Purification, Characterization, Gene Cloning, and Expression of *Saccharomyces cerevisiae* Redoxyendonuclease, a Homolog of *Escherichia coli* Endonuclease III[†]

Laura Augeri,[‡] Yeuk-Mui Lee,[§] Arnold B. Barton,[||] and Paul W. Doetsch*,[‡,§]

Department of Biochemistry and Division of Cancer Biology, Department of Radiation Oncology, Emory University School of Medicine, Atlanta, Georgia 30322, and Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey 07103

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ABSTRACT: *Saccharomyces cerevisiae* redoxyendonuclease (Scr), a homolog of *Escherichia coli* endonuclease III, was purified from yeast deficient in the major apurinic/apyrimidinic endonuclease, Apn1. Studies of this highly purified preparation of Scr have revealed a number of similarities between this protein and endonuclease III as well as provided further evidence for a common mechanism of action for this class of DNA glycosylase/AP lyases. We have employed a sensitive and specific assay for Scr which utilizes oligonucleotide substrates containing a single 5,6-dihydrouracil base lesion or an abasic site. These substrates were utilized to investigate the mode of action of Scr on damaged DNA and to compare the kinetic properties of the yeast enzyme with its *E. coli* counterpart. Furthermore, we have identified two distinct genes, *SCR1* and *SCR2*, which encode highly homologous proteins with similar activities in yeast. Analysis of the deduced amino acid sequences of *SCR1* and *SCR2* suggests that *S. cerevisiae* possesses two similar enzymes encoded on separate chromosomes: one which bears an Fe-S binding motif, while the other does not. The potential biological roles of these two forms of Scr are discussed.

Saccharomyces cerivisiae is an attractive source from which to purify and characterize eukaryotic DNA repair proteins. It offers a system from which one can obtain large numbers of cells for the purification of low-abundance proteins. *S. cerevisiae* is also well-established as a genetically amenable model system for the study of DNA repair proteins and the pathways in which they participate (Friedberg, 1991; Prakash, 1993). In addition, the availability of the yeast genomic DNA sequence has greatly facilitated the identification of candidate genes encoding DNA base-excision repair proteins (Hilbert *et al.*, 1996; Roldan-Arjona *et al.*, 1996; Van Der Kemp *et al.*, 1996).

S. cerevisiae redoxyendonuclease (Scr)[1] is a functional homolog of the *Escherichia coli* enzyme, endonuclease III, and has been shown to act on damaged DNA via combined *N*-glycosylase/AP (apurinic/apyrimidinic) lyase activities as the initial step in the base excision repair (BER) pathway (Gossett *et al.*, 1988; Augeri *et al.*, 1994). Upon damage recognition, Scr cleaves the N-glycosidic bond of the damaged base to produce an AP (apurinic/apyrimidinic) site (Gossett *et al.*, 1988; Augeri *et al.*, 1994). This AP site may be further processed by Scr, which is postulated to cleave the phosphodiester backbone at the damage site via a β-elimination mechanism to generate a 3′-terminal α,β-unsaturated aldehyde (4-hydroxy-2,3-*trans*-pentenal) and a 5′-phosphoryl terminus (Gossett *et al.*, 1988; Augeri *et al.*, 1994). Scr is thought to recognize and process a wide variety of base damage products caused by oxidative and reductive processes including products generated by UV light, such as pyrimidine photohydrates, and ionizing radiation products, such as thymine glycol, dihydrothymine, and dihydrouracil (Gossett *et al.*, 1988; Augeri *et al.*, 1994). Scr will act on DNA base lesions characterized by loss of aromaticity, including ring saturation, ring contraction, and ring cleavage products (Gossett *et al.*, 1988; Augeri *et al.*, 1994). It is also suspected that this enzyme will process a monobasic guanine photoproduct produced by UV irradiation, now known to be 2,6-diamino-4-hydroxy-5-formamidopyrimidine (fapy-Gua) (Gossett *et al.*, 1988; Augeri *et al.*, 1994; Doetsch *et al.*, 1995).

In previous studies, Scr has been partially purified from dried bakers' yeast and was estimated to be a protein of approximately 40 kDa (Gossett *et al.*, 1988; Augeri *et al.*, 1994). Scr has no apparent requirement for divalent cations, and it is stable and active in 10 mM EDTA (Gossett *et al.*, 1988). Measurements of Scr specific activity during the purification of this enzyme have been imprecise due to the use of supercoiled plasmid nicking assays which are subject to interference by a variety of DNA processing proteins (Gossett *et al.*, 1988; Augeri *et al.*, 1994). In this study, we have utilized a duplex oligonucleotide substrate containing a single dihydrouracil (DHU) for specific activity measurements of Scr during purification and subsequent kinetic analysis. DHU is formed by deamination of cytosine with subsequent ring saturation upon exposure to ionizing radiation under anoxic conditions. Unlike many known substrates of Scr and endonuclease III, DHU is relatively stable to the pH conditions necessary for solid phase oligonucleotide

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* Corresponding author.

[‡] Department of Biochemistry, Emory University School of Medicine.

[§] Department of Radiation Oncology, Emory University School of Medicine.

[||] University of Medicine and Dentistry of New Jersey.

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[1] Abbreviations: DHU, 5,6-dihydrouracil; FPLC, fast-protein liquid chromatography; EIII, endonuclease III; NT, no treatment; Scr, *Saccharomyces cerevisiae* redoxyendonuclease; MWCO, molecular weight cutoff; IPTG, ispropyl β-D-thiogalactoside; PMSF, phenylmethanesulfonyl fluoride; fapy-Gua, 2,6-diamino-4-hydoxy-5-formamidopyrimidine; PAGE, polyacrylamide gel electrophoresis; LB, Luria broth; PCIA, phenol−chloroform−isoamyl alcohol; BER, base-excision repair; AP, apurinic or apyrimidinic; TE, Tris−EDTA; HE, HEPES−EDTA; YPD, yeast extract−peptone−dextrose growth media.

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synthesis, and it appears to be a specific substrate for this enzyme.

We have carried out an extensive purification of Scr and have compared its kinetic properties to those of *E. coli* endonuclease III. Inhibition studies were conducted with modified bases as well as NaCN and $NaCNBH_3$ in order to probe the potential mechanism of action of Scr. We also report the identification of two *S. cerevisiae* genes (*SCR1 and SCR2)* that encode proteins Scr1 and Scr2, which possess a high degree of amino acid sequence similarity to each other and include hallmark features of endonuclease III and its homologs, both structurally and functionally (Hilbert *et al.*, 1996). Parallel purifications of Scr activity from extracts generated from wild-type and from *scr1* mutant cells show that there are two distinct activities in yeast which cleave dihydrouracil-containing DNA in a similar fashion. The gene product encoded by *SCR1* (and presumably, *SCR2*) shows enzymatic activity similar to both endonuclease III and the activity we have purified from *S. cerevisiae*.

MATERIALS AND METHODS

Yeast Strains. Δ*APN1* mutants (*MAT*α *his3*Δ*200 ura3-52 leu2*Δ*1* Gal^+) were obtained from the late Dr. Davis Chen (Emory University). Wild type yeast (*SJR328*) was obtained from Dr. Sue Jinks-Roberstson (Emory University). *FUN33* (*SCR1)* disruption mutant (*Dfm::leu2 MAT*α *leu2-3 his3-11 ade1 trp1*) and the corresponding parental, wild-type strain (*MAT*α *leu2-3 his3-11 ade1 trp1*) were generated as previously described (Barton & Kaback, 1994). All yeast strains were grown at 30 °C until the start of late log phase in YPD media. Large-scale growth of yeast was carried out at the University of Georgia (Athens) Fermentation Facility by Dr. Ronald Makula.

