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Rapid Identification of Homeodomain Binding Sites in the *Wnt-5a* Gene Using an Immunoprecipitation Strategy

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Here we describe an immunoprecipitation approach for identifying homeodomain binding sites within uncharacterized genomic sequences of a putative downstream target gene, *Wnt-5a*. Immunoprecipitation of *Wnt-5a* genomic fragments was performed using a purified Msx1 homeodomain polypeptide (Msx1) and its corresponding antisera (α-Msx1). This resulted in isolation of three fragments containing multiple DNA binding sites for Msx1, as confirmed by DNA binding studies. The three fragments were contiguous within a 3.4 kb intronic sequence of *Wnt-5a*. Moreover, at least one of the Msx1 sites has been conserved throughout evolution, suggesting that these sites may comprise or contribute to a regulatory element for *Wnt-5a*. We propose that the immunoprecipitation strategy permits a rapid, initial approach for identifying functionally-relevant homeodomain binding sites within target genes whose regulatory sequences have not yet been previously elucidated.

Various methods have been employed to identify genomic DNA binding sites for homeodomain proteins within the transcriptional control sequences of downstream target genes (reviewed in 1). For the most part, these approaches search for binding sites within total genomic DNA (2,3) or soluble (i.e., transcribed) chromatin (4,5). Therefore, subsequent to their identification, much additional work is required to define the putative target gene in which the binding site resides, and to determine whether it is indeed a regulatory component or merely a fortuitous binding site. We have shown previously that immunoprecipitation provides a useful strategy to identify high affinity and functionally-relevant homeodomain binding sites from the transcriptional control regions of a potential target gene, *Wnt-1* (6). Here we extend these observations to explore whether this approach may also be used to identify such sites in the uncharacterized genomic sequences of potential target genes.

We have chosen for this study the murine *Wnt-5a* gene since its pattern of expression during embryogenesis overlaps spatially and temporally with that of several homeobox genes, in particular, *Msx1* (7, 8, 9, 10, 11). Since *Wnt* genes are known to be downstream targets for homeodomain control (6), we predicted that *Wnt-5a* may be regulated by Msx1 or by another homeobox gene with which it is co-expressed. However, the regulatory sequences that underlie the restricted expression of *Wnt-5a* have not yet been characterized. As a first step to identify such sequences, we have screened *Wnt-5a* genomic DNA for homeodomain binding sites using the Msx1 protein. We find that *Wnt-5a* contains three fragments each containing multiple Msx1 binding sites. These fragments are contiguous within a 3.4 kb intron of *Wnt-5a*, and at least one of the sites has been evolutionarily conserved. These findings suggest that the immunoprecipitation strategy may provide a rapid initial approach to identify potential regulatory elements within the uncharacterized genomic sequences of putative homeodomain target genes.

