A Novel Structure-Specific Endonuclease Activity Associated with Polypyrimidine-Tract Binding (PTB) Related Protein from Rat Testis

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ABSTRACT: Nucleases are involved in the processing of various intermediates generated during crucial DNA metabolic processes such as replication, repair, and recombination and also during maturation of RNA precursors. An endonuclease, degrading specifically single-stranded circular DNA, was identified earlier in rat testis nuclear extract while purifying a strand-transfer activity. We are now reporting the purification of this endonuclease, which is a monomeric 42 kDa protein, from rat testis to near-homogeneity. In addition to degrading single-stranded circular DNA, it nicks supercoiled plasmid DNA to generate relaxed DNA and does not act on linear single-stranded or double-stranded DNA. It also makes specific incisions at the single-strand/duplex junction of pseudo-Y, 3′- and 5′-overhangs and 3′- and 5′-flap structures. Other structures such as mismatch, insertion loop, and Holliday junction are not substrates for the testis endonuclease. In contrast to FEN1, the testis endonuclease makes asymmetric incisions on both strands of the branched structures, and free single-stranded ends are not necessary for the structure-specific incisions. Neither 5′−3′ nor 3′−5′ exonuclease activity is associated with the testis endonuclease. The amino acid sequences of tryptic peptides of the 42 kDa endonuclease show near-identity to polypyrimidine-tract binding protein (PTB) that is involved in the regulation of splicing of eukaryotic mRNA. The significance of the results on the association of structure-specific endonucleae activities with PTB-related protein is discussed.

Nucleases play an important role in many of the DNA metabolic processes such as replication, repair, and recombination as well as RNA processing and turnover (*1*, *2*). During replication, the 3′−5′ exonuclease activity of DNA polymerase or polymerase-associated exonucleases is essential for proofreading any misincorporated nucleotide (*3*). Second, the processing of Okazaki fragments in the lagging strand involves participation of either Rnase H (*4*, *5*) or Dna 2 nuclease (*6*). Nucleases also form an important component of most of the DNA repair machinery, acting in a variety of structural frameworks ranging from site-specific (e.g., abasic endonuclease) to structure-specific (e.g., FEN1). In the base excision repair (BER) pathway, an altered base in DNA is excised by a DNA glycosylase, and the resulting abasic site is corrected by the concerted action of an AP endonuclease, DNA polymerase, and ligase (*7*). In mammalian cells, completion of BER, following DNA backbone cleavage at the AP site, can occur by either short patch repair in which 1 nucleotide is repaired, or long patch repair in which 2−13 nucleotides are replaced. A flap structure is generated in long patch repair which is processed by FEN1 (*8*). On the other hand, nucleotide excision repair (NER), which removes a variety of bulky and helix-distorting lesions from DNA, is a multistep process involving damage recognition, DNA unwinding, and dual incision to remove damage by nucleases such as Uvr B and Uvr C in *E. coli* (*9*), Rad 1/Rad 10 in yeast (*10*), and XPG and ERCC1/XPF in higher eukaryotes (*11*). The removal of mismatches from DNA in the mismatch repair pathway involves nucleases such as Mut H (*12*), following which the incised strand is processed by exonucleases (*13*, *14*). During recombination, nucleases such as *E. coli* exonuclease VIII act on the ends of double-strand breaks to generate 3′ overhangs (*15*). The branched intermediates generated at later stages of recombination are processed by nucleases such as Ruv C resolvases (*16*). In eukaryotic cells, double-strand break (DSB) repair pathways require the nuclease activities of Rad 50, MRE 11, and XRS2/NBS complex for DSB end processing (*17*−*20*).

Branched intermediates formed during most of the DNA metabolic processes, having unusual structures, act as substrates for various nucleases (*21*). Among the structure-specific nucleases reported so far, the RAD 2 family of nucleases includes a large number of nucleases possessing a range of nuclease activities that contribute to repair, replication, and recombination (*22*). The RAD 2 class I family consists of XPG-like proteins that function in NER to make incision on the target strand to the 3′ side of the bubble-like damage-containing structure (*23*). The RAD 2 class II family is comprised of the FEN1-like proteins. These enzymes exhibit a flap-specific endonuclease activity for bifurcated DNA structures produced during replication and recombination (*24*). FEN1 also appears to be involved in preventing the expansion/contraction of DNA repeat elements, a phenomenon associated with neurodegenerative disorders and cancer (*25*−*28*). The RAD 2 class III family

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members consist of the EXO I-like enzymes found in yeast, fly, and mammals. EXO I interacts with MSH2 and hence is implicated to have an important role in mismatch repair and recombination (*29*). Mre 11, in addition to its 3′−5′ exonuclease activity, also exhibits endonuclease activity on DNA-containing hairpin loops, making an asymmetric cleavage (*30*).

Ribonucleases (endo and exo) are essential both for nonspecific RNA degradation (turnover) and for RNA maturation in various cellular compartments (reviewed in *1* and *31*). Most of the RNAs are synthesized as precursor molecules which must be processed to generate the functional form of RNA. Such processing steps are obligatory steps in the processing of ribosomal precursor RNA, multimeric tRNAs, and messenger RNAs. The precise 5′ and 3′ termini are generated by endonuclease cleavage or trimming by exonuclease reactions. Several specific endoribonucleases have been described that take part in several of these RNA processing steps both in prokaryotic and in eukaryotic systems (*32*). Removal of intronic sequences from both tRNA precursors and hnRNA in eukaryotic cells is an important posttranscriptional event wherein there is an active participation of endoribonucleases. In the case of tRNA precursors, a specific splicing endonuclease cleaves pre-tRNA at both ends of the intron. Rnase P plays an important role in the processing of tRNA precursors (*33*, *34*). Rnase MRP, which is structurally related to Rnase P, is involved in the processing of precursor ribsosomal RNA in eukaryotes (*35*). In bacteria, several examples are known wherein polycistronic messenger RNAs are cleaved in intercistronic regions involving both Rnase III and Rnase E (*36*). Maturation of messenger RNA in eukaryotes involves two important steps, namely, splicing out of introns and poly(A) addition. Polyadenylation requires the action of cleavage factors and a host of other accessory factors (*37*, *38*). More recently, DNA topoisomerase I has been shown to possess endoribonuclease activity, suggesting a potential role in RNA processing (*39*). The splicing of intronic sequences is a complex process involving two transesterification reactions during which the two exons are brought together and the intron is released in a lariat configuration. Pre-mRNA splicing takes place in macromolecular complexes, spliceosomes, composed of five smaller ribonucleoprotein complexes (snRNPs) and a large number of polypeptides that are tightly associated with snRNPs (*40*). The development of an in vitro splicing system has facilitated identification of several cis- and trans-acting components necessary for pre-mRNA splicing. Most important among the required cis-acting elements are a consensus sequence at the 5′ splice site, a branch point sequence and adjacent polypyrimdine tract, and the 3′ splice site AG dinucleotide (*41*). A number of RNA binding proteins have been identified, some of which exhibit sequence-specific binding to pre-mRNA, and are associated with the spliceosome. The critical pyrimidine strectch has specific interactions with a polyprimidine-tract binding protein (PTB, *42*).

PTB was first purified as a protein that binds to the polypyrimidine tract preceding 3′ splice sites of higher eukaryotic mRNA introns (*42*). PTB was also identified as hnRNP1, a member of a family of proteins that bind to nascent RNAs and play multiple roles in splicing (*43*). Many eukaryotic genes employ alternate splicing as means of generating protein diversity. This differential incorporation of exons into the mature mRNA is often under developmental and/or tissue-specific control and enables the cell to tailor the proteins to suit its own particular requirements. PTB has an important role in the regulation of tissue-specific splicing and is implicated in the regulation of several alternatively spliced genes (*44*−*47*). PTB is also necessary for the initiation of translation from internal ribosome entry sites in some of the viral mRNAs (*48*, *49*).

We have previously observed the presence of an endonuclease that specifically degraded single-stranded circular DNA during the course of purification of strand-transfer activity from rat testis (*50*). We are now reporting here the purification of this endonuclease to homogeneity and show that this is a unique structure-specific endonuclease, which is distinct from all known structure-specific DNA endonucleases reported so far. Surprisingly, amino acid sequence analysis of the tryptic peptides of this protein revealed that it is related to polypyrimidine-tract binding protein (PTB). The identification of the testis endonuclease to be a PTB-like protein, with amino acid sequence nearly identical to the C-terminal two-thirds of PTB, is surprising. The possible significance of the structure-specific endonuclease activity associated with the PTB-related 42 kDa testis protein is discussed.

EXPERIMENTAL PROCEDURES

Endonuclease Assay. The assay used for enzyme purification and characterization is based on the degradation of single-stranded circular M13 phage DNA. The reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM DTT, 100 ng (or 40 fmol) of circular M13 mp19 single-stranded DNA, and 1 μL of the enzyme fraction in a total volume of 30 μL was incubated at 37 °C for 30 min. The reaction was stopped by adding 50 mM EDTA, 0.5 μg/mL proteinase K, and 0.1% SDS. The degradation of DNA was monitored by agarose gel electrophoresis (1%). A unit of endonuclease activity is defined as the amount of enzyme required to degrade 50% of the 100 ng of substrate (single-stranded circular M13mp19) DNA under standard endonuclease assay conditions.

