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A 55-kDa protein isolated from human cells shows DNA glycosylase activity toward 3,N^4-ethenocytosine and the G/T mismatch

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ABSTRACT **Etheno adducts in DNA arise from multiple endogenous and exogenous sources. Of these adducts we have reported that, 1,N^6-ethenoadenine (εA) and 3,N^4-ethenocytosine (εC) are removed from DNA by two separate DNA glycosylases. We later confirmed these results by using a gene knockout mouse lacking alkylpurine-DNA-*N*-glycosylase, which excises εA. The present work is directed toward identifying and purifying the human glycosylase activity releasing εC. HeLa cells were subjected to multiple steps of column chromatography, including two εC-DNA affinity columns, which resulted in >1,000-fold purification. Isolation and renaturation of the protein from SDS/polyacrylamide gel showed that the εC activity resides in a 55-kDa polypeptide. This apparent molecular mass is approximately the same as reported for the human G/T mismatch thymine-DNA glycosylase. This latter activity copurified to the final column step and was present in the isolated protein band having εC-DNA glycosylase activity. In addition, oligonucleotides containing εC·G or G/T(U), could compete for εC protein binding, further indicating that the εC-DNA glycosylase is specific for both types of substrates in recognition. The same substrate specificity for εC also was observed in a recombinant G/T mismatch DNA glycosylase from the thermophilic bacterium, *Methanobacterium thermoautotrophicum* THF.**

The four etheno adducts of DNA and RNA have been of considerable interest to organic chemists and physical scientists due to their physical, chemical and spectroscopic properties which had broad applications in protein-nucleic acid interactions and DNA structure [reviewed by Leonard (1, 2) and refs therein]. These adducts became of major interest when they were found to be formed by a variety of environmental agents, as well as produced endogenously (3–7). Mutagenesis studies have shown a wide range of mutagenic frequency depending on the type of the adduct, type of mutation and the system used for detection and quantitation (8–19).

For more than one decade, this laboratory has focused on studies on the repair (20–26) and replication/transcription (8–12) of etheno derivatives of dA, dC, and dG. The differing structures of the etheno adducts and their effect on base pairing and base stacking (27–31) influences both repair and replication. Much of the data has been obtained by using prokaryotic systems, which are not always identical to those now found in the more widely used mammalian systems. Although no model experiment can reproduce exactly what occurs in human cells, there is considerable progress on understanding repair of mutagenic lesions by using human cells and tissues (reviewed in ref. 32 and refs. therein).

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It was established earlier in repair studies, by using a human system, that all four etheno adducts were released by HeLa cell-free extracts, indicating that they are substrates for DNA glycosylases (24). After partial purification from HeLa cells, 3,N^4-ethenocytosine (εC) repair activity was found to be separate from 1,N^6-ethenoadenine (εA) repair activity (25), which is a function of alkylpurine-DNA-*N*-glycosylase (APNG) (22). A knockout mouse lacking APNG was then used as a genetic approach to verify these *in vitro* data (26). Under these conditions, of the two etheno adducts tested, only εC was released by the cell-free extracts of such mice, indicating that there was a different gene product for εC repair (26). We also found that the glycosylase responsible for εC recognition had an unusually high molecular mass for a DNA glycosylase, as judged by size exclusion chromatography (32). The calculated value was close to that reported by Jiricny and coworkers (33, 34) for the human G/T mismatch thymine-DNA glycosylase.

In this work, we report that further purification of the εC glycosylase activity from HeLa cells showed that the εC activity resided in a 55-kDa polypeptide. This protein also had a coexisting G/T mismatch activity. Other glycosylase substrates tested were not cleaved by this purified human εC-DNA glycosylase. Our conclusion agrees with that of Saparbaev and Laval (35), who, after our experiments were completed, reported a similar finding by using a purified recombinant human G/T mismatch thymine-DNA glycosylase.

MATERIALS AND METHODS

Oligonucleotide Substrates. The sequences of oligonucleotides used in this study are listed in Fig. 1. The synthesis of the 3,N^4-ethenodeoxycytidine phosphoramidite and its incorporation into oligomers has been described elsewhere (36, 24). The sequence of the 45-mer duplex containing G/T (sequence 4) was previously described by Sibghat-Ullah *et al.* (37). The oligomers were synthesized by using an Applied Biosystem model 392 DNA synthesizer and purified by HPLC and/or PAGE.