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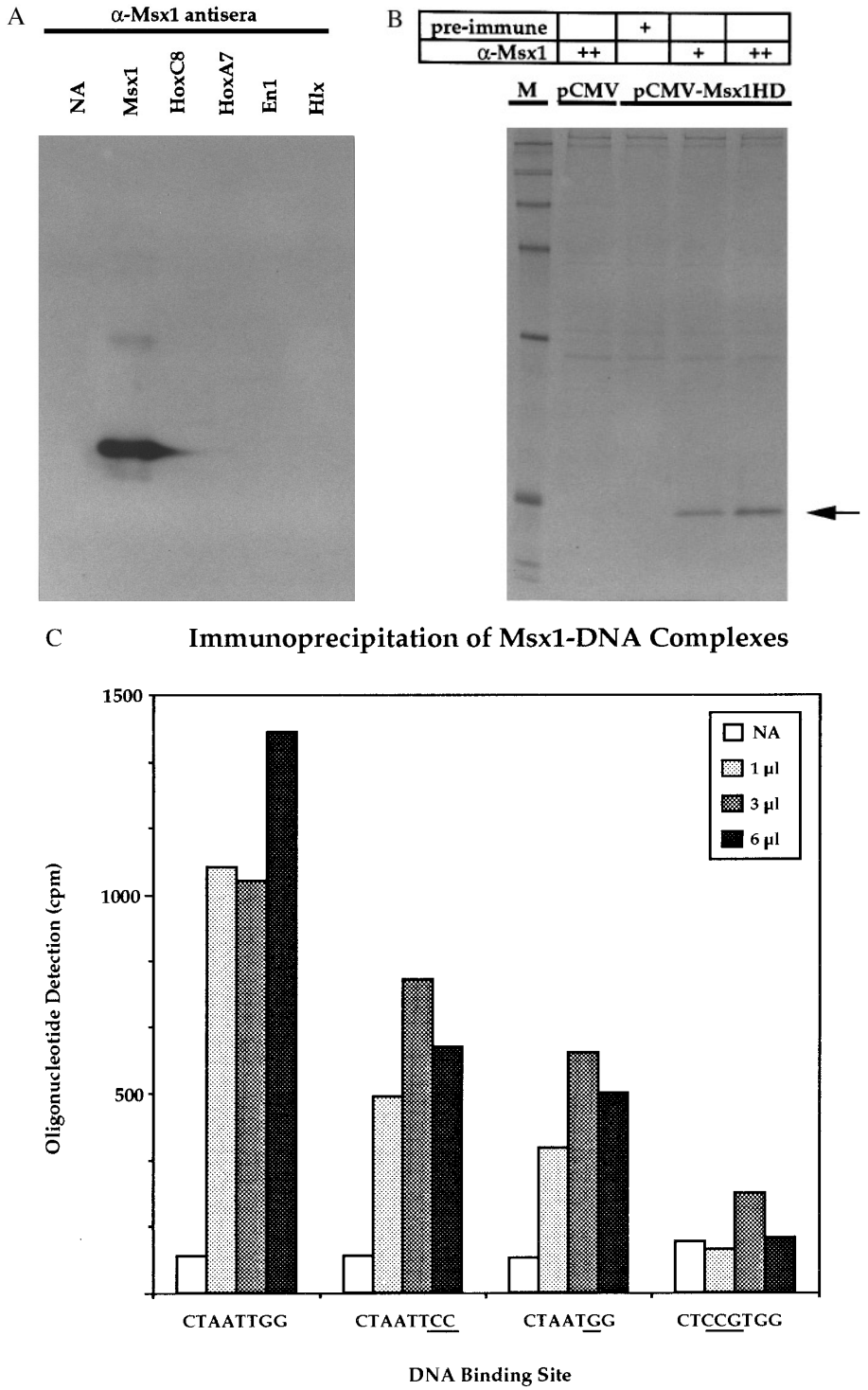

FIG. 1. Characterization of α-Msx1 antisera. (A) Western blot analysis. 50 ng of bacterially-expressed homeodomain polypeptides (Msx1, HoxC8, HoxA7, En1, Hlx) were mixed with a crude protein lysate (50 μg) and resolved on a 13.5% SDS-polyacrylamide gel. Western blot analysis was performed using a 1:1000 dilution of α-Msx1 and antigen-antibody complexes were visualized by chemiluminescence. NA indicates with no addition of homeodomain

MATERIALS AND METHODS

Antisera production and characterization. Expression and purification of Msx1 and other homeodomain polypeptides have been described previously (12, 13). Polyclonal antisera directed against the Msx1 homeodomain polypeptide (amino acids 157 to 233, as in 12) was prepared in rabbits (Cocalico Biologicals, PA). Western blotting was performed by standard techniques using a 1:1000 to 1:5000 dilution of antisera and antigen-antibody complexes were visualized using a chemiluminescence reagent (ECL immunoblotting kit, Amersham).

For immunoprecipitation from metabolically-radiolabeled cells, COS-1 cells were transfected with 2.5 μg/60 mm dish of an expression vector encoding the Msx1 homeodomain (pCMV-Msx1HD) or a control plasmid (pCMV) which were described in (12). 48 hours post-transfection, cells were radiolabeled by addition of ^{35}S-methionine (400 μCi/ 60 mm dish), and lysates prepared by addition of RIPA buffer. Immunoprecipitation was performed by incubating lysates with α-Msx1 for 1 hour at 4°C, followed by addition of Staph A cells. Proteins were resolved on a 13.5% SDS-polyacrylamide gel and visualized by autoradiography.

For immunoprecipitation with Msx1 binding sites, oligonucleotides containing three tandem DNA sites were radiolabeled with γ^{32}P-ATP in the presence of T4 polynucleotide kinase. Msx1 (0.25 μM) was incubated with the radiolabeled oligonucleotides, and DNA-protein complexes were precipitated with α-Msx1 in PBS containing 0.1% NP40. The cpms in the precipitated oligonucleotides were measured using a scintillation counter.