Preparation and Fractionation of Rat Testes Nuclear Extract. All the steps were carried out at 4 °C. Frozen rat testis (from male albino Wistar rats, aged 38−42 days), previously stored at −70 °C, were thawed on ice. Nuclei were isolated using the method of Rao et al. (*51*) with minor modifications. Briefly, the tunica was removed, and the tissue was gently teased and homogenized by hand (in a Teflon Potter Elvehjem homogenizer) in 0.34 M sucrose in buffer A (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.5 mM PMSF). The homogenate was filtered through 4 layers of cheesecloth. The filtrate was centrifuged at 1500*g* for 10 min at 4 °C in a Beckman JA-20 rotor. The supernatant was carefully decanted and the pellet resuspended in 1.0 M sucrose in the same buffer. The suspension was centrifuged at 7800*g* for 10 min at 4 °C. The pellet obtained was resuspended in 0.34 M sucrose in buffer A containing 0.2% Triton X-100 and recentrifuged at 1500*g* for 10 min at 4 °C, resulting in purified nuclear pellet. The nuclear pellet was resuspended in buffer B (20 mM potassium phosphate buffer, pH 7.5, 0.1 mM EDTA,

10% glycerol, 10 mM β-mercaptoethanol, and 0.5 mM PMSF) containing 0.5 M KCl (extraction buffer) for 4 h on ice with intermittent gentle homogenization for extraction of proteins. The resulting suspension was centrifuged at 150000*g* for 12 h at 4 °C in a Beckman Ti 50.2 rotor. Streptomycin sulfate solution (20%, neutralized with solid Tris) was added dropwise to the supernatant to a final concentration of 2% with continuous gentle stirring. After 1 h, the precipitated nucleic acids were removed by centrifugation at 12000*g* for 15 min at 4 °C. The clear supernatant was dialyzed against buffer B containing 150 mM KCl. The dialysate was centrifuged at 12000*g* for 10 min at 4 °C to pellet down the precipitate formed during dialysis. The clear supernatant thus obtained is referred to as crude nuclear extract.

The nuclear extract was adjusted to 30% saturation with solid ammonium sulfate with stirring and was kept on ice for 30 min. The precipitated proteins were collected by centrifugation at 12000*g* for 10 min. The supernatant recovered was adjusted to 60% saturation with ammonium sulfate. The precipitated proteins collected after centrifugation were resuspended in buffer C (buffer B with 150 mM KCl) and dialyzed against buffer C. The dialyzed protein fraction was loaded on a phosphocellulose (P-11) column (50 mL bed volume, 3 cm inner diameter) equilibrated with buffer C at a flow rate of 20 mL/h. The column was washed with 3 column volumes of buffer C. Bound proteins were eluted with a linear gradient of 150−800 mM KCl in buffer C. Two milliliter fractions were collected and assayed for the endonuclease activity. The fractions exhibiting the activity eluted between 280 and 460 mM KCl. Active fractions were pooled and dialyzed against buffer D (buffer B with 30 mM KCl). The dialysate was then applied to a S-Sepharose column (10 mL bed volume, 1.5 cm inner diameter) equilibrated with buffer D at a flow rate of 10 mL/h. After being washed with 3−4 column volumes of buffer D, the bound proteins were eluted with a linear gradient of 30−300 mM KCl in buffer D. Two milliliter fractions were collected and assayed for the endonuclease activity. The active fractions were pooled, dialyzed against buffer E (buffer B with 100 mM KCl), and concentrated by 0−60% ammonium sulfate precipitation. It was then fractionated on a Sephacryl S-200 column (100 mL bed volume, 1.5 cm inner diameter) equilibrated with buffer E. One milliliter fractions were collected and assayed for the nuclease activity. The active fractions were pooled and dialyzed against buffer F (buffer B with 50 mM KCl). The dialyzed protein solution was then loaded on a Bio-Rex 70 column (5 mL bed volume, 1.5 cm inner diameter) equilibrated with buffer F at a flow rate of 10 mL/h. The column was washed with 3−4 column volumes of buffer F, and the bound proteins were eluted with a linear gradient of 50−300 mM KCl in buffer E. One milliliter fractions were collected and assayed for the nuclease activity.

Purification of Recombinant FEN1. The expression clone containing FEN1 cDNA-encoding plasmid was a generous gift from Dr. Y. Chang of Los Alamos National Laboratory, Los Alamos, NM. The overexpressed protein was purified as described by Nolan et al. (*52*). Briefly, the bacterial lysate obtained after sonication in buffer S (50 mM Tris-HCl, pH 7.9, and 100 mM sorbitol) was centrifuged at 30000*g* for 1 h in a Beckman L7-55 ultracentrifuge. The recombinant FEN1 was purified by passing the supernatant over a Ni^{2+}-agarose column (Qiagen) under nondenaturing conditions. The column was extensively washed with buffer S1 (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, and 5 mM imidazole). After being washed with the buffer containing 60 mM imidazole, the FEN1 protein was eluted with buffer containing 1 M imidazole. The eluted protein was dialyzed against phosphate buffer saline. Purity of the protein was analyzed on SDS−PAGE (*53*).

Analysis of the Ends Generated by Endonuclease. The reaction products obtained in a typical endonuclease assay were processed further for determining the nature of the 5′ and 3′ ends generated. For 3′-end labeling, mock-treated and endonuclease-treated M13mp19 DNAs (2 μg of each) were incubated with 25 units of calf thymus TdT and 20 μCi of [α-^{32}P]dATP at 37 °C for 30 min. For 5′-end-labeling, mock-treated and endonuclease-digested DNAs (3 μg of each) were first incubated for 60 min at 37 °C with or without 1.5 units of calf thymus alkaline phosphatase. After phenol extraction, DNA was precipitated with ethanol, redissolved, and incubated with T4 polynucleotide kinase and 30 μCi of [γ-^{32}P]-ATP at 37 °C for 30 min. DNA samples from both the 3′- and 5′-end-labeling reaction mixtures were purified by two rounds of phenol/chloroform extraction and ethanol precipitation and subjected to agarose gel electrophoresis. The labeling pattern was monitored by autoradiography.

In-Gel Assay for Endonuclease Activity. The procedure described by Lacks and Springhorn (*54*) for in-gel assay was followed with minor modifications. SDS−PAGE of Fraction IV (Sephacryl S-200 active fraction) was carried out according to the method of Laemmli (*50*). M13mp19 single-stranded circular DNA was incorporated into the separating gel at a concentration of 8 μg/mL. Electrophoresis was carried out at 100 V. The gel was subsequently washed 4 times (30 min each) with 40 mM Tris-HCl, pH 7.5, to remove SDS and partially renature the proteins. It was then incubated for 24 h at 37 °C in 40 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$. The gel was stained with ethidium bromide and photographed under ultraviolet illumination. The proteins with nuclease activities can be identified as dark bands, which appear as a result of degradation of the DNA impregnated in the gel, in the background of the entire gel being fluoresced due to staining of impregnated DNA with ethidium bromide.

Preparation of Substrates Having Various Structures. The substrates used in this study were prepared according to the methods described by Harrington and Lieber (*24*) with minor changes. Oligonucleotides of defined sequences shown in Table 1 were synthesized by Bangalore Genei, India. They were purified by running on 15% urea−PAGE and eluting from the gel by UV-shadowing. The 5′-end-labeling of 300 ng of oligonucleotides (on which cutting was to be measured) was carried out using polynucleotide kinase (PNK) and [γ-^{32}P]ATP (BARC, India) according to Sambrook et al. (*55*). PNK and unincorporated nucleotides were removed by phenol extraction followed by spin column gel filtration through a 1 mL Sephadex G-25 column. The labeled oligonucleotides were then annealed to their complementary oligonucleotides at a 1:1.5 molar ratio in 10 mM Tris-HCl, pH 7.5, and 0.1 M NaCl by heating to 95 °C, followed by cooling gradually to room temperature. The annealed products were separated on 10% native PAGE and eluted from

the gel. The resulting duplexes, having the various desired structures indicated in Table 3, were used in the endonuclease assays.

Endonuclease Assay with Model Substrates. Endonuclease activity was measured in a 30 μL reaction containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 5 mM DTT and 10 fmol of 5′-end-labeled substrate. Incubation was done with or without the endonuclease (Fraction V) at 37 °C for 1 h. The reaction was terminated by adding 50 mM EDTA, 0.5 μg/mL proteinase K, and 0.1% SDS and was extracted with phenol/chloroform and precipitated with ethanol. The DNA pellet was resuspended in 80% formamide containing 0.1% xylene cyanol and 0.1% bromophenol blue, heated to 95 °C for 5 min, quick-chilled on ice, and loaded onto a 15% polyacrylamide gel containing 7 M urea and 1× TBE. Reaction products were visualized by autoradiography.

