Molecular characterization of hNRP, a cDNA encoding a human nucleosome-assembly-protein-I-related gene product involved in the induction of cell proliferation

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We have isolated from a human thymus cDNA library a cDNA clone encoding a potential protein with 54% amino acid similarity to that encoded by a previously identified cDNA for yeast nucleosome assembly protein I (NAP-I). The deduced amino acid sequence for this newly identified cDNA, designated hNRP (human NAP-related protein), contains a potential seven-residue nuclear localization motif, three clusters of highly acidic residues and other structural features found in various proteins implicated in chromatin formation. When expressed as a fusion protein in *Escherichia coli*, hNRP reacted specifically with a monoclonal antibody raised against human NAP-I. The hNRP transcript was detected in all tissues and cell lines studied, but levels were somewhat increased in rapidly proliferating cells. Moreover, levels of both hNRP mRNA and protein increased rapidly in cultured T-lymphocytes induced to proliferate by incubation with phorbol ester and ionomycin. Phorbol 12-myristate 13-acetate/ionomycin-induced increases in both hNRP mRNA and mitogenesis, as measured by thymidine incorporation, were markedly inhibited, however, in cells treated with an hNRP antisense oligonucleotide. These results demonstrate a correlation between induction of hNRP expression and mitogenesis and taken together with the structural similarities between hNRP and yeast NAP-I suggest that the hNRP gene product participates in DNA replication and thereby plays an important role in the process of cell proliferation.

INTRODUCTION

In eukaryotic chromosomes, DNA is packaged in the form of chromatin, a complex of DNA and histones organized into distinct regularly spaced structural units known as nucleosomes [1,2]. Characterization of the nuclear proteins involved in nucleosome and chromatin assembly has been of long-standing interest both in relation to understanding the process of DNA replication and also in terms of deciphering the relationship between DNA topology and gene transcription. To elucidate the mechanisms involved in nucleosome assembly, efforts have been made to purify specific factors that participate in nucleosome formation in either cellular or cell-free systems. Among the factors identified to date are two acidic proteins, nucleoplasmin and N1, which were initially detected in *Xenopus* egg extracts and which are now known to facilitate nucleosome formation by binding to histones H2A/H2B and H3/H4 respectively [3–5]. The functions of nucleoplasmin and N1 have been assessed extensively in the *Xenopus* system, but the relevance of these specific proteins to nucleosome assembly in mammalian cells is not yet clear. More recently, however, several factors that appear to play a similar role in relation to chromatin synthesis have been identified in human cells [6,7]. One such factor is nucleosome assembly protein (NAP-I), a 58 kDa protein isolated from HeLa cells and shown to facilitate nucleosome histone core formation and transfer to DNA [7,8]. By taking advantage of a monoclonal antibody reactive with this HeLa-cell protein, a yeast counterpart of the NAP-I gene was isolated and a role for the recombinant expressed protein in nucleosome assembly confirmed [9]. However, the mammalian NAP-I gene has not been previously identified. In the course of characterizing several newly isolated human T-cell cDNA clones, our group identified a cDNA with a predicted amino acid sequence similar to that deduced for yeast NAP-I. This cDNA, herein designated human hNRP (NAP-related protein), appears to belong to a multigene family and, when expressed *in vitro*, produces a protein that specifically reacts with antibodies to HeLa-cell-derived NAP-I. Analysis of hNRP expression in phorbol ester/ionomycin-stimulated human T-lymphoid cells indicates that levels of hNRP RNA and protein increase rapidly in conjunction with induction of cellular proliferation as assessed by thymidine incorporation. Both the increases in hNRP expression and induction of proliferation following treatment with these mitogenic agents are markedly inhibited by exposure of the cells to hNRP antisense oligonucleotide. These data provide evidence that the hNRP gene product plays a role in cell proliferation, as is consistent with its structural similarity to a yeast protein implicated in nucleosome assembly.

MATERIALS AND METHODS

Cells and cell activation

The following human cell lines were used in these studies: an Epstein–Barr virus (EBV)-transformed lymphoblastoid cell line (EBV-LP) derived from a healthy individual; Jurkat and SIT,

Abbreviations used: NAP, nucleosome assembly protein; hNRP, human NAP-related protein; EBV, Epstein–Barr virus; PBMC, peripheral blood mononuclear cells; XLR, X-linked, lymphocyte-regulated; GST, glutathione *S*-transferase; PMA, phorbol 12-myristate 13-acetate; 1 × SSC, 0.15 M NaCl + 0.015 M sodium citrate; IPTG, isopropyl β-D-thiogalactoside; TBS, Tris-buffered saline.

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The sequence data in Figure 1 will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession number M86667.

two T-leukaemic cell lines; HeLa, a cervical carcinoma cell line; and HEY, an ovarian carcinoma cell line. Cells were maintained in culture in complete medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine and 5 mg/ml gentamicin).

Fresh human peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation over Ficoll/Hypaque (Pharmacia, Piscataway, NJ, U.S.A.) and resuspended in complete medium. Enriched T-cell populations (> 98% CD3+) were obtained as previously described [10]. Briefly, PBMCs (10^6/ml) were cultured for 72 h in complete medium with phytohaemagglutinin (Difco, Detroit, MI, U.S.A.; 10 μg/ml). The cells were then washed, split at 1:10 ratios and propagated for 72 h in complete medium containing 2 nM human recombinant interleukin 2 (a gift from the Cetus Corporation, Emeryville, CA, U.S.A.). Before exposure to oligonucleotides and phorbol ester/ionomycin, cells were washed three times and incubated for 48 h in complete medium in the absence of exogenous interleukin 2.

