The catalytically competent transcription complex of RNA polymerase II from the fission yeast Schizosaccharomyces pombe was affinity labeled with photoreactive nucleotide analogues incorporated at 3' termini of nascent RNA chains. To locate the catalytic site for RNA polymerization, the labeled subunits were separated by SDS-polyacrylamide gel electrophoresis and subjected to partial proteolysis. After microsequencing of proteolytic fragments, a complex multidomain organization was indicated for both of the two large subunits, Rpb1 and Rpb2, with the most available sites of proteolysis in junctions between the conserved sequences among RNA polymerase from both prokaryotes and eukaryotes. The cross-linking studies indicate the following: (i) the 3' termini of growing RNA chains are most extensively cross-linked to the second largest subunit Rpb2 between amino acids 825 and 994; (ii) the regions 298–535 of Rpb2 and 614–917 of Rpb1 are cross-linked to less extents, suggesting that these regions are situated in the vicinity of the catalytic site. All these regions include the conserved sequences of RNA polymerases, and the catalytic site of Rpb2 belongs to an NH$_2$-terminal part of its conserved sequence II.

Eukaryotic RNA polymerase II is a multifunctional and multisubunit enzyme consisting of more than 10 putative subunits (1, 2). In contrast to prokaryotic RNA polymerases, little is known about the molecular architectures of eukaryotic counterparts except the following: (i) the two largest subunits, homologous to bacterial $\beta$ and $\beta'$ subunits, are involved in the binding of DNA template, the polymerization of RNA chains, and the association of nascent RNA chains (1, 2); and (ii) the subunit 3, a homologue of bacterial $\alpha$ subunit, plays a role in the assembly of RNA polymerase (3). Knowledge of the structure and function of the individual subunits is essential for understanding of the molecular mechanisms of transcription and regulation of the protein-coding genes in eukaryotes. Toward this ultimate goal, we have studied the structure-function relationship of Schizosaccharomyces pombe RNA polymerase II.

The S. pombe RNA polymerase II is composed of 11 subunits lacking subunit 4 (4, 5). Analyses of the subunit-subunit contact network using different approaches indicate that the two large subunits provide the platform for assembly of other small subunits (6–9). In this study, we tried to locate the active center for RNA polymerization using the method of affinity labeling with photoreactive nucleotide analogues. This technique has been used to identify the active sites of Escherichia coli RNA polymerase (10–13), calf thymus RNA polymerase II (14), Saccharomyces cerevisiae RNA polymerase I(A), II(B), and III(C) (15–18), HeLa RNA polymerase II (19), wheat germ RNA polymerase II (18, 20), and influenza virus RNA polymerase (21). Depending on experimental systems such as the type of enzymes and templates, the structure of affinity reagents and the length of nascent RNA chains, the cross-linking has been observed at different portions of the respective RNA polymerases, but always on one or both of the two large subunits, i.e. prokaryotic $\beta$ and $\beta'$ subunits or their eukaryotic homologues.

Here we tried to cross-link transcriptionally competent elongation complexes of S. pombe RNA polymerase II with photoreactive nucleotides incorporated at 3' termini of growing RNA chains. The 3'-ends of nascent RNA chains were found to be cross-linked to both of the two large subunits, Rpb1 and Rpb2, in good agreement with the previous findings with the S. cerevisiae RNA polymerase II (22, 23). Furthermore, after proteolytic cleavage of the subunit proteins cross-linked with radiolabeled nascent RNA chains at the 3' termini, we proceeded to locate the contact sites of the growing RNA 3'-ends on the primary sequences of the two large subunits of S. pombe RNA polymerase II.

**EXPERIMENTAL PROCEDURES**

**RNA Polymerase**—RNA polymerase II was purified from S. pombe by either the standard procedure in this laboratory (24) or nickel affinity column chromatography (3).

**Templates**—DNA templates (Templates 1 and 2) used in this study have the following structure (the underlined sequences represent the tetranucleotides which direct the incorporation of UMP or UMP analogues and CMP residues).

