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Human uracil DNA N-glycosidase: studies in normal and repair defective cultured fibroblasts

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ABSTRACT

Uracil DNA N-glycosidase, an enzyme which participates in the excision of uracil from DNA, was measured in extracts from fibroblast lines cultured from normal subjects, from several subjects with the genetic disease xeroderma pigmentosum, and from a subject with ataxia telangiectasia. The cell lines representative of complementation groups A and D of xeroderma pigmentosum and of ataxia telangiectasia had roughly the same level of activity as did the normal cells. On the other hand, cells from two xeroderma pigmentosum variants (XP4BE and XP13BE) had roughly half the normal level of activity, and cells from the heterozygous mother of XP4BE had an intermediate level of activity. In spite of these quantitative differences, no systematic alterations in reaction characteristics, apparent K_m for substrate, or purification characteristics were noted for enzyme from any of the lines. Thus a causal relationship, if any, between levels of activity and the disease symptoms is equivocal.

INTRODUCTION

Uracil residues apparently can be introduced into DNA by two mechanisms: the spontaneous deamination of DNA-cytosine, or the incorporation of deoxyuridylate from dUTP in place of thymidylate during DNA synthesis. The deamination of cytosine can occur under physiological conditions (1,2) and would, of course, require correction in order to eliminate a resulting transition mutation. The incorporation of deoxyuridylate from dUTP might occur at a high frequency, but experimental evidence is indirect. First, dUTP is efficiently incorporated into DNA by DNA polymerase *in vitro* (3); second, mutants of *E. coli* which have a decreased dUTPase[1] activity with a consequent higher pool of dUTP have abnormally small Okazaki fragments. Such fragments are thought to be intermediates in the pathway for the excision of uracil from DNA (4).

A candidate for an enzyme involved in the excision of uracil introduced into DNA by either process is uracil DNA N-glycosidase. The enzyme

cleaves the glycosidic bond between uracil and the deoxyribose moiety of the DNA (5,6) and the resulting apyrimidinic site is then presumably removed from the DNA by excision repair utilizing an apurinic/apyrimidinic endonuclease to incise the DNA. On the assumption that a defect in the glycosidase might result in a propensity toward transition mutations, and in the case of mammals toward carcinogenesis, we have initiated a study of this enzyme in cells cultured from individuals with so-called repair deficiency diseases. By a similar approach we had shown that certain lines from persons with the disease xeroderma pigmentosum (XP) have apurinic/apyrimidinic endonuclease activity with an abnormal substrate affinity (7), and that this defect is due to the absence of one of two isozymic species of the enzyme (Kuhnlein, U., Lee, B., Penhoet, E.E., and Linn, S., unpublished).

In this report we analyze uracil DNA N-glycosidase activity in extracts of human fibroblasts obtained from normal subjects, from subjects with XP, and from a subject with ataxia telangiectasia (AT). XP and AT patients have a high risk of developing cancer and their cultured cells are deficient in repair of DNA lesions caused by UV irradiation (XP) or X-rays (AT) (9,10). The UV excision repair deficiency in the case of XP falls into at least five genetic complementation groups (8), and, in addition, another "variant" form exists in which subjects have the clinical symptoms of XP, but cultured fibroblasts have normal excision repair. The latter cells are thought to be deficient in post-replication repair (11).

In a preliminary report Sekiguchi, et al. (12) have studied and measured the glycosidase in extracts from a normal cell line and from an XP line of the A group, finding no differences between the two.

MATERIALS AND METHODS

Growth of cells and preparation of extracts: Cell line 424 is a foreskin fibroblast cell line obtained from the Naval Biological Labs, Oakland, California; the other fibroblast lines are from skin biopsies and were obtained from the American Type Culture Collection (ATCC). Cell line numbers and complementation groups are listed in Table 1 below.

Cells were grown in 32 ounce prescription bottles with 50 ml of Dulbecco's modified Eagle's medium (Gibco) with 4.5 mg/ml dextrose and 10% fetal calf serum (Irvine Scientific); no antibiotics were added. Incubation was at 37°C in a CO_2 incubator. Confluent cells were "split" 1:4 after treatment with trypsin and periodic checks for mycoplasma were made either by determining the uptake ratio of uridine to uracil (13) or by

staining with "supermouse" (14).

