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Cruciform cutting endonucleases from *Saccharomyces cerevisiae* and phage T4 show conserved reactions with branched DNAs

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We have purified a cruciform DNA resolving endonuclease (Endo X3) >1000-fold from crude extracts of mitotically growing *Saccharomyces cerevisiae*. The enzyme shows high specificity for DNAs with secondary structures and introduces characteristic patterns of staggered 'nicks' in the immediate vicinity of the structure. The following substrates were analyzed in detail: (i) naturally occurring four-way X junctions in cruciform DNA of a supercoiled plasmid; (ii) synthetic four-way X junctions with arms of 9 bp; (iii) synthetic three-way Y junctions with arms of 10 bp; and (iv) heteroduplex loops with 19 nucleotides in the loop. Cleavages were always found in the double stranded portion of the DNA, located immediately adjacent to the junction of the respective structure. The Endo X3 induced cleavage patterns are identical or very similar to the cleavage patterns induced in the same substrates by endonuclease VII (Endo VII) from phage T4. Furthermore, the activity of Endo X3 is completely inhibited in the presence of anti-Endo VII antiserum. Endo X3 has an apparent mol. wt of 43 000 daltons, determined by gel filtration and of ~18 000 daltons in SDS-polyacrylamide gels. Maximum activity of the enzyme was obtained in the presence of 10 mM $MgCl_2$ at 31°C in Tris-HCl buffer over a broad pH range with a maximum ~8.0. About 70% of maximal activity was obtained when Mg^{2+} was replaced by equimolar amounts of Mn^{2+} or Ca^{2+}.

Key words: cruciform DNA/endonuclease/Holliday structure/recombination/repair

Introduction

The Holliday structure is the central intermediate in molecular models of homologous recombination (Holliday, 1964; Meselson and Radding, 1975; Szostak *et al.*, 1983). This structure occurs when, after synapsis, two DNA molecules cross-exchange strands of the same polarity. The molecular link, called Holliday junction, physically connects the recombining molecules and must be resolved before faithful segregation of the chromosomes can be achieved.

It has been tacitly assumed that the resolution of the Holliday structure at its junction requires a specific endonuclease. The discovery of endonuclease VII (Endo VII) from phage T4 and the finding that it resolves Holliday structures *in vitro* strongly supported this idea (Minagawa and Ryo, 1978; Kemper and Garabett, 1981; Kemper *et al.*, 1981; Mizuuchi *et al.*, 1982). Since then, similar properties were ascribed to other enzymes from a broad range of organisms. These include, for example, endonuclease 1 from phage T7 (de Massy *et al.*, 1987; Dickie *et al.*, 1987), Int-protein of bacteriophage lambda (Hsu and Landy, 1984), FLP-protein of the 2 μm plasmid of yeast *Saccharomyces cerevisiae* (Meyer-Leon *et al.*, 1988), an activity from HeLa cells (Waldman and Liskay, 1988), an activity from human placenta (Jeyaseelan and Shanmugam, 1988) and two activities from mitotic cells of yeast *S.cerevisiae* (Symington and Kolodner, 1985; West and Körner, 1985). Since these enzymes share the novel property of resolving four-way junctions in X-branched DNAs *in vitro*, we propose to give them the common name 'X-solvase(s)'.

We report here the purification and enzymatic characterization of a new X-solvase from yeast *S.cerevisiae* and compare its activity on branched DNAs with reactions of Endo VII from phage T4 on the same substrates.

For the search of X-solvase activities in crude cell extracts and during the development of a purification procedure we used cruciform DNA as an X-branched substrate instead of a 'true' Holliday structure (Holliday, 1964), because the recombination intermediates are difficult to obtain in sufficient quantities (Dressler and Potter, 1982). Although cruciform DNA is considered to be structurally equivalent to a Holliday junction, the homology situation between the two structures is markedly different. The pairwise homology characteristic for a 'true' Holliday junction is not present in cruciform DNA. Since Endo VII from phage T4 was shown to cleave both substrates with nearly the same efficiency (Mizuuchi *et al.*, 1982; Kemper *et al.*, 1984; Lilley and Kemper, 1984) the replacement seemed justifiable.

Cruciform DNA was obtained from two sources, one from natural supercoiled plasmid DNA with a perfect palindrome cloned into a predetermined position (homology in one pair of arms) and one made by *in vitro* hybridization of four synthetic oligonucleotides (lack of any homology) (Seeman, 1982; Kallenbach *et al.*, 1983). When we compared cleavage patterns made by Endo X3 or Endo VII in cruciforms, Y junctions and in heteroduplex loops, they were indistinguishable with respect to location and usage of cleavage sites. Furthermore, both enzymes were inhibitable in their reactions with cruciform DNA by an anti-Endo VII antiserum, suggesting a close relationship between the prokaryotic and eukaryotic X-solvases.

This is the third X-solvase described for yeast, and in this report we shall call these enzymes Endo X1 (West and Körner, 1985), Endo X2 (Symington and Kolodner, 1985) and Endo X3 (described here). Circumstantial evidence strongly suggests that Endo X3 is indeed different from Endo X1 and Endo X2.

Results

Purification of Endo X3

Cruciform DNA from phage M13mp2IR62E was used as a substrate during the search in crude extracts for X-solvase activity and during its purification. The plasmid carries a perfect palindrome of 2 × 31 bp cloned into its unique *Eco*RI restriction site in position 6232 (see Figure 1a). The inverted repeat adopts a cruciform configuration in supercoiled DNA. In different DNA preparations the fraction of molecules with a cruciform structure was usually >85%. This was determined with Endo VII from phage T4 which cleaves specifically at the base of cruciform structures (Kemper *et al.*, 1984; Lilley and Kemper, 1984). For the localization of cleavage sites the DNA was restricted with *Bam*HI after exposure to X-solvase containing samples. *Bam*HI cleaves the DNA once in position 2220 converting circular substrate molecules into linear molecules of 7.2 kb and molecules already linearized at the cruciform structure into two fractions of 4.0 and 3.2 kb respectively (Figures 1 and 2).

Since the formation of cruciform structure depends largely on supercoil (Lilley, 1980; Gellert *et al.*, 1983; Panayotatos and Wells, 1988), the assay is sensitive to factors reducing supercoil density of the substrate. This includes topoisomerases as well as endonucleases. In fact, the assay was not reliable until a *top1* mutant (kindly provided by R.Sternglanz) was used. Inhibition of a salt sensitive endonuclease with 200 mM NaCl and lowering the $MgCl_2$ concentration from 10 mM, the optimum for purified Endo X3, to 1 mM, further increased the specificity of the assay. The interfering endonuclease has not been further characterized yet. Supercoiled cruciform DNA could not be replaced by synthetic cruciform DNA during the search for Endo X3 in crude cell extracts because of unspecific degradation.

Endo X3 was routinely purified from 50 g of yeast cells. Strain RS190 was grown at 30°C in YEP medium and harvested at mid log growth phase. The purification procedure is described in detail in Materials and methods and a summary of a typical purification is given in Table I.

