Cleavage, but Not Read-through, Stimulation Activity Is Responsible for Three Bio logical Functions of Transcription Elongation Factor S-II*

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Toshiharu Ubukata‡, Tomoko Shimizu‡, Nobuki Adachi, Kazuhisa Sekimizu§, and Toshiyuki Nakanishi¶‡
From the ‡Frontier Project 3, Proteome Research Laboratory, Daiichi Pharmaceutical Company, Ltd.,
519 Shimo-Ishibashi, Ishibashi-machi, Shimotsuga-gun, Tochigi 329-0512, Japan and §Graduate School
of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Transcription elongation factor S-II stimulates cleavage of nascent transcripts generated by RNA polymerase II stalled at transcription arrest sites. In vitro experiments have shown that this action promotes RNA polymerase II to read through these transcription arrest sites. This S-II-mediated cleavage is thought to be necessary, but not sufficient, to promote read-through in the in vitro systems. Therefore, Saccharomyces cerevisiae strains expressing S-II mutant proteins with different in vitro activities were used to study both the cleavage and the read-through stimulation activities of S-II to determine which S-II functions are responsible for its biologic functions. Strains expressing mutant S-II proteins active in both cleavage and read-through stimulation were as resistant as wild type strains to 6-azaaracil and mycophenolic acid. 6-Azaaracil also induced IMD2 gene expression in both these mutant strains and the wild type. Furthermore, strains having a genotype consisting of one of these S-II mutations and the spt4 null mutation grew as well as the spt4 null mutant at 37 °C, a restrictive temperature for a strain bearing double null mutations of spt4 and S-II. In contrast, strains bearing S-II mutations defective in both cleavage and read-through stimulation had phenotypes similar to those of an S-II null mutant. However, one strain expressing a mutant S-II protein active only in cleavage stimulation had a phenotype similar to that of the wild type strain. These results suggest that cleavage, but not read-through, stimulation activity is responsible for all three biologic functions of S-II (i.e. suppression of 6-azaaracil sensitivity, induction of the IMD2 gene, and suppression of temperature sensitivity of spt4 null mutant).

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† To whom correspondence should be addressed. Tel.: 81-285-51-2382; Fax: 81-285-51-2226; E-mail: nakanfut@daichipharm.co.jp.
‡ The abbreviations used are: 6-AU, 6-azaaracil; MPA, mycophenolic acid.
importance of cleavage stimulation activity of S-II

Table 1 Yeast strains used in this study

| Strain   | Genotype                                      |
|----------|----------------------------------------------|
| HKY01    | MATa ura3-5 lys2-801amber ade-2-101htr           |
| HKY02    | MATa ura3-5 lys2-801amber ade-2-101htr          |
| TU100    | MATa ura3-5 lys2-801amber ade-2-101htr          |
| TU101    | MATa ura3-5 lys2-801amber ade-2-101htr          |
| TU102    | MATa ura3-5 lys2-801amber ade-2-101htr          |
| Mt1      | MATa ura3-5 lys2-801amber ade-2-101htr          |
| Mt2      | MATa ura3-5 lys2-801amber ade-2-101htr          |
| Mt3      | MATa ura3-5 lys2-801amber ade-2-101htr          |
| Mt4      | MATa ura3-5 lys2-801amber ade-2-101htr          |
| Mt5      | MATa ura3-5 lys2-801amber ade-2-101htr          |
| Mt6      | MATa ura3-5 lys2-801amber ade-2-101htr          |
| Mt7      | MATa ura3-5 lys2-801amber ade-2-101htr          |
| Mt8      | MATa ura3-5 lys2-801amber ade-2-101htr          |
| Mt9      | MATa ura3-5 lys2-801amber ade-2-101htr          |
| sp4A     | MATa ura3-5 lys2-801amber ade-2-101htr          |
| sp4ptA   | MATa ura3-5 lys2-801amber ade-2-101htr          |
| sp4ptptA | MATa ura3-5 lys2-801amber ade-2-101htr          |
| sp4ptptptA | MATa ura3-5 lys2-801amber ade-2-101htr         |
| pMt5     | MATa ura3-5 lys2-801amber ade-2-101htr          |
| pMt6     | MATa ura3-5 lys2-801amber ade-2-101htr          |
| pMt7     | MATa ura3-5 lys2-801amber ade-2-101htr          |
| pMt8     | MATa ura3-5 lys2-801amber ade-2-101htr          |
| pMt9     | MATa ura3-5 lys2-801amber ade-2-101htr          |

