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Aberrant melanogenesis and melanocytic tumour development in transgenic mice that carry a metallothionein/*ret* fusion gene

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We generated four independent transgenic mouse lines that showed severe melanosis of the whole body by introducing the *ret* oncogene fused to the mouse metallothionein (MT)-I promoter–enhancer (MT/*ret*). Whereas melanogenesis was accelerated without distinct proliferative disorders in one line, melanocytic tumours frequently developed in the other three lines. Northern hybridization and *in situ* hybridization analyses showed that tumour cells and non-tumorous melanin-producing cells expressed the transgene at high levels. The aberrant melanogenesis and tumour development were influenced by genetic and environmental factors. Furthermore, crossbreeding experiments between the transgenic mice and W^v mice suggested that the *ret* gene product can partially compensate for the defect of melanocyte development in W^v mice. This is a novel mammalian model in which melanosis and melanocytic tumours develop stepwise, triggered by a single transgene.

Key words: c-*kit*/melanocytic tumour and melanogenesis/metallothionein/*ret*/transgenic mice

Introduction

To date, various kinds of transgenic mice have been produced to investigate the function of oncogenes in the process of differentiation of cells *in vivo* (for reviews see Hanahan, 1988; Cory and Adams, 1988). The tissue specificity of lesions occurring in transgenic mice was principally determined by the regulatory elements combined with oncogenes. In some cases, however, the oncogene itself influenced the type of tumours even though the same regulatory unit was used. For example, while B cell lymphomas frequently developed in the transgenic mice carrying the hybrid gene of mouse c-*myc* and immunoglobulin enhancer (Adams *et al.*, 1985), H-*ras* and L-*myc* induced lung carcinomas and T cell lymphomas under the control of the same immunoglobulin enhancer, respectively (Suda *et al.*, 1987; Möröy *et al.*, 1990). In addition, when the metallothionein promoter, which is known to function in almost all tissues (Palmiter *et al.*, 1983), was used, various pathological changes occurred in mice depending on the introduced oncogenes (Messing *et al.*, 1985; Rüther *et al.*, 1987; Heisterkamp *et al.*, 1990). These experiments suggested the specific functions of oncogene products that could be controlled by cellular or microenvironmental factors in living organisms.

While most documented studies of transgenic mice theorize that a high expression of oncogenes is closely related to proliferative or dysplastic disorders *in vivo*, one recent article reported that the *ras* oncogene driven by a suprabasal keratin promoter induced hyperkeratosis of the skin and forestomach without marked hyperplasia (Bailleul *et al.*, 1990). This article supported a current view that oncogenes may be able to induce the differentiation of cells rather than their proliferation with competent environmental factors.

The *ret* oncogene was activated by DNA rearrangement of the *ret* proto-oncogene with other cellular sequences during the process of NIH3T3 transfection assay (Takahashi *et al.*, 1985; Takahashi and Cooper, 1987; Ishizaka *et al.*, 1989). The *ret* proto-oncogene encodes a receptor-type tyrosine kinase (Takahashi *et al.*, 1988, 1989; Tahira *et al.*, 1990) and is often expressed in human cell lines or tumours of neuroectodermal origin, such as neuroblastoma, pheochromocytoma and thyroid medullary carcinoma (Nagao *et al.*, 1990; Ikeda *et al.*, 1990; Santoro *et al.*, 1990; Takahashi *et al.*, 1991). Recently, rearrangement of the *ret* proto-oncogene has been detected *in vivo* in ~25% of human thyroid papillary adenocarcinoma (Grieco *et al.*, 1990). Correspondingly, we have proved the oncogenicity of the activated *ret* gene in transgenic mice, using the mouse mammary tumour virus (MMTV) LTR as a regulatory element. In this case, mammary and salivary gland adenocarcinomas and benign tumours of the Harderian glands and male reproductive tracts developed in a stochastic fashion (Iwamoto *et al.*, 1990).

To investigate further the action of the ret protein in various tissues, we chose the mouse metallothionein (MT)-I promoter–enhancer as an alternative regulatory unit. Surprisingly, four transgenic lines showed hyperpigmented skin due to aberrant melanogenesis. Melanocytic tumours developed in three of the four lines. Here we observe that each step of proliferation and transformation of the melanin-producing cells is under the controls of certain genetic and environmental factors. Furthermore, we provide evidence that the introduced *ret* oncogene partially compensates for the defect of melanocyte development in W^v mice bearing a mutation of a receptor-type tyrosine kinase, c-*kit*.

Results

Establishment of transgenic mouse lines with aberrant melanogenesis

Figure 1 shows a construct that contains the *ret* oncogene cDNA fused to the mouse MT-I promoter–enhancer (designated pMT/*ret*). By injecting a 6.9 kb *Tth*111I–*Pvu*I fragment into the fertilized eggs of (BALB/c×C57BL/6)×BALB/c mice, we obtained a total of 17 founder mice carrying the MT/*ret* fusion gene. Three of them developed salivary gland adenocarcinoma, hepatocellular carcinoma or

a retroperitoneal tumour. Unexpectedly, four mice exhibited severe pigmentation in their whole skin by 5 days after birth (Table I).

We then established transgenic lines 192, 242, 304 and 319 from the four mice with pigmented skin. The founder mice were crossed with BCF1 (BALB/c×C57BL/6) mice and the F1 transgenic mice were subsequently crossed with C57BL/6 mice (coat colour: black; genotype: *a/a*, *B/B*, *C/C*), BCF1 mice (agouti; *A/a*, *B/b*, *C/c*) and BALB/c mice (albino; *A/A*, *b/b*, *c/c*) to get sublines A, B and C, respectively (Table II). As a consequence of segregation of the coat colour loci, all mice in subline A had pigmented hairs (non-albino). On the other hand, 16, 69, 19 and 44% of transgenic mice were albinos in sublines 192-B, 192-C, 304-B and 304-C, respectively. The skin pigmentation was observed in all progeny with pigmented hairs carrying the *ret* transgene (Figure 2A) but not in albino mice, suggesting that tyrosinase, which is the key enzyme in the production of melanin, is necessary for this phenotype. Consistent with this observation, histological analysis of the pigmented skin revealed that significant numbers of melanin-containing cells were present in the basal layer of the epidermis and in the dermis of transgenic infants (Figure 3A). In the skin of adult transgenic mice, melanin-containing cells were predominantly detected in the dermis (Figure 3B). Immunohistochemical studies indicated that these cells consisted of S-100$^+$ melanin-producing cells and S-100$^-$ melanin-laden macrophages as described below. Although albino mice carrying the *ret* transgene did not show the skin pigmentation, 'amelanotic cells' that morphologically resembled the melanin-producing cells and that were reactive with the anti-S-100 antibody appeared in their dermis (data