At early steps during the purification, a factor inhibiting restriction enzymes obstructed the assay. The inhibitor became measurable during heparin–agarose chroma-

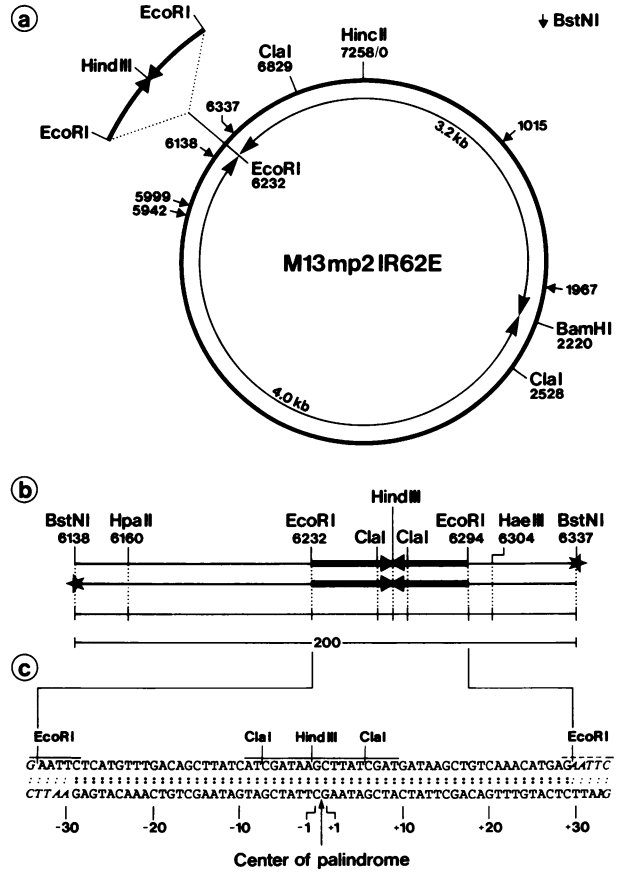

Fig. 1. Map of plasmid M13mp2IR62E. (**a**) Restriction sites relevant to this communication are indicated. The inverted repeat (IR) of the plasmid is depicted by the two arrows outside the circle. Arrows inside the circle show two fragments of 3.2 and 4.0 kb which are generated by concomitant cuts at the inverted repeat and the *Bam*HI restriction site. (**b**) Expanded map of the 200 bp *Bst*NI fragment covering the IR is shown with restriction sites. * = 3′ end label. (**c**) Complete sequence of the IR.

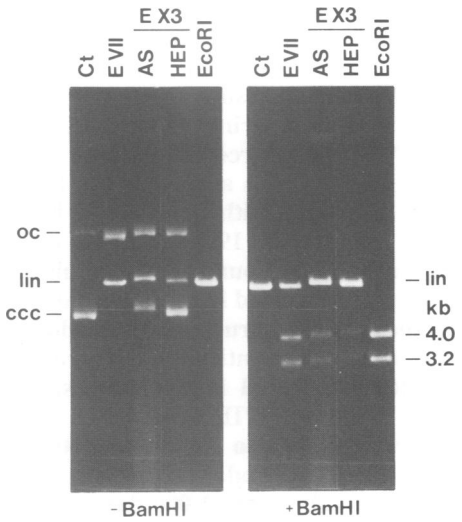

Fig. 2. Endo X3 cleaves supercoiled DNA from M13mp2IR62E at the cruciform structure. 200 ng of supercoiled DNA from M13mp2IR62E was treated with Endo X3 from fraction II (AS, ammonium sulfate precipitate) and fraction III (HEP, heparin–agarose eluate) under standard assay conditions as described in Materials and methods. After the reaction the samples were split and one half was further digested with *Bam*HI (+BamHI). All samples were analyzed on a 1% agarose gel. Purified Endo VII (EVII) from phage T4 and *Eco*RI were used in control fractions. ct = DNA without enzymatic treatment; OC = relaxed open circular DNA; lin = linear DNA; ccc = covalently closed circular DNA.

Table I. Purification of Endo X3

Step	Fraction	Total units	Units/ mg/protein	Yield %	Purification fold
S30	I	26 400	8.3	100	1
ASP	II	15 580	6.6	59	0.8
Heparin	III	43 200	655	164	79
Mono Q	IV	5250	1163	19.9	140
Mono S	V	3000	8333	11.4	1000
Superose 12[a]	VI	–	11 000	–	1325

Purification of Endo X3 from 50 g of cells was performed as described in Materials and methods. The activity was determined in the combination assay under standard conditions as described there.
[a]An aliquot of fraction V was used.

tography and its elution peak overlapped to some extent the activity peak of Endo X3. The factor could be completely separated from Endo X3 during Mono Q chromatography.

Purity of Endo X3

Fraction VI is essentially free of contaminating endo- and exonucleolytic activities, since prolonged incubation of the reaction mixture with 5′ end labeled cruciform DNA J9 did not alter the specific cleavage pattern induced by Endo X3 nor did it cause loss of radioactive end label. Separation of proteins of fraction VI on 8–25% polyacrylamide–SDS gels, however, revealed up to six bands with mol. wts between 15 000 and 70 000.

Stability of Endo X3

Fractions I (S30) and II (dissolved ammonium sulphate precipitate) are rather unstable and cannot be stored for >12 h at 4°C without considerable loss of activity. Fraction III (heparin–agarose eluate) and fraction IV (Mono Q eluate) can be stored at −20°C for at least 1 week without loss of activity. This applies also to fraction V (Mono S eluate) which is stable at −20°C for at least 5 months if BSA is added for stabilization. Any purification of Endo X3 beyond fraction V requires the addition of pure protein like BSA to a final concentration of 0.5 mg/ml. Fraction VI can be stored on ice for >1 month. It should not be frozen. For longer storage, dialysis against buffer E and storage at −20°C is advisable.

Requirements of Endo X3

Endo X3 (fraction VI) gave full activity with cruciform DNA under standard assay conditions as described in Materials and methods. The pH optimum determined with biological buffers, replacing Tris–HCl in the above mixture, showed a broad profile between pH 5.5 and 10.0. At these pH values 50% of full activity was measured. Maximum activity was obtained with 10 mM $MgCl_2$. The activity was reduced to 75 and 70% with 10 mM $MnCl_2$ and 10 mM $CaCl_2$ respectively. Low activity (<10%) was found with $CoCl_2$ and no activity (<1%) with $ZnCl_2$ at 10 mM each. In the presence of 10 mM $MgCl_2$, the enzyme is salt sensitive, exhibiting 60, 25 or <2% in buffer containing 100, 200 or 500 mM NaCl respectively. If the concentration of $MgCl_2$ was lowered to 1 mM, maximum activity was observed in the presence of 200 mM NaCl and reduced levels were found in the absence (50%) or in the presence of 300 mM (20%) or 500 mM (<2%) NaCl. ME was not required for activity.

