

[14] Strategies and Methods of Cross-Linking of RNA Polymerase Active Center

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RNA polymerase (RNAP) is one of the largest and complex enzymes in the living cell, which poses a big challenge in studies of its structure and function. Cross-linking as a complementary approach to X-ray crystallography has been successfully used to study the mechanism of RNA synthesis. A few main issues were addressed in these studies: (1) super-selective labeling and identification of the residues that contribute to the active center and its closest environment^{1,2}; (2) spatial arrangement of priming substrate, antibiotic rifampicin, and template DNA in the active center, which revealed the mechanism for rifampicin inhibition of RNA synthesis;³ (3) determination of the size of DNA–DNA heteroduplex in transcription elongation complex (TEC), which resolved a long-term controversy about the existence and functional role of the hybrid^{4,5}; (4) construction of a high-resolution model of TEC by mapping of the nucleic acid–protein contacts in TEC and projection of these contacts on the structure of RNAP core enzyme^{6,7}; (5) mapping of RNA contacts in the active center of transcription complex, which suggested the structural models for transcriptional arrest,⁸ substrate entry to the active center, and RNAP translocation.⁹ In

¹ M. A. Grachev, T. I. Kolocheva, E. A. Lukhtanov, and A. A. Mustaev, *Eur. J. Biochem.* **163**, 113 (1987).

² A. Mustaev, M. Kashlev, J. Lee, A. Polyakov, A. Lebedev, K. Zalenskaya, M. Grachev, A. Goldfarb, and V. Nikiforov, *J. Biol. Chem.* **266**, 23927 (1991).

³ A. Mustaev, E. Zaychikov, K. Severinov, M. Kashlev, A. Polyakov, V. Nikiforov, and A. Goldfarb, *Proc. Natl. Acad. Sci. USA* **91**, 12036 (1994).

⁴ E. Nudler, A. Mustaev, E. Lukhtanov, and A. Goldfarb, *Cell* **89**, 33 (1997).

⁵ N. Korzheva, A. Mustaev, E. Nudler, V. Nikiforov, and A. Goldfarb, in “CSH Symposia for Quantitative Biology,” Vol. 63, p. 337. Cold Spring Harbor Press, Cold Spring Harbor, New York, 1998.

⁶ N. Korzheva, A. Mustaev, M. Kozlov, A. Malhotra, V. Nikiforov, A. Goldfarb, and S. A. Darst, *Science* **289**, 619 (2000).

⁷ N. Korzheva and A. Mustaev, *Curr. Opin. Microbiol.* **4**, 119 (2001).

⁸ V. Markovtsov, A. Mustaev, and A. Goldfarb, *Proc. Natl. Acad. Sci. USA* **93**, 3221 (1996).

⁹ V. Epshtein, A. Mustaev, V. Markovtsov, O. Bereshchenko, V. Nikiforov, and A. Goldfarb, *Mol. Cell* **10**, (2002).

this article we present the basic strategies and methods used in some of these studies.

Labeling of the Priming Substrate Binding Site of the Active Center by Autocatalysis

The validity of affinity labeling depends on its selectivity, that is, on the ratio of specific to nonspecific modification (outside the binding site). It is hard to achieve high selectivity with large enzymes, especially when the binding constant of the ligand is low. It is not surprising, therefore, that numerous attempts to study the functional topography of RNAP substrate binding sites by means of traditional cross-linking have given relatively poor results.

In order to achieve the high selectivity of affinity labeling for RNA polymerase, we took advantage of "catalytic competence." This phenomenon reflects the ability of a substrate residue cross-linked at the active center of an enzyme to convert into a cross-linked product by the same enzyme molecule according to the normal mechanism of catalysis. This principle is illustrated later for RNAP^{1,10} (Fig. 1A). At the first stage RNAP is treated in the binary complex with a promoter by affinity reagent, which is an analog of initiating substrate. This results in the cross-linking of affinity reagent residues both inside and outside the active center. At the second stage the modified enzyme is supplemented with the second radioactive substrate complementary to the next base of DNA template. Reagent residues tethered at the active center could be "catalytically competent" and elongated by radioactive nucleotide because of the catalytic action of the active center. Residues covalently bound outside the active center do not attach radioactivity at the second stage and remain "invisible" during subsequent analysis.

This method initially developed for *Escherichia coli* RNAP^{1,10} has been extended to all kinds of NTP polymerizing enzymes, including representatives of eucaryotes,¹¹ procaryotes,¹² and phages¹³ (for review see^{12,14}).

¹⁰ Y.-V. Smirnov, V. M. Lipkin, Y.-A. Ovchinnikov, M. A. Grachev, and A. A. Mustaev, *Bioorg. Khim.* (Russian), **7**, 1113 (1981).

¹¹ M. Riva, A. R. Schaeffner, A. Sentenac, G. Hartmann, A. A. Mustaev, and E. F. Zaychikov, *J. Biol. Chem.* **262**, 14377 (1987).

¹² G. R. Hartmann, C. Biebricher, S. J. Glaser, F. Gross, M. J. Katzamaeyer, A. J. Lindner, H. Mosig, H. P. Hasheuer, L. B. Rothman-Dennis, A. R. Shaffner, G. J. Schneider, K. O. Stetter, and M. Thomm, *Biol. Chem. Hoppe-Seyler* **369**, 775 (1988).