Repair Enzymes. Recombinant thermostable G/T mismatch glycosylase was purchased from Trevigen (Gaithersburg, MD). Uracil-DNA glycosylase was obtained from GIBCO/BRL.

Preparation of εC-DNA Affinity Matrix. The εC-containing 16-mer (Fig. 1, sequence 2, upper strand) was annealed to a complementary strand to produce sticky ends. Then 260 mg of the duplex was lightly labeled with 5 μCi [γ-^{32}P]ATP (specific activity 6,000 Ci/mmol; 1 Ci = 37 GBq; Amersham). The ligation was carried out in the buffer containing 50 mM Tris·HCl (pH 7.8), 10 mM DTT, 10 mM $MgCl_2$, 1 mM

Abbreviations: εA, 1,N^6-ethenoadenine; εC, 3,N^4-ethenocytosine; m^{3}A, 3-methyladenine; U, uracil; AP, apurinic/apyrimidinic; APNG, alkylpurine-DNA-*N*-glycosylase; FPLC, fast protein liquid chromatography.

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For detection of εC activity:

1. 5'-CCGCTAGεCGGGTACCGAGCTCGAAT
   GGCGATC G CCCATGGCTCGAGCTTA-5'

For εC-DNA affinity matrix:

2. 5'-GATCGCTAGεCGGGTAC
   CGATC G CCCATGCTAG-5'

For detection of G/T(U) mismatch activity:

3. 5'-CCGCTAGTGGGTACCGAGCTCGAAT
   GGCGATCGCCCATGGCTCGAGCTTA-5'

4. 5'-ATTACGAATGCCCACACCGCTGGCGCCCACCACC
   TAATGCTTACGGGTGTGGCGGCCGCGGGTGGTGG

   ACTAGCTGGCC
   TGATCGACCGG-5'

5. 5'-CCGCTAGUGGGTACCGAGCTCGAAT
   GGCGATCGCCCATGGCTCGAGCTTA-5'

Unmodified oligomer duplex for competition:

6. 5'-CCGCTAGCGGGTACCGAGCTCGAAT
   GGCGATCGCCCATGGCTCGAGCTTA-5'

7. 5'-GATCGCTAGCGGGTACGATCGCTAGCGGGTACGA
   CTAGCGATCGCCCATGCTAGCGATCGCC CATGCT

   TCGCTAGCGGGTAC
   AGCGATCGCCCATG-5'

FIG. 1. Oligodeoxynucleotides used in this study. The numbers are referred to in the text. Mismatches are indicated in bold type.

hexamine cobalt chloride, 0.25 mM spermidine, 1 mM ATP, 25 μg/ml BSA, and 30 Weiss units of T4 DNA ligase (6,000 units/ml, New England Biolabs) for 7.5 hr at 15°C. The extent of ligation was checked by naturing PAGE after a 4-h incubation, at which point >90% of the 16-mer duplex was elongated to heterogeneous multimers.

Coupling the εC-DNA to the cyanogen bromide (CNBr)-activated Sepharose CL-4B (Pharmacia) was performed essentially as described (38). The εC-DNA-resin was equilibrated in PC buffer (25 mM Hepes-potassium hydroxide, pH 7.8/0.5 mM EDTA/0.125 mM phenylmethylsulfonyl fluoride/3 mM β-mecaptoethanol/10% glycerol). A 1.5-ml bed volume column was then poured and stored at 4°C before use.

Purification of εC-DNA Glycosylase. All steps were performed at 0–4°C. The starting material was 80 liters (105 g) of HeLa S3 cells (Cell Culture Center, Endotronics, Minneapolis, MN). The whole cell extract (Table 1, fraction I) and 65% $(NH_4)_2SO_4$ precipitate (Table 1, fraction II) were prepared as previously described by Rydberg *et al.* (20, 21).

The $(NH_4)_2SO_4$ precipitate was desalted by using Ultrafree-15 centrifugal filter devices (MWCO:15,000, Millipore). The sample was then divided into three parts and each was then passed through a phosphocellulose (P11) column (12 cm × 2.5 cm), which was packed and pretreated as directed by the manufacturer (Whatman) and equilibrated in PC buffer. After sample loading, the column was washed with 20 ml of PC buffer + 0.1 M KCl followed by 30 ml of PC buffer + 0.2 M KCl. A linear gradient (110 ml) from 0.2–1 M KCl in PC buffer was then used to elute the εC activity.

