Ada Protein-RNA Polymerase σ Subunit Interaction and α Subunit-Promoter DNA Interaction Are Necessary at Different Steps in Transcription Initiation at the Escherichia coli ada and aidB Promoters*

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Paolo Landini‡§, Jonathan A. Bown‡, Michael R. Volkert¶, and Stephen J. W. Busby‡

From the ‡School of Biochemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom, and the ¶Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

The methylated form of the Ada protein (meAda) binds the ada and aidB promoters between 60 and 40 base pairs upstream from the transcription start and activates transcription of the Escherichia coli ada and aidB genes. This region is also a binding site for the σ subunit of RNA polymerase and resembles the rrnB P1 UP element in AT content and location relative to the core promoter. In this report, we show that deletion of the C-terminal domain of the σ subunit severely decreases meAda-independent binding of RNA polymerase to ada and aidB, affecting transcription initiation at these promoters. We provide evidence that meAda activates transcription by direct interaction with the C-terminal domain of RNA polymerase σ70 subunit (amino acids 574–613). Several negatively charged residues in the σ70 C-terminal domain are important for transcription activation by meAda; in particular, a glutamic acid to valine substitution at position 575 has a dramatic effect on meAda-dependent transcription. Based on these observations, we propose that the role of the α subunit at ada and aidB is to allow initial binding of RNA polymerase to the promoters. However, transcription initiation is dependent on meAda-σ70 interaction.

Transcription activation is one of the principal strategies used by bacteria to respond to external stimuli and to adapt to a changing environment. Most Escherichia coli activators stimulate transcription by establishing protein-protein interaction with RNA polymerase. Different subunits of RNA polymerase can be a target for transcription activators; however, the majority of activators interact with either the α or the σ70 subunits (1, 2). α and σ70 are also the subunits of RNA polymerase responsible for specific DNA binding to promoters; σ70 contacts the −35 and −10 promoter elements (core promoter elements), whereas α interacts with UP elements. At the strong rrnB P1 promoter, an UP element stimulates transcription initiation 30-fold through direct interaction with the α subunit C-terminal domain (αCTD), in the absence of any other protein factors (3).

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‡ To whom correspondence should be addressed. Tel.: 0044-121-414-5434; Fax: 0044-121-414-7366; E-mail: p.landini@bham.ac.uk

§ The abbreviations used are: αCTD, α subunit C-terminal domain; MMS, methyl methanesulfonate; “Ada,” the methylated form of the Ada protein.

Exposure of E. coli to sublethal concentrations of methylating agents such as methyl methanesulfonate (MMS) activates expression from three promoters: the ada promoter (which also controls expression of the alkB gene), the alkA promoter, and the aidB promoter. This process is called the adaptive response (4–7). The product of the ada gene, the Ada protein, plays a dual role in the adaptive response; Ada transfers methyl groups from DNA to two of its cysteine residues, thereby functioning as a DNA repair protein. Upon self-methylation, Ada is converted into an activator able to stimulate transcription of the adaptive response genes, including its own (8–10). Ada is a 39-kDa protein organized in two independently structured domains, each with one methyl-acceptor cysteine (11). Methylation of Cys-69, in the N-terminal domain, triggers specific DNA binding by Ada and is required for transcription activation. In contrast, Ada protein singly methylated at Cys-321 is not capable of specific DNA binding activity. However, the Ada C-terminal domain does play a role in transcription activation; deletions in Ada CTD affect transcription of ada, even though they do not affect specific DNA binding (12–14). The methylated Ada protein (meAda) recognizes the sequence AAT(N)6GCAA, which at ada and aidB is located 5 and 7 base pairs upstream of the −35 sequence, respectively (10, 15).

In the model for meAda activation previously proposed (16), meAda contacts RNA polymerase through protein-protein interaction with αCTD and recruits RNA polymerase to the promoter region (15, 16). However, in a previous report (17), it was shown that RNA polymerase binds the ada and aidB promoters via αCTD, regardless of the presence of Ada. In the absence of meAda, RNA polymerase binds to the −60 to −40 region, which also includes the meAda binding site. This region closely resembles the UP element of the rrnB P1 promoter in AT content, in location, and in its function as a binding site for the σ subunit of RNA polymerase. Mutations in αCTD that abolish binding to the rrnB P1 UP element also affect binding to the −60 to −40 region of ada and aidB (17) as well as transcription activation by meAda (16, 18). Due to these similarities, the −60 to −40 regions of ada and aidB can be considered as “UP-like elements.” UP-like elements promote RNA polymerase binding to the ada and aidB promoters in the absence of meAda, but the resulting RNA polymerase-promoter binary complexes can only initiate transcription with poor efficiency. Binding of meAda promotes the formation of a ternary complex that is proficient in transcription initiation (17).

