Identification of the Region in Yeast S-II That Defines Species Specificity in Its Interaction with RNA Polymerase II*

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Yeast S-II was found to stimulate yeast RNA polymerase II only and not mouse RNA polymerase II. To identify the molecular region of S-II that defines species specificity, we constructed six hybrid S-II molecules consisting of three regions from yeast and/or Ehrlich cell S-II and examined their activity in terms of RNA polymerase II specificity and suppression of 6-azauracil sensitivity in the yeast S-II null mutant. We found that the region 132–270 (amino acid positions) of yeast S-II is indispensable for specific interaction with yeast RNA polymerase II in vitro and for suppression of 6-azauracil sensitivity in vivo. The corresponding region of Ehrlich cell S-II, the region 132–262, was also shown to be essential for its interaction with mouse RNA polymerase II. This region is known to be less conserved than the N- and C-terminal regions in the S-II family suggesting that it is important in the interaction with transcription machinery proteins in a tissue and/or species-specific manner.

Transcription initiation is a complex process that involves protein–protein and protein–nucleic acid interactions, and factors participating in this process have been extensively characterized (1–4). On the other hand, regulation of gene expression at the transcription elongation level has been less thoroughly studied. Recently, it has become evident that various cellular and viral genes are regulated at the level of transcription elongation (5–8). Thus, transcription elongation is likely to be a crucial step for eukaryotic gene expression that involves various transcription elongation factors such as S-II(TFIIS) (9), 10, Elongin (SHI) (11, 12), TFIIF (13–15), and ELL (16).

Transcription elongation factor S-II was originally purified from mouse Ehrlich ascites tumor cells as a specific stimulatory protein of RNA polymerase II, and it was thought to participate in eukaryotic transcription (9, 10, 17–19). Subsequently, S-II was purified from various organisms and was shown to enable RNA polymerase II to read through blocks of transcription units of many eukaryotic genes by promoting cleavage of the 3′ end of the nascent RNA by RNA polymerase II (20–30).

Previously, we purified and characterized S-II from Saccharomyces cerevisiae (31, 32). Yeast S-II was found to contain an N-terminal region of 73 residues and a C-terminal region, including a zinc ribbon motif, that are relatively well conserved in the S-II proteins of many other species (33, 34). A gene disruption experiment revealed that the S-II null mutant is viable but becomes sensitive to 6-azauracil.

During the study of yeast S-II, we found that there is a strict species specificity in the combination of S-II and RNA polymerase II. Yeast S-II did not stimulate mouse RNA polymerase II and vice versa. To identify the region of the S-II molecule that prescribes species specificity, we constructed various hybrid clones between yeast and Ehrlich cell S-II. We found that the region between Pro-131 and Phe-270 is needed for yeast S-II to interact with yeast RNA polymerase II and thus to suppress 6-azauracil sensitivity of S-II null mutant in vivo. The N- and C-terminal regions were shown to be interchangeable between yeast and Ehrlich cell S-II without loss of function.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Purification of Recombinant Hybrid S-II**—All plasmids for hybrid S-II molecules with restriction sites at the desired junction points were created by the polymerase chain reaction using pYSlII-2 (yeast S-II gene) (31) and pSlII-3 (CDNA for Ehrlich cell S-II) (35) as templates. Blunt sites were introduced at nucleotide 392, which corresponds to residue 131 (Pro), of yeast and Ehrlich cell S-II, and PsmCI sites at nucleotide 808, which corresponds to residue 270 (Thr), of yeast S-II and nucleotide 765, which corresponds to residue 262 (Thr), of Ehrlich cell S-II. The resulting constructs were ligated with an expression vector pET-3d, transfected into E. coli BL21(DE3)pLYsE, and recombinant proteins were expressed (36). Each recombinant hybrid S-II was purified to homogeneity exactly as described previously (32). Recombinant hybrid S-II proteins were detected by SDS-polyacrylamide gel electrophoresis followed by immunoblotting using anti-yeast S-II antibody (31) or anti-Ehrlich cell S-II antibody (37).

**Assay of Stimulation of RNA Polymerase II by Hybrid S-II**—The activity of hybrid S-II proteins to stimulate yeast and mouse RNA polymerase II was assayed exactly as described by Nakanishi et al. (31) and Sekimizu et al. (9), respectively. One unit of RNA polymerase II was defined as the amount incorporating 1 pmol of UTP into the acid-insoluble fraction under these standard assay conditions.

