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Alteration of the Carboxyl-Terminal Domain of Ada Protein Influences Its Inducibility, Specificity, and Strength as a Transcriptional Activator

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The *ada* gene of *Escherichia coli* K-12 encodes the regulatory protein for the adaptive response to alkylating agents. A set of plasmids carrying ordered deletions from the 3′ end of the *ada* gene were isolated and characterized. These *ada* deletions encode fusion proteins that derive their amino termini from *ada* and their carboxyl termini from the downstream vector sequence that occurs before an in-frame stop codon. Several of these *ada* deletions encode Ada derivatives that constitutively activate *ada* transcription to very high levels. A second class of *ada* deletions encode Ada derivatives that are dominant inhibitors of the inducible transcription of *ada* but are inducible activators of *alkA* transcription. In addition, we found that two Ada derivatives containing the same *ada* sequences but fused to different vector-derived tails have strikingly different properties. One Ada derivative constitutively activates both *ada* and *alkA* expression to very high levels. In contrast, the other Ada derivative is an inducible activator of *ada* expression, like the wild-type Ada protein, but is not an inducible activator of *alkA* transcription. Our data suggest that the carboxyl terminus of the Ada protein plays a key role in modulating the ability of the Ada protein to function as a transcriptional activator.

When *Escherichia coli* cells are exposed to low levels of methylating and ethylating agents, they acquire an induced resistance, termed the adaptive response, that protects them against the mutagenic and toxic effects of much higher doses of these alkylating agents (36). The product of the *ada* gene is a 39-kilodalton protein that plays three pivotal roles in the adaptive response (7, 21, 25, 37, 47). It not only encodes two different activities that repair alkylated DNA but also functions as a transcriptional activator of the *ada* gene itself, as well as of the *alkA*, *alkB*, and *aidB* genes (17, 27, 28, 43, 45).

The two repair activities of the Ada protein reside in two different domains. The domains can be separated either by cleavage of the Lys-178–Cys-179 bond which occurs in cell extracts (25, 42, 43) or by mild proteolysis of the purified Ada protein in vitro (38). One of the DNA repair activities of Ada is a methyltransferase that irreversibly transfers methyl groups from the mutagenic lesion O^6-methylguanine to the Cys-321 residue in the carboxyl-terminal domain of Ada (6, 7). The other activity is a methyltransferase that irreversibly transfers methyl groups from one of the stereoisomers of DNA-methylphosphotriesters to the Cys-69 residue in the amino-terminal domain of Ada (22, 23, 38, 48). After cleavage, each domain of Ada retains its respective DNA repair activity (38). Recent work has indicated that *E. coli* encodes a second methyltransferase protein of 19 kilodaltons that repairs O^6-methylguanine and O^4-methylthymine lesions in DNA (33, 34, 39). The last 93 residues of this recently identified methyltransferase show 49% homology with the last 94 residues of the carboxyl-terminal domain of Ada (33).

The mechanism by which Ada becomes activated is related to its capacity to repair alkylated DNA. In vitro studies have indicated that the methylated form of the Ada protein is much more efficient than the unmethylated Ada protein at binding to the *ada* promoter and activating transcription (29, 44). Teo et al. (44) have shown that methylation of the Cys-321 residue of Ada, which results from the repair of an O^6-methylguanine or O^4-methylthymine lesion, has no effect on the ability of Ada to promote *ada* transcription in vitro. However, methylation at the Cys-69 residue of Ada, which results from the repair of DNA methylphosphotriesters, converts the Ada protein into an efficient transcriptional activator.

The mechanism of transcriptional activation by Ada is particularly interesting because it differs substantially from that of other transcriptional activators that are involved in regulating the responses of *E. coli* to environmental change or stress. For example, Ada is a one-component system that both senses an environmental stimulus and functions as a transcriptional activator and, in this respect, differs from the numerous two-component systems that have been described (e.g., *ntrB*/*ntrC* and *envZ*/*ompR*), in which the sensing and activating functions are carried out by different molecules (30). Ada also differs from the catabolite activator protein (CAP). CAP interacts with cyclic AMP and then binds a specific DNA sequence in the promoter region to stimulate transcription (4), while the Ada protein is irreversibly converted to the active form by a covalent modification. There is no evidence at present to suggest that Ada functions as an alternative sigma subunit of RNA polymerase as does the *rpoH* (*htpR*) gene product that regulates the heat shock response (12).

In a previous study (19), we made the striking observation that a deletion that removed the 3′-terminal nucleotides of the *ada* coding sequence resulted in an Ada derivative that functions as a very strong constitutive activator of *ada* transcription. To explore this phenomenon further and to gain insights into the mechanism of positive activation by Ada, we have undertaken a detailed analysis of a set of Ada derivatives generated by 3′ deletions of the *ada* gene.

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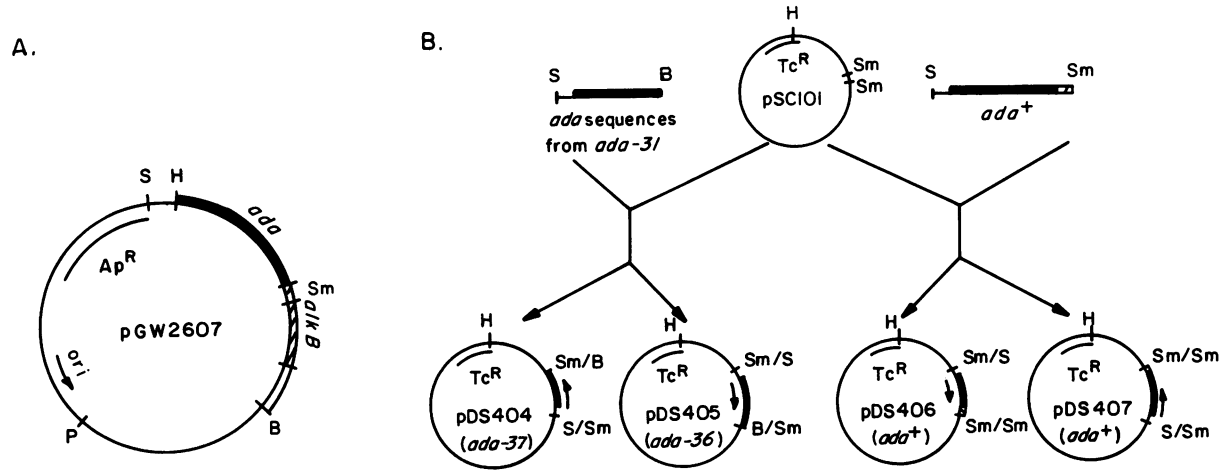