The active fractions from the phosphocellulose columns were pooled (Table 1, fraction III) and desalted by the same filter device described above to lower the conductivity to be equivalent to a KCl concentration of < 200 mM. The sample was then applied to a Blue Sepharose CL-6B column (8 × 2.5 cm) (Pharmacia), which was packed and equilibrated with three volumes of the PC buffer. The column was rinsed with 30 ml of PC buffer + 0.2 M KCl and developed with a linear gradient (100 ml) of 0.2–1 M KCl in PC buffer.

After the Blue Sepharose chromatography, the active fractions were pooled (Table 1, fraction IV), concentrated, diluted with PC buffer, and loaded onto a 1-ml Mono-S HR5/5 FPLC column (Pharmacia) equilibrated in PC buffer. The column was washed with 10 ml of PC buffer and eluted with a gradient of 30 ml from 0.1–0.5 M KCl in the buffer followed by a 3-ml gradient from 0.5–1 M KCl at a flowrate of 0.4 ml/min. Seven milliliters of active fractions was collected (Table 1, fraction V).

Fraction V was concentrated to 4 ml and diluted 1:3.5 with PC buffer. Then 30 μg of poly(dI-dC)·(dI-dC) was added to the fraction as a nonspecific competitor. After incubation at 4°C for 10 min, the sample was loaded, in aliquots, to the 1.5-ml εC-DNA affinity column (see previous section). Approximately 0.5 ml of the sample was allowed to be absorbed into the matrix each time and to stay for 5 min for binding. The column was then washed with 8 ml of PC buffer and developed with a 12-ml gradient from 0.1–0.8 M KCl in PC buffer at a flowrate of 0.3 ml/min. Fractions of 0.5 ml were collected and stored in siliconized tubes. The second εC-DNA affinity chromatography was carried out by using as competitor a 48-bp unmodifed oligomer duplex composed of three repeats of the 16-mer εC-oligomer used for the affinity matrix (Fig. 1, duplex 7). A linear gradient (11 ml) from 0.2–0.8 M KCl in PC buffer was applied to elute a 1-ml εC-DNA affinity column. Protein concentrations were determined by Bradford method (39).

SDS/PAGE and Silver Staining. Discontinuous SDS/PAGE was carried out according to Laemmli (40). The gels were stained by using a silver-staining kit according to the manufacturer's instructions (Pharmacia). SDS protein molecular mass markers were from BioRad (size range: 14.4–94.0 kDa).

Isolation and Renaturation of the εC Glycosylase from SDS/PAGE. Sixty microliters of the active fractions from the first εC-DNA affinity column was electrophoresed on a SDS/PAGE (5% stacking and 10% separating gel). The molecular mass markers and 5 μl of the same active fraction were run also in the side lanes and later silver stained. The unstained lane containing the bulky sample was cut into appropriate slices that were then each incubated in an elution buffer (33) overnight with vigorous shaking at 30°C. The denaturation with 6 M guanidine HCl (Sigma) and subsequent renaturation was carried out essentially as described by Hager and Burgess

Table 1. Purification of εC-DNA glycosylase from HeLa cells

Purification step	Volume, ml	Total protein, mg	Total εC activity, units*	Specific activity, units/mg	Yield, %	Purification fold, %
I. Whole cell extract	533	5,785	50,816	8.8		
II. Ammonium sulfate precipitate	286	3,639	40,841	11.2	80.4	1.3
III. Phosphocellulose (P11)	161	444	17,124	38.6	33.7	4.4
IV. Blue Sepharose	30	63.3	5,398	85.3	10.6	9.7
V. Mono-S	7	9	2,945	327.2	5.8	37
VI. εC-DNA affinity (1)	4.5	0.2	1,838	9,190	3.6	1,044
VII. εC-DNA affinity (2)	3.5	nd†	—	—	—	—

*One unit of εC-DNA glycosylase is defined as 1 fmol of the εC-oligomer cleaved after 10 min at 37°C.
†No measurable protein was recovered but there was sufficient εC activity to determine that G/T mismatch activity remained.

(41). The renatured protein samples were concentrated by using Centricon concentrators (MWCO:10,000, Millipore), which were pretreated with 0.5 mg/ml BSA solution.