**Glycerol Gradient Centrifugation**—This was done essentially as described by Horikoshi et al. (38). RNA polymerase II (30 pmol) and S-II (60 pmol) were incubated under the same conditions as for the RNA polymerase II stimulation assay but in the absence of DNA and nucleoside triphosphates. To separate S-II from mouse RNA polymerase II complex, the mixture was loaded onto a 5.2-ml linear gradient of glycerol (12.5–25%) containing 50 mM Tris/HCl, pH 7.9, 0.1 mM EDTA, 10 mM (NH4)2SO4, 0.3 mM diithiothreitol, and 0.04% Triton X-100. To separate S-II from yeast RNA polymerase II complex, the glycerol gradient was formed in 50 mM Tris/HCl, pH 7.9, 1.6 mM MnCl2, and 10 mM 2-mercaptoethanol. After centrifugation at 290,000 × g for 150 min at 0 °C, fractions were collected from the bottom of the tube. The RNA polymerase II and S-II in each fraction were detected by the enzyme assay and immunoblotting, respectively.

**Suppression of 6-Azauracil Sensitivity of Yeast S-II Null Mutant**—This was done essentially as reported previously (32). Briefly, plasmids containing hybrid S-II genes were introduced into the S-II null yeast mutant TNY14. Transformed cells were cultured in EMD medium (0.67% yeast nitrogen base without amino acids, 0.5% casamino acids technical, 2% glucose) with an appropriate supplement(s) at 30 °C until the optical density at 600 nm reached about 2.0. Then 2.5 × 106 cells
were transferred to 0.5 ml of fresh medium and incubated at 30 °C for 2 h. The cell suspension was diluted 1000-fold with sterilized water, and 120 ml of the diluted cell suspension was spread on YNBGS (0.67% yeast nitrogen base without amino acids, 5% galactose, 0.2% sucrose) plates containing an appropriate supplement(s) with or without 100 \( \mu \)g/ml 6-azauracil. Colonies on the plates were examined after incubation at 30 °C for 5 days.

Other Methods—Yeast and Ehrlich cell RNA polymerase II were purified as described previously (39, 40). Antibodies against yeast and Ehrlich cell S-II were prepared as described previously (9, 32). SDS-polyacrylamide gel electrophoresis was performed by the methods of Laemmli (41), and protein concentrations were determined by the method of Bradford (42). Affinity purification of antibodies and immunoblotting were performed according to the method described by Homma et al. (43). The immunofluorescence study was done with an affinity purified antibody against yeast S-II according to the standard procedure (44). DNA manipulations including restriction enzyme digestion, gel electrophoresis, DNA ligation, amplification of DNA by polymerase chain reaction, plasmid isolation, and \textit{E. coli} transformation were carried out by standard methods (45).

RESULTS

Recombinant Hybrid Molecules of Yeast and Ehrlich Cell S-II—During the study of yeast S-II, we found that recombinant yeast S-II stimulated only yeast and not mouse RNA polymerase II and vice versa. Experiments using various deletion mutants of yeast S-II showed that the last 168 C-terminal residues are sufficient for both stimulation of RNA polymerase II \textit{in vitro} and suppression of 6-azauracil sensitivity of the S-II null mutant \textit{in vivo} (32). To define the region responsible for species specificity of S-II, we constructed various hybrid molecules between yeast and Ehrlich cell S-II. For this, we divided the yeast S-II molecule into three parts, region 1–131 (amino acid positions), region 132–270, and region 271–309, and exchanged them with the corresponding regions of Ehrlich cell S-II to form six hybrid molecules.

The sequences of yeast and Ehrlich cell S-II and a schematic illustration of the hybrid molecules are shown in Fig. 1. To

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The sequences of yeast and Ehrlich cell S-II and a schematic illustration of the hybrid molecules are shown in Fig. 1. To
construct the hybrid molecules we chose two residues conserved in both yeast and Ehrlich cell S-II as junction points. These are Pro at position 131 and Thr at position 270 (position 262 in Ehrlich cell S-II).

After constructing cDNAs for these hybrid molecules, we expressed them in an E. coli expression system and purified them. As shown in Fig. 2A, each hybrid gave a single band of the expected size on SDS-polyacrylamide gel electrophoresis. A single character E or Y indicates one region of wild type Ehrlich cell S-II or yeast S-II and three aligned characters represents a hybrid molecule as shown in Fig. 1. Immunoblotting revealed that EYE, EY, and EYY strongly cross-reacted with an antibody against Ehrlich cell S-II, whereas YEY, YYE, and YEE strongly cross-reacted with an antibody against yeast S-II, indicating that they were in fact hybrid molecules (Fig. 2B). These antibodies seem to preferentially recognize the N-terminal regions of the corresponding S-II molecules.

