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Nuclear DEAF-1-related (NUDR) Protein Contains a Novel DNA Binding Domain and Represses Transcription of the Heterogeneous Nuclear Ribonucleoprotein A2/B1 Promoter*

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Nuclear DEAF-1-related (NUDR) protein is a novel transcriptional regulator with sequence similarity to developmental and oncogenic proteins. NUDR protein deletions were used to localize the DNA binding domain between amino acids 167 and 368, and site-specific DNA photocross-linking indicated at least two sites of protein-DNA contact within this domain. The DNA binding domain contains a proline-rich region and a region with similarity to a Myc-type helix-loop-helix domain but does not include the zinc finger motif at the C terminus. Deoxyribonuclease I protection assays confirmed the presence of multiple NUDR binding motifs (TTC(C/G)G) in the heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) promoter and also in the 5′-untranslated region (UTR) of hNUDR cDNA. NUDR produced a 65–70% repression of the hnRNP A2/B1 promoter activity, and NUDR binding motifs in the 5′-UTR were found to mediate this repression. NUDR-dependent repression was also observed when the 5′-UTR of NUDR was placed onto a heterologous thymidine kinase promoter in an analogous 5′-UTR position but not when placed upstream of transcription initiation. These results suggest that NUDR may regulate the *in vivo* expression of hnRNP A2/B1 and NUDR genes and imply that inactivation of NUDR could contribute to the overexpression of hnRNP A2/B1 observed in some human cancers.

NUDR[1] is a transcriptional regulatory protein that was initially identified in a monkey kidney cell (CV-1) cDNA library through protein expression and binding to a radiolabeled retinoic acid response element (RARE) based on the sequence in human retinoic acid receptor β2 gene (1). The encoded protein had 46% overall amino acid similarity to *Drosophila* Deformed epidermal autoregulatory factor-1 (DEAF-1) (2) and was therefore named nuclear DEAF-1 related (NUDR) (1). DEAF-1 has been shown to bind to TTCG-containing motifs located adjacent to DNA binding sites for the Deformed homeodomain protein that occur in the promoter regions of *Deformed* and other Deformed-regulated genes, indicating that DEAF-1 may act as a transcriptional cofactor of Deformed (2). NUDR was also shown to recognize TTCG-containing motifs (1), and the combination of sequence and functional similarities suggests that NUDR may be the mammalian homolog of DEAF-1.

In our previous report, NUDR was shown to transcriptionally activate a minimal proenkephalin promoter, and activation was increased by the addition of synthetic RAREs placed 5′ of the promoter (1). Because we were unable to demonstrate NUDR binding to proenkephalin sequences in either DNase I protection assays or mobility shift assays, we concluded that the activation of the proenkephalin promoter was likely to occur through protein-protein interactions (1).

Using a yeast two hybrid system, Sugihara *et al.* (3) identified the mouse homolog of NUDR (called mDEAF-1) through interaction with LMO-4, a new member of the LIM-only (LMO) family. LMOs contain two tandem repeats of the LIM zinc finger domain, which can associate tightly with another family of cofactors called Clims (also referred to as Ldb or NLI) to activate (4) or inhibit (5) transcription. Since LMO and Clim complexes have not been demonstrated to bind directly to DNA, they have been postulated to regulate transcription through the recruitment of DNA-binding proteins and the assembly of transcriptional complexes (3, 6, 7). Mouse NUDR was shown to interact with LMO-4, LMO-2, and Clim-2 in both *in vitro* and *in vivo* assays, and it was proposed that NUDR could provide the critical DNA binding function to LMO-Clim complexes (3).

Because NUDR showed only moderate affinity for the RARE sequence, higher affinity sequences were selected from a library of random oligonucleotides through binding to recombinant NUDR protein and amplification by PCR (1). Analysis of the selected sequences revealed the presence of one or more copies of TTCG and/or TTTCCG, and multiple sequence alignment suggested a NUDR binding consensus sequence of TTCGGGNNTTTCCGG (1). Comparison of the NUDR binding motifs and the RARE sequence suggested that the original identification of NUDR was likely through the fortuitous binding of NUDR protein to the TTCGG sequence found between the RARE half-sites. The similarity in DNA recognition sequences between NUDR and *Drosophila* DEAF-1 implied that

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s)AF049459 (human NUDR cDNA), AF049461 (African green monkey NUDR cDNA), AF049460 (human NUDR8 cDNA), and D28877 (HNRPA2B1, human hnRNP A2/B1 gene).

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[1] The abbreviations used are: NUDR, nuclear DEAF-1-related; hNUDR, human NUDR; sNUDR, monkey NUDR; DEAF-1, Deformed epidermal autoregulatory factor-1; hnRNP A2/B1, heterogeneous nuclear ribonucleoprotein A2/B1; 5′-UTR_{DNA}, cDNA region corresponding to the 5′-untranslated region; RARE, retinoic acid response element; LMO, LIM-only; PCR, polymerase chain reaction; NLS, nuclear localization signal; TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; and HLH, helix-loop-helix; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); CMV, cytomegalovirus; AB-dUTP, 5-[*N*-(4-azidobenzoyl)-3-aminoallyl]-deoxyuridine triphosphate.

the DNA binding domain may be in a region of greater amino acid homology between the proteins, namely, the zinc finger homology region at the C terminus (56% similarity) and/or the nuclear domain (1)/KDWK domain (2) located in the central region of the proteins (70% similarity). The distantly related zinc finger region of the progesterone receptor has been shown to be involved directly in the DNA binding domain, whereas the more homologous zinc finger region of MTG8 (ETO) has recently been shown to be involved in protein-protein interaction and the recruitment of nuclear corepressors and histone deacetylases (8, 9). The nuclear/KDWK domains of NUDR and DEAF-1 have similarity with proteins from the SP100 family (1, 10). SP100 proteins are localized to subnuclear structures termed "nuclear bodies" and are thought to play a role in the etiology of acute promyelocytic leukemia (reviewed in Ref. 11). Recently it was demonstrated that SP100B associates with non-histone chromatin components that behave as transcriptional silencers, and when fused to a GAL4 DNA binding domain, SP100B can repress transcription (12, 13).

In this report, we identify the DNA binding domain in the central region of NUDR that includes the nuclear/KDWK domain and a Myc-type helix-loop-helix structure, and we demonstrate that there are at least two sites of protein contact with the DNA. The *hnRNP A2/B1* gene, a potential early biomarker of lung cancer (14–17), is identified as a potential target gene of NUDR regulation by the presence of a NUDR binding consensus sequence within the promoter region. We show that NUDR represses transcription of the hnRNP A2/B1 promoter through a DNA binding-dependent mechanism and that NUDR binding motifs within the 5′-UTR are involved in this regulation. We hypothesize that elevated levels of hnRNP A2/B1 found in some cancers may be a consequence of the inactivation or deregulation of NUDR.

EXPERIMENTAL PROCEDURES

Construction of Bacterial Expression Plasmids—The bacterial expression and purification of recombinant proteins for full-length human NUDR (hNUDR) and monkey NUDR (sNUDR) have been described previously (1). To facilitate the construction of various deletion proteins and peptides, sNUDR was used to derive the peptide constructs G, H, I, J, K, and L, whereas hNUDR was used to derive all other constructs. The full-length proteins of sNUDR and hNUDR differ by only five amino acids and have virtually indistinguishable binding characteristics. For the deletion constructs B, C, D, and E, cDNA fragments of hNUDR were excised from the parent vector pBSSK (Stratagene, La Jolla, CA) with *Bsp*EI and *Sph*I (B), *Bsp*EI and *Hin*cII (C), *Bsp*EI and *Aat*II (D), and *Xcm*I and *Eco*RI (E) followed by T4 DNA polymerase fill-in, ligation of *Bam*HI linkers, and *Bam*HI digestion. The resulting DNA fragments were ligated into the *Bam*HI-digested pET-16b vector (Novagen, Inc. Madison, WI) for production of N-terminal histidine-tagged proteins. For the internal deletion construct F, the cDNA in pBSSK was digested with *Nco*I and *Afl*II, filled in with T4 DNA polymerase, and religated. For the internal deletion construct G, a portion of the cDNA was excised with *Eco*NI and *Aat*II and replaced with an SV40 nuclear localization signal (18) formed by hybridization of the following two oligonucleotides: 5′-cCCAAAAAAGAAGAGAAAGGTAgacgt-3′ and 5′-cTACCTTTCTCTTCTTTTTTGGgct-3′, with the lowercase letters denoting *Bsm*I and *Aat*II cohesive ends. The cDNAs for constructs F and G were excised with *Bsp*EI and *Eco*RI and treated as described above to add *Bam*HI linkers and then subcloned into the pET-16b vector. Recombinant histidine-tagged fusion proteins were purified as described previously for the full-length proteins except that the pH of the renaturation buffer was changed from 8.0 to 9.1 to adjust for differences in the isoelectric points of the deletion proteins.