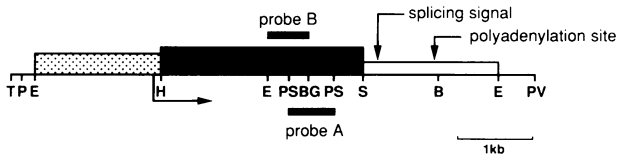

Fig. 1. Construction of the MT/*ret* fusion gene for microinjection. Dotted box, closed box, open box and line represent MT-I promoter–enhancer, *ret* cDNA, SV40 sequence and vector sequence, respectively. Restriction endonucleases shown are *Bam*HI (B), *Bgl*II (BG), *Eco*RI (E), *Hin*dIII (H), *Pvu*II (P), *Pst*I (PS), *Pvu*I (PV), *Sma*I (S) and *Tih*111I (T). A horizontal arrow indicates the predicted transcriptional start site located 84 bp upstream of the junction *Hin*dIII site.

Table I. Pathology of the MT/*ret* transgenic founder mice

Founder mice	Sex	Predicted copy number of the transgene[a]	Onset of tumour appearance (age in months)	Pathology
108[b]	F	6	1	Hepatocellular carcinoma
192	F	14	4	Melanosis and melanocytic tumour
207[b]	F	4	7	Salivary gland adenocarcinoma
242	M	4	No tumour	Melanosis
301[b]	M	5	3	Retroperitoneal tumour
304	M	5	3.5	Melanosis and melanocytic tumour
319	M	5	6.5	Melanosis and melanocytic tumour

[a]Copy number was determined by densitometric scanning of the autoradiogram after Southern blot analysis of genomic DNA.
[b]These mice were sterile.
M, male; F, female.

Table II. Distribution of tumours occurring in mice of lines 192 and 304

Line [subline]	Tumour incidence at Face[a]		Leg muscle[b]	Mediastinum[b]	Retroperitoneal cavity[b]
	Type I[c]	Type II[d]			
192					
[A]	0/28	17/28(19.9 ± 10.6)	0/10	0/10	0/10
[B]	0/25	10/25(12.2 ± 6.0)	0/10	0/10	0/10
[C]	0/16	1/16(7.0)	0/4	0/4	0/4
304					
[A]	57/59(6.1 ± 3.3)	0/59	19/29	18/29	17/29
[B]	31/36(11.4 ± 6.1)	0/36	9/16	6/16	5/16
[C]	10/36(21.0 ± 9.0)	2/36(17.5)	0/17	0/17	0/17

Tumours that occurred in F2 and F3 progeny are presented.
[a]The number of mice that developed tumours/the number of mice observed.
[b]The number of mice that developed tumours/the number of mice autopsied: these tumours were noticed only at autopsy. The mean ages of tumour onset and the standard deviation are indicated by weeks in parenthesis. Autopsy was done 4–8 weeks after tumours were noticed on the face. All other mice were observed at least for 34 weeks.
[c]Type I; exophthalmus, which resulted from the choroidal tumours, was the first sign of tumour development; tumours were preferentially found around the nose and neck at the progressive stage.
[d]Type II; tumours developed around the eye and nose and on the ear but not in the choroid. No differences were observed between males and females.

not shown). These 'amelanotic cells' seem to be the counterpart of the melanin-producing cells in albino mice.

Proliferation of melanin-producing cells and development of melanocytic tumours

In addition to the dermis of the skin, melanin-producing cells ('amelanotic cells' in albino mice) were abundant in the skeletal muscle and the choroid of the eyes of line 304 (100%) and in the Harderian glands of lines 304 (82%) and 192 (45%). These cells frequently formed small nests (Figure 3C) and some of them developed into melanocytic tumours at various ages (4−39 weeks) (Table II).

By 150 days of age, 93% of mice of lines 304-A and 304-B characteristically showed exophthalmus, resulting from tumour formation in the choroid of their eyes (Figures 2B, 3D and 3F). In particular, we noticed exophthalmus in 47% of mice of line 304-A by 30 days after birth. Tumours also arose frequently in the dermis of the face (around the nose) (Figures 2B, 3E and 3G) and neck, the leg muscles, the mediastinum and the retroperitoneal cavity (Table II). Mice of lines 192-A (61%) and 192-B (40%) developed tumours predominantly in the dermis of the face (around the eye and nose and on the ear). In contrast, in the mice of sublines crossed with the BALB/c strain (sublines 304-C and 192-C), the incidence of melanocytic tumours was low (Table II), indicating that the genetic background of mice affected their development. Nevertheless, there was no significant difference in tumour incidence between albino and non-albino mice. For example, the incidence of tumours in the face was 31% and 25% in the albino and non-albino mice of subline 304-C, respectively (data not shown). In the mice of line 319, the pattern of tumour development was similar to that in line 192, although nine transgenic mice have been observed to date (data not shown).

The melanocytic tumours which developed in the three lines consisted of round cells that contained little melanin and showed a high n/c (nucleus/cytoplasm) ratio (Figure 3G). In albino mice, these melanocytic tumours were completely amelanotic (data not shown). Although the tumours grew slowly and did not metastasize to any tissues of the mice, they were transplantable into nude mice. In addition, we have succeeded in establishing a cell line with fully malignant features from a transplanted tumour (data not shown).

