Negative Regulation of the Major Human AP-Endonuclease, a Multifunctional Protein[†,‡]

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ABSTRACT: Abasic sites in DNA are generated either spontaneously or after removal of altered bases during the base excision repair process. These as well as 3′ damaged ends of DNA at single-strand breaks induced by reactive oxygen species are repaired by AP-endonucleases. The major human AP-endonuclease (named APE-1) has two unrelated activities. It may function as an activator of c-Fos and c-Jun transcription factors and as a repressor of the parathyroid hormone (PTH) gene by binding to the negative Ca^{2+}-response elements (nCaRE) in its promoter. Preliminary studies indicate that the h-APE-1 gene is highly regulated. Analysis of its promoter activity by transient expression of the luciferase reporter gene in human, HeLa and TK6 cells suggested the presence of a negative regulatory element in the promoter. Two nCaRE-like sequences were identified in the promoter segment responsible for inhibiting reporter gene expression. Competitive electrophoretic mobility shift assay with HeLa nuclear extract indicated that the nCaRE sequences of the APE-1 and PTH genes are recognized by the APE-1 polypeptide. These results suggest that the APE-1 gene may be down-regulated by its own product.

Apurinic/apyrimidinic (AP)[1] sites are ubiquitous in DNA, and possibly the most abundant genomic lesions that are generated either by spontaneous depurination or as intermediates during cellular repair of modified and unnatural bases. It is estimated that about 10^4 abasic sites are generated per human cell per day (Lindahl, 1993). Reactive oxygen species (ROS), including $H_2O_2^{\bullet -}$, $OH^{\bullet}$, O_2, HOCl, and various free radicals, induce a wide variety of lesions in DNA ranging from oxidized base lesions, such as 8-oxoguanine and thymine glycol, to base loss and DNA strand breaks with modified ends such as 3′-phosphate and 3′-phosphoglycolate (Wallace, 1994; Ward, 1994; Breen & Murphy, 1995). The AP sites, strand breaks, and some base lesions block DNA replication and may also be mutagenic (Loeb & Preston, 1986; Neto *et al*., 1992; Grollman & Moriya, 1993; Wallace, 1994). This array of lesions is repaired via the base excision repair (BER) pathway (Friedberg *et al*., 1995; Wallace, 1994). Repair of modified bases is initiated by their removal by specific DNA glycosylases resulting in AP sites (Friedberg *et al*., 1995; Wallace, 1994). Repair of AP sites involves multiple steps starting with the cleavage of the DNA strand adjacent to the AP sites, removal of the deoxyribose phosphate residue, followed by gap-filling synthesis and ligation (Friedberg *et al*., 1995). The DNA strand cleavage is catalyzed by a multitude of AP-endonucleases (APE) present in both bacteria and mammals. The major enzyme cleaves 5′ to the AP site (Doetsch & Cunningham, 1990; Demple & Harrison, 1994). This enzyme, both from *Escherichia coli* (exonuclease III) and mammalian cells (where it was variously named HAP1, APEX, or APE and is called APE-1 here), has an associated 3′ exonuclease activity that removes 3′-*trans*-α,*β*-unsaturated aldehyde generated by AP lyases, as well as 3′-phosphate and 3′-phosphoglycolate generated in DNA by reaction with ionizing radiation (Doetsch & Cunningham, 1990; Chen *et al*., 1991; Robson *et al*., 1991; Seki *et al*., 1991). Thus, APE-1 is also responsible for the removal of 3′-blocked termini at the site of strand breaks generated by ROS.

We have recently observed that the human APE-1 is activated transiently 3- to 5-fold by nontoxic levels of only ROS, and that this increase in APE-1 is associated with a significant increase in cellular resistance to ROS such as H_2O_2 and bleomycin (C. V. Ramana, I. Boldogh, T. Izumi, and S. Mitra, unpublished observation). Furthermore, APE-1 may be the rate-limiting protein in the multi-component base excision repair system. *In vivo* reduction of APE-1 level by antisense RNA sensitized the cells to various DNA damaging agents, and to hypoxic and hyperoxic stress. (Ono *et al*., 1994; Walker *et al*., 1994).

