Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 9730–9734, October 1994
Biochemistry

The Ada protein acts as both a positive and a negative modulator of *Escherichia coli*'s response to methylating agents

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Communicated by Boris Magasanik, May 23, 1994

ABSTRACT **The adaptive response of *Escherichia coli* protects the cells against the toxic and mutagenic effects of certain alkylating agents. The major effector molecule regulating this response is the 39-kDa Ada protein, which functions as both a DNA repair protein and a transcriptional activator. Ada removes methyl groups from phosphotriester and O^6-methylguanine lesions in DNA, irreversibly transferring them to cysteine residues at positions 69 and 321, respectively. When methylated at Cys-69, Ada is converted into a potent activator of *ada* and *alkA* transcription and binds to a sequence (Ada box) present in both promoters. We have found that physiologically relevant higher concentrations of unmethylated Ada are able to inhibit the activation of *ada* transcription by methylated Ada, both *in vitro* and *in vivo*. In contrast, the same concentrations of unmethylated Ada do not inhibit the activation of *alkA* transcription by methylated Ada, either *in vitro* or *in vivo*. Deletion of the carboxyl-terminal 67 amino acids of Ada abolished the ability of the unmethylated form of the protein to inhibit activation of *ada* transcription but not the ability of the methylated form to activate *ada* or *alkA* transcription. Our results suggest that the Ada protein plays a pivotal role in the negative modulation of its own synthesis and therefore in the down-regulation of the adaptive response. Elements present in the carboxyl terminus of Ada appear to be necessary for this negative regulatory function.**

The adaptive response of *Escherichia coli* is an inducible DNA repair system that helps prevent the mutagenic and cytotoxic effects caused by exposure to alkylating agents, particularly methylating agents (1–3). The Ada protein removes methyl groups from the premutagenic lesions O^6-methylguanine and O^4-methylthymine, transferring them irreversibly to Cys-321, a cysteine located in its carboxyl-terminal domain (4–6), and also removes methyl groups from the *S* diastereoisomers of DNA methylphosphotriesters, transferring them irreversibly to Cys-69, which is located in its amino-terminal domain (7–10). Methylation at Cys-69 converts Ada into a strong transcriptional activator of its own gene (*ada*) and also of several other genes (*alkB*, *alkA*, and *aidB*) (11–15). The *alkA* gene encodes a DNA glycosylase that removes 3-methyladenine and several other alkylated bases from DNA (16–18).

Transcription of the *ada* and *alkA* genes promoted by the methylated form of Ada involves the binding of the Ada protein to a sequence referred to as an "Ada box." In the case of the *ada* gene, the Ada box is located upstream of the −35 region of the promoter, while in the case of *alkA*, the Ada box overlaps the −35 region of the promoter (13, 17, 19). It has been shown that the *ada* and *alkA* genes are differentially regulated (20–22). Recently, Ada has been classified as a class I transcription factor due to its requirement for the carboxyl-terminal domain of the α subunit of RNA polymerase for transcription activation (23).

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Genetic studies have demonstrated that Ada derivatives lacking 10–20% of the carboxyl terminus promote high levels of expression of the *ada* gene in the absence of a methylating agent but their ability to activate *alkA* expression remains inducible by methylating agents just as with wild-type Ada (20). Moreover, the amino-terminal half of the Ada protein is capable of activating transcription at *alkA* in the presence of a methylating agent but is not sufficient to activate transcription at *ada* (20, 22, 24). When methylated at Cys-69, the amino-terminal half of Ada will bind to the Ada box of the *ada* gene but activation of transcription does not occur (22). These observations indicate that the activation of *ada* differs from the activation of *alkA* in requiring elements in the carboxyl-terminal half of Ada and that the carboxyl-terminal 10–20% of the protein plays an important negative role in Ada's regulatory functions.

