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Characterization of the Nonmuscle Myosin Heavy Chain IIB Promoter: Regulation by E2F

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To identify DNA sequences important for the transcriptional regulation of the nonmuscle myosin heavy chain IIB (NMMHC-IIB) gene we isolated and sequenced genomic clones that contain the promoter of the gene for both human and mouse. In addition to considerable homology in the first (untranslated) exon (91%) we found 80% sequence identity in the 700 base pairs immediately upstream of the major start of transcription (+1) as well as significant homologies as far as 1500 base pairs upstream. The promoter region was characterized using luciferase reporter constructs transiently transfected into NIH3T3 cells. Consensus binding sites for several known transcription factors are present that are completely conserved between the mouse and human genes, including CRE/ATF, Sp1, CAAT, and the cell-cycle transcription factor E2F. Gel shift assays indicated that E2F can bind to its putative binding site in vitro. To test whether this site is functional we cotransfected NMMHC-IIB promoter constructs driving luciferase with a vector expressing E2F-1.The E2F-1 vector stimulated luciferase activity from an intact promoter whereas mutation of the site eliminates binding and diminishes transactivation. These data provide strong evidence that E2F or an E2F-related transcription factor is involved in the regulation of nonmuscle myosin expression.

Promoter Nonmuscle myosin E2F transcription factor Transactivation

MYOSINS comprise a large family of cytoskeletal proteins found in all organisms and cell types examined. Myosins involved in muscle contraction belong to the myosin II class of conventional myosins and consist of a pair of heavy chains (200 kDa) and two pairs of nonidentical light chains (15-20 kDa) (60). These two-headed molecules form bipolar filaments that generate contractile force through ATP hydrolysis and their interaction with actin filaments. Vertebrate cells also contain a diverse array of unconventional (apparently nonfilamentous) myosins that have been classified as myosins I and myosins III to IX (2). Conventional myosins, however, are also found in nonmuscle cells and these molecules are thought to be required for a number of contractile functions inside cells. These so-called nonmuscle myosins have been implicated in such cellular contractile processes as cytokinesis, morphogenetic changes, capping of surface receptors, cell motility, and secretion (54,61). Myosin II is likely to be responsible for constriction of the contractile ring by a sliding filament mechanism, like that which drives muscle contraction (48). Experiments performed in *Dictyostelium* demonstrated that disruption of the NMMHC gene (6) or inactivation of the gene by antisense RNA (25) produced cells that were defective in cytokinesis. Antibodies to myosin microinjected into echinoderms were also found to block cytokinesis (24,32). Conventional nonmuscle myosin has been shown to be essential in *Drosophila*. A mutation in the nonmuscle myosin regulatory light chain blocks cytokinesis (18) and embryos that lack NMMHC also have severe

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defects of morphogenesis (62). Inhibition of NMMHC with antisense oligonucleotides in cultures of rat aortic smooth muscle cells suppressed cell proliferation (52).

Evidence from cDNA cloning (45,53,58) indicates that there are at least two major isoforms of conventional myosin that contain heavy chains derived from different genes. Genes for nonmuscle myosin heavy chain types IIA and IIB (NMMHC-IIA and NMMHC-IIB) were mapped to human chromosomes 22 and 17, respectively (45,53,58). Multiple subtypes for both NMMHC-IIA and NMMHC-IIB isoforms have been demonstrated that are also differentially expressed in different tissues and cell types (14,38,39,40,56). Both NMMHHC-IIA and NMMHC-IIB have been shown to be localized to the cleavage furrow of dividing human cultured cells (34). Intestine, spleen, and thymus contain mostly NMMHC-IIA compared with NMMHC-IIB whereas in brain and testes NMMHC-IIB is the dominant isoform, and these differences appear to be mediated by changes in the levels of the mRNAs encoding the two isoforms (20,22,53). Proliferation of vascular smooth muscle cells is associated with a major shift in myosin isoform distribution (23) and changes in nonmuscle/smooth muscle myosin content have been demonstrated in atherosclerotic rabbits (26,63). The significance of these changes in myosin isoforms is currently unclear, but such tissue and cell type-specific expression suggests that each isoform performs a distinct cellular function. In a previous study (27), we reported that the level of NMMHC-IIB mRNA is increased in restenotic lesions (following balloon angioplasty) compared with primary atherosclerotic plaque (i.e., not previously treated by percutaneous revascularization). To identify the transcriptional controls that regulate the expression of the myosin heavy chain isoforms it is necessary to clone and characterize the relevant gene promoters. The promoters of the human NMMHC-IIA gene (21) and the rabbit smooth muscle myosin heavy chain gene (1,19) have been previously described.

In this report we describe the cloning of the promoter region of the NMMHC-IIB gene. Because important regulatory sequences (e.g., DNA motifs that bind transcription factors) are usually evolutionarily conserved between species, we cloned both the human and mouse promoter sequences for comparative analysis. We established that this region has promoter activity by transfection experiments and determined the origin of transcription. Inspection of the NMMHC-IIB promoter sequence revealed a potential binding site for the E2F transcription factor that is completely conserved between mouse and human genes. E2F was originally identified as a DNA binding protein that mediated the transcriptional stimulation of the adenovirus early region 2 (E2), and has now been shown to be involved in the transcription of a number of cellular genes that are important for cell cycle control and proliferation (41). These include the c-*myb*, c-*myc*, and N-*myc*, proto-oncogenes, thymidine kinase, DNA polymerase a, dihydrofolate reductase, cdc2, and the retinoblastoma susceptibility gene (Rb) (3,9, 41,42,57). It is now known that E2F activity derives from a family of polypeptides each of which has different properties and is encoded by distinct genes. For example, E2F-1, E2F-2, and E2F-3 are inhibited by binding of Rb in vivo whereas E2F-4 and E2F-5 are not (29,47). E2F-4 and E2F-5 are apparently regulated by the Rb-related proteins p107 and p130 (8,47,59). Each member of the E2F family exhibits a unique pattern of expression with respect to the cell cycle. Thus, E2F1 mRNA is expressed in late G1 (17) whereas E2F-4, E2F-5, and DP-1 mRNA expression is maximal in mid-G1 phase before E2F-1 is detectable (47). In this article we present evidence that E2F is involved in the transcriptional regulation of the NMMHC-IIB gene. This finding supports the idea that NMMHC-IIB is required for cell proliferation.

