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Study on the Specificity Alteration of Mammalian UV Endonuclease III

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Abstract : A mammalian DNA repair enzyme, UV endonuclease III which also functions as a ribosomal protein S3 (rpS3), was purified from mouse cells and characterized. UV endonuclease III was previously cloned and known to yield a peptide of 32 kDa upon expression in *E. coli* [Kim *et al.*, (1995) *J. Biol. Chem.* **270**, 13620-13629]. However, biochemically purified UV endonuclease III, which has a sedimentation coefficient of 3.2S, appears to have an additional peptide of 28 kDa. It appears that two bands were derived from one complex, judging from the comparison of the nuclease activity on the native and SDS-gel electrophoreses. UV endonuclease III becomes non-specific upon purification and this phenomenon is more significant in the case of pure fractions of the enzyme. Non-specific activity was not influenced by pH or any salt conditions.

Key words : DNA repair, endonuclease, specificity, ribosomal protein S3, UV damage.

UV light induces DNA lesions in cells, causing mutagenesis (Brash and Haseltine, 1982; Protic-Sabljic *et al.*, 1986), carcinogenesis and inhibition of DNA replication (Berger and Edenberg, 1986). These deleterious effects are corrected to a large extent by cellular DNA repair systems such as excision repair. Excision repair is an error-free repair system that removes bulky lesions in DNA. These include pyrimidine dimers produced by UV-irradiation and oxidative DNA damages introduced by reactive oxygen species or ionizing radiation, mismatched bases or base modifications in DNA.

Previous study has shown that there are three such excision DNA repair endonucleases from murine plasmacytoma cells that specifically nick DNA which was heavily irradiated with ultraviolet (UV) light - UV endonucleases I, II and III (Kim and Linn, 1989; Kim *et al.*, 1995). Murine UV endonuclease III, whose sequence has 100% identity with rat ribosomal protein S3 (rpS3), was cloned and expressed in *E. coli* to yield a peptide of M_r=32,000. Both human and murine UV endonucleases III have associated apurinic/apyrimidynic (AP) endonuclease activity which cleaves 3' side of AP site *via* a β-lyase mechanism, thus generating an inefficient primer for DNA polymerases (Kim and Linn, 1988; Kim *et al.*, 1995).

Another set of data recently revealed that the enzyme cleaves the phosphodiester bond between the pyrimidine-dimer as the very first step of excision repair in mammalian systems (Kim *et al.*, 1995). UV endonuclease III appears to be a missing factor of XP group D cells and identical to human AP endonuclease I which has a sedimentation coefficient of 3.2S, an enzyme reported previously by Kuhnlein *et al.* (1978).

XP is a rare, autosomal recessive human disease that is characterized clinically by hypersensitivity to UV irradiation, resulting in a 2000- to 4800- fold increase in frequency of sunlight-induced skin cancers (Kraemer *et al.*, 1984). UV-induced skin lesions and, occasionally, progressive neurologic degeneration are also observed in XP-A and XP-D cells (Kraemer *et al.*, 1987). Purified UV endonuclease III appears to lose its substrate specificity upon purification or storage.

In this study, we purified murine UV endonuclease III to homogeneity and studied the non-specific activity of the protein.

Materials and Methods

Materials

Murine plasmacytoma cell strain MPC-11 was ob-

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tained from American Type of Culture Collection. Supercoiled PM2 phage [^{3}H-thymidine] DNA (5000~16000 cpm/nmol) was purified as described (Kuhnlein *et al*., 1976). Sephacryl S-200 was from Pharmacia, phosphocellulose from Sigma, DE-52 from Whatman, hydroxylapatite from Bio-Rad. Heparin agarose resin was prepared according to Davidson *et al*. (1979) using heparin and CNBr from Sigma and agarose from Bio-Rad. BA85S597 0.45 mm nitrocellulose membrane filters were from Schleicher and Schuell. [Methyl-^{3}H] Thymidine was from Amersham.

