DNA-nuclease activity of the single-chain ribosome-inactivating proteins dianthin 30, saporin 6 and gelonin

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Abstract The single-chain ribosome-inactivating proteins (sc-RIPs) from plant origin are antiviral and antiproliferative agents employed in the preparation of immunotoxins. Similarly to the A-chains of ricin, sc-RIPs act as rRNA *N*-glycosidases. We demonstrate here that dianthin 30, saporin 6 and gelonin exert a specific nuclease activity on supercoiled DNA. Four specific sites of cleavage introduced by dianthin 30 and by saporin 6 and two specific sites of cleavage introduced by gelonin have been identified and mapped in pBR322.

Key words: Ribosome-inactivating protein; Saporin 6; Dianthin 30; Gelonin; DNAse activity; Supercoiled DNA

1. Introduction

RIPs from plant origin are single-chain proteins [1] similar to the A-chains of ricin and related toxins [2], which are known to depurinate the ribosomal RNA by cleaving the *N*-glycosidic bond in the A_{4324} position of the 28S RNA fraction, so rendering it incapable of binding to the elongation factors, with the consequent arrest of protein synthesis [3,4]. These proteins have attracted interest in the construction of immunotoxins after conjugation with monoclonal antibodies for the selective killing of neoplastic cells [5–7].

To better understand perspectives and limits of the sc-RIPs in clinical trials, we previously focused our attention on the antiproliferative effect of RIPs as native molecules or in combination with other drugs. We have demonstrated that the native form of the sc-RIP saporin 6 exerts an antiproliferative effect in vitro in human breast cancer, leukemia and melanoma cells to a very different extent and with diverse kinetics, dependent on the specific cell target analyzed [8–10]. The effect may be potentiated by other drugs: a strong synergism has been obtained by combination of saporin 6 with lonidamine in breast cancer cells in vitro [11]. More recent results obtained by others also suggest a possible penetration of the sc-RIPs into cells [12,13]. In the light of our results and of data concerning a possible nuclease activity exerted by the antiviral sc-RIP trichosanthin [14], the present study was undertaken to investigate the action of sc-RIPs on DNA in vitro.

Results indicate that the sc-RIPs dianthin 30, saporin 6 and gelonin introduce highly selective cleavage into supercoiled covalently closed circular DNA molecules of plasmids pBR322 and ΦX174. This DNAse activity is also evident in single-stranded M13 phage DNA, but not in linear double-stranded DNAs. The DNase activity of the HPLC fractionated sc-RIPs was evident here at a protein concentration 500-fold lower than that reported by Li et al. for trichosanthin [14].

2. Materials and methods

2.1. Materials

The sc-RIPs asparin 1, dianthin 30, gelonin, momordin, PAP-S (pokeweed antiviral protein from *Phytolacca americana* seeds) and saporin 6 were highly purified as described previously [15]. HPLC-purified saporin 6, whose specific *N*-glycosidase activity had been certified, was a kind gift of Prof. F. Stirpe (University of Bologna). The double-chain RIP ricin [3] and α-sarcin, from the mould *Aspergillus giganteus* [16], were kind gifts of Prof. Lucio Montanaro (University of Bologna).

pBR322 and ΦX174 plasmid DNA, lambda phage DNA, single-stranded M13 phage DNA, restriction endonucleases and DNA polymerase I Klenow fragment were purchased from Boehringer Mannheim (Germany). [α-^{32}P]dATP was purchased from Amersham (UK). Agarose and acrylamide/bis-acrylamide for gel electrophoresis were purchased from Clontech Laboratories, Palo Alto, CA (USA). All other chemicals were of analytical grade.

