Cis-acting regulatory sequences governing Wnt-1 expression in the developing mouse CNS

Yann Echelard†, Galya Vassileva and Andrew P. McMahon*
Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110 USA

*Present address and address for all correspondence: Department of Cellular and Developmental Biology, The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA
†Present address: Genzyme Transgenics Corp., One Mountain Road, Framingham, MA 01701-9322, USA

SUMMARY
The protooncogene Wnt-1 encodes a short-range signal which is first expressed in, and appears to demarcate, the presumptive midbrain. Absence of Wnt-1 expression leads to the loss of this region of the brain. By the end of neural tube closure, expression of Wnt-1 extends down much of the dorsal midline of the central nervous system (CNS). Expression is exclusively limited to the CNS at this and later stages. We have investigated the regulation of Wnt-1 during mouse development. Analysis of the embryonic expression of Wnt-1-lacZ reporter constructs spanning nearly 30 kb of the Wnt-1 locus identified a 5.5 kb cis-acting 3' enhancer element which confers correct temporal and spatial expression on the lacZ gene. Interestingly embryos express Wnt-1-lacZ transgenes in migrating neural crest cells which are derived from the dorsal CNS. Ectopic expression of the Wnt-1-lacZ transgenes may result from perdurance of β-galactosidase activity in migrating neural crest cells originating from a Wnt-1-expressing region of the dorsal CNS. Alternatively, ectopic expression may arise from transient de novo activation of the transgenes in this cell population. These results are a first step towards addressing how regional cell signaling is established in the mammalian CNS. In addition, transgene expression provides a new tool for the analysis of neural crest development in normal and mutant mouse embryos.

Key words: Wnt-1-lacZ fusions, transgenic mice, midbrain, neural crest

INTRODUCTION
The Wnt-1 protooncogene was identified in several independently arising mammary tumors in which proviral insertion of Mouse Mammary Tumor Virus (MMTV) resulted in activation and ectopic expression of an apparently normal Wnt-1 gene product (Nusse and Varmus, 1982; Nusse et al., 1984; van Ooyen and Nusse, 1984). Cell culture (Brown et al., 1986; Rijsewijk et al., 1987; Jue et al., 1992) and transgenic mouse (Tsukamoto et al., 1988; Kwan et al., 1992) studies have clearly demonstrated that ectopic expression of Wnt-1 in mammary epithelium leads to morphological transformation in vitro and dramatic hyperplasia in vivo. Thus, a causal relationship between ectopic expression of Wnt-1 and the development of mammary adenocarcinomas has been established (reviewed by Nusse and Varmus, 1992). More recently, studies in a number of developmental systems, most notably Drosophila and the mouse, have started to address the normal role of Wnt-1.

Wnt-1 in the mouse, and its Drosophila counterpart the segment polarity gene wingless (wg), encode cysteine-rich, secreted glycoproteins (Papoff et al., 1987; Brown et al., 1987; van den Heuvel et al., 1989; Gonzalez et al., 1991) which are members of a large family of putative signaling molecules (reviewed in McMahon, 1992; Nusse and Varmus, 1992). In Drosophila, analysis of multiple alleles indicates that wg is required throughout embryonic and larval development (Baker, 1988). The best studied aspect of wg-mediated regulation lies in patterning of the embryonic segment where wg is necessary to maintain expression of the homeobox-containing transcription factor, engrailed, in posterior cells (DiNardo et al., 1988; Martinez-Arias et al., 1988; Heemskerk et al., 1991; Gonzalez et al., 1991; Bejsovec and Martinez-Arias, 1991) and in the subsequent regulation of cell fate choices adopted by these cells (Dougan et al., 1991). In the absence of correct wg signaling, posterior pattern elements in each of the segments are lost, partly by cell death (Perrimon and Mahowald, 1987), and partly by cells adopting more anterior fates (Nüsslein-Volhard and Wieschaus, 1980; Dougan et al., 1991). Although later functions are less well understood, wg appears to be essential for development and/or patterning of the limb (Cohen, 1990; Couso et al., 1993; Struhl and Basler, 1993), wing (Couso et al., 1993; Williams et al., 1993) and Malpighian tubules (Skaer and Martinez-Arias, 1992). Thus, wg is used in many different contexts during insect development.

In contrast, in the mouse and all other vertebrate embryos studied to date, Wnt-1 is only expressed during development in the central nervous system (CNS; Wilkinson et al., 1987; Davis et al., 1988; Molven et al., 1991; McMahon et al., 1992;
DNA constructs

Genomic Wnt-1 fragments were obtained by screening a λ-GEM12 (Promega) 129/Sv mouse genomic library with a 375 bp MluI-BglII fragment derived from the fourth exon of the murine Wnt-1 gene. Two overlapping clones W1-5.1 and W1-15.1 (Fig. 1) were used in this study. The genomic inserts were excised with NotI digestion and subcloned into pGEM-SZI" (Promega) generating pW1-5.1 and pW1-15.1. Throughout, we distinguish plasmid-containing genomic inserts from the isolated genomic inserts by the prefix "p" for the former.

