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Analysis of the *Kit* and *Pdgfra* genes in the patch-extended (Ph^e) mutation†

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Summary

The patch (*Ph*) locus allele, patch-extended (Ph^e), has significantly less pigmentation than the original mutation and homozygotes have been known to survive to term. Analysing inter-subspecific F1 hybrids, we were able to demonstrate that Ph^e is a deletional mutation encompassing the platelet-derived growth factor receptor alpha subunit (*Pdgfra*). The deletion does not appear to extend into the coding sequence of the *Kit* gene (a related tyrosine kinase receptor). However, we were able to demonstrate that, while the *Kit* gene is transcribed, it does not encode a functionally active receptor.

1. Introduction

Mouse spotting mutants have received considerable attention over the past few years following the initial observation of an association between the KIT tyrosine kinase cell surface receptor and the dominant spotting (*W*) locus on chromosome 5 (Chabot *et al.*, 1988; Geissler *et al.*, 1988). Some studies have focused on the identification of the specific molecular defects in the *Kit* gene associated with mutant alleles (Nocka *et al.*, 1989; 1990; Reith *et al.*, 1990; Tan *et al.*, 1990; Duttlinger *et al.*, 1993; Nagle *et al.*, 1995), whereas others have examined the developmental consequence of *Kit* gene expression (Nishikawa *et al.*, 1991; Duttlinger *et al.*, 1993). In addition to this, the ligand for the KIT receptor was shown to be encoded by the steel (*Sl*) locus (Copeland *et al.*, 1990; Flanagan & Leder, 1990; Huang *et al.*, 1990; Williams *et al.*, 1990; Zsebo *et al.*, 1990), providing a direct link between these phenotypically similar but genetically distinct loci.

Closely associated with the *Kit* gene, on chromosome 5, is the patch (*Ph*) locus (Grüneberg & Truslove, 1960), which was shown to be a deletional mutation encompassing the platelet-derived growth factor receptor alpha subunit (*Pdgfra*) by Smith *et al.* (1991) and Stephenson *et al.* (1991). While the *Kit* gene appears to be intact (Stephenson *et al.*, 1991) subsequent investigations have indicated that *Kit* expression is disrupted in the *Ph* mutant (Duttlinger *et al.*, 1995; Wehrle-Haller *et al.*, 1996). It is the disruption of *Kit* gene expression that may account for the spotting phenotype associated with the *Ph* mutation. This is substantiated, in part, by the genomic rearrangement alleles at the *Kit* locus itself (Duttlinger *et al.*, 1993; Nagle *et al.*, 1994, 1995; Klupple *et al.*, 1997) and the genetically engineered 'knock-out' mutant of the *Pdgfra* gene (Soriano, 1997). While the knock-out homozygotes exhibit some of the characteristics of *Ph* homozygotes, knock-out heterozygotes do not have the spotting phenotype of the *Ph* heterozygotes.

The *Ph* locus is characterized by two alleles: the original mutant *Ph* (Grüneberg & Truslove, 1960) and patch-extended, Ph^e (Truslove, 1977). Both mutations are semi-dominant; the *Ph* heterozygote has a variable-sized mid-ventral white spot (Fig. 1*A*), whereas the Ph^e heterozygote has a more extensive spotting phenotype encompassing most of the body

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† Dedicated to Bruce Cattanach on the occasion of his retirement as director of the MRC Mammalian Genetics Unit. Bruce, as Jean-Louis Guénet put it, was part of a unique group of scientists at Harwell, who not only made a substantial contribution to our understanding of genetics, but was more than willing to share resources with the community at large. Like Verne, Bruce was known to have just the right mutant mouse to answer the burning genetic issues. A true gentleman and friend.

