Site-directed Mutagenesis of Arginine 246, Glutamic Acid 247, and Histidine 248 in the Eukaryotic Transcription Factor S-II*

Nobuo Horikoshi, Kazuhisa Sekimizu, and Shunji Natori†
From the Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

When His248 of the transcription factor S-II was replaced by alanine or tyrosine, the activity of the resulting mutants was less than 30% of that of the wild-type S-II. When Arg246, Glu247, and His248 were all replaced by leucine, the resulting mutant showed complete loss of activity. These results indicate that the amino acid sequence Arg-Glu-His at positions 246–248 of S-II is important for its stimulatory activity. The mutant S-II with no activity could not form a complex with RNA polymerase II, unlike wild-type S-II, but retained ability to interact with DNA.

Transcription factor S-II was originally purified as a protein stimulating RNA polymerase II from Ehrlich ascites tumor cells (1, 2). Subsequently, this factor was shown to be important in nuclear RNA synthesis (3). The same factor, cross-reactive immunologically with Ehrlich cell S-II, was purified from calf thymus (4). Recently, S-II was found to influence the ability of RNA polymerase II to read through a transcription pause site within the adenovirus genome (4, 5). Furthermore, Reines et al. (6) found that S-II enables RNA polymerase II to read through intrinsic terminator sequences present in the human histone gene intron.

Northern blotting experiments using S-II cDNA showed that transcription of the S-II gene of CD4-positive cells was enhanced by infection with human immunodeficiency virus under conditions in which transcription of the actin gene was repressed (7). Incidentally, the human immunodeficiency virus genome was found to contain intrinsic terminator sequences (8). Thus, it is likely that S-II is also involved in the transcription of the human immunodeficiency virus genome.

S-II consists of two domains of 21 and 18 kDa which can be separated by chymotryptic digestion in the presence of DNA (9). The 21-kDa domain, located in the carboxyl-terminal half of the S-II protein, is sufficient alone for the stimulation of RNA polymerase II (9). The 18-kDa domain contains a phosphorylation site, and phosphorylated S II, termed S-II', was found to have no appreciable activity to stimulate accurate transcription in an Ehrlich cell lysate although it stimulated purified RNA polymerase II (10, 11).

Analysis of cDNA of S-II showed that S-II consists of 301 amino acid residues and contains only 1 histidine residue at position 248 (12). This paper describes the results of site-directed mutagenesis, showing that at least 3 amino acid residues including this His248 are important for stimulation of RNA polymerase II.

EXPERIMENTAL PROCEDURES

Construction of Plasmids Containing S-II cDNA and Their Expression in Escherichia coli—E. coli HB101 was used as a host for expression vectors. The NdeI-Dral fragment of pSII 3 (a cDNA clone of S-II (12)), which contains the entire coding region of S-II, was inserted into the SmaI site of an expression vector PKK233-3 containing trp-lac (lac) promoter and named pSIKK. As this plasmid does not contain a signal peptide, we constructed another plasmid designed to produce a hybrid molecule of S-II and the signal peptide of Bacillus subtilis alkaline phosphatase. For this, we first inserted a synthetic linker P12 with the sequence CATGGAAGCTTC into the KpnI site of SIIKK to add the Ala-Ser sequence to the amino-terminal methionine of S-II and to create a HindIII site; we named this plasmid pSIKKH. Then we isolated a BamHI-HindIII fragment of pSIKKH containing the S-II sequence with the modified amino terminus and inserted it between the BglII and HindIII sites of pEAP92-3. This plasmid was originally designed by Murakami et al. (13) as an expression vector for E. coli and contains a gene for B. subtilis alkaline phosphatase. This plasmid was expected to produce a hybrid molecule of S-II and the signal peptide of B. subtilis alkaline phosphatase, and the signal peptide was expected to be cleaved between Ala-Ser in E. coli, releasing S-II protein with an extra serine residue in its amino terminus.

For production of S-II, E. coli HB101 carrying the expression vector of S-II was cultured in a medium containing 1.6% tryptone, 1% yeast extract, 0.2% glycerol, and 0.5% NaCl at 37 °C. Cells were harvested when the optical density at 650 nm reached 0.6-0.7.

Site-directed Mutagenesis of S-II—cDNA for mutant S-II with a substituted amino acid(s) in the vicinity of the histidine residue at position 248 was constructed by oligonucleotide-directed in vitro mutagenesis with an Amersham Corp. mutation kit. The synthetic mutagenic primers used for mutant construction were 5'-CACGAGGCTCAGATGGCGCAAG-3' for histidine to alanine substitution (mutant HA), 5'-GCCATCAGGGATTCGATAGGCGCAAG-3' for histidine to tyrosine (mutant HY), and 5'-AAAGAAGGGCATCCCTACTACGAGAAGAGCAG-3' for Arg-Glu-His to Leu-Leu-Leu (mutant RRH). Base changes in cDNA were confirmed by dideoxy sequencing using synthetic or universal oligonucleotide sequence primers (14). The HindIII-BamHI fragment containing mutant S-II cDNA was inserted into expression vector pEAP92-3 as described above.