FIG. 1. Plasmids. (A) pGW2607. (B) Construction of pDS404, pDS405, pDS406, and pDS407. The solid bars represent *ada* sequences, the hatched bars represent *alkB* sequences, and the open bar represents chromosomal DNA. See Materials and Methods for a detailed description of the constructions. Symbols: B, *Bam*HI; H, *Hin*dIII; P, *Pvu*II; S, *Ssp*I; Sm, *Sma*I.

MATERIALS AND METHODS

Bacterial strains and plasmids. An ordered set of deletions from the 3′ end of *ada* was made by cutting pGW2607 (Fig. 1A) randomly with DNase I (15) and then digesting the plasmid with *Bam*HI and *Pvu*II. The DNA ends were filled in with the Klenow fragment of DNA polymerase I and then phosphatase treated. DNA in the correct size range was eluted from an agarose gel, ligated to *Bam*HI linkers, and transformed into an *ada-3* strain (PJ3). Thirty-five plasmids that appeared by restriction digest analysis to have lost at least the *alkB* sequences and that contained a *Bam*HI site were selected for further study.

All strains are listed in Table 1 and were grown at 37°C unless otherwise noted. Strains were constructed by P1-mediated transductions as described by Miller (24). The *ada-10*::Tn*10 del-16 del-17* allele (19) has a transposition-defective Tn*10* derivative (11) inserted into the *ada* gene. pDS408 was constructed by digesting pBR322 with *Hin*dIII and *Sal*I, treating the cut plasmid with the Klenow fragment of DNA polymerase I, and religating. The plasmids pDS405 and pDS404 were constructed by inserting, in both orientations, the *Ssp*I-*Bam*HI fragment carrying the *ada* sequences of *ada-31*, from pGW3508, into the *Sma*I site of pSC101 (Fig. 1B). pDS406 and pDS407 were constructed in a fashion similar to that for pDS405 and pDS404 except that we used a *Ssp*I-*Sma*I fragment, carrying the intact *ada*$^+$ gene, from pGW2607 (Fig. 1). pGW3509 was constructed by digesting pGW3508 with *Bam*HI and religating.

Two plasmids, pGW2610 and pGW3508, had unexpectedly low copy numbers and deletions in the vector backbone (see below) and were also unstable in cells that were in the late log or stationary phase. Strains containing these plasmids were grown by inoculating liquid medium containing antibiotic with a dilution of cells such that 12 to 15 h of incubation was required for the culture to reach early log phase. These strains were always assayed before entering late log phase. All pBR322-derivative plasmids that were compared with these two plasmids were treated in the same manner.

Restriction enzymes and the Klenow fragment of DNA polymerase I were purchased from New England BioLabs. DNase I and phosphatase were purchased from Boehringer Mannheim Biochemicals.

Patch mutagenesis. A quick screen for the mutability and sensitivity of strains to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was performed by patching Arg$^-$ cells in sectors on a minimal plate containing a limiting amount of arginine. The limiting amount of arginine allows the MNNG-induced Arg$^+$ revertants to express the proteins required for arginine biosynthesis so that the cells can continue to grow once the arginine supplied in the plates is exhausted. A 10-μg sample of MNNG was applied to the filter disk in the center of the plate. After 2 to 3 days, the number of Arg$^+$ revertants was scored and the killing radius was determined (19).

Sequencing. DNA sequence determination of the *ada* deletion mutants was performed by the dideoxy-chain termination procedure on plasmid DNA as previously described (2). Two plasmids were found to contain deletions in the vector backbone. pGW2610 has the pBR322 sequences 2066 to 2352 deleted. pGW3508 has the pBR322 sequences 2066 to 2426 deleted. Because of multiple *Bam*HI linkers in the *ada-28*, *ada-29*, *ada-31*, and *ada-34* alleles, we were not able to unambigously read through the entire linker region. The last remaining base from the *ada* gene, the first base from the vector sequence, and the total number of *Bam*HI linkers were unambiguously determined. In our calculations, we assumed that all of the *Bam*HI linkers were intact. In all instances in which the linker region was successfully sequenced, there were no deviations from the expected linker sequence. Results from maxicell analysis and methyltransferase assays of strains containing plasmids encoding the Ada-29 and Ada-31 derivatives show molecular weights for these derivatives that are consistent with those predicted from DNA sequencing results (data not shown).

β-Galactosidase assays. β-Galactosidase assays were performed as previously described (24). Cells were grown in supplemented M9-glucose medium, and treatments were done at an optical density at 600 nm of 0.12 to 0.22 for GW5354 and GW5356 and of 0.15 to 0.30 for GW7102 and GW7103. Induction by MNNG was at a concentration of 1 μg/ml. MNNG was added at 0 h and remained in the samples throughout the experiment.