Molecular weight of Endo X3

Two independent methods were used to determine the native mol. wt of Endo X3. A Superose 12 gel filtration column was developed with buffer F and the activity eluted in a single peak with the same elution volume as ovalbumin, suggesting a mol. wt of 43 000 daltons (Figure 3a and b). Sedimentation analyses in sucrose gradients under the same high salt conditions, however, showed a more complex profile with the bulk of the activity sedimenting between ribonuclease A and ovalbumin with an apparent mol. wt of ~23 000 daltons. Two additional peaks containing ~25% of the total activity recovered from the gradient were found at two positions beyond BSA closer to the bottom of the tube, indicating a tendency of Endo X3 to aggregate with itself or with other proteins in the preparation (Figure 3c,d).

The difference between the mol. wts observed by the two methods may reflect a monomer–dimer relationship of

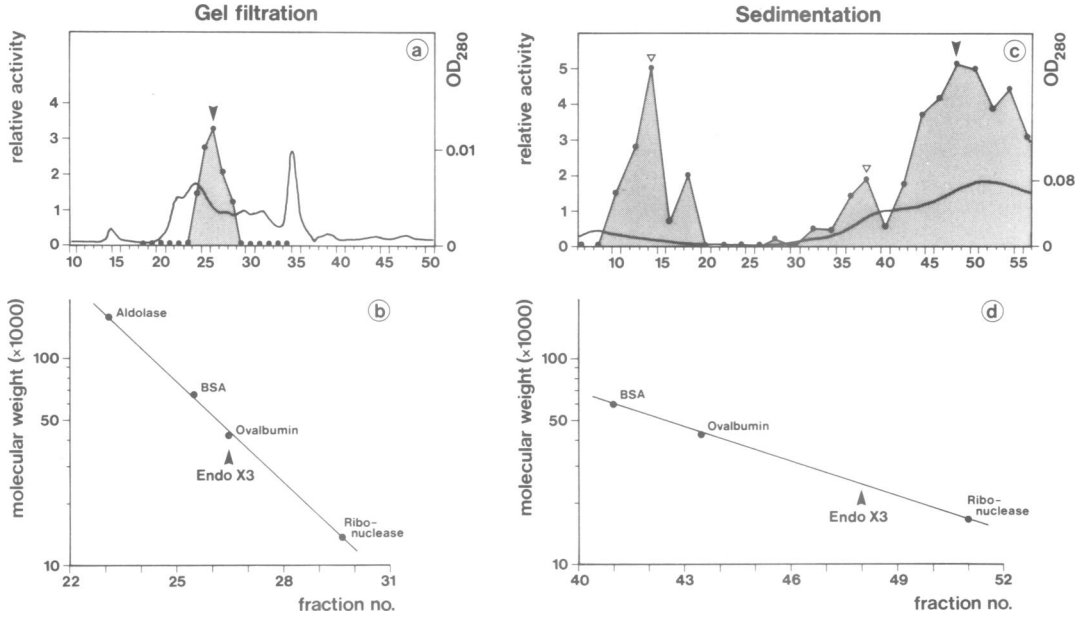

Fig. 3. Determination of the native mol. wt of Endo X3 by gel filtration and sedimentation. (**a**) Endo X3 from fraction V was applied to a FPLC superose 12 filtration column in buffer F. Samples of 1 ml were collected and numbered after the void volume (V_0). Endo X3 activity was determined under standard assay conditions (·−·−·). The protein elution profile was recorded during the run by measuring OD_{280} (——). (**b**) The elution volumes of marker proteins with known mol. wts were determined under identical conditions in separate runs. Endo X3 elutes in the same fraction as ovalbumin (arrow). (**c**) A sample of Endo X3 from fraction V was loaded onto a linear preformed gradient of 15.5–33% sucrose in buffer F and centrifuged for 21 h at 240 000 *g*. The gradient was fractionated from the bottom of the tube. Endo X3 activity was determined as indicated above (·−·−·). (**d**) The mol. wt of native Endo X3 was estimated by relating its migration distance to the migration distances of marker proteins determined in parallel tubes. Closed arrows indicate the main peak of Endo X3, open arrows point to side fractions.

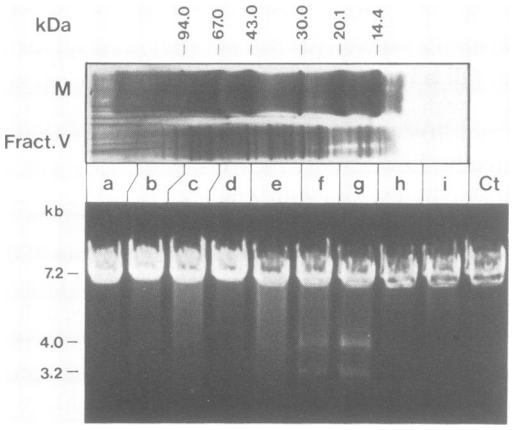

Fig. 4. Determination of mol. wt of Endo X3 by SDS−PAGE. 1 μl (1.5 U Endo X3) of fraction V was placed into SDS sample buffer without heat treatment and separated on an 8−25% polyacrylamide−SDS gel. Proteins were visualized by silver staining. Slices, indicated (a−j), were cut from unstained portion of the gel loaded with an excess of fraction V (250 U Endo X3). Each slice was put into 50 μl of buffer B and exhaustively dialyzed against the same buffer (20 h) at 4°C. The dialyzed supernatant was vacuum concentrated and then assayed for X-solvase activity under standard conditions with a prolonged incubation time of 20 h. M = marker proteins; ct = control containing cruciform DNA without enzyme; arrows point to positions of linearized cruciform DNA (7.2 kb) and the fragments indicative for X-solvase activity (4.0 and 3.2 kb).

protein subunits. This was supported by the observation that Endo X3 activity could be recovered from a denaturing SDS−polyacrylamide gel overloaded with fraction V, eluting from the adjoined slices containing polypeptides in the range of 14−21 kd and 21−31 kd respectively (Figure 4). Most of the recovered activity was obtained in the slice with polypeptides <21 kd. No activity was found in the slices with polypeptides ranging from 31 to 45 kd and higher. Furthermore, in Western blot analyses of SDS−polyacrylamide gels using anti-Endo VII antiserum, Endo X3 (fraction VI) shows one single band with a mol. wt of ~18 000 daltons.

It is worthwhile to note that Endo VII, the authentic X-solvase from phage T4, when analyzed in parallel under the same conditions, shows nearly the same mol. wt of 43 000 on Superose 12 filtration column and 36 000 in sedimentation analysis on sucrose gradients (results not shown). This is despite the fact that it does indeed consist of subunits with a mol. wt of 18 000, as deduced from the size of major open reading frame of the isolated gene (Barth *et al.*, 1988; Tomaschewski, 1988) and from SDS−PAGE analyses of the purified protein (H.Kosak, to be published).

Endo X3 is not induced by UV

Attempts to stimulate X-solvase activity in mitotic yeast cells by UV irradiation were not successful. In a typical experiment, UV light from a germicidal lamp was applied until ~75% of the cells were inactivated. The cells were harvested either immediately after irradiation or after an additional growth period ranging from 1 to 5 h. Changes of the specific activity measured in crude extracts were within the limits of experimental error. Under the same conditions mitotic recombination determined between the two trp5 markers in strain D7 was 80-fold stimulated.