Wolda et al., 1993). Wnt-1 expression is initiated at neural plate stages throughout the presumptive midbrain, then becomes rapidly restricted to a tight circle lying just anterior of the mid/hindbrain isthmus, by neural tube closure. In addition, Wnt-1 expression persists at the dorsal midline of the midbrain and extends anteriorly at the dorsal midline into the diencephalon and continues down the dorsal midline of the caudal hindbrain (myelencephalon) and spinal cord. Transient ventral expression is also seen in the diencephalon and midbrain shortly after neural tube closure. Thus, the earliest Wnt-1 expression demarcates the presumptive midbrain suggesting that Wnt-1 signaling may regulate the development of this brain region and later expression is consistent with a potential role in establishing dorsal-ventral pattern in much of the CNS.

Analysis of mutant alleles of Wnt-1 generated either spontaneously (Thomas et al., 1991) or by gene targeting (Thomas and Capecci, 1990; McMahon and Bradley, 1990) has clearly demonstrated that Wnt-1 is primarily required for development of the midbrain (Thomas and Capecci, 1990; McMahon and Bradley, 1990; Thomas et al., 1991; McMahon et al., 1992); the loss of the midbrain leading to a secondary loss of anterior hindbrain regions (McMahon et al., 1992). Although it has not been determined if Wnt-1 controls cell fate decision in the midbrain or some other process such as cell growth or survival (for review see McMahon, 1992), these studies demonstrate that activation of Wnt-1 is essential for normal brain patterning. Moreover, the establishment of a specific domain of Wnt-1 expression as early as neural plate stages strongly suggests that activation of Wnt-1 may be regulated by regional patterning signals that are thought to be initiated by the midline mesoderm (for review see Slack and Tannahill, 1992; McMahon, 1993). Interestingly, although Wnt-1 shows dorsally restricted expression in much of the CNS by neural tube closure, there is no apparent phenotype in null mutant embryos. A second Wnt-1 gene, Wnt-3a, appears to be coexpressed with Wnt-1 in these regions (Roelink and Nusse, 1991; McMahon et al., 1992) and it has been suggested that Wnt-3a signaling may be functionally redundant with that of Wnt-1 (McMahon et al., 1992).

To address the question of how regional expression of Wnt-1 is initiated in the mammalian CNS, we have studied the cis-acting regulatory sequences governing embryonic Wnt-1 expression in transgenic mice. We demonstrate that temporal and spatial expression of Wnt-1 is in the presumptive midbrain and at the dorsal midline of the CNS is controlled by a 3' enhancer. Finally, our experiments highlight a possible link between Wnt-1 expression and the neural crest cell lineage in the mouse.

MATERIAL AND METHODS

As an initial step towards the generation of fusion constructs between Wnt-1 genomic sequences and the E. coli lacZ gene, a polylinker was inserted upstream of a lacZ-SV40pA cassette contained in the plasmid pBl-actin PSKDLacZpA (J. Rossant, unpublished data). This plasmid was completely digested with HindIII, excising the human β-actin promoter fragment. The oligonucleotides lac1 and lac2 were annealed in 1× ligation buffer (Maniatis et al., 1982), then ligated into the HindIII cut vector to generate pPSK-DklacZpA1, which contains the following restriction sites: SalI, NotI, SphI, BclI, AarII, HindIII, and BglII just upstream of the Ncol site located at the translation initiation site of the lacZ gene.

pWZT7 was generated by ligating a 1.3 kb KpnI-Ncol fragment containing 5' untranslated sequence including the minimal Wnt-1 promoter (Nusse et al. 1990) and 5' transcribed but untranslated sequence up to and including the initiation methionine codon (Fig. 1) into BglII-Ncol-cut pPSK lacZpA1. To generate other Wnt-1-lacZ fusion constructs, pWZT7 was digested with BamHI, which cuts 3' of the human polyadenylation cassette, and ligated into this vector. This plasmid was propagated in E. coli lacZ (Kothary et al. 1989) to place this 3' Wnt-1 genomic sequence upstream of a 600 bp hsp68 promoter generating pWZTHsp5.

