Identification of a Nuclear Respiratory Factor-1 Binding Site within the Core Promoter of the human polio virus receptor/CD155 Gene*

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In this report we describe a cis-acting element within the core promoter of the CD155 gene specifying the polio virus receptor that is bound by the nuclear respiratory factor-1 (NRF-1) transcription factor. DNase I footprint analysis identified a nuclear protein binding site from −282 to −264 nucleotides upstream of the translation initiation codon of the CD155 gene, which we have called foot print IV (FPIV). Linker scanning mutagenesis revealed that a tandem repeat motif, GGCAGGCGGCA, located within FPIV was essential for basal activity of the CD155 core promoter. The results of the electrophoretic mobility shift assay experiments suggested that identical FPIV binding activities were present in a variety of nuclear extracts and that the tandem repeat was essential for binding. A one-hybrid screen was then carried out using FPIV as bait to clone the cDNA of the FPIV binding factor. The sequences of the cDNAs that were cloned from the screen were identical to NRF-1, a result that was confirmed by further electrophoretic mobility shift assay experiments. Overexpression of full-length NRF-1 and a dominant-negative form of NRF-1 modulated reporter gene expression driven by the core promoter. Remarkably, CD155 is the first gene shown to be regulated by NRF-1 that possesses an expression profile during embryogenesis correlating with this factor’s proposed role in the development of the vertebrate optic system. We propose that NRF-1, which has been shown by others to be expressed during embryogenesis in animal systems, may be involved in regulating the expression of CD155 at specific stages of central nervous system development.

The human polio virus receptor protein, which has recently been given the designation CD155, is a highly glycosylated 80-kDa type Ia single pass transmembrane cell surface protein that belongs to the immunoglobulin superfamily (1–5). CD155 was originally cloned based on its ability to serve as the cellular receptor for poliovirus (6) (reviewed in Refs. 6 and 7). CD155 belongs to a subgroup of genes within the immunoglobulin superfamily; these genes share a V-C2-C2 domain structure as well as primary sequence identity. Thus far, cDNAs have been cloned that encode two new human molecules related to CD155, named PRR1 and PRR2 (polio virus receptor related) (8, 9). Other CD155-related proteins possessing the V-C2-C2 immunoglobulin domain structure appear to be relatively conserved during evolution (10). Koike et al. (10) have shown that African green monkeys possess polio virus receptors encoded by two related but distinct genes AGMo1 and AGMo2 (African Green Monkey receptor). Two genes, mPRR2, also known as MPH (mouse polio virus receptor homologue), and Tage4 that are related to CD155, also exist in the mouse (11–13).

Members of the new CD155 gene family have emerged as cellular receptors for animal viruses. As pointed out earlier, CD155 is the receptor for all three serotypes of the polio virus. There is no available evidence for an alternative receptor except, perhaps, for mouse-adapted polio viruses (14). hPRR1 has recently been identified as a receptor for the α-herpes viruses (15, 16), and hPRR2 and mPRR2 have been identified as receptors for the pseudo-rabies virus (17).

The biological importance of CD155 and its relatives has slowly begun to be elucidated. CD155, as well as mPRR2, are expressed during embryogenesis. Recently, immunohistochemistry was used to determine that CD155 is expressed in regions of the developing human central nervous system, such as the notochord, floor plate, neural tube and the optic system. Surprisingly, in situ hybridization analysis of mPRR2 mRNA indicated that this gene was also expressed in these structures of embryonic mice. Many cell adhesion molecules belonging to the immunoglobulin superfamily important for the development of the CNS have been expressed during embryogenesis in the floor plate and optic system (19–21). Recently, it has been reported that both mPRR2 (22) and hPRR2 (23) possess activity as homotypic adhesion molecules. In addition, cell adhesion activity has also been demonstrated for hPRR1 (24). This may suggest that some of the members of the CD155 gene family may be adhesion molecules involved in the development of the CNS.

We have cloned the promoter region of the CD155 gene (25). Our initial analyses have mapped a 280-bp core promoter fragment that is high in GC nucleotide content, lacks TATA and CAAT boxes, harbors a region of multiple transcriptional start sites, and appears to contain determinants required for cell type-specific promoter activity (25, 26). Indeed, we have used a transgenic mouse system to determine that the 3.0-kilobase

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2 M. Gromeier and E. Wimmer, unpublished results.

3 The abbreviations used are: CNS, central nervous system; bp, base pairs; AP, activator protein; NRF, nuclear respiratory factor; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; DTT, dithiothreitol; RT, reverse transcriptase; E, embryonic day; FPIV, footprint IV; TBE, Tris/borate/EDTA.
CD155 promoter is capable of directing reporter gene expression to the appropriate anatomical structures where CD155 is expressed during embryogenesis. We have been interested in identifying the cis-acting elements and trans-acting factors that could potentially play a role in the tissue-specific activity of the CD155 promoter. Moreover, we have hoped that our studies would identify target elements or factors that could be involved in orchestrating the activity of the CD155 promoter during embryonic development. We have recently reported the presence of three cis-acting elements within the CD155 core promoter region referred to as FPI, FPII, and FPIII (25). Members of the activator protein-2 (AP-2) transcription factor have been found to bind to FPI and FPII and are able to activate reporter gene expression driven by the CD155 core promoter.