Band Shift and Enzymatic Assay. For testing binding and enzymatic activities, the oligomers were 5′-end labeled with [γ-^{32}P]ATP and annealed to the complementary oligonucleotides as previously described (20). For duplexes 3, 4, and 5 in Fig. 1, the upper strands containing T or U were 5′-labeled. The 3′-end labeling was the same as described by Hang *et al.* (42).

The DNA binding and nicking assays were essentially carried out as described by Rydberg *et al.* (20, 21). For testing activities of the thermostable G/T mismatch *N*-glycosylase (43), the reactions were carried out at 65°C in a buffer containing 50 mM Tris·HCl (pH 7.50), 100 mM KCl, and 5 mM EDTA (pH 8.0).

The specific activity of εC active fractions was measured as follows: The εC-containing 25-mer (Fig. 1, sequence 1) was incubated at 37°C for 10 min with increasing amounts of pooled fractions from each column step. The ^{32}P-labeled uncleaved 25-mer and the 7-mer resulting after cleaving were scanned and integrated by using a PhosphorImager system (Molecular Dynamics). The relative activities were calculated from a linear portion of the curve. One unit of εC-DNA glycosylase is defined as 1 fmol of the εC-oligomer cleaved after 10 min incubation at 37°C.

RESULTS

Purification of Human εC-DNA Glycosylase. We had earlier partially purified human εC-DNA glycosylase by using three conventional chromatographic steps, which completely separated εC activity from εA activity (25). To further identify and characterize this activity, we extended the purification scheme by introducing εC-DNA affinity chromatography. Table 1 summaries the steps used in the purification. The relative activities of εC fractions were monitored by using a nicking assay described in *Materials and Methods*.

The current purification scheme started with whole cell extracts from 105 g of HeLa cells. The initial stability study using the $(NH_4)_2SO_4$ fraction showed that εC activity is relatively stable with remaining activity of 95% (−20°C), 81% (4°C), 57% (20°C), and 24% (37°C) after 24 hr in PC buffer (activity at −75°C is treated as 100%). $(NH_4)_2SO_4$ precipitation and filtration through a cation-exchange phosphocellulose column removed 92% of the protein, and there was a 4.4-fold enrichment of the specific activity (Table 1). The εC protein bound strongly to the cation P11 matrix, and one single peak of activity was observed (Fig. 2*A*). The use of a dye affinity column, Blue Sepharose CL-6B, yielded a further 10-fold enrichment of the activity. Moreover, the volume of the sample was reduced to 30 ml, which allowed the use of 1-ml Mono-S FPLC column in the next step (Table 1, step V). The distribution profile of εC activity from this column is shown in Fig. 2*B*. Mono-S FPLC cation-exchange chromatography produced an overall 37-fold increase in purification. This step removed contaminating 5′ AP endonuclease(s) as judged by the cleavage pattern on denaturing PAGE (data not shown).

The next step of purification, εC-DNA affinity (1) chromatography (Table 1, step VI), produced the greatest increase in specific activity, 1,044-fold. We previously demonstrated an εC-binding protein in HeLa crude extracts and partially purified fractions (24, 25). In this work, the binding activity was found to copurify with the εC-nicking activity as shown in Mono-S fractions (Fig. 3*A*). The protein-DNA complex was shown to be stable for at least 1 hr at 4°C and was damage (εC)-specific (Fig. 4). We then could take advantage of these properties and prepare the εC-DNA affinity matrix. In the first

