Characterization of a Nuclear Deformed Epidermal Autoregulatory Factor-1 (DEAF-1)-Related (NUDR) Transcriptional Regulator Protein

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A monkey kidney cDNA that encodes a nuclear regulatory factor was identified by expression and affinity binding to a synthetic retinoic acid response element (RARE) and was used to isolate human placental and rat germ cell cDNAs by hybridization. The cDNAs encode a 59-kDa protein [nuclear DEAF-1-related (NUDR)] which shows sequence similarity to the *Drosophila* Deformed epidermal autoregulatory factor-1 (DEAF-1), a nonhomeodomain cofactor of embryonic *Deformed* gene expression. Similarities to other proteins indicate five functional domains in NUDR including an alanine-rich region prevalent in developmental transcription factors, a domain found in the promyelocytic leukemia-associated SP100 proteins, and a zinc finger homology domain associated with the AML1/MTG8 oncoprotein. Although NUDR mRNA displayed a wide tissue distribution in rats, elevated levels of protein were only observed in testicular germ cells, developing fetus, and transformed cell lines. Nuclear localization of NUDR was demonstrated by immunocytochemistry and by a green fluorescent protein-NUDR fusion protein. Site-directed mutagenesis of a nuclear localization signal resulted in cytoplasmic localization of the protein and eliminated NUDR-dependent transcriptional activation. Recombinant NUDR protein showed affinity for the RARE in mobility shifts; however it was efficiently displaced by retinoic acid receptor (RAR)/retinoid X receptor (RXR) complexes. In transient transfections, NUDR produced up to 26-fold inductions of a human proenkephalin promoter-reporter plasmid, with minimal effects on the promoters for prodynorphin or thymidine kinase. Placement of a RARE on the proenkephalin promoter increased NUDR-dependent activation to 41-fold, but this RARE-dependent increase was not transferable to a thymidine kinase promoter. Recombinant NUDR protein showed minimal binding affinity for proenkephalin promoter sequences, but was able to select DNA sequences from a random oligonucleotide library that had similar core-binding motifs (TTCG) as those recognized by DEAF-1. This motif is also present between the half-sites of several endogenous RAREs. The derived consensus- binding motif recognized by NUDR (TTCGGGNNTTTCCGG) was confirmed by mobility shift and deoxyribonuclease I (DNase I) protection assays; however, the consensus sequence was also unable to confer NUDR-dependent transcriptional activation to the thymidine kinase promoter. Our data suggests that NUDR may activate transcription independently of promoter binding, perhaps through protein-protein interaction with basal transcription factors, or by activation of secondary factors. The sequence and functional similarities between NUDR and DEAF-1 suggest that NUDR may also act as a cofactor to regulate the transcription of genes during fetal development or differentiation of testicular cells. (Molecular Endocrinology 12: 1619–1639, 1998)

INTRODUCTION

The final differentiated state of organs, tissues, and cells that make up an adult animal is the end result of a developmental program of gene expression that is initiated at fertilization and elaborated during embryogenesis. Molecules such as retinoic acid (RA) must sometimes occur in concentration gradients to produce regional gene expression leading to appropriate morphological changes, such as those observed in limb bud development (1). Alternatively, regional expression of transcription factors may allow combinatorial interactions of the factors at target genes, which then result in relevant signaling cascades. For exam-

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ple, in male gonadogenesis, the Wilms' tumor 1 (WT1) gene product appears to act as a dose-dependent cofactor with steroidogenic factor 1 (SF-1) to produce regulated expression of the Mullerian-inhibiting substance (MIS) gene (2). The synergistic action of WT1 and SF-1 can be antagonized by the X-linked, sex-reversal gene product, Dax-1, most likely through direct interaction with SF-1 (2).

Precise regional expression and interaction of specific transcription factors are also likely to be relevant to the mechanisms by which a master set of 'selector' genes termed *HOM-C*/*Hox* can determine the final morphological characteristics of an organism (reviewed in Ref. 3). The *HOM-C* gene cluster in *Drosophila* encodes a group of proteins that govern the anterior-to-posterior (A-P) segmentation patterning of developing fly embryos, and the homologous *Hox* gene clusters have been identified in all metazoans that have been examined (4, 5). In vertebrates, there are four *Hox* gene clusters, which show not only sequence homology to *Drosophila HOM-C*, but also display the linear chromosomal arrangement and 3′-to-5′ temporal expression pattern observed during development. Mutations in these genes lead to the substitution of one body part for another (homeotic transformation). The *HOM*/*Hox* genes encode proteins that share a highly conserved 60-amino acid DNA-binding domain called the homeodomain (6) and are thought to exert their broad range of regulatory effects through DNA binding and transcriptional regulation of downstream target genes (7).

The high degree of amino acid similarity in the homeodomains of these proteins also appears to produce similar DNA-binding specificities in both *in vitro* and *in vivo* experiments (see reviews in Refs. 8 and 9). The dilemma of achieving precise regulatory specificity with homeodomain proteins could be resolved if interactions with additional cofactors supplied the required specificity (3, 10). Indeed, a growing number of cofactors have been shown to provide functional enhancement to homeodomain proteins. For example, the human Oct-1 homeodomain protein will form high-affinity complexes with certain octamer motifs only in the presence of the coactivator VP16 (11, 12). The homeodomain protein extradenticle in *Drosophila* and its mammalian counterpart Pbx have also been shown to cooperatively bind DNA in the presence of some homeodomain proteins (13–15). More recent studies suggest that protein cofactors are required to switch some homeodomain proteins into a transcriptionally active state (3, 16).

Deformed epidermal autoregulatory factor-1 (DEAF-1) was identified as a nonhomeodomain protein that interacted as a cofactor with the Deformed protein from the *HOM-C* gene cluster in *Drosophila.* Deformed (Dfd) is a homeodomain protein that is expressed in the mandibular and maxillary segments of the embryonic head and is required for the subsequent development of structures derived from these segments (17–19). A 120-bp region of the *Dfd* promoter, referred to as module E, is capable of driving embryonic expression of a reporter gene in a pattern similar to endogenous Dfd expression (20). Module E was further dissected into a 24-bp Dfd-binding site and a 51-bp sequence (called region 5–6) that was required for appropriate Dfd expression (20). Thus, Dfd is capable of autoregulating its own expression but requires additional cofactors to provide segment-specific expression. DEAF-1 was identified as a protein cofactor that bound the 5–6 region in gel mobility shift and deoxyribonuclease I (DNase I) protection assays (21). Mutations in region 5–6 that improved DEAF-1 binding *in vitro* increased expression in transgenic embryos, indicating DEAF-1 or a similar protein is a required cofactor in *Dfd* expression. In addition, DEAF-1 was shown to bind multiple regions of the human *HOXD4* promoter, a homolog of *Dfd* (21), suggesting a mammalian counterpart of DEAF-1 may exist to assist in the regulation of paralogous group 4 *Hox* genes.

Retinoic acid receptors (RARs) are nuclear transcription factors that, along with retinoid X-receptors (RXRs), bind to retinoic acid (RA) response elements (RAREs) of target genes to mediate developmental events such as RA-dependent regulation of embyrogenesis and cellular differentiation (22). We had previously shown that the catalytic subunit of cAMP-dependent protein kinase [protein kinase A (PKA)] could phosphorylate recombinant RARα *in vitro*, and that PKA potentiated RA signaling in transfected cells (23). Rochette-Egly and coworkers (24) went on to show that serine 369 of RARα was phosphorylated upon cotransfection of PKA or by forskolin treatment of F9 cells. Surprisingly, mutation of this serine to alanine or glutamate did not eliminate PKA potentiation of RARα signaling (Ref. 24 and our own unpublished studies have confirmed this result). To support a mechanism for the PKA-dependent regulation of RA signaling, we hypothesized that other PKA-regulated transcription factors were binding to RAREs or interacting with RAR/RXR dimers. In a search for binding proteins that might recognize RAREs, we have identified a protein with significant homology to DEAF-1 which we have designated as NUDR (for nuclear DEAF-1 related protein). We present evidence that NUDR is a nuclear protein that activates transcription from the human proenkephalin promoter, a gene that is expressed in many neuroendocrine and reproductive tissues. NUDR protein is expressed at elevated levels in testicular germ cells and developing fetus and will likely function to regulate gene expression in these tissues. The sequence and functional similarities between NUDR and DEAF-1 suggest that NUDR is a potential mammalian homolog of DEAF-1 and may therefore serve as a transcriptional cofactor of homeodomain proteins in rapidly dividing or differentiating tissues.

RESULTS

Isolation of Human and Monkey cDNAs for NUDR

In an attempt to identify proteins other than RAR and RXR that may interact at the RARE, we used a ^{32}P-labeled oligonucleotide containing a RARE to screen a CV-1 monkey kidney cDNA library that was induced to produce β-galactosidase fusion proteins. The RARE sequence AG**GGTTCA**CCGAA**AGTTCA** (RARE half-sites are shown in *bold* and the pentameric nucleotide spacer is *underlined*) was based upon the DR5-RARE motif that occurs in the transcriptional promoter of the RARβ gene (25). Of approximately 700,000 plaques that were screened, a single plaque remained positive through tertiary screening. Sequencing of the 1604-bp cDNA (sNUDR1.6) confirmed that an appropriate fusion-protein to the *lacZ* gene product was produced. The cDNA was then used to rescreen the CV-1 cDNA library by hybridization, to obtain a second clone of 2405 bp (sNUDR, accession no. AF049461), which contained 395 bases of 5′-sequence before the first methionine codon and a continuous open reading frame of 565 amino acids. We have designated the encoded protein as NUDR (see *Discussion*). Preliminary Northern blot analysis had indicated the presence of similar transcripts in the human choriocarcinoma cell line JEG-3. A cDNA library was produced from this cell line and screened by hybridization with the monkey cDNA clone. Two human clones of 2065 bp (hNUDR, accession no. AF049459) and 2329 bp (hNUDR8, accession no. AF049460) were chosen for further characterization. Human (h) NUDR contained 50 bases of 5′-sequence before the first methionine codon and an open reading frame that, similar to the monkey clone, encoded a protein of 565 amino acids. The monkey and human conceptual proteins showed 99% sequence similarity when compared with each other, with only five amino acid changes (Fig. 1). hNUDR8 contained 356 bases of 5′-untranslated sequence that was 95% identical to the monkey sequence. The hNUDR8 coding sequence contained a 42-bp deletion relative to hNUDR, which eliminated amino acids 16–29 and converted amino acid 15 from glutamate to aspartate. Presumably, this difference arises from differential splicing of a primary transcript, perhaps to alter the functionality of an alanine-rich motif that occurs in this region of the protein. Directly after the protein-coding region and before polyadenylation, the human and monkey cDNAs displayed 320 and 315 bases of 3′-untranslated sequence, respectively, and were 96% identical. The 3′-untranslated sequence of hNUDR contained a 105-bp region with 95% identity to a sequence-tagged site (dbSTS 40925), strongly suggesting that the NUDR gene maps to human chromosome 11.

A rat NUDR cDNA clone of 2120 bp (rNUDR, accession no. AF055884) was identified by hybridization screening of a Sprague Dawley rat testicular germ cell cDNA library. Based on the homology to the primate clones, the rat clone also encodes a 565-amino acid protein with 102 bases of 5′-untranslated sequence and 320 bases of 3′-untranslated sequence. Comparison of the rat and human conceptual proteins showed 94% sequence identity with 34 amino acid differences (Fig. 1).

Comparison of Sequences with Similarities to NUDR

Comparison of the NUDR sequences to the nonredundant GenBank databases identified two cDNAs, designated as rat suppressin (1882 bp, accession no. U59659) and human suppressin (1888 bp, accession no. AF007165), with significant nucleotide and amino acid homologies. The rat suppressin clone was identified by screening of a pituitary cDNA library (26) with an antibody to bovine suppressin, a protein isolated and characterized from bovine pituitary (27). The suppressin protein has been shown to be synthesized and secreted by the GH_3 rat pituitary cell line and to inhibit proliferation of immune cells (27). Analysis of the rat suppressin and rat (r) NUDR sequences suggest that suppressin is a partial NUDR cDNA that would produce a protein lacking 68 amino acids (Fig. 1) if the suggested protein initiation site of suppressin were used. Comparison of the conceptual proteins showed they shared 94% amino acid identity while the nucleotide sequences were more than 99% identical. Comparison of human suppressin with hNUDR suggests it is also a partial NUDR cDNA that lacks the 5′-sequence coding for the first 48 amino acids and differs in sequence to produce four additional amino acid changes. As presented in the *Discussion*, we believe that it is unlikely the protein encoded by NUDR is the equivalent of the secreted protein characterized as suppressin.

The second most similar sequence identified in computer comparisons was the *Drosophila* DEAF-1. For this reason, we have designated the primate protein as nuclear DEAF-1 related (NUDR) protein. hNUDR shows 29% identity and 46% overall similarity with the 576 amino acids of DEAF-1. Other proteins identified in the database comparison show sequence similarity to specific regions of hNUDR and suggest the presence of five distinct functional domains (Fig. 2). The alanine-rich region near the amino terminus of NUDR is followed by an acidic-rich region, and together these regions (Alanine-Acidic, AA) produce matches primarily with proteins recognized as developmental transcription factors (Fig. 2A). Of the 17 proteins shown, 10 are homeodomain-containing proteins and another four are putative transcription factors closely associated with embryonic development. The three remaining proteins with AA similarity include the transcription factor JUN-D, human progesterone receptor (hPR), and a protein (MLL) that is associated with malignant transformation in t(11;19) leukemias (28).

