Amplified Expression of the tag+ and alkA+ Genes in Escherichia coli: Identification of Gene Products and Effects on Alkylation Resistance

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We have constructed plasmids which overproduce the tag and alkA gene products of Escherichia coli, i.e., 3-methyladenine DNA glycosylases I and II. The tag and alkA gene products were identified radiochemically in maxi- or minicells as polypeptides of 21 and 30 kilodaltons, respectively, which are consistent with the gel filtration molecular weights of the enzyme activities, thus confirming the identity of the cloned genes. High expression of the tag+-coded glycosylase almost completely suppressed the alkylation sensitivity of alkA mutants, indicating that high levels of 3-methyladenine DNA glycosylase I will eliminate the need for 3-methyladenine DNA glycosylase II in repair of alkylated DNA. Furthermore, overproduction of the alkA+-coded glycosylase greatly sensitizes wild-type cells to alkylation, suggesting that only a limited expression of this enzyme will allow efficient DNA repair.

N3-methylated adenine is quantitatively one of the major products formed in DNA exposed to simple alkylating agents such as methyl methanesulfonate (MMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (1, 15). This modified base is rapidly excised in both prokaryotic and eukaryotic cells by 3-methyladenine DNA glycosylases, which release the alkylated base in a free form (14, 17, 18, 29). The widespread existence from bacteria to human cells of such DNA repair enzymes suggests that alkyl groups in the N-3 position of adenines severely impair the metabolic function of the DNA.

Direct evidence for the importance of 3-methyladenine removal has been obtained from studies of Escherichia coli mutants lacking 3-methyladenine DNA glycosylase activity (11). E. coli has two enzymes of this type, one that is constitutively expressed, TagI (17, 25, 32), and another that is induced during exposure of the cells to alkylating agents, TagII (3, 7, 10, 23). Mutants lacking one or the other of these enzymes are slightly to moderately sensitive to alkylation, whereas double mutants show extreme sensitivity (3, 7). These results are direct evidence for the cytotoxic effects of persisting 3-methyladenines in DNA.

TagI and TagII have different substrate specificities. TagI appears to be specific for the removal of 3-methyladenines, whereas TagII can release 3-methylguanine, O2-methylcytosine, O6-methylthymine, and 7-methylguanine in addition to 3-methyladenines (10, 20, 32). It has therefore been suggested that TagI functions to remove 3-methyladenines from DNA, whereas the primary role of TagII is to remove other quantitatively minor lesions. Nevertheless, TagII can, once induced, effectively replace TagI in 3-methyladenine repair (6, 7).

The cloning of genes encoding TagI and TagII, tag and alkA, was recently reported (3, 23, 33). We selected these genes on the basis of their ability to complement the alkylation-sensitive phenotype of a tag ada double mutant (3). This strain is mutated in the structural gene of TagI (tag) and in the ada gene, which positively regulates the induction of TagII (4, 7, 11, 16, 22). The mutant therefore phenotypically appears as TagI+ TagII-, and its alkylation sensitivity provides a strong selection for recombinant plasmids expressing one or the other of the glycosylase activities. In this work the tag and alkA genes have been subcloned, yielding smaller plasmids, which results in elevated expression of the glycosylase genes. These plasmids have been used for the radiochemical identification of the tag and alkA gene products and to investigate the effect of glycosylase overproduction on alkylation survival.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

Enzymes. Restriction endonucleases, T4 DNA polymerase, and DNA ligase were purchased from Boehringer Mannheim Biochemicals or New England Biolabs, Inc., and were used according to the manufacturers' instructions.

Construction of plasmids. Plasmid pBK121 alkA+ was derived from pBK111 (3) carrying a y6 insertion about 2 kilobase pairs away from the alkA gene. Plasmid pBK111 has a BamHI-HindIII chromosomal insert of 6.2 kilobase pairs. The y6 insert has a BamHI site close to the end toward the alkA gene, thus allowing recloning of a BamHI fragment carrying the alkA gene into pBR322. The other plasmids used in this study were constructed as described in the legends to Fig. 1 and 3.

Labeling of plasmid-coded proteins. Polypeptides encoded by pBK202 and derivatives were labeled with [35S]methionine by the maxicell method of Sancar et al. (26), and those encoded by pBK161 and derivatives were labeled by the minicell method of Meagher et al. (21). Insertional inactivation of the glycosylase genes was carried out as previously described (8, 26).

SDS-polyacrylamide gel electrophoresis. Labeled proteins were analyzed on sodium dodecyl sulfate (SDS)–15% polyacrylamide slab gels by the method of Laemmli (13). The gels were treated with Enhance (New England Nuclear Corp.), dried, and subjected to fluorography at -70°C. [14C]-labeled protein markers were purchased from Amer sham Corp.