Here, we report the characterization of a fourth cis-acting element (FPIV) within the CD155 core promoter that is essential for basal promoter activity. This element is bound by a nuclear protein that is present in the nuclear extracts prepared from murine embryos of gestational stages where the CD155 promoter is active in vivo. We have determined that the transcription factor is nuclear respiratory factor-1 (NRF-1). NRF-1, a regulatory protein present in the nuclear extracts of murine embryos and many established cell lines, binds to FPIV and functions as a potent transcriptional activator of the CD155 core promoter. Interestingly, NRF-1 belongs to a family of developmentally expressed transcription factors (27). In view of these observations, we will discuss the potential significance of the NRF-1/FPIV interaction relative to the in vivo activity of the CD155 promoter.

**Materials and Methods**

**Cell Culture**

The HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), HEK293 (embryonic kidney), SK-N-MC (neuroblastoma), HTB15 (glioblastoma), and Ntera-2/clone D1 (teratocarcinoma) cell lines were grown in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. The HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), and Ntera-2/clone D1 (teratocarcinoma) cell lines were maintained in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum.

**Nuclear Extract Preparation and DNAse I Footprint Assays**

Mouse embryonic nuclear extracts were prepared using a modification of the method of Tamura et al. (28). Briefly, embryos of defined gestational age were harvested and homogenized in a solution containing 10 mM Hepes, 15 mM KCl, 1 mM EDTA, 2.2 mM sucrose, and 5% glycerol. The homogenate was layered over a solution containing 10 mM Hepes, 15 mM KCl, 1 mM EDTA, 2.0 mM sucrose, and 10% glycerol and centrifuged at 24,000 rpm for 1 h. The resulting pellet of nuclei was harvested and resuspended in a solution containing 10 mM Hepes, 550 mM KCl, 0.1 mM EDTA, 3 mM MgCl₂, and 10% glycerol to lyse the nuclei and strip the chromatin of DNA-binding proteins. After the chromatin was pelleted by centrifugation at 40,000 rpm for 1 h, the supernatant containing the nuclear proteins was harvested and concentrated by ammonium sulfate precipitation. Nuclear extracts from human cell lines for use in EMSA experiments were prepared according to the procedure by Schreiber et al. (29). DNase I footprinting was performed as described previously (25). Briefly, end-labeled DNA probes were generated via the polymerase chain reaction (PCR), using an oligonucleotide primer carrying a 5’-terminal [γ-32P]ATP label. PCR was performed under standard conditions using 10 ng of pGGL2-H template, 25 pmol of labeled and unlabeled primers, and 1.2 units Taq polymerase. Radiolabeled PCR products were subjected to electrophoresis on a 10% native polyacrylamide gel; the bands were visualized by autoradiography, and a selected band was excised from the gel and passively eluted.

**Transfection and Harvest of Cells for Dual Luciferase Assays**

All cell lines were transfected by the calcium phosphate procedure. Each transfection mixture for the linker scan series of constructs was composed of 18 μg of wild type or mutant BE reporter constructs and 1 μg of pRl-TK (standard to the measure of efficiency of transfection). The compositions of the co-transfection experiments was 9.0 μg of the BE or BE ΔTR, mixed with up to 1.5 μg of pcDNA3(NRF-1). Co-transfections were supplemented with empty pcDNA3 to keep the amount of backbone plasmid constant for each experiment. 50 μg of 2.5 mM CaCl₂ was added to the DNA mixtures that were subsequently diluted to a total volume of 500 μl with Tris/EDTA buffer. These solutions were then separately combined dropwise with 500 μl of ice-cold 2x HBSS and incubated ten minutes at room temperature. Half of the precipitates were then added to a separate 6-cm plate of tissue culture cells (~10⁶ cells), and the plates were incubated at 37 °C. 4 h later the medium was removed, and a solution of 20% glycerol in HBSS added. Following a 3-min incubation at 37 °C, 3 ml of medium was added and the supernatant was removed again and replaced by fresh medium with serum. All transfected cells were harvested 18 h post-transfection, and cell extracts (usually 200–400 μl) were made using the reporter lysis buffer from Promega.

**Electrophoretic Mobility Shift Assays**

The oligodeoxynucleotides used for EMSA were: FPIVs, 5’-GGGAC-TCGGCGAGGCGAGGCGG-3’; FPIVas, 5’-GGCACCGTCGGCCGCTCAGT-3’; IV (1), 5’-CCGCGCGCTGACTAGTCGCAGGTCTCTC-3’; and ΔTR, 5’-GGCCTCCCCGGCGAGGTGCTCTCCGGCGG-3’. (30).