MATERIALS AND METHODS

PCR Amplification of 5′ End of Human NMMHC-IIB mRNA

To obtain the 5′ sequence of human NMMHC-IIB mRNA, which was not previously available from screening of cDNA libraries, we used the procedure for rapid amplification of cDNA ends (RACE) (7) using a kit (Life Technologies) according to manufacturer's instructions. Poly(A)$^+$ RNA was isolated from K562 cells (a human erythroleukemia cell line) using the mRNA isolation system from Life Technologies. A specific primer for NMMHC-IIB from the known cDNA sequence (53) was used to make single-stranded cDNA. The sequence of this primer (T15) was 5′-ACGCTTCTTCCCTCTGTA-3′ (nucleotides 412–429 of the published sequence). The cDNA was tailed at the 3′ end with cytidine residues using terminal transferase. PCR amplification was performed using the Universal Anchor Primer (BRL) and a nested primer specific to NMMHC-IIB (T16) with the sequence 5′-GGCGAATT

CCTCCACCTTGGAAAACTTAG-3′ (nucleotides 233–253 plus an Eco RI linker). PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Initial amplification for 45 cycles yielded a smear of DNA around 400 bp. Two subsequent rounds of gel purification, dilution, and reamplification yielded a single band. The PCR product was subcloned into pCRII (Invitrogen) and sequenced. The insert was excised from the vector with Eco RI and purified by agarose gel electrophoresis for use as a hybridization probe.

Isolation of Genomic Clones

A human genomic library (#HL 1006d, Clontech Laboratories, Inc.) made in λ vector EMBL3 was screened with the 400 bp RACE probe using standard methods (46). Restriction analysis and Southern blotting revealed that a 2.6-kb Sal/RI fragment and a 2.0-kb Eco RI/Bam HI fragment contained sequences hybridizing to the RACE probe. A mouse genomic library (#ML1009d) made in λ vector EMBL3 was screened with the same probe. A 1.8-kb Eco RI/Bam HI fragment and a ~700-bp Hind III/Eco RI fragment hybridized to the RACE probe. Hybridizing fragments were subcloned into pBluescript KS(−) (Stratagene) for further analysis.

DNA Sequencing

Sequencing of PCR cDNA clones and genomic clones was performed partly by manual methods and partly on an automatic sequencer (Applied Biosystems model 373). Double-stranded DNA was sequenced manually by the dideoynucleotide termination method using Sequenase II (Amersham). Deletion subclones for sequencing were generated with restriction enzymes that cleave the polylinker and at least once in the insert, followed by ligation. Determined sequence was extended using synthetic oligonucleotide primers. Single-stranded DNA was generated from these subclones for sequencing on the automatic sequencer. All sequence was determined on both strands. Analysis of DNA sequence was performed using MacVector software (Eastman Kodak Company). DNA sequences reported in this article have been deposited with the Genbank/EMBL Databases with accession Nos. U34301 (human promoter), U34302 (mouse promoter), U34303 (mouse exon 2), U34304 (human cDNA).

RNase Protection Assay

A 753-bp Xma I fragment from the 5′ end of the human NMMHC-IIB gene was cloned into the polylinker of pBluescript KS(−). This plasmid was linearized within the insert with Pvu II (at nucleotide −338) and used to make a RNA probe of 509 nucleotides (including 77 nucleotides of vector) using T3 polymerase and [α-^{32}P]CTP. A smaller probe of 293 nt was made by digestion of the same plasmid with BssH II (at nucleotide −122). The probes were purified by polyacrylamide gel electrophoresis, hybridized to total RNA, and treated with RNase A and T1 as described (46). Protected fragments were analyzed on urea-6% polyacrylamide gels.

Construction of Reporter Plasmids

A 1.5-kbp BstY1 fragment originally from genomic clone ϕ101 was subcloned into the Bgl II site of a promoterless luciferase vector pGLBasic (Promega Biotec) to make the initial promoter construct. This construct contains 16 bp from the first (nonocoding) exon and 1492 bp of 5′ flanking DNA. Deletion constructs were made by digestion of the −1492 construct by restriction enzymes that cleave at least once in the inserted promoter sequence and once in the polylinker (but not in the rest of the vector) followed by ligation. Thus, −1038 was made by digestion of −1492 with SacI followed by ligation, joining the Sac I site in the polylinker to the Sac I site at position −1038. Similarly, constructs −663, −389, −338, and −122 were made by digestion with Sma, Apa, PvuII and Bss HII, respectively. All other constructs were made by PCR as follows: a 3′ PCR primer was synthesized from the sequence within the 5′ untranslated region with a Hind III site added: 5′-CCCCAAGCTTCAGTCCCAAACCC ACCGCTGCCT-3′ (Hind III site underlined). Various 5′ primers were synthesized with Xho I sites added. PCR products from each pair of primers were digested with Xho I and Hind III and cloned into the polylinker of pGLBasic between these two sites. To mutate the putative E2F binding site we used the fact that it overlaps with a Bss HII site (GCGCGC). Construct D4 (−389) was digested with Bss HII, treated with S1 nuclease to remove single-strand extensions, then blunt-ended with Klenow fragment and deoxynucleotides. This procedure removed 4 base pairs (bp) (CGCG from −122 to −119 from the start of transcription) to produce construct D18. Constructs were confirmed by direct sequencing of double-stranded plasmid DNA.