- **Template I**
  
  5'–AA AGA GGA AAA GAG GGG AAA GGA GTC CCA GGA AAC CCG G–3'
  
  3'–(C)$_n$ TT TCT CTT TTT CTC TCC TTT CCT CAG GGT CCT TTG GCC CC–5'

- **Template II**
  
  5'–TCC CAA AGA GGA AAA GAG GGG AAA GGA GAG GAA ACC CCG G–3'
  
  3'–(C)$_n$ AGG GTT TCT CTT TTT TTT CTC TTT TTT GTC TTT TTT GCC C–5'
ambient temperature for annealing. Annealed full-length duplexes were separated from shorter, incomplete chains and unannealed oligonucleotides by non-denaturing 20% PAGE and recovered from gel slices by diffusion in 0.3 M sodium acetate (pH 5.2). The duplexes were concentrated by repeated extraction with n-butanol, precipitated with ethanol, and dissolved in water. DNA concentrations were determined by measuring the absorbance at 260 nm.

**Photoactive UTP Analogues—SOC1 (1 ml)** was added dropwise to 5-ml methanol solutions of γ-aminobutyric acid, ε-aminoacrylic acid, or ω-aminoacrylic acid (2 mmol each), which are the precursors of analogues 2, 3, and 4, respectively (see Fig. 1). Methyl ethers of the corresponding aldehydes obtained were evaporated after treatment with N-hydroxysuccinimide esters of p-azidotetrafluorobenzoic acid (334 mg, 1 mmol) which was prepared according to the published procedure (25). The products were separated by flash chromatography on SiO2 (hexane:chloroform:methanol, 5:5:1). The compounds obtained were dissolved in methanol (4 ml) and treated with NaOH (1 ml of 10% solution) for 2.5 days. The reaction mixtures were acidified by adding 2 ml HCl to pH below 1 and extracted three times with equal volumes of chloroform. The chloroform solutions were dried over Na2SO4 and evaporated. The derivatives of p-azidotetrafluorobenzoic acid with the attached residues of linear amino acids obtained were converted into N-hydroxysuccinimide esters. Compounds were dissolved in 65.5 ml of dry CHCl3 and treated with dicyclohexylcarbodiimide (206 mg, 1 mmol) and N-hydroxysuccinimide (115 mg, 1 mmol) overnight. The products were purified by chromatography on silica gel (hexane:chloroform:methanol, 5:5:1). Fractions containing the product were pooled and evaporated in vacuo.

5-(Aminopropenyl)-1-uridine-5'-triphosphate was synthesized as described (26). The photoactive derivatives were obtained by overnight treatment of this compound with an excess amount of N-hydroxysuccinimide ethers of p-azidotetrafluorobenzoic acid derivatives described above in a solvent of dimethylformamide:water (3:1) mixture in the presence of triethylamine added as a catalyst. The products were subjected to chromatography on DEAE-cellulose DE52 (250 × 10 mm) and eluted with a 0–0.3 M linear gradient of triethylammonium bicarbonate (pH 7.0). The final step of purification was achieved by reverse phase chromatography (Lichroprep RP-18, 250–10 mm) with a 0–50% linear gradient of acetonitrile in 0.05 M triethylammonium bicarbonate (pH 7.0). The products were evaporated, dissolved in the minimum volume of water, and precipitated with 2% LiClO4 in acetone.

**Chemicals and Enzymes—α-[32P]CTP and [α-32P]GTP were purchased from Amersham Pharmacia Biotech. RNAsin, Staphylococcus aureus V8 protease, subtilisin, and papain were products of Takara (Japan), Sigma, Boehringer Mannheim (Germany), and Sigma, respectively. Benzonase and RNase T1 were from Sigma and TCI Biomedicals, respectively. γ-Aminobutyric acid, ε-aminoacrylic acid, and ω-aminoacrylic acid were products of Sigma. All other chemicals used in this work were commercially available products of the highest quality.**

**Analysis of RNA Products—** Transcription assay was carried out at 37°C for 10 min in 70 µM Tris-HCl (pH 8.0), 75 µM (NH4)2SO4, 5 mM MgCl2, 0.15 mM DTT, 5 mM spermidine, 2 µM template I or II, 0.4 mM each of ATP, CTP, UTP or UTP analogues, 0.05 mM GTP, 0.15 µCi/µl [α-32P]GTP, 2 units/µl RNasin, and 0.04 µg/µl RNA polymerase II. After the reaction, an equal volume of 2× formamide loading buffer (27) was added followed by heat treatment for 2 min at 95°C. RNA products were analyzed on 13.5% PAGE in the presence of 6 M urea.