pWZT10AC was constructed by a multistep approach. The pW1-15.1 subclone was completely digested with restriction enzymes AarII and ClaI, and the 2,774 bp Wnt-1 AarII-ClaI fragment isolated (Fig. 1). This fragment was ligated into the pGEM-7Zf' vector (Promega) digested with AarII and ClaI, generating pW1-18. This plasmid was digested at the unique Ncol site located at the Wnt-1 translation initiation codon, and the annealed oligonucleotides NB1 and NB2 were inserted, regenerating only the 5' Ncol site and introducing a new 3' BglII site. This plasmid, pW1-33, was cut with Ncol-BglII and ligated into the Ncol-BglII lacZ-SV40pA expression cassette from pWZT7* to generate pWZT17NB. Finally, pWZT17NB was partially digested with ClaI and ClaI (AarII and ClaI) both cut within the lacZ gene) and a 6.5 kb partial fragment isolated containing 5' and 3' Wnt-1 sequences surrounding the lacZ-SV40 expression cassette. This fragment was subcloned into AarII-ClaI digested pW1-15.1 to generate pWZT10AC. pWZT8C was obtained by ligating a 1.3 kb Wnt-1 lacZ fragment isolated from pWZT10AC into NotI-cut pWZT7* or into NotI-cut pWZT8C by generating a 3' proximal 5.5 kb BglII fragment from pW1-5.1, located just downstream of the Wnt-1 polyadenylation sequence and ligating this into BglII-cut pWZT7*.

pWZT73 and pWZTHsp5 were obtained by the addition of NolI linkers on to the 5.5 kb proximal 3' BglII fragment and ligating into either NotI-cut pWZT7* to generate pWZT73 or into NotI-cut phspPTlacZpA (Kothary et al. 1989) to place this 3' Wnt-1 genomic sequence upstream of a 600 bp hsp68 promoter generating pWZTHsp5.

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Oligonucleotide sequences are as follows:

Production and genotyping of transgenic mice For mapping Wnt-1 regulatory elements, transgenic mice were generated by microinjection of linear DNA fragments, separated from plasmid vector sequences into the male pronucleus of B6CBAF1/J (C57BL/6J × CBA/J) zygotes as described by Hogan et al. (1986). B6CBAF1/J females were also used as recipients for injected embryos and for all breeding studies. Wnt-1-lacZ transgenic embryos were identified by PCR analysis of proteinase-K digests of yolk sacs. Briefly, yolk sacs were carefully

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dissected free from maternal and embryonic tissues, avoiding cross-contamination between littermates, then washed once in PBS. After overnight incubation at 55°C in 50 µl of PCR protease-K digestion buffer (McMahon and Bradley, 1990), 1 µl of heat-inactivated digest was subjected to lacZ-specific polymerase chain reaction (PCR), 94°C/30 minutes → 55°C/30 minutes → 72°C/1 minute at 40 cycles in a 20 µl total reaction volume. The oligonucleotides, 5′ - TAC-CACAGCGGATGGTTCGG - 3′ and 5′ - GTTGGTGT-TATGCCGATGC - 3′ amplify a 352 bp lacZ-specific product. As an internal control for the presence of mouse genomic DNA in the yolk sac digests, ZP3 primers (Lira et al. 1990), which amplify a 511 bp fragment, were used in the same reactions.

**Whole-mount β-galactosidase staining**

Embryos from transgenic experiments at 7.5 to 12.5 days post coitum (dpc) were stained with X-Gal as described in Whiting et al. (1991). The embryos were stained for periods ranging from 30 minutes to 12 hours according to the strength of Wnt-1-lacZ transgene expression. The reaction was stopped by washing the embryos in PBS, and immediately postfixing in 4% paraformaldehyde (Sigma). Whole embryos were photographed on an Olympus SZH microscope with an Olympus diaphot microscope. Photomicrographs were obtained on a Leitz Orthoplan 2 microscope under Nomarski optics using Fujichrome 64T film.

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization to 9.5 dpc embryos was performed essentially as described by Wilkinson (1992) using single-strand digoxigenin-UTP labeled RNA probes. The expression was initiated in the early mammalian CNS, we have started to dissect the cis-acting regulatory sequences that govern expression of Wnt-1. Two overlapping genomic fragments covering a 30 kb area encompassing the Wnt-1 gene at 9.5 to 10.5 dpc are located within the lacZ regulatory region (Table 1). These results support the conclusion that regulatory sequences lie 3′ of the Wnt-1 polyadenylation site. To test this hypothesis directly, transgenic G0 embryos were generated with a third construct, pWZT9B (Fig. 1), which contains 1.3 kb upstream of the Wnt-1 translation initiation codon, including the minimal Wnt-1 promoter, together with 5.5 kb of 3′ Wnt-1 flanking sequences lying just downstream of the Wnt-1 polyadenylation site. Eleven transgenic mice were generated using the WZT9B construct; among these, five were established as stable lines, and the remainder were examined directly as G0 embryos. These WZT9B transgenics, eight expressed the lacZ reporter gene with a pattern similar to that of the endogenous Wnt-1 gene (Table 1) and WZT8C transgenic embryos. Transgenics containing just the 1.3 kb Wnt-1 promoter region showed no lacZ expression as expected (WZT7 in Fig. 1, Table 1). Moreover, the 3′ regulatory region conferred correct expression on a heterologous heat-shock promoter (WZTWhsp5 in Fig. 1 and Table 1). These results demonstrate that sequences that are sufficient for spatial regulation of the Wnt-1 gene at 9.5 to 10.5 dpc are located within a 5.5 kb region just downstream of the Wnt-1 polyadenylation signal.