Immunoprecipitation from genomic DNA. Wnt-5a DNA was prepared for immunoprecipitation as follows: a 14.3 kb fragment was isolated by *Eco*RI digestion of the pGEM4Z (Promega) derivative, p437 (kindly provided by A. McMahon), and then digested with *Hae*III. 'Catch linkers' contained internal *Hae*III and *Cla*I restriction sites flanked by *Not*I half-sites. Catch linker A (5′-CCGCAGGCCATCGATGCGG-3′) and Catch linker B (5′-CCGCATCGA TGGCCTGCGG-3′) were phosphorylated and radiolabeled using T4 polynucleotide kinase and γ^{32}P-ATP. Dephosphorylated *Hae*III fragments were ligated overnight to annealed 'catch linkers' (1:6 ratio). Following inactivation of ligase, DNA was over-digested twice with *Not*I enzyme to disrupt self-ligated linkers. For immunoprecipitation, DNA was radiolabeled using α^{32}P-dCTP in the presence of Klenow, and incubated with 0.13, 0.40, or 1.2 μM of Msx1 or 0.04, 0.12, or 0.40 μM full-length Msx1 protein (14). Protein-DNA complexes were precipitated with α-Msx1 (10 μl) followed by Staph A cells (40 μl). Bound DNA was isolated, and DNA fragments were resolved by gel electrophoresis and identified by autoradiography. Precipitated DNA was eluted from gel fragments and amplified by PCR, and the products cloned into the polylinker of pGL2-promoter vector (Promega) for further analysis.

Electrophoretic mobility shift assays and DNaseI footprinting. DNA binding assays were performed as described in (12). For competition assays, radiolabeled fragments were pre-incubated with increasing concentrations (5 - 100 ng) of cold competitor oligonucleotide (20 bp) containing a single copy of the Msx1 consensus DNA site (12). For DNase I footprinting, oligonucleotides corresponding to flanking sequences in the plasmid were used to PCR-amplify fragment A3. The products were purified using Microcon-100 columns (Amicon), and radiolabeled. Footprinting was performed as described (15).

RESULTS AND DISCUSSION

Characterization of Anti-Msx1 Antisera (α-Msx1)

Besides its coincident co-expression with *Wnt-5a* during embryogenesis (10, 11), Msx1 is a good choice of homeodomain proteins to develop the immunprecipitation strategy since its DNA binding properties have been well characterized and since it exhibits a relatively high affinity and specificity for DNA (12). For use in the immunoprecipitation assay, we produced a polyclonal antisera (α-Msx1) directed against the bacterially-expressed, purified Msx1 homeodomain polypeptide (Msx1) (12). Western blot analysis demonstrated that α-Msx1 recognizes Msx1, but does not cross react with the other homeodomain-containing proteins tested, i.e., HoxC8, HoxA7, En1 and Hlx (Fig. 1A). Antisera specificity was further confirmed by immunoprecipitation from metaboli-

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protein. (B) Immunoprecipitation from metabolically-labeled cells. COS-1 cells were transfected with an expression plasmid encoding Msx1, pCMV-Msx1HD, or a control plasmid, pCMV. Cells were radiolabeled by addition of ^{35}S-methionine and cell lysates were incubated with the indicated amount of α-Msx1 followed by addition of Staph A cells. Immunoprecipitation products were resolved on a 13.5% SDS-polyacrylamide gel and visualized by autoradiography. Markers, M, correspond to ^{14}C-labeled protein markers; + indicates 1.5 μl of α-Msx1 or pre-immune sera; ++ indicates 3 μl of α-Msx1. Arrow corresponds to Msx1. (C) Immunoprecipitation of Protein/DNA Complexes: 0.25 μM Msx1 was incubated with 50,000 cpm of each oligonucleotide containing a three copies of the DNA sites shown. α-Msx1 (1, 3, or 6 μl) was incubated for 1 hour, followed by addition of Staph A cells to pellet the immune complexes. After washing, bound oligonucleotide was detected as counts per minute (cpm) using a scintillation counter. Underscore highlights nucleotides that differ from the consensus Msx1 binding site.