Restriction nucleases employed were: *Pvu*II (from *Proteus vulgaris*) incubation buffer: Tris-HCl 10 mM, NaCl 100 mM, $MgCl_2$ 10 mM, dithioerythritol 1 mM, pH 7.5 at 37°C; *Pst*I (from *Providencia stuartii)* and *Hin*fI (from *Haemophylus influenzae* R_f*)* incubation buffer: Tris-HCl 50 mM, NaCl 10 mM, $MgCl_2$ 10 mM, dithioerythritol 1 mM, pH 7.5 at 37°C; *Bam*HI (from *Bacillus amyloliquefacens H)* and *Hin*dIII (from *Haemophylus influenzae Rd com-10)* incubation buffer: Tris-HCl 10 mM, NaCl 100 mM, $MgCl_2$ 5 mM, 2-mercaptoethanol 1 mM, pH 8 at 37°C.

2.2. DNase activity assay

pBR322 and ΦX174 plasmid DNA (250 ng), single-stranded M13 phage, lambda phage DNA, linearized plasmid DNA (250 ng) were incubated with or without various amounts (250–0.25 ng) of the diverse proteins in a final volume of 10 μl at 37°C for various times. The reactions were carried out in a range of 5–60 mM NaCl (or $MgCl_2$), 1 mM Tris-HCl, 0.1 mM EDTA. The range of pH was 6.5–8. At the end of the incubations, all the samples were extracted with phenol followed by ether and precipitated [17]. Digestion with restriction endonucleases was carried out as specified above. The samples were analyzed in 0.8% agarose and/or in 6% polyacrylamide gel electrophoresis. DNA bands were visualized by staining with ethidium bromide [17].

2.3. End-labelling of DNA

DNA was incubated with the proteins as described above, extracted with phenol followed by ether and precipitated, and then digested with restriction nucleases. The samples were then end-labelled by DNA polymerase I Klenow fragment and [α-^{32}P]dATP [17]. For the autoradiography, the gel was exposed to Kodak X-Omat RP film at −70°C.

3. Results

Double-stranded supercoiled pBR322 DNA (Fig. 1a) and ΦX174 DNA (Fig. 1b) were selectively cleaved by the highly

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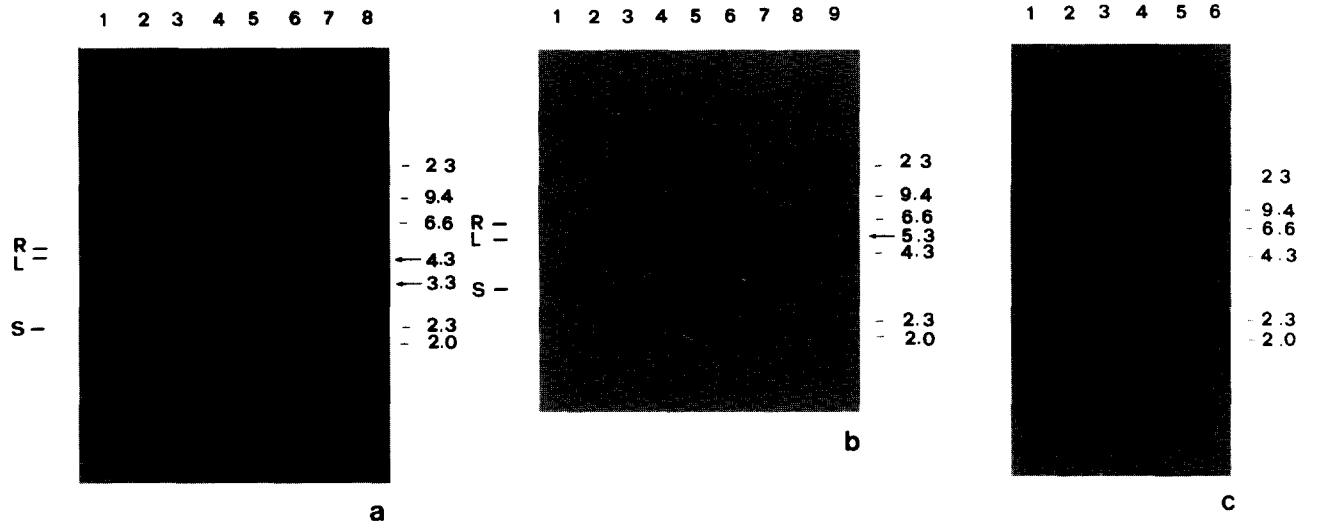

