Hepatocyte Nuclear Factor-3 Homologue 1 (HFH-1) Represses Transcription of Smooth Muscle-specific Genes*

Received for publication, June 26, 2000, and in revised form, July 6, 2000
Published, JBC Papers in Press, July 13, 2000, DOI 10.1074/jbc.M005595200

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Results show that smooth muscle-specific promoters represent novel downstream targets of the winged helix factor hepatocyte nuclear factor-3 homologue 1 (HFH-1). HFH-1 strongly represses telokin promoter activity when overexpressed in A10 vascular smooth muscle cells. HFH-1 was also found to repress transcription of several other smooth muscle-specific promoters, including the SM22α promoter. HFH-1 inhibits telokin promoter activity, by binding to a forkhead consensus site located within an AT-rich region of the telokin promoter. The DNA-binding domain alone was sufficient to mediate inhibition, suggesting that binding of HFH-1 blocks the binding of other positive-acting factors. HFH-1 does not disrupt serum response factor binding to an adjacent CArG box within the telokin promoter, implying that HFH-1 must compete with other unidentified trans-activators to mediate repression. The localization of HFH-1 mRNA to the epithelial cell layer of mouse bladder and stomach implicates HFH-1 in repressing telokin expression in epithelial cells. This suggests that cell-specific expression of telokin is likely mediated by both positive-acting factors in smooth muscle cells and negative-acting factors in nonmuscle cell types. We propose a model in which the smooth muscle specificity of the telokin promoter is regulated by interactions between positive- and negative-acting members of the hepatocyte nuclear factor-3/forkhead family of transcription factors.

Unraveling the mechanisms regulating the expression of smooth muscle-specific genes is an important step toward understanding the development and differentiation of smooth muscle. The differentiation state of smooth muscle is altered under many pathological conditions, such as atherosclerosis, restenosis following angioplasty, and chronic asthma (1–3). The changes that occur during these pathological conditions result in down-regulation of many proteins characteristic of adult smooth muscle (4). Although the extracellular signals that influence the growth and differentiation state of smooth muscle have been studied extensively, little is known about the nuclear factors that control these processes (5). To begin to identify proteins that regulate the differentiation state of smooth muscle we initiated an analysis of mechanisms regulating expression of the telokin gene. We have previously shown that telokin mRNA is transcribed from a second promoter located within an intron that interrupts the exons encoding the calmodulin-binding domain of the smooth muscle myosin light chain kinase (6). Unlike the smooth muscle myosin light chain kinase which has been detected in all adult tissues examined thus far, telokin protein and mRNA expression is restricted to smooth muscle tissues and cells (6–8). Although its physiological function is unclear, telokin has been shown to bind to unphosphorylated myosin filaments and to stimulate myosin mini-filament assembly in vitro. Consequently, it has been proposed that telokin may play an important role in maintaining the stability of unphosphorylated myosin filaments in vivo (9, 10). Recently, telokin has also been reported to mediate smooth muscle relaxation through the activation of myosin light chain phosphatase (11).

The regulatory regions of several smooth muscle-specific genes including the telokin, smooth muscle myosin heavy chain, SM22α, smooth muscle α- and γ-actin genes have been studied in order to identify transcription factors that regulate their expression. Analysis of these genes in transgenic mice has revealed that each transgene exhibits a distinct pattern of expression in different smooth muscle tissues (6, 12–17). Both the telokin and γ-actin transgenes are expressed at high levels in visceral smooth muscle and lower levels in vascular smooth muscle (6, 13). In contrast, the mouse SM22α promoter directs transgene expression specifically to arterial smooth muscle in adult mice (14, 15) and the smooth muscle myosin and α-actin promoters direct high levels of transgene expression to all smooth muscle tissues (12, 16). The pattern of expression of these transgenes in various smooth muscle tissues suggests that distinct regulatory elements, and presumably distinct transcription factors, are required for expression of a single gene in different smooth muscle tissues. To date no transcription factors have been identified that are expressed only in smooth muscle cells, however, several more generally expressed factors have been shown to be important for the expression of smooth muscle proteins. These include positive-acting factors present in smooth muscle cells, such as SRF,1 MEF2B, TEF-1, b53 (12, 14, 18–25), and negative-acting factors, such as Purα, Purβ, and MSY1 present in other cell types that help restrict expression of proteins to smooth muscle (26–28). Of the factors currently identified SRF appears to play a central role in the expression of many different smooth muscle-specific genes including the smooth muscle myosin heavy chain gene, smooth muscle α- and γ-actin genes, calponin, SM22α, and telokin genes (14, 18, 21, 25, 29–31). Although SRF is

