Long-range genomic rearrangements upstream of Kit dysregulate the developmental pattern of Kit expression in W\textsuperscript{57} and W\textsuperscript{banded} mice and interfere with distinct steps in melanocyte development

Michael Klüppel\textsuperscript{1,2}, Deborah L. Nagle\textsuperscript{3,†}, Maja Bucan\textsuperscript{3} and Alan Bernstein\textsuperscript{1,2,*}

1Program in Molecular Biology and Cancer, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario, M5G 1X5, Canada
2Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario, Canada
3Departments of Psychiatry and Genetics, University of Pennsylvania, 415 Curie Boulevard, Philadelphia, Pennsylvania 19104, USA
*Author for correspondence
†Present address: Millennium Pharmaceuticals, Inc., 640 Memorial Drive, Cambridge, Massachusetts 02139, USA

SUMMARY

Mutations in the murine dominant white spotting (W) locus cause pleiotropic developmental defects that affect hematopoietic cells, melanocytes, germ cells and the interstitial cells of Cajal in the gut. W mutations either alter the coding sequence of the Kit receptor tyrosine kinase, resulting in a receptor with impaired kinase activity, or affect Kit expression. Here we describe the molecular and cell-type-specific developmental defects of two of the latter class of regulatory W alleles, W\textsuperscript{57} and W\textsuperscript{banded(bd)}. In both mutants, the temporal and spatial patterns of Kit expression are dysregulated during embryogenesis and in adult animals. In W\textsuperscript{bd} mice, ectopic expression of Kit in the dermatome of the somites at days 10.8 and 11.8 of development seemed to interfere with melanoblast development. In contrast, the W\textsuperscript{57} allele leads to an intrinsic pigmentation defect by downregulating developmental Kit expression in trunk melanoblasts, but not melanoblasts around the otic vesicle. Both mutations affect transcriptional initiation of the Kit gene. The W\textsuperscript{57} allele is associated with a 80 kb deletion 5’ of the Kit-coding region while W\textsuperscript{bd} is associated with a 2.8 Mb genomic inversion of chromosome 5 with the distal breakpoint between Kit and the platelet-derived growth factor receptor alpha (Pdgfra) gene, and the proximal breakpoint between the genes for the GABA receptor beta 1 (Gabrb1) and the Tec tyrosine kinase, juxtaposing the Kit and Tec tyrosine kinase genes. Neither W\textsuperscript{57} nor W\textsuperscript{bd} affect genomic sequences previously suggested in in vitro experiments to control cell-type-specific expression of Kit. These results link specific mechanisms of cellular and developmental defects to long-range genomic rearrangements that positively and negatively affect Kit transcription in different cell lineages as well as in different subpopulations of the same lineage.

Key words: Dominant white spotting, Kit, Steel, melanogenesis, genomic rearrangement, transcriptional regulation, mouse, melanocyte

INTRODUCTION

Mutations in the dominant white spotting (W) and Steel (Sl) loci on mouse chromosomes 5 and 10, respectively, result in pleiotropic developmental defects affecting primordial germ cells, hematopoietic cells, melanocytes and interstitial cells of Cajal in the small intestine (Reith and Bernstein, 1991; Galli et al., 1994; Huizinga et al., 1995; Ward et al., 1994). The W locus encodes the Kit receptor tyrosine kinase (Chabot et al., 1988; Geissler et al., 1988) whereas Sl encodes the ligand for the Kit receptor, Steel factor, also termed Kit ligand, mast cell growth factor and stem cell factor (Anderson et al., 1990; Copeland et al., 1990; Flanagan and Leder, 1990; Huang et al., 1990; Williams et al., 1990; Zsebo et al., 1990).

Of the numerous W alleles analyzed thus far, the majority affect the Kit-coding region, resulting in a mutant receptor with diminished or undetectable kinase activity (Reith et al., 1990; Nocka et al., 1990). In contrast to these structural mutations, there are W alleles, which affect the expression, rather than the structure, of the Kit receptor. For example, W\textsuperscript{57} is a spontaneous mutation that gives rise to an irregular white band in the trunk region, a white head spot and a very mild anemia in homozygotes. Heterozygous animals have a white spot in the ventral trunk region. The W\textsuperscript{57} allele does not affect fertility. The coding sequence of Kit is not altered by the W\textsuperscript{57} mutation (A. Reith and A. B., unpublished results), but both the levels of the Kit protein and Kit kinase activity are reduced to the same extent in mast cells (Reith et al., 1990). Heterozygous W\textsuperscript{banded(bd)} mice display a white band in the trunk region, while W\textsuperscript{bd} homozygotes are fertile, black-eyed and white, with occa-
sional pigmentation of the ears and the snout. Erythropoiesis is unaffected in $W^{bd}/W^{bd}$ homozygotes (Beechey et al., 1986). The $W^{bd}$ mutation results in a similar phenotype to that seen in $W^{ash(sh)}$ animals (Lyon and Glenister, 1982), another regulatory allele of $W$ (Duttlinger et al., 1993).

Consistent with their multiple functions in many distinct cell types, both Kit and Sl genes, whose protein products interact directly to activate an intracellular signal transduction pathway, are expressed in contiguous cell layers (Motro et al., 1991; Keshet et al., 1991). Biological, genetic and biochemical findings showed that the membrane-bound form of Steel factor is a more potent ligand than the processed soluble protein in activating the Kit receptor (Bernstein, 1993). Together, these observations suggest that the transcriptional mechanisms that regulate Kit and Sl expression must ensure the proper spatial, developmental and temporal patterns of expression of both genes in very distinct cell lineages.

To understand the molecular mechanisms that regulate Kit expression, we have analyzed the developmental defects and molecular alterations associated with the $W^{57}$ and $W^{bd}$ regulatory mutations. Here, we show that the $W^{57}$ and $W^{bd}$ mutations lead to distinct cellular dysfunctions and dysregulation of Kit expression in adults and during embryogenesis. In particular, we were interested in possible mechanisms of interference with normal melanocyte development in both $W$ mutants. Around day 11 of embryonic development, neural-crest-derived trunk melanoblasts commence their migration along a dorsolateral pathway in a rostral-to-caudal sequence from the dorsal aspect of the neural tube towards the dermatome (Rawles, 1947; Mayer, 1973; Wehrle-Haller and Weston, 1995). Shortly thereafter, melanoblasts migrate ventrally through the developing dermis and, at around 14 to 15 days of embryogenesis, from the demins into the overlying epidermis, where they differentiate into mature melanocytes (Mayer, 1973; Rawles, 1947; Serbedzija et al., 1990). Several alternating stages of Kit-dependency and Kit-independency have been described for melanoblasts throughout development (Nishikawa et al., 1991; Yoshida et al., 1996).

Here we show that the $W^{bd}$ allele leads to ectopic expression of Kit in the dermatome of the somites, the mesenchyme around the otic vesicle and the floorplate at embryonic days 10.8 and 11.8, recapitulating the spatial, but not the temporal expression of its ligand, Steel factor. Early melanoblasts expressed approximately normal levels of Kit but disappeared shortly after emigration out of the neural tube, at the same time that Kit is ectopically expressed in the dermatome.

In contrast to $W^{bd}$, the $W^{57}$ allele does not lead to ectopic Kit expression. In $W^{57}$ animals, embryonic Kit expression is downregulated in early trunk melanoblasts, but not melanoblasts around the otic vesicle. This observation suggests that the $W^{57}$ mutation leads to an intrinsic defect in the development of a subpopulation of neural-crest-derived melanoblasts.

These distinct developmental defects in melanogenesis are associated with long-range genomic rearrangements upstream of the Kit-coding region that affect transcriptional initiation of the Kit gene. $W^{57}$ is associated with a 80 kb deletion, whereas genomic DNA from $W^{bd}$ mice contains an inversion encompassing 2.8 Mb of chromosome 5.

