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A human DNA editing enzyme homologous to the *Escherichia coli* DnaQ/MutD protein

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Mammalian DNA polymerases α and β lack 3′ exonuclease activity and are unable to edit errors after DNA synthesis. However, editing exonucleases can be functions of separate polypeptides. We isolated a widely distributed DNA-specific 3′ exonuclease from rabbit liver nuclei, sequenced tryptic peptides by mass spectrometry, and identified the corresponding human open reading frame. The protein expressed from the cloned human sequence exhibits 3′ exonuclease activity. The human clone shares sequence homology with the editing function of the *Escherichia coli* DNA polymerase III holoenzyme, i.e., the DnaQ/MutD protein, and weakly with the editing 3′ exonuclease domain of eukaryotic DNA polymerase ε. The gene maps to human chromosome 3p21.2–21.3. In a reconstituted human DNA repair system containing DNA polymerase β and DNA ligase III-XRCC1, accurate rejoining of a 3′ mismatched base residue at a single-strand break is dependent on addition of the exonuclease.

Keywords: 3′ exonuclease/mammalian DNase III/mass spectrometry protein sequencing/repair and replication fidelity

Introduction

DNA polymerases typically incorporate one mismatched base per 4000–20 000 newly synthesized residues (Osheroff *et al*., 1999). Such a high error frequency would not be acceptable during DNA synthesis *in vivo*, and most DNA polymerases have a distinct 3′ exonuclease domain which edits the nascent DNA and immediately excises mismatched residues (Brutlag and Kornberg, 1972). Unexpectedly, two of the mammalian nuclear DNA polymerases, α and β, do not have such an intrinsic editing capacity (Wang, 1996; Wilson and Singhal, 1998). Instead, a separate 3′ exonuclease may work in concert with those proteins. A precedent for this situation is found in the replicative DNA polymerase III holoenzyme of *Escherichia coli* and other Gram-negative bacteria, where two different genes, *dnaE* and *dnaQ* (also called *mutD*), encode the separate functions for polymerization and exonucleolytic editing (Scheuermann and Echols, 1984).

Mammalian DNA polymerase β (Pol β) is required for gap-filling during base excision-repair of endogenous DNA damage, and enzyme-deficient mice exhibit an embryonic lethal phenotype (Sobol *et al*., 1996). A single nucleotide is usually replaced in this short-patch repair process (Kubota *et al*., 1996) with a misincorporation frequency of 1 residue in 4500 (Osheroff *et al*., 1999). Non-enzymatic hydrolytic depurination of DNA causes the loss of 9000 bases per day from the DNA of a mammalian cell (Nakamura *et al*., 1998), and a similar amount of DNA damage may occur as a consequence of intracellular oxidation and methylation events (Lindahl, 1993). Thus, the slow DNA turnover resulting from continuous repair of endogenous DNA damage by base excision-repair should be associated with misincorporation of several nucleotide residues into the genome by Pol β per cell per day. This situation seems incompatible with the ~10-fold lower spontaneous mutation frequency of mammalian cells (Drake *et al*., 1998). Consequently, a separate, unidentified DNA editing function is required during base excision-repair to compensate for error-prone synthesis by Pol β. Editing is obviously also needed during mammalian DNA replication; in this case the processive DNA polymerases δ and ε (Zlotkin *et al*., 1996) both have associated 3′ exonuclease activity, whereas editing during replication initiation by Pol α is poorly understood.

In an early survey, two distinct exonucleases in mammalian cell nuclei were observed to account for the major part of total exonucleolytic activity on DNA by cell extracts (Lindahl *et al*., 1969; Lindahl, 1971). They were identified as a 3′ exonuclease which acted preferentially on single-stranded DNA, and a 5′ exonuclease specific for double-stranded DNA that could also release single-stranded 5′ overhangs containing damaged residues as oligonucleotides. The enzymes were called DNase III and DNase IV, respectively. DNase IV has also been described as an essential replication factor required for trimming 5′ ends of Okazaki fragments during lagging-strand DNA replication (Ishimi *et al*., 1988; Waga and Stillman, 1998) and as a nuclease that could remove single-stranded flap structures by cleavage at the bifurcation point (Harrington and Lieber, 1994). In connection with the latter studies, DNase IV was renamed flap endonuclease 1/5′ exonuclease 1 (FEN-1), and the roles of this activity in recombination, repair, and trinucleotide expansion events have been investigated in several laboratories (Lieber, 1997). In contrast, there has been no recent interest in the properties of DNase III. Since this enzyme could potentially have an editing activity, we have purified it further to allow for cloning of a human cDNA encoding the protein. Human DNase III shows sequence homology with the proofreading DnaQ protein of the *E.coli* Pol III

