The RNA Polymerase α Subunit Carboxyl-terminal Domain Is Required for Both Basal and Activated Transcription from the alkA Promoter*

(Received for publication, February 25, 1997, and in revised form, April 16, 1997)

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Expression of the Escherichia coli adaptive response genes (ada, aidB, and alkA) is regulated by the transcriptional activator, Ada. However, the interactions of RNA polymerase and Ada with these promoters differ. In this report we characterize the interactions of Ada, methylated Ada (meAda), and RNA polymerase at the alkA promoter and contrast these interactions with those characterized previously for the ada and aidB promoters. At the alkA promoter, we do not detect the RNA polymerase α subunit-mediated binary complex detected at the ada and aidB promoters. In the presence of either of these two activators, RNA polymerase protects the alkA core promoter, including the elements at −35 and −10, and is more efficient in transcription initiation in vitro. RNA polymerase holoenzyme containing the α subunit mutation R265A is severely impaired in Ada-independent basal alkA transcription, shows no activation by Ada or meAda, and fails to bind the alkA promoter in vitro. Binding of the purified wild type α subunit to alkA was not detected, but a complex of promoter DNA, Ada or meAda, and α was observed in gel shift assays. These observations suggest that both forms of Ada protein activate alkA transcription by enhancing RNA polymerase holoenzyme and α subunit binding.

In bacteria, transcriptional activation plays a pivotal role in adaptation processes. Changes in the cell’s environment stimulate transcriptional activators to bind their target promoters and to induce their expression. This results in adaptation of gene expression to the environmental requirements. Transcriptional activators have recently been classified on the basis of their mechanism of interaction with RNA polymerase, leading to the definition of Class I and Class II activators (1). According to this definition, Class I transcriptional activators work by contacting the α subunit of RNA polymerase, whereas Class II activators interact with the σ factor. α and σ are also the subunits of RNA polymerase involved in specific DNA binding and promoter recognition. α is responsible for interaction with the −35 and −10 sequences, whereas σ is able to bind a third element of the bacterial promoter identified at the rrrB P1 promoter region and termed the UP element (2).

The Escherichia coli adaptive response to DNA methylation damage is induced when cells are exposed to sublethal doses of alkyllating agents (3–5). ada is the regulatory gene of the adaptive response. The product of the ada gene, Ada, is a methyltransferase able to transfer methyl groups from damaged DNA to two of its own cysteine residues (6, 7). Methylation of Cys-69 converts Ada into a transcriptional activator. The Cys-69-methylated Ada protein (meAda) is able to recognize specific sequences upstream of its own promoter and at least two other promoters, alkA and aidB, and activate their transcription (8–10). meAda has been proposed as a Class I transcriptional activator, since deletion of the carboxyl-terminal domain of the RNA polymerase α subunit results in the loss of transcriptional activation by meAda at the ada promoter (11).

We have recently shown that the α subunit of RNA polymerase binds to the promoters of the adaptive response genes ada and aidB in the absence of Ada protein (12). RNA polymerase holoenzyme binds the two promoter regions between −60 and −40 via its α subunit; this area overlaps the meAda binding site and closely resembles the UP element of the rrrB P1 promoter with respect to location, high A/T content, and recognition by α (2). This RNA polymerase-promoter binary complex shows only basal levels of transcription and is modified by meAda into a ternary complex competent in transcription initiation at induced levels (12). The single amino acid substitution in the α DNA binding domain, R265A, which is a change from arginine to alanine at residue 265, prevents α from binding the rrrB P1 promoter (13) as well as the −60 to −40 region of the ada and aidB promoters (12).