For construction of the glutathione *S*-transferase (GST) fusion peptides H, I, J, K, and L, cDNA fragments of sNUDR were excised from the parent plasmid with *Eco*NI and *Aat*II (H), *Nco*I and *Aat*II (I), *Apa*I and *Aat*II (J), *Nco*I and *Afl*II (K), and *Apa*I and *Afl*II (L), treated as described above to add *Bam*HI linkers, and subcloned into the *Bam*HI site of pGEX-2T (Amersham Pharmacia Biotech). Recombinant GST fusion proteins were purified as described previously for GST-sNUDR (1). To determine the concentration of each protein preparation, the recombinant proteins were subjected to SDS-PAGE, stained with Coomassie Blue, and compared with a bovine serum albumin standard curve using a Densitometer SI (Molecular Dynamics, Sunnyvale, CA).

Electrophoretic Mobility Shift Assays—Recombinant proteins were incubated on ice with either nonspecific or specific oligonucleotide competitors (as indicated) in a 20-μl reaction containing 500 ng of poly(dI-dC), 100 mM KCl, 20 mM HEPES (pH 8.1), 2 mM dithiothreitol, 7% glycerol, and 0.05% Tween 20. The glucocorticoid response element (GRE) oligonucleotide used for nonspecific competitor (Fig. 2*B*) was formed by hybridization of two synthetic oligonucleotides, 5′-TCGACTGTACAGGATGTTCTAGCTACT-3′ and 5′-TCGAAGTAGCTAGAACATCCTGTACAG-3′ (19), and the N42–78 oligonucleotide was formed by hybridization of 5′-cgggatccTTCGGACTGATTCGGCTTCCCACTTCG-3′ and 5′-cgggatccCGAAGTTCCCCGAAGTGGGAAGCCGAA-3′. The lowercase letters denote *Bam*HI restriction sites used for subcloning. Radioactive oligonucleotide probes were produced by fill-in reactions with Klenow and [α-^{32}P]dATP. After 15 min, the reactions were mixed with 120–240 fmol of ^{32}P-labeled probe and incubated an additional 15 min at 25 °C. Protein-DNA complexes were separated on 4% nondenaturing polyacrylamide gels (acrylamide:bis, 40:0.8, in 1× Tris-borate EDTA) at 120 volts for 3 h, and results were imaged using a 445 SI PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

DNase I Protection Assays—The following DNA fragments were isolated and radiolabeled by fill-in reaction with [α-^{32}P]dATP and Klenow DNA polymerase: *Eco*RI/*Bsp*MI fragment of hNUDR8 cDNA (Fig. 2*A*), *Eco*RI/*Hpa*I fragment of N42–78 that was inserted in the *Bam*HI site of pBLCAT5 (Fig. 2*C*), *Hin*dIII/*Sma*I fragment of the hnRNP A2/B1 gene from hnRNPCAT (Fig. 5*A*), and *Eco*RI/*Hin*cII fragment of the hnRNP A2/B1 gene from hnRNP in pBSKSII (Stratagene) (Fig. 5*B*). DNase I protection assays were performed as described (1), except that poly(dI-dC) was not used in Fig. 5.

Protein-DNA Photocross-linking—The synthesis of the photoreactive nucleotide analog AB-dUTP (Fig. 4*A*) has been described elsewhere (20, 21). The N42–78 oligonucleotide of hNUDR8 was subcloned into the *Bam*HI site of pBLCAT5 and then excised with *Hin*dIII and *Eco*RI. After biotinylating the DNA fragment, one of the DNA strands was selectively immobilized on paramagnetic beads and used as the template in the synthesis of the photoaffinity probe (22). Approximately one pmol of template was used in a reaction containing 6 pmol of the specific oligonucleotide 5′-CGGCTTCCCACTTCGGGG-3′, ~4.5 pmol [α-^{32}P]dCTP, 0.6 μM AB-dUTP, 0.6 μM dATP, and 0.25 units of exonuclease-free Klenow fragment of DNA polymerase I (Amersham Pharmacia Biotech) in a final volume of 20 μl. After 5 min at 37 °C, 2.5 μl of 5 mM unlabeled dNTPs were added and incubated at 37 °C for an additional 10 min. A second oligonucleotide, complementary to the multiple cloning region of pBLCAT5 and 5′ of the first oligonucleotide, was annealed to the immobilized DNA, and double-stranded DNA was synthesized with T4 DNA polymerase and subsequent treatment with T4 DNA ligase to seal the nicks. The double-stranded DNA photoaffinity probe was removed from the solid support by digestion with *Hin*cII.

The binding reaction conditions for cross-linking were identical to those described for electrophoretic mobility shift assays (EMSAs), except that 2 fmol of the photoreactive probe were used. The cross-linking of the DNA and protein was performed by irradiation with UV light at 380 μW/cm^2 for 2 min at a distance of 20 cm. The cross-linked samples were treated with DNase I and S1 nuclease as described (23) to remove all but the four labeled pyrimidines attached to the protein (labeled *Intact*, Fig. 4*D*). An aliquot of the cross-linked sample was treated with 70% formic acid and 2% diphenylamine at 70 °C for 20 min to cleave the acid-labile Asp-Pro linkage at position 195–196 (labeled Asp-Pro cleavage, Fig. 4*D*). Samples were separated by SDS-PAGE on 10% polyacrylamide gels, followed by autoradiography.

Construction of Reporter Plasmids and Mammalian Expression Plasmids—A 743-bp DNA fragment containing the hnRNP A2/B1 promoter (positions 1844–2586) was amplified by 35 cycles of PCR (GeneAmp 9600, Perkin-Elmer) using 600 ng of genomic DNA isolated from the human JEG-3 cell line, and the primers 5′-ACTTTCAGCAGCGAACTCTCC-3′ and 5′-AGTCGCTTCAGCCCGATTTC-3′. The PCR product was subcloned into the *Eco*RV site of pBSKSII (Stratagene) before excision with *Bam*HI and *Hin*dIII and ligation into the *Bam*HI/*Hin*dIII site of pBLCAT6 (24) to produce the reporter plasmid, hnRNPCAT. The hnRNP PCR product in pBSKSII was digested with *Bsp*EI, followed by a fill-in reaction and ligation of *Bam*HI linkers. The DNA fragment containing the hnRNP promoter was excised with *Bam*HI and *Hin*dIII and ligated into the *Bam*HI/*Hin*dIII site of pBLCAT6 to produce the reporter hnRNPΔICAT. The reporter plasmids hnRNPΔIICAT and hnRNPΔI,IICAT were produced by excision of a *Xho*I DNA frag-

ment from the plasmids hnRNPCAT and hnRNPΔICAT and subsequent religation.

The 5′-UTR DNA was excised from hNUDR8 with *Eco*RI/*Bsp*EI, followed by a fill-in reaction and the addition of *Bam*HI linkers. The 356-bp DNA fragment was ligated into the *Bam*HI site or *Bgl*II site of pBLCAT5 (24) to produce the reporter constructs (h8N1–356)TKCAT and TK(h8N1–356)CAT, respectively. The 121-bp *Eco*RI/*Bsp*MI fragment of hNUDR8 was treated similarly and ligated into the *Bgl*II site of pBLCAT5 to produce TK(h8N1–121)CAT.

To achieve high levels of protein expression in mammalian cells, the cDNAs for hNUDR, sNUDR, and hNUDR-R302T/K304T were subcloned into an expression plasmid that utilized the human cytomegalovirus immediate early gene promoter (CMV), as described previously (1). The zinc finger homology region of hNUDR was deleted by *Hpa*I and *Bsu*36I digestion of the cDNA in pBSSKII, followed by fill-in and religation of the plasmid. The cDNA was excised with *Eco*RI, and *Bam*HI linkers were added, digested with *Bam*HI, and subcloned into the *Bgl*II site of pCMVNeo for the construct, CMVhNUDRaa1–505. The *Nco*I/*Eco*RI DNA fragment containing the C-terminal portion of hNUDR was subcloned into the *Bgl*II site as above to produce the construct CMVhNUDRaa243–565. The primers 5′-CGCGGATCCACCATGGCAGCTCCCCTCAC-3′ and 5′-CTACCGGATCCTAGACGTCGCCCTGGGC-3′ were used in a 20-cycle PCR reaction with hNUDR as the template. The PCR product was digested with *Bam*HI and subcloned into the *Bgl*II site of pCMVNeo for the construct, CMVhNUDRaa167–368. An *Eco*RI fragment of hNUDR from construct G in pBSSK was treated as above for the construct CMVhNUDRΔ255–367/SV40NLS. For each construct, the orientation and DNA sequence of the sites flanking an insertion or deletion were determined. Protein expression was confirmed in transfected CV-1 cells by immunofluorescence detection of NUDR, and the percentage of cells showing nuclear or cytoplasmic localization was estimated: hNUDRaa243–565 was 100% nuclear; hNUDR, hNUDRaa1–505 and hNUDRaa167–368 were 80% nuclear; hNUDRΔ255–367/SV40NLS was 64% nuclear; and hNUDR-R302T/K304T was 100% cytoplasmic (immunofluorescence data not shown).