In order to determine whether the spatial restriction conferred by the 5.5 kb Bg/Il fragment on the lacZ-SV40pA reporter gene is position- or orientation-dependent, this element was inverted (WZT14B) or placed upstream of the Wnt-1 promoter (WZT73; Fig. 1). Five of sixteen WZT14B and two of seven WZT73 G0 transgenic embryos showed correct expression of β-galactosidase in the Wnt-1 expression domain (Table 1). Thus, although altering the position and orientation of the 3′ regulatory element resulted in a decrease in the percentage of transgenics expressing lacZ (72% to 29-36%), correct spatial expression was maintained. In summary, the relative insensitivity of the Bg/Il fragment to position, orientation and promoter sequences indicates that this element contains a cis-acting Wnt-1 enhancer.

**RESULTS**

**Identification of a Wnt-1 enhancer**

As a first step towards understanding how regional gene expression is initiated in the early mammalian CNS, we have started to dissect the cis-acting regulatory sequences that govern expression of Wnt-1. Two overlapping genomic fragments covering a 30 kb area encompassing the Wnt-1 gene (Fig. 1) were used to generate all the Wnt-1-lacZ fusion constructs analyzed in our transgenic studies. The first fusion construct, WZT8C, contained 7.0 kb of genomic sequence upstream of the Wnt-1 transcription start sites fused directly to an E. coli lacZ-SV40pA reporter gene cassette. All Wnt-1 exons and introns (4 kb) and approximately 17.5 kb of non-transcribed 3′ flanking region were placed downstream of the reporter, WZT8C was injected into the pronucleus of fertilized mouse eggs and generation 0 (G0) embryos, examined at 9.5 dpc to 11.5 dpc for presence of the transgene and expression of β-galactosidase. A total of 126 generation 0 (G0) embryos were analyzed. Of these, only two were transgenic (Table 1), an unusually low frequency. However, in both cases, transgene expression was similar to the previously reported distribution of Wnt-1 RNA (Wilkinson et al., 1987; Parr et al., 1993).

The lacZ reporter gene was expressed at the dorsal midline of the diencephalon, midbrain and spinal cord; in a circle just anterior to the mid/hindbrain border and in the ventral midbrain close to, but excluding, the floor plate (data not shown). Moreover, a characteristic gap of expression occurred in the metencephalon, as previously reported (Wilkinson et al., 1987; McMahon et al., 1992; Parr et al., 1993). Thus, it is clear that Wnt-1 regulatory regions are contained on this large reporter construct. Interestingly, these two transgenic embryos both showed ectopic β-galactosidase activity in newly emerging neural crest derivatives along the trunk and in the branchial arches. In addition, ectopic activity was detected in a small region of the ventral telencephalon.

The poor transgenic frequency precluded our establishing stable transgenic lines with this construct. Thus we decided to test subfragments for cis-acting regulatory sequences. 26 G0 transgenics containing a reporter construct, WZT10AC (Fig. 1), lacking 3′ flanking sequence but retaining 7.5 kb of upstream sequence and the Wnt-1 transcription unit, were examined. Only two of these showed β-galactosidase expression and, in both instances, expression was weak and did not occur within the normal Wnt-1 expression domain (Table 1). These results support the conclusion that regulatory sequences lie 3′ of the Wnt-1 polyadenylation site. In summary, the relative insensitivity of the Bg/Il fragment to position, orientation and promoter sequences indicates that this element contains a cis-acting Wnt-1 enhancer.

**The 3′ Wnt-1 enhancer drives correct temporal and spatial expression of a lacZ transgene**

The establishment of stable transgenic lines with the WZT9B construct allowed us to examine temporal and spatial expression of the transgene in detail. Five independent transgenic males (lines 97, 135, 188, 195, 206) were generated, and
several litters from each line analyzed from 7.5 dpc to 11.5 dpc. F1 embryos from each of the WZT9B stable lines showed similar reporter gene expression with minimal ectopic expression. Strong histochemical staining was obtained in as little as 5 minutes (line 135) at 37°C, although most lines required 15 to 30 minutes to reveal the normal Wnt-1 expression domain. Differences between lines may reflect position effects rather than transgene copy number as the weakest expression was obtained in offspring from the line with the highest transgene copy number (line 188).