The mechanism by which the adaptive response shuts off has remained in question. Since the methylation of Cys-69 is apparently irreversible, it has been postulated that methylated Ada is simply diluted out during cell growth subsequent to removal of alkylated DNA damage. An alternative hypothesis is that the Ada protein can be inactivated *in vivo* by proteolytic cleavage at its Lys-178–Gln-179 bond (4, 5, 25–28). However this hypothesis has received no support since *in vivo* cleavage of Ada has not been detected and observed *in vitro* cleavage is apparently due to the OmpT gene product, an outer membrane protease (6, 29).

In this paper, we report *in vitro* and *in vivo* evidence suggesting that, once all repairable methylphosphotriester lesions have been repaired, the unmethylated Ada that accumulates plays a role in shutting off the adaptive response. Furthermore, we suggest that the carboxyl-terminal 10–20% of Ada is important for this particular negative regulatory role.

MATERIALS AND METHODS

Protein Purification. Purification of wild-type Ada, Ada-287 (containing the first 287 amino acids), and Ada-178 (containing the first 178 amino acids) proteins was performed using a modification of the procedure by Akimaru *et al.* (22). Briefly, GW7105 cells containing the Ada gene or its derivatives under the control of the *lac* promoter (20) were cultured at 37°C in 500 ml of LB broth with appropriate antibiotic selection to an OD_{600} = 0.3. After addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 1 mM, the culture was incubated for an additional 2–4 hr. The cells were harvested, lysed, treated with Polymin P to 0.5%, and precipitated by the addition of ammonium sulfate to 52% saturation. The precipitate was dissolved in 0.5 ml of buffer A/150 mM NaCl, spun in a Microfuge, filtered, and loaded onto a Superose-75 column (Pharmacia). Eluted fractions containing Ada protein were pooled and loaded onto a Mono S ion-exchange column (Pharmacia) and eluted with a linear gradient from 75 mM to 500 mM NaCl in buffer A. Peak

Abbreviations: IPTG, isopropyl β-D-thiogalactoside; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

activity was determined using a methyltransferase assay as described by Akimaru *et al.* (22). Methylated *Micrococcus luteus* DNA substrate utilized for the methyltransferase assay was prepared using the procedure described by Nakabeppu and Sekiguchi (12).

***In Vitro* Transcription.** Methylation of proteins and *in vitro* transcription experiments were performed exactly as described by Sakumi and Sekiguchi (19). Template DNAs used for *in vitro* transcription experiments were purified using a Qiaex (Qiagen, Chatsworth, CA) gel extraction kit as follows: a 175-bp *Eco*RI/*Hin*dIII *ada'* promoter from pGW2609 (26), a 265-bp *Mlu* I/*Acc* I *alkA'* promoter from pBK161 (30), and a 203-bp *Eco*RI *lacUV5'* promoter from p0032 (31). PhosphorImager scanning was performed using a Molecular Dynamics PhosphorImager scanner.

RESULTS

Unmethylated Ada Protein Can Activate Transcription at the *ada* Promoter *In Vitro* but Inhibits *ada* Transcription at Higher Concentrations. Previous analyses of transcriptional activation at the *ada* promoter *in vitro* had led to the conclusion that only the methylated form of the Ada protein was capable of activating transcription at *ada* (12, 19, 22). However, these studies were performed using relatively low concentrations of unmethylated Ada (up to 6 pmol in a standard reaction volume of 35 μl). Assuming an intracellular volume of 0.64 μm^3 (32), the highest concentration of Ada tested in these previous experiments corresponds to ≈60 molecules of unmethylated Ada per cell. Since an *E. coli* cell exposed to a methylating agent will accumulate up to 3000 Ada molecules per cell (33, 34), the levels tested *in vitro* correspond to the lower end of the concentration range that is physiologically relevant. We found that 20–50 pmol of unmethylated Ada protein per reaction clearly activated transcription at *ada* (Fig. 1). Furthermore, above 50 pmol of unmethylated Ada protein per reaction, there was an unexpected inhibition of *ada* transcription. The *lacUV5* transcript, used as our control, was unaffected by increasing the amounts of unmethylated Ada in the reaction.

