Purification and Characterization of the SRS2 DNA Helicase of the Yeast Saccharomyces cerevisiae*

Lei Rong and Hannah L. Klein

From the Department of Biochemistry and Kaplan Cancer Center, New York University Medical Center, New York, New York 10016

The SRS2 gene of Saccharomyces cerevisiae was identified through mutational analysis as a suppressor of radiation-sensitive mutations in the error-prone repair pathway and by a hyper-recombination phenotype. Comparison of the derived amino acid sequence revealed the gene to have high homology to the bacterial DNA helicases UvrD and Rep (Aboussekhra, A., Chanet, R., Zgaga, Z., Cassey-Cravat, C., Heude, M., and Fabre, F. (1989) Nucleic Acids Res. 17, 7211–7219). We have purified the SRS2 protein from Escherichia coli extracts by tagging the SRS2 gene with 6 carboxyl-terminal histidine residues and overexpressing the tagged protein in a pET-3c vector. Extracts were passed over a metal-chelating affinity chromatography column followed by gel filtration to obtain an enriched protein fraction. Sephacryl gel filtration of pooled fractions containing the SRS2 protein yielded purified SRS2 protein by Coomassie Blue stain of SDS-polyacrylamide gel electrophoresis gels. The purified SRS2 protein was found to have in vitro DNA-dependent ATPase and DNA helicase activities. The polarity of the helicase activity was determined to be 3' to 5', the same polarity as that found for the UvrD and Rep proteins. The carboxyl-terminal region of the protein is shown to contain a sequence for nuclear localization. Expression of the SRS2 in yeast was examined and found to be extremely low.

DNA helicases are enzymes that unwind a region of duplex DNA through the disruption of the hydrogen bonds that hold the two strands of the duplex together. These enzymes usually act in concert with other enzymes in a DNA metabolic activity such as replication, repair, or recombination and are thought to open the DNA helix in preparation for the DNA replication or repair complex to act on regions of single-stranded DNA (for review see Matson and Kaiser-Rogers, 1990; Matson, 1991). The role of DNA helicases in recombination is more speculative, but these enzymes could be part of a repair step in recombination or aid in branch migration of a Holliday junction. Several DNA helicases have been identified in Escherichia coli through genetic and biochemical approaches, with 11 DNA helicases thus far identified (Matson and Kaiser-Rogers, 1990; Matson, 1991; Umezu et al., 1990) and several more candidate genes under study. The fact that mutations in these genes result in a phenotype indicates that these helicases do not have redundant functions and demonstrates the importance of the helicase reaction in DNA metabolism.

The DNA helicases described in eukaryotes is limited, although these enzymes may have overlapping functions and hence a lack of discernible phenotype. One of the most studied helicases is the simian virus 40 (SV40) large tumor (T) antigen which is a multifunctional protein with ATPase, helicase, and DNA binding activities (for reviews see Stillman (1988), Matson and Kaiser-Rogers (1990), and Borowiec (1990)). In the yeast Saccharomyces cerevisiae the RAD53 gene, which is involved in excision repair (for reviews see Friedberg (1988) and Friedberg et al. (1991)), has been shown to encode a DNA helicase with a 5' to 3' polarity (Harosh et al., 1989; Sung et al., 1987a, 1987b). The PIF1 gene encodes a DNA helicase, also with a 5' to 3' polarity (Lahaye et al., 1991), which is involved in mitochondrial DNA repair and recombination (Foury and Kolodynski, 1983; Foury and Van Dyck, 1985). Other genes including RAD54 and CHL1, have been reported to have amino acid sequence homology to DNA helicases, but no activity has been demonstrated (Emery et al., 1991; Gerring et al., 1991).