In this report, we show that although αCTD is responsible for the formation of the initial RNA polymerase-promoter binary complex, transcription activation requires protein-protein interaction between meAda and the C-terminal domain of the
The minimal sequence requirement for Ada-dependent activation of the aidB promoter is shown in Fig. 1. This fragment was subcloned into the multiple cloning site of the plasmid pSL1180 (Amersham Pharmacia Biotech), producing plasmid pPL115. The substitutions of 10 and 35 promoter elements to consensus sequences, and of the aidB 60 to 40 region to the rrnB P1 UP element (see “Results”), were introduced by using double strand oligodeoxynucleotides. Plasmid pPL115 was digested with either BsmHI and NcoI or NcoI and MluI and religated in the presence of complementary double strand oligodeoxynucleotides carrying the desired substitutions and the corresponding restriction sites. The ligation mixtures were used to transform E. coli strain RB791 (19), and recombinant plasmids were sequenced using the T7 sequencing kit (Pharmacia). The promoters were then tested for their in vivo activity by β-galactosidase assays in the rpoD strain MV792. Deletion of the rpoD gene completely abolishes ada-independent regulation of aidB (20, 21). Strains were grown to 0.2 A600nm in LB medium supplemented with 0.2% glucose, 20 μg/ml tetracycline, and 80 μg/ml ampicillin and then re diluted 1:50. At an A600nm of about 0.02, the cultures were divided in two aliquots, and one was supplemented with 0.04% MMS to activate the adaptive response. Samples were taken 2 h after induction, and β-galactosidase activity was measured as described in Ref. 22. For β-galactosidase experiments with wild type and mutant rpoD alleles, strain MV3766 (alkB +; ΔPSG1 camR lacZ) was used. β-Galactosidase activity was measured as described above, except that 25 μg/ml chloramphenicol was added to the medium, and MMS induction was started at 0.1 A600nm.

In Vitro Transcription—Reconstitution of RNA polymerase with histidine-tagged full-length α or histidine-tagged truncated α-235 was performed as in Ref. 23. No contamination from wild type α was detectable by SDS-polyacrylamide gel electrophoresis in the α-235 RNA polymerase preparation. For reconstitution of RNA polymerase with wild type α factor or the E575V mutant, histidine-tagged α was purified using Ni-NTA columns (Quiagen), using the standard protocol provided by the manufacturer. Purified α factors were added at a 4:1 ratio to core RNA polymerase (Epitope). Ada was purified as in Ref. 14 and methylated prior to use by the method reported in Ref. 9; when necessary, 0.2 μM of mAdA was added to the transcription reaction mixture. Single-round in vitro transcription experiments from linear templates were performed as in Ref. 18. 5 pmol (0.1 μM) of reconstructed RNA polymerase was used, except for a-235 RNA polymerase, when 12.5 pmol (0.25 μM) was used. For the experiments in Fig. 3, the DNA templates were EcoRI-ScaI fragments from pPL115 (wild type aidB promoter sequence) or from pPL116 (aidB derivative with a perfect consensus -10 sequence). Both fragments are 166 base pairs long and produce a RNA transcript of 40 nucleotides. A DNA fragment carrying the lacUV5 promoter was used as internal control. The fragment is 205 base pairs long and produces a transcript of 65 nucleotides (9). For the experiment in Fig. 8, the template was a HindIII-EcoRI fragment from pYN3066 for the ada promoter (9) and an EcoRI-ScaI fragment from pPL115, described above, for aidB. The amount of transcription was quantified after normalization to the lacUV5 transcript using a phosphorimager (Molecular Dynamics).

Gel Retardation Assays—Fragments for gel retardation assays were the same as those used for the in vitro transcription experiments. Fragments were labeled, and 5,000 cpm/sample was used in 20 μl final volume of reaction buffer (50 mM Tris-HCl, pH 7.6, 50 mM NaCl, 2.5 mM dithiothreitol, 6.25% glycerol, 10 μg/ml herring sperm DNA). Wild type and mutant RNA polymerases, α factors (purified as in Ref. 24), and mAdA were added as described in the figure legends. Samples were incubated 20 min at 37 °C and loaded either on to a 4% (for experiments with RNA polymerase) or a 6% (for experiments with α factors) native polyacrylamide gel. Gels were run at 10 V/cm in 0.25 × TBE (22.5 mM Tris borate, 0.5 mM EDTA), 1.25% glycerol. Bands were visualized by autoradiography.