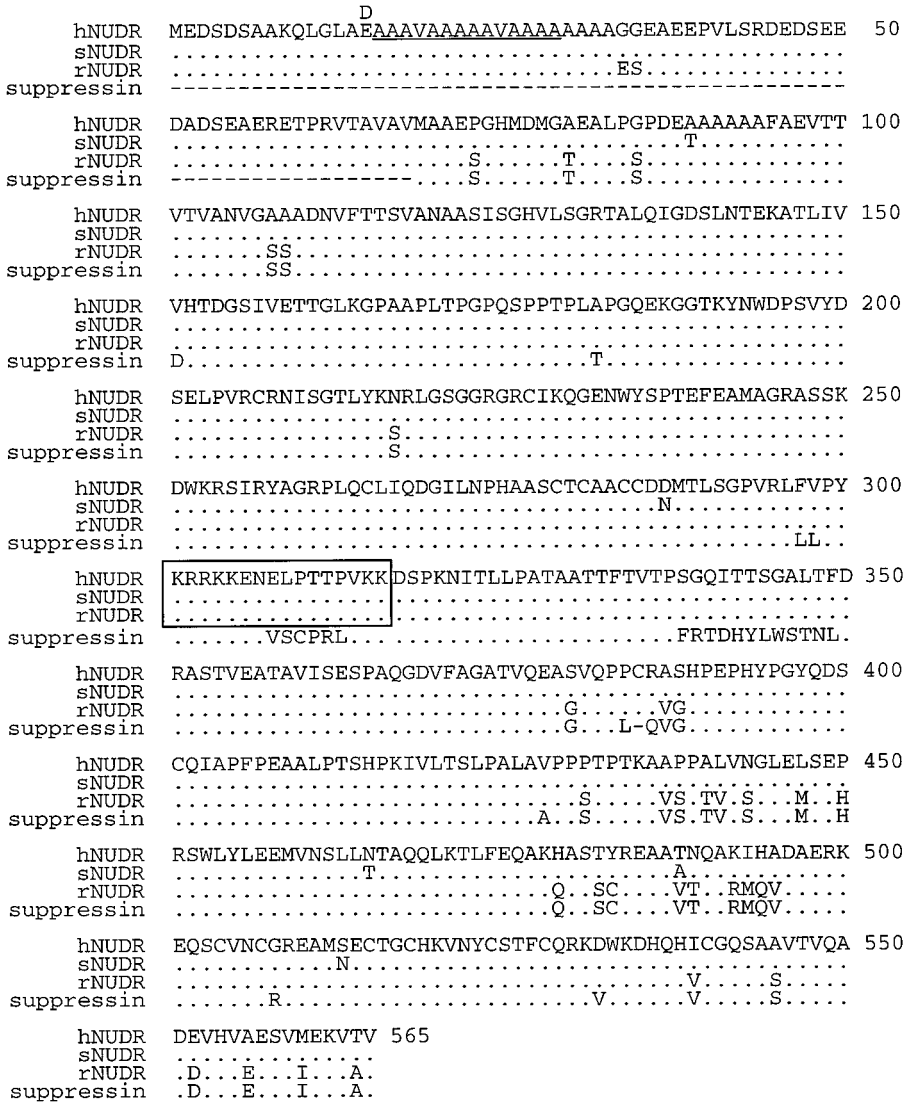


Fig. 1. Amino Acid Sequence Comparison of Human, Monkey, and Rat NUDR with Rat Suppressin

The amino acid sequences for human, monkey, and rat NUDR were deduced from the cDNA sequences and compared with rat suppressin (accession no. U59659). The *upper line* shows the one-letter amino acid sequence of human NUDR (hNUDR, accession no. AF049459). Amino acid identity of monkey NUDR (sNUDR, accession no. AF049461), rat NUDR (rNUDR, accession no. AF055884), and rat suppressin with hNUDR are indicated by *dots*, and amino acid differences are shown as *letters*. The initiator methionine indicated for rat suppressin corresponds to methionine at position 69 of NUDR, and *dashed lines* show missing sequence. A second human NUDR clone (hNUDR8, accession no. AF049460) contains a 42-bp deletion (*underlined*) that resulted in the deletion of 14 amino acids (between amino acids 16 and 29) and a change of a glutamic acid to aspartic acid at position 15 (denoted by a D *above* the hNUDR sequence) as the deletion occurred within two codons. A potential bipartite NLS is *boxed*.

The nuclear domain (ND) region of hNUDR displays the highest homology to DEAF-1 (70%) and also has similarity to three other proteins that localize to the nucleus (Fig. 2B). The ND region was previously referred to as the "KDWK" domain in DEAF-1 (21) because of a conserved amino acid motif occurring in several proteins with unknown function (21), including one derived from a presumed partial cDNA of hNUDR (dbest R19688). The homology between NUDR and DEAF-1 in this extended comparison includes a nuclear localization signal (NLS, described below), indicating this region may also constitute a NLS for DEAF-1. The SP100-B protein also displays homology to the NUDR ND region and has been shown to colocalize with the promyelocytic leukemia (PML) gene product at distinct subnuclear structures termed PML nuclear bodies (for recent review see Ref. 29). In acute promyelocytic leukemia (APL), a t(15;17) translocation produces a PML-RARα fusion oncoprotein that dis-

rupts normal localization to PML nuclear bodies and potentially contributes to APL pathogenesis (30–33). The LYSP100-B protein is similar to SP100-B, but its expression is restricted to lymphoid cells, and it localizes to subnuclear structures that are distinct from PML nuclear bodies (34). Phosphoprotein 41 (p41) is one of two highly related nuclear phosphoproteins that display similarity to the ND region of NUDR and that are inducible by interferons (35), a property shared with SP100-B (36).

The carboxy terminus of hNUDR contains a zinc finger homology (ZFH) region with 56% similarity to the analogous region in DEAF-1 and also shows similarity to a functionally diverse set of proteins through the conserved spacing of cysteine and histidine residues (Fig. 2C). The spacing of these residues in the *nervy* gene product was previously suggested to resemble proteins that coordinate zinc through zinc-finger domains, while probably lacking the necessary arrangement for DNA binding (37). However, similarities of the NUDR ZFH domain with the DNA binding domains of hPR and HNF-4 [a member of the nuclear hormone receptor superfamily (38)], suggest a potential role of this region in DNA binding. The AML-1/MTG8 fusion protein arises from a t(8:21) translocation occurring in acute myeloid leukemias (AML), and its homology to the ZFH domain suggests a third potential link of hNUDR to oncogenic proteins produced in leukemias. Four other proteins with ZFH similarity have potential roles in cell signaling: RACK7 is a protein kinase C-binding protein (39); t-BOP is a zinc-finger protein expressed in T cells and muscle (40); BS69 is an inhibitor of adenovirus E1A transactivation (41); and PDCD2 is associated with the process of programmed cell death (42). The conservation of the ZFH pattern can also be seen in proteins from lower eukaryotes (celeganF23, yeast72kd in Fig. 2C), suggesting evolutionary conservation of a functional motif. In summary, the database similarities shown in Fig. 2 suggest that NUDR contains functional domains often found in nuclear transcription factors with developmental or oncogenic potential.

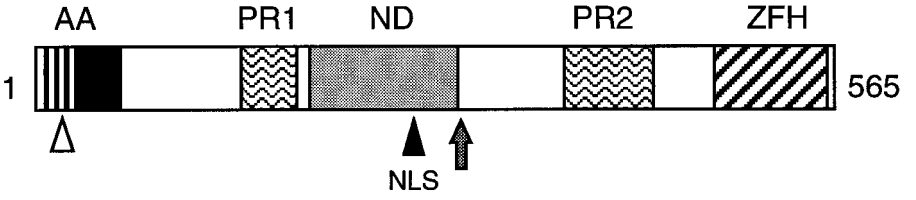
Tissue Distribution of NUDR mRNA

Northern blot analysis shows that the predominant NUDR mRNA form in CV-1 cells has a molecular size of 2.4 kb, indicating that the monkey cDNA that has been isolated is likely to be full length (Fig. 3A). Examination of various rat tissues for NUDR RNA expression showed the 2.4-kb mRNA in all tissues, with highest levels of expression in brain, adrenal, and lung (Fig. 3B). A second hybridizing band of RNA, with an estimated size of 6 kb, was observed in most tissues and represented the more abundant form in lung. Whether this longer RNA represents an alternative splice variant of a single NUDR gene or an RNA transcript from a highly related gene is not currently known.

Detection of NUDR Protein in Testis, Fetus, and Cell Lines

The first ATG codon encountered in the sNUDR cDNA occurs at nucleotide position 396; in the hNUDR cDNA, the first ATG codon occurs at nucleotide position 51. The next in-frame ATG codon occurs 204 nucleotides (68 codons) downstream from this first occurrence. Utilization of the first AUG codon as the initiator methionine would produce a protein with a calculated molecular mass of 59 kDa. To determine the molecular size of the protein encoded by these cDNAs, an *in vitro* transcription/translation system was used to produce ^{35}S-labeled sNUDR and hNUDR proteins, and the translation products were separated by SDS-PAGE (Fig. 4). A single major band of radiolabeled protein was produced from both plasmids with an estimated molecular size of 72 kDa relative to the mobilities of hRXRα and protein molecular mass standards. The discrepancy between the observed 72 kDa and the calculated molecular size of 59 kDa suggests NUDR has an anomalous mobility in SDS-PAGE. To further investigate this discrepancy, recombinant sNUDR protein was produced in bacteria from a plasmid in which the sequence encoding the first 22 amino acids of the His-Tag vector was fused in frame to the ATG at position 396 of sNUDR cDNA (see *Materials and Methods*). A polyclonal antibody to recombinant NUDR was produced and used to detect the levels of recombinant sNUDR and endogenous proteins in CV-1 cells, JEG-3 cells, and various rat tissues by Western blot analysis (Fig. 5A). Recombinant sNUDR produced using the designated ATG showed a similar mobility to the *in vitro* NUDR translation product and also to an endogenous 72-kDa protein observed in CV-1, JEG-3 cells, and rat testis. This indicates that the first methionine codon is used for NUDR protein initiation in both *in vitro* translations and endogenous tissues. Proteins of lower molecular mass were observed in muscle, brain, and heart samples. The protein band occurring at 28 kDa was observed on Western blots incubated with preimmune serum, indicating the band is nonspecific (see Fig. 5C). The other lower molecular mass proteins could represent NUDR degradation products, tissue-specific proteolysis, alternative translation start sites, or proteins with similar antigenic epitopes.

To identify which cell type(s) in the testis was responsible for NUDR protein production, cell populations enriched for Sertoli, myoid, and Leydig cells were isolated from rat testis. Total germ cells were also isolated and fractionated on a Staput gradient to obtain cell populations enriched in spermatocytes, round spermatids, and elongating spermatids. Proteins extracts were produced from each of these cell fractions as well as epididymal sperm. As shown in the Western blot in Fig. 5B, the 72-kDa NUDR protein is expressed primarily in developing germ cells, and at diminished levels in Sertoli cells. NUDR was not detected in Leydig cells, myoid cells, or epididymal spermatozoa. A



A. Alanine-Acidic (AA) Region

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hNUDR      2 EDSDSAAKQLGLAEAAAVAAAAAVAAAAAAAAGGEAEEPVLSRDEDSEEDADSEAERETPRVTAVAVMAAEPG
nervy     22 ..............AAAAAAAAVAAGRALAVASAAAVQVPPASSSSAGSASSAAAAATNNTTSAAATAAAIS
Cbfa1     14 ..............AAAAAAAAAAAAAAAAVPRLRPPHDNRTMVEIIADHPAELVRTDSPNFLCSVLPSHW.
BF-2     280 ......LQLPPCAPPSALFAAAAAAAAAAFHPHSPPPPPPPPPPPPPGAAAELARTAFGYRSHALAAALPGPLQ
shn       30 ..............ATAAAAAAAAAAATTTVTTAATPTKKRTYRETATATTVTQRSTNKANIAAAIALAAA
HOXA13   115 ............SAAAAAAAAAAAAAAAAASSSGGPGPAGPAAAEAAKQCSPCSAAAQSSSGPAALPYG..
oct1     222 ...AAAAHHGAAAAAAAASAGQVAAASAAAAVVSAAGLASICDSDTDPRELEAFAERFKQRRIKLGVTQADVG
POUIII    85 ..............AAAAAAAAAAAAAAEAGSPWSSSPVGMASSPQQQDVKSSSNRE..............
MLL       46 ...VGCGGPGAPPSPPAVAAAAAAAGSSGAGVPGGAAAASAASSSSASSSSSSSSSASSGPALLRVG......
HME1     181 DGSQPAAAGAGASKAGNPAAAAAAAAAAVAAAAAAAA..................................
Evx2     205 ....AAAAGLNSAASAAAAAAAAAAAASSAAAAGAPP..................................
BarH1    220 ...AAAAAAAAARNSAAAAAAAAAAAASAAAAGGGGGGGLGVGSAPAGAELDDSSDYHEE..........
BarH2     67 ............GAAAASAAAAAAAAALAAASSGGGRGSPTDSEREQS.......................
hPR      231 ESAGPLLKGKPRALGGAAAGGGAAACPPGAAAGGVALVPKEDSRFSA.........................
Arx      261 .......RRCSVATTGTVAAAAAAAAAAVATEGGE.....................................
HB9      117 ............AHPGAAAAAAAAAAAAAAAGGLA.....................................
JUN-d     49 ............QVAAALKAPGAAAAAAAAAGGEATAGLLGSPE...........................
HoxD8     15 ..................AAAAAAAAAAAAAAGEAINP.....................................
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B. Nuclear Domain (ND) Region