The tails of mice of lines 192 and 319 often became thick due to the remarkable proliferation of melanin-producing cells or 'amelanotic cells' in the dermis (Figure 3H). The incidence of this thickening was similar in sublines 192-A (39%), 192-B (44%) and 192-C (50%). In addition, the choroids of all examined mice of subline 304-C were also thick due to the proliferation of these cells, despite the low incidence of melanocytic tumour development. These results suggested that the genetic background affected the neoplastic transformation of cells of melanocyte lineage rather than their proliferation in our transgenic mice.

Characterization of melanin-containing cells

In order to confirm the origin of tumour cells, we stained several tissues, including the skin, the choroid and the leg muscles, with the anti-S-100 antibody that is known to react with melanoma and nevus cells. Most of the tumour cells and melanin-containing cells in the transgenic mice were strongly positive by this test (Figure 4A). However, some of melanin-containing cells did not stain with this antibody, indicating that they were melanin-laden macrophages. In addition, electron microscopic examination showed melanosomes in melanocytic tumour cells developed in mice with the pigmented skin (Figure 3I).

Mice of line 242, 39 mice of line 242-A and 12 mice of line 242-B developed no tumours, while they displayed hyperpigmentation in the skin to the same extent as the other three lines. To characterize melanin-containing cells in the dermis of the mice of line 242, we stained the skin of these mice with anti-S-100 antibody after bleaching melanin granules. As a result, most melanin-containing cells were not stained, indicating that they were primarily melanophages (Figure 4B, C and D) and that melanin-producing cells did not actively proliferate in the dermis of the mice of line 242.

Expression of the transgene

Total RNAs were isolated from various tissues of transgenic mice, and analysed by Northern blotting using a *ret* cDNA fragment as a probe (probe A) (see Figure 1). In several mice of lines 192 and 304, all of the tumours expressed a

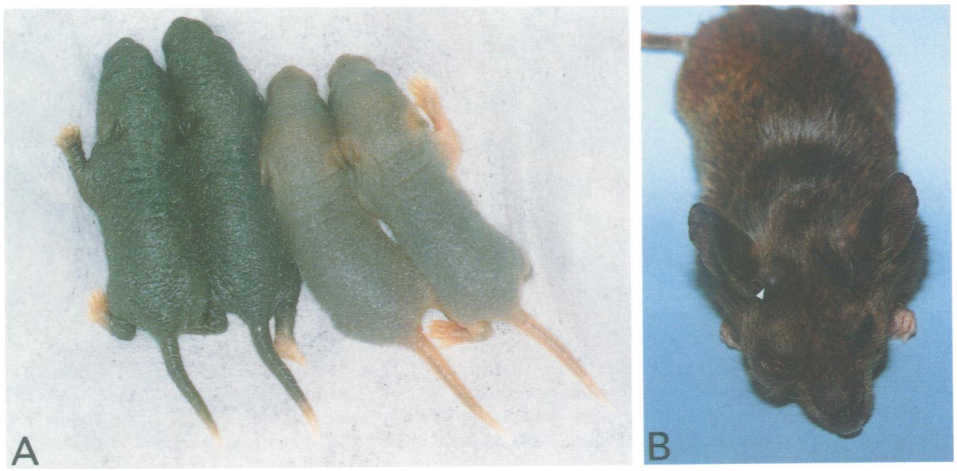

Fig. 2. Gross features of the MT/*ret* transgenic mice. (**A**) Progeny of line 304-B at 5 days of age. The two on the right are non-transgenic and the two on the left are transgenic mice. These four mice were littermates and exhibited agouti coat colour. (**B**) A 13 week-old male of line 304-A. Note a tumour on the nose and exophthalmus caused by tumours in the choroid of the eyes. A small nodule of tumour is observed in the pigmented skin (indicated by an arrowhead).