The alkA gene encodes a glycosylase responsible for recognition and excision of methylated DNA bases (14). A number of observations strongly suggest that the mechanism of Ada action on the alkA promoter differs from that of the other adaptive response genes. First, methylation of Ada is not necessary for alkA induction. Overexpression of the unmethylated Ada in vitro is sufficient to induce alkA (i.e. in the absence of alkylation damage) (14). Both Ada and meAda proteins are able to activate transcription of alkA in vitro (9), although higher concentrations of Ada are required (15, 16). In contrast, the ada and aidB promoters are only activated by meAda protein (6, 8), and high concentrations of Ada protein inhibit activation by meAda (17). Second, the location of the Ada binding site in the alkA promoter region differs from that in the aidB and ada promoters. At the alkA promoter, meAda protects residues from −54 to −29 (16, 18), overlapping the −35 region. In both ada and aidB, meAda binds further upstream, protecting sequences from −62 to −38 in DNase I protection experiments (10, 19) (see Fig. 1).

* This work was supported in part by National Institutes of Health, American Cancer Society-Massachusetts Division, Inc. Grants GM37052 (to M. R. V.) and GM37048 (to R. L. G.), and by research funds from the University of Massachusetts Medical Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡¶ The abbreviations used are: meAda, methylated Ada; bp, base pair(s).
Third, Ada mutants capable of activating alkA but not ada, and vice versa, have been isolated (15, 20). Likewise, the isolated, methylated amino-terminal domain of Ada acts differently on the two types of promoters, activating alkA but inhibiting ada (18).

In this report we investigate the interaction between RNA polymerase and the alkA promoter and the effects of Ada and meAda on this interaction. We find that 1) the RNA polymerase α subunit plays an important role in both basal and Ada-activated transcription, 2) both Ada and meAda stimulate specific binding of both RNA polymerase holoenzyme and the purified α subunit of RNA polymerase, and 3) the Ada-RNA polymerase-alkA promoter ternary complex differs from the ternary complex formed by meAda in the extent of upstream DNA binding.

MATERIALS AND METHODS

Chemicals—Restriction endonucleases were from Boehringer Mannheim. [α-32P]dATP and [α-32P]CTP were from Amersham Life Science. E. coli RNA polymerase and RNase-free DNase I were from Pharmacia Biotech Inc.

DNase I Protection and Gel Retardation Experiments—An NruI/PstI (−60 to +296 of the alkA region) fragment from pYN3072 (9) was subcloned into pSL1180 (Pharmacia) using the corresponding restriction sites to obtain plasmid pMV447. To obtain pMV465, PMV447 was cut with AccI, and the vector fragment was re-ligated to delete 253 bp from the polylinker of pSL1180. For the coding strand, a HindIII/AccI (145 bp) fragment from pMV447 that contained the alkA promoter region from −60 to +24 plus an additional 61 bp of upstream vector DNA was labeled at the AccI site with [α-32P]dATP by an end-filling reaction with Klenow enzyme. For the template strand, a HindIII/EcoRI (169 bp) fragment from pMV465 that contained the same 145-bp fragment as pMV447 plus an additional 24 bp of downstream vector DNA was labeled at the HindIII site. The region downstream of −60 contained all the necessary elements for transcription and regulation of alkA (Ref. 16 and Fig. 1). Deletion of the region upstream of −60 resulted in the loss of a second promoter that reads in the opposite direction (14). This promoter is not regulated by the Ada protein and does not affect levels of alkA transcription either in vivo or in vitro.2 The Ada protein was purified as in Saget and Walker (17), and methylation of the Ada protein was obtained as in Nakabeppe and Sekiguchi (9).

DNase I protection and gel retardation experiments were performed in 20 μl final volume of binding buffer (12). Samples were incubated for 20 min at 22 °C and processed as described previously (12).