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hNUDR     188 GGTKYNWDPSVYDSELLPVRCRNISGTLYKNRLGSGGRGRCIKQGE.NWYSPTEFEAMAGRASSKDWKR
DEAF-1    205 GSSGWSENPSTQHNEVFQIRCKTTCAELYRSKLGSGGRGRCVKYKD.KWHTPSEFEHVCGRGSSKDWKR
LYSP100-B 590 ..............LPVTCGGVKGILHKKKLQQGILVKCIQTEDGKWFTPTEFEIKGGHARSKNWRL
SP100-B   603 ............SELPVTCGEVKGTLYKERFKQGTSKKCIQSEDKKWFTPREFEIEGDRGASKNWKL
pp41      161 ............SKSPVTCGEAKGILYKKKMKHGSSVKCIRNEDGTWLTPNEFEVEGKGRNAKNWKR

hNUDR     255 SIRYAGRPLQCLIQDGILNPHAASCTCAACCDDMT...LSGPVRLFVPYKRRKKEN...ELPTTPVKK
DEAF-1    273 SIKYGGKSLQSLIDEGTLTPHATNCSCTVCCDDEAGESASGPVRLFTPYKRRKRNQTDLDMESGPKRK
LYSP100-B 643 SVRCGGWPLRWLMENGFL.....................................................
SP100-B   658 SIRCGGYTLKVLMENKFL.....................................................
pp41      216 NIRCEGMTLGELLKSGLL.....................................................
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C. Zinc Finger Homology (ZFH) Region

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hNUDR     471 KTLFEQAKHASTYREAATNQAKIHADAERKE..QSCVNCGREAMSECT
DEAF-1    510 ..........................LHGNEIISA..KKCANCNREALAECS
RACK7     326 RQSLEQERDRLIAEVKKQLELEKQQAVDETKKKQWCANCKKEAIFYC.
nervy     558 KFFMEMSRHSSGERDLDNKSPSMASAQNGSNLQQQCWNCGRKATETCS
AML1-MTG8 639 ..........AEAKRQAAEDALAVINQQEDSS..ESCWNCGRKASETCS
BS69      501 ..............EELKKLATQHKQLISQTKKKQWCYNCEEEAMYHC.
hPR       548 ..............DSEASQSPQYSFESLPQK....ICLICGDEASGCHY
HNF-4      83 ..............SSTNSSQQQLQQQQQQQSPTVCAICGDRATGKHY
t-BOP      25 ..........................................QEKLHRCG
PDCD2     135 ..........................................CRVCGCLGPKTCS
celeganF23 116 RADLENKHRENLKMESAKLNEKHKRELVASRKKQWCWSCDNEAIYHC.
yeast72kd 510 ........................RQCANFSCGKWEDFPRQFAKCR

hNUDR     517 SCHKVNYCSTFCQRKDWK.DHQHICGQSAAVTVQADEVHVAESVMEK
DEAF-1    534 LCRKTPYCSEFCQRKDWN.AHQVECTRNPQTETQ.............
RACK7     372 .CWNTSYCDYPCQQAHWPEHMKSCTQSATAPQQEADAEVNTETLNK.
nervy     606 GCNMARYCSASCQYRDWD.SHHQVCGNTRASELSAKHLHSASNL...
AML1-MTG8 676 GCNTARYCGSFCQHKDWE.KHHHICGQTLQAQQQGDTPAVSSSV...
BS69      534 .CWNTSYCSIKCQQEHWHAEHKRTC.....................
hPR       580 GVLTCGSCKVFFKRAMEGQHNYLC.....................
HNF-4     117 GASSCDGCKGFFRRSVRKNHQYTCRFARNCWVDKDK.........
t-BOP      33 QCKFAHYCDRTCQKDAWLNHKNECAAIKRYGKVPNENIRLAARIMWR
PDCD2     148 RCHKAYYCSKEHQTLDWRLGHKQACAQ.....................
celeganF23 162 .CWNTAYCSVECQQGHWQTHRKFC.....................
yeast72kd 532 RCKRTKYCSRKCQLKAWGYHRY......WCHEVGSSHMRS.......
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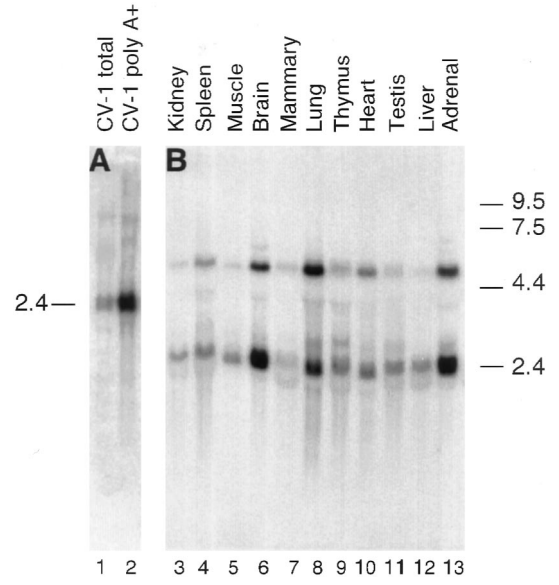


Fig. 3. Tissue Distribution of NUDR mRNA Expression in CV-1 Cells and Rat Tissues

A, Ten micrograms of total RNA (lane 1) and poly A+ RNA (lane 2) from CV-1 cells were electrophoresed in a 1% denaturing agarose gel, transferred to a nylon membrane, and hybridized with radiolabeled sNUDR probe. B, Ten micrograms of poly A+ RNA from various rat tissues (lanes 3–13) were subjected to Northern blot analysis as in panel A except the blot was hybridized with a radiolabeled rat cDNA probe. The blots were exposed to film and the film was scanned with a densitometer. The mobility of NUDR is shown on the *left* and the RNA size standards (in kilobases) are shown on the *right*.

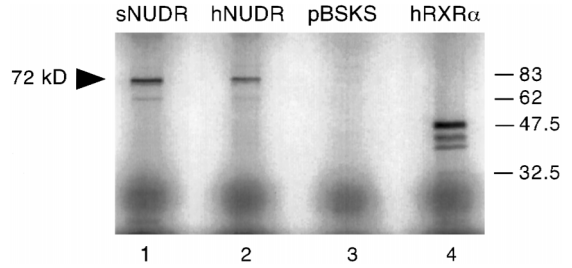


Fig. 4. *In Vitro* Synthesis of NUDR Proteins

^{35}S-Labeled proteins were produced by incubating 1 μg of the indicated plasmid DNA with 40 μCi of [^{35}S]methionine in an *in vitro* transcription/translation system (TNT, Promega) and separated on 10% gel by SDS-PAGE. The gel was dried and imaged with a Molecular Dynamics' PhosphorImager. Lane 1, Monkey NUDR cDNA in pBSSK (sNUDR); lane 2, human NUDR cDNA in pBSSK (hNUDR); lane 3, control plasmid (pBSKS); and lane 4, human RXRα cDNA in pBSKS (hRXRα). The mobility of NUDR and the prestained protein size markers (in kilodaltons) are shown on the *left* and *right*, respectively.

minor protein species of approximately 80 kDa was also observed, perhaps indicating a protein isoform or a protein modification of NUDR. The absence of NUDR in spermatozoa suggests that NUDR protein will be eliminated at some point after elongating spermatid formation.

Since DEAF-1 had been characterized in the developing fly embryo, we sought to determine whether NUDR protein was synthesized during vertebrate development. Total proteins were prepared from 14-, 15-, and 17-day mouse fetuses and subjected to Western blot analysis (Fig. 5C). In each age of fetus tested, the 72-kDa NUDR protein was detected with the immune serum, as well as one or two proteins of higher molecular mass.

Although all tissues had shown the presence of the 2.4 kb NUDR mRNA in Northern blots (Fig. 3), detectable amounts of the 72-kDa NUDR protein were only observed in testis, fetus, and the cell lines. This may indicate that NUDR protein is unstable in most tissues and that significant levels of the protein may occur only in cells that are rapidly dividing or undergoing differentiation.

Cellular Localization of Endogenous and Transfected NUDR

To confirm that NUDR was a protein distinct from the protein characterized as the secreted protein suppres-

Fig. 2. Regional Homology of NUDR with Other Proteins

The *diagram at the top* depicts approximate locations of potential functional domains in NUDR determined by sequence alignments to proteins shown in subsequent panels. The *open triangle* represents a naturally occurring deletion observed in the hNUDR8 cDNA; the *solid triangle* represents the NLS, and the *arrow* indicates the peptide region used for antibody production. Regions of similarity were identified by the BLAST program (National Center for Biotechnical Information, Bethesda, MD) and then aligned using the PILEUP program from the Wisconsin sequence analysis package by Genetics Computer Group, Inc. (GCG, Madison, WI). *Shading* represents mutationally conserved amino acids with scores above 0.7 in the default GCG scoring matrix. The alignments are: panel A, an alanine-acidic-rich (AA) region; panel B, an ND region; and panel C, a region with potential ZFH. Not shown are the alignments that indicated two conserved proline-rich regions (PR1, PR2). The following are the names and Entrez Protein Accession numbers used: hNUDR; DEAF-1, 1209883; nervy, 790600; cbfα1 (core binding factor α subunit 1), 735898; BF-2, 603460; shn, 1079151; HOXA13, 1832353; oct1 (octamer binding transcription factor 1), 2135847; POUIII (POU domain protein), 1730449; MLL, 2160396; HME1 (homeobox engrailed-1), 462291; Evx2, 106292; BarH1, 103026; BarH2, 419958; hPR (human progesterone receptor), 130894; Arx, 2317259; HB9, 1082461; JUN-d, 135307; HoxD8 (Hox4.3), 110005; LYSP100-B, 1173654; SP100-B, 1173656; pp41 (phosphoprotein 41), 1362889; RACK7, 1199659; AML1-MTG8, 407727; BS69, 1362759; HNF-4 (homolog), 1708276; t-BOP, 1809322; PDCD2, 998901; celeganF23 (*c. elegans* conceptual), 2088857; and yeast72KD (hypothetical), 2497149.

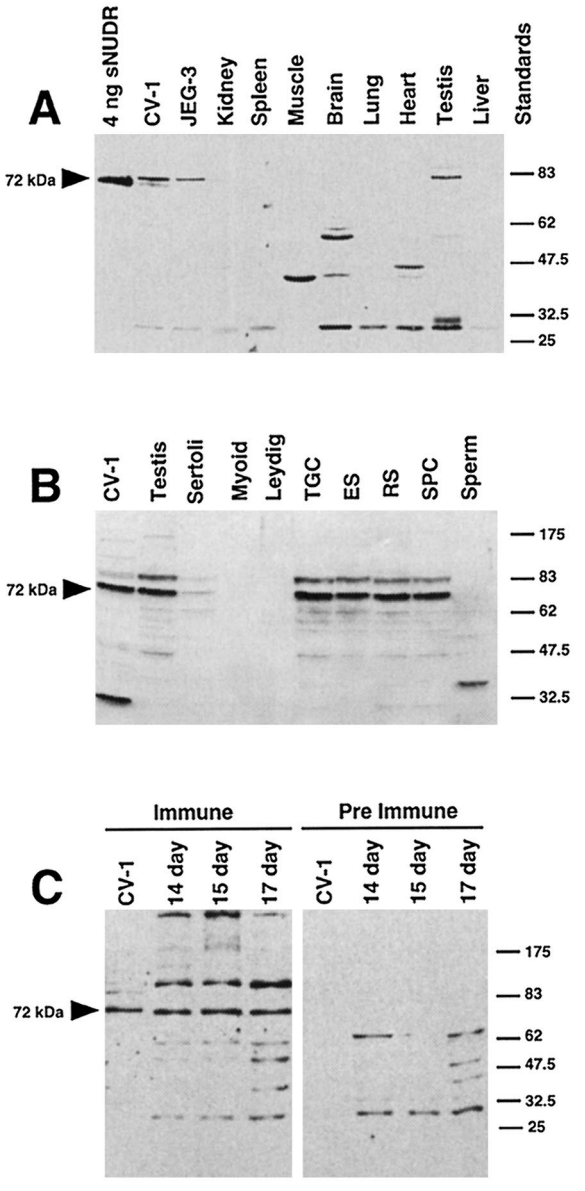


Fig. 5. Western Blot Analysis of NUDR Proteins

A, Four nanograms of recombinant sNUDR with an amino-terminal histidine tag were compared with 100 μg of total protein from untransfected monkey CV-1 cells, human JEG-3 cells, and the indicated adult rat tissues. Proteins were separated by SDS-PAGE on 10% gels, and immunoreactive proteins were detected with a polyclonal antibody to NUDR by Western blot analysis (see *Materials and Methods*). B, Total proteins (100 μg) from untransfected CV-1 cells were compared with proteins isolated from adult rat testis; cultured Sertoli, myoid, and Leydig cells; total germ cells (TGC); elongating spermatids (ES); round spermatids (RS); and spermatocytes (SPC). Western blot analysis was performed as in panel A. C, Total proteins (50 μg) from untransfected CV-1 cells were compared with total proteins (25 μg) isolated from 14-, 15-, and 17-day-old mouse embryos by Western blot analysis as in panel A using a polyclonal antibody to NUDR (*left*) or preimmune serum (*right*) on duplicate blots. The mobility of the prestained protein size markers (in kilodaltons) are shown on the *right* of each panel.