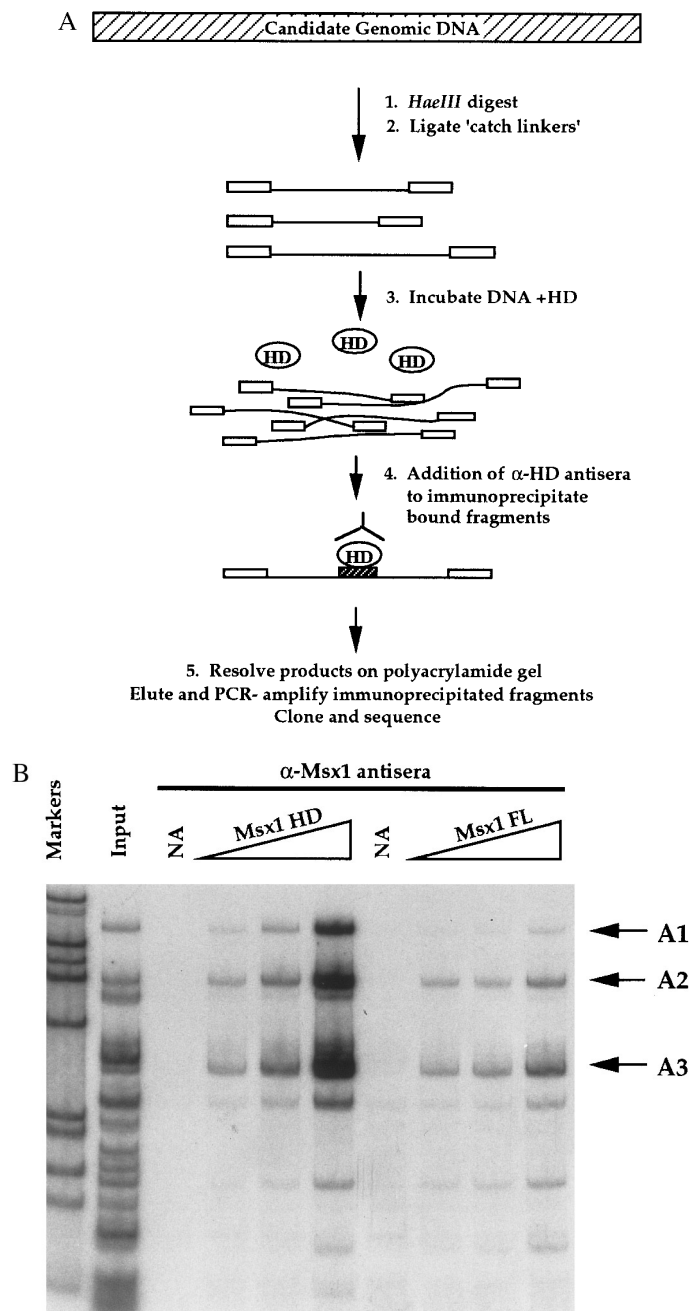

FIG. 2. Three fragments are immunoprecipitated from *Wnt-5a*. (A) Strategy for immunoprecipitation of genomic homeodomain binding sites. Candidate genomic DNA (i.e., *Wnt-5a*) was digested with *Hae*III and 'catch linkers' ligated to ends. 'Catch linked' fragments were incubated with bacterially expressed, highly purified homeodomain polypeptide using DNA binding conditions (Catron et al., 1993). α-Msx1 was added, followed by Staph A cells to immunoprecipitate Msx1-DNA complexes. DNA fragments were eluted, resolved on a 6% polyacrylamide gel, isolated and PCR-amplified using 'catch linkers' as primers. HD=homeodomain polypeptide. (B) Immunoprecipitation of fragments from *Wnt-5a* genomic clone. *Wnt-5a* genomic DNA was digested with HaeIII (lane marked input), radiolabelled fragments were incubated in absence of protein (NA) or with increasing amounts of Msx1 (0.13, 0.40, or 1.2 μM) or the full length Msx1 protein (0.04, 0.12, or 0.40 μM). Markers correspond to λ-*Hin*dIII and ϕX174-*Hae*III digest. The positions of fragments A1, A2, A3 are shown by the arrows.

cally-radiolabeled cells expressing Msx1 (Fig. 1B). α-Msx1 immunoprecipitated a single polypeptide corresponding to the correct molecular size of Msx1 from cells transfected with a plasmid encoding the protein (pCMV-Msx1HD) but not from cells transfected with a control plasmid (pCMV) (Fig. 1B). To test whether α-Msx1 also recognized Msx1-DNA complexes, we used an immunoprecitation assay. Radiolabeled oligonucleotides containing DNA sites known to have varying affinities for Msx1 (12) were incubated with the protein, followed by precipitation with α-Msx1 (Fig. 1C). As shown, the relative degree of binding (evident from the precipitated cpms) paralleled the relative affinity of Msx1 for the various DNA sites. The availability of antisera specific for Msx1 and which does not disrupt the DNA-protein complex provides an essential tool for immunoprecipitation of Msx1 binding sites from genomic DNA.