* This work was supported by National Institutes of Health Grant HL-58571 (to B. P. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: SRF, serum response factor; MEF2, myocyte enhancer factor-2; HFH-1, hepatocyte nuclear factor-3 homologue 1; bp, base pair(s); DMEM, Dulbecco’s modified Eagle’s medium; GI SMC, gastrointestinal smooth muscle cell.
expressed in all tissues, its expression is greatest in muscle tissues (32). In addition to being important for growth factor regulation of genes, SRF has been shown to be important for the tissue-specific expression of the skeletal and cardiac muscle α-actin genes as well as several smooth muscle-specific genes (33, 34). SRF has also been shown to be important for the differentiation of proepicardial cells into coronary vascular smooth muscle cells (35). For the cardiac muscle α-actin gene SRF has been shown to interact with other tissue-restricted transcription factors to mediate cell-specific expression (36). The mechanism by which SRF regulates the cell-specific expression of smooth muscle-specific genes has not yet been resolved. For the telokin gene we have shown that an AT-rich region adjacent to the CARG element that binds SRF is important for promoter activity. The AT-rich region in the telokin promoter has been shown to bind both TATA-binding protein and myocyte enhancer factor-2 (MEF2), although disruption of MEF2 binding had no effect on reporter gene activity in A10 cells (18). In contrast, mutation of the telokin TATA sequence to the TATA sequence of the thyidine kinase gene abolished promoter activity suggesting that this region is important, yet may not be simply functioning as a TATA box. Together these data suggest that there are likely to be transcription factors that bind to the AT-rich region of the telokin promoter and regulate promoter activity, perhaps through an interaction with SRF bound to the adjacent CARG element.

In the current study we utilized a yeast one-hybrid screening procedure to identify factors that can bind to the AT-rich core of the telokin promoter. From this analysis we isolated a transcription factor of the forkhead family, HFH-1, that binds to the AT-rich region of the telokin promoter. HFH-1 is expressed at high levels in adult stomach and bladder and at lower levels in several other smooth muscle tissues. In stomach HFH-1 expression was found to be largely restricted to the epithelial cells of the mucosa. HFH-1 was found to repress the transcription of telokin and other smooth muscle-specific reporter genes. The repressor of reporter gene activity is mediated by the forkhead domain of HFH-1 and does not appear to involve inhibition of SRF binding to the promoter. These data suggest that the cell-restricted expression of telokin and other smooth muscle-specific genes is likely to be controlled by the activity of positive-acting factors in smooth muscle cells together with negative regulatory factors such as HFH-1 in other cell types.

MATERIALS AND METHODS

Yeast One-hybrid Screen—A core fragment of the telokin promoter that includes a putative E box, an AT-rich region, and a CARG box was generated by annealing sense and antisense oligonucleotides. The sequence of the sense oligonucleotide was AATTTCGAGTTGGTCTTATATACATTCCCTTTTATGGGAC. Three tandem copies of the core fragment were ligated into pHSi-1 and pLaCZi (CLONTECH). Sequencing confirmed that each of the fragments were present in the 5′-3′ orientation relative to the minimal yeast promoter. The core-pHSi-1 and core-pLaCZi plasmids were linearized at Xhol and NotI sites, respectively, and sequentially integrated into the host yeast strain YM4271 to generate a dual yeast reporter strain. Growth of the telokin core promoter yeast reporter strain was suppressed by the addition of 45 mM 3-aminothiazole. The yeast strain exhibited no background β-galactosidase activity. A cDNA activation domain fusion library was generated in pGAD10 from polyclonal mRNA isolated from adult mouse bladder using a two-hybrid cDNA library construction kit (CLONTECH). Plasmid DNA was obtained from the library according to the manufacturer's directions. Purified plasmid DNA (20 μg) was transformed into the core telokin promoter yeast reporter strain and positive colonies were initially selected based on the absence of histidine and uracil and in the presence of 45 mM 3-aminothiazole. The yeast screening plates were then transferred onto filter paper and analyzed for β-galactosidase activity. Positive colonies were isolated and replated and the process was repeated until each colony represented a single clone. Plasmid DNA was isolated from the yeast clones by standard procedures (37, 38) and used to transform electrocompetent DH5α E. coli. Plasmids were recovered from the bacteria by standard procedures and subjected to automated DNA sequencing.