Methyltransferase extracts. Cultures were grown in 100 ml of LB broth with the appropriate drugs to an optical density at 600 nm of 0.5 to 0.7. The cells were washed with assay buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Parental strain or plasmid vector	Source or reference
E. coli strains			
AB1157	F' *thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL-31 supE37*		8
GW5354	*ada'-lacZ*	AB1157	19
GW5356	*ada'-lacZ* λ *ada*$^+$ *alkB*$^+$	GW5354	19
GW5352	*ada-10*::Tn*10 del-16 del-17*	AB1157	19
GW7101	Δ*ada-25*	AB1157	39
PJ3	*ada-3*	AB1157	18
MV1571	*argE3 his-4 leu-6 proA2 thr-1 ara-1 galk2 lacY1 mtl-1 xyl-5 thi-1 rpsL31 supE44 tsx-33 rfa-550 alkA51*::Mu-d1(Ap^r *lac*)		45
MV1902	*alkA104*::λpSG1	MV1571	46
GW7102	*ada-10*::Tn*10 del-16 del-17*	MV1902	This paper
GW7103	Δ*ada-25*	MV1571	This paper
Plasmids			
pBR322	Ap^r Tc^r		Laboratory stock
pSC101	Tc^r		Laboratory stock
pGW2607	*ada*$^+$ *alkB*$^+$ Ap^r	pBR322	19
pGW2609	*ada*$^+$ Ap^r	pBR322	19
pGW2610	*ada-33* Ap^r	pBR322	19
pGW3503	*ada-26* Ap^r	pBR322	This paper
pGW3504	*ada-27* Ap^r	pBR322	This paper
pGW3505	*ada-28* Ap^r	pBR322	This paper
pGW3506	*ada-29* Ap^r	pBR322	This paper
pGW3507	*ada-30* Ap^r	pBR322	This paper
pGW3508	*ada-31* Ap^r	pBR322	This paper
pGW3509	*ada-32* Ap^r	pBR322	This paper
pGW3511	*ada-34* Ap^r	pBR322	This paper
pGW3512	*ada-35* Ap^r	pBR322	This paper
pDS404	*ada-37* Tc^r	pSC101	This paper
pDS405	*ada-36* Tc^r	pSC101	This paper
pDS406	*ada*$^+$ Tc^r	pSC101	This paper
pDS407	*ada*$^+$ Tc^r	pSC101	This paper
pDS408	Ap^r	pBR322	This paper

ethanesulfonic acid]-potassium hydroxide [pH 7.8], 10 mM dithiothreitol, 1 mM EDTA, 5% glycerol) and suspended in 1 ml of assay buffer. The cells were lysed by sonication and centrifuged. The supernatants were immediately frozen at −70°C. Protein concentrations were determined by using the Bio-Rad protein microassay.

Methyltransferase assays. *N*-[^{3}H]methyl-*N*-nitrosourea ([^{3}H]MNU) (Amersham)-treated *Micrococcus luteus* DNA (180 cpm/μg of DNA) and [^{3}H]MNU-treated poly(dT)·(dA) (140 cpm/μg DNA) were generously provided by Leona Samson. Cell extracts (1.2 mg) were incubated with 1,300 cpm of alkylated *M. luteus* DNA or 1,000 cpm of alkylated poly(dT)·(dA) for 10 min at 37°C. The reaction mixture was then subjected to sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis. The gel was cut into 2-mm slices which were incubated overnight at 55°C in nonaqueous scintillation fluid containing 5% protosol (New England Nuclear Corp.). The samples were assayed for tritium by liquid scintillation counting.

Copy number determination. The *rop* gene is deleted in all of the pBR322-derivative plasmids in this study. The deletion of the *rop* gene generally results in an increased copy number of the altered plasmid compared with that of the parent plasmid, pBR322 (1). We performed dot blots to compare the copy numbers of the vector, the *ada*$^+$ plasmid, and the mutant plasmids. Strains were grown in supplemented M9-glucose medium to an optical density at 600 nm of 0.1 to 0.2 or of 0.9 to 1.0. The transfer of the DNA and the high-stringency hybridization and washes were performed as described in the manual for using the GeneScreen Plus filter (New England Nuclear Corp.). The filter was probed with a *Pst*I-*Hin*dIII fragment derived from pBR322 since this fragment was contained in all of the plasmids that were being assayed. We also probed the filter with a *Bgl*II fragment containing only *umuC* sequences as a control for cell lysis. This fragment should hybridize only to chromosomal DNA. There was no significant difference in cell lysis among the strains. By using a serial dilution series for comparison, pGW2610 and pGW3508 were found to have at least a twofold-lower copy number than the parent pBR322 vector. However, the signal from these two plasmids was close to the background level (cells containing no plasmid).

We also compared plasmid copy numbers by lysing overnight cultures by using the alkaline lysis procedure (20) and then performing agarose gel electrophoresis to compare the amounts of plasmid DNA from different strains. Analysis of the DNA preparations suggested that pGW2610, pGW3508, pGW3509, and pGW3512 have copy numbers equal to or slightly lower than that of pSC101 (data not shown). There are about eight copies of pSC101 per chromosome (13).

Primer extension. RNA for primer extension was isolated as previously described (31). Primer extension was performed as previously described (32) by using a primer containing the *ada* sequences 200 to 184. The sample, suspended in gel loading buffer, was loaded onto an 8% sequencing gel.