In Vitro Transcription—Single-round in vitro transcription experiments were performed using the linear DNA templates as follows. For the alkA promoter, a HindIII/EcoRI fragment from pMV465 (169 bp) was used. This fragment contained the same 169-bp alkA promoter fragment used in DNase I protection experiments of the template strand. The RNA transcript obtained from this fragment was 48 nucleotides in length. As a control, we used the lacUV5 promoter, a 205-bp fragment from pYN3077 (9), to produce an RNA transcript of 65 nucleotides. In the experimental conditions, a second RNA transcript approximately 10 nucleotides shorter was also produced, possibly from a cryptic promoter on the same DNA fragment. The sizes of the RNA transcripts were confirmed by DNA sequencing ladders run on the same gels, assuming an electrophoretic mobility 10% slower for RNA than DNA. 0.3 pmol of each promoter region were preincubated for 5 min at room temperature with 4 pmol of either wild type RNA polymerase holoenzyme or α R265A-RNA polymerase (reconstituted as in Ref. 13) in 40 mM Tris-HCl, pH 8.0, 30 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol, and 20 μg/ml bovine serum albumin. When required, 10 pmol of unmethylated or methylated Ada were added, and the mixture was incubated for 15 min at 37 °C. Methylation of Ada was performed as in Ref. 9, except that the amount of methylated calf thymus DNA was lowered to 0.06 μg. Transcription reactions were started by the addition of 0.1 mM each ATP, GTP, and UTP, 0.001 mM CTP, 10 μCi of [α-32P]CTP, 250 μg/ml heparin (final reaction volume, 50 μl). After 5 min of incubation at 37 °C, 1 μl unlabeled CTP was added, and the reaction was allowed to continue for 3 min and then blocked with 50 μl of stop solution (600 mM NaCl, 50 mM EDTA, pH 8.0, 30 μg/ml salmon sperm DNA). Samples were precipitated with 2 volumes of ethanol, resuspended in gel loading buffer, and analyzed on a 6% polyacrylamide, 8 M urea gel.

RESULTS

Effects of the α Subunit Mutation R265A on RNA Polymerase Function at alkA—Consistent with previous reports (8, 9, 16), we observed a low level of transcription of the alkA promoter with wild type RNA polymerase in vitro in the absence of any activator protein (Fig. 2, lane 2). Both the unmethylated and the methylated forms of Ada (Ada and meAda) stimulated in vitro transcription, although the highest levels of transcription occurred with meAda (Fig. 2, lanes 3 and 4). We and others have previously found that the carboxyl-terminal domain of the RNA polymerase α subunit plays a role at other Ada-activated promoters (11, 12). However, the binding site for meAda in the alkA promoter (−52 to −30, partially overlapping the −35 region) differs from that at the aidB and ada promoters that have meAda binding sites extending from −36 to −60 and −40 to −64, respectively. This result combined with the results from the genetic and biochemical studies of others (11, 17, 18, 20) suggests that the mechanisms at these promoters may differ. We therefore asked whether the carboxyl-terminal domain of the RNA polymerase α subunit is essential for activation of transcription by Ada at the alkA promoter.

We used an RNA polymerase containing the α R265A mutation. This mutant α subunit was previously shown to be defective in DNA binding and, when assembled into the RNA polymerase holoenzyme, was defective in UP element function at the rrrB P1 promoter (13). Purified a R265A was also shown to be defective in the binding of the upstream regions of the Ada-dependent aidB and ada promoters (12). The α R265A-RNA polymerase was reconstituted in vitro and used in transcription of alkA in the presence or absence of Ada and meAda (Fig. 2, lanes 5–8). The activator-independent, basal level of transcription of alkA was reduced substantially by the R265A mutation (lane 6), and no activation of transcription was detected by either Ada or meAda (lanes 7 and 8). The R265A-RNA polymerase transcribed the lacUV5 promoter as efficiently as

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wild type RNA polymerase in these reactions (compare lanes 1–4 with lanes 5–8). Another transcript from the placUV5 fragment was also detected with wild type RNA polymerase (lanes 1–4) although not with R265A-RNA polymerase (lanes 5–8). This transcript is approximately 52 nucleotides in length and may be the result of either initiation from a weak promoter-like sequence or premature termination.

