cDNA cloning of rat major AP endonuclease (APEX nuclease) and analyses of its mRNA expression in rat tissues.

Yunshan Tan∗
Hajime Wakabayashi**

Yuko Nakagawa†
Altaf H. Sarker††

Kosuke Akiyama‡
Shuji Seki‡‡

∗Okayama University,
†Okayama University,
‡Okayama University,
**Okayama University,
††Okayama University,
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Yunshan Tan, Yuko Nakagawa, Kosuke Akiyama, Hajime Wakabayashi, Altaf H. Sarker, and Shuji Seki

Abstract

APEX nuclease is a mammalian DNA repair enzyme having apurinic/apyrimidinic (AP) endonuclease, 3'-5'-exonuclease, DNA 3' repair diesterase and DNA 3'-phosphatase activities. It is also a redox factor (Ref-1), stimulating DNA binding activity of AP-1 binding proteins such as Fos and Jun. In the present paper, a cDNA for the enzyme was isolated from a rat brain cDNA library using mouse Apex cDNA as a probe and sequenced. The rat Apex cDNA was 1221 nucleotides (nt) long, with a 951-nt coding region. The amino acid sequence of rat APEX nuclease has 98.4% identity with mouse APEX nuclease. Using the rat Apex cDNA as a probe for Northern blot analysis, the size of rat Apex mRNA was shown to be approximately 1.5 kb. Its expression was compared in 9 rat organs on postnatal days 7 and 28. Although Apex mRNA was expressed ubiquitously, the levels varied significantly, suggesting organ- or tissue-specific expression of the Apex gene. The highest level was observed in the testis, relatively high levels in the thymus, spleen, kidney and brain, and the lowest level in the liver. The level of expression at postnatal day 28, with the exception of the testis, was almost the same as or lower in respective organs than that at postnatal day 7. Postnatal developmental changes of Apex mRNA expression in the testis and thymus were further studied. The expression in testis was markedly increased on postnatal days 21 and 28. The expression in thymus increased once at postnatal day 14, and then decreased. The developmental changes of Apex mRNA expression in testis and thymus suggest that APEX nuclease is involved in processes such as recombinational events.

KEYWORDS: Apurinic/apyrimidinic endonuclease; APEX nuclease; Repair enzyme; Apex mRNA; Northern blot; Development; testis; rat

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cDNA Cloning of Rat Major AP Endonuclease (APEX Nuclease) and Analyses of Its mRNA Expression in Rat Tissues

Yunshan Tan, Yuko Nakagawa, Kosuke Akiyama, Hajime Wakahayashi, Altaf H. Sarker and Shuji Seki*

Department of Molecular Biology, Institute of Cellular and Molecular Biology, Okayama University Medical School, Okayama 700, Japan

APEX nuclease is a mammalian DNA repair enzyme having apurinic/apyrimidinic (AP) endonuclease, 3'-5'-exonuclease, DNA 3' repair diesterase and DNA 3'-phosphatase activities. It is also a redox factor (Ref-1), stimulating DNA binding activity of AP-1 binding proteins such as Fos and Jun. In the present paper, a cDNA for the enzyme was isolated from a rat brain cDNA library using mouse Apex cDNA as a probe and sequenced. The rat Apex cDNA was 1221 nucleotides (nt) long, with a 951-nt coding region. The amino acid sequence of rat APEX nuclease has 98.4% identity with mouse APEX nuclease. Using the rat Apex cDNA as a probe for Northern blot analysis, the size of rat Apex mRNA was shown to be approximately 1.5 kb. Its expression was compared in 9 rat organs on postnatal days 7 and 28. Although Apex mRNA was expressed ubiquitously, the levels varied significantly, suggesting organ- or tissue-specific expression of the Apex gene. The highest level was observed in the testis, relatively high levels in the thymus, spleen, kidney and brain, and the lowest level in the liver. The level of expression at postnatal day 28, with the exception of the testis, was almost the same as or lower in respective organs than that at postnatal day 7. Postnatal developmental changes of Apex mRNA expression in the testis and thymus were further studied. The expression in testis was markedly increased on postnatal days 21 and 28. The expression in thymus increased once at postnatal day 14, and then decreased. The developmental changes of Apex mRNA expression in testis and thymus suggest that APEX nuclease is involved in processes such as recombinational events.