Cells were harvested in batches of 8 to 12 bottles and suspended in 40 ml of phosphate-buffered saline (25 mM potassium phosphate, pH 7.0 - 0.15 M NaCl - 0.015 M sodium citrate) by scraping from the bottle surface. They were washed twice in phosphate-buffered saline by centrifugation and resuspension and finally resuspended in 2 ml of 50 mM Tris-HCl, pH 7.5, and stored in liquid nitrogen. For extraction, the thawed cell suspension was sonicated for three 15-sec intervals with a Biosonic sonicator (needle probe, 50 W). The sonicate was centrifuged for 60 min at 45,000 rpm in a Beckman Type 50 rotor and the pellet discarded. This "high speed supernatant" fraction was stored in liquid nitrogen. Ten prescription bottles generally yielded between 3 and 4 mg of extract protein, though double this level could be obtained by prolonged culturing of the cells.

Column chromatography: All operations were carried out at 2° or on ice. The high speed supernatant was made 0.4 M in NaCl by the addition of 5 M NaCl and the extract was passed through 0.7 ml of type 40 DEAE-cellulose which had been equilibrated with 0.4 M NaCl - 50 mM Tris-Cl, pH 7.5. Fractions of 0.75 ml were collected, and the four fractions containing significant activity were pooled and dialyzed overnight against two 200-ml aliquots of 10 mM potassium phosphate, pH 7.4. The dialyzed DEAE-pool was then applied to a 3.5 ml column of Whatman P-11 phosphocellulose in a plastic syringe which had been equilibrated with the dialysis buffer. The column was washed with 5 ml of 10 mM potassium phosphate, pH 7.4, then 3 ml of 50 mM potassium phosphate. It was finally eluted with a 40-ml linear gradient between 50 mM and 275 mM potassium phosphate, pH 7.4. Fractions of 1 ml were collected into plastic tubes and 10 µl of 10 mg/ml acetylated BSA were added to each fraction in order to stabilize the enzyme. Activity did not appreciably decrease during storage of the fractions on ice for several days. The recovery of both activity and protein from DEAE-cellulose was 70 - 100%; that of activity from phosphocellulose was roughly 50%.

Assay of uracil DNA N-glycosidase: Activity was measured upon [^{32}P, uracil-^{3}H]DNA obtained from the Bacillus subtilis phage, PBS-2. The DNA naturally contains uracil in place of thymine, and was prepared as described by Gates and Linn (15). It had 28,000 ^{3}H cpm per nmol and, initially, 17,300 ^{32}P cpm per nmol. The reaction mixture (0.1 ml) contained 3.3 µM PBS-2 DNA (expressed as total DNA nucleotide), 50 mM Tris-HCl, pH 7.5, 4 mM EDTA and 100 µg/ml acetylated BSA. After incubation at 37° for 30 min

the reactions were chilled and 0.1 ml 5 mg/ml BSA and 0.25 ml of 7% trichloroacetic acid were added. After 5 min on ice, the reactions were centrifuged at 10,000 X g for 5 min and the radioactivity in 0.3 ml of the supernatant fluid was counted by liquid scintillation. One unit of activity releases 1 pmole/min of uracil from the DNA. PBS-2 DNA contains 36% uracil (16).

Other methods: Protein was determined by the procedure of Lowry et al. (17). Buffer pH was routinely measured at 50 mM at room temperature and salt concentrations were determined with a Radiometer conductivity meter. BSA was acetylated with acetic anhydride according to Dowhan (18).

RESULTS

Analysis of the activity in crude extracts: Uracil DNA N-glycosidase can be determined by measuring the release of uracil from the DNA of phage PBS-2. The DNA of this Bacillus subtilis phage contains uracil in the place of thymine and can be specifically labelled in the uracil residues by using a uracil auxotrophic host-strain (19). Figure 1 shows the time dependence of the specific release of uracil catalyzed by a high speed centrifugation supernatant of an extract from normal human fibroblasts. The initial reaction velocity was proportional to the concentration of extract, and non-specific exonuclease activity did not obscure the glycosidase activity as noted by the relatively slight release of phosphate from the DNA.

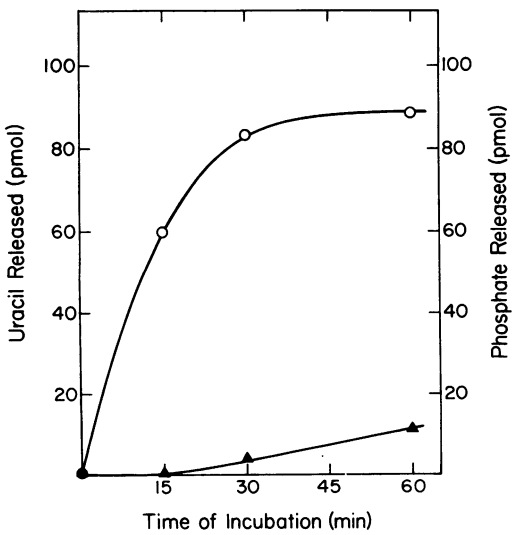

Figure 1: Time course of the uracil DNA N-glycosidase reaction. Assays were as described in Materials and Methods with 0.38 mg/ml extract protein of a high speed supernatant fraction of 424 foreskin fibroblasts. Uracil released (o) and phosphate released (▲) were measured as counts rendered acid-soluble.