Chloramphenicol Acetyl Transferase Assay—CV-1 cells were transfected with various reporter constructs and expression plasmids, cell extracts were prepared in 250 μl of homogenization buffer, and chloramphenicol acetyl transferase (CAT) activities were determined and normalized as described previously (1). The normalized CAT activity determined for the indicated reporter construct was set at 100% and the effects of cotransfecting different expression plasmids with the reporter are shown relative to this activity.

RESULTS

NUDR Binding Sequences Are Found in the 5′-UTR$_{DNA}$ of NUDR cDNAs—In a search for genes that contain the NUDR binding consensus sequence or TTCG motifs, we noted that multiple TTCG motifs occurred in the cDNA corresponding to the 5′-untranslated region (5′-UTR$_{DNA}$) of human NUDR8 and monkey NUDR. Computer analysis of the NUDR cDNA for TTCG motifs demonstrates the presence of 14 motifs in the 5′-UTR$_{DNA}$ of hNUDR8, four motifs in the coding region, and the absence of motifs in the 3′-UTR$_{DNA}$ (Fig. 1*A*). To test whether NUDR protein could bind these motifs, we performed an EMSA using radiolabeled DNA sequences from the 5′-UTR$_{DNA}$ (99 bp) and 3′-UTR$_{DNA}$ (130 bp) of hNUDR8. Low mobility complexes were observed with the 5′-UTR$_{DNA}$ probe when combined with 10 and 30 pmol of recombinant hNUDR protein, whereas no complexes were formed with the 3′-UTR$_{DNA}$ probe (Fig. 1*B*). These data indicate that NUDR protein could potentially bind multiple TTCG motifs within its own 5′-UTR$_{DNA}$ *in vivo*.

To examine the specific sequences within the 5′-UTR$_{DNA}$ that NUDR was binding, we utilized DNase I protection assays. In the presence of NUDR protein, a large 74-base pair region in the upper half of the 5′-UTR$_{DNA}$ was protected from nuclease digestion (Fig. 2*A*). This protected region contained, but was not limited to, the TTCG motifs. Within this large region was a smaller area that was protected by the lowest protein concentration and contained two sets of TTCG pairs separated by six nucleotides (shown in *bold capitals letters* in Fig. 2*D*). Comparison of the two sets showed that 9 of the 15 nucleotides of each

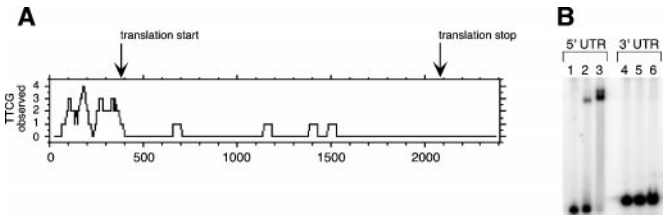

FIG. 1. **NUDR protein binds to sequences within the 5′-UTR$_{DNA}$ of hNUDR8.** *A*, the cDNA sequence for hNUDR8 was analyzed for TTCG sequences by the WINDOW program and plotted with the STATPLOT program from the Wisconsin sequence analysis package by Genetics Computer Group, Inc. (GCG, Madison, WI). The window size was 50 bp, and the shift increment was 3 bp. *B*, A 99-bp *Eco*RI/*Bsp*MI fragment (*5′UTR*) and a 130-bp *Sma*I/*Eco*RI (*3′UTR*) fragment of hNUDR8 were radiolabeled by fill-in reaction, and each fragment was incubated with no protein (*lanes 1* and *4*) or with 10 pmol (*lanes 2* and *5*) and 30 pmol (*lanes 3* and *6*) of recombinant hNUDR protein before separation of DNA-protein complexes and free probe on a 4% nondenaturing polyacrylamide gel. Results were visualized with a PhosphorImager.

set were identical to each other and can be represented by the sequence, TTCGGNNNNNTTCGN. In addition, 9 nucleotides within each set of TTCG pairs (15 nucleotides in length) were identical to the derived NUDR binding consensus sequence, TTCGGGNNTTTCCGG. The six-nucleotide spacing between a pair of TTCGs would align the TTCG sequences on the same face of the DNA double helix within one turn and may allow optimal binding or interaction of one or more NUDR molecules.

Other areas in the lower half of the 5′-UTR$_{DNA}$ of hNUDR8 were also protected from nuclease digestion by NUDR binding; some of these contained TTCG motifs whereas others did not (data not shown). Because the sequences and boundaries of the DNA protected by NUDR binding were not limited to TTCG or TTCG-like motifs, protein-protein interactions may extend the protection from DNase I to flanking sequences or may alter the DNA binding specificity.

To facilitate subsequent studies, an oligonucleotide spanning the two sets of TTCG pairs from the 5′-UTR$_{DNA}$ of hNUDR8 (nucleotides N42–78, shown in *bold capital letters* in Fig. 2*D*) and including *Bam*HI restriction sites at both ends was synthesized. In EMSA, radiolabeled N42–78 oligonucleotide was shifted by the addition of recombinant NUDR protein (Fig. 2*B*). DNA binding specificity of NUDR was shown by DNA binding competition with an excess of unlabeled N42–78, whereas no competition was observed with an excess of unlabeled oligonucleotide containing a glucocorticoid response element (Fig. 2*B*). The N42–78 oligonucleotide was subcloned into a plasmid, and a DNA fragment containing this sequence was used in DNase I protection assays. As shown in Fig. 2*C*, NUDR protein protected the entire N42–78 sequence from nuclease digestion. In addition, NUDR protein binding also produced DNase I hypersensitive sites in the sequence flanking N42–78 (Fig. 2*C*) and in small regions of the 5′-UTR$_{DNA}$ of hNUDR8 (data not shown).

Characterization of the DNA Binding Domain of NUDR—We examined NUDR for a potential DNA binding domain, and the cysteine-rich, C terminus of the protein appeared as the most likely candidate. There are at least 20 protein sequences in the GenBank™ data base that have homology to this region of NUDR (1), and several investigators have suggested that this arrangement of cysteines and histidines may constitute a zinc finger motif capable of interacting with DNA (2, 25–29).

To investigate the region(s) of the protein responsible for DNA binding, we constructed various N-terminal, C-terminal, and internal deletions of NUDR (Fig. 3) and inserted them into bacterial expression vectors to produce fusion proteins with GST or an N-terminal histidine tag (see "Experimental Procedures"). Recombinant proteins were purified and assayed for their ability to bind the radiolabeled N42–78 sequence in