The human APE-1 has two other intrinsic activities. It was independently identified as Ref-1, responsible for reductive activation of c-Fos and c-Jun proteins (Xanthoudakis *et al*., 1992). Interaction of oxidized APE-1 with thioredoxin predicted for its reactivation has recently been demonstrated (Qin *et al*., 1996). Furthermore, two negative

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[‡] The nucleotide sequence reported in this paper has been submitted to GenBank with accession number U59695.

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[1] Abbreviations: AP, apurinic/apyrimidinic; APE, AP-endonuclease; *β*-gal, *β*-galactosidase; BER, base excision repair; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; IL-4, interleukin-4; LDH-A, lactate dehydrogenase A subunit; luc, luciferase; nCaRE, negative calcium response element; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PTH, parathyroid hormone.

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Ca^{2+}-response elements (nCaRE-A and nCaRE-B) were identified in the promoter of the parathyroid hormone (PTH) gene which is regulated by extracellular Ca^{2+}; the nCaRE-binding protein was identified as APE-1/Ref-1 (Okazaki *et al*., 1994). However, recent studies indicate requirement of additional protein(s) for this binding (Chung *et al.*, 1996).

The results of the study reported here suggest that the APE-1 gene is autoregulated through the interaction of APE-1 protein with nCaRE sequences located upstream of its own promoter.

MATERIALS AND METHODS

Reporter Plasmids. Isolation of the human APE-1 genomic DNA was described previously (Zhao *et al.*, 1992). The pGL2-basic plasmid (Promega), a promoterless vector containing the firefly luciferase (luc) reporter gene, was used as the negative control and also for cloning promoter fragments. DNA was purified by either CsCl-banding twice (Ausubel *et al*., 1993) or by chromatography on Qiagen Med Column. Serial deletions of the promoter region were constructed by using PCR-amplified DNA. The amplified DNA sequences were confirmed by direct sequencing analysis. The pSV-β expression plasmid (Promega), encoding *E. coli* β-galactosidase (β-gal), was purified twice by CsCl-banding and the same batch of DNA was used as an internal control in order to normalize variation in transfection efficiency.

Cell Culture and Transfection. A human lymphoblastoid cell line, TK6 (Skopek *et al*., 1978), was obtained from Dr. W. Thilly and grown in RPMI 1640 (Gibco) supplemented 10% bovine serum (Hyclone). HeLa S3 cells were grown in Dulbecco-modified essential medium containing 10% fetal bovine serum (Hyclone); or in suspension in S-MEM medium supplemented with 10% fetal bovine serum. For transient transfection experiments, exponentially growing cells (5×10^5/mL) were harvested, washed twice in phosphate-buffered saline (PBS), and then resuspended in PBS at 4×10^7 cells/mL. After mixing a 250 μL suspension with plasmid DNA (20 μg of luc plasmid and 10 μg of pSV-β plasmid); the cells were incubated on ice for 10 min and then electroporated (Bio-Rad gene pulser, 250 V and 960 μF). After 20 min incubation on ice, the cells were transferred into 10 mL of complete medium and incubated for 16 h, washed twice with PBS, and finally lysed in lysis buffer (Promega). The β-gal and luc activities were measured in assay mixtures containing their substrates (Promega) by absorbance at 420 nm and with a chemiluminescence luminometer (Tatsuka *et al*., 1995), respectively. The luc activity was normalized to the β-galactosidase activity. The universal unit of β-gal and luc activities were calculated by standard enzyme reaction supplied by Promega and Boehringer Mannheim respectively.

Gel Retardation Assay. The following oligonucleotides were used for EMSA in which the nCaRE motifs are underlined. PTH-nCaRE-B, TATGTTTTTGAGACAGGGTCTCACTCTGA; nCaRE-B1, GATCCTTTTGAGACAGTCTCAGCTCTG; nCaRE-B2, GATCCTTTTTGAGACAGAGTTTCACTCTTG.