Finally, we show that genomic DNA sequences recently implicated in in vitro studies (Yasuda et al., 1993; Tsujimura et al., 1996) in the control of Kit expression in mast cells are not affected in $W^{57}$ and $W^{bd}$ mice; nevertheless, Kit expression is severely affected in mast cells derived from both mutants. These observations suggest that the regulation of Kit expression is complex, involving cis-acting elements immediately proximal to Kit, as well as sequences located at some distance upstream of the Kit-coding region.

**MATERIALS AND METHODS**

**Mice and embryos**

C57BL/6J and $W^{57}/W^{57}$ mice were purchased from the Jackson Laboratory. $W^{bd}/W^{bd}$, C3H and 101 mice were provided by Dr C. Beechey, MRC Radiobiology Unit, Chilton, UK. $W^{bd}/W^{bd}$ mice were also kindly provided by Dr V. Chapman, Roswell Park, Buffalo, NY. Embryos were derived from appropriate matings of C57BL/6J and C3H/101(F1) for wild-type controls. Mutant embryos were derived from matings of homozygous $W^{57}$ and $W^{bd}$ and from C3H/101(F1) x $W^{bd}/W^{bd}$ mice. The noon after vaginal plug was considered day 0.5 p.c.

**Cell culture**

Primary bone marrow-derived mast cell cultures were prepared as previously described (Reith et al., 1990).

**Flow cytometry**

BMMC were first incubated with ACK2, a monoclonal antibody directed against the extracellular domain of Kit (generously provided by Dr S. Nishikawa) for 30 minutes at 4°C. The cells were then rinsed and stained with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat Ig serum (Cedarlane, Hornby, Ontario). Cells were rinsed and analyzed on a FACScan flow cytometer (Becton Dickinson).

**RNA in situ hybridizations**

RNA in situ hybridizations on sections and whole mounts were performed essentially as previously described (Motro et al., 1991). The Kit, Trp-2 and Sl probes have been described previously (Motro et al., 1991; Steel and Davidson 1992). All experiments were performed on at least three embryos from at least two different litters.

Double labeling RNA in situ hybridizations were performed with a digoxigenin-labelled Trp-2 probe and a 35S-labelled Kit probe. Initial experimental procedures were identical to single probe experiments, except that the hybridization solution contained a mix of both probes. After the post-hybridization washes, subsequent steps were performed as previously described (Marks et al., 1992; Miller et al., 1993).

**RNA isolation and northern blot analysis**

These procedures were basically performed as previously described (Sambrook et al., 1989).

**Nuclear run ons**

The experiment was performed twice as previously described (Lassam and Jay, 1989) with different preparations of nuclei.

**Preparation of mouse genomic and high molecular weight genomic DNA, Pulsed-field gel electrophoresis and Southern blot analysis**

All techniques and probes have been described previously (Brunkowski et al., 1995; Nagle et al., 1994).

**Analysis of Kit upstream sequences by Polymerase Chain Reaction (PCR)**

Genomic DNA fragments from C57BL/6J, C3H/101(He)F1, $W^{57}/W^{57}$ and $W^{bd}/W^{bd}$ were amplified using the following primer pairs: Exon 1/Intron 1 (+28 to +171, relative to transcriptional start site); Kit#6
Dysregulation of Kit expression in W57 and Wbd mutant mice

We also analyzed the steady-state levels of Kit transcripts in various tissues and mast cells from Wbd/Wbd mice. As shown in Fig. 1A, Kit expression was undetectable in lung, gut and mast cells, and downregulated in testis; in contrast, the brain expressed approximately wild-type Kit RNA levels (Fig. 1A).

To investigate if the reductions in Kit expression in mast cells were associated with a corresponding decrease in the levels of Kit protein, we analyzed Kit protein expression in bone-marrow-derived mast cells (BMMC) by fluorescence-activated cell sorting (FACS), using a monoclonal antibody directed against the extracellular domain of the Kit receptor (Nishikawa et al., 1991). Surface expression of the Kit receptor was not detected in Wbd/Wbd BMMC; in contrast, BMMC from W57/W57 expressed Kit at about 30% of wild-type levels (Fig. 1B). We used toluidine blue staining of skin and lung sections from adult mice to investigate if this reduction in Kit mRNA and protein levels correlated with a reduction in mast cell numbers; indeed, mast cell numbers in skin and lung of adult W57/W57 animals were reduced to about 30%, whereas mast cells were completely absent from the skin and lung of Wbd/Wbd mice (data not shown). These data demonstrate that the W57 and Wbd mutations exert distinct cell-type-specific effects on the levels of Kit transcripts.

Both W57 and Wbd are associated with dysregulation of embryonic Kit expression during mid- and late gestation

We next analyzed Kit expression during embryonic development by RNA in situ hybridization analysis on sectioned embryos at different developmental stages. At 14.5 days of gestation, Kit was expressed in various areas in the brain, including the cortex, midbrain and medulla oblongata, in dorsal root ganglia (drg), olfactory epithelium, digestive tract, gonads, liver, lung, kidney and skin of wild-type embryos, as described previously (Orr-Urtreger et al., 1990; Motro et al., 1991; Keshet et al., 1991) (Fig. 2A,D). The levels and patterns of Kit expression in W57 homozygous embryos were indistinguishable from wild-type embryos at the level of RNA in situ hybridization (Fig. 2B,E). Interestingly, Kit expression in the lung mesenchyme was normal at day 14.5 (Fig. 2B), whereas expression was completely absent at day 18 (data not shown) and in adults (Fig. 1A). Similarly, Kit expression in the skin was clearly present at day 14.5 (Fig. 2E), but significantly downregulated at day 18 (data not shown).

In contrast to the results described above for W57/W57 embryos, Kit expression was undetectable in Wbd/Wbd embryos in the lung, the developing external muscle layer of the gut and the skin at both day 14.5 (Fig. 2C,F) and day 18 (data not shown). Expression in the olfactory epithelium was also severely reduced, whereas expression in the brain appeared only slightly reduced. Expression in drg and liver was normal (Fig. 2C). The fetal liver is the major site of hematopoiesis at day 14.5; thus, normal Kit expression in this organ in both mutants is consistent with the absence of an obvious anemic phenotype in these mice. In addition, we observed ectopic Kit expression in the heart of Wbd/Wbd embryos at day 14.5 (Fig. 2C) and at day 18 (data not shown).

Disturbed Kit expression in Wbd embryos during somite differentiation

We next compared Kit expression at embryonic days 10.8 and

---

**RESULTS**

**Tissue-specific dysregulation of Kit expression in both W57 and Wbd adult animals**

We first examined the effects of the W57 and Wbd mutations on Kit expression in adult mice by Northern blot analysis on a variety of tissues. W57/W57 mice expressed approximately normal steady state levels of Kit mRNA in brain, gut and testis (Fig. 1A); in contrast, there was a 3- to 4-fold reduction in the levels of Kit mRNA in W57/W57-derived mast cells. Transcripts corresponding to the Kit gene were not detectable in lung, a tissue that also normally expresses Kit.

We also analyzed the steady-state levels of Kit transcripts in various tissues and mast cells from Wbd/Wbd mice. As shown in Fig. 1A, Kit expression was undetectable in lung, gut and mast cells, and downregulated in testis; in contrast, the brain expressed approximately wild-type Kit RNA levels (Fig. 1A).

To investigate if the reductions in Kit expression in mast cells were associated with a corresponding decrease in the levels of Kit protein, we analyzed Kit protein expression in bone-marrow-derived mast cells (BMMC) by fluorescence-activated cell sorting (FACS), using a monoclonal antibody directed against the extracellular domain of the Kit receptor (Nishikawa et al., 1991). Surface expression of the Kit receptor was not detected in Wbd/Wbd BMMC; in contrast, BMMC from W57/W57 expressed Kit at about 30% of wild-type levels (Fig. 1B). We used toluidine blue staining of skin and lung sections from adult mice to investigate if this reduction in Kit mRNA and protein levels correlated with a reduction in mast cell numbers; indeed, mast cell numbers in skin and lung of adult W57/W57 animals were reduced to about 30%, whereas mast cells were completely absent from the skin and lung of Wbd/Wbd mice (data not shown). These data demonstrate that the W57 and Wbd mutations exert distinct cell-type-specific effects on the levels of Kit transcripts.