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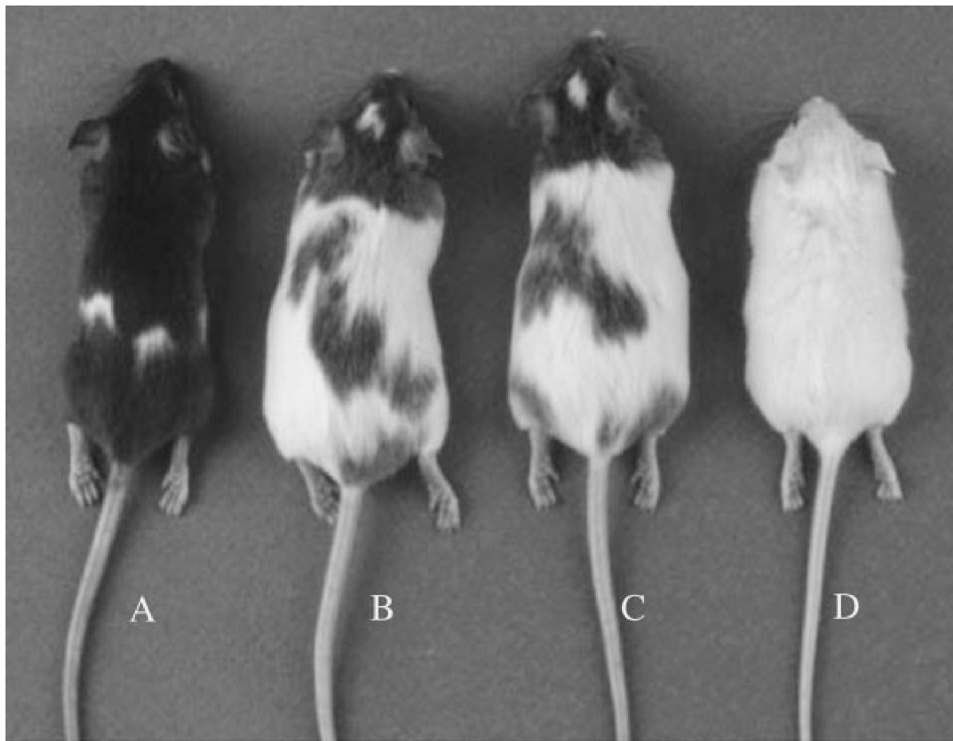

Fig. 1. Pigmentation phenotype of $Ph/+$ heterozygote (*A*); $Ph+/+Kit^W$ compound heterozygote (*B*); $Ph^e/+$ heterozygote (*C*); and $Ph^e+/+Kit^W$ compound heterozygote (*D*). Both the $Ph^e/+$ heterozygote and $Ph+/+Kit^W$ compound heterozygote have a similar but significantly enhanced white spotting phenotype compared with that observed in the $Ph/+$ heterozygote. However, the $Ph^e+/+Kit^W$ compound heterozygote has the black-eyed white coat phenotype characteristic of that produced in complementation involving different *Kit* alleles.

(Fig. 1*C*). While most homozygotes for both alleles usually die around mid-gestation with gross anatomical abnormalities, one presumptive Ph^e homozygote with a split face did survive to term. Although complementation studies suggested allelism with the *Ph* locus, the phenotype of the $Ph^e+/+Kit^W$ compound heterozygote (Fig. 1*D*) would also suggest allelism with *Kit* as well. Given this situation we decided to ascertain the molecular defect associated with the Ph^e allele.

2. Methods

(i) *Mice*

The patch-extended mutation was obtained from Dr Gillian Truslove at University College, London. The dominant spotting mutation was purchased from the Jackson Laboratory, Maine. The wild-derived *Mus musculus* PWK stock was originally established from animals trapped in Czechoslovakia and has been maintained as an inbred line at Roswell Park for more 20 generations of sib matings.

(ii) *Crosses*

Inter-subspecific F1 hybrids were produced by crossing a patch-extended heterozygous female to a PWK male. Patch-extended dominant spotting ($Ph^e+/+Kit^W$) compound heterozygotes were obtained from crosses between the patch-extended mutant stock and the dominant spotting mutant stock and identified on the basis of their characteristics (i.e. black-eyed white coat phenotype). Dominant spotting homozygotes (Kit^W/Kit^W) were obtained by crossing heterozygotes together and collecting fetuses 16 days *post-coitum*. They were also identified on the basis of phenotype (i.e. an anaemic-looking liver).