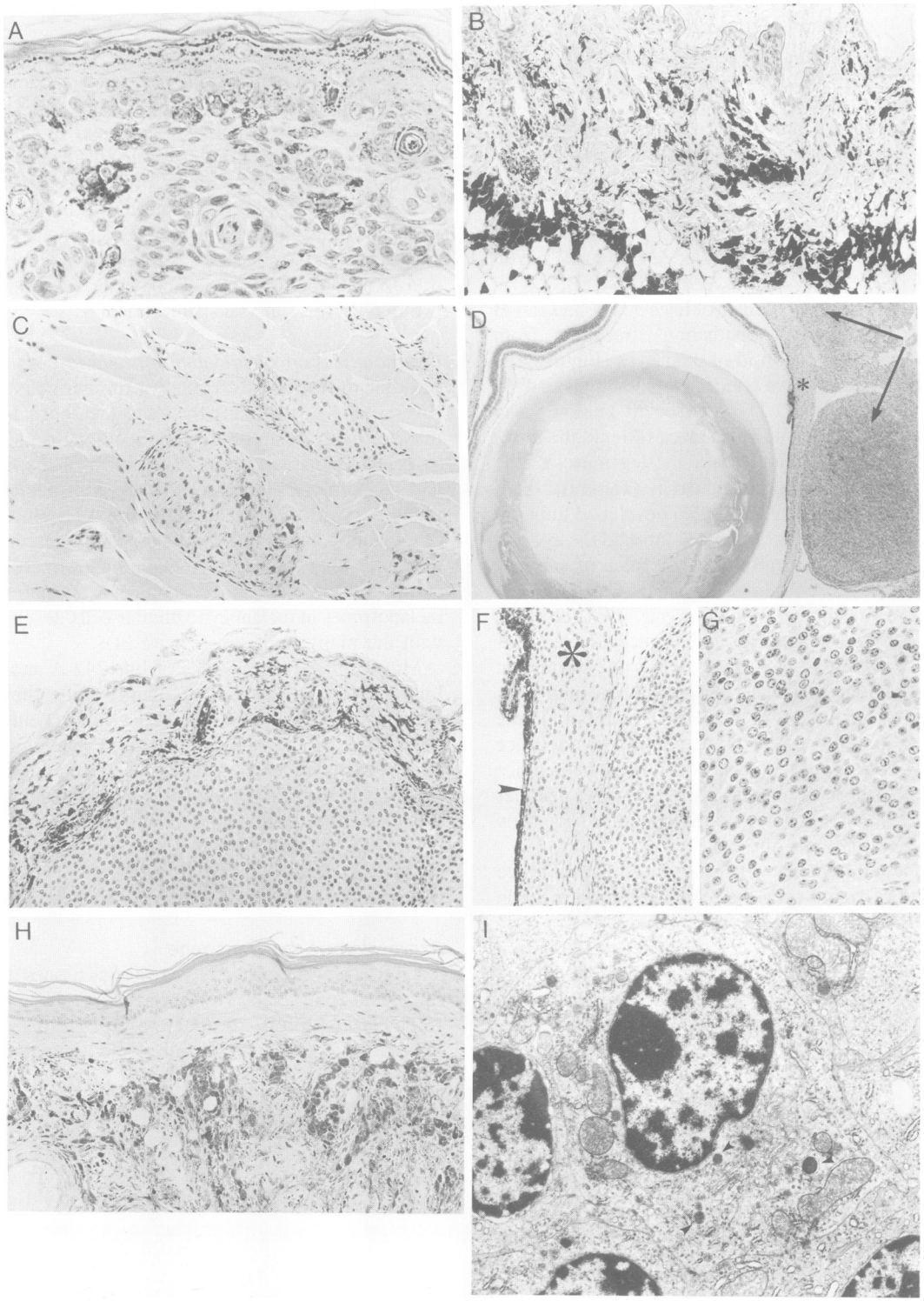

Fig. 3. Histopathology of the MT/*ret* transgenic mice. (**A**–**H**) Haematoxylin and eosin staining. (A) The pigmented skin of a 5 day-old mouse of line 304-A. Considerable melanin-containing cells are present in the basal layer of the epidermis and in the dermis (×270). (B) The pigmented skin of a 17 week-old male of line 304-B. Numerous melanin-containing cells are present in the dermis (×95). (C) Femoral muscle of a 13 week-old female of line 304-A. Small nests of melanin-producing cells are seen in the skeletal muscle. Considerable amounts of melanin are present in them (×95). (D) Choroidal tumours of a 13 week-old female of line 304-A. Melanin-producing cells occupy the choroid (indicated by asterisk). Nodular lesions (indicated by arrows) are composed of melanocytic tumour cells (×20). (E) A skin tumour of a 13 week-old female of line 304-A. The deep dermis shows a sharply circumscribed nodule consisting of melanocytic tumour cells. The upper dermis contains dark melanophages with abundant melanin (×95). (F) High power view of (D). The retinal pigment epithelium (indicated by an arrowhead) is intact (×95). (G) High power view of (E). The tumour is composed of a dense mass of round cells with round nuclei, prominent nucleoli and scanty cytoplasm (×225). (H) The tail skin of a 43 week-old male of line 192-B. The deep dermis is predominantly occupied by melanin-producing cells with variable amounts of melanin and by melanophages with large amounts of melanin (×95). (**I**) Electron micrograph of melanocytic tumour of a 13 week-old female of line 304-A. Tumour cells contain a small number of dense melanosomes (indicated by arrowheads) (×3800).