Purification of S-II from E. coli Cells—All operations were conducted at 0 °C unless otherwise stated. Freshly harvested E. coli cells (9–10 g) were lysed by lysozyme-EDTA treatment. Solid ammonium sulfate was added to the lysate to a concentration of 0.5 g/ml, and the resulting precipitate was collected by centrifugation. The precipitate was dissolved in 20 ml of buffer 1 (10 mM Tris-HCl, pH 7.9, 5 mM 2-mercaptoethanol, 0.1% Triton X-100) and dialyzed against the same buffer. The sample was diluted 2-fold and applied to a column of DEAE-cellulose (2.5 x 5 cm) equilibrated with buffer 1. Unadsorbed materials were pooled and applied to a column of phosphocellulose (1.5 x 3.5 cm) equilibrated with buffer 2 (20 mM Tris-HCl, pH 7.9, 5 mM 2-mercaptoethanol, 0.1% Triton X-100). The column was washed extensively, and then S-II was eluted with 50 ml of a gradient of 0.3–0.5 M NaCl. Fractions containing S-II were pooled, dialyzed against buffer 2, and subjected to fast protein liquid chromatography on a Mono S column (Pharmacia LKB Biotechnology...
RESULTS

Isolation of S-II Mutants with Substitutions for Histidine Residues

As shown by cDNA analysis, S-II contains only 1 histidine residue in its 21-kDa domain. To determine the function of this His\textsuperscript{248}, we treated S-II with diethyl pyrocarbonate or p-bromophenacyl bromide, both of which are known to modify histidine residues selectively (17, 18), and tested the activity of the modified S-II. As shown in Fig. 1, 0.1 mM diethyl carbonate or 5 mM p-bromophenacyl bromide inhibited the activity of S-II almost completely whereas RNA polymerase II itself was not affected appreciably under these conditions. These results suggested that His\textsuperscript{248} is important for the activity of S-II. However, we could not exclude the possibility that other amino acid residues were also modified by these reagents under these conditions. Therefore, to confirm the role of His\textsuperscript{248} in S-II, we constructed mutant proteins in which His\textsuperscript{248} was replaced by other amino acid residues.

We first tried to produce S-II in E. coli. For this, the coding sequence of S-II cDNA pSII-3 was ligated to the signal peptide of B. subtilis alkaline penicillinase in the expression vector pEAP82-3. With this construction, the S-II produced should have an extra serine residue in its amino terminus judging from the specificity of the signal peptidase.

Using this system, it was possible to produce the recombinant S-II in E. coli, and it could be purified to homogeneity. As shown in Fig. 2A, the final preparation contained a single protein with the same mobility as authentic S-II. Moreover, this protein reacted with anti-S-II antibody on immunoblotting, as shown in Fig. 2B. Table I summarizes the purification of recombinant S-II from E. coli. About 20 μg of pure S-II was obtained from 9 g of cells.

The molecular activity and enzyme specificity of S-II produced by E. coli were compared with those of authentic S-II. As shown in Fig. 3, the two preparations of S-II stimulated RNA polymerase II from Ehrlich ascites tumor cells similarly depending on the doses of S-II used but had almost negligible effect on wheat germ RNA polymerase II. From these results, we concluded that S-II produced by E. coli has the same activity and specificity as authentic S-II.

Next we constructed cDNAs for two mutants, HA and HY, with substitutions for histidine residues. Site-directed mutagenesis of S-II was carried out using RNA polymerase II purified from Ehrlich ascites tumor cells, as described before (15, 16).

Fig. 1. Effects of two reagents modifying histidine residues on the RNA polymerase II stimulatory activity of S-II. Authentic S-II (2 ng) was incubated with various concentrations of diethyl pyrocarbonate (A) or p-bromophenacyl bromide (B) for 1 h at 37 °C. Then bovine serum albumin was added at a final concentration of 0.1% to trap the reagents, and the stimulatory activity of S-II was measured using 20 units of purified RNA polymerase II. O—O, RNA polymerase II + reagent (control); ●—●, RNA polymerase II + S-II.

Fig. 2. Analyses of S-II produced by E. coli. A, electrophoretic profiles of proteins in various fractions obtained during purification of recombinant S-II. The gel was calibrated with various molecular mass markers and stained with Coomassie Brilliant Blue. Lane 1, authentic S-II (0.15 μg); lane 2, lysozyme-EDTA extract (20 μg); lane 3, ammonium sulfate fraction (32 μg); lane 4, DEAE-cellulose fraction (9.9 μg); lane 5, phosphocellulose fraction (0.25 μg); lane 6, Mono S fraction (0.08 μg). B, Western blot analysis of the same gel with affinity-purified anti-S-II antibody. Proteins cross-reacting immunologically with S-II were located using a radiolabeled second antibody.

Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity</th>
<th>Protein</th>
<th>S-II</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEA-cellulose</td>
<td>ND\textsuperscript{a}</td>
<td>80</td>
<td>μg</td>
<td>units/μg protein</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>13,600</td>
<td>0.35</td>
<td>μg</td>
<td>39,000</td>
</tr>
<tr>
<td>Mono S</td>
<td>3,900</td>
<td>0.015</td>
<td>μg</td>
<td>243,000</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Not determined.