Transfection Experiments

NIH3T3 cells, a continuous cell line derived from mouse fibroblasts, were grown in Dulbecco's

modified eagle medium (DMEM) supplemented with 10% calf serum and antibiotics. Cells were plated into six-well plates 1 day before transfection such that the cells were almost confluent before treatment (2×10^6 cells per six wells). The promoter construct (2 μg) plus 0.5 μg of the control alkaline phosphate plasmid pSV2Apap (12) were used for each transfection. For cotransfection studies, 0–200 ng of an E2F-1 expression vector was included and 2 μg of CMV-βgal (Clontech) as control. The total amount of DNA in each transfection was kept constant using a promoterless plasmid. Transfection was performed as previously described using positively charged liposomes (DOTAP from Boehringer Mannheim) (44). Expression of luciferase was measured using an assay kit from Promega (Madison, WI). Cell extracts were prepared with 0.2 ml cell lysis reagent per well. Extract (20 μl) was added to 100 μl of luciferase assay reagent containing beetle luciferin and light emission integrated over 5 s was measured using a Berthold Lumat LB 9501 luminometer. For the alkaline phosphatase assay, the remaining extract was heated for 20 min at 65°C then chilled on ice. Residual debris was pelleted by centifugation in a microfuge for 3 min at 15,000 rpm. Extract (20 μl) was added to 200 μl reaction buffer containing 100 mM diethylolamine, pH 9.8, 50 mM $MgCl_2$, 1% CHAPS, and 1 mM CSPD chemiluminescent substrate (Tropix, Bedford, MA). The reaction was incubated for 60 min at room temperature, then light emission was measured for 5 s. β-Galactosidase activity was determined using a chemiluminescent assay system from Tropix, Inc. and measured by luminometer.

Gel Shift Assays

Gel shift assays were performed using the conditions described previously for E2F-1 (10). Binding reactions contained 10 μg of HeLa cell extract (Promega), 20 mM HEPES, (pH 7.4), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.1% NP40, and 2 μg of sonicated salmon sperm DNA. Extracts were preincubated with the carrier DNA and unlabeled competitor DNA in a total volume of 9 μl for 10 min at room temperature. Unlabeled competitor oligonucleotide was included, where appropriate, at a 100 times molar excess. After addition of 1 μl of ^{32}P-labeled, double-stranded probe (0.2–0.5 ng, 30,000 cpm), the reaction was incubated for a further 20 min, then products were separated on a 4% polyacrylamide gel run in 0.25 × Tris-borate-EDTA. The gel was then dried and exposed to film overnight at −70°C with intensifying screens.

Oligonucleotides for Gel Shift Assays

Oligonucleotides for gel shift assays containing a binding site for E2F or a mutation of that site were as previously described by Helin et al. (10). The sequences of oligonucletides derived from the NMMHC-IIB gene were as follows:

NMBwt: GGA GAT GGG CGC GCA AAC CAA CCA G

NMBmut: CCT TGG AGA TGG GCA AAC CAA CCA G

Note that the sequence of NMBmut corresponds to the sequence of the mutated promoter used in the transactivation experiments.

RESULTS

Cloning of the 5′ End of the Human and Mouse NMMHC-IIB Gene

To isolate the promoter of the NMMHC-IIB gene we required a probe corresponding to the 5′ end of the mRNA. The previously published cDNA sequence of human NMMHC-IIB in fact began at amino acid 59 (53). To obtain the 5′ sequence we used the polymerase chain reaction (PCR) technique for rapid amplification of cDNA ends (RACE) (7). Three RACE clones were analyzed and confirmed as NMMHC-IIB by identification of 59 bp at the 3′ end identical to that published previously. These clones differed in the lengths of 5′ sequence: containing 96, 71, and 57 bp of 5′ untranslated region. The sequence of these clones (not shown) matches 100% the cDNA sequence recently published by Phillips et al. (43), which started 79 nucleotides upstream from the ATG start codon. The Eco RI insert from the largest RACE clone was used as a hybridization probe to screen human and mouse genomic libraries. Restriction maps of the isolated λ phage clones from the human and mouse libraries are shown in Fig. 1A and B, respectively. In the case of the human gene, Southern blot analysis (not shown) revealed that the RACE probe hybridized to a 2.0-kb Eco RI/Bam HI fragment and this fragment was completely sequenced. Part of this sequence is presented in Fig. 2. We identified the 5′-most exon of the NMMHC-IIB gene by comparison of its sequence with that of the RACE cDNA clones. The 3′ end of the sequence, which matches the cDNA sequence, coincides with the appearance of a consensus splice junction marking the end of the exon as indicated. As has been found with other myosin genes, the 5′-most exon contains no coding region. In the case of the

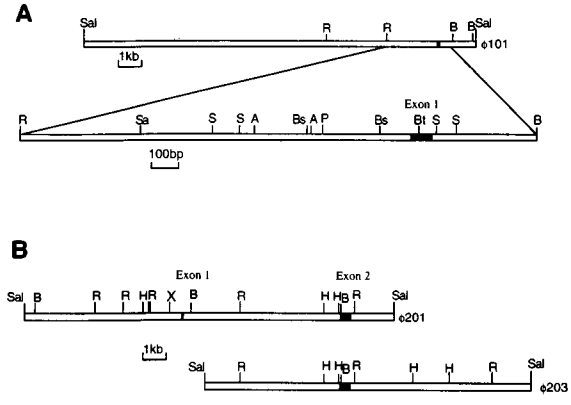

FIG. 1. Restriction maps of λ phage clones containing the 5′ end of the NMMHC-IIB gene. (A) Restriction map of λ clone containing 17 kb of the 5′ region of the human NMMHC-IIB gene. The position of exon 1 is indicated. (B) Restriction map of λ clones containing the 5′ region of the mouse NMMHC-IIB gene. The positions of exons 1 and 2 are indicated. Abbreviations used for restriction enzyme sites: A, Apa I; B, Bam HI; Bs, Bss HII ; Bt, Bst YI; R, Eco RI; H, Hind III; P, Pvu II; Sa, Sac I; S, Sma I; X, Xho I.