Exonuclease Assay with Branched Substrates. For 3′-end-labeling, complementary oligonucleotides were annealed and incubated with Klenow fragment of DNA polymerase I and [α-^{32}P]dTTP under standard reaction conditions. 5′-End-labeling was carried out using T4 polynucleotide kinase and [γ-^{32}P]ATP. Excess ATP was removed using a Sephadex G-25 spin column, and the labeled oligonucleotide was then annealed to its complementary oligonucleotide at a 1:1.5 molar ratio in 10 mM Tris-HCl, pH 7.5, and 0.1 M NaCl by heating to 95 °C, followed by cooling gradually to room temperature. The 3′- and 5′-end-labeled duplexes were separated on 10% native PAGE and eluted from the gel. The 3′- and 5′-labeled linear duplex DNA substrates were incubated with endonuclease, and the release of labeled mononucleotide was monitored on 15% urea−PAGE. The T7 exonuclease and exonuclease III, which release mononucleotides from 5′- and 3′-labeled duplex DNA, were used as positive controls.

RESULTS

Identification and Purification of an Endonuclease from Rat Testis. Spermatogenesis in mammals is a unique process of differentiation and involves both mitotic and meiotic events and extensive alternate splicing of precursor pre-mRNAs (*56*). While purifying a strand-transfer activity from rat testis, we had observed previously that some fractions separating away from the strand-transfer peak specifically degraded single-stranded circular DNA, one of the substrates in the strand-transfer assay (*50*). This endonuclease activity has now been purified from rat testis nuclear fraction. The chromatographic behavior of the endonuclease during the purification steps was monitored using the same property (i.e., degradation of single-stranded circular DNA) under standard endonuclease assay conditions. The strategy employed for its purification is summarized in Figure 1. The crude nuclear extract was first subjected to ammonium sulfate fractionation, and the activity was detected in the 30−60% fraction (Fraction I). The dialyzed protein was then subjected to ion-exchange chromatography on phosphocellulose. The protein profile is shown in Figure 2A while the activity profile is shown in Figure 2B. The activity as seen by the disappearance of single-stranded circular M13mp19 DNA eluted between fractions 28 and 64, that corresponded to a concentration of 280−460 mM KCl. The most active fractions (Fraction II) were pooled and processed for further

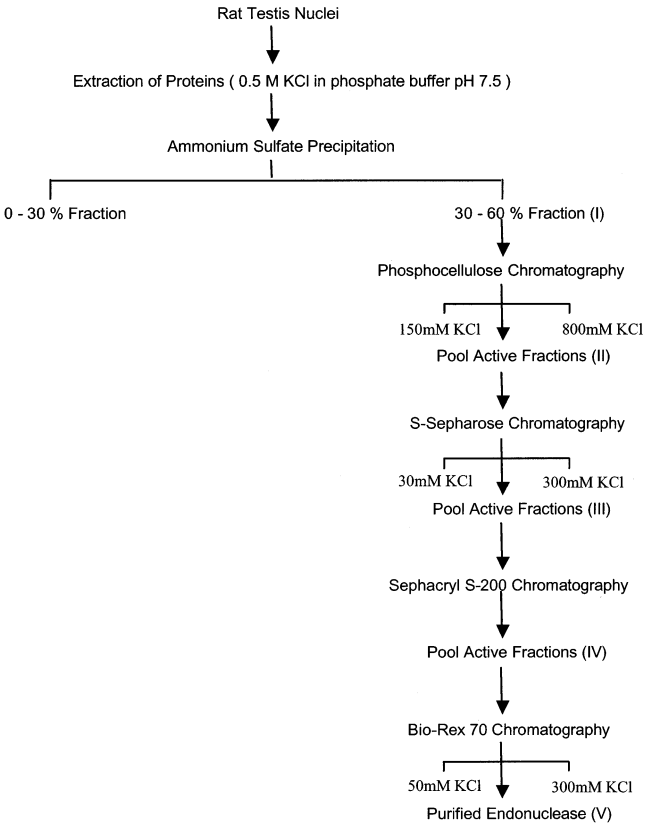

FIGURE 1: Purification of testis endonuclease from rat testis nuclear extract. The fractionation scheme of the testis endonuclease from rat testis nuclear extract is summarized in the flowchart.

fractionation on S-Sepharose. The absorbance and the activity profiles of the eluted fractions are shown in Figure 2C and Figure 2D, respectively. The activity eluted between 50 and 100 mM KCl. These fractions were pooled (Fraction III) and subjected to 0−60% ammonium sulfate precipitation and subsequently to gel permeation chromatography on Sephacryl S-200. The absorbance and the activity profiles are shown in Figure 2E,F, respectively. The activity eluted between fractions 64 and 88, coinciding with the first half of the major protein peak. The most active fractions were pooled (Fraction IV) and fractionated by Biorex 70 chromatography. The endonuclease activity eluted between 135 and 185 mM KCl (Fraction V). At this stage of purification, the amount of protein eluted from the column was too low to be monitored by absorbance; therefore, only the activity profile is shown in Figure 2G. The results of the purification are shown in Table 1. Starting with about 74 mg of nuclear proteins (salt-extractable), the final preparation obtained after Biorex 70 chromatography yielded about 1.6 μg of protein having very high specific activity. A purification of approximately 300-fold was achieved. There was a major loss of protein at the last step. As the specific activity of the endonuclease at this step was very high, the amount of protein obtained was sufficient for its detailed characterization, and hence alternative strategy for purification was not attempted.

Molecular Mass of the Endonuclease. The pooled active fractions from each step of purification (crude nuclear extract and Fractions I−V) were analyzed by SDS−PAGE, and proteins were visualized by silver staining (Figure 3A). As shown in Figure 3A, Fraction V contained a nearly homogeneous preparation containing a 42 kDa protein. To

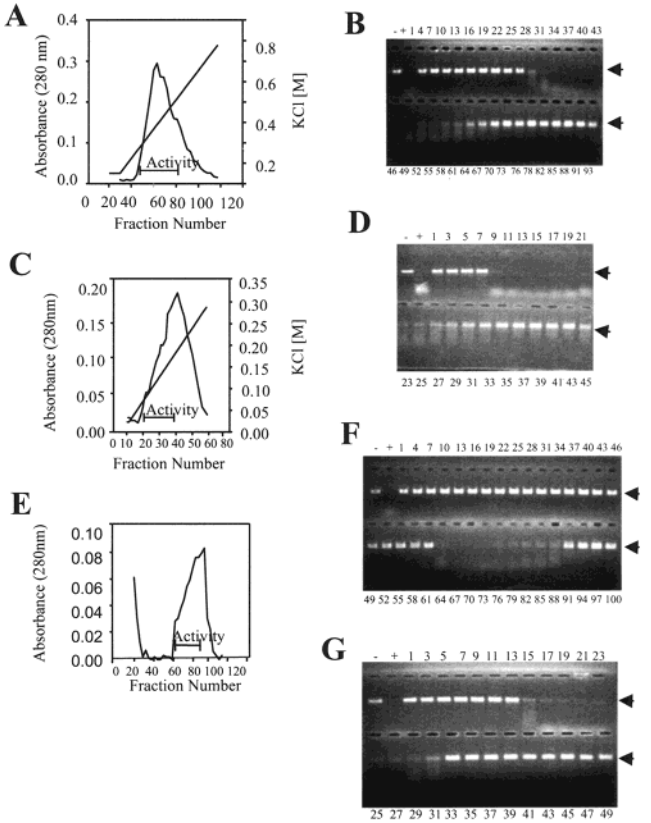

FIGURE 2: Fractionation of Fraction I by sequential chromatographic steps. (A) Elution profile of proteins on the phosphocellulose column. Elution was carried out with a linear gradient of 150−800 mM KCl. Two milliliter fractions were collected, and the absorbance at 280 nm was recorded. (B) Agarose gel (1%) showing the endonuclease activity profile (degradation of single-stranded circular DNA) eluted from the phosphocellulose column. (C) Elution profile of proteins from the S-Sepharose column. Elution was done with a linear gradient of 30−300 mM KCl. Absorbance of the 2 mL fractions collected was recorded at 280 nm. (D) Agarose gel (1%) showing the endonuclease activity profile of alternate S-Sepharose fractions. (E) Fractionation profile of proteins from Sephacryl S-200. One milliliter fractions were collected, and their absorbance was recorded at 280 nm. (F) Agarose gel (1%) showing the endonuclease activity profile of every third fraction collected from Sephacryl S-200. (G) The active fractions from Sephacryl S-200 were further fractionated on Biorex 70. Elution was done with a linear gradient of 50−300 mM KCl, and 1 mL fractions were collected. Agarose gel (1%) showing the endonuclease activity profile of alternate Biorex 70 fractions. Lanes (−) and (+) indicate reaction done in the absence and presence of 2 μL of the pooled active fraction from the previous chromatographic step, respectively.

determine the native molecular mass of the endonuclease, Fraction V was concentrated using 0−60% ammonium sulfate precipitation and loaded onto a Sephacryl S-200 gel filtration column. The endonuclease was eluted at a position representing a calculated molecular mass of 42 kDa (Figure 3B), indicating that the enzyme purifies as a 42 kDa polypeptide. Fraction V was used in subsequent experiments for its further characterization.