Yeast strains used in this study

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Importance of Cleavage Stimulation Activity of S-II

rupted by URA3 or HIS3 gene introduction in these strains, respectively. Plasmid pH1180 was constructed by inserting the 3.4-kb PvuII fragment of the S. cerevisiae S-II gene into plBluescript KS (+) (Stratagene, La Jolla, CA). To screen for mutant clones, silent base-exchange mutations were introduced to form the following new restriction enzyme recognition sites in each mutant: KasI site for Mt1 and Mt2; SacII sites for Mt3, Mt4, Mt5, and Mt6; an SpI site for Mt5, a BglII site for Mt6; and a PvuII site for Mt7. pMt7 was constructed by inserting the PstI-NcoI fragment of the S-II gene amplified by PCR with the primers 5'-AAATCGGAGATCGGGCGACGAGCTTTAGGTTAAG-3' and 5'-GGGGGCGAGGTTTCACTTACGTGATCC-3' into the PstI-NcoI site of pH1180. pMt1 was constructed by inserting the PstI-KosI fragment of the S-II gene amplified by PCR with the primers 5'-AAATCGGAGATCGGGCGACGAGCTTTAGGTTAAG-3' and 5'-GGGGGCGAGGTTTCACTTACGTGATCC-3' into the PstI-NcoI site of pH1180. pMt1 was constructed by inserting the PstI-NcoI fragment of the S-II gene amplified by PCR with the primers 5'-AAATCGGAGATCGGGCGACGAGCTTTAGGTTAAG-3' and 5'-GGGGGCGAGGTTTCACTTACGTGATCC-3' into the PstI-NcoI site of pH1180. The primers used were 5'-CCATCAGATGTCATGCAGCCATGCAAATCAACCGAGGAAAAGGCTGTCG-3' and 5'-GATCGACAGCTTTCTGTGAGTCGAGTCAG-3' followed by restriction enzyme digestion. The introduction of the mutation sequences into the S-II locus was confirmed by Southern blot analysis. S-II genes were amplified from each S-II mutant strain, and sites of mutation were confirmed by DNA sequencing. Each mutant strain bore only the introduced mutation.

A PCR-based gene disruption method (25) was used to prepare all spt4A strains. PCR with the plasmid pH5416 (Stratagene) as a template was used to prepare the DNA fragment containing the URA3 gene used to disrupt the S-II gene. The primers used were 5'-ATTAGTTACTA-TTAAATGTCATGCAACGAAGGAAAGGTATGCTGACTGAGGTG-3' and 5'-TTTACACTCTGCACATCCATTAGCTTTGCAAAGGACTGATGACCC-3' followed by restriction enzyme digestion. The resulting fragment was separately introduced into YPH499, HKY02, and Mt1 to -9 using an EZ Yeast Transformation Kit. URA3 transformants were selected on SD (+) agar plates (0.67% [w/v] yeast nitrogen base without amino acids; 2% [w/v] glucose; 0.1 mg/ml each histidine, adenine sulfate, tryptophan, and lysine; and 0.25 mg/ml leucine; 2% [w/v] 20% baagotaer), and Southern blot analysis was used to confirm gene disruption.

Anti-yeast S-II Antibodies—Recombinant yeast wild type S-II protein was expressed in Escherichia coli and purified as described elsewhere (2). Anti-yeast S-II antisera was prepared at Asahi Techno Glass Corp. (Chiba, Japan) by injecting a New Zealand White rabbit with 0.3 mg of the purified recombinant yeast S-II protein. Anti-yeast S-II antibodies were affinity-purified from the antisera as previously described (26).
Hybond-P membrane (Amersham Biosciences). The blot was incubated with affinity-purified anti-yeast S-II antibodies at 4 °C for 16 h and then with anti-rabbit IgG (donkey) conjugated to horseradish peroxidase (Amersham Biosciences) at 20 °C for 1 h. ECL Plus (Amersham Biosciences) was used for detection. A Kaleidoscope Prestained Standard protein ladder (Bio-Rad) was used to estimate molecular weights.

