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Wnt-1-Inducing Factor-1: a Novel G/C Box-Binding Transcription Factor Regulating the Expression of *Wnt-1* during Neuroectodermal Differentiation

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The *Wnt-1* proto-oncogene is essential for proper development of the midbrain and is expressed in a spatially and temporally restricted manner during central nervous system development in mice. In vitro, the gene is specifically transcribed during the retinoic acid (RA)-induced neuroectodermal differentiation of the P19 line of embryonal carcinoma cells. The P19 cells differentiate into neurons, astrocytes, and fibroblast-like cells when treated with RA. Treatment of the cells with dimethyl sulfoxide leads to differentiation along mesodermal lineages, including skeletal and cardiac muscle. We have used the P19 cell line to study the *Wnt-1* promoter and identify and characterize the transcription factor(s) that regulates the differentiation-specific transcription of *Wnt-1* in RA-treated P19 cultures. Transient-transfection assays have revealed that a 230-bp region comprising positions −278 to −47 of the 5′ upstream *Wnt-1* sequence was sufficient to direct RA-specific transcription. This promoter fragment was shown to contain a binding site for a nuclear factor that was not detected in undifferentiated P19 stem cells or their dimethyl sulfoxide-treated derivatives but was induced in differentiating RA-treated cells. This factor was termed *Wnt-1*-inducing factor-1 (WiF-1). DNase I footprinting analysis has identified the G/C-rich WiF-1 binding site, and UV cross-linking studies have shown that WiF-1 is a protein with an M_r of 65,000. WiF-1 binding activity was also detected in postpubertal mouse testis, the only tissue that expresses *Wnt-1* in adults. Site-directed mutations that inhibited WiF-1 binding to the *Wnt-1* promoter concomitantly abolished the activity of the promoter in RA-treated P19 cells. The active WiF-1 protein was purified by DNA affinity chromatography. Our data suggest that WiF-1 is a novel G/C box-binding transcription factor and support a physiological role for WiF-1 in the developmentally regulated expression of *Wnt-1*.

The *Wnt-1* (formerly *int-1*) proto-oncogene was initially identified as an integration site for mouse mammary tumor virus in mouse mammary tumor virus-induced mammary carcinomas (38; see reference 39 for a review). The gene encodes a protein bearing the hallmarks of a secreted signaling molecule, such as a signal peptide, putative glycosylation sites, and numerous cysteine residues having the potential to form disulfide bonds (12). Recent studies have revealed that *Wnt-1* is a member of a relatively large gene family, with at least 10 identified members in mice (13, 30, 44).

The demonstration that *Wnt-1* is the murine homolog of the *wingless* (*wg*) segment polarity gene of *Drosophila melanogaster* (42, 54) immediately suggested the involvement of the *Wnt-1* gene product in differentiation and development. Indeed, studies of *Xenopus laevis* have shown that ectopic expression of *Wnt-1* mRNA in fertilized eggs can lead to perturbation of the development of the embryonic axis ranging in severity from a split axis to induction of a complete secondary axis, depending on the time and site of injection of the exogenous RNA (29, 49).

Perhaps the most dramatic and convincing demonstration of the role of *Wnt-1* in development comes from studies of mice homozygous for targeted inactivating mutations in *Wnt-1* (27, 52). Such mice show a range of phenotypes in which the development of specific parts of the central nervous system (CNS) is more or less severely affected. Some animals die around the time of birth and lack the entire cerebellum and a significant portion of the midbrain (27), whereas others survive into adulthood and are only affected in the anterior half of the cerebellum (52). The reason for the variable penetrance of the *Wnt-1* mutations is unclear, but factors such as genetic background may be implicated. It has been recently postulated that *Wnt-1* primarily regulates midbrain development (28) and maintains the expression of the mouse *engrailed* (*En*) genes through an inductive event in adjacent tissue, just as *wg* regulates the expression of *en* in *D. melanogaster* (10). The loss of cerebellar structures in *Wnt-1* null mutants would then be an indirect effect mediated through the loss of expression of *En* genes (28). It should be noted that a naturally occurring mouse mutant, *swaying*, carries a single nucleotide deletion mutation in the *Wnt-1* gene that causes premature termination of the translation of the Wnt-1 protein (53). The phenotypes of the *swaying* homozygote mice are identical to those of the mice in which *Wnt-1* has been inactivated through homologous recombination (53).

A remarkable finding of those studies is that the additional structures of the CNS which express *Wnt-1* (8, 56) (see below) remain completely intact. Redundancy of function of some *Wnt* family members has been invoked to explain these findings (28). In particular, *Wnt-3A* is expressed in some of the same areas of the CNS that express *Wnt-1* (28, 43) and could substitute for *Wnt-1* function in these areas (28).

The pattern of the expression of *Wnt-1* during mouse embryogenesis correlates well with the affected structures in the *Wnt-1* mutants. The gene is expressed between embryonic days 8.5 and 14.5 in restricted areas of the developing

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CNS (8, 16, 47, 56). Initially, *Wnt-1* mRNA is detected across the mesencephalic neural plate at embryonic day 8.5 (56). After neural tube closure, *Wnt-1* transcripts are expressed in a circle of cells in the most posterior portion of the midbrain, just anterior to the junction with the hindbrain, as well as along the dorsal midline of the brain ventricles and spinal cord (56). A similar pattern of *Wnt-1* RNA expression in zebrafish embryos has also been described (31). In amphibians, *Xwnt-1* is expressed transiently during the neurula stages of early *Xenopus* development (36). Interestingly, *Wnt-1* is also expressed in the round spermatid stage of spermatogenesis in adult male mice (47).