**Both W57 and Wbd are associated with dysregulation of embryonic Kit expression during mid- and late gestation**

We next analyzed Kit expression during embryonic development by RNA in situ hybridization analysis on sectioned embryos at different developmental stages. At 14.5 days of gestation, Kit was expressed in various areas in the brain, including the cortex, midbrain and medulla oblongata, in dorsal root ganglia (drg), olfactory epithelium, digestive tract, gonads, liver, lung, kidney and skin of wild-type embryos, as described previously (Orr-Urtreger et al., 1990; Motro et al., 1991; Keshet et al., 1991) (Fig. 2A,D). The levels and patterns of Kit expression in W57 homozygous embryos were indistinguishable from wild-type embryos at the level of RNA in situ hybridization (Fig. 2B,E). Interestingly, Kit expression in the lung mesenchyme was normal at day 14.5 (Fig. 2B), whereas expression was completely absent at day 18 (data not shown) and in adults (Fig. 1A). Similarly, Kit expression in the skin was clearly present at day 14.5 (Fig. 2E), but significantly downregulated at day 18 (data not shown).

In contrast to the results described above for W57/W57 embryos, Kit expression was undetectable in Wbd/Wbd embryos in the lung, the developing external muscle layer of the gut and the skin at both day 14.5 (Fig. 2C,F) and day 18 (data not shown). Expression in the olfactory epithelium was also severely reduced, whereas expression in the brain appeared only slightly reduced. Expression in drg and liver was normal (Fig. 2C). The fetal liver is the major site of hematopoiesis at day 14.5; thus, normal Kit expression in this organ in both mutants is consistent with the absence of an obvious anemic phenotype in these mice. In addition, we observed ectopic Kit expression in the heart of Wbd/Wbd embryos at day 14.5 (Fig. 2C) and at day 18 (data not shown).

**Disturbed Kit expression in Wbd embryos during somite differentiation**

We next compared Kit expression at embryonic days 10.8 and

---

**Fig. 1.** Tissue- and cell-type-specific perturbances of Kit expression patterns in W57/W57 and Wbd/Wbd adult mice. (A) Northern blot analysis of Kit mRNA in adult tissues and cell types from wild-type, W57/W57 (57) and Wbd/Wbd (bd) mice. 15 μg of total RNA was loaded per lane; for gut, 3 μg of poly(A) RNA was loaded per lane. Hybridization of the same blot to a Tubulin cDNA probe was used as a loading control. Sizes of 28 S and 18 S ribosomal RNAs are indicated on the right. (B) FACS analysis of Kit receptor surface expression on +/-, W57/W57 and Wbd/Wbd BMMC using the Ack2 monoclonal antibody. Fluorescence values for levels of expression are indicated in the upper right corners. Omission of the secondary antibody Ack2 as a negative control resulted in fluorescence values between 0.2 and 0.4 (not shown).

---

**Table 1.** Solid expression of Kit during gestation in W57 and Wbd cultures. (A) Northern blot analysis of Kit mRNA in tissue and cell types from wild-type, W57/W57 (57) and Wbd/Wbd (bd) mice. 15 μg of total RNA was loaded per lane; for gut, 3 μg of poly(A) RNA was loaded per lane. Hybridization of the same blot to a Tubulin cDNA probe was used as a loading control. Sizes of 28 S and 18 S ribosomal RNAs are indicated on the right. (B) FACS analysis of Kit receptor surface expression on +/-, W57/W57 and Wbd/Wbd BMMC using the Ack2 monoclonal antibody. Fluorescence values for levels of expression are indicated in the upper right corners. Omission of the secondary antibody Ack2 as a negative control resulted in fluorescence values between 0.2 and 0.4 (not shown).
11.8 in wild-type and mutant embryos. At these stages, Kit is normally expressed in early postmitotic neurons in the neural tube (Fig. 2G,L), in the liver as a hematopoietic organ, in the epithelial lining of the gut, in primordial germ cells and in migrating neural-crest-derived melanoblasts (data not shown and discussed below) (Orr-Urtreger et al., 1990; Motro et al., 1991; Keshet et al., 1991). No qualitative differences in Kit expression between wild-type and W<sup>57</sup>/W<sup>57</sup> embryos were detectable (Fig. 2H and data not shown), except that the onset of Kit expression in the posterior neural tube appeared to be delayed at day 11.8 (Fig. 4E).

In contrast, at day 10.8, Kit was ectopically expressed in W<sup>bd</sup> heterozygous (data not shown) and homozygous embryos in the dermatome of the somites, the floorplate (Fig. 2I), and the mesenchyme around the otic vesicle (data not shown), in a pattern indistinguishable from that previously reported for the W<sup>bd</sup> mutation at this stage (Duttlinger et al., 1993). In addition, all three tissues expressed Steel factor normally (Fig. 2J and data not shown) (Matsui et al., 1990; Duttlinger et al., 1993). However, in contrast to the transient ectopic expression of Kit in the floorplate of W<sup>bd</sup> embryos, evident at day 10.5 but not at day 11.5 (Duttlinger et al., 1993), the ectopic pattern of Kit expression in the floorplate, as well as in the dermatome and the mesenchyme around the otic vesicle, continued on day 11.8 in W<sup>bd</sup> embryos (Figs 2N, 4J,K, expression of Kit and Steel factor around the otic vesicle). By day 15.5, Kit expression was no longer observed in the floorplate of W<sup>bd</sup> embryos (data not shown). This ectopic pattern of Kit expression in the dermatome recapitulates the spatial, but not temporal, expression of Steel factor, the Kit ligand. Steel factor expression ceases during the rostral-to-caudal epithelial-mesenchymal transition of dermatomal tissue at around day 11 to day 12, but reappears at later stages of dermis development (data not shown), (Duttlinger et al., 1993; Matsui et al., 1990; Wehrle-Haller and Weston, 1995). The ectopic expression of Kit does not cease in W<sup>bd</sup> embryos during this transition and was observed during the expansion of the developing dermis, at times when Steel factor is not expressed. Fig. 2O shows the downregulation of Steel factor in the dermatome of a day 11.8 W<sup>bd</sup> homozygous embryo at the level of the forelimb, while Steel factor expression at more posterior levels,