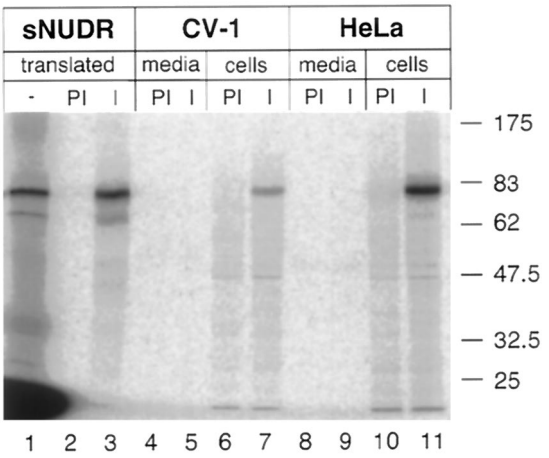


Fig. 6. *In Vivo* Labeling of NUDR Proteins

CV-1 cells and HeLa cells were transfected with hNUDR and incubated with ^{35}S-labeled methionine/cysteine for 4 h before collecting the culture media and harvesting the cells. Media (lanes 4, 5, 8, and 9) and cell extracts (lanes 6, 7, 10, and 11) were treated with either preimmune serum (PI) or immune serum to full-length NUDR (I) followed by immunoprecipitation with protein A agarose beads. ^{35}S-labeled sNUDR produced by *in vitro* transcription/translation was loaded directly in lane 1; or subjected to immunoprecipitation with preimmune serum (lane 2) or immune serum (lane 3). Immune complexes were separated on 9% gels by SDS-PAGE, and ^{35}S-labeled proteins were detected with a PhosphorImager. The mobility of the prestained protein size markers (in kilodaltons) are shown on the *right*.

sin, we tested whether NUDR was secreted into the media of CV-1 and HeLa cells that were transfected with an expression vector for hNUDR. Cells were incubated with ^{35}S-labeled methionine/cysteine, and the proteins in the culture media and cells were immunoprecipitated (Fig. 6). ^{35}S-labeled NUDR protein was observed in the cell extracts of CV-1 and HeLa cells but was not detected in the culture media. These results indicate that, under our experimental conditions, NUDR is not a secreted protein.

Antibodies to the full-length recombinant protein were used to identify the cellular localization of endogenous NUDR protein in CV-1 cells by fluorescence microscopy. All cells showed immunofluorescence in the nucleus and minimal immunofluorescence in the cytoplasm (Fig. 7B), indicating that NUDR is primarily a nuclear protein. Nuclear localization was also observed for endogenous NUDR in HeLa cells (not shown). To test whether NUDR could confer nuclear localization to a cytoplasmic protein, the hNUDR cDNA was subcloned into a mammalian expression vector to produce a green fluorescent protein-NUDR (GFP-NUDR) fusion protein. CV-1 cells were transfected with either the parent vector (pEGFP-N3) or pEGFP-hNUDR and examined by fluorescence microscopy. Cells expressing GFP showed the fluorescence distributed throughout the cell (Fig. 7C), while cells overexpressing the GFP-hNUDR fusion protein

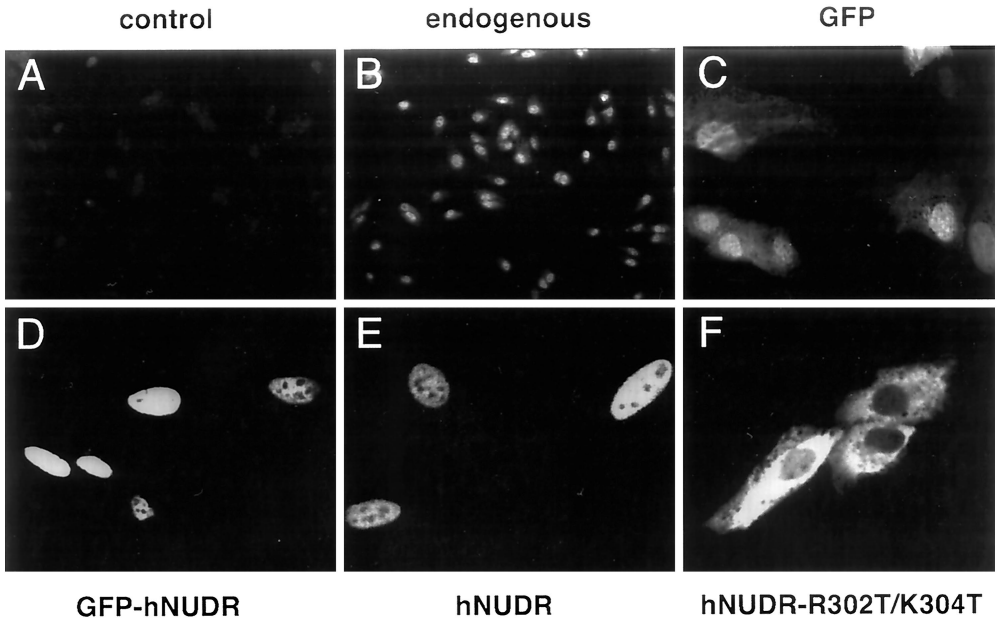


Fig. 7. Localization of Endogenous NUDR and Overexpressed NUDR

To detect endogenous NUDR protein, CV-1 cells were fixed on slides and incubated with preimmune serum (panel A, control) or serum containing antibodies to full-length NUDR (panel B, endogenous) followed by a biotinylated second antibody and fluorescein-conjugated avidin. CV-1 cells were transfected with pEGFP-N3 (panel C, GFP) and pEGFP-hNUDR (panel D, GFP-hNUDR) and fixed, and cells expressing GFP were visualized by fluorescence microscopy. CV-1 cells were transfected with pCMVhNUDR (panel E, hNUDR) and pCMVhNUDR-R302T/K304T (panel F, hNUDR-R302T/K304T) and incubated with an antibody to NUDR peptide followed by a fluorescein-labeled second antibody. Fluorescence was visualized with fluorescein filters using an Olympus IMT-2 microscope (panels A and B, 40× magnification) or Olympus Fluoview Confocal Imaging System attached to an Olympus IX70 microscope (panels C–F, 400× magnification).

exhibited fluorescence predominantly in the nucleus (Fig. 7D). These observations indicate that a signal for nuclear import must be present in NUDR, and sequence analysis indicated a potential NLS in the ND region (see Figs. 1 and 2). In this region of NUDR, two clusters of basic amino acid residues are separated by nine amino acids, a motif that is similar to the bipartite NLSs of nucleoplasmin and glucocorticoid receptor (43). To test whether these residues contributed to a NLS in NUDR, *in vitro* mutagenesis was used to change arginine 302 and lysine 304 to threonines (hNUDR-R302T/K304T). Expression vectors for the wild-type hNUDR and hNUDR-R302T/K304T were transfected into CV-1 cells, and the cellular location of the proteins was examined with a peptide antibody specific for NUDR, but with minimal ability to detect endogenous levels of protein. The combined use of the peptide antibody and hNUDR overexpression reduced the possibility of detecting cross-reacting antigens and low levels of endogenous NUDR and ensured the detection of NUDR protein produced from transfected plasmid DNA. NUDR-transfected cells showed the protein concentrated in the nucleus (Fig. 7E), confirming the endogenous NUDR localization (Fig. 7B). The substitution of two amino acids in the putative NLS resulted in hNUDR-R302T/K304T being localized almost exclusively to the cytoplasm (Fig. 7F). The single mutations, hNUDR-R302T and hNUDR-K304T, were also transfected into CV-1 cells, and each mutation abolished nuclear import and resulted in cytoplasmic localization similar to the double mutant (data not shown). These results confirm that this cluster of basic amino acids is critical for targeting NUDR to the nucleus and suggest that the protein may function in the nucleus, potentially as a DNA- binding protein/transcription factor.

NUDR Is a DNA-Binding Protein

Since the original partial sNUDR clone was identified by expression screening with a radiolabeled RARE oligonucleotide, we sought to verify the DNA-binding ability of the NUDR protein in an electrophoretic mobility shift assay (EMSA). In the absence of other factors, recombinant NUDR protein was capable of altering the mobility of a radiolabeled DR5 RARE (Fig. 8). However, moderate concentrations of unlabeled and nonspecific poly dI-dC, poly dA-dT, and salmon sperm DNA were able to displace the RARE bound by NUDR (Fig. 8A). This suggests that NUDR has only a modest affinity for the RARE DNA sequence that was used to identify the clone. To further examine the interaction of NUDR at the RARE, DR5 and DR2 RAREs were incubated with the recombinant RAR, RXR, and/or a molar excess of NUDR (Fig. 8B). NUDR

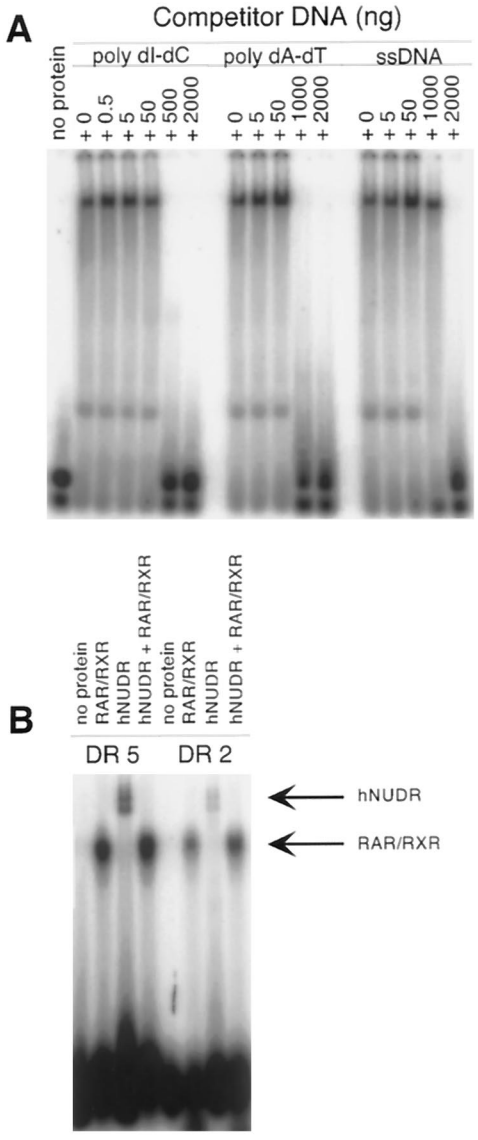


Fig. 8. Electrophoretic Mobility Shift Assay

A, A ^{32}P-labeled DR5 RARE oligo (120 fmol) was incubated with 35 pmol of recombinant hNUDR protein with increasing quantities (0–2000 ng) of nonspecific competitor DNA: either poly dI-dC, poly dA-dT, or salmon sperm DNA (ssDNA). B, Either ^{32}P-labeled DR5 RARE or DR2 RARE oligos (240 fmol) were incubated with 38 pmol of recombinant hNUDR protein and/or 1 pmol of recombinant hRARα and *in vitro* translated hRXRα. Protein-DNA complexes were separated from free probe on a 4% nondenaturing gel. Results were imaged with a PhosphorImager. In panel B, the *arrows* indicate hNUDR-shifted RARE complex and RAR/RXR-shifted RARE complex.

produced a complex with both RARE motifs that was distinct in mobility from the complex formed by RAR/RXR. Incubation of RAR, RXR, and NUDR eliminated the NUDR complex from both RARE motifs, suggesting that RAR/RXR can displace NUDR from the RAREs. We conclude from these experiments that in the absence of other factors, NUDR binding to a RARE

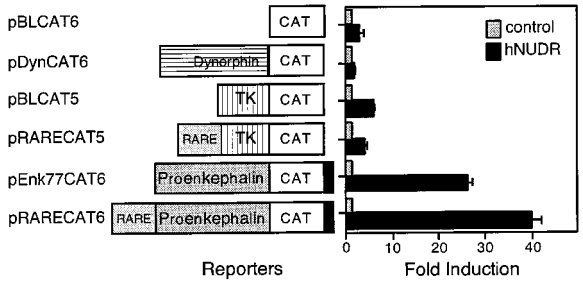


Fig. 9. Selective Transcriptional Activation by NUDR

CV-1 cells were cotransfected with 2 μg of reporter plasmid (pBLCAT6, pDynCAT3, pBLCAT5, pRARECAT5, pEnk77CAT6, or pRARECAT6) and either 1 μg of CMVNeo (control) or 1 μg of CMVhNUDR (hNUDR) as described in *Materials and Methods*. Reporter names and schematic representations of the constructs are shown to the *left of the graph* (not to scale). The fold induction due to cotransfection of NUDR is relative to each reporter, which is set to a value of 1. The results from this experiment are representative of three experiments and are expressed as the average ± SD of triplicate plates.

sequence occurs with only low-to-moderate affinity relative to RAR/RXR binding.

NUDR Regulates Transcription from the Proenkephalin Promoter

Since NUDR was found to be a nuclear protein that was able to bind to a RARE-containing DNA sequence, we investigated the transactivation potential of NUDR with several promoters linked to the reporter gene, chloramphenicol acetyl transferase (CAT). The reporter construct, pRARECAT6, consists of two copies of the DR5 RARE sequence inserted 5′ of a minimal human proenkephalin promoter and linked to CAT. We have previously shown this reporter construct to be useful in the analysis of RAR function (23). Cotransfection of a hNUDR expression vector with pRARECAT6 produced a 41-fold increase in CAT activity over the reporter alone, demonstrating that hNUDR can potentiate transcriptional activation (Fig. 9). However, hNUDR was also able to increase CAT activity 26-fold from the minimal proenkephalin promoter (pEnk77CAT6). These results suggested that the majority of the activation by hNUDR occurs through sequences in the proenkephalin promoter rather than through the RARE sequence. Since the endogenous proenkephalin gene (44) and the human proenkephalin transgene (45) are expressed at elevated levels in rodent germ cells, the proenkephalin promoter may be a potential target for NUDR activation.

To further examine the contribution of the RARE sequence to hNUDR transactivation, the RARE sequence was inserted 5′ to the thymidine kinase promoter in the reporter pRARECAT5. The pRARECAT5 reporter showed a small 4-fold NUDR-dependent in-

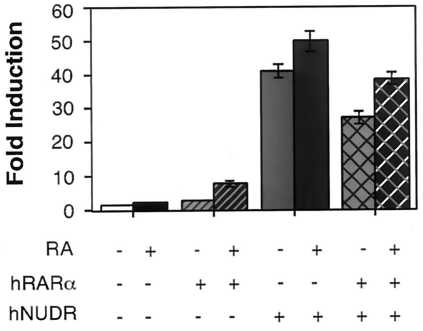


Fig. 10. Transactivation of pRARECAT6 by RARα and NUDR

CV-1 cells were transfected with 2 μg of the reporter plasmid pRARECAT6 and 1 μg of the indicated expression vector, CMVhRARα (hRARα) and/or CMVhNUDR (hNUDR). The cells were treated in the absence (−) or presence (+) of 1 μM retinoic acid (RA) for 24 h before harvest. Fold inductions are relative to the reporter alone. The results from this experiment are representative of three experiments and are expressed as the average fold induction ± SD of triplicate plates.