Endo X3 is not induced during meiosis

Cells of the diploid strain RS/RS were induced by starvation in acetate medium to undergo meiosis. About 50% of the cells had developed asci when measured 48 h after the induction by microscopic inspection. Crude extracts prepared from samples collected from 2 to 8 h after the induction, however, did not show a measurable change in X-solvase activity.

Cleavage of natural cruciforms

As shown above, Endo X3 linearizes supercoiled plasmid DNA at a cruciform structure. In the described combination assay with *Bam*HI the cleavage site(s) were mapped to the cruciform structure with an accuracy of about ±50 bp.

A precise mapping of Endo X3 induced cleavage sites was achieved by 3′ end labeling the reaction products at two *Bst*NI restriction sites, 200 bp apart flanking the cruciform structure (positions 6138 and 6337 in Figure 1). This allows simultaneous detection of all fragments derived from the top and bottom strands of this piece of DNA. Endo X3 induced fragments therefore appear in the same lane of the gel (Figure 5a). The allocation of fragments to either strand was determined by digesting aliquots with either *Hpa*II or *Hae*III. The *Hae*III site is located 10 bp to the right of the base of the cruciform structure and the *Hpa*II site 72 bp to its left (Figure 5b). Fragments disappearing after *Hae*II digestion therefore span the distance between the right *Bst*NI site and the cruciform structure, while fragments from the left side will disappear after *Hpa*II digestion. As shown in Figure 5a and b, this procedure allowed the assignment of five fragments to the right side of the cruciform structure and four fragments to its left side.

When we compared the Endo X3 induced cleavage pattern with an Endo VII induced cleavage pattern, a surprisingly high degree of similarity was observed. As shown in Figure 5a, not only size and origin of the fragments were the same but also the usage of individual cleavage sites as deduced from the amount of radioactivity in each band.

The exact size of each of the X-solvase induced fragments was determined at the nucleotide level by running them side by side with sequence ladders of chemically degraded DNA from either side of the cruciform structure, i.e. the top strand sequence for fragments spanning the right side of the junction and the bottom strand sequence for fragments spanning the left side of the junction (not shown). Assuming that the X-solvase induced fragments were exclusively labeled at their *Bst*NI ends, we deduced the cleavage pattern for both enzymes at the cruciform structure. They are summarized in Figure 5c. The major cuts are symmetrically arranged and appear in identical locations if seen in directions from top left to bottom right or top right to bottom left across the junction.

Cleavage of synthetic cruciform DNA J9

Synthetic cruciform DNA with arms of 9 bp and an immobile junction was assembled from synthetic oligonucleotides (Figure 6b). For the experiments reported here, each of the four strands was 5′ end labeled and incorporated into four separate structures. Each structure was treated in parallel with Endo X3 and Endo VII, and the reaction products were analyzed by denaturing PAGE. As shown in Figure 6a, Endo X3 introduces defined fragment patterns by cleaving the four strands 3′ of the junction within four