HeLa nuclear extracts were prepared as described (Ausubel *et al*., 1993), and about 2 mg of protein (6 mg/mL) was present in the extract of 2×10^8 cells. Oligonucleotides were labeled with [γ-^{32}P]ATP (Amersham, 3000 Ci/mmol) and T4 polynucleotide kinase (Pharmacia) and purified by gel filtration. The binding reaction mixture (20 μL) contained 15 μL of buffer (0.35 mM HEPES-KOH, pH 7.8, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, and 10% glycerol), 1 μg of poly (dI-dC) (Sigma), ^{32}P-labeled oligonucleotide, nuclear extracts (12 −18 μg of protein) and competitors as described in the figure legend. After incubation for 20 min at 25 °C, 10 μL samples were loaded on to a 5% polyacrylamide gel for electrophoresis in Tris-borate at 4 °C. The gel was dried and exposed to Kodak X-AR5 film overnight for autoradiography.

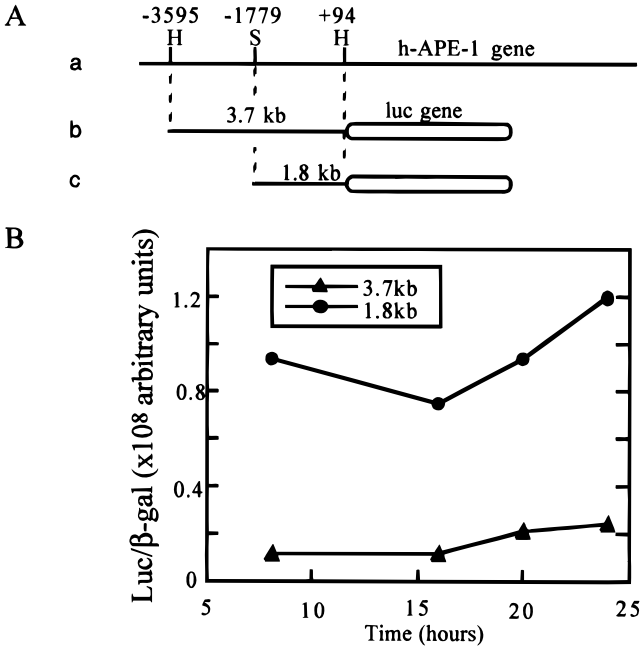

FIGURE 1: Analysis of the h-APE-1 promoter by luciferase reporter expression. (A) Two plasmid DNAs for luciferase (luc) gene expression are shown in b and c with 3.7 and 1.8 kb promoter fragments, respectively, aligned with the h-APE-1 gene (line a). H, *Hin*dIII; S, *Sma*I. The numbers above line a indicate the gene map position relative to the transcription start site (Zhao *et al*., 1992). **(B)** Kinetics of luciferse expression in TK6 cells after transfection with the reporter plasmids. Luciferase activity was normalized for β-galactosidase expressed from a co-transfected plasmid.

DNA Sequencing. The sequencing was carried out in recombinant pBluescript (Stratagene) plasmids in UTMB's Recombinant DNA Laboratory using T7 and T3 universal primers. Consensus sequences were obtained from the NIH transcriptional factor data base (TFD).

RESULTS AND DISCUSSION

The first indication for the presence of a negative regulatory element in the human APE-1 promoter was provided by our observation that transient expression of the luciferase gene in TK6 cells, under the control of the APE-1 promoter, was significantly inhibited when the expression plasmid contained a 1.9 kb *Hin*dIII/*Sma*I fragment located upstream of the promoter (Figure 1). When normalized with respect to coexpressed β-galactosidase, the level of luciferase activity encoded by the plasmid with a 3.7 kb promoter was 6−10-fold lower than that encoded by the 1.8 kb promoter-containing plasmid; this difference was not significantly affected by the time of expression after transfection. The negative regulatory role of the 1.9 kb fragment was also found in transfected HeLa cells (data not shown).