Before examining the effect of higher concentrations of methylated Ada on *ada* transcription, we tried a variety of strategies for purifying the methylated form of Ada. However, since the methylated protein strongly aggregated, we instead generated methylated Ada *in situ* (19) by treatment of small quantities of Ada with methylnitrosourea-methylated *M. luteus* DNA prior to using it in the *in vitro* transcription assays. Once Ada had been methylated, strong activation of *ada* transcription was observed with as little as 1 pmol of methylated Ada protein per reaction as previously reported (Fig. 1). The higher levels of "competitor" *M. luteus* DNA resulted in a decrease in the levels of both *ada* and *lacUV5* transcription at the highest concentrations of methylated Ada tested. However, in contrast to unmethylated Ada, there was only a modest inhibition of *ada* transcription relative to *lacUV5* transcription as methylated Ada was increased to 200 pmol per reaction.

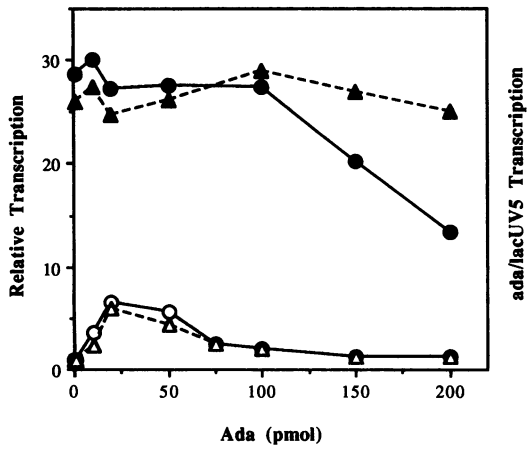

FIG. 1. *In vitro* transcription at the *ada* promoter by unmethylated and methylated Ada. *In vitro* transcription at the *ada* promoter was performed as described in the text. The transcript run-off reaction contained 0.3 pmol of template DNA, 3 pmol of RNA polymerase, and 0–200 pmol of unmethylated or methylated Ada protein. PhosphorImager analyses were performed on the autoradiogram for quantification of the transcripts. ○, Unmethylated Ada-promoted *ada* transcription; △, ratio of *ada*/*lacUV5* transcription; ●, methylated Ada-promoted *ada* transcription; ▲, ratio of *ada*/*lacUV5*.

Activation of Transcription by Methylated Ada at the *ada* Promoter Is Inhibited by Unmethylated Ada. We directly examined whether higher concentrations of unmethylated Ada protein would inhibit transcriptional activation at the *ada* promoter mediated by methylated Ada protein. We performed an *in vitro* transcription competition experiment using a constant amount (1 pmol per reaction) of methylated Ada protein while increasing the level of unmethylated Ada. This level of methylated Ada protein strongly activates transcription at *ada* (Figs. 1 and 2). As shown in Fig. 2, the presence of >20 pmol of unmethylated Ada per reaction inhibited *ada* transcription activated by 1 pmol of methylated Ada with transcription being mostly inhibited by 100 pmol of unmethylated Ada per reaction. Thus, concentrations of unmethylated Ada corresponding to >200 molecules per cell (20 pmol per reaction) inhibited *ada* transcription promoted by a concentration of methylated Ada (1 pmol per reaction) corresponding to ≈10 molecules per cell. This observation raised the possibility that unmethylated Ada might play a key role in inhibiting transcription of the *ada* gene *in vivo* and that this inhibition may be a mechanism(s) for down-regulation of the adaptive response after completion of DNA damage repair.

Unmethylated Ada Inhibits *ada* Transcription *In Vivo* Promoted by Methylated Ada. To explore the physiological relevance of this hypothesis, we took advantage of a strain

