Cloning and Characterization of the Human Activity-Dependent Neuroprotective Protein*

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*This study was supported in part by the US-Israel Binational Science Foundation (BSF to I.G. and D.E.B) and by the Israel Science Foundation (ISF).

The nucleotide sequence reported in this paper has been submitted to the GenBank TM/EBI Data Bank with accession number AF250860.
We have recently cloned the mouse activity-dependent neuroprotective protein (mADNP). Here, we disclose the cloning of human ADNP (hADNP) from a fetal brain complementary DNA library. Comparative sequence analysis of these two ADNP orthologs indicated 90% identity at the mRNA level. Several single nucleotide polymorphic sites were noticed. The deduced protein structure contained 9 zinc fingers, a proline-rich region, a nuclear bipartite localization signal and a homeobox domain profile, suggesting a transcription factor function. Further comparative analysis identified an ADNP paralog (identity 33%, similarity 46%) indicating that these genes belong to a novel protein family with 9-zinc finger motif followed by a homeobox domain. The hADNP gene structure spans ~40Kb and includes five exons and four introns with alternative splicing of a non-translated second exon. The hADNP gene was mapped to chromosome 20q12-13.2, a region associated with aggressive tumor growth, frequently amplified in many neoplasias, including breast, bladder, ovarian, pancreatic, and colon cancers. Human ADNP mRNA is abundantly expressed in distinct normal tissues and high expression levels were encountered in malignant cells. Down regulation of ADNP by antisense oligodeoxynucleotides up regulated the tumor suppressor p53 and reduced the viability of intestinal cancer cells by 90%. Thus, ADNP is implicated in maintaining cell survival, perhaps through modulation of p53.
INTRODUCTION

Mouse activity-dependent neuroprotective protein (mADNP)\(^1\), a novel vasoactive intestinal peptide (VIP) responsive gene, was recently cloned (1). The relative enrichment of mADNP transcripts in the cerebellum, cortex, hippocampus, medulla and midbrain and the increases found in the presence of VIP, an established neuroprotective substance (2) implied a potential function in brain metabolism. Specifically, mADNP mRNA increased two-three-fold in astroglial cells incubated for three hours in the presence of nanomolar amounts of VIP (1). Another tissue containing increased mADNP transcripts was the mouse testis, a highly proliferative tissue, suggesting the involvement of ADNP in cell division.

As deregulation of oncogenes has been associated with neurodegeneration (3), pathways that regulate neuronal survival may impinge upon cancer proliferation. VIP regulates both neuronal survival and cell division (2). A system whereby labeled VIP is suggested as a tumor marker has been proposed, localizing in vivo tumors of patients with gastrointestinal neuroendocrine cancers as well as pancreatic and colonic adenocarcinomas (4). Other studies have identified a very high incidence of VIP receptor binding in breast, ovarian, endometrial, prostate, bladder, lung, esophageal, colonic, and pancreatic tumors, as well as in neuroendocrine and brain tumors (5). However, the VIP effect on cancer growth depends on the specific tumor and may be stimulatory (6,7) or inhibitory (8). In view of the high incidence of tumors containing VIP receptors, a potential intervention in tumor growth may employ a gene downstream of VIP’s action that is directly associated with stimulation of cell proliferation and survival.

The present report mapped the human ADNP gene\(^2\) to a chromosomal region amplified in cancer and ADNP mRNA expression was found to increase in
proliferative tissues. Inhibition of ADNP protein expression by antisense oligodeoxynucleotides resulted in marked reduction in metabolic activity in the target cells coupled with increases in the tumor suppressor p53 (3). Furthermore, a paralogous protein was discovered, suggesting a novel protein family containing zinc fingers and a homeobox domain.
EXPERIMENTAL PROCEDURES

RNA preparation- Neuroblastoma cells (6) were incubated in the presence of 25 nM VIP in phosphate-buffered saline (PBS) for three hours. Total RNA was prepared using RNAzol B solution (Tel-Test, Inc., Friendwood, TX, USA). A similar extraction method was used for tumor tissues, obtained fresh, post-surgery and frozen immediately on liquid nitrogen.

