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Regulatory mechanisms for induction of synthesis of repair enzymes in response to alkylating agents: Ada protein acts as a transcriptional regulator

(adaptive response/*Escherichia coli*/*ada* gene/*alkA* gene)

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ABSTRACT **Expression of the *ada* and *alkA* genes, both of which are involved in the adaptive response of *Escherichia coli* to alkylating agents, is positively controlled by Ada protein, the product of the *ada* gene. Large amounts of *ada*- and *alkA*-specific RNA were formed in cells treated with a methylating agent, whereas little such RNA was produced in untreated cells. The *in vivo* transcription-initiation sites for the two genes were determined by primer-extension cDNA synthesis. In an *in vitro* reconstituted system, both *ada* and *alkA* transcripts were formed in an Ada protein-dependent manner. However, responses of the two transcription reactions to methylating agents differed; *ada* transcription was stimulated by methylnitrosourea, while *alkA* transcription was suppressed. We prepared a methylated form of Ada protein by an *in vitro* reaction and compared the activity with that of the normal, unmethylated form. The methylated form was more effective in promoting *ada* transcription than was the unmethylated form, but the effects of both forms were much the same with regard to *alkA* transcription. Based on these findings, we propose a model for the molecular mechanism of adaptive response.**

Escherichia coli cells exposed to a low concentration of a methylating agent, such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MeNNG) or methyl methanesulfonate (MeMes), show a markedly increased resistance to both the mutagenic and the killing effects of the same or other alkylating agents (1, 2). This response is known as the adaptive response to alkylating agents and is related to the induced synthesis of at least two enzymes, O^6-methylguanine-DNA methyltransferase and 3-methyladenine-DNA glycosylase II (3–7). The former enzyme repairs O^6-methylguanine, O^4-methylthymine, and methylphosphotriester by direct transfer of the methyl group to one of the cysteine residues of the enzyme (4, 5, 8, 9), whereas the latter excises 3-methyladenine, 3-methylguanine, and 7-methylguanine from the alkylated DNA (6, 7).

Studies with mutants have revealed that at least two genes of *E. coli*, *alkA* and *ada*, are involved in the adaptive response (6, 10, 11). We and others have shown that *alkA* is the structural gene for 3-methyladenine-DNA glycosylase II (12–14) and that *ada* encodes O^6-methylguanine-DNA methyltransferase (8, 15, 16). In addition, there is evidence that the expression of both *alkA* and *ada* genes is controlled by the *ada* gene per se (11, 13, 17–19). Since the adaptive response takes place in ada^+, but not ada^-, cells that have been treated with methylating agents (6, 11, 13, 17–19), it seemed likely that Ada protein, which itself is a methyltransferase and accepts the methyl group, would be a transcriptional regulator.

To elucidate the role of the Ada protein in regulation of expression of the two inducible genes, we performed *in vivo* and *in vitro* transcription experiments. Using a reconstituted system with purified components, we obtained evidence that the Ada protein indeed functions as a transcriptional activator, for both the *alkA* and *ada* genes.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Phages. *E. coli* strains YN3 (16, 19) and DH1 (20) are derivatives of strain K-12. Recombinant plasmids pYN1000 (12) and pYM3 (19), which carry the *alkA* gene and the *ada'-lacZ'* fused gene, respectively, are pBR322 derivatives. The plasmid pYN3059 used for overproduction of Ada protein was as described (16). The following plasmids are pUC9 derivatives (21) and were used for the preparation of truncated DNA templates carrying a specific promoter: pYN3066, which carries a 176-base-pair (bp) *Hin*dIII–*Eco*RI fragment containing the *ada* promoter region (16); pYN3072, which carries a 844-bp *Eco*RI–*Hin*cII fragment containing the *alkA* promoter (13); and pYN3077, which carries three copies of a 205-bp *Eco*RI fragment containing the *lacUV5* promoter and which was derived from pKB252 (22). M13 phages mYN9 and mYN11, which carry the noncoding strand of a 176-bp *Hin*dIII–*Eco*RI fragment of the *ada* gene and the noncoding strand of a 2-kbp *Eco*RI fragment containing the *alkA* gene (12, 13), respectively, are mWB2342 derivatives (23).