Relative Binding Affinities of Ada and mAda at the alkA Promoter—Since unmethylated Ada was found to stimulate transcription by wild type RNA polymerase, but less effectively than mAda (Fig. 2 and Ref. 9), we tested the possibility that there are differences in the affinity of the two forms of the activator for the binding site in the alkA promoter. We used a gel shift assay to compare the concentrations of Ada or mAda required to observe formation of a complex with an alkA promoter fragment (Fig. 3). A mAda-DNA complex was formed that was detectable at a relatively low mAda concentration (0.015 μM) and required a concentration of approximately 0.05 μM for complete site occupancy. Complexes of higher mobility were also formed at high concentrations of mAda, but the nature of the interactions in these complexes is not understood. In contrast, the unmethylated form of Ada formed complexes with alkA only at a relatively high concentration (0.17–0.50 μM), and full site occupancy was not detected at the maximum concentration used. Thus, the methylated form of Ada binds the alkA site more efficiently.

Formation of Complexes of alkA, RNA Polymerase, and Either Ada or mAda—We previously observed that RNA polymerase alone, in the absence of mAda, protected the upstream region of the aidB and ada promoters in a complex detected by DNase I footprinting. The basal level of alkA transcription by the wild type RNA polymerase (Fig. 2) indicates that RNA polymerase is also able to specifically recognize the alkA promoter in the absence of activator. However, we were unable to detect this RNA polymerase-alkA complex in a DNase I footprint (Fig. 4, compare lane 1 with 4), presumably reflecting the instability of the complex under these conditions. Consistent with the reduced ability of the α R265A mutant RNA polymerase to carry out basal transcription of alkA (Fig. 2), we were also unable to detect a complex of this mutant RNA polymerase with the promoter (Fig. 4, lane 7).

A stable ternary complex of alkA promoter, RNA polymerase, and either Ada (Fig. 4, lane 5) or mAda (Fig. 4, lane 6) was observed. The boundaries of protection conferred by the activator protein alone (Δ52 to −30) are seen in the footprint of mAda (Fig. 4, lane 3), consistent with previous results (18). Although the unmethylated form of Ada does not bind well enough to confer clear protection in the DNase I footprints (Fig. 4, lane 2; see also Figs. 3 and 5), it does support the formation of a ternary complex with RNA polymerase and the promoter. The upstream boundaries of protection observed with Ada and mAda containing ternary complexes differ (compare lane 5 with 6). The complex containing mAda had an upstream boundary of −52, whereas the complex with Ada had a boundary at −43. Increasing the concentration of Ada to 2.5 μM did not convert the protection pattern exhibited by Ada to that of mAda (data not shown), suggesting that the difference in protection patterns reflects differences in the structure of the complexes, rather than degree of occupancy of the promoter. The Ada or mAda concentration dependence of formation of these complexes is examined further in Fig. 5.

Consistent with the inability of the mutant R265A-RNA polymerase to be activated by Ada or mAda at the alkA promoter (Fig. 2), we did not observe a ternary complex of R265A-RNA polymerase, the alkA promoter, and Ada or mAda in DNase I footprint assays (Fig. 4, lanes 8 and 9). (The protected region in lane 9 reflects binding of mAda (compare with lane 3).) Thus, the arginine 265 residue in the carboxyl-terminal domain of the α subunit is required for formation of the Ada- or mAda-stimulated ternary complexes.

