c-kit Gene Is Expressed by Skin Mast Cells in Embryos But Not in Puppies of W^{th}/W^{th} Mice: Age-Dependent Abolishment of c-kit Gene Expression

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The W^{th} is a mutant allele at the W (c-kit) locus of mice, but no significant abnormalities are found at the coding region of the W^{th} allele. Since cultured mast cells derived from the spleen of W^{th}/W^{th} mice do not express messenger RNA (mRNA) of c-kit, we studied the interrelation between the number of mast cells and the magnitude of c-kit mRNA expression in the skin of W^{th}/W^{th} mice of various ages. The number of mast cells in the skin of W^{th}/W^{th} embryos of 18 days postcoitum (pc) was approximately 40% that of normal control (+/+ embryos, but the number of mast cells decreased exponentially after birth; the number dropped to 0.6% that of +/+ mice at day 150 after birth. A weak but apparent signal of c-kit mRNA was detectable in the skin of 18-day pc W^{th}/W^{th} embryos by RNase protection assay but not in the skin of 5-day-old W^{th}/W^{th} mice. The number of c-kit protein-containing cells was significantly greater in the skin of 18-day pc W^{th}/W^{th} embryos than in the skin of 5-day-old W^{th}/W^{th} mice. The abolishment of c-kit mRNA expression appeared to be specific, because the expression of mast cell carboxypeptidase A mRNA but not of c-kit mRNA was detectable by in situ hybridization in skin mast cells of 5-day-old W^{th}/W^{th} mice. Taken together, the expression of c-kit mRNA was abolished first, then the content of c-kit protein dropped to undetectable levels, and then the disappearance of W^{th}/W^{th} mast cells themselves followed.

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MATERIAL AND METHODS

Mice and embryos. The original stock of W^{th}+/+ mice was obtained from Dr J.F. Loutit (MRC Radiobiology Unit, Harwell, Didcot, UK). The W^{th} mutant gene was maintained by repeated backcrosses to our own inbred colony of the C57BL/6+/+ strain. At the time of this experiment, the backcrosses exceeded more than 25 generations. Mice of W^{th}+/+ genotype were mated together to obtain embryos of W^{th}/W^{th} genotype. C57BL/6+/+ (+/+ mice) were also mated together to obtain +/+ embryos. Mice of W^{th}/W^{th} genotype were identified by the absence of melanocytes in the skin. For embryonic staging, the day of vaginal plug observation was counted as the first day postcoitum (pc).

For demonstration of melanocytes in embryos and neonatal mice, melanin synthesis from L-3,4-dihydroxy-phenylalanine (L-DOPA, Sigma Chemical Co, St Louis, MO) was examined histochemically. Skin pieces of the head region were fixed in ice-cold 4% paraformaldehyde (PFA) solution containing 2% calcium acetate. After washing with distilled water, they were incubated in L-DOPA at 20°C overnight. They were washed with distilled water, dehydrated with ethanol buffer (pH 7.0, PB) containing 0.1 mol/L phosphate buffer (PB) containing 0.1% L-DOPA at 20°C overnight. They were washed with distilled water, dehydrated with ethanol buffer (pH 7.0, PB) containing 0.1% L-DOPA at 20°C overnight. They were washed with distilled water, dehydrated with ethanol buffer (pH 7.0, PB) containing 0.1% L-DOPA at 20°C overnight. They were washed with distilled water, dehydrated with ethanol buffer (pH 7.0, PB) containing 0.1% L-DOPA at 20°C overnight.

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also killed by over-inhalation of ether. Skin pieces were removed in the embryo and skin was divided by the length from the back and smoothed onto a piece of filter paper to keep the head and another near the tail; mast cells in the subcutaneous connective tissue between these two points were counted under the microscope. In the section of skin pieces, mast cells between epithelium and connective tissue were counted under the microscope. The numbers represent the nucleotide numbers on the complementary strands of each cDNA sequence, ie, c-kit cDNA reported by Qiu et al.\(^5\) FcεRI β-subunit cDNA by Ra et al.\(^6\) and MC-CPA cDNA by Reynolds et al.\(^7\) Ten microliters of the PCR products were electrophoresed in agarose gel. Membrane-transferred DNA was hybridized with each random-primed probe of c-kit cDNA, FcεRI β-subunit cDNA, or MC-CPA cDNA labeled with digoxigenin deoxyuridine 5'-triphosphate. Hybridized filters were washed and signals were detected using a Nucleic Acid Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions.