Complementary DNA isolation and sequencing- Oligodeoxynucleotide primers were synthesized in accordance with the mouse ADNP cDNA sequence (1). These primers (ACCTGCAGCAAAACAACTAT and GCTCGTTACAGATTGTAC, sense and antisense, respectively, for the mouse ADNP cDNA) were thereafter used for reverse transcriptase (RT) -polymerase chain reaction (PCR) with human neuroblastoma RNA, including murine-mammary leukemia virus RT (Gaithersburg, MD, USA) and AmpliTaq DNA Polymerase (Perkin Elmer, Branchburg, New Jersey, USA). The resulting PCR product was sequenced automatically (Applied Biosystems, The Weizmann Institute of Science Core facilities, Rehovot, Israel). A human neuroblastoma ADNP-RT-PCR product utilizing the primers 5′ ATCTGTAGGCC AGGGTTACA 3′ and 5′ TTGAGGAAGTGTTACCTGGG 3′, sense and antisense, respectively (1350-1369, sense; 1653-1672, antisense, Fig. 1) was labeled with α-32P-dCTP (Amersham, Little Chalfont, UK; 3000 Ci/mmol). The labeled product was used to screen a cDNA library derived from human whole fetal brain (male-female pooled, Caucasian, 19-23 week gestation, cloned unidirectionally into Uni-ZAP™XR vector, Stratagene, La Jolla, California, USA).

Northern blot hybridization- RNA (10-12 μg) was subjected to electrophoresis
followed by Northern blot hybridization on Nitrans 0.45µm filters (Schleicher and Schuell, Dassel, Germany). For probe labeling, the cDNA was subjected to PCR as above. Ribosomal RNA stained with ethidium bromide and actin mRNA amounts were used as internal standards (e.g. 1).

**Chromosomal mapping** - The chromosomal localization of hADNP was performed using several methods, as follows. 1] Radiation hybrid mapping (Stanford Human Genome Center). 2] Fluorescent in situ hybridization (FISH) with a genomic human contig. 3] FISH with hADNP. The H7 cDNA (Fig. 1) was nick-translated with biotin-14-dATP and hybridized in situ at a final concentration of 20 ng/µl to metaphases from two normal males. The FISH was modified from that previously described (9) in that no pre-reassociation was necessary and chromosomes were stained before analysis with both propidium iodide (as counterstain) and the fluorescent DNA stain 4,6-diamidino-2-phenylindole (DAPI) for chromosome identification. Images of metaphase preparations were captured by a cooled CCD camera using the ChromoScan image collection and enhancement system (Applied Imaging Int Ltd). FISH signals and DAPI banding were merged for figure preparation.

**Western analysis** - For ADNP analysis, cultures were washed with phosphate buffered-saline (PBS) and subjected to lysis (15 min. 4°C) in a buffer containing 1 mM EDTA, 150 mM NaCl, 0.1 mM ZnCl₂, 1mM MgCl₂, 50 mM Tris, pH 8.5, 0.1% SDS, 0.1% Triton). Nuclear DNA was fragmented by sonication and supernatants (10,000Xg, 10 min.) were collected and frozen until further measurements. For p53 analysis, cells were washed PBS and cell lysis (10 min. 4°C) was conducted in a buffer containing 5 mM EDTA, 150 mM NaCl, 10 mM Tris, pH 7.4, 1% Triton, 0.23 units/ml Aprotonin, 10 mM Leupeptin, 1mM phenylmethylsulfonylfluoride and 1mM
Human ADNP. Protein supernatants were collected following sonication by centrifugation (16,000g, 20 min. 4°C). Five µg of the soluble proteins were separated by electrophoresis on a 10% polyacrylamide gel and electro-transferred to nitrocellulose filters. Membranes were treated with 10% milk + PBS/Tween 0.2% for 1 hr and incubated O/N at 4°C in 2% milk + PBS/Tween 0.2% and the appropriate antibody. After incubation with peroxidase-conjugated secondary antibodies (Boehringer Mannheim, Indianapolis, IN, USA) signals were revealed by chemiluminescence using the ECL kit (Amersham, Arlington Heights, IL, USA).