In situ hybridization studies on sections (McMahon et al., 1992) and in whole mounts (Parr et al., 1993) of neural plate-stage mouse embryos indicate that Wnt-1 transcripts are first detected in the presumptive midbrain at the 1-somite stage. Analysis of transgenic lines first detected β-galactosidase activity restricted to the presumptive midbrain prior to formation of the first somite, presumably reflecting the high sensitivity of histochemical staining (Fig. 3A,B). Thus, activation of Wnt-1 in the midbrain is one of the first signs of regional specialization within the brain. Whilst expression does not occur in all cells at this time, expressing cells are widely distributed across the lateral-medial (future dorsal-ventral) axis as documented for Wnt-1 itself (McMahon et al., 1992; Parr et al., 1993). Thus, activation of the reporter construct under the control of the 3′ Wnt-1 enhancer mimics in vivo activation.

β-galactosidase expression extends progressively more caudal along the dorsal aspects of the hindbrain from three somites, whilst broad midbrain expression is maintained (Fig. 2C-F). Coupled with the progressive caudal extension of Wnt-1 expression at the dorsal midline, there is an anterior extension into the diencephalon and a loss of broad midbrain expression. By 9.5 dpc midbrain expression is limited to the dorsal midline (continuous with the diencephalon), a circle anterior of mid/hindbrain isthmus and the ventral midline either side of the floor plate. This final distribution of Wnt-1 transcripts, which is established by 9.5 dpc, is maintained for several days (Wilkinson et al., 1987). As expected, lacZ expression in the WZT9B lines also shows correct temporal and spatial modifications by 9.5 dpc (Fig. 3A,B) and is maintained until at least 11.5 dpc (Fig. 3C,D). Thus, the cis-acting Wnt-1 regulatory

### Table 1. Transgenic analysis of Wnt-1 expression

<table>
<thead>
<tr>
<th>Reporter construct</th>
<th>Number of embryos examined</th>
<th>Number showing correct reporter gene expression†</th>
<th>Number showing ectopic expression‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>WZT8C</td>
<td>126</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>WZT10AC</td>
<td>93</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>WZT9B</td>
<td>34</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>WZT14B</td>
<td>79</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>WZT7</td>
<td>48</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>WZT73</td>
<td>46</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>WZT8hsp5</td>
<td>30</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

*G0 embryos were examined in all cases except for five WZT93 transgenics that were used to generate permanent transgenic lines.
†All G0 embryos were examined at 9.5 to 10.5 dpc. F1 embryos from the WZT9B lines were analyzed between 7.5 and 12.5 dpc.
‡Excluding ventral telencephalic expression observed in WZT9B and WZT8C transgenic embryos.
element confers all aspects of the dynamic regulation of \( Wnt-1 \) gene expression on the reporter gene construct.

**\( Wnt-1 \) expression in newly emerging neural crest**

Although we observed a strong concordance between the reported distribution of \( Wnt-1 \) transcripts and the observed expression of \( \beta \)-galactosidase in WZT9B transgenic offspring at each developmental stage, some differences were observed. For example, all lines showed strong ectopic \( lacZ \) expression in the ventral telencephalon at 9.5 and 10.5 dpc (Figs 3A,C, 5D, 6B). Ectopic expression was also seen in WZT8C transgenics, suggesting that some region not present in 30 kb of genomic DNA, which includes the \( Wnt-1 \) gene and 26 kb of flanking sequences, is necessary for telencephalic repression of
Wnt-1 expression. More interestingly, we observed widespread expression of lacZ in neural crest derivatives throughout development, both in WZT9B and WZT8C transgenic embryos. This is well illustrated by examining WZT9B transgenic embryos at 9.5 dpc. Littermates (from line 206) were removed and incubated in the β-galactosidase substrate X-Gal for varying lengths of time. Short incubations resulted in histochemical staining restricted to the normal Wnt-1 expression domains except in more caudal regions of the spinal cord where weakly stained cells were visible with a segmental arrangement (Fig. 4, 30'). With progressively longer incubation, broad expression of Wnt-1 was detected in cells of the emerging cranial and trunk neural crest (Fig. 4, 60-120'). These include neural crest cells around the eye, in branchial arches, cranial ganglia, dorsal root ganglia and developing enteric nervous system (Figs 4, 5). β-galactosidase activity was highest in the most recently formed neural crest. Thus, in contrast to normal Wnt-1 transcription, which is not seen in migrating neural crest, β-galactosidase activity is apparent in several neural crest derivatives. The midbrain also showed more extensive β-galactosidase activity on long incubation (Fig. 4, 120') reflecting the previously widespread distribution of lacZ expression at earlier stages.