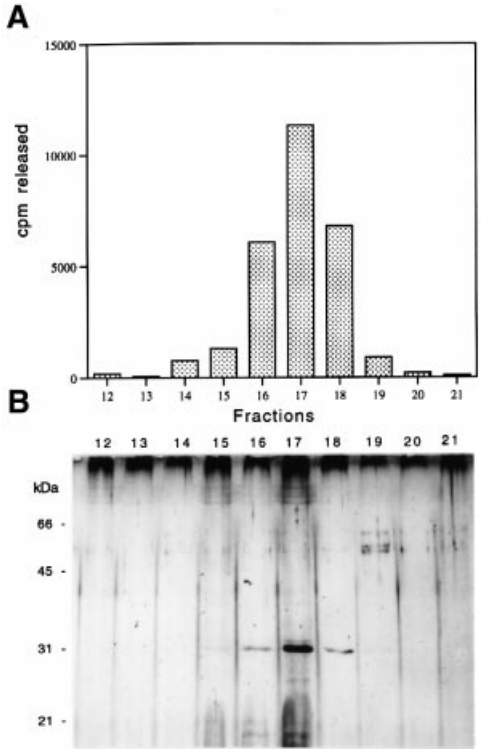

Fig. 1. Final purification of DNase III from rabbit liver nuclei by Mono S chromatography. Partially purified DNase III protein was applied to a SMART Mono S column. Adsorbed proteins were eluted from the column with a linear salt gradient, and all fractions assayed for 3′ exonuclease activity. Fractions containing enzyme activity are shown. (**A**) Aliquots (1 µl) of the fractions indicated were assayed for 3′ exonuclease activity as described in Materials and methods. (**B**) Aliquots (10 µl) of the fractions indicated were separated by SDS–PAGE (10%) and proteins detected by silver-staining. The positions and masses of protein size markers are indicated.

holoenzyme, as well as with the proofreading 3′ exonuclease domain of eukaryotic DNA polymerase ε, and has biochemical properties consistent with a role in DNA editing.

Results

Isolation of DNase III from cell nuclei

DNase III was first detected as a nuclear enzyme present in several rabbit tissues (Lindahl *et al*., 1969). Using rabbit liver nuclei and similar initial purification steps, followed by DNA–cellulose and mono S chromatography, an ~10 000-fold purified protein preparation has now been obtained with a single major polypeptide of ~31 kDa co-fractionating with the enzyme activity (Figure 1). In gel filtration experiments in the presence of 1 M NaCl, DNase III activity appeared in the 55–60 kDa region, indicating that native DNase III is a dimer.

Peptide sequencing by mass spectrometry and identification of a human cDNA sequence

After SDS–PAGE, the major stained band of purified protein (Figure 1) was excised and digested with trypsin. Peptide sequences of the small amounts of material available were then determined by *de novo* sequencing by low-energy collision-activated dissociation (CAD) using an ion-trap mass spectrometer after peptide derivatization with *N*-succinimidyl-2-morpholine acetate (Sherman *et al*., 1995). The complete sequence of a 10-residue peptide was SYS[L/I]GS[L/I]YTR and a partial sequence of a 13–14mer was PMYGATAT...SPR. BLAST 2 homology searches of a 6-frame translation of the dbEST database of expressed sequence tags (ESTs) (Boguski *et al*., 1993; Altschul *et al*., 1997) identified a human cDNA clone (IMAGE ID:1651428, accession No. AI091422) that encoded both homologous sequences within a large open reading frame (ORF). Further database searches revealed an apparently full-length cDNA clone (IMAGE ID:306966, accession No. N91973) which encoded a 32.3 kDa polypeptide.

Sequence homology with known DNA editing enzymes

The peptide sequence of 304 amino acids encoded by the human cDNA clone (Figure 2) showed weak but significant homology with the editing 3′ exonuclease domain of Pol ε (Kesti *et al*., 1993), but not detectably with any other known mammalian or yeast proteins in the current database. The homology with Pol ε was largely restricted to three short sequence motifs previously shown to be conserved among editing enzymes, containing magnesium-binding Asp and Glu residues required for 3′ exonuclease activity (Blanco *et al*., 1992; Barnes *et al*., 1995; Huang *et al*., 1997).

Surprisingly, more extensive homology with the human DNase III sequence was shown by the *E.coli dnaQ*/*mutD* gene product and related DNA editing functions from other Gram-negative bacteria (Figure 2). In this case, homology was particularly clear within the three exonuclease sequence motifs but also occurred throughout most of the protein sequences, except for the C-terminal regions. The C-terminal region of DnaQ is probably employed for protein–protein interactions, as deduced from the presence of a BRCT domain in this part of the DnaQ protein from *Mycobacterium tuberculosis* (Aravind *et al*., 1999), and this domain would be distinct from the catalytic region. These data indicate that DNase III has an editing 3′ exonuclease function similar to that of *E.coli* DnaQ and, like the bacterial enzyme, might act in concert with a DNA polymerase.

A TBLASTN (Altschul *et al*., 1997) search of the 6-frame translation of the DDBJ/EMBL/GenBank database using the DNase III ORF as a query revealed a second human peptide sequence ~35% identical with DNase III. This was part of a larger ORF located in a genomic clone from chromosome Xq28 (accession No. AF002998). Analysis of the exon/intron structure using GENSCAN (Burge and Karlin, 1997) predicted a gene comprising 15 exons, spanning 26 kb and encoding a protein of 853 residues. The same search also revealed a *Drosophila melanogaster* homologue (accession No. AC004375) with ~20% identity. Similar analysis predicts a 1.2 kb gene with two exons, encoding a protein of 351 residues.