Enzymes. *E. coli* RNA polymerase holoenzyme, ribonuclease inhibitor (from human placenta) and avian myeloblastosis virus (AMV) reverse transcriptase were obtained from Pharmacia, Wako Pure Chemical Industries, and Life Sciences (St. Petersburg, FL), respectively. Restriction endonucleases and T4 polynucleotide kinase were obtained from Takara Shuzo and Nippon Gene.

DNA and Synthetic Oligonucleotides. Truncated DNA templates were prepared as follows. Plasmid DNAs were digested with appropriate restriction endonucleases. DNA fragments were separated by electrophoresis in a 6% polyacrylamide gel and purified (24). The 27-mer and 30-mer oligodeoxynucleotides were synthesized by an automated DNA synthesizer (Applied Bio Systems, model 380A).

Methylated DNA was prepared as follows (25). A reaction mixture containing 18 mM Amediol buffer (pH 10), 20 mg of *Micrococcus luteus* DNA (Sigma), 9% dimethyl sulfoxide, and 90 mM *N*-methyl-*N*-nitrosourea (MeNU) was incubated for 30 min at 37°C. After ethanol precipitation, the DNA was dissolved in 10 ml of 50 mM Tris·HCl (pH 7.6), dialyzed against four changes of 2 liters of the same buffer at 4°C for 24 hr, and stored at −80°C.

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Abbreviations: MeNU, *N*-methyl-*N*-nitrosourea; MeNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; MeMes, methyl methanesulfonate; bp, base pair(s).

RNA Preparation. Total cellular RNA was prepared by the hot phenol procedure (26) and purified by centrifugation with a cushion of 5.7 M CsCl to remove traces of DNA (24).

Primer-Extension cDNA Synthesis. The 5′ ends of synthetic oligodeoxynucleotide primers were phosphorylated with T4 polynucleotide kinase and [γ-^{32}P]ATP (5000 Ci/mmol; 1 Ci = 37 GBq). Hybridization and cDNA synthesis were carried out as described by Treisman *et al.* (27).

***In Vitro* Transcription.** *In vitro* transcription was carried out essentially as described by Kajitani and Ishihama (28).

Preparation of Normal and Methylated Forms of Ada Protein. Ada protein was purified from YN3 cells harboring pYN3059, as described (16). To obtain a methylated form of Ada protein, a crude extract from YN3 cells harboring pYN3059 was prepared in 70 mM Hepes/KOH, pH 8.0/10 mM dithiothreitol/1 mM EDTA/5% (vol/vol) glycerol. MeNU-treated DNA (3.5 mg) was added to 5 ml of the crude extract, which contained ≈130 nmol of Ada methyltransferase, and the mixture was incubated at 37°C for 30 min. In this reaction, 98% of the methyltransferase activity in the crude extract was inactivated, indicating that most of the Ada protein had been methylated. A parallel experiment with labeled DNA revealed that the Ada protein was singly methylated. The reaction mixture was subjected to 0.5% Polymin P and the methylated Ada protein was purified as described (16). The purified preparations of the either normal or methylated forms were stored in 10 mM Tris·HCl, pH 8.5/0.1 M $(NH_4)_2SO_4$/0.5 mM EDTA/0.5 mM 2-mercaptoethanol/50% glycerol at −20°C. The concentration of Ada protein was determined spectrophotometrically, and the amounts were calculated using $\varepsilon_{280} = 3.8 \times 10^4\ M^{-1}\ cm^{-1}$.