**RNase protection assay.** The procedure has been described in detail by Nomura et al.\(^8\) A 288 bp DNA fragment encoding c-kit cDNA (nucleotide 11 to 277) was obtained by reverse transcription modification of PCR (RT-PCR) technique and subcloned into EcoRI-BamHI site of Bluescript I KS (−) (Stratagene, La Jolla, CA). The plasmid (hereafter called pkit288) was linealized with PvuII, and the 639 base single-strand RNA containing a 351 base sequence of vector and a 288 base sequence of antisense of c-kit mRNA was uniformly labeled with [α-32P]UTP (Amersham Radiolabeled Chemicals, Inc, St Louis, MO) using T7 RNA polymerase (GIBCO BRL, Gaithersburg, MD). An 8 × 10⁵ cpm (1760 pg) probe was hybridized with 15 μg of total RNA at 50°C in 80% formamide solution (pH 6.8) containing 40 mmol/L piperazine-N,N'-bis (2-ethane-sulfonic acid), 400 mmol/L NaCl and 1 mmol/L ethylenediaminetetraacetic acid (EDTA) for 16 hours, and then digested with a mixture of 20 μg/mL RNase A (Boehringer Mannheim) and 2 μg RNase T1 (Boehringer Mannheim) at 30°C for 1 hour. The protected fragment was analyzed on a 6% acrylamide-6 mol/L urea gel followed by autoradiography, using [32P] end-labeled MspI-digested pBR322 DNA as a size marker.

**Preparation of RNA probes.** Digoxigenin-labeled single strand RNA probes were prepared by a DIG RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's instructions. For generation of mouse c-kit probes, five fragments (nucleotides 1-1064, 1065-1620, 1621-2548, 2549-3564, and 3564-4244; the number represents the region of the sequence described by Qiu et al.) of mouse c-kit cDNA were subcloned into the HindIII site of Bluescript I KS (−). These plasmids were either linealized with ClaI and transcribed with T7 RNA polymerase to generate antisense probes or linealized with XhoI and transcribed with T7 RNA polymerase to generate sense probes. A fragment of MC-CPA gene (nucleotides 688-1377, the number represents the region of the sequence described by Reynolds et al.) was obtained by RT-PCR technique and subcloned into the EcoRI site of Bluescript I KS (−). This plasmid was either linealized with HindIII and transcribed with T7 RNA polymerase to generate antisense probes or linealized with EcoRI and transcribed with T3 RNA polymerase to generate sense probes.

**In situ hybridization.** Hybridization was carried out as described by Nomura et al.\(^9\) Tissues were fixed with 4% PFA in 0.1 mol/L PB overnight. After dehydration with ethanol series, tissues were embedded in paraffin. Sections (4 μm thick) were counterstained with nuclear fast red and examined under the microscope.

**Number of mast cells.** Pregnant mice were killed by over-inhalation of ether and embryos were removed. Mice of various ages were also killed by over-inhalation of ether. Skin pieces were removed from the back and smoothed onto a piece of filter paper to keep them flat. The embryos and skin pieces were fixed in Carnoy's solution and embedded in paraffin. Sections (4 μm thick) were stained with alcian blue and nuclear fast red. In the sagittal section of embryos, two points were marked in ink on the back, one near the head and another near the tail; mast cells in the subcutaneous connective tissue between these two points were counted under the microscope. In the section of skin pieces, mast cells between epithelium and connective tissue were counted. The number of mast cells thus obtained in the embryo and skin was divided by the length of the portion in which mast cells were counted, and expressed as mast cells per centimeter.\(^9\)

**cDNA synthesis, polymerase chain reaction (PCR), and Southern blot analysis.** Various amounts of total RNA (5.0, 0.5, 0.05, and 0.005 μg) obtained from skin tissues of 18-day pc embryos, 5-day old mice, and 150-day-old mice were reverse transcribed in 20 μL of the reaction mixture containing 20 μL of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) and 50 pmol downstream antisense primers for c-kit, high affinity receptor of IgE (FcεRI) β-subunit, and mouse mast cell carboxypeptidase A (MC-CPA) genes.\(^9\) One microliter of each reaction product was amplified in 25 μL of PCR mixture containing 0.125 U of Taq DNA polymerase (Takara Shuzou, Kyoto, Japan) and 12.5 pmol each of sense and antisense primers for c-kit, FcεRI β-subunit, and MC-CPA genes by 20 cycles of 30-second denaturation at 94°C, 30-second annealing at 55°C, 1-minute synthesis at 72°C. The following oligonucleotide primers were used for the reverse transcription and PCR: the mouse c-kit, sense primer 5'-GCCCACCCGTTCATTACAGA-3' (2039 through 2059) and antisense primer 5'-GATCCCTGATCATCTGTC-3' (2678 through 2654); the mouse FcεRI β-subunit, sense primer 5'-GAGCAGGACAGTCTGTC-3' (40 through 59) and antisense primer 5'-AAAAAGCCGAGGTGGACAG-3' (575 through 551); the MC-CPA, sense primer 5'-ACA- CAGGATCGAATGTGGAGG-3' (688 through 707) and antisense primer 5'-TAATGCAGACTTCAAGGCC-3' (1358 through 1377). The numbers represent the nucleotide numbers on the complementary strands of each cDNA sequence, ie, c-kit cDNA reported by Qiu et al., FcεRI β-subunit cDNA by Ra et al., and MC-CPA cDNA by Reynolds et al. Ten microliters of the PCR products were electrophoresed in agarose gel. Membrane-transferred DNA was hybridized with each random-primed probe of c-kit cDNA, FcεRI β-subunit cDNA, or MC-CPA cDNA labeled with digoxigenin deoxyuridine 5'-triphosphate. Hybridized filters were washed and signals were detected using a Nucleic Acid Detection Kit (Boehringer Mannheim) according to the manufacture's instructions.