![Fig. 2. RNA in situ analysis of Kit and Steel factor expression during embryogenesis using radiolabelled probes shows a tissue-specific deregulation of Kit expression in W<sup>bd</sup>/W<sup>bd</sup> embryos. Dark-field photomicrographs of sections from day 14.5 (A) +/+, (B) W<sup>57</sup>/W<sup>57</sup> and (C) W<sup>bd</sup>/W<sup>bd</sup> embryos. oe, olfactory epithelium; co, neocortex; mb, midbrain; mo, medulla oblongata. Dark-field picture of Kit expression in the developing skin at day 14.5 in (D) +/+, (E) W<sup>57</sup>/W<sup>57</sup> and (F) W<sup>bd</sup>/W<sup>bd</sup> embryos. Note the reduced expression of Kit in olfactory epithelium, lung, gut mesenchyme and skin and the ectopic expression of Kit in the heart of W<sup>bd</sup>/W<sup>bd</sup> embryos. Expression of Kit in (G) wild-type, (H) W<sup>57</sup>/W<sup>57</sup> and (I) W<sup>bd</sup>/W<sup>bd</sup> embryos at day 10.8. Note the lack of Kit expression in the neural tube (large arrow) and the ectopic expression of Kit in the dermatome (small arrow) and the floorplate (arrowhead) of W<sup>bd</sup>/W<sup>bd</sup> embryos. (J) This ectopic expression recapitulates Steel factor expression in dermatome (small arrow) and floorplate (arrowhead), shown here in an adjacent section to I, hybridized to a Steel factor probe. (K) Bright-field picture of I. Expression of Kit in (L) wild-type and (N) W<sup>bd</sup>/W<sup>bd</sup> embryos at day 11.8 at forelimb level (main frame) and in the tail (insert). Note apparently normal expression in the neural tube (large arrow) and continued ectopic expression in dermatome (small arrow) and floorplate (arrowhead) at both axial levels in W<sup>bd</sup>/W<sup>bd</sup> embryos. Expression of Steel factor in M wild-type and (O) W<sup>bd</sup>/W<sup>bd</sup> embryos at day 11.8 in adjacent sections to the sections in L and N, respectively. Note the lack of Steel factor expression in the dermatome at the forelimb level (small arrow), whereas this expression is still present in the tail (small arrow). Scale bar represents 1 mm in A-C, 100 μm in D-K, 200 μm in L-O.](image-url)
e.g. in the tail, was still apparent (Fig. 2O, insert) Ectopic Kit expression in the dermis was obvious at both levels at this stage (Fig. 2N), and thus appears to be independent of the epithelial-mesenchymal transition. When Steel factor was again expressed in the developing dermis at day 14.5, ectopic wild-type and embryos of the same age. At day 11.8, comparison between temporal effect of the expression in the neural tube (Fig. 2L,N), suggesting a epithelial-mesenchymal transition. When stage (Fig. 2N), and thus appears to be independent of the expression in the dermatome was obvious at both levels at this tail, a few melanoblasts were found in the MSA migration towards the dermatome at more tail, followed by their proliferation and development were most distinct at the hindlimb level: in normal and W57/homozygous embryos, we observed melanoblasts migrating towards the MSA at the base of the tail, followed by their proliferation and migration towards the dermatoe at more anterior axial levels (Fig. 3A,B). Melanoblast numbers were reduced at the hindlimb level in W57/W57 embryos (Fig. 3B); this reduction was more apparent at the forelimb level (Fig. 3F), compared to wild-type embryos (Fig. 3E). The numbers of Trp-2-positive melanoblasts around the otic vesicle were normal (compare Fig. 3K,J), as were numbers in other areas of the head (data not shown).

In contrast to these observations on W57/W57 embryos, Trp-2-positive cells were severely reduced at day 10.8 around the otic vesicle (data not shown) and undetectable in the trunk (Fig. 3H), around the otic vesicle (Fig. 3M) and in other areas of the head (data not shown) of Wbd/Wbd embryos at day 11.8. At the base of the tail, a few melanoblasts were found in the MSA over a length of four to five segments (Fig. 3D,I). Cells at the hindlimb level at day 11.8 of development represent cells that have recently migrated out of the neural crest towards the migration staging area (MSA) between neural tube and somites (Wehrle-Haller and Weston, 1995). These cells were initially present in Wbd/Wbd embryos, but disappeared shortly thereafter, as indicated by the absence of melanoblasts at more anterior levels.

We also analyzed melanoblast development in heterozygous Wbd/+ embryos by whole-mount RNA in situ. At day 10.8, Trp-2-positive melanoblasts were present around the otic vesicle, although the numbers were reduced compared to wild-type embryos (data not shown). At day 11.8, melanoblasts were present at the hindlimb (Fig. 3C) and the forelimb levels (Fig. 3G) and around the otic vesicle (Fig. 3I), but again the numbers were reduced. Thus, the Wbd allele exerts a dominant

The effects of the W57 and Wbd mutations on morphogenetic behaviour and fate of developing melanoblasts

To investigate the developmental stages at which the pigmentation defects in W57 and Wbd are established, we analyzed the number and distribution of trunk melanoblasts during development in normal and mutant animals. To follow the development of melanoblasts, we utilized RNA probes specific for Kit and for tyrosinase-related-protein-2 (Trp-2) as early melanoblast markers (Steel et al., 1992; Wehrle-Haller and Weston, 1995) in whole-mount and section RNA in situ experiments.

At day 10.8, normal numbers of Trp-2-positive melanoblasts were observed in the mesenchyme around the otic vesicle in W57/W57 embryos (data not shown). At day 11.8, we observed Trp-2-positive melanoblasts in the trunk lateral to the neural tube. The temporal differences between various stages of melanoblast migration and development were most distinct at the hindlimb level: in normal and W57 homozygous embryos, we observed melanoblasts migrating towards the MSA at the base of the tail, followed by their proliferation and migration towards the dermatoe at more anterior axial levels (Fig. 3A,B). Melanoblast numbers were reduced at the hindlimb level in W57/W57 embryos (Fig. 3B); this reduction was more apparent at the forelimb level (Fig. 3F), compared to wild-type embryos (Fig. 3E). The numbers of Trp-2-positive melanoblasts around(471,855),(630,885)

![Fig. 3. Expression analysis of Trp-2 at day 11.8 reveals a reduction in trunk melanoblast numbers in both W57/W57 and Wbd mutant embryos. (A) RNA whole-mount in situ analysis using a digoxigenin-labelled Trp-2 probe identifies Trp-2-positive melanoblasts in the hindlimb area: single melanoblasts appear first at the base of the tail (right side of picture) in the MSA, followed by proliferation and migration towards the dermatoe at more anterior axial levels (left side of picture); hl, base of hindlimb. (B) W57/W57 embryos have normal numbers of Trp-2-positive melanoblasts in the MSA, whereas melanoblast numbers do not expand to the same degree as in wild-type embryos during the proliferation and migration toward the dermatoe. (C) Wbd/+ embryos display a marked reduction of melanoblasts in the hindlimb area. (D) Wbd/Wbd embryos have a few Trp-2-positive melanoblasts in the MSA (arrows), but the cells disappear shortly after; no cells migrating towards the dermatoe can be observed. The asterisk marks the group of melanoblasts magnified in H. (E) Trp-2-positive melanoblasts at the forelimb level of a wild-type embryo. (F) Numbers of Trp-2-positive melanoblasts are severely reduced in W57/W57 embryos. (G) Trp-2-positive melanoblasts are reduced in numbers and are not randomly distributed anymore. The arrow marks an area devoid of melanoblasts. (H) No Trp-2-positive melanoblasts are found in Wbd/Wbd embryos. Normal numbers of Trp-2-positive melanoblasts are seen in J wild-type and (K) W57/W57 embryos, whereas in L Wbd/+ embryos melanoblast numbers were severely reduced and in M Wbd/Wbd embryos melanoblasts are absent. Scale bar represents 150 μm in A-D, 100 μm in E-H and J-M and 40 μm in I.]{fig3.png}
effect early during melanocyte development, that also included a modification of the migration characteristics of melanoblasts in Wbd/Wbd embryos.

Melanoblasts normally migrate over the entire surface of the dermatome, not distinguishing between the anterior and posterior part of somites (Loring and Erickson, 1987) (Fig. 3E). In contrast, neural crest cells that give rise to sympathetic ganglia and epinephrine-secreting cells of the adrenal medulla only migrate through the anterior, not the posterior, parts of each somite (Bronner-Fraser, 1986). Trunk melanoblasts in the MSA of the tail region of Wbd heterozygous embryos, although reduced in numbers, had a normal, random distribution (Fig. 3C), whereas melanoblasts at older, more anterior axial levels formed clusters of cells located only over one part of the somites (Fig. 3G). Although we did not determine the anterior and posterior boundaries of the somites, the observed pattern was strikingly similar to the patterns of other neural crest lineages and different from the normal pattern of melanoblast migration. We do not know the influence this altered migration pattern might have on the development of melanoblasts in Wbd/Wbd embryos.