Cell culture

MPC-11 cells were grown and split 1:2 daily in suspension medium containing 50% Dulbecco's modified Eagle's medium (DME), 50% RPMI 1640, 20% heat-inactivated horse serum, 2 mM λ-glutamine, 100 U/ml each of penicillin and streptomycin, 0.02 M Hepes (pH 7.2) and 50 mM 2-mercaptoethanol.

Preparation of damaged DNAs

PM2 DNA was UV-irradiated in 10 mM Tris-HCl (pH 7.5) and 0.02% glycerol at 2.5 J/m^2/sec for 3.5 min with a Westinghouse gemicidal lamp (G15T8). This total dose of 525 J/m^2 produces approximately 2 sites sensitive to *E. coli* endonuclease III per PM2 DNA duplex (Demple and Linn, 1982). Depurination of PM2 DNA was carried out at 700C in 10 mM sodium citrate (pH 4.5), 100 mM NaCl for 10 min so as to generate 1.5 AP sites per DNA duplex.

Endonuclease assays

The filter-binding nicked circle assay with nitrocellulose filters and PM2 DNA was used to determine endonuclease activity. Standard reaction mixtures for UV endonuclease contained 40 mM Tris-HCl (pH 8.0), 70 mM KCl, 0.01% Triton X-100, 3 mM EDTA, 10 mM 2-mercaptoethanol and 20 mM UV-irradiated PM2 [^{3}H]DNA substrate. AP endonuclease assays were similar except that depurinated PM2 [^{3}H]DNA was the substrate. For either substrate, the reaction mixtures (100 ml) were incubated for 15 min at 37°C and then stopped by the addition of 100 ml of 0.3 M K_2HPO_4 (pH 12.4), which partially denatures the PM2 DNA. For total denaturation, 100 ml of 0.3 M K_2HPO_4 (pH 13.2) was added or the reaction mixture was spotted directly on the filter. After 5 min, the samples were neutralized with 50 ml of 1 M KH_2PO_4 (pH 4.0). Five minutes later, 100 ml of 5 M NaCl was added. This treatment results in the renaturation of Form I but not Form II DNA. Each sample was finally diluted to 3 ml with 50 mM Tris-HCl (pH 8.2), 1M NaCl and filtered through a Schleicher and Schuell nitrocellulose filter which selectively retains the denatured DNA. The filters had been previously equilibrated with the same buffer. After being washed with 5 ml of 50 mM Tris-HCl (pH 8.2), 1 M NaCl and then with 5 ml of 0.3 M NaCl, 0.02 M sodium citrate, the filters were dried and counted by liquid scintillation.

The average number of nicks introduced per PM2 genome was calculated from the percentage of total PM2 [^{3}H]DNA bound to each filter assuming a Poisson distribution of endonucleolytic sites as described by Kuhnlein *et al*. (1976). One unit of endonuclease activity specifically introduces 1 fmol of nicks into damaged DNA per min at 37°C.

Purification of UV endonuclease III activity from MPC-11 cells

UV endonuclease III was purified to homogeneity to study the non-specific activity as reported recently by Kim *et al*. (1995). All procedures were carried out at 0~4°C and all chromatography buffers were sterilized by autoclaving. Purification procedures were adopted from Kim *et al*., (1995).

Ninety-six liters of culture (1.1×10^{11} cells) in logarithmic growth were harvested − 8 liters at a time, washed once with phosphate-buffered saline (PBS), frozen as a pellet in liquid nitrogen and stored at −70°C for upto 30 days.

Cells were thawed and resuspended in a final volume of 200 ml of 10 mM Tris-HCl (pH 8.0), 0.7 M KCl, 1 mM EDTA, 5.0% sucrose and 10 mM 2-mercaptoethanol and then the suspension was immediately sonicated five times for 15s with a Branson sonifier and a large probe. After adjustment to a final concentration of 0.3 M KCl with 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM 2-mercaptoethanol, the extract was further sonicated four times for 15s, then gently stirred for 2 h at 0°C and centrifuged twice at 12,000 rpm for 20 min using a Sorvall GSA rotor at 4°C.