Alkylation survival. Alkylation survival was measured...
either by exposing exponentially growing cells to MMS (20 mM) or MNNG (0.33 mM) in phosphate buffer (pH 6.0) for various periods of time at 37°C or by plating the cells on nutrient agar containing various amounts of alkylating agent (7). The buffer exposure measures the survival of unadapted cells, whereas the plate exposure is an indirect measure of survival of adapted cells, since the low concentration of MMS used in the plates will not immediately kill the cells and hence allows for some degree of induction. This is indicated by the response of tag mutant cells, which are essentially wild-type MMS resistant when scored by the plate method presumably because TagII is induced and can replace TagI in repair (3, 7). Upon buffer exposure, tag mutant cells are significantly more sensitive than wild-type cells.

**Glycosylase assays.** Cell extracts for enzyme analysis were prepared by sucrose plasmolysis and lysozyme treatment as previously described (28). 3-Methyladenine DNA glycosylase activity was measured essentially as described by Riazuddin and Lindahl (25). The cells were adapted by growing the cells in K medium for 90 min in the presence of 0.5 μg of MNNG per ml (7).

**RESULTS**

**Identification of the tag gene product.** Mapping of ?8 insertions in pBK201 (tag?) indicated that the entire tag gene was located in between the EcoRI and the HindIII sites near the EcoRI site of pBR322. The tag gene was subcloned by cutting with EcoRI and reinserter into the EcoRI site of pBR322 (Fig. 1). The resulting plasmid, pBK202, harbors the tag gene flanked by two 31-base-pair HindIII-EcoRI fragments originating from pBR322 (31). In this plasmid the tag gene is present as a cartridge which can be moved to other vectors by cuts with either one of three different enzymes, HindIII, Clal, and EcoRI. Much more TagI enzyme is produced from pBK202 than from the original multicopy plasmid, pBK201 (Table 2). This appears to be not only the result of reduction in plasmid size and higher copy number; sequence data indicate that a fusion promoter has been formed which is stronger than the natural one (30).

Polypeptides encoded by pBK202 and derivatives having three different ?8 insertions within the tag gene were examined by the maxicell method (26). Only three major protein species appeared on the autoradiogram, the tet and the amp gene products and a third polypeptide of approximately 20 kilodaltons (Fig. 2). This band was absent from extracts isolated from cells carrying plasmids with ?8 insertions in the tag gene. We conclude that this protein is the tag gene product. A new faint band appears on the autoradiogram expressed from one of the insertion plasmids (pBK202-1), and this seems likely to represent a truncated polypeptide from the tag gene. Of the three insertion plasmids, pBK202-1 had the insertion mapped closest to the amp side on pBR322. Since larger truncated peptides were not seen in lanes from the other insertion plasmids, it may suggest that transcription is counterclockwise on the map in Fig. 2. This agrees with the nucleotide sequence analysis of the tag gene (30). From the insertion plasmid pBK202-3 the tet gene product is not expressed (Fig. 2). F-mediated transfer of nonconjugative plasmids is known to generate deletions with high frequency (8), and restriction enzyme analysis confirmed that pBK202-3 was deleted for the tet gene (data not shown).

In the autoradiogram shown in Fig. 2, which is heavily

**TABLE 1. Strains and plasmids used in this work**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1157</td>
<td>Wild type</td>
<td>P. Howard-Flanders</td>
</tr>
<tr>
<td>BK2106</td>
<td>tag-2 ada</td>
<td>Karren et al. (11)</td>
</tr>
<tr>
<td>BK2118</td>
<td>tag-2 alkA</td>
<td>Evensen et al. (6)</td>
</tr>
<tr>
<td>MS23</td>
<td>alkA</td>
<td>Yamamoto et al. (34)</td>
</tr>
<tr>
<td>CSR603</td>
<td>wvrA recA phr</td>
<td>Sancar et al. (26)</td>
</tr>
<tr>
<td>DS410</td>
<td>minA minB</td>
<td>Dougan and Sherratt (5)</td>
</tr>
<tr>
<td>AB4014</td>
<td>F&lt;sup&gt;+&lt;/sup&gt;</td>
<td>B. Low</td>
</tr>
<tr>
<td>pBR322</td>
<td>Ap, Tc&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Bolivar et al. (2)</td>
</tr>
<tr>
<td>pBK201</td>
<td>tag&lt;sup&gt;+&lt;/sup&gt; M&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Clarke et al. (3)</td>
</tr>
<tr>
<td>pBK202</td>
<td>tag&lt;sup&gt;+&lt;/sup&gt; Ap&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Clarke et al. (3)</td>
</tr>
<tr>
<td>pBK121</td>
<td>alkA&lt;sup&gt;-&lt;/sup&gt; Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>pBK132</td>
<td>alkA&lt;sup&gt;-&lt;/sup&gt; Ap&lt;sup&gt;-&lt;/sup&gt;</td>
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</tr>
<tr>
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<td>pBK141</td>
<td>alkA&lt;sup&gt;-&lt;/sup&gt; Ap&lt;sup&gt;-&lt;/sup&gt;</td>
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<td>pBK151</td>
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<td>pBK161</td>
<td>alkA&lt;sup&gt;-&lt;/sup&gt; Tc&lt;sup&gt;-&lt;/sup&gt;</td>
<td>This work (Fig. 3)</td>
</tr>
</tbody>
</table>