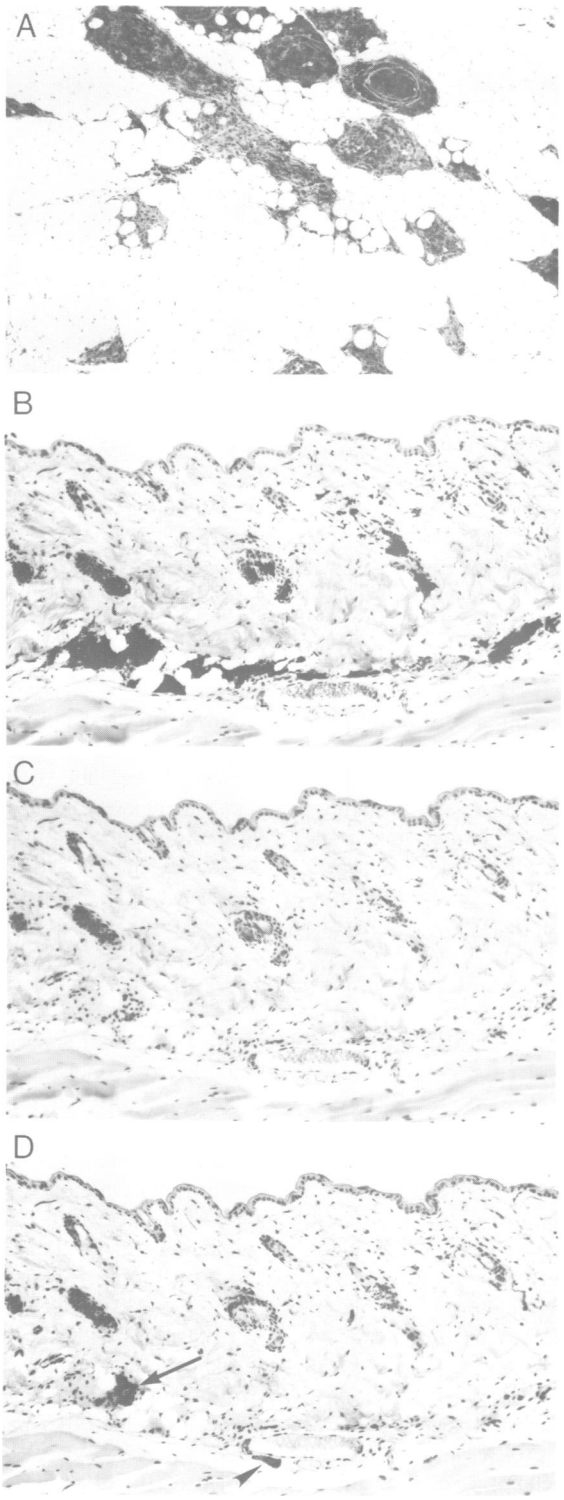

Fig. 4. Immunohistochemical analysis of tissues of the MT/*ret* transgenic mice. (**A**) Femoral muscle of a 9 week-old male of line 304-A. The muscle was stained with anti-S-100 antibody by the immunoperoxidase method (Hidaka *et al.*, 1983). Nests of melanin-producing cells in the muscle were clearly stained (×55). (**B–D**) The pigmented skin of a 16 week-old male of line 242-A. B: haematoxylin and eosin staining. A large number of melanin-containing cells are present in the dermis (×55). C: section B was bleached according to the method of Frangioni and Borgioli (1988). Melanin granules completely disappeared. D: section C stained with anti-S-100 antibody by the immunoperoxidase method. Hair follicular and perifollicular melanocytes, perifollicular nerves, nerve bundles (indicated by an arrowhead) and some melanin-containing cells (indicated by an arrow) in the deep dermis were stained. In contrast, most melanin-containing cells were negative.

4.3 kb transcript of the MT/*ret* gene at high levels (Figure 5). Its expression level was variable in all tissues containing melanin-producing cells, such as the skin, muscle and Harderian glands, but was low or undetectable in other normal tissues examined. In mice of line 242, we detected low levels of the transgene expression in RNA of the skin showing hyperpigmentation (Figure 5C). On the other hand, the endogenous expression of the *ret* proto-oncogene was undetectable in various tissues from non-transgenic mice (data not shown).

To confirm the specific expression of the transgene in melanin-producing cells, we performed *in situ* hybridization using a probe specific to the tyrosine kinase domain of the *ret* gene (probe B) (see Figure 1). We hybridized frozen sections of the skeletal muscle of a mouse of line 304 with the digoxigenin-labelled RNA probe. As shown in Figure 6A, we were able to detect strong signals with the antisense *ret* probe in the melanin-producing cells scattered in the muscle as well as in the melanocytic tumour cells. The level of these signals dramatically decreased when a high dose of the non-labelled antisense *ret* probe was added for inhibition (Figure 6B). In addition, hybridization of the sequential sections with the sense *ret* probe showed almost no staining (Figure 6C). These results indicated that the *ret* transgene was expressed not only in tumour cells but also in the non-tumorous melanin-producing cells.

The ret gene product can partially compensate for the defect of melanocyte development in W^v/W^v mice

Recently, it was revealed that the proto-oncogene c-*kit*, which encodes another transmembrane tyrosine kinase receptor, maps to the murine *dominant white-spotting* locus (*W*) and that *W* mutations represent point mutations or deletion of the c-*kit* coding sequence (Chabot *et al.*, 1988; Nocka *et al.*, 1990). Since *W* mutations affect proliferation and/or migration of melanoblasts during embryonic development, it was interesting to investigate how the melanoblasts of the MT/*ret* transgenic mice behave in *W* mice. We therefore crossed transgenic mice of line 192 with C57BL/6 mice heterozygous for the W^v allele, one of the mutational alleles of the *W* locus. These heterozygotes have variable numbers of white spots and slightly diluted coat colour. Homozygotes have completely white coats and black eyes (Figure 7B, right) although some of them have a small patch of pigmented skin on one or both ear pinnae (Figure 7B, middle) (Silvers, 1979).

In $W^v/+$, *ret*/+ F1 heterozygotes, melanin-containing cells were not detected in the epidermis and dermis of white spots. Melanogenesis in the pigmented area of these mice was reduced as compared with that in the *ret*/+ littermates without W^v alleles (Figure 7A). Thus, melanin-producing cells of transgenic mice seemed to be controlled by the *W* locus.

We further mated these $W^v/+$, *ret*/+ F1 progeny to produce W^v/W^v homozygotes. Interestingly, some of the W^v/W^v homozygotes carrying the *ret* transgene showed severe pigmentation of the ears on one or both sides (Figure 7B, left). This was due to the appearance of a large number of melanin-producing cells in the dermis and epidermis. Furthermore, pigmented hair was found around the ear (Figure 7B, left), on the face or the trunk. Histologically, mature melanocytes existed in the follicles of pigmented hair (data not shown). These features were not observed in any W^v/W^v homozygotes without the *ret* transgene (Table III and Figure 7B, middle and right).

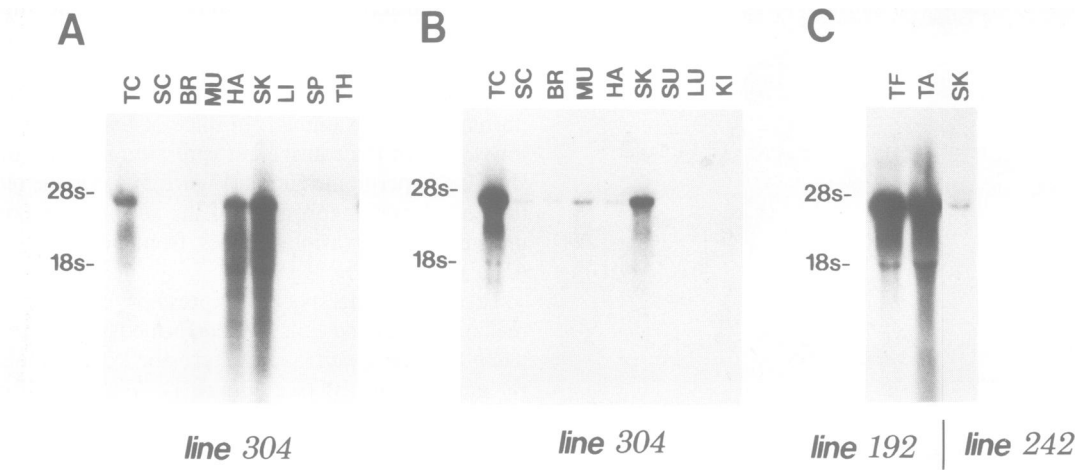

Fig. 5. Expression of the *ret* transgene in various tissues of the MT/*ret* transgenic mice. (**A**) A 12 week-old female of line 304-A. (**B**) An 18 week-old male of line 304-B. (**C**) A 30 week-old male of line 192-B (TF and TA) and an 18 week-old transgenic male of line 242-A (SK). mRNA levels of the transgene were assayed by Northern blot analysis, using probe A (see Figure 1). Migration of 28S and 18S ribosomal RNA is indicated. Abbreviations are: TC, tumour of the choroid; SC, spinal cord; BR, brain; MU, muscle; HA, Harderian gland; SK, skin; LI, liver; LU, lung; KI, kidney; SP, spleen; TH, thymus; SU, suprarenal gland; TF, tumour in the face and TA, tail skin. Variable numbers of proliferating melanin-producing cells were present in HA, SK, MU and TA but not in SC, BR, LI, LU, KI, SP, TH or SU.

