Role of a small RNA pol II subunit in TATA to transcription start site spacing

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ABSTRACT
The yeast shi mutation affects the spacing between the TATA promoter element and transcription initiation sites; for the H2B and ADH1 genes, a series of start sites located -50-80 bp downstream of TATA is used in addition to the wild-type initiation sites located at around 100 bp from TATA (1). Here, the yeast SHI wild-type gene has been isolated by complementation and shown to be identical to RPB9, the gene encoding a small subunit of RNA polymerase II. A point mutation in the shi gene, changing a cysteine residue in a putative zinc ribbon motif into a phenylalanine residue, was demonstrated to permit the observed usage of upstream initiation sites. Deletion of the non-essential SHI gene also results in usage of upstream initiation sites and causes conditional growth defects.

INTRODUCTION
The major positioning signal for initiation of pre-mRNA transcription by eukaryotic RNA polymerase II (pol II) is the TATA sequence. In addition to the well-documented binding of TBP to this element, pol II start site selection involves other proteins, including the initiation factor TFII B and one or more integral pol II subunits (reviewed in 2). Recently, Li et al. (3) analyzed biochemically the interplay between these components in specifying the transcription start. They took advantage of the inherent difference in TATA to start distance between Saccharomyces cerevisiae (40-120 bp) and Schizosaccharomyces pombe (~30 bp). The species-specific determinants of transcription starting proved to be the transcription factor TFII B and RNA polymerase II itself. When TFII B and RNA polymerase II from S. cerevisiae were replaced with their S. pombe homologs in a system in which the remaining components were derived from S. cerevisiae, S. pombe-specific starts were seen. The role of TFII B was not unexpected, since it was shown earlier that a mutation in the SUA7 gene, which was identified as a yeast homolog of human TFII B, affects the location of start sites (4). Since RNA polymerase II consists of at least 11 subunits in yeast (5), these in vitro studies raise interesting questions regarding the identity and mode of function of pol II apoenzyme subunits that participate in transcription initiation and start site specification. In view of their strong evolutionary conservation in prokaryotic and eukaryotic RNA polymerases, the two largest pol II subunits are likely participants in these steps. Indeed, mutations in RPB1, the yeast gene encoding the largest pol II subunit, have recently been reported to shift transcription initiation sites away from the TATA element (6). We describe here mutant effects upon the TATA to start distance that act in the opposite manner, by decreasing the TATA to start distance. Surprisingly, the protein encoded by the gene which is mutated is a small and non-essential subunit of RNA polymerase II. The yeast gene we have previously named SHI is shown to be identical to RPB9. We demonstrate that a single base pair mutation in a cysteine motif as well as deletion of the gene lead to the observed shift in RNA initiation sites.

MATERIALS AND METHODS
Yeast strains, media and transformation

The originally isolated shi strain (EG1), used for cloning the wild-type SHI gene and for primer extension analysis, has genotype MATa shi ade2 ade4 trp1 adh1Δ adh2Δ adh3 SUP4::URA3-Δ50. Except for the shi mutation, this strain is otherwise identical to the wild-type SHI parent strain (EG2) used for primer extension analysis. [Identical to strains designated shi and Δ50, respectively, as described in (1).] For determining the minimal complementing piece of the SHI clone, a MATα shi cyh2 leu2 trp1 adh1 adh2 adh3 SUP4::URA3-Δ50 strain (EG3), derived by a cross of the original shi strain EG1, was used. A diploid strain (EG5) derived from a cross of SHI strains EG2 and EG4 (MATα SHI cyh2 leu2 trp1 ura3) was used for disruption of the SHI allele. Strain EG6, containing a deletion of the SHI locus, is a Ura+ haploid segregant of strain EG5, with genotype MATα ade2 trp1 leu2 cyh2 adh1Δ adh2Δ adh3 SHI::URA3. Yeast media, matings, sporulation, transformation and genetic techniques were as described in (1). Medium lacking inositol was prepared as described in (7).

Plasmids
Plasmid pCEN-SHI/1.5 was constructed by inserting the 1.5 kb PstI-HincI fragment encoding the putative SHI gene (see Fig.

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2) into the corresponding restriction sites of single copy vector pRS314 (8). pCEN-SHI/4.1 is a corresponding construct containing the 4.1 kb SalI–BamHI fragment of the wild-type SHI gene. The SnaBI–BglII fragment of the wild-type SHI allele contained in pCEN-SHI/4.1 was replaced by the corresponding fragment of the shi mutant allele, containing the mutation of cysteine 7 to phenylalanine, thereby generating plasmid pCEN-shi/4.1. The two plasmids differ only by a single base pair change, as verified by sequencing both strands in the region SnaBI–BglII for both plasmids. For testing the ability of the putative SHI gene to target homologous recombination to the correct locus, the 3.2 kb PstI–BamHI fragment was fused next to the TRP1 selective marker in integrating plasmid pRS304 (8) to create plasmid pINT-SHI/3.2.