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A purinic/apyrimidinic (AP) sites resulting from loss of bases are the most frequent DNA lesion in cells, and are generated in DNA by spontaneous hydrolysis, radiation, oxidative damage and action of DNA glycosylases on modified bases (1-4). Single-strand breaks with 3'-blocked termini are also frequently produced by radical-induced DNA damage (3-5). The resulting AP sites and 3'-blocked single-strand breaks, if they can not be repaired, block transcription and replication of DNA, and have cytotoxic and mutagenic effects on cells (2-6). Repair of these lesions is thought to be mostly initiated by AP endonucleases having 5' AP endonuclease and DNA 3' repair diesterase activities (2-5). Although multiple forms of AP endonucleases have been reported in mammalian cells, so far only one form (the major 5' AP endonuclease designated as APEX nuclease, or HAP1, APE or Ref-1 protein) of AP endonuclease/DNA 3' repair diesterase has been clearly identified (7-11). In vitro studies have shown that the major AP endonuclease (APEX nuclease) is multifunctional. It shows 5' AP endonuclease, 3'-5' exonuclease, DNA 3' repair diesterase, DNA 3'-phosphatase and RNase H activities as well as reductive activation of AP-1-binding proteins such as Fos and Jun (4, 7-15). However, at present, we have not enough information on actual in vivo functions and regulation of APEX nuclease.

Studies on changes of its expression in vivo depending on specific DNA damage, cell proliferation, cell differentiation or organ development are thought to be helpful to elucidate in vivo functions of the enzyme. We

* To whom correspondence should be addressed.
† The nucleotide sequence data reported in this paper appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases under accession number D44495.
considered that rats are the well usable laboratory animal with appropriate organ sizes for studying difference of its expression in various conditions, organs or developmental stages. In the present paper, therefore, we cloned and sequenced rat Apex cDNA, and studied Apex mRNA expression in various rat tissues using the cDNA as a probe.

Materials and Methods

**Materials.** The reagents used in these experiments were obtained from the following sources: restriction enzymes from Toyobo Biochem., Osaka, Japan; T4 DNA ligase from Promega Corp. (Madison, WI, USA); Taq DNA polymerase from Wako Pure Chemical Industries (Osaka, Japan); human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA and a rat brain cDNA library in phage λgt10 from Clontech Lab., CA, USA; PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit from Applied Biosystems (Mountain View, CA, USA); Megaprime DNA Labeling System, \([^{32}P] \text{dCTP} \) (3000 Ci/mmold) and positively charged nylon membrane (Hybond-N+) from Amersham Japan, Tokyo; RNAzol B from Biotec Lab., Texas, USA.

**Cloning and DNA sequencing.** The recombinant phages in the rat brain cDNA library (Clontech) were plated with *Escherichia coli* C500H11, and three replica nylon membranes per plate were prepared. The coding region of mouse Apex cDNA was amplified by polymerase chain reaction using a mouse cDNA clone (pEXE2) (7) and appropriate oligonucleotide primers. A \(^{32}P\)-labeled probe was prepared using the amplified DNA fragments as the template and \([^{32}P] \text{dCTP} \) (3000 Ci/mmol) as the labeled nucleotide by the random priming method (16) using the Megaprime DNA Labeling System. About 7.5 \times 10^5 independent colonies of the rat brain cDNA library were screened with the \(^{32}P\)-labeled probe by the plaque hybridization technique (17).

DNA sequences were determined by the dyeoxy-termination method (18) using an ABI 373A DNA Sequencer (Applied Biosystems, Japan).