**Oligo Association—** One nmol of each dye and noncoding oligodeoxynucleotide were reassociated in a volume of 50 μl using a thermocycler. Settings were 5 min at 95 °C and 1 h each at 65 °C, 60 °C, 55 °C, 50 °C, 45 °C, and 40 °C. The oligodeoxynucleotides were allowed to form aggregates in this temperature sequence.

**Labeling—** Ten pmol of reassociated oligodeoxynucleotide was end-labeled by a fill in reaction using CombiPol Polymerase (InViTek). In a volume of 20 μl, the buffer, 0.5 μl of enzyme, 50 μCi of [α-32P]dCTP, 1 μl of 25 mM MgCl₂, and the oligodeoxynucleotide were incubated at 40 °C for 10 min, 45 °C for 10 min, and 50 °C for 20 min. The labeled oligodeoxynucleotide was purified by Sephadex G50 chromatography (Nick columns, Amersham Pharmacia Biotech). Usually more than 50% of label were found to be incorporated into the oligodeoxynucleotide.

**Shift Assay—** A binding reaction containing 1 μl of 10x incubation buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 5 mM DTT, 25% glycerol), 1.5 μg of poly(dI-dC)(dI-dC), various concentrations of competitor or antibody (goat anti-NRF-1 and rabbit anti-Beclin 1 were the kind gift of Richard Scarpulla), and 4 μl of cell extract was prepared and preincubated for 10 min at room temperature. After preincubation 1 μl of labeled oligodeoxynucleotide corresponding to 100 fmol were added, and the incubation continued for another 20 min. Samples were loaded onto a 6% 12% TBE polyacrylamide gel. After the electrophoresis (200 V) the gel was fixed in 10% acetic acid/30% methanol for 30 min and dried.
RNA Isolation, RT-PCR, Cloning of NRF-1 Expression Vectors, and In Vitro Transcription/Translation

Total cellular RNA for use in RT-PCR was isolated from 3 × 107 SK-N-MC or HeLa cells according to the TRIzol protocol (Life Technologies). The identity of the cloned insert was verified by sequence analysis. The four tandem repeats were then subcloned into the yeast integration vectors pHISi-1 and pLacZi. These resulting vectors, p4xFPV-YES1-1 and pl4xFPV-LacZi, were then linearized, transformed sequentially into the YM4271 yeast strain, and grown on the proper medium to select for colonies harboring the plasmids that had recombined in their proper genetic loci. The resulting doubly integrated yeast strain, YM4271 (His, LacZ; 4xFPV), was then streaked onto the above amplification conditions. The full-length product was cut out and the products were separated on a 1.0% agarose gel. The full-length product was then cloned by amplifying the full-length cDNA with the NRF-1 RT, 5′-NTR, and 5′-portion of the CD155 promoter. The resulting library clones that encoded proteins that could bind to the 4xFPV-target element were subjected to a series of linker scan mutations from the library plasmids from the expression of the luciferase reporter gene was determined. Strikingly, an 80% reduction of promoter activity was observed when the 50 bp in the vicinity of the border construct were removed (Fig. 1C) or replaced with the HeLa and Hep-2 cell lines, and its ability to direct the expression of the luciferase reporter gene was determined. Therefore, nuclear extracts of embryos from the E10.5–E14.5 gestational stages could conceivably contain the full complement of trans-acting factors required to produce this expression pattern. Because our main aim was to identify transcription factors that could potentially contribute toward the in vivo activity of the CD155 promoter, we used nuclear extracts prepared from E10.5 murine embryos for DNase I footprint analysis. When footprinting experiments were performed with 75 μg of extract, a single protected region, called FPIV, was observed that was located from −282 to −264 base pairs upstream of the initiator ATG of the CD155 gene (see Fig. 2, for relative locations see Fig. 1A, B). Interestingly, FPIV is located within a 280-bp genomic DNA fragment, named BE (see the borders of the BE fragment, Fig. 1A), that harbors full ability to direct the expression of a reporter gene when transfected into tissue culture cell lines that naturally express CD155 (25, 26). Serial deletion analysis of the CD155 promoter was used in our original experiments to map the functional boundaries of the BE fragment (26). The exact 5′-borders of two of the serial deletion constructs, named B and C, are shown in Fig. 1, A and B. The promoter activity of the C construct has been found to be greatly reduced when compared with that of B (26), an observation suggesting the existence of a fourth cis-acting element within this area of the CD155 promoter. To investigate this possibility further, we utilized two BssHII restriction sites that flank the B–C region to generate a finite scale deletion within this area (for location of the BssHII restriction sites see Fig. 1B). The ΔBssHII construct was transiently transfected into the HeLa and Hep-2 cell lines, and its ability to direct the expression of the luciferase reporter gene was determined. Serial deletion analysis of the CD155 promoter was used in our original experiments to map the functional boundaries of the BE fragment (26). The exact 5′-borders of two of the serial deletion constructs, named B and C, are shown in Fig. 1, A and B. 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When footprinting experiments were carried out using nuclear extracts prepared from human cell lines, a partial footprint was also observed overlapping with that produced from E10.5 extract (data not shown). These results suggest that a nuclear protein binding site exists within the 5′ of the CD155 core promoter. Several data base searches for transcription factor binding motifs using the FPIV region yielded no significant homology to known transcription factor binding sites. However, a 12-base pair tandem repeat sequence, GCGCAGGCGCAG, showed significant homology to identical motifs found in a variety of other human promoters (data not shown). Linker Scanning Mutagenesis of the FPIV Region—The DNase I footprint experiment (Fig. 2) identified a segment of the CD155 upstream sequence that is likely to harbor a cis-element required for promoter function. To more precisely map this element, a series of linker scan mutations was generated.
transfections were 103,870 for HEp-2 and 73,153 for HeLa. The activity of the construct was transfected into the HeLa and HEp-2 cell lines. Reporter gene expression of the wild type BE fragment was normalized to 100%, and the activity of the ΔBssHII deletion construct was expressed relative to that level. The average Renilla luciferase corrected RLU values of these transfections were 103,870 for HEp-2 and 73,153 for HeLa.