**Affinity Labeling of RNA Polymerase II—** RNA polymerase II (3 µg) was incubated for 4 min at 37°C in 40-µl reaction mixtures containing 70 µM Tris-HCl (pH 8.0), 75 µM (NH4)2SO4, 5 mM MgCl2, 0.15 mM DTT, 5 mM spermidine, 0.4 mM ATP, 0.4 mM one of the photoreactive UTP derivatives, 0.4 mM GTP, and 6 µM template I or II. The transcription mixture was then UV-irradiated at 100 µJ/cm2 for 1 min with Funa UV Linker (λ = 256 nm). After the addition of 1 µl of [α-32P]CTP (10 µCi/µl, final concentration 0.25 µCi/µl), the incubation was continued for 15 min at 37°C, and then 10 µl of 10% SDS and 2.6 µl of 1 M DTT were added to make final concentrations of 2% and 60 mM, respectively. The mixtures were heated 6 min at 95°C and immediately cooled on ice. The reaction products were treated with a mixture of nuclease (benzonase A, 0.02 mg/ml and RNase T1, 7.5 μg/ml) for 20 min at 37°C to hydrolyze RNA. After addition of 56 µl of 2× SDS loading buffer (22), the mixtures were heated for 3 min at 100°C and analyzed on SDS-7.5% PAGE.

**Mapping of the Labeled Sites—** Gel pieces containing radioactive RNA polymerase II subunits were excised and subjected to peptide mapping by limited proteolysis essentially according to the procedure of Cleveland (28, and see Ref. 21). Gel pieces containing labeled subunits were soaked for at least 1 h in the equilibration buffer (0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 20% glycerol, 1 mM EDTA, 42.7 mM 2-mercaptoethanol, and 0.1% bromophenol blue). Each gel slice was put on the bottom of a sample well of SDS-15% PAGE, and then the overlay buffer (0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 20% glycerol, 1 mM EDTA, 42.7 mM 2-mercaptoethanol) was added. Finally, 20 µl of a freshly diluted protease solution in the equilibration buffer was overlaid. Electrophoresis was performed at low voltage (1.5–2 V/cm) during the first several hours and then at 15 V/cm for sample separation. Proteolysis took place during the initial period when the labeled subunits and proteases remain in 4.5% stacking gel. The duration of the proteolytic cleavage was 4 h for S. aureus V8 protease, 2.5 h for papain, and 3 h for subtilisin. After electrophoresis, the peptides generated were transferred onto ProBlott membrane (Applied Biosystems), and the membranes were stained with Cooamassie Brilliant Blue. Electroblotting was fulfilled at a constant current of 0.8 mA/cm2 at room temperature overnight using a semi-dry blotting system. The peptide bands were excised and subjected to NH2-terminal sequencing (model 491, Applied Biosystems).

**RESULTS**

**Synthesis of Photoactive UTP Derivatives—** To map the catalytic site of RNA polymerization in S. pombe RNA polymerase II, photoactive nucleotide analogues were added only to the 3′ termini of growing RNA chains, and then transcription elongation complexes containing such transcripts were exposed to UV light for cross-linking. In this study, we used four types of photoactive base-substituted analogues of UTP with spacers of different lengths (1.5–3 nm) connecting the base and the photoactive arylazido group (Fig. 1A).

The synthesis of these nucleotide analogues was fulfilled starting from 5-(aminopropenyl)-1-uridine-5'-triphosphate (26). This compound was allowed to react with excess amounts of four different N-hydroxysuccinimide ethers of p-azidotetrafluorobenzoic acid, each containing an aliphatic chain (X) of different length. Reactions proceeded quantitatively in the presence of triethylamine as a catalyst and dimethylformamide:water (3:1) as a solvent. The products were purified by ion-exchange and reverse phase high pressure liquid chromatography and characterized by NMR spectra (data not shown).

The substances have several characteristic features. First, the photoactive groups are attached at position C-5 of uracil which is not involved in hydrogen bonding with adenine. The substitution may not give significant effects in the conformation of ribose moiety, and therefore the modified UTP derivatives may function as substrates in RNA synthesis. Second, upon exposure to UV irradiation, the p-azidotetrafluorobenzoic acid group is able to generate the highly active intermediate particle, singlet nitrene, which can react with any molecules in surroundings. The generation of singlet nitrene proceeds very rapidly, and full decomposition of the photoreactive groups takes place within only 1 min upon exposure at 256 nm at an energy 100 µJ/cm2. Probably aromatic nitrenes will be rearranged to form inactive 7-atomic ring. The substitution of all protons in benzene ring by fluorine atoms allows the avoidance of this undesirable side reaction. Third, since the efficiency of affinity labeling often depends on the structure of nucleotide analogues, the use of spacers (X) with different lengths allows us to choose the best compound characteristic of the enzyme to be studied.