RESULTS

***In Vivo* Transcription of *alkA* and *ada* Genes.** To specify the promoter regions of *alkA* and *ada* genes, we determined the transcription-initiation sites for the two genes by primer-extension cDNA synthesis. Synthetic oligodeoxynucleotide primers, a 27-mer corresponding to the *alkA* coding strand (from the 19th to the 27th codon) and a 30-mer corresponding to the *ada* coding strand (from the 4th to the 13th codon), were labeled at their 5′ ends and hybridized with RNAs extracted from untreated and adapted cells of YN3, of YN3 harboring pYN1000 (*alkA*), and of YN3 harboring pYM3 (*ada′-lacZ′*). After cDNA synthesis by avian myeloblastosis virus reverse transcriptase, the length of the cDNA products was determined by comparison with products of dideoxy sequence reactions using the same primers and single-stranded DNAs of mYN11 (*alkA*) and mYN9 (*ada*). The results are shown in Fig. 1.

A single band for *alkA* cDNA was present at the same position for adapted cells both of YN3 and of YN3 harboring *alkA*$^+$ plasmid (Fig. 1*a*). Since 5 times more RNA was applied to the slots for the YN3 sample as compared with the YN3(pYN1000) sample, the actual amount of RNA synthesized in the adapted cells carrying *alkA*$^+$ plasmids was about 20-fold that of the adapted cells without plasmid. The same band was also found in untreated cells harboring *alkA*$^+$ plasmid, although the intensity of the band for untreated cells was <10% of that for adapted cells. From these results we concluded that the transcription-initiation site for *alkA* is guanine, 19 bases upstream from the translation-initiation site (13), in either normal or adapted conditions, and that expression of the *alkA* gene is controlled at the transcriptional level.

Similarly, the transcription-initiation site for *ada* was determined to be adenine or thymine, 22 or 23 bases upstream from the translation-initiation site (16) (Fig. 1*B*). This result is consistent with the transcription-initiation site determined by nuclease S1 mapping (16). There was no band in samples from untreated cells of both YN3 and YN3 harboring pYM3

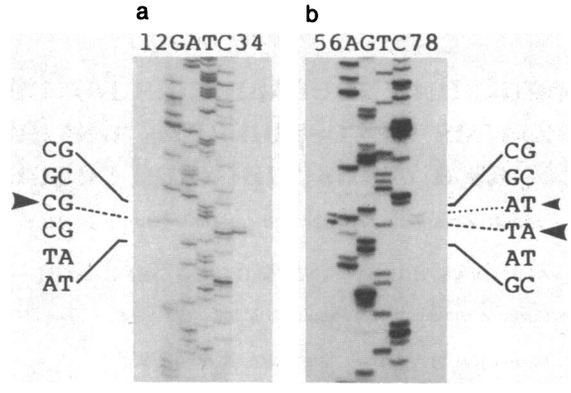

FIG. 1. Mapping of the 5′ ends of the *alkA* and *ada* mRNAs formed *in vivo*. (*a*) *alkA*. 5′-^{32}P-labeled primer (27-mer) was hybridized with 100 μg of RNA isolated from untreated (lane 1) or adapted (lane 2) YN3 cells or with 20 μg of RNA isolated from adapted (lane 3) or untreated (lane 4) cells of YN3 harboring the *alkA*$^+$ plasmid pYN1000. (*b*) *ada*. 5′-^{32}P-labeled primer (30-mer) was hybridized with 100 μg of RNA isolated from untreated (lane 5) or adapted (lane 6) YN3 cells or with 20 μg of RNA isolated from adapted (lane 7) or untreated (lane 8) cells of YN3 carrying the *ada′-lacZ′* construct pYM3. cDNA was synthesized and analyzed by electrophoresis in an 8% polyacrylamide gel with 8.3 M urea (27). Nucleotide sequencing ladders are shown in parallel lanes (G, A, T, and C); sequencing reactions were performed by the dideoxy method (23, 29) with the labeled primers and single-stranded DNA of mYN11 (*alkA*) and mYN9 (*ada*). Parts of the sequences deduced from the ladders are shown at left or right, along with sequence of each complementary strand. Transcription-initiation sites are shown by arrowheads.