The full-length human cDNA encodes an active 3′ exonuclease

The ORF encoding DNase III was amplified by PCR from the full-length cDNA clone. The PCR product was sub-cloned into an expression vector under control of an arabinose-inducible promoter, verified by DNA

Fig. 2. Alignment of the amino acid sequences of human DNase III, human and yeast DNA polymerase ε, and bacterial DnaQ/MutD proteins. Regions of homology of DNase III with three conserved core motifs present in editing 3′ exonucleases are indicated by asterisks. The two tryptic peptides in rabbit DNase III sequenced by mass spectrometry correspond to residues 166–175 (100% identity) and 221–234 (~70% identity) of the human sequence and are denoted by lines above the sequence. SwissProt accession Nos: Human DNA polymerase ε Q07864, *S.cerevisiae* DNA polymerase ε P21951, *E.coli* DNA polymerase III P03007, *S.typhimurium* DNA polymerase III P14566, *H.influenzae* DNA polymerase III P43745, *B.aphidicola* DNA polymerase III Q08880.

sequencing, and expressed in *E.coli*. An inducible system was chosen because of concerns that overexpression of an active nuclease could be lethal for the host cell. For efficient manipulation, the expressed protein was fused with a C-terminal Myc epitope and $(His)_6$ tag, yielding a fusion protein of 36.3 kDa. Upon arabinose induction, only small amounts of the protein were produced, possibly because of translational inefficiency; the polypeptide was readily detectable with antibodies to the Myc epitope but was difficult to see directly by Coomassie Blue staining after SDS–PAGE (Figure 3A and B). Moreover, the expressed protein was 98–99% insoluble in bacteria grown at 37°C, as well as at 25 or 15°C. The soluble fraction of the His-tagged protein was purified by chromatography on a nickel affinity column and was detected by staining at this stage (Figure 3A and B). The protein expressed from the cDNA sequence co-chromatographed with a 3′ exonuclease activity not present in extracts of uninduced *E.coli* cells, or containing the vector alone. Moreover, after mixing these DNase III fractions with either anti-His or anti-Myc antibody, the enzyme could be further purified as an active immune complex using protein A–Sepharose beads (Figure 3C). In further studies of the substrate specificity of this 3′ exonuclease, the enzyme expressed from the human cDNA sequence had apparently identical catalytic properties to the enzyme isolated from rabbit liver nuclei with a variety of substrates (see below and Figures 4 and 5).

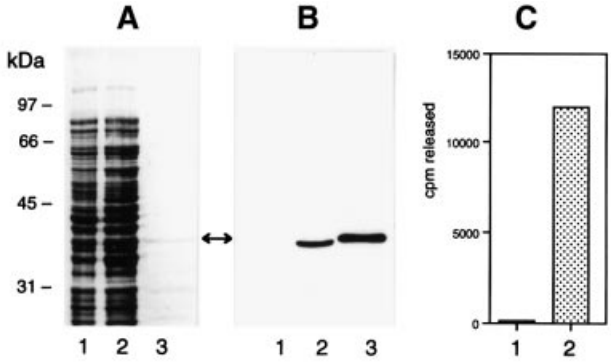

Fig. 3. Purification and assay of recombinant Myc-His-tagged human DNase III. The DNase III protein was overexpressed in *E.coli*, and the small amount of soluble material purified by chromatography on a Ni–NTA resin column. (**A**) Proteins separated by SDS–PAGE (10%) and detected by Coomassie Blue staining. Whole cell lysate (20 µg protein) from bacteria harbouring the expression construct before induction (lane 1) and after induction by 0.01% arabinose for 4 h at 37°C (lane 2). Lane 3 shows the peak fraction (0.2 µg) eluted with 0.25 M imidazole from a Ni–NTA column loaded with a crude extract from an induced culture (see Materials and methods). DNase III is indicated with an arrow. The positions and masses of protein size markers are indicated. (**B**) Immunoblotting with antibody against the Myc epitope. Protein fractions as in (A) were resolved by SDS–PAGE (10%) on a separate gel, transferred onto a nitrocellulose membrane, and probed with anti-Myc antibody. Antigen–antibody complexes were detected by enhanced chemiluminescence. DNase III and protein size markers are indicated as in (A). (**C**) Exonuclease activity. Lysates of bacteria harbouring the DNase III expression construct before induction (column 1) and after induction with arabinose (column 2) were affinity-purified by Ni–NTA chromatography, and further immunopurified using anti-Myc antibody and protein A–Sepharose beads (see Materials and methods). The immunocomplexes were assayed for exonuclease activity under standard conditions with 3′-^{32}P-labelled poly (dA) as substrate.

Chromosomal mapping and tissue distribution of DNase III

EST matches containing the DNase III sequence have been located on the current Human Genome Map (GM98; Deloukas *et al*., 1998). The gene encoding DNase III maps at chromosome 3p21.2–21.3. Flanking microsatellite

