Cross-Linking of *Escherichia coli* RNA Polymerase Subunits: Identification of β′ as the Binding Site of ω†

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ABSTRACT: The ω protein is a peptide found in near-stoichiometric levels in highly purified *Escherichia coli* RNA polymerase. In order to determine the binding site of ω to RNA polymerase, we cross-linked ω to RNA polymerase with the hetero-bifunctional cross-linker *N*-hydroxysuccinimidyl 4-azidobenzoate and analyzed for cross-linked partners using antibodies raised against each of the subunits. Our analysis indicates that ω cross-links predominately with the β′ subunit, while a very low level of cross-linking was detected to the α subunit. We did not detect cross-linking to either the σ^{70} or the β subunits. This report demonstrates the utility of combining cross-linking and immunological techniques to determine interactions between RNA polymerase subunits.

The omega (ω) protein (molecular weight 10 105) is an often ignored polypeptide present in highly purified preparations of *Escherichia coli* RNA polymerase (Burgess, 1969). Despite its abundance, ω is not required for in vitro activity. Reconstituted RNA polymerase that lacks ω has no known alteration in its transcription behavior (Heil & Zillig, 1970). This appears also to be true in vivo since mutants that lack ω are viable under all conditions tested (Gentry & Burgess, 1989; Sarubbi et al., 1989). It is thus likely that ω is a factor required for some regulatory function or catalytic activity overlooked by in vitro assays and not immediately obvious by the behavior of mutants in vivo. By way of example, the NusA (Greenblatt & Li, 1981) and GreA (Borukhov et al., 1992) proteins both bind RNA polymerase and are required for efficient transcription termination/antitermination and elongation, respectively, but they play no role in simple RNA synthesis. Igarashi et al. (1989) claim that ω is required for the stringent response on the basis of the results of an assay designed to mimic the stringent response in vitro. The stringent response occurs following amino acid starvation, is characterized by an abrupt decrease in the transcription of stable RNA genes, and is thought to occur by direct inhibition of stable RNA synthesis by the nucleotide guanosine 3′,5′-bispyrophosphate (ppGpp)[1] (Cashel & Rudd, 1987). The claim that ω is required for the stringent response is not consistent with the finding that strains lacking *rpoZ*, the gene encoding ω, retain a normal stringent response (Gentry et al., 1991).

Strains carrying the *rpoZ* deletion also have a defect in the degradation of ppGpp (Gentry & Burgess, 1989; Sarubbi et al., 1989). This defect in ppGpp degradation is due to polarity on the expression of the *spoT* gene, which is immediately downstream of *rpoZ* (Gentry & Burgess, 1989; Sarubbi et al., 1989). *spoT* encodes for the major ppGpp pyrophosphohydrolase in the cell (Cashel & Rudd, 1987) as well as for a ppGpp synthetase (Xiao et al., 1991; Hernandez & Bremer, 1991). The genetic location of *rpoZ* is what prompted Igarashi et al. to test the effect of ω on ppGpp-regulated processes since proteins encoded in the same operon are generally involved in the same cellular function. *spoT* and *rpoZ* share an operon with *gmk* (encoding guanylate kinase) (Gentry et al., 1993), *recG* (encoding a resolvase) (Kalman et al., 1992; Lloyd, 1991), and an open reading frame of unknown function called *spoU* (Xiao et al., 1991). Because of this diversity of function, it is impossible to infer a function for ω by the function of the products of genes which share its operon. Using antibodies raised against ω to probe fractions of an *E. coli* lysate fractionated by gel filtration, we found that the majority of ω is bound to RNA polymerase, making it less likely that it is merely an abundant contaminant (Gentry, 1990). Furthermore, using the same antibody, we found that all of a number of enteric bacteria tested also possess a peptide of identical size which reacts with the anti-ω antibody. In the case of *Serratia marcescens* and *Proteus vulgaris*, we have determined that the cross-reacting peptide copurifies with RNA polymerase (D. R. Gentry and R. R. Burgess, unpublished results). These data confirm that, despite it not having an obvious function, ω is evolutionarily conserved.