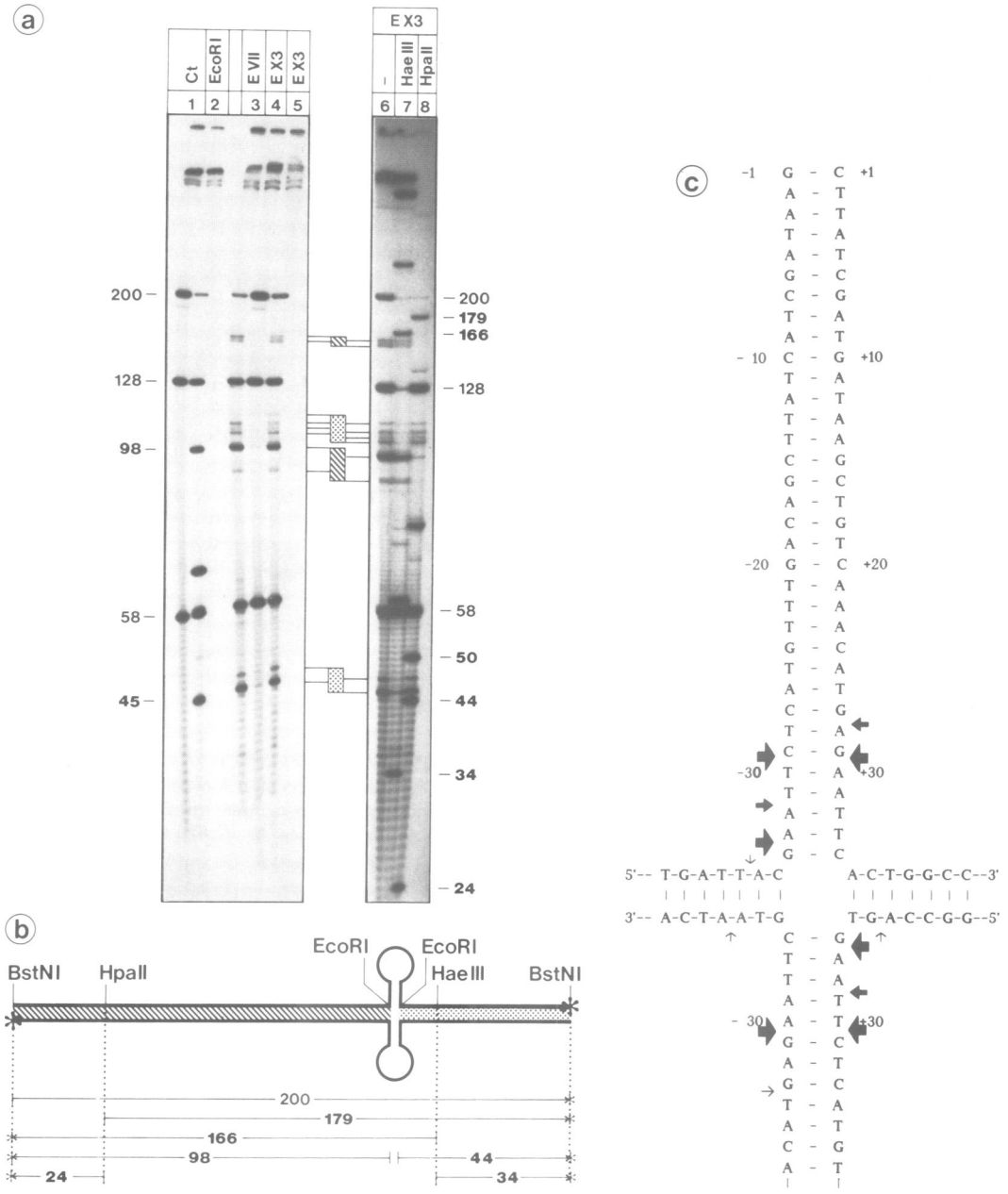

Fig. 5. Mapping of cleavage sites in natural cruciform DNA. Supercoiled DNA of M13mp2IR62E was treated with 0.6 U (**lane 4**) or 4 U (**lane 5**) Endo X3 from fraction VI (X3), Endo VII (EVII) or *Eco*RI under standard assay conditions. The DNA was then digested with *Bst*NI and the generated 3′ ends were labeled by fill-in reactions with [α-^{32}P]thymidine. The fragments were then separated by denaturing PAGE (8%). Numbers in medium type indicate the *Bst*NI fragments which are used here as markers. Numbers in bold type indicate fragments obtained after further digestions with restriction enzymes *Eco*RI, *Hpa*II or *Hae*III (see **b**). X-solvase induced fragments are marked with lines connected to stippled or cross hatched boxes symbolizing DNA from the right and left side of the cruciform structure. (**b**) Sketch of the 200 bp *Bst*NI fragment covering the cruciform structure. Fragment sizes relevant to this experiment are indicated. The labeled ends are marked with *. (**c**) Summary of Endo X3 and Endo VII induced cleavages. The cruciform is shown in an idealized form. Parts of the lower arm and the circular molecule are cut off. Arrows point to the cleavage sites and their difference in size reflects relative usage of sites as seen in the gel under (**a**).

nucleotides from the branchpoint. Endo VII uses the same sites for cleavage with the same relative efficiency. Some additional cleavage sites, away from the junction on its 5′ side, are recognized by Endo VII, but not or only faintly by Endo X3.

The exact location of cleavages was determined by comparing the enzyme induced fragments with a sequence ladder obtained from each strand by chemical degradation reactions (not shown). The results are summarized in Figure 6b. It should be noted that the cleavage patterns in each strand of J9 are slightly different. This reflects sequence specificity of the X-solvases, which was studied in greater detail using a set of substrates with systematically varied sequence (Pottmeyer and B.Kemper, unpublished observations). If the sequence is the same in two of the arms, as we found it in natural cruciforms like M13mp2IR62E, the patterns are the same showing 2-fold symmetry at the junction.

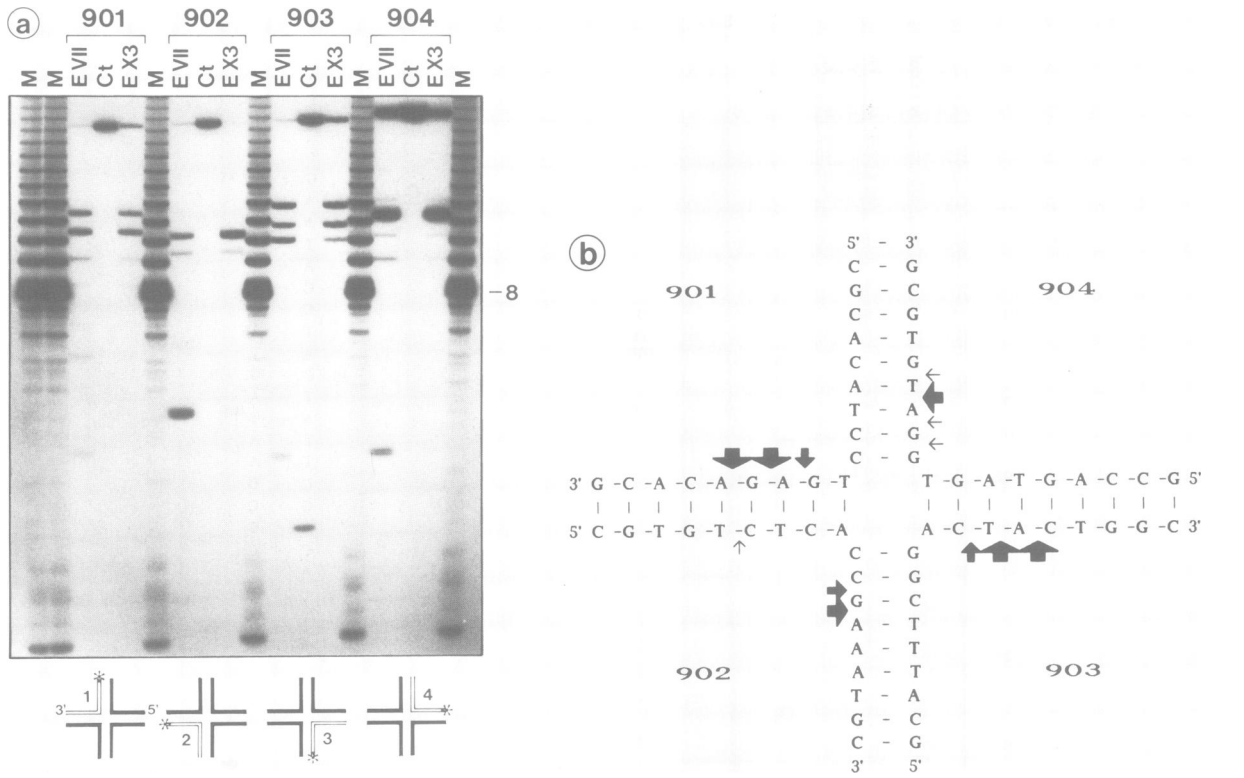

Fig. 6. Mapping of cleavage sites in synthetic cruciform DNA J9. (**a**) 40 ng of DNA J9 were treated with 4 U of Endo X3 or with 1000 U Endo VII at 18°C for 120 min. The reaction was terminated by drying in the vacuum and resuspension in 5 μl of formamide stop mix and samples were analyzed by denaturing PAGE (20%). The sketches below the figure show the labeled strand in each substrate. (**b**) Complete sequence of cruciform J9. The arrows indicate Endo X3 induced cleavages. Their size reflects differences in the usage of sites as deduced from the appearance of bands in the autoradiograph shown in (**a**). M = marker.

Cleavage of Y junctions

Y-branched DNA with three 10 bp long arms formed by a single 70 nt oligonucleotide and containing an internal ^{32}P label was constructed. A series of fragments corresponding to cleavage sites at or very close to the junction was found after digestion of the DNA with Endo X3 or Endo VII (data not shown). Fragments were indistinguishable in size and similar in relative amount, indicating that both enzymes cleave Y-branched DNA in a very similar way.

Cleavage of heteroduplex-loop DNA

Synthetic heteroduplex-loop DNA was made *in vitro* by hybridizing circular plus strands from phage M13mp19IN19 with linear minus strands from phage M13mp19 as described before (Kleff and Kemper, 1988). The 19 bp insert formed the single stranded loop in the plus strand of the heteroduplex. The sequence of the loop region between a *Hin*dIII and an *Eco*RI restriction site in the polylinker of M13mp19 is shown in Figure 7.

In separate experiments, each of the strands was 3′ end labeled at the *Hin*dIII or the *Eco*RI restriction site and then digested with Endo X3 or Endo VII. As shown in Figure 7, the structure is recognized by both enzymes and cleaved at the same positions with similar efficiencies. There is one major site 3′ of the loop in the looping strand and one 3′ of the base of the loop in the non-looping strand. Both are flanked by two or three minor sites.