**Transcription with the Use of Photoactive UTP Derivatives—** In order to incorporate photoactive UTP analogues only at 3′-ends of transcripts, we constructed two kinds of 40-base pair-long DNA duplexes that consist of two segments, 3′-proximal segment with a sequence of TC mixtures and 5′-proximal segment of G clusters, both being connected by a
single A residue (Fig. 2; for detailed sequences see “Materials and Methods”). The two single A templates carried essentially the same sequence except that one containing A in the middle (I) and the other near the 3'-end (II). For use as templates for transcription in vitro, an extra sequence of 10 C residues was attached to the 3'-ends of the template strands. On such templates, transcription is preferentially initiated within the single-stranded protruded region (29) at 3–5 residues upstream from the junction between single-stranded and double-stranded regions (30). Thus, transcripts should carry heterogeneity in the number of 5'-terminal G residues arising from the initiation at variable positions on the single-stranded C tail. Since both templates contain a single A residue, UTP should be incorporated only at this unique site. In both templates, this unique A is followed by three G residues that are absent in the upstream regions. Thus, in the absence of CTP, transcription should be stalled after the incorporation of UMP or UMP analogues.

To test the above possibilities, RNA synthesis was carried out using the template I and in the presence of ATP, UTP, and [α-32P]GTP. Polyacrylamide gel electrophoresis of transcripts indicated that in the absence of CTP, transcription complexes become stalled after UMP incorporation, resulting in the formation of transcripts (RNA_{ST}) of 25–30 nucleotides in length (Fig. 1B, –CTP lane), whereas in the presence of CTP, template-sized read-through transcripts (RNA_{RT}) were synthesized (Fig. 1B, +CTP lane). When UTP analogues were used in place of UTP, RNA synthesis was also stalled, forming stalled transcripts with apparently the same sizes as the transcripts observed with UTP (Fig. 1B, +UTP analogue lanes).

To confirm the incorporation of UTP analogues into the RNA products, the single nucleotide addition experiment was performed (Fig. 1C), in which RNA synthesis was carried out in the presence of ATP and [α-32P]GTP (without UTP) to form the stalled complexes, and then UTP or UTP analogues were added to allow the addition of a single U residue. The incorporation of nucleotide derivatives bearing arylazido groups into RNA significantly decreases the mobility in gel electrophoresis (31). We then analyzed RNA products under improved conditions of gel electrophoresis. In fact, we could distinguish RNA products with regular UMP and UMP analogues based on the difference in the mobility on PAGE (Fig. 1C; the arrow indicates RNAs with modified UTP analogues). The efficiency of UTP analogue incorporation was higher for analogues 1 and 4 (the difference in the incorporation of UMP analogues is discussed below).

**Fig. 1.** Structures and activities of the photoreactive UTP analogues. A, chemical structures of the photoreactive UTP analogues. X indicates a spacer between the nucleotide and the photoreactive arylazido group. Structures of the spacers for UTP analogues 1–4 are shown in the table. B, RNA synthesis was carried out using the template I and in the presence of ATP, GTP, and UTP or UTP analogues (1–4). In addition, CTP was added in one reaction shown as CTP+. Transcripts were analyzed by denaturing 13.5% PAGE. The migration positions of read-through (RNA_{RT}) and stalled (RNA_{ST}) transcripts are indicated on the left side. C, transcription was carried out by the single nucleotide addition system using template I. In the first-step reaction, RNA synthesis was carried out for 1 min at 37 °C in the presence of GTP and ATP, and then the second-step reaction was continued for 4 min at 37 °C after the addition of UTP or UTP analogues (1–4). The arrow indicates RNA with the modified UTP incorporated at the 3'-end of transcripts.
The nucleotide sequences of templates I and II are the same except that the single A residue is positioned either in the middle (template I) or at the 3′-end (template II) of template strand. In both cases, this single A is followed by three G residues (underlined). The absence of CTP, RNA polymerase should stall after the incorporation of three G residues, that is, after the formation of the catalytic site of RNA polymerization or at the right side, whereas the bands of RNA polymerase subunits with covalently cross-linked with radioactive RNA are marked on the left side.