λ Library Screening—DNA prepared from mouse bladder as described above was ligated to λgtI arms and packaged into phage particles using Giga Pack GII (Promega). The λgt11 library was amplified and screened by standard procedures (39). Nitrocellulose filters were hybridized at 65 °C overnight with a 22P-probe corresponding to the NotI fragment of the HFH-1 clone obtained from the yeast library screen. This fragment corresponds to nucleotides 2502 to 3252 bp of the published Hfh-1L genomic clone sequence. Filters were then washed in 2 × SSPE + 0.1% SDS at 65 °C for 15 min followed by 0.2 × SSPE + 0.1% SDS at 65 °C for 10 min. λDNA was isolated using Lambdaorb (Promega), digested with NotI, and the resulting fragments were subcloned into pGEM 5Z (Promega) and sequenced by automated sequencing.

Northern Blotting—Total RNA was isolated from adult tissues using a single step guanidinium isothiocyanate procedure (39) and 15 μg were separated on a 1.2% formaldehyde-agarose gel and transferred to a nylon membrane under vacuum. Hybridization was carried out at 65 °C overnight with the same probe used for λ library screening. Final wash conditions were 2 × SSPE + 1.0% SDS for 10 min 55 °C.

RNase Protection Assays—A 171-bp fragment of the HFH-1 cDNA (corresponding to nucleotides 2502–2673) was subcloned into pGEM T7 (Promega). The plasmid was linearized with Sall and a 32P-labeled antisense riboprobe was generated using SP6 polymerase and a MaxiScript In Vitro Transcription kit according to the manufacturer's directions (Ambion). The full-length HFH-1 transcript (213 bases) was gel purified on a 6% polyacrylamide, 8 μm urea gel and eluted overnight at 37 °C. Ribonuclease protection assays were then performed according to the manufacturer's directions (Standard RPA II kit; Ambion). Briefly, 1 × 105 cpm of gel purified HFH-1 riboprobe was co-purified with 20 μg of RNA and hybridized overnight at 42 °C. Samples were digested with RNase A/T1 at 1:150 dilution for 30 min at 37 °C and then inactivated and precipitated. Samples were solubilized in 8 μl of gel loading buffer and one-half volume was loaded onto a 6% polyacrylamide, 8 μm urea gel run at 55 watts for 2 h. 35S-Sequencing reactions were run alongside samples to verify the size of the probe and protected fragments.

In Situ mRNA Hybridization—mRNA in situ hybridization was performed on 10-μm crossections of mouse bladder and stomach as described previously (40, 41). The HFH-1 probe used was identical to the riboprobe used for RNase protection analysis, except that 35S-nucleotides were labeling. The 180-nucleotide mouse telokin probe used corresponds to residues 53–233 of the mouse telokin cDNA. This probe is specific for telokin and does not cross-react with myosin light chain kinase.2 Antisense probes were generated using T7 RNA polymerase and sense probes using SP6 RNA polymerase. Hybridization was carried out at 50° for 16–18 h. Final wash conditions were 0.1 × SSC at 37 °C.

HFH-1 Mammalian Expression and Promoter-Reporter Gene Assays—All promoter reporter genes were constructed by cloning fragments of promoters into the pGL3-B luciferase vector (Promega). The rabbit telokin promoter-luciferase reporter gene used includes nucleotides –256 to +147 of the telokin gene as described previously (6). The SM22α-luciferase reporter gene includes nucleotides –475 to +61 of the mouse SM22α gene (43). The smooth muscle α-actin promoter fragment was extended from nucleotide –1075 to +46 (44) and the smooth muscle myosin heavy chain promoter from nucleotide –1175 to +47. The minimal TK promoter used comprised nucleotides –113 to +20 of the thymidine kinase gene. The AT/CARG TK construct contained two copies of the AT-rich region-CARG box from the telokin promoter (–90 to –51) upstream of the minimal TK promoter.