Immunoprecipitation of Wnt-5a Genomic DNA Yields Three Fragments with Multiple Msx1 Binding Sites

The main distinction between the immunoprecipitation strategy we have employed and those previously described (2, 3) is that rather than using total genomic DNA, we have focused our study on uncharacterized DNA from a gene (i.e., *Wnt-5a*) considered to be candidate for homeodomain control. This distinction permits us to examine interactions of Msx1 with genomic sites without the necessity of identifying the resident gene containing such sites. The general strategy is outlined in Fig. 2A. As shown, genomic DNA (*Wnt-5a*) is digested with *Hae*III, to yield fragments of 100-2000 bp. Linkers are ligated to the fragments to facilitate subsequent PCR amplification, and the fragments incubated with limiting amounts of homeodomain protein (Msx1), followed by addition of antisera (α-Msx1) to immunoprecipitate the protein-bound fragments. The fragments are analyzed by polyacrylamide gel electrophoresis and cloned for further characterization.

The results of such an experiment performed using *Wnt-5a*, Msx1 and α-Msx1 are shown in Figure 2B. Immunoprecipitation of *Wnt-5a* genomic DNA using either Msx1 (Msx1 HD) or the full-length Msx1 protein (Msx1 FL) resulted in the isolation of three fragments, termed A1, A2 and A3; one of 1.5 kb (A1), one of 800 bp (A2), and one of 400 bp (A3) (Fig. 2B). Sequence analysis of A1, A2 and A3 revealed the presence of multiple Msx1 binding sites (Fig. 3B; discussed below). The *Wnt-5a* genomic clone used in this study contained the coding sequence (summarized in Fig. 3A), but not sequences upstream that include the promoter (Iler and Abate-Shen, unpublished). Similar immunoprecipitation studies performed using a 3 kb genomic clone containing the *Wnt-5a* promoter failed to isolate any DNA fragments (Iler and Abate-Shen, unpublished). Therefore, *Wnt-5a* has three fragments containing Msx1 binding sites, and these are located downstream of its promoter.

The Three Fragments Map to a Single 3.4 kb BamHI Fragment within a Wnt-5a Intron

To define the location of A1, A2, and A3 with respect to each other and to the *Wnt-5a* coding sequence, a combination of Southern blot analysis and PCR were performed. Since the *Wnt-5a* genomic sequence is largely uncharacterized, a crude restriction map was constructed to aid in the localization of the fragments (Fig. 3A). Southern blot analysis showed that A1, A2, and A3, were all contained within a single 3.4 kb *Bam*HI fragment (summarized in Fig. 3A). Furthermore, PCR using various mixtures of oligonucleotides corresponding to the 5′ and 3′ sequences of the individual fragments revealed that A1, A2, and A3 were contiguous and that they were located primarily in an intron between exons 3 and 4 (summarized in Fig. 3A). Complete sequence of the *Bam*HI fragment (shown in Fig. 3B) confirmed these results and defined the position of exon 3 within A1 (Fig. 3A and 3B). Sequence analysis also revealed the presence of multiple Msx1 binding sites within all three fragments; for example, A1 contained seven sites, A2 fragment contained nine sites; and A3 contained six sites (Fig. 3B; indicated by the TAAT core or underscored ATTA core). We noted that of the 29 putative Msx1 binding sites in the *Bam*HI fragment, 15 were clustered in the 3′ end in the A3 and A2 fragments.