Consistent with the gel shift results of Fig. 3 indicating that only a small fraction of DNA molecules were bound by Ada, we did not detect an Ada footprint at concentrations ranging from 0.02 to 0.5 μM (Fig. 5, lanes 2–4). Over a comparable concentration range, mAda fully occupied its alkA promoter binding site (Fig. 5, lanes 5–7). Although binding of unmethylated Ada protein alone was not detectable, it stimulated the formation of a ternary RNA polymerase-alkA-Ada complex at each concentration tested (Fig. 5, lanes 9–11), as did mAda (lanes 12–14). Thus, it appears that there is cooperative interaction of RNA polymerase and Ada at this promoter.
**FIG. 4.** DNase I protection experiments of the *alkA* promoter template strand by wild type or α R265A mutant RNA polymerase in the presence or absence of Ada and "Ada" protein. Lanes 1–3, *alkA* promoter, no RNA polymerase; lanes 4–6, wild type RNA polymerase (5 pmol, 0.25 μM); lanes 7–9, α R265A mutant subunit containing RNA polymerase (5 pmol, 0.25 μM); lanes 1, 4, and 7, no Ada protein; lanes 3, 5, and 8, Ada (10 pmol, 0.50 μM); lanes 3, 6, and 9, "Ada" protein (10 pmol, 0.50 μM). The dashed line indicates the region protected by the "Ada" alone. The thick solid line indicates the region protected by the unmethylated Ada-RNA polymerase complex. The dashed and solid line marks the region protected by the complex "Ada"-RNA polymerase. The numbering of the bases of the *alkA* promoter region is indicated on the left and is relative to the transcription initiation site defined by Furuichi et al. (16).

**DISCUSSION**

In a previous report (12), we showed that RNA polymerase is able to contact the promoters of the adaptive response genes *ada* and *aidB* via binding of the α subunit to the −40 to −60 region in the absence of the transcriptional activator "Ada". At these promoters, binding of "Ada" converts the RNA polymerase-promoter complex into a ternary complex competent for induced levels of transcription initiation. At the *ada* and *aidB* promoters, only "Ada" functions as an inducer, and its role in activation is to isomerize a binary complex of RNA polymerase bound to an upstream site via α subunit contacts; binding of "Ada" alters the nature but not the affinity of RNA polymerase binding, leading to complete promoter protection and induced transcription (12). The *alkA* promoter differs from *ada* and *aidB* promoters in several respects. Both forms of the Ada protein activate *alkA* transcription (9) and stimulate complete protection of the *alkA* promoter region by RNA polymerase (Fig. 4, lanes 5 and 6). RNA polymerase alone binds the *alkA* promoter too weakly to afford clear DNase I protection, but both forms of Ada activator enhance RNA polymerase binding to the *alkA* promoter. Thus, the role for "Ada" at the
The ternary complex is increased at least 25-fold (Fig. 5) by affinity of unmethylated Ada to the alkA promoter spans between the part of the sequence that varies (10), which in the (Ref. 18 and Fig. 4, allow a determination of its binding site. The unmethylated Ada in the absence of RNA polymerase does not extend the area of protection provided by "mAda (data not shown), suggesting that the binding site overlaps the "mAda binding site and is therefore indistinguishable in DNase I protection experiments. Overlapping sites for a and "mAda have already been observed at both the ada and aidB promoters (12).

The R265A mutation of a impairs RNA polymerase binding to the alkA promoter in the presence of Ada, as determined by DNase I protection experiments (Fig. 4) and almost completely abolishes basal and activated transcription from the alkA promoter (Fig. 2). These observations suggest that the subunit plays an important role in the formation of the RNA polymerase-alkA promoter binary complex and that a binding is necessary for uninduced, basal levels of alkA transcription. This finding is apparently a contradiction with the lack of binding observed with the purified a subunit (Fig. 6). However, additional interactions between the promoter and the other subunits of RNA polymerase may enhance or stabilize a binding when it is part of the holoenzyme. Indeed, differences in concentration needed for DNA binding by the subunit alone and a assembled into RNA polymerase have already been described (2, 12). At the alkA promoter, the binding of RNA polymerase subunits in other areas of the promoter might provide stabilization of a binding. Moreover, the requirement of Ada or "mAda for a subunit binding suggests that the different role for Ada in activation of alkA predicted from genetic studies (9, 14–17) may be that at alkA, Ada and "mAda function to enhance RNA polymerase holoenzyme binding by stimulating a binding, whereas at the ada and aidB promoters, only "mAda functions as an activator, and a binding is unaffected by "mAda; it has been proposed that at ada and aidB, "mAda is required for binding of RNA polymerase to the core promoter elements at −10 and −35 (12).