The specific activities of uracil DNA N-glycosidase in extracts from some normal cell lines, several xeroderma pigmentosum (XP) cell lines, and an ataxia telangiectasia (AT) cell line are listed in Table I. The activities of the cells of XP complementation groups A and D appeared to be near normal. (These were the groups shown to have altered apurinic/apyrimidinic endonuclease in a previous study (7).) The AT cell line also had normal activity, but two XP variant cell lines had consistently lower levels of activity. When the measurement was repeated with a subclone of the XP4BE variant cell line which had been cultured independently for several passages, the same activity was found as in the first extract (51 vs. 44

TABLE I Specific Activities of Various Extracts

Cell Line[a]	Genotype	Specific Activity (units/mg)
424	normal foreskin fibroblast line	79[b] 95[b]
CRL 1262	"normal"[c]	110
CRL 1343 (Se Pan)	Ataxia telangiectasia	70
CRL 1223 (XP12BE)	XP Complementation Group A	69
CRL 1160 (XP5BE)	XP Complementation Group D	86
CRL 1200 (XP7BE)	XP Complementation Group D	74
CRL 1258 (XP13BE)	XP variant	45
CRL 1162 (XP4BE)	XP variant	44[b] 51[b]
CRL 1172	mother of XP4BE, presumed heterozygote	65

(a) CRL numbers are those of the American Type Culture collection. Patient codes of Robbins, et al. (20) are also included where applicable.

(b) These were measured with two separate extracts.

(c) CRL 1262 is derived from a patient with the unrelated disease, osteogenesis imperfecta, but is considered normal for these studies.

Specific activities were determined as described in Materials and Methods with an incubation of 30 min at 37° and 0.07 - 0.19 mg extract protein per ml. All cells were between passages 10 and 20, except for CRL 1160 which was at passage 25 and the second determination of line 424 which was at passage 38. The cell protein obtained per culture flask was generally 0.3 - 0.4 mg with the exception of the CRL 1258, CRL 1223 and the second determination of line 424 which had 0.05, 0.67 and 0.88 mg protein per flask, respectively. These results imply that there is no dependence of the specific activities measured upon either the passage number or the cell density within the ranges utilized.

units/mg). In addition, the cells from the heterozygous mother of XP4BE had a level of activity intermediate between XP4BE and normal. The activity levels did not appear to correlate with the cell density (as measured as amount of extract protein obtained per culture flask) nor with the passage levels in the interval used. (See legend to Table I.)

By analogy with our studies for the apurinic/apyrimidinic endonuclease, the apparent K_m for substrate of the glycosidase was measured with extracts from representative cell lines. The enzyme followed Michaelis-Menten kinetics (Fig. 2), and no significant differences were noted among extracts of the various lines (Table II), even when comparing an extract derived from an XP-variant with a relatively low specific activity to that of a normal line (Fig. 2). The values obtained were generally in the range of 0.35 - 0.55 μM DNA-deoxyuridylate when PBS-2 DNA was used as substrate (Table II). Hence, the K_m for the uracil DNA N-glycosidase is several orders of magnitude greater than that for apurinic endonuclease in the unfractionated extracts (approximately 1 nM depurinated sites (7)) or for the two major isozymic species of endonuclease after purification (5 and 44 nm, respectively (Kuhnlein, U., Lee, B., Penhoet, E.E., and Linn, S., unpublished)).

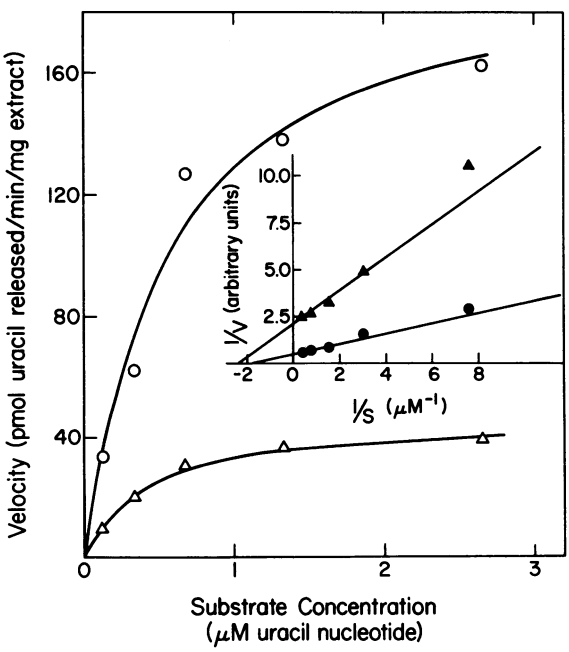

Figure 2: Apparent K_m of uracil DNA N-glycosidase. Reaction mixtures contained 0.15 mg/ml CRL 1262 "high speed supernatant" fraction (○, ●) or 0.29 mg/ml of the same fraction from XP4BE (△, ▲). Incubations were for 15 min, and curves were fitted, and apparent K_m values calculated (see Table II) according to the method of Wilkinson (21).