**Indirect Immunofluorescence**—The same yeast strains used in the Western blot analysis were separately incubated in SD (ura−) medium at 30 °C until they reached midlog phase growth, and then 0.18 volume of formaldehyde and 0.13 volume of 1 M potassium acetate buffer (pH 6.5) were added to the medium. Each culture was incubated at 23 °C for 1 h. Cells were then collected by centrifugation and resuspended in 1 M potassium acetate buffer (pH 6.5) containing 5% formaldehyde and incubated at 23 °C for 1 h. The cells were then collected by centrifugation and washed once with SHA buffer (1 M sorbitol, 0.1 M HEPES-Na, pH 7.2, and 5 mM NaN3) and resuspended in SHA. Cells were then treated with Zymolyase 100T (Seikagaku Corp., Tokyo, Japan) and transferred onto ADCELL slides (Erie Scientific Co.; Portsmouth, NH). Cells were fixed in methanol at −20 °C for 6 min and then in acetone at −20 °C for 30 s. Fixed cells were incubated with affinity-purified anti-yeast S-II antibodies or preimmune rabbit IgG followed by incubation with anti-rabbit IgG (goat) conjugated to the Alexa-488 fluorescent dye and then with anti-rabbit IgG (donkey) conjugated to horseradish peroxidase. The plates were incubated at 30 °C for 60 min. The plates were incubated at 30 °C (restrictive temperature for dst1) and 37 °C (restrictive temperature for dst1 Spt42A double null mutant) for 5 days.

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**RESULTS**

**Construction of S-II Mutant Strains**—The *S. cerevisiae* S-II protein consists of three domains: domains I, II, and III. Domains II and III are joined by a short linker region (30). Awrey et al. (31) constructed a variety of the *S. cerevisiae* S-II mutant proteins that span domains II and III. They then determined the in vitro cleavage and read-through-stimulating activities of these mutant S-II proteins with an assay that used a human histone H3.3 gene fragment, which contains well-characterized transcriptional arrest sites, as the template (31). To see whether both cleavage and read-through-stimulating activities of S-II were responsible for its biologic functions, nine of these mutant S-II proteins, designated Mt1 to Mt9, were selected for further tests. Results from the in vitro assays conducted by Awrey et al. (31) show that four of the mutant proteins had strong cleavage stimulation but different read-through-stimulating activities (Mt1, -4, -5, and -7); four others (Mt2, -6, -8, and -9) had moderate cleavage stimulation but different read-through-stimulating activities, and Mt3 had little of either activity (Table II). Mt6 has a mutation in domain II, which binds to RNA polymerase II. Mt1, -2, and -7 have mutations in the linker region, and the other mutant proteins have mutations in domain III, which is essential for transcript cleavage and read-through-stimulating activities as well as 6-AU sensitivity suppression (31, 32). These mutations were introduced into the S-II locus of *S. cerevisiae* by homologous recombination.

First, Western blot analysis was used to determine the amount of mutant S-II proteins expressed, and indirect immunofluorescence was used to determine the cellular localization of the mutant S-II proteins; both methods used anti-yeast S-II specific antibodies. Results of the Western blot analysis show that all S-II mutant strains have expression patterns similar to that of the wild type strain (Fig. 1). In addition to a major band of 42 kDa, which was as large as the recombinant S-II, a faint band (35 kDa) was observed in all strains. Since this signal was not detected in the S-II null mutant HKY01 (Fig. 1), it is

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**Table II**  
**Summary of the phenotypes of S-II mutants**  
**In vitro activities** of the mutants were determined by Awrey et al. (31). 0, inactive or partially active at 500:1 (S-II protein:RNA polymerase II); 1, active at 500:1; 2, active at 100:1; 3, active at 5:1 for *in vitro* activities. +, resistant or strong induction; −, sensitive; **−−**, very sensitive or marginal induction for *in vivo* activities.