We have reported the isolation of hyper-recombination mutants of yeast, among which was the gene HPR5 (Aguilera and Klein, 1988). Genetic studies have indicated that this gene functions in the error-prone repair pathway in yeast during mitotic growth and is also required for meiotic viability (Rong et al., 1991). The HPR5 gene was independently identified in two other studies as a suppressor of rad18 mutations in the error prone repair pathway and called SRS2 (Lawrence and Christensen, 1979) and RADH (Aboussekhra et al., 1989). Genetic studies have shown that these three genes are the same (Rong et al., 1991). The amino acid sequence of the RADH gene as reported by Aboussekhra et al. (1989) showed a high degree of identity to the bacterial DNA helicases UvrD and Rep, both of which have a 3' to 5' polarity (Matson, 1986; Duguet et al., 1978). In this report we describe the purification of the SRS2 protein and present the characterization of the wild type activity. The SRS2 protein has a region of 300 amino acids carboxyl-terminal to the bacterial DNA helicase homology. Data on one function of this region, the presence of a nuclear localization signal (NLS), is also presented in this report.

MATERIALS AND METHODS

Plasmids

pRL101—The 1-kb SacI-Sac1 fragment of pHK201 (Rong et al., 1991) was subcloned into M13mp18. A NdeI site was created at the start codon of the SRS2 coding region (as deduced by Aboussekhra et al. (1989)) by site-directed mutagenesis (Kunkel et al., 1987) using

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1The abbreviations used are: kb, kilobase(s); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); AMP-PNP, adenosine 5'-[(y-g-imino)triphosphate. 1252
the oligonucleotide 5'-GGATAGCATATGTGTC-3'. The mutagenized plasmid was screened by digestion with NdeI and verified by DNA sequencing. The SacI-Sacl fragment bearing the NdeI site was substituted for the original SacI-Sacl fragment of pHK201 to form plasmid pRL101. The entire SRS2 coding region together with the 3'-untranslated region from the CiaI to BamHI sites was excised as a 770-bp SacI-BamHI fragment and subcloned into the NdeI-BamHI site of the pET-3c (Studier et al., 1990) to form pRL102.

pRL103—In order to maximize expression of the cloned SRS2 gene the XhoI-BamHI fragment at the 3'-end of the nontranslated region in plasmid pRL102 was deleted by digestion with XhoI and BamHI and the ends filled in with Klenow enzyme. The blunt ends were religated to form plasmid pRL105.

pRL104—The 1.5-kb fragment between the PvuII site at nucleotide 2797 and the PvuII site at nucleotide 4345 of the SRS2 coding region was deleted from plasmid pRL102 to form plasmid pRL105. This deletion results in a frameshift of the SRS2 coding sequence 3' to the PvuII site at position 4345 and creates a stop codon at the 12th codon carboxyl-terminal to this site. The open reading frame of pRL105 thus only encodes the NH2-terminal 625-amino acid residues plus 11 extra residues at the carboxyl terminus, creating a protein of 69 kDa. This plasmid was used to overexpress the 69-kDa truncated form of the SRS2 protein for use as an antigen in raising anti-SRS2 antisera.

Expression of truncated SRSS protein (SRS~(H~S)~ Protein)

PET-3c-derived expression vector PETH containing the full SRS2 coding region was subcloned into the SacI-XhoI site of the yeast expression vector strain pRL105 (Johnson, 1991) downstream of the GAL10 promoter to create pRL106. The protein was expressed at 30°C in the yeast strain of E. coli strain HB101 transformed with plasmid pRL105 and the ends filled in with Klenow enzyme. The blunt ends were religated to form plasmid pRL105.

Immunoblot Analysis

Immunoblot analysis of the SRS2 protein and the various mutant derivative forms was performed by electrophoresis of protein extracts on a 10% SDS-PAGE followed by electrotransfer of the protein to nitrocellulose and then antibodies to the SRS2 protein, the anti-SRS2 sera and preimmune sera were purified by stepwise precipitation (McKinney and Parkinson, 1987) with subsequent protein A affinity chromatography (Harlow and Lane, 1988) to obtain pure IgG fractions.