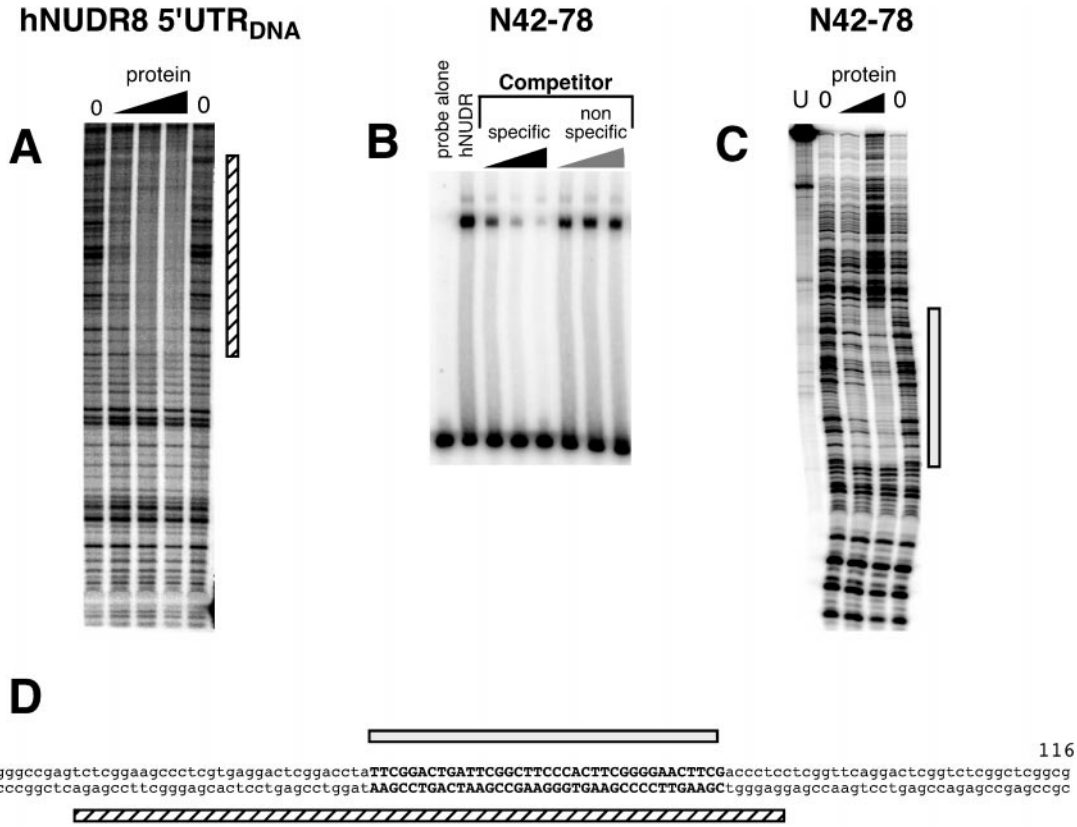

FIG. 2. **NUDR protein binds with high affinity to nucleotides 42–78 of hNUDR8.** *A*, the lower strand of an *Eco*RI/*Bsp*MI fragment of hNUDR8 was radiolabeled and treated with DNase I in the absence (*0*) or presence of increasing amounts of recombinant hNUDR protein (15, 50, and 100 pmol, indicated by the *wedge*). Samples were separated on denaturing 6% acrylamide gels, and the results were visualized with a PhosphorImager. The NUDR-protected sequence is indicated by the *striped bar* to the right of *panel A* and below the corresponding nucleotide sequence in *D*. *B*, a double-stranded oligonucleotide consisting of nucleotides 42–78 of hNUDR8 cDNA (*bold capitals letters* in *D*) was radiolabeled (probe) and incubated with 10 pmol of recombinant hNUDR protein alone (*hNUDR*) and in the presence of increasing amounts of unlabeled oligonucleotide (indicated by the *wedge*), that was either the N42–78 sequence (*specific*) or a glucocorticoid response element (*nonspecific*). Samples were analyzed as in Fig. 1*B*. *C*, the upper DNA strand of N42–78 oligonucleotide was radiolabeled and either untreated (*U*) or treated with DNase I in the absence (*0*) or presence of increasing amounts of recombinant NUDR protein (10 and 145 pmol indicated by the *wedge*) and analyzed as in *A*. The NUDR-protected sequence is indicated by the *gray bar* to the right of the *panel* and above the nucleotide sequence in *D*. *D*, the nucleotide sequence of the *Eco*RI-*Bsp*MI fragment of hNUDR8 is shown, with the *bars* indicating the sequences protected from DNase I by NUDR protein binding. Nucleotide positions 42–78 are shown in *bold capital letters*.

EMSAs. We found deletion of the last 84 amino acids, which includes the potential zinc finger motif, had little effect on the DNA binding of NUDR (Fig. 3, *construct B*). Similarly, deletion and site-directed mutations of the zinc finger motif in DEAF-1 had no effect on its DNA binding properties (2). Furthermore, removal of up to 195 amino acids from the C terminus of NUDR (Fig. 3, *constructs B-D*) and up to 138 amino acids from the N terminus (data not shown) had little effect on NUDR binding of the N42–78 probe. DNA binding was compromised but not abolished by the deletion of the first 187 amino acids from the N terminus of NUDR (Fig. 3, *construct E*), suggesting that the area between amino acids 138 and 187 of NUDR may be involved in DNA binding. The recombinant proteins produced with internal deletions of amino acids 242–289 and 254–368 showed reduced binding (Fig. 3, *constructs F* and *G*). These data indicate that the region of NUDR between amino acids 242–368 are also important for DNA binding.

In an attempt to transfer the DNA binding properties of NUDR to a non-DNA-binding protein, we constructed a series of NUDR peptides fused to GST. A peptide containing amino acids 267–368 (Fig. 3, *H*) was not sufficient for DNA binding, but the inclusion of an additional 25 N-terminal amino acids (amino acids 243–368) conferred some binding activity (Fig. 3, *peptide I*). Full DNA binding activity was achieved by peptide J, composed of amino acids 167–368. The N-terminal half of peptide J, amino acids 167–289 (peptide L), showed similar DNA binding as the C-terminal half, amino acids 243–368 (peptide I), with both peptides having reduced binding capacities compared with peptide J. The region shared by peptides L and I did not constitute the DNA binding domain, since construct F, which lacks this region, was still able to bind DNA, and a shorter peptide K (amino acids 243–328), which includes this region, was unable to bind DNA. The reduced binding activities of the peptides (I and L) and the deletion constructs (F and G) suggest there may be cooperativity or synergy among the peptide regions to achieve the stronger DNA binding activity displayed by peptide J and the full-length protein (Fig. 3). Alternatively, the reduced binding activities of the peptides could be a consequence of improper folding of the recombinant proteins and not simply an elimination of the amino acids involved in binding. Nonetheless, these results indicate that the DNA binding domain resides in the central region of the protein and is represented by peptide J.

To verify that this central region of the protein was in direct contact with DNA, we performed DNA-protein photocross-linking. A DNA photoaffinity probe was synthesized by incorporating the photoreactive deoxyuridine analog AB-dUTP (Fig. 4*A*) and radioactive deoxynucleotides into the N42–78 sequence (Fig. 4*B*). A short-chain-length tether between the photoreactive aryl azide and the deoxyuridine (AB-dUTP, ~10.0 Å) was used to label the protein at sites of DNA contact (20, 21, 30). The double-stranded DNA photoaffinity probe was used in an