| S-II         | Cleavage | Read-through | 6-AU resistance | MPA resistance | IMD2 induction | Temperature resistance |
|--------------|----------|--------------|-----------------|----------------|----------------|------------------------|
| Wild type    | 3        | 3            | +               | +              | +              | +                      |
| Null mutation| 0        | 0            | −               | −              | −              | −                      |
| Mt1 (K249A/Q243A) | 2       | 2            | +               | +              | +              | +                      |
| Mt2 (N252A/Q255A) | 1       | 1            | +               | +              | +              | +                      |
| Mt3 (R287E/Q291L) | 0       | 0            | −               | −              | −              | −                      |
| Mt4 (F296A)  | 2        | 2            | +               | +              | +              | +                      |
| Mt5 (K307A)  | 0        | 0            | −               | −              | −              | −                      |
| Mt6 (R298A)  | 2        | 2            | +               | +              | +              | +                      |
| Mt7 (F298A)  | 1        | 1            | +               | +              | +              | +                      |
| Mt8 (R287E/Q291N) | 1       | 0            | −               | −              | −              | −                      |
| Mt9 (R287Q)  | 1        | 1            | +               | +              | +              | +                      |

(0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, and 0.06% (w/v) complete supplement mixture (−his, −leu, −trp) from Qiagen Inc., 0.1 mg/ml each leucine, tryptophan, and lysine, and 0.04 mg/ml adenine sulfate) at 30 °C for 16 h and then diluted to A600 = 0.085 with YPDA medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, and 0.04 mg/ml adenine sulfate). The diluted cultures were incubated until the A600 reached 0.17 and then diluted 10-, 100-, 1000-, and 10,000-fold; 7 μl of each culture was then spotted onto YPDA plates. Plates were then incubated at either 30 or 37 °C (restrictive temperature for dst1 Spt42A double null mutant) for 5 days.

**Other Methods**—DNA manipulation was performed as described elsewhere (28). The method of Bradford (29) was used to determine protein concentration; bovine serum albumin was used as a standard.

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**Importance of Cleavage Stimulation Activity of S-II**

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**Temperature Sensitivity Assay**—*S. cerevisiae* strains were separately incubated in SC (ura−) plates containing 75 μg/ml MPA, or no drug. The plates were incubated at 30 °C until they reached midlog phase growth, and then 0.18 volume of formaldehyde and 0.13 volume of 1 M potassium acetate buffer (pH 6.5) were added to the medium. Each culture was incubated until the A600 reached 0.17 and then 0.0425. The weights.

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**Drug Sensitivity Assay**—The same yeast strains used in the Western blot analysis were separately incubated in SD (ura−) medium at 30 °C until midlog phase, and then 6-μl to give 75 μg/ml was added. After 0, 0.5, or 2 h, cells were collected by centrifugation, and a hot phenol solution of S-II were responsible for its biologic functions, nine of these mutant S-II proteins, designated Mt1 to Mt9, were selected for further tests. Results from the in vitro assays conducted by Awrey et al. (31) show that four of the mutant proteins had strong cleavage stimulation but different read-through-stimulating activities (Mt1, -4, -5, and -7); four others (Mt2, -6, -8, and -9) had moderate cleavage stimulation but different read-through-stimulating activities, and Mt3 had little of either activity (Table II). Mt6 has a mutation in domain II, which binds to RNA polymerase II. Mt1, -2, and -7 have mutations in the linker region, and the other mutant proteins have mutations in domain III, which is essential for transcript cleavage and read-through-stimulating activities as well as 6-AU sensitivity suppression (31, 32). These mutations were introduced into the S-II locus of *S. cerevisiae* by homologous recombination.

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thought that the smaller band represents a degradation product of S-II. Results of preliminary studies suggest that the smaller form lacks the carboxyl terminus region that is essential for RNA polymerase II stimulation, read-through stimulation in vitro, and 6-AU sensitivity suppression in vivo (32). The band intensity of the Mt4 S-II protein is reproducibly weaker than the S-II proteins from the other mutant strains (Fig. 1). These data suggest that, compared with the S-II proteins from the other mutant strains, the amount of S-II protein expressed by the Mt4 strain was lower, or the antigenicity of Mt4 S-II protein is weaker. However, it should be noted that results presented below indicate that the amount of Mt4 S-II protein expressed was apparently sufficient to perform its biologic functions (Table II).