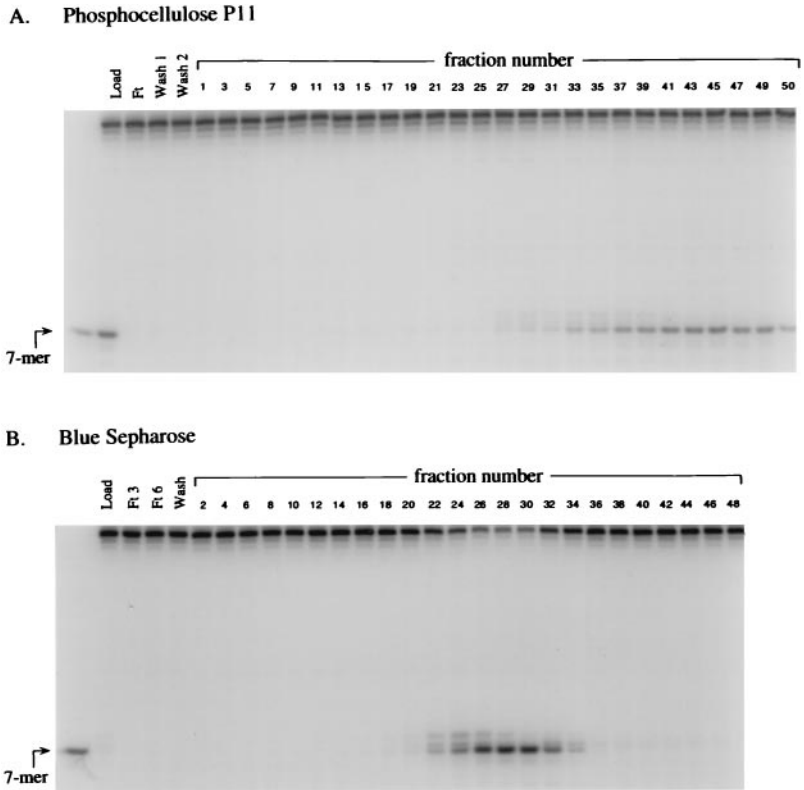

FIG. 2. Autoradiogram of 12% denaturing PAGE showing εC activity profile from two column steps of purification. (*A*) Fractionation of εC activity by using phosphocellulose P11 chromatography (Table 1, step III). The cleavage of the 25-mer εC-oligomer (Fig. 1, seq. 1) is shown as indicated by the 7-mer size marker on the left. The peak of the activity elutes at 0. 9 M KCl. (*B*) εC activity profile from Blue Sepharose CL-6B chromatography (Table 1, step IV). The peak of the activity elutes at 0.7 M KCl. In both *A* and *B*, 3-ml fractions were collected and 2.5 μl of each was tested in a total of 10 μl of reaction at 37°C for 1 hr as described in *Materials and Methods*. Ft, flow through.

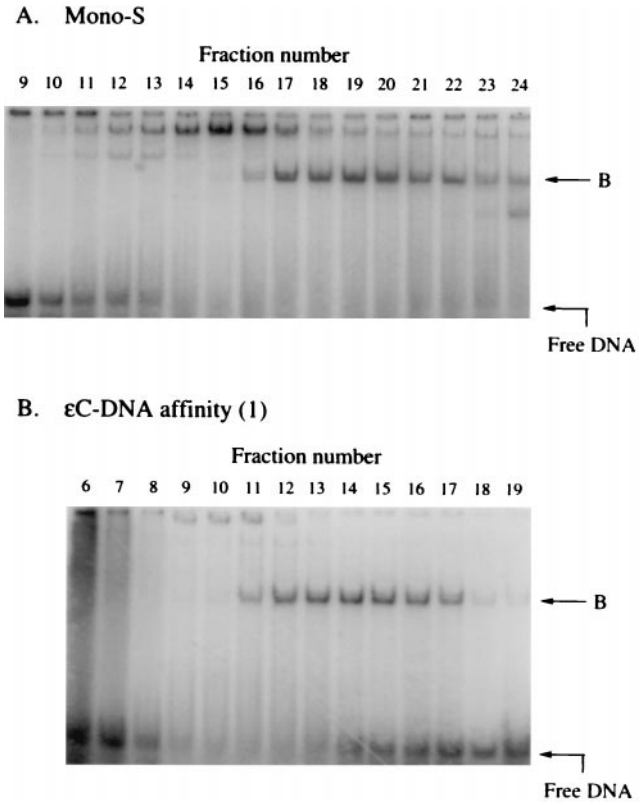

FIG. 3. εC binding activity in fractions from Mono-S (Table 1, step V) (*A*) and εC-DNA affinity (1) (Table 1, step VI) (*B*). Two and one-half microliters of fractions were used to react with 20 fmol εC-oligomer (Fig. 1, seq. 1) at room temperature for 30 min in a band-shift assay as described by Rydberg *et al.* (20). On the right of the gels, B indicates the binding band that correlates with the nicking pattern of the same column fractions (Fig. 5). The unbound DNA also is indicated.

round of purification, εC nicking activity was detected in fractions eluted from 0.45–0.65 M KCl (Fig. 5*B*) and was in agreement with the profile of the εC-binding activity in the same fractions (Fig. 3*B*). The second εC-DNA affinity chromatography (Table 1, step VII) led to a very low recovery of the εC activity so that the protein concentrations in all fractions were undetectable even under the silver staining. However, there was detectable εC activity at the same KCl concentration eluting εC activity in the first affinity column.