Construction of the shi deletion mutant strain

The URA3 marker gene was fused between the two BglII sites within the SHI gene, thereby removing the coding sequence for the N-terminal 69 amino acids along with 290 bp of 5′-upstream sequences (Fig. 2B and C). A linear fragment containing the inserted URA3 marker flanked by remaining SHI sequences was used to transform ura3/bura3 diploid strain EG5. Eight Ura+ diploids were sporulated and the resulting tetrads dissected. For each of the dissected diploids, 4 spored tetrads could be obtained; for the ura3/bura3 marker segregated 2:2. Southern blot analysis on Ura+ haploid segregants from 4 of the dissected diploids showed that the SHI locus in these segregants was disrupted and that the deleted BglII fragment was indeed absent (not shown).

RNA isolation, primer extension, and PCR reactions

Preparation of total RNA and primer extensions were performed as described previously (9). Two oligonucleotides were used for primer extension experiments. One is complementary to the S.cerevisiae PGK gene between nucleotides +27 and +48 (5′-GTCCTTTCAAGTCCAAATCTG-3′) and the other is complementary to the S.cerevisiae histone H2B-1 gene in the region between +29 and +46 (5′-CATGCTGGGTCTTTGG-3′). Primer extensions with the PGK-specific oligonucleotide were done with 400 units M-MLV reverse transcriptase (BRL) at 37°C for 1 h. Extensions with the H2B-specific oligonucleotide were done with 15 units AMV reverse transcriptase (Promega) at 42°C. For the PCR amplification of the shi allele, an oligonucleotide 5′-GGGAAAACA-CATTATTGG-3′ homologous to the region 180 bp downstream of the DraI site, and a second oligonucleotide 5′-CCAGATA-GAAGAGATCCCTTGCTAGTATAAG-3′, spanning the BglIII and SnaBI sites, were used.

RESULTS

The isolated SHI gene is identical to RPB9

The shi mutation was recovered in a genetic selection designed to identify proteins involved in initiation site selection in S.cerevisiae. The selection relied on the use of a defective reporter gene for which the location of the initiation sites must be shifted upstream in order to produce a functional mRNA. For this purpose, we used an alcohol dehydrogenase allele (adhΔ50) with an artificially short distance between the TATA sequence and ATG. In wild-type S.cerevisiae, this gene yielded only non-functional message that initiated from sites downstream of the first ATG codon (Fig. 1; see also 1). Trans-acting mutations were identified that activated the expression of an adhΔ50 allele integrated in a S.cerevisiae strain devoid of its endogenous ADH genes. The resulting mutant strains could then be tested for their effects on transcription initiation. In strains containing the shi gene, named for shift of initiation, RNA start sites for adhΔ50 are shifted upstream, closer to TATA (Fig. 1). In addition to its effects on initiation sites chosen for the adhΔ50 allele, the shi gene permits initiation sites closer to TATA to be used for several other genes (1).

The wild-type SHI gene could be cloned by complementation, due to the recessivity of the shi allele. Transformation of a shi adhΔ50 strain with a plasmid containing the dominant wild-type SHI allele would be expected to change the cellular phenotype from Adh+ to Adh−. A plasmid with an 8.5 kb DNA insert (pCEN-SHI/8.5) that behaved in this manner was identified by transforming a trpl shi adhΔ50 mutant strain (Adh+ phenotype, strain EG1) with a library of yeast sequences contained on a CEN TRP1 vector (10), and screening for an Adh− transformant. After a passage through Escherichia coli, pCEN-SHI/8.5 was retransformed into strain EG1, resulting in Adh− transformants only. The 8.5 kb insert included a 1.5 kb PstI–HincII fragment (Fig. 2) which conferred the dominant Adh− phenotype when expressed from the single copy plasmid pCEN-SHI/1.5 in a leu2 trpl shi adhΔ50 (Adh+) strain (strain EG3).