The results of indirect immunofluorescence experiments to locate S-II are shown in Fig. 2. As shown in Fig. 2A, anti-S-II staining of the wild type strain TU100 showed strong fluorescence that overlapped with nuclear staining, as previously reported (33). In contrast, no fluorescence was observed in S-II null mutant strain HKY01 stained with anti-S-II (Fig. 2B) or in wild type (Fig. 2B) and Mt1 to Mt9 (data not shown) cells stained with the same concentration (1.7 μg/ml) of preimmune rabbit IgG. The staining patterns of Mt1 to Mt9 with anti-S-II were indistinguishable from that of the wild type (Fig. 2C) and overlapped with nuclear staining (data not shown). These results indicate that all of the mutant S-II proteins are localized in the nucleus as is the wild type S-II protein.

**Drug Sensitivity in S-II Mutant Strains**—The *S. cerevisiae* S-II null mutant is known to be sensitive to 6-AU (2) and MPA (20). To investigate the biologic effects of S-II mutant proteins, the sensitivity of each S-II mutant strain to 6-AU and MPA was tested. Fig. 3 shows that mutant strains Mt1, -2, -4, -5, -6, -7, and -9 are as resistant to 6-AU as the wild type is; in contrast, Mt3 and Mt8 are as sensitive to 6-AU as is the S-II null mutant. Interestingly, despite its inability to promote read-through in vitro (Table II), Mt5 is as resistant to 6-AU as is the wild type.

**IMD2 Gene Induction in S-II Mutant Strains**—The IMD2 gene encodes IMP dehydrogenase, a key enzyme in the de novo synthesis of guanine nucleotides (34). The activity of IMP dehydrogenase is inhibited by MPA and by 6-azauridine monophosphate, an active metabolite of 6-AU (20). IMD2 gene transcription can be induced by the addition of 6-AU or MPA to the culture medium (22), and this induction depends on S-II. This induction is thought to be important for the development of 6-AU and MPA resistance. Therefore, the ability of 6-AU to induce IMD2 gene transcription in the S-II mutant strains was tested. The wild type TU100 strain, the S-II null mutant HKY01 strain, and the mutant strains Mt1 to Mt9 transformed with pRS416 were incubated in a synthetic medium in the presence or absence of 75 μg/ml 6-AU for up to 2 h, and Northern blot analysis was used to determine the amount of IMD2 mRNA produced by each strain. As shown in Fig. 4,}

### FIG. 1. Expression of S-II mutant proteins. Yeast strains were incubated in SD (ura-) medium at 30 °C until they reached midlog phase, and then cells were collected, lysed, and used for Western blot analysis with anti-yeast S-II antibody. Purified recombinant S-II was used as a positive control. The numbers represent the S-II mutant strain numbers 1–9. WT, wild type TU100 strain; KO, S-II null mutant HKY01 strain.

| S-II mutant | Recombinant S-II (ng) |
|-------------|------------------------|
| 1           | 42 kDa                 |
| 3           | 35 kDa                 |

### FIG. 2. Cellular localization of S-II mutant proteins. S-II proteins and yeast nuclei were visualized as described in “Experimental Procedures.” DAPI, nuclear staining with 4',6-diamidino-2-phenylindole. Bars, 10 μm. A, wild type cells stained with anti-S-II antibody. B, S-II null mutant cells stained with anti-S-II antibody and wild type cells stained with preimmune rabbit IgG as negative controls. C, results for S-II mutant strains Mt1 to Mt9. Only S-II stainings are shown.

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mutant strains Mt1, -2, -4, -5, -6, -7, and -9 induced IMD2 gene expression as well as the wild type strain did, whereas mutant strains Mt3 and Mt8 were transcriptionally inactive as the S-II null mutant. These data illustrate that the biologic ability to induce the IMD2 gene transcription correlates positively with the in vitro cleavage stimulation activity exhibited by the S-II mutant proteins.

