied Enzymology (Vilnus).

Supercoiled DNA was purified from E.coli as described by Maniatis [19].

Polyclonal antibodies against topoI were kindly provided by Dr.A.Belyavskii of Institute of Molecular Biology. Monoclonal antibodies against topoI were isolated and characterized as published previously [20].

DNA topoisomerase I was purified from calf thymus using the previously published procedure [21].

The nuclei were collected from thymus homogenate by centrifugation at 1500g for 10 min and washed several times with buffer,containing 0.25 M sucrose, 50 mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 25mM KCl, 1.4 mM 2-ME (2-mercaptoethanol), 1mM PMSF (phenylmethylsulfonylfluoride). In the last wash,0.2% Triton X-100 was included. The nuclei were lyzed in a 1M KCl solution. Nucleic acids were precipitated with polyethyleneglycol 6000 and the supernatant was loaded on a hydroxylapatite column(5×15 cm),which was eluted with a 1.2 l linear gradient(from 0.2 to 0.8 M) of potassium phosphate buffer (KPB), pH 7.3, containing 1.4 mM 2-ME and 1mM PMSF.The fractions comprising topoI activity were combined, diluted to 0.2 M KPB and loaded on a second hydroxylapatite column (2.5×10 cm). The same linear gradient of KPB was used for elution,but glycerol was added to 20% (v/v). Active fractions were combined, diluted to a concentration of 0.05 M KPB and then loaded on a heparin-Sepharose (1.5×12 cm) column and eluted with a linear KPB gradient (from 0.05 to 0.5 M) containing 20% glycerol, 1.4 mM 2-ME, 1 mM PMSF. The active material which eluted at 0.3 M KPB was diluted and applied on a Bio-Rex 70 column (0.8×16 cm), and the elution was carried out with a 60 ml linear gradient of KPB (0.2 M to 0.8 M). The two separated fractions of TopoI were finally concentrated on the Bio-Rex 70

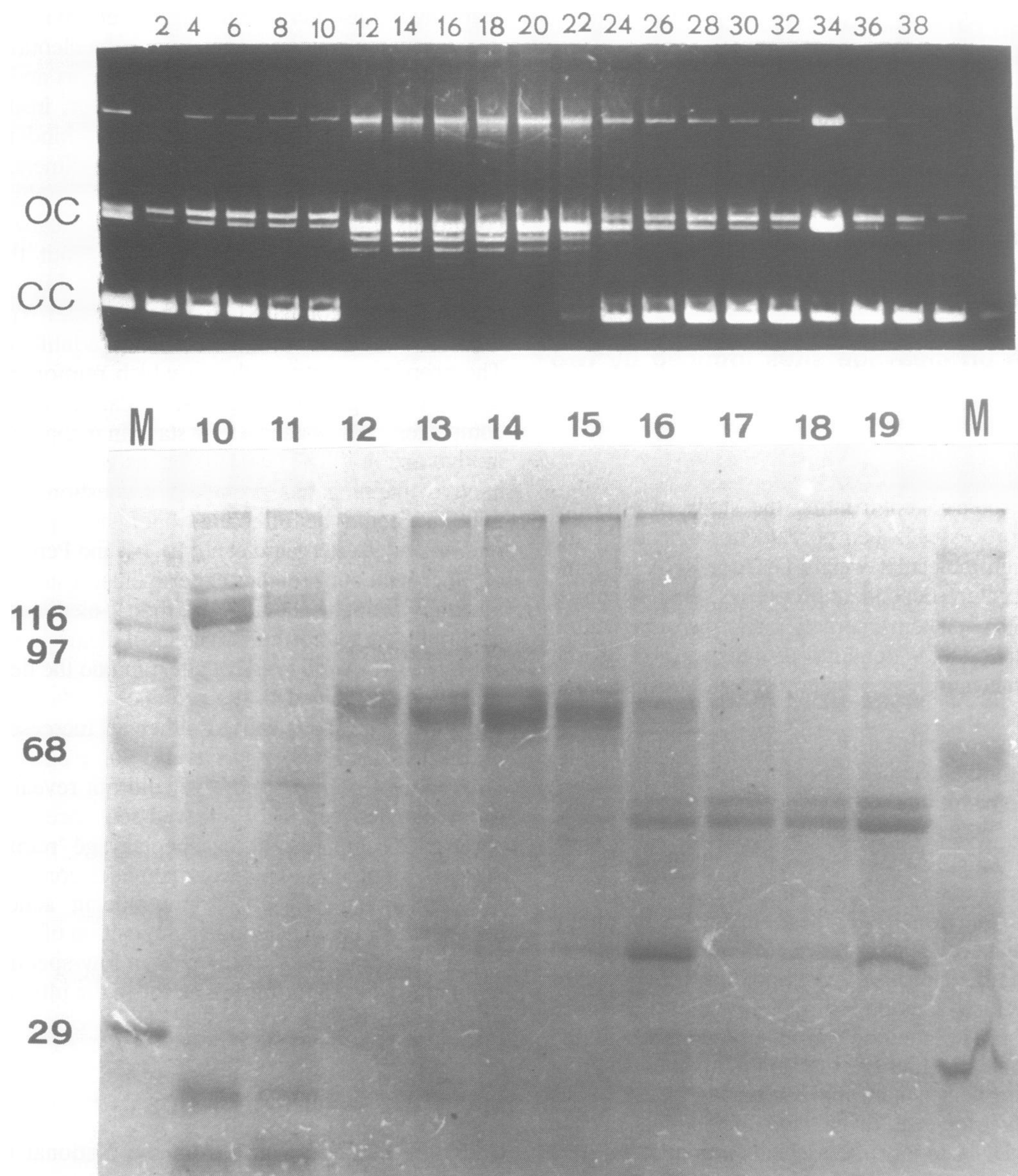

Fig.1 A. Relaxation activity in fractions eluting from the Bio-Rex column. oc—open circular DNA pUC19. cc—supercoiled DNA pUC19. B. Electrophoresis of the protein fractions, eluting from the Bio-Rex column. M—Molecular weight markers.