Fig. 1. Effect of sc-RIPs on DNA. (a) pBR322. Lane 1: pBR322 untreated; lane 2: pBR322 linearized and treated with saporin 6; lane 3: pBR322 treated with momordin; lane 4: pBR322 treated with PAP-S; lane 5: pBR322 treated with gelonin; lane 6: pBR322 treated with dianthin 30; lane 7: pBR322 treated with saporin 6; lane 8: λ-*Hin*dIII. (b) ΦX174. Lane 1: ΦX174 untreated; lane 2: ΦX174 linearized and treated with saporin 6; lane 3: ΦX174 treated with saporin 6; lane 4: ΦX174 treated with dianthin 30; lane 5: ΦX174 treated with gelonin; lane 6: ΦX174 treated with asparin 1; lane 7: ΦX174 treated with momordin; lane8: ΦX174 treated with PAP-S; lane 9: λ-*Hin*dIII. (c) Single-stranded M13. Lane 1: M13 untreated; lane 2: M13 treated with saporin 6; lane 3: M13 treated with dianthin 30; lane 4: M13 treated with gelonin; lane 5: M13 treated with asparin 1; lane 6: λ-*Hin*dIII. R = relaxed DNA, L = linear DNA, S = supercoiled DNA. The sizes in kb of fragments are indicated.

purified sc-RIPs gelonin, dianthin 30 and saporin 6 (Fig. 1a,b). Previously linearized plasmid DNAs and double-stranded lambda phage DNA were unaffected by the treatment with the sc-RIPs (Fig. 1a,b and not shown), while single-stranded M 13 phage DNA was completely degraded by dianthin 30 and saporin 6 or partially by gelonin (Fig. 1c). No activity was found on single- and double-stranded DNAs of asparin 1, momordin and PAP-S (Figs. 1c and 1a,b respectively and not shown). The disappearance of the supercoiled form of the plasmid DNAs treated with the active RIPs was accompanied by the appearance of the linearized form (Fig. 1a,b). In pBR322 plasmid DNA treated with dianthin 30 and saporin 6 the appearance of a new smaller fragment was evident at approximately 3300 bp (Fig. 1a).

The supercoiled DNAs treated with the active sc-RIPs remained unaffected at 4°C, partially disappeared at 21°C and disappeared at 37°C (Fig. 2a). Fragments of DNA were partially visualized within 10 min of incubation and were clearly evident after 1 h of incubation at 37°C (Fig. 2b). The optimum of activity was at ionic strength 5–60 mM NaCl (Fig. 2c) and at pH 7.2–7.4. $MgCl_2$ was not required (not shown).

The same results were obtained with different preparations of the active RIPs. The enzymatic activity was unaffected by storage at −20°C and by repeated freezing.