Antibody preparation- Commercial antibodies used were: mouse monoclonal IgG anti human p53 antibodies (Santa Cruz Biotechnology, CA, USA); rabbit anti β-actin antibodies (Sigma, Rehovot, Israel); goat anti mouse IgG, peroxidase-conjugated AffiniPure (Jackson ImmunoResearch Laboratories, Bar Harbor, Maine, USA); donkey anti rabbit Ig-horse radish peroxidase linked (Amersham). Anti ADNP antibody was prepared against a synthetic peptide 989-CEMKPGTWSDESSQSEDARSSKPAAKK-1015 fused to keyhole limpet hemocyanin through the N-terminal cysteine moiety. In a parallel experiment, the carrier protein was bovine serum albumin. Affinity chromatography was performed on the peptide attached to Sepharose as before (1).

Cell culture and inhibition of growth by antisense oligodeoxynucleotides- The human colon cancer cell line HT29 (10) was cultured in Dulbecco-modified Eagle’s medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine and 1% Pen-Strep-Nystatin (Biological Industries, Beit Haemek, Israel). The adherent cells were split when a sub-confluent monolayer was formed following trypsin/EDTA treatment (0.25 units/0.02%) and naturalization with serum containing medium. For growth inhibition experiments, sub-confluent adherent cells were
washed with phosphate-buffered saline (PBS), treated with trypsin as above and re-suspended in DMEM containing 5% FCS to a final concentration of 50,000 cells/ml. 100μl aliquots were seeded into individual wells of a 96-well microtiter plates (Nunclon, Nunc Brand Products, Roskilde, Denmark). Each plate had a blank column and the appropriate controls. Plates were incubated for 24 hours in a humidified atmosphere containing 95% air/ 5% CO₂, at 37°C, medium was then replaced to contain an antisense oligodeoxynucleotide (10 μM) in DMEM without FCS. Following an additional 24-hour-incubation period the medium was replaced again to contain DMEM/5% FCS and cells were subjected to a further 48-hour-incubation period. Viable cell number was determined by a 3-hour-incubation period with the MTS reagent (CellTiter 96 AQueous cell proliferation kit, Promega, Madison WI, USA). The MTS reagent is oxidized by active mitochondria, resulting in increases in light absorbance at 490 nm (evaluated by a multiscan plate reader). For protein preparation cells were harvested (as described above) after a 30-hour incubation period.

Statistical analysis- ANOVA with Student-Neuman-Kuel’s multiple comparison of means test was used to assess the results.
RESULTS

*Human ADNP structure*- To isolate and characterize hADNP, the human ortholog of mADNP (1), a cDNA library derived from human fetal brain (19-23 week gestation) was screened and eight clones were isolated. Complete sequence of two cDNA clones (clone H7 and clone H3) indicated 90% identity to the mADNP at the mRNA level. Fig. 1 shows the sequence of hADNP (clone H7) with additional deduced upstream sequences EST sequences (AW453069, AW452644, AW139427, AW17331 and ref. 11), human genomic contig sequences containing ADNP (dJ914P20 contig ID 02099 and the genomic clone AL034553). Table 1 shows the exon-intron junctions of the five exons of the gene. The estimated gene size is 40647bp. A CpG island that stretches over 1135 bases as predicted by grail was observed around exon 1 (GC 69%). As particularly rich -CG dinucleotides have been previously associated with promoter regions we tested this sequence using promoter prediction programs TSSW and TSSG (http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html). Results gave low scoring promoter (TSSW at base 106 with LDF 5.69), TSSG gave no promoter. Alternative splicing of the second axon has been observed in ESTs (AI827420; AW007743). Only the three 3’ exons are protein coding. The proximal gene upstream to the ADNP gene is DPM1 (dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit) separated by 3438 bp.