In order to achieve specific cross-linking of RNA within the catalytically active elongation complexes, the first-step RNA synthesis was carried out in the presence of ATP, GTP, and photoreactive UTP derivatives, and the stalled complexes thus formed were exposed to UV light. The cross-linking was found to take place with only two large subunits. We tried to remove ribonucleotide moieties by nuclease treatment of the cross-linked samples prior to SDS-PAGE analysis. Migration positions of the marker proteins are indicated by vertical lines. Controlled cleavage at various protease concentrations led to the generation of mixtures of different peptides, as observed after SDS-PAGE analysis. Since some peptides with similar sizes co-migrate on SDS-PAGE, the complete separation of all peptides was not always possible. Peptides thus separated were transferred onto ProBlot membranes and subjected to NH2-terminal sequencing. Based on the sequence of Rpb1 and Rpb2 subunits (Figs. 6 and 7; the number of Rpb1 and Rpb2 subunits (Figs. 6 and 7; the number of proteolytic sites was determined by controlled cleavage by V8 protease. For in this prediction, the intensity of Rpb2 labeling increased with the increase in hydrophobicity of UTP derivatives (2 → 3 → 1 → 4).

When the regular UTP was used in place of the UTP analogues or when the reaction was carried out in the absence of GTP, we detected no radioactivity associated with the RNA polymerase II subunit bands. For this purpose, we first performed limited digestion of isolated Rpb1 and Rpb2 with six different proteases, i.e. chymotrypsin, elastase, papain, subtilisin, trypsin, and V8 protease. Among these proteases, papain, subtilisin, and V8 protease. The limited proteolysis at various protease concentrations led to the generation of mixtures of different peptides, as observed after SDS-PAGE analysis (Figs. 4 and 5). Since some peptides with similar sizes co-migrate on SDS-PAGE, the complete separation of all peptides was not always possible. Peptides thus separated were transferred onto ProBlot membranes and subjected to NH2-terminal sequencing. Based on the sequence knowledge, we could locate the peptides on the primary sequence of Rpb1 and Rpb2 subunits (Figs. 6 and 7; the number of peptides was only for those sequenced). Controlled cleavage of Rpb1 and Rpb2 subunits was carried out in the presence of ATP, [α-32P]CTP, and the indicated UTP analogue. The RNA polymerase with (+) and without (−) UV irradiation was analyzed by SDS-PAGE. Migration positions of the marker proteins are indicated by vertical lines. The bands of RNA polymerase subunits that were covalently cross-linked with radioactive RNA are marked on the left side.
cleaved preferentially after Asp, whereas the specificity of papain became broader, with nonspecific cleavage activities at Ala, Gly, Leu and Thr.

Analysis of the proteolytic fragments of Rpb1 (Fig. 5) revealed 7 protease-sensitive regions, correspondingly generating 7 distinctive protease-insensitive domains plus the carboxyl-terminal domain (see Fig. 7). As in the case of Rpb2, the cleavage sites are located approximately on the junctions between the conservative motifs (see Fig. 11).

Identification of Segments Cross-linked with Nascent RNA 3’-End—By using the Rpb1 and Rpb2 that were cross-linked with nascent RNA chains containing UTP derivative 1 (with the shortest linker) by the catalytically competent technique, we next tried to identify the structural elements in the vicinity of the active site of RNA polymerase II. After the nuclease hydrolysis and separation on SDS-7.5% PAGE, the radioactively labeled Rpb2 and Rpb1 subunits were cut from the gel and subjected to the digestion by the three proteases, V8, papain, and subtilisin, in 4.5% stacking gel in the presence of 0.1% SDS, and the peptides generated were separated on SDS-15% PAGE. After electrophoresis, the gels were stained with a silver reagent, dried, and exposed to phosphorimage plates. Comparison of the silver-stained gels and the patterns of phosphorimage allowed us to identify a set of peptides bearing the radioactive label. Some of the results are shown on Figs. 8 and 9. The nucleotide cross-linked segments were identified only in the case when the peptides were well separated from other peptides on SDS-PAGE and only when the peptides gave only a single unique amino acid sequence.