Despite the recent accumulation of stimulating data on the importance of *Wnt-1* in the proper development of the CNS, relatively little is known about the regulation of the exquisitely restricted pattern of expression of *Wnt-1* during embryogenesis and in adults. We (50) and others (46) have shown that *Wnt-1* is specifically expressed during the neuroectodermal differentiation of the P19 line of mouse embryonal carcinoma (EC) cells. Retinoic acid (RA) induces P19 cells to differentiate into neurons, astrocytes, and fibroblast-like cells (18, 26), whereas treatment of the same population of EC cells with dimethyl sulfoxide (DMSO) induces differentiation into a variety of nonneural cell types, including cardiac and skeletal muscle cells (11, 25). Our own work has shown that *Wnt-1* expression in RA-treated P19 cells is controlled at the level of gene transcription (50). Nusse et al. (37) have sequenced the mouse *Wnt-1* 5′-upstream region and demonstrated that a 1-kb upstream fragment is able to confer differentiation-specific expression to a heterologous gene in RA-treated P19 cultures.

We have used the P19 line of EC cells to identify and characterize the transcription factors binding to the *Wnt-1* promoter region to control its developmentally regulated expression. We report the purification of *Wnt-1*-inducing factor-1 (WiF-1), a novel transcription factor binding a G/C box motif. Mutations that abolished WiF-1 binding to the *Wnt-1* promoter also completely inhibited the activity of the promoter in RA-treated P19 cells. Moreover, WiF-1 binding activity was detected in adult mouse testis, supporting a physiological role for WiF-1 in the expression of *Wnt-1* in relevant target tissues.

MATERIALS AND METHODS

Plasmids. Plasmid pMT26, containing about 6 kb of upstream *Wnt-1* sequence, was a gracious gift from John Mason and Harold Varmus (Department of Microbiology and Immunology, University of California, San Francisco). *Kpn*I-to-*Sfi*I (nucleotides −1052 to +176; numbering per Nusse et al. [37]), *Bss*HII-to-*Sfi*I (−277 to +176), and *Bss*HII-to-*Pst*I (−277 to −48) fragments from pMT26 were subcloned into convenient sites in the polylinker of plasmid pGL2-Basic (Promega Corp., Madison, Wis.) by standard techniques to generate plasmids pKSluc, pBSluc, and pBPluc, respectively. Plasmid pKSmu luc was obtained by site-directed mutagenesis of pKSluc with a T7-Gen In Vitro mutagenesis kit (United States Biochemical, Cleveland, Ohio) by following the instructions of the manufacturer. The mutated primer oligonucleotide (WiF mu) had the sequence 5′ GTCGGGGCTCCCCCCAGCCCCCTG 3′; the underlined residues represent the engineered mutations in the WiF-1 binding site. The presence of the mutation in pKSmu luc was ascertained by sequencing both strands with a Sequenase Version 2.0 kit (United States Biochemical) as directed by the manufacturer. Plasmid pSV2CAT was obtained from the American Type Culture Collection (Rockville, Md.).

Transient-transfection assays. P19 cells were maintained as described by Rudnicki and McBurney (45). The cells were plated at low density (5×10^4 cells per ml) in 60-mm-diameter dishes and then transfected the next day by a modification of the calcium phosphate precipitation technique described by Chen and Okayama (5). Pairs of duplicate dishes were transfected with 9.5 μg of test plasmid together with 0.5 μg of plasmid pSV2CAT as an internal control for the efficiency of transfection. Half of the dishes were then treated with 5×10^{-7} M RA for 48 h, and then all of the cells were harvested in lysis buffer (25 mM Tris-phosphate [pH 7.8], 2 mM dithiothreitol [DTT], 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton X-100). Chloramphenicol acetyltransferase activity was measured by the phase-extraction assay of Neumann et al. (35). Volumes of cell lysates showing comparable chloramphenicol acetyltransferase activities were then assayed for luciferase activity by the luciferase assay system (Promega) according to the instructions of the manufacturer.

Gel retardation and DNase I footprinting assays. Nuclear extracts from P19 EC cells and their differentiated derivatives were prepared essentially as described by Dignam et al. (9). Nuclear extracts from whole mouse testes were prepared by the protocol of Bunick et al. (3). The fragment probe used in all binding assays was a 230-bp 5′ *Wnt-1* fragment comprising nucleotides −277 to −48 (*Bss*HII-to-*Pst*I subfragment of pMT26) labeled on the noncoding or coding strand by Klenow fragment fill-in or kinase labeling, respectively, by standard procedures (1, 51). Other probes used include a 36-mer double-stranded oligonucleotide encoding the WiF-1-binding site (nucleotides −214 to −178 of the *Wnt-1* promoter) and the double-stranded WiF mu oligonucleotide described above. For gel retardation assays, 5 μg of nuclear extract was incubated with 4×10^3 cpm of probe (approximately 2 ng) in gel retention mix (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol) in the presence of nonspecific competitor DNA [6 to 8 μg of poly(dI-dC) for the fragment probe and 2 μg of poly(dI-dC) for the oligonucleotide probes]. After incubation for 30 min at room temperature, the bound and free probes were separated on a 4% polyacrylamide gel under nondenaturing conditions by using band shift buffer (6.7 mM Tris-HCl [pH 7.5], 3.3 mM Na acetate, 1 mM EDTA) with buffer recirculation. The gels were dried and subsequently autoradiographed. DNase I footprinting assays were performed essentially as described by Jones et al. (17), except that the binding reaction was carried out at room temperature for 30 min in Z^{et} buffer (25 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 1 mM DTT, 20% glycerol, 0.1% Nonidet P-40).