To determine if the melanoblasts in mutant embryos express normal levels of Kit, we hybridized adjacent sections of day 11.8 wild-type, W57/W57 and Wbd/Wbd embryos to radiolabelled Kit and Trp-2 RNA probes. Trp-2-positive trunk melanoblasts were readily detectable lateral to the neural tube at the hindlimb level of wild-type embryos (Fig. 4A); these cells express high levels of Kit (Fig. 4B). Melanoblasts around the otic vesicle of the same embryo also expressed high levels of Kit (Fig. 4C). Trp-2-positive trunk melanoblasts, albeit reduced in numbers, were also clearly present in W57/W57 embryos at this stage (Fig. 4D); surprisingly, we did not observe any Kit expression in these trunk melanoblasts (Fig. 4E), whereas melanoblasts around the otic vesicle of the same embryo expressed apparently normal levels of Kit (Fig. 4F). The insert in Fig. 4E shows strong expression of Kit in the endothelium of the gut and the liver of the same embryo displayed in the main frame, showing that W57 does not lead to a general downregulation of Kit expression. The insets in Fig. 4C,F show individual melanoblasts from the main frames at higher magnification: the density of silver grains in these melanoblasts around the otic vesicle from wild-type and W57/W57 embryos was indistinguishable. Very few Trp-2-positive melanoblasts were present in the hindlimb area of Wbd/Wbd embryos at day 11.8 (Fig. 4G); these cells expressed normal levels of Kit (Fig. 4H,I). Kit-positive melanoblasts around the otic vesicle could not be identified due to the strong ectopic expression of Kit in the mesenchyme (Fig. 4J), compare to Steel factor expression in the same area in Fig. 4K). We showed above that melanoblasts are absent at this stage around the otic vesicle of Wbd homozygous embryos.

Similar results on the differential effects of the W57 and Wbd mutations on Kit expression in trunk melanoblasts were also obtained when using a digoxigenin-labelled Kit probe on day 11.8 whole-mount embryos (data not shown).

The surprising result that W57 trunk melanoblasts seemed not to express detectable levels of Kit at day 11.8 led us to perform double labelling experiments in which a digoxigenin-labelled Trp-2 probe and a radiolabelled Kit probe were hybridized to the same section. This enabled us to visualize Kit expression in unequivocally identified melanoblasts. Fig. 4L,N shows melanoblasts from the hindlimb region of a wild-type embryo at day 11.8, labelled with Trp-2. These melanoblasts expressed high levels of Kit, as visualized by the scattered silver grains overlaying the cells (Fig. 4M,O). In contrast, Kit expression was undetectable in Trp-2-positive melanoblasts from the hindlimb region of a day 11.8 W57/W57 embryo (Fig. 4P), with the density of silver grains barely above background (Fig. 4Q).

These results confirm that the W57 mutation downregulates Kit expression in a subpopulation of melanoblasts migrating along the dorsolateral pathway in the trunk, without affecting Kit expression in melanoblasts around the otic vesicle.

To determine the morphogenetic behaviour of melanoblasts at later stages in development, we analyzed the fate of Trp-2-positive melanoblasts during organogenesis, at day 14.5 and day 18 of embryogenesis. At day 14.5, formation of the dermis is complete and melanoblasts begin to migrate from the dermis into the developing epidermis. At this stage, W57/W57 embryos had variable numbers of melanoblasts in the trunk. Some embryos had almost normal numbers of Trp-2-positive cells (compare Fig. 5A and B), whereas the numbers in other embryos were clearly reduced (Fig. 5C). In all W57/W57 embryos analyzed, we detected one or a few melanoblasts in the mid-trunk region per section (Fig. 5F), comparable to the numbers in wild-type embryos (Fig. 5E). These observations suggest that the pigmentation defect, i.e. the absence of melanocytes in the mid-trunk region of W57/W57 adults, has not yet been established at day 14.5, although melanoblast numbers were already reduced from day 11.8 on. These observations on Trp-2-positive melanoblasts confirm and extend previous findings (Duttlinger et al., 1993) that Kit-positive cells in the epidermis of wild-type embryos at midgestation show a graded distribution, with high density in the tail and face, and low density in the trunk region. The number of Trp-2-positive melanoblasts in the developing inner ear as well as other areas of the head were unaffected in W57/W57 embryos at this stage (data not shown).

At day 18 of development, large areas in the lower trunk and head were completely devoid of Trp-2-positive melanoblasts in W57/W57 embryos (Fig. 5H and data not shown), corresponding to the location of future depigmentation in the adult. In contrast, wild-type embryos at day 18 displayed a continuous layer of Trp-2-positive melanoblasts in the skin of the lower trunk (Fig. 5G) and head (data not shown). Again, the number of Trp-2-positive melanoblasts in the developing inner ear was not affected in W57/W57 embryos (data not shown).

We did not observe any Trp-2-positive melanoblasts in Wbd/Wbd embryos at day 14.5 or day 18 (Fig. 5D and data not shown).

To investigate if Kit expression is still downregulated in trunk melanoblasts of W57/W57 embryos at day 14.5 or day 18, we performed double labeling RNA in situ experiments utilizing a digoxigenin-labelled Trp-2 probe and a 35S-radiolabelled Kit probe. This double-labelling technique was utilized to distinguish between two Kit-expressing cell types, melanoblasts and mast cells, present in the developing skin from midgestation onwards. Trp-2-positive melanoblasts in wild-type (day 14.5, Fig. 6A; day 18, Fig. 6E) and W57/W57 embryos (day 14.5, Fig. 6C; day 18, Fig. 6G) expressed approximately equal levels of Kit (Fig. 6B,D,F,H).
Both \(W^{57}\) and \(W^{bd}\) affect transcriptional initiation of Kit

The results presented above demonstrate that the \(W^{57}\) and \(W^{bd}\) mutations differentially affect the temporal and spatial expression patterns of Kit, leading to distinct cellular and developmental defects. To understand the molecular basis for these effects on Kit expression, we first performed nuclear run-on assays to determine whether these mutations act at the level of transcriptional initiation or mRNA stability. We measured the rates of transcriptional initiation of Kit in homogeneous populations of BMMC from +/-, \(W^{57}/W^{57}\) and \(W^{bd}/W^{bd}\) mice. The results were quantitated by comparing the intensity of the Kit hybridization signal to the corresponding signals for Tubulin and GAPDH, two housekeeping genes whose expression is not expected to be affected by \(W\) mutations.

While BMMC from wild-type mice expressed high levels of Kit mRNA, transcriptional initiation of Kit was essentially undetectable in \(W^{bd}/W^{bd}\) mast cells (6.3% of wild-type levels) and reduced to 29% of wild-type levels in mast cells derived from \(W^{57}/W^{57}\) mice (Fig. 7A,B). These results demonstrate that both \(W^{57}\) and \(W^{bd}\) affect transcriptional initiation of Kit and that the decrease in the steady state levels of Kit mRNA and protein in mast cells from these mutant mice (see Fig. 1) can be entirely accounted for by effects on transcriptional initiation.

The \(W^{57}\) mutation is associated with a 80 kb deletion 5' of Kit

The Kit nucleotide sequence in \(W^{57}\) DNA is identical to the Kit cDNA sequence in C57BL/6J DNA (A. D. Reith and A. B., unpublished results) and Southern blot analysis with six different restriction enzymes failed to reveal any gross structural alteration in \(W^{57}\) and \(W^{bd}\) genomic DNA. In addition, we determined by PCR that regulatory sequences located up to 400 bp upstream of the first coding exon of Kit which have recently been implied in vitro experiments to play an important role in transcriptional regulation of Kit (Yasuda et al., 1993; Tsujimura et al., 1996) are not affected by both mutations. In \(W^{bd}\), sequences up to approximately 624 bp upstream of Kit are present and linked to the first coding exon. In \(W^{57}\), sequences up to 4221 bp are intact (data not shown).