E. coli RNA polymerase is a complex enzyme containing multiple subunits. The core enzyme contains three subunits, β, β′, and α (stoichiometry $\alpha_2\beta\beta'$) (Burgess, 1969), and is responsible for RNA synthesis. The β subunit (molecular weight 150 619) contains the active site, is the site of interaction of the antibiotics rifampicin and streptolydigin (Zillig et al., 1976), and is by far the best characterized subunit of the core enzyme. Mutations in the *rpoB* gene, encoding β, have been isolated that affect the expression of a variety of genes and transcriptional processes (Jin & Gross, 1988). The β′ subunit (molecular weight 155 162) is much less well characterized. It is thought to aid in nonspecific binding of DNA (Zillig et al., 1976), while some mutations have been isolated that affect transcription termination (Yarulin & Garlenko, 1985). The α subunit (molecular weight 36 511) is the source of considerable recent interest as it is implicated as being required for some transcription activators to function (Russo &

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[1] Abbreviations: Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; BSA, bovine serum albumin; CNBr, cyanogen bromide; ppGpp, guanosine 3′,5′-bispyrophosphate; SDS, sodium dodecyl sulfate; UV, ultraviolet.

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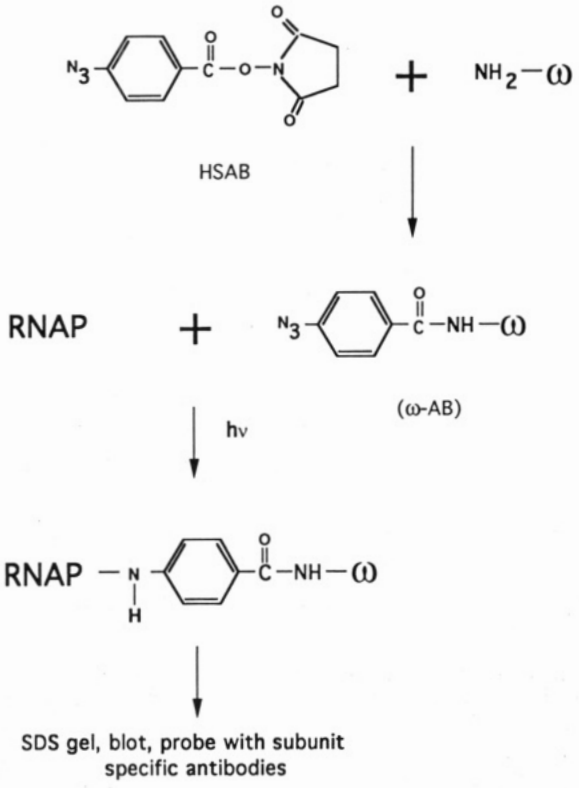

FIGURE 1: Scheme used for the modification of ω, cross-linking, and analysis of cross-linked products. RNAP, RNA polymerase; HSAB, *N*-hydroxysuccinimidyl 4-azidobenzoate.

Silhavey, 1992). Transcription initiation occurs at specific sequences on the DNA template called promoters. RNA polymerase core enzyme is directed to initiate at a promoter by a σ factor which binds RNA polymerase (Helmann & Chamberlin, 1988). A number of σ factors exist in *E. coli*, each one recognizing specific promoter sequences. The major σ factor in *E. coli* is σ^{70} (molecular weight 70 262).

Despite considerable biochemical and genetic study, little is known of the structure of DNA polymerase. Electron microscopy (Tichelaar et al., 1983), neutron scattering (Heumann et al., 1988), and studies of two-dimensional crystals (Darst et al., 1989) have provided low-resolution three-dimensional models of overall structure. These studies do not allow the assignment of subunit interactions at the amino acid or subunit level. Such interactions should be at least partially decipherable by protein–protein cross-linking. Previous cross-linking studies of RNA polymerase have painted a complex and ambiguous portrait of the interaction of the individual subunits (Coggins et al., 1977; Hillel & Wu, 1977). This ambiguity is partially due to the lack of a satisfactory means of identifying the members of cross-linked partners other than the comparison of the observed molecular weights of cross-linked products with all possible products. With the generation of antibodies to the individual subunits, some of this ambiguity is easily reduced. In an attempt to better understand both ω and the structure of RNA polymerase, we have undertaken a cross-linking study to localize the binding site for ω on RNA polymerase using a protein–protein cross-linking technique. In this article, we report the localization of the ω binding site on RNA polymerase to β′.