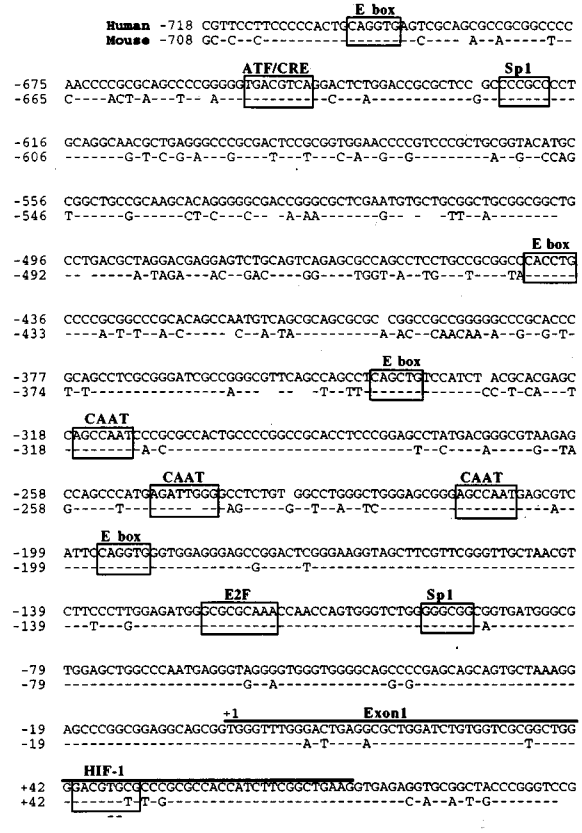

FIG. 2. Conservation of sequence in the promoter region of the NMMHC-IIB gene between human and mouse. The nucleotide sequences of the human and mouse NMMHC-IIB promoter regions were aligned for comparison. The position of several conserved putative binding sites for DNA binding proteins are indicated. Identical nucleotides are indicated by dashes and spaces indicate gaps inserted to optimize homology. Exon 1 is overlined and the major transcription start site is numbered as +1. Note that only part of the determined sequences are shown: the sequence of the entire 1959-bp Eco RI/Bam HI fragment from the human NMMHC-IIB gene (−1492 to +467) and the sequence of the 1814-bp Eco RI/Bam HI fragment from the mouse NMMHC-IIB gene (−1383 to +431) were submitted to Genbank (accession Nos. U34301 and U34302, respectively).

mouse clones, the RACE probe hybridized to two different regions: 1.8 kb Eco RI/Bam HI fragment and a Hind III/Eco RI fragment of 700 bp, which were also completely sequenced and turned out to contain the first and second exons, respectively.

Conservation of Sequence in the NMMHC-IIB Promoter Between Human and Mouse

Figure 2 shows a sequence alignment between the human and mouse NMMHC-IIB genes in the 5′-flanking and untranslated regions. The two sequences are surprisingly conserved well beyond the start of transcription into the promoter: 80% sequence identity in the 700 bp immediately upstream of the major start of transcription (+1) as well as significant homologies as far as 1500 bp upstream. Inspection of the sequence indicated that there was no TATA box, but there were several interesting putative transcription factor binding sites conserved between the two species including three CAAT boxes (33), two Sp1 sites (16), an ATF/CRE (activating transcription factor/cAMP response element) (13,36), four E boxes (35,50), and sequences matching the consensus binding sites for the E2F transcription factor (41) and hypoxia-inducible factor 1 (HIF-1) (49). The putative E2F site in fact matches the class 2 consensus binding site described by Chittenden et al. (4). The conservation of these putative regulatory elements between mouse and human is very suggestive of their functionality. The sequence AGCCAAT appears three times in the human sequence at −421, −319, and −213 with almost equal spacing (105 bp v. 104 bp) but only the last two of these sites are completely conserved in mouse. A perfect 8-bp palindrome TGACGTCA, which matches the well-characterized CRE/ATF, is completely conserved (36). The region from −325 to −301 (which also contains a CAAT box) is very similar to a sequence found in the promoter of a brain-specific rat α-tropomyosin gene (30). Interestingly this α-tropomyosin sequence is in a similar position with respect to the start of transcription (−300 to −275) and this gene also contains no TATA box but multiple CAAT boxes. It is note-

worthy that the human NMMHC-IIB 5′ untranslated region is very similar to that of the mouse (91% sequence identity) and yet has virtually no similarity to the chicken NMMHC-IIB (56) (28% identity) or rat neuronal NMMHC (55) (27%).

The sequence of the second exon from the mouse NMMHC-IIB gene together with the deduced amino acid sequence is shown in Fig. 3. We did not isolate the second exon of the human gene, but the mouse clones allowed us to confirm that the second exon contains the translational start codon together with 31 bp of 5′ untranslated region. The second exon also encodes amino acids 1 to 130, which are identical in mouse and human (by comparison with cDNA sequences). The intron between the first and second exons was found to be approximately 10 kb. Although this is quite large for an intron it is considerably smaller than the 37-kb first intron of the human NMMHC-IIA gene (21). Restriction mapping data obtained by genomic Southern blots (not shown) agreed with data obtained from the isolated clones for both the mouse and human genes and indicated the existence of only one gene for NMMHC-IIB. It is therefore unlikely that the isolated clones were derived from a pseudogene.