Northern blot analysis showed that the *ret* transgene was expressed in the pigmented ear but not in the non-pigmented one (Figure 7C).

Discussion

Why did the disorders of pigment cells occur?

Although several oncogenes have been introduced into mice using the human or mouse MT promoter (Messing *et al.*, 1985; Ruther *et al.*, 1987; Heisterkamp *et al.*, 1990), the expression of transgenes in pigment cells and their neoplastic transformation has never been reported. It is interesting to note that mice carrying the appropriately modified SV40 early region gene linked with the MT−human growth hormone fusion gene (MGH/SV40-T gene) developed demyelinating peripheral neuropathies, resulting from abnormalities of the Schwann cells (Messing *et al.*, 1985). Since both melanocytes and Schwann cells originate from the neural crest cells and are very close ontogenically, the MT enhancer may function efficiently in these cell types. In this context, Krauter *et al.* (1989) reported that melanoma cells contain large amounts of metallothionein.

On the other hand, the fact that the *ret* proto-oncogene is specifically expressed in some kinds of human tumours of neural crest origin, such as neuroblastoma, pheochromocytoma and thyroid medullary carcinoma, suggests a possible role for this proto-oncogene in the differentiation and proliferation of neural crest cells. Although we were not able to detect its expression in the human and murine melanoma cell lines and mouse embryos (T.Iwamoto *et al.*, unpublished observation), it is possible that the *ret* proto-oncogene is involved in melanocyte development during embryogenesis. The specific function of the activated *ret* oncogene may therefore be important for proliferation of melanin-producing cells in our transgenic mice.

However, it seems difficult to attribute the development of aberrant melanogenesis and melanocytic tumours entirely to the function of either the metallothionein promoter or the *ret* oncogene product. Novel expression patterns of the transgenes have been reported in some other transgenic mice. Swanson *et al.* (1985) proposed that the unexpected tissue specificity of expression of the metallothionein-I−growth hormone (MGH) fusion genes could be due to the combinations of *cis*-acting regulatory sequences. Thus it is conceivable that the expression of the transgene in pigment cells of the MT/*ret* transgenic mice was the consequence of the unique combination of the sequences present in the chimeric MT/*ret* transgene.

Genetic and environmental control of ret transgene action

The present data show that the action of the *ret* transgene is under the control of genetic and environmental factors. The incidence and location of the tumours differed among the four transgenic lines. In mice of lines 192 and 319, almost all proliferative features of pigment cells were detected in the dermis of the skin, while the tumours developed in a variety of tissues of mice of line 304. In mice of line 242, melanogenesis was accelerated without tumour development. These differences could be due to a position effect on the transgene.

When the transgenic mice were crossed with BALB/c mice (subline C in Table II), the incidence of melanocytic tumours was significantly lower than in the progeny of C57BL/6 mice (subline A in Table II). On the other hand, the proliferation of S-100$^+$ melanin-producing cells or 'amelanotic cells' in the tail of mice of line 192 was not suppressed by crossing with BALB/c mice. Since the *ret* transgene was expressed in both tumour cells and non-tumorous melanin-producing cells at similar levels, these results suggest that genetic factors carried by C57BL/6 mice are closely related to additional events necessary for the development of melanocytic tumours. Alternatively, BALB/c mice may carry factors that suppress the development of these tumours. Tyrosinase was not a crucial factor since the development of melanocytic tumours in mice of subline C was suppressed irrespective of the hair colour of the mice. Identification of factors concerning these events could greatly promote the understanding of the mechanisms of neoplastic transformation of pigment cells. Transgenic mouse studies