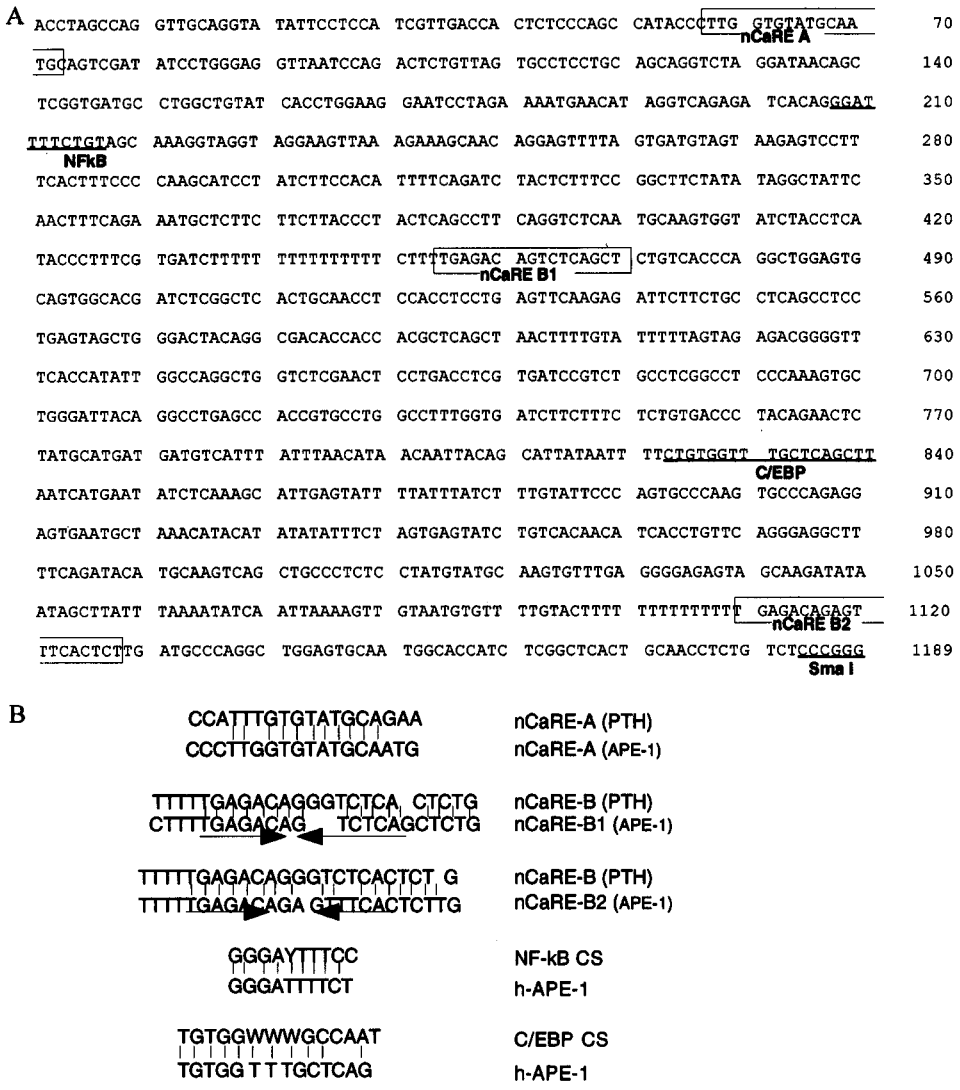

FIGURE 2: Characterization of potential regulatory elements in the h-APE-1 promoter. (A) DNA sequence of the region upstream of the *Sma*I site (shown in Figure 1) responsible for down-regulation. Candidate sequences for nCaRE's, NF-κB, and C/EBP are indicated. (B) Sequence similarity of NF-κB and C/EBP between consensus sequences (CS) and those found in the h-APE-1 promoter, and of nCaRE's in PTH and h-APE promoters. Arrows within nCaRE-B's indicate inverted repeats (Okazaki *et al.*, 1994).