The human and mouse NMMHC-IIB promoters have a similar overall structure with respect to base composition. In both genes the GC content of the first exon and upstream region is very high

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nt                                                          aa
  1 AAGCTTGTAAATTGTTTTACATGTCCCAAGCATCTAATTCTGCCCTTCTC
 51 TTCCAG /AGGCAATTACTTTTGGGTCCTTCTGTTTACAATGGCCCAGAGAA
                                              M  A  Q  R   4
101 CTGGACTGGAGGATCCCGAGAGGTATCTCTTTGTGGACAGGGCTGTCATC
    T  G  L  E  D  P  E  R  Y  L  F  V  D  R  A  V  I   21
151 TACAACCCTGCCACTCAAGCTGACTGGACAGCTAAAAAGCTGGTGTGGAT
    Y  N  P  A  T  Q  A  D  W  T  A  K  K  L  V  W  I   38
201 TCCATCGGAACGCCATGGTTTTGAGGCAGCTAGTATTAAAGAAGAGCGGG
    P  S  E  R  H  G  F  E  A  A  S  I  K  E  E  R      54
251 GCGATGAAGTTATGGTGGAGCTGGCAGAGAATGGGAAGAAAGCAATGGTC
    G  D  E  V  M  V  E  L  A  E  N  G  K  K  A  M  V   71
301 AACAAAGATGACATTCAGAAGATGAACCCACCAAAGTTCTCCAAGGTGGA
    N  K  D  D  I  Q  K  M  N  P  P  K  F  S  K  V  E   88
351 GGATATGGCAGAGCTGACATGCTTGAACGAAGCCTCCGTCTTACATAATC
    D  M  A  E  L  T  C  L  N  E  A  S  V  L  H  N     104
401 TGAAGGACCGCTACTATTCAGGACTTATCTAC/ GTGAGTATTTCTTCTCAC
    L  K  D  R  Y  Y  S  G  L  I  Y                    115
451 TATCCGTGGGAGTTGTACCTGAGAAGTAGTCTAATTATATATGCATAATG
501 TTTAACCACTTGAGAACAGAGGTTCCCAATATCCTGCTTGACATTATATT
551 TCTGATAAAAATAGGAAGGTATTTGCTTAAGCAGCTTCTGCTTTCTTTCC
601 CATCGTTTCTCTGTATAACAAGTCCTGGTTGTCCTGGACTCTCTTTGTAG
651 ACCAGGCTGGCCTTGAATTC
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FIG. 3. Sequence of exon 2 of mouse NMMHC-IIB gene with deduced amino acid sequence. The complete sequence of the Hind III/Eco RI frament containing the second exon is presented. Positions of exon/intron junctions are indicated by slash marks.

(between 60% and 80%) and this region is flanked on the 5′ side by an AT-rich region. There is an abrupt end to the high degree of sequence identity between the two species at −723 in the human and −713 in the mouse, which begins again at −913 in the human and −836 in the mouse. The mismatched region therefore contains an additional 67 bp in the human sequence compared to the mouse. The region of homology corresponding to the first exon extends through the splice site at position +75 into the first intron. Smaller islands of homology also exist in the first intron. Major homology extends upstream as far as −1287 in the human (−1187 in the mouse) and additional smaller stretches of homology exist up to −1500. Both the upstream Eco RI site and the downstream Bam HI sites were conserved.

Identification of the Transcriptional Initiation Site

To map the initiation point of transcription we performed RNase protection analysis (Fig. 4A, B). These assays indicated that there are a number of transcriptional initiation sites as might be expected of a gene lacking a TATA box, which is thought to direct RNA polymerase II to a specific start site (15). Because the most intense protected band appeared at 75 nucleotides we designated the corresponding start site as nucleotide +1, which happens to be 10 bp upstream of the 5′ end of the longest RACE clone that we obtained. Although there is no classical TATA box it should be noted that this start site is 21 bp downstream of the sequence TAAA (−25 to −22), which was conserved between human and mouse. Using probe I (which contains 432 nucleotides of the gene) we also detected a prominant band at 153 (corresponding to position −88) and four bands between 220 and 280 nucleotides. These bands appear to be produced by the specific hybridization of RNA because they did not appear in the control lanes containing probe alone, probe with RNase, or probe hybridized to yeast RNA. The same pattern of protected bands was seen in a number of independent assays and with RNA from all sources examined, including human endothelial cells, vascular smooth muscle cells, K562 erythroleukemic cells, and with NIH3T3 cells using an equivalent mouse-specific probe (data not shown). For the analysis shown in Fig. 4B we used a smaller probe (probe II) that also produced a protected band of 75 nucleotides but also an extra band at 73, which might be the result of nibbling of the heteroduplex ends by the RNase. The band