column. They were eluted with 2 M NaCl in 50 mM Tris-HCl (pH 7.5), containing 20% glycerol and 1.4 mM 2-ME. The concentration of protein in the final enzyme preparation estimated by Bradford's method [22] was from 0.5 to 1 mg/ml. One unit of topoI was the amount of enzyme yielding 50% of relaxed 0.5 μg pUC19 DNA in 10 min at 37°C.

The human c-Ha-ras gene (6.6 Kb) [23] cloned in the Bam HI site of pBR322 was kindly provided by Dr.V.Prassolov (Institute of Molecular Biology, Acad.Sci,Moscow).

The 0.54 Kb fragment of the human p53 oncogene was cut out of plasmid p06H [24].

Double-stranded DNA preparations were 3'end- labeled with the Klenow fragment of E.coli DNA polymeraseI [19]. The DNA substrate preparation is further specified in the legends to figures.

Topoisomerase I cleavage reactions were performed in a final volume of 20 μl in a cleavage buffer containing the following solutes: 10 mM Tris-HCl (pH 7.5),40 mM NaCl, 1mM dithiothreitol,1mM EDTA, 20 ng of the analyzed end- labeled DNA,and 200 ng of Topo I. In some experiments, camptothecin was added to the reaction mixture. The samples were incubated at 30°C for 30 min (the incubation period over the range from 2 to 60 min gave identical results). The reaction was terminated by adding SDS up to 1%. DNA samples were electrophoresed in 2% alkaline-agarose gels. For fine mapping of the topoI cleavage sites,proteinase K-treated and deproteinized products of the cleavage reaction were precipitated with ethanol, dissolved in a sample buffer containing 80% formamide, denatured by heating to 100°C and electrophoresed in urea-polyacrylamide sequencing gels. The products of DNA cleavage formed in the sequencing reactions [25] were run in parallel slots.

For the formation of the topoI-oligonucleotide complex, the cleavage reaction mixture containing 1μg of nick-translated DNA and 5 μg of protein from a Bio-Rex column,fraction 12–17, was incubated for 30 min at 30°C. To trap the protein-DNA covalent intermediate, NaOH was added to a final concentration of 50 mM. After heating for 5 min at 37°C,the mixture was neutralized with 1 M Tris-HCl (pH 7.5) and 1 M HCl to pH 8.0. DNA was digested overnight at 37°C with micrococcal nuclease (30 u/ml) in the presence of 1 mM $CaCl_2$.

Protein samples were analyzed on SDS/polyacrylamide gels (10% acrylamide, 0.3% N,N-methyl-bisacrylamide, 0.1% SDS) according to Laemmli [26], followed by Coomassie R-250 staining or Western blotting.

Autoradiography was performed at −70°C with a RM-V film. The relative frequency of cleavage was determined by densitometric scanning of the autoradiograms.