Earlier studies on promoter analysis of the human APE-1 gene did not include characterization of the 3.7 kb fragment, particularly the 1.9 kb upstream fragment that apparently contains the negative regulatory element (Harrison *et al.*, 1995). We determined its complete sequence and established its physical map, which allowed us to construct a series of luciferase expression plasmids containing various regions of the 1.9 kb fragment (Figure 2A). Furthermore, the fragment was transposed to the 3′ terminus of the luciferase gene which is still *cis* to the transcription unit or placed *trans* in an independently co-transfected plasmid (Figure 3A, lines 3 and 4). The results on the normalized expression of luciferase show that the putative negative regulatory element is active even when it is placed downstream to the luciferase gene. However, the repressor activity is lost when the element is present in *trans* in a separate plasmid. There are many examples of negative regulatory elements on silencer sequences in the promoters of mammalian genes. The position-independent effect of a silencer was also observed in negative regulation of human IL-4 and rat LDH-A genes (Li-Weber *et al.*, 1993; Chung *et al.*, 1995). Characterization of the human APE-1 promoter and analysis of its function have been reported previously, but the presence of a negative regulatory element was not detected (Harrison *et al.*, 1995). While we cannot explain this discrepancy, it is possible that the disparate results were due to differences in the methods of transfection and in the reporter genes.

Attempts to identify the exact repressor sequence by using overlapping deletion constructs yielded complex results (Figure 3B). It is evident that even a 275 bp fragment at the *Sma*I boundary conferred silencer activity (Figure 3B, line 5), which disappeared by further 93 bp deletion (Figure 3B, line 6). However, the silencer activity also disappeared for the 606 bp fragment-containing plasmid and reappeared with the presence of 445 bp fragment (Figure 3B, lines 3 and 4). These results are reproducible as indicated in the standard errors and the multiple number of times (shown in parenthesis in the right hand column in Figure 3B) these transfection assays were performed. Our observations suggest the presence of multiple silencer and enhancer elements located in the region upstream in the APE-1 promoter. It is interesting that a similar situation exists in some other genes in that a negative regulatory element functions by directly suppressing the activity of an enhancer element (Li-Weber *et al.*, 1993; Chung *et al.*, 1995). Although these elements are located close to each other in the case of IL-4 promoter,