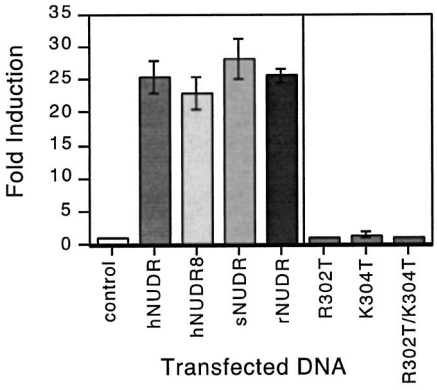


Fig. 11. Nuclear Localization Is Critical for NUDR Activity

CV-1 cells were transfected with 2 μg of the reporter plasmid, pEnk77CAT6, and 1 μg of a CMV-promoter driven expression plasmid [containing no insert (control) or the cDNAs for the following: human NUDR (hNUDR), hNUDR with a 14-amino acid deletion (hNUDR8), monkey NUDR (sNUDR), rat NUDR (rNUDR), and hNUDR with mutations (R302T, K304T, and R302T/K304T) in the NLS]. The results from this experiment are representative of two experiments and are expressed as the average fold induction over control (set at 1) ± SD of triplicate plates.

crease (Fig. 9) that was similar to the 6-fold increase for thymidine kinase promoter alone (pBLCAT5). This indicates that the RARE does not increase NUDR-dependent activation in the context of the thymidine kinase promoter. Cotransfection of hNUDR did not increase transcription from all promoters, since the dynorphin promoter (pDynCAT3) was unaffected by hNUDR addition (Fig. 9).

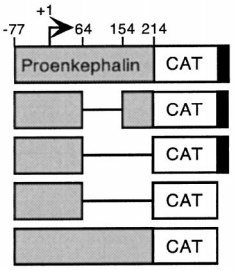
NUDR activation of the pRARECAT6 reporter was compared with the activation by the ligand-inducible retinoic acid receptor (hRARα) to ascertain the effectiveness of NUDR as a transcriptional activator at the RARE and to investigate potential interactions between the two proteins *in vivo*. CV-1 cells have low endogenous levels of RARs as evidenced by a 1.8-fold increase in reporter activity with retinoic acid (RA) treatment (Fig. 10). Cotransfection of cells with RARα showed a minimal increase in CAT activity in the absence of ligand (2-fold), which was increased to 7-fold in the presence of RA. In contrast, hNUDR increased transcription 41-fold in the absence of RA, which was further elevated to 50-fold with RA treatment. The RA-dependent increase is most likely due to low levels of endogenous RA-activated factors and not due to hNUDR binding of RA. These observations demonstrate the potency of NUDR as a transcriptional activator relative to RARα.

In cotransfections of both hNUDR and RARα, additive effects were not observed, but rather, intermediate levels of transcriptional activation were observed [27-fold in the absence of RA and 38-fold in the presence of RA (Fig. 10)]. Since NUDR is the stronger transcriptional activator and the RAR/RXR complex has greater affinity for the RARE than NUDR (Fig. 8B), the decrease in transcriptional activity from 41-fold (NUDR alone) to 27-fold (NUDR plus RARα) is likely a result of competition at the RARE in which RAR displaces NUDR binding. This premise is further supported by the similarity in activations produced in cotransfections of hNUDR and RARα with pRARECAT6 (27-fold), compared with transfections in the absence of the RARE (hNUDR with pEnk77CAT6 in Fig. 9, 26-fold). The combination of EMSA and transfection assays suggests that although NUDR is able to bind to a RARE *in vitro* and can activate transcription from the RARE in pRARECAT6, the RARE-dependent activation by NUDR is not generally transferable to other promoters.

Nuclear Import of NUDR Is Essential for Transcriptional Activation from the Proenkephalin Promoter

We compared the transactivation potential of monkey NUDR (sNUDR), rat NUDR (rNUDR), and the human NUDR deletion variant (hNUDR8) to the previously tested hNUDR. As shown in Fig. 11, sNUDR, rNUDR, and hNUDR8 activated transcription from the proenkephalin promoter to a similar extent as hNUDR; however, in some assays the naturally occurring deletion of the alanine-rich region in hNUDR8 showed a trend for reduced transactivation by as much as 33% (not shown).

To further validate that transcriptional activation requires the presence of NUDR in the nucleus, we tested the hNUDR expression vectors with single (R302T or K304T) and double mutations (R302T/K304T) in the NLS. All three mutants were unable to activate transcription from the proenkephalin reporter plasmid (Fig. 11). Since NUDR proteins mutated in the NLS are located exclusively to the cytoplasm (Fig. 7F), we con-



Reporter	Normalized CAT Activity		Fold
	basal	+hNUDR	Change
pEnk77CAT6	1462 ± 264	32472 ± 1617	22.2
pEnk77CAT6Δ65-153	154 ± 57	9382 ± 944	60.9
pEnk77CAT6Δ65-213	207 ± 13	6754 ± 1040	32.6
pEnk77CAT6Δ65-213, Δ3'UTR	226 ± 13	6060 ± 795	26.8
pEnk77CAT6Δ3'UTR	1049 ± 85	14574 ± 188	13.9

Fig. 12. Analysis of Enkephalin Sequences Involved in NUDR Regulation

Schematic representations and names of the reporters used in the transfections are shown to the *left of the table*. CV-1 cells were cotransfected with 2 μg of indicated reporter plasmid, 5 μg of SV2βgal, and either 1 μg of CMVNeo (basal) or 1 μg of CMVhNUDR (hNUDR), as described in *Materials and Methods*. Normalized CAT activity is the ratio of CAT activity to β-galactosidase activity, and the results are expressed as the average ± SD from triplicate plates. Fold change is the ratio of normalized CAT activity of +hNUDR to basal. The results are representative of two experiments.

cluded that nuclear import of NUDR is critical for its transcriptional activation of the proenkephalin promoter.

NUDR Activation of Proenkephalin Promoter Regions Appears to be Independent of DNA Binding

In an attempt to identify the sequences through which NUDR activated transcription of the pEnk77CAT6 reporter, enkephalin sequences in the 5′-untranslated region (UTR) and 3′-UTR were deleted, and the resulting reporter constructs were tested in CAT assays. Deletion of nucleotides 65–153 of the intron (position 71–157) resulted in a 60.9-fold increase in activation by NUDR (Fig. 12), but much of this fold change can be attributed to a significant decrease in basal activity (relative to pEnk77CAT6), as shown by the normalized CAT activity data. The deletion of additional 5′-UTR sequences (Δ65–213) decreased NUDR activation (32.6-fold), indicating that sequences between 153 and 213 may contribute to activation by NUDR. Additional sequences in the 3′-UTR (1081 bp) may also contribute slightly to activation by NUDR as seen by the decreased fold change with the reporters, pEnk77CAT6Δ65–213,Δ3′-UTR and pEnk77CAT6Δ3′-UTR. The normalized CAT activities are shown for each reporter to indicate that there are changes in both basal and NUDR-stimulated CAT activities. In all of these constructs, the majority of the NUDR-dependent activation appeared to map to a minimal promoter region (position −77 to +65), suggesting that NUDR may act in close proximity to the basal transcriptional machinery.

All of the proenkephalin DNA regions indicated in Fig. 12 were tested for interaction with recombinant NUDR protein by EMSA and DNase I protection assays. NUDR failed to bind any of the proenkephalin DNA sequences with affinities that were equal to the minimal binding shown for the RARE in Fig. 8 (data not shown). It seems unlikely that low-affinity binding to DNA would mediate NUDR transactivation of the proenkephalin promoter, and our results suggest that protein-protein interactions of NUDR with promoter-specific or basal transcriptional machinery is the more likely mechanism. Alternatively, NUDR-dependent induction of downstream transcription factors could also produce regulated expression from the proenkephalin promoter.

Identification of NUDR-Binding Sequences

To identify DNA sequences to which NUDR might bind with high affinity, we used recombinant NUDR protein to select oligonucleotides from a library of double-stranded degenerate oligonucleotides and then amplified the selected sequences based on the method described by Lu *et al.* (46). Briefly, a set of oligonucleotides were synthesized that contained 30 random bases (1×10^{18} potential sequences), flanked on each end by different primer-specific sequences. Glutathione-*S*-transferase-sNUDR (GST-sNUDR1.5) fusion protein was immobilized on glutathione-agarose beads and used to select DNA sequences from the random set of oligonucleotides for which NUDR had affinity. Primers to each of the flanking sequences were then used to amplify the selected internal sequences by PCR, and the processes of affinity binding and amplification were repeated six times to select for DNA sequences that were consistently bound to NUDR protein. The DNA sequences were cloned into pBLCAT5 and sequenced to produce a collection of NUDR-binding sequences or NBSs. The alignment of 23 sequences from the approximately 100 oligonucleotides sequenced is shown in Fig. 13A. Analysis of the sequences showed 52% of the 23 NBSs contained one or more copies of the sequence TTCG, previously identified as the DEAF-1 core-binding sequence (21). Twenty-two percent and 61% of the NBSs contained one or more copies of the consensus motifs TTCGGG and TTTCCG, respectively.

To increase the specificity of interaction of NUDR with DNA sequences obtained after six rounds of selection, a final round of DNA sequence selection was

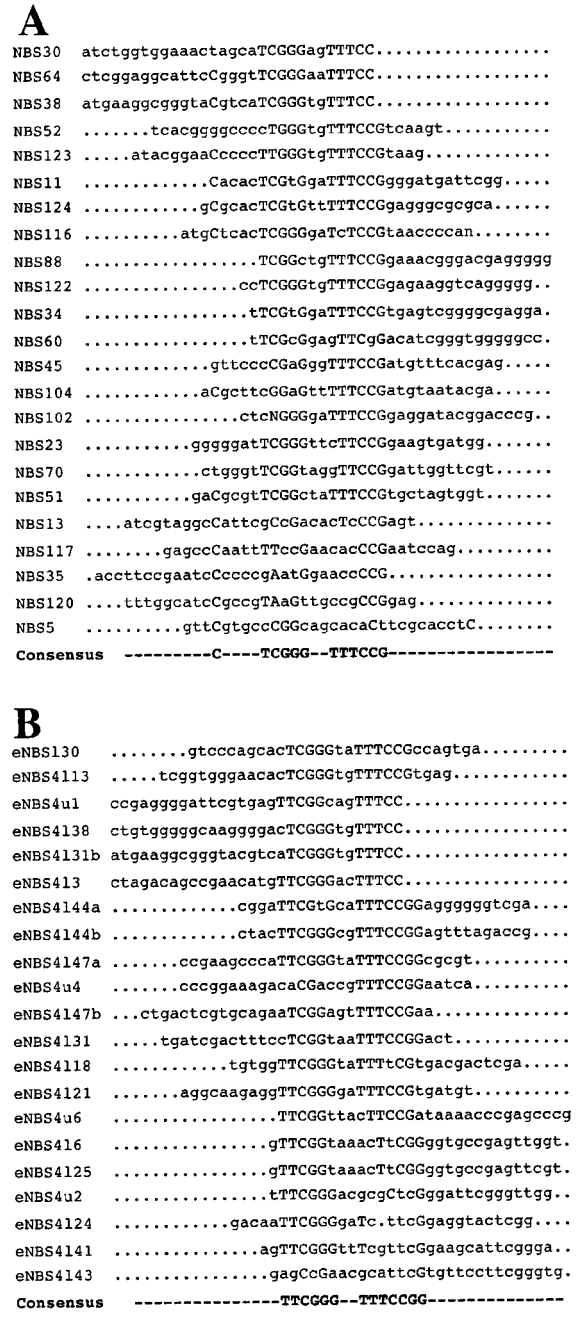


Fig. 13. Alignment of NBSs

A, NBSs were selected from a library of double-stranded oligonucleotides (70 mers, 30 random nucleotides flanked on either side by 20 nucleotides with different primer-specific sequences) by affinity to recombinant NUDR. After six cycles of binding to NUDR and DNA amplification, the oligonucleotides were cloned and sequenced. A consensus was derived from the alignment of 23 of the sequences. B, Recombinant NUDR was used in EMSA to shift a subset of the oligonucleotides from the sixth round of selection. A consensus sequence was derived from the alignment of these eNBSs. The consensus derived from each alignment corresponds to a plurality of greater than 50%.

performed using EMSA. The NUDR-shifted oligonucleotides were isolated, amplified, cloned into pBLCAT5, and sequenced to produce a collection of sequences called enhanced NBSs or eNBSs (Fig. 13B). Of the 20 eNBSs shown, 75% contained at least one copy of TTCG, and 50% of these contained two to four copies of this motif. The 25% of NBSs that lacked a TTCG sequence contained one or more copies of the second motif, TTTCCG. Sixty-one percent of the eNBS contained at least one copy of TTTCCG. Alignment of the eNBSs suggested the consensus motif TTCGGGNNTTTCCGG as the DNA sequence required for efficient binding by NUDR.

To confirm the DNA-binding activity of NUDR, EMSA and DNase I protection assays were performed on DNA fragments derived from the NBS consensus sequence (Fig. 14). In both assays, poly dI-dC was maintained at a level that had eliminated NUDR binding to the RARE and proenkephalin sequences (500 ng). Increasing levels of NUDR protein produced increased band intensity of the shifted consensus sequence in an EMSA (Fig. 14A). In DNase I protection assays, increasing levels of NUDR protein provided increased protection to three different radiolabeled DNA fragments (Fig. 14, B–D), verifying NUDR binding to the entire length of the consensus sequence. NUDR also extended its protection into the flanking vector sequence gatccgg, which resulted from the ligation of the consensus sequence into the *Bam*HI site of pBLCAT5. Similar results were obtained for NUDR binding to several of the individual NBS sequences in EMSA and DNase I protection assays (data not shown).