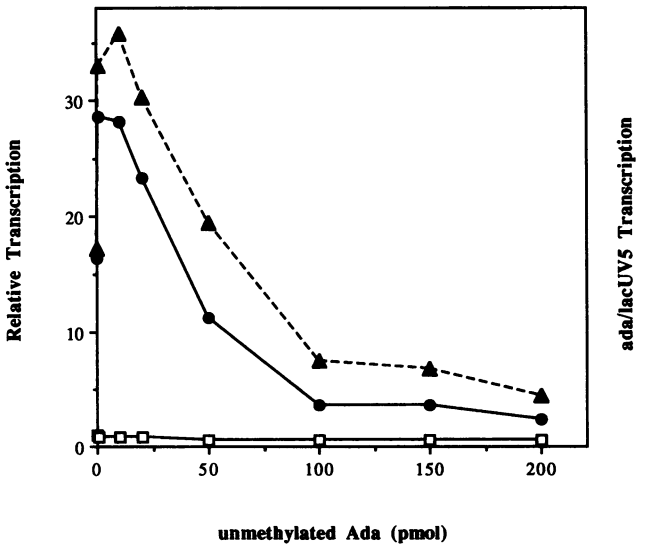

FIG. 2. Inhibition of methylated Ada-promoted *ada* transcription by unmethylated Ada protein. *In vitro* transcription at the *ada* promoter was performed as described in the legend to Fig. 1 (see text). The transcript run-off reaction contained 0.3 pmol of template DNA, 3 pmol of RNA polymerase, 1 pmol of methylated Ada, and 0–200 pmol of unmethylated Ada protein. ●, *ada* transcript; □, *lacUV5* transcript; ▲, ratio of *ada*/*lacUV5* transcription.

that contains a plasmid carrying the *ada* gene under the control of the *lac* promoter and in which the chromosomal *ada* gene has been replaced with an *ada–lacZ* transcriptional fusion (20). In this strain, the levels of the Ada protein can be controlled by varying the IPTG concentration and are therefore not affected by exposure to methylating agents. The chromosomal *ada–lacZ* transcriptional fusion was used to monitor transcription at the *ada* promoter.

The strain was grown in the presence of different concentrations of IPTG to achieve different intracellular levels of Ada protein. We then exposed the cells to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) at a dose of 1 μg/ml and assayed for β-galactosidase 1 hr later. This low dose of MNNG causes some induction of the adaptive response in a wild-type cell but does not saturate an induced wild-type cell's capacity to remove methyl groups from its DNA (3). All cells grown in the presence of very low levels of IPTG, 0–50 μM, exhibited increased levels of transcription from the *ada* promoter after treatment with 1 μg of MNNG per ml as compared to cells not treated with MNNG (Fig. 3*A*). However, the extent of β-galactosidase induction by MNNG treatment displayed an interesting dependence on IPTG concentration. Up to 20 μM, increasing concentrations of IPTG in the growth medium resulted in increased *ada–lacZ* expression after exposure to 1 μg of MNNG per ml. In contrast, slightly higher concentrations of IPTG in the growth medium resulted in decreased *ada–lacZ* expression after exposure to 1 μg of MNNG per ml. The low dose of MNNG used in these experiments should permit only a limited number of Ada molecules to become methylated. Thus, once sufficient IPTG has been added to induce enough Ada molecules to repair all the phosphotriesters induced by 1 μg of MNNG per ml, any further increases in IPTG concentration will result in the synthesis of more Ada molecules than can be methylated. We suggest that, at concentrations of IPTG of 30 μM and higher, unmethylated Ada molecules are inhibiting activation of transcription at the *ada* promoter by methylated Ada molecules just as they do *in vitro*.