TABLE 2. Patch mutagenesis assays of representative *ada* derivatives

Plasmid	*ada* allele	Result[a] of assay with strain:		
		AB1157 (*ada*$^+$)	PJ3 (*ada-3*)	GW5352 (*ada-10*)
pBR322		0	0	0
pGW2609	*ada*$^+$	+	+	+
pGW3508	*ada-31*(Act)	+	+	0
pGW2610	*ada-33*(Act)	+	+	0
pGW3511	*ada-34*(Act)	+	+	0
pGW3512	*ada-35*(Act)	+	+	0
pGW3505	*ada-28*(Inh)	−	−	0
pGW3506	*ada-29*(Inh)	−	−	0
pGW3503	*ada-26*(Def)	0	0	0
pGW3504	*ada-27*(Def)	0	0	0
pGW3507	*ada-30*(Def)	0	0	0

[a] Symbols: 0, no difference in the phenotype of the strain with or without the plasmid; +, the strain with the plasmid was more resistant to the mutagenic and killing effects of MNNG than the strain without the plasmid; −, the strain with the plasmid was more sensitive to the mutagenic and killing effects of MNNG than the strain without the plasmid.

RESULTS

Isolation of 3′ deletions of the *ada* gene. In a previous study (19), we made the unexpected observation that a deletion that removed 3′-terminal nucleotides of the *ada* coding sequence resulted in an Ada derivative that functions as a strong constitutive activator of *ada* transcription. To investigate further the role of the carboxyl terminus in Ada function, we isolated and characterized a set of plasmids carrying ordered deletions from the 3′ end of the *E. coli* K-12 *ada* gene (Fig. 1A). Thirty-five such plasmids were separated into three phenotypic classes on the basis of a patch mutagenesis assay using strains AB1157 (*ada*$^+$), PJ3 (*ada-3*), and GW5352 (*ada-10*::Tn*10 del-16 del-17*) (Table 2). The first class consists of several plasmids that make strains containing the leaky *ada-3* allele or the *ada*$^+$ allele more resistant to the mutagenic and killing effects of MNNG. As we show below, these plasmids carry *ada* alleles whose gene products resemble that of our previously described *ada* deletion derivative (which we now term *ada*-33) on plasmid pGW2610 (19) and constitutively activate *ada* transcription. We refer to these alleles as activators (Act). The second class of plasmids makes strains carrying the leaky *ada-3* or *ada*$^+$ alleles more sensitive to the mutagenic and killing effects of MNNG. We demonstrate below that these plasmids encode dominant inhibitors of the wild-type *ada* gene and we refer to these alleles as inhibitors (Inh). The third class consists of many plasmids carrying *ada* deletion derivatives that have no detectable phenotype in any of the strains tested, and we refer to these alleles as defective (Def). None of the plasmids in any of the three classes had any observable effect on the phenotype of the *ada-10* strain. We chose nine representative mutant plasmids and our previously reported deletion derivative, *ada-33* (19), for further study.

These plasmids carrying 3′ deletions of the *ada* gene encode fusion proteins that derive their amino termini from *ada* and their carboxyl-terminal tails from the downstream vector (pBR322) sequence that occurs before an in-frame stop codon. For each mutant, we sequenced the junction between the *ada* DNA and the vector DNA. The junction points and the number and sequence of amino acids from the vector sequences are listed in Table 3 along with the equivalent information for other *ada* alleles discussed below. All of the mutants that we characterized have lost the cysteine residue (Cys-321) of Ada that accepts a methyl group from O^6-methylguanine or O^4-methylthymine residues. However, the Ada fusion proteins encoded by the activator and inhibitor alleles tested [*ada-31*(Act), *ada-33*(Act), *ada-29*(Inh)] retain phosphotriester-DNA methyltransferase activity (data not shown).

Activator derivatives constitutively activate *ada* transcription. To further characterize the plasmids that make an *ada*$^+$ strain more resistant to the mutagenic and toxic effects of MNNG, we introduced these plasmids carrying activator alleles into a strain containing a chromosomal *ada′-lacZ* operon fusion integrated at the *ada* locus. We then measured the β-galactosidase activity of these strains, grown with and without MNNG. Although *ada* transcription is induced by MNNG if the cell carries a single copy of the *ada*$^+$ gene, we observed that *ada* transcription is constitutively activated in a strain containing the *ada*$^+$ gene on a very high-copy-number plasmid (>50 copies per cell) and is not induced further upon exposure to MNNG (Table 4). The gene products of the two activator alleles, *ada-31*(Act) and *ada-33*(Act), constitutively activate *ada* transcription to levels three- to fourfold above the level produced by the *ada*$^+$ gene on a very high-copy-number plasmid (Table 4). The gene products of two other truncated *ada* derivatives, *ada-34*(Act) and *ada-35*(Act), constitutively activate *ada* expression to levels close to or slightly lower than the level produced by the *ada*$^+$ gene on a very high-copy-number plasmid (pGW2609). The *ada-32*(Act) allele is discussed in Table 3.

It was difficult to compare the effects of these Ada derivatives to that of the wild-type Ada protein, however, because DNA dot blots and agarose gel electrophoresis of DNA preparations revealed that the plasmids containing the activator alleles *ada-31*, *ada-33*, and *ada-35* have much lower copy numbers than that of the vector containing the *ada*$^+$ gene (data not shown). In the course of sequencing, we also discovered that the plasmids carrying the *ada-31*(Act) and *ada-33*(Act) alleles each have a deletion in the vector sequence. Although different, both deletions extend from the end of the truncated *ada* gene to near the origin of replication of the vector, deleting about 300 base pairs (bp). It seems likely that the deletions in the vector backbone are responsible for the reduction in copy number of these altered plasmids. Although the plasmid carrying the *ada-35*(Act) allele has no obvious deletion in the vector backbone, it probably contains a small deletion or point mutation that reduces its copy number. The fact that the strongest *ada*(Act) alleles were isolated on plasmids with a reduced copy number suggests that our procedure was biased against the isolation of strong activator alleles. Even with the reduced copy number, cells containing plasmids with strong activator alleles grew poorly, suggesting that the presence of such an allele on a high-copy-number plasmid would be lethal.