The supernatant was loaded onto a DEAE-cellulose column(a) (5×16 cm) which had been equilibrated with 0.4 M NaCl, 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 10 mM 2-mercaptoethanol to remove nucleic acids and then the column was eluted with the same buffer at a flow rate of 100 ml/h. Flow-through fractions (400 ml) which had significant UV absorbance at 280 nm were dialyzed against 20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA and 10 mM 2-mercaptoethanol (Buffer A).

The dialysate was loaded onto a 1.2-liter phosphocellulose column at a flow rate of 50 ml/h which was equilibrated with Buffer B (5 mM potassium phos-

phate (pH 8.1), 0.01% Triton X-100, 0.1 mM EDTA and 10 mM 2-mercaptoethanol). It was washed with the same buffer, and flow-through fractions (UV endonuclease III) were collected.

UV endonucleases I and II were also eluted from this column with a 4-liter linear gradient from 0 M to 1.2 M KCl in Buffer A. Twenty-four-ml fractions were collected and assayed for endonuclease activity directed against UV-irradiated and undamaged DNAs.

The UV endonuclease III phosphocellulose fraction was loaded without dialysis onto a 400-ml of the second DEAE-cellulose column(b) which was equilibrated with Buffer B. The column was washed with Buffer A and then eluted with a 1.2-liter linear gradient from 5 to 250 mM potassium phosphate (pH 8.1) in 0.01% Triton X-100, 0.1 mM EDTA and 10 mM 2-mercaptoethanol. UV endonuclease III, which was collected in 10-ml fractions, eluted at 90 mM potassium phosphate. Active fractions were pooled and dialyzed in Buffer B.

The dialysate of 164 ml was subject to 30~70% ammonium sulfate fractionation. Powdered ammonium sulfate (26.8 g) was added very slowly with constant stirring on ice, then left for 10 min on ice, then centrifuged in a GSA Sorvall rotor for 15 min at 20,000×*g* at 4℃ and the supernatant was collected. After the addition of 40 g of ammonium sulfate to the supernatant, the precipitate was collected as described above and dissolved in a final volume of 8-ml Buffer B.

The dissolved ammonium sulfate precipitate was loaded onto a Sephacryl S-200 column (140 ml bed volume) which had been equilibrated with 25 mM potassium phosphate, 0.01% Triton X-100 and 10 mM 2-mercaptoethanol. The eluent was collected in 8-ml fractions. Activity separated into 2 peaks and the second peak was dialyzed against 5 mM potassium phosphate (pH 8.1), 0.01% Triton X-100 and 10 mM 2-mercaptoethanol (Buffer C).

A 0.5-ml hydroxylapatite column was equilibrated with Buffer C and the dialysate was applied onto the column, washed with the same buffer then eluted with a linear gradient from 5 to 300 mM potassium phosphate (pH 8.1) in Buffer C and collected in 0.5-ml fractions. UV endonuclease III activity eluted at 70 mM potassium phosphate, and the active fractions were pooled and dialyzed against Buffer C.

The hydroxylapatite fraction was loaded onto a 1.5 ml heparin agarose column equilibrated with Buffer C and 12-ml of a 50~300 mM potassium phosphate (pH 8.1) gradient in 0.01% Triton X-100, 0.1 mM EDTA and 10 mM 2-mercaptoethanol was applied. Enzyme was collected in 0.6-ml fractions and fractions were assayed for endonuclease activity. For sucrose gradient ultracentrifugation, 200 ml of heparin agarose fraction was layered onto a 4.8-ml sucrose gradient (5~20%) in 25 mM potassium phosphate (pH 8.1), 0.02% Triton X-100 and centrifuged in a Beckman SW 50.1 rotor at 49,000 rpm for 20 h at 4℃.

Native gel electrophoresis

10% acidic native gel was prepared according to Reisfeld *et al.* (1962) with a slight modification. Solution A (100 ml) contained 48 ml of 1 N potassium hydroxide, 17.2 ml of glacial acetic acid and 4.0 ml of TEMED. Solution B (100 ml) contained 40 g of acrylamide and 0.8 g of bis-acrylamide. Solution C (100 ml) contained 0.8 g of bis-acrylamide. The running gel (10%) was composed of 1 part of A, 2 parts of B, 4 parts of 2.8 mg/ml ammonium persulfate and 1 part of water. Stacking gel (5%) contained 1 part of A, 1 part of B, 1 part of C, 1 part of water and 4 parts of 2.8 mg/ml ammonium persulfate. The gel was pre-run for 40 min at 200 V before applying 20 ml-samples of the sucrose gradient fractions. The gel running buffer (pH 4.5) contained 3.12% β-alanine and 0.8% glacial acetic acid.