throughout this protected sequence. Each mutant promoter construct contained 6 base pairs of wild type CD155 promoter sequence replaced by a SpeI restriction enzyme site within the context of the BE fragment (for locations see Fig. 1B). The panel of mutant promoter constructs was transfected into the HEp-2, HEp-2, SK-N-MC, and HTB15 cell lines (Fig. 3). Of the three linker replacement mutations, the IV (3) mutant construct displayed a greater than 80% reduction in promoter activity in all the cell lines tested. This result indicated that this mutation disrupts a sequence required for the basal activity of the CD155 core promoter. Interestingly, the IV (3) mutation is located in the center of the tandem repeat motif mentioned above (GCGCAGGCGCAG to GCGactagtCAG, see Fig. 1B) that we found by homology searching to be contained in other human promoters. We also generated a construct that lacked this 12-base pair motif. When this construct, called ΔTR, was transfected into the cell lines, the reporter gene activity was as severely reduced as in the IV (3) mutant promoter (see Fig. 3). We conclude that a functional cis-acting element resides within boundaries of the FPIV region. Linker scanning and fine scale deletion mutations suggest that a 12-base pair tandem repeat motif is likely to represent this important regulatory element.

**EMSA Analysis of FPIV Binding Activity**—To further study protein binding to FPIV, we performed EMSA experiments. For these studies, nuclear extracts of human cell lines were used to analyze the characteristics of the FPIV binding activities. All the nuclear extracts tested possessed several binding activities for the FPIV probe (see Fig. 4A, lanes 2–10). Competition analysis using a 250-fold molar excess of unlabeled FPIV probes (containing either a wild type or mutated tandem repeat sequence) was used to dissect which of the observed complexes were specifically binding to the tandem repeat motif. One set of the complexes showed a competition profile expected for a nuclear protein binding to the FPIV probe (see Fig. 4A, complex marked with arrows). The addition of a 250-fold molar excess of the wild type cold competitor reduced the intensity of this complex, whereas the addition of an equal amount of a FPIV probe that harbored the IV (3) linker mutation did not. Interestingly, this specific complex was present in all of the cell line nuclear extracts tested. When electrophoresed for a longer time, this complex resolved into a doublet of two closely migrating bands (see Fig. 5A).

The combination of EMSA experiments and our genetic analyses suggested that we had identified an as yet unknown transcription factor or factors required for the activity of the CD155 core promoter in tissue culture cell lines. Nuclear extracts prepared from embryos of the E10.5, E12.5, and E14.5
gestational stages possessed FPIV binding activities that appeared to have a similar mobility to those observed with those from human cell lines (see Fig. 4A, lanes 11–19). Importantly, these complexes also shared identical competition characteristics when exposed to a 250-fold molar excess of the wild type or mutant FPIV probes. These data suggest that murine embryo nuclear extracts possess FPIV binding activity and that this binding activity is identical in migration and competition characteristics to those observed in human cell lines. This result represents an important link between our genetic and biochemical analyses of the core promoter in tissue culture, on one

hand, and CD155 promoter activity in vivo, on the other. The mutations within the FPIV cis-acting element greatly reduce reporter gene expression indicating that the transcription factor or factors interacting with this region are required for the basal activity of the CD155 promoter. Because this binding activity is present in embryonic nuclear extracts, we conclude that this factor may play an important role in the regulation of CD155 promoter activity during embryogenesis.