UV cross-linking of WiF-1. UV cross-linking of WiF-1 to its cognate site was performed as described by Wu et al. (58). The 70-bp probe was labeled with [^{32}P]dCTP by Klenow fragment fill-in of a coding strand oligonucleotide (positions −231 to −162) annealed to a noncoding strand 12-bp primer (−173 to −162) in the presence of 5′bromo-2′-dUTP (BrdU). Binding conditions were identical to those of the gel retardation assay, and UV irradiation was for 20 min at 4°C with a 5-cm sample-to-lamp separation. Nuclease digestion of the bound probe to generate the smallest possible cross-linked fragment was carried out as previously reported (58). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed on a 10% polyacrylamide gel as described by Laemmli (22).

Purification of WiF-1. Preparative-scale quantities of P19

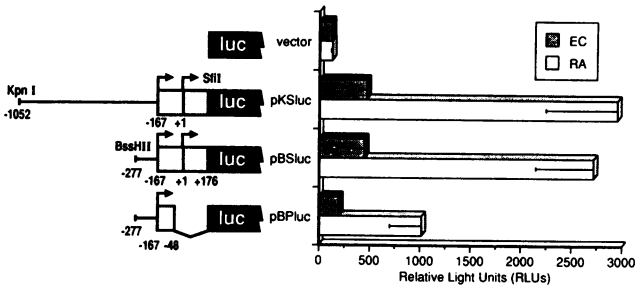

FIG. 1. Activity of deleted *Wnt-1* promoter expression vectors in differentiating P19 cells. Pairs of duplicate dishes were cotransfected with the illustrated test plasmids together with an internal control expression vector to monitor for fluctuations in the efficiency of transformation. After transfection, half of the dishes were treated with 5×10^{-7} M RA. Cells were harvested 48 h later and luciferase activity was measured and standardized for the activity of the internal control as described in Materials and Methods. Results are expressed as means of at least 6 experiments; error bars indicate standard errors of the mean.

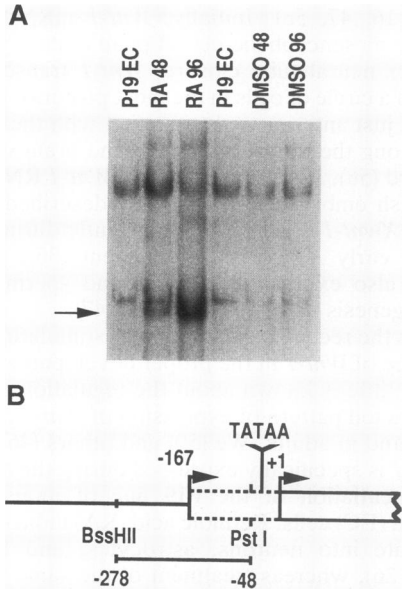

FIG. 2. Induction of a nuclear factor binding the *Wnt-1* promoter in RA-treated P19 cells. (A) Nuclear extracts were prepared from P19 EC cells and at various times after differentiation with RA or DMSO. Binding of nuclear factors to a 230-bp *Wnt-1* promoter fragment was analyzed by the gel retardation assay. The arrow points to the complex specifically induced in RA-treated P19 cultures. (B) Partial map of the *Wnt-1* promoter region. The probe used (−278 to −48) is indicated below the map.

cells (12 to 18 liters seeded at 10^5 cells per ml) were grown in microcarrier spinner flasks (Bellco Glass, Vineland, N.J.) in the presence of 5×10^{-7} M RA for 4 days to induce WiF-1. A crude nuclear extract was prepared (9) and chromatographed on a wheat germ agglutinin column (15) to remove endogenous Sp1. The wheat germ agglutinin column flowthrough was precipitated in 53% ammonium sulfate, and the pellet was resuspended in TM buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride and 1 mM sodium metabisulfite) prior to chromatography on a Sephacryl-300 (S-300) column (Pharmacia-LKB Biotechnology, Baie d'Urfé, Québec, Canada). S-300 column fractions showing WiF-1 binding activity on a gel retardation assay were pooled, and WiF-1 was purified by DNA affinity chromatography as described by Kadonaga (19), except that Z^{et} buffer was substituted for Z buffer and the binding to the affinity resin was carried out for 30 min at room temperature. The DNA coupled to the affinity column was the concatenated double-stranded 36-mer described above. Analysis of the pure protein by SDS-PAGE was performed on a 10% polyacrylamide gel as described by Laemmli (22), and proteins were visualized by staining with silver (33).

RESULTS

Deletion analysis of the 5′-upstream sequence of *Wnt-1*. The *Wnt-1* gene is transcribed from two major transcription initiation start sites (37). The sequence upstream of the more 3′ start site is reminiscent of a typical promoter with a well-defined TATAA box. The second start site, located 167 bp upstream, does not show a canonical TATAA box and is preceded by a very GC-rich stretch of DNA. To identify the region(s) of the *Wnt-1* promoter which is required for proper expression of *Wnt-1* in differentiating P19 cells, we constructed a series of expression vectors in which various portions of the *Wnt-1* promoter are linked to the luciferase reporter gene. Figure 1 shows that a 1-kb upstream fragment from the *Kpn*I site at −1052 to the *Sfi*I site at +176 (just upstream of the translation initiation AUG codon) confers RA-dependent expression to the reporter gene (plasmid pKSluc). In all experiments, the expression of the reporter gene was induced approximately fivefold.

These experiments confirm the data of a study done by Nusse et al. (37) in which an identical *Wnt-1* promoter fragment was linked to the chloramphenicol acetyltransferase reporter gene and stably transfected into P19 cells. A similar construct containing *Wnt-1* sequences from −1052 to −48 fused to the *lacZ* reporter gene was also silent in undifferentiated P19 EC cells as well as DMSO-treated cells, whereas it was expressed in RA-treated P19 cells (data not shown). This pattern of expression is analogous to the expression profile of the endogenous gene (46, 50), suggesting that most if not all of the 5′ sequence information required for proper differentiation-specific expression of *Wnt-1* in P19 cells is contained in this 1-kb upstream fragment.