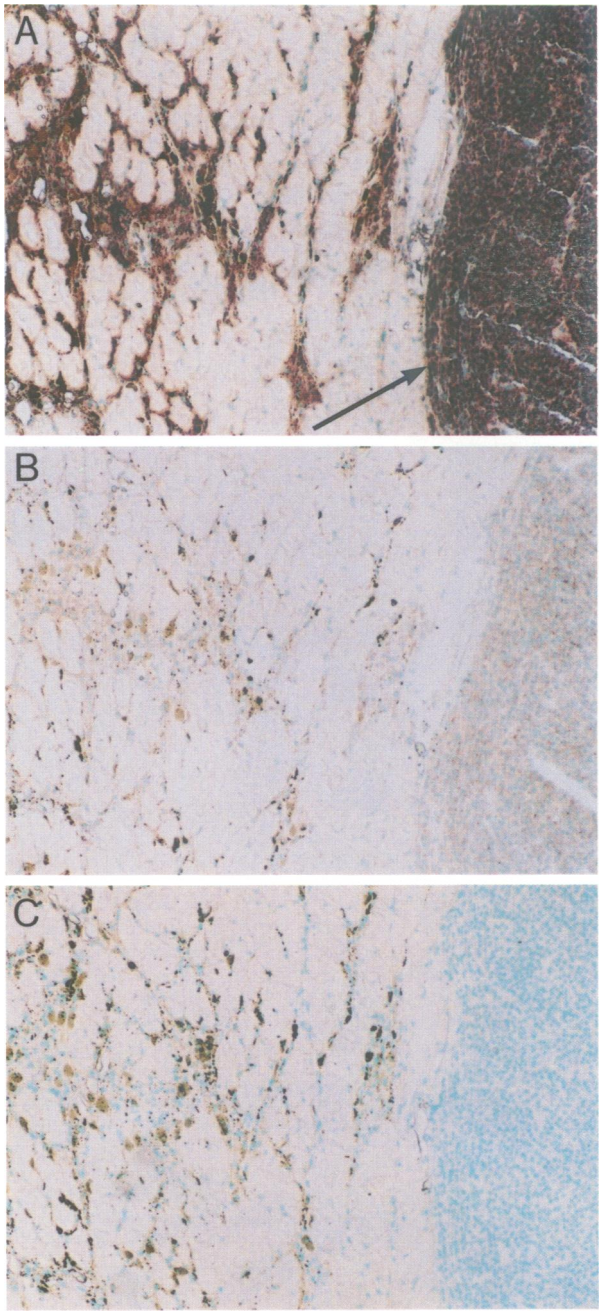

Fig. 6. *In situ* hybridization analyses of the *ret* transgene in skeletal muscle. (**A**) A frozen section of muscle of a 14 week-old male of line 304-B. The section was hybridized with a digoxigenin-labelled antisense *ret* probe. Strong signals were detected in melanocytic tumour cells (indicated by an arrow) and melanin-producing cells scattered in the muscle (×70). (**B**) Hybridization with digoxigenin-labelled antisense *ret* probe in the presence of excess amounts of non-labelled antisense probe for cold inhibition assay. Signals were much weaker than those in (A). (**C**) Hybridization with a digoxigenin-labelled sense *ret* probe. Signals were hardly detected.

about genetic background effects on tumorigenesis have also been reported by other investigators (Harris *et al.*, 1988, Yukawa *et al.*, 1989).

Environmental factors appeared to affect the actions of the *ret* transgene. No proliferative disorders of melanin-producing cells were observed in the epidermis, although mature melanocytes were present in the hair follicles as observed in non-transgenic mice. In addition, we found many melanin-producing cells in the basal cell layer of the epidermis of transgenic infants. These observations suggest that once melanin-producing cells migrate into the epidermis, they could differentiate into normal melanocytes under the microenvironmental control in the epidermis. Consistent with this, Dotto *et al.* (1989) reported that proliferation of melanocytes transformed by the *neu* oncogene was suppressed by concomitant grafting of keratinocytes in a skin reconstitution experiment *in vivo*.

Melanogenesis and melanocyte development in transgenic mice

Aberrant melanogenesis of the whole body was a common phenotype observed in the four transgenic mouse lines. Especially, in mice of line 242, melanin synthesis was increased without distinct proliferation of melanin-producing cells (see Figure 4B, C and D). The production of melanin is a marker of melanocyte differentiation. Thus it is possible that the *ret* oncogene product promoted the differentiation of melanocytes in our transgenic mice. In agreement with this view, we frequently found pigmented hair in homozygous W^v/W^v mice carrying the *ret* transgene, resulting from the development of mature melanocytes in the hair follicles. Such features have never been found in W^v/W^v mice without the *ret* transgene. These observations suggested that the *ret* oncogene product supported the differentiation/proliferation of some melanoblasts during embryogenesis in W^v mice with mutated c-*kit*.

Unfortunately, we do not have direct evidence for expression of the transgene in embryos; this could be due to the restricted expression of the transgene in melanin-producing cells. Analysis by *in situ* hybridization will be necessary to understand how the *ret* transgene contributes to melanocyte development during embryogenesis.

A mouse model of melanosis and melanocytic tumours

We had few mammalian models in which melanocytic tumours systematically developed, although melanoma and nevus are rather common human diseases. Recently, Bradl *et al.* (1991) reported a transgenic mouse model for malignant melanoma that carried the fusion gene (Tyr-SV40E) of the mouse tyrosinase promoter and SV40 early region. Interestingly, the origins of the tumour cells differed between their transgenic mice and ours. For example, while the ocular tumours in the Tyr-SV40E mice originated chiefly in the retinal pigment epithelium derived from the neural tube, all ocular tumours in the MT/*ret* mice originated in the choroidal pigment cell derived from the neural crest (see Figure 3F). In addition to these ocular tumours, melanocytic tumours arose frequently in the dermis in MT/*ret* mice but not in Tyr-SV40E mice. Moreover, the skin colour of the Tyr-SV40E mice was apparently unchanged and their coat was even hypopigmented, which contrasted to the severe skin hyperpigmentation of the MT/*ret* mice (see Figure 2). All of these differences, which are probably linked to distinct natures of the regulatory elements and the oncogenes used, would clearly distinguish the two animal models from each other.

It is noteworthy that the pathological changes in the MT/*ret* transgenic mice developed stepwise as the mice aged. This process appears to resemble that of human giant congenital melanocytic nevus that is present at birth and sometimes gives rise to malignant melanoma (From, 1991). The novel