The distribution of labeled neural crest cells was examined in more detail in sectioned material from WZT9B transgenics (Fig. 5). In the midbrain, neural crest is generated from 4- to 7-somite stages (see Discussion). In midbrain sections at 3 somites, broad β-galactosidase activity is apparent in the neural plate; no activity is visible elsewhere.

**Fig. 3.** Expression of β-galactosidase in WZT9B transgenics following neural tube closure. (A) Lateral; (B) dorsal views of reporter gene expression in the 9.5 dpc CNS, including the diencephalon (DI), midbrain (MB), myelencephalon (MY) and spinal cord (SC), but not in the dorsal telencephalon (TE), or metencephalon (MT). In addition, expression is detected in an extensive circle just anterior of the midbrain/metencephalic junction (large arrows) and ventral midbrain and diencephalon (small arrow). Consistent ectopic expression is seen in this and other independently derived WZT9B transgenic lines in the ventral telencephalon (arrowhead). Correct reporter gene expression is maintained at 10.5 dpc (C) and 11.5 dpc (D). At all stages, lacZ-positive neural crest cells are emerging in the caudalmost areas of the spinal cord (open arrow).
In contrast, β-galactosidase-expressing midbrain neural crest cells can be seen in the head mesenchyme by the lateral (future dorsal) aspects of the midbrain at the 5-somite stage (Fig. 5B). By 9.0 dpc, cranial neural crest is found in anterior head regions in both the first and second branchial arch and surrounding the rostral spinal cord (Fig. 5C). At 9.5 dpc, cranial crest cells still expressing β-galactosidase are condensing to form cranial sensory ganglia (Fig. 5E). By 10.5 dpc, new neural crest cells are only generated in trunk regions of the CNS. In caudalmost areas of the spinal cord, β-galactosidase expression is restricted to the dorsal midline in a distribution indistinguishable from endogenous Wnt-1 RNA expression (Fig. 5F). The trunk crest, which emerges (Fig. 5G) and migrates ventrally (Fig. 5H) at progressively more rostral levels in the spinal cord, continues to express β-galactosidase.

Previous cell lineage studies in the chick (Bronner-Fraser and Fraser, 1988) injecting single cells at the dorsal midline of the spinal cord have shown that these cells will give rise to progenitors which form the dorsal midline of the CNS (roof plate) and neural crest derivatives, suggesting that these cells share a common lineage. Wnt-1 expression at the dorsal midline, which precedes emigration of neural crest cells, is confined, at the RNA level, to the CNS. The observed expression of the lacZ reporter gene may simply reflect abnormal ectopic activity of the regulatory elements in the neural crest. However, given the normal location of Wnt-1 expression and the cell lineage studies in the chick, it seems as likely that neural crest cells and the roof plate share a Wnt-1-expressing precursor
Fig. 5. Analysis of lacZ expression in sections through WZT9B transgenic embryos. (A,B) Transverse sections through the midbrain at 3- and 5-somite stages. Broad expression is seen throughout much of the midbrain. By 5 somites, lacZ-positive neural crest cells are emerging from the lateral aspects of the midbrain (arrow). (C) Section through a 9.25 dpc embryo reveals lacZ-positive cells in the dorsal spinal cord and in cranial neural crest derivatives in the mesenchyme surrounding the forebrain and branchial arches I and II. (D) Sagittal section through 9.5 dpc embryo reveals dorsal midline expression in all the CNS except the telencephalon and metencephalon as previously reported for Wnt-1. Ectopic expression occurs in the ventral telencephalon (arrowhead). (E) Cranial neural crest cells in condensing cranial ganglia express the Wnt-1 lacZ fusion gene. (F) In the spinal cord prior to somite formation, expression of the reporter lacZ gene is restricted to the dorsal midline, as is expression of the endogeneous Wnt-1. (G) Moving rostrally, lacZ-expressing neural crest cells (arrowhead) start to emerge from the dorsal midline and more rostrally still, migrate ventrolaterally (H). For key, see legend to Fig. 3.
cell. Perdurance of reporter gene RNA or residual enzyme activity thereby would mark the neural crest cells arising from these dorsal midline precursor cells. As would be predicted if this explanation is correct, most recently emerging neural crest cells stain strongest and little or no neural crest cell staining is visible by 11.5 dpc except in the most caudal region of the trunk where the newest crest cells have recently emerged (Fig. 3D). This explanation gains support from whole-mount in situ hybridization analysis of Wnt-1 and lacZ RNA expression in transgenic and non-transgenic embryos (Fig. 6). Transcripts show similar spatial distribution within the CNS although there is strong ectopic expression of lacZ RNA in the ventral telencephalon (Fig. 6B), and possibly stronger expression of lacZ RNA than expected in the ventral midbrain (compare Fig. 6A with 6B). However, even after extensive incubation, lacZ RNA was only detected in the most recently arising neural crest cells of the trunk as they migrate through the sclerotomal component of the somite. In contrast, long periods of staining for lacZ expression detected β-galactosidase activity in forming cranial and dorsal root ganglia and in crest derivatives within the branchial arches (Fig. 4).