To verify that the isolated DNA fragment was indeed derived from the SHI locus, we showed that the putative SHI gene could target homologous integration at or very near to the SHI locus. Integrating plasmid pINT-SHI/3.2 containing the putative SHI gene fused next to a TRP1 marker (see Materials and Methods)
was linearized at the unique HincII restriction site within the PstI-BamHI piece. After integrative transformation of shi trp1 adhΔ50 strain EG1, Adh- Trp+ prototrophs were obtained. Such a transformed strain was mated to shi trp1 cyh2 adhΔ50 strain EG3, and the segregation of the integrated TRP1 marker was monitored by tetrad dissection with respect to the cyh2 (scored by resistance to cycloheximide) and shi loci (scored by growth on antianycin-containing plates). No recombination was observed between the integrated TRP1 marker and the SHI locus in a total of 30 dissected, viable tetrads, indicating that the cloned DNA could target homologous recombination to the SHI locus. Furthermore, the TRP1 marker mapped 25 cm away from the cyh2 locus, in agreement with the map distance between shi and cyh2 determined previously (1).

DNA sequencing of the PstI-HincII fragment revealed a single open reading frame that encodes a predicted protein of 122 amino acids (Fig. 2B and D). Comparison with known sequences showed that the SHI gene is identical to RPB9, the yeast gene encoding the 12.6 kDa subunit of RNA pol II. The RPB9 gene had previously been cloned using oligonucleotides designed from tryptic fragments of the purified subunit (11).

Deletion of the SHI gene produces the same effects as the shi mutation

To produce a yeast strain lacking the chromosomal SHI allele, a defective SHI allele lacking codons for the first 69 amino acids and marked with a URA3 gene (Fig. 2C) was integrated by homologous recombination in one homolog of diploid strain EG5. After sporulation and tetrad dissection, the resulting haploid strain harboring the shi deletion allele was able to grow at 30°C, indicating that the SHI/RPB9 gene is not essential at this temperature, as has also been reported earlier (11). However, several growth related phenotypes were observed in the shi deletion strain. Germination of tetrads from strains heterozygous for the disrupted shi locus was reduced. In 48% of the tetrads from strains in which the SHI gene was disrupted on one homolog, only 1-3 of the spores were able to germinate; in the 52% of tetrads showing germination of all 4 spores, microcolonies were frequently observed from one or two of the spores. Furthermore, haploid strains with the disrupted shi allele grew more slowly on complete medium at 30°C, were severely defective for growth at 37°C and 18°C, and were auxotrophic for inositol. Interestingly, the analogous deletion in Drosophila of the fly homolog of RPB9, which is 46% identical to its yeast counterpart at the amino acid level, caused lethality (12). This suggests that the Drosophila homolog has either acquired some additional, essential functions within the polymerase holoenzyme that the yeast subunit lacks, or, more likely, that the effect of abolishing the function of this polymerase subunit is more deleterious to Drosophila than to yeast, due to the stricter spacing regimen between TATA and start sites in higher eukaryotes.

The absence of the SHI protein led to a similar effect on RNA initiation sites as was seen with the shi allele. Start sites for the endogenous yeast PGK and H2B genes were mapped using the

Figure 2. Restriction maps and sequence information for the shi complementing DNA. (A) Originally isolated DNA piece which complemented the shi mutation when expressed from single copy vector M111. (B) Detailed map of the sequenced, complementing PstI-HincII piece. The open reading frame of the SHI gene is indicated by an open arrow. (C) The URA3 gene was used to replace part of the SHI gene and its promoter to construct a SHI:URA3 deletion mutant by homologous recombination. (D) The nucleotide sequence and corresponding amino acid sequence of the 122 amino acid open reading frame of the SHI/RPB9 protein. The SHI and RPB9 DNA sequences were identical throughout the coding region, with the exception of the third position of codon glutamine 82 (marked with *; GAG in RPB9), not resulting in an amino acid change. Upstream of the ATG (not shown), 5 scattered nucleotide differences were found, reflecting differences in the yeast strains from which the respective genes were isolated. Cysteine residues are shown in bold. The site of the TGT—TTT, Cys—Phe change in the shi allele is shown with an asterisk.
primer extension technique for RNA isolated from the \textit{shi} and \textit{shi} deletion strains. In both strains, prominent upper bands were seen for the \textit{PGK} gene (Fig. 3A, position \(-81\)) as well as for the \textit{H2B} gene (Fig. 3B, positions \(-90\) and \(-76\)), corresponding to start sites that were only weakly used in wild-type strains. These initiation sites, which are located between 48 and 69 bp downstream of TATA, are used in addition to the wild-type sites at distances of 102 (\textit{H2B}) and 112 (\textit{PGK}) bp downstream. By inference, transcription starts on the \textit{adh}\textit{\textDelta 50} allele are also shifted upstream by the deletion of \textit{SHI}; like the \textit{shi} strain, the \textit{shi} deletion strain was \textit{Adh}\textsuperscript{+} in the presence of the \textit{adh}\textit{\textDelta 50} allele.