MATERIALS AND METHODS

Immunological Methods. Transfers of proteins to nitrocellulose from SDS polyacrylamide gels were performed as described by Towbin et al. (1979). In all Western blots, 0.2-μm nitrocellulose (Schleicher and Schuell) was used. Filters were blocked with 2% nonfat dry milk (Blotto) in phosphate-buffered saline (PBS) for 1 h before the addition of antibody (Johnson et al., 1984). Blocked filters were incubated with ascites fluid or whole serum diluted 1:500 in PBS/0.2% Blotto for at least 1 h. Following incubation with the first antibody, the filters were washed with PBS with several changes for 20 min. Goat anti-rabbit IgG–alkaline phosphatase conjugate or goat anti-mouse IgG–alkaline phosphatase conjugate (Boehringer-Mannheim) was added at a dilution of 1:1000 in 0.2% Blotto/PBS and incubated for at least 30 min. After incubation with the second antibody, the filters were washed with PBS with several changes for 20 min. The blots were developed with NBT/BCIP (Blake et al., 1984). After development, the filters were washed with tap water and dried at room temperature. Antibodies against an ω–BSA conjugate were raised in rabbits as described previously (Gentry, 1990).

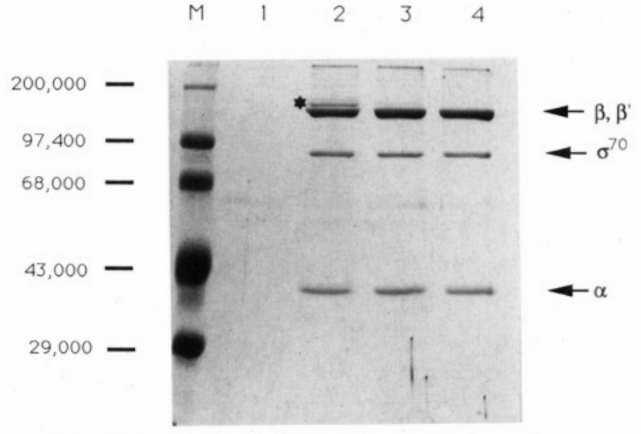

FIGURE 2: Total protein detection of the cross-link reaction. ω was cross-linked to RNA polymerase as described in Materials and Methods. Subunits were separated on a 12% polyacrylamide gel and stained with Coomassie brilliant blue: lane M, BRL prestained molecular weight markers; lane 1, ω–AB only; lane 2, ω–AB + RNA polymerase holoenzyme; lane 3, ω–AB + RNA polymerase + unmodified ω competitor (added at the same time as ω–AB); lane 4, RNA polymerase holoenzyme. The sizes of the molecular weight markers are indicated on the left side of the figure. The β′–ω cross-linked product is indicated by a star directly to the left of it in lane 2.

Protein Purification. ω-free RNA polymerase was purified by the method of Hager et al. (1990) from the *rpoZ* insertion mutant *DG2* (Gentry & Burgess, 1989), which lacks detectable ω. ω was purified from an ω-over-producing strain as described by Gentry and Burgess (1990).

Cross-Linking ω to RNA Polymerase. ω was modified with the hetero-bifunctional cross-linking reagent *N*-hydroxysuccinimidyl 4-azidobenzoate (HSAB, Pierce) as follows. Thirty-two micrograms of ω in 1 mL of a buffer consisting of 10 mM Bicine (pH 8.0) and 0.5 M NaCl was brought to 50 μg/mL HSAB (the stock solution of HSAB was prepared in dimethylformamide at a concentration of 3 mg/mL) and incubated at 37 °C for 2.5 h. One hundred microliters of 1.0 M glycine (pH 8.0) was added followed by an additional incubation of 4 h to block unreacted HSAB. The modified ω,ω–AB, was then added directly to RNA polymerase without further treatment. To cross-link ω–AB to RNA polymerase, 65 μg of ω-free holoenzyme was mixed with 8 μg of ω–AB in a buffer consisting of 20 mM Tris-HCl (pH 7.6), 0.25 M NaCl, and 0.1 mM DTT in a final volume of 0.5 mL. Forty micrograms of unmodified ω was added at the same time as ω–AB when needed as a competitor. The ω–AB and RNA polymerase was incubated for 2 h at 37 °C. The tubes were