In the case of Rpb2, the labeled peptides include V8 segments, p1, p2, p4, p7, and p11 (Fig. 8A), papain segment p3 (Fig. 8B), and subtilisin segments, p1, p2, and p3 (Fig. 4C). For Rpb1, the labeling was observed for V8 peptides p1 and p2 (Fig. 9A), papain segment p8 (Fig. 9B), and subtilisin segments p4 and p5 (Fig. 9C). When the cross-linking reaction was carried out using the template II, the patterns of labeled peptides were essentially the same with those obtained with the template I.
The locations of these radioactive peptides on the primary sequence of Rpb2 and Rpb1 polypeptides are summarized in Figs. 10 and 11, respectively. The peptides of the most intense labeling are located between amino acids 825 and 994 of Rpb2, the region including the conserved sequences H and G (Fig. 10). The less intensive labeling also occurs in the region between amino acids 298 and 535 of Rpb2. This region contains the conserved sequences C and D. The extent of Rpb1 labeling was lower than that of Rpb2, and all the cross-linked peptides are located at the region between amino acids 614 and 917, which contains the conserved sequence F (Fig. 11).

**DISCUSSION**

For precise identification of the catalytic site on RNA polymerase II, we took special care by employing several new approaches. (i) The templates were designed so as to allow the incorporation of photoreactive substrate analogues only into 3'-ends of initiation oligonucleotide products ("initiation complex") or 3'-growing ends of nascent RNA chains ("elongation complex"). (ii) Only the catalytically active transcription complexes were detected by labeling with a single radioactive substrate added next to the cross-linked nucleotides (stalled complexes tend to isomerize into dead-end complexes where 3'-ends of RNA have different contacts with the RNA polymerase (33)). (iii) A number of small radioactive peptide fragments were isolated from each of the labeled Rpb2 and Rpb1 by treatment with three different proteases to narrow down the cross-linked regions. As a result, we found that both the initiation and elongation complexes gave essentially the same cross-linked peptides, indicating that the same region of RNA polymerase II is always located close to the 3' termini of both initiation oligonucleotides and nascent RNA chains.

Several lines of evidence indicated that the cross-linking observed under the reaction conditions employed were specific to the growing ends of nascent transcripts, because of the following: (i) when the transcription was carried out using UTP in place of the UTP analogues or in the absence of GTP, we detected no radioactivity associated with the RNA polymerase II subunit bands; (ii) likewise, we could not detect protein-bound radioactivity when the reaction was carried out in the absence of template DNA; and (iii) upon exposure to UV light, the UTP analogues used are rapidly degraded with a half-life of...
less than 1 min (34). It should be noted, however, that the RNA polymerase isomerizes during the transition from initiation to elongation complexes (35, 36). In this study, we used duplex DNA templates I and II with a tail of 10 C residues attached at 3′-end of the template strand. On such templates, transcription is efficiently initiated on the protruded single-stranded tails about 3–5 bases upstream from the single- and double-strand junction (29, 30). The initiation complexes we analyzed were associated with 3–5 G residues transcribed by this single-stranded C tail. The template II was designed as to cross-link the initiation complexes, in which the initiated transcripts with the sequence of G\textsubscript{3–5}UPhotoC\textsubscript{1–3} might not be displaced. On the other hand, on the elongation complex formed on the template I, the nascent transcripts with the expected sequence of G\textsubscript{3–5}(AG)\textsubscript{24}UPhotoC\textsubscript{1–3} could be displaced at their 5′ termini. The elongation complexes formed on such templates as employed in this study could be different from the typical elongation complexes formed on the complete duplex DNA. For instance, Kadesch and Chamberlin (29) reported that approximately half of transcripts on the elongation complexes initiated from the same type of synthetic template remained bound to the template without being displaced. The site of RNA displacement is, however, far from the catalytic site of RNA polymerization, with these two sites being separated by at least several nucleotides. Thus, the difference in the site of RNA displacement between the synthetic and native DNA templates might not influence the location of 3′-end of nascent transcripts.