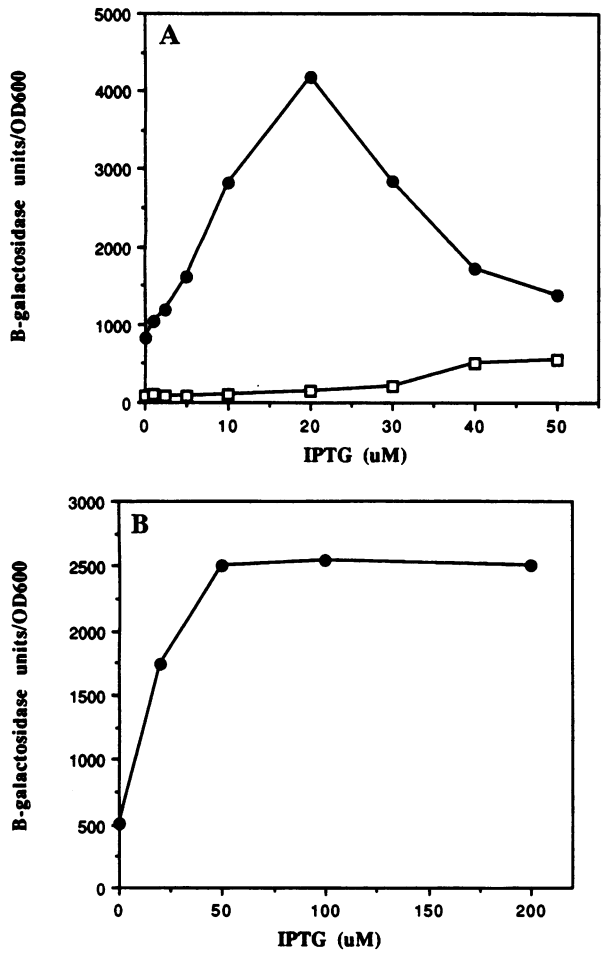

FIG. 3. Regulation of *ada* transcription *in vivo* by increasing cellular concentrations of Ada. β-Galactosidase assays were performed exactly as described (20). Briefly, GW7105 (pDS411) cells were grown in M9 minimal medium (35) to an OD_{600} of 0.2–0.3. Various concentrations of IPTG were added for 1 hr; cells were then grown for another hour with or without the addition of MNNG. GW7105 (pDS411) is an *ada*-null mutant of a Δ*lacU169 pro*$^+$ derivative of AB1157 (36) with an *ada–lacZ* operon fusion, an F'[*proA*$^+$*B*$^+$ *lacI*^{q}*lacZ* Δ*M15* Tn*10* (Tc^R)], and a pBR322 derivative containing the *ada* gene under the regulatory control of the *lac* promoter. All strains are *E. coli* K-12. (*A*) ●, Addition of 1 μg of MNNG per ml; □, without MNNG. (*B*) ●, Addition of 5 μg of MNNG per ml.

Higher Concentrations of Unmethylated Ada Do Not Inhibit Transcription of *alkA in Vitro* or *in Vivo*. *In vitro* transcription experiments (Fig. 4*A*) indicated that unmethylated Ada could activate *alkA* transcription when present at sufficiently high concentrations, but higher levels of unmethylated Ada did not inhibit *alkA* transcription. An *in vitro* transcription competition experiment analogous to that described above revealed that higher concentrations of unmethylated Ada do not inhibit methylated Ada protein-promoted *alkA* transcription (Fig. 4*B*).

We then carried out *in vivo* experiments analogous to those described above (Fig. 3*A*) except that, in this case, the expression of an *alkA–lacZ* fusion was monitored instead of an *ada–lacZ* fusion (the chromosomal copy of the *ada* gene was inactivated) (Fig. 5). Growing these cells in increasing concentrations of IPTG in the absence of a methylating agent led to increased levels of expression of the *alkA–lacZ* fusion with maximal expression being observed by a level of 50 μM IPTG as reported previously (20). Treatment of the cells with a dose of 1 μg of MNNG per ml further induced the expression of the *alkA–lacZ* fusion. As in the case of the *ada–lacZ* fusion, the maximal induction induced by 1 μg of MNNG per ml occurred with cells grown in the presence of 20 μM IPTG. However, in this case, growth of the cells in higher concentrations of IPTG resulted in approximately the same levels of MNNG-induced β-galactosidase expression rather than the decrease seen with the *ada–lacZ* fusion (Fig. 3*A*).