Synthesis of human X-linked lymphocyte-regulated (hXLR) cDNA

The hXLR cDNA used for isolation of the human NAP gene was initially obtained in conjunction with a search for human homologues to members of the murine, XLR gene family [11,12]. For this purpose, cDNA synthesis was carried out using 3 μg of poly(A)-enriched RNA from the EBV-B cell line, EBV-LP, oligo(dT) primer and murine leukaemia reverse transcriptase. PCR amplification of the cDNA was performed using two 37-mer oligonucleotide primers containing restriction sites at their 5′ ends and corresponding to sequences from the initial 28 nucleotides (5′-primer: 5′-GTGAAGCTTATGGAAAACTGG-ACTTGTCAAGTGATC-3′) and the terminal 28 nucleotides (3′-primer: 5′-GTGCTGCAGTTAGTCTGAAGATGGGAA-ACTAGAAGAG-3′) respectively, of the cDNA encoding the XLR gene, pM1 [12]. For amplification, 1 μl of cDNA was mixed with 2 μl of each primer (at a final concentration of 1 μM), all four deoxynucleotide triphosphates, *Taq* polymerase (Cetus–Perkin–Elmer, Emeryville, CA, U.S.A.) in a reaction buffer containing 1.5 mM Mg^{2+}. The final mixture was incubated for 30 cycles, each cycle consisting of 1 min denaturation at 94 °C, 2 min annealing at 45 °C and 3 min extension at 72 °C. The amplified product was restriction digested with *Hin*dIII–*Pst*I and subcloned into the *Hin*dII–*Pst*I site of pUC19.

Isolation of the hNRP clone

The hXLR cDNA was ^{32}P-labelled by random priming and used to screen a human thymocyte cDNA library constructed in the λ ZAPII vector (Stratagene, La Jolla, CA, U.S.A.). Hybridizations were carried out in a solution containing 1 M NaCl, 5% dextran sulphate and 1% SDS at 60 °C and filters were washed in 0.2× SSC/0.1% SDS at 55 °C. Positive clones were subcloned into a Bluescript plasmid vector for sequence analysis.

Nucleotide sequencing and analysis

The hXLR cDNA as well as hXLR-hybridizing thymic-derived cDNA clones were sequenced in both directions directly from plasmid DNA by the dideoxy-chain termination method [13] using [^{35}S]dATP (Amersham, Arlington Heights, IL, U.S.A.). The cDNA inserts of clones T2 and T4 were sequenced using Bluescript primers as well as oligonucleotides homologous to sequences from within the inserts. The computer programs of the University of Wisconsin Genetics Computer Group were used to assemble and analyse the sequence data [14].

Oligonucleotides and cell activation

To examine the effects of hNRP expression on cell proliferation, an hNRP antisense oligomer complementary to the 15 nucleotides beginning at the translation initiation codon (5′-GTCAATGT-CTGCCAT-3′) and the corresponding sense oligomer (5′-ATGGCAGACATTGAC-3′) were obtained from the Regional DNA Synthesis Facility at the University of Calgary. PBMC or T-cell cultures were established in 96-well microtitre plates at 10^5 cells/200 μl (for proliferation assays) and in culture plates at 15×10^6 cells/30 ml (for Northern and immunoblotting assays) in complete medium. At the onset of the culture period, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co., St. Louis, MO, U.S.A.) at a final concentration of 50 nM, 1 μM ionomycin (Calbiochem, San Diego, CA, U.S.A.) and the specified concentrations of hNRP antisense or sense oligonucleotides.

Construction of pGEX-hNRP expression plasmid

An 800 bp *Eco*RI fragment from within the T2 clone and extending from nucleotide 572 to nucleotide 1369 of the hNRP coding region was subcloned into the *Eco*RI sites of the pGEX-2T vector [15] so as to yield a fusion protein in frame with glutathione *S*-transferase (GST). The recombinant plasmid was used to transform *Escherichia coli* JM109, and the bacterial cells were grown in Luria–Bertani broth at 37 °C to A_{600} 0.4–0.7 before the addition of isopropyl β-D-thiogalactoside (IPTG) to 1 mM. To obtain lysates for immunoblotting assays, cells were grown for a further 3 h and portions were then pelleted, lysed in Laemmli reducing buffer [16] supplemented with 7 M urea, and boiled for 10 min.

Immunoblotting assays

T-lymphocytes (15×10^6) were stimulated for 0, 4, 8 or 24 h with 50 nM PMA, 1 μM ionomycin and 5 μM sense or antisense oligomer as described above. Cells were then pelleted and lysed in 0.5 ml of cold lysis buffer (50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 1% Nonidet P40, 0.1% SDS, 0.1% sodium deoxycholate, 2 mM EDTA, 10 μM orthovanadate and 50 μM $ZnCl_2$). The lysates were centrifuged at 10000 ***g*** and the supernatants boiled in reducing Laemmli buffer for 10 min. These lysates as well as the bacterial cell lysates prepared as described above were then subjected to SDS/PAGE (12% gels) and the separated proteins transferred to a nitrocellulose filter (Schleicher and Schuell, Keene, NH, U.S.A.). The membranes were blocked overnight with 5% BSA (Calbiochem) in 10 mM Tris/HCl buffer, pH 8.3, containing 140 mM NaCl and 0.01% NaN_3 and then incubated overnight with monoclonal anti-(HeLa-cell NAP-I) antibody (1:10 dilution of tissue culture supernatant collected from the 4A8 hybridoma) [7]. Filters were sequentially washed twice with 1× Tris-buffered saline (TBS), once with 1× TBS containing 0.5% Nonidet P40 and twice again with 1× TBS and were then incubated for 1 h in a solution containing rabbit anti-mouse immunoglobulin (Western Blotting Associates, Mississauga, Ont., Canada). After further washing, the filters were incubated with 1 μCi of ^{125}I-Protein A (Amersham Corp., Oakville, Ont., Canada) in 4 ml of blocking solution for 1 h at room temperature. Unbound ^{125}I-Protein A was washed away and the immuno-

reactive proteins were detected by autoradiography at −70 °C. Filters containing bacterial protein extracts were later stripped in 1 × TBS/1 % SDS, blocked as described above, and incubated overnight with 4 μl of rabbit polyclonal anti-GST antibody in 4 ml of blocking solution. The blots were then incubated with 1 μCi of ^{125}I-Protein A as described above and again exposed to Kodak XAR-5 film at −70 °C.

RNA isolation and Northern-blot hybridization

Total RNA was extracted by the guanidium thiocyanate/caesium chloride method [17] from untreated cells and cell lines and from T-cells stimulated for 0, 4, 8 or 24 h with 50 nM PMA, 1 μM ionomycin and 5 μM hNRP sense or antisense oligomer. For Northern-blot analyses, 10 μg of RNA was size-fractionated over formaldehyde gels and transferred to nitrocellulose paper (Schleicher and Schuell). The probes used for hybridization were the full-length T2 cDNA and the amplified hXLR cDNA. Probes were ^{32}P-labelled by random priming and the hybridizations were performed in solution containing 50 % formamide, 5 × SSC, 2 % SDS and 5 × Denhardt's solution at 42 °C. The filters were washed in 2 × SSC/1 % SDS for 30 min at room temperature and in 0.1 × SSC/0.1 % SDS for 30 min at 60 °C followed by autoradiography at −70 °C. To control for amounts of RNA per lane, filters were stripped and rehybridized with a β-actin cDNA (ATCC, Rockville, MD, U.S.A.), and the hNRP and β-actin band intensities were compared using scanning densitometry.