Cleavage of VFS-DNA

The similarity between the reactions of Endo X3 and Endo VII with the described constructs prompted us to also test

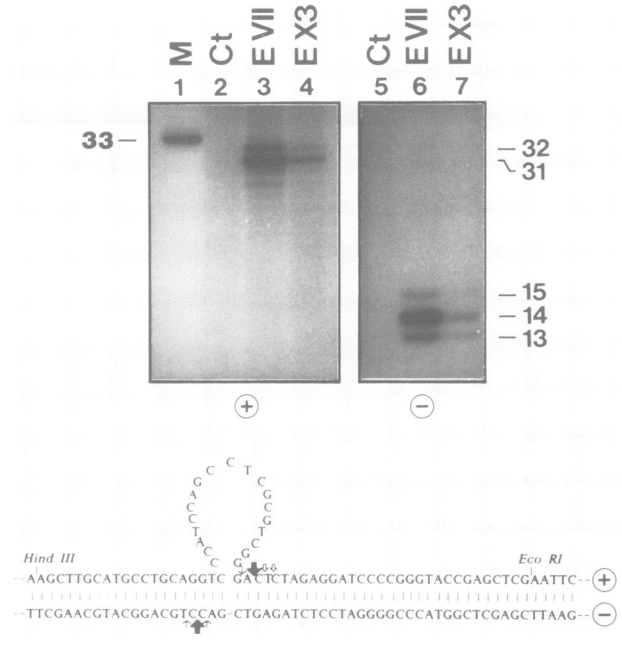

Fig. 7. Mapping of cleavage sites in heteroduplex-loop DNA. 50 ng of heteroduplex-loop DNA was linearized by *Eco*RI (**lanes 2–4**) or *Hin*dIII (**lanes 5–7**), 3′ end labeled by Klenow polymerase, digested with 4 U Endo X3 or 1000 U Endo VII under standard assay conditions and then analyzed by denaturing 8% PAGE. The sketch shows the sequence of the heteroduplex-loop region between the *Hin*dIII and the *Eco*RI restriction site of the polylinker of phage M13mp19. The positions of X-solvase cleavage sites are marked by arrows.

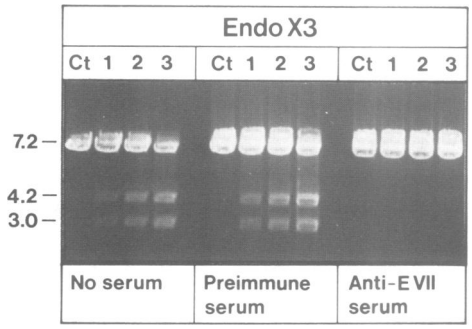

Fig. 8. Reaction of Endo X3 with anti-Endo VII antiserum. Purified Endo X3 (10 U from fraction V) were incubated overnight at 4°C with undiluted anti-Endo VII antiserum, preimmune serum or without serum additions in 10 μl Endo X3 dilution buffer. After that, the mixture was diluted into reaction buffer with supercoiled cruciform DNA of plasmid M13mp2IR62E as substrate. X-solvase activity was determined as described in Materials and methods and in the legend to Figure 2.

natural VFS-DNA, which is a highly branched and unpackageable replicative DNA intermediate obtained from cells after infections with T4 gene 49$^-$ mutants (Kemper and Brown, 1976). It is the presumed natural substrate for Endo VII *in vivo* because it can be converted *in vitro* by purified Endo VII into smaller units of one-third to one-quarter the length of unit T4 DNA. Endo X3 degrades VFS-DNA into products of exactly the same size. Hydroxymethyl cytosine (HMC) replacing the regular cytosine in T4 DNA did not affect the reactions with either enzyme and cytosine-containing VFS-DNA was degraded with the same kinetics giving the same reaction products (results not shown).

Anti-Endo VII antiserum inhibits Endo X3

The similarities between Endo VII and Endo X3 reactions with branched DNA substrates prompted us to investigate whether antiserum raised against Endo VII would inhibit Endo X3 *in vitro*. Endo X3 was incubated overnight at 4°C in enzyme dilution mixture with crude rabbit antiserum or purified IgG. After dilution into reaction mixture containing supercoiled DNA from M13mp2IR62E as substrate, the remaining activity was determined. As shown in Figure 8, the activity was completely inhibited in the presence of anti-Endo VII antiserum, while there was no response with the preimmune serum.

Discussion

We have reported here the purification of Endo X3 from crude extracts of yeast *S.cerevisiae*. The activity was detected by an assay diagnostic for cuts at a cruciform structure in supercoiled DNA. Since formation and maintenance of cruciform structures in circular DNA depends largely on superhelical density (Lilley, 1980; Gellert *et al.*, 1983; Panayotatos and Wells, 1988) the assay is sensitive to loss of supercoil by topoisomerases and endonucleases. We therefore worked with a topoisomerase I$^-$ mutant and made the assay conditions unfavorable for an unspecific endonuclease which was present in crude extracts and found enriched in the pellet of the high speed spin (fraction I in Table I).

Purified Endo X3 from fraction VI (Superose 12 pool in Table I) was used in studies with a series of DNA substrates with secondary structures. These included four-way junctions, three-way junctions and single stranded loops. Any of these DNA substrates was efficiently cut by Endo X3 and staggered nicks were placed 3′ from the junction in synthetic branched DNAs or 3′ of the loop in heteroduplex-loop DNA. In contrast to synthetic cruciform DNA, the cleavage patterns in natural cruciform DNA looked different and major cuts were found opposite each other on the same side of the junction as depicted in Figure 5c. The junction, however, can theoretically be located at any position within the homologous sequence of the short arms, and cleavage by Endo X3 may have taken place in these molecules only, where the sites for cleavage were located 3′ of the junction. Why then do we not find cleavages 3′ of the junction and outside of the palindrome when this is completely extruded as it is drawn in Figure 5c? One plausible explanation would be that the nucleotides in this region are not a substrate for the enzyme. It was recently shown that Endo VII specifically cleaves cross-over strands in Holliday junction analogs and the nucleotides next to the junction determine which of the strands cross over (Duckett *et al.*, 1988; Mueller *et al.*, 1988). According to this, it is possible that the supercoiling distorts the 2 domain motif of relaxed junctions (Churchill *et al.*, 1988; Chen *et al.*, 1988), so that the crossover strands do not have the proper structure for cleavage.

Endo VII, the X-solvase from bacteriophage T4 which had earlier been shown to react with the same set of substrates (Jensch and Kemper, 1986; Duckett *et al.*, 1988; Kleff and Kemper, 1988; Mueller *et al.*, 1988) was used here in a comparative study and found to induce very similar cleavage patterns in each of the substrates. Location, number and usage of the cleavage sites were almost identical. In addition, we found that Endo X3 is also able to resolve the highly branched replicative DNA from phage T4 which accumulates in cells infected with gene 49$^-$ mutants (Endo VII$^-$). These results comprise an interesting example of functional analogy between two specialized enzymes from a prokaryote and a eukaryote. The similarity is further stressed by the findings that (i) both proteins exhibit the same mol. wt of 43 000 daltons when determined by gel filtration through a molecular sieve (Superose 12 chromatography) and (ii) an antiserum raised against highly purified Endo VII inhibits Endo X3 in reactions with cruciform DNA or VFS-DNA (results for VFS-DNA not shown). In Western blot analyses, both enzymes show single protein bands of ~18 000 daltons on SDS−PAGE. Since the isolated gene 49 is flanked by direct and inverted repeats, suggesting transpositional origin (Barth *et al.*, 1988), it is tempting to speculate that the function of the protein was conserved after genetic exchange between phage T4 and yeast had occurred. Evidence for the occurrence of such exchange was recently discussed for intron coded proteins of phage T4 and filamentous fungi (Michel and Dujon, 1986).

