Formation of positive supercoiled DNA by a nuclear factor from myeloma cells

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The formation of positive supercoiled DNA by an activity from a hypermutating myeloma line is reported. This activity forms positive supercoils from negative supercoiled DNA, it does not use positive supercoils to form negative ones and does not require an exogenous source of energy. The linking number changes by steps of 1, suggesting a type-I mechanism of action, and there seems to be an upper limit to the degree of positive supercoiling that can be achieved. Positive supercoiled DNA has to be taken into account as a possible structure of DNA *in vivo* for those functions where torsional stress is involved.

INTRODUCTION

The lymphoid system has several cell-specific recombination processes required to form functional variable regions of immunoglobulin genes and to switch the class of the heavy-chain constant region (Tonegawa, 1983). In addition, productively rearranged immunoglobulin genes are able to mutate in a localized gene segment at a very high rate (McKean *et al.*, 1984; Clarke *et al.*, 1985). All these processes will require at some stage an endonuclease as well as a ligase activity as part of their mechanism.

Topoisomerase activities modulate the conformational state of DNA molecules (Cozzarelli, 1980; Gellert, 1981, 1982; Wang, 1982*a,b*; Drlica, 1984), and they have been implicated in several physiological processes, such as recombination (either general or specific) and replication (Gellert, 1982; Wang, 1982*b*; Drlica, 1984). In eukaryotic cells, topoisomerase I has been found associated with nucleosomes (Fleischmann *et al.*, 1984) and topoisomerase II with the nuclear matrix skeleton, but their physiological functions within cells are not yet clearly established. Supercoiling seems to be required to maintain transcriptional activity (Villeponteau *et al.*, 1984), but a transcriptional block by supercoiled DNA has also been reported (Peck & Wang, 1985; Brahms *et al.*, 1985).

During the search for nuclease activities in a hypermutable line, myeloma S107 (Cook & Scharff, 1977), an activity that generated positive supercoiled DNA was detected. This is the first observation of such an activity in mammalian cells, and this property is not usually associated with any of the topoisomerase activities from mammalian cells so far known (Gellert, 1982; Wang, 1982*a*). We need to be aware that the role of most nuclease activities in cells is probably not to degrade nucleic acids, even though that is the way these activities are usually assayed. In most cases, the role of these nucleases is *in vivo* is not understood.

EXPERIMENTAL

To prepare nucleases, nuclei were isolated from 1 g of S107 myeloma cells by using the protocol of Klocker *et al.* (1983). The only modification was the addition of

5 mM-PMSF at all stages of the purification in order to prevent proteolytic degradation as much as possible. The nuclear pellet was extracted with 0.5 M-KCl (low salt) and cleared by centrifugation at 10000 *g* for 10 min. The supernatant was dialysed overnight against 10 mM-Tris/HCl/10 mM-NaCl/1.5 mM-MgCl₂/3 mM-β-mercaptoethanol and 30% (v/v) glycerol, pH 7.4, to make the nuclear extract. This nuclear extract was applied to a DEAE-cellulose column (DE-52, Whatman) and eluted with a 40 ml linear salt gradient (10–500 mM-NaCl) in the same buffer (Fig. 1*a* below). Binding assays were performed at room temperature in 40 μl of a mixture containing 10 mM-Hepes, pH 7.4, 100 mM-KCl, 10 mM-NaCl, 0.1 mM-EDTA, 5 mM-MgCl₂, 2 mM-β-mercaptoethanol, bovine serum albumin (5 μg/ml) and aprotinin (2.5 k.i.u./ml), as well as the DNA substrate and a 1 μl portion of protein sample. The mix was applied to a nitrocellulose filter (BA-85; Schleicher and Schuell, Keene, NH, U.S.A.) and washed with 5 ml of 10 mM-Hepes (pH 7.4)/10 mM-NaCl/5 mM-MgCl₂ (Nagata *et al.*, 1983). The retained fragments were recovered from the filters as described by Siebenlist *et al.* (1984) to determine possible specificity. Alternatively filters were dried and radioactivity was counted to determine total DNA retention.

To assay for nuclease activities, several plasmids carrying segments of mouse immunoglobulin genes were used. Plasmid pVhJ558 contains the V_H gene of myeloma J558 productively rearranged to J_{H1} (Brodeur & Riblet, 1984). Plasmid pJ11 contains unrearranged J_{H3} and J_{H4} genes with their respective recombination sequences as well as the heavy-chain enhancer (Marcu *et al.*, 1980). Plasmid pJ14 contains the μ-switch region of the heavy chain (Marcu *et al.*, 1980). Plasmid pBR322 and phages λ and M13mp8 were also used as DNA substrates. Plasmids were grown in *Escherichia coli* HB101, and negative supercoiled DNA was prepared by purification in a CsCl gradient (Maniatis *et al.*, 1982).