Carboxyl-terminal tails modulate the transcriptional activation properties of the Ada derivatives. To accurately compare the effects of the wild-type Ada protein and the gene product of an activator allele on *ada* transcription, we subcloned the *ada*$^+$ gene and the truncated *ada* gene from the *ada-31*(Act) allele onto the low-copy-number plasmid, pSC101, in both orientations (Fig. 1B). In the case of the plasmids carrying sequences from *ada-31*(Act), the two orientations result in two different tails for the truncated Ada protein. We refer to

TABLE 3. Deletion endpoints and carboxyl-terminal amino acid sequences of the truncated Ada fusion proteins

Plasmid	Parent vector	*ada* allele	No. of amino acids		Junction and vector-encoded carboxyl terminus[a]
			ada specified	Vector specified	
pGW2609	pBR322	*ada*$^+$	354		
pDS406	pSC101	*ada*$^+$	354		
pDS407	pSC101	*ada*$^+$	354		
pGW3512	pBR322[b]	*ada-35*(Act)	315	13	K/ACLARFGDDGENL
pGW3511	pBR322[b]	*ada-34*(Act)	309	55	E/RIRGSAASRVSVMTVKTSDTCSSRRRSQLVCKRMPG ADKPVRARQRVLAGVGAQP
pGW2610	pBR322[c]	*ada-33*(Act)	308	27	L/ALPLPRSLTRCARSFGCGERYQLTQRR
pGW3509	pBR322[c]	*ada-32*(Act)[d]	278	5	A/LGSGR
pGW3508	pBR322[c]	*ada-31*(Act)	278	26	A/LGSADPRIRAVIRLSTESGDNAGKNM
pDS405	pSC101[e]	*ada-36*(Act)	278	70	A/LGSGGRFETDINRLCPLTDTGVIRAAQRNVHQGKN GINKTLRSPQGQPEYAFNHQNGGDGEIRIALGPSS
pDS404	pSC101[e]	*ada-37*(Ind)	278	133	A/LGSGLIRQTHRKIRAAAADGAYDTRLCHDELRRKK ISELIPPRKGAGYWPGGYADRNRAVANQRMTGSNAR WKWTTDYNRRSIAETAMYRVKQLFGGSLTLRDYDG QVAEAMALVRALNLMTKAGMPESVRIA
pGW3506	pBR322[b]	*ada-29*(Inh)	233	55	A/RIRGSAASRVSVMTVKTSDTCSSRRRSQLVCKRMPG ADKPVRARQRVLAGVGAQP
pGW3505	pBR322[b]	*ada-28*(Inh)	149	55	Q/RIRGSAASRVSVMTVKTSDTCSSRRRSQLVCKRMPG ADKPVRARQRVLAGVGAQP
pGW3507	pBR322[b]	*ada-30*(Def)	236	ND[f]	ND
pGW3504	pBR322[b]	*ada-27*(Def)	120	ND	ND
pGW3503	pBR322[b]	*ada-26*(Def)	3	ND	ND

[a] The last amino acid specified by *ada* sequences is separated by a slash from the amino acid sequence of the vector-derived carboxyl terminus. ND, Not determined.

[b] The vector-encoded carboxyl termini begin at the *Pvu*II site in pBR322 for the *ada-26*, *ada-27*, *ada-28*, *ada-29*, *ada-30*, *ada-34*, and *ada-35* alleles. These plasmids have high copy numbers.

[c] The vector-encoded carboxyl termini begin at position 2427 in pBR322 for the *ada-31* and *ada-32* alleles and at positive 2353 in pBR322 for the *ada-33* allele. These plasmids have low copy numbers, similar to that of pSC101 (see Materials and Methods).

[d] Since the tail sequences appeared to modulate the activity of the Ada derivatives, we constructed another derivative of the *ada-31*(Act) allele, termed *ada-32*. The *ada-32*(Act) allele contains the identical vector backbone and the same *ada* sequences as *ada-31*(Act). pGW3509, which carries the *ada-32* allele, also has the same low copy number as pGW3508, which carries the *ada-31* allele. However, *ada-32*(Act) has had two *Bam*HI linkers removed and thus, as a consequence of a shift in reading frame, has acquired a new tail.

[e] The vector-encoded carboxyl termini begin at the *Sma*I sites in pSC101 for the *ada-36* and *ada-37* alleles (Fig. 1B). These plasmids have low copy numbers.

[f] ND, The vector-encoded carboxyl termini for these alleles could not be accurately sequenced because of multiple linker insertions between the *ada* and vector sequences.

the two new *ada* alleles generated by this process as *ada-36* and *ada-37*. These alleles share the same amino-terminal sequences derived from *ada-31*(Act) but have different vector (pSC101)-derived carboxyl-terminal sequences. The vector backbone and copy number are also the same for the plasmids carrying the *ada*$^+$, *ada-36*, and *ada-37* alleles, allowing for a direct comparison.

We observed a striking difference in the effects of the gene products produced by the *ada-36* and *ada-37* alleles on *ada* transcription (Table 4). The product of the *ada-36* allele constitutively activates *ada* transcription to levels 200-fold over that seen with the uninduced *ada*$^+$ gene. In contrast, the effect of the *ada-37* gene product on *ada* expression resembles that of the wild-type Ada protein. We refer to the *ada-37* allele as inducible (Ind). The effect of the *ada*$^+$ gene on transcription was the same regardless of its orientation in pSC101. These data suggest that the vector-derived carboxyl-terminal sequences of the mutant proteins and not the orientation of the genes in pSC101 affect the ability of the altered proteins to activate *ada* transcription.