**FIG. 1.** Subcloning of tag. Plasmid pBK202 was constructed by recloning the EcoRI fragment from pBK201 (3) into the EcoRI site of pBR322. kbp, Kilobase pairs.

**TABLE 2.** 3-Methyladenine DNA glycosylase activity in E. coli transformed with tag and alkA plasmids

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>Enzyme activity*</th>
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<tr>
<td></td>
<td>Noninduced cells</td>
</tr>
<tr>
<td>BK2118 (tag alkA)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>AB1157 (wild type)</td>
<td>21</td>
</tr>
<tr>
<td>BK2118(pBK201)</td>
<td>300</td>
</tr>
<tr>
<td>BK2118(pBK202)</td>
<td>80</td>
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<tr>
<td>BK2118(pBK131)</td>
<td>140</td>
</tr>
<tr>
<td>BK2118(pBK132)</td>
<td>300</td>
</tr>
<tr>
<td>BK2118(pBK133)</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>BK2118(pBK141)</td>
<td>70</td>
</tr>
<tr>
<td>BK2118(pBK151)</td>
<td>54</td>
</tr>
<tr>
<td>BK2118(pBK161)</td>
<td>44</td>
</tr>
</tbody>
</table>

* Activity is expressed as picomoles of alkylated base released per milligram of protein in extract.

*Adaptation of wild-type cells does not always result in a substantial total increase in 3-methyladenine DNA glycosylase activity. However, the activity in the adapted cells is refractory to inhibition by free 3-methyladenine and hence represents TagII activity (10). Therefore, it seems that TagI activity sometimes is being reduced or partly consumed during adaptation.
overexposed to show any truncated polypeptides, there is a faint remaining band of 20 kilodaltons present in the lanes from cells with the insertion plasmids. We believe that this is caused by a small fraction of the cell population carrying plasmids where the insertion has been lost and hence represents normal tag gene product. This view is supported by survival experiments, which show that cultures of tag alkA mutant cells with the insertion plasmids contain a small fraction (less than 0.1%) of apparently MMS-resistant clones.

Identification of the alkA gene product. We were unable to identify the alkA gene product in maxicells carrying the original plasmid, pBK101. Initially, we thought this could be due to insufficient expression of the alkA gene product from this plasmid, which has a large chromosomal insert of more than 10 kilobase pairs. To improve expression, smaller plasmids were constructed (pBK132 and pBK151; Fig. 3) which yielded elevated levels of TagII from both in uninduced and in alkylation-induced (adapted) cells (Table 2). Still, we could not identify a polypeptide band on autoradiograms of SDS gels from minicells which was absent from cells carrying plasmids with insertions in the alkA gene.

All of the plasmids examined thus far carried inserts within the tet gene of pBR322 and expressed high levels of β-lactamase from the amp gene. Since the $M_i$ of TagII from the biochemical characterization had been characterized to about 30,000 (28), the possibility remained that the alkA gene product was masked by the strong 30-kilodalton β-lactamase band on the gels. Therefore, another plasmid was constructed with the alkA gene fragment within the amp gene (pBK161; Fig. 3). Specific radioactive labeling of polypeptides expressed in minicells carrying this plasmid demonstrated the synthesis of a protein of 30 kilodaltons which was absent from minicells carrying plasmids with γδ insertions within the alkA gene (Fig. 4). Polypeptides synthesized in minicells carrying the tag$^+$ plasmid were examined on the same SDS gels. Plasmid pBK202 tag$^+$ produces β-lactamase, which comigrates with the alkA gene product. Also the tag gene product is expressed, which comigrates with the 21-kilodalton marker on the gels. This marker was not included in the gels from the maxicell experiments (Fig. 2), and the comigration of tag and the marker more precisely determines 21 kilodaltons as the molecular mass of the tag gene product. This agrees very well with the calculated molecular mass of 21,104 daltons as determined by nucleotide sequence analysis.