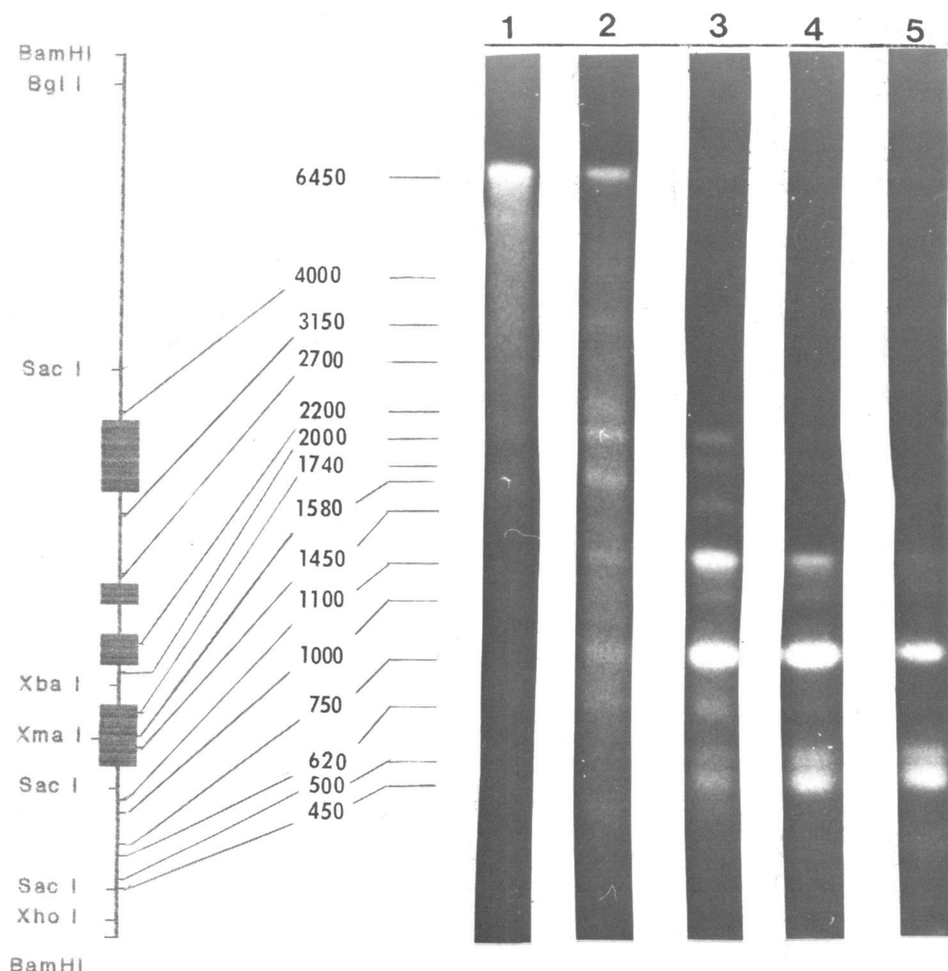

Fig.2 The low resolution separation of cleavage products yielded of human c-Ha-ras gene cleavage by topoI in the presence of different camptothecin concentrations. The 6.6 kb c-Ha-ras gene cut out of plasmid pVP (see Materials and Methods) was ^{32}P -labeled at its 3'-ends, and digested with Bgl II which removes a 160 bp fragment from the 3' end of the gene. 1.DNA incubated without enzyme; 2. DNA incubated with 200 ng topoI/82; 3,4,5-DNA incubated with topoI in the presence of 1,10 and 50 μm camptothecin,respectively (see Materials and methods for reaction conditions). Restriction map of c-Ha-ras gene is shown at the left side of the photo (exon sequences are indicated by black boxes and the intron sequences with lines).

Statistical analysis is conducted as follows. The sequences of each group are aligned according to the cleavage point. Then the probabilities of the observed occurrences being due to chance are calculated for nucleotides, dinucleotides, unique dinucleotide steps, different purine-pyrimidine sequences and pairs like (A,T) (G,C) or (T,G) (A,C). Both positive and negative preferences are calculated and the confidence ranges are estimated. A detailed description of the method is published elsewhere [27].

RESULTS

Electrophoretic analysis of the calf thymus proteins, eluted from Bio-Rex 70 column, by the Schmitt's method shows that two fractions can be isolated from the active region possessing relaxation activity (Fig 1). Fraction 14 consists of 2 or 3, rarely 4, polypeptides of Mr ranging from 100 to 70 kDa. Fraction 18 has predominantly two polypeptides with Mr of 55−57 kDa. Both fractions were rechromatographed on Bio-Rex 70, concentrated, and utilized for running cleavage reaction. Several criteria were applied for the reference of the polypeptides obtained to topoisomerase I:

(i) specific enzyme activity estimated by the reaction of supercoiled DNA relaxation: fraction of p82-p75 polypeptides and fraction p55-p57 have values of 2.0×107 and 1.8×107 U/mg respectively. The respective yield amounted to 1 mg and 200 μg. In view of the close specific activities of the electrophoretically homogenous fractions, the relaxation activity revealed in both fractions can be attributed to the activity of major polypeptides in each fraction;

(ii) both preparations are characterized by the capacity to form a covalent DNA-protein complex which, subjected to a special treatment (see Materials and Methods), can be recorded as the oligonucleotide-labeled protein (data not shown);

(iii) relaxation activity of the two analyzed preparations was inhibited by camptothecin, a topoI specific inhibitor, under conditions providing for the distributive mechanism of the reaction (0.2 M NaCl) and this enzyme:substrate ratio when $[E] < [S]$;

(iv) immunochemical analysis using anti-topoI polyclonal and monoclonal antibodies demonstrated common epitopes for intrafractional and interfractional polypeptides (data not shown);

(v) both preparations are capable of DNA cleavage. Unlike the relaxation reaction, cleavage is usually performed by the processive mechanism of topoI action (0.05 M NaCl or even lower), and in this case $[E] > [S]$.

Therefore, the preparations employed for DNA cleavage in our experiments contain polypeptides exhibiting DNA relaxing and cleaving activities.

Fig.2 represents the electrophoregram of cleavage products of the c-Ha-ras gene coding strand. Notwithstanding a high molar excess of the enzyme and low ionic strength (40−50 mM), some molecules remain uncleaved. A topoI/82-mediated cleavage of the c-Ha-ras gene DNA fragment induced by camptothecin eliminates integrity and the majority of high molecular weight fragments. Fragments 1100, 750, and 450 nucleotide remain prevalent for a long time at the such cleavage. The effect of camptothecin become apparent at a concentration as low as 1 μM and tends to increase with a rise in concentration. Even electrophoresis in agarose gel markes a minor change noticeable in the electrophoretic mobility of the fragments in the presence of camptothecin, and induces new fragments, such as fragments of 1450 and 1740 nucleotides. Genomic localization of DNA breaks at the nucleotide level in two comparatively small regions of c-Ha-ras gene and a p53 gene region suggested that the change in site specificity of topoI-mediated DNA cleavage under the effect of camptothecin causes the observed phenomena (Fig 3−6). Camptothecin affects site specificity such that cleavage intensity in each site can be either enhanced, e.g.: 331, 454 (Fig.3), 1780, 1846, 1963 (Fig.4), 306, 324, 522 (Fig.6) or weakened e.g.: 222, 290, 454 (Fig.6), or else remain unaffected, e.g. 426 (Fig.3), 1740 (Fig.4) and 198, 447 (Fig.6). The most typical sites of those weakened under the action of camptothecin, i.e., the sites completely free of cleavage, were characterized by a peculiar pentanucleotide sequence GACTG near the cleavage site. Sites which occur rarely are those which can have an enzyme-induced break between any two, three or sometimes more existing adjacent nucleotide residues to form a set of fragments differing in length by one nucleotide. Breaks in the region of 363 −367 nucleotides in c-Ha-ras gene (Fig.3) and a break at position 252 (Fig.5,6) are of this type there is an equal probability of breakage in neighbouring positions. Camptothecin produces an effect on the breakage position by promoting the enzyme to interact with one of the two adjacent residues which are equally recognized in the absence of camptothecin.