To provide further evidence that the 42 kDa protein, as seen in SDS−PAGE, is indeed having the endonuclease activity and it is not due to any minor contaminants that are not visualized in the silver-stained polyacrylamide gel, we carried out an in-gel activity assay. For this purpose, we impregnated a polyacrylamide gel with circular single-stranded M13mp19 DNA, and after electrophoresis, the proteins were renatured, and the gel was incubated in buffer containing $MgCl_2$. The degradation of impregnated DNA can be detected as negative fluorescence under ultraviolet light after staining the gel with ethidium bromide. Results of such an experiment show the presence of a dark band only at the position of a 42 kDa protein (Figure 3C, lane 2). As a result of degradation of DNA, this region will be detected as a dark band in a fluorescent background. DNase I was used as a positive control in this experiment which also showed a clear negative stain (dark band) at the ~30 kDa region (Figure 3C, lane 1). This experiment, therefore, confirms that the endonulcease activity is associated with the 42 kDa polypeptide and not due to any contaminant in Fraction V.

Table 1: Summary of Purification of the Endonuclease from Rat Testis

fraction	protein (mg)	activity (units)	sp act. (units/mg)	purification (*x*-fold)
nuclear extract	74.00	8.8×10^6	1.18×10^5	−
ammonium sulfate (Fraction I)	47.76	6.8×10^6	1.42×10^5	1.2
phosphocellulose (Fraction II)	16.14	5.0×10^6	3.09×10^5	2.6
S-Sepharose (Fraction III)	6.60	4.4×10^6	6.60×10^5	5.6
Sephacryl S-200 (Fraction IV)	1.00	2.4×10^6	2.40×10^6	20.3
Bio-Rex 70 (Fraction V)	0.0016	5.6×10^4	3.50×10^7	296.6

Properties of the Endonuclease. The protein concentration and time dependence of endonuclease activity are shown in Figure 4A and Figure 4B, respectively. Linearity was observed both with respect to enzyme concentration and with respect to time. Initially, a broad streak was observed over a wide range of sizes (lanes 3, 4, and 5). At intermediate concentrations of enzymes and time, a more defined product of ~250−300 nucleotides (lanes 7 and 8) was observed which was further degraded at higher enzyme concentrations and longer incubation times (Figure 4A, lane 10, and Figure 4B, lane 13). The endonuclease showed an absolute requirement of divalent metal ions for enzymatic activity (Figure 4C). Magnesium supported the activity over a wide range of concentrations (5−50 mM). Among other divalent ions, only manganese could substitute for magnesium, 5 mM being the optimal concentration. Calcium and zinc, however, could not substitute for magnesium. The endonuclease activity was sensitive to monovalent cations. The enzyme activity did not require either NaCl or KCl, and higher concentrations of these monovalent cations (above 75 mM) inhibited the enzymatic activity (Figure 4D) as observed by the generation of intermediate sized products.

The endonuclease was purified from rat testis based on the degradation of circular single-stranded DNA, and, therefore, it was of interest to test the activity of the enzyme on other types of DNA substrates. As can be seen from Figure 5A, the purified endonuclease created a nick in the supercoiled pUC19 DNA, resulting in the formation of nicked circular DNA (lane 2). The enzyme also did not have any effect on linear single-stranded DNA (49mer oligonucleotide) (Figure 5B) or linear duplex DNA (Figure 5A, lanes 3 and 4).

In most of the pathways involving nucleases, the final step is the ligation of the processed ends by DNA ligase. In this context, it is necessary to find out what kinds of ends are

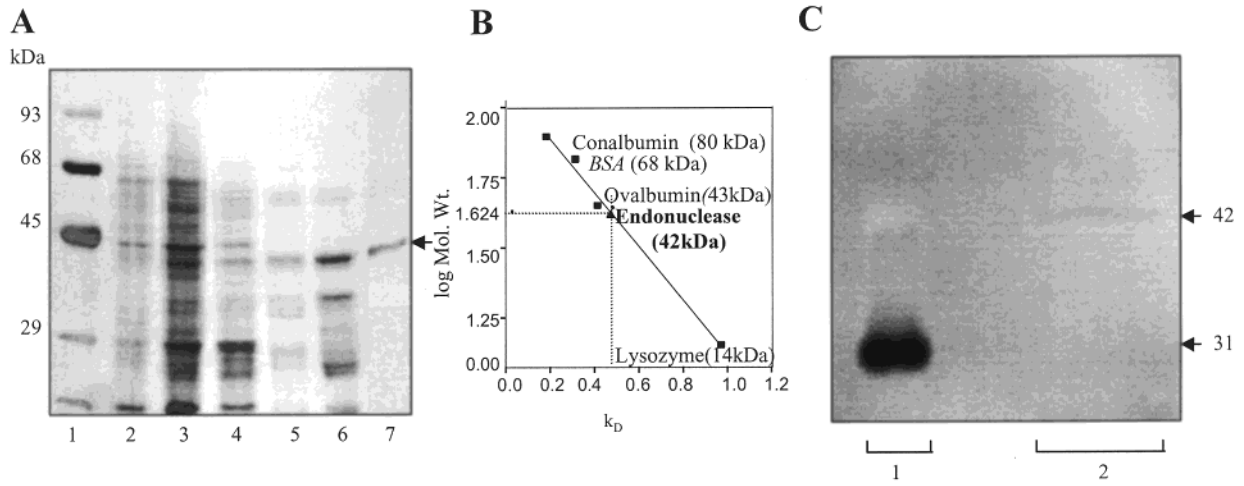

FIGURE 3: Molecular mass of the testis endonuclease. (A) SDS−PAGE analysis of the testis endonuclease purification fractions. Aliquots of each purification fraction were loaded onto a 10% SDS−polyacrylamide gel. Proteins were visualized by silver staining. Lane 1, low molecular mass PAGE standards; lane 2, crude nuclear extract (3 μg); lane 3, Fraction I (30−60% ammonium sulfate fraction) (5 μg); lane 4, Fraction II (phosphocellulose pool) (3 μg); lane 5, Fraction III (S-Sepharose pool) (1 μg); lane 6, Fraction IV (Sephacryl S-200 pool) (1 μg); lane 7, Fraction V (Biorex 70 pool) (100 ng). Numbers on the left indicate the size of the molecular mass standards in kilodaltons. (B) Native molecular size of the testis endonuclease. An aliquot of Fraction IV was concentrated and loaded onto a Sephacryl S-200 column. The arrow indicates the position at which the testis endonuclease activity was found. The column was calibrated with molecular mass standard proteins (conalbumin, 80 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; and lysozyme, 14 kDa). (C) In-gel assay for endonuclease activity. Electrophoresis was carried out in a SDS−polyacrylamide gel containing M13 mp19 single-stranded circular DNA (8 μg/mL). Following electrophoresis, the gel was washed several times and incubated for 15 h at 37 °C in 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, and 5 mM DTT. The gel was stained with ethidium bromide and photographed under ultraviolet illumination. Lane 1, bovine pancreatic DNase I; and lane 2, partially purified endonuclease (Fraction IV).

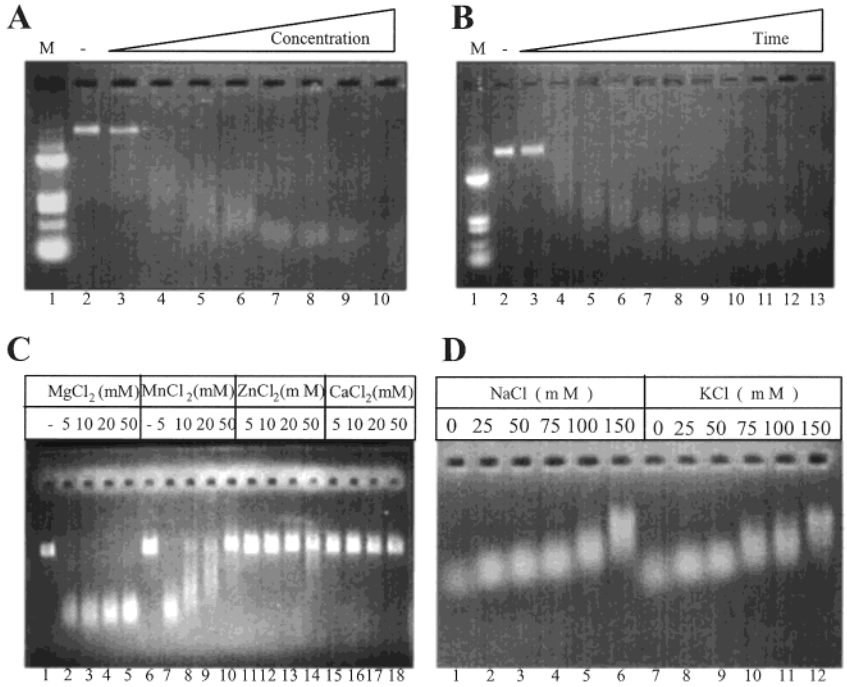

FIGURE 4: Effect of enzyme concentration, time, and divalent and monovalent cations on testis endonuclease activity. (A) Endonuclease assay with single-stranded circular DNA as substrate was performed using different concentrations of testis endonuclease. Lane 1, molecular size DNA standards (lambda *Mlu*I digest); lane 2, no enzyme; lanes 3−10, 0.05, 0.25, 0.5, 0.75, 1.25, and 2 units of enzyme, respectively. (B) Time course of testis endonuclease activity was carried out using 1 unit of testis endonuclease with single-stranded circular DNA as substrate. Lane 1, molecular size DNA standards (lambda *Mlu*I digest); lane 2, no enzyme; lanes 3−13, 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min, respectively. (C) Endonuclease assay was performed with 1 unit of testis endonuclease and single-stranded circular DNA as substrate using different concentrations of divatent cations. Lanes 1 and 6, no divalent cation; lanes 2−18, different concentrations of $MgCl_2$, $MnCl_2$, $ZnCl_2$, and $CaCl_2$ as indicated above its corresponding lane. (D) Endonuclease assay was performed with 1 unit of testis endonuclease and single-stranded circular DNA as substrate using different concentrations of monovalent cations. The concentration of NaCl and KCl has been indicated above its corresponding lane.