Unmethylated Ada-287 Does Not Inhibit Transcription of the *ada* Promoter. In light of our observation that higher concentrations of unmethylated Ada protein inhibited transcription at the *ada* promoter, we wondered whether Ada derivatives lacking 10–20% of the carboxyl-terminal end are constitutive activators of *ada* transcription, at least in part, because they have lost their ability to cause this inhibition. Ada-287 contains the first 287 amino acids of the protein and therefore lacks O^6-methylguanine methyltransferase activity but still possesses phosphotriester methyltransferase activity (data not shown). *In vitro* transcription experiments (Fig. 6*A*) demonstrated that unmethylated Ada-287 is a considerably stronger activator of *ada* transcription *in vitro* than wild-type unmethylated Ada but is not as effective an activator as methylated Ada (Fig. 1). At higher levels of protein (up to 200 pmol per reaction), Ada-287 did not display the pronounced inhibition observed with higher levels of unmethylated Ada. When methylated, Ada-287

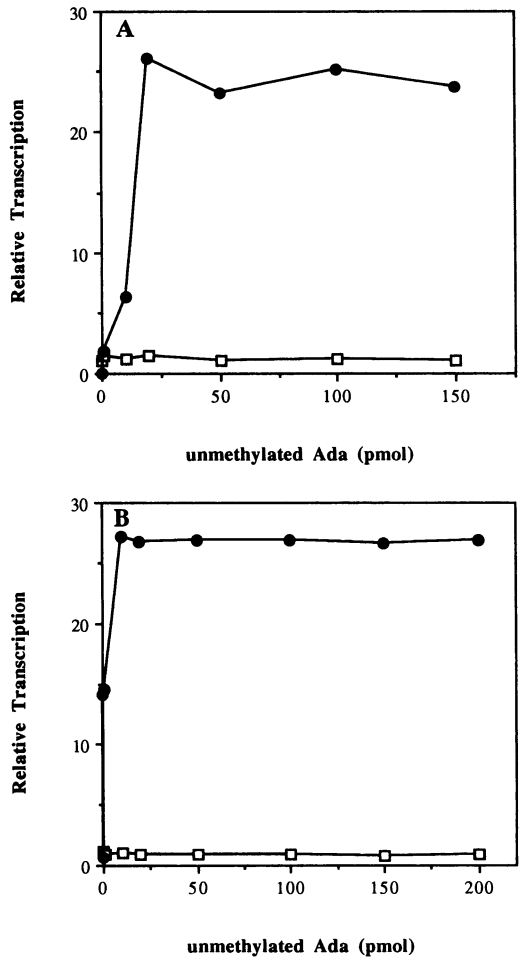

FIG. 4. *In vitro* transcription at the *alkA* promoter. Reaction conditions for *in vitro* transcription at the *alkA* promoter were as described in the legend to Fig. 1 (see text). ●, *alkA*; □, *lacUV5*. (*A*) Unmethylated Ada-promoted *alkA* transcription (experiment is analogous to that in Fig. 1*A*). (*B*) Increasing concentrations of unmethylated Ada do not inhibit *alkA* transcription promoted by 1 pmol of methylated Ada (experiment is analogous to that in Fig. 2).

behaved essentially like methylated wild-type Ada (data not shown). These results suggest that the elements of the Ada protein responsible for the inhibition of *ada* transcription seen with unmethylated Ada reside, at least in part, in the 67 carboxyl-terminal amino acids that have been deleted in Ada-287.

We compared the *in vivo* action of Ada-287 to wild-type Ada protein using the same *in vivo* system as described above and found that they were consistent with the results observed in our *in vitro* experiments. In the absence of MNNG, Ada-287 was a more effective activator of *ada* (Fig. 6*B*) than was wild-type unmethylated Ada (Fig. 3*A*), but it became a more effective activator of *ada* transcription after the cells were treated with 1 μg of MNNG per ml. However, in contrast to the situation with wild-type Ada, cells grown at IPTG concentrations of >20 μM and then treated with 1 μg of MNNG per ml expressed the *ada–lacZ* fusion at higher levels rather than lower levels.

We also tested the ability of the Ada-287 protein to activate transcription at the *alkA* promoter *in vitro* and found its behavior to be indistinguishable from that of the wild-type protein (data not shown). These results were consistent with our earlier observations of the *in vivo* properties of Ada-287 (20) and indicate that these 67 carboxyl-terminal amino acids of Ada do not play a role in the regulation of *alkA* transcription.