¹³ T. G. Maximova, A. A. Mustaev, E. F. Zaychikov, D. L. Lyakhov, V. L. Tunitskaya, A. K. Akbarov, S. V. Luchin, V. O. Rechinsky, B. K. Chernov, and S. N. Kochetkov, *Eur. J. Biochem.* **195**, 841 (1991).

¹⁴ M. A. Grachev, A. A. Mustaev, and F. F. Zaychikov, "Chemical Modification of Enzymes" (B. I. Kurganov, N. K. Nagradova, and O. I. Lavrik, eds.), p. 309. Nova Science Publishers, Inc., New York, 1997.

Unusually high selectivity of the labeling allows visualization of polymerase molecules in partially purified preparations² or even in crude cellular extracts.^{15,16}

Autocatalytic Affinity Labeling of RNAP at the Active Center

A mixture (9 μ l) containing 50 mM Tris-HCl, pH 8.5, 0.1 mM 2-mercaptoethanol, 50 mM NaCl, 10 mM MgCl₂, 2 pmol Bsp-1462 DNA fragment of T7 DNA containing promoters A₀-A₃, and 4 pmol RNAP is incubated for 5 min at 37°. 1 μ l of 10 mM solution of one of the reagents I-V (Fig. 1D) is added. After incubation for 20 min at room temperature, the reaction mixtures containing reagents II-V are supplemented by 1 μ l of immediately prepared 0.1 M NaBH₄ and the incubation continues for another 20 min. Then [α -³²P]UTP (3 μ l of 1000-3000 Ci/mmol, 1 μ Ci/ μ l) is added to all the mixtures. In 20 min the mixtures are supplemented with 3 μ l solution containing 5% SDS, 5% 2-mercaptoethanol, 50% glycerol, and 0.1% bromophenol blue, and after 30 min incubation at 37°, analyzed by (SDS)-PAGE. Labeled subunits are revealed by autoradiography.

RNA-Protein Cross-linking in the Active Center of Initiating Complex

As soon as during initiation short RNA products easily fall off the active center studies RNA-protein contacts therein (i.e., in the active center) is a complicated task. To overcome this difficulty the initiating Rif-GCU complex was constructed on the T7 A2 promoter using as a primer the chimerical compound Rif-GTP in which rifampicin is covalently linked to GTP.³ Such compounds bind to RNAP bifunctionally so that Rif occupies its natural pocket, and the nucleotide enters the i site (Fig. 1B). Rif-GTP was used to prime the incorporation of the two next nucleotides. In the resulting ternary complex, the trinucleotide product is flexibly tethered to RNAP via the Rif moiety and serves to mimic the abortive product, with the difference that it is prevented from dissociating out of the enzyme.³ The following is an example of efficient cross-linking in this system, which occurs at Methionine (Met)932 of β' subunit.

¹⁵ O. Morozova, A. Mustaev, N. Belyavskaya, E. Zaychikov, E. Kvetkova, Yu. Wolf, and A. Pletnev, *FEBS Lett.* **277**, 75 (1990).

¹⁶ V. Rait and F. Seifart, in "Nucleic Acid Symposium Series," Vol. 36, p. 162. Oxford University Press, Oxford, UK 1997.