Expression vectors in which 5′ *Wnt-1* sequences were deleted to position −277 upstream of the more 3′ start site retained full RA-specific promoter activity (Fig. 1, plasmid pBSluc). Further deletion of sequences from −47 to +176 (plasmid pBPluc) yielded a 230-bp promoter fragment that was still specifically active in RA-treated P19 cultures (Fig. 1). The lower basal and stimulated activities of plasmid pBPluc were probably due to the removal of the downstream start site and its TATAA box; however, the activation of the expression of pBPluc in RA-treated P19 cells was identical to that measured with the other *Wnt-1* promoter expression vectors (approximately fivefold induction [Fig. 1]). In all experiments, the vector plasmid containing the luciferase reporter gene alone was silent in both EC and RA-treated P19 cell cultures (Fig. 1, vector).

Nuclear factors binding the *Wnt-1* promoter. We have used the minimal 230-bp *Wnt-1* promoter fragment (−278 to −48 [Fig. 2B]) as a probe in a gel retardation assay to identify nuclear factors that could be implicated in the differentiation-specific transcription of *Wnt-1*. Figure 2A reveals that this promoter fragment contains a binding site for a nuclear

protein that was not detected in undifferentiated P19 EC cells or their DMSO-treated derivatives but was induced in P19 cells treated with RA for 48 or 96 h. The presence of the factor always correlated with the detection of *Wnt-1* transcriptional activity as assessed by in vitro nuclear run-on assays as well as with the detection of *Wnt-1* mRNA in the RA-treated cells (data not shown). The *Wnt-1* probe also bound two other factors that appeared ubiquitously expressed in undifferentiated and differentiated P19 cells (Fig. 2A). The other retarded complexes detected in Fig. 2 were not reproducibly observed (not shown).

The binding site of the RA-induced factor was determined by DNase I footprinting. As shown in Fig. 3, partially purified nuclear extracts from P19 cells treated with RA for 96 h protected a region comprising positions −206 to −186 of the *Wnt-1* promoter. An identical region was also protected on the noncoding strand of the probe (see Fig. 7A, lane 2). This region is extremely GC rich. Binding and competition studies with synthetic oligonucleotides in gel retardation assays (not shown) established the optimal binding site for the factor as 5′ GGGGGCGGGGGTGAGCCCCGA 3′ (−206 to −186), although the minimal binding site was 5′ GGGGGCGGGGGTGAG 3′. A partial footprint could also sometimes be observed between positions −221 and −214 (Fig. 3). This footprint was not consistently observed with different nuclear extract preparations (data not shown).

The 5′ half of the major binding site centered at position −196 shows extremely high sequence similarity to the consensus binding site for the Sp1 transcription factor (2). However, use of anti-Sp1 antibodies (kindly supplied by S. Jackson and R. Tjian, University of California, Berkeley) in gel retardation assays revealed that the RA-induced factor binding the *Wnt-1* promoter was not Sp1 (not shown). We therefore tentatively named the *Wnt-1* promoter binding activity *Wnt-1*-inducing factor-1 (WiF-1).

WiF-1 is necessary for regulated *Wnt-1* expression in P19 cells. To assess the importance of WiF-1 in differentiation-specific transcription of *Wnt-1*, we mutated the WiF-1-binding site within the context of the full *Wnt-1* promoter region using site-directed mutagenesis. Figure 4A shows that the engineered mutation (see Materials and Methods) completely abolished WiF-1 binding. When the mutated promoter fused to the luciferase reporter gene (plasmid pKSmu luc) was introduced into P19 cells in a transient-transfection assay, the WiF-1 mutation totally inhibited the RA-induced transcriptional activation (Fig. 4B). These data demonstrate that WiF-1 is the major factor controlling *Wnt-1* expression in differentiating P19 cells and strongly suggest that WiF-1 is a transcription factor. Moreover, subcloning of synthetic oligonucleotides encoding the WiF-1 binding site upstream of a heterologous minimal promoter fused to a reporter gene showed specific copy number-dependent activation of transcription in RA-treated P19 cells in a transient-transfection assay (not shown).

WiF-1 is expressed in adult mouse testes. Since *Wnt-1* is expressed in the testes of adult male mice (47), we tested for the presence of WiF-1 binding activity in nuclear extracts from testis tissue using the gel retardation assay. *Wnt-1* mRNA has been detected at the round spermatid stage of spermatogenesis (47). Figure 5A shows that WiF-1 binding activity was almost undetectable in testis tissue of 16-day-old animals. WiF-1 could easily be detected at 21 days of age, which coincides with the appearance of spermatids in the testes (34). WiF-1 binding activity then increased with age (Fig. 5A) as the relative proportion of round spermatids in the testis increased. The minor differences between the migration patterns observed in Fig. 2 and 5 are due to the different nuclear extract preparation techniques used for testis tissue and shorter migration times. The identity of the factor binding the *Wnt-1* probe in the testis extracts was ascertained by competing away the binding activity with an excess of an unlabeled synthetic oligonucleotide encoding the WiF-1 binding site (Fig. 5B). WiF-1 binding activity could not be detected in nuclear extracts from HeLa cells or osteoblastic cells (data not shown).