**Animals and RNA extraction.** Male Donryu rats were purchased from Shizuoka Laboratories Center (Shizuoka, Japan), and maintained on a laboratory diet (RC4, Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*. Rats were sacrificed under ether anesthesia on postnatal days 1, 7, 14, 21, 28, 147 (21 w) or 224 (32 w). At least 3 rats were used for each experimental point.

Rat organs to be used for total RNA isolation were flash-frozen in liquid nitrogen immediately after sacrifice and stored at −80°C. The extraction of total RNA was performed using RNAzol B as described previously (19, 20). The RNA concentration was determined spectrophotometrically, and the amount and property of RNA electrophoresed on a denaturing 1% agarose gel were monitored after ethidium bromide staining.

**Northern blot hybridization.** The RNA fractions were denatured with 6% formaldehyde, and electrophoresed at 30 μg/lane at 20 V for 18 h on a 1% agarose gel in the presence of 6% formaldehyde. After the electrophoresis, the gel was treated with 0.25 M ammonium acetate, and then RNA on the gel was transferred overnight onto a positively charged nylon membrane (Hybond-N+) by the capillary blotting technique (17). To analyze the results of Northern blotting, three or four blotted membranes were prepared for each set of samples. The membranes were prehybridized at 42°C in a prehybridization buffer consisting of 5 × SSPE (1 × SSPE: 150 mM NaCl, 10 mM NaH₂PO₄, 1 mM EDTA, pH 7.6), 5 × Denhardt’s reagent, 0.5% SDS, 50% formamide and 100 μg/ml denatured salmon sperm DNA. Hybridization was performed by incubating the membranes at 42°C for 18 h in the prehybridization buffer supplemented with a denatured \(^{32}P\)-labeled probe prepared using the cloned rat Apex cDNA. After removing the excess probe by washing, the membranes were processed for autoradiography.

The preparation procedure for the \(^{32}P\)-labeled G3PDH probe was the same as that for the Apex probe. After removal of the Apex probe, the RNA-blotted membranes were incubated at 65°C for 20 h for hybridization with the denatured, \(^{32}P\)-labeled G3PDH probe in a buffer containing 6 × SSC, 5 × Denhardt’s solution, 0.5% SDS and 100 μg/ml salmon sperm DNA. The membranes were processed in the same way as that for hybridization with the Apex probe.

**Analysis of autoradiograms.** Quantitative analysis of the autoradiograms was performed using a computerized imaging analysis system using Fujix BioImaging Analyzer BAS2000 (Fuji Photo Film, Tokyo, Japan) as described previously (21). The hybridized membranes were exposed for about 40 h at room temperature to phosphor imaging plates. The exposed imaging plates were inserted into an image reading unit and
scanned with a fine laser beam. To compare Apex mRNA expression in various tissues, a mean value (arbitrary units), which was obtained by the analyzer, of Apex mRNA in 7-day-old rat livers was used as the standard for normalization of the data obtained in separate experiments and for comparing the values in different tissues and different developmental stages. Each datum is expressed as a percentage of the mean value in 7-day-old rat livers.

Results

Cloning of rat Apex cDNA. A rat brain cDNA library was screened with 32P-labeled mouse Apex cDNA. Seven positive clones were isolated from 7.5 × 10⁶ plaques. The insert of the positive clone was subcloned into the EcoRI site of the pUC18 plasmid vector. Among these positive clones, two clones having inserts of roughly the Apex mRNA size were selected and the inserts were sequenced. The inserts of the two clones consisted of an identical region in both clones and a non-identical region. The nucleotide sequence of the identical region was highly homologous to that of mouse Apex cDNA, and was thought to be downstream of the 5' non-coding sequence and the complete sequence of the coding and 3' non-coding regions. However, the upstream sequences of the two inserts were not identical to each other and were not homologous to that of mouse Apex cDNA. The Sma I/PmaCI and EcoRI fragment, which belongs to the identical region, was subcloned into pUC18 plasmid to generate the rat Apex cDNA clone pUC18-APEX80. The cDNA sequence of the clone pUC18-APEX80 was determined for both strands from overlapping DNA regions. The DNA sequencing identified a coding region of 951 nucleotides (nt) with 115 nt of 5' flanking and 155 nt of 3' flanking regions followed by a poly(A) tail at 17 nt downstream of an authentic polyadenylation signal, AATAAA (22) (Fig. 1). The cDNA for rat Apex nuclease (951 nt) encodes 317 amino acids.