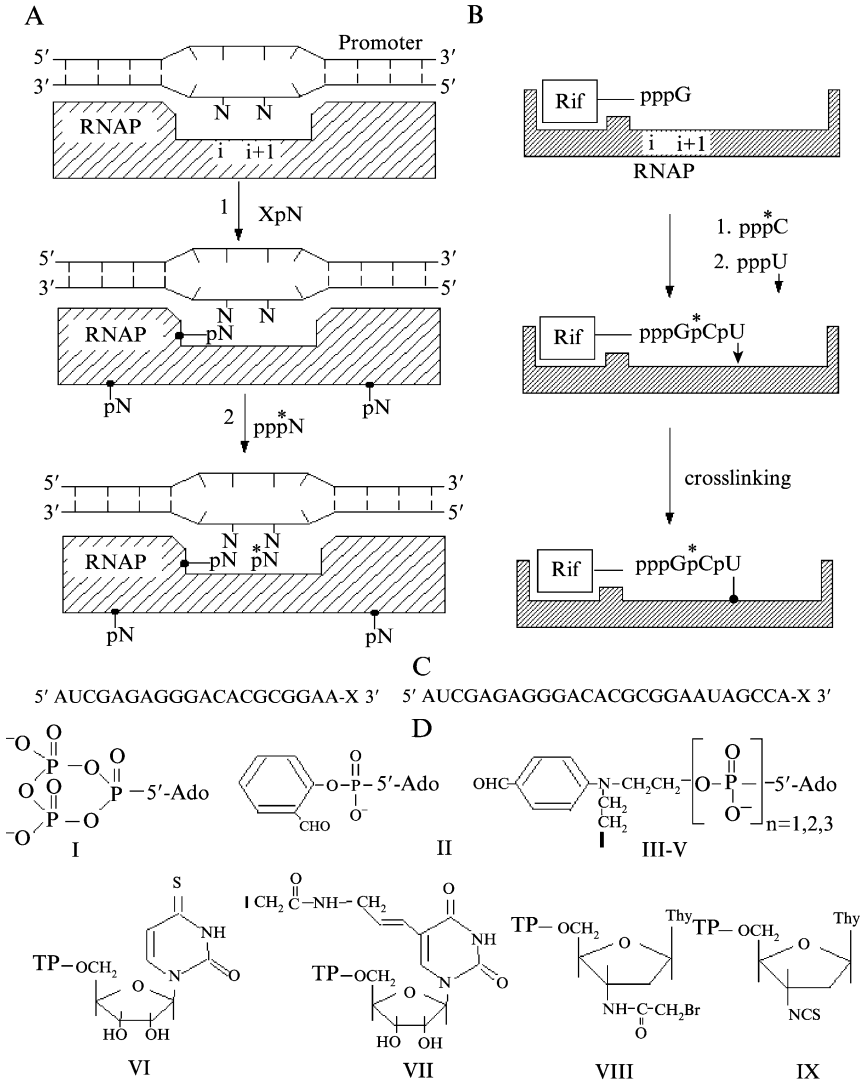


FIG. 1. Strategies used for cross-linking studies of RNAP active center. (A) Autocatalytic affinity labeling of RNAP in initiating complex. X-pN represents cross-linking derivative of initiating substrate; (*), radioactive phosphate. (B) Cross-linking in initiating complex. Rif symbolizes rifampicin residue tethered to GTP; arrow, reactive group; filled circle, a cross-link. (C) Sequence of RNA synthesized from T7A1 promoter. X-reactive nucleotide residue used for cross-linking in TEC. (D) Substrate analogs used in cross-linking studies. TP stands for triphosphate residue.

*Cross-linking in Rif-GCU Complex*⁹

Reaction mixture contains 4 pmol RNAP, 4 pmol T7A2 template, and 10 μM Rif-(CH₂)₅-GTP in suspension of 15 μl of Ni-NTA agarose and 15 μl of transcription buffer (TB 20 mM HEPES-HCl, pH 7.9, 50 mM NaCl, 10 mM MgCl₂). After 5 min incubation at 37° the mixture is washed with the same buffer (3 \times 1 ml) and [α -³²P]CTP (3000 Ci/mmol) is added. The reaction is allowed to proceed for 10 min at 37° and washed (3 \times 1 ml) by TB containing 2.5 mM MnCl₂ instead of 10 mM MgCl₂. The reagent VIII (Fig. 1D) is added to the final concentration of 200 μM . After 30 min incubation at 37°, the reaction is quenched and cross-linked products analyzed as before.

RNA-protein Cross-linking in the Active Center of Elongation Complex^{8,9}

To study the contacts of RNA in the active center of elongation complex, we obtained TECs containing 20 and 26 nt RNA (Fig. 1C) on T7 A1 promoter by “walking” protocol using RNA primers and an incomplete set of NTPs. Reactive derivatives of UTP (VI-IX; Fig. 1D) are used to introduce the cross-linking group at 3' RNA terminus in the previous TECs. Derivative VI is photoreactive and can react with any residues, VII and VIII are alkylating compounds able to cross-link to nucleophilic residues, whereas IX forms stable adducts exclusively with lysine. As soon as the elongation complexes are stable, the cross-links therein are highly selective.

*Cross-linking Protocol for TEC21*⁹

Fifteen pmol of RNAP and T7A1 template are mixed with suspension of 15 μl preequilibrated Ni-NTA agarose and 15 μl of the transcription buffer, incubated for 5 min at 37°, and washed with the same buffer (3 \times 1 ml). The volume is adjusted to 30 μl by TB, and AUC primer (10 μM) together with GTP and ATP (25 μM each) are added. The mixture is incubated at 37° for 5 min and washed again (3 \times 1 ml) by the same buffer. ATP and GTP (25 μM each) are added with 2 μl [α -³²P]CTP (3000 Ci/mmol), incubated for 5 min at 20°; nonradioactive CTP (25 μM) is introduced, and incubation continues for another 2 min. The buffer is changed to TB containing 2.5 mM MnCl₂ instead of MgCl₂, the dUTP-cross-linking analogs VIII and IX are added to each mixture to final concentration 100 μM , and incubation continues for 30 min at 37°. The reaction is quenched and analyzed as before.

Mapping of Cross-linking Sites

To map the cross-linking sites, we used the method developed earlier in one of our labs.¹⁷ Assume that a polypeptide is labeled by radioactive affinity adduct at a single site. Single-hit degradation of this polypeptide (e.g., at Met residues by CNBr) will generate the products, which possess C or N terminus of the original polypeptide. It is obvious that upon the cleavage at each particular site, only one product will contain radioactivity. Separation of the products according to their sizes by SDS-PAGE will give characteristic pattern of radioactive products, which depends on the position of the radioactive label on the polypeptide and distribution of Met residues in the sequence. Resulting patterns are compared with theoretical ones calculated under the assumption that the label resides close to either the C or N terminus of the polypeptide.