**DISCUSSION**

**Wnt-1 regulation**

Our results demonstrate that correct temporal and spatial expression of the mouse Wnt-1 gene in the CNS depends upon a cis-acting regulatory region lying 3' of the Wnt-1 polyadenylation
site. Sequences contained within a 5.5 kb fragment can act independent of orientation, and on a heterologous promoter, to activate, modify and maintain expression of a reporter gene construct in the normal Wnt-1 expression domain. In this study, it is impossible to quantitate rigorously the effects of position and direction on the Wnt-1 enhancer, which would require the generation of many stable transgenic lines. However, constructs in which the enhancer is displaced give rise to a smaller proportion of G0 embryos, which express correctly the reporter, indicating an increased sensitivity to position effect. Whilst reporter gene expression largely mimics the normal Wnt-1 expression domain, consistent ectopic expression in the ventral telencephalon was observed suggesting that negative regulatory elements responsible for repression of Wnt-1 expression in the telencephalon are missing from the test constructs. However, it is clear that expression is substantially normal. Although the Wnt-1 gene has been cloned from a number of species including zebrafish (Molven et al., 1991), Xenopus (Noordemeere et al., 1989), man (van Ooyen et al., 1985) and Drosophila (Baker, 1987; Risjewijk et al., 1987; Cabrera et al., 1987; Uzvölgyi et al., 1988), our results constitute the first identification of cis-acting regulatory regions governing normal embryonic expression.

Currently, there is considerable interest in the role of Wnt-1 in vertebrate and invertebrate development where Wnt-1 signaling plays key regulatory roles. In vertebrates, Wnt-1 expression is initiated at early neural plate stages in the presumptive midbrain and is essential for subsequent development of this and the surrounding metencephalic region (Thomas and Capecci, 1990; McMahon and Bradley, 1990; McMahon et al., 1992; Thomas et al. 1991). Thus, activation of Wnt-1 is one of the earliest signs of regional organization of the neural plate. A number of studies (reviewed in Slack and Tannahill, 1992; McMahon, 1993) have led to the conclusion that regional gene expression in the anterior CNS is initiated in response to mesodermally derived signals from the dorsal mesoderm operating vertically and/or in the plane of the neural plate. Thus transcriptional activation of the Wnt-1 gene, which encodes a putative signaling molecule, may be a direct response to reception of mesodermally initiated signal or signals that regulate anterior-posterior patterning of the vertebrate brain. Elsewhere in caudal regions of the hindbrain and spinal cord, Wnt-1 expression at the dorsal midline is one of the first examples of dorsal-ventral polarity in the CNS (Wilkinson et al., 1987; McMahon et al., 1992) suggesting that Wnt-1 signaling may potentially regulate some aspect of dorsal neural tube development. Although there is no apparent phenotype in these regions in Wnt-1 mutant embryos (Thomas and Capecci, 1990; McMahon and Bradley, 1990) a second Wnt-gene, Wnt-3a, is coactivated in this domain suggesting that these members may be functionally redundant (McMahon et al., 1992), a conclusion supported by biological assays of Wnt-properties (Wolda et al., 1993; G. Wong, B. Gavin and A.P. McMahon, unpublished data). Thus, as previously mentioned, the regulatory pathway that controls Wnt-1 expression in the vertebrate CNS will lead to a better understanding of how CNS pattern is regulated and may potentially identify aspects of these pathways which regulate other Wnt genes such as Wnt-3a. Recent experiments indicate that establishment of dorsal expression of Wnt-1 may depend on signaling from the non neural ectoderm (Takada et al., 1994) It will be interesting to determine where these ectoderm-responsive regulatory regions lie in the Wnt-1 enhancer.

At present, there are few clues as to the nature of cis-acting regulatory factors. Several studies (St-Arnaud et al., 1989; Schuuring et al., 1989) have demonstrated that P19 embryonal carcinoma cells when induced to differentiate along neural pathways by application of retinoic acid (Jones-Villeneuve et al., 1982; McBurney et al., 1982) activate endogenous Wnt-1 expression. Activation of reporter gene fusion constructs transfected into P19 cells has identified an activity that binds to a GC-rich site (~206 to ~186) (St-Arnaud and Moir 1993) in the Wnt-1 minimal promoter. This factor, WiF-1, is only present following retinoic acid treatment and its appearance correlates well with activation of reporter constructs as well as the endogenous Wnt-1 gene. However, our experiments indicate that Wnt-1 promoter sequences are not essential for normal embryonic expression. Thus, it seems unlikely that WiF-1-mediated regulation plays a significant role in vivo.