A comparison of the cleaving activity of two topoI forms (82 and 55 kDa) has shown that as a rule they produce DNA cleavage at the same sites in the absence of camptothecin. Among the cleavage site patterns demonstrated in Figs 4, and 6, a substantial difference between topoI/82 and topoI/55 can be seen only at position 1846 in Fig.4. In all the remaining sites, breaks coincide and it is the intensity of cleavage that differs. The introduction

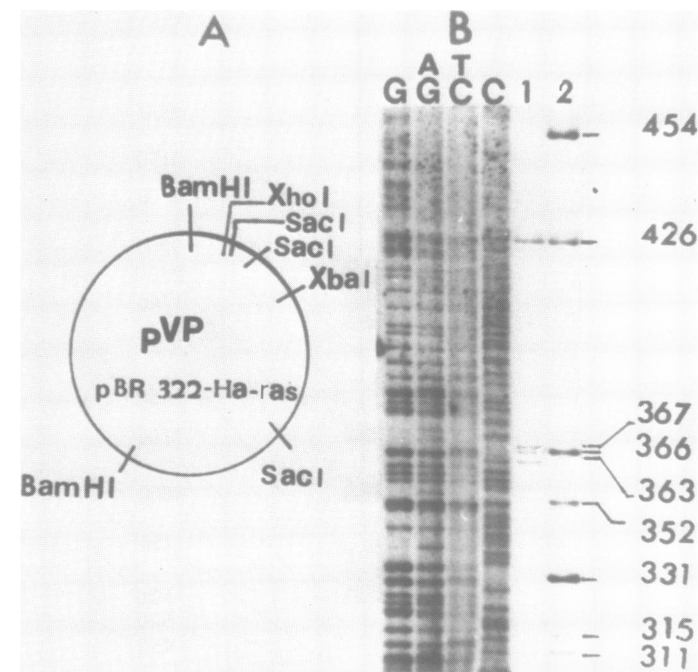

Fig.3 The effect of camptothecin on the high-resolution localisation of topoI generated cleavages in the coding strand of a c-Ha-ras gene fragment. A. Diagram of the plasmid pVP. B-Electrophoresis of the topoI cleavage products of the 1700 bp XhoI-XbaI fragment of c-Ha-ras-gene labeled at the 3 end of its transcribed strand. After treating the plasmid with XhoI,the DNA was 3'-end labeled and treated with XbaI. The single-end labeled 1700 bp fragment formed was purified by electrophoresis and incubated with topoI. 1. The XhoI-XbaI fragment incubated with topoI/82; 2—same in the presence of 50 μm camptothecin (see Materials and methods for reaction conditions). G,A+G,C+T, and C,the Maxam-Gilbert cleavage products of the same DNA fragment,run concurrently with the TopoI cleavage products.

of camptothecin drastically changes the DNA cleavage pattern. It is worth noting that the degree of change differ according the region of one or another gene. The most dramatic effect is seen in the second fragment of the untranscribed strand of c-Ha-ras gene. A large number of sites reinforced by camptothecin also characterized this region. Earlier [15] we determined the degree of probability of detecting nucleotide residues at various positions from the cleavage site. Within the interval from −6 to +5 nucleotides, the strong sites unaffected by camptothecin had a characteristic consensus. In camptothecindependent sites