Proliferation assay

T-cells and PBMCs were cultured in replicates of five for 4, 8, 24 or 48 h in 96-well microtitre plates at 10^5 cells/200 μl in complete medium containing 50 nM PMA, 1 μM ionomycin and various concentrations (0.5, 5 and 10 μM) of either sense or antisense hNRP oligomer. At 4 h before termination of the incubation, cultures were pulsed with 2 μCi/well of [^{3}H]thymidine (Amersham International, Amersham, Bucks., U.K.) and the levels of thymidine incorporation were subsequently determined by liquid-scintillation counting.

RESULTS AND DISCUSSION

Isolation of hXLR-related thymocyte cDNAs

As part of an effort to identify genes that influence human haemopoietic differentiation, we undertook studies aimed at isolation of a human homologue to a murine cDNA sequence, pM1. The pM1 cDNA represents the best characterized member of the murine XLR gene family, a family of sequences which map to the X chromosome and which appear to be expressed largely in lymphoid cells [11,12]. Accordingly, oligonucleotides corresponding to the 5′ and 3′ regions of the pM1 cDNA sequence were used in conjunction with PCR to amplify human XLR cDNA from EBV-transformed B-lymphoblastoid cells. The amplified products were subcloned into pUC19, and analysis of the sequences recovered from four pM1-hybridizing subclones revealed the isolation of the same 346 bp sequence in each instance. This sequence, designated human XLR (hXLR), contained a single open reading frame of 85 amino acids and was found to be expressed in human lymphoid tissues and at particularly high levels in thymocytes. Because the size of the transcript identified by the amplified hXLR cDNA (1.7 kb) was longer than the cDNA sequence, the latter was used to screen a human thymocyte cDNA library and isolate longer cDNAs. Of 5×10^5 recombinants, five positively hybridizing clones were identified in tertiary screening and were subcloned into the Bluescript plasmid for further characterization.

Sequence analysis of these cDNAs revealed that two were entirely identical in sequence whereas the others (clones T2–T5) overlapped with one another extensively. As shown in Figure 1, alignment of these sequences, as well as that of hXLR cDNA, revealed that these cDNAs represent various portions of a single gene and together comprise a 1560 bp transcript encompassing the entire coding region as well as a large 3′ untranslated sequence.

Sequence analysis of T2–T5 cDNAs

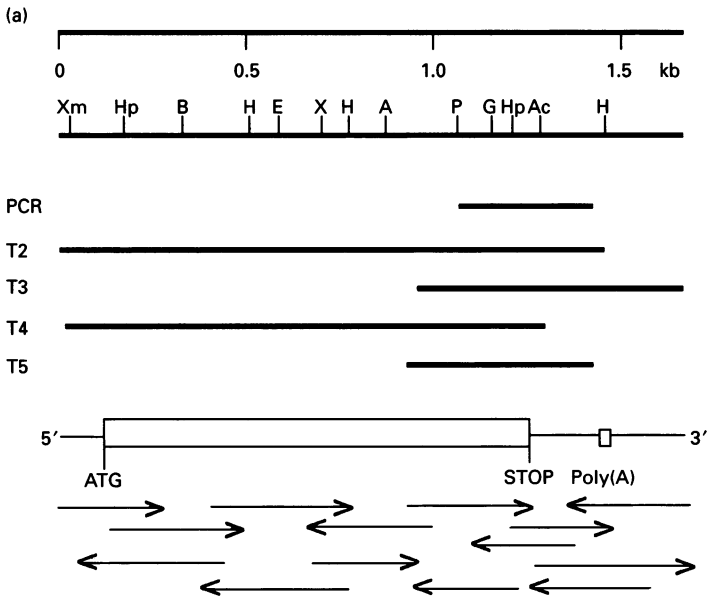
The complete nucleotide and predicted amino acid sequences of the gene represented by the four cDNAs T2–T5 are shown in Figure 1(b). An open reading frame extends 1241 bp from nucleotide 7 through to nucleotide 1248. The first in-frame ATG, which occurs at amino acid position 26 of the open reading frame, is flanked by sequence which closely matches the consensus sequence for eukaryotic translation initiation [18] and probably represents the initiation codon. Thus the open reading frame encodes a 391-amino acid protein of approximately 45 kDa. The termination codon, TGA, is followed by a stretch of 309 nucleotides which contains a consensus polyadenylation signal motif (ATAAA) at nucleotide positions 1392–1396 and appears enriched in A and T sequence. High AT content has been frequently observed within the 3′ untranslated regions of mRNAs for growth factors and their receptors as well as for oncogenes, and is thought to adversely influence transcript stability and half-life [19]. Interestingly, the 3′-untranslated region of the pM1 gene is also remarkably rich in AT sequences [12].

Comparisons of the deduced amino acid sequence of this newly isolated gene with those in GenBank revealed its striking structural similarity to the protein sequence predicted for two recently identified cDNA clones. One of these is the yeast cDNA encoding NAP-I which displays 29 % amino acid identity with the sequence identified here [9]. However, as shown in Figure 2, when conservative amino acid replacements are taken into consideration, the proteins potentially encoded by these respective nucleotide sequences match at 54 % of their amino acid residues. This degree of sequence similarity between gene homologues in such evolutionarily distant organisms as man and yeast is remarkable and suggests the isolation of a human counterpart, hNRP, to the yeast NAP-I gene.

The hNRP cDNA sequence is also strikingly similar to that of a murine cDNA, DN38, which was isolated from a murine brain cDNA library by subtraction hybridization and which has not previously been functionally characterized [20]. The deduced polypeptide sequences for DN38 and hNRP are identical over 322 of 327 amino acids and it therefore appears that DN38 may also encode a protein involved in nucleosome assembly.