Reagents and Kits. SP Sepharose fast-flow resin was obtained from Pharmacia. Phosphocellulose (P11) and DEAE-cellulose (DE52) were obtained from Whatman. Single-stranded DNA−cellulose was obtained from Sigma. Yeast extract and peptone were obtained from Difco. α-D-Glucose (dextrose), NaCN, and $NaCNBH_3$ were obtained from Aldrich. Radioisotopes, [α-^{32}P]ddATP (3000 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham. All other chemicals were of the highest grade commercially available**.** TA cloning kit was purchased from Invitrogen. The pET expression system was obtained from Novagen, Inc.

Oligonucleotide Substrates. The dihydrodeoxyuridine dimethoxytrityl-blocked phosphoramidite building block was synthesized by Glen Research, Sterling, VA. Dihydrouracil (DHU)-containing oligomer (DHU-37mer) with the sequence 5′-CTT-GGA-CTG-GAT-GTC-GGC-AC**X**-AGC-GGA-TAC-AGG-AGC-A-3′ (X = DHU) and its complementary strand (G is opposite to DHU) were synthesized by Research Genetics, Birmingham, AL. Undamaged 37mer (UND-37mer, X = C) and uracil-containing 37mer (URA-37mer, X = uracil) of the same sequence as the DHU oligomer were synthesized by the Emory University Microchemical Facility.

Oligonucleotides were either 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (Tabor, 1989) or 3′-end-labeled with terminal transferase and [α-^{32}P]ddATP as previously described (Tu *et al.*, 1980). End-labeled single-stranded DNA was gel-purified on a denaturing, 7 M urea, Tris/borate/EDTA 20% polyacrylamide gel (Maniatis *et al.*, 1982). Purified single-stranded oligonucleotide was annealed in 10 mM Tris, pH 7.5, 1 mM EDTA (TE) plus 100 mM NaCl by heating to 70 °C and cooling slowly to room temperature in a heat block. Duplex oligonucleotides were gel-purified on a 20% nondenaturing polyacrylamide gel. DNA was resuspended in either TE buffer or 10 mM HEPES−KOH, pH 8.0, 2 mM EDTA (HE) and stored at −20 °C.

End-labeled, duplex URA-37mer (20−50 pmol) was incubated with uracil−DNA glycosylase (6 units) for 30 min at 37 °C in UDG buffer (30 mM HEPES−KOH, pH 7.5, 1 mM EDTA, 50 mM NaCl) to generate the abasic site-containing oligonucleotide, AP-37mer. AP-37mer was extracted with PCIA (phenol−chloroform−isoamyl alcohol, 29:19:1, v/v/v, equilibrated with HE buffer and 0.1% 8-hydroxyquinoline) and was evaluated for its AP site content by cleavage with alkali (1.0 M piperidine) at 90 °C for 20 min. Reaction products were electrophoresed on 20% denaturing (7 M urea) polyacrylamide gels (DNA sequencing gels), which were subjected to autoradiography. DNA strand scission product formation was determined by phosphorimaging (Molecular Dynamics Model 445 SI).

Enzymes. E. coli endonuclease III was a gift from from Dr. Richard Cunningham (SUNY−Albany). Restriction enzymes and terminal transferase were obtained from Promega. T4 polynucleotide kinase was obtained from New England Biolabs. Uracil−DNA glycosylase was obtained from Epicentre Technologies.

Expression of FUN33 Protein in E. coli. The *FUN33* (*SCR1*) gene sequence was amplified by PCR from plasmid DNA containing the *TBD3-DEP1* intergenic region of chromosome I in yeast (Barton & Kaback, 1994) using the following primers: 5′-ATGATTCATCATATGTTTGTATC-3′,5′-ACAACTAGGCCTGAATTCTTTTC-3′. The resulting PCR products were subsequently inserted into the TA cloning vector and electroporated into *E. coli* JM109 competent cells. Recombinant plasmids of the correct orientation were selected, purified, and restricted with *Nde*I and *Eco*RI. The resulting product was ligated into the pET22b expression vector to generate pET22b-*FUN33* which was used to tranform *E. coli* BL21 (DE3) cells. Recombinant clones were selected for the *FUN33* insert, and a single colony was selected and grown in a 10 mL culture overnight. This culture was used to inoculate a 1 L culture in LB plus ampicillin (50 mg/L). Growth was monitored hourly and induced with 1.0 mM isopropyl thiogalactoside (IPTG) when cells reached an optical density of 0.4 AU at 600 nm. Samples were collected hourly during the induction period up to 4 h, after which the remaining cells were harvested by centrifugation and the cell pellets were resuspended in 20 mL of buffer B plus 50 mM NaCl. Cells were lysed by sonication, and extracts from cells expressing *FUN33* and those which contained the pET22b vector alone (without insert) were fractionated by Mono S FPLC. Proteins were eluted with a 0.05−1.0 M (20 mL × 20 mL) NaCl linear gradient, and fractions were assayed for Scr activity using DHU-37mer.

Enzyme Assays. (A) Identification of Scr Activity. End-labeled damaged DNA substrates were incubated with various amounts of Scr or endonuclease III in buffer B (15 mM KH_2PO_4, pH 6.8, 10 mM EDTA, and 10 mM β-mercaptoethanol plus 40 mM KCl) for 45 min at 37 °C. DNA was extracted with PCIA and ethanol-precipitated. DNA digested with either Scr or endonuclease III was resuspended in loading dye containing 80% deionized formamide and

electrophoresed on DNA sequencing gels.

(B) Specific Activity Assays. Parameters for specific activity assays were determined utilizing endonuclease III as a model base excision repair enzyme in order to establish the conditions and reproducibility of this method. Duplex DHU-37mer (500 fmol) was incubated with varying amounts of Scr or endonuclease III (25 pg) in a 50 μL reaction containing buffer B plus 40 mM KCl at 37 °C for 20 min (endonuclease III) or 30 min (Scr). Peak Scr fractions pooled at each stage of purification were titrated in order to identify an enzyme amount that would cleave 10−30% (in the linear range of the assay) of DHU-37mer. Reactions were performed in triplicate with the amount of enzyme specified by titration results. Endonuclease III reactions containing 25 pg of enzyme were used as an internal control for each assay to ensure consistent reaction conditions and electrophoretic separations were attained with each assay performed. One unit of enzyme activity is defined as 1 fmol of DNA strand scission product generated/min under these assay conditions.

(C) Kinetic Assays. Enzyme incubations were carried out with 33.6 ng of Scr fraction VI-B, 6.3 μg of Scr fraction VI-A, or 25 ng of endonuclease III in a final reaction volume of 25 μL. Substrate concentrations were varied (50−300 nM, 3′-end-labeled DHU-37mer or AP-37mer); incubations were carried out in buffer B plus 40 mM KCl for 0−10 min. Reactions were terminated either by PCIA extraction and ethanol precipitation or by the addition of formamide loading dye for direct loading onto DNA sequencing gels. Data points from a single reaction time (60 s) were plotted and analyzed using the Enzfitter kinetic program (Leatherbarrow, 1987).