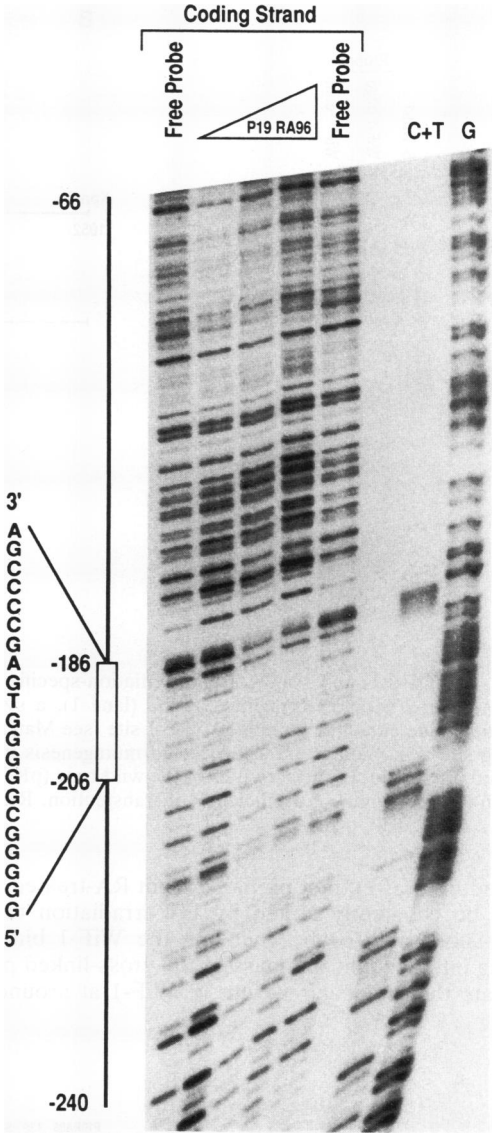

FIG. 3. Identification of the WiF-1 binding site. A nuclear extract was prepared from P19 cells treated with RA for 96 h and purified as described in Materials and Methods. The S-300 fractions showing WiF-1 binding activity were pooled and analyzed by DNase I footprinting with the 230-bp *Wnt-1* promoter fragment as a probe. The probe was labeled at the *Bss*HII site on the coding strand by kinase labeling. The protected region and its corresponding sequence are represented diagrammatically on the left.

UV cross-linking of WiF-1 to its binding site. We used UV cross-linking (photoaffinity labeling) with a BrdU-substituted labeled probe (58) to estimate the molecular weight of WiF-1. Figure 6, lane 1, shows that WiF-1 from a partially

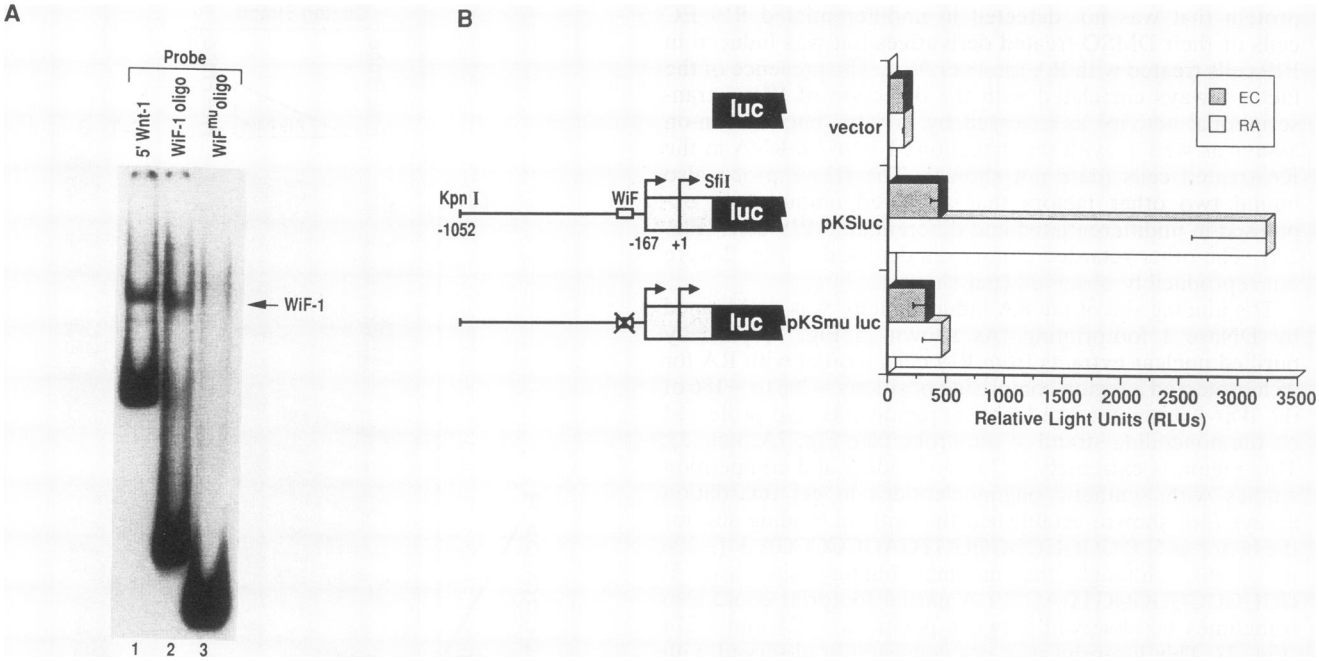

FIG. 4. WiF-1 is necessary for differentiation-specific *Wnt-1* expression. (A) Gel retardation assay of an RA-treated P19 nuclear extract with the *Wnt-1* promoter fragment probe (lane 1), a synthetic oligonucleotide (oligo) encoding the WiF-1 binding site (lane 2), and an oligonucleotide encoding a mutated WiF-1 site (see Materials and Methods). The arrow points to the WiF-1–DNA complex. (B) The WiF-1 binding site was mutated by site-directed mutagenesis in an expression vector fusing the *Wnt-1* promoter to the luciferase reporter gene (plasmid pKSmu luc). The activities of the wild-type (pKSluc) and mutated expression vectors were analyzed by transient-transfection assay with an internal control for efficiency of transfection. Results are means (n = 8), with standard errors of the mean indicated by error bars.

purified nuclear extract prepared from RA-treated P19 cells could be covalently linked by UV irradiation to a 50-bp BrdU-substituted probe encoding the WiF-1 binding site. Taking into account the mass of the cross-linked probe, we estimate the molecular weight of WiF-1 at around 65,000.