This is illustrated in Fig. 2A, where RNAP β subunit was cross-linked to either the initiating substrate or RNA product. In this case the distribution of CNBr degradation products on the autoradiogram is very close to the theoretically inferred C-terminal degradation pattern and does not match the N-terminal one. The position of the tag is located between CNBr cleavage sites, which give rise to the shortest labeled cleavage product and the next shortest product, which is not radioactive. Thus it is seen that in the presented cases the label resides within the intervals 951–1066 (reagent II), 1232–1242 (reagent I), and 1304–1315 (TEC14). Besides CNBr, other specific cleaving agents can be used:

1. Hydroxylamine—selectively cleaves Asn–Gly bonds at pH 9–10.
2. *N*-chlorosuccinimide (NCS) and *N*-bromosuccinimide (NBS)—at single-hit conditions perform cleavage at Trp residues.
3. Bromine—cleaves at Trp and Tyr with preference to Trp.
4. 2-nitro-5-thiocyanobenzoic acid (NTCBA)—cleaves at Cys residues.

Limited single-hit proteolysis at particular types of residues can also be used (Lys-C, Glu-C, Asp-N, trypsin, etc.) for mapping in combination with chemical degradation. As soon as the cleavage rates at a particular site differ significantly because of unequal accessibility to the protease (even in the presence of SDS) and different neighboring residues adjacent to the cleaved bond, the conditions of proteolysis must be carefully chosen to avoid multiple-hit degradation. An application of single-hit trypsinolysis for mapping is described.¹⁷

¹⁷ M. Grachev, E. Lukhtanov, A. Mustaev, E. Zaychikov, M. Abdukajumov, I. Rabinov, V. Richter, Y. Skoblov, and P. Chistyakov, *Eur. J. Biochem.* **180**, (1989).

For the mapping, different cleavage strategies may be used either in parallel or consequently (e.g., cleaving the proteolytic fragment with CNBr¹⁷ or single-hit cleavage of complete CNBr product by NH₂OH¹⁴). Supplementary data obtained by independent means (e.g., determination of the size of product of complete cleavage by SDS-PAGE¹⁴ or mass spectrometry,¹⁸ partial sequencing of cleavage products,¹⁴ and their affinity purification or separation using His-tags¹⁹) can help in providing and refining the results obtained by single-hit cleavages.

There are a few requirements that must be obeyed to avoid the misinterpretation of the mapping results. (1) There must be equal susceptibility of the particular peptide bonds to the cleaving reagent to obtain the pattern representing all expected bands, which is achieved by denaturation of the protein before cleavage. (2) It is very important to distinguish between single- and multiple-hit products. To this end, the cleavage must be performed to a low extent when the main part of the protein remains uncleaved. Single-hit cleavage products must follow first-order time course, which can be judged by quantitation of the cleavage products. (3) Interpretation of the data may be complicated by the abnormal electrophoretic mobility of the cleavage products. For instance, three peptides (Fig. 2A, lane 6) corresponding to cleavage at Met 1107, 1119, and 1131 migrate as a single band, which can be resolved, although on a longer run (lane 10). It should be stressed, however, that the method is based on the analysis of the whole pattern of the bands rather than on determination of the exact sizes of the peptides from their electrophoretic mobility.

The interpretation of the degradation patterns is straightforward for those cases where the cross-linking site is located close to one of the termini, because the two sets of nested radioactive fragments corresponding to the NH₂ and COOH termini do not overlap on the autoradiogram. For cross-links located closer to the middle of the subunit, a mixture of NH₂- and COOH-degradation products has to be interpreted. As reference, degradation patterns of terminally labeled polypeptide can be used in this case. An example is given in Fig. 2C where the cross-link resides in the middle part of the β' subunit as revealed by single-hit CNBr cleavage (bottom panel). As a reference, CNBr degradation products of the β' subunit are labeled at the kinase site engineered at the C terminus.⁹ Comparison of the patterns allows unequivocal positioning of the cross-linking site between Met 747 and Met 821. This difficulty may be also avoided by

¹⁸ K. Severinov, D. Fenyo, E. Severinova, A. Mustaev, B. Chait, A. Goldfarb, and S. A. Darst, *J. Biol. Chem.* **269**, 20826 (1994).

¹⁹ K. Severinov, A. Mustaev, E. Severinova, M. Kozlov, S. A. Darst, and A. Goldfarb, *J. Biol. Chem.* **270**, 29428 (1995).

precleaving of the labeled polypeptide (e.g., by limited proteolysis in non-denaturing conditions that give a limited number of products at the initial stage), followed by single-hit chemical degradation of purified proteolytic fragment.^{14,17}

Single-Hit Degradation of Polypeptides at Particular Residues

Preparation of Starting Material

When only one subunit is labeled after the cross-link, the material can be used directly in degradation reactions. However, if more than one subunit is a target, each labeled polypeptide must be isolated and treated separately.

After SDS-PAGE separation of the labeled enzyme, the radioactive bands are excised from the gel and placed in Eppendorf tubes. After washing with water (2×1 ml) for 5 min with shaking, the gel pieces are crashed and soaked in ~ 0.6 ml of 0.03% SDS. After 1 h elution at 37° with shaking, the tubes are centrifuged and the eluate is carefully collected, avoiding the uptake of any gel pieces. The gel phase is additionally washed by $100 \mu\text{l}$ of 0.03% SDS, and the solutions are combined. The remaining gel and solution are checked by radioactivity monitor. If the recovery of radioactivity is less than 50%, the elution is continued. The lesser the mobility of the corresponding product in the separation gel, the more time is required for elution. The eluate is either freeze-dried or placed into Eppendorf tubes ($200\text{--}300 \mu\text{l}$ portions), supplemented with 3 volumes of acetone, and kept at -20° for an hour. After centrifugation, the precipitate is dried under diminished pressure, dissolved in 1% SDS, and used in degradation reactions.