At the protein structure level (Fig.1), 9 potential zinc finger motifs that are identical between hADNP and mADNP (1) were identified. These zinc finger domains (12), a proline-rich region (12), a nuclear bipartite localization signal (13) and a partial homeobox domain profile (14), suggest nuclear localization (12-14).
Furthermore, a glutaredoxin active site (15) as well as a leucine-rich nuclear export sequence were found (16). One striking difference between mouse and human was a polyglutamic acid stretch of nine residues in mouse (1) shortened to one residue in human (position 931, Fig. 1).

The second cDNA clone (H3) was identical to H7 except for several polymorphic regions (Table 2) and utilization of a different polyadenylation site (Fig. 1). Moreover, H3 contained a frame-shift mutation (an additional A nucleotide at position 3393, Fig. 1), with a premature termination codon at 3408 (Fig. 1). Unexpectedly, the H3 cDNA contained an additional protein coding sequence downstream a short poly (A) stretch, encoding the human immunodeficiency virus tat transactivator protein 1 (TBP1, references 17,18).

Comparative analysis utilizing blast identified part of the rat ADNP (accession no. AAF40431) (Fig. 2, identity 90%). Further analysis revealed a 33% identity and 46% similarity with the paralogous brain protein KIAA0863 (19). This protein revealed similar 9 Zinc finger domains and a similar homeobox domain as found in ADNP, suggesting a new gene family (Fig. 2).

*Human ADNP expression*—Northern blot hybridization utilizing mADNP (1) and hADNP identified one major mRNA band (5.5 kb, Fig. 3A). This mRNA showed increased expression in the heart, skeletal muscle, kidney and placenta. As ADNP was originally cloned from embryonic brain tissue (please see above and also ref. 1) further analysis of different brain regions (Fig. 3B) was performed. Results indicated increased expression in the cerebellum and cortex (Fig. 3B). Data from serial analysis of gene expression (SAGE, http://www.ncbi.nlm.nih.gov/SAGE/) was also performed. Results obtained suggested an increased expression in tumor tissues, adenocarcinoma (breast and ovaries), medulloblastoma (brain) and glioblastoma (brain) and colon
cancer. In normal tissues, ADNP sequences were found in microvascular endothelial cells and in brain (mostly white matter). SAGE of the related KIAA0863 (cDNA isolated from human brain)\(^8\) revealed increased expression in tumors (colon and prostate) and in brain white matter as well as in kidney and testis (http://zearth.kazusa.or.jp/huge/gfpage/KIAA0863/).

**Chromosomal localization-** Twenty metaphases from a normal male were examined by fluorescent in situ hybridization (FISH). All of these metaphases showed signal on one or both chromatids of chromosome 20 in the region q12-q13.2; 40% of this signal was at 20q12, 32% was at 20q13.1 and 28% was at 20q13.2 (Fig. 4). Similar results were obtained, utilizing public databases localizing the gene to chromosome 20q13.2 [with identity to the ordered markers: G30243, W45435 in linkage to the genome data base (GDB) locus D20S831\(^9\) and to 20q13.13-13.2 utilizing a human contig sequence containing the hADNP gene.

KIAA0863 was localized to human chromosome 18 using public databases.

**Human ADNP and cancer-** Since SAGE analysis identified increased ADNP expression in cancer cell lines and since the chromosomal region 20q12-20q13 is amplified in a wide variety of tumors (19-23), we investigated the association of hADNP with cancer growth. Three lines of experimental studies were conducted. 1) hADNP mRNA was quantitated in human primary cancer tissue (breast and colon) in comparison to adjacent normal tissue and was shown to be significantly increased in the cancer. A 2.5-3.5-fold increase was observed in colon cancer (data not shown). The increased expression was most evident with breast cancer and ranged 14.4 \( \pm \) 4.6 fold (mean \( \pm \) SEM). When the ADNP mRNA content was compared to actin mRNA content, in the same breast cancer samples, the increase ranged 10.9 \( \pm \) 5 fold (Fig. 5).