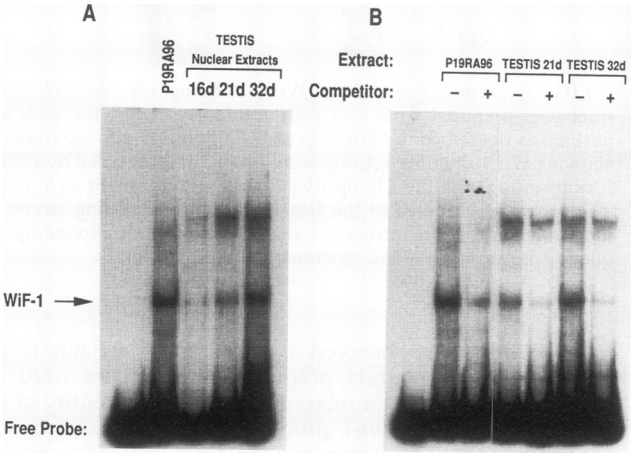

FIG. 5. WiF-1 is expressed in the adult mouse testis. (A) Total nuclear extracts were prepared from testes of 16-, 21-, or 32-day (d)-old mice and analyzed by a gel retardation assay with the 230-bp *Wnt-1* promoter fragment probe. The arrow points to the WiF-1–DNA complex. (B) The extracts were preincubated with (+) or without (−) an excess (200 ng; approximately 100-fold molar excess) of an unlabeled synthetic oligonucleotide encoding the WiF-1 binding site before the gel shift assay. P19RA96 indicates extract from P19 cells treated with RA for 96 h.

The binding specificity of the cross-linked protein was ascertained by adding an excess of unlabeled oligonucleotide to the binding reaction prior to UV irradiation. An oligonucleotide containing the wild-type WiF-1 binding site was able to compete for the binding of the 65,000-M_r protein, whereas the oligonucleotide encoding the mutant WiF-1 binding site (Fig. 4A) was incapable of competing for WiF-1 binding to the labeled probe (Fig. 6, lanes 2 and 3, respectively). Incubation of the probe in the absence of proteins or omission of the UV irradiation did not yield any cross-linked species (data not shown).

Purification of WiF-1. To further characterize the WiF-1 protein, we substantially enriched it in preparations of RA-treated P19 nuclear extracts, using a purification scheme based on DNA affinity chromatography. After preparation of the crude nuclear extract, lectin column chromatography on wheat germ agglutinin was performed. This type of column has been shown to retain certain glycosylated transcription factors such as Sp1 and CCAAT-binding transcription factor (15). WiF-1 flowed through the lectin column and could therefore be easily separated from Sp1 (data not shown). The lectin column flowthrough was then subjected to size-exclusion chromatography on S-300, and fractions showing WiF-1 binding activity were pooled and loaded onto a WiF-1 DNA affinity column. Figure 7A illustrates that the S-300 pool as well as the 0.4 M salt fraction from the first round of DNA affinity chromatography bound the WiF-1 site in the *Wnt-1* promoter in a DNase I footprinting assay (lanes 2 and 4 to 6). The 0.4 M fraction was diluted back to 0.1 M salt and subjected to a second round of WiF-1 affinity chromatography. The fraction eluted with 0.4 M salt after the second DNA affinity column also retained WiF-1 footprinting activity (Fig. 7A, lanes 9 and 10). The partial protection of the

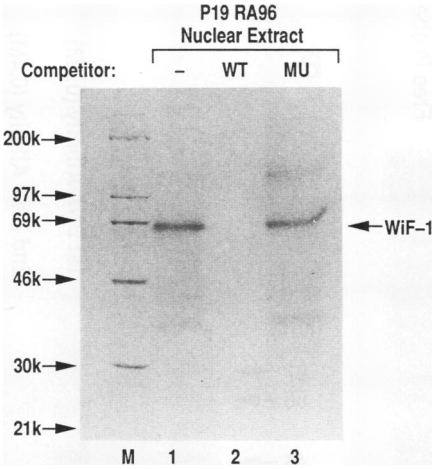

FIG. 6. UV-cross-linking of WiF-1. Partially purified nuclear extract from P19 cells treated with RA for 96 h was incubated with a BrdU-substituted labeled probe encoding the WiF-1 binding site in the presence or absence of competitor oligonucleotides. The bound WiF-1 protein was then covalently linked to the probe by UV irradiation. Lane M, molecular weight markers (indicated in thousands at left); lane 2, wild-type (WT) oligonucleotide competitor; lane 3, mutant (MU) oligonucleotide competitor. The arrow points to the 65,000-M_r cross-linked WiF-1 protein.

binding site in the footprint assay of this fraction was most likely due to its low protein concentration.

We analyzed some of the fractions generated through the purification procedure by silver staining of the proteins separated by SDS-PAGE. Figure 7B reveals that the active 0.4 M fraction from the second round of DNA affinity chromatography contained a major protein band at an M_r of 65,000. These data correlate with the UV cross-linking data showing an M_r of 65,000 for WiF-1 (Fig. 6). Thus, two rounds of DNA affinity chromatography have allowed us to purify the active 65,000-M_r WiF-1 protein to near homogeneity. Moreover, analysis of the available data on the molecular weight of previously characterized G/C box-binding factors (Table 1), coupled to their binding site specificities and patterns of expression (see Discussion), strongly supports the notion that WiF-1 is a novel G/C box-binding transcription factor.