Expression of the truncated SRS2 protein from E.coli and Purification of Anti-SRS2 Antibody

The plasmid pRL105 was used to transform strain BL21(DE3) which contains a lysogenized copy of the T7 RNA polymerase gene under control of the T7 promoter (Studier et al., 1980). Expression of the SRS2 gene was induced in midlogarithmic growth of an E. coli culture by the addition of isopropyl-D-thiogalactoside. Cells were then centrifuged, and the pellet was resuspended in cold TEN buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.3 M NaCl). Lysis was achieved by sonication (30 s, with 2-s pulses). Lysis was continued in 6 M guanidine for overnight with end-to-end rocking at 4°C. The cell debris was removed by centrifugation and further clarification was achieved by passing the extracts through a Nalgen 0.45-pm filter.

The extracts were subjected to isopropyl-D-thiogalactoside. Lysis was carried out in TEN buffer at 30°C until the cell debris was removed by centrifugation and further clarification was achieved by passing the extracts through a Nalgen 0.45-μm filter.

The extracts were subjected to nickel-chelate affinity chromatography as described (Genta et al., 1989; Hochuli et al., 1987). Fractions containing SRS2 protein as determined by SDS-PAGE and Western blotting were pooled and applied to a S200-Sephacryl gel filtration column (50 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 15% glycerol) and loaded onto an 8% polyacrylamide gel. The prominent 69-kDa band was excised. The gel slice was briefly destained in distilled water and then was subjected to electrotransfer to an 80 V buffer at 80 V in dialysis tubing. Gel debris was removed, and the eluted protein was dialyzed against 0.1 M MOPS, pH 7.0. The protein was concentrated by absorption with AQUACIDE (Calbiochem) and was sent to the Pocono Rabbit Farm Laboratory for injection into New Zealand White rabbits. The antisera was affinity-purified using nitrocellulose filter antibody purification (Towin et al., 1985) and used in immunoblotting and in direct immunofluorescence experiments. For inhibition of the ATPase and helicase activities of the SRS2 protein, the anti-SRS2 sera and preimmune sera were purified by stepwise precipitation (McKinney and Parkinson, 1987) with subsequent protein A affinity chromatography (Harlow and Lane, 1988) to obtain pure IgG fractions.
treated with Centricron-100 (Amicon) to 250 μl. The protein could be stored at -70 °C for 1 month without loss of activity.

Expression of the SRS2 Wild-type Protein and Derivative Forms 

in Yeast

The yeast strain SC340, provided by Dr. J. Hopper (Pennsylvania State University, Hershey, PA), was used to achieve maximal expression of the SRS2 insertions into the pSJ101 vector (Mylin et al., 1990). Transformants of this strain using the pSJ11 constructs were selected on synthetic complete medium lacking leucine. Transformants were grown in preculture medium (Mylin et al., 1990) to saturation at 30 °C. The cultures were used to inoculate noninducing medium and grown until reaching O.D600 of 0.4. Induction was achieved by the addition of galactose to a final concentration of 2%.

After 5-6 h of incubation at 30 °C the cells were collected, and extracts were prepared as described (Mylin et al., 1990).

Indirect Immunofluorescence

SC340 yeast strains transformed with various pSJ101 plasmid constructs (see Fig. 8) were used to express the SRS2 wild-type and derivative forms as described above. Cells were harvested and fixed by formaldehyde treatment. The fixed cells were subjected to indirect immunofluorescence microscopic analysis as described by Rose et al. (1991). Affinity-purified anti-SRS2 antibody was diluted 300-fold with PBS before use. Monoclonal anti-β-galactosidase was purchased from Sigma and used at 1:1000 dilution. Yeast nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride at a concentration of 1 μg/ml. Anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody and anti-mouse fluorescein isothiocyanate-conjugated secondary antibody were purchased from Sigma. The cells were visualized with a Nikon MICROPHOT EPI-FL microscope using a 100× objective and oil immersion and results recorded with a Nikon F2S5A camera using Kodak TMAX400 film.

Preparation of Helicase Substrates

A substrate with a 18-bp duplex region was made by annealing the 17-nucleotide M13 universal (−40) sequencing primer (United States Biochemicals (USB)) to circular single stranded M13mp18 DNA and extending the 3′-end for one nucleotide using 5 units of Klenow polymerase and 10 μCi of [γ-32P]dGTP (800 Ci/mmol, Du Pont) for 20 min at room temperature. The reaction was chased by the addition of 1 mM cold dGTP with further incubation at room temperature for 20 min. Samples were chilled on ice for 10 min and then passed through a Bio-Rad Spin30 column to remove unincorporated nucleotides.