While this work was in progress, Nakabeppu et al. (23, 24) reported the cloning of the alkA gene and the identification of the alkA gene product as a 30-kdalton protein; our results agree with their data.

Overproduction of TagII sensitizes wild-type cells to alkylation. We were initially puzzled by the observation that the alkA$^+$ plasmid first isolated did not fully suppress the alkylation-sensitive phenotype of alkA mutant cells (3). This was not caused by incomplete expression of TagII activity from the plasmid, since TagII activity was fully restored, even overproduced, by the alkA$^+$ plasmid in the mutant. After subcloning, we again tested the ability of the various alkA$^+$ plasmids to complement alkA mutants with respect to alkylation survival (Fig. 5). There appeared to be large variability between the different plasmids in the capability to restore alkylation resistance of alkA mutant cells. When wild-type

FIG. 2. SDS-polyacrylamide gel electrophoresis of polypeptides expressed in maxicells carrying pBK202 (tag$^+$) and derivative plasmids. The left lane contains marker proteins (30-kilodalton carboxic anhydrase, 14-kilodalton lysozyme). pBK202 derivatives pBK202-1 through -3 contain γδ insertions within the tag gene. Note that the third insertion also eliminates the tet gene product and has generated a deletion of the tet gene.
cells were transformed by the same plasmids, cells became much more sensitive to alkylation (Fig. 5B). Survival of the various transformed bacteria depended not on the genotype of the host (alkA+ or alkA−), but on the type of plasmid present (Fig. 5).

TagII activity is overproduced from all the different alkA+ plasmids investigated, some more than others, as judged from glycosylase assays of crude extracts from transformed cells (Table 2). The plasmids which were most effective in sensitizing wild-type cells to MMS also yielded the highest enzyme levels. We have plotted the level of enzyme activity in cell extracts from noninduced or in alkylation induced (adapted) cells versus cell survival of transformed alkA mutant cell (Fig. 6). There appears to be a correlation between the extent of overproduction and cell survival, with an optimum level of alkA+ expression for maximum survival. We hence conclude that overproduction of TagII enzyme sensitizes wild-type cells to MMS exposure. In control experiments, the plasmid pBK132 was deleted for the alkA gene, and the resulting plasmid (pBK133) did not sensitize wild-type cells (Fig. 5), nor did plasmids with γ6 insertions in the alkA gene (data not shown).

**Overproduction of TagI suppresses the alkylation sensitivity of alkA mutants.** We also transformed the tag+ plasmid into alkA mutant cells and measured survival to alkylation. Surprisingly, in contrast to the alkA+ plasmids, the tag+ plasmid pBK202 completely suppressed the alkylation-sensitive phenotype of the alkA mutant (Fig. 7). This was true both when survival was measured by the MMS-agar plate method (data not shown) and when survival was measured after exposing the cells for various periods of time in buffer to either MMS or MNNG (Fig. 7). With this method we also tested the sensitizing effect of the alkA+ plasmid pBK131 on wild-type survival, with results similar to those obtained with the MMS-agar plate method.

The complementation by the tag+ plasmid of alkA sensitivity must be due to overproduction of TagI by the multicopy plasmid, since alkA mutant cells have a tag+ gene and express TagI at normal levels (34). The original tag+ plasmid pBK201 yields less TagI than pBK202 and also suppresses the alkylation sensitivity of alkA1 to a lesser extent. Plasmid pBK202 also suppresses the MMS sensitivity of an alkA deletion mutant, implying that the complementing effects are not allele dependent (data not shown).

**DISCUSSION**

The tag and alkA genes have been subcloned, and their gene products have been identified radiochemically as polypeptides of 21 and 30 kilodaltons, respectively. These molecular masses correspond to those determined previously for TagI and TagII enzyme activities by gel filtration (25, 32) and confirm that the tag and alkA genes as cloned indeed are the structural genes for these enzymes. The size correlation between the identification and the activity determination also indicates that both enzymes are active as monomers.