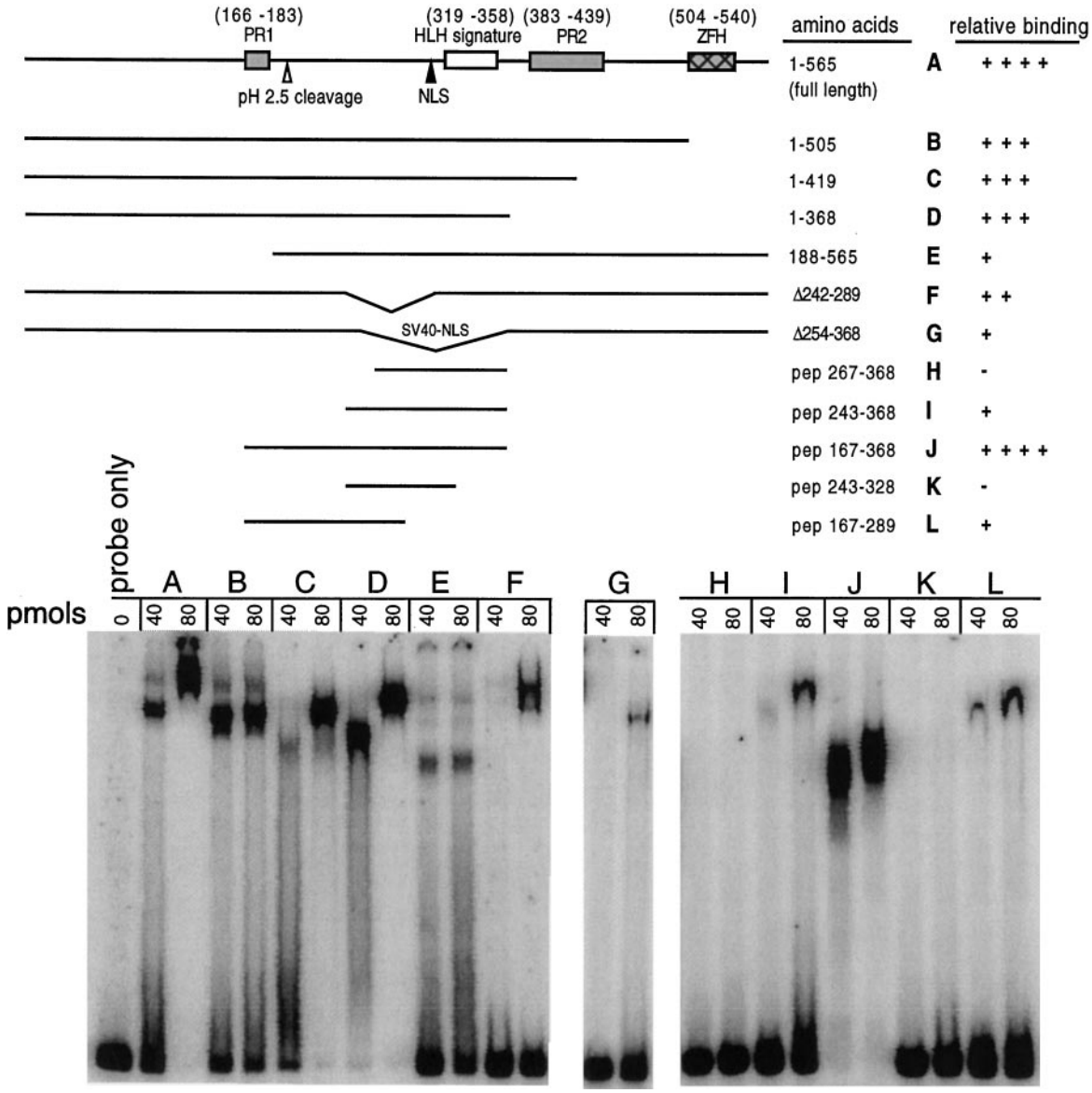

FIG. 3. **Deletion analysis to identify the NUDR DNA binding domain.** At the top is a schematic representation of the full-length NUDR protein with salient features and potential functional domains indicated including two proline rich regions (PR1, 44% proline and PR2, 28% proline), a zinc finger homology domain (*ZFH*), and a nuclear localization signal sequence (*NLS*) (1). The *open rectangle* is a region with similarity to Myc-type, helix-loop-helix dimerization domain signature (*HLH signature*) that was identified in a search of the PROSITE data base (32). The *open triangle* denotes an Asp-Pro linkage that is susceptible to acid cleavage. The *line drawings* represent various deletions and peptide portions of recombinant NUDR protein that were produced as bacterial fusion proteins with either glutathione *S*-transferase or an N-terminal histidine tag (see "Experimental Procedures"). The numbers listed to the right of each construct indicate the amino acids of NUDR included in the fusion protein, and those preceded by a Δ are amino acids deleted from full-length NUDR. Forty and 80 pmol of each recombinant protein (*constructs A-L*) were assayed for their ability to bind radiolabeled N42–78 in an EMSA, as in Fig. 1*B*. Note that in some cases increased levels of protein produced decreased mobility of complexes, thus indicating potential multimerization of NUDR proteins.

EMSA under reduced lighting conditions to show that NUDR protein was able to bind the modified DNA (Fig. 4*C*). After photocross-linking with UV light, the protein-DNA complex was treated with DNase I and S1 nuclease to reduce the DNA attached to the protein to the four radiolabeled and modified nucleotides. The radioactive protein was cleaved with formic acid, and the peptide fragments were resolved by SDS-PAGE (Fig. 4*D*). Two radiolabeled peptides were observed with a combined molecular mass that approximately equaled the uncut protein (Fig. 4*D*). Because NUDR contains only one pH 2.5 acid-labile bond at amino acid position 195/196 (shown schematically in Fig. 3), the cross-linking data indicates that amino acids on both sides of the cleavage site are in contact with the DNA. Considering the EMSA results, the cross-linking of the 37-kDa N-terminal peptide would indicate that DNA is in contact with NUDR between amino acids 167 and 195, and this may be further refined to a position between amino acids 167 and 187 based on the decreased DNA binding of deletion construct E (amino acids 188–565) compared with peptide J (Fig. 3). The cross-linking of the 57-kDa C-terminal peptide would be consistent with additional protein-DNA contacts between amino acids 195 and 368. Together these data indicate that the DNA binding domain of NUDR, as delineated by amino acids 167–368 (peptide J), consists of at least two regions of the protein in contact with the DNA that interact together to enhance DNA binding.

NUDR Protein Binds and Regulates the hnRNP A2/B1 Promoter—A data base search for genes that may contain the NUDR binding consensus sequence revealed an exact match within the human hnRNP A2/B1 promoter (see sequence in

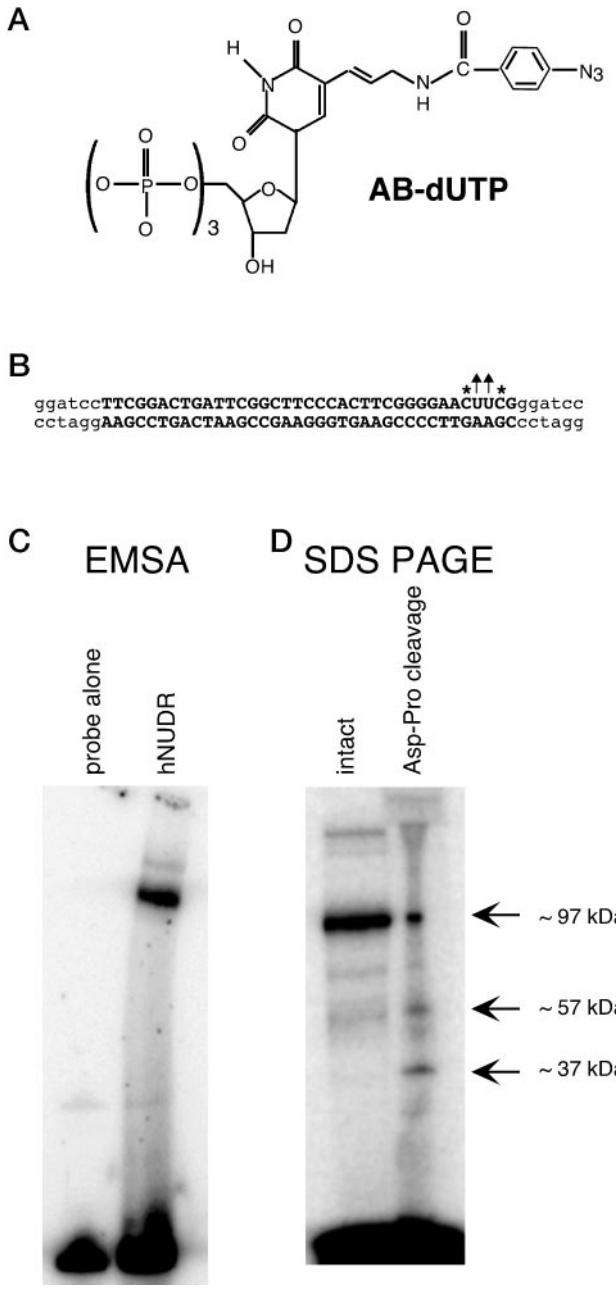

FIG. 4. **Photocross-linking of NUDR protein to radiolabeled DNA indicates at least two DNA contacts.** *A*, the chemical structure of the photoreactive deoxyuridine analog AB-dUTP consists of a photoreactive aryl azide group attached to the C-5 position of deoxyuridine triphosphate. *B*, the sequence of the N42–78 oligonucleotide was modified by incorporation of [α-^{32}P]dCTP (shown by an *asterisk*) and AB-dUTP (shown by a verticle arrow) at the positions indicated. *C*, the modified N42–78 double-stranded oligonucleotide probe (2 fmol) was incubated under low light conditions in the absence or presence of hNUDR protein, and binding was analyzed by EMSA as in Fig. 1*B*. *D*, the modified N42–78 double-stranded oligonucleotide probe (6 fmol) was incubated with hNUDR protein, irradiated with UV light, and either left intact or incubated with 70% formic acid before separation by 10% SDS-PAGE. The results were visualized with a PhosphorImager. Approximate molecular masses of the labeled proteins were estimated from the mobility of prestained molecular weight markers (not shown).