Results

Purification of UV endonuclease III from MPC-11 cells

When UV endonuclease III was partially purified from MPC-11 cells by chromatography upon Sephacryl S-200, three activities (UV endonucleases I, II & III) were initially resolved by the Sephacryl S-200 column as reported previously and of the three, only UV endonuclease III did not bind to phosphocellulose (Kim and Linn, 1989). Because the Sephacryl S-200 gel-filtration step early in the purification was difficult to scale-up, an alternative scheme was utilized. In this scheme, the crude extract was passed through DEAE-cellulose (a) in 0.4 M NaCl to remove nucleic acids. When a phosphocellulose column was used as the next column, UV endonucleases I and II eluted between 0.65~0.85 M and between 0.38~0.45 M KCl respectively. The flow-through UV endonuclease III activity pool was loaded onto the second DEAE-cellulose column(b) and then UV- and AP endonuclease activities co-eluted at 90 mM potassium phosphate as expected (UV endonuclease III is AP endonuclease I) (Fig. 1). The dialysate was subject to 30~70% ammonium sulfate fractionation. The precipitate was collected and the dissolved precipitate was loaded onto a Sephacryl S-200 column and the activity was separated in 2 peaks ranging from fractions 4~10 and 12~15 (Fig. 2).

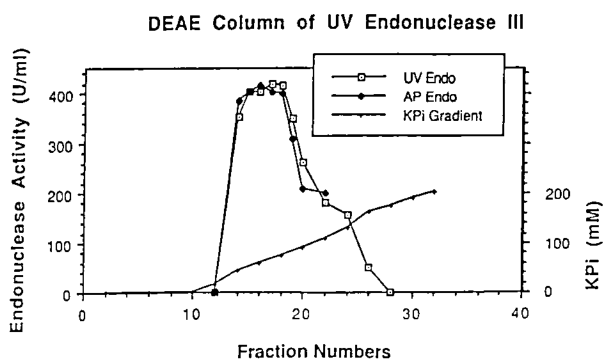

Fig. 1. DEAE-cellulose column chromatography of UV endonuclease III.

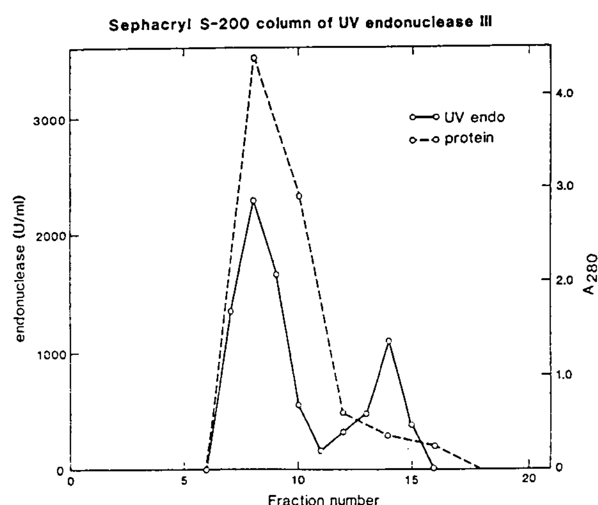

Fig. 2. Sephacryl S-200 column chromatography of UV endonuclease III.