Although the DNA binding function of the subunit is required for binding of RNA polymerase to the alkA promoter, this requirement does not exclude the possibility that transcription is activated by Ada through a Class I transcriptional activator mechanism, as proposed (1, 11). At the alkA promoter, methylated Ada binding is required for a binding, a role consistent with the general model proposed for Class I transcriptional activators (23). It is possible that interaction of the Ada protein with the subunit triggers an extensive conformational change in RNA polymerase, so that the −35 and −10 elements are accessible to the factor, and transcription initiation can occur. However, direct interaction of Ada and "mAda with other subunits such as cannot be ruled out; the location of the Ada binding site in the alkA promoter would favor such interaction, which might not be possible in the other Ada-dependent promoters where the Ada binding site is 6–8 base pairs upstream of the −35 sequence.

Acknowledgment—We thank Richard L. Gourse for critical reading of the manuscript.

REFERENCES
1. Ishihama, A. (1993) J. Bacteriol. 175, 2483–2489
2. Ross, W., Gosink, K. K., Salomon, J., Igarashi, K., Zou, C., Ishihama, A., Severinov, K., and Gourse, R. L. (1993) Science 262, 1407–1413
3. Samson, L., and Cairns, J. (1977) Nature 267, 281–283
4. Jeggo, P. (1979) J. Bacteriol. 139, 783–791
5. Lindahl, T., Sedgwick, B., Sekiguchi, M., and Nakabeyashi, Y. (1988) Annu. Rev.
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6. McCarthy, T. V., and Lindahl, T. (1985) *Nucleic Acids Res.* 13, 2663–2668
7. Demple, B., and Linn, S. (1982) *Nucleic Acids Res.* 10, 3781–3789
8. Tee, I., Sedgwick, B., Kilpatrick, M. W., McCarthy, T. V., and Lindahl, T. (1986) *Cell* 45, 315–324
9. Nakabeppu, Y., and Sekiguchi, M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 6297–6301
10. Landini, P., and Volkert, M. R. (1995) *J. Biol. Chem.* 270, 8285–8289
11. Sakumi, K., Igarashi, K., Sekiguchi, M., and Ishihama, A. (1995) *J. Bacteriol.* 175, 2455–2457
12. Landini, P., and Volkert, M. R. (1995b) *EMBO J.* 14, 4329–4335
13. Gaal, T., Ross, W., Blatter, E. E., Tang, H., Jia, X., Krishnan, V. V., Assa-Munt, N., Ebright, R. H., and Gourse, R. L. (1996) *Genes Dev.* 10, 16–26
14. Nakabeppu, Y., Miyata, T., Kondo, H., Iwanaga, S., and Sekiguchi, M. (1984) *J. Biol. Chem.* 259, 13730–13736
15. Saget, B. M., Shevell, D. E., and Walker, G. C. (1995) *J. Bacteriol.* 177, 1268–1274
16. Furuichi, M., Yu, C. G., Anai, M., Sakumi, K., and Sekiguchi, M. (1992) *Mol. Gen. Genet.* 236, 25–32
17. Saget, B., and Walker, G. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 9730–9734
18. Akiyama, K., Sakumi, K., Yoshikai, T., Anai, M., and Sekiguchi, M. (1990) *J. Mol. Biol.* 216, 291–273
19. Sakumi, K., and Sekiguchi, M. (1989) *J. Mol. Biol.* 205, 373–385
20. Shevell, D., and Walker, G. C. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 9001–9005
21. Volkert, M. R., Gately, F. H., and Hajec, L. I. (1989) *Mutat. Res.* 217, 109–115
22. Attey, A., Belyaeva, T., Savery, N., Hoggett, J., Fujita, N., Ishihama, A., and Busby, S. (1994) *Nucleic Acids Res.* 22, 4375–4380
23. Busby, S., and Ebright, R. H. (1994) *Cell* 79, 743–746
24. Melanson, P., Burgess, R. R., and Record, M. T. (1982) *Biochemistry* 21, 4318–4331