(D) Enzyme Inhibition Enzyme Assays and Enzyme−Substrate Complex Formation. Scr (16.8 ng fraction VI-A) or endonuclease III (100 pg) was reacted with 3′-end-labeled duplex DHU-37mer in buffer B plus variable amounts of the following potential inhibitors: dihydrodeoxyuridine (0.1−50 mM), dihydrouracil (0.1−50 mM), thymine glycol (0.1−50 mM), NaCl (40−300 mM), NaCN (40−300 mM) or $NaCNBH_3$ (50 mM). Reactions (200 μL) using $NaCNBH_3$ to generate irreversibly cross-linked enzyme−substrate complexes were carried out for 1 h at 37 °C. Products were concentrated and resuspended in water several times in Spin X-UF filters (Corning-Costar, MWCO = 3000) to remove $NaCNBH_3$. Recovered samples were loaded onto 12% SDS−PAGE gels (Bio-Rad Protean II). Gels were fixed in 10% acetic acid, 40% methanol, and 50% water (v/v/v), silver-stained (Bio-Rad Silver Stain Plus), and subjected to autoradiography or phosphorimager analysis.

Purification of Scr. (A) Generation of Crude Extracts for Large-Scale Purification, Small-Scale Purification, and Microscale Lysis of Yeast. A large-scale growth of Δ*APN1* yeast yielded 3 kg of packed cells from a 400 L culture grown to the onset of late log phase. Yeast cells were suspended in an equal volume of buffer containing 50 mM KH_2PO_4, pH 8.7, 0.2 M phenylmethanesulfonyl fluoride (PMSF), and 1.0% dimethyl sulfoxide (buffer Y) plus 0.7 M NaCl. This cell suspension was pumped through a continuous-flow Dynomill (Glenn Mills, Inc.) which contains an enclosed chamber of glass beads. The cell extract (fraction I) was collected and stored at 4 °C. Small batches of yeast cell suspension (10−200 g of cells) were processed in a Bead Beater (Biospec Products, Bartlesville, OK) in which an equal volume of 0.5 mm glass beads was added to the cell suspension. The mixture was subjected to four, 1 min spin bursts and held on ice for 5 min between bursts. Crude extracts generated from small yeast cultures (<1.0 g) were disrupted by vortexing (six, 30 s rounds) cell suspensions with an equal volume of glass beads in microfuge tubes and held on ice for 60 s between bursts. Cell debris was removed by centrifugation as previously described (Gossett *et al.*, 1988; Augeri *et al.*, 1994).

(B) Ammonium Sulfate Precipitation. Fraction I, obtained from the large-scale preparations, was subjected to a 65% ammonium sulfate precipitation. The resulting precipitate contains the majority of Scr activity. The ammonium sulfate precipitate was divided into separate batches and stored at −80 °C where it is stable for at least 1 year. This material (fraction II) was processed in 100 g batches.

(C) DEAE-Cellulose Removal of Nucleic Acids. DEAE batch separation was performed to remove nucleic acids. Batches of fraction II were resuspended in buffer A (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mM β-mercaptoethanol) plus 0.3 M NaCl and extensively dialyzed against this buffer. The dialyzed preparation was mixed for 1 h with preswelled DE52 resin which was removed by low-speed centrifugation. Under these conditions, the majority of proteins, including Scr, will remain unbound while nucleic acids are removed (Gossett *et al.*, 1988; Augeri *et al.*, 1994). The supernatant (fraction III) was utilized for phosphocellulose chromatography.

(D) Phosphocellulose Chromatography. Fraction III (55 g) was dialyzed extensively against buffer A plus 50 mM NaCl and applied to a phosphocellulose column (5.0 × 75.0 cm) equilibrated with the same buffer. The column was washed until the protein absorbance measurements at 280 nm were close to zero. Protein that was retained by the column was eluted with a 0.05−1.0 M NaCl linear gradient (1000 mL × 1000 mL) in buffer A. Scr elutes from the column between 0.2 and 0.4 M NaCl, and peak fractions were pooled and concentrated (fraction IV).

(E) SP−Sepharose Chromatography. Fraction IV (6.5 g) was dialyzed against buffer B plus 50 mM NaCl and applied to an SP−Sepharose fast-flow column (3.75 × 60.0 cm) equilibrated in the same buffer**.** The flow-through material was collected, and the column was washed until the absorbance at 280 nm was close to zero. Protein that was retained by the column was eluted with a 0.05−1.0 M NaCl linear gradient (500 mL × 500 mL). Fractions containing Scr activity eluted between 0.35 and 0.6 M NaCl and were pooled and concentrated (fraction V).

(F) Single-Stranded DNA−Cellulose Chromatography. Fraction V was dialyzed against buffer B plus 50 mM NaCl and applied to a single-stranded DNA−cellulose column (2.5 × 20 cm) equilibrated in the same buffer. The column was washed until the absorbance at 280 nm was near zero. Protein retained by the column was eluted with step washes of buffer B plus 0.3, 0.5, 0.7, and 1.0 M NaCl. Two pools of Scr activity of different specific activity were collected at 0.7 and 1.0 M NaCl (fractions VI-B and VI-A, respectively).

SDS−PAGE. SDS−PAGE was performed in a discontinuous reducing buffer system according to the methods described (Laemmli *et al.*, 1970).

RESULTS

Determination of Yeast Strain for SCR Purification. For large-scale yeast growth, a number of yeast strains were

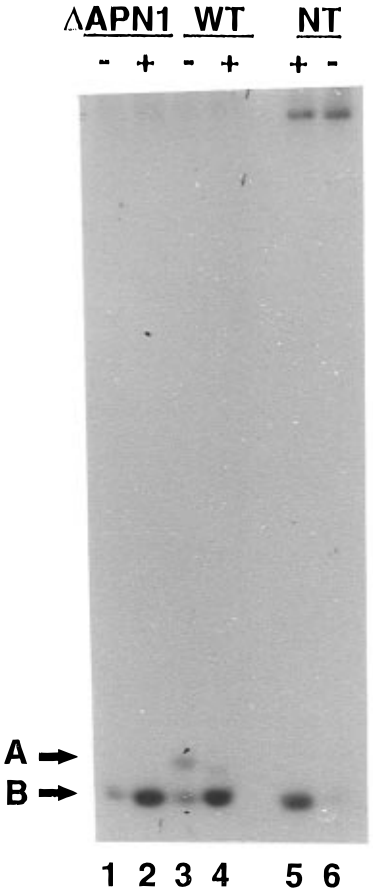

FIGURE 1: Scr activity in wild type and Δ*APN1* yeast. Gel autoradiogram of Scr activity monitored by incubation of 3′-end-labeled DHU-37mer with 50 μg of crude extract from either wild type (lanes 3 and 4) or Δ*APN1* (lanes 1 and 2) yeast or reaction buffer alone (lanes 5 and 6) with (+ lanes) or without (− lanes) subsequent treatment with hot alkali. Reaction products were analyzed on a DNA sequencing gel as described under Materials and Methods. Scr activity is revealed as the production of a specific DNA strand scission product occurring at the DHU site which contains a 5′-terminal phosphoryl group resulting from Scr-mediated AP lyase (β-elimination reaction) activity at the abasic site (arrow B). In wild-type cells, the AP site produced by Scr DNA glycosylase can be subsequently processed either by the AP endonuclease activity of Apn1 which produces a DNA scission product containing a 5′-terminal deoxyribose phosphate group (lane 3, arrow A) or by the AP lyase activity of Scr (lane 3, arrow B). The 5′-terminal deoxyribose phosphate is converted to 5′-phosphoryl after treatment with hot alkali (lane 4). DHU is hot alkali-labile and results in a DNA strand scission product which also contains a 5′-phosphoryl group (lane 5).