(iii) *DNA extraction and purification*

High-molecular-weight genomic DNA was prepared from either fresh or frozen kidneys using standard extraction techniques described previously (Mullins *et al*., 1988).

(iv) *RNA extraction and purification*

Fresh brain tissue was homogenized in a solution containing 4 M guanidine thiocyanate, 20 mM sodium acetate, 0·1 mM dithiothreitol (DTT) and 0·5% *N*-lauryl sarcosine. The RNA was collected as a pellet after ultracentrifugation on a caesium chloride gradient. After resuspension, the RNA was further purified by ethanol precipitation.

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(v) *Southern blot analysis*

Approximately 5 μg of DNA was digested with either *Bgl*II or *Pst*I restriction endonuclease in accordance with the manufacturer's recommendations (either Bethesda Research Laboratories, Promega or Stratagene). The digested DNA was resolved on an 0·8% agarose gel (SeaKem, FMC) in TAE buffer (40 mM Tris-acetate and 2 mM EDTA, pH 7·5). After denaturation, the DNA was transferred to Zetabind membrane (AMF, Cuno) using the Southern blot technique (Southern, 1975). Blots were hybridized with either *Kit* or *Pdgfra* using previously defined conditions (Stephenson *et al*., 1991).

(vi) *Northern blot analysis*

Approximately 5 μg of total RNA was resolved on a 1·2% agarose gel containing 1% formaldehyde with MOPS running buffer (200 mM 3[*N*-morpholino]-propane sulphonic acid, 50 mM sodium acetate, 10 mM EDTA, pH 7). The RNA was blotted onto Zetabind membrane and hybridized overnight at 50 °C with a radioactively labelled cDNA probe to the *Kit* gene in a solution containing 5% SDS, 0·5 M phosphate buffer pH 7·4. The blot was washed several times in 4×SSC 0·1% SDS at 50 °C then exposed to X-ray film at −70 °C. The film was developed 12 h later.

(vii) *RT-PCR*

RNA from the compound heterozygote together with suitable controls was reverse transcribed using the Moloney murine leukaemia virus (M-MuLV) reverse transcriptase (New England BioLabs) from an oligo-dT primer. The conditions used were those recommended by the manufacturer. An aliquot of the reversed transcribed reaction mix was subsequently used in two separate PCR reactions. The first involved amplification of a fragment spanning the transmembrane domain from the forward (KIT1550: CAAGAGTTCCGCCTTCTTTA) and reverse (KIT1851: ATCCGACTTAATCAAGCCAT) primers. The second involved the amplification of the cytoplasmic domain from a forward (KIT1649: GATGGGGATCATTGTGATGG) primer within the transmembrane domain and a reverse (KIT3000: ATCACAGAAGCCAGAAGGACG) primer at the C-terminal end. The same conditions were used for both reactions: buffer supplied by the manufacturer was used with the *Taq* polymerase (Boehringer–Mannheim, Indianapolis, IN) supplemented with 4 mM $MgCl_2$ and 25 μM dinucleotide triphosphates (dNTPs). Amplification was performed on an MJ thermocycler machine (MJ Research, Watertown, MA) according to the following protocol: one cycle of 94 °C for 4 min, 55 °C for 1 min, 72 °C for 5 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min; and a final cycle of 94 °C for 4 min, 55 °C for 1 min, 72 °C for 5 min. The amplification product was examined on a 1% agarose gel.