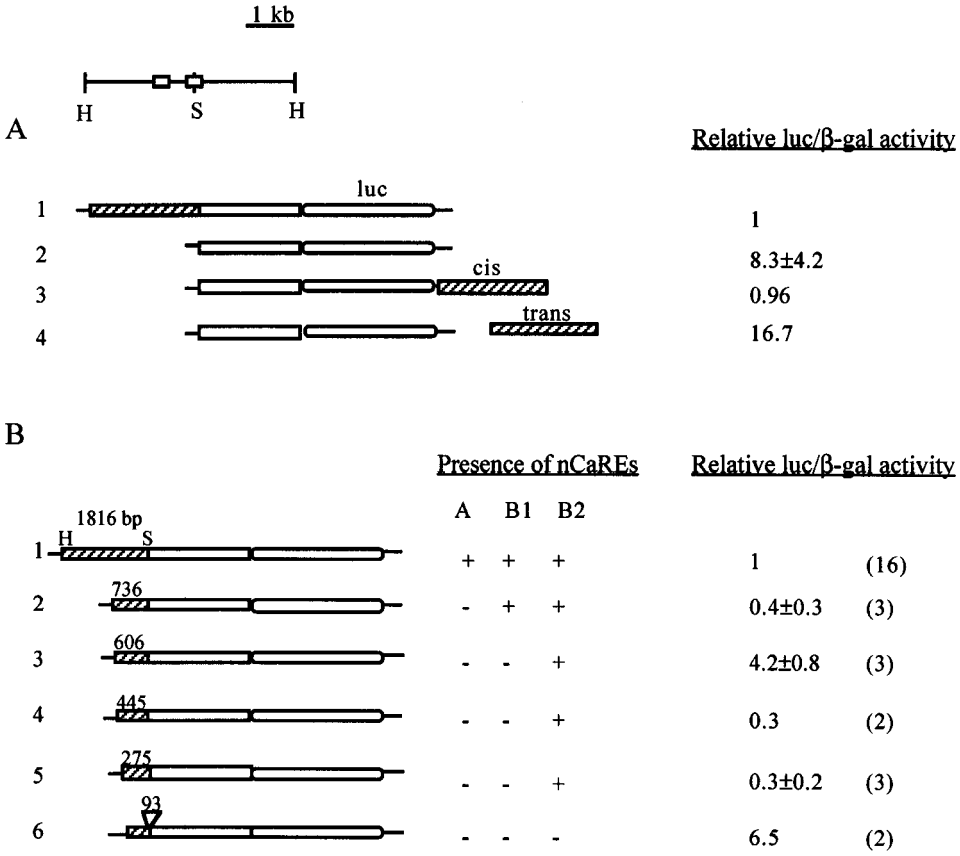

FIGURE 3: Deletion analysis of the h-APE-1 promoter. (A) Schematic diagram of 3.7 kb *Hin*dIII (H) fragment containing the h-APE-1 promoter and showing the Alu sequences (boxes). S, *Sma*I site, same as in Figure 1. Bars represent reporter constructs containing the luciferase gene (luc) linked to promoter segments including regions responsible for repression (hatched), and aligned with the 3.7 kb fragment. (B) Fine structure mapping of the repressor element (hatched bar) line 1, same as in A. Nested deletions of the sequence starting at the 5′ terminus *Hin*dIII (H) site and ending at the *Sma*I (S) site in plasmid 1 were generated by PCR for aligned plasmids 2 through 5 with the size (bp) of the fragments given above. Plasmid 6 contains a 93 bp internal deletion including the nCaRE-B2 element. Measurement of expression of luciferase activity was described in Figure 1. Numbers of independent assays are given in parentheses.

the silencer functions in a position-independent fashion (Li-Weber *et al*., 1993).

Sequence analysis of the 0.9 kb region showed the presence of two *Alu* repeat sequences (Figure 3). More surprisingly, there are three nCaRE sequences in this region, two of them located within the *Alu* repeat sequences. The APE-1 promoter has one nCaRE-A type sequence which is identical in 11 out of 13 internal bases to the nCaRE-A sequence of the PTH gene. However, since repression of the luciferase reporter was observed in the absence of nCaRE-A (Figure 3B), this element may not be important for the negative regulation. The APE-1 promoter has two nCaRE-B sequences, denoted nCaRE-B1 and nCaRE-B2, with a 2 base pair gap, and a gap along with a mismatch relative to the nCaRE-B sequence of the PTH gene, respectively (Figure 2B). Unlike the nCaRE-A type sequence, the presence or absence of these two elements had significant effect on the activity of the promoter (Figure 3B). Specifically, dramatic repression of luciferase expression due to the presence of a 93 bp segment of the APE-1 promoter is evident from a comparison of luciferase activities in lines 5 and 6. A similar increase in luciferase expression was observed as a result of deletion of a 35 bp segment spanning the nCaRE-B2 from the reporter plasmid represented in line 5 (data not shown). Thus this down-regulation may be attributed to the presence of nCaRE-B2 element in the APE-1 promoter.

Okazaki *et al*. (1994) showed that both nCaRE-A and nCaRE-B oligonucleotides formed complexes with the nuclear extracts of various cells as indicated by electrophoretic mobility shift. We inquired whether the APE-1 nCaRE-B1 and nCaRE-B2 oligonucleotides also complexed with HeLa nuclear extracts. Our results show that not only did the oligonucleotides form specific complexes with HeLa proteins, but these complexes could be competed with PTH nCaRE-B, but not with nonspecific plasmid DNA (Figure 4A).