tested for optimal Scr activity, including those which were DNA repair-deficient. One of these, Δ*APN1*, is deficient in the major yeast AP endonuclease, Apn1, and had the same levels of Scr-mediated cleavage activity on 3′-end-labeled DHU-37mer compared to wild-type yeast (Figure 1, lanes 1 and 3). However, in wild-type yeast, there is a second activity which is not present in the Δ*APN1* mutant extracts. In contrast to the Δ*APN1* cell extract, wild-type yeast extracts generate a second, slower migrating product that is dependent on the presence of Apn1. Apn1 is a hydrolytic, 5′ AP endonuclease which, upon processing an abasic site (such as those generated by the action of Scr), produces a DNA strand scission product containing a deoxyribose 5′-phosphate terminus (Johnson & Demple, 1988). In order to determine the nature of the Apn1-dependent activity, the reaction products were further treated with hot alkali which removes the deoxyribose 5′-phosphate (Maxam & Gilbert, 1980).

Table 1: Purification of *S. cerevisiae* Redoxyendonuclease

step	protein (mg)[a]	specific activity[b]	percent yield
(I) crude extract	90000	ND[c]	100
(II) ammonium sulfate	78000	0.011	87
(III) DEAE-cellulose	55000	ND[c]	ND[c]
(IV) phosphocellulose	6500	0.14	92
(V) SP−Sepharose fast-flow	420	1.94	82
(VI-B) single-stranded DNA−cellulose (1.0 M NaCl)	0.56	50.1	2.8
(VI-A) single-stranded DNA−cellulose(0.7 M NaCl)	0.02	1364	2.7

[a] Protein determination by the Bio-Rad (Richmond, CA) dye binding assay. [b] Specific activity (units) determined by DNA cleavage assay with DHU-37mer as described under Materials and Methods. [c] Not determined.

Subsequent treatment of wild-type extract-generated reaction products with hot alkali eliminates the slower migrating species and results in products with the same mobility as those produced with the Δ*APN1* strain (Figure 1, lane 4). Because Scr activity was monitored as a composite of its *N*-glycosylase and AP lyase activities, the Δ*APN1* strain was chosen for the large-scale purification. Elimination of Apn1 activity abrogated potential complications for Scr specific activity measurements, particularly at the early stages of purification where Apn1 would compete with Scr for AP site recognition and processing.

Purification of Scr. Scr was purified by a series of batch separations and column chromatographic steps which are summarized in Table 1. Utilization of DHU-37mer, which contains a single base damage site, allowed for measurements of Scr activity that was both sensitive and quantitative. For example, Figure 2 shows the identification of Scr activity following SP−Sepharose chromatography. Scr eluted as a relatively broad peak of activity between 0.35 and 0.55 M NaCl (fractions 35−55). This chromatographic step resulted in an approximately 15-fold purification of Scr. The level of Scr specific activity correlated well with the removal of total protein through the phosphocellulose (fraction IV) and SP−Sepharose (fraction V) chromatographic steps. However, recovery of Scr activity after single-stranded DNA−cellulose chromatography was low. Scr was pooled as two fractions (VI-B and VI-A) which correspond to activity collected from the 0.7 M and 1.0 M NaCl step elutions, respectively. The measurement of specific activity in fraction II is probably underestimated due to the presence of inhibitory factors in the crude extracts such as nucleic acids which are removed by DEAE-cellulose (Gossett *et al.*, 1988; Augeri *et al.*, 1994). The most highly purified Scr preparation (fraction VI-B) was very dilute, and it was necessary to concentrate the sample in the presence of a carrier protein. Ubiquitin was utilized as a carrier and blocking agent due to its low molecular mass (~8 kDa), neutral p*I*, high stability (Hunter & Cary, 1985), and negligible effects on Scr activity (not shown). The resulting fractions, VI-A and VI-B concentrate, were utilized for studying the mechanistic and kinetic properties of Scr.

NaCN Inhibition of Scr and Endonuclease III. NaCN inhibition experiments were conducted in order to gain insight into the mechanism of action of Scr. For example, the DNA glycosylase/AP lyase T4 endonuclease V (T4 endo V) forms an imino intermediate during glycosylase catalysis (Dodson *et al.*, 1993). This imino/Schiff base intermediate is formed with the N-terminal α-amino group of this enzyme.

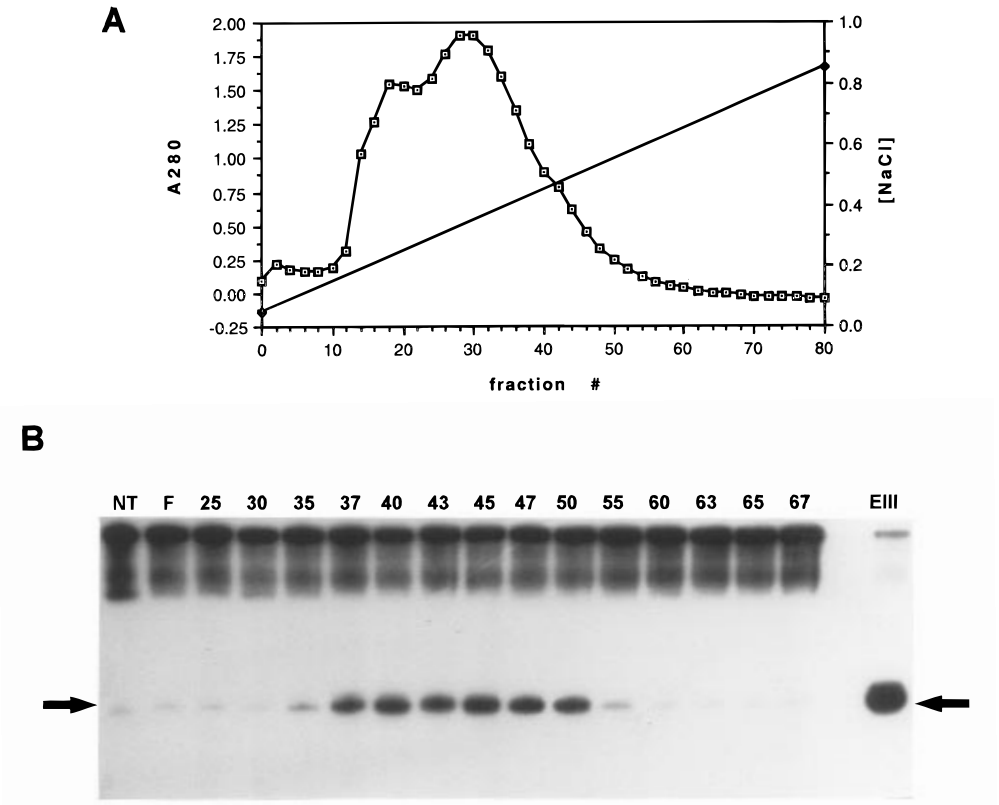

FIGURE 2: SP−Sepharose chromatography purification step of Scr. Fraction IV (6.5 g) was subjected to SP−Sepharose fast-flow chromatography as described in the text. (A) UV (280 nm) absorbance profile (open symbols) versus fraction number following elution with the NaCl gradient (solid line). (B) Gel autoradiogram of Scr activity present in corresponding fractions (25−67) or the unbound, flow-through (lane F) on 3′-end-labeled DHU-37mer. DHU-37mer was also incubated with buffer alone (lane NT) or endonuclease III (lane EIII). The arrow indicates the DNA strand scission product at the position of DHU corresponding to Scr activity.