Fig. 3. Activity and specificity of recombinant S-II. The stimulatory activities of authentic S-II and recombinant S-II were compared using Ehrlich cell RNA polymerase II (open symbols) or wheat germ RNA polymerase II (closed symbols). Assays were done in the absence of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} to increase the sensitivity, as reported before. ○, authentic S-II; ■, □, recombinant S-II.
FIG. 4. Stimulatory activities of S-II mutants obtained by site-directed mutagenesis. A, activities of HY and HA mutants and wild-type S-II. △, HY mutant; ●, HA mutant; ○, wild-type S-II. B, activities of the REH mutant and wild-type S-II. ●, REH mutant; ○, wild-type S-II.

Characterization of S-II Mutants—We first examined the activity of mutant S-IIs. As shown in Fig. 4A, the molecular activities of HA and HY mutants were less than 30% of that of the wild-type S-II, but these mutants clearly had activity to stimulate RNA polymerase II. However, as shown in Fig. 4B, the REH mutant had almost no activity. Thus, we concluded that the amino acid residues in the vicinity of His\(^{246}\) are important and that at least the sequence of the 3 amino acid residues Arg-Glu-His at position 246–248 is essential for the activity of S-II.

Two distinct characters of S-II are that it forms a complex with RNA polymerase II (19) and that it can be separated into two domains by digestion with chymotrypsin in the presence of DNA (9). We examined whether the REH mutant, which had no activity to stimulate RNA polymerase II, showed these two characters.

Wild-type S-II and the REH mutant were each incubated with purified RNA polymerase II (20) and three nucleoside triphosphates in the presence of template DNA, as described before (9). The resulting complex formed was subjected to glycerol density gradient centrifugation, and the fractions containing RNA polymerase II were analyzed by SDS-polyacrylamide gel electrophoresis. As shown in Fig. 5, with the REH mutant no band corresponding to S-II was detected (lane 3) whereas a stoichiometric amount of wild-type S-II was recovered in the RNA polymerase II fraction (lane 2). This finding indicated that the REH mutant was not able to form a complex with RNA polymerase II. Thus, the Arg-Glu-His sequence is essential for interaction of S-II with RNA polymerase II.

Although S-II alone is not susceptible to chymotrypsin, it is cleaved into 18-and 21-kDa domains by this enzyme in the presence of DNA, suggesting that the conformation of S-II changes on interaction with DNA. We examined the cleavage of the mutant S-II. As shown in Fig. 6, like wild-type S-II the REH mutant was cleaved into 21- and 18-kDa fragments in the presence of DNA but was resistant to chymotryptic digestion in the absence of DNA. Thus, mutant S-II without the Arg-Glu-His sequence interacted with DNA with a change in conformation in the same way as wild-type S-II did.

DISCUSSION

S-II consists of 301 amino acid residues as shown by the analysis of its cDNA (12). Site-directed mutagenesis revealed
that at least 3 amino acid residues in the vicinity of His$^{248}$ are essential for its stimulatory activity. The 21-kDa domain of S-II is sufficient alone for stimulation of RNA polymerase II (9), and the Arg-Glu-His sequence is located in the 21-kDa domain (12). Of these 3 amino acid residues, we found that histidine could be replaced by alanine or tyrosine although the resulting mutants showed significantly lower activity than wild-type S-II. This means that His$^{248}$ is important but not essential for the activity of S-II.

The charge in the vicinity of His$^{248}$ probably contributes greatly to the activity of S-II because the REH mutant with Leu-Leu-Leu instead of Arg-Glu-His in this region showed no activity to stimulate RNA polymerase II. This loss of stimulatory activity of the REH mutant was shown to be due to loss of the ability to interact with RNA polymerase II, so we predicted that the structure of Arg-Glu-His is important for the interaction of S-II with RNA polymerase II.

The site in the S-II molecule which is essential for interaction with DNA is not directly related to the Arg-Glu-His sequence because the REH mutant became susceptible to chymotryptic digestion in the presence of DNA like wild-type S-II. Thus, S-II probably has independent binding sites for RNA polymerase II and DNA.

In this study we developed an expression vector to produce S-II in E. coli. This vector was constructed by combining the signal sequence of B. subtilis alkaline penicillinase and the coding sequence of S-II. Theoretically, the recombinant S-II molecule should contain an extra serine residue in its amino terminus. However, as the activity, antigenicity, and mobility on SDS gel of the recombinant S-II were exactly the same as those of authentic S-II, we did not analyze the amino-terminal amino acid residue of the recombinant S-II. This recombinant S-II should be useful in analysis of the function of S-II.

Acknowledgments—We are grateful to Drs. K. Horikoshi and C. Kato for providing us with expression vectors.

REFERENCES

Site-directed mutagenesis of arginine 246, glutamic acid 247, and histidine 248 in the eukaryotic transcription factor S-II.
N Horikoshi, K Sekimizu and S Natori


Access the most updated version of this article at http://www.jbc.org/content/265/20/11854

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/20/11854.full.html#ref-list-1