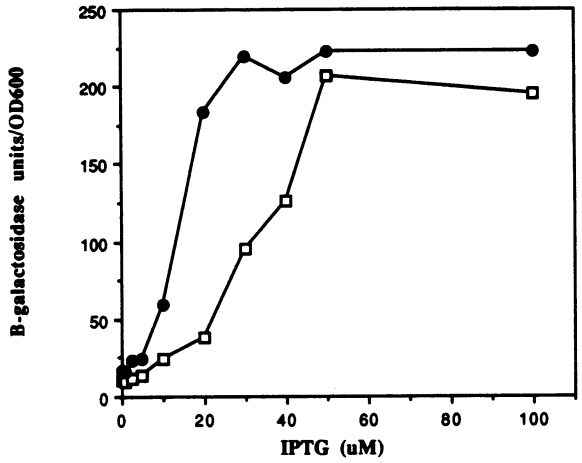

FIG. 5. Effects of *alkA* induction by increasing cellular concentrations of Ada. β-Galactosidase assays were performed on strain GW7106 (pDS411) exactly as described in the legend to Fig. 3. GW7106, a *Δada-25 ΔlacU169 pro*$^+$ derivative of MV1902, also contains an F′[*proA*$^+$*B*$^+$ *lacI*^{q} *lacZ* Tn*5* (Kan^R)]. MV1902 is a *alkA104* λpSG1 *rfa-550* derivative of AB1157 (37). ●, Addition of 1 μg of MNNG per ml; □, without MNNG.

DISCUSSION

Uninduced *E. coli* contain very low levels of Ada protein (≈1 molecule per cell) (33) but, after exposure to a methylating agent, they accumulate up to 3000 molecules of Ada per cell. Previous *in vitro* analyses of the roles of methylated Ada in transcriptional activation have utilized concentrations of Ada protein (corresponding to ≈10–60 molecules per cell) that are at the lower end of the concentration range that is physiologically relevant (12, 19, 22). The experiments presented here show that concentrations of unmethylated Ada corresponding to ≈200–500 molecules per cell activate *ada* transcription *in vitro*, but at higher concentrations, *ada* transcription is unexpectedly inhibited. We also found that methylated Ada, a more potent activator of *ada* transcription, does not exhibit this inhibition. Furthermore, in *in vitro* experiments we found that concentrations of unmethylated Ada corresponding to >200 molecules per cell strongly inhibited *ada* transcription promoted by levels of methylated Ada corresponding to ≈10 molecules per cell. To test the physiological relevance of these results, we utilized a system that enabled us to alter the ratio of unmethylated Ada to methylated Ada molecules in a living cell and examined the effects of this on transcription at the *ada* promoter. The results of these studies indicated that unmethylated Ada could inhibit the ability of methylated Ada to activate transcription at the *ada* promoter *in vivo* as well as *in vitro*. Furthermore, no inhibition was demonstrated at the *alkA* promoter by higher concentrations of unmethylated Ada *in vitro* or *in vivo*.

Collectively, these *in vitro* and *in vivo* observations support our conclusion that concentrations of unmethylated Ada within the physiologically relevant concentration range can inhibit transcription at the *ada* promoter activated by methylated Ada protein. We suggest that this phenomenon could play an important role in regulating the shutoff of the adaptive response once the damage has been removed from a cell that has been previously exposed to a methylating agent. When all of the repairable methylphosphotriester lesions have been repaired via methyltransfer to Ada, methylated Ada molecules will continue to activate transcription at *ada*, generating higher levels of unmethylated Ada. The pools of unmethylated Ada molecules per cell will then increase but the concentration of methylated Ada will not. As a result, on the basis of our *in vitro* and *in vivo* observations, it appears that sufficient levels of unmethylated Ada will inhibit methylated