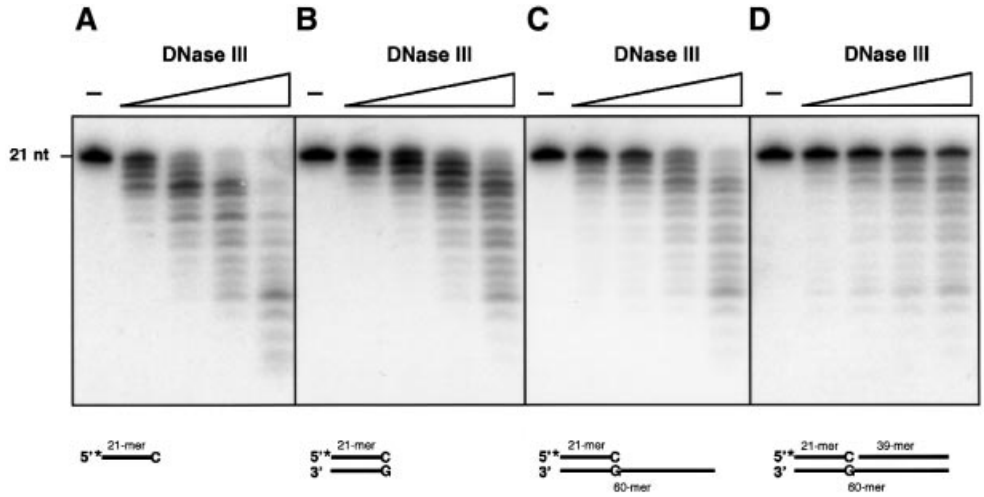

Fig. 4. Attack on various DNA end structures by DNase III. 5′-Radiolabelled single- and double-stranded DNA oligonucleotides with different conformations at the 3′-ends were incubated with increasing amounts of DNase III purified from cell nuclei (0.6, 1.5, 3 and 6 fmol). Reaction products were analysed on a phosphorimager after electrophoretic separation on denaturing 20% polyacrylamide gels. Degradation of the 21 nucleotide (nt) radiolabelled strand was measured compared with a control reaction containing no DNase III (–). The structure of the substrate oligonucleotide is depicted below each panel (**A**–**D**). Asterisks indicate the position of the ^{32}P-label.

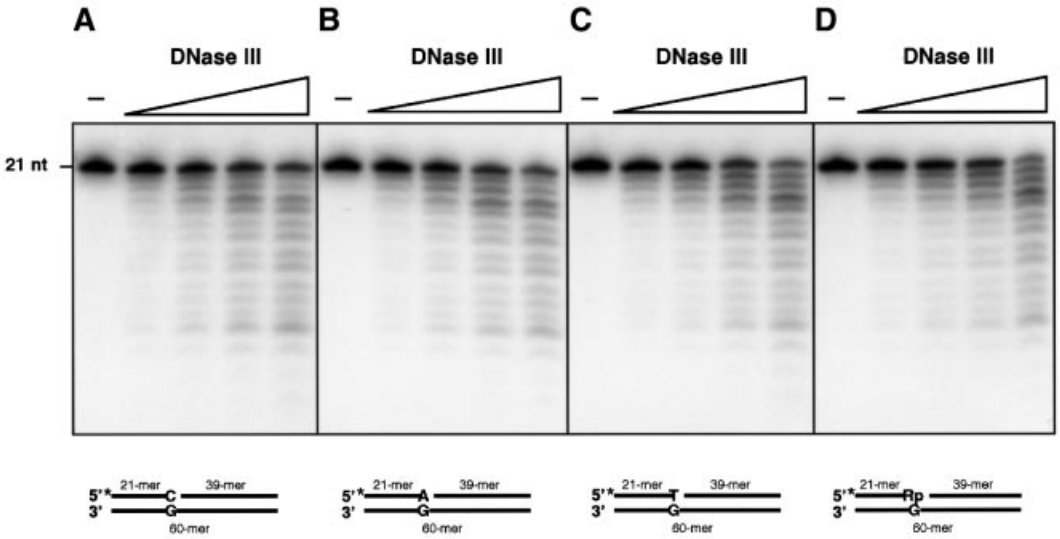

Fig. 5. Degradation of different matched and mismatched 3′-terminal residues at DNA single-strand interruptions by DNase III. The assay procedure was as in Figure 4. 'Rp' refers to a 3′-terminal deoxyribose-phosphate residue.

anchor markers are D3S3582 and D3S1588. The DNase III gene is located close to the gene encoding the ubiquitin protease proto-oncogene at 3p21.3 (Gray *et al.*, 1995). However, no known human oncogene or tumour suppressor gene seems to co-localize with the DNase III gene.

The human tissue distribution of DNase III transcripts was investigated by Northern blots (Clontech) using a radiolabelled DNase III cDNA probe. The mRNA of 1.15 kb was present at similar levels in thymus, spleen, liver, brain, heart, small intestine and colon (data not shown). Thus, DNase III transcripts were present in similar amounts both in proliferating and non-proliferating tissues.

The 3′ exonuclease function

DNase III degrades single-stranded DNA in a non-processive way from the 3′ terminus (Figure 4A). The enzyme also hydrolyzes double-stranded DNA at a rate three times slower using a substrate with a blunt or a recessed 3′-end (Figure 4B and C) and at a rate nine times lower using a substrate with an internal 3′-end (Figure 4D). Similar results were obtained with human DNase III expressed in *E.coli* and enzyme purified from rabbit liver nuclei. The enzyme could excise either a matched or a mismatched 3′-terminal residue at a single-strand break effectively (Figure 5A–C). A slight preference for a mismatched residue was apparent, which would be in agreement with the more efficient degradation of single-stranded DNA. DNase III does not detectably degrade RNA or poly(rA) (data not shown). Furthermore, it is unable to incise DNA endonucleolytically at abasic sites (<1% of the 3′ exonuclease activity, data not shown). However, the enzyme can excise a 3′-terminal base-free sugar-phosphate residue from DNA at a rate similar to a 3′-terminal mismatched nucleotide (Figure 5D). DNase III might be

one of several mammalian enzyme activities, in addition to the more abundant human apurinic-apyrimidinic endonuclease (HAP1/APE), which release such base-free 3′-terminal residues during repair of oxidized DNA (Klungland *et al*., 1999).