A One-hybrid Screen Identifies Nuclear Respiratory Factor-1 Binding to FPIV—We next attempted to determine the identity of the protein(s) that could bind to FPIV. To accomplish this task, we used the yeast one-hybrid system, a method suitable to isolate the cDNAs of a DNA-binding protein whose binding site has been characterized by biochemical methods. Briefly, a yeast strain was generated where the expression of the HIS3 and LacZ reporter genes was placed under control promoters in which the essential upstream activator site was replaced by four tandem repeats of the FPIV sequence (for details see “Materials and Methods”). In the absence of protein binding to the synthetic upstream activator site, the promoter controlling the expression of the reporter was inactive (data not shown). The reporter strain was then transformed with a library consisting of cDNAs from an E9/E10.5 mouse embryo fused to the HSV VP16 activation domain. Any cDNA that possessed an open reading frame encoding a protein with the capability to bind FPIV would lead to the activation of the HIS3 and LacZ genes. The one-hybrid screen yielded seven His3+ and LacZ+ colonies (data not shown) that were candidates to harbor cDNAs encoding DNA-binding protein(s) with FPIV binding activity. The library plasmids of these seven colonies were isolated, transformed into E. coli DH5α cells, and produced in bulk for sequencing of the cDNA inserts. The inserts of those four colonies that were most robustly positive for LacZ expression during the library screen all contained overlapping regions of an identical sequence (data not shown). This sequence was then entered into BLAST (31) searches to determine if it harbored homology with any known open reading frames. The

![DIAGRAM](http://www.jbc.org/)
results of BLAST homology searches indicated that the sequences of the cDNA inserts were identical to the DNA binding domain of murine NRF-1. This strongly suggested that NRF-1 might indeed represent a FPIV-binding protein. The optimal binding site of NRF-1 has been determined to be GCGCAT-GCGCAG (32), which differs by only one base pair from the tandem repeat sequence of FPIV. As we had mentioned earlier, the tandem repeat sequence of FPIV was identical to motifs in other human promoters. Indeed, NRF-1 has been shown to bind to motifs in the promoters of the human ubiquinone-binding protein and GPAT-AIRC, two sequences that are identical to our FPIV tandem repeat (33, 34).

Confirmation of NRF-1 Binding to FPIV—To biochemically corroborate the result of our yeast one-hybrid experiment, the full-length human NRF-1 cDNA was cloned by RT-PCR (see “Material and Methods”). The NRF-1 cDNA was subcloned into a mammalian expression vector that also contained a phage T7 promoter for in vitro transcription by T7 RNA polymerase. This cDNA was then used to program an in vitro transcription/translation system to produce NRF-1 protein for EMSA experiments. Indeed, the NRF-1 cDNA produced binding complexes with a FPIV probe that migrated in a manner identical to the native FPIV binding activity in HeLa nuclear extracts (see Fig. 5A, compare lanes 2 and 5). Interestingly, the in vitro translated NRF-1 protein produced both bands of the doublet that we had previously observed, a result suggesting both complexes contain NRF-1. The reason for the formation of the doublet bond is as yet unknown but appears to relate to post translational protein modification. In vitro translated NRF-1 protein also displayed competition characteristics identical to the native FPIV binding activity of HeLa nuclear extract when exposed to a 250-fold molar excess of cold wild type and mutant FPIV probes (see Fig. 5A, compare lanes 3 and 4 to lanes 6 and 7). Experiments with radiolabeled mutant FPIV competitors as probes for EMSA corroborated these results. Neither the in vitro translated NRF-1 nor the native binding activity present...
in nuclear extracts could bind to probes that possessed alterations in the GGCGAGGCCGAG motif (data not shown).

Further evidence supporting the hypothesis that NRF-1 binds to FPIV was obtained by supershift analysis. Anti-NRF-1 polyclonal serum was added to EMSA binding reactions, and the resulting complexes were then resolved by native polyacrylamide gel electrophoresis (see Fig. 5B). Both bands of the doublet were supershifted by the addition of the antiserum, an observation indicating that both complexes were antigenically related to NRF-1 (Fig. 5B, see lanes 3, 5, 7, 9, 11, and 13). Taken together the results of our competition and supershift experiments provide direct biochemical evidence confirming the findings from our one-hybrid system screen.

The Importance of the NRF-1 Interaction for CD155 Promoter Function—Our results suggest that NRF-1 binds to the CD155 promoter and that binding is detectable in cell lines and murine embryos where the CD155 promoter is active. We next wanted to determine the functional significance of this interaction on core promoter activity. Our first attempt to address this question was to overexpress the NRF-1 transcription factor in the presence of the core promoter in co-transfection experiments. In these experiments the BE construct was transfected with 750 ng of an NRF-1 expression vector into the HepG2, HTB15, SK-N-MC and Ntera-2 cell lines. Remarkably, overexpression of NRF-1 stimulated CD155 core promoter activity 3–5-fold in all four of the cell lines tested (see Fig. 6B). The ΔTR mutant construct was also co-transfected with 750 ng of NRF-1 expression vector and was activated to a much lower extent by the overexpression than that we had observed for the wild type BE construct (see Fig. 6B). These results confirm the interaction of NRF-1 with the CD155 core promoter. Moreover, they suggest that NRF-1 is a potent activator of CD155 promoter activity and that full activation of the core promoter requires an intact NRF-1 binding site.