To determine whether the cellular and regulatory defects in \(W^{57}\) mice might result from long-range genomic rearrangements, we analyzed high molecular weight spleen DNA from wild-type, heterozygous \(W^{57/+}\) and homozygous \(W^{57}/W^{57}\) mice by pulsed-field gel electrophoresis (PFGE). We have previously established that the genes encoding two other RTKs, Pdgfra and Flk1 are closely linked to Kit in the order Pdgfra-Kit-Flk-1 with Pdgfra and Kit in the same transcriptional orientation (Brunkow et al., 1995). Therefore, genomic DNAs were analyzed with probes for all three genes. Using four rare cutting restriction enzymes (NotI, MluI, NarI, Pmbl), we identified novel DNA fragments that were reduced in size by approximately 80 kb in all digests of \(W^{57}\) DNA (Fig. 8A). For example, while the Kit cDNA probe hybridized to a 290 kb Pmbl fragment in wild-type DNA, this probe detected both 290 kb and 210 kb DNA fragments in \(W^{57/+}\) DNA and a 210 kb DNA fragment in \(W^{57}/W^{57}\) DNA. Similarly, the Pdgfra cDNA probe detected a 580 kb NotI fragment in wild-type DNA and a novel 500 kb fragment in \(W^{57}/W^{57}\) DNA; NotI fragments of both sizes were present in \(W^{57/+}\) DNA. Only DNA fragments of normal sizes were observed when \(W^{57}\) DNA was analyzed with the Flk-1 cDNA probe (data not shown). Using single and double digests (data not shown), we established a long-range restriction map of the rearranged \(W^{57}\) chromosome. By comparing this restriction map to the wild-type long-range restriction map previously described (Nagle et al., 1994; Brunkow et al., 1995), we conclude that the \(W^{57}\) mutation is associated with a 80 kb deletion, located in a 160 kb Pmbl/NotI genomic fragment 5' to Kit, between the Kit and Pdgfra genes (Fig. 8B). The Buc3 probe (see Fig. 9C) is not affected by the \(W^{57}\) mutation. The addition of the Pmbl restriction sites to the existing genomic map significantly narrows down the genomic region that contains the \(W^{57}\) deletion as well as the genomic sequences that contain the distal breakpoint in the \(W^{bd}\) and \(W^{sh}\) mutations (see below).

The \(W^{bd}\) mutation is associated with a 2.8 Mb inversion upstream of Kit

The phenotypic similarity of \(W^{bd}\) and \(W^{sh}\) mice prompted us to compare the Kit-Pdgfra intergenic region in both \(W^{bd}\) and \(W^{sh}\) DNA by PFGE. We observed novel DNA fragments of the same size in \(W^{bd}/W^{bd}\) and \(W^{sh}/W^{sh}\) DNA digested with MluI or Pmbl after hybridization to the Kit probe (Fig. 9A and data not shown), whereas the Pdgfra probe detected novel DNA fragments following digestion with BssHII, NotI, MluI or NruI (Fig. 9B and data not shown). To compare further the genomic rearrangements in the \(W^{bd}\) and \(W^{sh}\) alleles, the same PFGE blots were hybridized with cDNA probes for Kit and Tec as well as Pdgfra and Gabrb1. Tec and Gabrb1 are located 3 Mb proximal to Kit and known to flank the proximal breakpoint of the \(W^{sh}\) inversion (Nagle et al., 1995). The Kit and Tec probes detected a novel DNA fragment of identical size in \(W^{bd}/W^{bd}\) and \(W^{sh}/W^{sh}\) DNA digested with Pmbl (Fig. 9A), while the Pdgfra and Gabrb1 probe detected the same altered DNA fragments in \(W^{bd}/W^{bd}\) and \(W^{sh}/W^{sh}\) DNA digested with the enzymes BssHII, NotI, MluI and NruI (Fig. 9B). These results establish the structural similarities between the \(W^{bd}\) and \(W^{sh}\) alleles and demonstrate that \(W^{bd}\), an independently isolated allele, is associated with an inversion in the same chromosomal region as \(W^{sh}\). Fig. 9C summarizes the physical map of the rearranged \(W^{bd}\) and \(W^{sh}\) chromosomes. Our results demonstrate the approximate location of the distal breakpoint of both the \(W^{bd}\) and \(W^{sh}\) inversions in the same 160 kb Pmbl/NotI genomic fragment that also harbours the \(W^{57}\) deletion (Fig. 8B). In both \(W^{bd}\) and \(W^{sh}\), the proximal breakpoint is located in the intergenic region between Tec and Gabrb1.

DISCUSSION

In this paper, we have described the cellular, developmental and molecular defects associated with two regulatory alleles of the W locus, \(W^{57}\) and \(W^{bd}\). \(W^{57}\) and \(W^{bd}\) differentially affect the temporal and spatial expression of Kit in both the adult and during embryogenesis. Reduction of Kit mRNA expression in BMMC correlated directly with the reduction of Kit surface receptor expression in BMMC and reduction in mast cell numbers in the skin and lung of \(W^{57}/W^{57}\) and \(W^{bd}/W^{bd}\) mice.

Embryos homozygous for the \(W^{57}\) mutation displayed an
Fig. 4. RNA in situ analysis of *Trp-2* and *Kit* expression in melanoblasts (arrows) of wild-type, *W*<sup>57</sup>/*W*<sup>57</sup> and *W*<sup>bd</sup>/W*<sup>bd</sup> embryos at day 11.8. (A) *Trp-2* expression and (B) *Kit* expression (adjacent sections) in melanoblasts migrating on a dorsolateral pathway at the hindlimb level of a wild-type embryo. (C) *Kit* expression in melanoblasts around the otic vesicle of a wild-type embryo. The three melanoblasts marked with the arrow are shown at higher magnification and in bright field. (J) *Trp-2*-positive melanoblasts at the hindlimb level of *W*<sup>bd</sup>/W*<sup>bd</sup> embryos. (E) In the adjacent section, no *Kit* expression could be observed in melanoblasts, whereas *Kit* expression in liver (li) and gut endothelium (arrowhead) appeared normal (inset). (F) *Kit* expression in melanoblasts around the otic vesicle appeared to be at wild-type levels. The two melanoblasts marked with the arrow are shown at higher magnification and in bright field in the inset. We observed only very few *Trp-2*-positive melanoblasts at the hindlimb level of *W*<sup>bd</sup>/W*<sup>bd</sup> embryos (G), which expressed normal levels of *Kit* (H). (I) The melanoblast from H at higher magnification and in bright field. (J) *W*<sup>bd</sup>/W*<sup>bd</sup> embryos display ectopic *Kit* expression in the floorplate (white arrowhead) and in the mesenchyme around the otic vesicle (black arrowhead), which recapitulates (K) Steel factor expression in the same structures. (L-Q) Double labelling RNA in situ analysis of wild-type and *W*<sup>57</sup>/*W*<sup>57</sup> melanoblasts at the hindlimb level. The same section was hybridized to a digoxigenin-labelled *Trp-2* probe and a radiolabelled *Kit* probe. Cells and silver grains are in different focal plains due to a Parlodion coating of the slides before exposure to prevent a color reaction between color product and emulsion. (L-N) *Trp-2*-positive melanoblasts in wild-type embryos express high levels of *Kit* (M,O), as judged by the density of silver grains overlaying the melanoblasts, whereas *Trp-2*-positive melanoblasts in *W*<sup>57</sup>/W*<sup>57</sup> embryos (P) show a severe reduction in *Kit* expression: silver grain density overlaying the melanoblasts is barely above background (Q). (nt, neural tube; ot, otic vesicle; cv, anterior cardinal vein; drg, dorsal root ganglion; dm, dermatome). Scale bar represents 200 μm in A-G, 30 μm in the inlets of C, F and I, 100 μm in H, J, K, and 15 μm in L-Q.

Almost wild-type *Kit* expression pattern. Between day 14.5 and day 18 of embryogenesis, *Kit* expression in both the lung and skin was greatly diminished, suggesting that the *W*<sup>57</sup> mutation leads to the dysregulation of proper temporal expression of *Kit* in the two *Kit*-expressing cell types normally present in the skin, melanoblasts and mast cells. Since the numbers of mast cells in adult lung and skin are reduced, the loss of *Kit* expression in embryonic lung and skin of *W*<sup>bd</sup>/W*<sup>bd</sup> embryos between day 14.5 and day 18 most likely reflects a reduction in mast cell numbers; however, it is possible that other cell types in the lung that also express *Kit* are also affected, as we have shown for melanoblasts in the skin. In contrast to these marked effects on *Kit* expression in lung and skin, the levels of *Kit* mRNA in brain, olfactory epithelium, drg, kidney and liver were normal at these stages.