(viii) *KIT immunoprecipitation and* in vitro *kinase assay*

Antibody raised against the KIT receptor was the gift of Dr Alan Bernstein, Mount Sinai Research Institute, Toronto. Brain tissue was homogenized in RIPA buffer and processed according to Reith *et al*. (1990). Briefly, 500 μl of brain lysate, cleared by centrifugation, was mixed with 5 μl pre-immune serum plus 50 μl of 10% protein A sepharose and incubated for 2 h at 4 °C. After centrifugation, the supernatant was mixed with 5 μl rabbit anti-KIT plus 50 μl of 10% protein A sepharose and incubated for a further 2 h at 4 °C. The immunoprecipitate–protein A sepharose complex was washed three times with RIPA buffer and two times with 50 mM Tris-HCl (pH 7·5) and 1% Triton X-100. The immune complex was resuspended in 10 μl of kinase reaction buffer containing 10 mM $MnCl_2$, 1% Triton X-100 and 10 μCi [γ-^{32}P]ATP. The kinase reaction was incubated at 30 °C for 10 min then mixed with an equal volume of loading buffer. After electrophoresis on 7% SDS-acrylamide gel, the gel was fixed in 10% acetic acid, 30% methanol, followed by treatment with 1 M KOH prior to autoradiography as described by Reith *et al*. (1990).

3. Results

Genomic DNA from inter-subspecific F1 hybrids between the Ph^e mutant and the wild-derived *Mus musculus* PWK strain was digested with *Pst*I and hybridized with a full-length cDNA clone to the mouse *Pdgfra* gene. Previous studies (Stephenson *et al*., 1991) had established that this restriction endonuclease identified a useful polymorphism in the two alleles of the *Pdgfra* gene. The results of this analysis are present in Fig. 2. F1 hybrids carrying the Ph^e allele lacked some of the fragments detected in their wild-type sibs (i.e. 5·2, 4·9, 3·5, 2·6, 2·0 kb). These fragments are diagnostic of the laboratory allele for the *Pdgfra* gene. However, fragments defining the PWK allele (i.e. 7·9, 4·8, 2·8, 2·5 kb) were present. These results demonstrate that Ph^e, like *Ph* itself (Stephenson *et al*., 1991; Smith *et al*., 1991), carries a deletion of the *Pdgfra* gene.

Analysis of the *Kit* gene, also using a previously defined polymorphism (Stephenson *et al*., 1991), failed to reveal any abnormalities (data not shown). This would indicate that the Ph^e deletion, like *Ph*

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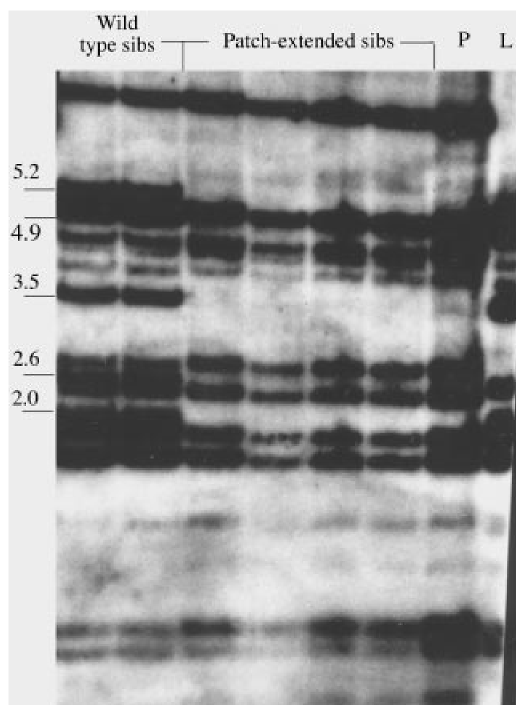

Fig. 2. Southern blot analysis of genomic DNA from inter-subspecific F1 hybrids between the Ph^e mutant stock (L) and the wild-derived *Mus musculus* PWK stock (P) with a cDNA clone to the *Pdgfra* gene. The wild-type sibs exhibit a banding pattern that combines the pattern of both parents, whereas the Ph^e sibs have a banding pattern highly indicative of the PWK parent only (i.e. they lack the unique fragments of the Ph^e parent).