We also investigated the requirement for NRF-1 on core promoter activity using a dominant-negative approach. The first 304 amino acids of NRF-1 have been proposed to comprise the DNA binding, dimerization, and nuclear localization signal domains, but they lack the bipartite hydrophobic activation domains of this protein (see Fig. 6A). Virbasius et al. (27) and Gómez-Cuadrado et al. (35) have shown that this N-terminal portion of NRF-1 possesses functional DNA binding properties. Therefore the NRF-1 protein that lacks the C terminus could display a dominant-negative activity if it were to competitively inhibit the wild type protein from binding to a NRF-1 binding site. Indeed, Gugneja et al. (36) have shown that overexpression of this portion of NRF-1 could inhibit luciferase expression driven by four tandem repeats of an NRF-1 binding site. We transfected the BE construct with an increasing amount of a vector expressing this putative dominant-negative NRF-1 into the HepG2, HTB15, and SK-N-MC cell lines. Overexpression of the truncated NRF-1 decreased CD155 core promoter activity in a dose-dependent manner in the three cell lines tested to a level 50–60% of wild type activity (see Fig. 6C). These results suggest that the truncated NRF-1 protein used in this set of experiments exerts a dominant-negative activity, a finding that may be of use in the elucidation of the biological functions of the NRF-1 transcription factor. In addition, they support our hypothesis that the binding of NRF-1 is needed for optimal activity of the CD155 core promoter. These data are in agreement with the activities of our FPIV linker scan mutation constructs, which lack NRF-1 binding to FPIV.

CONCLUSIONS

We have uncovered a new genetic element of the CD155 promoter that, together with the previously identified AP-2-responsive elements (25), contributes to the control of the expression of CD155 polypeptides. These CD155 proteins of which the splice variants CD155a and CD155b serve as receptors for polio virus in humans (1, 2, 7) belong to a new group of Ig-like polypeptides with the general extracellular structure V-C2-C2. These proteins are found in humans, monkeys and rodents.1 A protein named hPRR1 (8), which is related to CD155, has recently been shown to function as a receptor for α-herpes viruses (15, 16). The viral receptor functions of CD155 and of the other CD155-related polypeptides is the best understood aspect of the biology of these molecules, whereas little is known about their nonpathogenic functions. It has recently come to light that both the PRR1 and PRR2 relatives of CD155 possess cell adhesion activity. However, the function(s) of CD155 is unknown. Our studies that have focused on the mechanism of regulation of expression of the CD155 gene are also aimed at gaining insight into the nonpathologic function of the CD155 polypeptide.

CD155 is expressed in the developing human CNS; the anatomical location of expression includes the notochord, floor plate, neural tube, and optic system.1 Furthermore, making use of transgenic mice expressing a reporter gene, we have recently shown that the CD155 promoter is active during embryogenesis.1 In this system, the CD155 promoter directed the expression of β-galactosidase to regions of the developing CNS that midgestation that matched the authentic expression of the CD155 protein. Interestingly, the expression was observed only between E10 and E12.5 postconception, and it subsequently disappeared to an undetectable level.1 This expression profile of CD155 is strikingly similar to the expression of other molecules of the immunoglobulin superfamily that are known to be cell adhesion molecules with functions in the developing CNS (19–21). Therefore, given the fact that other members of the CD155-related gene family are cell adhesion molecules, it is conceivable that CD155 may possess similar functions. This possibility is now being investigated in our laboratory.

Of special interest to us were cis-acting elements and trans-acting factors that could participate in the tissue-specific activity of the CD155 promoter. The core promoter region of the CD155 gene harbors basal and cell type-specific activities (26). In the past, we have characterized three cis-acting elements (FPI, -II, and -III) that are located within the core promoter that are likely to be involved in the regulation of expression of the CD155 gene (25). Two of these elements (FPI and FPII) are bound by the developmentally expressed protein AP-2, a transcription factor that has been described to participate in the regulation of gene expression in the developing lens, retinal ganglion cell layer, and neural tube (37–39). The developmentally regulated expression of AP-2 overlaps temporally and spatially with that of CD155, an observation we interpret to suggest that the AP-2 transcription factors may influence the CD155 expression profile during embryogenesis.

Further studies have now revealed that reporter gene expression was lost upon deletion of sequences in the 5′-terminal part of the core promoter (constructs B, C, and ΔBsaIII, see Fig. 1, A and B). This suggested the presence of a fourth cis-acting element within the first 50 bp of the core promoter that was essential for basal activity. A DNase I footprint, called FPIV, was detected within this region –282 to –264 nucleotides upstream of the ATG translation initiation codon of the CD155 gene. It was of particular interest that FPIV could also be readily detected when footprinting experiments were carried with nuclear extracts prepared from E10.5 murine embryos. This observation suggested that a trans-acting factor existed in the extract prepared during this phase of embryogenesis and that this factor could interact with FPIV of the core promoter. Mutagenesis of FPIV identified a 12-base pair tandem repeat,
The overexpression of full-length and dominant-negative NRF-1 modulates reporter gene expression driven by the CD155 core promoter. 