Results

In Vivo Transcription from aidB Promoter Derivatives—Fig. 1 shows the sequence of the ada and aidB promoters. The −60 to −40 regions of the two promoters show a high degree of similarity to the UP element of the rrnB P1 promoter. This region is also the binding site for mAdA, and its deletion abolishes ada-dependent activation (Ref. 26; Fig. 2). Although both promoters have recognizable −35 and −10 sequences, they differ from the consensus hexamers TTGACA and TATAAT. At positively controlled promoters, one of the functions of activator proteins is to improve recognition of one or more weak promoter elements by RNA polymerase through protein-protein interaction or modification of local DNA structure. To understand which of the weak promoter elements is the target of mAdA activation, we constructed a set of derivatives of the aidB promoter in which either the −35 or the −10 elements were substituted by perfect consensus hexamers, and the UP-like element was substituted by the UP element of rrnB P1. We investigated the effects of these substitutions on both basal and ada-activated levels of transcription in vivo.

Results shown in Fig. 2 suggest that at the rrnB UP element aidB hybrid promoter (aidB “rrnB UP”), ada-independent transcription levels are only slightly higher than for the wild type aidB promoter (1.6-fold, Fig. 2). Thus, mAdA does not activate
transcription by converting the α binding site of aidB into a better UP element. Because this substitution also replaces the Ada binding site, ada-dependent activation is almost completely abolished. Changes to consensus −35 or −10 sequences did have more pronounced effects on transcription; the consensus −10 increased ada-independent transcription levels by almost 5-fold, and introduction of a consensus −35 resulted in a more than 12-fold increase. However, neither substitution completely relieved the dependence on mAda for optimal promoter expression; mAda activates the aidB promoter derivative with a consensus −10 element (aidB −10 con) by 5-fold, and the derivative with a consensus −35 element (aidB −35 con) by 2.3-fold (Fig. 2). These observations suggest that mAda activates transcription by affecting RNA polymerase interaction with both the −35 and −10 elements of aidB.

In Vitro Transcription—Sakumi et al. (16) had proposed that mAda activates transcription by direct contact with the CTD of the α subunit. We further investigated the role of CTD in activation by mAda by performing in vitro transcription experiments using two forms of reconstituted RNA polymerase different with respect to their α subunits: one form carried wild type α, the other a mutant α deleted of the C-terminal 94 amino acids (α-235). Although α-235 RNA polymerase is impaired in UP element utilization and in transcription from some activator-dependent promoters, it is proficient in transcription from factor-independent and promoters dependent on activators that do not interact with α (1).

We tested the two forms of reconstituted RNA polymerase for transcription from the wild type aidB promoter (aidB wt) as well as from an otherwise identical promoter in which the −10 sequence was changed to consensus (aidB −10 con). The latter promoter shows 5-fold higher basal transcription level in vivo compared with the wild type aidB promoter but is still dependent on mAda for maximal promoter expression (Fig. 2). Deletion of the CTD dramatically affects both mAda-dependent and independent transcription from the wild type aidB promoter, even though activation by mAda is not totally abolished (Fig. 3, lanes 5 and 6). Transcription from aidB −10 con by α-235 RNA polymerase is also affected; however, at this promoter mAda activates transcription by both forms of RNA polymerase to a similar extent (5.2-fold for wild type α- and 4-fold for α-235 RNA polymerase; Fig. 3, lanes 7–10). These results strongly suggest that, although CTD is necessary for efficient transcription at the wild type aidB promoter, it is not required for activation by mAda.

The results of in vitro transcription experiments raise the possibility that α-235 RNA polymerase is not proficient in carrying out transcription from the wild type aidB promoter because it is affected in RNA polymerase-promoter interaction, rather than interaction with mAda. To investigate this possibility, we tested both wild type and α-235 RNA polymerase for their ability to bind the wild type aidB promoter in the absence of mAda. As shown in Fig. 4, wild type RNA polymerase binds aidB wt at 0.04–0.08 μM, whereas α-235 RNA polymerase fails to bind the promoter at concentrations up to 0.32 μM. A similar result was obtained for the wild type ada promoter (data not shown). Both forms of RNA polymerase were equally as efficient in binding both the lacUV5 and the gapP promoters under the same experimental conditions (data not shown). The above results show that CTD promotes recruitment of RNA polymerase to the ada and aidB promoters independently of mAda.