Structural features of hNRP

The predicted protein sequence for hNRP has a number of interesting characteristics. The first of these is a clustering of acidic amino acid residues in three regions of the molecule. As shown in Figure 2, two of these regions are located near the N-terminus of the molecule between amino acid positions 13 and 26 and 129 and 141 and contain 10 of 14 and 9 of 13 acidic amino acid residues respectively. Similarly, 22 acidic residues occur within a 29 amino acid stretch mapping between positions 348



(b)

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CCCTGAGTCACTGCCTGCGCACGTCCGGCCGCCTGGCTCCCCATACTAGTCGCCGATATTTGGAGTTCTTACAACATGGCAGACATTGAC      90
                                                                  MetAlaAspIleAsp                  5

AACAAAGAACAGTCTGAACTTGATCAAGATTTGGATGATGTTGAAGAAGTAGAAGAAGAGGAAACTGGTGAAGAAACAAAACTCAAAGCA    180
AsnLysGluGlnSerGluLeuAspGlnAspLeuAspAspValGluGluValGluGluGluGluThrGlyGluGluThrLysLeuLysAla     35

CGTCAGCTAACTGTTCAGATGATGCAAAATCCTCAGATTCTTGCAGCCCTTCAAGAAAGACTTGATGGTCTGGTAGAAACACCAACAGGA    270
GlnLeuThrValGlnMetMetGlnAsnProGlnIleLeuAlaAlaLeuGlnGluArgLeuAspGlyLeuValGluThrProThrGlyTyr     65

TACATTGAAAGCCTGCCTAGGGTAGTTAAAAGACGAGTGAATGCTCTCAAAAACCTGCAAGTTAAATGTGCACAGATAGAAGCCAAATTC    360
TyrIleGluSerLeuProArgValValLysArgArgValAsnAlaLeuLysAsnLeuGlnValLysCysAlaGlnIleGluAlaLysPhe     95

TATGAGGAAGTTCATGATCTTGAAAGGAAGTATGCTGTTCTCTATCAGCCTCTATTTGATAAGCGATTTGAAATTATTAATGCAATTTAT    450
TyrGluGluValHisAspLeuGluArgLysTyrAlaValLeuTyrGlnProLeuPheAspLysArgPheGluIleIleAsnAlaIleTyr    125

GAACCTACGGAAGAAGAATGTGAATGGAAACCAGATGAAGAAGATGAGATTTCGGAGGAATTGAAAGAAAAGGCCAAGATTGAAGATGAG    540
GluProThrGluGluGluCysGluTrpLysProAspGluGluAspGluIleSerGluGluLeuLysGluLysAlaLysIleGluAspGlu    155

AAAAAGGATGAAGAAAAAGAAGACCCCAAAGGAATTCCTGAATTTTGGTTAACTGTTTTTAAGAATGTTGACTTGCTCAGTGATATGGTT    630
LysLysAspGluGluLysGluAspProLysGlyIleProGluPheTrpLeuThrValPheLysAsnValAspLeuLeuSerAspMetVal    185

CAGGAACACGATGAACCTATTCTGAAGCACTTGAAAGATATTAAAGTGAAGTTCTCAGATGCTGGCCAGCCTATGAGTTTTGTCTTAGAA    720
GlnGluHisAspGluProIleLeuLysHisLeuLysAspIleLysValLysPheSerAspAlaGlyGlnProMetSerPheValLeuGlu    215

TTTCACTTTGAACCCAATGAATATTTTACAAATGAAGTGCTGACAAAGACATACAGGATGAGGTCAGAACCAGATGATTCTGATCCCTTT    810
PheHisPheGluProAsnGluTyrPheThrAsnGluValLeuThrLysThrTyrArgMetArgSerGluProAspAspSerAspProPhe    245

TCTTTTGATGGACCAGAAATTATGGGTTGTACAGGGTGCCAGATAGATTGGAAAAAAGGAAAGAATGTCACTTTGAAAACTATTAAGAAG    900
SerPheAspGlyProGluIleMetGlyCysThrGlyCysGlnIleAspTrpLysLysGlyLysAsnValThrLeuLysThrIleLysLys    275

AAGCAGAAACACAAGGGACGTGGGACAGTTCGTACTGTGACTAAAACAGTTTCCAATGACTCTTTCTTTAACTTTTTTGCCCCTCCTGAA    990
LysGlnLysHisLysGlyArgGlyThrValArgThrValThrLysThrValSerAsnAspSerPhePheAsnPhePheAlaProProGlu    305

GTTCCTGAGAGTGGAGATCTGGATGATGATGCTGAAGCTATCCTTGCTGCAGACTTCGAAATTGGTCACTTTTTACGTGAGCGTATAATC   1080
ValProGluSerGlyAspLeuAspAspAspAlaGluAlaIleLeuAlaAlaAspPheGluIleGlyHisPheLeuArgGluArgIleIle    335

CCAAGATCAGTGTTATATTTTACTGGAGAAGCTATTGAAGATGATGATGATGATTATGATGAAGAAGGTGAAGAAGCGGATGAGGAAGGG   1170
ProArgSerValLeuTyrPheThrGlyGluAlaIleGluAspAspAspAspAspTyrAspGluGluGlyGluGluAlaAspGluGluGly    365

GAAGAAGAAGGAGATGAGGAAAATGATCCAGACTATGACCCAAAGAAGGATCAAAACCCAGCAGAGTGCAAGCAGCAGTGAAGCAGGATG   1260
GluGluGluGlyAspGluGluAsnAspProAspTyrAspProLysLysAspGlnAsnProAlaGluCysLysGlnGln *              391

TATGTGGCCTTGAGGATAACCTGCACTGGTCTACCTTCTGCTTCCCTGGAAAGGATGAATTTACATCATTTGACAAGCCTATTTTCAAGT   1350

TATTTGTTGTTTGTTTGCTTGTTTTTGTTTTTGCAGCTAAAATAAAAATTTCAAATACAATTTTAGTTCTTACAAGATAATGTCTTAATT   1440

TTGTACCAATTCAGGTAGAAGTAGAGGCCTACCTTGAATTAAGGGTTATACTCAGTTTTTAACACATTGTTGAAGAAAAGGTACCAGCTT   1530

TGGAACGAGATGCTATACTAATAAGCAAGT                                                                 1560
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Figure 1 The nucleotide and predicted amino acid sequence for the hNRP cDNA