At midgestation, *Kit* was not expressed in the lung, gut and skin of *W*<sup>bd</sup>/W*<sup>bd</sup> embryos. In addition, expression in the olfactory epithelium was greatly reduced, whereas *Kit* was ectopically expressed in the heart. The effects on *Kit* expression in skin and lung most likely reflect the absence of melanoblasts and tissue mast cells due to the *W*<sup>bd</sup> mutation. During somite formation and differentiation at day 10.8 and day 11.8, *Kit* was ectopically expressed in *W*<sup>bd</sup> embryos in a variety of structures that also express Steel factor, including the dermatome of the somites, the floorplate and the mesenchyme around the otic vesicle.

To gain insights into the cellular and molecular mechanisms of action of the *W*<sup>57</sup> and *W*<sup>bd</sup> alleles, we followed melanoblast development throughout embryogenesis, using *Trp-2* as a melanoblast marker. This analysis demonstrated that the pigmentation defect in the mid-trunk of *W*<sup>57</sup>/W*<sup>57</sup> animals is a late defect that is not manifested in all embryos before late gestation, although melanoblast numbers are reduced in the trunk from day 11.8 onward. At day 18, melanoblast numbers are reduced everywhere and large areas in the trunk region, corresponding to the regions of depigmentation in adult *W*<sup>57</sup>/W*<sup>57</sup> animals, are devoid of melanoblasts. This defect in melanoblast development was associated with a marked reduction in *Kit* expression in trunk melanoblasts, but not melanoblasts around the otic vesicle, at day 11.8 of embryo-
Dysregulation of Kit expression in W57 and Wbd mutant mice

Fig. 5. Dark-field photographs of RNA in situ experiments using a radiolabelled Trp-2 probe on sections of day 14.5 and day 18 embryos. Trp-2-positive melanoblasts at the base of the tail of day 14.5 (A) wild-type, (B), (C) W57/W57 and (D) Wbd/Wbd embryos. Note the moderate (B) and severe (C) reduction in melanoblast numbers in two different W57/W57 embryos, whereas no Trp-2-positive melanoblasts are present in Wbd/Wbd embryos (D). Very few Trp-2-positive melanoblasts are present at the mid-trunk level at day 14.5 in both (E) wild-type and (F) W57/W57 embryos. Melanoblast numbers at the mid-trunk level at day 18 are markedly reduced in (H) W57/W57 embryos, compared to (G) wild-type embryos, leaving large areas devoid of Trp-2-positive melanoblasts. Arrows mark single melanoblasts. Scale bar represents 150 μm.

Fig. 6. Double-labeling RNA in situ analysis on sections of day 14.5 and day 18 embryos using a digoxigenin-labelled Trp-2 probe and a radiolabelled Kit probe. Trp-2-positive trunk melanoblasts of day 14.5 (A) and day 18 (E) wild-type embryos are stained blue and express Kit (B,F). Trp-2-positive melanoblasts at the base of the tail of day 14.5 (C) and day 18 (G) W57/W57 embryos also express Kit at approximately wild-type levels (D,H). Scale bar represents 10 μm.

 genesis, whereas at later stages W57 trunk melanoblasts expressed approximately normal levels of Kit. These data suggest that W57 causes an intrinsic, cell-autonomous defect in a subset of neural-crest-derived melanoblasts through the temporal downregulation of Kit expression during early development of trunk melanoblasts. Because melanoblasts display a graded distribution along the embryonic body axis, with highest numbers in the tail and face and lowest numbers in the trunk region (Duttlinger et al., 1993), an overall reduction, but not complete absence, of melanoblasts would lead to selective depigmentation in the trunk region, while tail and face would still have sufficient melanocyte numbers to ensure pigmentation.

It has recently been shown that melanoblasts undergo several alternating stages of Kit-dependency and Kit-independency between day 9.5 and birth (Yoshida et al., 1996). The temporal downregulation of Kit expression in trunk melanoblasts of W57/W57 embryos might at least partially overlap with a phase of Kit-independency, therefore causing only a partial depigmentation. Alternatively, some residual expression of Kit, not necessarily detectable in our assays, might enable some trunk melanoblasts to survive through the period of Kit downregulation.

W57/W57 animals display a white head spot; we showed that this phenotype is established between day 14.5 and day 18. Although we observed approximately normal expression of Kit in craniofacial melanoblasts around the otic vesicle at day 11.8 as well as in trunk and head melanoblasts at day 14.5 and day 18, we cannot exclude that the W57 mutation affects Kit expression and therefore development of a subset of craniofacial melanoblasts, therefore leading to depigmentation of parts of the head. Alternatively, craniofacial melanoblasts might be in general more sensitive to small changes in Kit expression, which we might have not detected. We would argue against this latter point, since Trp-2-positive melanoblast numbers in the developing inner ear appear normal throughout embryogenesis in W57/W57 embryos.

Melanoblasts in Wbd/Wbd embryos never migrate to the dermatome, although they express apparently normal levels of Kit. During the rostral-to-caudal sequence of dorsolateral melanoblast migration from the neural crest towards the somites, melanoblasts are stalled at the MSA, lateral to the neural tube, and disappear shortly thereafter. Thus, the pigmentation defect is established early, coincidentally at the same time that Kit is ectopically expressed in the dermatome of the somites. Interactions between Kit-expressing
Fig. 7. (A) Nuclear run on analysis comparing transcriptional initiation of Kit in nuclei from BMMC derived from adult wild-type, W<sup>57</sup>/W<sup>57</sup> and W<sup>bd</sup>/W<sup>bd</sup> mice. Controls include the transcriptional initiation of Tubulin and GAPDH, two housekeeping genes, PDGFRα, a gene not expressed in mast cells and the pKS vector, which does not have any transcriptional initiation sites.

(B) Quantitation of the results shown in A by comparing transcriptional initiation of Kit to that of the two housekeeping genes Tubulin and GAPDH shows that in W<sup>57</sup>/W<sup>57</sup> BMMC, the rate of transcriptional initiation of Kit is only 29% of that of wild-type BMMC; whereas in W<sup>bd</sup>/W<sup>bd</sup> BMMC, the rate of transcriptional initiation is only 6.3% of wild-type levels.

Melanoblasts on the dorsolateral pathway and Steel factor-expressing dermrand have been suggested to play an important role in early melanoblast migration from the MSA towards the dermrand (Wehrle-Haller and Weston, 1995). Duttingler et al. (1993, 1995) have previously suggested that the ectopic expression of Kit in the dermrand of W<sup>sh</sup> and Patch (Ph) embryos might sequester functional soluble Steel factor, thereby reducing the amount of ligand available to the migrating melanoblasts and affecting their survival by a non-cell autonomous mechanism. Interestingly, stimulation of human fetal melanoblasts in vitro with Steel factor affects the expression of several integrin subunits and changes the attachment of those cells to extracellular matrix (ECM) molecules (Scott et al., 1994). The failure of melanoblasts in W<sup>bd</sup> and W<sup>sh</sup> embryos to be exposed to Steel factor from the dermrand could lead to a change in integrin expression and subsequently to an inability of these cells to recognize and bind to the ECM. In this scenario, melanoblast migration would be stalled at the MSA, as we observed in W<sup>bd</sup>/W<sup>bd</sup> mutant embryos.

In contradiction to this non-cell autonomous model, Huszar et al. (1991) have shown that transplantation of normal neural crest cells into the amniotic cavity of W<sup>sh</sup>/W<sup>sh</sup> embryos results in the formation of pigmented areas in the skin, suggesting a cell autonomous defect in W<sup>sh</sup>-mutant animals. Thus, it is not clear if the ectopic expression of Kit in the dermrand is causally related to the pigmentation defects in W<sup>bd</sup> and W<sup>sh</sup> mice.