FIG. 2. In vivo transcription of aidB promoter derivatives (performed as in “Experimental Procedures”). Open bars, β-galactosidase levels in the absence of MMS. Filled bars, β-galactosidase levels in cells treated with 0.04% MMS. Data are the average of four independent experiments. The standard deviation was <15%.

FIG. 3. In vitro transcription with reconstituted wild type (0.1 μM) and α-235 (0.25 μM) RNA polymerases. The filled arrow head shows the position of the transcript from the lacUV5 promoter (lacUV5). The open arrow head indicates the position of transcripts from either the wild type aidB promoter (paidB wt, lanes 3–6) or the aidB −10 con′ promoter (pαidB −10 con, lanes 7–10). Fold-activation by mAda is shown below the transcripts.

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\[
\begin{array}{cccccccc}
\alpha_{\text{wt-RNAP}} & + & + & + & + & + & + & + \\
\alpha_{\text{235-RNAP}} & + & + & + & + & + & + & + \\
\text{p} & \text{aidB} & \text{wt} & + & + & + & + & + \\
\text{p} & \text{aidB} & \text{−10 con} & & & & & + \\
\text{m} & \text{Ada} & & & & & & + \\
\end{array}
\]

\[
\begin{array}{cccccccc}
2 & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 \\
\alpha_{\text{wt-RNAP}} & + & + & + & + & + & + & + & + & + & + \\
\alpha_{\text{235-RNAP}} & + & + & + & + & + & + & + & + & + & + \\
\text{p} & \text{aidB} & \text{wt} & + & + & + & + & + & + & + & + \\
\text{p} & \text{aidB} & \text{−10 con} & & & & & + & + & + & + \\
\text{m} & \text{Ada} & & & & & & + & + & + & + \\
\end{array}
\]

22.1 2.8 5.2 4.0
Transcription Activation at Ada-dependent Promoters

Transcription activation is driven by the ada promoter. Over 1700 colonies were screened and four mutant colonies were isolated. The mutant rpoD alleles were sequenced; three carried nonsense mutations, which resulted in truncations of σ70 at amino acid 560 (1 clone) or at amino acid 584 (2 clones). The fourth candidate carried a missense mutation, resulting in a glutamic acid to valine change at position 575 of σ70 (E575V).

In addition to polymerase chain reaction-directed mutagenesis, we also tested a set of plasmids (kindly given by C. Gross, UCSF) carrying rpoD alleles with single alanine substitutions at 17 amino acids of σ70CTD (Fig. 7). β-Galactosidase assays were performed to quantify the effect of the alanine substitutions, as well as the E575V mutation, on transcription from the ada promoter. In strain MV3766, wild type σ70 from the chromosomal rpoD gene is normally expressed, and mutated σ factors are present in only a slight excess to wild type σ70 (data not shown). Nevertheless, expression of several mutant rpoD alleles resulted in altered levels of in vivo transcription at the ada promoter: E574A, E575V, I590A, E591A, E605A, and D613A substitutions significantly decreased expression, and mutated σ factors are present in only a slight excess to wild type σ70 (data not shown). Nevertheless, expression of several mutant rpoD alleles resulted in altered levels of in vivo transcription at the ada promoter: E574A, E575V, I590A, E591A, E605A, and D612A substitutions significantly decreased ada-dependent transcription, with E575V having the most severe effect (Fig. 7). Some mutations, such as R596A, K597A, R608A, and D613A, resulted instead in an increased level of ada-dependent transcription (120–150%, Fig. 7).

To verify that inhibition of ada-dependent transcription in vivo is indeed due to disruption of adaσCTD interaction, we purified both wild type and E575V σ factors and reconstituted RNA polymerase in vitro. As shown in Fig. 8, E575V σ70-RNA polymerase was able to carry out transcription from the lacUV5 promoter with the same efficiency as wild type σ70-RNA polymerase, which suggests that the E575V mutation does not affect either core enzyme-σ70 interaction or factor-independent transcription; however, ada-dependent transcription by the mutant RNA polymerase at both ada (Fig. 8) and aidB (data not shown) was drastically impaired.