A substrate containing a 145-bp duplex region was constructed using circular single stranded M13mp18 DNA and a 145-bp PCR product. The PCR reaction was carried out using the −40 universal sequencing primer (Boehringer) and the −48 reverse sequencing primer (Boehringer) and used according to the manufacturer's specification. After the PCR reaction the Taq polymerase was purchased from Perkin-Elmer/Cetus. The annealing temperature was 55 °C, and the extension reaction was performed at 72 °C. Twenty-five cycles of amplification were done using an Ericomp TwinBlock thermal cycler (Ericomp, Inc.) as described (Mullis and Faloona, 1984). Protein analysis was carried out on SDS-PAGE according to Laemmli (1970). Following electrophoresis, the gels were stained with Coomassie blue or transferred to nitrocellulose sheets for immunoblot analysis. Protein concentration was determined using a Bio-Rad dye binding assay.

RESULTS

Expression and Purification of the SRS2 Protein—To facilitate the purification of the SRS2 protein, the pET-3c vector system (Studier et al., 1990) was used to increase protein expression. The level of expression obtained was extremely low, and the expected band of 130 kDa was barely detectable by Coomassie Blue staining (data not shown). However, a deletion derivative of SRS2, which is missing the carboxy-terminal 550 amino acids, expressed a protein in E. coli of the expected molecular mass of 69 kDa. This protein was expressed to a sufficient extent to be used as an antigen for the production of anti-SRS2 antibody, which was used primarily in Western analysis to monitor the purification of the SRS2 protein.

Purification of the SRS2 protein from E. coli was achieved through metal-chelating affinity chromatography. A short polypeptide containing 6 histidine residues was attached in-frame to the carboxy terminus of the SRS2 coding region to form a derivative protein called SRS2(His)6. The SRS2(His)6 protein was expressed in E. coli using the pET-3c vector system. Analysis of cell extracts by SDS-PAGE and immunoblotting revealed that the SRS2 protein was predominantly in the insoluble fraction (data not shown). Therefore, cell extracts were solubilized in 6 M guanidine prior to application to a NTA-nickel column. The SRS2 protein eluted from this column at pH 5.0 under denaturing conditions (see "Materials and Methods") as a greatly enriched fraction (Fig. 1A). Fractions containing the SRS2 protein were pooled and passed over a 2S00-Septahcyl gel filtration column to remove the low molecular weight contaminants.

As the purified SRS2 protein was still in a denatured form, protocols that renature proteins were applied (Marston and Hartley, 1990). Dialysis of a dilute sample against successive reduced guanidine concentrations was used in the purification of the yeast PIF1 DNA helicase (Lahaye et al., 1991). The
SRS2 protein was renatured as detailed under "Materials and Methods" at neutral pH, keeping the protein concentration below 10 μg/ml. After removal of the guanidine the protein was concentrated and used directly in ATPase and DNA helicase assays. The renatured protein was shown to be a single band of 130 kDa by Coomassie Blue staining (Fig. 1B) and was positively identified by anti-SRS2 antibody (Fig. 1C).

SRS2 Is a DNA-dependent ATPase—Since the predicted SRS2 protein sequence had shown homology to the bacterial DNA helicases UvrD and Rep (Aboussekra et al., 1989), we anticipated that the purified protein would have ATPase and DNA helicase activities. Therefore, assays to measure these types of activities were applied to the purified SRS2 protein. We found that the purified SRS2 protein contained significant ATPase activity when Mg²⁺ and M13mp18 single stranded DNA were used as cofactors (Fig. 2). The $K_v$ of the activity was measured to be 285 μM, and the $V_{max}$ was 11.1 μM min⁻¹. The turnover number of the SRS2 protein for ATP hydrolysis was determined to be 191 μM min⁻¹ μM⁻¹ of SRS2 protein, indicating that the enzyme is very efficient in the hydrolysis of ATP.