Exonucleases are not usually product-inhibited by mononucleotides, but the proof-reading *E.coli* DnaQ function is partly inhibited by millimolar concentrations of deoxyribonucleotides, with dGMP being most effective (Scheuermann and Echols, 1984). To investigate whether DNase III also has this unusual property, standard enzyme assays were performed in the presence and absence of nucleotides. Similarly to DnaQ, mammalian DNase III was also inhibited by mononucleotides, with ~40% inhibition by 2 mM dAMP or dGMP, whereas TMP and dCMP were less effective (data not shown). These results indicate that the partly homologous *E.coli* DnaQ and mammalian DNase III enzymes have retained similar active sites for interactions with their DNA substrates.

Reconstituted system for editing during DNA repair

A double-stranded oligonucleotide substrate with a 3′ mismatch at a single-strand break was employed for DNA rejoining and repair reactions *in vitro*. Addition of DNA ligase III-XRCC1 (Kubota *et al*., 1996) resulted in inefficient rejoining (Figure 6). Less than 0.2% of the nicked product was ligated under these experimental conditions (Figure 6, lane 3) which is in marked contrast to a 70% ligation efficiency when a similar substrate with a matched 3′ base pair was employed (Figure 6, lane 2). Supplementation of the reaction mixture containing the mismatched substrate with Pol β resulted in a slight increase of re-ligation efficiency to ~1% (Figure 6, lane 4). However, addition of increasing amounts of DNase III improved the ligation efficiency by up to 60-fold over that of DNA ligase III-XRCC1 and Pol β alone (Figure 6, lanes 5–9). This increase was due to excision of the mismatched residue followed by gap-filling with the correct nucleotide, as shown by digestion of the repaired substrate with the *Asp*718 restriction enzyme (Boehringer Mannheim) which requires a C/G match at position 21 of the substrate. The rejoined oligonucleotide had become sensitive to *Asp*718 cleavage, whereas the small amount of rejoined material observed in the absence of DNase III (Figure 6, lane 4) was refractory to restriction enzyme cleavage (data not shown). The maximum efficiency of rejoining of the substrate with a 3′ mismatch in the presence of DNase III (Figure 6, lane 8) was ~60% of that obtained with the substrate having a 3′ matched base-pair at the strand interruption (lane 2). These data show that DNase III excised the 3′ mismatched residue at the nick, and in some cases also one or a few additional nucleotides in a non-processive way (lanes 5 and 6), followed by gap-filling by Pol β and ligation. This mode of action by DNase III would be well suited to correcting incorporation errors by Pol β during DNA gap-filling.

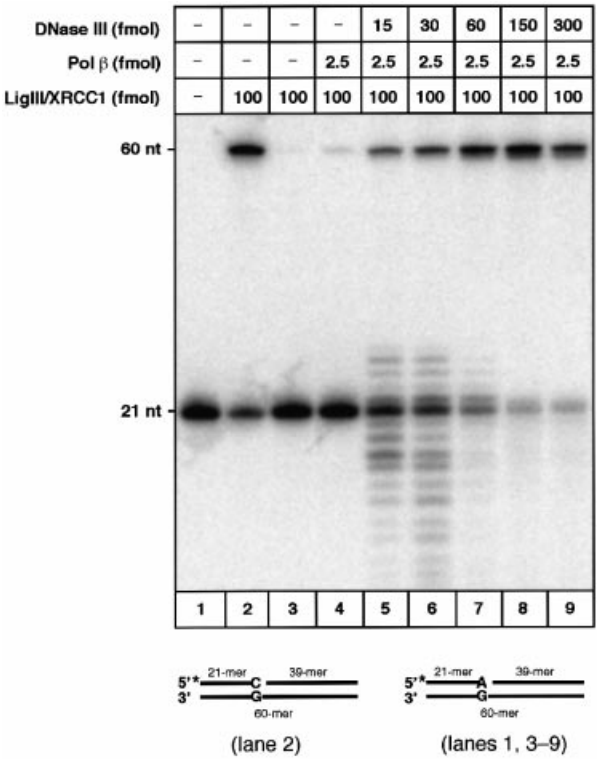

Fig. 6. *In vitro* proofreading activity of DNase III. 5′-Radiolabelled 60mer double-stranded DNA oligonucleotides containing an internal single-strand break in the top strand after position 21 were incubated with the human DNA ligase III-XRCC1 heterodimer, human Pol β, and increasing amounts of nuclear DNase III, as indicated. In lane 2, the DNA substrate contained a matched C/G base-pair at the internal strand break. For all other lanes, an oligonucleotide containing an A/G mismatch on the 3′ side of the strand interruption was used. The joining of the radiolabelled 21mer with the unlabelled 39mer to generate a labelled 60mer was analysed on a phosphorimager after electrophoretic separation on denaturing 15% polyacrylamide gels. The sizes (nt) of the reaction products are indicated.