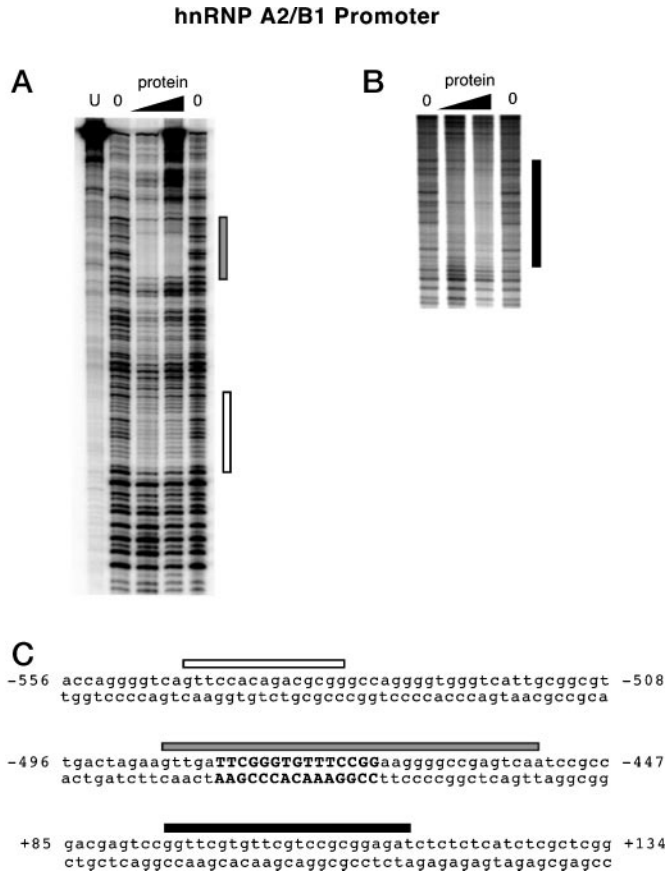

FIG. 5. **NUDR protein binds to sequences in the human hnRNP A2/B1 promoter.** *A*, a *Hin*dIII/*Sma*I fragment from the hnRNPCAT plasmid was radiolabeled and either untreated (*U*) or treated with DNase I in the absence (*0*) or presence of increasing amounts of recombinant NUDR protein (20 and 148 pmol, indicated by the *wedge*) and analyzed as in Fig. 2*A*. The NUDR-protected sequences are indicated by the different *bars* adjacent to the *panel*, and their corresponding *bars* above the nucleotide sequences in *C*. *B*, a *Eco*RI/*Hin*cII fragment from the 5′-UTR$_{DNA}$ of hnRNP was radiolabeled and treated as in *panel A* (10 and 50 pmol, indicated by the *wedge*). *C*, the numbering of the human *hnRNP A2/B1* gene sequences has been adjusted so that the transcription initiation site is (+1) (based on exon 1 starting at position 2427 in GenBank™ accession number D28877). The NUDR binding consensus sequence is shown in *bold*, *capital letters*.

bold, Fig. 5*C*). DNA fragments containing various regions of the hnRNP A2/B1 promoter were used in DNase I protection assays to determine whether NUDR protein would bind. As shown in Fig. 5, NUDR bound three specific regions of the promoter. The first region (*shaded bar*, Fig. 5*A*) included the NUDR binding consensus sequence, a second region (*open bar*, Fig. 5*A*) appeared to have limited homology to the consensus (6 nucleotides matched out of the 15 defined nucleotides), and a third region located 3′ of the transcription initiation site included two closely spaced TTCG motifs (*closed bar*, Fig. 5*B*). Closer examination of the first region revealed that the entire protected sequence (34 nucleotides in length) was a large inverted repeat, whereas the third protected region occurs in the 5′-UTR$_{DNA}$ of hnRNP, a position analogous to the NUDR binding sequences found in the 5′-UTR$_{DNA}$ of NUDR.

To determine whether NUDR might regulate the expression of hnRNPA2/B1, a 742-bp DNA fragment containing the promoter was ligated to the reporter gene CAT in the construct, hnRNPCAT. Cotransfection of hnRNPCAT with the expression vector for full-length hNUDR resulted in a 65–70% reduction in CAT activity compared with the reporter alone (Fig. 6 and 7). The transcriptional repression of this promoter was somewhat surprising, since previous transfection studies had shown that NUDR activated transcription of the proenkephalin promoter by 26-fold (1). However, in contrast to the current studies, we had been unable to demonstrate direct NUDR binding to proenkephalin sequences, and we therefore suggested that transcriptional activation by NUDR may occur through additional protein-protein interactions.

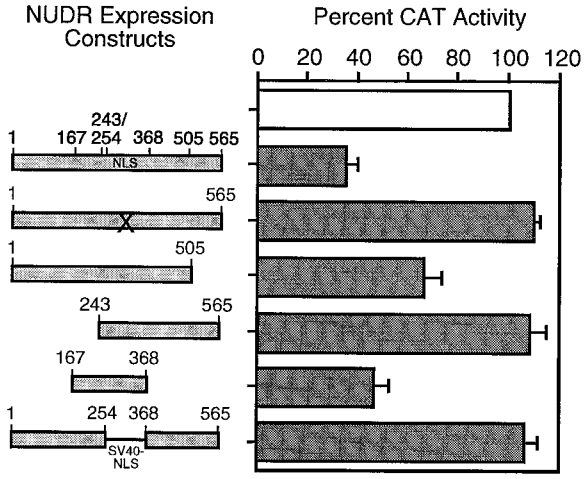

FIG. 6. **NUDR represses expression from the hnRNP A2/B1 promoter.** CV-1 cells were cotransfected with 2 μg of the hnRNPCAT reporter and 1 μg of a CMV expression vector without (*open bar*) or with a NUDR cDNA for wild-type or one of the deletion constructs (*shaded bars*) and shown schematically to the left of the graph. The nuclear localization signal is indicated by NLS, and an *X* indicates a double mutation (R302T/K304T) in the NUDR NLS. The internal deletion construct that removed the NUDR NLS was modified to include the NLS from the SV40 large T-antigen (see "Experimental Procedures"). The results are presented as percent CAT activity, with the activity of the hnRNPCAT reporter alone set at 100%, and are the average of triplicate measurements from two independent experiments ±S.D.

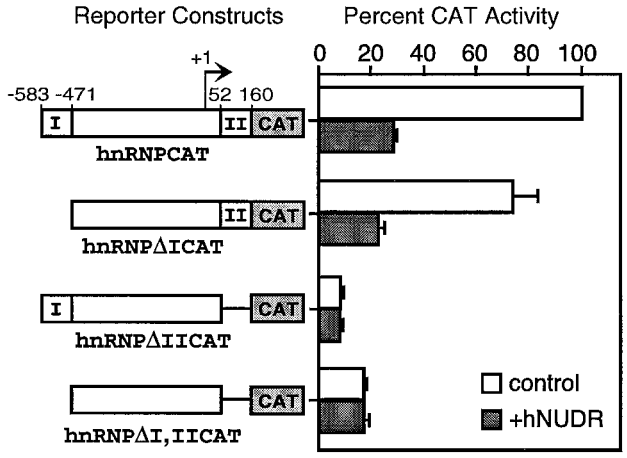

FIG. 7. **NUDR repressor activity on the hnRNP A2/B1 promoter is through sequences downstream of transcription initiation.** CV-1 cells were cotransfected with 2 μg of the indicated hnRNP promoter containing CAT reporter and 1 μg of either CMVNeo (*control*) or CMVhNUDR (+*hNUDR*) expression vectors. Regions I and II represent regions of the hnRNP promoter that were protected from DNase I by NUDR protein (see Fig. 5) and have been deleted where indicated. The results are presented as percent CAT activity with the activity of the hnRNPCAT reporter alone set at 100% and are the average of triplicate measurements from two independent experiments ±S.D.

The establishment of NUDR binding to sequences in the hnRNP promoter provided the rationale to use transcriptional repression of hnRNP as an *in vivo* approach to assess the functional activity of NUDR proteins with various mutations and deletions (see "Experimental Procedures," Fig. 3). Mutations in the NLS of NUDR (R302T/K304T) that resulted in the protein being localized exclusively to the cytoplasm (1) failed to repress transcription of the hnRNP promoter (Fig. 6). Since point mutations in the NLS did not impair the DNA binding activity of NUDR (data not shown), these results indicate that nuclear localization is required for transcriptional repression. Similarly, the N-terminal half of NUDR (amino acids 1–242) also had no effect on transcription (data not shown). A C-terminal-truncated NUDR protein (amino acids 1–505) that lacked the zinc finger homology region was less effective as a repressor compared with the full-length protein (34% reduction *versus* 65%). This result suggests a potential role of the zinc finger homology region in repressor function but may also reflect the somewhat reduced DNA binding activities observed for recombinant NUDR proteins that lack this region (Fig. 3). The C-terminal half of NUDR (amino acids 243–565) was unable to repress transcription of the hnRNP promoter. Since recombinant NUDR proteins and peptides missing amino acids 167–243 show reduced DNA binding activities (Fig. 3), the lack of repression by the C-terminal protein may also be a consequence of reduced DNA binding. We tested peptide J (amino acids 167–368) for repressor activity, because this peptide was shown to have DNA binding activity similar to full-length NUDR (Fig. 3). Peptide J repressed transcription of hnRNPCAT to a similar level as the full-length protein, suggesting this region of NUDR is sufficient for repression of the hnRNP promoter. To further establish that the central region of NUDR was involved in transcriptional repression, we constructed a chimeric protein of NUDR in which the NLS of NUDR and part of the DNA binding domain (deletion of amino acids 254–368, similar to *construct G* in Fig. 3) were replaced with the NLS from the SV40 large T-antigen (see "Experimental Procedures"). The chimeric protein was localized to the nucleus (not shown) but was unable to repress the expression of hnRNPCAT. Together these results indicate that the repressor domain colocalizes with the DNA binding domain in the central portion of NUDR (amino acids 167–368) and that DNA binding is required for repressor function.