How different are the X-solvases from yeast? Although a final answer requires isolation of the the respective genes, several observations suggest that the three enzymes are indeed different. (i) The mol. wt of >200 000 for Endo X1 (West and Körner, 1985), and 43 000 for Endo X3 (this communication) differ greatly from one another. (ii) Endo X1 does not react with anti-Endo VII antiserum in Western blot experiments and shows full activity *in vitro* in its presence (S.West, personal communication). (iii) Endo X3 reacts with cruciform DNA in a way different from the other

two enzymes. Endo X3 cleaves cruciform structures at exactly the same positions as Endo VII from phage T4, while Endo X1 cleaves cruciform DNA in positions different from Endo VII (cf. Figure 5 in Parsons and West, 1988). Furthermore Endo X3 can efficiently cleave cruciform DNA with short arms of 9 bp (this communication) while Endo X2 was reported not to cleave a cruciform with armlengths of 9, 11, 15 and 17 bp in the same structure (Evans and Kolodner, 1987). Endo X2 did not cleave three-armed junctions made from cleavable four-armed structures (Evans and Kolodner, 1987) and single stranded DNA from several phage M13 derivatives including M13mp2IR62E, which due to their secondary structures are good substrates for Endo X3. Furthermore Endo X2 is not active with Ca^{2+}, replacing Mg^{2+} in the reaction (R.Kolodner and R.Reenan, personal communication; results with single stranded DNA will be published elsewhere).

Another clue suggesting the existence of more than one X-solvase in yeast was found when highly mutagenized stocks were screened for X-solvase defective mutants and one mutant with a reduced level of total X-solvase activity was found exhibiting full Endo X3 activity as determined by our assay and purification procedure (S.Kleff and R. Sternglanz, personal communication).

What is the function of Endo X3 *in vivo*? Since the enzyme activity was not inducible by UV irradiation or induction of meiosis we believe that Endo X3 belongs to housekeeping functions and is expressed at all times during cell growth. If we assume that the similarity between Endo X3 and Endo VII in their reactions with branched DNAs *in vitro* also reflects similarity of their functions *in vivo*, Endo X3 can be seen as a specialized factor related to the processes organizing the mass of intracellular DNA. This is in analogy to the function of Endo VII which is responsible for the maintenance of the newly replicated DNA in a packageable conformation late during infection. In the absence of Endo VII (gene 49^- mutants), the DNA cannot be packaged to completeness, and X- and Y-branched DNA molecules accumulate in the cell (Frankel, 1968; Kemper and Brown, 1976; Kemper and Janz, 1976). An estimated number of three to four branches on average per unit length of T4 DNA at the time when packaging begins is in agreement with the view that these structures directly block packaging by steric hindrance (Kemper and Brown, 1976). In analogy, one can envisage how a single Holliday structure blocks cell division by linking homologous chromosomes in a eukaryotic cell and that a constant level of an X-solvase like Endo X3 is required for guarding the DNA throughout the cell cycle, even when the frequency of recombination is very low.

An alternative explanation for the lack of inducibility of Endo X3 is that the enzyme is already induced due to the *top1* mutation which has been shown to increase the superhelical density of plasmids with transcriptionally active genes (Brill and Sternglanz, 1988). Changes in superhelical density have frequently been observed to influence gene expression in prokaryotic and eukaryotic systems (Weintraub, 1985).

Materials and methods

Materials, buffers and media

Agarose was from BRL (Eggenstein, FRG). Heparin–agarose was from Sigma GmbH (Deisenhofen, FRG). Prefilled columns Mono Q (HR5/5), Mono S (HR 5/5) and Superose 12 (HR 10/30) were from Pharmacia (Freiburg, FRG) and operated by automated programs with equipment for fast protein liquid chromatography (FPLC) from the same company.

Restriction endonucleases, polynucleotide kinase, DNA polymerase I 'Klenow fragment', DNA-ligase and proteinase K were from New England Biolabs (Schwalbach, FRG) or Boehringer (Mannheim, FRG); Endo VII was purified in our laboratory from phage T4 infected cells following a published procedure (Kemper and Garabett, 1981) or from *Escherichia coli* harboring a plasmid system overexpressing Endo VII (Barth *et al.*, 1988; purification to be published). [α -^{32}P]dNTP and [γ-^{32}P]ATP were from Amersham (Braunschweig, FRG).

Buffer A contained 50 mM Tris–HCl, pH 8.0, 10 mM β-mercaptoethanol (ME), 5 mM EDTA, 10% (v/v) glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF). Buffer B contained 10 mM Tris–HCl, pH 8.0, 10 mM ME, 1 mM EDTA and 10% (v/v) glycerol. Buffer C contained 10 mM K-phosphate, pH 6.0, 10 mM ME, 1 mM EDTA and 10% (v/v) glycerol. Buffer D contained 10 mM Tris–HCl, pH 7.6, and 600 mM NaCl. Buffer E contained 10 mM Tris–HCl, pH 8.0, 0.1 mM glutathione and 50% (v/v) glycerol. Buffer F contained 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 2 mM dithiotreitol (DTT) and 1 M KCl. TAE buffer contained 40 mM Tris–acetate, pH 8.0, and 2 mM EDTA. Stop mix contained 30 mM EDTA, 1.6 mM Tris–HCl, pH 8.0, 48% (v/v) glycerol, 0.1% bromophenol blue, 0.01% xylene cyanol and 7.5 mg/ml proteinase K. Formamide stop mix contained 90% formamide in TAE buffer. Growth media were prepared as described (Sherman *et al.*, 1986).

Strains and DNA

Phage M13mp2IR62E, a derivative of phase M13mp2 (Gronenborn and Messing, 1978), and M13mp19IN19 were constructed in our laboratory as described before (Jensch and Kemper, 1986; Kleff and Kemper, 1988). Phage M13mp19 was purchased from Boehringer (Mannheim, FRG), yeast strain D7 (a/α, *ade2-40/ade2-119, trp5-12/trp5-27, ilv1-92/ilv1-82*) was kindly provided by F.K.Zimmermann (University of Darmstadt, FRG) (Zimmermann *et al.*, 1975). Yeast strains RS190 (*a,top1-8, ade2, ura 3, his3, trp1, can*^{r}) and RS401 (α, *top1-1, ade2, ura3, his3, leu2*) were kindly provided by R.Sternglanz (State University of New York at Stony Brook, USA). A diploid *top1*$^-$ strain, termed RS/RS, was selected as *trp*$^+$ *leu*$^+$ colonies after mating strains RS190 and RS401.