(*ada′-lacZ′*). It seems that the amount of *ada* mRNA in untreated cells is too small to detect with this procedure, reflecting the fact that the enzyme activity in untreated cells of YN3 harboring pYM3 is ≈0.1% that of the adapted cells (19).

Fig. 2 shows the nucleotide sequences of the transcriptional control regions for the *alkA* and *ada* genes (13, 16). Promoter-like DNA sequences were found immediately upstream from the transcription initiation sites. GCGCAG (for the −35 region) and TATGCT (for the −10 region), separated by 15 bases, and TTGCGT (for the −35 region) and TAAAGG (for the −10 region), separated by 18 bases, may be functional promoters for *alkA* and *ada*, respectively. Although there is another promoter-like sequence in the *alkA* DNA (13), it is too far from the initiation site and probably is not functional.

Requirement of Ada Protein for *in Vitro* Transcription at *alkA* and *ada* Promoters. Having acquired accurate *in vivo* transcription-initiation sites for the two genes, we went on to do *in vitro* transcription experiments using a reconstituted system. A 265-bp *Mlu* I–*Acc* I fragment containing the *alkA* promoter and a 176-bp *Hin*dIII–*Eco*RI fragment containing the *ada* promoter region (Fig. 2) were used as DNA templates. As a control, a 205-bp *Eco*RI fragment containing the *lacUV5* promoter was also used (22, 28). To either one or a mixture of DNA templates, *E. coli* RNA polymerase holoenzyme was added, and the preparation was incubated at 37°C for 1 hr in the presence or absence of a purified preparation of Ada protein, in order to produce a transcription-initiation complex. Then a mixture of ATP, GTP, CTP and [α-^{32}P]UTP was added together with heparin, which prevents further initiation, and the reaction mixture was incubated at 37°C for 5 min. If accurate transcription does occur in this reconstituted system, 23-, 98-, and 63-base-long RNAs should be produced from the *alkA*, *ada*, and *lac* promoters, respectively.

When the products were analyzed by polyacrylamide gel electrophoresis, the result shown in Fig. 3 was obtained. The