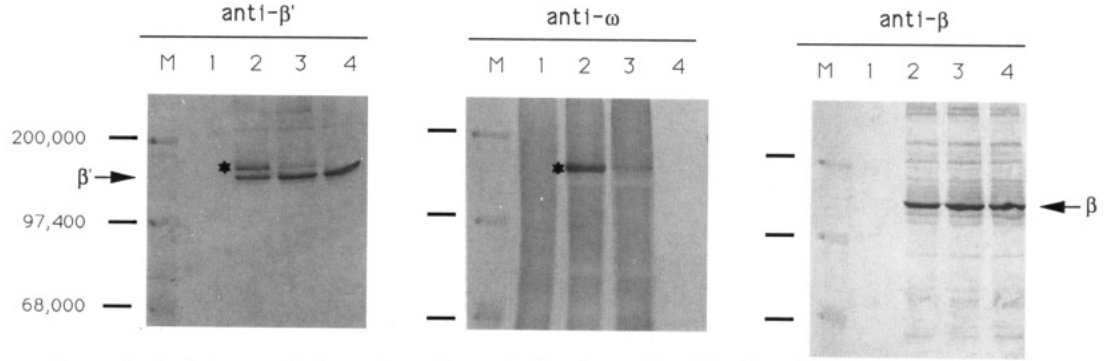

FIGURE 3: Western blot analysis of the cross-link reaction using anti-β', anti-ω, and anti-β. Samples of the cross-link reaction were run, in identical order to Figure 2, on a 6% polyacrylamide gel, blotted onto nitrocellulose, and probed with the antibodies indicated above each panel. The positions of unmodified β' and β are indicated by arrows. The β'–ω cross-linked product is indicated by a star, as in Figure 2. Sizes of molecular weight markers are indicated on the left side of the figure. The positions of the same markers on individual blots are indicated by the bars shown to the left of each blot.

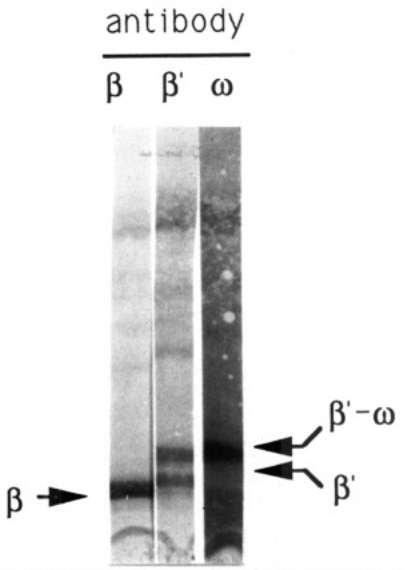

FIGURE 4: Western analysis of the cross-link reaction run in a single lane. A sample of the cross-link reaction was run in a single wide lane on a 4% polyacrylamide SDS gel, blotted onto nitrocellulose, cut into strips, and probed with the antibodies indicated above each strip. The positions of β, β', and β'–ω proteins are indicated by arrows.

then placed on a ultraviolet illuminator for 5 min to activate the ω–AB. We have found that all common ultraviolet light sources, hand-held or flat-bed, common to most laboratories have sufficient energy to activate HSAB. No detectable cross-linking is observed in the absence of ultraviolet irradiation (not shown). Cross-linked products were then analyzed by blotting onto nitrocellulose and probing with antibodies specific for the different subunits of RNA polymerase.

RESULTS

Cross-Linking ω to RNA Polymerase. Cross-linking ω to RNA polymerase involves two steps: modifying ω with the hetero-bifunctional cross-linker HSAB, followed by incubating the modified ω with RNA polymerase and exposing the mixture to UV light (see Figure 1). The first step, the modification of ω, adds an azidobenzoate moiety to free amino groups on ω to make ω–AB. Four residues on ω can be modified in this way (ω has three lysines and the amino terminus). The reactivity of ω toward HSAB is pH-dependent, with optimal cross-linking occurring above pH 8.0 while very little is apparent at pH 7.0 (data not shown).

The second step is the actual cross-linking of ω to RNA polymerase. Exposure of the phenyl azide group attached to ω–AB to UV light results in the formation of a highly reactive phenylnitrene, which will form covalent bonds with a variety of molecules. Cross-links to RNA polymerase subunits following exposure to UV light are detected by the appearance of a new protein species on SDS gels which is recognized by both anti-ω and anti-RNA polymerase subunit antibodies. The size of the observed species should be approximately the molecular mass of ω plus that of the specific subunit. Furthermore, if the mode of binding is specific, then the amount of cross-linking should be reduced if excess unmodified ω is included in the reaction as a competitor.

As shown in lane 2 of Figure 2, when ω is cross-linked to RNA polymerase, a polypeptide with MW of approximately 180 000 appears. When unmodified ω is added as a competitor, this polypeptide is not detectable by Coomassie blue staining (Figure 2, lane 3). The anti-ω and the anti-β', but not the anti-β, antibodies detect this new polypeptide, as shown in Figure 3. To confirm that the peptides detected by the anti-ω and β antibodies are the same molecular weight, a sample of the cross-linked RNA polymerase was run in a single large well of a 4% SDS polyacrylamide gel, blotted onto nitrocellulose, cut into strips, and probed with anti-ω, β, or β' antibodies (Figure 4).