Analysis of deduced amino acid sequence of rat APEX nuclease. The amino acid sequence of rat APEX nuclease is highly homologous to that of mouse APEX nuclease (98.4% identity/317 residues), human APEX nuclease (93.1% identity/318 residues), and bovine AP endonuclease 1 (92.1% identity/318 residues). Amino acid variation in these sequences is mostly in the N-terminal 60 amino acids (Fig. 2). Recently, Wilson et al. (23) reported the amino acid sequence of a rat AP endonuclease (rAPEN), deduced from the cDNA cloned by using a rat testis gt11 cDNA library and mouse Apex cDNA. The rAPEN amino acid sequence is 99.1% identical with that of rat APEX nuclease.

Expression of Apex mRNA in various rat organs. The levels of Apex mRNA in various rat

Fig. 1  Nucleotide sequence and the deduced amino acid sequence of the cDNA for rat Apex nuclease. The deduced amino acid sequence in the standard one-letter code is shown beneath the nucleotide sequence. Amino acids are numbered, starting with the first in-frame methionine as number 1. The translation termination codon and polyadenylation signal are indicated with triple asterisks and double underline, respectively.
Fig. 2  Comparison of the amino acid sequence of rat APEX nuclease (rAPEX) with those of mouse APEX nuclease (mAPEX) (7), bovine AP endonuclease I (BAPI) (27) and human APEX nuclease (hAPEX) (8). Gaps (indicated by dashes) are introduced at position 10 of the rat and mouse APEX nucleases. Amino acids identical to rat APEX nuclease are shown as a dot.

Fig. 3 (Left)  Northern blot analyses of mRNA expression of Apex and G3PDH genes in various organs of 7-day-old rats. Thirty micrograms of total RNA from various rat organs were electrophoresed on a 1% agarose gel containing 6% formaldehyde, blotted onto a nylon membrane, and hybridized first with the 32P-labeled rat Apex cDNA probe (A), and then with 32P-labeled G3PDH probe after detaching the Apex probe (B). To monitor the RNA samples, total RNA of each sample was electrophoresed at 1 µg/lane and stained with ethidium bromide (C). Numbers to the right in Figs. 3A and 3B indicate sizes of transcripts in thousands of nucleotides.

Fig. 4 (Right)  Northern blot analyses of mRNA expression of Apex (A) and G3PDH (B) genes in various organs of 28-day-old rats. The Northern blotting, hybridization and monitoring of the RNA samples (C) were performed as described in Materials and Methods and in the legend to Fig. 3.
Figure 5  Levels of Apex mRNA in various organs of 7-and 28-day-old rats. Quantitative analyses of the autoradiograms were performed as described in Materials and Methods. To compare Apex mRNA expression in various organs, its expression level in livers in 7-day-old rats was used as the standard for normalization of the data obtained in separate experiments, and the percentage of Apex mRNA expression in an organ to the expression in livers were calculated. Each point indicates the mean values of 3 independent determinations. Vertical bars indicate the standard errors of the mean values.

Fig. 5  Levels of Apex mRNA in various organs of 7-and 28-day-old rats. Quantitative analyses of the autoradiograms were performed as described in Materials and Methods. To compare Apex mRNA expression in various organs, its expression level in livers in 7-day-old rats was used as the standard for normalization of the data obtained in separate experiments, and the percentage of Apex mRNA expression in an organ to the expression in livers were calculated. Each point indicates the mean values of 3 independent determinations. Vertical bars indicate the standard errors of the mean values.

The expression patterns of the G3PDH gene, which was used as an internal control for Northern blot hybridization, show that the relative expression levels in these organs at postnatal day 28 are similar to those at postnatal day 7 and 28.