The mutation in the \textit{shi} allele is a Cys to Phe change in a \textit{Cys}\textsubscript{4} zine ribbon

To find the molecular basis for the observed shift of RNA initiation caused by the \textit{shi} allele, we used the polymerase chain reaction to amplify the coding region of the \textit{shi} gene from several independent DNA samples prepared from the \textit{shi} strain. The entire amplified \textit{SnaBl}–\textit{DraI} fragment (Fig. 2B) was sequenced for one PCR product and was found to be identical to the wild-type \textit{SHI} gene with the exception of a single base pair change.

Partial sequencing of the other independently isolated PCR products showed that all contained the identical single base pair change, altering the cysteine residue at amino acid position 7 (encoded by a TGT codon) to a phenylalanine residue (encoded by a TTT codon). This cysteine residue is located in the first of two cysteine-rich (\textit{Cys}\textsubscript{4}) domains with sequences \textit{CX}_{2}\textit{CX}_{16}\textit{CX}_{3}\textit{C} and \textit{CX}_{2}\textit{CX}_{23}\textit{CX}_{2}\textit{C} (where \textit{C} denotes cysteine and \textit{X} any other amino acid) as noted by Woychik \textit{et al.} (11) for the \textit{RPB9} gene.

To show that this sequence alteration is solely responsible for the shift of initiation sites seen in the \textit{shi} mutant strain, we demonstrated that a plasmid containing this single base pair change could not restore the wild-type growth phenotype or RNA initiation pattern when transformed in the \textit{shi} deletion strain. The \textit{shi} deletion mutant strain was transformed with plasmids p\textit{CEN-SH1/4.1} and p\textit{CEN-shi/4.1}, which are identical except for the mutation causing the cysteine to phenylalanine residue change. Transformation of the wild-type \textit{SHI} gene into the \textit{shi} deletion strain complemented not only the inositol requirement for growth but also reversed the ability of the \textit{shi} deletion strain to activate the \textit{adh}\textit{\textDelta 50} allele. In addition, the RNA initiation patterns for the \textit{PGK} and \textit{H2B} genes in these transformants were very similar to those seen in the wild-type strains (Fig. 3); the prominent upper start site (position \(-81\)) of the \textit{PGK} gene and the upper start sites (positions \(-76\) and \(-90\)) for \textit{H2B} were essentially unused.

Transformation of the wild-type \textit{SHI} gene into the \textit{shi} mutant strain led to a similar restoration of the wild-type RNA initiation patterns for these genes (not shown). This demonstrates that the wild-type \textit{SHI} gene complements the shift in initiation site phenotype. In contrast, transformants of the mutant \textit{shi} gene in the \textit{shi} deletion strain could not grow in the absence of inositol, and could not complement the \textit{Adh}\textsuperscript{+} phenotype of the \textit{shi} deletion strain that was due to activation of the \textit{adh}\textit{\textDelta 50} allele. Additionally, the pattern of start sites was unaffected by introduction of the mutant \textit{shi} gene by transformation (Fig. 3). This result identifies the cysteine to phenylalanine change as responsible for the observed shift of initiation sites in the \textit{shi} mutant strain.

**DISCUSSION**

Mutations in the \textit{S.cerevisiae} \textit{TFIIB} gene (\textit{SU47}) and the gene encoding the largest subunit of pol II (\textit{SU48}) have been identified in a selection scheme requiring a downstream shift in RNA initiation sites (4, 6). Our independent selection for proteins involved in RNA start site selection in \textit{S.cerevisiae} yielded a mutation in another subunit of RNA polymerase II, the 12.6 kDa subunit. The wild-type \textit{SHI} gene was cloned through complementation of the \textit{shi} mutant phenotype, and the mutant \textit{shi} allele was subsequently isolated by PCR amplification. The mutant allele was found to contain a single base pair mutation, changing a cysteine to a phenylalanine. This single base pair mutation led to the same molecular phenotype as deletion of the entire gene, resulting in increased usage of upstream initiation sites for the \textit{PGK} and \textit{H2B} genes.

The finding that substitution of a cysteine residue in one \textit{Cys}\textsubscript{4} motif leads to an apparent loss of function of the \textit{SHI} protein underscores the functional importance of this motif. The two \textit{Cys}\textsubscript{4} motifs in \textit{SHI/RPB9} belong to a family of sequences analogous to the \textit{Cys}\textsubscript{4} motif seen in the transcription elongation factor TFIIS; the solution structure of TFIIS has been shown by
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