Promoter fragments were isolated by polymerase chain reaction using mouse genomic DNA as a template and the following oligonucleotides; SM22α sense, GTTTGCATAGTGGTGTGATGGAACCC; SM22α antisense, GCTTGGTCGTTTGTGGACTGGAAGGAGAG; smooth muscle α-actin sense, CCGTGACCCCACTATACATTCCCTTTTATGGGAC; smooth muscle α-actin antisense, CTAAGCTTGAGCAGCGACGGTCTGTTCGCTTCTC; smooth muscle myosin heavy chain sense, GAGCGCCGGTCTGTTCGCTTCTC; smooth muscle myosin heavy chain antisense, GAGCTCGGGATCTGACGGTCATGGCAAGAGGAGG; muscle myosin light chain antisense, GAGCTCGGGATCTGACGGTCATGGCAAGAGGAGG. Polymerase chain reaction fragments were cloned into pCRBlunt (Invitrogen), sequenced and then subcloned into pGL3-B (Promega).
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For expression in mammalian cells a fragment of the HFH-1 cDNA encoding the coding region was amplified by polymerase chain reaction and cloned into pcDNA 3.1 His-C (Invitrogen). This results in the expression of HFH-1 fused in-frame at the amino terminus to 6×His and X-press epitope tags. Truncated forms of HFH-1 were generated by subcloning partial cDNA clones into pcDNA 3.1 His. The resultant plasmids were sequenced to verify the integrity of the inserts.

Plasmids were transfected into rat A10 vascular smooth muscle cells using Fugene (Roche Molecular Biochemicals). A10 cells were grown in high glucose DMEM containing 50 units/ml penicillin, 50 µg/ml streptomycin, and 20% fetal bovine serum. Cells to be transfected were seeded at 1.4 × 10^5 cells/dish in 35-mm dishes. 16–18 h post-seeding each dish was washed once with phosphate-buffered saline, pH 7.4, replaced with 2 ml of complete media, incubated with a total of 2 µg of plasmid DNA (1 µg of telokin-luciferase, 0.5 µg of HFH-1 expression plasmid, and 0.5 µg of pRL-luciferase as an internal control) and 3 µl of Fugene in 0.1 ml of DMEM (Life Technologies, Inc.). 24 h later 10 µl of clarified extracts (400 µl/dish) were prepared for dual luciferase assays. Assays were performed using a dual luciferase reporter assay system according to the manufacturer’s directions (Promega). Reporter gene luciferase activities were normalized to the luciferase activity of the internal control.

Expression and Purification of Recombinant SRF and HFH-1 from Bacteria—Full-length human SRF and mouse HFH-1 were expressed in bacteria using the pET expression system (Novagen). The coding region of each cDNA was isolated by polymerase chain reaction, sequenced, and cloned into pET28a. SRF and HFH-1 protein expression was induced by addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 1 h. SRF lysates were prepared by sonicating bacterial pellets in phosphate-buffered saline containing 0.1% Triton X-100, 500 µg/ml leupeptin, 10 µg/ml lysozyme. HFH-1 lysates were prepared by sonicating bacterial pellets in 20 mM Tris, pH 8, 100 mM NaCl, 8 µM urea, 500 µg/ml phenylmethylsulfonyl fluoride. Cleared lysates were incubated with Talon beads (CLONTECH), pre-equilibrated in lysis buffer. Bound proteins were washed with lysis buffer containing 10 mM imidazole and the purified proteins eluted with 500 mM imidazole. Eluted proteins were dialyzed against phosphate-buffered saline containing 0.1% Triton X-100, 500 mM imidazole, and stored at −80 °C.

Gel Mobility Shift Assays—Mobility shift assays were performed in a final volume of 15 µl. Binding mixes contained 0.2 ng (1.5 × 10^4 cpm) of end-labeled double stranded DNA probe, 200 ng of salmon sperm DNA, 4.5 µg of bovine serum albumin, and various amounts of expressed, recombinant protein, purified from E. coli as indicated, in a binding buffer containing 12 mM HEPES, pH 7.9, 60 mM KCl, 4 mM MgCl2, 10%
glycerol, 1 mM dithiothreitol. All binding reactions were incubated for 20 min at room temperature then loaded onto a 4% polyacrylamide gel (containing 6.75 M Tris, pH 7.9, 3.3 M Na acetate, pH 7.9, 1 M EDTA, 2.5% glycerol). A 200-fold excess of unlabeled double stranded oligonucleotide competitors was included in some reactions as indicated in the figure legends. Sections reacted with sense probes to telokin exhibited similar background staining as those reacted with sense probes to HFH-1. M, mucous layer; E, epithelial cells; SM, submucosa; ME, muscularia externa; MM, muscularia mucosa. Scale bar represents 100 μm. Arrows point out the high levels of HFH-1 expression in the upper left panel and high levels of telokin expression in the lower left panel.