The ectopic expression of Kit in the dermrand of W<sup>bd</sup> embryos recapitulates the spatial, but not temporal, expression pattern of Steel factor. Steel factor expression is turned off during the epithelial-mesenchymal transition of the dermrand (Wehrle-Haller and Weston, 1995), but is re-expressed at day 14.5 in the skin. We observed continuous ectopic expression of Kit in the dermrand of W<sup>bd</sup> embryos at day 11.8, even at axial levels where Steel factor expression was turned off during the epithelial-mesenchymal transition. While Steel factor is re-expressed in the skin at day 14.5, ectopic Kit expression ceases between day 11.8 and day 14.5.

Other structures that express Kit ectopically in W<sup>sh</sup> mutant embryos (Duttingler et al., 1993) were also affected in W<sup>bd</sup> embryos, including the mesenchyme around the otic vesicle and in the floorplate, both structures that normally express Steel factor. However, unlike W<sup>sh</sup> embryos, which cease to express Kit in the floorplate after day 10.5, W<sup>bd</sup> embryos sustained ectopic Kit expression in the floorplate on day 11.8, but not day 15.5. In addition, W<sup>bd</sup> embryos expressed Kit ectopically in the heart at mid- and late gestation, which has not been described for W<sup>sh</sup>.

Both W<sup>57</sup> and W<sup>bd</sup> involve long-range genomic rearrangements only detectable by PFGE. The W<sup>57</sup> mutation is associated with a 80 kb deletion, located in a 160 kb genomic region between the upstream Pmel site and the 5’ end of the Kit-coding sequence. W<sup>bd</sup> is a 2.8 Mb inversion, with one breakpoint 5’ to

Fig. 8. (A) PFGE analysis of high molecular weight DNA from wild-type (C57BL/6J), W<sup>57</sup>/+ and W<sup>57</sup>/W<sup>57</sup> mice. Restriction digests with NotI, NalI and MluI resulted in novel DNA fragments in W<sup>57</sup> DNA when hybridized to a PDGFRα cDNA probe, whereas digests with Pmel and MluI resulted in novel DNA fragments in W<sup>57</sup> when hybridized to a Kit cDNA probe. All novel fragments detected were reduced in size by approximately 80 kb. (B) Long-range restriction map of wild-type and rearranged W<sup>57</sup> chromosome. The arrow indicates the position of the 80 kb deletion 5’ to Kit in W<sup>57</sup> DNA and the approximate location of the proximal breakpoint of the W<sup>bd</sup> and W<sup>sh</sup> inversions. Molecular weights are indicated.
the Kit-coding sequence in the same 160 kb fragment that harbours the W57 mutation and one breakpoint between the Tec and the Gabrb1 genes. This inversion juxtaposes the Pdgfra and Gabrb1 genes on the proximal side as well as Kit and Tec on the distal side of the inversion. Surprisingly, the Wbd inversion appeared indistinguishable from the Wsh inversion (this study; Duttlinger et al., 1993, 1995; Nagle et al., 1995), although both are spontaneous and independent mutations (Beechey et al., 1986; Lyon and Glenister, 1982). The Buc3 probe was affected by Wbd and by Wsh, but not by W57, indicating genomic sequences that are uniquely affected in these mutations. The breakpoints of both the W57 deletion and the Wbd inversion have not yet been cloned; therefore, only an approximate location of their breakpoints can be given. Interestingly, two genomic regions upstream of Kit, a binding site for the Mi transcription factor, encoded by the microphthalmia (mi) locus (Tsujimura et al., 1996), and the region immediately upstream of the Kit transcriptional initiation site (Yasuda et al., 1993), have recently been shown to be necessary for expression of Kit reporter plasmids in vitro. Neither of these regions are affected by the W57 and Wbd mutations. Together, these results demonstrate that important regulatory elements, distinct from the two cis-acting regions described previously and located further upstream, contribute significantly to the complex temporal and spatial pattern of Kit expression in vivo.

In both mutations, these large genomic rearrangements differentially affect Kit transcriptional initiation in different cell lineages during development. The W57 and Wbd mutations might disrupt a transcriptional regulatory region that normally drives Kit expression in a cell-type-specific manner. Interestingly, Kit expression is only perturbed in trunk melanoblasts in W57/W57 embryos, but not in melanoblasts around the otic vesicle, suggesting that distinct cis-acting elements regulate Kit expression in different subpopulations of neural-crest-derived melanoblasts. The Kit and Steel factor genes are expressed in cell layers that are immediately contiguous (Motro et al., 1991; Keshet et al., 1991). Thus, the mechanisms that regulate expression of this gene pair must ensure their coordinate expression in space and time during development and in the adult. It is striking, therefore, that the ectopic Kit expression in Wbd animals recapitulates in part the expression pattern of its ligand, Steel factor. If the mechanisms that regulate Kit and Steel factor expression are related, it is conceivable that negative cis-elements ensure that Kit is not expressed in cells normally expressing its ligand. Disruption of these silencer elements might then result in

Fig. 9. PFGE analysis showing that the Wbd mutation is associated with a chromosomal inversion. (A) PFGE analysis of the distal breakpoint of the Wbd inversion. Wild-type (C3H/He), Wsh/Wsh and Wbd/Wbd DNA were digested with PmeI. The Kit probe detected novel DNA fragments of identical size in Wsh and Wbd DNA. In addition, the Kit and Tec probes hybridized to novel DNA fragments of identical size. (B) PFGE analysis of the proximal breakpoint. With all enzymes tested (BssHII, NruI and NruI/NotI), both the Pdgfra and Gabrb1 probes detected novel DNA fragments of identical size in both Wsh/Wsh and Wbd/Wbd DNA. Molecular weights (in kb) and a zone of limited mobility (LM) are indicated. (C) Long-range restriction map of the central portion of mouse chromosome 5 of wild-type (C57BL/6J and C3H/He), Wbd and Wsh chromosomes. The map of the wild-type chromosome and Wsh chromosome has previously been reported (Nagle et al., 1995). The map indicates sites for the restriction enzyme BssHII (B). Partial cutting sites are shown in parentheses. Positions of loci are indicated by solid rectangles above the map. Scale in kb is shown at top of map. The approximate location of the inversion breakpoints are indicated by the zig-zag lines.
involved in ensuring proper Kit expression must have both spatial and temporal aspects, of which only the former is perturbed by the W°bd inversion.

The genomic rearrangements in W°bd and W°47, respectively, might also bring regulatory sequences not normally located at some distance from Kit into close proximity of this gene, positively or negatively affecting its expression. According to this model, Kit might acquire aspects of the expression pattern of another gene, whose regulatory elements have no influence on Kit transcription in wild-type mice.

The Kit RTK is expressed in the early stem cells and lineage-committed progenitor cells of very different cell lineages (Reith and Bernstein, 1991; Galli et al., 1994). Thus, insights into the mechanisms that govern the regulation of Kit expression should provide insight into the early events involved in lineage specification. The analysis of W mutants that disrupt normal Kit transcription would be an important first step towards the characterization of factors controlling lineage determination and elaboration.

This paper is dedicated to the memory of Verne Chapman. We thank S. Vesel for help with mast cell preparations, Y. Kitamura for generously providing us with the Ack2 antibody and V. Chapman for generously providing us with W°bd/W°bd mice, Diane Poslinski and Debbie Swiatek for animal care, and William Brown and Mary Lyon for providing PFGE plugs with W°bd DNA from the Harwell colony. These studies were supported by grants from the Medical Research Council of Canada, the National Cancer Institute of Canada (to A. B.) and NIH grant HD 28410 (to M. B.). M. K. was supported by a long-term Government of Canada Award fellowship and D. L. N. by a fellowship in Immunology 55, 1-96.

REFERENCES


Reith, A. D., Rottapel, R., Giddens, E., Brady, C., Forrester, L. and Bernstein, L. (1990). W mutant mice with mild or severe developmental defects contain distinct point mutations in the kinase domain of the c-kit receptor. Genes Dev. 4, 390-400.