Discussion

Physiological roles of 3′ exonucleases in human cell nuclei

Several mammalian 3′ exonucleases with intriguing functions have been cloned and investigated recently. The hMre11 component of the hRad50–hMre11–Nbs1 complex shows 3′ exonuclease activity on double-stranded DNA (Paull and Gellert, 1998) and is involved in repair of DNA double-strand breaks. Defects in the Nbs1 subunit of the protein complex results in the cancer-prone inherited human disease Nijmegen breakage syndrome, characterized by cellular hypersensitivity to ionizing radiation (Carney *et al*., 1998). The separate Werner syndrome gene, which was identified by a large-scale positional cloning effort (Yu *et al*., 1996), encodes a protein with DNA helicase and 3′ exonuclease activities (Kamath-Loeb *et al*., 1998) which plays an important role as a human tumour suppressor function also required to prevent premature cellular ageing. The *Schizosaccharomyces pombe* RAD1/*Saccharomyces cerevisiae* RAD17/*Ustilago maydis* REC1 protein has cell-cycle control and 3′ exonuclease functions; the recently isolated human homologue may also contain 3′ exonuclease activity (Freire *et al*., 1998; Parker *et al*., 1998). Moreover, the tumour suppressor gene product p53 has been reported to have intrinsic 3′ exonuclease activity (Mummenbrauer *et al*., 1996; Janus *et al*., 1999).

Several preliminary accounts of various partly purified 3′ exonucleases from mammalian cells have also appeared. Activities of particular interest are those that have been

observed to co-purify initially with DNA polymerases α and β. Bialek and Grosse (1993) detected and purified a form of Pol α from calf thymus extracts that was associated with a 3′ exonuclease, and both enzyme activities appeared to be contained within a 66 kDa polypeptide. In an earlier study, Skarnes *et al*. (1986) observed a similar 69 kDa exonuclease activity associated with human Pol α from HeLa cells. However, more recent investigations after cDNA cloning and expression of the 165, 70, 58 and 49 kDa subunits of Pol α have not confirmed the occurrence of an intrinsic exonuclease activity (Wang, 1996). A different exonuclease activity, termed DNase V, is due to an ~15 kDa protein that initially co-purifies with Pol β but chromatographs differently on DNA–cellulose. DNase V is a double-strand-specific bidirectional activity that degrades DNA both in the 3′→ 5′ and 5′→3′ directions (Mosbaugh and Meyer, 1980; Mosbaugh and Linn, 1983; Randahl *et al*., 1988). The enzyme can promote DNA nick-translation by Pol β *in vitro* but its physiological role appears uncertain, since this reaction is performed better by FEN-1/DNase IV together with proliferating cell nuclear antigen (PCNA) (Gary *et al*., 1999). The specificity of DNase V for accurately base-paired termini does not support a putative function as an editing enzyme.

When a direct biochemical approach was employed to isolate the major 3′ exonuclease acting on DNA from mammalian nuclear extracts, we found DNase III rather than hMre11 or other activities. In an independent and parallel effort, Mazur and Perrino (1999) recently purified and partly sequenced the major 3′ exonuclease activity from calf thymus extracts and identified the same homologous human ORF as the one investigated here. They also encountered similar difficulties in expressing the enzyme in soluble and active form in *E.coli*.

DNA editing by DNase III

The distinct sequence homology between mammalian DNase III and the DnaQ protein of the *E.coli* DNA polymerase III holoenzyme, as well as the 3′ exonuclease domain of eukaryotic Pol ε, indicates that DNase III serves an editing function. Gene knockout mice have not yet been constructed; however, they might be anticipated to have an increased spontaneous mutation frequency (Fijalkowska and Schaaper, 1996). Attempts to partly suppress the mutator phenotype of *E.coli dnaQ/mutD* mutants by transformation with the human cDNA encoding DNase III were not successful, probably because of the low level of expression of soluble DNase III and the dominant negative phenotype of *mutD* mutants (B.Sedgwick and T.Lindahl, unpublished data). The biochemical properties of DNase III are fully consistent with the properties expected of an editing enzyme, because the enzyme acts in a non-processive way on its DNA substrates, acts only unidirectionally at 3′-ends, and preferentially degrades single-stranded DNA. Double-stranded oligonucleotides are surprisingly stable in crude nuclear extracts of mammalian cells, but the 3′-terminal one or two nucleotide residues turnover relatively rapidly; a likely model for such idling is that the 3′-terminal residue(s) is excised by DNase III, followed by immediate resynthesis by a DNA polymerase. Similarly, a mismatched 3′-terminal residue can be excised at a DNA-strand interruption by DNase III, allowing for correct resynthesis and DNA ligation (Figure 6). By the same mechanism, a damaged 3′-end with a base-free residue can be removed (Figure 5D), and this might be a useful strategy for excision of 3′-terminal phosphoglycolates and similar lesions at single-strand breaks generated by oxygen free-radicals.

DNase III is present in similar amounts in the nuclei of non-growing cells, e.g. adult liver, as in rapidly proliferating tissues and cells. This is a very different distribution from that of replication factors such as Pol α, and strongly suggests that DNase III is not an editing function active exclusively during DNA replication. The more likely alternative is that DNase III fulfils the requirement for an editing function during base excision-repair by Pol β. In this regard, it is of interest that the latter steps of base excision-repair are quite different between yeast and mammalian cells (Klungland and Lindahl, 1997), and the apparent absence of a direct counterpart in *S.cerevisiae* of mammalian DNase III is also true for mammalian Pol β, XRCC1 and DNA ligase III.

Studies of DNA replication and repair in mammalian cell-free systems have, so far, mainly been concerned with the core reactions of synthesis, and excision of primers or damaged residues. The accessory factors that ensure high fidelity *in vivo* largely remain to be characterized. The identification here of a nuclear protein with the expected properties of an editing enzyme should be a step in this direction.