Because DNase I protection assays had demonstrated NUDR protein binding to DNA sequences within the hnRNP promoter (Fig. 5), we examined the importance of these sequences for transcriptional activity and NUDR repression. Deletion of region I from the hnRNP A2/B1 promoter (nucleotides −583 to −471) removed a major portion of the two binding sequences identified upstream of transcription initiation, whereas deletion of region II (nucleotides 51 to 160, hnRNPΔIICAT) removed NUDR binding sequences identified in the 5′-UTR_{DNA}. As shown in Fig. 7*A*, deletion of region I (hnRNPΔICAT) resulted in a slight reduction (25%) in basal CAT activity compared with hnRNPCAT; however NUDR repressed transcription of hnRNPΔICAT as effectively as full-length hnRNPCAT (30% of basal activity). These results indicate that NUDR repression is not mediated through sequences in region I. Deletion of region II from the hnRNP promoter in the reporter constructs, hnRNPΔIICAT and hnRNPΔI,IICAT resulted in significantly reduced basal CAT activities when compared with the parent construct hnRNPCAT (Fig. 7). However, the transcriptional activities of these promoters were not eliminated and were approximately 10-fold higher than those produced by the thymidine kinase (TK) promoter in pBLCAT5 (data not shown). The reduced activity could signify the removal of a *cis*-acting element in the 5′-UTR_{DNA} region of the promoter. Importantly, the overexpression of NUDR did not repress the basal activities of these two reporters. These data support the hypothesis that NUDR repression of the hnRNP promoter occurs through NUDR binding to sequences located 3′ and proximal to the transcription initiation site.

Because NUDR repression mapped to the 5′-UTR_{DNA} of the hnRNP promoter, and since we had also observed multiple NUDR binding motifs in the 5′-UTR_{DNA} of the hNUDR8 cDNA clone, we sought to determine whether placement of NUDR sequences on a heterologous promoter and in positions comparable with those found in the hnRNP promoter would affect basal transcription and confer NUDR protein regulation. We

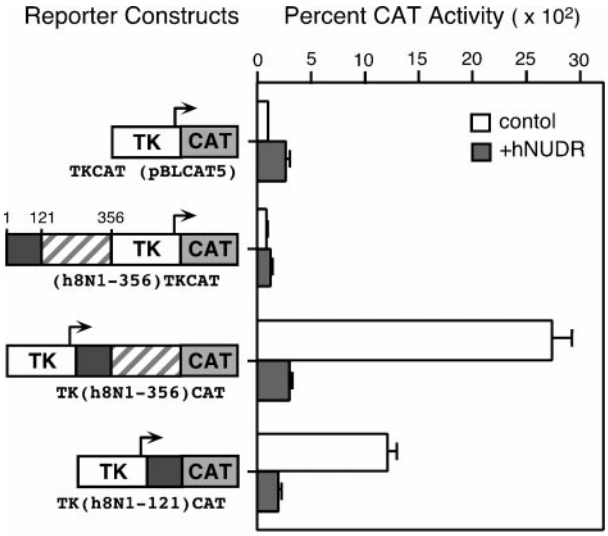

FIG. 8. **Analysis of hNUDR8 5′-UTR$_{DNA}$ sequences using the thymidine kinase promoter.** Sequences from the 5′-UTR$_{DNA}$ of hNUDR8 were inserted into pBLCAT5 as described under "Experimental Procedures." CV-1 cells were cotransfected with 2 μg of the indicated reporter constructs, drawn schematically at the left, and 1 μg of CMV-Neo (*control*) or CMVhNUDR (+*hNUDR*) expression vectors. The results are presented as percent CAT activity with the activity of pBLCAT5 alone set at 100% (multiplied by 10^2) and are the average of triplicate measurements from two independent experiments ±S.D.

inserted sequences from the 5′-UTR$_{DNA}$ of hNUDR8 into pBLCAT5 in positions 5′ of the TK promoter ((h8N1–356)TKCAT) and 3′ of the transcription initiation site of the CAT gene (TK(h8N1–356)CAT and TK(h8N1–121)CAT) and examined the effects of NUDR coexpression. The transcriptional activity of (h8N1–356)TKCAT showed low basal activity similar to pBLCAT5 and was not regulated by NUDR (Fig. 8). We also found no effect on CAT activity when the 5′-UTR$_{DNA}$ sequences were placed in a promoterless CAT vector, pBLCAT6 (data not shown). In contrast, the reporter TK(h8N1–356)CAT with the 5′-UTR$_{DNA}$ sequences inserted 3′ of the TK transcription initiation site produced significant increases in the basal CAT activity, and this activity was repressed by NUDR protein overexpression (89% reduction). Since we had localized the majority of NUDR binding to sequences in the upper third of the 5′-UTR$_{DNA}$ of hNUDR8 (Fig. 2), we tested a second reporter, with nucleotides 1–121 of the h8NUDR cDNA inserted 3′ of the TK transcription initiation site (TK(h8N1–121)CAT). The TK(h8N1–121)CAT reporter also showed an elevated basal level of CAT activity, and this activity was repressed by NUDR overexpression (84% reduction). Although TK(h8N1–356)CAT and TK(h8N1–121)CAT produced high levels of CAT activity relative to pBLCAT5, it should be noted that these levels are modest relative to hnRNPCAT. These results are analogous to those observed for the hnRNP A2/B1 promoter and support the hypothesis that NUDR represses transcription through DNA binding at sites located 3′ proximal to the transcription start site.

DISCUSSION

In this report, we continue the characterization of NUDR as a DNA-binding protein and transcriptional regulator. We have localized the DNA binding domain of NUDR to the central region of the protein by demonstrating that a peptide comprised of amino acids 167–368 (*peptide J*, Fig. 3) has *in vitro* DNA binding activities comparable with the full-length protein. The DNA binding domain appears to be composed of smaller regions that individually have weak DNA interactions yet can interact cooperatively to achieve full binding activity. The DNA binding domain overlaps with the previously identified nuclear domain (1)/KDWK domain (2) and is the region that displays the highest homology between NUDR and *Drosophila* DEAF-1 (70%) (1). Consequently, it is not surprising that the DNA sequences recognized by NUDR and DEAF-1 are similar and consist of at least one and usually multiple TTCG motifs (1, 2). Although we have derived a NUDR binding consensus of TTCGGGNNTTTCCGG from the analysis of oligonucleotides selected by NUDR binding and PCR amplification (1), we have also noted that NUDR binds preferentially to sequences with mulitiple copies of TTC(C/G)G without a strict requirement for specific spacing between the motifs.

The observations that NUDR protects large DNA regions in DNase I protection assays and the presence of multiple, low mobility complexes in EMSA are indicative of potential protein-protein interactions (see Figs. 1 and 2). The concept of NUDR homodimerization or multimerization is supported by the observation that *Drosophila* DEAF-1 may form multimers (2). In addition, MTG8 (also known as ETO) has homology to NUDR in the zinc finger region and has been observed to form homomeric complexes (31), whereas the mouse homolog of NUDR (mDEAF-1) was shown to interact with itself, albeit weakly, in a yeast two-hybrid system (3). A difficulty in resolving this issue is that we have yet to resolve a minimal DNA element recognized by NUDR. NUDR binds DNA fragments with multiple TTC(C/G)G-like sequences with greater affinity and at lower protein concentrations than DNA fragments with fewer TTC(C/G)Gs (data not shown). And, at least *in vitro*, NUDR appears to bind somewhat promiscuously and with low affinity to other sequences flanking these motifs, especially at higher protein concentrations (Fig. 2 and 5; Ref. 1). We suggest that optimal binding of NUDR to DNA occurs when multiple binding elements are in close proximity to one another to enhance the cooperativity among NUDR multimers.