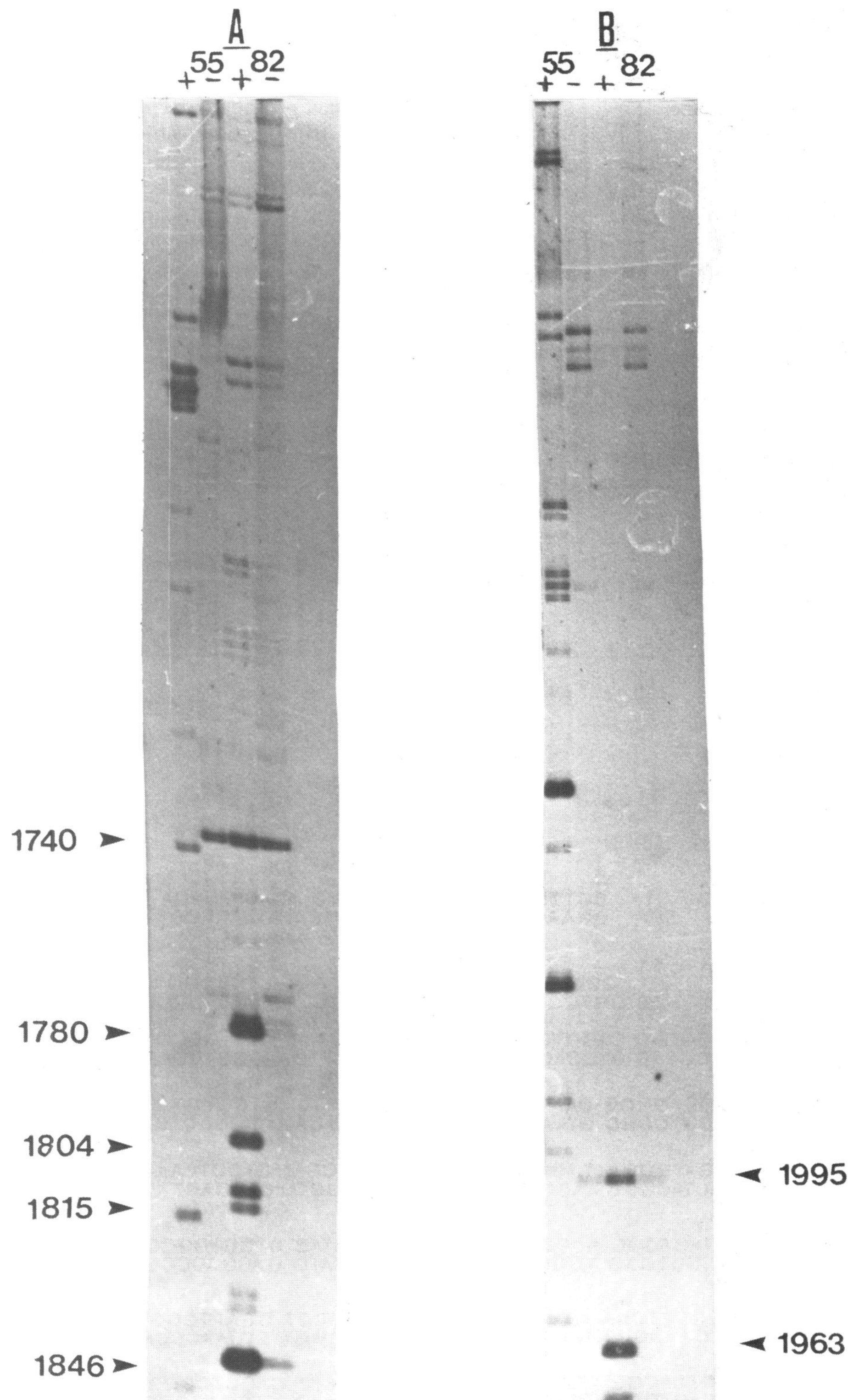

Fig.4 Sequencing electrophoresis of topoI generated cleavage products of the untranscribed strand of the SacI-XbaI fragment and of the transcribed strand of the XbaI-SacI fragment of c-Ha-ras gene. pVP DNA (see fig.3),cleaved by XbaI, was 3′end labeled,and digested with SacI. Single-end labeled 2037 and 856 bp fragments formed were purified by electrophoresis ,and incubated with topoI. A—topoI generated cleavage products separated in 6% PAAG. 1.The non-coding strand of the 856 bp SacI-XbaI fragment treated with topoI/82 and topoI/55 in the presence of 50 μm of camptothecin (+) and in the absence of it (−). B—the transcribed strand of the 2037 bp XbaI-SacI fragment treated with topoI/82 and topoI/55 in the same conditions.

nucleotide distribution was similar to the distribution pattern reported by Been et.al for so called 'weak sites' [6]. The conversion of these sites into 'strong' ones in the presence of camptothecin raised the question why some 'weak' sites become reinforced or still more weakened, while the others remain unchanged. One possible conclusion was that the modulating effect of camptothecin could be related to the influence produced by sequences flanking the cleavage site. An order to establish regularities of nucleotide distribution in sequences located within the distance of ±25 nucleotides from the cleavage site, the sites identified in the p53 gene fragment under analysis were divided into three groups, depending on the effect generated by camptothecin. The first group included 17 sequences with high efficiency of cleavage: more than two- fold in the presence of camptothecin. It was noted that one site in this group had a 130-fold reinforcement degree. The second group contained 13 sites characterized by the degree of reinforcement ranging from 1 to 2, i.e.,so called camptothecin-independent sites. The third group consisted of 8 weakening sites.

Computer analysis has shown that these groups have peculiar flanking sequences and are characterized by common features specific to each group.

The results are shown in Figure 7 . In all three cases the analysis reveals positions with certain preferences,while the patterns differ somewhat . All cases imply a certain preference for thymine at −1 position, although it is only pronounced is that in camptothecin- independent sites (9 of 11 , $P=10^{-3.9}$) The remaining positions in camptothecin-independent sites display no probabilities less than $10^{-2,5}$ although this may be due to insufficient statistics. In contrast, the camptothecin-dependent sites have positions which deviate slightly from random distribution. In camptothecin-reduced sites there is a stretch of pirimidines at −24-(−19) positions ($P=10^{-2.9}-10^{-3.9}$) the most significant probability being noticed at −23-(−21) positions (7 of 11 trinucleotides are of YYY type, $p=10^{-4}$) ; GC dinucleotide at −8 position ($P=10^{-3.5}$) . In the case of a camptothecin-induced site the nonrandom positions are mainly situated in the downstream flank, except YYY/RRR stretch at −8 -(−6) (12 of 18 sequences contain this stretch, $P=10^{-3.6}$) Among downstream positions are: TG at +1+2 position ($P=10^{-4.5}$ for G); in +2 R-R dinucleotide at +14−+15 and R-R/Y-Y +21−+22 position ($P=10^{-2.9}$).