Antibody inhibition studies were used to verify that the ATPase activity resulted from action of the SRS2 protein. Purified anti-SRS2 IgG inhibited the ATPase activity to 93.5% inhibition, while the preimmune IgG serum gave no inhibition of activity (Fig. 3).

SRS2 Is a DNA Helicase—DNA helicase activity of the SRS2 protein was measured by the capacity of the protein to unwind a partially duplex DNA consisting of a circular single stranded M13 DNA annealed to a complementary 18-nucleotide oligonucleotide or a 145-nucleotide oligonucleotide. The SRS2 protein was able to promote the unwinding of both substrates in the presence of Mg²⁺ and ATP (Fig. 4). The unwinding was inhibited by the addition of anti-SRS2 IgG to the reaction. Unwinding of the 18-bp duplex was inhibited by 55% (Fig. 4A). As shown in Fig. 5, unwinding of the 18-bp duplex substrate occurred only in the presence of Mg²⁺ and ATP or dATP. Other nucleotide triphosphates, ADP, and the nonhydrolyzable ATP analog AMP-PNP could not be substituted in the reaction. Very low helicase activity was detected when Mg²⁺ was substituted by Mn²⁺ or Ca²⁺. These results show that, as predicted from the protein sequence, SRS2 is a DNA helicase and has ATPase activity.

Polarity of the SRS2 Helicase—The direction or polarity of a helicase has been defined as the direction in which the helicase unwinds with respect to the strand of DNA to which the enzyme is bound. To determine the direction of the SRS2 helicase a linear DNA substrate with duplex ends of different lengths was constructed as initially designed by Matson...
Fig. 4. DNA helicase activity of the SRS2 protein. Purified SRS2(His)6 protein was used to unwind partial duplex DNA substrates. The scheme of the assay is depicted on the left. Dissociation of the labeled oligonucleotides is visualized by fractionating the reaction products on a 15% nondenaturing polyacrylamide gel followed by autoradiography. The partial duplex helicase substrates consisting of either an 18-bp duplex region or a 145-bp duplex region were used in the assay. Construction of the substrates and helicase assays are detailed under “Material and Methods.” SRS2(His)6 protein was used with the 18-bp partial duplex substrate. Lane 1, DNA substrate without SRS2(His)6 protein; lane 2, 50 ng of purified SRS2(His)6 protein was used in the presence of 4 mM MgCl2 and 1 mM ATP; lane 3, as lane 2 except 150 ng of protein was used; lane 4, as lane 2 but 30 ng of purified anti-SRS2 IgG was incubated with the SRS2(His)6 protein at 4 °C for 1 h prior to the helicase reaction; lane 5, as lane 2 with MgCl2 omitted from the reaction mixture; lane 6, as lane 2 with ATP omitted from the reaction mixture; lane 7, heat-denatured substrate. Reaction time was 10 min. In this experiment 15% of the substrate was displaced by 150 ng of SRS2 protein in 10 min at 37 °C (lane 3). B, helicase activity of the SRS2 protein with the 145-bp partial duplex substrate. Lane 1, DNA substrate without SRS2(His)6 protein; lane 2, 150 ng of purified SRS2(His)6 protein was added to the complete reaction mixture; lane 3, heat-denatured substrate. Reaction time was 30 min. In this reaction 33% of the substrate was displaced by 150 ng of SRS2 protein in 30 min at 37 °C (lane 2).

Fig. 5. Cofactor requirements of the DNA helicase activity of the SRS2 protein. 50 ng of purified SRS2(His)6 protein was used in each reaction. Complete reaction mixture for helicase assay was as described under “Material and Methods.” Each lane is designated by either the omission of a particular factor or substitution of another factor. Divalent cations substituting for Mg2+ were used at 4 mM, the same molarity as the Mg2+ cation in the complete reaction mixture. Nucleotide triphosphates, deoxyribonucleotide triphosphates, ADP, and AMP-PNP replacing ATP were all used at 1 mM. The reaction was performed and visualized as described under “Material and Methods.” Control lane represents DNA substrate without protein. Reaction time was 30 min.