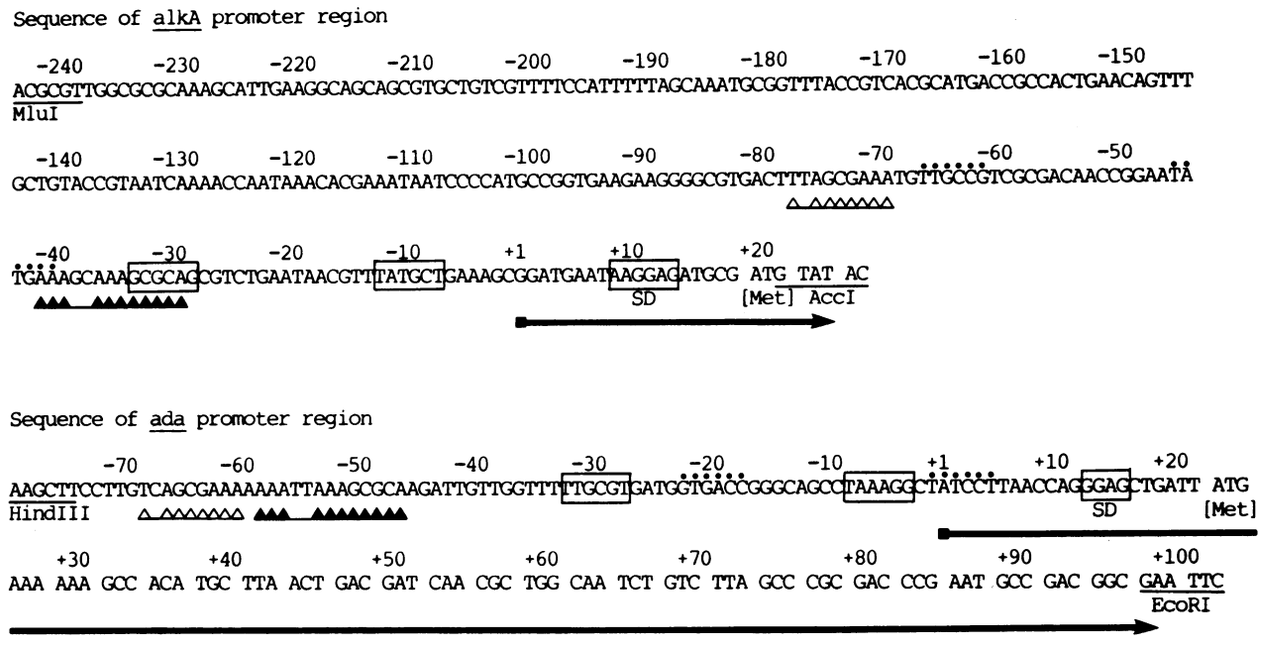

FIG. 2. Nucleotide sequences of the promoter regions of the *alkA* and *ada* genes. Nucleotide sequence of the noncoding strands of a 265-bp *Mlu* I–*Acc* I and a 176-bp *Hin*dIII–*Eco*RI fragment are given from 5′ to 3′. Nucleotides are numbered from the transcription-initiation sites (+1). Plausible −35 and −10 sequences for functional promoters and Shine–Dalgarno (SD) sequences (ribosome binding sites) are boxed. Initiation codons are shown by [Met]. Additional promoter-like sequences are dotted. Two conserved sequences between both promoter regions are shown by the open and closed triangles. Arrows indicate transcripts expected from the DNA fragments (23 bases long for *alkA* and 98 bases long for *ada*).

23-base *alkA* transcript and 98-base *ada* transcript were produced from the corresponding promoters when Ada protein was present in the reaction mixture. No band or only a faint band was found when the reaction was performed without Ada protein. In the case of *ada*, a 90-base RNA was formed in addition to the 98-base transcript. Formation of this 90-base RNA as well as of the control 63-base *lac* transcript was not significantly affected by the presence of Ada protein. These results clearly indicate that Ada protein acts on the *alkA* and *ada* promoters as a positive regulator to enhance transcription.

Effects of Alkylating Agents on *in Vitro* Transcription. Methylating agents, such as MeNNG, MeNU, and MeMes, effectively induce expression of the *ada* and *alkA* genes *in vivo* (13, 19, 30). Therefore, we examined the effects of MeNU on the formation of the three types of RNA in the reconstituted system. When increasing amounts of MeNU were added, formation of the 98-base *ada* transcript was preferentially enhanced while formation of the 23-base *alkA* and 63-base *lac* transcripts was suppressed (Fig. 4). The suppressing effects of MeNU may be due to inactivation by methylation of the components in the reaction mixture, thereby implying that the actual enhancement of *ada* transcription by MeNU is much higher than that observed. When a similar experiment was performed with ethylnitrosourea (0.25–10 mM), little enhancement was observed (data not shown), consistent with *in vivo* data (30). Enhancement of *alkA* transcription by methylating agents *in vivo* may be due to overproduction of Ada protein, as will be discussed below.

Promotion of *ada* Transcription by the Methylated Form of Ada Protein. The data described above suggest that transfer of a methyl group from DNA to Ada protein can activate Ada as a transcriptional regulator. To obtain support for this assumption, we prepared a methylated form of Ada protein and compared its transcription-promoting activity with that of normal, unmethylated Ada protein. These experiments were performed in the presence of a restricted amount (enzyme/DNA = 1:1) and an excess amount (enzyme:DNA = 10/1) of RNA polymerase (Fig. 5). Under either condition, the methylated form of Ada protein promoted the formation of 98-base *ada* RNA more efficiently than did the normal, unmethylated form of Ada protein. On the other hand, both forms of Ada protein were equally active in promoting transcription of *alkA*, when excess RNA polymerase was present in the reaction mixture (Fig. 5*b*). The methylated Ada protein did not promote *alkA* transcription in the presence of a restricted amount of RNA polymerase (Fig. 5*a*). Because the formation of *lac* RNA drastically decreased with increasing amounts of the methylated Ada protein, it seems that most of the active enzymes were associated with the *ada* promoter.