*CNBr Degradation at Met Residues*¹⁷

The reaction mixture after the cross-link to RNAP β or β' subunit is adjusted by water (if necessary) to $24 \mu\text{l}$, supplemented with $3 \mu\text{l}$ of 10% SDS (if the radioactive polypeptide is purified as before, this stage is omitted), and kept at 37° for 30 min for denaturation. $1.5 \mu\text{l}$ of each 1 M HCl and 1 M CNBr in water are added. At time intervals 0, 5, and 10 min, $9\text{-}\mu\text{l}$

cross-linking tag. *Far right:* Degradation products resolved on the longer gel. Solid lines link identical cleavage products; dashed lines indicate the position of the next cleavage product, which is not radioactive. (B) The scheme indicating the positions of Met residues in the C-terminal half of β subunit. (C) *Top:* Positions of Met residues in the sequence of β' subunit; *bottom:* CNBr degradation patterns of C-terminally labeled β' subunit (M) and of the same subunit cross-linked in initiation complex according to scheme B (Fig. 1) using reagent IX.

aliquots are withdrawn and quickly mixed with 3 μl of solution containing 5% 2-mercaptoethanol, 0.5 M Tris-HCl, pH 9.0, 0.2% bromophenol blue, and 50% glycerol. These mixtures are subjected to SDS-PAGE in a Lämmli system on 13% or 8–20% gradient gel.

NTCBA Degradation at Cys Residues^{8,9}

To a solution (30 μl) of labeled polypeptide in 8 M urea and 50 mM Tris-HCl, pH 8.3, 1 μl of 150 mM 2-mercaptoethanol is added and the mixture is incubated for 30 min at 37°. 3 μl of 100 mM freshly prepared NTCBA in methanol is added, and the incubation continues at 37°. In 15 min the pH of the mixture is adjusted to 9–9.5 by 1 M NaOH. In 30 and 60 min the aliquots (10 μl) are withdrawn and mixed with 3 μl of solution containing 5% SDS, 50% glycerol, and 0.2% bromophenol blue. The samples are analyzed as before.

Hydroxylamine Degradation at Asn-Gly Sites

To the solution of labeled enzyme or its purified subunit SDS and 2-mercaptoethanol is added a final concentration of 1%. After incubation at 37° for 30 min an equal volume of 2 M NH₂OH/0.2 M Na₂CO₃ pH 10.0 (prepared from NH₂OH◆HCl by titration with NaOH to pH 10.0 and mixing with Na₂CO₃, pH 10.0) is added, and incubation continues at 37°. In 30 and 60 min, aliquots are withdrawn and mixed with 1:5 (v/v) solution of 50% glycerol and 0.1% bromophenol blue.

Cleavage by NBS and NCS at Trp Residues and by Br₂ at Trp and Tyr Residues

The labeled enzyme is denatured by 1% SDS as before and mixed with Na-formate, pH 4.0, to final concentration 0.1 M. The mixture is supplemented by freshly prepared water solutions of NCS, NBS, or Br₂ to final concentrations 3 and 10 mM (or 1 mM in the case of bromine) and kept for 10 min at 20°. 1:3 (v/v) of 5% 2-mercaptoethanol, 0.5 M Tris-HCl, pH 9.0, 0.2% bromophenol blue, and 50% glycerol is added, and the mixture is analyzed as before.

Detection and Mapping of Multiple Cross-linking Sites

As soon as the active sites are usually formed at the interface of different subunits or structural domains, multiple cross-linking sites could be expected for the reactive ligands. If the cross-link occurs at different subunits, this could easily be seen after separation of the labeled polypeptides. More complicated is the situation when two or more cross-linking sites

reside within the same subunit. The following are a few examples of the detection and mapping of such sites.

Multiple cross-linking sites can be revealed by quantitative analysis of single-hit degradation products. Fig. 3 shows CNBr degradation pattern for cross-linked β subunit according to scheme B (Fig. 1) using reagent IX (lanes 1–3) and the reference degradation pattern (lane 4). In both cases the shortest degradation product corresponds to cleavage at Met 1232, which defines the cross-linking site in the region 1232–1243. Also, in both cases the intensity of the products on the upper part of the gel is fairly equal. However, in one case (lane 3) the products starting from 1066 and lower are much weaker than those in the reference pattern (lane 4). Indeed, quantitation of the corresponding products (B) shows that in one case the intensity of the weaker products is about three times less than in the reference. These