RESULTS

Isolation of HFH-1 Clones—Two positive clones were obtained from a one-hybrid screen of a mouse bladder cDNA library using the telokin core promoter yeast reporter strain. Both clones contained identical 750-bp inserts. Comparison of the sequences with the GeneBank database revealed a 100% identity to the previously identified mouse forkhead transcription factor Hfh-1L (Fig. 1) (45). The clones isolated spanned nucleotides 2502–3252 of the published intronless-Hfh-1L genomic clone (accession number AF010405), which includes the region encoding the forkhead DNA-binding domain. To verify that the clones isolated were responsible for activating the yeast reporter strain the purified plasmids were retransformed into the yeast reporter strain. Following transformation the resultant yeast colonies stained positive for β-galactosidase activity (data not shown), indicating that the cDNAs encoded by the plasmids were indeed responsible for activating the reporter strain.

In order to obtain full-length cDNAs for expression studies, the clones were used to screen a λgt11 cDNA library generated from mouse bladder. Twenty-five positive clones were obtained from this screen all of which encoded various portions of Hfh-1L. The longest clone obtained was 2.65 kilobases and represented an approximately full-length clone as determined from Northern blotting (Fig. 2A). Sequencing confirmed that this clone was identical to the published Hfh-1L cDNA sequence and extended this sequence to include the putative polyadenylation sequence identified in the genomic clone (45). As described previously, mouse Hfh-1L is 93% homologous to rat HFH-1 at the nucleotide level in both the untranslated region and the coding region. We noted that five separate, single nucleotide deletions in the rat HFH-1 cDNA (46) alter the reading frame of this cDNA resulting in divergence of rat and mouse HFH-1 proteins carboxyl-terminal of the winged helix domain. It is likely that these deletions result from sequencing or cloning artifacts as the high degree of nucleotide homology suggests that Hfh-1L is the mouse homologue of rat HFH-1, hence we will refer to it as mouse HFH-1.

Expression of HFH-1 mRNA—Northern blot analysis of total RNA isolated from adult mouse tissues revealed a 2.7-kilobase transcript in bladder and stomach (Fig. 2A). To examine the expression of HFH-1 mRNA in more detail ribonuclease protection assays were performed (Fig. 2B). In agreement with the results of the Northern blots the highest level of expression was seen in stomach and bladder, weaker signals were detected in ileum, proximal and distal colons, vas deferens, liver, lung,
HFH-1 Repression of Telokin Expression

**HFH-1 Binds to the AT-rich Region in the Core of the Telokin Promoter**—To directly determine the binding site for HFH-1 within the core of the telokin promoter, gel mobility shift assays were performed. A gel mobility shift assay using a probe that included both the AT-rich region and CArG box of the telokin promoter demonstrated that recombinant HFH-1 binds specifically to this fragment (Fig. 6). The HFH-1 mobility shifted complex could be competed away by unlabeled fragments encompassing the AT-rich region and CArG box or by a fragment that included only the AT-rich region, but not by a fragment that included only the CArG box or by a homeodomain (HOX) consensus site. Similarly HFH-1 formed a specific mobility shifted complex on a probe that included only the AT-rich region but did not form a mobility shifted complex on a probe that encompassed only the CArG box (Fig. 6). Together these data suggest that HFH-1 binds to the AT-rich region of the telokin promoter. However, it was noted that CArG and HOX fragments could partially compete for HFH binding to probes derived from the AT-rich region alone but these fragments did not compete for HFH-1 binding to a longer probe encompassing both the AT-rich region and the CArG box (Fig. 6). This suggests that although the AT-rich region is sufficient for HFH-1 binding adjacent sequences increase the binding affinity.