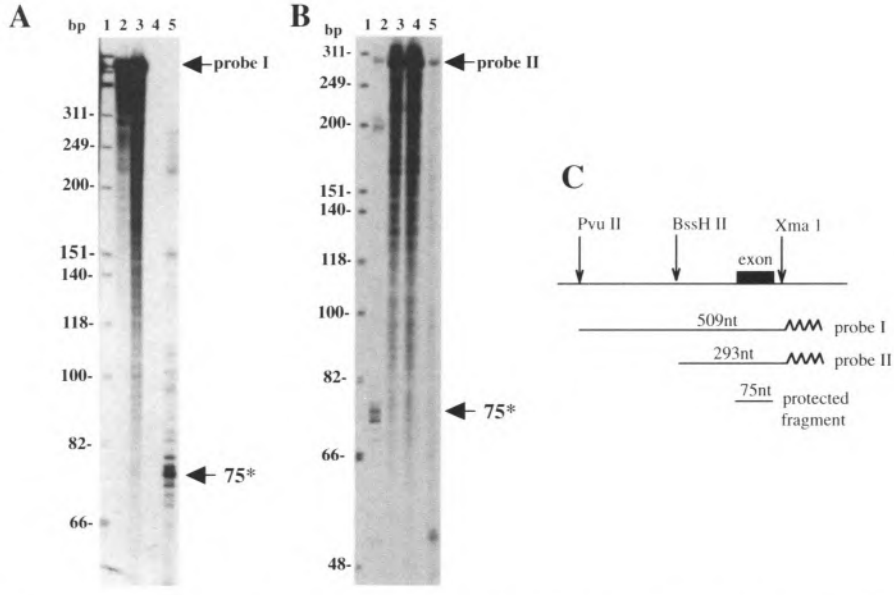

FIG. 4. Mapping of transcriptional start sites by ribonuclease protection. RNA probes containing the 5′-flanking sequence of the NMMHC-IIB gene including the first exon and part of the first intron were hybridized to total RNA and treated with RNase A and T1. Protected fragments were analyzed on urea-6% polyacrylamide gels. Probe I was 509 nucleotides in length including 432 nucleotides of NMMHC-IIB sequence and 77 nucleotides of vector sequence as indicated in (C). Probe II was 293 nucleotides in length including 216 nucleotides of NMMHC-IIB sequence and 77 nucleotides of the vector. (A) Lane 1: molecular weight markers ϕX174 × Hinf I; lane 2: probe I alone; lane 3: probe I hybridized to yeast tRNA without RNase digestion; lane 4: negative control—probe I hybridized to yeast tRNA with RNase; lane 5: probe I hybridized to 50 μg total RNA from human endothelial cells. The major protected band of 75 nucleotides corresponding to the transcriptional initiation point designated as nucleotide +1 is indicated. A number of other protected bands are also detected. (B) Lane 1: molecular weight markers ϕX174 × Hinf I; lane 2: probe II hybridized to 50 μg total RNA from human endothelial cells; lane 3: probe II alone; lane 4: probe II hybridized to yeast tRNA without RNase digestion; lane 5: negative control—probe hybridized to yeast tRNA with RNase digestion. The major protected band of 75 nucleotides corresponding to the transcriptional initiation point designated as nucleotide +1 is indicated. Note the prominant band at 197 nt corresponding to protection of the whole of probe II except for 19 nucleotides of the first intron and 77 nt of vector.

at 197 represents full protection of the 5′ end of the probe up to the splice site with removal only of the intron and vector sequences. The 197 band, therefore, confirms the existence of longer transcripts derived from upstream initiation sites. The functional significance of the apparent upstream start sites is still uncertain, but these are clearly minor compared to the major start site at position +1. We were unable to obtain unequivocal confirmation of transcriptional start sites by primer extension, presumably due to multiple strong stop sites within this GC-rich region (data not shown).

Promoter Activity of the 5′ Sequence

To demonstrate that the isolated clones contained the promoter of the gene for NMMHC-IIB we tested the ability of this segment of DNA to initiate transcription using luciferase reporter constructs. A series of DNA fragments from the putative promoter was inserted upstream of the luciferase coding region and the resulting plasmids were transfected into NIH3T3 cells. Relative luciferase activities, normalized for transfection efficiency with a cotransfected control plasmid expressing alkaline phosphatase, are shown in Fig. 5. These plasmids were transiently transfected into NIH3T3 cells and the efficiency of transcription was monitored by measurement of luciferase activity (Fig. 5). These experiments indicated that constructs which contained between 1492 and 305 bp of the 5′-flanking region all had essentially similar promoter activity. Because the −305 construct does not contain the first CAAT box it appears that this sequence is not required for basal promoter activity in these cells. It is notable that luciferase activity drops to approximately 50% when sequences up to −220 are deleted and this

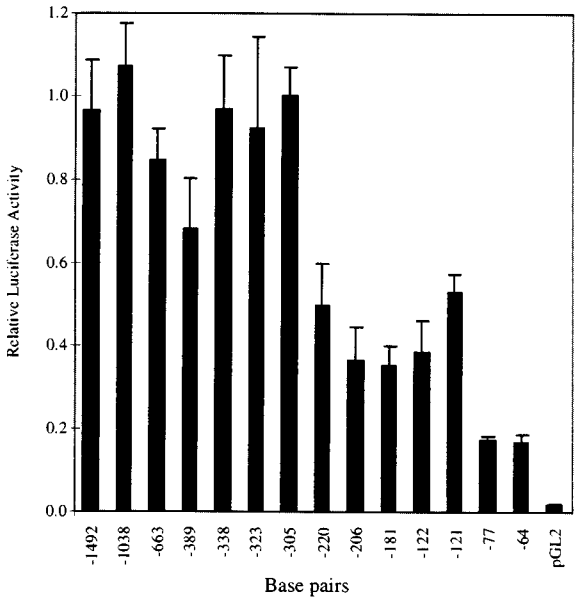

FIG. 5. Promoter activity in the 5′ region of the human NMMHC-IIB gene. Constructs containing portions of the human NMMHC-IIB promoter driving expression of luciferase were transfected into NIH3T3 cells along with a plasmid expressing alkaline phosphatase to control for transfection efficiency. The minus numbers indicate the endpoint of each deletion with respect to the major start site of transcription. The graph shows the relative luciferase activity produced by each construct normalized to the alkaline phosphatase activity. For comparison each result is expressed as a proportion of that obtained by construct −305, which contains the smallest promoter insert that still gave maximal activity. Each data point is the mean of at least three experiments ± SE. The luciferase vector with no promoter (pGL-2Basic) was transfected as a control.

coincides with the loss of the second CAAT box. Luciferase activity stays at about the same level in subsequent deletions up to −121, then another precipitous drop occurs with construct −74. The smallest construct with only 64 bp of the 5′-flanking region still retains significant promoter activity at 8.5 times the background obtained with the promoterless contruct. Construct −181 has similar activity to −206, indicating that the deletion of the E box at −195 has no effect in these cells.