generated by the testis endonuclease. We have, therefore, analyzed the nature of the 5′ and 3′ ends generated following endonucleolytic cleavage of single-stranded DNA. The products of the endonuclease reaction were labeled using polynucleotide kinase or terminal transferase. As can be seen from Figure 5C, the endonuclease reaction products could

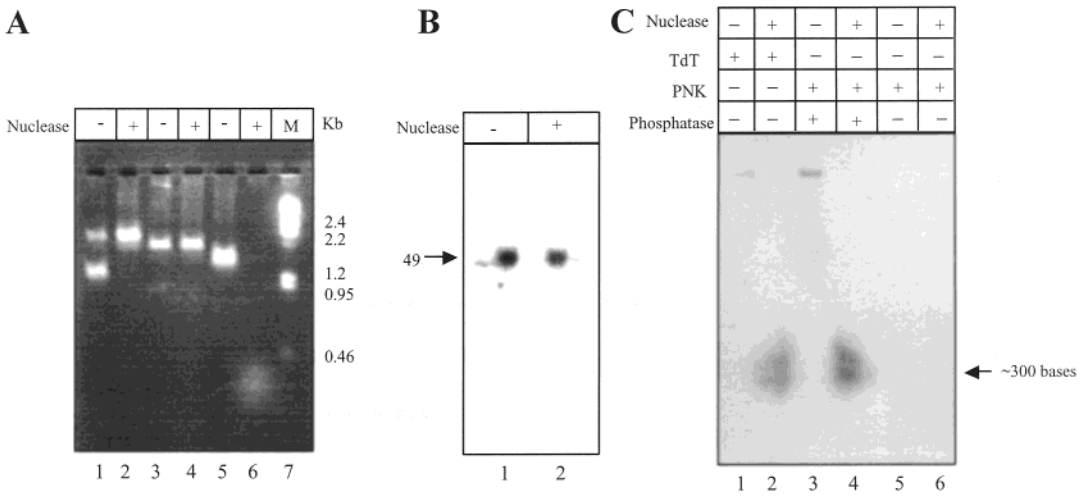

FIGURE 5: Activity of the endonuclease on various DNA substrates. Using different kinds of DNA as substrates, endonuclease assay was performed under standard reaction conditions, and the reaction mix was run on a 1% agarose gel. Lanes 1 and 2, supercoiled DNA in the absence and presence of 1 unit of enzyme, respectively; lanes 3 and 4, linear duplex DNA in the absence and presence 1 unit of enzyme, respectively; lanes 5 and 6, single-stranded circular DNA in the absence and presence of enzyme, respectively; lane 7, *Mlu*I digest of lambda DNA. (B) Endonuclease assay with linear single-stranded DNA (49mer oligonucleotide). 5′-End-labeled oligonucleotide was used as the substrate, and the reaction mixture was run on a 15% urea−PAGE and visualized by autoradiography. (C) Testis endonuclease produces 3′-hydroxyl and 5′-phosphate groups. The endonuclease-digested single-stranded circular M13 mp19 DNA (lanes 2, 4, and 6) and mock-treated DNA (lanes 1, 3, and 5) were reacted with calf thymus terminal transferase in the presence of [α-^{32}P]dATP (lanes 1and 2) or with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP without (lanes 5 and 6) or with (lanes 3 and 4) prior dephosphorylation by calf thymus alkaline phosphatase.

Table 2: Nucleotide Sequences for Substrate Oligonucleotides and PCR Primers

DNA	Nucleotide Sequence
A1, 37mer	5'- ATGTGAATCAGTATGGTTCCTATCTGCTGAAGGAATT-3'
A2, 37mer	5'- AATTCCTTCAGCAGATAGGAACCATACTGATTCACAT-3'
A3, 37mer	5'- ATGTGAATCAGTATGGTTTCTATCTGCTGAAGGAATT -3'
A4, 45mer	5'- ATGTGAATCAGTATGCACACACAGTTTCTATCTGCTGAAGGAATT - 3'
A5, 37mer	5'- ATGTGAATCAGTATGGTTGTTGGCTCATCAGAAGGCC - 3'
A6, 22mer	5'- GTTCCTATCTGCTGAAGGAATT- 3'
A7, 20mer	5'- ATGTGAATCAGTATGGTTCC - 3'
A8, 30mer	5'- GGACTCTGCCTCAAGACGGTAGTCAACGTG -3'
A9, 34mer	5'- GATGTCAAGCAGTCCTAACTTTGAGGCAGAGTCC-3'
A10, 16mer	5'- CACGTTGACTACCGTC-3'
A11, 34mer	5'- CACGTTGACTACCGAGAATCCTGACGAACTGTAG- 3'
A12, 16mer	5'- TCTTGAGGCAGAGTCC- 3'
A13, 14mer	5'- GACTGCTTGACATC- 3'
A14, 14mer	5'- CTGCTGAAGGAATT- 3'
A15, 14mer	5'- GGCCTTCTGATGAG- 3'
A16, 14mer	5'- ATGTGAATCAGTAT- 3'
A17, 14mer	5'- CTGCTGAAGGAATT- 3'
A18, 49mer	5'- GACGCTGCCGAATTCTGGCGTTAGGAGATACCGATAAGCTTCGGCTTAA-3'
P1, 27mer	5'-GCGCATATGGACGGCATCGTCCCAGAC-3'
P2, 34mer	5'-CGCAAGCTTCTAGATGGTGGACTTGGAAAAGGAC-3'

be efficiently labeled with [α-^{32}P]dATP using terminal transferase. However, 5′ ends could be labeled by T4-polynucleotide kinase only after prior treatment with calf intestinal phosphatase (lanes 4 and 6). Thus, the endonuclease products possess 5′-phosphate and 3′-hydroxyl groups.

Ability of the Rat Testis Endonuclease To Cleave Branched Structures. Although purification of the endonuclease was carried out using single-stranded circular DNA as the substrate, the occurrence of such a DNA molecule has not been reported until now in mammalian cells. However, it is well-known that different branched structures arise transiently in DNA during various DNA metabolic processes. It was of interest, therefore, to test whether the endonuclease cleaves such branched structures. For this purpose, various branched structures were prepared by annealing different sets of complementary oligonucleotides whose sequences are given in Table 2. The set of oligonucleotides and the nature of structures generated are given in Table 3. In the first set of experiments, linear duplex DNA, mismatch containing heteroduplex, and DNA containing insertion loop were used as substrates for endonuclease activity. Theoretically, cleavage of the G/T mismatch and insertion loop should result in the generation of products of approximately 18 nucleotides on a denaturing polyacrylamide gel. As can be seen from Figure

Table 3: DNAs Used for Testis Endonuclease Substrate-Specificity Studies

DNA	Nucleotide Sequence	Structure generated
Linear duplex	A1, A2	
Linear duplex	A1, A2	
Linear single stranded	A18	
(G/T) mismatched duplex	A2, A3	
Insertion loop	A2, A4	
Pseudo-Y	A2, A5	
Pseudo-Y	A2, A5	
3'- overhang	A2, A6	
5'- overhang	A2, A7	
5'- flap	A8, A9, A10	
3'- flap	A8, A11, A12	
End blocked 5'-flap	A8, A9, A10, A13	
End blocked pseudo-Y	A2, A5, A14	
End blocked pseudo-Y	A2, A5, A15	
End blocked 5'- overhang	A2, A5, A16	
End blocked 5'- overhang	A2, A5, A17	

6A, the expected products were not obtained, showing that these substrates, including the linear duplex DNA, are not substrates for the enzyme. Next, we examined whether the testis endonuclease cleaves branched structures such as pseudo-Y, 5′ and 3′ overhangs.

These substrates were prepared and used as substrates in the endonuclease reaction. Incision of pseudo-Y, 5′ and 3′ overhang structures near the single-strand/double-strand junction would result in the generation of approximately 19, 18, and 22 nucleotides, respectively, on a denaturing acrylamide gel. These structures were indeed cleaved by the endonuclease around the junction to generate products of the expected sizes (Figure 6B). Cleavage of the pseudo-Y substrate generated a major 19 nucleotide product and a minor 18 nucleotide product. Cleavage of the 3′ overhang generated a 25, a 24, and a 29 nucleotide product. In addition to these two products, faint cleavage products of 19 nucleotides and 18 nucleotides were also observed. The endonuclease reaction with the 5′ overhang as the substrate resulted in products of 19 and 18 nucleotides, which were very similar to the ones generated with pseudo-Y structure as the substrate. Thus, it is apparent that the endonuclease cleaves the three branched structures at or near the single-strand/double-strand junction. We also examined whether the cleavage of the pseudo-Y structure occurs on one or both of the unpaired single strands. If the pseudo-Y structure labeled at the opposite strand was used as the substrate, a 22nt product was generated (Figure 6B, lane 8, as opposed to a 19nt product observed in Figure 6B, lane 2). This result indicates that there is an asymmetric cleavage on both strands of the pseudo-Y substrate at the single-strand/duplex junction.