(**a**) A restriction map of the hNRP cDNA is shown at the top. Overlapping clones T2, T3, T4 and T5 were isolated from a human thymocyte cDNA library by screening with the amplified hXLR cDNA. The thin lines indicate the length and positions of the hXLR (PCR) and T2–T5 cDNAs in relation to the full-length hNRP gene. The position of the ATG initiation codon, the TGA termination codon, the poly(A) adenylation motif and the open reading frame (open box) are shown at the bottom. The arrows indicate the strategy for sequence determination. Restriction sites are: A, *Ava*II; Ac, *Acc*I; B, *Bsm*I; E, *Eco*RI; G, *Gsu*I; H, *Hae*III; Hp, *Hph*I; P, *Pst*I; X, *Xmu*I; Xm, *Xma*III. (**b**) The nucleotide sequence of the T2 and T3 cDNAs. The deduced amino acid sequences of the open reading frame is shown below the DNA sequence. The first three underlined sequences correspond to polyacidic regions within the hNRP-predicted protein and the last underlined sequence represents the polyadenylation signal. The boxed Cys at position 258 indicates a conserved cysteine residue which represents a potential site for disulphide bonding.

and 376 at the C-terminus of the molecule. As clusters of highly acidic regions are similarly located within the yeast NAP-I sequence [9] as well as that of nucleolin, another protein implicated in chromatin structuring [21,22], it appears that their presence may be of functional importance, possibly in relation to formation of the nucleosome histone core. The acidic nature of the amino acid sequence predicted for hNRP is also reflected by the hydropathicity profile (Figure 3) which indicates that the hNRP cDNA, like yeast NAP-I, encodes a highly hydrophilic protein [9,23]. Similarly, highly hydrophilic regions containing clusters of acidic residues have been observed in most of the human nucleolar proteins characterized to date, including nucleolin [24], high-mobility-group protein (HMG1) [25], and human nucleolar transcription factor [26]. As noted above, it is believed that the polyacidic stretches facilitate binding of these molecules to histones or to other basic proteins, such as SL1 in the case of human nucleolar transcription factor [26,27].

Comparisons of the hNRP- and yeast NAP-I-deduced amino acid sequences also revealed several other regions of similarity. For example, sequences almost identical with the hNRP sequences DSFFNFFAPP (295–304) and KGIPEFWLT (165–173) are found in yeast NAP-I [9], suggesting that these regions represent functionally important domains. Seven amino acid residues from this latter conserved region (i.e. GIPEFWL) are also present in the amino acid sequence predicted for the murine DN38 cDNA [20], as is consistent with the suggestion that this sequence encodes a nucleosome assembly-related protein.

Other notable features of the hNRP sequence include the presence of two serine residues (positions 10 and 237) and one threonine residue (position 128) at consensus sites for phosphorylation by casein kinase II, an enzyme implicated in phosphorylation of nuclear proteins such as the high-mobility-group proteins [28–30]. Similarly, phosphorylation sites have been recognized in nucleoplasmin and in the nucleolar proteins, nucleolin and B23 [31], and recent data suggest that the phosphorylation state of these molecules may affect their role in modulating chromatin structure. For example, phosphorylated nucleoplasmin binds to histone more effectively and is more active in nucleosome assembly than is the unphosphorylated form [22]. Glycosylation may also influence hNRP function, as the predicted protein structure includes two possible sites for *N*-glycosylation (at positions 267–269 and 293–295).

Also of interest in relation to the hNRP-predicted protein sequence is a seven-residue sequence, IKKKQKH, located between the second and third hyperacidic domains (272–279). This sequence closely resembles nuclear localization motifs found in nucleosome assembly proteins such as nucleoplasmin [32] and N1 [33], as well as in other nuclear proteins, including p53, c-abl and simian virus 40 T-antigen [34,35]. Whether other sequences within the hNRP with fewer similarities to the consensus motif also play a role in nuclear localization of this protein remains to be determined.

Finally, with the exception of yeast NAP-I and DN38, protein sequences of other nucleosome assembly proteins show relatively little overall similarity to that of hNRP. For example, comparisons of the hNRP-deduced amino acid sequence with those of *Xenopus* nucleoplasmin, *Xenopus* N1 protein and human nucleolin revealed sequence similarities of only 38% over 52 residues [32], 67% over 12 residues [33] and 28% over 39 residues [24] respectively. The hNRP-predicted protein also lacks some of the nucleic acid-binding motifs found in these other nuclear proteins. For example, hNRP does not have zinc-finger-binding domains [36], the ribonucleoprotein-binding consensus sequence found in RNA-binding proteins such as nucleolin [37] or the 100% Glu/Asp stretch as well as other DNA-binding domains present in high-mobility-group protein [25].

One other point of note concerning the hNRP-deduced amino acid sequence is its lack of similarity to that of the murine XLR gene, pM1. This latter cDNA is thought to encode a lamin-like nuclear protein and, as such, like hNRP, may play a role in assembly of certain nuclear components during mitosis [12,38]. However, although a significant stretch of sequence similarity does exist between the hNRP and pM1 nucleotide sequences, the similarity is actually between the hNRP cDNA sequence and the complement of the pM1 sequence. Thus, while the proteins encoded by the hNRP and pM1 cDNAs may be functionally related, they lack any significant amino acid similarity and in this context the isolation of the hNRP cDNA in conjunction with a search for human homologues to XLR/pM1 appears somewhat coincidental.

The hNRP fusion protein is recognized by monoclonal anti-(NAP-I) antibody

To examine further the relationship between the protein encoded by the hNRP cDNA and NAP-I, an 800 bp *Eco*RI fragment from the longest cDNA clone (T2) was expressed in *E. coli* as a fusion protein with GST, and the binding of the fusion protein to a monoclonal antibody against HeLa-cell NAP-I [7] was assessed by immunoblotting. The 810 bp segment of T2 was selected for expression because it contains the two highly conserved peptide sequences (165–173 and 295–304) alluded to earlier as well as the sequences encoding the hyperacidic tail of the hNRP molecule and was therefore thought to represent the most functionally relevant regions of hNRP. As shown in Figure 4(a), after induction with IPTG, the bacterial clones carrying the pGEX-hNRP plasmid expressed a fusion protein that bound to monoclonal anti-(NAP-I) antibody and had the molecular mass expected for the full-length GST-hNRP fusion protein. In contrast, the antibody did not react with GST alone or with protein extracts obtained from pGEX-hNRP-carrying *E. coli* cultured in the absence of IPTG. To demonstrate the specificity of this result, the blot was stripped and then reprobed with an anti-GST antibody. As shown in Figure 4(b), this latter antibody bound to both GST and GST–hNRP fusion protein, a finding that indicates that the recognition of hNRP by monoclonal anti-(NAP-I) antibody is specific and does not reflect non-specific reactivity to *E. coli* proteins or GST. Furthermore, these immunological data are consistent with the structural analogies apparent between the yeast NAP-I- and hNRP-predicted protein sequences and support the contention that the hNRP cDNA encodes a human NAP-I counterpart.