DNA Sequences Recognized by NUDR Do Not Behave as Transferable Transcriptional Response Elements

The NBS motifs that had been selected by recombinant NUDR protein were cloned 5′ of the thymidine kinase promoter in the pBLCAT5 vector to facilitate analysis of their potential transcriptional activation by NUDR. The plasmids were transiently transfected into CV-1 cells either in the absence or presence of hNUDR, and CAT assays were performed. Table 1 shows the results from selected representative plasmids. While some of the NBS plasmids showed increased basal activity relative to the parent plasmid (pBLCAT5), many also showed decreased basal activity. Upon cotransfection with the hNUDR expression vector, all of the NBS-containing reporters showed less than a 2-fold increase in CAT activity, which was less than that observed for pBLCAT5 (3-fold). These data indicate that the presence of NBS motifs within a promoter are not sufficient to reconstitute the greater than 20-fold induction observed for the proenkephalin promoter, and that NUDR transcriptional activation may occur through spacing-dependent or multiple motifs, or through mechanisms independent of NUDR binding to DNA.

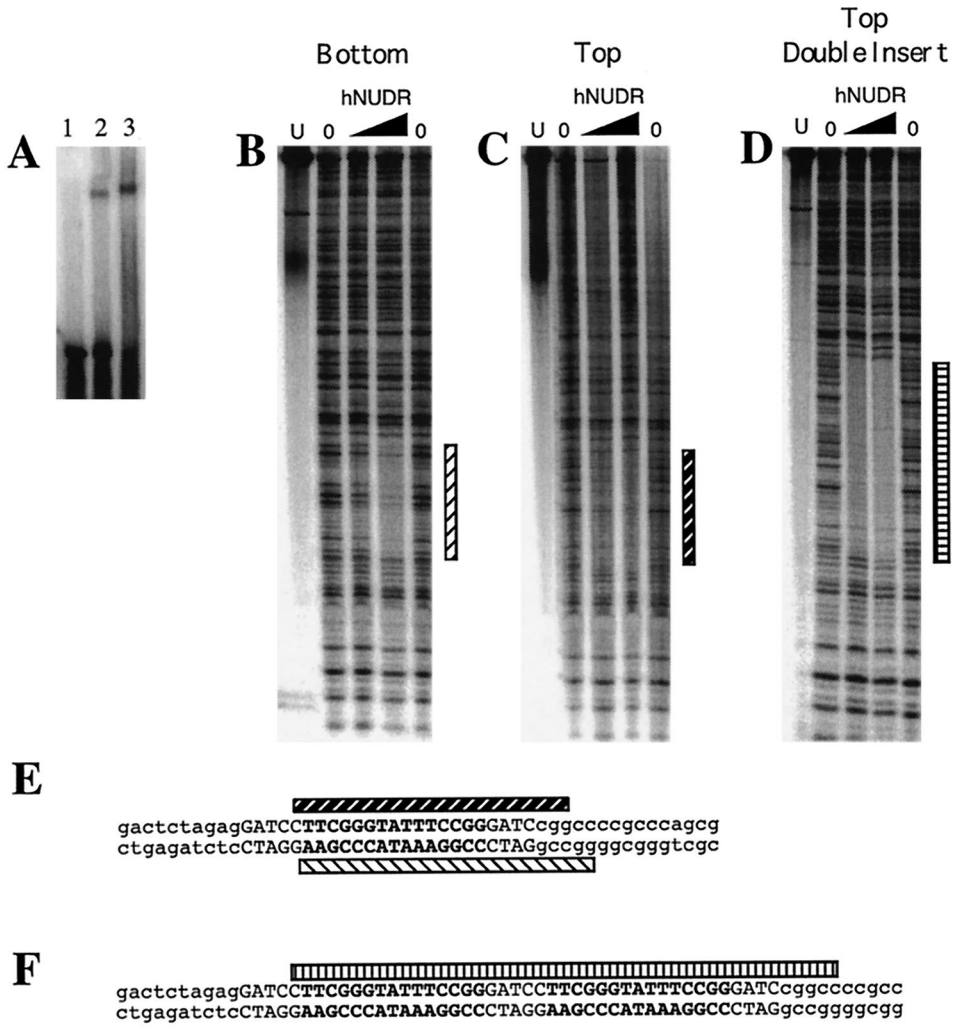


Fig. 14. EMSA and DNase I Protection Assays of the NBS Consensus

A, Radiolabeled DNA containing the NBS consensus (sequence shown in panel E) was incubated alone (no protein) or with 10 pmol and 30 pmol of recombinant hNUDR. Samples were separated on a 4% nondenaturing polyacrylamide gel and analyzed by autoradiography. B–D, Radiolabeled double-stranded DNA containing one copy of the NBS consensus (B, bottom strand labeled and C, top strand labeled) or two copies of the NBS consensus (D, top strand labeled) was left untreated (U), or was treated with DNase I in the absence (O) or presence of increasing amounts of recombinant hNUDR (indicated by the *wedge*). Samples were separated on 6% denaturing sequencing gels and analyzed by autoradiography. The protected regions in panels A–C are indicated by *hatched bars to the right of each panel*, and the nucleotide sequences protected are shown by the corresponding *hatched bars above or below* the sequences shown in panels E and F. The NBS consensus sequences are shown in *bold capital letters*.

DISCUSSION

We have presented the isolation of rat, two monkey, and two human cDNAs and have initiated the characterization of the encoded proteins. We have designated the protein NUDR for its similarities in both sequence and potential function to *Drosophila* DEAF-1. Comparison of the nucleotide sequence of hNUDR to the GenBank databases showed highest homology (83%) to a rat cDNA called suppressin. The conceptual protein of the putative suppressin cDNA showed 88% identity to hNUDR (Fig. 1), suggesting that they are rat and human homologs. However, several lines of evidence suggest that neither is the suppressin entity described by LeBoeuf *et al.* (27).

Depicted as a secreted protein and a novel inhibitor of cell proliferation, suppressin was initially purified from bovine pituitary and was identified as a monomeric polypeptide with an isoelectric point (pI) of 8.1 and a molecular mass of 63 kDa (27). A polyclonal antibody to bovine suppressin was used to screen a rat pituitary cDNA library, and a partial cDNA (691 bp) of rat suppressin was obtained. The 691-bp clone was subsequently used to isolate a 924-bp cDNA by rescreening the library by hybridization, and additional 5′-sequence was obtained by 5′-RACE (26). The

Table 1. NUDR Overexpression Does Not Enhance Expression of Reporters Containing NBSs

Reporter	Normalized CAT Activity		Fold Change
	Basal	+hNUDR	
pBLCAT5	5,024 ± 324	15,250 ± 618	3.0
pNBS23CAT5	2,260 ± 415	2,704 ± 340	1.2
pNBS30CAT5	6,818 ± 284	10,726 ± 1313	1.6
pNBS45CAT5	14,023 ± 207	21,635 ± 1797	1.5
pNBScons2.8CAT5	1,768 ± 291	2,094 ± 164	1.2
pNBScons2.11CAT5	1,843 ± 204	2,048 ± 198	1.1
pNBScons2.12CAT5	2,097 ± 291	1,907 ± 89	0.9
pEnk77CAT6	1,462 ± 264	32,472 ± 1617	22.2

CV-1 cells were transfected with reporter constructs in the absence or presence of hNUDR, and the results are reported as normalized CAT activity ± SD and fold change as described in Fig. 12 and *Materials and Methods*.

1882-bp cDNA called rat suppressin in the GenBank (U59659) is most likely a compilation of these partial sequences. Comparison of the conceptual protein encoded by the 1882-bp rat suppressin cDNA (26) and purified bovine suppressin (27) shows little similarity in amino acid composition and pI. Based on the 99% nucleotide homology to rat NUDR (Fig. 1), we suggest that the 1882-bp rat sequence represents a partial cDNA with a downstream methionine selected as the initiator methionine. Furthermore, the amino acids that follow the indicated initiator methionines in either suppressin or NUDR do not conform to the motifs required of signal peptides (47), making it highly unlikely that these proteins would be secreted. Using *in vivo* labeling, we were able to detect ^{35}S-labeled NUDR protein in CV-1 cell and HeLa cell extracts by immunoprecipitation, but were unable to detect any secreted proteins in the culture media (Fig. 6). As detailed in the experimental results, we have determined that the encoded NUDR proteins localize to the nuclei of cells and behave as transcription factors, making them unlikely candidates for secreted regulatory factors. And although the secreted protein characterized as suppressin has been shown to inhibit cell proliferation, it remains to be demonstrated that the protein encoded by the suppressin rat cDNA produces a secretory product that can inhibit cell proliferation.

Using antibodies to NUDR in Western blot analysis (Fig. 5), we observed three proteins in rat brain extracts that were lower in molecular mass than full-length NUDR but approximated the size of bovine pituitary suppressin (63 kDa). The antibody also detected several proteins in muscle and heart in the 35–45 kDa range that either share similar antigenic determinants with NUDR or are NUDR derivatives. Thus, it is conceivable that NUDR has antigenic determinants that could be recognized by anti-suppressin antibodies, potentially enabling the identification of a rat NUDR clone by antibody screening.

The second most similar sequence to NUDR identified by computer comparisons was the *Drosophila* DEAF-1. DEAF-1 has been shown to be an important cofactor in *Deformed* (*Dfd*) gene expression during embryonic development. A 120-bp region of the *Dfd* promoter, referred to as module E, is capable of driving embryonic expression of a reporter gene in a pattern similar to endogenous *Dfd* expression (20) and contains binding sites for DEAF-1 and Dfd, through which Dfd can autoregulate its own expression (21). hNUDR showed 46% similarity overall with DEAF-1 and contained regions of higher homology. DEAF-1 was initially purified from embryonic nuclear extracts by DNA affinity chromatography and migrated as a 85-kDa protein in SDS gels (21). DEAF-1, like NUDR, shows anomalous migration in protein gels, as the calculated molecular mass of DEAF-1 is 62 kDa and the protein produced by *in vitro* transcription/translation migrates like a 85-kDa protein (noted in Ref. 21).

An alanine-rich region in the amino terminus of NUDR may contribute to its transactivation as suggested by the decrease in CAT activity with hNUDR8, which has a naturally occurring deletion of the alanine-rich region (Fig. 11). A decrease in transactivation was also obtained using an amino-terminal deletion of sNUDR that lacked the first 75 amino acids (data not shown). The sequence similarity of this region to numerous homeodomain factors suggests that it may mediate an important transactivation function during development.

NUDR shows high regional similarity to SP100 proteins that colocalize with PML to subnuclear dot-like structures termed PML nuclear bodies. In APL, the normal pattern of PML localization to nuclear bodies is disrupted by a t(15;17) translocation, which produces a PML-RARα fusion protein. The oncoprotein shows aberrant localization and potentially contributes to APL pathogenesis (33). Treatment of APL-derived cells with RA restores the nuclear bodies (33), and RA can produce clinical remission of APL patients through differentiation of leukemic cells into mature granulocytes with associated decreases in cell proliferation (for reviews see Ref. 48). While a uniform nuclear presence of NUDR is observed in HeLa and CV-1 cells, the similarities to the SP100 proteins suggest interaction at PML nuclear bodies might potentially occur in appropriate cell types. Because NUDR also shows homology to leukemic oncogenes at its AA and ZFH domains, its precise subnuclear localization in normal and transformed lymphoid cells should be investigated.

The ZFH domain located at the carboxy terminus of both NUDR and DEAF-1 contains the sequence Cys-X_2-Cys-X_7-Cys-X_2-Cys-X_5-Cys-X_3-Cys-X_7-His-X_3-Cys ($Cys_6HisCys$). While cysteine residues frequently form disulfide bonds in extracellular proteins, cysteine and histidine residues are often used to bind metal ions, such as zinc, and stabilize structural folds of intracellular proteins (49). Although the exact spacings between cysteine residues and the histidine in NUDR and DEAF-1 are somewhat unusual, the motif is cognate of forming a zinc-binding domain (49) that may contrib-

ute to protein-protein interactions, DNA binding, and transcriptional activation. Gross and McGinnis (21) suggest this domain serves a non-DNA-binding role, since they were unable to obtain DNA binding in EMSA and DNase I protection assays with an amino-terminal truncated DEAF-1 that consisted of only the last 84 amino acids. In addition, they state that altering the second and third cysteines to serines in this domain did not affect the ability of full-length DEAF-1 to bind DNA in EMSA. Similarly, we have found that deletion of the last 60 amino acids of hNUDR resulted in only slight decreases in DNA binding by EMSA and transactivation in CAT assays (data not shown). Unlike many zinc finger domains that are critical for DNA binding, these data suggest that the ZFH region does not appear to be an independent DNA-binding module.

NUDR's affinity for DNA was used to select specific DNA-binding sequences, and a direct repeat of TTC(G/C)GG was derived as a NUDR-binding motif. Interestingly, the sequence TTCGG is found between the RARE half-sites in hRARβ2 (50), mRARβ2 (51), and the RA-responsive *mHoxa-1* (52) and may contribute to the higher levels of NUDR-induced CAT activity from pRARECAT6 over pEnk77CAT6. However, NUDR may also bind to some DNAs with low affinity and/or recognize additional sequences, since NUDR was able to bind the DR2 RARE (Fig. 8B), which lacks a TTCGG sequence, and because moderate levels of nonspecific competitor DNA readily displaced NUDR binding at the RAREs. NUDR binding of DNA is not synonymous with transactivation since constructs containing one and two copies of a RARE, NBS, or the NBS consensus were unable to confer NUDR-dependent activation to the thymidine kinase promoter in CAT assays. If NUDR DNA-binding activity exists to promote transactivation, then these studies indicate that the promoter context and/or the presence of multiple binding motifs of specific spacing may be important for NUDR transcriptional activation of target genes. However, as already noted, we have been unable to demonstrate high-affinity binding of NUDR to any proenkephalin sequences, which implies that NUDR may regulate proenkephalin transcription through mechanisms other than DNA binding, such as protein-protein interaction.