**Temperature Sensitivity of the Yeast Strains Bearing S-II Mutations and spt4 Null Mutation**—SPT4 gene encodes a subunit of the transcription elongation factor DSIF (DRB sensitivity-inducing factor) (35). A null mutant of the DST1 gene, which encodes S. cerevisiae S-II, shows temperature sensitivity when combined with the spt4 null mutation, whereas the spt4 null mutant is not temperature-sensitive (17). To see whether mutant S-II proteins can suppress the temperature-restricted growth of the dst1/H9004 spt4/H9004 double null mutant strain, S-II mutant strains carrying the spt4 null mutation were constructed and tested for growth at 37 °C. The results are shown in Fig. 5. The dst1/H9004 spt4/H9004 double null mutant strain showed clear temperature sensitivity. Mt1spt4/H9004, Mt4spt4/H9004, Mt5spt4/H9004, Mt7spt4/H9004, and Mt9spt4/H9004 grew as well as spt4/H9004 at 37 °C, indicating that the Mt1, -4, -5, -7, and -9 S-II mutant proteins suppress the temperature sensitivity as well as does the wild type S-II protein. However, the read-through activities of these five S-II mutant proteins vary and do not correlate with their suppression activities. Mt2spt4/H9004 and Mt6spt4/H9004 showed moderate temperature sensitivity, indicating that the suppression activities of Mt2 and Mt6 proteins are weaker than that of the wild type S-II protein. Mt3spt4/H9004 and Mt8spt4/H9004 were temperature-sensitive, showing that the Mt3 and Mt8 proteins lack suppression activity. These results indicate that the biologic ability to suppress temperature-restricted growth correlates positively with the in vitro cleavage stimulation activities of the S-II mutant proteins.

**DISCUSSION**

At a transcriptional arrest site in a gene, S-II stimulates the nuclease activity of RNA polymerase II into cleaving several bases from the nascent transcript and causes the realignment of the 3' end of the nascent transcript with the active site of the polymerase, and the transcription elongation complex tries to read through the arrest site again (11–14). Results from previous studies have shown that this cleavage stimulation activity of S-II is necessary, but not sufficient, to promote read-through in vitro (15). To clarify this observation, S. cerevisiae S-II mutant strains were used to investigate the roles of the cleavage
and read-through stimulation activities of S-II in vitro. A 6-AU sensitivity assay (Fig. 3), RNA analysis for IMD2 induction by 6-AU (Fig. 4), and rescue from temperature-dependent growth inhibition of separate strains carrying one of the S-II mutations and an spt4 null mutation (Fig. 5) were used to assess S-II function. The results show that S-II mutant proteins that were active in both cleavage stimulation and read-through in vitro also suppressed 6-AU sensitivity, induced IMD2 gene expression, and suppressed delta1/kappaDelta temperature-dependent growth inhibition. In contrast, those S-II mutant proteins in an spt4 null mutation (Fig. 3), RNA analysis for inhibition of separate strains carrying one of the S-II mutations 6-AU (Fig. 4), and rescue from temperature-dependent growth inhibition of separate strains carrying one of the S-II mutations were not caused by insufficient protein expression or by cellular mislocalization. These results, summarized in Table II, strongly suggest that the cleavage stimulation activity of S-II alone is responsible for its biologic functions, whereas the read-through stimulation activity of S-II is dispensable. Previously, S-II has been regarded as a transcriptional read-through factor, and its read-through activity has been assumed to be important for its biologic functions; however, the results of the present study do not support this notion. It is possible, however, that the S-II mutant proteins that are inactive in an in vitro read-through assay have sufficient read-through activity in the cell.

The results of the present study also imply the presence of some as yet unidentified transcriptional read-through factors. Since the mutant S-II protein Mt5, which lacks read-through activity in vitro, exhibited full biologic activity, it is possible that once the nascent transcript is cleaved by RNA polymerase II, other read-through factors might promote read-through in place of a read-through-incompetent S-II protein in Mt5 strain. Awrey et al. (31) suggested that a conformational change in the ternary elongation complex (RNA polymerase II-DNA template-nascent transcript) is required for read-through after the cleavage of the nascent transcript. It has been suggested that S-II and Rpb9 influence the conformational change necessary for read-through (31). Thus, Rpb9 is a good candidate for a read-through factor other than S-II. Mutant yeast strains sensitive to 6-AU that bear the Mt5 mutation may be good tools for identifying this putative read-through factor. Another possible approach would be to use an in vitro read-through assay system containing the S-II Mt5 protein to isolate read-through-promoting factors biochemically. These studies, which are currently under way, are the next logical steps in understanding the regulation of transcription elongation by RNA polymerase II in eukaryotic cells.

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Importance of Cleavage Stimulation Activity of S-II 8585

39.
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