Expression of hNRP occurs in multiple cell lineages

To examine the pattern of hNRP expression, the amplified hXLR and T2 cDNAS were used in Northern-blot hybridizations with a variety of tissues and cell lines. As shown in Figure 5, both these probes identify a single 1.7 kb transcript, a size that is consistent with that of the full-length hNRP cDNA (1.5 kb). This transcript was detected in all tissues studied including several lymphoid as well as non-lymphoid tissues. However, levels of hNRP transcript appeared to be somewhat increased in acute lymphoblastic and myeloblastic leukaemia cells compared with other tissues and were also relatively high in the two carcinomas and two T-cell leukaemia lines studied here (Figure 5b). On the basis of these data, it appears that hNRP expression is ubiquitous, but may be up-regulated to some extent in rapidly proliferating cells such as neoplastic tissues. These results are

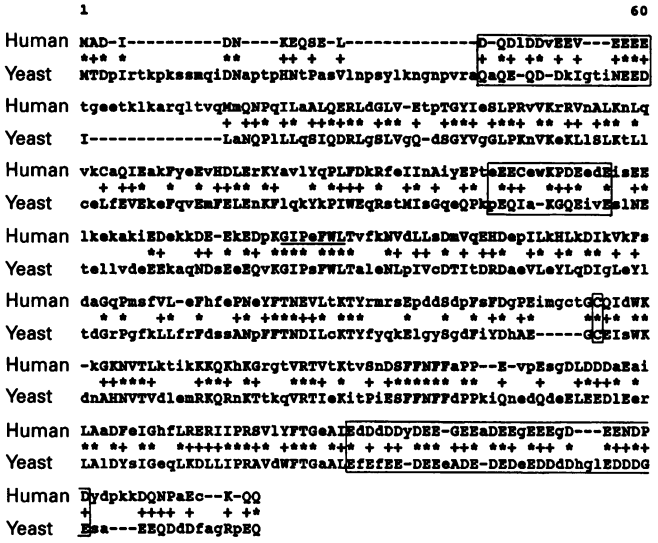

Figure 2 Comparison of the hNRP and yeast NAP-I predicted protein sequences

The hNRP and yeast NAP-I amino acid sequences were aligned by computer analysis with gaps (indicated by dashes) inserted to maximize identity. Capital letters denote identity or functional similarity of residues. Three conserved acidic amino acid stretches and a conserved cysteine residue are boxed. The GIPEFWL peptide found in the murine DN38 sequence is underlined. * indicates identical; 128 of 439 amino acids = 29%. † indicates conserved; identical + conserved: 235 of 439 amino acids = 54%.

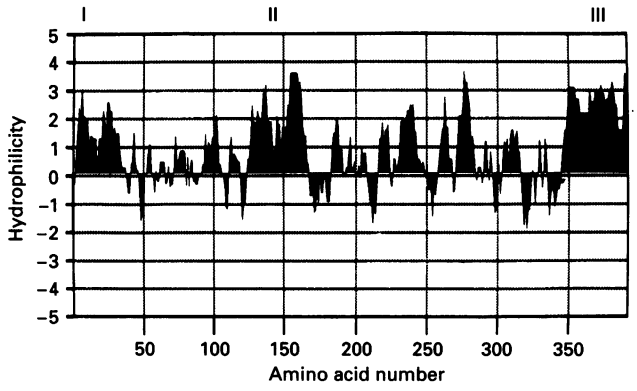

Figure 3 Hydropathic character of the hNRP predicted amino acid sequence

Relative hydrophilicity was analysed by the method of Kyte and Doolittle [23]. Amino acid positions are shown below the profile. Three regions that are highly hydrophilic are designated I, II and III.

consistent with the hNRP gene product participating in some manner in cell proliferation as would be expected for a nuclear protein involved in chromatin formation.

Antisense hNRP oligonucleotide inhibits T-cell proliferation *in vitro*

As indicated above, the structural similarities between the hNRP gene product and various nuclear proteins implicated in chromatin assembly suggest that hNRP may also play a role in chromatin formation and, by extension, in the cell proliferation process. To

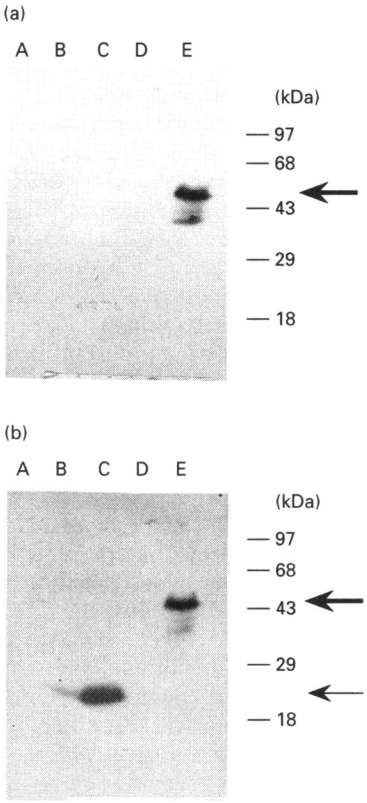

Figure 4 Reactivity of monoclonal anti-(NAP-I) antibody with the hNRP fusion protein

Samples of *E. coli* protein extracts were prepared from: (A) *E. coli* alone; (B) *E. coli* transformed with pGEX-2T expression vector and cultured in the absence of IPTG; (C) *E. coli* transformed with pGEX-2T and cultured in the presence of IPTG; (D) *E. coli* transformed with the pGEX-hNRP construct and cultured in the absence of IPTG; (E) *E. coli* transformed with pGEX-hNRP and cultured in the presence of IPTG. Samples were boiled for 10 min and subjected to SDS/PAGE (12% gels). (**a**) Immunoblots were initially probed with monoclonal HeLa-cell NAP-I antibody [7]. The arrow identifies the hNRP fusion protein (lane E). Numbering on the right indicates apparent molecular mass (kDa). (**b**) The blot shown in (**a**) was stripped and reprobed with an anti-GST antibody. The thin arrow identifies GST (lane C) and the darker arrow indicates the GST–hNRP fusion protein (lane E).