However, in the case of endonuclease III, it is known that the active site residue is a lysine (K120), found in the amino acid sequence LPGVGRK (Kuo *et al.*, 1992; Hilbert *et al.*, 1996) (Figure 5, region I), which is highly conserved among all known endonuclease III homologs identified to date (Hilbert *et al.*, 1996). The involvement of K120 in catalysis has been demonstrated by the ability of an endonuclease III−substrate intermediate to be trapped upon treatment with NaCN, $NaCNBH_3$, or $NaBH_4$. NaCN forms a reversible complex while reduction results in the formation of a dead-end, irreversible complex (Dodson *et al.*, 1994; Hilbert *et al.*, 1996). Based upon the properties of T4 endo V and endonuclease III, we wished to determine if a Scr−substrate intermediate could be trapped in a similar fashion. Inhibition of cleavage activity of Scr (fraction VI-A) in the presence of NaCN was measured utilizing 3′-labeled DHU-37mer. Endonuclease III (Figure 3A, lane 1) and Scr (lanes 5−9) are inhibited to similar extents by NaCN but are relatively resistant to inhibition by similar concentrations of NaCl (lanes 2−4 and 10−12, respectively). This inhibition of enzymatic activity suggests that these enzymes form an *N*-acylamine intermediate, a property of several different DNA glycosylase/AP lyases (Dodson *et al.*, 1994; Tchou *et al.*, 1995; Hilbert *et al.*, 1996; Nash *et al.*, 1996). Such Schiff base intermediates can be stabilized by chemical reduction to secondary amines, and complex formation can be visualized by gel shift assay on native gels or on SDS−PAGE gels (Hilbert *et al.*, 1996; Nash *et al.*, 1996). Based upon these observations, we conducted gel shift analysis to determine whether complex formation occurred with Scr. Trapping of an enzyme−substrate complex between DHU-37mer and either purified endonuclease III or Scr (fraction VI-A) was observed when $NaCNBH_3$ was included in the reaction (Figure 3B). These results indicate that endonuclease III generates two species: protein complexed to either double-stranded or single-stranded DNA. Similarly, Scr preparations yield a similar set of species of higher molecular size. Our results also confirm that at this stage of purification, Scr preparations appear to contain a single activity which acts on the DHU-containing oligomer.

Previous studies have demonstrated that endonuclease III is inhibited by RNA and single-stranded DNA as well as relatively high concentrations of thymine glycol and other modified bases and nucleotides (Radman *et al.*, 1976; Kow *et al.*, 1987). We have observed a similar pattern of Scr inhibition by mRNA and single-stranded DNA. In addition, thymine glycol (5 mM) was found to inhibit (∼50%) Scr and endonuclease III-mediated cleavage of DHU-37mer (data not shown).

Kinetic Comparison of Scr and Endonuclease III. The V_{max} and K_m were measured for purified preparations of Scr and endonuclease III using DHU-37mer as substrate for both enzymes. Figure 4 shows the Michaelis−Menten kinetics and Lineweaver−Burk plots of the DHU-37mer cleavage reactions. The apparent K_m for DHU-37mer was calculated to be 109 nM for Scr (fraction VI-B) and 91 nM for endonuclease III. The values obtained for both enzymes are similar, indicating that each enzyme is comparably effective in processing dihydrouracil-containing DNA. It should be noted that both enzymes require relatively high concentrations of this particular substrate in order to reach their V_{max}. The ability of Scr preparations to process the same oligonucleotide containing an abasic site (AP-37mer) was also determined. Relatively high concentrations, in excess of 250

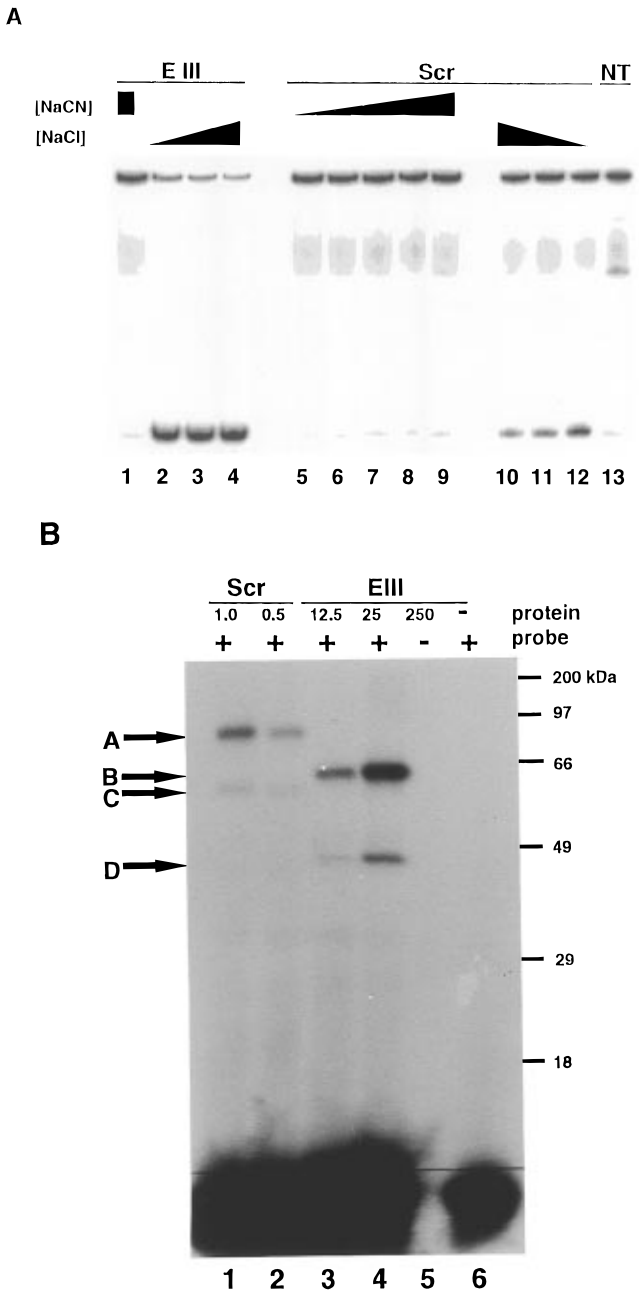

FIGURE 3: Inhibition of Scr by NaCN and trapping of Scr−substrate complex. (A) Phosphorimager picture showing inhibition of Scr (lanes 5−12) or endonuclease III (lanes 1−4) cleavage of DHU-37mer by 40−300 mM (lanes 5−9) and 300 mM (lane 1) NaCN or 40−300 mM NaCl (lanes 2−4 and 12−10, respectively). Lane 13, DHU-37mer incubated with buffer alone (no treatment). Reactions with Scr or endonuclease III and DHU-37mer and analysis of DNA strand scission products on DNA sequencing gels were carried out as described in the text. (B) SDS−PAGE analysis (gel autoradiogram) of enzyme−substrate complexes formed with 3′-end-labeled DHU-37mer (probe) and Scr (lanes 1 and 2) or endonuclease III (lanes 3 and 4) in the presence of $NaCNBH_3$. Reactions were carried out as described in the text. Lanes 5 and 6 are endonuclease III and probe alone, respectively. Protein amounts are shown for Scr (μg) and endonuclease III (ng). Enzyme−substrate complexes for Scr on single-stranded (arrow C) and duplex (arrow A) DHU-37mer or endonuclease III on single-stranded (arrow D) and duplex (arrow B) DHU-37mer. Positions for molecular mass markers shown on the right. Uncomplexed endonuclease III migrates to a position corresponding to 24 kDa (not shown).