DISCUSSION

Our data show that *Wnt-1* transcription in differentiating RA-treated P19 cells is regulated through the interaction of the WiF-1 transcription factor with its cognate GC-rich binding site centered at position −197 of the *Wnt-1* promoter. WiF-1 binding activity is specifically induced in RA-treated P19 cells but is undetectable in undifferentiated EC stem cells or their DMSO-treated mesodermal derivatives. However, WiF-1 activity can be measured in the postpubertal male testis, the only tissue that expresses *Wnt-1* in adult mice. Our results further suggest that the purified 65,000-M_r WiF-1 protein is a novel G/C box-binding transcription factor.

A number of proteins binding GC-rich sequences have been previously identified and characterized (Table 1 and references therein). The genes for some of them have also been cloned. The WiF-1 binding site bears the highest similarity to the Sp1 binding site as well as the target site for the Krox (or Egr) family of transcription factors. We have ascertained that WiF-1 is not Sp1 through the use of antibodies directed against the Sp1 protein in the gel retardation assay (not shown) as well as through the purification scheme that we have selected, which separates Sp1 from WiF-1 early in the procedure (see Results). The molecular weight of the purified protein is also significantly different from the reported molecular weight of the Sp1 protein (2).

The molecular weight of WiF-1 also appears different from those reported for all the Krox family members (Table 1; these also include nerve growth factor-induced early response gene (NGF-1C) and WT-1, the product of the Wilms' tumor gene, on the basis of their sequence similarity and the similitude of their binding sites). However, some of those protein masses have been deduced solely from the cDNA sequence of their genes; it is possible that the actual gene products are posttranslationally modified and migrate differently in SDS-PAGE. Recent studies have shown that Krox-20 and Krox-24 are expressed in undifferentiated P19 EC cells (7, 23). Since WiF-1 binding activity is undetectable in EC stem cells (Fig. 2A), WiF-1 is most likely distinct from those two proteins. Perhaps the most convincing evidence that WiF-1 is not a Krox family member comes from studies of the consensus binding site for this family of factors. In every case examined in detail, substitution of the penultimate 3′ C residue in the Krox binding site by a T residue has abolished binding (24, 41). However, alignment of the WiF-1 binding site with the Krox binding sequence reveals that the WiF-1 site has a T residue precisely at that position (Table 1). Moreover, binding and competition studies with synthetic oligonucleotides in gel retardation assays have revealed that this T residue is essential for WiF-1 binding (49a). Thus, WiF-1 and Krox family members display different sequence specificity for binding and are likely distinct.

The late SV40 factor (LSF) purified by Huang et al. (14) is the G/C box-binding factor that has the molecular weight closest to that of WiF-1 (Table 1). However, the LSF binding site does not show very high sequence similarity with the WiF-1 binding site (Table 1). Moreover, LSF was originally purified from HeLa cell nuclear extracts (14), and we have failed to detect WiF-1 binding activity in HeLa cell extracts (data not shown). On the basis of these observations, we believe that WiF-1 represents a novel DNA-binding factor. The formal demonstration that WiF-1 possesses an actual transcriptional activation domain will require the cloning of the *WiF-1* gene and in vitro transcription studies with the purified protein. Nevertheless, our data strongly suggest that WiF-1 is indeed a transcription factor (see below).

WiF-1 is necessary for the differentiation-specific expression of *Wnt-1* in P19 cultures. WiF-1 binding activity is specifically induced in RA-treated P19 cells with kinetics that correlate with the induction of *Wnt-1* transcription in those cells (Fig. 2A) (49a, 50). Moreover, mutations in the *Wnt-1* promoter region that inhibit WiF-1 binding also completely abolish *Wnt-1* promoter-directed transcriptional activation in RA-treated P19 cells (Fig. 4). This result indicates that the numerous putative Sp1 binding sites in the *Wnt-1* promoter region (37) are not implicated in the RA-induced transcription of *Wnt-1* in P19 cells.

Wnt-1 is transcribed from two major transcription initiation start sites (37). Both start sites appear to be used indiscriminately in every tissue so far examined, including P19 cells (24a, 37). Our experiments with the mutated WiF-1 binding site (Fig. 4), which show complete inhibition of RA-induced transcription, suggest that WiF-1 controls the expression of *Wnt-1* from both start sites concomitantly.