Cruciform DNA J9 and Y-DNA were made *in vitro* from synthetic oligonucleotides. The individual strands of J9 were designed to minimize freedom of branch migration at the junction. The protocol of hybridization and purification of the structure followed published procedures (Seeman, 1982; Kallenbach *et al.*, 1983). Heteroduplex-loop DNA was made as described earlier (Kleff and Kemper, 1988). Tritium labeled very fast sedimenting DNA (VFS-DNA) was isolated from *E.coli* cells infected with mutants in gene 49 (am E727) of phage T4, following a published procedure (Kemper and Janz, 1976).

Methods

Yeast cells were grown in YPD medium at 30°C under steady state growth conditions in a fermenter. Cells were harvested at a density of $5-8 \times 10^7$ cells/ml, pelleted in a refrigerated centrifuge, washed with TE buffer, recentrifuged and frozen at −20°C. Cells were stored for >6 months without detectable loss of activity.

To induce sporulation, cells were grown in presporulation medium to 4×10^7 cells/ml, centrifuged, washed and resuspended in sporulation medium.

Agarose gels for DNA separation were run in TAE buffer and stained with ethidium bromide (1 μg/ml). Ready-to-use SDS–polyacrylamide gels (8–25%) for protein separation were purchased from Pharmacia (Freiburg, FRG) and run in automated equipment (Phast system) from the same company. Silver staining was carried out as recommended by the manufacturer. Protein was determined with a kit from Biorad (München, FRG), using bovine serum albumin (BSA) as standard).

Purification of Endo X3

All procedures were performed at 4°C unless otherwise stated. A summary of the purification is given in Table I.

Crude extracts. Frozen cells (50 g) were covered with 125 ml of buffer A in the plastic beaker of a household mixer (Braun Multipractic Plus Electronic) and gently thawed on ice. After the addition of 400 g of acid washed cold glass beads (Braun Melsungen, 0.5 mm) the slurry was agitated at a speed setting of 1.5 using metal kneading hooks protected by silicone tubing. Temperature was not allowed to exceed 8°C. Clearing of the solution was followed by measuring the OD_{600} and agitation was stopped when the density had dropped by a factor of five. At this stage, >99% of the cells

were broken as determined by light microscopy. Glass beads were separated from the crude extract by centrifugation. During the beginning of the search for X-solvases, crude lysates were additionally sonicated but, since this step did not improve the yield of Endo X3, it was omitted from later purifications.

After centrifugation for 45 min at 30 000 *g*, 130 ml of a cleared extract (S30) were obtained. This is fraction I. When the pellet was extracted with 0.3% deoxycholate in the presence of 1 M KCl, about the same amount of cruciform resolving activity could be eluted. This activity was highly contaminated with unspecific endonuclease activity and has not been characterized yet.

Ammonium sulfate precipitation. Ground solid ammonium sulfate was added slowly to 55% saturation (351 g/l) and the precipitate was collected by centrifugation. The pellet was resuspended in 50 ml of buffer B and dialyzed against the same buffer until the solution reached the conductivity corresponding to 200 mM NaCl in buffer B. Precipitated protein was removed from the extract by centrifugation for 10 min at 10 000 *g*. The opalesque supernatant is fraction II.

Heparin–agarose chromatography. Fraction II (90 ml) was loaded on a heparin–agarose column (diameter 2.5 cm, height 11 cm) equilibrated with buffer B + 200 mM NaCl. A constant flow rate of 2 ml/min was maintained and fractions of 10 ml were collected. The loaded column was washed with equilibration buffer until the base line of the recorder was reached. An elution step of 240 ml of buffer B + 350 mM NaCl was applied, followed by a linear gradient of 50 ml from 350 to 700 mM NaCl in buffer B. Elution of the activity occurred at ~700 mM NaCl, slightly delayed from the bulk of protein. Peak fractions were pooled (~200 ml) and dialyzed against buffer B + 200 mM NaCl. During this step a substantial amount of protein precipitates without affecting X-solvase activity. After removal of the precipitate, the protein concentration of the eluate was reduced by a factor of 5–10 which contributes to the overall purification of the enzyme.

Concentration of the enzyme from the cleared eluate was achieved by another run over a small heparin–agarose column (diameter 1.5 cm, height 5.5 cm) equilibrated with 200 mM NaCl in buffer B. Full activity was recovered in one step by elution with 1 M NaCl in buffer B. Active fractions (~20 ml) were pooled and dialyzed against buffer B until the conductivity reached a value of 150 mM NaCl in buffer B. This is fraction III.

Mono Q FPLC chromatography. A column prepacked with 1 ml of Mono Q was equilibrated with 150 mM NaCl in buffer B. The FPLC system was set at a flow rate of 1 ml/min and programmed to load 20 ml of fraction III, wash with 4 ml of equilibration buffer, apply the first gradient of 3 ml until 200 mM NaCl, wash with 4 ml of the same buffer, apply a second gradient of 10 ml until 400 mM NaCl, wash with 4 ml of the same buffer, and finally apply a third gradient of 1 ml until 1 M NaCl in buffer B. Fractions of 1 ml were collected. The activity eluted during the second gradient with ~300 mM NaCl.

After the main peak of activity, a protein inhibitory for restriction enzymes eluted from the column which interfered with the assay for X-solvase. Active fractions were pooled as fraction IV (5–10 ml).

Mono S FPLC chromatography. Fraction IV was dialyzed against buffer C until the salt concentration was <100 mM KCl as determined by conductivity measurement. The whole sample was loaded on a FPLC Mono S column (1 ml) equilibrated with 100 mM KCl in buffer C. After washing the column with 4 ml of the equilibration buffer, a step of 4 ml of 300 mM KCl in buffer C and a linear gradient of 5 ml from 300 mM to1 M KCl in the same buffer was applied. The gradient was followed by two additional washing steps of 4 ml, each containing 1 and 1.5 M KCl in buffer C. The activity eluted midway through the gradient at ~650 mM KCl. Active fractions were pooled and BSA (0.5 mg/ml) was added to stabilize the activity. This is fraction V.

Superose 12 chromatography. A sample of fraction V was dialyzed against buffer D and loaded onto a prepacked FPLC column (type HR 10/30) equilibrated with buffer D. The column was developed with the same buffer at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected in tubes with 0.5 mg/ml BSA. This is fraction VI.

Endo X3 assay and definition of units. A mixture of 10 μl total volume contained 200 ng of supercoiled RFI-DNA from phage M13mp2IR62E, 10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM ME and 100 μg/ml of BSA. After the addition of enzyme and following an incubation period of 90 min at 29°C, 2 units of *Bam*HI were added and the incubation continued for another 45 min at 37°C. 5 μl of stop mixture with proteinase K was added, and after 15 min at 37°C the sample was separated on a 1% agarose gel. The relative amount of DNA in each band was determined by scanning the photographic negative.

One unit of Endo X3 is defined as that amount of enzyme which linearizes 50 ng of cruciform DNA in a standard assay with 200 ng of total supercoiled substrate DNA.

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