Primer extension experiments have shown that the products of at least two of the activator alleles, *ada-33*(Act) and *ada-36*(Act), promote *ada* transcription to initiate at the same start site (22 to 23 bases upstream of the translational start site) as the wild-type protein (data not shown). These data agree with the primer extension studies carried out by Nakabeppu and Sekiguchi (29).

Activator mutants show promoter specificity. To determine how these activator proteins affect the transcription of other *ada*-regulated genes, we examined their effect on *alkA* expression. The *alkA* gene encodes 3-methyladenine-DNA glycosylase II, which protects the cell against the toxic effects of alkylating agents (3, 10, 16, 26). We performed β-galactosidase assays using a strain with an *alkA*::λpSG1(Cm^r) fusion integrated at the *alkA* locus and a chromosomal *ada-10* mutation or a strain with a chromosomal *alkA*::Mu-d1(Ap^r *lac*) fusion and a Δ*ada-25* allele. These strains also contained a plasmid carrying either the *ada*$^+$ gene or one of the *ada* derivatives (Table 4). Although *alkA* transcription is induced by MNNG if the cell carries a single copy of the *ada*$^+$ gene, the *alkA* gene is expressed constitutively in a strain containing the *ada*$^+$ gene on a very high-copy-number plasmid (pGW2609).

Plasmids carrying two of the strongest activator alleles, *ada-31* and *ada-33*, have a much smaller effect on *alkA* expression than they do on *ada* expression (Table 4). Only the activator derivative, Ada-36(Act), is able to constitutively activate *alkA* as well as *ada* transcription to levels higher than the levels seen with the induced *ada*$^+$ gene. Interestingly, the Ada-37(Ind) derivative, while inducible for *ada* transcription, cannot activate *alkA* transcription under induced or uninduced conditions.

Dominant inhibitor and defective mutants. As mentioned above, we also identified a set of plasmids encoding truncated Ada fusion proteins which make the wild-type or leaky *ada-3* strain more sensitive to the effects of MNNG. These Ada derivatives inhibit the ability of a strain containing the wild-type *ada* gene to induce *ada* transcription. We per-

TABLE 4. Effect of Ada derivatives on *ada* and *alkA* transcription

Plasmid	*ada* allele	Parent vector	β-Galactosidasee activity[a] in strain with fusion:					
			ada'-lacZ[b]		*alkA-lacZ*[c]		*ada'-lacZ*, λ *ada*$^+$ *alkB*$^+$[d]	
			Uninduced	Induced	Uninduced	Induced	Uninduced	Induced
pBR322		pBR322[e]	80	70	9	9	200	16,000
pGW2609	*ada*$^+$	pBR322[e]	3,300	3,800	760	760	1,200	3,300
pGW3512	*ada-35*(Act)	pBR322[e]	1,700	1,500	20	50		
pGW3511	*ada-34*(Act)	pBR322[e]	2,500	2,700	110	220		
pGW2610	*ada-33*(Act)	pBR322[f]	15,000	14,000	50	110	14,400	16,200
pGW3509	*ada-32*(Act)	pBR322[f]	4,100	5,300				
pGW3508	*ada-31*(Act)	pBR322[f]	11,000	11,000	20	40	1,500	11,900
pGW3506	*ada-29*(Inh)	pBR322[e]	100	100	10	30	170	1,600
pGW3505	*ada-28*(Inh)	pBR322[e]	100	110	10	130	250	1,700
pGW3507	*ada-30*(Def)	pBR322[e]	110	100	9	10	240	4,100
pGW3504	*ada-27*(Def)	pBR322[e]	100	110	8	10	230	3,900
pGW3503	*ada-26*(Def)	pBR322[e]	100	100			170	8,200
pSC101		pSC101[f]	80	80	50	40		
pDS406	*ada*$^+$	pSC101[f]	100	3,400	50	190		
pDS407	*ada*$^+$	pSC101[f]	100	3,600	40	190		
pDS405	*ada-36*(Act)	pSC101[f]	24,000	22,000	200	300		
pDS404	*ada-37*(Ind)	pSC101[f]	100	2,700	40	50		

[a] β-Galactosidase activity is expressed as units per optical density at 600 nm (24).

[b] β-Galactosidase assays were performed on strains containing a chromosomal *ada'-lacZ* operon fusion (GW5354) and a plasmid carrying one of the *ada* derivatives. Induced cultures were assayed 2 h after the addition of 1 μg of MNNG per ml. Uninduced cultures were assayed after 2 h of growth without MNNG.

[c] β-Galactosidase assays were performed on strains containing a chromosomal *alkA-lacZ* fusion, an *ada* null mutation, and a plasmid carrying one of the *ada* deletion derivatives. Plasmids of pBR322 origin were assayed in a GW7102 (*alkA*::λpSG1 *ada-10*) background, and plasmids of pSC101 origin were assayed in a GW7103 [*alkA*::Mu-d1(Ap^r *lac*) Δ*ada-25*] background to accommodate the drug-resistant markers on the plasmids being assayed. The background β-galactosidase level varies with the particular strain so that only qualitative comparisons can be made between the two strains. Induced cultures were assayed 1 h after the addition of 1 μg of MNNG per ml. Uninduced cultures were assayed after 1 h of growth without MNNG. GW7103 derivatives were grown at 30°C.

[d] β-Galactosidase assays were performed on strains containing a chromosomal *ada'-lacZ* operon fusion, λ*ada*$^+$ *alkB*$^+$ (Tc^r) (GW5356), and a plasmid carrying an *ada* derivative. Induced and uninduced cultures were treated as described in footnote *a*, except that GW5356 derivatives were grown at 30°C to maintain the lambda vector. pDS408 (Ap^r Tc^s) was used as a control instead of pBR322 (Ap^r Tc^r) in experiments involving GW5356, which is Km^r Sp^r Tc^r.

[e] High-copy-number plasmid.