Materials and methods

Oligonucleotide and polynucleotide substrates

Poly(dA) (Pharmacia Biotech) was 3′-end labelled with [α-^{32}P]dATP and terminal transferase (Boehringer Mannheim). Oligonucleotides were prepared on a commercial DNA synthesizer, purified on a denaturing 20% polyacrylamide gel and annealed to complementary oligonucleotides as required (see Figures 4–6). To obtain the structures depicted in Figure 4, the 21mer 5′-TAGACATTGCCCTCGAGGTAC-3′ was 5′-end labelled by incubation with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs) and either used in its single-stranded form or annealed to the 21mer 5′-GTACCTCGAGGGCAATGTCTA-3′, the 60mer 5′-CGGAATTCGTCTAGGTTTGAGGTCGAAATCGGATCC-ATGGTACCTCGAGGGCAATGTCTA-3′, or the 39mer 5′-CATGGA-TCCGATTTCGACCTCAAACCTAGACGAATTCCG-3′, as indicated. To construct the DNA substrates shown in Figure 5, the 21mers 5′-TAGACATTGCCCTCGAGGTAC-3′, 5′-TAGACATTGCCCTCGAGG-TAA-3′, or 5′-TAGACATTGCCCTCGAGGTAT-3′ were 5′-end labelled and annealed to the 60mer and the 39mer, as indicated. The oligonucleotide containing a 3′-terminal deoxyribose-phosphate residue was made by 5′-end labelling of the 21mer 5′-TAGACATTGCCCTCGAGGTAU-3′, annealing to the 60mer and the 39mer as above and incubating with 100 fmol uracil–DNA glycosylase at 37°C for 5 min.

Purification of DNase III from cell nuclei

Livers (~1 kg) from 20 adult rabbits were removed and immediately immersed in 4 vol ice-cold 0.25 M sucrose/3.3 mM $CaCl_2$. Cells were disrupted in a Waring Blendor, the homogenate was filtered through several layers of gauze, and the crude nuclear fraction was collected by centrifugation at 2000 *g* for 20 min. The pellets were washed three times with 0.25 M sucrose/3.3 mM $CaCl_2$, resuspended in 0.25 M sucrose, and centrifuged at 6000 *g* for 10 min. The nuclei were resuspended in 2 vol 450 mM NaCl, 75 mM Tris–HCl pH 7.5, 1.5 mM EDTA, 3 mM dithiothreitol (DTT), 15% glycerol, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 25 μg/ml tosyl-lysine chloromethyl ketone (TLCK), 5 μg/ml chymostatin, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, and homogenized in a Dounce homogenizer before centrifugation at 25 000 *g* for 1 h. This crude nuclear extract was recovered and diluted with 2 vol buffer A (50 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol). A slurry of phosphocellulose P11 (Whatman) pre-equilibrated with

buffer A (0.3 vol) was added and the suspension was stirred slowly for 1 h. The material was poured into a column, washed extensively with buffer A containing 100 mM NaCl, and the enzyme activity eluted with buffer A containing 400 mM NaCl. Ammonium sulfate was added to the protein solution to 55% saturation, followed by gentle stirring for 30 min. After centrifugation at 20 000 *g* for 20 min, the pelleted protein was recovered and resuspended in buffer B (100 mM NaCl, 50 mM Tris–HCl pH 7.5, 1 mM DTT, 1 mM potassium phosphate, 10% glycerol) to a concentration of ~5 mg/ml, and dialysed against buffer B. Insoluble material was removed by centrifugation and the protein solution was passed through a Bio-Gel HT hydroxyapatite column (Bio-Rad; 1 ml bed per 5 mg protein) pre-equilibrated with buffer B. Most of the protein adsorbed to the hydroxyapatite, but the exonuclease activity was recovered in the flow-through fraction and loaded directly onto a denatured DNA–cellulose column (Pharmacia Biotech, one-twentieth the volume of the previous column) which had been pre-equilibrated with buffer B. The column was washed with buffer A containing 100 mM NaCl and then with buffer A containing 200 mM NaCl. An active exonuclease fraction was eluted with buffer A containing 800 mM NaCl, and then dialysed against buffer A containing 50 mM NaCl. The protein was recovered and loaded onto a SMART Mono S PC 1.6/5 column (Pharmacia Biotech) pre-equilibrated with buffer A containing 50 mM NaCl. Proteins were eluted with a 2 ml linear gradient of NaCl (50–400 mM) in buffer A. The exonuclease activity was eluted at 190 mM NaCl. The purified enzyme was stored in aliquots at –80°C and was stable for at least 6 months.

Bacterial expression and purification of cloned human DNase III

The human ORF encoding DNase III was amplified by PCR using *Pfu* DNA polymerase (Stratagene) and the oligonucleotides 5′-GAAGATCTCAGACCCTCATCTTTTTCGACATG-3′ (sense) and 5′-GCTCTAGAAACTCCCCAGGTGTGGCCAGGGATAG-3′ (antisense). The amplified fragment was subcloned in-frame as a *Bgl*II–*Xba*I restriction fragment (restriction sites underlined) into the multiple cloning site of the pBAD/Myc-His vector (version B; Invitrogen) with the 5′-end proximal to the araBad promoter and verified by DNA sequencing. The plasmid carrying the DNase III ORF was used to transform *E.coli* TOP10 (Invitrogen).