Transactivation of the NMMHC-IIB Promoter by E2F-1

To investigate whether the putative E2F-1 site is capable of confering E2F-1 inducibility on the NMMHC-IIB promoter, cotransfection experiments were performed using an E2F-1 expression vector, pCMV-E2F-1 (10). We used a reporter construct (D4) in which 389 bp of the 5′-flanking region of the human NMMHC gene was inserted into the promoterless, plasmid pGL2-Basic, upstream of the luciferase coding region. To show that transactivation of the NMMHC-IIB promoter was mediated by the presence of the E2F binding site we made a new construct in which the E2F site was mutated. Construct D18 contains a deletion of 4 bp within the E2F element but is otherwise identical to D4. These plasmids were cotransfected into NIH3T3 cells along with increasing amounts of pCMV-E2F-1. Figure 6 shows that expression of E2F-1 resulted in a large increase in luciferase activity derived from promoter construct D4. The increase in luciferase activity was dependent on the amount of the E2F vector used, rising to an approximately eightfold increase with 200 ng of the E2F-1 vector. As was reported previously for the c-*myc* gene (57), mutation of the E2F-1 site reduced the basal activity of the NMMHC-IIB promoter to approximately 26%. E2F-1 expression produced a modest increase in luciferase activity, which could be the result of an indirect effect mediated by other genes upregulated by E2F-1. These findings were consistent between separate transfection experiments, and similar results were obtained when the control plasmid used was an alkaline phosphatase expression vector driven by the SV40 promoter rather than the MMTV-βgal plasmid. In addition, a mutated version of E2F-1 (containing a deletion of amino

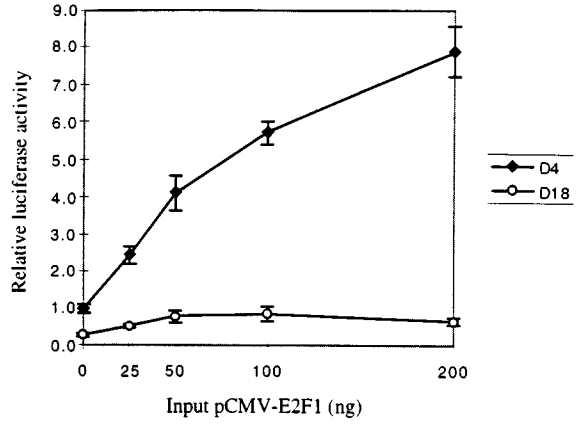

FIG. 6. Transcriptional activation of the NMMHC-IIB promoter by E2F-1. In each transient tranfection experiment 2 μg of construct D4 or D18 was cotransfected with increasing amounts of the E2F-1 expression vector into NIH3T3 cells. Construct D4 contains 389 bp of the NMMHC-IIB promoter driving luciferase. D18 contains a 4 bp deletion within the putative E2F binding site. To control for transfection efficiency a plasmid expressing β-galactosidase driven by the CMV promoter was cotransfected and luciferase results were normalized. All luciferase values are expressed relative to that obtained for D4 with carrier DNA alone. Each data point is the mean of three experiments ± SE.

acids 285 to 416) failed to transactivate the myosin promoter (data not shown).

Binding of E2F to the NMMHC-IIB Promoter

To test whether the putative E2F binding site in the NMMHC-IIB promoter is capable of binding E2F we performed gel mobility retardation assays (Fig. 7). A 25-bp oligonucleotide containing the sequence in question from the human NMMHC-IIB gene was used as a probe to test for E2F binding with a HeLa cell extract. A number of bands were shifted by the extract, several of which were lost when an oligonucleotide containing the E2F consensus binding site (10) was used as a competitor in the assay. These bands also disappeared when the NMMHC-IIB oligonucleotide was itself used as a competitor but not when a mutated version of this sequence (corresponding to the sequence in construct D18 used in the transactivation experiments described below) was used in the assay. A previously described mutated version of the E2F oligonucleotide (10) also failed to compete for these bands. Comparison with the shift obtained with the E2F consensus probe (10) confirmed that these bands have the mobility of the E2F complex. When the E2F oligonucleotide was used as the probe, however, the NMMHC-IIB oligonucleotide did not completely compete for the E2F complex. We conclude that the putative E2F element from the NMMHC-IIB promoter binds the E2F activity in HeLa cell extract but at a lower affinity than the consensus E2F site.

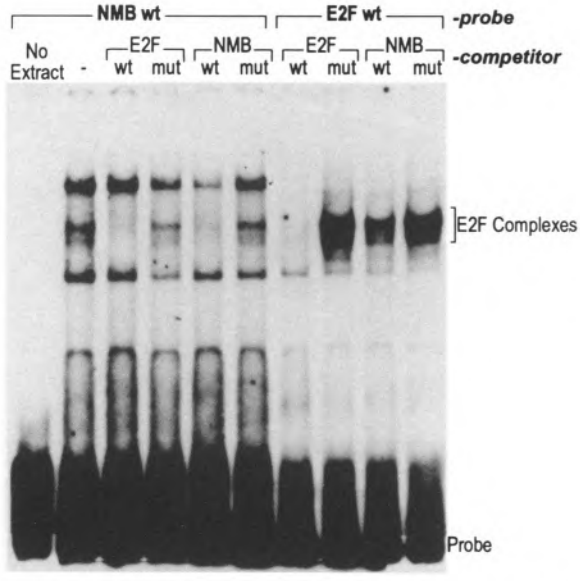

FIG. 7. Gel shift assay: the putative E2F element from the NMMHC-IIB promoter binds to the E2F activity in HeLa cell extract. Gel mobility retardation assays with a HeLa cell extract were used to test whether the sequence in the NMMHC-IIB promoter is a functional binding site for E2F. Oligonucleotides (25 bp) containing the sequence in question from the NMMHC-IIB gene (NMB wt) and the E2F consensus previously described by Helin et al. (10) were used as probes. Unlabeled competitor oligonucleotide was included, where indicated, at a 100 times molar excess. Products were separated on a 4% polyacrylamide gel run in 0.25 × Tris-borate-EDTA. The gel was dried and exposed to X-ray film overnight at −70°C. The experiment was performed three times with similar results.