The second activity was taken because it had higher specific activity. The dialysate was applied onto a hydroxylapatite column. UV endonuclease III activity eluted at 70 mM potassium phosphate and the hydroxylapatite fraction was loaded onto a heparin agarose column and eluted at 150 and 200 mM potassium phosphate. Fractions were assayed for endonuclease activity. For sucrose gradient ultracentrifugation, 200 ml of heparin agarose fraction eluted at 200 mM was layered onto a 4.8-ml sucrose gradient (5~20%) in 25 mM potassium phosphate (pH 8.1), 0.02% Triton X-100 and centrifuged in a Beckman SW 50.1 rotor at 49,000 rpm for 20 h at 4℃. Twelve-drop fractions were collected from the tube bottom and assayed for UV endonuclease activity directed against UV-irradiated DNA. Molecular weight markers [hemoglobin (4.3 S) and cytochrome C (2.1 S)] were layered onto a parallel gradient. The sedimentation coefficient of UV endonuclease III was 3.2 S, essentially the same value as that obtained from human AP endonuclase I

Table 1. Purification of UV endonuclease III

	Vol. (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)
Crude extract	300	15.000	2,400,000	164
DEAE-cellulose (a)	375	11.250	3,900,000	350
Phosphocellulose	250	3,000	300,000	100
DEAE-cellulose (b)	164	334	210,000	638
Ammonium sulfate	8	181	120,000	663
Sephacryl S-200	17.2	4.8	12.000	2,500
Hydroxyapatite	2.0	0.50	3,800	7,500
Heparin agarose	0.5	0.077	1.656	21,500
Sucrose gradient[a] (the peak fraction)	1.0	0.006	310	50,000

Purification was performed as described in Methods.
Proteins were quantitated according to Bradford (1976).
[a] Values is corrected for the fact that only 0.2 ml of the heparin agarose fraction had been sedimented.

(human UV endonuclease III) which was known to be lacking in xeroderma pigmentosum group D cells (Kunlein *et al*., 1978; Kim *et al*., 1995). This new purification scheme resulted in a 300-fold purification as shown in Table 1.

Native and SDS gel electrophoresis of sucrose gradient fractions of UV endonuclease III

Generally it is hard to predict the molecular weight of the peptide from its S value, even-though 3.2S indicates approximately 43 Kd assuming a globular configuration of the peptide. This would be somewhat larger than the 32 Kd peptide of UV endonuclease III, indicating the possibility of subunit(s). The sucrose gradient fractions of murine UV endonuclease III were analyzed by electrophoresis upon a 10% SDS-polyacrylamide gel and protein staining with silver. However, no peptides seemed to correspond to the UV endonuclease activity (Fig. 3). Nevertheless, the only possible combination which could agree with the observed activity is the combined action of 28 and 32 kDa peptides with 32 KDa band probably playing a major role. In order to test whether two bands were derived from one complex, native gel electrophoresis was applied. When the same fractions were subject to a basic native gel electrophoresis, no peptides appeared even-though marker proteins were visible, indicating that peptides are basic. Then, the same fractions were loaded on an acidic native gel electrophoresis, and a protein group, which happened to correspond to endonuclease activity, was visible, even-though it was not a sharp band (Fig. 3). This result indicates that this group is basic protein (s). The fact that antibodies directed against the 32