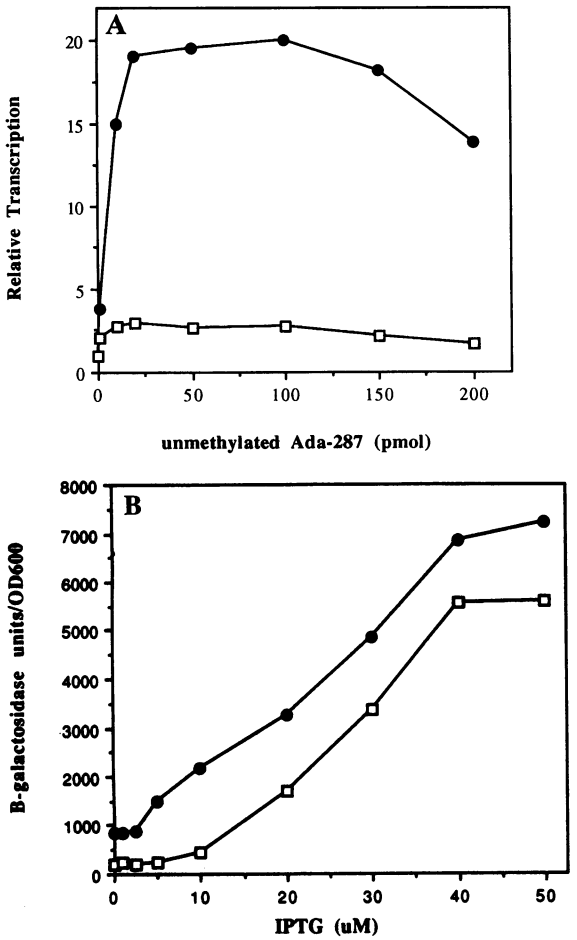

FIG. 6. Effects of Ada-287 on *ada* transcription *in vitro* and *in vivo*. (*A*) Reaction conditions for *in vitro* transcription at the *ada* promoter were performed as described in the legend to Fig. 1 (see text) except that 0–200 pmol of Ada-287 was used as the activator. ●, *ada*; □, *lacUV5*. (*B*) β-Galactosidase assays were performed on strain GW7105 (pDS418) (20) exactly as described in the legend to Fig. 3. ●, Addition of 1 μg of MNNG per ml; □, without MNNG.

Ada-promoted transcription resulting in a decrease in *ada* transcription.

The fact that approximately the same concentration of unmethylated Ada is required to activate maximally both *ada* (Fig. 1) and *alkA* (Fig. 4*A*) suggests that unmethylated Ada binds approximately equally to the *ada* and *alkA* Ada boxes despite the small differences in sequence between the two boxes. A more complicated mechanism of inhibition is also suggested by the fact that higher concentrations of unmethylated Ada inhibit the transcription at *ada* that is promoted by lower concentrations of unmethylated Ada (Fig. 1).

The inhibition at the *ada* promoter could be explained by mechanisms that involve the interaction of one or more unmethylated Ada molecules with a methylated Ada molecule. If the formation of a complex between unmethylated Ada and methylated Ada is important for transcription inhibition, the fact that it occurs at the *ada* promoter but not at the *alkA* promoter could be due to (*i*) the different spacing of the Ada boxes relative to their promoters and the apparently different mechanisms used to activate the two promoters (20), (*ii*) some additional DNA sequence element that is present near the *ada* promoter but not present near the *alkA* promoter, or (*iii*) the differences in the sequences of the Ada boxes at the two promoters. The fact that apparently the same inhibition phenomenon can be observed not only *in vivo* but also *in vitro* using linear templates and RNA polymerase suggests that the mechanism of inhibition does not require a supercoiled template or any accessory proteins. Whatever the mechanism, it appears that it will involve elements located within the carboxyl-terminal 67 amino acids of Ada. This is an extremely interesting portion of the molecule since the recent determination of the crystal structure of the carboxyl-terminal half of the Ada protein has led to the proposal that the mechanism of methyl removal from O^6-methylguanine, which involves a transfer of the methyl group to cysteine 321 (38), involves a movement about residues 325–335 of Ada that would reposition the carboxyl-terminal helix (residues 340–350).

We thank the members of the Walker laboratory for their helpful discussions and Alan Grossman for both technical suggestions and perspectives. This work was supported by Grant 5-P01-ES03926 from the National Institutes of Environmental Health Sciences.

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