**Wnt-1 and neural crest**

Much of the skeletal structures of the head, peripheral nervous system of the head and trunk, and pigment of the body is derived from the neural crest (for review, see LeDouarin 1982). These cells, which have their origin in the dorsal aspects of the neural plate in all vertebrates, are formed in a rostral-caudal gradient during development. The first crest cells arise in the midbrain/hindbrain regions at 4-somites in the mouse (Nichols, 1981; Chan and Tam, 1988; Serbedzija et al., 1992) and migration is completed by the 7-8 somite stage. In the trunk, crest cells start to emerge from the neural tube 2 to 4 somites rostral to the most recently formed somite and continue to emigrate from this region for 36 to 48 hours (Rawles, 1947; Erickson and Weston, 1983; Serbedzija et al., 1990). The pathway of crest migration and the contribution of crest cells to specific derivatives has been extensively studied in avian species and to a lesser extent in the mouse (for review, see Bronner-Fraser, 1993). These studies indicate that there is a ventral-to-dorsal progression in migratory pathways and that some, if not all, crest cells are multipotent during this migratory phase. Stemple and Anderson (1992) have isolated neural crest cells and demonstrated in vitro, that single cells can generate neural and non-neural crest derivatives. Thus, the diversity of cell types formed in the trunk region may be controlled by environmental factors encountered along the migratory pathway or at the end points of migration.

Although the migratory and terminal differentiation phases of neural crest development have been extensively investigated, relatively little is known of the lineage relationships and mechanisms by which these cells are segregated from the neural tube. The observed expression of active β-galactosidase enzyme within migrating and differentiating neural crest cells in our studies suggests that Wnt-1-expressing cells generate neural crest cells and the roof plate in both cranial and caudal regions. Alternatively, lacZ expression in the neural crest may result, in transgenic mice, from the absence of a repressor element which keeps the gene shut off in the neural crest lineage, or from de novo activation. There is some evidence in support of the former explanation. Firstly, high levels of lacZ RNA are only detected in the normal Wnt-1 expression domain. Low levels of lacZ RNA are restricted to the newly
emerging neural crest. Even after long periods of incubation, transcripts are not detected in more mature crest population. Thus expression is transient, consistent with the gradual decay of a more stable lacZ RNA. At the protein level, β-galactosidase activity is only strong in the normal Wnt-1 expression domain and in the newly emerging crest. Staining in earlier formed crest lineages shows a strict temporal-spatial relationship, strongest in the most caudal and recently formed neural crest, weakest in the most rostral and earliest crest cells. Thus the data appears most consistent with the gradual loss of lacZ RNA and β-galactosidase enzyme activity in the neural crest rather than an extremely transient de novo activation. Given the previous results from clonal analysis of cell lineage in the dorsal neural plate, which demonstrated that the roof plate and neural crest shared a CNS Precursor (Bronner-Fraser and Fraser, 1988), the simplest explanation of our results is that this precursor normally expresses Wnt-1. If this is the case, down regulation of Wnt-1 is presumably an early step in the genesis of neural crest cells. Thus, the perdurance of high levels of lacZ RNA and β-galactosidase enzyme activity in transgenic mice may act as in vivo lineage tracer identifying recent ancestral relationships between the CNS and neural crest in cranial and trunk region. This issue may be best addressed by the introduction of the lacZ gene into the normal context of the Wnt-1 locus. However, this data is not currently available.

There are several significant aspects to this observation. By isolating populations of β-galactosidase-expressing cells using fluorescent sorting techniques, premigratory crest precursors may be purified and their differentiation program studied in the mouse embryo using wheat germ agglutinin-gold conjugate as the cell marker. Development 102, 427-442.

REFERENCES


Epstein, D. J., Vekemans, M. and Gros, P. (1991). Splotch (sp[+]) mutation affecting development of the mouse neural tube, shows a deletion within the pair-2 homeodomain of Pax-3 gene (Epstein et al., 1991), we may be better able to understand the nature of the neural crest deficiencies in such mutants. Finally, these results may point to new phenotypes, which we may expect in compound Wnt-1 mutants. As discussed earlier, Wnt-1 and Wnt-3a, which are co-activated at the dorsal midline of much of the CNS, may share equivalent biological properties. Like Wnt-1 mutants, Wnt-3a null mutant embryos show no obvious phenotype in the dorsal CNS or PNS derivatives (Takada et al., 1994). Thus, the generation of compound Wnt-1/Wnt-3a mutant embryos may be required to reveal complete phenotypes for these members. If Wnt-1/Wnt-3a regulate the development of a roof plate and neural crest precursor cell, loss of both of these genes may lead to an additional neural crest phenotype.

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