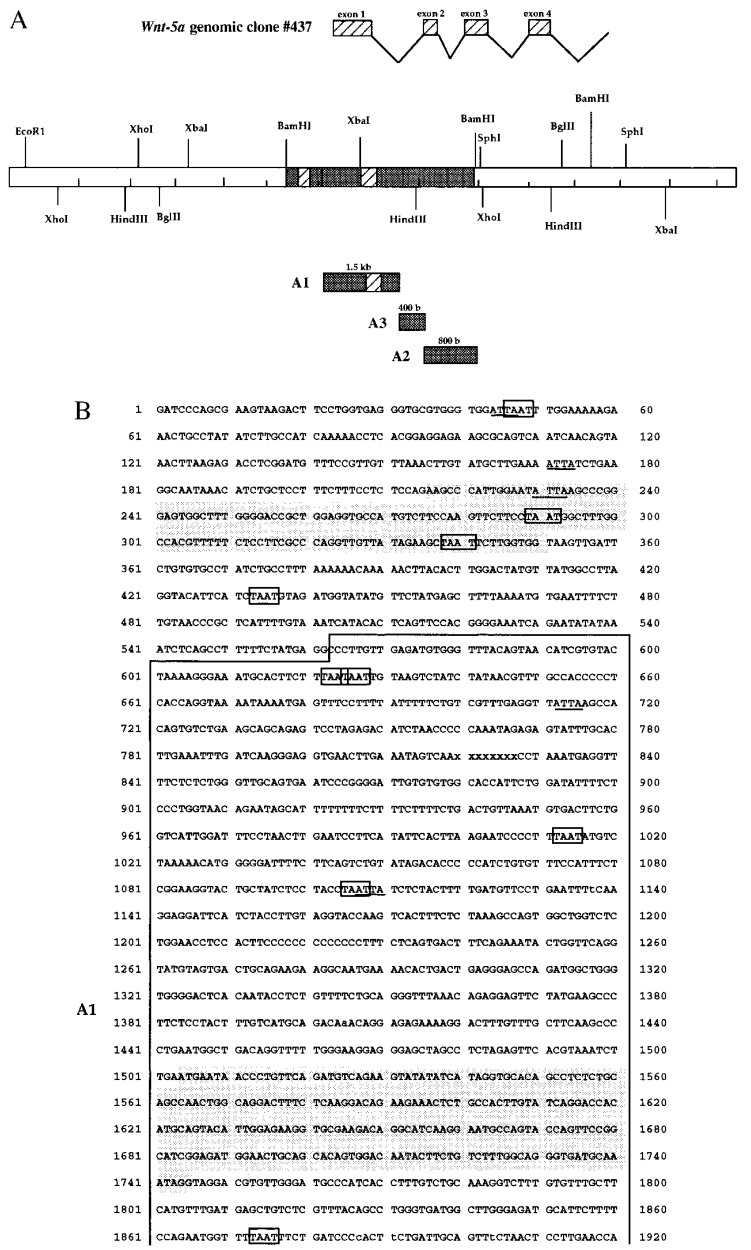

FIG. 3. The *Wnt-5a* immunoprecipitation fragments map to a 3.4 kb intronic *Bam*HI fragment. (A) Location of immunoprecipitation fragments within the *Wnt-5a* genomic clone, p437. Top: *Wnt-5a* genomic map with coding regions indicated as slashed boxes. Middle: Crude restriction map of *Wnt-5a* genomic clone, with location of *Bam*HI fragment shown as stippled box, and pertinent exons (140 bp and 240 bp, respectively) as indicated. Bottom: Location of A1, A2 and A3 fragments shown separately to display their order within the *Bam*HI fragment and to highlight their proximity to each other in context of the entire 14.3 kb genomic clone. (B) Sequence of the 3.4 kb *Bam*HI fragment. TAAT motifs are boxed, complementary ATTA motifs are underscored. Light stippled regions indicate exons (140 bp and 240 bp); dark stippled region indicates DNA sequence used in DNaseI footprinting (see Figure 4B) and also sharing 71% identity with a 190 bp region of the 3′ UTR of human *Wnt-5a.* A1, A2, and A3 fragments are boxed as indicated. Plus strand is shown.