As mentioned in the introduction, flap structures are generated as intermediates in a variety of events including recombination (*1*), DNA replication (*2*), and DNA end-joining (*57*) which are processed by the flap endonuclease (FEN1). We were interested to know whether the rat testis endonuclease that we have purified also cleaves flap structures in a strand-specific manner as FEN1. To test this, we designed and generated 5′-flap and 3′-flap structures, which were used as substrates in the endonuclease assay. Results of these experiments are presented in Figure 6C,D, respectively. The results show that the 5′-flap strand was cleaved, generating major and minor products of 27 and 19 nucleotides, respectively (Figure 6C, lane 2). This shows that there is a minor cleavage site at the duplex/single-strand junction similar to the one observed with FEN1, but the major cleavage site happens to be 3′ to the junction. It can also be seen from this figure that there is no strand specificity as the cleavage was observed in the Fbr (bridge) strand also. The cleavage sites are just opposite to the duplex/single-strand junction on the bridge strand (Figure 8A), which generates an 18 nucleotide major product and a 16 nucleotide minor product (Figure 6C, lane 4). This property of the rat testis endonuclease is distinct from that of FEN1 (*21*). RAG1/RAG2 is the only 3′-flap structure processing endonuclease reported so far (*58*). As RAG1/RAG2 is specific for V(D)J recombination, the question still remains as to how the 3′-flap structures are handled by the cell in general. Therefore, an obvious question in the present context is whether the testis endonuclease cleaves 3′-flap structures as well as 5′-flap structures. So, we tested the activity of the endonuclease on 3′-flap structures, the results of which are shown in Figure 6D. Indeed, the endonuclease was able to cleave 3′-flap structures at the junction and 3′ to the junction, generating 18nt and 22nt products (lane 2). Just like in the case of the 5′-flap, the bridge strand of the 3′-flap was also cleaved opposite to the duplex/single-strand junction by this endonuclease (lane 4). But the adjoining strands of both the 5′- and 3′-flap structures were not cleaved by this enzyme. Thus, the cleavage pattern of the branched structures by the testis endonuclease is distinctly different from those of FEN1. To ascertain the validity of such differences, it was necessary to show that recombinant FEN1 cleaves the substrates used in the present study, generating the expected sized products. For this purpose, we have purified recombinant FEN1 (see Experimental Procedures) and used it in the endonuclease reactions. The SDS−PAGE showing the purified FEN1 is shown in Figure 7B. The purified FEN1 was used in the positive control experiments. As can be seen in Figure 7C (lane 2), the 5′-flap structure was cleaved by FEN1 to generate the expected sized 19 and 21 nucleotide products. FEN1, on the other hand, did not cleave the 3′-flap structure

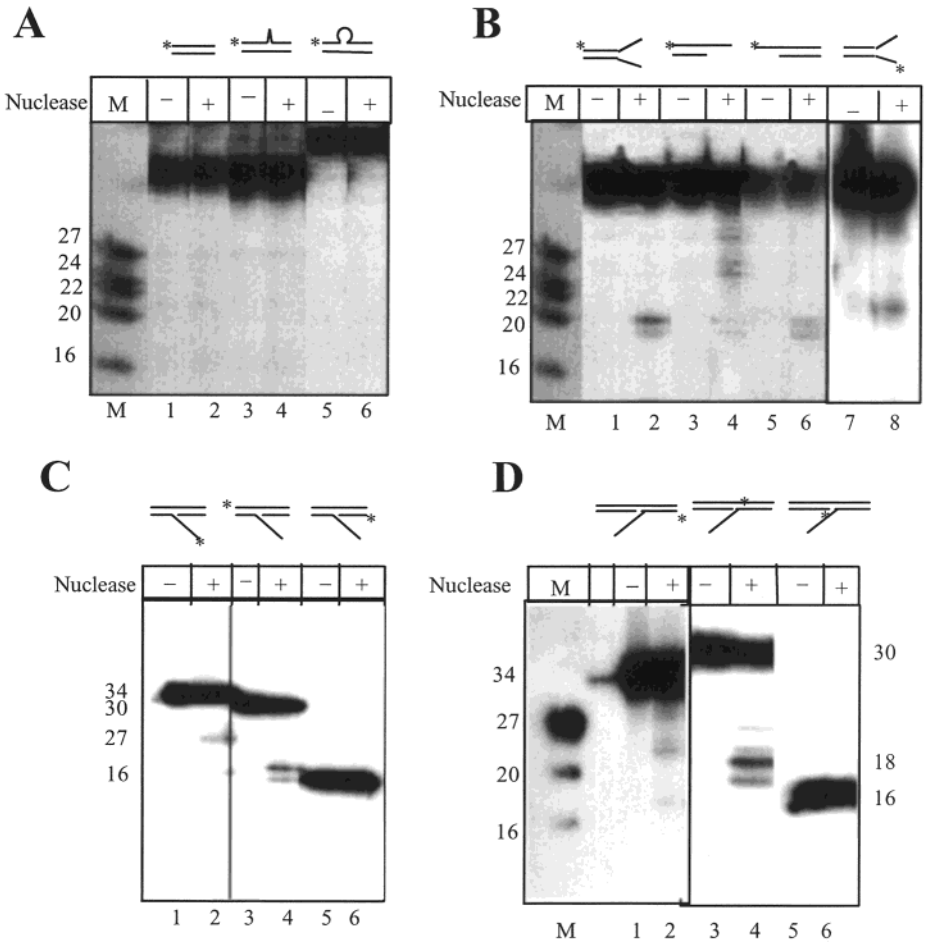

FIGURE 6: Analysis of testis endonuclease substrate specificity using various branched DNA substrates. Purified testis endonuclease (Fraction V) was assayed for its ability to cleave various types of branched DNA structures. The structure of each substrate is shown above the corresponding lanes. The asterisks indicate the position of the 5′-end-label. Following the reaction, products were separated on a 15% denaturing polyacrylamide gel and visualized by autoradiography. M denotes molecular size markers. The numbers on the left indicate the position and size of oligonucleotide standards. (A) Reactions were carried out in the absence (lanes 1, 3, 5) and presence (lanes 2, 4, 6) of 1 unit of testis endonuclease. Lanes 1 and 2, perfect duplex; lanes 3 and 4, duplex with a G/T mismatch; lanes 5 and 6, insertion loop. (B) Reactions were carried out in the absence (lanes 1, 3, 5, 7) and presence (lanes 2, 4, 6, 8) of 1 unit of testis endonuclease. Lanes 1 and 2, pseudo-Y; lanes 3 and 4, 3′-overhang; lanes 5 and 6, 5′-overhang; lanes 7 and 8, pseudo-Y. (C) Strand specificity of testis endonuclease cleavage. Purified testis endonuclease (Fraction V) was assayed for its ability to cleave the Fbr strand and Fadj strand of the 5′-flap structures. Reactions were carried out under standard testis endonuclease assay conditions in the absence (lanes 1, 3, 5) and presence (lanes 2, 4, 6) of 1 unit of testis endonuclease. (D) Purified testis endonuclease (Fraction V) was assayed for its ability to cleave the Fbr strand and Fadj strand of the 3′-flap structures. Reactions were carried out in the absence (lanes 1, 3, 5) and presence (lanes 2, 4, 6) of 1 unit of testis endonuclease.

(lanes 5 and 6). As reported earlier, FEN1 exhibited very poor activity on the pseudo-Y structure. All these activities of FEN1 agree well with those reported in the literature. This positive control, therefore, not only proves the distinctive nature of the testis endonuclease but also validates the structure of the substrates we have used in all our studies. A summary of cleavage sites of the endonuclease on different structures is shown in Figure 8A.

Effect of End-Blocking on Endonuclease Activity. FEN1 has been shown to require the free single-stranded end of the flap for cleavage to occur (*59*). It is believed that the enzyme loads on the free single-stranded end and then tracks down the single-stranded tail until it reaches the junction, following which a nick is made at the junction (*60, 61*). To check whether the single-stranded ends are necessary for the testis endonuclease to exert its endonucleolytic activity, we hybridized 5′-flap, the two unpaired arms of pseudo-Y, 3′- and 5′-overhang structures with oligonucleotide complementary to the single-stranded ends (Table 3) to prepare the end-blocked substrates and then used them in the endonuclease assay. The results of this experiment presented in Figure 7A show that the endonuclease did act upon these substrates even though the single-stranded tails have been converted into duplex structures. In the control experiment, end-blocked substrates were not cleaved by FEN1 as reported in the literature (Figure 7C, lanes 3 and 4), reconfirming the distinctive features of the testis endonuclease and FEN1.