We failed, as did Okazaki *et al*. (1994), in showing gel mobility shift of nCaRE-oligonucleotide with purified h-APE-1 polypeptide (data not shown). The lack of APE-1 binding to nCaRE can be explained by the requirement of a heterodimerization partner for interaction with the response element. However, the presence of APE-1 in the shifted complex could be shown by preincubating the HeLa nuclear extract with rabbit anti-APE-1 antisera. While the control nonimmune sera had no effect on complex formation, it was prevented after preincubation with the anti-APE-1 antibody (Figure 4B).

The gel mobility shift experiments are completely reproducible and their results strongly suggest that the APE-1 polypeptide binds to its own promoter in the same way as it does to the PTH promoter and thus may act as a silencer. However, the expression of PTH is affected by altering extracellular Ca^{2+} level in parathyroid cells. Intracellular

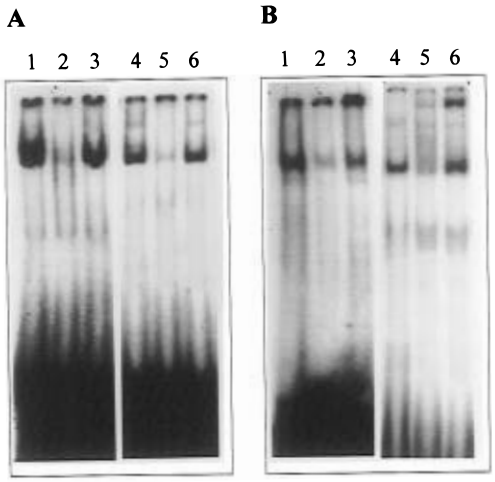

FIGURE 4: APE-1 binding activity of nCaRE-B sequences in the h-APE-1 promoter. Aliquots of HeLa nuclear extracts (12 μg protein) were incubated with 50 fmol of ^{32}P-labeled nCaRE-B1 (lanes 1−3) or nCaRE-B2 (lanes 4−6) oligonucleotides. (A) Lanes 1 and 4, control with no addition; lanes 2 and 5, addition of 250-fold excess oligonucleotide corresponding to PTH nCaRE-B; lanes 3 and 6, addition of nonspecific (plasmid, pBluescript) DNA (1 μg). (B) Lanes 1 and 4, control without preincubation; lanes 2 and 5, preincubation with anti-h-APE-1 antisera (1:20); lanes 3 and 6, preincubation with preimmune sera (1:20). The experimental details are described in Materials and Methods.

Ca^{2+} concentration may be significantly modulated by extracellular Ca^{2+} level in parathyroid cells but not in other cells, including HeLa (Carafoli, 1987). Thus whether the APE-1 protein binds to its promoter in a Ca^{2+}-dependent fashion needs to be tested by indirect experiments which are under way. It should be noted in this connection that unknown factor(s), in addition to APE-1, are still required for complex formation with nCaREs. Chung *et al*. (1996) reported recently that Ku80 (and Ku70) appeared to be the other factors involved in binding to the nCaRE-A sequence. The same proteins, however, failed to form complex with nCaRE-B and the APE-1 protein (Chung *et al*., 1996). Nevertheless, both our results and those of Okazaki *et al*. (1994) strongly suggest that unknown factor(s) are also required for the binding of APE-1 to nCaRE-B sequence.

In summary, the human APE-1 gene appears to contain multiple positive and negative regulatory elements. The presence of two nCaRE-B sequences among these elements suggests that APE-1 may regulate its own expression by binding to these sequences, in particular to the nCaRE-B2 element. Such autoregulation, if confirmed, will be the first such example of regulation of a mammalian DNA repair gene. Furthermore, in view of its multiple roles in transcription, autoregulation of APE-1 should have significant impact on expression of many other genes.

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