Partial proteolysis by three proteases, V8, papain, and subtilisin, indicated complex multidomain organizations for both Rpb1 and Rpb2. Rpb1 contains 8 major structural domains followed by 27 repetitions of the carboxyl-terminal domain sequence of 7 amino acid residues, whereas Rpb2 includes 7 major domains (see Figs. 10 and 11). From the sequence comparison, subunits 1 and 2 of RNA polymerases from both prokaryotes and eukaryotes are known to contain 8 (A–H plus carboxyl-terminal domain) and 9 (A–I) conserved sequences,

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**FIG. 7. Proteolytic cleavage pattern of the Rpb1 subunit.** A, cleavage pattern by V8 protease. The location of Asp (the potential cleavage site by V8) is shown on the primary sequence of Rpb1 indicated by the horizontal thick line. Triangles above the line indicate the cleavage sites by V8 as analyzed in this study. The sensitivity to V8 is shown by the size of triangles. The NH\textsubscript{2} termini of V8 fragments were determined after sequencing (the number in brackets represent the NH\textsubscript{2}-terminal residue), whereas the carboxyl termini were estimated from the size of fragments. Squares under the line represent the approximate positions of carboxyl termini of each fragment. B, cleavage pattern by papain. C, cleavage pattern by subtilisin.
respectively (1, 2) (see Figs. 10 and 11). Each major structural domain of Rpb1 and Rpb2 identified in this study contains one or two of the respective conserved sequences.

Detailed mapping of the Rpb1 and Rpb2 regions cross-linked with 3’ termini of nascent transcripts was carried out on the basis of the cleavage map of these subunit polypeptides by these three proteases. In the case of S. pombe Rpb2, the most intensive cross-linking was found to occur in the region between amino acids 825 and 994, including the structural domains 5 and 6 and the conserved sequences G and H (see Fig. 10). In the case of S. cerevisiae RNA polymerase II, the region between amino acids 946 and 999 of the B150 (RPB2) subunit which corresponds to the region 935–988 of Rpb2 for S. pombe is cross-linked with nucleotide analogues polymerized into RNA chains (22). Thus, we concluded that the catalytic site...
for RNA polymerization is located on the subunit 2 and that the location of the catalytic site on the subunit 2 is essentially the same between *S. pombe* and *S. cerevisiae* RNA polymerase II. It may be worthwhile to note that essentially the same region of Rpb2 (amino acids 902 to 991) is involved in the molecular contact between Rpb2 and Rpb3 (9). One possibility is that the binding of Rpb3 induces the correct folding of Rpb2 leading to expression of its intrinsic activities. In fact, the β subunit of *E. coli* RNA polymerase expresses the binding activities to rifampicin, nucleoside 5'-triphosphates, and DNA only after the formation of αβ complex formation (37). Likewise, the Rpb2 (β homologue), Rpb3 (α homologue), and Rpb11 (α homologue) form a core subassembly with the DNA binding activity (3).

On Rpb2, the less intensive labeling was observed in the region between amino acids 306 and 542, including the structural domain 2 and the conserved sequences C and D (see Fig. 10). This region also includes the RNase-like domain (38). The isolated Rpb2 fragments containing this RNase-like domain,
however, did not show the activities of RNA cleavage in vitro, and site-directed mutagenesis of this domain did not affect growth of the mutant S. pombe (39).

In the case of E. coli RNA polymerase, the most sensitive sites for proteolytic cleavage on the β subunit (Rpb2 homolog) are located in two regions, one upstream of the conserved sequence D and the other upstream of the conserved sequence F (40). These sites may correspond to the cleavage between the domains 2 and 3 and between the domains 3 and 4 of Rpb2. The binding sites for substrates and rifampicin of E. coli β subunit overlaps the upstream labeling region of S. pombe Rpb2, whereas the catalytic site for RNA polymerization of the β subunit overlaps the downstream labeling region of Rpb2 (13). The overall organization of structure and function is similar between the prokaryotic Rpb2 (13). The overall organization of structure and function is similar between the conservative sequences B and C and the other available sites for trypsin are located in two regions, one between the conservative sequences B and C and the other between the conserved sequences F and G. Rpb1 also has the sites for proteolytic cleavage in these regions, but in addition, there are three additional sites exposed for proteases. The RNA-binding site on E. coli β′ subunit was reported to be within the conserved sequence D (13), but our cross-linking studies identified the RNA-binding site on Rpb1 near the conserved sequence F. This discrepancy remains to be solved. One possibility is that the location of β′ subunit relative to the catalytic site of the β subunit shifts during the process of RNA chain elongation.

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