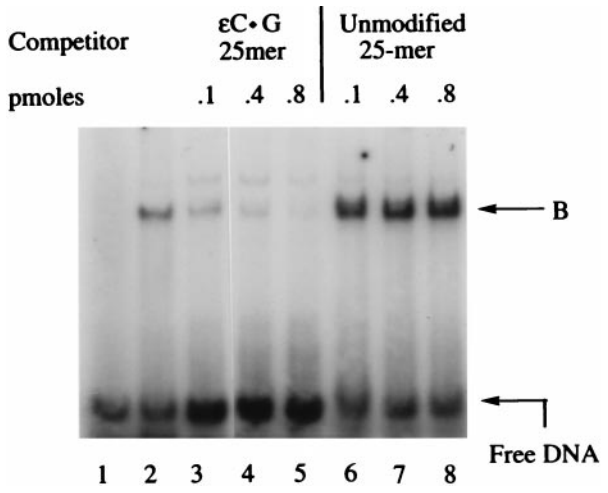

FIG. 4. Competition-binding assay of ^{32}P-labeled εC-oligomer (Fig. 1, seq. 1) with the same unlabeled εC-oligomer and with unmodified oligomer (Fig. 1, seq. 6); 0.02 pmol ^{32}P-labeled εC 25-mer was incubated at room temperature for 30 min with 2.5 μl of Mono-S fractions in the presence of increasing amounts of unlabeled εC- (lanes 3–5) or unmodified (lanes 6–8) oligomers. Lane 1 is the control without protein, and lane 2 is with protein but no competitor. For symbols, see Fig. 3. Note that the unmodified 25-mer lacking εC did not compete for εC binding (lanes 6–8).

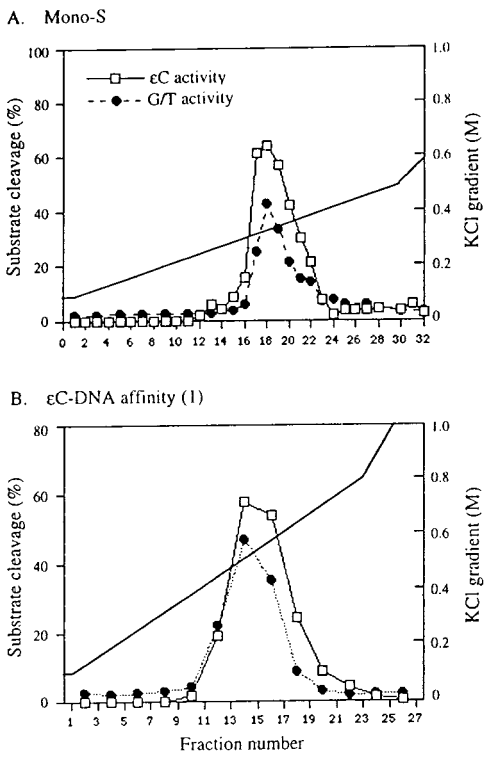

FIG. 5. Coelution of εC (—□—) and G/T (—●—) glycosylase activity from Mono-S (Table 1, step V) and εC-DNA affinity (1) chromatography (Table 1, step VI). The percentage of cleavage is shown on the left and the gradient on the right. The substrate used for εC glycosylase activity is oligonucleotide duplex 1 in Fig. 1 and for the G/T activity the probe is oligonucleotide duplex 4 in Fig 1. The assay conditions are the same as in Fig. 2. Note that assays for the two activities were carried out separately so that the absolute cleavage does not reflect the relative ratio of the two activities.

The apparent molecular mass of the εC activity was determined by isolating the protein bands from SDS/PAGE of the first εC-DNA affinity fractions and subsequent renaturation of the eluted proteins (*Materials and Methods*). The enzymatic test indicated that the target band is ≈55 kDa (Fig. 6, arrow). This size is unusual for DNA glycosylases, which are generally between 25–40 kDa (44). We had earlier reported a similarly large size (≈66 kDa) for the same activity by using gel filtration chromatography (32). This apparent discrepancy in size for the same activity is attributed to the methods used.