To further evaluate the residues important for HFH-1 binding additional competition experiments were performed using fragments that contained different portions of the AT-rich region as well as those containing specific mutations (Fig. 7). The sequence of the fragments used are shown in Fig. 8, together with a summary of their ability to inhibit promoter activity. Results from this analysis show that HFH-1 inhibits telokin promoter activity by 75 ± 2% (Fig. 4). To examine the specificity of the repression, the effects of HFH-1 on several other promoters was examined. HFH-1 repressed the activity of reporter genes driven by SM22α, smooth muscle myosin heavy chain, and smooth muscle α-actin promoters by 76 ± 1, 50 ± 5, and 57 ± 2%, respectively. In contrast, HFH-1 only repressed the activity of the thymidine kinase promoter by 30 ± 3%. The repression of all promoter constructs by HFH-1 was statistically significant with p values < 0.005 (Anova). Based on the ability of HFH-1 to bind to the core of the telokin promoter in yeast, it is likely that HFH-1 exerts its inhibitory effect by binding to this region of the telokin promoter. To demonstrate that this region is responsible for mediating repression it was placed upstream of the minimal thymidine kinase promoter (AT/CArG-TK; Fig. 4). HFH-1 was found to repress the activity of this chimeric promoter reporter gene by 92 ± 0.2%, demonstrating that the core fragment from the telokin promoter mediates the inhibitory effects of HFH-1. To begin to determine the mechanism by which HFH-1 inhibits telokin promoter activity a series of HFH-1 truncations were generated and analyzed for their ability to inhibit promoter activity. Results from this analysis revealed that the inhibitory activity of HFH-1 was contained within the forkhead DNA-binding domain. Truncated HFH-1 proteins that lack the entire forkhead domain were unable to repress telokin promoter activity, in contrast expression of the forkhead domain alone was able to repress promoter activity (Fig. 5). Western blot analysis confirmed that each deletion mutant of HFH-1 was expressed at similar levels and immunohistochemical analysis revealed that each mutant was present in the nucleus of transfected cells (data not shown).

**HFH-1 Represses the Activity of Smooth Muscle-specific Promoters**—Results described above demonstrated that HFH-1 is able to bind to the telokin promoter in yeast. To determine the functional consequence of HFH-1 binding to the telokin promoter, a telokin promoter fragment (400 bp)-luciferase reporter gene was co-transfected together with an HFH-1 expression vector into A10 smooth muscle cells. Previously we have shown that this fragment of the rabbit telokin promoter is sufficient to produce high levels of luciferase activity in A10 cells (6). Results from this analysis show that HFH-1 represses telokin promoter activity by 75 ± 2% (Fig. 4). To examine the specificity of the repression, the effects of HFH-1 on several other promoters was examined. HFH-1 repressed the activity of reporter genes driven by SM22α, smooth muscle myosin heavy chain, and smooth muscle α-actin promoters by 76 ± 1, 50 ± 5, and 57 ± 2%, respectively. In contrast, HFH-1 only repressed the activity of the thymidine kinase promoter by 30 ± 3%. The repression of all promoter constructs by HFH-1 was statistically significant with p values < 0.005 (Anova). Based on the ability of HFH-1 to bind to the core of the telokin promoter in yeast, it is likely that HFH-1 exerts its inhibitory effect by binding to this region of the telokin promoter. To demonstrate that this region is responsible for mediating repression it was placed upstream of the minimal thymidine kinase promoter (AT/CArG-TK; Fig. 4). HFH-1 was found to repress the activity of this chimeric promoter reporter gene by 92 ± 0.2%, demonstrating that the core fragment from the telokin promoter mediates the inhibitory effects of HFH-1. To begin to determine the mechanism by which HFH-1 inhibits telokin promoter activity a series of HFH-1 truncations were generated and analyzed for their ability to inhibit promoter activity. Results from this analysis revealed that the inhibitory activity of HFH-1 was contained within the forkhead DNA-binding domain. Truncated HFH-1 proteins that lack the entire forkhead domain were unable to repress telokin promoter activity, in contrast expression of the forkhead domain alone was able to repress promoter activity (Fig. 5). Western blot analysis confirmed that each deletion mutant of HFH-1 was expressed at similar levels and immunohistochemical analysis revealed that each mutant was present in the nucleus of transfected cells (data not shown).

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taining a 2-base pair mutation within the TATA core of AT-rich region was unable to compete for HFH-1 binding (AT-MUT). Together, these results suggest that the TATA sequence within the AT-rich region of the telokin promoter is important for HFH-1 binding. These data are also consistent with the winged helix consensus binding sequence (ATAAAT) being the important site for HFH-1 binding to the telokin promoter (Fig. 8).