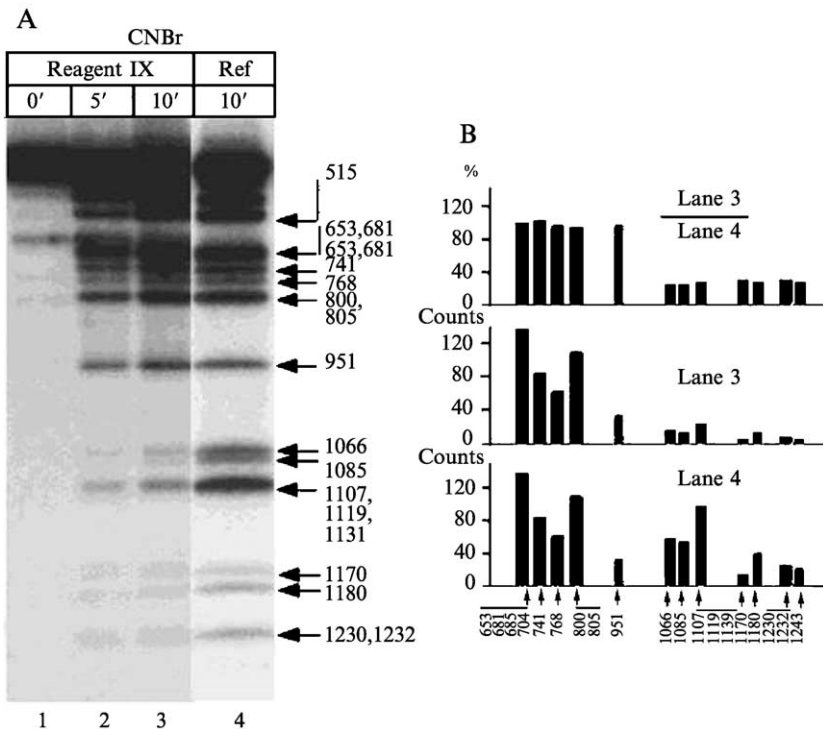


FIG. 3. Detection of the double cross-linking site by quantitative phosphorimager. Limited CNBr degradation patterns of RNAP β subunit labeled according to Fig. 1C using reagent IX (A, lanes 1–3), and reference degradation pattern for the β subunit labeled according to Fig. 1A using reagent I (lane 4). (B) Histograms of the degradation products.

data point to two cross-linking sites, one of which (major) resides in the region 951–1066 and the other (minor) in the region 1232–1243.

Multiple cross-linking sites also can be detected by limited proteolysis (Fig. 4). The major cross-link in the active center using derivatives III–V according to scheme A (Fig. 1) occurred to β subunit.¹⁹ Limited trypsinolysis of the labeled enzyme (which is known to occur between the residues 903 and 904 of the β subunit²⁰) gave two radioactive fragments of the expected size (Fig. 4A,B), which is indicative for at least two cross-linking sites. Using the enzyme containing insertion in β subunit (see Fig. 6C and Fig. 4A), which is much more sensitive to trypsin, allows quantitative cleavage of the modified subunit (Fig. 4C). Purified proteolytic fragments were used for further refining of the mapping.

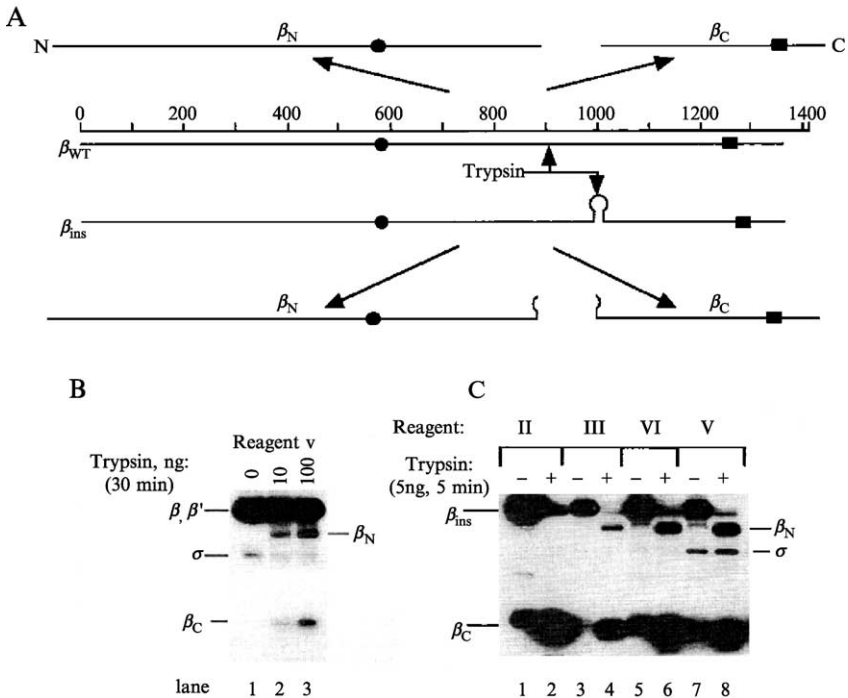


FIG. 4. Detection of the double cross-linking sites by limited proteolysis. (A) The scheme of tryptic cleavage of WT and insertion containing β subunit in the context of cross-linked initiating complex. Filled circle and box indicate the position of cross-linking tags. (B) Trypsinolysis of the cross-linked RNAP in the case of WT β subunit. (C) The same with β_{ins} .

²⁰ S. Borukhov, K. Severinov, M. Kashlev, A. Lebedev, I. Bass, G. C. Rowland, P. P. Lim, R. E. Glass, V. Nikiforov, and A. Goldfarb, *J. Biol. Chem.* **266**, 23921 (1991).