The TTCG motif has been identified as the core binding sequence of DEAF-1 and is found in region 5–6 of the *Dfd* promoter (21). Multiple copies of the motif and/or mutations that improved DEAF-1 binding *in vitro* generally increased the expression of the corresponding transgene in *Drosophila* embryos (21). Since the *Dfd* binding site and region 5–6 were required for segment- specific expression of *Dfd* in the embryo (20), DEAF-1 or a similar protein was postulated to be a cofactor of the homeodomain protein *Dfd* and required for *Dfd* expression (21). DEAF-1 is one of a growing number of nonhomeodomain proteins that have been identified as cofactors of homeodomain proteins. Two models have been proposed in which protein cofactors may interact with Hox and homeodomain-containing proteins to achieve greater target gene binding specificity. The coselective binding model envisions cofactors selectively targeting homeodomain proteins to different DNA sites, and the widespread binding model envisions cofactors altering the activity of homeodomain proteins that are already bound to the DNA (3). The close proximity of the binding sites for DEAF-1 and the homeodomain protein Dfd in the *Dfd* promoter would suggest a likely interaction among these proteins. However, Gross and McGinnis indicated that, at least in mobility shift assays, they had failed to detect cooperative interaction between DEAF-1 and Dfd and postulated that additional factor(s) may be required to form an activating transcription complex (21). The demonstration that DEAF-1 was also able to bind multiple regions in the promoter of human *HOXD4* gene, a vertebrate homolog of *Dfd* (21), implies the existence of a mammalian counterpart of DEAF-1. The expression of NUDR during mouse fetal development and the strong sequence and functional similarities between NUDR and DEAF-1 would indicate that NUDR is a potential vertebrate homolog of DEAF-1 and, by analogy, may serve as a potential cofactor to homeodomain proteins and regulate the expression of the paralogous group 4 *Hox* genes and other downstream target genes. Future investigations will focus on the possible mediating role of NUDR in these developmental processes.

MATERIALS AND METHODS

Cloning of Monkey and Human NUDR cDNAs

A partial monkey NUDR cDNA was identified during expression screening of a CV-1 cell cDNA library (cDNA library 936111 in λ ZAP II vector, Stratagene, La Jolla, CA). Protein replica filters were prepared as described (53): 7×10^5 pfu were plated (20 $\times$ 150-mm plates) on a lawn of *E. coli* strain XL-1 Blue MRF′. Plates were incubated 3.5 h at 37 C until pinpoint size plaques appeared and then overlayed with nitrocellulose filters that had been soaked in 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The filters were incubated at 37 C for 5 h to induce β-galactosidase-fusion protein production before being placed in blocking solution [25 mM KCl, 25 mM HEPES (pH 7.3), 0.05% Triton X-100, 2% nonfat milk, 1 mM dithiothreitol (DTT)] at 4 C. The filters were then screened for proteins capable of binding to the DR5 RARE found in the human RARβ promoter (25). The oligo 5′-GATC-C**AGGGTTCACCGAAAGTTCA**CTG-3′ was hybridized with the complementary oligo 5′-GATCCAG**TGAACTTTCGGTGAACCCT**G-3′ (the RARE-containing sequence is shown in bold, with flanking *Bam*HI sites) and 50 pmol of this sequence were radiolabeled by a fill-in reaction with T4 DNA polymerase and ^{32}P-α-dATP (ICN Pharmaceuticals, Costa Mesa, CA), followed by phosphorylation and ligation of the DNA to form multimers. The radiolabeled probe was added to the filters in 75 ml of binding buffer (blocking buffer with nonfat milk reduced to 0.25%, and 10 μg/ml salmon sperm DNA) and incubated overnight with shaking at 4 C. The filters were washed over a 30-min period in 2 $\times$ 250 ml binding buffer with salmon sperm DNA and 2 $\times$ 500 ml binding buffer without salmon sperm DNA, and then exposed to x-ray film overnight. Initial screening with the RARE sequence showed

two positive plaques, but only one continued to show binding through tertiary screening. After excision from the λ ZAP II vector and *Eco*RI digestion, the pBluescript plasmid was found to contain a 1.6-kb insert, which, upon further sequence analysis, was identified as a partial clone (sNUDR1.6).

A human choriocarcinoma cell line (JEG-3) cDNA library was constructed from cDNA synthesized from JEG-3 mRNA primed with oligo-dT using the SuperScript Choice System (Life Technologies, Gaithersburg, MD). The cDNA was ligated into λ Zap II and packaged with Gigapack III Gold packaging extracts (Stratagene). The JEG-3 cDNA library contained 3×10^6 independent clones.

A 1.5-kb *Eco*RI/*Sma*I fragment of the monkey NUDR cDNA (sNUDR1.6) was radiolabeled by random priming and used to rescreen the CV-1 cDNA library (10^6 clones) and the JEG-3 cDNA library (10^6 clones) by hybridization. DNA from the plaques was lifted onto nitrocellulose filters and hybridized to the radiolabeled probe in 5 × SSPE (750 mM NaCl, 50 mM sodium phosphate, 5 mM EDTA), 0.5% SDS, 10 μg/ml salmon sperm DNA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, and 50% formamide, overnight with shaking at 50 C. Filters were washed in 2 × SSPE for 45 min at 50 C, dried, and exposed to x-ray film to identify hybridizing clones. A monkey clone (2405 bp, sNUDR) and two human clones (2065 bp, hNUDR; 2328 bp, hNUDR8) were isolated and sequenced using Thermo Sequenase cycle sequencing kit (Amersham Corp., Arlington Heights, IL). Sequence comparison of the 1.6-kb and 2.4-kb monkey clones revealed a single nucleotide difference in the coding region that resulted in the substitution of an aspartic acid in the shorter clone relative to the asparagine (codon 287) in sNUDR.

A Sprague Dawley rat testicular germ cell library was constructed from cDNA synthesized in a similar manner to the JEG-3 cDNA library and contained 1×10^6 independent clones. Oligonucleotide primers corresponding to position 1227–1245 and 1677–1660 of rat suppressin (accession no. U59659) were used to amplify a 434-bp DNA fragment from rat testis cDNA, which was cloned into pBSKS and sequenced to confirm the identity. The DNA fragment was radiolabeled and used to screen the germ cell library for full-length rat NUDR clones using conditions similar to those stated above.

Bacterial Expression Plasmids and Recombinant Protein Production

The cDNAs for sNUDR and hNUDR were subcloned into the pET-16b vector (Novagen, Inc. Madison, WI) for production of recombinant proteins in bacteria. The cDNA fragments containing sNUDR and hNUDR were excised from pBSSK by *Bsp*EI and *Eco*RI digestion, followed by T4 DNA polymerase fill-in, ligation of *Xho*I linkers, and digestion with *Xho*I. The 2.0-kb *Xho*I fragments were subcloned into the *Xho*I-digested pET-16b vector. DNA sequencing was used to confirm the correct insertion of the cDNAs in the vector. Resulting fusion proteins have an amino-terminal extension of 10 histidines followed by a factor Xa cleavage site.

hNUDR and sNUDR in pET-16b plasmids were introduced into *E. coli* strain BL21(DE3), and the expression of His-Tag-hNUDR and His-Tag-sNUDR proteins was induced by the addition of 1 mM IPTG during the last hour of bacterial growth. Bacterial pellets were sonicated in 5 ml of buffer A [6 M guanidine HCl, 0.1% IGEPAL CA-630 (Sigma, St. Louis, MO), 100 mM KCl, 20 mM Tris (pH 8.0)] and shaken for 1 h to solubilize proteins. Insoluble material was removed by centrifugation at 15,000 × *g* at 15 C for 20 min, and the supernatant was loaded onto a column containing 1 ml of His-Bind metal chelation resin (Novagen). The column was washed with buffers and recombinant His-Tag proteins were eluted from the column with buffer D [8 M urea, 100 mM KCl, 20 mM Tris (pH 6.8), 500 mM imidazole]. Proteins were renatured by five successive rounds of dialysis at 4 C in buffers that reduced the urea and increased glycerol to a final buffer of 15 mM Tris (pH 7.5), 50 mM KCl, 50% glycerol, 10 μM $ZnCl_2$, and 1 mM DTT.

A GST-sNUDR (1.5) fusion protein was produced in bacteria from a plasmid constructed by ligation of a 1.5-kb *Sma*I fragment of sNUDR1.6 into the *Sma*I site of pGEX-2T (Pharmacia Biotech, Piscataway, NJ). The expression of the GST-sNUDR1.5 fusion protein in *E. coli* strain CAG 748 (New England BioLabs, Beverly, MA) was induced by the addition of IPTG, and the recombinant protein was purified by affinity chromatography on glutathione-agarose.

hRARα was excised from the plasmid pGEMhRARα (kindly provided by Dr. R. Evans, Salk Institute) by *Msc*I digestion, followed by ligation of *Bam*HI linkers, digestion with *Bam*HI, and ligation of the DNA fragment into the *Bam*HI site of pGEX-2T. GST-hRARα fusion protein was purified by affinity chromatography and treated with thrombin to cleave the GST moiety from hRARα immediately before use in the EMSA.

Purified recombinant proteins and BSA protein standards were separated on SDS-PAGE, stained with Coomassie blue, and scanned with a Densitometer SI (Molecular Dynamics, Sunnyvale, CA) to determine protein concentrations.

In vitro transcription/translation was used to produce recombinant proteins using the TNT Coupled Reticulocyte Lysate System (Promega, Madison, WI) and 1 μg of the plasmids, sNUDR in pBSSK, hNUDR in pBSSK, and hRXRα (the cDNA equivalent to position 76–1866 in Ref. 54) in pBSKS, according to the supplied instructions.

Mammalian Expression Plasmids

The cDNAs for NUDR were subcloned into pCMVNeo to obtain high levels of expression from the human cytomegalovirus immediate early gene promoter (CMV). The cDNAs for hNUDR, sNUDR, rNUDR, and hNUDR8 were excised from pBSSK by *Eco*RI digestion, followed by T4 DNA polymerase fill-in, ligation of *Bam*HI linkers, and digestion with *Bam*HI. The *Bam*HI fragments were subcloned into the *Bgl*II site of eukaryotic expression vector pCMVNeo (55). Sequencing pCMVhNUDR, pCMVsNUDR, pCMVrNUDR, and pCMVhNUDR8 confirmed that the orientation of the 5′-end of the cDNAs was adjacent to the CMV promoter.

To examine subcellular localization of hNUDR by a nonimmunological method, hNUDR was also subcloned into pEGFP-N3 vector (CLONTECH, Palo Alto, CA) to produce a fusion protein with GFP at the amino terminus. Expression is under the control of the CMV promoter. The cDNA fragment containing hNUDR was excised from pBSSK by *Bsp*EI and *Bsu*36I digestion, followed by Klenow fill-in, ligation of *Bam*HI linkers (10 mers, New England BioLabs), and digestion with *Xho*I. The 2.0-kb *Xho*I fragments were subcloned into *Bam*HI-digested pEGFP-N3. Sequencing pEGFP-hNUDR confirmed the 5′-end of the cDNA was in the same reading frame as GFP.

Site-directed mutagenesis was used to produce *in vitro* mutations in the NLS of NUDR using a megaprimer PCR method (56). To generate the mutant megaprimers, 20 cycles of amplification were performed using the 2.0-kb *Eco*RI fragment of NUDR as the template, a reverse primer (TGATAGCCGGGATAGTGAG), and either the mutant R302T primer (GTGCCTTACAAAACGCGC) to change Arg 302 (AGG) codon to a Thr (ACG) or the mutant K304T primer (GCGCACGAAGGAGAATG), which changes the codon for Lys 304 (AAG) to a Thr (ACG). The resultant 289-bp and 302-bp DNA products were gel isolated and used as mutant megaprimers in the second round of amplification using the same template and a forward primer (TTAAACCCTCACGCTGCCTC). The PCR products were gel isolated and digested with *Afl*II and *Aat*II, and the fragments containing the mutations were substituted for the corresponding region in hNUDR in pBSSK. The double mutation R302T/K304T was generated using the R302T mega primer, the reverse primer, and a 2.0-kb *Eco*RI fragment of hNUDR with the K304T mutation as the template.

DNA sequencing confirmed the presence of each of the three mutations before the subcloning of the 2.0-kb *Eco*RI fragments into the pCMVNeo vector as above. Oligonucleotides were synthesized by Operon Technologies, Inc. (Alameda, CA).