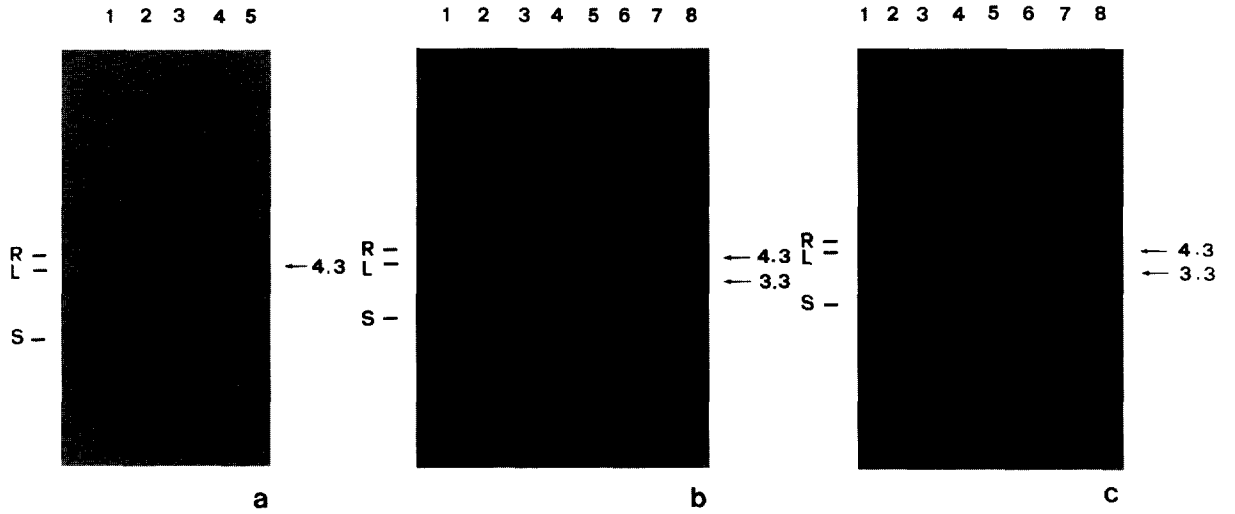

Fig. 2. Characterization of the enzymatic activity exerted by the sc-RIPs on supercoiled DNA. Lane 1: pBR322 untreated; lane 2: pBR322 linearized. (a) Effect of temperature. Incubation of pBR322 DNA with gelonin at 4°C (lane 3), at 21°C (lane 4) and at 37°C (lane 5). (b) Effect of time. Incubation of pBR322 DNA with dianthin 30 for 60, 50, 40, 30, 15, 10 min (lanes 3–8 respectively). (c) Effect of ionic strength. Incubation of pBR322 DNA with saporin 6. Lanes 3–8: pBR322 treated with saporin 6 at ionic strength of 5, 10, 30, 50, 70, 100 mM NaCl respectively. R = relaxed DNA, L = linear DNA, S = supercoiled DNA. The sizes in kb of fragments are indicated.

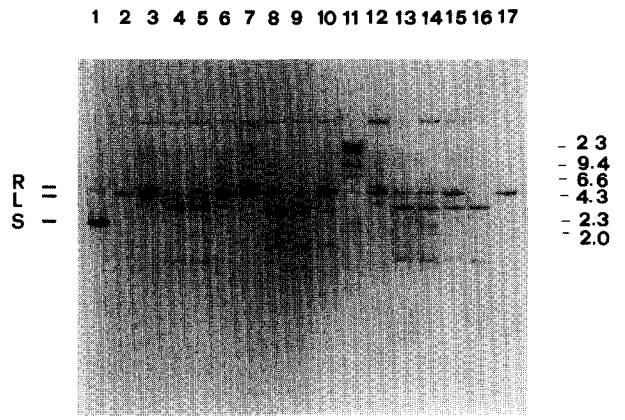

Fig. 3. Restriction analysis in 0.8% agarose of pBR322 treated with active sc-RIPs. Lane 1: pBR322 untreated; lane 2: pBR322 linearized; lane 3: pBR322 treated with dianthin 30; lane 4: pBR322 treated with dianthin 30 and digested with *Hin*dIII; lane 5: pBR322 treated with saporin 6 and digested with *Hin*dIII; lane 6: pBR322 treated with gelonin and digested with *Hin*dIII; lane 7: pBR322 treated with gelonin; lane 8: pBR322 treated with dianthin 30 and digested with *Bam*HI; lane 9: pBR322 treated with saporin 6 and digested with *Bam*HI; lane 10: pBR322 treated with gelonin and digested with *Bam*HI; lane 11: λ-*Hin*dIII; lane 12: pBR322 treated with saporin 6; lane 13: pBR322 treated with dianthin and digested with *Pvu*II; lane 14: pBR322 treated with saporin 6 and digested with *Pvu*II; lane 15: pBR322 treated with gelonin and digested with *Pvu*II; lane 16: pBR322 digested with *Pst*I/*Bam*HI; lane 17: pBR322 linearized. The sizes in kb of restriction fragments are indicated.