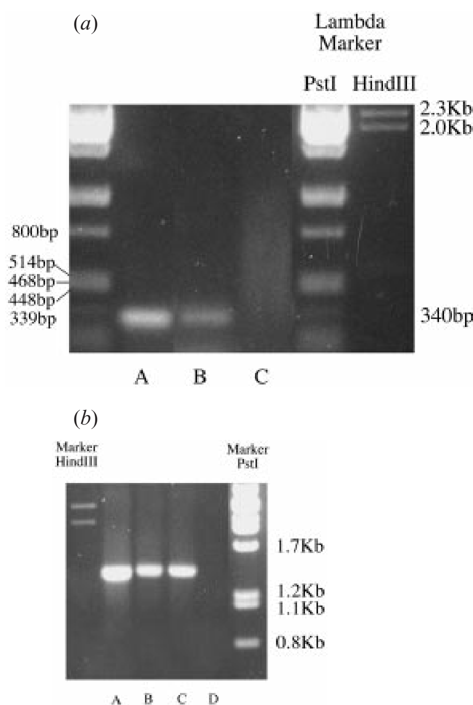

Fig. 3. (*a*) RT-PCR analysis of *Kit* mRNA extracted from the brain of a $Ph+/+Kit^W$ compound heterozygote (track A), $Ph^e+/+Kit^W$ compound heterozygote (track B) and Kit^W/Kit^W homozygote (track C) amplified with KIT1550/KIT1851. (*b*) Tracks B, C and D respectively correspond to A, B and C in (*a*), but amplified with KIT1649/KIT3000. Track A in (*b*) is a *Kit* cDNA control. As *Kit* mRNA encoded by the Kit^W allele lacks the exon containing the transmembrane domain (Nocka *et al.*, 1990), the presence of a 340 bp (*a*) and a 1·3 kb product (*b*) associated with RNA extracted from both compound heterozygotes implies that the *Kit* gene associated with both *Ph* alleles is transcribed.

(Stephenson *et al.*, 1991), does not appear to remove coding sequences associated with the *Kit* gene as defined by the cDNA probe.

We had hoped to establish whether the *Kit* gene encoded by the Ph^e deletion mutation was functionally transcribed using simple Northern blot analysis, by exploiting a slight difference in size between mRNA encoded by the wild-type and the dominant spotting (Kit^W) alleles (Nocka *et al.*, 1990). Brain RNA from a patch-extended/dominant spotting compound heterozygote (i.e. $Ph^e+/+Kit^W$) failed to produce the dual hybridization signal anticipated for this sample (data not shown). However, RT-PCR analysis did provide evidence indicating that the *Kit* gene was transcriptionally active from the deletional chromosome identified by the Ph^e mutation. A 340 bp product, identifying a normal transmembrane domain, was detected in the sample from the compound heterozygote (Fig. 3*a*, track B) but not in a sample from the Kit^W/Kit^W fetus (Fig. 3*a*, track C) with the primers KIT1550 and KIT1851. Amplification of contaminating genomic DNA can be precluded because the primers would encompass a region containing two or more introns (Andre *et al.*, 1997). As a consequence, the transmembrane fragment would be more than twice the size of that detected off the mRNA. Thus, the presence of a 340 bp product is consistent with an mRNA species containing the full-length transmembrane domain. This result was confirmed by the detection of a 1·3 kb (Fig. 3*b*, track C) product generated by the use of the transmembrane oligo (KIT1649) and an oligo-nucleotide primer from the 3′ end of the open reading frame (KIT3000). Amplification of contaminating genomic DNA would have a dramatic impact on the size of the fragment as this region contains 11 introns (Andre *et al.*, 1997), which, if amplified, would add a further 10 kb to the product size. A similar-sized product was not detected in the Kit^W/Kit^W fetal control (Fig. 3*b*, track D).

The results of the autophosphorylation analysis are presented in Fig. 4. Autophosphorylation signal at approximately 121 kDa is highly consistent with that associated with the KIT receptor (Nocka *et al.*, 1990). This signal is present in samples taken from the *Ph*, Ph^e and $Ph+/+Kit^W$ compound heterozygotes as well as the Kit^W heterozygote. However, no signal was detected in the $Ph^e+/+Kit^W$ compound heterozygote.