nM substrate, were necessary to reach V_{max} (data not shown). Most reported K_m values for endonuclease III and another *N*-glycosylase/AP lyase, Fpg protein, in studies utilizing

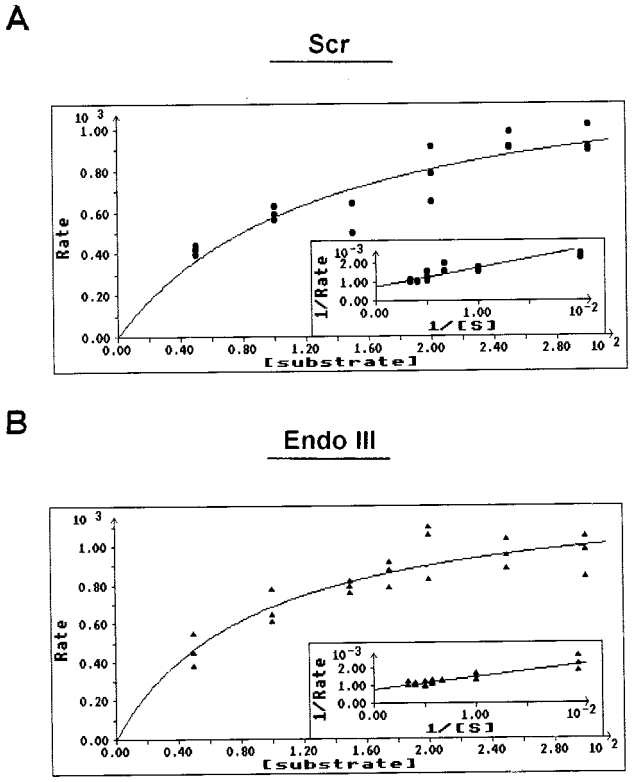

FIGURE 4: Kinetic analysis of DHU-37mer cleavage by Scr and endonuclease III. Scr (A) and endonuclease III (B) were reacted with increasing amounts (50−300 nM) of 3′-end-labeled DHU-37mer and analyzed for strand scission activity as described in the text. Substrate concentrations presented as nM × 10^2. Values for triplicate reactions are plotted. Inset: Lineweaver−Burk plots of kinetic data.

oligonucleotide substrates are in the range of 1−200 nM (Tchou *et al.*, 1994; R. P. Cunningham, personal communication).

Identification of SCR1 and SCR2. Two distinct genes were identified in the yeast genome database that possessed extensive sequence similarity to various regions of endonuclease III. One sequence, designated *FUN33*, encodes a protein of 45.6 kDa which is close to the size estimate for Scr (Gossett *et al.*, 1988; Augeri *et al.*, 1994). The predicted, translated product of the *FUN33* gene (Figure 5, line Scr1) has significant homolgy to endonuclease III in its active site region (Figure 5, region I) but does not contain the Fe-S cluster found in endonuclease III and other homologs (region II) (Hilbert *et al.*, 1996). Several approaches were taken to determine whether *FUN33* encodes the Scr protein. Extracts from a strain, *fun33*, which contains a disruption of the *FUN33* gene (Barton & Kaback, 1994) possess lower activity on DHU-37mer compared to extracts generated from the parental yeast strain (data not shown). Since we had previously determined several chromatographic properties of Scr (Gossett *et al.*, 1988; Augeri *et al.*, 1994), we performed comparative fractionations (Mono S FPLC) of extracts generated from wild-type and *fun33* strains. Wild-type yeast possess two activities (peaks 1 and 2) which cleave DHU-37mer in a similar manner and which overlap when cell extracts are subjected to this chromatographic step (Figure 6). Extract from *fun33* possesses an activity which can cleave DHU-37mer, but is missing the activity (peak 1) which is present in the earlier eluting fractions from wild-type extract. This result suggests that the total Scr activity observed in wild-type yeast is comprised of two separate activities and one of these activities is dependent on *FUN33*