DISCUSSION

The above results as well as data reported by other authors [14,16] convincingly prove that camptothecin can affect the site specificity of topoI. To a great extent the degree and character of the

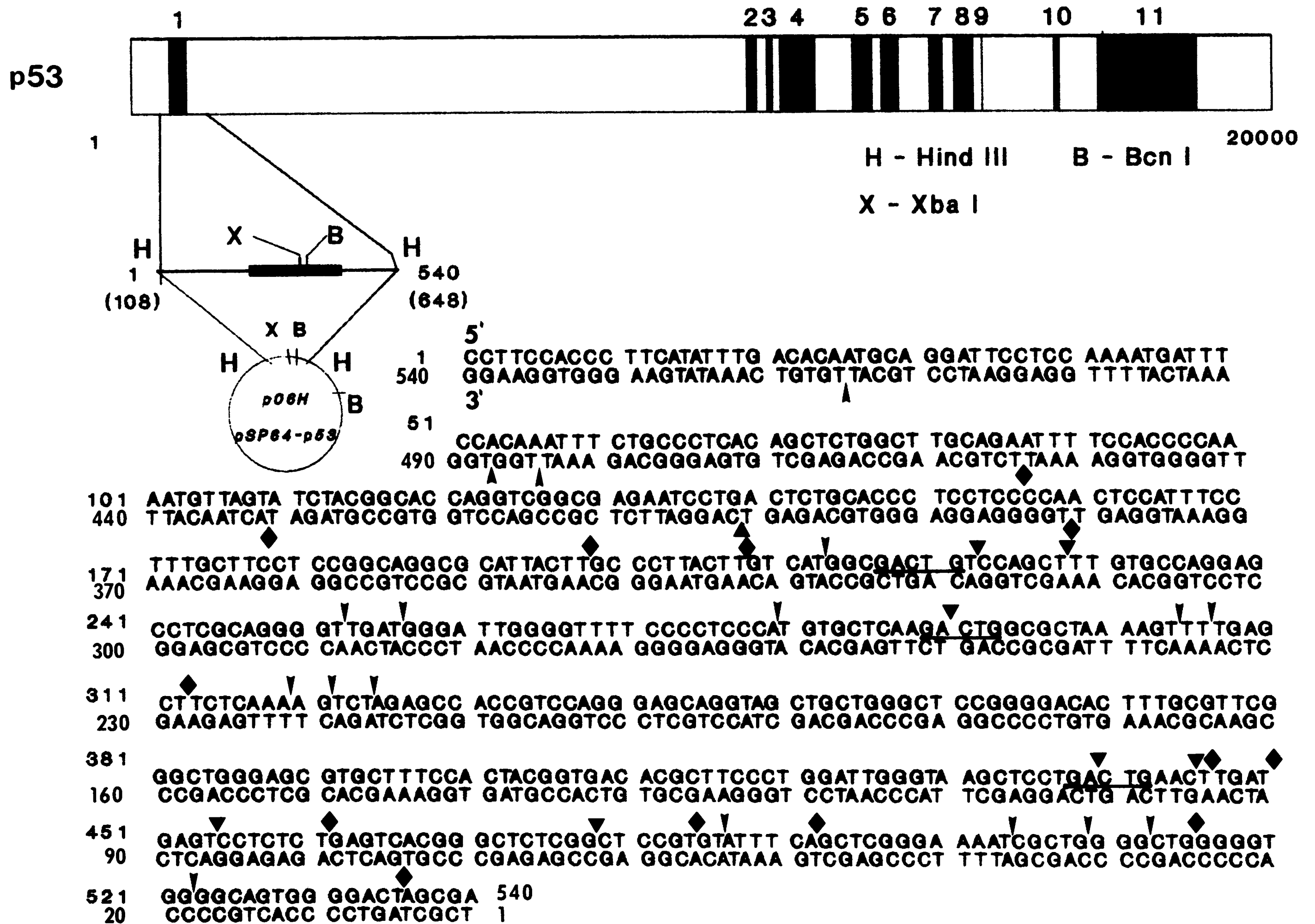

Fig.5 The fine mapping of topoI-induced single-strand cleavages in a fragment of the p53 gene. Exon sequences are indicated by black boxes; camptothecin induced cleavages are indicated by arrows; camptothecin reduced cleavages are indicated by angles; camptothecin independent sequences are indicated by rhombs.

produced effect are determined by a number of interrelated factors. This presumptive dependence may account for the diversity by conclusions drawn about topoI site-specificity changes under the action of the inhibitor [17]. Above all this concerns the use of DNA in the analysis of the site-specificity of topoI. It is not mere coincidence that all data indicating an