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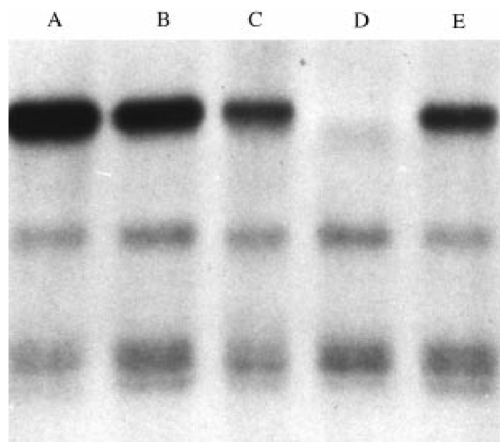

Fig. 4. Autophosphorylation analysis of immunoprecipitate of KIT protein extracted from the brain of a *Ph*/+ heterozygote (track A), $Ph+/+Kit^{W}$ compound heterozygote (track B), $Ph^{e}/+$ heterozygote (track C), $Ph^{e}+/+Kit^{W}$ compound heterozygote (track D) and $Kit^{W}/+$ heterozygote (track E). The presence of autophosphorylation signal at approximately 121 kDa in every sample except for the $Ph^{e}+/+Kit^{W}$ compound heterozygote (track D) suggests that there is a functionally active KIT protein in each sample. The lack of signal in the $Ph^{e}+/+Kit^{W}$ compound heterozygote (track D) implies that there is no functionally active KIT protein in this sample.

Similarly, no signal was detected in the Kit^{W}/Kit^{W} fetal control (not shown). Previous studies (Nocka *et al*., 1990) have demonstrated that the KIT receptor encoded by Kit^{W} is not retained by the cell because it lacks the transmembrane domain; the presence of autophosphorylation in the $Ph+/+Kit^{W}$ compound heterozygote would imply that the *Kit* gene associated with the *Ph* mutation is functionally active. The absence of signal in the $Ph^{e}+/+Kit^{W}$ compound heterozygote would imply that the *Kit* gene associated with the Ph^{e} mutation is functionally inactive. Quantitative measurements of the autophosphorylation signal are reasonably consistent with the level of a functionally active receptor (unpublished observation). Taken together, these observations establish that, while the *Kit* gene from the Ph^{e} chromosome is transcribed, it does not give rise to a functionally active receptor.

4. Discussion

In the present study we have established that the Ph^{e} allele at the *Ph* locus is a deletional mutation encompassing the *Pdgfra* gene but not the *Kit* gene. In this respect, it is similar to the *Ph* allele (Stephenson *et al*., 1991). While there is transcriptional activity from the *Kit* gene encoded by the deletional chromosome of the Ph^{e} allele, the translational product is not functionally active. This is in contrast to the *Kit* gene encoded on the deletional chromosome associated with the *Ph* allele.

When describing the origins of the Ph^{e} allele, Truslove (1977) indicated that it arose from a promiscuous mating between a *Ph*/+female and a *Ph*/+ or $Kit^{Wv}/+$male. The phenotypic similarities between Ph^{e} heterozygotes and the $Ph+/+Kit^{W}$ compound heterozygote, as well as the observation that Ph^{e} does not complement mutations at the *Kit* locus, would tend to suggest that the Ph^{e} mutation also carries a defect in the *Kit* gene. The results of the present study confirm this by demonstrating that, although present, the *Kit* gene associated with the Ph^{e} is functionally inactive. Abolition of functional kinase activity is a characteristic of several *Kit* alleles including Kit^{Wv} (Nocka *et al*., 1990). In conjunction with this and the observation that the $Ph^{e}+/+Kit^{Wv}$ compound heterozygote is slightly anaemic and sterile (Truslove, 1977), it would suggest that the illegitimate mating probably involved the $Kit^{Wv}/+$male. If this were the case, these two alleles, which were in repulsion in the original cross, should have segregated in subsequent matings. As they did not, it would imply that the *Kit* gene associated with the original *Ph* mutant acquired a mutation abolishing its activity. Several mechanisms suggest themselves as possible explanation for this event: (i) a spontaneous mutational event; (ii) a gene conversion event; or (iii) an intergenic recombinational event. While it is probably almost impossible to distinguish between these possibilities, they are not without precedence in nature (see review by Pittman & Schimenti, 1998).

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