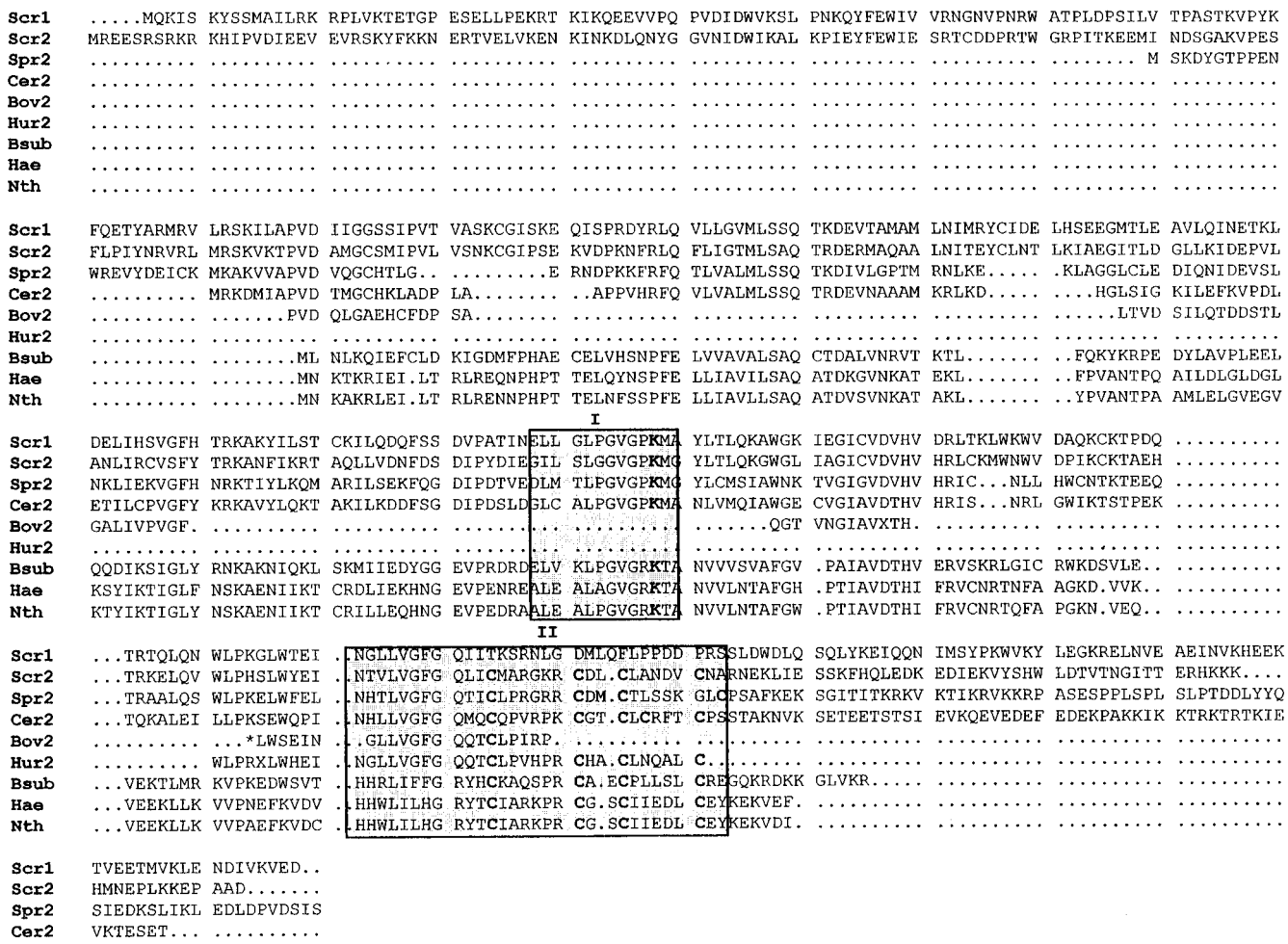

FIGURE 5: Amino acid sequence alignment of Scr1, Scr2, and related proteins. Translated sequences obtained from the sequence database or the indicated references are presented for Scr1 (line Scr1; Barton & Kaback, 1994), Scr2 (line Scr2; Accession No. S66728), and Scr homologs from *S. pombe* [line Spr2 (Nth-Spo); Roldan-Arjona *et al.*, 1996], *C. elegans* (line Cer2; Accession No. Z05874), *Bovus sp.* (line Bov2; Hilbert *et al.*, 1996), *H. sapiens* (line Hur2, Accession No. F04657), *B. subtilis* (line Bsub; Accession No. U11289), *H. influenzae* (line Hae, Accession No. U32842), and *E. coli* endonuclease III (line Nth; Asahara *et al.*, 1989). Region I designates the active site region for this family of proteins, and region II designates the segment containing the conserved Fe-S cluster in all proteins except Scr1.

expression. To confirm that the gene product of *FUN33* possesses the ability to recognize and cleave DHU-37mer, *FUN33* was subcloned into pET22b to generate pET22b-*FUN33* and expressed in *E. coli* (Materials and Methods). Extracts from cells containing vector alone (pET22b) and those containing pET22b-*FUN33* were subjected to comparative fractionation by Mono S FPLC. A prominent, 46 kDa band is present in fraction 28 and flanking fractions but not in the corresponding fractions from the control extracts (Figure 7). Fractions containing this 46 kDa protein demonstrated a high level of Scr activity on DHU-37mer. In contrast, the corresponding fractions from control cell extract were devoid of Scr activity. We conclude that *FUN33* encodes Scr1, and we have renamed this gene *SCR1* to denote this fact. Although the amino acid sequence similarity of Scr to its homologs from bacteria, bovine, *C. elegans*, and *S. pombe* sources is significant, it is different in one important respect: the translated Scr1 sequence does not possess a Fe-S cluster characterized by the amino acid sequence C-X_6-C-X-X-C-X_5-C (Figure 5, region II) (Kuo *et al.*, 1992). In addition, when first identified, the *FUN33* sequence was postulated to encode a mitochondrial transit peptide (Shore *et al.*, 1995). These observations, taken together with the data which show that *fun33* mutants are deficient but not devoid of Scr activity, indicate that there should exist a second gene which encodes another protein functionally similar to Scr1. An extensive search of a complete yeast genomic sequence database revealed a sequence containing an open reading frame homologous to *FUN33 (SCR1)*. This sequence, encoded on chromosome XV, bears the Fe-S binding region similar to other endonuclease III homologs (Figure 5, region II). We have designated this gene as *SCR2* and predict that the Scr2 protein represents the DHU-37mer cleavage activity observed in fractionated extracts from *fun33* mutants (Figure 6).

DISCUSSION

We have developed a sensitive assay for the purification of Scr which utilizes a duplex synthetic oligonucleotide (DHU-37mer) containing a single, centrally located base lesion. DHU-37mer should be useful for the functional analysis of other Scr and endonuclease III homologs, several of which have been recently identified (Hilbert *et al.*, 1996; Roldan-Arjona *et al.*, 1996).

The K_m values obtained for Scr (109 nM) and endonuclease III (91 nM) with DHU-37mer are quite similar. However, these measurements are relatively high compared to the K_m values obtained for endonuclease III with other types of DNA substrates containing different base damages in various sequence contexts (Kow *et al.*, 1987; R. P. Cunningham, personal communication). We believe that this

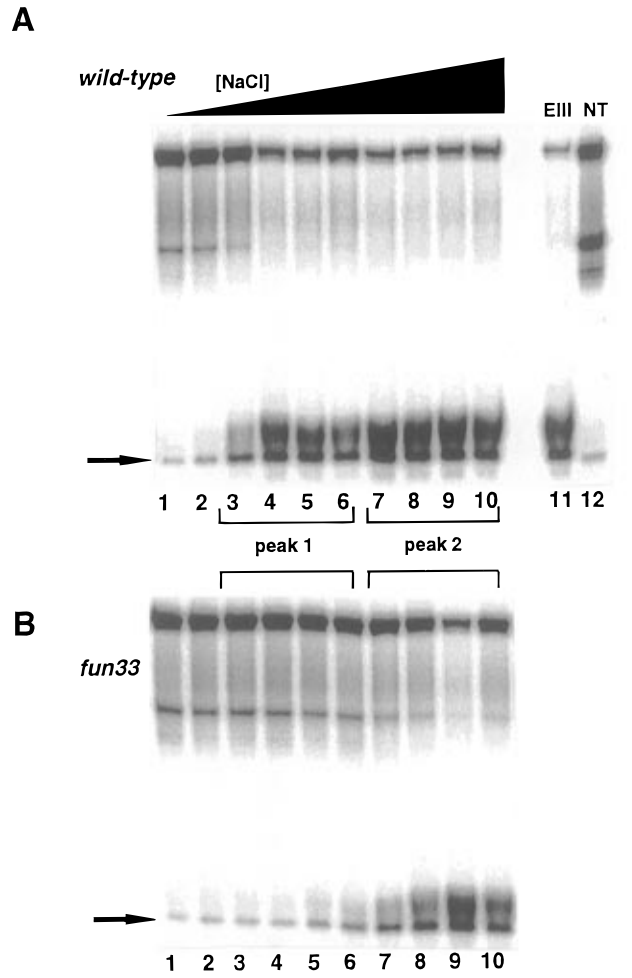

FIGURE 6: Scr activity in wild-type and *fun33* yeast strains. Phosphorimager picture showing Scr activity present in Mono S FPLC-fractionated extracts from wild-type and *fun33* yeast. Aliquots of corresponding column fractions (lanes 1−10) from wild-type (A) or *fun33* (B) extracts eluted with a 0.05−1.0 M NaCl linear gradient as previously described for wild-type yeast (Augeri *et al.*, 1994) were monitored for Scr activity via cleavage of 3′-end-labeled DHU-37mer and generation of DNA strand scission product (arrows) as described in the text. Wild-type and *fun33* extracts produced two peaks (peak 1, fractions 4, 5; and peak 2, fraction 9) and one peak (peak 2, fraction 9) of Scr activity, respectively. Lanes 11 and 12 correspond to DHU-37mer reaction with endonuclease III and buffer alone (no treatment), respectively.