The effects of double-chain RIP ricin, whose A-chain cleaves the A_{4324} of 28S rRNA, and of α-sarcin, which cleaves the phosphodiester bond between G_{4325} and A_{4326} in rat 28S rRNA, were also investigated on pBR322 and on ΦX174 plasmids. In both cases, no effect was present under the same experimental conditions (not shown).

To determine if the cleavages introduced by the sc-RIPs gelonin, dianthin 30 and saporin 6 into supercoiled plasmids were at specific or random loci, the pBR322 DNA was treated with the proteins and then specifically cleaved with a series of restriction enzymes. The products after *Hin*dIII, *Bam*HI and *Pvu*II digestion of the sc-RIP treated pBR322 were separated by agarose gel electrophoresis (Fig. 3). While three fragments were evident after *Pvu*II digestion of pBR322 treated with dianthin 30 and with saporin 6, two fragments were evident after *Pvu*II digestion of pBR322 treated with gelonin, suggesting the presence of a diverse number of cleavage sites (Fig. 3).

Detailed mapping of pBR322 was achieved by polyacrylamide gel electrophoresis and confirmed by autoradiography obtained by end-labelling the fragments arising from the restriction analysis (Fig. 4a–c). The results indicate the presence of four specific sites of cleavage introduced by dianthin 30 and saporin 6 into pBR322 DNA (at 4227, 4124, 3312, 3148 ± 10 bp) and of two specific sites of cleavage introduced by gelonin (at 3312 and 3148 ± 10 bp). The restriction map, showing the location of the specific sites of cleavage introduced by dianthin 30, saporin 6 and gelonin into pBR322 supercoiled DNA, is presented in Fig. 5.

4. Discussion

Present results indicate that sc-RIPs dianthin 30, saporin 6 and gelonin act directly on DNA by introduction of selective cleavage into supercoiled covalently closed circular DNA molecules of pBR322 and ΦX174 plasmids. They also degrade DNA of single-stranded M13 phage. The linear double-stranded DNAs were unaffected.

According to the analysis of the nucleotide sequence of the pBR322 DNA molecules [18], present results indicate that three of the cleavage sites introduced by dianthin 30, saporin 6 and gelonin (at 3312, 4124 and 4227 bp) are comprised in the A+T rich regions, while the fourth (at 3148 bp) is very near to one of them (3180 bp). The presence in those regions of weak hydrogen bonds [19] may facilitate the DNase action of the sc-RIPs.

The fact that sc-RIPs degrade single-stranded M13 phage DNA, but not double-stranded DNAs, may suggest a single-stranded nuclease activity for these proteins. Our finding that this DNase activity is fully dependent on the supercoiling density of the nucleic acid is consistent with this hypothesis: it is well known, in fact, that supercoiled DNA generates single-stranded DNA molecules in the A+T rich regions [20].

It is known that the sc-RIPs do release the A_{4324} residue located in a strongly conserved, purine rich, single-stranded loop structure (the α-sarcin domain) on the surface of the ribosomal 28S fraction [4,21]. Through this *N*-glycosidase activity, they do not catalyze hydrolysis of RNA, but instead modify a specific nucleotide (the A) and this renders the surrounding phosphodiester bonds highly susceptible to hydrolysis after treatment with aniline [3,4]. On the other hand, it has been recently hypothesized that some sc-RIPs may induce the release of adenine residues not only from rRNA but also from other RNAs, DNA (herring sperm) and Poly(A) [22]. The recognition sites on supercoiled DNA (A+T rich regions) and on rRNA (purine rich loop) are substantially similar. Most likely, therefore, the DNAse activity observed in the present experiments is a consequence of an *N*-glycosidase activity towards the single-stranded DNA molecules generated in the A+T rich regions, which modifies the torsional stress of the supercoiled DNA with subsequent break of the DNA strand [20]. The cleavage of DNA, unlike that of RNA, does not here require any chemical treatment to be evident.