Absence of Associated Exonuclease Activity in the Rat Testis Endonuclease. Although some of the experiments described above indicated that the testis endonuclease did not possess any associated exonuclease activity, we examined whether the testis endonuclease has any 5′ to 3′ exonuclease activity by following the generation of labeled mononucleotide from 5′-end-labeled duplex DNA. The data presented in Figure 8B (lane 2) show that no labeled mononucleotide was released, indicating that the enzyme has no 5′ to 3′ exonuclease activity. Similarly, no labeled mononucleotide was released from 3′-end-labeled duplex DNA, showing that

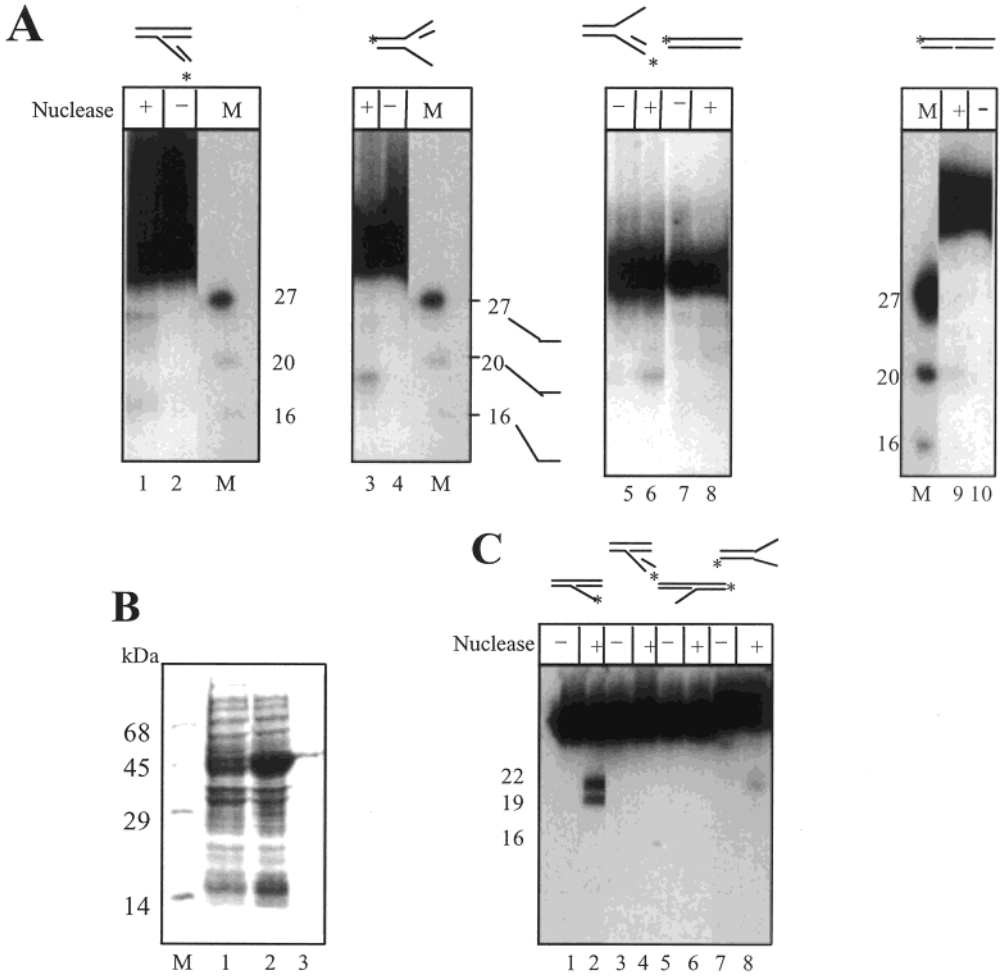

FIGURE 7: (A) Activity of testis endonuclease on substrates with end-blocked single-stranded ends. Purified testis endonuclease (Fraction V) was assayed for its ability to cleave various types of DNA structures in which the free single-stranded ends have been blocked. The structure of each substrate is shown above the corresponding lanes. The asterisks indicate the position of the 5′-end-label following the reaction; products were separated on a 15% denaturing polyacrylamide gel and visualized by autoradiography. M denotes molecular size markers. The numbers on the left indicate the position and size of oligonucleotide standards. Reactions were carried out in the absence (lanes 2, 4, 5, 7, 10) and presence (lanes 1, 3, 6, 8, 9) of 1 unit of testis endonuclease. (B) Expression and purification of recombinant FEN1. 10% SDS−PAGE showing expression and purification of FEN1. M indicates low molecular mass protein standards; lanes 1 and 2, *E. coli* (BL21) cells with pET28b containing FEN1 cDNA before and after induction with IPTG; lane 3, purified FEN1. (C) Analysis of FEN1 substrate specificity. Purified FEN1 was assayed for its ability to cleave various types of DNA structures. The structure of each substrate is shown above its corresponding lane. The asterisks indicate the position of the 5′-end-label. Following the reaction, products were separated on a 15% denaturing polyacrylamide gel and visualized by autoradiography. The numbers on the left indicate the position and size of oligonucleotide standards. Reactions were carried out under standard FEN1 endonuclease assay conditions in the absence (lanes 1, 3, 5, 7) and presence (lanes 2, 4, 6, 8) of 1 unit of testis endonuclease. Lanes 1 and 2, 5′-flap; lanes 3 and 4, end-blocked 5′-flap; lanes 5 and 6, 3′-flap; lanes 7 and 8, pseudo-Y.

the enzyme also did not possess 3′ to 5′ exonuclease activity (lane 5). T7 exonuclease and exonuclease III, which release mononucleotides from 5′- and 3′-end-labeled duplex DNA, were used as positive controls (lanes 3 and 6), respectively.

Sequence Analysis of the Endonuclease. The amino acid sequence of the tryptic peptides derived from the 42 kDa rat testis endonuclease was determined by LC/MS (microsequencing). The amino acid sequence of the peptides is shown in Figure 9A. These sequences, when compared with the existing protein data bank, matched to a great extent with stretches of polypyrimidine-tract binding protein (PTB), which is involved in pre-mRNA splicing in eukaryotic cells. The sequence of rat PTB corresponding to each of the peptide sequences obtained from the 42 kDa testis endonuclease is shown in Figure 9B. All the peptides were found to match stretches of PTB sequence (pink colored regions). However, there were a few changes observed in the sequence of the peptides, which are highlighted by green color. The extensive similarity observed (starting from residue 215) indicates that the 42 kDa protein must be related to PTB if not identical to PTB (assuming an error in the peptide sequencing). It is possible that the 42 kDa endonuclease may either be a proteolytically derived product of PTB or be its alternate spliced product. PTB is known to exist in at least three different isoforms termed PTB1, PTB2, and PTB4 generated by alternative splicing (*62*, *63*). To check whether there are any alternatively spliced mRNA species in rat testis which could account for the 42 kDa size of the testis endonuclease, we carried out northern blot analysis using total testicular RNA with rat PTB cDNA as the probe. This result presented in Figure 9C revealed only one mRNA species. Thus, no shorter RNA was detected. Therefore, it is very likely that the 42 kDa endonuclease is proteolytically derived from PTB.