A, domain structure of NRF-1 proteins used in overexpression studies. The full-length NRF-1 protein contains 503 amino acids (aa). The N terminus of NRF-1 harbors DNA binding, dimerization, and nuclear localization signal domains, whereas the C terminus harbors the bipartite hydrophobic activation domain. The putative dominant-negative dominant-negative NRF-1 used in these studies is comprised of only the first 304 amino acids of the NRF-1 protein.

B, effect of co-transfection of a full-length NRF-1 expression vector on the promoter activity of the wild type and a FPIV mutant BE promoter construct in the HepG2, SK-N-MC, HTB 15, and Ntera-2 cell lines. Each cell line was seeded in 6-well tissue culture plates and were transfected by the calcium phosphate method with 4.5 μg of the BE or ΔTR promoter construct with 750 ng of pcDNA3(NRF-1). Transfections were filled in with pcDNA3 to keep the amount of expression vector backbone in each reaction at a constant level for all experiments. Transfected cells were harvested 18 h post-transfection and the luciferase activity contained within the cytoplasmic extract of transfected cells was determined using the luciferase reporter system (Promega). The activity of the promoter construct co-transfected with only pcDNA3 was set to 100% (control promoter activity) and the level of activation caused by co-transfected of pcDNA3(NRF-1) is expressed relative to that 100%. The absolute level of reporter gene activities directed by the BE and ΔTR promoters was as described in Fig. 3. Results are the mean ± S.D. of triplicate transfections for HepG2, SK-N-MC, HTB 15, and Ntera-2 cell lines. Each cell line was seeded in a 6-well tissue culture plate and was transfected by the calcium phosphate method with 4.5 μg of the BE and up to 2.0 μg of pcDNA3ΔNRF-1. Transfected cells were harvested 18 h post-transfection, and the luciferase activity contained within the cytoplasmic extract of transfected cells was determined using the luciferase reporter system (Promega). The activity of the BE construct co-transfected with only pcDNA3 was set to 100% (control promoter activity), whereas the activity of each construct co-transfected with the indicated amount of pcDNA3ΔNRF-1 is expressed relative to that 100%. Results are the mean ± S.D. of triplicate transfections. The average Renilla luciferase corrected RLU values of the BE core promoter fragment in these sets of transfections was 137,128 for HEp-2, 95,501 for SK-N-MC, and 581,303 for HTB15.

Fig. 6. The overexpression of full-length and dominant-negative NRF-1 modulates reporter gene expression driven by the CD155 core promoter. A, domain structure of NRF-1 proteins used in overexpression studies. The full-length NRF-1 protein contains 503 amino acids (aa). The N terminus of NRF-1 harbors DNA binding, dimerization, and nuclear localization signal domains, whereas the C terminus harbors the bipartite hydrophobic activation domain. The putative dominant-negative dominant-negative NRF-1 used in these studies is comprised of only the first 304 amino acids of the NRF-1 protein. B, effect of co-transfection of a full-length NRF-1 expression vector on the promoter activity of the wild type and a FPIV mutant BE promoter construct in the HepG2, SK-N-MC, HTB 15, and Ntera-2 cell lines. Each cell line was seeded in 6-well tissue culture plates and were transfected by the calcium phosphate method with 4.5 μg of the BE or ΔTR promoter construct with 750 ng of pcDNA3(NRF-1). Transfections were filled in with pcDNA3 to keep the amount of expression vector backbone in each reaction at a constant level for all experiments. Transfected cells were harvested 18 h post-transfection and the luciferase activity contained within the cytoplasmic extract of transfected cells was determined using the luciferase reporter system (Promega). The activity of the promoter construct co-transfected with only pcDNA3 was set to 100% (control promoter activity) and the level of activation caused by co-transfected of pcDNA3(NRF-1) is expressed relative to that 100%. The absolute level of reporter gene activities directed by the BE and ΔTR promoters was as described in Fig. 3. Results are the mean ± S.D. of triplicate transfections for HepG2, SK-N-MC, HTB 15, and Ntera-2 cell lines. Each cell line was seeded in a 6-well tissue culture plate and was transfected by the calcium phosphate method with 4.5 μg of the BE and up to 2.0 μg of pcDNA3ΔNRF-1. Transfected cells were harvested 18 h post-transfection, and the luciferase activity contained within the cytoplasmic extract of transfected cells was determined using the luciferase reporter system (Promega). The activity of the BE construct co-transfected with only pcDNA3 was set to 100% (control promoter activity), whereas the activity of each construct co-transfected with the indicated amount of pcDNA3ΔNRF-1 is expressed relative to that 100%. Results are the mean ± S.D. of triplicate transfections. The average Renilla luciferase corrected RLU values of the BE core promoter fragment in these sets of transfections was 137,128 for HEp-2, 95,501 for SK-N-MC, and 581,303 for HTB15.
GGCGAGGCAG, required for basal promoter activity.