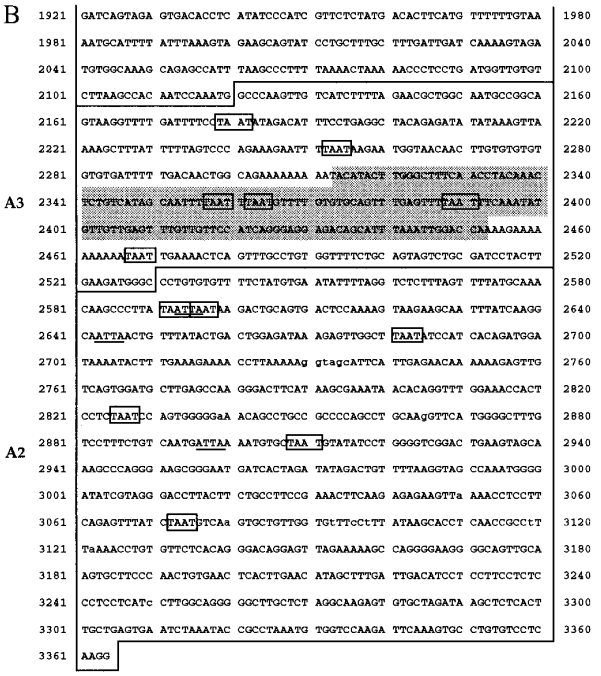

FIG. 3. —*Continued*

Msx1 Interacts with the Homeodomain Binding Sites in A1, A2 and A3

To examine the interaction of Msx1 with its putative binding sites, we used A1, A2, and A3 as probes in electrophoretic mobility shift analysis. All three fragments were bound specifically by Msx1 (Fig. 4A). Moreover, increasing amounts of Msx1 resulted in the formation of multiple protein-DNA complexes, indicating the presence of multiple DNA binding sites (Fig. 4A). Interaction of Msx1 with A1, A2, and A3 was inhibited by addition of unlabeled oligonucleotides containing the Msx1 consensus binding site (Fig. 4A), but not by an oligonucleotide containing a nonspecific DNA site (data not shown). To further examine the Msx1-DNA interactions, we performed DNAse I footprinting on A3. Both Msx1 and the full-length Msx1 protein protected A/T-rich regions contained within this fragment (Fig. 4B and C). As shown in Figures 4B and C, three regions are protected or altered: Region A contains two sites in tandem (TTTAATTTAATGT) that are bound by both Msx1 and the full-length protein; Region B (TTTTAATTTCA) is protected by Msx1 and partially by the full-length Msx1; Region C (ATTTAAATT) is a hypersensitive site. Together with the electrophoretic mobility shift assays, these studies demonstrate that Msx1 binds specifically to multiple sites within the *Wnt-5a* genomic sequence.

The BamHI Fragment Contains Multiple Putative Transcription Factor Binding Sites, as Well as a Conserved Msx1 Binding Site

A computer-assisted search for regulatory elements within the 3.4 kb *Bam*HI fragment revealed a large number of putative transcription factor binding sites, including an octamer site (1846), NF-κB site (1031), GCN4 sites (938, 1267, 2608, 2657), Sp1 site (2848), AP-1 site (2894), AP-3 site (2487), c-mos binding motifs (1867, 2384, 2978), and 19 putative E box elements (CANNTG). We also performed comparative analyses of the *Wnt-5a* fragment with putative regulatory regions of the human homologue of *Wnt-5a, hWnt-5a.* This gene