No DNase effect was present in plasmid DNAs treated with the double-chain RIP ricin or with the phosphodiesterase mould toxin α-sarcin.

The DNAse activity was exerted by HPLC fractionated dianthin 30, saporin 6 and gelonin to an equimolar DNA:-protein ratio in the absence of Mg^{2+}, so discounting the presence of nuclease contaminants [14].

The different nuclease activity exerted by dianthin 30 and saporin 6 (four sites of cleavage) and by gelonin (two sites of cleavage) together with the absence of activity by other sc-RIPs (momordin, asparin 1, PAP-S) may be due to intrinsic diversities of the molecules, purified from plants taxonomically related (*Saponaria officinalis* and *Dianthus caryophyllus* belong to Caryophyllaceae) or not (the others). It has been definitely demonstrated that RIPs also behave differently on the various ribosomal substrates tested and on different cellular targets [8–10]. It is possible that experimental conditions too may play a role in such diversity: a DNase-like activity has been recently demonstrated by the abortifacient and HIV inhibitor sc-RIP tricosanthin [14], by which the linear form from supercoiled DNA is produced, together with nicked circular one, at a very high concentration of the protein [DNA:

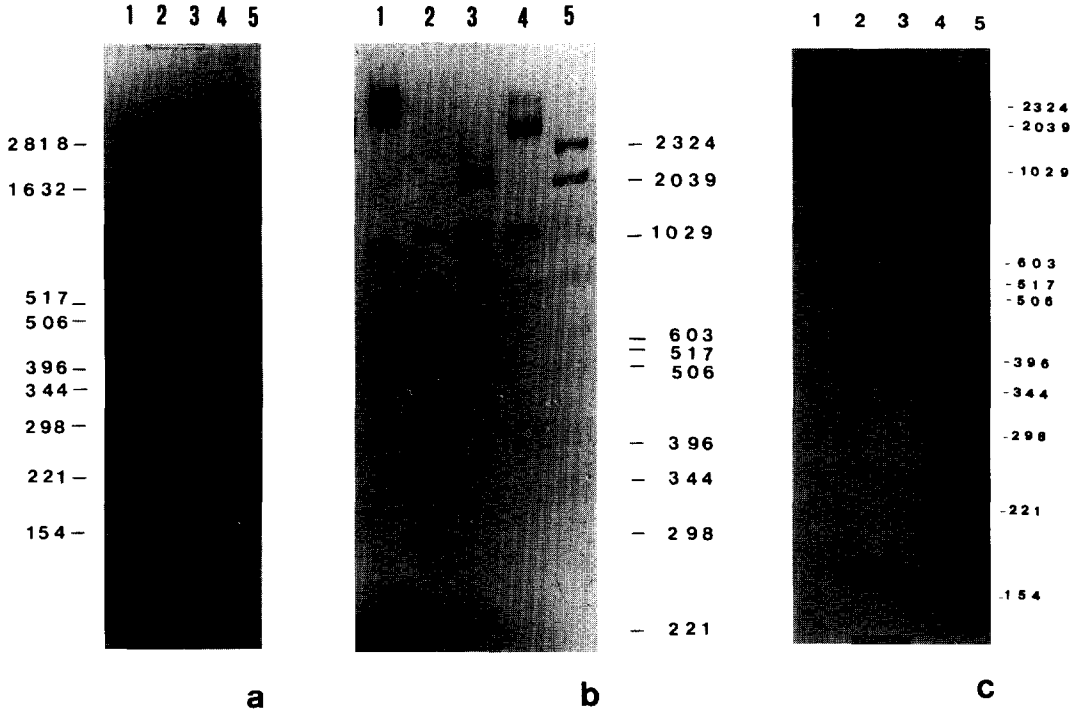

Fig. 4. Gel electrophoresis in 6% acrylamide of pBR322 DNA treated with active sc-RIPs. (a) Lane 1: pBR322 digested with *Pst*I/*Pvu*II; lane 2: pBR322 treated with saporin 6 and digested with *Pst*I/*Pvu*II; lane 3: pBR322 digested with *Hin*fI; lane 4: pBR322 digested with *Pst*I/*Hin*dIII; lane 5: pBR322 treated with saporin 6 and digested with *Pst*I/ *Hin*dIII. (b) Lane 1: pBR322 treated with saporin 6; lane 2: pBR322 digested with *Hin*fI/ *Hin*dIII; lane 3: pBR322 treated with saporin 6 and digested with *Hin*dIII/*Pvu*II; lane 4: pBR322 treated