2) Six antisense oligodeoxynucleotides were synthesized (Fig.1) and further utilized
to inhibit cell proliferation. The oligodeoxynucleotides were chosen as complementary to the most 5′ methionines (indicated in Fig. 1). Results have shown that the antisense oligodeoxynucleotide 1 inhibited cell division (measured as metabolic activity) of the human intestinal cancer, HT-29 (Fig. 6, P< 0.001). A similar inhibition was observed with the antisense oligodeoxynucleotide 8 (Fig. 6, P< 0.001). Furthermore, the antisense oligodeoxynucleotide 9 inhibited by about 37.5 ± 3 %, and the antisense oligodeoxynucleotide 68 also inhibited growth (by 45 ± 3%, P < 0.001). In contrast to 8 and 9, the sequence of 68 is shared by other cDNA sequences, hence it may not be specific. Further specificity was determined with a control sense oligodeoxynucleotide complementary to antisense 8 and with an antisense 8 with all internucleotide bonds of the phosphorothionated type (Fig. 6, thio). In addition, the antisense oligodeoxynucleotide 07 and 67 did not inhibit growth.

3) To determine that indeed the antisense oligodeoxynucleotides inhibited ADNP expression, western blot analyses were performed with actin and the tumor suppressor p53 as internal standards. Results (Fig. 7) show that ADNP (114,000 M.W.) was decreased by about 3-fold in comparison to actin (densitometric scan results: 1.11 ± 0.23 vs. 0.31 ± 0.11, respectively, P<0.023, n=3), while p53 levels showed an apparent increase (1.04 ± 0.04 vs. 2.41 ± 0.41, P<0.029, n=3).
DISCUSSION

The present report characterizes human activity-dependent neuroprotective protein (hADNP) gene, encoding an mRNA that is abundantly expressed in distinct normal tissues and may be alternatively spliced. The 5′ UTR of the mRNA is GC rich as has been recently shown for several other genes (e.g. 24-26). Human ADNP was found to contain zinc fingers and a homeobox domain. Furthermore, a family including at least two genes of significant homologies is described.

Based on cDNA and deduced protein sequence (12-14), hADNP and KIAA0863 may represent nuclear DNA-binding proteins, putative transcription factors. The thiotransferase/glutaredoxin active site (15) found in ADNP (Fig 1) may modulate its own DNA binding activity or that of other DNA-binding proteins in response to oxidative stress and signal transduction pathways implicated in the redox state of the cell (27). We have previously hypothesized that mADNP is a secreted protein (1). To reconcile this discrepancy, one hypothesis may involve alternate utilization of the 7 putative initiator methionine residues at the N-terminal of hADNP (Fig. 1) resulting in processing pathways that may yield secreted portions. An alternative hypothesis was put forward by us in a recent report suggesting the existence of a nuclear-export signal within the ADNP mRNA (Fig. 1, 16, 28). A similar sequence was discovered in the engrailed transcription factor (16) as well as in the ADNP-related protein KIAA0863.

The ADNP containing locus, the 20q12-13.2 chromosomal region is amplified in many tumors (19-23). In breast tumors, comparative genomic hybridization revealed approximately 20 regions of recurrent increased DNA sequence copy number (23, 29-31). These regions are predicted to encode dominant genes that may play a role in tumor progression or response to therapy. Three of these regions have been
associated with established oncogenes: ERBB2 at 17q12, MYC at 8q24, and CCND1 and EMS1 at 11q13. Amplification at 20q13 occurs in a variety of tumor types, but up-to-date does not involve a previously known oncogene (20).

Another aspect of ADNP/cancer/neuroprotection interaction is the fact that ADNP and p53 expression may be interrelated, as shown here, and both proteins may influence tumor growth as well as brain function (1,3).

The hADNP (h3) cDNA contained the TBP1 cDNA sequence downstream of the coding region of ADNP. Previously, the TBP1 gene was localized to chromosome 11p12-p13 (18) and the TBP1 gene product was associated with the cell cycle. The finding of TBP1 downstream of hADNP may either be trivial, resulting from molecular cloning manipulations, or may indicate translocation involved with cancer abnormalities.