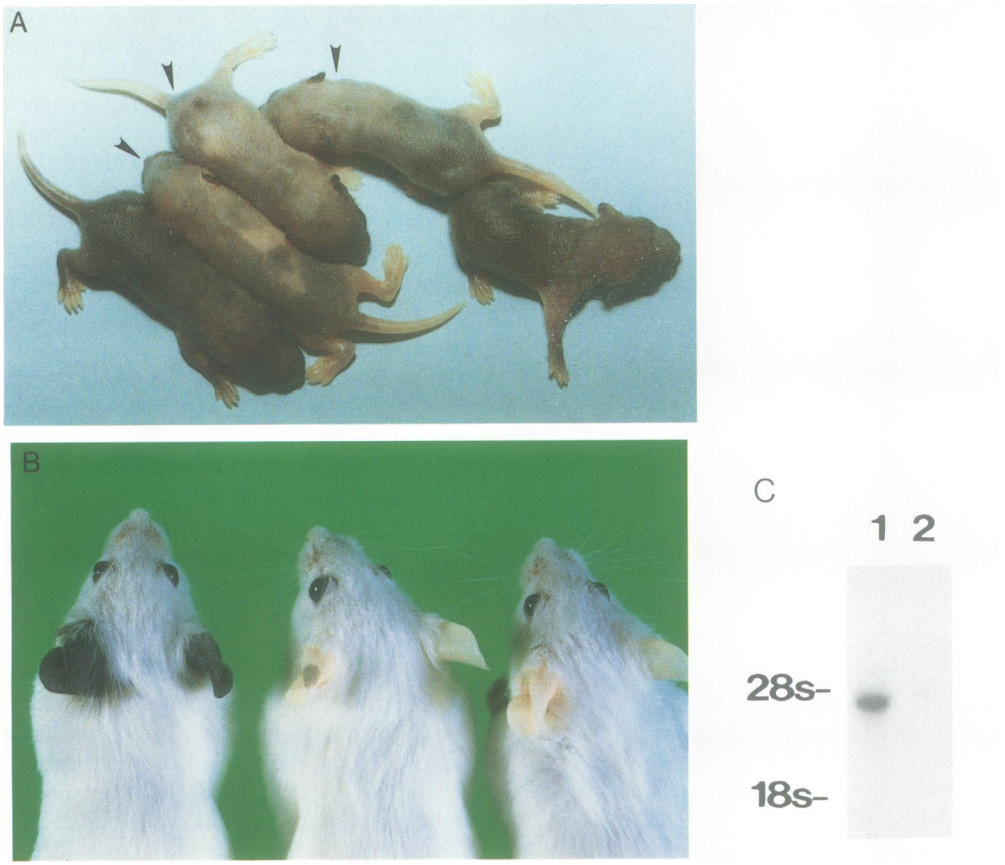

Fig. 7. Gross features of W^v mice carrying the *ret* transgene and expression of the transgene. (**A**) F1 progeny of line 192 mice crossed with C57BL/6-W^v/+ mice. Three W^v/+, *ret*/+ mice (indicated by arrowheads) and two +/+, *ret*/+ mice (others) are shown. Note that the pigmentation in the W^v/+, *ret*/+ mice is weaker than in the +/+, *ret*/+ mice. In addition, two of the W^v/+, *ret*/+ mice show clear spots lacking pigmentation. (**B**) Phenotype of transgenic W^v/W^v mice. Note severe pigmentation of both ears and pigmented hair around the left ear of a transgenic W^v/W^v mouse (left). A small patch of pigmentation is observed on the left ear pinnae of one non-transgenic W^v/W^v mouse (middle) and no pigmentation is observed in another non-transgenic W^v/W^v mouse (right). (**C**) Transgene expression in a transgenic W^v/W^v mouse that contained one pigmented ear (lane 1) and one non-pigmented ear (lane 2). Total RNAs were extracted from each ear and assayed by Northern blot analysis, using probe A (see Figure 1).

mouse lines we have presented here would provide a new avenue for the analysis of the mechanisms of the multistep transformation of pigment cells.

Materials and methods

Mice

BALB/c, C57BL/6, BCF1 and C57BL/6-W^v/+ mice were supplied by the Institute for Laboratory Animal Research, Nagoya University School of Medicine.

Construction of plasmids

A 1.7 kb *Eco*RI–*Bgl*II fragment containing mouse metallothionein-I (MT-I) promoter–enhancer was excised from plasmid pMK (Brinster *et al.*, 1981) and subcloned into the polylinker site of pUC8. Then, a 340 bp *Pvu*II–*Hin*dIII fragment corresponding to the SV40 regulatory region of the pSV2retT plasmid (Takahashi *et al.*, 1988) was replaced by a *Pvu*II–*Hin*dIII fragment containing the MT-I promoter–enhancer subcloned into pUC8 to give a construct designated pMT/*ret*.

Production of the transgenic mice

The eggs injected with the *Tth*111I–*Pvu*I pMT/*ret* fragment (Figure 1) were implanted into pseudopregnant DDY females. The method of microinjection, embryo transfer and DNA analysis have been described elsewhere (Hogan *et al.*, 1986).

Northern hybridization

Total cellular RNA (15 μg) was isolated by a single step method (Chomczynski and Sacchi, 1987), separated on agarose–formaldehyde gels and transferred to nylon membranes (Amersham). Prehybridization, hybridization and washes were performed as described previously (Iwamoto *et al.*, 1990).

Table III. Phenotypes of transgenic and non-transgenic W^v/W^v mice

Genotype	Number of mice with severely pigmented ear and pigmented hair/total number of mice
ret homozygotes[a]	4/5
ret heterozygotes[a]	4/8
Non-transgenic[b]	0/11

[a]These were obtained by mating W^v/+, *ret*/+ F1 heterozygotes.
[b]Six mice were obtained by mating W^v/+, *ret*/+ F1 heterozygotes and others by mating W^v/+, +/+ F1 heterozygotes.

In situ hybridization

In situ hybridization was carried out as described by Cox *et al.* (1984) with slight modifications. Frozen sections of the skeletal muscle of transgenic mice were fixed with 4% paraformaldehyde at room temperature for 15 min and were acetylated with 0.25% acetic anhydride at room temperature for 10 min. After preincubation in 2×SSC containing 50% formamide at 51°C for 20 min, hybridizations were performed at 51°C in 2×SSC containing 50% formamide, 10 mM Tris–HCl (pH 7.5), 4% blocking reagent, 0.1% sarkosyl, 0.02% SDS, 10% dextran sulphate, 500 μg/ml tRNA, 100 μg/ml poly(A) and digoxigenin-labelled RNA probes (2 μg/ml) for 12–16 h. For the competitive inhibition assay, reaction mixtures containing 70 μg/ml non-labelled antisense RNA were used. After washing, immunological detection was done according to the recommended protocol with certain modifications

(Boehringer Mannheim). Following colour development in nitroblue-tetrazolium/5-bromo-4-chloro-3-indolylphosphate, sections were stained with 0.2% methyl green. Single-stranded sense and antisense *ret* RNA probes labelled with digoxigenin were synthesized *in vitro* with SP6 or T7 RNA polymerase from the *Eco*RI−*Bgl*II fragment of *ret* cDNA (probe B in Figure 1). They were hydrolysed to an average length of 120 bases.

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