[f] Low-copy-number plasmid.

formed β-galactosidase assays on strains containing an *ada'-lacZ* chromosomal operon fusion, the *ada*$^+$ gene on a low-copy-number lambda-derived vector (9), and plasmids carrying either the *ada*$^+$ gene or one of the *ada* derivatives. These strains were grown at 30°C to maintain the lambda vector. Interestingly, *ada* transcription is still inducible in this background when the *ada*$^+$ gene is present on a high-copy-number plasmid. The unmethylated Ada protein may be less active in promoting *ada* transcription at 30°C. The results of these assays show that in an *ada*$^+$ background, the gene products of the *ada-28*(Inh) and *ada-29*(Inh) alleles decrease the MNNG-induced levels of *ada* transcription to 50% of that seen with a plasmid carrying the *ada*$^+$ gene (Table 4). This inhibition is not simply due to the titration of the methylated Ada protein by excess copies of the *ada* promoter. A plasmid carrying the *ada-26* allele, which contains the *ada* promoter and encodes only three *ada*-specified amino acids, does not reduce the level of induction of *ada* transcription compared with the level observed with a plasmid carrying the *ada*$^+$ gene. However, the plasmid carrying *ada-26* does reduce the level of induction of *ada* transcription compared with the level seen with a plasmid carrying no *ada* sequences.

The effects of the gene products of two of the strongest activator alleles in this background are drastically different. The gene product of the dominant *ada-33* allele constitutively activates *ada* transcription, while the gene product of the *ada-31* allele is only a moderate transcriptional activator in the presence of an uninduced *ada*$^+$ gene.

None of the products of the defective alleles were able to activate *ada* expression under either uninduced or induced conditions (Table 4). Some of them do interfere slightly with the induction of the *ada* gene in an *ada*$^+$ strain (GW5356) when compared with a plasmid carrying a truncated *ada* gene containing only the *ada* promoter and specifying 3 amino acids of *ada* but not when compared to a strain containing the *ada*$^+$ plasmid (Table 4).

Dominant inhibitors of *ada* activate *alkA* transcription upon MNNG induction. Surprisingly, while a strain with a plasmid carrying the *ada-28*(Inh) allele cannot activate *ada* transcription under uninduced or induced conditions, a strain containing an *alkA*::λpSG1 chromosome fusion, an *ada-10* mutation, and a plasmid carrying the *ada-28*(Inh) allele can increase *alkA* expression 10-fold upon exposure of the cells to MNNG (Table 4). The same background strain containing a plasmid carrying the *ada-29*(Inh) allele can increase *alkA* expression threefold when the cells are induced with MNNG. Moreover, these inhibitor derivatives, unlike the wild-type Ada protein, cannot induce their own expression, so that there is probably less inhibitor protein present in an induced cell containing a plasmid with an inhibitor allele than

TABLE 5. Classes of Ada derivatives

Class	Plasmid	*ada* allele	Parent vector	Phenotype of the Ada derivative as a transcriptional activator[a]	
				ada	*alkA*
Wild type	pGW2609	*ada*$^+$	pBR322	Wild type, constitutive	Wild type, constitutive
1	pGW3508	*ada-31*(Act)	pBR322	Strong, constitutive	Weak to moderate, slightly inducible
	pGW2610	*ada-33*(Act)	pBR322	Strong, constitutive	Weak to moderate, slightly inducible
2	pGW3511	*ada-34*(Act)	pBR322	Moderate wild type, constitutive	Moderate, slightly inducible
	pGW3512	*ada-35*(Act)	pBR322	Moderate wild type, constitutive	Moderate, slightly inducible
3	pGW3505	*ada-28*(Inh)	pBR322	No activation	Weak moderate, inducible
	pGW3506	*ada-29*(Inh)	pBR322	No activation	Weak moderate, inducible
4	pGW3504	*ada-27*(Def)	pBR322	No activation	No activation
	pGW3507	*ada-30*(Def)	pBR322	No activation	No activation
Wild type	pDS406	*ada*$^+$	pSC101	Wild type, inducible	Wild type, inducible
	pDS407	*ada*$^+$	pSC101	Wild type, inducible	Wild type, inducible
5	pDS405	*ada-36*(Act)	pSC101	Strong, constitutive	Strong, constitutive
6	pDS404	*ada-37*(Ind)	pSC101	Wild type, inducible	No activation

[a] The strength of the Ada derivative as a transcriptional activator is listed first as weak, moderate, wild type, or strong (in order of increasing strength). The inducibility of the Ada derivative is listed second.

there would be wild-type Ada protein in induced cells containing a plasmid with an *ada*$^+$ allele.

DISCUSSION

In this work, we have shown that the nature of the carboxyl terminus of the Ada protein can strongly influence the ability of Ada to function as a positive regulator of transcription. A family of fusion proteins was generated whose amino termini consist of different lengths of Ada sequences and whose carboxyl termini consist of diverse amino acid sequences encoded by plasmid DNA. These Ada derivatives can be separated into six classes on the basis of the inducibility, specificity, and strength of the Ada derivative as a transcriptional activator (Table 5). Unlike many eucaryotic transcriptional activators, which appear to require an excess of acidic residues in the activating domain of the protein for proper function, most of the Ada activator derivatives have an excess positive charge overall and in the carboxyl-terminal domain (40). None of the Ada derivatives characterized retains the Cys-321 residue of Ada that serves as the methyl acceptor in the repair of O^6-methylguanine and O^4-methylthymine lesions. The absence of this repair activity accounts for the failure of these *ada* derivatives to complement the increased sensitivity of *ada-10* strains to the toxic and mutagenic effects of MNNG.