Agarose-gel electrophoresis was performed in both one and two dimensions as described by Peck & Wang (1983) in 90 mM-Tris/boric acid (pH 8.3)/2.5 mM-EDTA. Before the second-dimension run was started, the gel was soaked for 7 h in 2 μM-chloroquine. Chloroquine was present

while the second-dimension run was performed. All reagents were of the highest purity commercially available from Sigma, New England Biolabs or Bethesda Research Laboratories.

RESULTS AND DISCUSSION

The nuclear extract from myeloma S107 was fractionated by DEAE-cellulose chromatography. The fractionation was characterized by the binding of 1 μ l of each fraction to DNA. This binding assay (Fig. 1a), performed with linearized plasmids pVhJ558 and pJ11, showed that there are two fractions that bind to DNA, namely peaks F2 and F8, which are eluted at 10 mM- and 200 mM-NaCl respectively. More than 80% of the protein applied to the column was eluted in the F2 peak. Since these two plasmids share the DNA of plasmid pBR322, another binding assay was performed by using labelled pBR322 digested with the restriction enzyme *DdeI*. The nature of the retained pBR322 fragments was also analysed by recovering the DNA from the filters and detecting them by separation in a 3.5% (w/v)-polyacrylamide gel in Tris/borate (Maniatis *et al.*, 1982). As shown in Fig. 1(b), F2 seems to bind more strongly to the smaller *DdeI* restriction fragments of pBR322, mainly the 162 bp and 166 bp species, on the basis of relative intensity of the bands. When the DNA sequences of these two fragments were compared, the only significant homology found was in the sequence 5'-GGATCTCA-3' localized at positions 1666-1674 and 3212-3220, forming a direct repeat. There is no direct evidence that this sequence is recognized by F2, and there might be more than one binding factor in the F2 fraction. By contrast, F8 seems to bind in a non-specific way to DNA, since all the *DdeI* restriction fragments of pBR322 were recovered. The binding activities were lost if the fractions were boiled for 10 min before the assay was performed, suggesting that they are proteins. The binding activity of F2 was lost during attempts to purify it further.

Both F2 and F8 fractions were also analysed for nuclease activities by using as substrates supercoiled DNA (plasmids), double-stranded linear DNA (phage λ) and single-stranded DNA (phage M13). The assays were done with and without 1-5 mM-ATP, in case the activity might require an exogenous energy source. F2 has a nicking activity with supercoiled DNA (all the supercoiled plasmid was converted into nicked relaxed DNA) and a slightly activated ATP-dependent exonuclease activity with linear double-stranded DNA as substrate. F8 generated a pattern of topoisomers with negative

Fig. 1. Fractionation of myeloma nuclear proteins

(a) Profile of DEAE-cellulose DE-52 fractions analysed by a nitrocellulose binding assay, with 1 μ l from each fraction. Plasmids pJ11(□) and pVhJ558 (○) were used as substrates. The radioactivity shown in the Figure is the total retained by each fraction. ----, [NaCl] gradient. (b) Polyacrylamide gel of DNA fragments retained in the binding assay. The specificity of DNA binding was analysed by using 500 ng of plasmid pBR322, nick-translated followed by *DdeI* digestion (a), or pBR322 digested first with *DdeI* followed by end-labelling (b). Control experiments were done without DE-52 fractions to determine non-specific retention by the filters. The reference lanes (R) contain 500 ng of pBR322 digested with *DdeI* and end-labelled.

Fig. 2. Topoisomer mixture generated by F8 topoisomerase activity analysed in one (b) and two (c) dimensions (the plasmid control is a)

The gel used was 0.8% agarose. The spot in the upper-left-hand corner corresponds to nicked relaxed DNA. The top of the smooth set of topoisomers is the relaxed form, the topoisomers to its right are positive supercoils and those to the left are negative supercoils (Peck & Wang 1983). In this experiment 1 μ g of plasmid pJ14 was used.

supercoiled DNA, and these tended to accumulate in the upper half of the region on an agarose gel between relaxed and supercoiled DNA (Fig. 2b).

The topoisomer pattern of F8 activity was compared in an agarose gel with those of *E. coli* topoisomerase I and bovine topoisomerase II from commercial sources. The spacing between topoisomers generated by F8 is similar to that observed by relaxing the same plasmid with *E. coli* topoisomerase I, suggesting that the linking number varies by steps of 1. The topoisomer distribution with both enzymes was different, however, and the typical 'ladder' was not seen (Wang, 1982b).