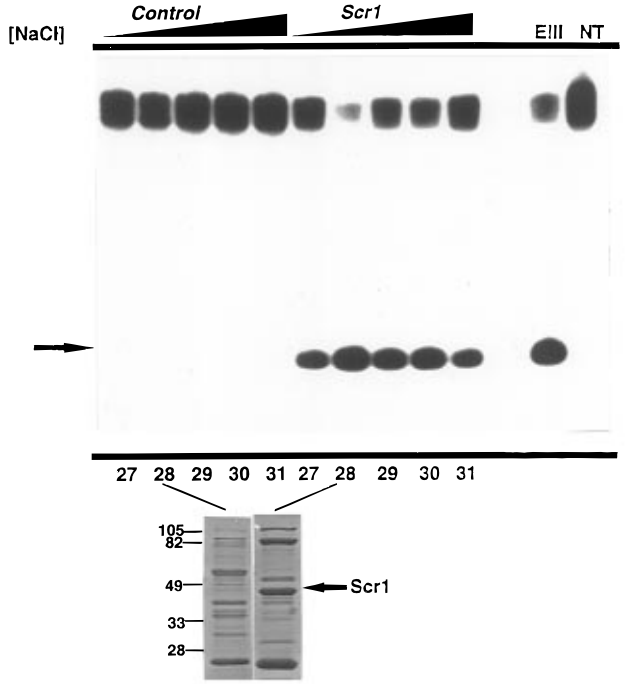

FIGURE 7: DHU-37mer cleavage activity of expressed Scr1. Extracts from cells containing pET22b-*FUN33* (expressing Scr1) and pET22b (control, vector alone) were fractionated via Mono S FPLC as described in the text. Proteins were eluted with a 0.05−1.0 M NaCl linear gradient, and equivalent fractions from control and Scr1-expressing cells were monitored for Scr activity by incubation with 3′-end-labeled DHU-37mer. Lanes EIII and NT are DHU-37mer incubations with endonuclease III and buffer alone (no treatment), respectively. The unlabeled arrow indicates the DNA strand scission product at the position of DHU corresponding to Scr and endonuclease III activity. The gel autoradiogram shows Scr peak activity in Scr-expressing cells at fraction 28 and below; proteins present in this fraction (SDS−PAGE and Coomassie blue staining) were compared to the equivalent fraction from control cells. Scr1 (arrow) was detected in fraction 28 as a prominent band of approximately 46 kDa.

may be due, in part, to the duplex properties of DHU-37mer. DHU-37mer was designed to form a stable duplex with minimal capacity for secondary structures or mispairing with its complementary strand. As a result, we obtained a sequence which forms a very stable duplex that does not easily denature, even in the presence of 7 M urea and 80% formamide (Figures 2 and 6). These characteristics may greatly affect the activity of this type of enzyme, which is postulated to "flip-out" damaged bases into the active site of the enzyme in order for catalysis to occur (Thayer *et al.*, 1995). The effects of DNA sequence context and duplex stability on enzyme activity are illustrated by differences in the efficiency of the Fpg protein in its cleavage activity on a variety of oligonucleotide substrates (Tchou *et al.*, 1994). Studies with substrates containing various base damages with the same and different sequence contexts may also provide information which distinguishes Scr1 and Scr2 from each other as well as from endonuclease III. Recently, it was discovered that the *S. pombe* homolog of Scr is more efficient than endonuclease III for excision of urea from duplex polymers, but the converse situation exists for the removal of thymine glycol (Roldan-Arjona *et al.*, 1996).

The substrate specificity of Scr includes several base lesions including thymine glycol, pyrimidine photohydrates, abasic sites, and, from this study, dihydrouracil (Gossett *et al.*, 1988; Augeri *et al.*, 1994). In addition, Scr cleaves UV-irradiated DNA at positions of guanine (Gossett *et al.*, 1988; Augeri *et al.*, 1994) which most likely correspond to recognition of fapy-Gua (Doetsch *et al.*, 1995). A similar "G-cutter" activity has been observed for endonuclease III (Helland *et al.*, 1986).

Scr activity in crude extracts from wild-type and Δ*APN1 S. cerevisiae* cells has revealed that the combined *N*-glycosylase/AP lyase activity of Scr may not be concerted, in contrast to the reported concerted mechanism for endonuclease III (Kow *et al.*, 1987). Wild-type yeast display Apn1-dependent competition with Scr for an abasic site generated by the DNA glycosylase activity of Scr (Figure 1). Such competition between AP lyase (Scr) and AP endonuclease (Apn1) activities suggests that the DNA glycosylase/AP lyase activities of Scr are separable and not part of a concerted reaction mechanism. Furthermore, the observation that Apn1 can compete for an abasic site generated by Scr raises several issues concerning the events which occur at the site of damage during the base excision repair process. Scr must first remove the damaged base by its *N*-glycosylase activity before either Scr or Apn1 can act

on the abasic site that is generated. Apn1 can compete with Scr (already at the lesion site), suggesting that Scr may dissociate from the target DNA and reassociate for the subsequent lyase (β-elimination) step. A similar dissociation/reassociation event has been suggested for the DNA glycosylase/AP lyase T4 endonuclease V (Latham *et al.*, 1995). Alternatively, Scr may be in association with Apn1 in the cell, which would result in Apn1 being in close proximity to the AP site at the time it is generated. Such a situation would allow for effective competition for an abasic site by Apn1.

These studies have also provided information relevant to the chemical nature of Scr catalysis. Scr activity is completely inhibited by NaCN, and it was possible to trap the Scr−DHU-37mer reaction intermediate by reduction with $NaCNBH_3$. Such a result indicates Scr follows a reaction mechanism simliar to other *N*-glycosylase/AP lyases (Dodson *et al.*, 1994; Tchou *et al.*, 1995; Hilbert *et al.*, 1996; Nash *et al.*, 1996). If one considers the active site homology of the *SCR1* and *SCR2* gene products (Figure 5, region I) compared to endonuclease III and its homologs, this result is not surprising. Such a property of Scr further substantiates a universal reaction mechanism among this class of base excision repair enzymes (Dodson *et al.*, 1994; Tchou *et al.*, 1995; Hilbert *et al.*, 1996; Nash *et al.*, 1996).

Our studies show that there are two proteins in yeast (Scr1 and Scr2) which bear a close similarity to endonuclease III. Fractionation of Scr from wild-type and *scr1 (fun33)* cells shows that there are two activities which act on DHU-containing DNA in a similar manner (Figure 6). Both activities have partially overlapping chromatographic properties, and one of the two activities is absent in *scr1* yeast. The amino acid sequences of Scr1 and Scr2 are closely related (41% identity, 63% similarity) to each other. Endonuclease III also shows significant homology to Scr1 (24% identity, 46% similarity) and Scr2 (25% identity, 51% similarity) (Figure 5). Scr1 and Scr2 are encoded by genes present on chromosomes I and XV, respectively (Barton & Kaback, 1994; Dujon *et al.*, in preparation). One significant difference between Scr1 and Scr2 is the absence of an Fe-S center in Scr1. With the exception of Scr1, this Fe-S cluster is a feature common to all endonuclease III homologs identified thus far. A second difference between Scr1 and Scr2 is the presence of a putative mitochondrial targetting sequence (Shore *et al.*, 1995). We speculate that Scr1 and Scr2 represent mitochondrial and nuclear isoforms, respectively. Future studies should be directed toward determining the subcellular localization of these proteins. Whether or not other organisms contain multiple endonuclease III homologs with and without a Fe-S cluster should be revealed as additional genomic sequence information becomes available.

We note that during the submission of this paper, a report by Eide *et al.* (1996) appeared which also shows that the *FUN33* gene encodes a protein with activity similar to endonuclease III.

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