Comparison of the two autoradiograms (Fig. 5 *a* and *b*) further revealed that a 90-base *ada* RNA was formed more abundantly when a larger amount of RNA polymerase was used. In contrast to the 98-base *ada* RNA, formation of the 90-base RNA was not enhanced by either form of Ada protein but rather was suppressed when formation of the 98-base RNA was enhanced.

DISCUSSION

Expression of the *ada* and *alkA* genes, which are involved in adaptive response in *E. coli*, is positively regulated by Ada protein, which is encoded by *ada*. Large amounts of *ada*- and *alkA*-specific RNAs were produced in cells treated with a methylating agent, whereas small amounts of these RNAs were formed in normal cells. The transcription-initiation sites for the *ada* and *alkA* genes in adapted cells were determined by primer-extension cDNA synthesis, and the results are consistent with the data obtained by nuclease S1 mapping (16). However, no such determination was made with untreated cells, because the levels of *ada*- and *alkA*-specific RNAs in uninduced cells are too low to be detected by these procedures.

In *in vitro* transcription experiments, two types of RNAs were formed on the *ada* DNA template, one 98 bases long and

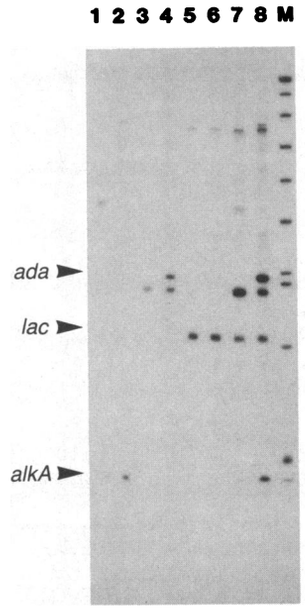

FIG. 3. Formation of *alkA*, *ada*, and *lac* transcripts in an *in vitro* reconstituted system. Either one or a mixture of DNA fragments (0.3 pmol each) containing *alkA*, *ada*, or *lacUV5* promoter was incubated with a 10-fold molar excess of *E. coli* RNA polymerase in the presence or absence of Ada protein. Reaction mixtures (35 μl final volume) also contained 50 mM Tris·HCl (pH 7.8), 3 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol, 50 mM NaCl, and 25 μg of nuclease-free bovine serum albumin per ml. Incubation was at 37°C for 60 min. Then 15 μl of a pre-warmed solution containing 50 μM [α-^{32}P]UTP (2 μCi per reaction); 160 μM each ATP, GTP, and CTP; and 200 μg of heparin per ml was added and the mixture was incubated at 37°C for 5 min. The samples were precipitated with ethanol, applied to an 8% polyacrylamide gel containing 8.3 M urea, and run at 65 W for 1.5 hr. Lanes: 1 and 2, *alkA* DNA without and with Ada protein (3 pmol); 3 and 4, *ada* DNA without and with Ada (3 pmol); 5 and 6, *lacUV5* DNA without and with Ada (3 pmol); 7 and 8, *alkA*, *ada*, and *lacUV5* DNA without and with Ada (9 pmol). Lane M: *Hap* II-digested pUC9 DNA for size markers (501/489, 404, 320, 242, 190, 147, 110, 101, 67, 34, and 26 bases, respectively). Note that RNA samples run slightly slower than DNA markers of the same length.