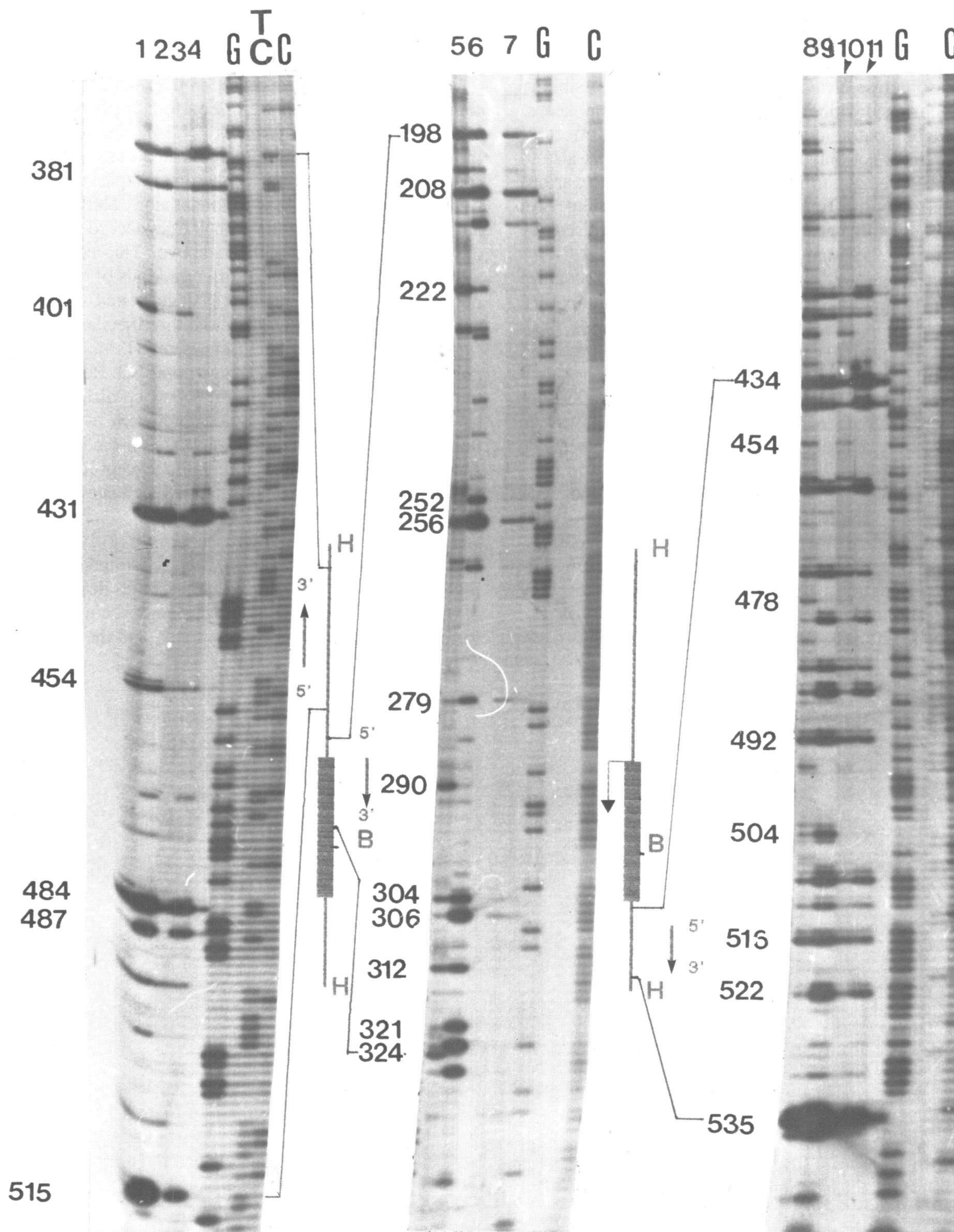

Fig.6 Sequencing electrophoresis of topoI generated cleavage products. Plasmid p06H was cut with Hind III,and 3'-ends were labeled with ^{32}P-dATP. THe labeled fragments were digested with BcnI. The resultant 360 bp and 180 bp fragments carried the label in the transcribed and untranscribed strands,respectively. In order to localise topoI cleavages in the opposite strands of DNA fragments,plasmid p06H was first digested with BcnI,then the 3'ends were labeled with ^{32}P- dGTP and digested with Hind III. This yields 360 bp and 180 bp fragments carrying the 3'-end label in the untranscribed and transcribed strands, respectively. The fragments were purified by electrophoresis and used for topoI and chemical degradation. 1,5,8—topoI/82 induced cleavages in the absence of camptothecin. 2,6,9—topoI/82 induced cleavages in the presence of 50 μm camptothecin. 3,10—topoI/55 induced cleavages in the absence of camptothecin. 4,7,11—topoI/55 induced cleavages in the presence of 50 μm camptothecin. First exon sequence is indicated by black box. The point of initiation and direction of transcription are indicated by black angle.

exclusively enhancing effect of camptothecin on cleaving intensity at constant sites, with a certain consensus sequence, have been obtained with DNA of SV40 virus [17,18], and not with various individual genes as in [15,16]. If the cleavage effectiveness depends not only on the 'center' of the cleaved sequence but on the flanks as well, it becomes clear that the absence of specific flanking sequences should bring about a unilateral camptothecin effect on topoisomerase cleaving activity. It is also possible to detect regions enriched with one type of site in individual genes. Another cause of contradictory data regarding the effect of camptothecin relates not only to flanking sequences but also to the structure of the enzyme proper which is subject to various posttranslational modifications in the in vivo system. An especially marked influence is likely to be exercised by phosphorylation [28,29] and proteolysis [20].

It was earlier suggested [30,31] that proteins can recognize certain general properties of a DNA coil as well as individual nucleotides. This recognition capability is called 'analogue' [32]. An example of the analogue recognition is the effect produced by sites flanking the consensus region on some or other characteristics of the DNA protein complex. Thus modifications in the fragments flanking recognition sites of restrictases can change the probability of breakage by several orders of magnitude [32].

In the case of the modulating effect of camptothecin,the DNA sequence has a determining significance.

Common features were observed in the flanks of camptothecin-dependent sites. Several preferential positions on the 5′ flank determined the disappearance of the cleavage in several sites when the drug was administered the presence of the drug. The nucleotide sequence at the 3′ side from the cleavage point is likely to affect the cleavage, which is reinforced in the presence of camptothecin.