Since P19 cells do not permit correlation of *Wnt-1* expres-

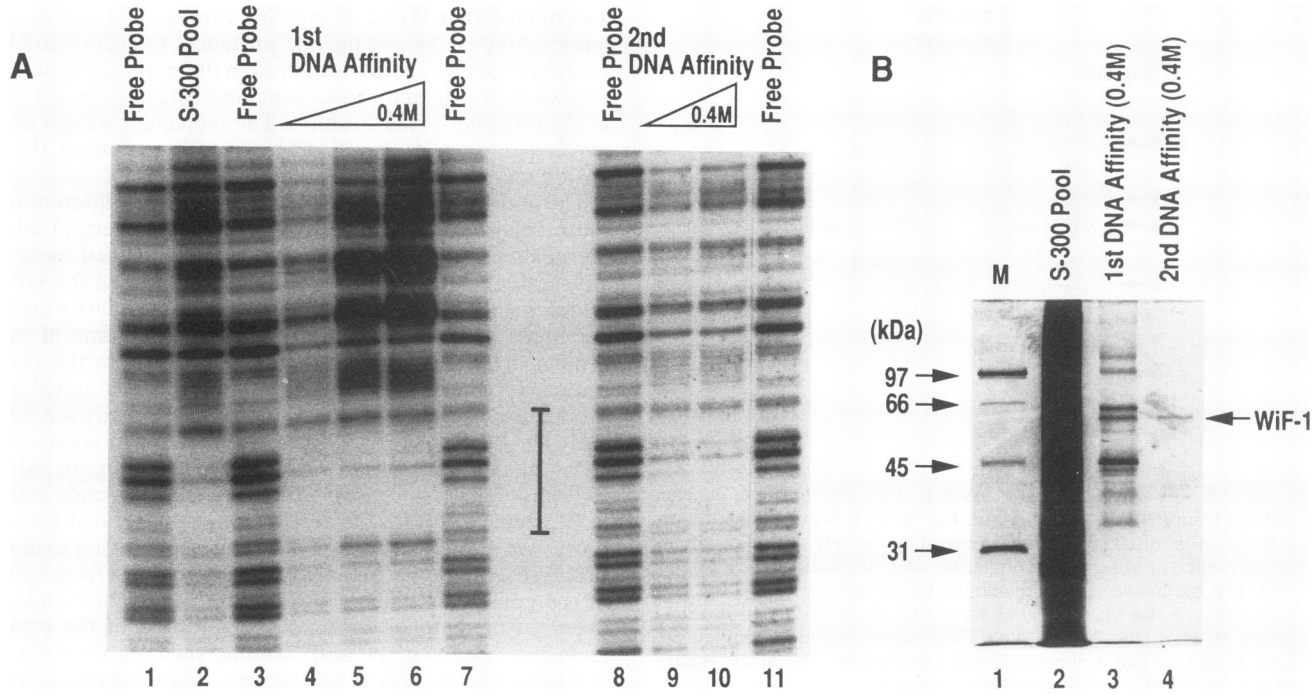

FIG. 7. Purification of WiF-1 by DNA affinity chromatography. (A) DNase I footprinting activity of the S-300 pool (lane 2) and of the affinity purified fractions eluted with 0.4 M salt in the first (lanes 4 to 6) and second (lanes 9 and 10) rounds of WiF-1 affinity chromatography. The probe used was the 230-bp 5′ *Wnt-1* fragment (−277 to −48; *Bss*HII to *Pst*I) labeled on the noncoding strand. The vertical bar delineates the protected area of the probe. (B) Silver-stained SDS-polyacrylamide gel containing the affinity-purified fractions, showing footprinting activity. The molecular mass markers are indicated on the left. The volumes of sample loaded on the gel were 3 μl for lane 2 and 25 μl for lanes 3 and 4. The arrow points to the purified WiF-1 protein (M_r, 65,000).

sion in vitro with its domains of expression in the developing embryo, our data do not preclude the involvement of other factors in the spatially and temporally restricted expression of *Wnt-1* in vivo. Experiments with various regions of the *Wnt-1* promoter driving a reporter gene in transgenic mice will be necessary to decipher the complete molecular requirements of the tissue-specific expression of *Wnt-1*. At any rate, our results suggest that WiF-1 is one of the key factors implicated in this process. Interestingly, the human *Wnt-1* gene has an entirely homologous WiF-1 binding site at the same relative position (−191 to −176) in its 5′-flanking domain (55). Moreover, preliminary experiments have revealed the presence of WiF-1 binding activity in *Xenopus* embryos (12a), thus suggesting evolutionary conservation of the mechanisms regulating *Wnt-1* expression.

Wnt-1 mRNA has been detected in the round spermatids of postpubertal male mice (47). Our results also show WiF-1 binding activity in nuclear extracts from adult testis (Fig. 5) at a time coincidental with the appearance of the round spermatids in the male reproductive gland (34). However, Northern blot analysis of testis RNA failed to detect *Wnt-1* transcripts before 24 days of age (47), a full 3 days after we can unequivocally detect WiF-1 binding (Fig. 5A). This discrepancy may be due solely to the low sensitivity of the Northern blot assay. Alternatively, WiF-1 expression may precede the activation of *Wnt-1* transcription. Such a 1- to 2-day delay between the expression of a transcription factor and the detection of the mRNA of the genes that it controls has already been reported for the Pit-1 transcription factor and the expression of the prolactin and growth hormone

TABLE 1. G/C box-binding transcription factors

Factor[a]	Binding site	M_r	Reference
Sp1	GGGGCGGGGC	95,000, 105,000	2
ETF[b]	GCCCCCGGCGC	120,000	20
GCF[b]	N G/C CG G/C G/C G/C CN	91,000	21
LSF	GGGCGGAACTGGGCGG	63,000	14
AP-2	GCCNNNGGC	50,000	57
Krox-20 (Egr 2)	GCGGGGGCG	48,000[a]	4
Krox-24 (Egr 1)	GCGGGGGCG	80,000	7
NGFI-C[b]	GCGGGGGCG	50,000[a]	6
Egr 3[b]	GCGGGGGCG	43,000[a]	40
WT-1	GCGGGGGCG	52,000	32
WiF-1	GGGGGCGGGGGTGAG	65,000	

[a] ETF, epidermal growth factor receptor-specific transcription factor; GCF, GC factor; NGFI-C, nerve growth factor-induced early response gene C; Egr 3, early growth response gene 3.
[b] Deduced from cDNA sequence.

genes during the development of the mouse pituitary gland (48). At any rate, the detection of WiF-1 binding activity in the testes supports a physiological role for WiF-1 in the regulation of *Wnt-1* expression. It shall prove interesting to investigate WiF-1 activity or expression in the developing brain.

The G/C box is an important and versatile regulatory motif. Both ubiquitous and regulated factors recognize identical or related sequences to control the transcription of a wide variety of genes. Our work has identified WiF-1, yet another member of this large class of DNA-binding transcription factors. The cloning of the gene encoding WiF-1 should provide further information on the structure and function of these molecules and on the regulatory cascade controlling CNS differentiation and development.

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