Expression of SRS2 in S. cerevisiae—An expression vector containing the SRS2 coding region regulated by the GAL10 promoter (plasmid FPPT, Fig. 7) was used to overproduce the SRS2 protein in yeast. As was the case for expression in E. coli, we found that expression in yeast was low even when an overproduction system was used. The SRS2 protein was identified as a 130-kDa band by immunoblotting using the anti-SRS2 antibody (Fig. 7). This reactive band was identified unambiguously as the SRS2 protein through the use of a deletion allele, srs2-166, which truncates the terminal 127 amino acid residues and results in an immunoreactive band of 116 kDa. Surprisingly, the level of expression of the truncated protein was at least 5-fold higher than that observed for the full length SRS2 protein, suggesting that the terminal

Fig. 6. Polarity of SRS2 DNA helicase. Substrate construction was described under “Material and Methods.” A schematic drawing of the substrate and the rationale of the assay is shown on the left. Lane 1, 150 ng of SRS2(His)6 protein was used in the reaction, resulting in the dissociation of the 80-nucleotide oligonucleotide; lane 2, DNA substrate without protein; lane 3, heat-denatured substrate. Reaction time was 30 min.
region of the protein inhibits high expression or enhances degradation of the protein.

We were unable to detect the endogenous SRS2 protein by immunoblot analysis. This corroborates our observations on the mRNA level of SRS2 as determined by Northern analysis (Palladino, 1992). The other yeast helicase genes RAD3 and PIF1 have been found to be expressed at very low levels (Sung et al., 1987a, 1987b; Lahaye et al., 1991).

No deleterious phenotype was observed when the SRS2 gene was overexpressed in yeast. We have shown that the overexpressed protein is functional by the ability of cells bearing the FPPT plasmid to complement the srs2-166 null allele phenotype on galactose medium, but not on glucose medium where the wild-type plasmid-borne SRS2 gene is not expressed.

Nuclear Localization of the SRS2 Protein Is Determined by a Carboxyl-terminal Nuclear Localization Signal—The SRS2 protein possesses approximately 300 amino acid residues carboxyl-terminal to the region of homology to the Rep and UvrD helicases. This region shows no significant homology to sequences in the data base (Aboussekhra et al., 1989). Inspection of this region has revealed several clusters of basic amino acid residues, particularly in the carboxyl-terminal 127 residues, where 24% are lysine or arginine residues. This is in contrast to the remainder of the protein sequence which possesses only 14% basic residues. Of particular note is the sequence KKKSK from residues 1117-1121 which closely resembles the SV40 large T antigen nuclear localization signal (Kalderon et al., 1984).

Indirect immunofluorescence using affinity-purified anti-SRS2 antibody to stain-fixed yeast cells showed that the SRS2 protein predominantly localizes to the nucleus (Fig. 8C). We were able to detect the protein by immunofluorescence only when it was overexpressed from the GAL10 promoter in the FPPT plasmid. The deletion derivative srs2-166 which lacks the carboxyl-terminal 127 residues (Fig. 8) encoded a protein which localized predominantly to the cytoplasm (Fig. 8F). Transformation of a null allele strain with a plasmid carrying the srs2-166 allele expressed from the SRS2 promoter resulted in the null phenotype with respect to hyper-recombination and suppression of rad18 UV sensitivity. The inability of the srs2-166 gene to complement the null allele suggests that nuclear localization of the protein is essential for wild-type activity. These results show that the carboxyl-terminal region of the SRS2 protein contains a nuclear localization signal and that deletion of a region encompassing this signal gives a null phenotype. They do not indicate, however, that the only function of this region is to determine nuclear localization.