Our computer analysis has revealed a pattern of positive and negative preferences in DNA sequences of both camptothecin-induced and camptothecin-reduced sites, which are important in the compilation of statistic. The sequences covered 25 base pairs flanking the cleavage point.

We have taken into consideration flanks of 25 bp since flanking regions by some length were seen to contribute to the binding efficiency in a number of cases [27,34]. The sequence patterns in these cases are more complicated and less pronounced compared to conserved nucleotides in a simple consensus, require a more sensitive analysis to find them. These patterns were analyzed by calculating the probabilities according to the method described in [27] and outlined above. This common measure allows comparison amongst different kinds of one, two or three nucleotide 'alphabets' including degenerated ones. Even with small numbers of sequences in the three sets analyzed, one can find a number of certain preferences ($P < 10^{-3}$) including positions remote from a cleavage point (see Results and Fig.7). Omitting the detailed picture of sequence differences found in sites unequally influenced by camptothecin, it should be noted that in camptothecin-induced sites the non-random positions are situated mainly in the upstream region, except RRR/YYY stretch at $-8|-6$, while in camptothecin-reduced sites the downstream flank seems more important.

The results indicating the essential role of flanking sequences for topoI-DNA interaction have been supported by some recently reported data. In particular, Kjeldsen et al. described the effect of the DNA 5′ region from the cleavage site upon the effective formation of the topoI-DNA complex, and the 3′ region on the

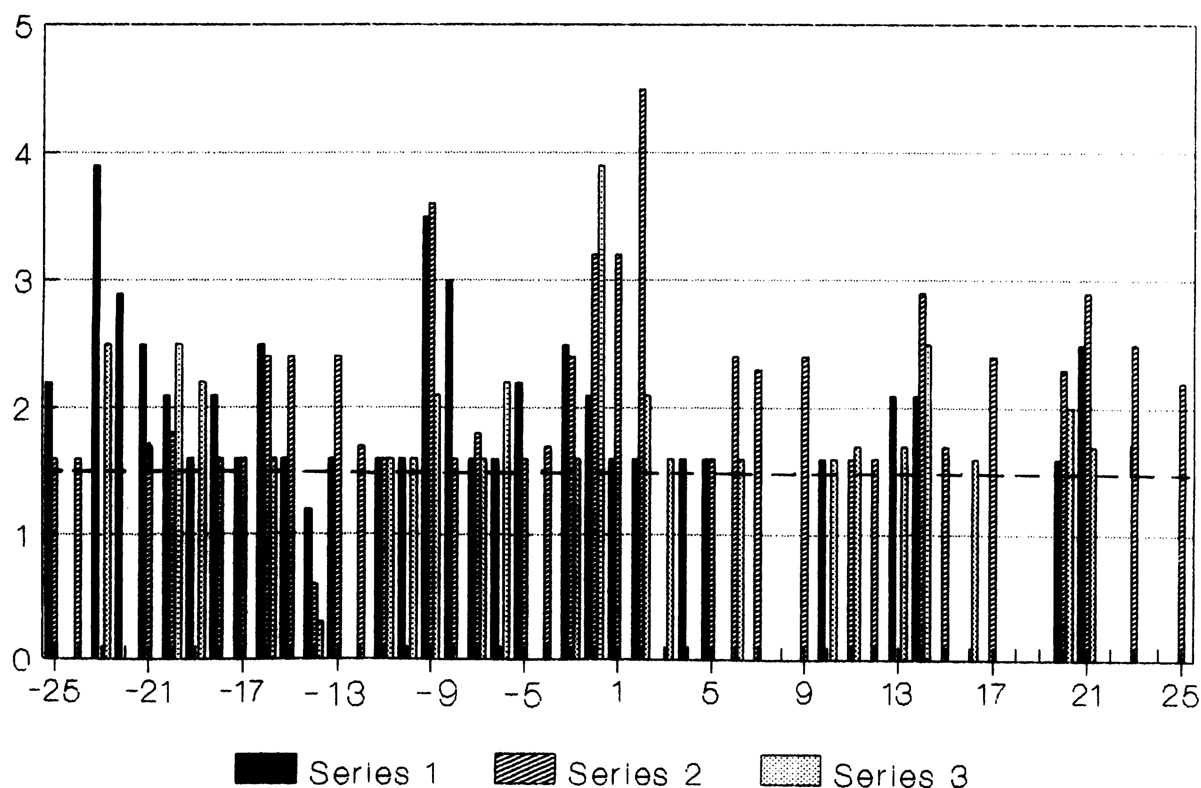

Fig.7 The diagram of sequence-preferences (see Methods) in camptothecin-reduced sites (Series 1) ,camptothecin-induced sites (Series 2) and camptothecin-independent sites (Series 3), is represented by 8, 17 and 13 sequences respectively. The flanking regions of 25 bp are analyzed. The values of $-\log(P)$, where P is the probability of random occurrence for a particular (di)nucleotide, are presented. The dashed line shows the $\pm 2\sigma$ noise range level calculated from random sequences.

nicking- closing reaction [35]. Champoux et al. [18] did more on the analysis of camptothecin-induced wheat germ topoI sites where non-random distribution of some nucleotides at positions +9 and +10 from the cleavage point was established.

In conclusion, we can say that the regulation of topoI activity is directly dependent on the nucleotide sequence, as one and the same enzyme modification (in our case, interaction with camptothecin) may lead to different results in different DNA regions.

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