Overproducing TagI and TagII plasmids has the opposite effect of what could be expected on alkylation survival of alkA mutant cells. Whereas some of the alkA+ plasmids only marginally complement the alkylation-sensitive phenotype of alkA mutant cells, the tag+ plasmid fully suppresses the alkylation sensitivity. The lack of effect by the TagII-overproducing alkA+ plasmids is accounted for by the sensitizing effect of TagII overproduction on wild-type survival. Maples and Kushner (19) have previously described a similar effect for the uvrD+ gene product. Wild-type cells carrying the uvrD+ gene on a multicopy plasmid are much more sensitive to UV irradiation and MMS exposure than cells without the plasmids. However, since the uvrD+ gene codes for a DNA helicase and an ATPase, one can easily imagine that excessive enzyme will cause excessive DNA unwinding, ATP consumption, and DNA degradation during repair and thus have a negative effect on survival. There is not such an obvious explanation for why overproduction of TagII should have a sensitizing effect. Because TagI does not have a similar effect, one cannot simply ascribe this to glycosylase action per se, but will have to consider the characteristics of TagII compared with TagI. TagI has a broader substrate specificity than TagI, which implies that TagII can initiate more repair events at the same time. Too many ongoing
repair events may be detrimental for the cells, since it will increase the possibility for creating double-strand breaks and causing DNA degradation. At doses of alkylation which are biologically relevant, the number of total alkylations is still only in the size order of thousands per chromosome, which means that each lesion on the average is several kilobases apart (11). About 60 to 70% of these are 7-methylguanines, which are very poor, if at all, substrates for TagII (10). It seems unlikely therefore that too many repair events should be the reason for the sensitizing effect of TagII amplification. More likely to us seems the possibility that the alkA gene or TagII enzyme has some other hitherto unknown property or function in the cell which causes the negative effects upon amplification.

From the sequence data published for the ada and alkA genes (4, 22, 24) we have calculated that both of these genes, in particular alkA, have an unusually high proportion of rare codons (30). The codon usage in E. coli is thought to be part of translational regulation of gene expression (9, 12). Genes with a high proportion of rare codons are limited in expression because of limiting amounts of tRNA available for rare codons. In cases with multicopy plasmids under adaptive conditions, it is plausible that too much of the tRNA for rare codons will be tied up in the expression of nonuseful amounts of these gene functions, thus limiting expression of other genes essential for repair. In other words, under such conditions other steps in repair might be deficient due to the lack of production of other enzymes. This could also explain the difficulty in the isolation of ade (ada constitutive) mutants from E. coli K-12, but not from strain B (27), since E. coli B may well have a different codon usage than strain K-12.

FIG. 6. Survival of alkA1 mutant cells transformed with alkA+ plasmids on nutrient agar containing MMS as a function of TagII activity in cell extracts. The data from Table 2 on the expression of TagII activity in cell extracts were plotted against survival of MS23 (alkA1) mutant cells on agar containing 5 mM MMS (Fig. 5). The ratio of enzyme activity for adapted (○) and nonadapted (●) cells in the figure axis was chosen as 1:6. This value corresponds to an average of that observed for cells containing the various alkA+ plasmids (except for pBK131 and pBK132, for reasons as given in Discussion).

FIG. 7. Survival of wild-type and alkA1 mutant cells transformed with pBK131 (alkA+*) and pBK202 (tag*) after exposure to MMS (A) or MNNG (B) as described in Materials and Methods. The exposure was stopped by diluting the cells at least 100-fold in buffer. Symbols: (▲) MS23 (alkA1) transformed with pBK202, (▼) AB1157 (wild type), (▲) AB1157 transformed with pBK131, (△) MS23 (alkA1).

Table 2 shows that the different alkA+ plasmids express TagII at different levels, even though all of them appear to respond to the ada+ dependent induction. Judging from the maps of the plasmids, sequences both upstream and downstream for the alkA gene are of importance for this variation. Nakabeppu et al. (24) have determined the direction of transcription for the alkA gene, which is clockwise on the maps drawn in Fig. 2 (except for pBK161). In the case of plasmids pBK131 and pBK132, which show high constitutive expression of alkA, it is plausible that the alkA gene can be transcribed from the tet promoter. EcoRV, used to make pBK131, cuts just behind the tet promoter in pBR322, whereas ClaI, used to make pBK141, cuts in the middle of the promoter (31). Therefore, the alkA gene cannot be transcribed from the tet promoter in pBK141 and pBK151, and these plasmids also show much less constitutive expression of alkA+ than pBK131 and pBK132.

The complementation of the alkA mutant sensitivity by tag+-overproducing plasmids emphasizes the overlapping roles of TagI and TagII in the repair of alkylated DNA. In the original cloning experiments, we selected for plasmids which would restore alkylase resistance of a tag ada double mutant and picked up the alkA gene as well as the tag gene. From the data presented here, one would expect that an alkA mutant could be used to select for recombinant plasmids carrying the tag gene. In fact, Yamamoto et al. (33) did isolate the tag gene in their cloning of the alkA+ gene using the alkA1 mutant for the selection.

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LITERATURE CITED