investigate this possibility, an antisense oligonucleotide complementary to the first 15 nucleotides of the hNRP-coding region was derived and used to examine the relationship between hNRP expression and induction of T-lymphocyte activation. A similar approach has been used successfully by several groups to demonstrate the requirement for up-regulated expression of other non-T-cell-specific genes, such as the nuclear proto-oncogenes c-*myc* [39] and *Bcl*-2 [40] in T-cell mitogenesis. In the current study, human PBMCs and purified T-lymphocytes were cultured in the presence of antisense or the corresponding sense hNRP phosphorothioate oligonucleotides and stimulated with both the phorbol ester, PMA, and the calcium ionophore, ionomycin. These latter reagents induce protein kinase activation and intracellular free Ca^{2+} increases respectively, and have been shown to evoke T-cell activation responses comparable with those elicited by ligation of the T-cell antigen receptor [41].

To confirm that the antisense oligomer used here inhibited hNRP expression, we initially evaluated the effects of the antisense versus sense oligonucleotides on T-cell hNRP mRNA and protein levels during the 24 h period after PMA/ionomycin treatment. As indicated by the densitometric analysis shown in Figure 6(a), PMA/ionomycin stimulation in the presence of

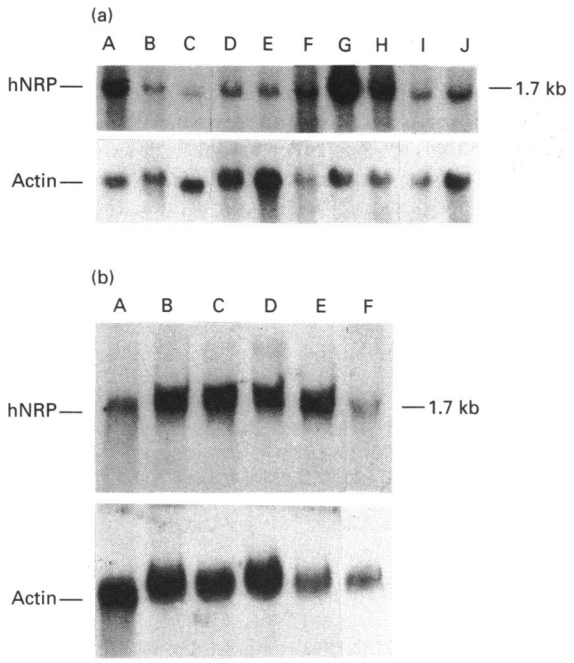

Figure 5 Northern-blot analysis of hNRP RNA in various human tissues and cell lines

Approx. 10 μg of total cellular RNA was loaded into each lane, electrophoresed in 1% formaldehyde/agarose gels and transferred to nitrocellulose. (**a**) RNA from freshly isolated (A) thymus, (B) oesophagus, (C) stomach, (D) kidney, (E) bowel, (F) uterus, (G) acute myeloblastic leukaemia cells, (H) acute lymphoblastic leukaemia cells, (I) lung cancer cells and (J) breast cancer cells was probed with the hXLR amplified cDNA (top) and the blots then stripped and reprobed with a β-actin probe (bottom). (**b**) RNA from (A) thymocytes, (B) the T-cell line, SIT, (C) the T-cell line, Jurkat, (D) the ovarian carcinoma cell line HEY, (E) the cervical carcinoma line, HeLa, and (F) peripheral blood lymphocytes was probed with the T2 cDNA (top) and the blots then stripped and reprobed with a β-actin probe (bottom).

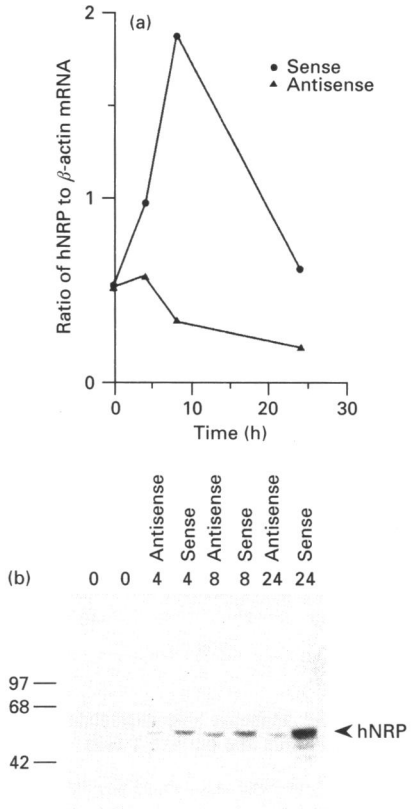

Figure 6 Effects of hNRP antisense oligonucleotide on hNRP mRNA and protein expression in human PMA/ionomycin-stimulated T-lymphocytes

(**a**) T-cells were stimulated with 50 nm PMA and 1 μM ionomycin and cultured in the presence of 5 μM sense (●) or antisense (▲) hNRP oligonucleotide as described in the Materials and methods section. Cell cultures were harvested at the indicated times (0, 4, 8, 24 h). Total cellular RNA samples (10 μg of each) were electrophoresed in 1% formaldehyde/agarose gels and transferred to nitrocellulose. The blots were then sequentially hybridized with ^{32}P-labelled T2 cDNA and β-actin probes, and the levels of hNRP and β-actin mRNA were measured by scanning optical densitometry of autoradiographs. The relative levels of hNRP to β-actin mRNA were calculated and are shown for each time period (0, 4, 8, 24 h). Values shown represent means of duplicate determinations.(**b**) Cell lysates were prepared for immunoblotting analysis, subjected to SDS/PAGE, transferred to nitrocellulose and the resultant blots were probed with anti-(Hela-cell NAP-I) antibody and ^{125}I-Protein A. The predominant hNRP protein band (arrowhead) is compared between antisense and sense oligonucleotide-treated cells for each time point indicated at the top of the panel. Size markers (kDa) are shown on the left.