Another approach that appears to be very helpful for the mapping is based on the usage of functionally active enzymes assembled from the fragments of RNAP subunits.²¹ A cross-link from 3'-RNA terminus using reagent VII (Fig. 1D) according to scheme B (Fig. 1) occurred to both β and β' subunit. CNBr degradation pattern of the purified β subunit was complex, suggesting two cross-linking sites in C- and N-terminal regions of the subunit. Indeed, when in the same experiment RNAP assembled from the individually expressed fragments of β subunit was used, three radioactive products were detected on the gel (Fig. 5B). One of them co-migrated with β' subunit, and the smaller two with the fragments of β subunit. CNBr degradation patterns of gel-purified smaller fragment of β subunit is shown in Fig. 5C and reveals the segment 1243–1273 as a cross-linking site.

Refining the Mapping

The precision of the described mapping depends on the distance between two cleavable sites flanking the cross-linking tag. The number of available cleavage reactions at specific residues is limited, which in unfortunate cases is a reason for low resolution of the mapping. The obvious way to increase that

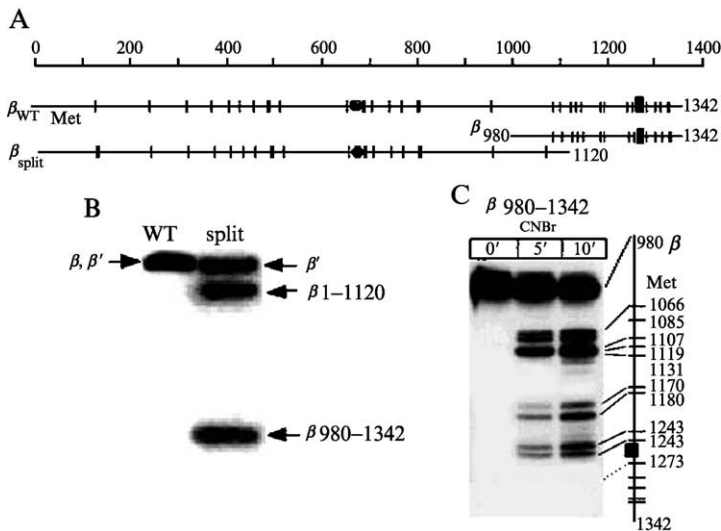


FIG. 5. Detection of the double cross-linking sites using RNAP containing split site in the β subunit. (A) Positions of Met residues in the intact β subunit and in the fragments of split β subunit used for cross-linking experiment. Rectangle and circle indicate the cross-linking tags. (B) Radioactive polypeptides cross-linked according to Fig. 1B with reagent VIII. (C) Single-hit CNBr degradation patterns of the purified fragment.

resolution is to engineer additional cleavage sites around the putative cross-link. This principle is illustrated in Fig. 6A. The cross-link at the i site of the active center by Lys-specific probe was mapped within the interval 1232–1243 of the β subunit.¹⁷ There are two lysine residues in the segment—Lys1234 and Lys1242. To distinguish between those two residues, we introduced conservative substitution L1238M between them and used the mutant enzyme in the same experiment. Compared with wild type (WT) enzyme, single-hit CNBr cleavage showed one extra band migrating slightly below the cleavage product at Met 1232, which appears as a result of cleavage at Met 1238 (Fig. 6A). Therefore the target of cross-link is Lys1242.

Resolution of mapping can be increased by conservative substitution of the presumed cross-linking residues to nonreactive ones unable to produce the cross-link with the used affinity probe. In our work this principle has been successfully used in a few cases.^{2,9} The environment of the catalytic center was probed by introducing the reactive UMP analogs VIII and IX (Fig. 1D) at RNA 3' terminus in the context of elongation complex⁹ (Fig. 1C). Analog IX is Lys-specific, whereas VIII can potentially react with all nucleophilic amino acid residues. In both cases β' subunit was the main target of cross-link (Fig. 6B). Single-hit chemical cleavage allowed mapping of the cross-linking area 747–821 in the case of reagent IX. There are three Lys residues in this segment: Lys749, Lys781, and Lys789. Lys749 and Lys781 cannot be the targets, because for the probe to reach them, dramatic distortions of the TEC structure would be required. That Lys789 is the cross-link site was confirmed using the K789R substitution (Fig. 6B, lane 2), which led to the dramatic reduction of the cross-link. Further single-hit degradation of the residual cross-linking product showed that it is due to the cross-link to some other region of the subunit. The same effect is observed when M932L substitution was used in the experiment with reagent VIII (Fig. 6B, lane 4), suggesting that this residue was a target.

In some cases, when the level of the expression of mutant subunit is not very high, the reconstituted enzyme used for the previous purposes is contaminated with WT subunits from the host strain, complicating the interpretation. A new assay developed to overcome this complication is presented in Fig. 6C, D. Active center-directed cross-link using Lys-specific reagent II (Fig. 1D) according to scheme A has been mapped in the segment 1036–1066 of the β subunit by limited CNBr degradation and trypsinolysis.¹⁷ This fragment contains four Lys residues. Lys1065 of this segment is highly conserved in evolution and therefore seems to be the most likely target of cross-link. Two substitutions were made, including

²¹ K. Severinov, A. Mustaev, E. Severinova, I. Bass, M. Kashlev, R. Landick, V. Nikiforov, A. Goldfarb, and S. A. Darst, *Proc. Natl. Acad. Sci. USA* **92**, 4591 (1995).