HFH-1 and SRF Can Simultaneously Bind to the Telokin Promoter—Gel mobility shift analysis demonstrated that HFH-1 binds sequences within the AT-rich region of the core of the telokin promoter and not to the CArG box required for SRF binding (Figs. 6 and 7), suggesting that both transcription regulators could bind the telokin promoter at the same time. HFH-1 is also known to bend DNA, leading to the possibility that it could indirectly prevent SRF from binding and transactivating the telokin promoter. In order to examine the possibility that HFH-1 and SRF are able to bind to the telokin promoter at the same time, the AT/CArG probe was incubated with increasing amounts of purified recombinant HFH-1 and a constant amount of purified, recombinant SRF. At low concentrations of HFH-1 (up to 200 ng), two gel mobility shifted complexes formed, corresponding to the individual SRF- and HFH-1-bound complexes (Fig. 9). As the amount of HFH-1 was increased there is slight upward shift of the SRF containing complex, together with a progressive decrease in the intensity of the HFH-1 complex, suggesting that a dimeric complex was being formed. At higher concentrations of both SRF and HFH-1 only the more slowly migrating dimeric complex was observed. Within this complex, SRF and not HFH-1 was competed by a CArG fragment, while HFH-1 but not SRF was competed by an AT fragment. These results demonstrate that HFH-1 binding...
HFH-1 binding. ^32P-Labeled double-stranded AT/CArG oligonucleotide and purified HFH-1 (100 ng) were incubated alone (NONE) or with a 200-fold excess of unlabeled oligonucleotide competitors as indicated. Reactions were incubated at room temperature for 20 min, separated on a 4% polyacrylamide gel and mobility shifted complexes visualized by autoradiography. Competitors used were: no competitor (NONE); AT/CArG, a probe encompassing the AT-rich element and CArG element of the telokin promoter; AT/CArG MUT, a mutated form of the AT-rich element and CArG element of the telokin promoter; AT, the AT-rich region alone; AT-MUT, a 2-bp mutation in the AT-rich region; CArG, the CArG element alone; MEF2 MCK, a consensus binding site for MEF2 from the creatine kinase gene; MEF2 MUT, the AT-rich element of the telokin promoter harboring a mutation that prevents MEF2 from binding to this region; E BOX, the E box element from the telokin promoter; OCT, a consensus octomer-binding site (Fig. 8).

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to the telokin promoter does not disrupt SRF binding and that both SRF and HFH-1 can bind to the telokin promoter at the same time.

DISCUSSION

These results show that the smooth muscle-specific telokin promoter is a downstream target of HFH-1 and provide evidence of a novel inhibitory role for HFH-1. HFH-1 inhibits telokin promoter activity, by binding to a forkhead consensus site located within an AT-rich region of the telokin promoter core and preventing the binding of transcription activators. HFH-1 was also found to repress transcription of several other smooth muscle-specific promoters, including the SM22α, smooth muscle myosin, and smooth muscle α-actin promoters. The localization of HFH-1 mRNA to the epithelial cell layer of mouse bladder and stomach may implicate HFH-1 in repressing telokin expression in epithelial cells. Previous studies on the smooth muscle α-actin and myosin heavy chain promoters have shown that the activity of these promoters is repressed in fibroblasts through the binding of negative-acting factors (28, 29, 47, 48). Taken together with our data, these results suggest a general paradigm in which the expression of smooth muscle-specific proteins is restricted to smooth muscle, not only through the action of positive-acting factors located in smooth muscle cells, but also through negative-acting factors in non-muscle cell types such as epithelial cells.

HFH-1 is a member of the hepatocyte nuclear factor/forkhead family of nuclear factors that share a conserved DNA-binding domain that structurally resembles a winged helix (49). Several of these factors have been shown to have important roles in early embryonic development and during development of many organ systems including the central nervous system (50), skeletal system (51), cardiovascular system (52), and the urogenital system (53) in addition to liver and lung development (54, 55). Within the conserved winged helix domain, a range of sequence diversity exists among family members such that family members can be subdivided into several distinct classes (46, 49). For example, the winged helix region of the rat HFH-1 is only 50% homologous to HNF-3α and has been thought to be a subgroup of the HNF-3/fkh family (46). It is likely that the sequence variation within the winged helix in addition to residues flanking this domain allows for the specific DNA site recognition that is required for the unique cellular functions of each HFH family member (56). HFH-1 has been previously proposed to play a role in kidney development because of its expression in the outer medulla of the kidney and transitional epithelium of the renal pelvis and ureter (45). The low levels of HFH-1 mRNA detected in kidney by our RNase protection analysis as compared with previous Northern blot data (45) likely result from differences in tissue preparation. Most of the renal pelvis and ureters were removed from our kidney samples during tissue collection and these structures were previously reported to express the majority of the HFH-1 present in the kidney. We also detected high levels of HFH-1 expression in the urinary bladder and stomach and lower levels of expression in ileum, colon, vas deferens, liver, lung, and kidney. The localization of HFH-1 to the epithelial cells of the renal pelvis, ureter (45), bladder, and stomach (Fig. 3) suggests that HFH-1 may play a general role in the differentiation of specific epithelial cell lineages. Initially the localization of HFH-1 to epithelial cells, rather than smooth muscle cells that express high levels of telokin, was surprising given this factor was cloned by its ability to bind to the smooth muscle-specific telokin promoter. This apparent contradiction can, however, be explained by the finding that HFH-1 actually inhibits the activity of smooth muscle-specific promoters (Fig. 4), and would therefore be anticipated to be absent from smooth muscle cells.