The F8 activity does not require an exogenous source of energy (ATP at 1 or 5 mM was used) and is not affected by oxolinic acid (an inhibitor of the relaxing activity of topoisomerase II in the absence of ATP) at 50 μ g/ml. Nalidixic acid and novobiocin at 50 μ g/ml had no effect. Although $MgCl_2$ was always present to at least 37.5 μ M (in the buffer containing F8), the addition of 50 mM-EDTA did not inhibit the reaction; EDTA only slightly displaced the set of topoisomers closer to the relaxed form, without accumulating more relaxed DNA, suggesting that this activity either does not require Mg^{2+} or binds to it very tightly. Relaxed DNA was not accumulated by increasing either the amount of F8 protein or the incubation time. Spermine at 5 mM had no effect. All these experiments failed to characterize the F8 activity as any of the known mammalian topoisomerases, though it seemed to use a type-I mechanism. Further attempts to purify this activity with phosphocellulose, heparin-cellulose or Cibacron Blue-cellulose resulted in its loss, suggesting that several proteins might be involved.

From the outset it seemed unusual that the F8 activity

accumulated topoisomers in the upper half of the gel region between relaxed DNA and the supercoiled DNA used as substrate (Fig. 2a), and that relaxed DNA never increased noticeably. This observation was made with the four plasmids mentioned above, arguing against any kind of sequence specificity by the immunoglobulin-gene segments. This led to the suspicion that there might be another explanation. So, to characterize further the topoisomer mixture generated by F8, two-dimensional agarose-gel electrophoresis was performed (Peck & Wang, 1983). This technique was originally used to detect Z-DNA. Surprisingly, this type of gel (Fig. 2) showed that the topoisomers were positively supercoiled, up to 18 bands of such isomers being observed, with plasmid pJ14 (9 kbp) the biggest of all of them. The transition was always from negative to positive supercoils, and there seemed to be a limit to the degree of positive supercoiling that could be achieved, hence the uneven distribution of topoisomers observed in one-dimensional gels (Fig. 2b). To check that this observation was not a technical artefact, the DNA was partially relaxed with *E. coli* topoisomerase I and analysed by using the two-dimensional gel system; the expected distribution of negative supercoiled topoisomers was observed.

Positive supercoiled DNA has not yet been identified in mammalian cells as a stable conformation of DNA. Positive supercoiled DNA can be made by using intercalating drugs such as ethidium bromide or lowering the salt concentration (Vosberg *et al.*, 1975; Wang, 1971). More recently it has been obtained enzymically by using *E. coli* DNA ligase and DNA gyrase (topoisomerase II) from *Micrococcus luteus*, in the presence of NAD^+ as an exogenous energy donor for the ligase (Liu & Wang, 1978). Although these enzyme sources are an unlikely

combination *in vivo*, such a reaction is possible enzymically. Induction of positive supercoiling has not been shown for any of the known mammalian enzymes. Actually what has been shown is the relaxation of chemically generated positive supercoiled DNA (Liu & Wang, 1978). The F8 activity seems to act by a type-I mechanism and is active in the absence of Mg^{2+} , even though it does not show properties of the topoisomerase I of mammalian cells [e.g. relaxing positive supercoils (Geider & Hoffmann-Berling, 1981)]. It is eluted at 0.2 M-NaCl instead of 0.4 M-NaCl from DEAE-cellulose DE-52, as is the case for topoisomerase I from 2A49 and L-cells (Vosberg *et al.*, 1975), although in this case it might have been a proteolytic fragment; since then the purification procedure has changed (Liu, 1983; Martin *et al.*, 1983) and elution profiles are no longer comparable. Alternatively, the F8 activity might be the result of a regulatory factor acting on a known topoisomerase activity.

The failure to observe enzymically generated positive supercoiled DNA up until now might be the result of several factors. Inactivation of the enzyme during the purification is a likely one; in the case of F8, the partial purification took a very short time and was always done with proteinase inhibitors. Although the amount obtained was very small, it has proved to be very stable at -70°C . Overpurification, resulting in the loss of a regulatory component of a known topoisomerase enzyme, might be another possible reason, since enzymes using DNA, e.g. polymerases, are composed of multiple subunits. Nothing is known about regulation of topoisomerases in mammalian cells. An alternative interpretation is that positively supercoiled DNA is an intermediate with a short life in a process not yet characterized and consequently extremely difficult to detect *in vivo*.

At this moment a functional role cannot be given to the F8 activity, and no relation can be made to any of the specific functions of the immune system that led to its accidental finding. But whatever its ultimate function, the important point is that positive supercoiled DNA can indeed be generated by mammalian proteins, and consequently it has to be considered as a possible intermediate in reactions in which topoisomerase activities are involved or torsional stress is required.

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