Utilizing EMSA and DNase I protection assays, we have demonstrated that NUDR protein binds to sequences within its own 5′-UTR$_{DNA}$ and to sequences within the hnRNP A2/B1 promoter both 5′ and 3′ of transcription initiation. In transient transfection assays, NUDR was shown to repress transcription from the hnRNP A2/B1 promoter. Furthermore, the peptide encompassing the DNA binding domain (peptide J, amino acids 167–368) was shown to be almost as effective (55% reduction) as the full-length protein in repressing hnRNPCAT activity, suggesting that the majority of NUDR repressor activity also appears to reside within this domain.

The finding that amino acids 167–368 of NUDR could contain a repressor domain is intriguing because this region has sequence homology with SP100B and LYSP100B (1) and was identified as a "Myc-type, helix-loop-helix dimerization domain signature" (amino acids 319–358) in a comparison of the PROSITE data base (32). SP100 proteins are localized to subnuclear structures termed nuclear bodies and are thought to play a role in the etiology of acute promyelocytic leukemia (reviewed in Ref. 11). SP100 was shown to associate with non-histone chromatin components that behave as transcriptional silencers, and when fused to a GAL4 DNA binding domain, SP100B was able to repress transcription (12, 13).

Helix-loop-helix (HLH) motifs are often dimerization domains, and when accompanied by an adjacent region rich in basic amino acids (basic helix-loop-helix or bHLH), they can interact directly with DNA (33). Typically the two helices in HLH domains are amphipathic and create a hydrophobic interface to stabilize the interaction between dimers. Although secondary structure algorithms (*e.g.* Chou and Fasman (34)) predict NUDR to have two helices separated by a turn in a region near the basic amino acids of the NLS, other programs

that plot the peptide sequence as a helical wheel do not recognize these helices as amphipathic (not shown). In mobility shift assays, most of the recombinant NUDR proteins that included the potential HLH motif displayed decreased mobility complexes with increased levels of protein (Figs. 1*B* and 3), indicating the possible multimerization of NUDR proteins. We have shown that the region encompassing the NLS and potential HLH is required for full DNA binding activity *in vitro* (Fig. 3), and the repressor activity of NUDR also maps to this region. We have not yet determined whether these activities are separable. It is interesting to note that the inhibitory domain of an *ets* oncogene family member also bears strong resemblance to a HLH motif (35), strengthening the potential contribution of the NUDR HLH region in repressor function.

The C-terminal, zinc finger homology region of NUDR shares amino acid homology with the repressor domain of MTG8/ETO (1, 8). The zinc finger region of MTG8/ETO (previously described as the MYND domain (2)) was established as a crucial site of interaction with the nuclear corepressor N-CoR, and deletion of this region impaired transcriptional repression (8). In acute myeloid leukemias, the chromosomal translocation t (8, 21) converts the transcriptional activator AML-1 into a transcriptional repressor by producing an AML-1/ETO fusion protein (28). This fusion protein most likely disrupts normal hematopoietic differentiation by recruitment of nuclear corepressors (*i.e.* N-CoR and Sin3), which then repress genes essential for normal differentiation (9, 36, 37). The high degree of homology between MTG8/ETO and NUDR suggests that the C terminus of NUDR may also be involved in the recruitment or interaction with corepressors. Deletion of the zinc finger domain of NUDR resulted in a protein that was not as effective as the full-length protein in transcriptional repression, implying that this region of NUDR may indeed recruit corepressors. However, since peptide J, which lacks the zinc finger domain, approached the level of repression achieved by the full-length protein, the majority of the repressor activity appears to reside within the central DNA binding domain of NUDR, with possible minor contributions by the C-terminal zinc finger domain.

The C-terminal region of the mouse homolog of human NUDR (mDEAF-1) has been shown to interact with LMO proteins (3). Using a yeast two-hybrid interaction assay, Sugihara *et al.* (3) showed that the region between amino acids 334 and 518 of mDEAF-1 interacted with the LIM domain. Although LMO proteins do not directly bind DNA, they are thought to interact with DNA-binding proteins and form complexes involved in transcriptional regulation (38). LMOs have been identified at sites of chromosomal translocations, and their ectopic expression has been associated with childhood T-cell acute leukemias (39). Since the LMO interaction and DNA binding domains overlap, it is interesting to speculate that LMO interaction with NUDR could alter the DNA binding specificity of NUDR, perhaps allowing recognition and transcriptional activation of the proenkephalin promoter.

The *hnRNP A2/B1* gene was first indicated as a potential NUDR regulated gene in a data base search by the identification of a perfect match of the NUDR binding consensus sequence upstream of the proximal promoter. Although NUDR does bind to this consensus sequence, NUDR binding sites downstream of the transcription initiation were shown to be responsible for the NUDR repression of transcription (Fig. 7). The position-dependent repression by NUDR was confirmed in a heterologous promoter by transferring NUDR binding sequences from the 5′-UTR_{DNA} of hNUDR8 into sites downstream of the TK promoter (Fig. 8). NUDR repressed transcription of TK(h8N1–356)CAT by 89% but did not repress transcription of the reporter with NUDR binding sequences inserted upstream of transcription initiation ((h8N1–356)TKCAT). The position-dependent repression by NUDR suggests that a potential mechanism of NUDR repression may be through blocking of the RNA polymerase II complex and inhibition of elongation rather than inhibition of transcription initiation. Pausing of RNA polymerase II at sites downstream of transcription initiation have been demonstrated for c-Myc (40, 41), c-Myb (42), c-Fos (43), adenosine deaminase (44), and *Drosophila* hsp70 (45). Recently, the 1,25-dihydroxyvitamin D3-induced transcriptional elongation block of c-Myc was found to be linked to HOXB4 binding site within intron 1 of the c-*myc* gene (46), suggesting a role for developmental factors in c-Myc regulation.

Multiple sequence elements in the 5′-UTR of the *bcl-2* gene have been shown to be responsible for the decreased expression from the bcl-2 P1 promoter, and transfer of these sequences to a 5′-UTR position in a CMVNeo construct resulted in decreased expression from the CMV promoter (47). In contrast, NUDR binding sequences in the 5′-UTR_{DNA} position of hnRNPCAT and TK(h8N1–356)CAT resulted in elevated levels of basal reporter activity, suggesting the possible recruitment of a position-dependent activator protein(s). Thus, an alternative explanation for NUDR-dependent repression may be through competition or inhibition of an activator at or near NUDR binding sites. Mechanisms of transcriptional repression by competition and displacement have been noted for several transcription factors, including Sp1, EGR-1, and WT1 (48), although in most cases the repression, both direct and indirect, occurs through sequences upstream of the promoter (49–52). Finally, the interactions of glucocorticoid receptor and the transcription factor AP-1 (Jun and Fos heterodimer) illustrate another possible mechanism for repression. Although in most cases glucocorticoid receptor and AP-1 behave as transcriptional activators, in some promoters the colocalization of glucocorticoid receptor and AP-1 on DNA sequences can repress transcription (53).

A comparison of DNA sequences recognized by NUDR and other transcription factors revealed that NUDR binding sequences may overlap with the DNA recognition sequence of some ETS domain-containing proteins, many of which have been shown to be transcriptional activators (54–57). The sequence recognized by several ETS domain proteins is 5′ A/G**C-CGGAA/**T 3′, with the ETS core binding sequence underlined (56). This sequence contains a potential NUDR binding motif (shown in bold), which is more readily visualized on the complementary strand, 5′ A/**TTCCGG**C/T 3′. Whether ETS domain proteins can bind to sequences in the 5′-UTR_{DNA} of *hnRNP A2/B1* or the NUDR gene and up-regulate their expression remains to be determined, as does the precise mechanism of NUDR repression.

The identification of NUDR binding sites within the hnRNP A2/B1 promoter *in vitro* and the demonstration that NUDR represses expression from this promoter in transactivation assays indicate a potential *in vivo* role for NUDR in the regulation of the *hnRNP A2/B1* gene. hnRNP A2/B1 mRNA and protein have been shown to display dynamic patterns of expression during mammalian lung development, with highest levels in primitive alveoli, and lowest levels in mature lung (58). Although low levels of hnRNP A2/B1 protein were detected in normal bronchial epithelium, elevated levels of protein were detected in a variety of lung cancer cell lines (15). hnRNP A2/B1 has been proposed as an early marker in the detection of lung cancer (14–17), and the results of ongoing clinical trials have shown that up-regulation of hnRNP A2/B1 expression can accurately predict the subsequent development of lung cancer (17, 59, 60). Based on our data, we would predict that deregu-

lation of NUDR expression and/or mutations in NUDR that inactivated the DNA binding or repressor activities could contribute to the higher expression levels of hnRNP A2/B1 found in some cancers.

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