Reporter Constructs

Previously (23), we had constructed a human basal proenkephalin reporter plasmid (ENK84CAT) that contained 84 bp of 5′-flanking sequence, 69 bp of exon I, 86 bp of intron A, and 55 bp of exon II, fused to the CAT gene and followed by 1081 bp of proenkephalin 3′-flanking sequence. This reporter plasmid displayed minimal induction by cAMP, the PKA catalytic subunit, and RA. Two copies of a DR5 RARE sequence were inserted into a *Bam*HI site 5′ to position −84 of the proenkephalin promoter, producing the plasmid RARECAT, which was then shown to be RA responsive (23). To remove a potentially problematic AP2 consensus sequence, these plasmids were digested with *Pst*I and *Nae*I, filled in with Klenow, and religated, resulting in plasmids that eliminated the AP2 site and reduced proenkephalin 5′-flanking sequence to position −77 to 213 (transcription start site at position +1 as defined by sequence accession no. J00122). To further reduce basal reporter activity, the regions defined above were removed as *Bgl*II/*Hin*dIII (blunt) cassettes from their parent pSP73 vectors and substituted into the *Bgl*II/*Sma*I region of pBLCAT6 (accession no. M80484). The resulting plasmids were termed pEnk77CAT6 and pRARECAT6, respectively. The plasmid pBLCAT5 (accession no. M80483) contains a 169-bp region of the thymidine kinase promoter inserted into pBLCAT6. The plasmid pRARECAT5 was constructed by placement of a *Bgl*II/*Sal*I fragment of RARECAT6 (containing two RARE motifs) into the *Bam*HI/*Sal*I site of pBLCAT5. The plasmid pDynCAT3 was constructed by placement of a 1.9-kb *Hin*dIII/*Nhe*I fragment of the rat prodynorphin promoter (57) into the *Hin*dIII/*Xba*I site of pBLCAT3 (accession no. X64409). The reporter plasmids, pEnk77CAT6Δ65–153 and pEnk77CAT6Δ65–213, are equivalent to deletion of bases 65–153 and 65–213 of the proenkephalin sequences (accession no. J00122) in pEnk77CAT6, respectively. The plasmids, pEnk77CAT6Δ3′-UTR and pEnk77CAT6Δ65–213,Δ3′UTR, are equivalent to the deletion of 1081 bases of enkephalin's 3′-UTR (position 1985–3065, accession no. K00489) from pEnk77CAT6 and pEnk77CAT6Δ65–213, respectively. All plasmid constructs were confirmed by DNA sequencing. pNBScons2.11CAT5 and pNBScons2.12CAT5 contain a single copy of the NBS consensus sequence shown in Fig. 14E but in opposite orientations, and pNBScons2.8CAT5 contains two copies of the NBS consensus sequence as shown in Fig. 14F. The plasmids, pBLCAT3, pBLCAT5, and pBLCAT6 (58), were kindly provided by Gunther Schutz, German Cancer Research Center, Heidelberg.

Selection of NUDR-Binding Sequences

GST-sNUDR (GST-sNUDR1.5) fusion protein was immobilized on glutathione-agarose beads and used to select DNA sequences with affinity to NUDR from a random set of double-stranded oligonucleotides (1×10^{18} potential sequences) that contained 30 random bases and were flanked on each end by different primer-specific sequences following the method described by Lu *et al.* (46). After six rounds of NUDR-affinity selection and PCR amplification, the oligonucleotides called NBSs were kinased, ligated into the *Bam*HI site of pBLCAT5, and sequenced. A final round of selection was performed using EMSA, and the eNBSs were isolated from the gel-shifted NUDR/DNA complex and cloned into pBLCAT5, as above.

RNA Isolation and Northern Blot Analysis

Total RNA from CV-1 cells and rat tissues was isolated using TRIzol reagent per supplier's instructions (Life Technologies) with subsequent isolation of poly A+ RNA by oligo-dT chromatography. Ten micrograms of total RNA or poly A+ RNA were separated on 1.0% agarose gels containing 6% formaldehyde, 20 mM HEPES (pH 7.8), and 1 mM EDTA and then transferred to nylon membranes. A 1.37-kb *Eco*RI/*Sma*I cDNA fragment of the 1.6- kb monkey clone was radiolabeled by random priming and used to probe the membrane containing the CV-1 RNAs. A DNA fragment of rNUDR (corresponding to position 1481–1931) was radiolabeled by random priming and used to probe a membrane containing the rat RNAs. After overnight hybridization in 400 mM sodium phosphate (pH 7.2), 5% SDS, 1 mM EDTA, 1 mg/ml BSA, and 50% formamide at 62 C, the membranes were washed in 0.1 × SET (1 mM Tris, pH 7.5, 0.5 mM EDTA, 0.1% SDS) at 70 C and autoradiographed. X-ray films were scanned and digitized with a Densitometer SI (Molecular Dynamics).

Antibodies

Antibodies to the full-length recombinant hNUDR and to the MAP-conjugated peptide VKKDSPKNITLLPAT (amino acids 314–328 of hNUDR) were produced in rabbits by Research Genetics, Inc. (Huntsville, AL).

In Vivo Labeling and Immunoprecipitation

HeLa and CV-1 cells were plated at a density of 300,000 cells on 6-cm plates in DMEM supplemented with 5% FBS and transfected with 5 μg CMVsNUDR per plate by calcium phosphate precipitation (59). Five hours after glycerol shock, the culture medium was replaced with 1 ml of F-12 medium deficient in methionine and cysteine plus 150 μCi ^{35}S translabel (ICN). The cells were cultured for 4 h in the labeling media before collection of the media for immunoprecipitation of secreted proteins. The cells were scraped in 1 ml of immunoprecipitation buffer [0.5% Triton X-100, 0.5% deoxycholate, 150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, and Complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN)] and lysed by freeze-thaw. Particulates were removed from cell extracts and media by centrifugation for 10 min at 30,000 × *g*. Aliquots (250 μl) of the cell extracts and media were mixed with 400 μl of immunoprecipitation buffer plus 2 μl immune serum (antibody to full-length NUDR) or preimmune serum and incubated overnight with shaking at 4 C. Ten microliters of protein A agarose beads (Life Technologies) were added and incubated for 1 h at 25 C. The immune complexes/beads were washed with 4 × 0.5 ml immunoprecipitation buffer at 25 C to remove nonspecific binding, boiled in SDS loading buffer for 10 min, and subjected to SDS-PAGE analysis. The gel was dried and imaged with a 445 SI PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Image analysis indicated that 85% of the *in vitro* translated NUDR (Fig. 6, lane 1) was immunoprecipitated (Fig. 6, lane 3).

Total Protein Preparation and Western Blot Analysis

Testicular cell fractions were either isolated directly or using short-term primary culture, as previously described (60). Frozen cell pellets were resuspended in 300 μl (50 mM HEPES (pH 7.3), 0.4 M NaCl, 2 mM DTT, 20% glycerol, and Complete protease inhibitor cocktail). Homogenization was performed by 30 strokes in a Dounce homogenizer with A-sized pestle and one freeze-thaw cycle on dry ice. The homogenate was centrifuged for 1 h at 30,000 × *g* at 4 C to obtain the soluble protein fraction. Frozen rat tissues were homogenized in 50 mM Tris (pH 7.4), 1% IGEPAL CA-630, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsul-

fonyl fluoride, 1 mM NaF, and 1 μg/ml of aprotinin, leupeptin, and bacitracin. Mouse embryos were homogenized in 8 M urea, 50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and 0.1% Tween 20. Protein concentrations of cell and tissue homogenates were determined from a protein standard curve using a BCA protein assay (Pierce, Rockford, IL). Total proteins were separated on a 10% SDS denaturing gel and electrophoretically transferred to a nitrocellulose membrane. The membrane blot was blocked with 1% BSA and 5% nonfat dry milk in 200 ml TBS (25 mM Tris, pH 7.4, 137 mM NaCl, 2.7 mM KCl) with shaking for 1–2 h, incubated with NUDR polyclonal antibody (diluted 1:5000) in TBS and 2% nonfat dry milk for 1–2 h, washed, incubated with a 1:10,000 dilution of goat antirabbit second antibody linked to horseradish peroxidase (Vector Labs, Burlingame, CA) in TBS and 2% nonfat milk for 1 h, washed, and detected using Super Signal ULTRA substrate (Pierce). All incubations were performed at 25 C, and washes consisted of 3 × 200 ml volumes of TBS and 0.01%–0.05% Tween 20 for 15 min. A detection limit of approximately 1 ng NUDR protein was achieved with the polyclonal antiserum, while the detection limit of 20 ng was achieved with the peptide antibody. Blots were exposed to x-ray film and the films were scanned with the Densitometer SI.

Electrophoretic Mobility Shifts

Recombinant hNUDR protein (35 pmol) was incubated on ice with either poly dI-dC, poly dA-dT, or salmon sperm DNA (0–2 μg) as nonspecific competitor in a 20-μl reaction containing 50 mM KCl, 20 mM HEPES (pH 7.3), 2 mM DTT, 5% glycerol, and 0.1% Triton X-100. After 15 min of incubation on ice, 120–240 fmol of ^{32}P-labeled oligonucleotide probe were added and incubated an additional 20 min at 25 C. Probes were labeled by Klenow fill-in reactions of double-stranded oligonucleotides formed by hybridization of complementary oligos for the DR5 RARE (sequences shown in the cloning section) and the DR2 RARE [5′GATCC**GATAGGTCAAA-AGGTCAGA**G-3′ and 5′-GATCC**TCTGACCTTTTGACCT-AC**G-3′ from mCRBP-I (61)]rsqb]; or an *Eco*RI and *Hpa*I DNA fragment excised from pNBScons2.11CAT5 for the NBS consensus probes (consensus sequence shown in Fig. 14E). In the competition experiment with RAR/RXR, 240 fmol of radiolabeled DR5 or DR2 oligo were incubated with 38 pmol of hNUDR and/or 1 pmol of hRARα plus 2 μl of hRXRα produced in a TNT reaction (Promega). Protein-DNA complexes were separated from free probe on a 4% nondenaturing acrylamide gel (acrylamide:bis, 40:0.8, in 80 mM Tris, pH 8.0, 1 mM EDTA) at 40 mA for 4 h. Results were imaged with a 445 SI PhosphorImager.

DNase I Protection Assay

DNA fragments containing the NBS consensus sequence (one or two copies) were radiolabeled by fill-in using Klenow and α-^{32}P-dNTP. After phenol/chloroform extraction and two ethanol precipitations, the radiolabeled probe was resuspended in 50 mM KCl, 20 mM Tris (pH 7.5) at 2000 cpm/μl. Recombinant His-Tag-hNUDR protein (40–120 pmol) was mixed with 0.5 μg poly dI/dC in binding buffer, (20 mM HEPES, pH 7.3, 75 mM KCl, 2 mM DTT, 7.5% glycerol, 0.1% Triton-X-100) in a final volume of 25 μl and incubated on ice for 15 min. The radiolabeled DNA probe (5 μl) was added and incubated at 25 C for 10–15 min before the addition of 3 μl of diluted (1:75) RQ1 DNase I (Promega) and $MgCl_2/CaCl_2$ to a final concentration of 5 mM. After digestion for 90 sec at 25 C, the reaction was terminated by the addition of 2 × STOP (0.2 M NaCl, 20 mM EDTA, 0.5% SDS, 0.25 mg/ml salmon DNA). Samples were phenol/chloroform extracted and ethanol precipitated before separation on 6% sequencing gels.

Cell Culture and Transfection

Cells were maintained in DMEM supplemented with 5% FBS (Summit Biotechnologies, Fort Collins, CO) in a humidified chamber maintained at 37 C and 5% CO_2. CV-1 cells were plated at a density of 300,000 cells per 60-mm dish and transfected with plasmid DNA by calcium phosphate precipitation (59), glycerol shocked, and harvested 36–40 h after the precipitates were placed on the cells (55). In all experiments, triplicate plates of cells were cotransfected with 2 μg of the indicated reporter plasmid; 5 μg of the internal control plasmid SV2βgal; 1 μg of expression plasmid driven by the CMV promoter [CMVNeo (control), human RARα (hRARα), monkey NUDR (sNUDR), human NUDR (hNUDR), hNUDR with a 14 amino acid deletion (hNUDR8), nuclear localization mutations (hNUDR-R302T, hNUDR-K304T, and hNUDR-R302T/K304T)]; and additional plasmid DNA to maintain the total amount of transfected DNA at 17 μg. For some experiments, cells were treated with 1 μM RA for the last 24 h before harvest.

CAT Assay

Cell extracts were prepared by sonication in 200 μl of homogenization buffer (10 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM DTT, and 250 mM sucrose). CAT activity in 25 μl of extract was assayed after incubation for 3 h at 37 C (62), and β-galactosidase activity in 25 μl of extract was measured, as previously described (63). CAT activity is the counts per min in the acetylated products, normalized to the β-galactosidase activity in each extract. In addition, the data are expressed as "fold induction" which is the ratio of CAT activity produced by a reporter plasmid cotransfected with a given expression vector, divided by the CAT activity produced by the reporter plasmid alone.

Localization of Proteins by Intrinsic Fluorescence and Immunofluorescence

Antibodies were used to detect endogenous NUDR expression in CV-1 cells or overexpressed NUDR in transfected CV-1 cells. For endogenous NUDR expression, cells were plated onto slides and cultured overnight before fixation and treatment as described below. For overexpressed NUDR, cells were plated at a density of 65,000 per well in a Lab-Tek II chamber slide (Nalge Nunc International, Naperville, IL) and transfected by calcium phosphate precipitation of 1.5 μg of plasmid DNA (CMV promoter driving the expression of the cDNAs for hNUDR, hNUDR-R302T/K304T, GFP, or GFP-hNUDR). The cells were rinsed with PBS, fixed for 15 min in 4% paraformaldehyde, rinsed with PBS, permeablized for 7 min in 100% methanol at −20 C, rinsed with PBS, and then incubated at 25 C in normal goat serum (Vector Laboratories) for 20 min. The normal goat serum was removed and the slides were incubated with 1:1000 dilution of either preimmune rabbit serum or anti-NUDR serum overnight at 4 C. The slides were rinsed with PBS, incubated for 1 h at 25 C with either a 1:100 dilution of goat antirabbit antibody conjugated to fluorescein isothiocyanate (Sigma, St. Louis, MO) or a biotinylated goat antirabbit second antibody and fluorescein-conjugated avidin, and rinsed with PBS, after which coverslips were mounted on the slides using VECTASHIELD Mounting Medium (Vector Laboratories). For endogenous NUDR, indirect immunofluorescence was observed using fluorescein filters with an Olympus IMT-2 microscope. For overexpressed NUDR, fluorescent images were obtained from an Olympus Fluoview Confocal Imaging System attached to an Olympus IX-70 inverted microscope.

Experimental Animals

Use of animals in this study was conducted in accordance with the principles and procedures outlined in "Guidelines for Care and Use of Experimental Animals" and also in accordance with protocols issued by the Southern Illinois University Animal Care Committee.

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