Fig. 7. Overexpression of the SRS2 protein in yeast. Plasmids FPPT (with full length SRS2 insert) and FRL166 (with a truncated SRS2 insert missing the carboxyl-terminal 127 amino acids) were used to transform a yeast strain with an intact endogenous SRS2 gene. Constructions of the overexpression plasmids are described under "Material and Methods." Schematic drawings of the plasmids are shown on the top, with only relevant restriction sites indicated. Overexpression was induced by adding galactose to the cell culture (see "Material and Methods"). Cell extracts were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Immunodetection was performed using the purified anti-SRS2 antibody. Lane 1, untransformed cells; lane 2, cells transformed with FRL166, overproducing srs2-166 truncated protein (116 kDa); lane 3, cells transformed with FPPT, overproducing the full length SRS2 protein (130 kDa).

Fig. 8. Subcellular localization of the full-length and derivative SRS2 proteins by indirect immunofluorescence. Cells overproducing the SRS2, srs2-166, srs2-167, and the SRS2-LacZ proteins were prepared for the indirect immunofluorescence experiments as described under "Material and Methods." Schematic drawings of the relevant inserts regulated by the GAL10 promoter are presented at the top. A, D, G, J are phase-contrast microphotographs of cells overproducing SRS2, srs2-166, srs2-167, and SRS2-LacZ proteins, respectively. B, E, H, and K are 4,6-diamidino-2-phenylinodole staining of the respective yeast strains. C, F, and I are immunofluorescence of SRS2, srs2-166, and srs2-167 proteins using affinity-purified anti-SRS2 antibodies. L is the immunofluorescence of the SRS2-LacZ chimeric protein using monoclonal anti-β-galactosidase antibody at a dilution of 1:1000.
nor that the null phenotype of the srs2-166 allele results from mislocalization.

To directly demonstrate a role of the KKKSK sequence in the nuclear localization of the SRS2 protein, an in-frame deletion of these five amino acids was made, forming the allele srs2-167 (Fig. 8). Indirect immunofluorescence showed that the protein encoded by this allele had a cytoplasmic localization (Fig. 8l). We next made a fusion protein in which the amino-terminal region of the E. coli β-galactosidase gene was fused to the region of SRS2 encoding the carboxyl-terminal 127 residues. As is shown in Fig. 8L, the fusion protein was localized to the nucleus, whereas the β-galactosidase protein with no fusion was localized in the cytoplasm (Hall et al., 1984). Taken together, these results suggest that the KKKSK sequence at the carboxyl terminus of the SRS2 protein functions as a nuclear localization signal and that the SRS2 protein is most likely localized in the nucleus for its action.

As mentioned above, the protein encoded by the srs2-166 allele, which is missing the NLS signal and is localized in the cytoplasm, is expressed 5-fold higher from the GAL10 promoter than the intact SRS2 protein. Similar observations have been made by Silver et al. (1984, 1988) for the localization and expression levels of the yeast GAL4 protein, where absence of the NLS signal results in a higher level of expression. The nature of this regulation is unknown.

**DISCUSSION**

We have purified the SRS2 protein from *E. coli* and demonstrated in vitro ATPase and DNA helicase activities, as predicted by the homology to the UvrD and Rep DNA helicases. The affinity of the protein for ATP is modest, but is within the range of that determined for other DNA helicases (Matson and Kaiser-Rogers, 1990). However, the turnover number for ATP hydrolysis is high, resulting in an efficient use of ATP. The turnover number we have determined is a lower limit as renaturation of the protein during the purification process may be less than 100%. The natural SRS2 protein may have higher affinity for ATP and be more efficient in the hydrolysis.

Two other DNA helicases, the RAD3 and PIF1 proteins, have been purified from *S. cerevisiae*. Both act in the 5' to 3' direction, which is the opposite polarity to the SRS2 DNA helicase. The RAD3 protein functions in excision repair (Wilkox and Prakash, 1981; Reynolds and Friedberg, 1981; Sung et al., 1988), while the PIF1 protein functions in mitochondrial DNA recombination (Fourny and Kolodny, 1983; Fourny and Van Dyck, 1985). Genetic data have indicated that the SRS2 protein acts in the error-prone repair pathway (Aboussekhar et al., 1989; Rong et al., 1991). In the absence of functional SRS2 protein, repair intermediates are channeled into a recombination repair pathway, while in the presence of functional SRS2 protein the repair substrates go through the error-prone repair pathway. We believe that SRS2 acts at the beginning of the repair process, but at this stage have no information as to whether the protein acts directly on the repair substrate. The RAD3 helicase cannot substitute in vivo for the SRS2 helicase, indicating that the two proteins act in different repair events.