The essential role of the tandem repeat was then confirmed by EMSA analyses, using authentic probes or mutated derivatives thereof and a variety of extracts. All nuclear extracts that we tested, including those that were prepared from murine embryos, possessed identically shifted complexes that bound to the tandem repeat motif of FPIV (see Fig. 4A). These results suggested that the FPIV binding activity was present at times when the CD155 promoter is active during embryogenesis.

A yeast one-hybrid system screen of a mouse embryo cDNA library then revealed a protein that was identical in nucleotide sequence to the murine NRF-1 transcription factor. Indeed, EMSA experiments using in vitro translated human NRF-1 or anti-NRF-1 polyclonal antiserum confirmed that the authentic NRF-1 protein was indistinguishable from the native FPIV binding activities that we had observed in gel shift experiments.

Evans and Scarpulla (33) originally cloned NRF-1 as a factor that could bind to the rat somatic cytochrome c promoter. These authors have suggested that NRF-1 plays an integral role in the transcription of mitochondrial genes that are encoded in the nucleus. Subsequently, NRF-1 has been shown to regulate many other genes, such as the genes for eIF2α, tyrosine aminotransferase, chicken hiitone h5, and CXXC4. This suggests a wider range of genes can be regulated by this transcription factor than originally envisioned (32, 35, 40–42). Indeed, Virbasius et al. (27) and Gómez-Cuadrado et al. (35) have both proposed that NRF-1 may be more accurately described as a regulator of cell growth, when considering this wider range of promoters it appears to regulate. NRF-1 is a member of a family of transcription factors (32, 35, 43). NRF-1 homologues have been cloned from human, mouse, rat, and zebrafish (35, 43, 44). Two proteins related to NRF-1, called Erect Wing and P3A2, have been cloned from drosophila and sea urchin, respectively (18, 45), although, so far, only P3A2 and NRF-1 have been directly shown to be DNA-binding proteins. The highest region of homology between NRF-1, Erect Wing, and P3A2 is located in their putative DNA binding domains, which share ~54% amino acid identity. Interestingly, both Erect Wing and P3A2 are developmentally expressed, an observation suggesting that they function during embryogenesis (18, 45). Recently, the expression pattern and possible function during embryogenesis of a protein termed “not really finished” (Nrf), the zebrafish homologue to NRF-1, has been studied in detail (44). The Nrf protein of zebrafish shares 91% amino acid identity with the human NRF-1, an observation illustrating that this protein family is highly conserved during vertebrate evolution. Becker et al. (44) have suggested that Nrf plays a critical role in the development of the retina of zebrafish. This was based on a genetic analysis of zebrafish whose nrf gene was inactivated; zebrafish that were homozygous for retroviral insertion at the nrf locus displayed a larval lethal phenotype with perturbations in the formation of the neural retina during development. At early stages of wild type zebrafish development, Nrf RNA is expressed strongly in the developing CNS, most intensely in the optic system.

Later in zebrafish development, Nrf RNA is expressed in the retinal ganglion cell layer, optic nerve, and optic tract regions of the developing eye (44). Genetic inactivation of the nrf locus inhibits Nrf RNA expression in mutant zebrafish leading to increased apoptosis in the retina and optic tectum. This, in turn results in the disruption of retinal formation. If mammalian NRF-1 RNA shares a similar expression profile with its zebrafish homologue, the report of Becker et al. (44) is highly informative for our finding that the CD155 promoter is regulated by this factor. The expression profile of zebrafish Nrf suggests its function in development is to regulate the expression of genes required for proper retinal development. The CD155 promoter is active in exactly these anatomical structures, suggesting that potential regulation by NRF-1 could play a role in directing promoter activity to these locations. Interestingly, Erect Wing is expressed in all developing neurons of drosophila embryos, an observation suggesting that expression in the CNS may also be an evolutionary conserved feature among NRF-1-related proteins (18).

The cell type- and tissue-specific activity of the CD155 promoter in transgenic embryos indicates that this promoter fragment harbors cis-acting elements specific for directing gene expression to a select subset of structures within the developing CNS. The CD155 promoter can be used as a model for understanding the mechanism of gene expression in the above mentioned embryonic structures. Our studies have elucidated some of the cis-elements of the CD155 promoter and have revealed the nature of some of the trans-acting factors, which could exert affects on the CD155 expression pattern. Concomitantly, a picture is emerging of how these factors may cooperate to orchestrate CD155 promoter activity, especially during embryogenesis. Therefore, it is of particular interest to learn about the significance of FPIV and the identification of its putative trans-acting binding partner(s). This problem is currently under investigation. We hope to build on our genetic analyses of this promoter by generating additional transgenic mouse lines where reporter gene expression is directed by mutant CD155 promoter fragments. These in vivo experiments should lead to an improved understanding of the roles that the NRF-1 and AP-2 transcription factors play in the transcriptional regulation of CD155 expression.

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Identification of a Nuclear Respiratory Factor-1 Binding Site within the Core Promoter of the human polio virus receptor/CD155 Gene

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