The result that the product of the *ada-37*(Ind) allele contains only the first 278 amino acids of Ada yet can be activated in response to DNA damage suggests that the terminal 76 amino acids of Ada are not necessarily required for Ada to become activated upon exposure to alkylated DNA. These in vivo results are consistent with the in vitro studies of Teo et al. (44) that suggest that the conversion of the Ada protein into an efficient activator of transcription does not require methylation of Cys-321. Although the vector-derived tail of the Ada-37(Ind) protein contains one cysteine residue, the neighboring amino acids differ from those surrounding the Cys-321 residue of Ada and the physiological properties of the *ada-37*(Ind) mutant indicate that the protein cannot repair O^6-methylguanine or O^4-methylthymine.

The fact that the only difference between the gene products of the *ada-36*(Act) allele (a strong constitutive activator of both *ada* and *alkA*), the *ada-37*(Ind) allele (an inducible activator of *ada* but not *alkA*), and the *ada-31* allele (a strong constitutive activator of *ada* but not *alkA* transcription) is the carboxyl-terminal domain of the proteins provides strong support for the importance of the carboxyl terminus in determining the transcriptional activating properties of Ada. Other studies are consistent with this conclusion. Demple (5) has shown that two *ada* mutants are simultaneously defective in positive regulation and the repair of O^6-methylguanine, while these mutants are not altered in phosphotriester-DNA methyltransferase activity. Also, Takano et al. (41) have found that replacing the Ada Cys-321 residue with alanine results in a protein that constitutively activates *ada* expression.

No obvious similarities or differences exist among the various vector sequences fused to activator, inhibitor, and inducible mutants. It is possible that the elimination of some element in the carboxyl terminus of the Ada protein results in a constitutively activated protein and that the carboxyl-terminal tails modify this effect. Alternatively, the tails could be required for activation. The carboxyl-terminal domain of the Ada activator derivatives could increase the stability of the mutant protein or RNA, increase the ability of the mutant protein to bind to the *ada* promoter, or improve interactions of the Ada derivative with RNA polymerase.

There are several differences between the *ada* and *alkA* promoter regions that could account for the differential regulation of the *ada* and *alkA* genes. The Ada box (29, 44), the sequence at which the Ada protein binds DNA in the promoter region, ends 42 bp before the RNA start site in the *ada* promoter, whereas it lies 30 bp from the RNA start site in the *alkA* promoter, overlapping the proposed −35 region (29, 44). Genes regulated by CAP also show differences in the distance between the CAP-binding site and the start site of transcription (4). Also, the proposed −35 region for *ada* (TTGCGT) (29) has better homology with the consensus sequence (TTGACA) (35) than the proposed −35 region for *alkA* (GCGCAG) (29). Finally, the dyad symmetry in the *ada*

promoter, which overlaps with the Ada box sequence and is absent in the *alkA* promoter region, could play a regulatory role. We have found it necessary to delete the *ada* promoter to put the expression of the *ada* gene under the control of the *lac* promoter that was initially placed only 100 bp upstream of the translational start site of *ada* (unpublished results).

The inhibitor derivatives of Ada may provide other insights into the mechanism of transcriptional activation by Ada. The apparent toxicity of Ada overproduction (unpublished results) or of the Ada activator derivatives suggests that the expression of the Ada protein may need to be tightly regulated. As mentioned above, upon cell lysis, the Ada protein is readily cleaved into two domains (25, 42, 43). Cleavage of the Ada protein, should it occur in vivo, could be a mechanism to switch off *ada* induction (44). We have found that some truncated *ada* derivatives inhibit the induction of *ada* transcription but still activate *alkA* transcription. These in vivo findings are consistent with those of Yoshikai et al. (T. Yoshikai, Y. Nakabeppa, and M. Sekiguchi, Abstract, J. Cell. Biochem. **124**:299), who found that the purified methylated N-terminal domain of the wild-type Ada protein could activate *alkA* transcription but not *ada* transcription in vitro. Proteolysis may not only degrade the transcriptional activator of the adaptive response but may also generate Ada fragments that inhibit the activity of the intact Ada protein at the *ada* promoter. The possibility of such a mechanism is particularly interesting since Ada is irreversibly activated by methylation at Cys-69. However, the ability of the inhibitor derivatives to activate *alkA* transcription upon induction with MNNG suggests that upon cleavage of the Ada protein in vivo, the amino-terminal domain could retain the ability to activate *alkA* and a subset of other *ada*-regulated genes. Such a mechanism would allow for differential temporal regulation of a group of gene products that are under the control of a single regulatory gene.

Several models exist to explain the ability of the inhibitor derivatives of Ada to interfere with the induction of *ada* transcription. The inhibitor derivatives could compete with the wild-type Ada for a limited amount of the inducing signal (methyl groups from DNA phosphotriesters). Consistent with this model, the *ada-29*(Inh) gene product still has methylphosphotriester DNA-methyltransferase activity. Alternatively, the inhibitory protein could compete with the wild-type Ada protein for binding at the *ada* promoter. Sedgwick et al. (38) have shown that the purified N-terminal domain of Ada can still bind the *ada* promoter. Another possibility is the formation of a mixed multimer, composed of altered and wild-type Ada protein, that would be less efficient at promoting *ada* transcription than the wild-type methylated Ada multimer. If the inhibitor proteins function in this manner, then only the first 149 amino acids of Ada would be required for oligomerization. Several examples of the two latter types of dominant negative mutations have been described in other systems (14). The ada^+ strains with a high-copy-number plasmid containing the ada^+ gene also have a reduction in the amount of *ada* transcription induced upon exposure to MNNG in comparison with a strain containing pBR322. If the inducing signal is limited, the overproduction of the Ada protein could result in the unmethylated Ada protein competing for the *ada* promoter or forming a mixed multimer that is less effective at activating *ada*.

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