The discovery of ADNP (1) as a VIP responsive gene in astroglial cells (a major component of brain white matter) is now extended to the SAGE finding of ADNP encoding sequences in brain (mostly white matter) as well as in microvascular endothelial cells. VIP binding sites have been described in astrocytes (32) as well as in endothelial cells (33). In both cases, developmental functions (33, 34) and proliferation (34-36)/survival (32, 37) functions have been hypothesized. The homeobox-containing protein, ADNP may thus mediate some of the VIP developmental/survival-associated effects involving normal growth and cancer proliferation. The abundance of ADNP mRNA in heart, skeletal muscle, kidney and placenta may represent, in part, an astrocyte-like cell population (38), or enrichment in blood microvessels (39). Indeed, the original characterization of VIP was as a vasodilator (40) and since endothelial cells play a major role in vasodilatation,
endothelial ADNP points toward a new avenue for research on potential VIP/ADNP interactions.

Our original findings related ADNP to VIP-mediated neuroprotection. Thus, ADNP mRNA increased in glial cells incubated with VIP and a very short peptide fragment derived from ADNP (NAPVSIPQ; termed NAP) provided potent neuroprotection (1). Given the abundant expression of ADNP, future experiments are aimed at further assessing the question of general normal cell protection and of secreted processed forms of ADNP providing cellular protection against external toxicity.

The increased ADNP mRNA expression in the cerebellum [a structure enriched in VIP binding sites (41)] suggests a further avenue of research dealing with tissue specific expression and function.

From a clinical perspective, the present report provides methods of using hADNP nucleic acid probes to detect and identify pathologically proliferating cells, including cancer cells. Furthermore, our results suggest that ADNP is important for cell survival, and the antisense ADNP oligodeoxynucleotides may be developed as anti tumor therapeutics.
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Footnotes:

1. GenBank accession numbers AF068198, NM_009628
2. GenBank accession number AF250860
3. ESTs (AW453069, AW452644, AW139427, AW17331)
4. Sanger genome center, The Sanger Blast Server dJ914P20 contig ID 02099
5. Genomic clone AL034553
6. ESTs (AI827420; AW007743)
7. Rat ADNP (accession no. AF234680)
8. KIAA0863 (accession no.: AB020670)
9. Genome Data Base (GDB) locus D20S831

The abbreviations used are: mADNP, mouse activity-dependent neuroprotective protein; VIP, vasoactive intestinal peptide; hADNP, human ADNP; RT, reverse transcriptase; PCR, polymerase chain reaction; FISH, Fluorescent in situ hybridization; DMEM, Dulbecco-modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; SAGE, Serial Analysis of Gene Expression.
Acknowledgements- We are grateful to Prof. Samuel Berkovic for his invaluable help with the chromosomal mapping. Prof. Illana Gozes is the incumbent of the Lily and Avraham Gildor Chair for the Investigation of Growth Factors. This work is in partial fulfillment of the Ph.D. requirements of Ms. Rachel Zamostiano and Mr. Albert Pinhasov. We are grateful to Mr. Joshua Steinerman and Ms. Sharon Furman for critical reading of the manuscript. Patents have been applied for hADNP and the antisense oligodeoxynucleotides.
Legends to the Figures:

FIG. 1. The human ADNP cDNA and gene. The human ADNP sequence (based on clone H7). Left side of the sequence denotes nucleotides no. and the right side denotes amino acid no. The beginnings of the exons are marked by arrows (↓). Alternative polyadenylation sites are marked by arrows and numbered(1↑-4↑): 1↑ - clone H4, 2↑ - clones H6, H2, 3↑ - clone H10, 4↑ - clones H3, H5, and H7. The calculated molecular weight of the protein was 123562.8 Dalton and the theoretical pI: 6.97. Marks on the Figure: Antisense oligonucleotides areas are **underlined** and marked by numbers: 1, 8, 9, 7, 67, 68. Motifs: **Zinc finger domains, bold and dotted underlined** (the second, sixth and seventh dotted Zinc Finger domains are designated as trusted by Pfam, the other domains are potential Zn binding domains). **Bipartite nuclear localization signal (bold and pattern underline); Homebox domain (bold and double line); Proline-rich region (bold and broken underline); Partial Glutaredoxin (thioltransferase) active site (bold and double dotted underlined).**

Leucine-rich nuclear export sequence -

↑KLASSLWLWSDIALSF↑

FIG. 2. The ADNP gene is conserved among species, comparative studies identified a new family member, KIAA0863. Dashed lines are zinc finger domains, solid line is a presumptive homeobox domain region.
FIG. 3. Patterns of expression of the human ADNP mRNA.  