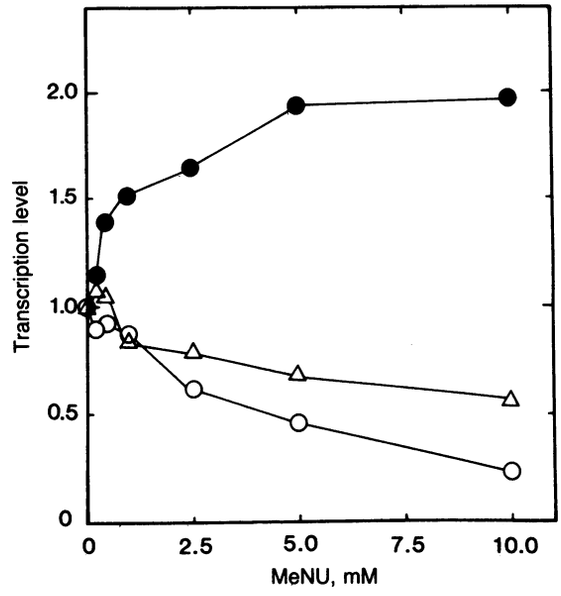

FIG. 4. Effects of MeNU on transcription of three types of DNA templates. Mixtures containing 0.3 pmol each of *alkA*, *ada*, and *lacUV5* fragments; 0.9 pmol of RNA polymerase holoenzyme (enzyme/DNA = 1:1) and 9 pmol of Ada protein were incubated for 60 min at 37°C in the presence of various concentrations of MeNU. *In vitro* transcription was initiated by simultaneous addition of substrates and heparin and was allowed to continue for 5 min at 37°C. RNA products were analyzed by 8% polyacrylamide gel electrophoresis. Autoradiograms were traced with a video densitometer (Bio-Rad model 620) and the levels of transcription for each template were plotted relative to the control reaction, without MeNU. ○, *alkA* RNA; ●, *ada* RNA; △, *lac* RNA.

the other 90 bases long. The length of the former corresponded well to that deduced from the *in vivo* initiation site, and the formation of this RNA was totally dependent on Ada protein. These observations indicate that the 98-base RNA represents the *ada* mRNA formed in the induced state *in vivo*. The *in vivo* initiation site for the latter RNA was not elucidated, though it is possible to assign a likely promoter for this transcription on the DNA sequence (see Fig. 2). The *in vitro* formation of the 90-base RNA was dependent on levels of RNA polymerase, but not on Ada protein. It is uncertain whether the 90-base RNA represents the RNA formed in the uninduced state *in vivo* or is merely an artifact of transcription *in vitro*.

Formation of the 98-base transcript was enhanced by the addition of MeNU to the reconstituted system, and a methylated form of Ada protein promoted formation of this RNA more efficiently than did an unmethylated form. No such effect of methylation was observed for *alkA* transcription. Recently, Lindahl and his associates have found that a methylated form of Ada protein acts on the *ada* promoter, thereby producing a large amount of Ada protein (T. Lindahl, personal communication).

Based on these findings, we propose a model for the adaptive response of *E. coli* to alkylating agents. (*i*) In normal cells, the *ada* gene and the *alkA* gene are expressed only weakly and only small amounts of Ada protein and AlkA protein are formed. (*ii*) When methylating agents attack the cells, the DNAs are rapidly methylated, and the O^6-methylguanine and methylphosphotriester formed are demethylated by the Ada protein. (*iii*) This repair reaction gives rise to the methylated form of Ada protein, which acts on the *ada* promoter to enhance transcription of the *ada* gene. (*iv*) Once the Ada protein is overproduced in this manner, the protein, in either methylated or unmethylated form, acts on both the *alkA* and *ada* promoters, thereby yielding a large amount of alkA protein (3-methyladenine-DNA glycosylase II) as well as of Ada protein (O^6-methylguanine-DNA methyltransferase). Thus, most of the mutagenic and lethal lesions of the DNA will be repaired by the actions of these two enzymes.

Increased resistance of cells to alkylating agents disappears when the treated cells are incubated for 2–3 hr after removal of the agents (1). This may be achieved by dilution of Ada protein by cell division or by inactivation of the protein. The Ada protein is cleaved by a specific cellular proteinase(s) (15, 16, 31), and this may be related to turn-off of the adaptive response.