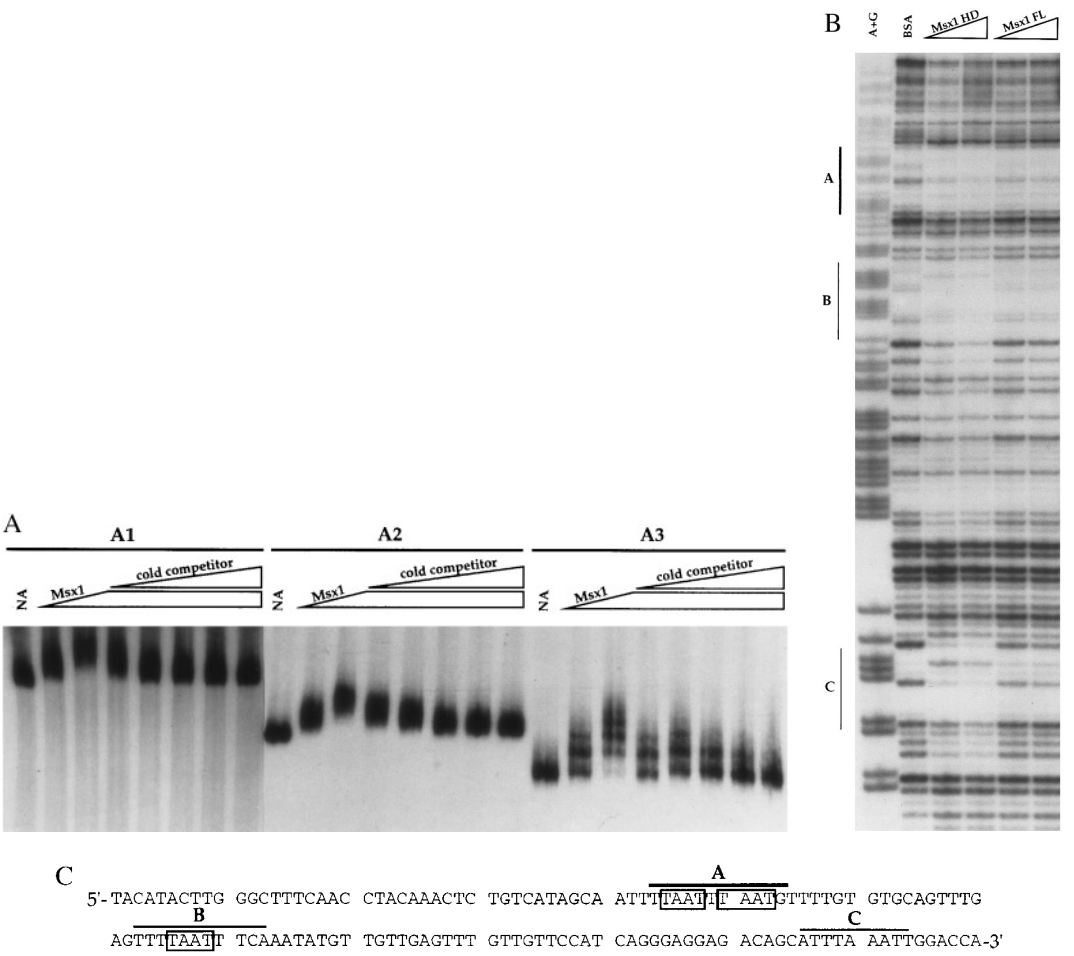

FIG. 4. Msx1 interacts with DNA sites within A1, A2, and A3 fragments. (A) Gel retardation assays were performed with radiolabeled A1 (1.5 kb), A2 (800 b), and A3 (400 b) fragments in the absence (NA) or presence of Msx1 homeodomain polypeptide (0.05 or 0.2 μM). For competition assays, increasing amounts of cold competitor oligonucleotide (20 bp) containing a consensus homeodomain binding site (CTAATTGG) were pre-incubated with radiolabeled fragments, followed by incubation with Msx1 (0.2 μM), as indicated. An autoradiograph of the gel is shown. (B) DNaseI footprinting was performed on the 400 bp A3 fragment in the presence of BSA (200 ng), Msx1, or full-length Msx1 (0.6 or 1.2 μM). Experiments were performed with top and bottom strands; results obtained with bottom strand are shown. Footprinted regions are indicated on the left of autoradiograph as vertical bars. A+G indicates Maxam-Gilbert sequencing reaction. (C) Sequence of the A3 fragment corresonding to the region subjected to DNaseI footprinting in (B). Regions protected from DNaseI cleavage are highlighted (A, B, C), as indicated; TAAT motifs are boxed.

encodes a protein product with >99% identity to its respective murine counterpart (16). The 3′ untranslated region of *hWnt-5a* is approximately 2.5 kb in length and contains 34 putative homeodomain binding sites. A computer-assisted sequence alignment revealed 71% identity within a 180 bp subregion of A3 (Fig. 3B; 2320-2496) and 190 bases of the *hWnt-5a* 3′ UTR (3655-3845). Within this conserved region, three of four TAAT motifs have been retained. Interestingly, of the footprinted regions in A3 (Fig. 4B), 10 of 11 nucleotides (90.9%) are conserved in region A; 8 of 11 (72.7%) are conserved in region B; and region C, the hypersensitive site, is considerably less conserved, 55.6% (5 out of 9). Conservation of A3 with regulatory

sequences contained within human *Wnt-5a* gene suggests that this fragment may contribute to a regulatory region of murine *Wnt-5a.*

CONCLUSIONS

We describe a rapid approach for screening high affinity homeodomain binding sites within candidate genomic DNA. This strategy relies on the use of antisera directed against the homeodomain of Msx1 that is specific and efficient in immunopurifying Msx1-DNA complexes. We found that *Wnt-5a* contains three fragments which have Msx1 binding sites. These fragments are linked and reside within a 3.4 kb *Bam*HI fragment of primarily intronic sequence. Furthermore, this fragment may exhibit regulatory activity, as suggested by its partial conservation with the 3′ UTR of human *Wnt-5a*. In other cases, immunoprecipitation experiments performed to isolate genomic targets have used soluble chromatin from lysed nuclei, as a source of DNA. While this strategy may prove quite effective (17), identification of the gene from which the immunoprecipitated fragment originates can be a laborious, and often, insurmountable task, particularly if multiple fragments result. By restricting our analysis to candidate genes we circumvent this problem, however, additional levels of regulation present in soluble chromatin are lost. Although the strategy we describe herein has been used quite successfully to identify a biologically relevant homeodomain binding site in the *Wnt-1* gene (6), this approach should be perceived as a useful starting point at which to further examine homeodomain-DNA interactions in the context of a cellular environment and to investigate the potential for specific interactions with identified sites *in vivo.*

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