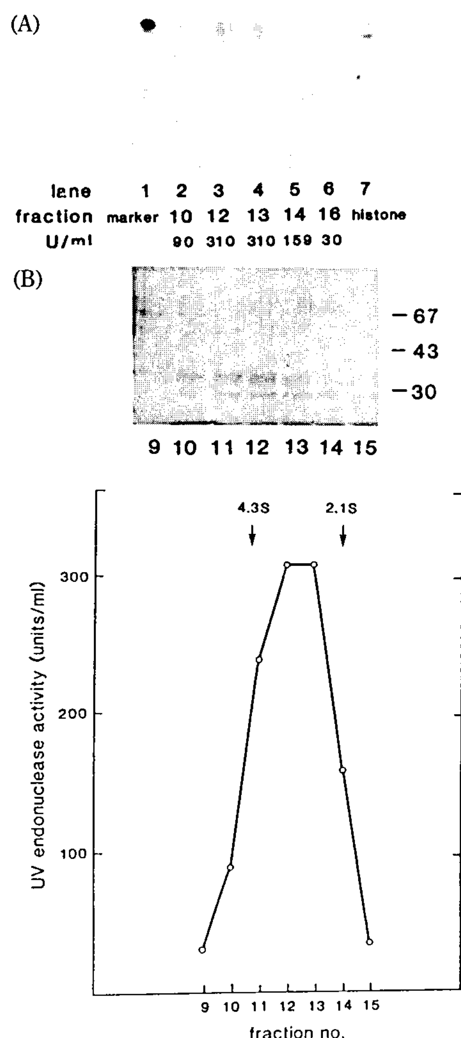

Fig. 3. 10% Native gel electrophoresis (Panel A) and 10% SDS-gel electrophoresis (Panel B) of sucrose gradient fractions of UV endonuclease III. 10% acidic native gel was prepared as described in Methods. For both of the gels, 20 μl-samples of the sucrose gradient fractions were subjected to gel elctrophoresis and silver-stained.

kDa protein did not recognize the other protein strongly indicated that the two peptides are distinct proteins. Nevertheless, it is very possible that the 32 and 28 kDa peptides were derived from one complex judging from the native gel electrophoresis and endonuclease assay results.

Appearance of non-specific activity of UV endonuclease III

On storage of pure enzyme or during purification, pure UV endonuclease III from mammalian cells or rpS3 protein expressed in *E. coli* tends to lose its preference for DNA damage- i.e., it becomes active upon undamaged DNA equally well (Kim *et al*., 1995). The purer the fractions of enzyme are, the more significant the phenomenon is. It also behaves as though it becomes less anionic during ion-exchange chromatography.

Table 2. Appearance of non-specific activity of UV endonuclease III

	Total units on UV irradiated DNA	Total units on nonirradiated DNA
Ammonium sulfate	169,000	49.000
Sephacryl S-200	18,300	7,100
Hydroxyapatite	4,560	740
Heparin agarose (a)	1,660	0
Heparin agarose (b)	1,900	1,740
Sucrose gradient (a)	310	0
Sucrose gradient (b)	0	0

Heparin agarose(b) and sucrose gradient (b) were the same fractions which had been frozen in liquid nitrogen and kept at −80°C before the assay. The assay was performed under the standard assay condition.

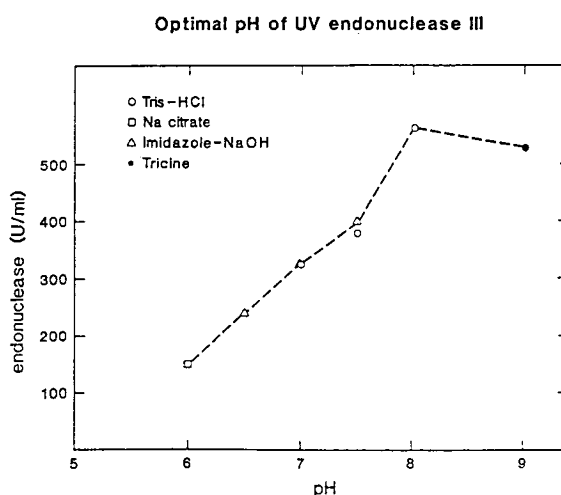

Fig. 4. Optimal pH of UV endonuclease III. Reactions were incubated for 15 min at 37°C under 40 mM Na-citrate (pH 6.0), imidazole (pH 6.5, 7.0 & 7.5), or Tris-HCl (pH 7.0, 7.5, 8.0 & 8.2), 70 mM KCl, 3 mM EDTA, 10 mM 2-mercaptoethanol and hydroxyapatite fractions of murine UV endonuclease which had been dialyzed against 5 mM potassium phosphate (pH 8.1), 0.1 mM EDTA, and 10 mM 2-mercaptoethanol.

The hydroxylapatite fraction remained essentially specific for UV-irradiated DNA. However, the sucrose fraction was UV-specific at first but lost its endonuclease activity on UV-irradiated or non-irradiated DNA after storage on ice or in −70°C overnight due to the unstability of the enzyme. On the other hand, when the heparin agarose fractions were assayed, a loss of specificity occurred reproducibly (Table 2). Heparin agarose fractions were kept in −70°C after being fro-

zen in liquid nitrogen. When these fractions were tested directed against UV-irradiated and undamaged DNAs later, the loss of UV-specificity in these fractions was observed. It is not clear when and how the heparin agarose fractions of UV endonuclease III lost their specificity. However, it is evident that the loss occurred after the heparin agarose column chromatography and that non-specific activity somehow increased. One of the possibilities is that it might have lost specificity upon further purification by diluting out a factor (or factors) which could confer specificity. Another possibility is that freezing and thawing could have caused this result, but hydroxylapatite or Sephacryl S-200 fractions did not show this phenomenon upon freezing and thawing. One can not rule out the possibility that post-translational modification such as phosphorylation of the protein changed the specificity of the enzyme. Also, a contaminating nuclease could have been concentrated during the purification by accident.