DISCUSSION

In a previous report, we showed that RNA polymerase binds to the ada and aidB promoters via the α subunit, independently of the Ada protein. Thus, ada does not recruit RNA polymerase to the promoters but rather converts the RNA polymerase-promoter complex into a ternary complex proficient in transcription initiation (17). The location of the ada binding site suggests the possibility of interactions with either αCTD or σ; alternatively, the mechanism for activation by ada could involve turning the α-binding sites of ada and aidB into more efficient UP elements. Our experiments with hybrid promoters show that when the UP-like element of aidB is substituted by the rrnB P1 UP element, no significant stim-
ulation of transcription occurs (Fig. 2). In contrast, changing either the ς35 or ς10 sequences of aidB to consensus results in a significant stimulation of ada-independent transcription in vivo, suggesting that meAda improves RNA polymerase interaction with the core promoter region (Fig. 2). Thus, meAda activates transcription either by improving initial binding of RNA polymerase to the ς35 or by facilitating a later step in transcription initiation, such as isomerization to open complex.

Deletion of ςCTD severely affects meAda-activated transcription (Ref. 16; Fig. 3) and prevents RNA polymerase binding to the ada and aidB promoters (Fig. 4). However, at an aidB derivative in which the ς10 sequence was changed to consensus (aidB “−10 con”), meAda is able to activate transcription by ς-235 RNA polymerase (Fig. 3) to roughly the same extent as wild type RNA polymerase. Thus, dependence on ςCTD for transcription at Ada-dependent promoters can be by-passed by strengthening the core promoter, presumably by providing an alternative binding site for ς-235 RNA polymerase that compensates for its loss of interaction with the UP-like element. Although it is possible that altering the −10 sequence of the aidB promoter also modifies the interaction between meAda and RNA polymerase, the results at the aidB ς10 con promoter clearly demonstrate that meAda can activate transcription by RNA polymerase containing an ς subunit deleted of its CTD. Although we cannot rule out the possibility of direct ς-meAda interaction, we propose that inefficient transcription by RNA polymerase deleted of its ςCTD results from the loss of the ς-UP-like element interaction and consequent inability of RNA polymerase to bind the promoter, rather than from lack of interaction with meAda.

Several lines of evidence show that meAda interacts with ς70. Gel retardation experiments (Figs. 5 and 6) indicate that the terminal 39 amino acids of ς70 are necessary for this interaction. This region contains determinants for the recognition of the ς35 element (region 4.2), followed by the so-called “basic cluster” (28). Several amino acids in both region 4.2 and in the basic cluster have been found to be important for interaction with transcription activators, such as PhoB, bacteriophage λ CI protein, and FNR (29, 30). A set of substitutions between amino acids 570 and 580 of ς70 severely impairs transcription activation by PhoB at the psfS promoter (31) and also affects activation by CI at λ PRM promoter (30). Mutational analysis of

\[ \text{Fig. 7. Effects of substitutions in } \sigma^{35}\text{CTD on } \sigma^-\text{-dependent transcription in vivo. Values are an average of three independent experiments and are given as a percentage of the wild type. The average value for the wild type was 903 Miller units.} \]

\[ \text{Fig. 8. In vitro transcription with RNA polymerases reconstituted with either wild type or E575V } \sigma \text{ at the } \sigma^- \text{ promoter. Fold-activation by } \sigma^- \text{ is shown below the transcripts.} \]
σ^70CTD suggests that meAda interacts with a set of negatively charged residues both in and downstream of region 4.2 (Fig. 7). With the exception of I590A, all the substitutions that significantly affect ada-dependent transcription involve negatively charged amino acids (Glu-574, Glu-575, Glu-605 and Asp-612); substitution to alanine of several positively charged residues (Arg-596, Lys-597, Arg-608) results in increased levels of transcription activation by PhoB (29), suggesting that the loss of meAda−σ^70 interactions act at separate but inter-dependent steps of transcription initiation. It is possible that Ada CTD is responsible for this interaction; deletions in Ada CTD abolish transcription activation at the ada promoter without affecting DNA binding by meAda (14).

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**Figure 9.** Model for transcription activation by meAda. The upper panel illustrates the specific interactions established between RNA polymerase and the ada or aidB promoters in the absence of meAda; the RNA polymerase-promoter complex results from protein-DNA interactions between σ^70 CTD and the UP elements. The lower panel shows the RNA polymerase-promoter-Ada ternary complex. Ada binds to its DNA site via its N-terminal domain (NTD) (12) and stimulates transcription initiation (black arrow) via protein-protein interaction between its C-terminal domain (14) and σ^70 CTD.