Recombinant Myc-His-tagged DNase III protein was expressed in *E.coli* TOP10 under control of the araBad promoter. Cultures were grown at 37°C to an OD_{600} of 0.5 and expression induced by addition of arabinose to a final concentration of 0.01% and incubation for a further 4 h at 37°C. After centrifugation at 10 000 *g* for 20 min, the cell pellet (4 g) was resuspended in 20 ml sonication buffer: 500 mM NaCl, 50 mM Tris–HCl pH 8.0, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM imidazole and 1 mM PMSF. The cells were disrupted by ultrasonic treatment and the extract clarified by centrifugation at 15 000 *g* for 20 min. Soluble proteins in the supernatant were batch adsorbed to 2 ml Ni–NTA resin (Qiagen) pre-equilibrated with sonication buffer at 4°C for 1 h. The resin was then packed into a column and washed extensively with sonication buffer. Proteins were eluted from the column stepwise with 50 mM Tris–HCl pH 7.0, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol containing increasing amounts of imidazole: 40 mM, 80 mM and 250 mM. Fractions of each elution were assayed for 3′ exonuclease activity and subjected to immunoblotting with anti-Myc antibody. Fractions with activity that were recognized by the antibodies (250 mM imidazole eluate) were pooled, aliquoted and frozen at –80°C.

For immunoblotting, proteins were subjected to SDS–PAGE (10%), transferred onto nitrocellulose membranes (Schleicher and Schuell) and detected by immunostaining with diluted antibody. The C-myc antibody (ICRF 9E10.F5, directed against the leucine zipper epitope) was used at 1:2000. Antigen–antibody complexes were detected by enhanced chemiluminescence using a derivatized secondary antibody (ECL; Amersham).

For immunopurification of DNase III (from 250 mM imidazole eluate, Ni–NTA) to remove remaining traces of *E.coli* nucleases, 0.5 ml aliquots were mixed with either 10 µl aliquots of anti-Myc antibody or anti-His antibody (Clontech, 1:4000), and incubated at 0°C for 1 h. Immunocomplexes were affinity-purified on protein A–Sepharose beads 4 Fast Flow (Pharmacia Biotech) at 4°C for 1 h with continuous mixing. The protein A–Sepharose beads were then washed extensively with 150 mM NaCl, 50 mM Tris–HCl pH 8.0 containing 1% Nonidet P-40. Protein A–Sepharose-bound immunocomplexes were subsequently assayed for 3′ exonuclease activity.

Peptide sequencing

Following SDS–PAGE (Figure 1) the major protein band was excised from the gel and digested with trypsin, essentially according to Shevchenko *et al*. (1996). Following overnight digestion, peptides were extracted with 5% formic acid and acetonitrile. The dried peptides were derivatized with *N*-succinimidyl-2-morpholine acetate to facilitate *de novo* sequence analysis by tandem mass spectrometry (Sherman *et al*., 1995). Dried peptide fractions were treated with 7 µl freshly prepared, ice-cold 1% *N*-succinimidyl-2-morpholine acetate in 1 M HEPES–NaOH pH 7.8, containing 2% acetonitrile. After 20 min on ice, the reaction was terminated by the addition of 1 µl heptafluorobutyric acid and diluted with an equal volume of water. The solution was then injected in 3× 5 µl aliquots onto a capillary reverse-phase column (300 µm×15 cm) packed with POROS R2/H material (Perseptive Biosystems, MA) equilibrated with 2% methanol/0.05% trifluoroacetic acid running at 3 µl/min. The adsorbed peptides were washed isocratically with 15% methanol/0.05% trifluoroacetic acid for 30 min at 3 µl/min to elute the excess reagent and HEPES buffer. Derivatized peptides were eluted with 75% methanol/0.1% formic acid and collected in two 3 µl fractions. The derivatized peptides were then sequenced *de novo* (Hunt *et al*., 1986) by low-energy CAD using a Finnigan MAT LCQ ion-trap mass spectrometer fitted with a nanoelectrospray source. CAD was typically performed with collisional offset voltages between –20 and –35 V.

Enzyme assays

For standard assays during enzyme purification, reaction mixtures (50 µl) contained 0.01 µg 3′-^{32}P-labelled poly(dA) (50 000 c.p.m.) in 50 mM Tris–HCl pH 8.5, 4 mM $MgCl_2$, 1 mM DTT, 5 µg bovine serum albumin (BSA), and a limiting amount of enzyme. After incubation at 37°C for 30 min, the samples were chilled to 0°C and 50 µg carrier DNA (denatured calf thymus DNA) added to each reaction. The DNA was then precipitated with ethanol, and the radioactivity in the supernatants was determined by scintillation counting.

In experiments with oligonucleotide substrates, reaction mixtures (10 µl) contained 140 fmol oligonucleotide, 45 mM HEPES–KOH pH 7.8, 70 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.4 mM EDTA, 1 µg BSA and enzyme as indicated. Incubations were at 37°C for 30 min, and were terminated by dilution to 100 µl with H_2O, addition of tRNA carrier to 0.5 mg/ml, phenol–chloroform extraction, and ethanol precipitation. This material was resolved by 20% denaturing polyacrylamide gel electrophoresis and visualized and quantified on a phosphorimager.

Reconstituted system for editing during DNA repair

Reaction mixtures (10 µl) were as above but contained 100 mM KCl and were supplemented with 2 mM ATP, 20 µM each of dATP, dGTP, dTTP and dCTP, and purified enzymes as indicated in Figure 6. The DNA ligase III-XRCC1 and Pol β reagent enzymes were described previously (Kubota *et al*., 1996). Reactions were carried out and analyzed as above, except 15% denaturing polyacrylamide gels were used for electrophoresis.

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