Although no cross-linked products were detected by total protein staining other than the approximately 180 000 MW product, we checked for minor cross-linked products containing α and σ^{70} by using higher percentage polyacrylamide gels and probing with the appropriate antibodies. The results are shown in Figure 5. While no cross-linking of ω to σ^{70} is detected, a faint band of protein is detected by both anti-ω and anti-α. This species is reduced by the addition of unmodified ω, satisfying our criteria for specific binding. The level of apparent cross-linking of ω to α is much lower than that of β'. Also apparent in Figure 5 is the extensive cross-linking of ω to itself, forming multimers of up to at least octamer size. This cross-linking is concentration-dependent and does not reflect the oligomeric state of free ω. ω will not cross-link to itself using either HSAB or diimido esters at lower concentrations of ω (D. R. Gentry and R. R. Burgess, unpublished results). This cross-linking of ω to itself obscures the already low detectability of the possible ω–α cross-linked product.

DISCUSSION

The purpose of the research presented in this article is to determine the binding site of ω to RNA polymerase. The data presented here indicate that ω binds the β' subunit and, to a much lower level, the α subunit. Given this result, it is tempting to speculate that ω binds β' near to where α binds to β'. Previous studies indicate that β' and α cross-link at a

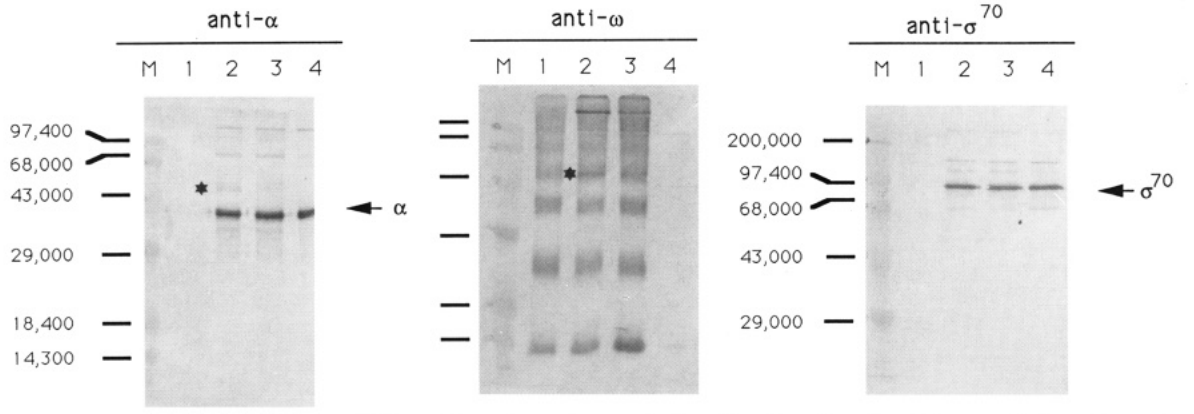

FIGURE 5: Western blot analysis of the cross-link reaction using anti-ω, anti-σ^{70}, and anti-α. Samples of the cross-link reaction were run on a 12% (for α and ω) or 10% (for σ^{70}) polyacrylamide SDS gel, blotted onto nitrocellulose, and probed with the respective antibodies. Lanes contain samples in the same order as in Figure 2. Sizes of molecular weight markers are indicated to the left of the blots probed with anti-α and anti-σ^{70}. The position of a possible α–ω cross-linked product is indicated by a star to its left in the left and center panels.

fairly high frequency. Hillel and Wu (1977) observe a doublet which could be α–β′ and α–β′–ω when the RNA polymerase core enzyme is cross-linked with 2-iminothiolane, consistent with this speculation. Although the results reported here are incremental toward an understanding of ω function, they are important because they demonstrate the ability to determine sites of interaction between RNA polymerase subunits. This ability is due, in part, to the clear results we are able to obtain in contrast with the results typically reported in the literature for complex protein mixtures. The clarity of analysis of cross-linked partners is aided by the availability of highly specific monoclonal antibodies to each of the subunits and by the ability to modify ω in the absence of RNA polymerase. The yield of cross-linked products is probably sufficient for further characterization. In preliminary experiments, we found that cleavage of the cross-link reaction with CNBr yields a peptide of about 30 000 MW that reacts with anti-ω antibody, in addition to what is probably the largest CNBr fragment of free ω. Future work will include further characterization of the 30 000 MW peptide. In addition, we are using the methodology outlined here to determine the sites of interaction of σ^{70} to the core enzyme.

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