It is of interest to learn how the Ada protein is activated by methylation. The methyl group of O^6-methylguanine in DNA is transferred to cysteine-321 of the Ada protein by its own methyltransferase activity (4, 31). We recently produced an altered form of Ada protein, in which this cysteine residue is replaced by alanine, by site-directed mutagenesis of the cloned *ada* gene (unpublished work). In cells carrying such a mutant *ada* gene, the Ada protein was overproduced without adaptive treatment (unpublished data). Thus, activation of the Ada protein seems to be induced by replacement of the cysteine residue by alanine as well as by formation of *S*-methylcysteine at the same site. This implies that such amino acid changes would cause a local alteration of conformational structure of the Ada protein so that the

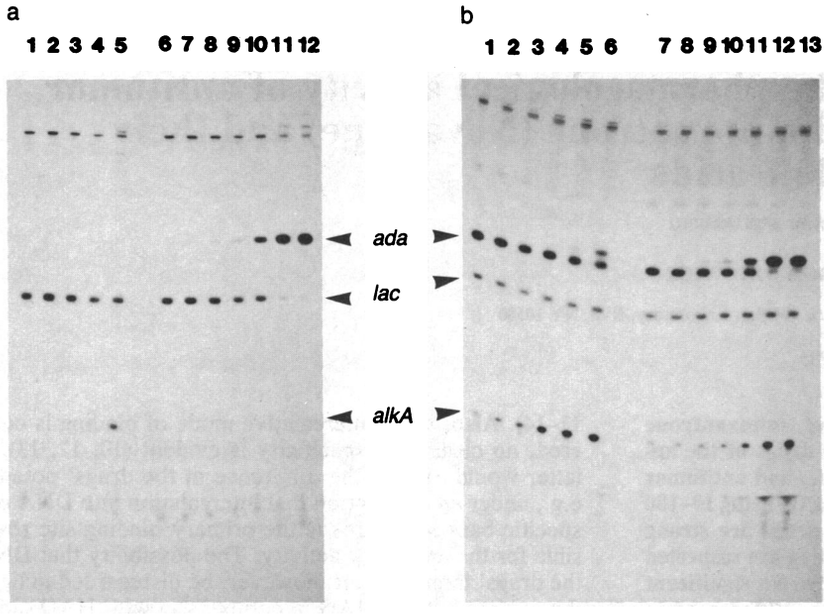

FIG. 5. Effects of the normal and methylated forms of Ada protein on transcription. The preincubation mixture contained 0.3 pmol each of *alkA*, *ada*, and *lacUV5* fragments; 0.9 or 9 pmol of RNA polymerase holoenzyme; and various amounts of the normal or methylated form of Ada protein. (*a*) In the presence of 0.9 pmol of RNA polymerase (enzyme/DNA = 1:1), normal Ada protein (0, 0.3, 0.9, 3.0, or 9.0 pmol, lanes 1–5, respectively) or methylated Ada protein (0, 0.09, 0.15, 0.3, 0.9, 3.0, or 9.0 pmol, lanes 6–12) was added. (*b*) In the presence of 9 pmol of RNA polymerase (enzyme/DNA = 10:1), normal Ada protein (0, 0.15, 0.3, 0.9, 3.0, or 9.0 pmol, lanes 1–6) or methylated Ada protein (0, 0.09, 0.15, 0.3, 0.9, 3.0, or 9.0 pmol, lanes 7–13) was added.

altered form of the protein would bind more readily to the *ada* promoter region or RNA polymerase.

To determine whether Ada protein interacts with the *ada* and *alkA* control regions of DNA, we performed foot-printing experiments by DNase I protection. We found that RNA polymerase could bind specifically to the promoters only in the presence of the Ada protein and that either RNA polymerase or Ada protein alone did not bind tightly to the promoters (unpublished result). Since the promoter sequences of the two genes deviate considerably from the consensus sequences for *E. coli* promoters (see Fig. 2) (32), it seems that Ada protein somehow functions in a manner so as to enhance RNA polymerase binding to such promoters. There are two common sequences in the control regions (see Fig. 2), and these sequences might play some role(s) in making an active complex for transcription.

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