Electrophoretic mobility shift assays restricted HFH-1 binding to the AT-rich region of the telokin promoter core. Within this region there are two overlapping sequences that partially conform to the HFH-1 consensus DNA-binding site (A(A/T)T-GTTTA(G/T)(A/T)T) determined from degenerate oligonucleotides (Fig. 8) (56). However, both sequences show significant divergence from the consensus HFH-1-binding sites (Fig. 8; −75 to −65, TTGCTTTATAT; −58 to −68 ATAGTTTATAT), similar sequences were also found in the SM22α promoter (−406 to −414, GCTTTAAA), the smooth muscle myosin heavy chain promoter (−22 to −29, GCTTTATA), and smooth muscle α-actin promoter (−30 to −22 CCTATAA; −235 to −226 ATGTTTATCT). Results from gel mobility shift assays suggest that the −58 to −68 consensus site in the telokin promoter is most important for HFH-1 binding to this promoter (Fig. 7). However, competition experiments suggest that sequences flanking the consensus site are also important for high affinity binding (Fig. 6). Interestingly the −58 to −68 site in the telokin promoter most closely matches the HFH-1 consen-
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Fig. 5. Oligonucleotide sequences and their ability to compete for HFH-1 binding to the telokin promoter. Names and sequences of the sense strands of oligonucleotides used in the gel mobility shift assays are aligned below the telokin promoter core. Underlined nucleotides are not present in the telokin promoter and lowercase nucleotides indicate mutations. Oligonucleotide sequences that do not align with the telokin promoter are listed below the horizontal line. Two overlapping, consensus winged helix-binding motifs are enclosed by boxes. Indicated to the left of the oligonucleotide name is their ability to compete for binding to HFH-1, + indicates competition, − indicates the inability to compete.

Fig. 9. HFH-1 and SRF bind the AT/CARG oligonucleotide simultaneously. Gel mobility shift assays were performed using 32P-labeled double-stranded AT/CARG oligonucleotide as a probe. The probe was incubated with various quantities (ng) of purified HFH-1 and SRF, expressed in bacteria, as indicated. A 200-fold excess of unlabeled competitor (COMP) was added to the reaction where indicated. C, CARG; A, AT-rich region; M, AT-rich region containing a two-base pair mutation (Fig. 8). 32P-Labeled double-stranded CARG and AT oligonucleotides were used as probes in the last two right lanes of the gel.

that the DNA-binding domain alone is sufficient to repress promoter activity, thus suggesting that inhibition is likely to be mediated by binding the loop of positive-acting factors. In further support if this mechanism alteration of residues within the HFH-1-binding site decreased rather than increased telokin promoter activity in A10 smooth muscle cells (18). Although HFH-1 does not block SRF binding to the telokin promoter these data suggest that it blocks the binding of another unidentified positive-acting factor. Previous studies on the CCSP promoter in HeLa cells demonstrated that HNF-3β inhibited promoter activity whereas HNF-3α activated the promoter (57). The opposing effects of these two members of the HNF-3 family suggest a model in which the transcription of telokin in smooth muscle cells requires the binding of an unidentified, positive-acting member of the HFH family. In other cells types this factor would either be absent and/or high levels of the inhibitory HFH-1, such as found in epithelial cells, would block its activity. Identification of other proteins that can bind to this region of the telokin promoter will be required to resolve these possibilities, based on previous reports possible candidates would include HNF3-β and HFH-8 (42, 58).

In summary, our results show that smooth muscle-specific promoters represent novel downstream targets of the winged helix factor HFH-1. HFH-1 strongly represses telokin promoter activity when overexpressed in A10 vascular smooth muscle cells. We propose a model in which the smooth muscle specificity of the telokin promoter is regulated by interactions between positive- and negative-acting members of the hepatocyte nuclear factor-3/forkhead family of transcription factors.

Acknowledgments—We thank Dr. R. Prywes for providing the human SRF cDNA and Dr. Patricia Gallagher for helpful comments on this manuscript.

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doi: 10.1074/jbc.M005595200 originally published online July 13, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M005595200

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