We also checked the optimal pH of UV endonuclease III which is around pH 8.0 (Fig. 4) and tested specificity change under different pH conditions or other salt conditions. However, changing the reaction conditions did not seem to alter the specificity.

Discussion

Kuhnlein *et al.* (1978) reported that AP endonuclease activity from cultured human fibroblasts was resolved into two species, AP endonucleases I and II, by phosphocellulose column chromatography. AP endonuclease I, with a sedimentation coefficient of 3.2S, passed through the phosphocellulose column. However, AP endonuclease II, with a sedimentation coefficient of 2.7S, was retained in the resin. Subsequent study revealed that AP endonuclease I cleaves on the 3' side of the AP site to produce 3'-deoxyribose and 5' phosphomonoester termini, which are not efficient primers for *E. coli* DNA polymerase I (class I AP endonuclease). On the other hand, AP endonuclease II cleaves on the 5'-side of the AP site to produce 3'-hydroxyl nucleotide- and deoxyribose 5-phosphate termini (class II AP endonuclease) (Mosbaugh and Linn, 1980). It has been known that normal human fibroblasts contain both AP endonucleases, whereas, fibroblasts from patients with XP-D lack AP endonuclease I activity, but contain a normal class II AP endonuclease (Kuhnlein *et al.*, 1978). However, it has been unsuccessful to purify this enzyme further due to the high nonspecific activity and instability under the former assay conditions. A newly developed assay condition, which includes Triton X-100 and EDTA without $MgCl_2$, enabled us to purify this enzyme further and to identify the associated UV endonuclease activity, named UV endonuclease III. Human AP endonuclease II does not have associated UV endonuclease activity. The first evidence that UV endonuclease III is AP endonuclease I came from the fact that both enzymes pass through a phosphocellulose column. Another evidence came with the realization that both enzymes have class I AP endonuclease activities which cleave on the 3' end of the AP site and are missing from XP-D cells (Kim *et al.*, 1995). In this paper, we are adding another evidence, namely, that both enzymes have virtually the same sedimentation coefficients. It is to be noted that UV endonuclase III also functions as ribosomal protein S3 in the cytoplasm and repair endonuclease in the nucleus. UV endonulcease is somehow lacking the endonuclease activity in XP-D cells but Northern blotting analysis revealed that mRNA of ribosomal protein S3 is expressed in XP-D cells (Kim *et al.*, 1995). Therefore, this strongly indicates that post-translational regulation might control the translocation of this protein either into the cytoplasm or nucleus. One cannot rule out the possibility that the loss of the endonuclease activity might be related to the post-translational modification.

In this regard, it is also to be noted that many ribosomal proteins are subject to methylation, acetylation and phosphorylation. In fact, the activity of UV endonuclease III is apparently decreased upon treatment with phosphatase (Kim, J., manuscript in preparation).

In addition to this, *E. coli* rpS3 was known to be a target of O-carboxyl-methylation (Kim et al., 1977). Therefore, it is possible that phosphorylation or methylation of mammalian rpS3 is somehow involved in the regulation of endonuclease activity and/or in the translocation of the protein from cytoplasm to nucleus or vice versa.

Tycowski *et al.* (1993) reported that human U15A RNA is coded within the intron I of the S3 gene. It is interesting, though, that the two-dimensional secondary structure of U15A RNA with its stem/loop/stem motif is similar to that of tRNA. It is also possible that specificity change and/or translocation of the protein could be conferred by an as yet unidentified ligand such as a tRNA-like species. The relationship of 28 kDa peptide with all these phenomena also remains to be investigated.

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