Based on the proposed molecular mechanisms of repair helicases in both yeast and *E. coli*, we suggest that the SRS2 protein could function in one of two ways. In the first model the SRS2 helicase has a damage scanning function similar to that of the *E. coli* UvrAB helicase (Van Houten, 1991) and yeast RAD3 helicase in excision repair (Naegeli et al., 1992). The SRS2 protein binds in the vicinity of the damaged site, most likely on the undamaged strand, and unwinds DNA in a 3' to 5' direction (similar to the mode of *E. coli* helicase II in the excision repair pathway). The single strand region spanning the damage site would be the substrate for the subsequent repair steps by a DNA polymerase activity. Alternatively, the SRS2 helicase could act as an antirecombinator, ensuring that the repair substrates are not channeled into the recombination repair pathway, by destabilizing damage or mismatch containing heteroduplexes. Such a model has been suggested to explain both the hyper-recombination phenotype of some *uvrD* mutants and the recombination barrier between *E. coli* and *Salmonella typhimurium*, where the homology between the genomes of the two organisms is about 80% (Radman, 1988; Rayssiguier et al., 1989). Measurement of binding of the SRS2 protein to mismatched or damaged substrates in vitro may help in examining a possible role of the SRS2 protein in antirecombination function.

The SRS2 protein contains a 300-amino acid region at the carboxyl terminus that shows no significant homology to any known protein. Included within this region is a sequence that results in nuclear localization of the SRS2 protein. When the carboxyl-terminal 127 amino acids contained within the 300-amino acid region are fused to *LacZ*, nuclear localization of β-galactosidase was observed. We have not yet tested whether a derivative form of the SRS2 protein that includes the complete homology to UvrD but lacks the 300 carboxy-terminal amino acids has ATPase and DNA helicase activity. Additional information has been obtained concerning this region of the protein by the recovery of a missense mutation in this region (Palladino and Klein, 1992). This mutation has a modest effect on recombination and no significant effect on the meiotic phenotype seen in some SRS2 alleles. The mutation contains a sequence for nuclear localization of the protein, but there was no change in the amino acids residues of the protein. It may therefore be important for interaction of SRS2 with other, as yet unidentified, repair/recombination factors. It will be of interest to determine the biochemical activities of this mutant protein.

Overexpression of the SRS2 protein in *E. coli* resulted in a low level of protein, while overexpression of a truncated form resulted in a higher level. The poor expression of the wild-type protein could result from a functional interference of the SRS2 protein with *E. coli* proteins, perhaps the UvrD protein. We are currently testing this hypothesis by examining whether the SRS2 gene can complement *uvrD*-deficient strains. In addition, the occurrence of rare codons in the SRS2 coding region could limit the expression level in *E. coli* and yeast.

In yeast, deletion of the NLS-containing carboxy-terminal region of the SRS2 gene resulted in higher expression. NLS sequences have been postulated to regulate the steady state levels of nuclear proteins in yeast (Silver et al., 1988), hence deletion of this sequence could result in higher levels of expression.

The SRS2 protein has been shown to possess both ATPase and DNA helicase activities, as predicted from the protein sequence. Whether the protein contains additional activities is unknown. The carboxy-terminal region of 300 residues contains a sequence for nuclear localization of the protein, but may also be involved in protein stability and protein-protein interactions. The recovery of mutations in this region that do not involve the NLS sequence suggest that this region is important for the normal function of the protein. The precise function of the protein is unknown. The genetic data have suggested that the SRS2 protein functions in the error-prone repair pathway at the beginning of the process. Exactly what the natural substrate for the SRS2 protein is and
whether SRS2 acts together with an exonuclease and DNA polymerase is unknown. Some of these issues may be resolved by characterization of mutant protein forms from the collection of srs2 alleles that we have isolated (Palladino and Klein, 1992).

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