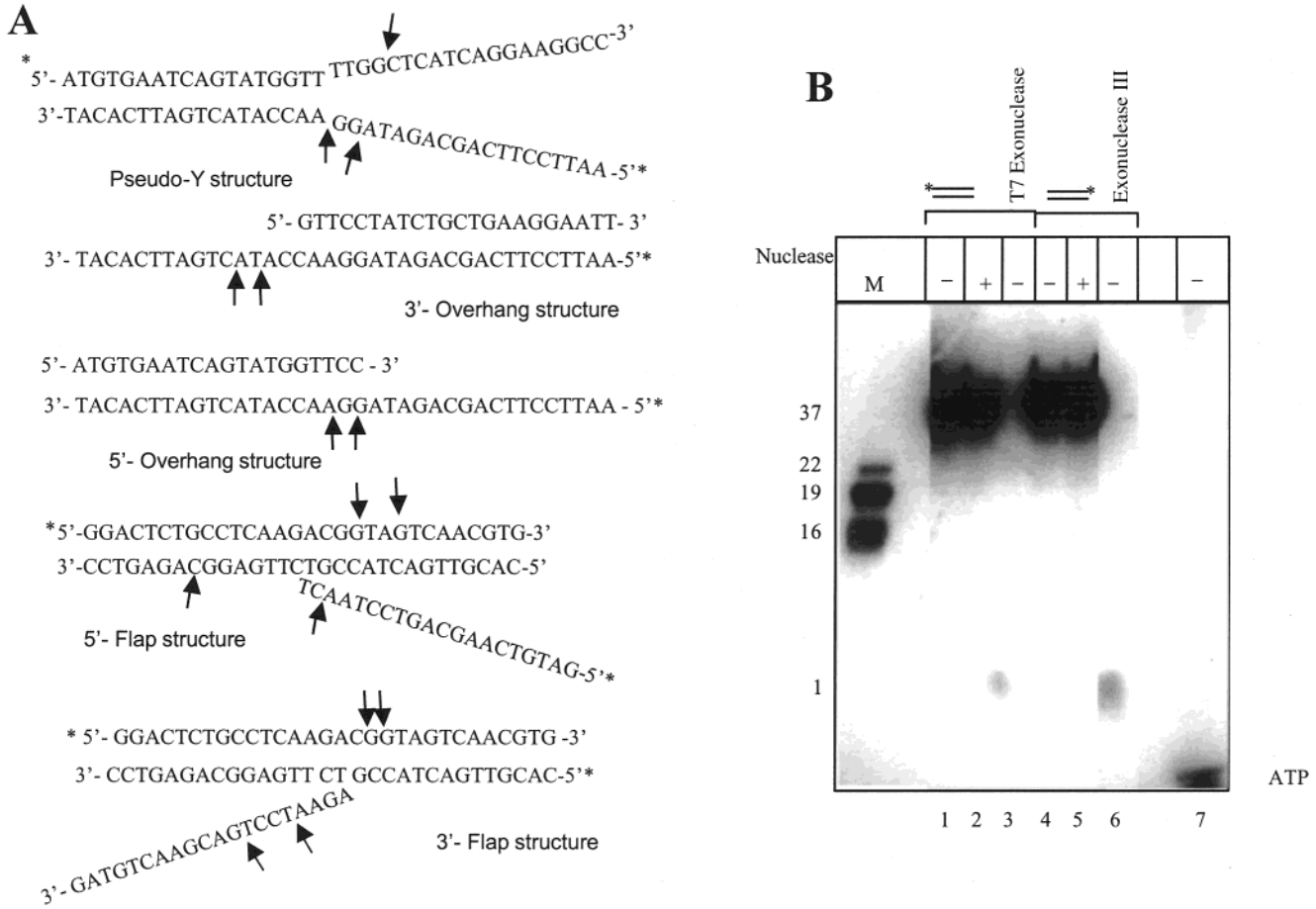

FIGURE 8: (A) Summary of cleavage sites. The arrow indicates the site of cleavage. The asterisks indicate the position of the 5′-end-label. (B) Absence of an exonuclease activity associated with testis endonuclease. Purified testis endonuclease (Fraction V) was assayed for its ability to cleave 5′- and 3′-end-labeled duplex DNA. The structure of each substrate is shown above its corresponding lane. The asterisks indicate the position of the end-label. Following the reaction, products were separated on a 15% denaturing polyacrylamide gel and visualized by autoradiography. M denotes molecular size markers. The numbers on the left indicate the position and size of oligonucleotide standards. Reactions were carried out in the absence (lanes 1, 4) and presence (lanes 2, 5) of 1 unit of testis endonuclease. Lanes 3 and 6 show reactions carried out with T7 exonuclease and exonuclease III, respectively, which act as positive controls. Lane 7 shows the position of ATP.

DISCUSSION

In this study, we have purified and characterized a novel structure-specific endonuclease from rat testis. Initially, it was identified as an enzyme activity which catalyzes the degradation of single-stranded circular DNA but is inactive on linear duplex DNA. Its purification has showed that the enzyme activity is associated with a 42 kDa polypeptide. It does not possess the associated 5′−3′ or 3′−5′ exonuclease activity and generates ends having a 3′-hydroxyl and a 5′-phosphate. Detailed analysis of substrate specificity has revealed several interesting properties. First, testis endonuclease nicks supercoiled DNA. Second, it does not act upon linear single-stranded and duplex DNA. Third, it cleaves pseudo-Y, 5′-flap, 3′-flap, and 5′- and 3′-overhangs at or around the single-strand/duplex junctions. Finally, other branched DNA structures such as the Holliday junction, insertion loop, and stem−loop were not cleaved by the testis endonuclease (data not shown). These results suggest that it might belong to the class of structure-specific endonucleases. Although the testis endonuclease shares some of its characteristics with the well-known structure-specific endonuclease FEN1, it is distinct from FEN1 in several ways. (a) It does not possess associated 5′−3′ exonuclease activity. (b) It acts on both 5′- and 3′-flap structures. (c) Unlike FEN1, this endonuclease can cleave pseudo-Y, 5′- and 3′-overhangs with an equal efficiency as the 5′-flap structure. In addition, two very distinctive features of this endonuclease are its ability to cleave the bridge strand (Fbr) of the flap structures apart from the flap strand, and it does not require free single-stranded ends for cleaving branched structures. These observations indicate that this endonuclease activity might be mechanistically different from that of FEN1. It has been well documented in the case of FEN1 that free single-stranded ends are absolutely essential for its activity as the enzyme loads on the free ends and tracks along till it reaches the single-strand/duplex junction where it makes the incision. The crystal structure of FEN1 also supports this mechanism of action (*64*, *65*). It is quite likely that the testis endonuclease probably recognizes the single-strand/duplex junctions directly in the 5′, 3′ and pseudo-Y and then cleaves asymmetrically on both strands. We would also like to highlight one important observation here that while the kinetics of degradation of single-stranded circular DNA are very fast, the activity of this enzyme on branched structures is very sluggish. Such sluggish activity is also a hallmark feature of FEN1. Among the accessory proteins which have been identified so far, recently it was shown that the Werner protein stimulates FEN1 activity by 8−10-fold, indicating

FIGURE 9: Relationship between testis endonuclease and PTB. (A) Peptide sequences obtained by sequencing the tryptic peptides of 42 kDa testis endonuclease. (B) Rat PTB sequence. The region highlighted with pink color shows the peptide sequences obtained from the 42 kDa rat testis endonuclease. The green colored regions show the differences in the sequence between the two proteins. (C) Northern blot analysis of rat testis total RNA using rat PTB cDNA as probe. Lanes 1 and 2 have 30 and 50 μg of total RNA, respectively.

that accessory proteins modulate the activities of structure-specific endonucleases (*66*). Therefore, it remains to be seen whether there exist any such accessory proteins which might modulate the activity of testis endonuclease. The observation that free single-stranded ends are not required for the endonucleolytic activity of the testis endonuclease is very similar to the activity of the RAG1/RAG2 complex. This complex also removes single-stranded extension from a number of branched DNA intermediates It has been postulated that the 3′-flap endonucleolytic activity of the RAG1/RAG2 complex plays an important role in generating junctional and combinatorial diversity during V(D)J recombination (*58*). It is interesting to note that bacterial transposon Tn10 also possesses 3′-flap endonuclease activity, which might take part in transpositional recombination. Thus, the possible functional roles of the testis endonuclease (having overlapping but distinctive properties as compared to FEN1 and RAG1/RAG2 complex) need to be addressed in future studies.

The distinctive features of the 42 kDa testis endonuclease from those of the known structure-specific endonucleases prompted us to analyze its amino acid sequence. The results of the sequencing of the tryptic peptides were rather surprising as most of the peptide sequences matched with the polypyrimidine-tract binding protein (PTB), ruling out any relationship with structure-specific nucleases. This observation was intriguing but at the same time exciting. PTB has been identified as a 60 kDa protein that binds to the polypyrimidine tract preceding the 3′ splice site in the introns present in the pre-mRNA. It is a multifunctional protein having indispensable roles in the regulation of alternate splicing (*44*, *47*) and enhancement of translation from the internal ribosome entry site (IRES) (*48*, *49*, *67*−*69*). At the outset, it may look puzzling as PTB, shown to be an RNA binding protein, shows single-strand-specific endonucleolytic activity with DNA as substrate. In this context, we would like to point out that PTB does bind to DNA containing polypyrimidine stretches (*70*). Even FEN1, which was originally described to act on DNA structures, was later on shown to possess RNase activity also (*71*). At present, it is becoming increasingly clear that many of the enzymes can act both on DNA and on RNA substrates and thus take part in both DNA and RNA metabolism including DNA topoisomerase I (*39*).

One very important point that needs to be addressed is the difference in the sizes of PTB (60 kDa) and the PTB-related testis endonuclease (42 kDa). The sequences of all the peptides derived from the testis endonuclease could be accounted for in the PTB sequence (Figure 9). However, there were several changes observed in the sequence of peptides from that of PTB, which might have arisen from sequencing errors. Since all the peptide sequences matched the C-terminal two-thirds of PTB (Figure 9B) and PTB is known to have isoforms (*62*, *63*), an obvious question is, whether the testis endonuclease is one of the known isoforms of PTB. PTB has been reported to exist in atleast three isoforms with predicted molecular masses of 57, 59, and 60 kDa named PTB2, PTB1, and PTB4, respectively (*62*, *63*). PTB2 and PTB4 arise from PTB1 by insertion of 19 and 26 amino acids, respectively, at nucleotide position 921. The

original report of PTB3 having a molecular mass of 42.8 kDa (*63*) has not been confirmed. However, the testis endonuclease cannot be PTB3, since PTB3 originates from PTB1 with a frameshift mutation altering the entire C-terminal amino acid sequence.

At this juncture, we would like to raise an important question. Is there any relationship between the endonucleolytic activity of PTB or PTB-like protein and the splicing process of precursor mRNA? According to the present model proposed for the sequence reactions involved in mRNA splicing, the reaction consists of two phosphoryl-transfer steps. Initially, the 5′-intron phosphate (5′-splice site) is attacked by a 2′-hydroxyl specified within the intron. Subsequently, the 3′-intron phosphate (3′-splice site) is attacked by the 3′-hydroxyl of the cleaved exon, yielding the ligated exons and the excised intron in a branched lariat form. At present, the question of how this lariat structure is processed has not been answered. Based on our observation and the above discussion, we are tempted to speculate that PTB, which is a component of splicing machinery, might also be involved in the processing of the lariat intermediate using its endonucleolytic activity. We have recently cloned and expressed rat PTB in *E. coli*. The recombinant PTB did show some of the structure-specific endonuclease activity reported here (data not shown). It would be very interesting to examine the endonucleolytic activity of recombinant PTB with several model RNA substrates and also to systematically study the domain structure of PTB in relation to its multifunctional properties.

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