5 μM sense oligomer caused marked augmentation in T-cell hNRP mRNA levels relative to those of the constitutively expressed β-actin gene, the ratio of hNRP to β-actin mRNA increasing as early as 4 h and peaking at about 8 h after stimulation. In contrast, no increases in the levels of hNRP relative to β-actin transcript were observed when PMA/ionomycin stimulation was carried out in the presence of 5 μM hNRP antisense oligomer. In fact, hNRP mRNA levels were found to be lower in the antisense-treated cells at 8 and 24 h after stimulation than in resting (i.e. non-stimulated) cells, an observation that may reflect oligonucleotide-mediated RNAase H-like cleavage of hNRP mRNA [42]. Similarly, immunoblotting analysis using the monoclonal anti-(NAP-I) antibody [7] revealed levels of hNRP to be markedly reduced in the antisense relative to sense hNRP-treated T-cells over the 24 h period after PMA/ionomycin stimulation, the most striking difference being evident at the 24 h time point (Figure 6b). Together these findings indicate that the antisense hNRP oligonucleotide used in this study can block PMA/ionomycin-induced increases in the synthesis of hNRP and therefore provides a potentially useful probe for evaluation of hNRP function.

As indicated by the data shown in Figure 6(a), the hNRP gene is induced rapidly after PMA/ionomycin stimulation, with increases in hNRP/β-actin mRNA levels evident within 4 h. This finding as well as the similar time course observed with respect to PMA/ionomycin-triggered induction of the interleukin 2 receptor gene, a critical event in T-cell activation [43], are consistent with a role for the hNRP gene product in cell proliferation. To address this issue, the effects of the antisense hNRP oligomer on cell proliferation were next examined. For these studies, unseparated PBMCs as well as enriched T-cell cultures were incubated with PMA/ionomycin alone or in the presence of various amounts of hNRP sense or antisense oligonucleotides and cell proliferation was assayed by measuring [^{3}H]thymidine uptake at 4, 8, 24 and 48 h. An example representative of the results obtained using an oligonucleotide concentration of 5 μM is shown in Figure 7. As indicated by this result, T-cells and PBMCs treated with the sense oligonucleotide showed peak proliferative responses to PMA/ionomycin at 24 and 48 h respectively. The basis for the different response kinetics observed in the unseparated versus purified T-cells is unclear, but this finding does not reflect artifact introduced by the sense oligonucleotide, as the same results were obtained in the absence of

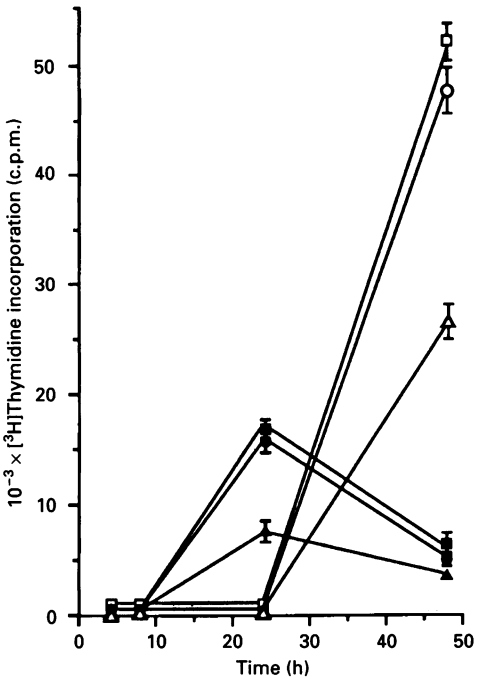

Figure 7 Effect of hNRP antisense oligonucleotide on PMA/ionomycin-induced proliferation of PBMCs and purified T cells

PBMCs or purified T-cells (10^5 cells/well) were cultured with PMA (50 nM) and ionomycin (1 μM) alone or in the presence of 5 μM sense or antisense hNRP oligonucleotide, and [^{3}H]thymidine incorporation was determined after 4, 8, 24 or 48 h of culture. Values shown represent the arithmetic mean $\pm$ S.D. of pentaplicate samples. ●, Sense, T-cells; ▲, antisense, T-cells; ■, no oligonucleotide, T-cells; ○, sense, PBMCs; △ antisense, PBMCs; □, no oligonucleotide, PBMCs.

oligonucleotide treatment (Figure 7). Proliferative responses were also detected in the T-cell and PBMC cultures treated with 5 μM antisense oligomer and again peaked at 24 and 48 h respectively. However, the maximal levels of PMA/ionomycin-induced proliferation observed in the antisense-treated cultures were reduced by about 50% from those detected in sense-treated cells or cells treated with either wholly irrelevant (results not shown) or no oligonucleotides. Although the most effective suppression of T-cell mitogenesis was obtained using an oligonucleotide concentration of 5 μM, 0.5 and 10 μM antisense oligomer also inhibited induction of T-cell proliferation (results not shown).

These observations demonstrate an association between the induction of T-cell proliferation and expression of elevated hNRP mRNA and protein levels and indicate that suppression of increases in hNRP expression significantly reduce induction of mitogenesis. The finding that hNRP antisense treatment reduced, but did not entirely abrogate PMA/ionomycin-induced proliferation may reflect incomplete inhibition of hNRP production in the antisense-treated cells, or alternatively the existence of other nuclear proteins which subserve functions similar to hNRP in relation to cell proliferation. Thus hNRP may play an important, but not prerequisite, role in the induction of cell proliferation, a possibility that is consistent with data showing that expression of the nucleosome assembly protein NAP-I is not essential for yeast survival (Y. Ishimi, unpublished work).

In conclusion, the hNRP cDNA identified here is structurally similar to yeast NAP-I and other nuclear proteins implicated in nucleosome assembly. As is consistent with a putative role in nuclear function, hNRP expression appears to be ubiquitous and increases rapidly after induction of T-lymphocyte mitogenesis. The observation that antisense-mediated suppression of hNRP mRNA and protein increases in cells exposed to mitogenic stimuli markedly inhibits cell proliferation also indicates a role for hNRP in some facet of mitogenesis. Whether this role involves NAP-like or some other activity related to chromatin assembly and DNA replication remains to be determined. However, on the basis of the structural data, the hNRP gene product encodes a human counterpart to yeast NAP-I and as such its further characterization should provide a framework for delineating the mechanisms whereby these proteins modulate chromatin formation and contribute to the regulation of cell proliferation.

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