Identification of the Domain Carrying Species Specificity in the Stimulation of RNA Polymerase II in Vitro—To identify the domain carrying species specificity, we examined the stimulatory activity of these six hybrid S-II molecules on yeast and mouse RNA polymerase II. As is evident from Fig. 3, region 132–270 (or 262 in the case of Ehrlich cell S-II) was shown to determine species specificity in terms of the stimulation of RNA polymerase II. When this region was derived from yeast S-II, the resulting hybrid molecules exclusively stimulated yeast RNA polymerase II in a dose-dependent manner. The same was true with Ehrlich cell S-II and mouse RNA polymerase II. These results indicate that residues within this approximate 140-amino acid region are essential for the interaction with RNA polymerase II of the same species, whereas the other two regions are exchangeable between yeast and Ehrlich cell S-II without loss of function.

Suppression of 6-Azauracil Sensitivity of the S-II Null Mutant by Hybrid S-II Expression—As described previously, the yeast S-II null mutant (TNY14) is viable but becomes sensitive to 6-azauracil and loses its colony-forming activity in the presence of 100 μg/ml 6-azauracil (32, 46). As the region 132–270 in S-II was shown to be crucial for its stimulation with RNA polymerase II in vitro the same was true with Ehrlich cell S-II and mouse RNA polymerase II. These results indicate that residues within this approximate 140-amino acid region are essential for the interaction with RNA polymerase II of the same species, whereas the other two regions are exchangeable between yeast and Ehrlich cell S-II without loss of function.

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As shown in Fig. 4A, YEY, YYE, and EYY transformants suppressed 6-azauracil sensitivity, whereas no significant suppression was detected when YEY, YYE, and YEE were expressed. Wild type Ehrlich cell S-II did not suppress 6-azauracil sensitivity either. These results also indicate that the region 132–270 is indispensable for yeast S-II activity in vivo and that the same region from Ehrlich cell S-II cannot compensate for the function of this region in yeast. To confirm that each trans-
formant did express the corresponding hybrid S-II molecule, we performed immunoblotting and detected EYE, EYE, and $\text{YEE} \text{ with anti-Ehrlich S-II antibody and YEE, YEE, and YEE with anti-yeast S-II antibody in the lysates of transformants tested (Fig. 4B).}

As S-II is a nuclear protein, it may not be able to function unless it is transported into the nuclei. To determine whether the hybrid S-II molecules with region 132–262 of Ehrlich cell S-II were transported into the yeast nuclei, we examined the subcellular distribution of YEE and YEE in the transformants by indirect immunofluorescence labeling. As is evident from Fig. 5, both hybrid molecules were found to localize in the nuclei as did wild type yeast S-II. Therefore, we concluded that although two hybrid S-II molecules examined were expressed and were localized in the nuclei in the respective yeast transformants, only YEE having the region 132–270 derived from yeast S-II was active in suppressing the 6-azauracil sensitivity of TNY14. This finding may be extended to other hybrid S-II molecules.

**DISCUSSION**

It is known that the N- and C-terminal regions of S-II are relatively well conserved among various species. In particular, the sequence identity of the first 30–40 C-terminal residues is over 70% among S-II family proteins. Previously, we demonstrated that yeast S-II and RNA polymerase II form a stoichiometric complex that can be detected by glycerol gradient centrifugation (38). Therefore, we examined whether the region 132–270 (or 262) of S-II is essential for specific interaction with RNA polymerase II of the same species by this method. A fixed amount of hybrid S-II together with yeast or mouse RNA polymerase II was incubated to accomplish complex formation, and then the mixture was subjected to glycerol gradient centrifugation to separate free S-II from the complex. After fractionation, the RNA polymerase II activity, and the amount of hybrid S-II, in each fraction was determined. As shown in Fig. 6, wild type S-II molecules detected by immunoblotting sedimented with RNA polymerase II of the same species only (A–D). The EYE and YEY S-II hybrids sedimented only with mouse and yeast RNA polymerase II, respectively. Alternative combinations did not form stable complexes (E–H). Thus, we concluded that the binding of S-II with RNA polymerase II occurs in a species-specific manner and that the region 132–270 (or 262) determines the species specificity of S-II in its interaction with RNA polymerase II.