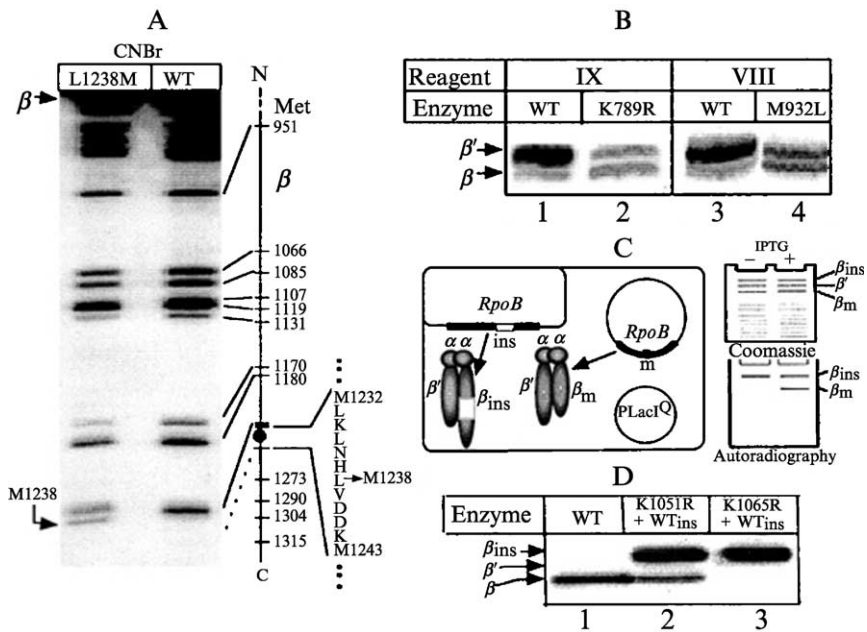


FIG. 6. Ways to increase precision of the mapping. (A) Engineering of the additional cleavage site between the potential targets. Limited CNBr degradation patterns of a wild type (WT) labeled RNAP and the enzyme containing L1238M substitution. B–D Conservative substitutions of the potential targets with nonreactive residues unable to produce the cross-link. (B) Cross-link from 3'-RNA terminus in TEC by reactive nucleotide analogs VIII and IX in TEC21. (C, left) Genetic system constructed to discriminate between the cross-links in the RNAP normal-sized mutant β subunit encoded by a plasmid, carrying a mutation (m) and WT chromosomal β_{ins} subunit containing insertion in nonessential region of *RpoB* gene. PLacIQ, expression plasmid carrying a gene for *lac* repressor used to “turn off” the production of a plasmid encoded β subunit. (C, right) A typical picture of Coomassie-stained SDS–PAGE of partially purified cross-linked RNAP preparations containing cross-linking (+) and noncross-linking β subunit and radioautography of the same gel. (D) Radioautography of cross-linked RNAP using reagent II according to Fig. 1A. Lane 1, WT RNAP with normal-sized β subunit; lane 2, partially purified RNAP from induced cells containing K1051R mutation in plasmid-borne β subunit; lane 3, the same but with K1065R substitution.

K1065R and K1051R as a control.² To determine whether the mutant β subunits in the context of holoenzyme would be labeled by using Lys-specific probe, β subunit expression plasmid containing each mutation (m) was placed into the host strain (Fig. 6C) harboring the insertion of 127 extra amino acids into the nonessential region of the β subunit. This insertion leads to elongated polypeptide β_{ins} easily distinguishable from the normal-sized plasmid-encoded β subunit on SDS–PAGE (Fig. C, right panel).

Crude RNAP fraction was prepared from the IPTG induced cells and subjected to affinity labeling according to the protocol presented in Fig. 1A using reagent II. It is seen (Fig. 6D) that both chromosomal β_{ins} and plasmid encoded β subunit were labeled in the case of mutant K1051R, but only β_{ins} subunit was labeled when the plasmid carried the substitution K1065R. This highly suggests that the target of cross-link was indeed Lys1065.

Concluding Remarks

The autocatalytic affinity labeling described previously is simple and highly selective and has a wide applicability. Besides initiation, it has been used to map the product binding site of RNAP.^{22,23} This method has been applied to more than 30 different NTP polymerases and allowed not only to get valuable information about the active sites, but also to address questions about their structure and function. In this way the contribution of the different RNAP subunits to the active center was determined²⁴ and the structural modules of the subunits that are able to assemble to a functional polymerase were defined.²¹ Another example of the application of this approach is identification of the genes coding for replicases of tick-borne encephalitis virus.¹⁵

As for the described mapping technique, it has a number of important advantages:

1. It is very sensitive and fast. The whole experiment can be performed with picomole amount of an enzyme during one working day.
2. It may be used with unstable labels that do not survive standard procedures of mapping.
3. It is very simple and does not depend on any expensive equipment.
4. It is potentially general and could be used for mapping of not only affinity, but also other kinds of labels and particular sites (e.g., epitopes,^{25,26} phosphorylation sites).

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