TABLE II Apparent K_m Values with Extracts from Various Cell Lines

Cell Line and Genotype	Apparent K_m (μM uracil nucleotide)
424 -- normal	0.45 ± 0.13
CRL 1262 -- normal	0.55 ± 0.13
CRL 1160 (XP5BE) -- XP-D	0.42 ± 0.29
CRL 1200 (XP7BE) -- XP-D	0.54 ± 0.13
CRL 1258 (XP4BE) -- XP-variant	0.42 ± 0.02
CRL 1343 (Se Pan) -- AT	0.34 ± .08

K_m values and standard errors were calculated by the regression analysis of Wilkinson (21) from assays similar to those of Fig. 2. Cell lines are further defined in Table I.

Analysis of the activity upon partial purification: High speed supernatant extracts were freed of nucleic acids by filtration through DEAE-cellulose in high salt, then partially purified by phosphocellulose chromatography as described in Materials and Methods. Neither the DEAE-cellulose column, nor a subsequent dialysis had a major effect upon the specific activity of the enzyme. Upon phosphocellulose chromatography a small fraction of the activity passed through the column, but the bulk of the activity eluted between 125 and 225 mM potassium phosphate. The enzyme often eluted in several peaks in this region, but no systematic differences in elution pattern were noted among the various cell lines. In particular, the XP variant lines showed similar elution characteristics to the others, in spite of the lower amount of activity that they contained. Recoveries of activity off of the phosphocellulose columns averaged about 50 percent and the purification was greater than 10-fold; exact purification factors are not available because the protein concentrations were too low (< 30 μg/ml). No changes in catalytic properties were noted after the chromatography.

DISCUSSION

Uracil DNA N-glycosidase activity is present in relatively high levels in the crude extracts of human fibroblasts. We have previously analyzed apurinic/apyrimidinic endonuclease activity in extracts from normal cells and from XP cells (7). This enzyme is thought to be involved in the

excision of apurinic or apyrimidinic sites from the DNA, some of which are probably generated by the removal of uracil from DNA by uracil DNA N-glycosidase (22,23). Apurinic/apyrimidinic endonuclease is present at a similar high level of activity as uracil DNA N-glycosidase, but as noted above it has an apparent K_m for substrate that is more than 100-fold lower. Thus, of the first two steps of the presumptive excision pathway for uracil, the cleavage of the glycosidic bond would appear to be rate limiting.

Uracil DNA N-glycosidase and apurinic/apyrimidinic DNA endonuclease are separated by phosphocellulose chromatography. The DNase elutes from phosphocellulose in two fractions, one in the pass-through of the column, and a second fraction eluting after glycosidase at 225 - 275 mM potassium phosphate (Kuhnlein, U., Lee, B., Penhoet, E.E., and Linn, S., unpublished). No major uracil DNA N-glycosidase activity is present in these fractions, indicating that the two activities are separable.

Although the XP variant cell lines had a somewhat reduced amount of uracil DNA N-glycosidase activity, the significance of the observation is equivocal. The enzyme from these lines did not prove to be more thermolabile than that from normal cells. Also, there is no obvious correlation between this reduction and the generally-accepted molecular defect in the variant cell lines -- the relatively slow rate of sealing of gaps in DNA that were induced by ultraviolet irradiation (11). XP variant cells are also sensitive to caffeine (24) and have a low level of photo-reactivating enzyme (25). Perhaps the clinical symptoms associated with the disease are due to a summation of minor defects in several repair pathways. At any rate, it would be of interest to extend the studies of the glycosidase to cells derived from individuals with other "repair-deficiency" diseases. However, such an extension might be premature until the complementation analyses for these diseases reach the same levels of sophistication as those obtained for XP (8).

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FOOTNOTES

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[1] Abbreviations: dUTPase, deoxyuridinetriphosphatase (EC 3.6.1.23); XP, xeroderma pigmentosum; AT, ataxia telangiectasia; BSA, bovine serum albumin; UV, ultraviolet light.

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