DISCUSSION

We have shown here that a sequence in the NMMHC-IIB promoter that is conserved between mouse and human can bind the transcription factor E2F in vitro and that coexpression of E2F-1 leads to transactivation of the NMMHC-IIB promoter. These findings provide strong evidence that the NMMHC-IIB gene is regulated by E2F or an E2F-related transcription factor. A wide variety of genes have been shown to contain binding sites for E2F. These include genes required for DNA synthesis and G1/S-regulatory factors. Proliferating cell nuclear antigen (PCNA) is also regulated by E2F but its expression does not fluctuate significantly during the cell cycle (37). Transient transfection assays have generally been used to measure induction of suspected target genes. It was recently demonstrated that E2F-1 expressed from a recombinant adenovirus was capable of activating the endogenous genes that were suspected to be targets of E2F (5). The mRNA for E2F-1 is low or undetectable during G0/G1 and increases as cells enter and proceed through S phase (17). Interestingly, mRNA abundance of E2F-4 and E2F-5 is also cell cycle regulated but is maximal in mid-G1 phase (47). Regulation by E2F may be linked to the requirement of nonmuscle myosin for cytokinesis (6,18,24,25,32). It is interesting to note that NIH3T3 cells, which constitutively express a mutant form of E2F-1, undergo a dramatic shape change to an extremely rounded morphology lacking focal contacts when cultured in media containing 10% serum (31). In low serum media these cells have the flattened morphology and cytoskeletal structure of normal NIH3T3 cells. It is striking that a number of cellular and viral promoters that are regulated by E2F also contain an ATF/CRE site (11,28,42,61) and this arrangement is reiterated in the NMMHC-IIB gene. This site apparently does not confer cAMP inducibility, because forskolin did not upregulate the NMMHC-IIB promoter and cAMP inhibitors had no effect (data not shown).

Sequence similarity in the 5′-flanking region between the human and mouse genes extends as far as 1500 bp upstream of the major start of transcription. Judging from this high degree of homology it is likely that the NMMHC-IIB promoter contains many sequences that may be involved in the regulation of this gene. Comparison of the mouse and human promoter sequences of the NMMHC-IIB gene allowed us to discount the significance of certain potential transcription factor binding sites that were not conserved. For example, an AP2 site in the human sequence (−81 to −89) does not appear in the mouse sequence. The human sequence had five potential Sp1 binding sites but only two of these were conserved in the mouse (Fig. 3). Similarly, although both sequences contain multiple potential CAAT boxes only three of these are conserved. On the other hand, several interesting putative transcription factor binding sites were completely conserved. Like the promoter for NMMHC-IIA (21), the NMMHC-IIB promoter has several features typically associated with housekeeping genes. Neither gene contains a sequence that strongly matches the consensus TATA box and the GC content of both promoter regions is high. No significant sequence similiarities were found between NMMHC-IIA and NMMHC-IIB promoters.

The major transcriptional start site was mapped to position +1 but there are clearly a number of upstream start sites the significance of which is currently unclear. We cannot currently rule out the possibility that alternative upstream promoter sequences and transcriptional start sites may predominate in cell types other than those tested so far. It is also possible that the conservation of sequence seen in the 5′-flanking region of the gene may in fact relate more to the function of the 5′ untranslated region of these upstream initiated transcripts rather than for binding of transcription factors. The transfection experiments presented here, however, demonstrate that this region does constitute a functional promoter. Transfections were performed in NIH3T3 cells because this is a well-characterized line that expresses NMMHC-IIB and is relatively easy to grow and transfect. It is possible that in other cell types sequence motifs that appear unimportant in NIH3T3 cells are in fact required for regulated expression. E-box-dependent mechanisms have been implicated in the regulation of cardiac and smooth muscle myosin heavy chain genes (19,35), but deletion of these sequences appeared to have no effect on NMMHC-IIB expression in NIH3T3 cells. The 25-bp sequence of NMMHC-IIB between −325 and −301, which is similar to the gene for rat brain α-tropomyosin (30), could conceivably be involved in coordinate regulation of these two genes in brain tissue. NMMHC-IIB mRNA has been shown to be differentially expressed in different tissues (20,22,53). Of particular interest is the expression of NMMHC isoforms in smooth muscle cells as they "dedifferentiate" from the quiescent to the synthetic phenotype. We showed that the level of NMMHC-IIB mRNA is increased in smooth muscle cells of the vascular wall in restenotic lesions (following balloon angioplasty) compared with primary atherosclerotic plaque (i.e., not previously treated by percutaneous revascularization) (27). Elevated levels of NMMHC-IIB mRNA in primary lesions appeared to correlate well with the subsequent development of restenosis (51). Transcriptional activation of the NMMHC-IIB may have a key role in the switch that takes place when vascular smooth muscle cells change from the contractile to the proliferative phenotype.

It is also noteworthy that the sequence of the first exon is remarkably well-conserved between mouse and human with only seven differences in 75 bp (91% homology). The sequence of the 5′ untranslated region of the NMMHC-IIB mRNA (106 nucleotides) is 89% identical to the mouse. This result was unexpected because this sequence has virtually no similarity to the chicken NMMHC-IIB (56) (28% identity) or rat neuronal NMMHC (55) (27% identity). If the sequence of the 5′ untranslated region was conserved between mouse and human because it performs an important function, why is that sequence not conserved in chicken? There are two major possibilities: a) this function is not required in avian species, or b) the chicken cDNA sequence derives from an alternatively spliced exon or an alternative promoter. The second possibility could be demonstrated by cloning of the chicken NMMHC-IIB gene. The 5′ untranslated region of NMMHC-IIB also shares no obvious sequence similarity with that of human NMMHC-IIA, indicating no conservation of function. These results also confirm that rat neuronal NMMHC (55) (which was classified as B-type) is not the rat homolog of the human and mouse NMMHC-IIB genes presented here.

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