with saporin 6 and digested with *Hin*dIII; lane 5: pBR322 digested with *Hin*dIII/*Pvu*II. (c) Autoradiography of restriction fragments 5′ end [α-^{32}P]dATP labelled pBR322. Lane 1: pBR322 digested with *Pvu*II/*Hin*dIII; lane 2: pBR322 treated with saporin 6 and digested with *Pvu*II/*Hin*dIII; lane 3: pBR322 treated with dianthin 30 and digested with *Pvu*II/*Hin*dIII; lane 4: pBR322 treated with gelonin and digested with *Pvu*II/*Hin*dIII; lane 5: pBR322 digested with *Hin*fI/*Hin*dIII. The sizes in bp of restriction fragments are indicated.

protein ratio of 1:5 (w/w)], about 500-fold higher than that found effective here.

Apart from the current interest in the pharmacological potential of the sc-RIPs, under study both as native molecules [8–10] or in combination with other drugs in vitro ([11] and unpublished results) and in the construction of immunotoxins for the selective killing of neoplastic and virus infected cells [5–7], the present evidence of a new DNA structure specific DNase may provide a novel mechanism for their biological action. Moreover, the DNase active proteins may represent a useful instrument for the study of the prokaryotic and eukaryotic biological processes in which the role exerted by DNA supercoiling has assumed considerable evidence [23]. The degree of supercoiling represents in fact a fundamental regulatory element in gene transcription and expression, both in normal and in cancer genetics [24–27]. In this field, recent works suggest the possibility to consider DNA supercoiling as a drug target and a good partner for even more specific conjugates [28,29].

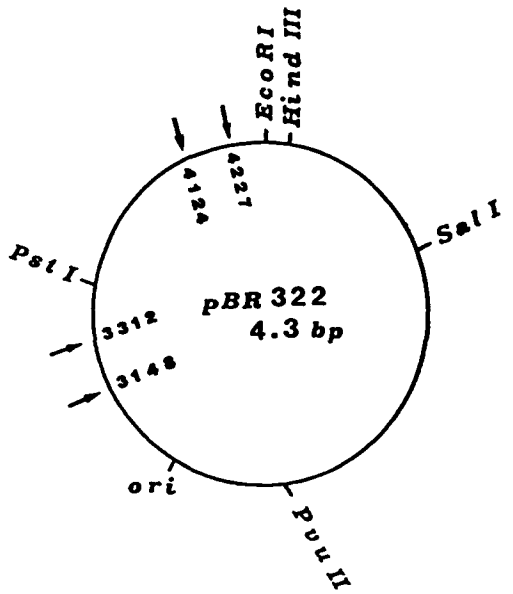

Fig. 5. Sites of cleavage in pBR322. The position of the sites of cleavage introduced by dianthin 30, saporin 6 and gelonin in pBR322 are indicated by the arrows at 4227 bp, 4124 bp, 3312 bp, 3148 bp.