We previously reported the εC release from DNA by HeLa cell-free extracts (24). In this study, a nicking assay by using 3′ end-labeled substrate oligomers and purified fractions was performed to confirm this glycosylase-mediated mechanism. Indeed, the same cleavage pattern was found when the εC oligomer was treated with purified fractions, as when a uracil-containing oligomer was treated with uracil-DNA glycosylase, suggesting that the same mechanism was used by εC activity as that for uracil-DNA glycosylase (data not shown).

Coexistence of εC and G/T Mismatch Repair Activity. As stated above, the molecular mass for εC activity was close to the unusually large size of the purified and cloned G/T mismatch thymine-DNA glycosylase (33, 34). We then tested the newly purified εC-DNA glycosylase for possible G/T mismatch activity in the course of purification. The coelution

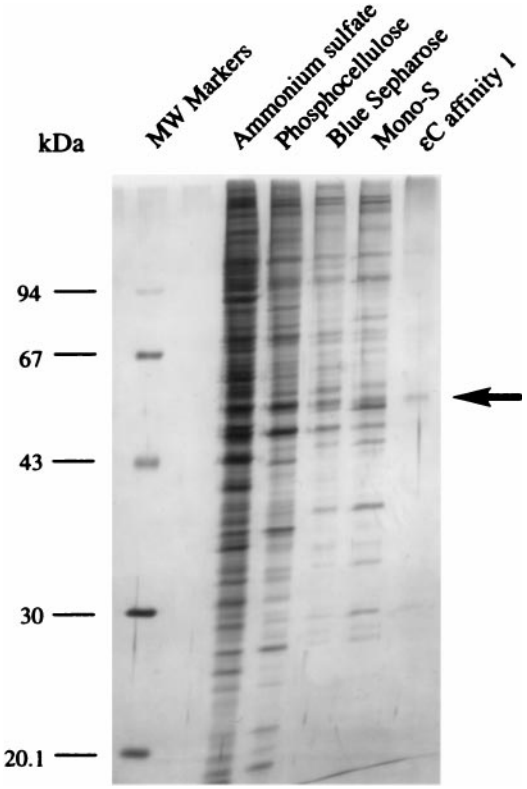

FIG. 6. Silver-stained SDS/PAGE of pooled fractions from purification steps of the HeLa εC-DNA glycosylase. On the left are molecular mass markers (BioRad) in kilodaltons, and on the right the arrow indicates the target protein band, which is ≈55-kDa. The gel slice containing this band, after protein elution, denaturation, and renaturation (see *Materials and Methods*), showed both εC and G/T mismatch cleavage activity.

of these two activities was found in the last three column runs. The superimposed peak activities for steps V and VI are shown in Fig. 5.

The isolated 55-kDa polypeptide in Fig. 6 also showed activity toward both εC and G/T mismatch substrates. To test the possibility that two polypeptides with same size coeluted, competition assays were performed in which the εC binding of first affinity fraction was competed with oligomers containing εC·G, G/T, or G/U. All the mismatches were specific competitors with εC·G and G/U the most efficient (data not shown). G/U, in double stranded form, is also a substrate for the human G/T mismatch glycosylase and showed better protein binding than G/T (33). We thus concluded that a single repair protein had both activities. Finally, a commercial bacterial recombinant thermostable G/T glycosylase originally found in *M. thermoautotrophicum* THF (43) was tested in the same manner. Again both εC and G/T mismatch oligomers were cleaved (Fig. 7).