(A) Master blot (Human 12-Lane MTN Blot #7780-1, Clontech, Laboratories Inc. Palo Alto, CA USA). Lane description: 1] brain; 2] heart; 3] skeletal muscle; 4] colon; 5] thymus; 6] spleen; 7] kidney; 8] liver; 9] small intestine; 10] placenta; 11] lung; 12] peripheral blood leukocytes.

(B) The human ADNP mRNA is in brain tissues. Human brain RNA master blot - was purchased from Clontech, Palo Alto, CA, USA # 7755-1. Hybridization was performed as described in the methods. Lane description: 1] cerebellum, 2] cerebral cortex, 3] medulla, 4] spinal cord, 5] occipital lobe, 5] frontal lobe, 6] temporal lobe, 7] putamen.

FIG. 4. Chromosomal localization of hADNP. Photographs and idiogram (insert) showing the hybridization sites of H7. A total of 2 non-specific background dots was observed in the 20 metaphases tested. A similar result was obtained from hybridization of the probe to 10 metaphases from a second normal male (not shown). Two representative pictures are shown.

FIG. 5. The ADNP mRNA content is increased in tumors. RNA was extracted from human primary tumors (breast) and from adjacent normal tissue and subjected to Northern blot hybridization. C = control tissue; T = tumor. Breast cancer from 48 year-old female. The figure shows autoradiogram ADNP and ACTIN mRNA and ethidium bromide staining, RNA.
FIG. 6. HT-29 cell growth is inhibited in the presence of antisense oligodeoxynucleotides specific for ADNP mRNA. Five oligodeoxynucleotides were synthesized (Fig. 1) and utilized to inhibit cancer growth. A representative figure is shown. 1] Control; 2] antisense 1; 3] sense 8; 4] antisense 8; 5] antisense 8 with all internucleotide bonds of the phosphorothionated type.

FIG. 7. Western blot analysis: Reduction in ANDP in HT29 cells in comparison to actin and p53. Experiments were performed as described in the method section. - no antisense oligodeoxynucleotide; + cells incubated in the presence of the antisense oligodeoxynucleotide.
### Table 1: Exon-intron junctions of the hADNP gene

| Exon no. | mRNA location | Gene location | Exon-Intron junctions (exon sequences are in upper case, introns are in lower case). |
|----------|---------------|---------------|----------------------------------------------------------------------------------|
| 1        | 1-165         | 1 - 165       | GTCAAGgtaagccggecgcegcgecg                                                     |
| 2        | 166-340       | 2166-2340     | TcctttgatttccatatagGTGTGA...ACACCGgtgggtatattcttttgga                           |
| 3        | 341-453       | 26990-27102   | taatatatatcaataaGACTAT...ATAGAAgtaagtagcatgttatttt                              |
| 4        | 454-546       | 28882-28974   | CttttacttttttctagGATT TT...AACCAGgtaagtaggcacagagactt                           |
| 5        | 547-4716      | 36479-40648   | agttttgtttacttttagGACTAT                                                         |
Table 2: Polymorphic sites in hADNP (Fig. 1A)

| Base # | polymorphism | Sequences found in comparison to H7: |
|--------|--------------|-------------------------------------|
| 2421   | G/A          | H7/ref.11                           |
| 2913   | C/T          | H7/ref.11                           |
| 3672   | A/G          | H7/H3, ref.11; Contig-dJ914P20.02099. Of the eight hADNP clones 50% had G and 50% A. |
| 3704   | C/A          | H7/contig- dJ914P20.02099            |

Comparison among different hADNP indicated a few polymorphic sites that result in no changes in the deduced protein.
Fig. 2
Fig. 5
Fig. 7
Cloning and characterization of the human activity-dependent neuroprotective protein
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J. Biol. Chem. published online September 29, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M007416200

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