Fig. 6  Northern blot analysis of Apex mRNA expression in the developing rat testes. The Northern blotting of total RNA isolated from developing rat testes and hybridization with the 32P-labeled rat Apex cDNA probe (A), and monitoring of electrophoresis of the RNA samples (B) were performed as described in Materials and Methods and in the legend to Fig. 3. The sample examined was testis at postnatal day 7 in lane 1, 14 in lane 2, 21 in lane 3, 28 in lane 4, 147 in lane 5 and 224 in lane 6. (C) The same experiment was repeated 3 times. The results were analyzed as described in the legend to Fig. 5.
rat testis was observed at a fairly high and constant level in 7-, 14- and 21-day-old rats. The expression increased sharply from postnatal day 21 to day 28, possibly in association with the increase of pachytene spermatocytes as discussed later, and finally attained the adult level which was almost 3 times and 1.5 times higher than the levels in testes of 7- and 21-day-old rats and of 28-day-old rats, respectively.

Postnatal changes of the Apex gene expression in the thymus are shown in Fig. 7. Although the variation in experimental data are relatively large, the result shows that the Apex gene expression in rat thymuses tends to be the highest in 14-day-old rats, and declines sharply at the beginning and then gradually to half of the highest expression.

Discussion

APEX nuclease (also designated HAP1 (9), APE (10), or Ref-1 (11) gene product) is a multifunctional DNA repair enzyme which may be involved in DNA repair of AP sites and 3’ blocked single-strand breaks. The enzyme is also known to function as a redox factor which stimulates the DNA binding activity of Fos-Jun heterodimers, Jun-Jun homodimers and Hela cell AP-1 protein as well as that of several other transcription factors including NF-κB, Myb and members of ATP/CREB family (11, 13, 24–26).

Previous studies on the enzyme have been mostly restricted to in vitro studies. In the present investigations, cDNA cloning of APEX nuclease from a rat cDNA library and its sequencing were performed for analyzing in vivo functions of the enzyme. Apex gene expressions in various rat tissues and in developmental stages of testis and thymus were studied using the cloned rat Apex cDNA.

The amino acid sequence of rat APEX nuclease deduced from the rat Apex cDNA sequence exhibits intensive homology with those of mouse and human APEX nuclease and of bovine AP endonuclease 1 (BAP1), especially with that of mouse APEX nuclease (7-10, 27). During this study, Wilson et al. (23) reported cDNA cloning for the multifunctional rat AP endonuclease (rAPEN)/redox factor using mouse Apex cDNA as a probe. The amino acid sequence reported for rAPEN was almost identical (99.1 % identity/317 amino acids) to that reported here for rat APEX nuclease. There are some differences in the cDNA and deduced
The present experiments also showed a similar developmental decrease in Aplex expression in most tissues. The expression pattern of the Aplex gene in most of the rat tissues examined suggested that its expression decrease slightly or markedly in accordance with the development and reached adult levels which are quite different from tissue to tissue. The developmental change of its expression in thymus is phenomenologically associated with the proliferative change of the organ (36).

It is well-known that specific cell types appear in the seminiferous tubules at a well-defined age during postnatal development of the testis (28, 37). In 23- and 26-day-old rats, spermatocytes are seen at all phases of the long meiotic prophase. Increasing numbers of pachytenic spermatocytes and spermatids are observed in all seminiferous tubules in 26-day-old rats. The adult cell pattern in seminiferous tubules is seen in 50-day-old rats (37). The Aplex mRNA expression in testis increased markedly from postnatal day 21 to day 28 in association with the increase of pachytenic spermatocytes, in which the recombination process occurs, and finally reached the adult high level.

The developmental changes and tissue or cellular variations of Aplex gene expression suggest that APLEX nuclease is multifunctional in vitro, as suggested by in vitro studies, and that it is involved in cellular processes such as growth, development, transcriptional regulation and recombination as well as base excision repair. To study further the in vitro functions of APLEX nuclease, the rat Aplex cDNA may provide a useful tool.

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