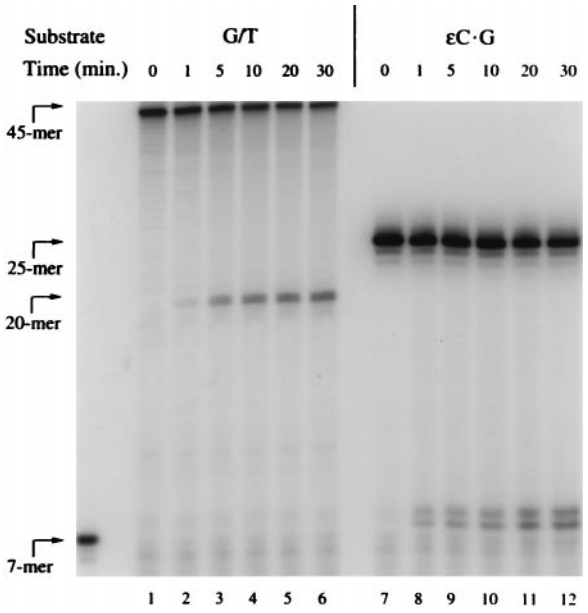

FIG. 7. Time course of cleavage of G/T (Fig. 1, seq. 4) and εC·G 25-mer (Fig. 1, seq. 1) by using DNA mismatch *N*-glycosylase of the thermophilic archaeon *M. thermoautotrophicum* THF; 0.05 unit of enzyme was incubated with 20 fmol oligomer substrates at 65°C for indicated times under the conditions described in *Materials and Methods*. In each case, the furthest migrating bands are the expected cleavage products. The 7-mer marker is shown on the left. The size of the substrate oligomers used are indicated also by their migration.

DISCUSSION

It is not unusual that repair enzymes can have a broad substrate range (reviewed in refs. 32, 44, 45). However, it is difficult to understand what the recognition signal(s) is for such enzymes. In this laboratory, it was unexpected that the human APNG could not only cleave εA but with greater efficiency than the original substrate, 3-methyladenine (23). A second recent example is the repair of bulky p-benzoquinone modified dA, dC, and dG by human and *Escherichia coli* 5′-AP endonucleases (32, 42, 46), inasmuch as the p-benzoquinone moiety adds two additional rings to the bases (47).

In the present work and that of Saparbaev and Laval (35), the two types of mismatches recognized by a single enzyme are εC·G and G/T(U). In the case of G/T(U) mismatches, there is a strong biochemical rationale for their repair because G/T(U) can be produced by deamination of 5-methylcytosine or cytosine, which is estimated to occur with a frequency of $\approx 1.7 \times 10^2$ events/day/10^{10} bases for deamination in rat liver (48). Thus, it would be expected that cells have developed a repair mechanism to ensure survival and genetic integrity.

εC is an environmentally induced modified base but also arises from metabolic processes (3–7). It differs from the effects of deamination of 5-methylcytosine or cytosine in that these latter bases are changed to another normal base, which directly changes the DNA sequence. In contrast, εC is a noninstructive base, which miscodes in replication, both *in vitro* and *in vivo*. *In vitro*, generally adduct-directed mutation frequency is higher than observed *in vivo* (reviewed in ref. 49). The reason is simple: *in vitro* experiments measure only base–base interactions in the presence of a purified polymerase, whereas *in vivo* there are multiple biochemical factors influencing replication. The primary ones are repair and the specificity and multiplicity of DNA polymerases, which are necessary for correction or prevention of miscoding. When qualitatively changed base pairing is the same *in vitro* and *in vivo*, it is usually clearer as to which normal bases can be inserted opposite modified bases.

In the case of εC there is a high frequency of pairing with A or T *in vitro*, in both transcription (8) and replication (10, 17, 18). The work of Singer and Spengler (10), Zhang *et al.* (17), and Shibutani *et al.* (18) all agree that there is little or no εC·C or εC·G pairing that occurs. *In vivo*, by using different systems and assays, the same preferences for εC pairing with A or T were reported by several groups (14–16). Mutation frequency varied but primarily εC → T transitions and εC → A transversions resulted. Lethality was generally high, but of the surviving cells, there were up to 80% mutants (16).

It therefore appears logical that a repair system in the cell is also necessary for the removal of the εC base. Indeed, εC is

efficiently cleaved by the human DNA glycosylase described in this work, as compared with the G/T, even though final proof that multiple activities reside in the same enzyme must await genetic approaches such as a gene knockout. The implication that the εC activity is evolutionarily preserved or results from adaptation also suggests a real *in vivo* role of the enzyme in counteracting those mutagenic effects resulting from εC.

The finding that one DNA glycosylase acts on two seemingly unrelated substrates, εC·G and G/T, poses an interesting structural problem. Do these two substrates appear the same to the enzyme in terms of adduct structure as well as local perturbation of the nucleic acid? Or can the glycosylase accommodate additional yet unknown substrate structures? If so, what are the limits?

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