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References

[1] Gasperi-Campani, A., Barbieri, L., Battelli, M.G. and Stirpe, F. (1985) J. Nat. Prod. 48, 446–454.
[2] Gasperi-Campani, A., Barbieri, L., Lorenzoni, E., Montanaro, L., Sperti, S., Bonetti, E. and Stirpe, F. (1978) Biochem. J. 174, 491–496.
[3] Endo, Y., Mitsui, K., Motizuki, M. and Tsurugi, K. (1987) J. Biol. Chem. 262, 5908–5912.

[4] Endo, Y., Tsurugi, K. and Lambert, J.M. (1988) Biochem. Biophys. Res. Commun. 150, 1032–1036.
[5] Funatsu, G., Islam, M.R., Minami, Y., Sung-Sil, K. and Kimura, M. (1991) Biochemie 73, 1157–1161.
[6] Pastan, I., Chandhary, V. and FitzGerald, D.J. (1992) Annu. Rev. Biochem. 61, 331–354.
[7] Perkins, A.C., Pimm, M.V. and Baldwin, R.W. (1987) Eur. J. Cancer Clin. Oncol. 23, 1225–1227.
[8] Gasperi-Campani, A., Zauli, G., Roncuzzi, L., Valvassori, L., Vitale, L., Gaggioli, L. and Bagnara, G.P. (1989) Exp. Hematol. 17, 755–759.
[9] Gasperi-Campani, A., Zoli, W., Volpi, A.L., Roncuzzi, L. and Amadori, D. (1991) Anticancer Res. 11, 1007–1012.
[10] Gasperi-Campani, A., Musa, A.R. and Roncuzzi, L. (1993) Melanoma Res. 3, 363–367.
[11] Roncuzzi, L., Zoli, W., Bajorko, P. and Gasperi-Campani, A. (1995) Anticancer Res. 15, 773–776.
[12] Battelli, M.G., Montacuti, V. and Stirpe, F. (1992) Exp. Cell Res. 201, 109–112.
[13] Cavallaro, U., Nykjaer, A., Nielsen, M. and Soria M.R. (1995) Eur. J. Biochem. 232, 165–171.
[14] Li, M.-X., Yeung, H.-W., Pan, L.-P. and Chan, S.I. (1991) Nucleic Acids Res. 19, 6309–6312.
[15] Barbieri, L., Stoppa, C. and Bolognesi, A. (1987) J. Chromatogr. 408, 235–243.
[16] Endo, Y. and Wool, I.G. (1982) J. Biol. Chem. 257, 9054–9060.
[17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Sutcliffe, J.G. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 77–90.
[19] Kowalski, D., Natale, D.A. and Eddy, M.J. (1988) Proc. Natl. Acad. Sci. USA 85, 9464–9468.
[20] Panayotatos, N. and Wells, R.D. (1981) Nature 289, 466–470.
[21] Endo, J., Gluck, A., Chan, Y.C., Tsurugi, K. and Wool, I.G. (1990) J. Biol. Chem. 265, 2216–2222.
[22] Barbieri, L., Gorini, P., Valbonesi, P., Castiglioni, P. and Stirpe, F. (1994) Nature 372, 624 (letter).
[23] Smith, G.R. (1981) Cell 24, 599–600.
[24] Goodburn S. (1990) Biochim. Biophys. Acta 1032, 53–77.
[25] Sun, L. and Fuchs, J.A. (1994) J. Bacteriol. 176, 4617–4626.
[26] Bazar,L., Meighen, D., Harris, V., Duncan, R., Levens, D. and Avigan, M. (1995) J. Biol. Chem. 270, 8241–8248.
[27] Cox, P.M. and Goding, C.R. (1991) Br. J. Cancer 63, 651–662.
[28] Kobets, N.D., Borissov, R.G., Chernolovskaya, E.L., Gorn, V.V. and Vlassov, V.V. (1994) Antisense Res. Dev. 4, 259–262.
[29] Corey, D.R., Munoz-Medellin, D. and Huang, A. (1995) Bioconjug. Chem. 6, 93–100.