**Binding of Hybrid S-II with RNA Polymerase II—Our in vitro and in vivo results strongly suggested that the region 132–270 (or 262) of S-II is essential for its specific interaction with RNA polymerase II of the same species. Previously, we demonstrated that Ehrlich cell S-II and RNA polymerase II form a stoichiometric complex that can be detected by glycerol gradient centrifugation (38). Therefore, we examined whether the region 132–270 (or 262) of S-II is essential for specific interaction with RNA polymerase II of the same species by this method. A fixed amount of hybrid S-II together with yeast or mouse RNA polymerase II was incubated to accomplish complex formation, and then the mixture was subjected to glycerol gradient centrifugation to separate free S-II from the complex. After fractionation, the RNA polymerase II activity, and the amount of hybrid S-II, in each fraction was determined. As shown in Fig. 6, wild type S-II molecules detected by immunoblotting sedimented with RNA polymerase II of the same species only (A–D). The EYE and YEY S-II hybrids sedimented only with mouse and yeast RNA polymerase II, respectively. Alternative combinations did not form stable complexes (E–H). Thus, we concluded that the binding of S-II with RNA polymerase II occurs in a species-specific manner and that the region 132–270 (or 262) determines the species specificity of S-II in its interaction with RNA polymerase II.

**FIG. 6. Binding of hybrid S-II with RNA polymerase II.** Hybrid S-II and RNA polymerase II were incubated to form a complex. Then the mixture was subjected to glycerol gradient centrifugation. After centrifugation, fractions were collected, and RNA polymerase II activity and the amount of S-II were determined for each fraction. A, C, E, and G represent binding with Ehrlich cell RNA polymerase II, and B, D, F, and H represent binding with yeast RNA polymerase II. The hybrid S-II molecules used were as follows: A and B, wild type Ehrlich cell S-II (control); C and D, wild type yeast S-II (control); E and F, YEE; G and H, YEY.

By constructing various hybrid molecules of yeast and Ehrlich cell S-II, we have demonstrated that the region 132–270 (or 262 in the case of Ehrlich cell S-II), consisting of 138 residues, determines the species specificity and that the above mentioned 113 residues are included in this region. Recent structural analysis of yeast S-II revealed that it is composed of three distinctive structural domains, termed domains I (1–105/124), II (106/125–246), and III (247–309) (47). We divided yeast S-II into three regions on the basis of previous deletion experiments (32) and sequence comparison with Ehrlich cell S-II. The region 132–270 is located between domain II and domain III and does not contain the zinc ribbon.
Agarwal et al. (48) showed that the region 100–230 of human S-II is required for binding to human RNA polymerase II in vitro. As human S-II and Ehrlich cell S-II are very similar, the region 100–230 of human S-II may correspond to the region 132–262 of Ehrlich cell S-II; however, it is not known whether Ehrlich cell S-II stimulates human RNA polymerase II. Cipres-Palacin and Kane (49) prepared two mutants of human S-II, named TFIIS5 (E174A, E175A) and TFIIS7 (K187A, E175A), and showed that those residues are critical for arrest release activity of S-II. As all these residues are located in the region corresponding to the region 132–262 of Ehrlich cell S-II, these mutants are likely to have lost the ability to interact with RNA polymerase II of the same species. This report presents two unique findings as follows: 1) yeast and Ehrlich cell S-II were not interchangeable in either in vitro or in vivo transcription systems, and 2) less conserved sequences in the region 132–270 (or 262) of S-II were found to define species specificity. These findings suggest that this region and possible S-II binding site of RNA polymerase II co-evolved in yeast and mice. The other two regions were shown to be interchangeable between yeast and Ehrlich cell S-II. The third region, region 271–309 (262–301 in the case of Ehrlich cell S-II), is crucial for the stimulation of RNA polymerase II (32), so the molecular mechanism of the stimulation of RNA synthesis by S-II is the same regardless of the species. Therefore, identifying the region of RNA polymerase II that interacts with the region 132–270 of S-II will provide a clue about the regulation of transcription at the level of elongation.

It is noteworthy that both the YEY and YVE hybrid molecules were expressed in yeast and migrated into nuclei. It is difficult to assign the nuclear transport signal to a region of S-II, but these nuclear localization experiments rule out the possibility that the failure of YEY to suppress 6-azauracil sensitivity of an S-II deletion strain is simply due to mislocalization of this hybrid protein.

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