**Fig 1. Number of mast cells in the skin of +/+ and Wsh/Wsh mice of various ages. Each point represents the mean of five to nine mice; bars show SE. Symbols plotted on the left end represent the number of mast cells in the skin of +/+ and Wsh/Wsh embryos of 18 days pc.**

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Fig 2. Expression of c-kit, FceRI β-subunit, or MC-CPA mRNA in the skin of +/- and W°/W° mice. Total RNAs from skin tissues of +/- and W°/W° mice of various ages were reverse transcribed with c-kit, FceRI β-subunit, and MC-CPA primers and then PCR-amplified with c-kit, FceRI β-subunit, or MC-CPA primers for 20 cycles. PCR products from RNAs of +/- mice (lanes 1 to 4) and from RNAs of W°/W° mice (lanes 5 to 8) were separated by electrophoresis. Amounts of RNA used for the reverse transcription were 5.0 pg (lanes 1 and 5), 0.5 pg (lanes 2 and 6), 0.05 pg (lanes 3 and 7), and 0.005 pg (lanes 4 and 8), respectively. DNA was transferred to nylon membrane and hybridized with each probe.

### RESULTS

Numbers of skin mast cells were counted in W°/W° and control +/- mice of various ages. Appreciable numbers of mast cells were detected in the skin of W°/W° 18-day pc embryos (40% that of +/- embryos, Fig 1). The number of mast cells in the skin of W°/W° mice exponentially decreased thereafter and the number dropped to 6% that of control +/- mice at day 60 and to 0.6% at day 150 after birth. In contrast with the remarkable and continuous decrease of mast cells in the skin of W°/W° mice, the number of mast cells in the skin of +/- mice decreased slightly in the suckling period but thereafter the number was nearly constant throughout the observation period (Fig 1).

In the previous study, we demonstrated that the c-kit mRNA was not expressed by CMC derived from the spleen of W°/W° mice. Since appreciable numbers of mast cells were found in the skin of W°/W° embryos, we examined the expression of c-kit mRNA in the skin of +/- and W°/W° mice of various ages. Mast cells express FceRI β-subunit and MC-CPA mRNAs, and thus we used these mRNAs as controls. The c-kit signal was detected in the skin tissue of +/- 18-day pc embryos when 0.05 µg of total RNA was used as the starting material, whereas 0.5 µg of total RNA from the skin tissue of W°/W° embryos was necessary to detect the c-kit signal (Fig 2). The magnitude of FceRI β-subunit and MC-CPA signals after applying 0.5 µg of total RNA from the skin tissue of +/- embryos was comparable with that of the signals after applying 5.0 µg total RNA from the skin tissue of W°/W° mice. In the skin of +/- mice, the magnitude of signals of c-kit, FceRI β-subunit, and MC-CPA did not change until day 150 after birth. On the other hand, the magnitude of signals dramati-
cally decreased with age in the skin of $W^{bo}/W^{bo}$ mice. First of all, the c-kit signal was hardly detectable at day 5 after birth. The signals of FcεRI β-subunit and MC-CPA were detectable at day 5 but not at day 150 after birth (Fig 2). The absence of all c-kit, FcεRI β-subunit, and MC-CPA signals at day 150 reflected the disappearance of mast cells in the skin of $W^{bo}/W^{bo}$ mice. However, the disappearance of the c-kit signal apparently occurred earlier than the disappearance of skin mast cells themselves (Figs 1 and 2).

Since the estimate of the reduction in c-kit mRNA expression using RT-PCR technique is at best only an approximation, we examined de novo transcription of c-kit mRNA with the more sensitive and quantitative method, i.e., the RNase protection assay. Total RNA samples prepared from the skin tissue of 18-day pc embryos or 5-day-old mice were hybridized with the 639 base single-strand RNA probe containing a 288 base sequence of c-kit antisense RNA. The intensities of the protected bands (288 base fragment) in skin tissues of $W^{bo}/W^{bo}$ mice were compared with those of +/+ mice of the same age (Fig 3). The intensity of the protected band did not change significantly between 18-day pc +/+ embryos and 5-day-old +/+ mice. In contrast, it decreased dramatically in $W^{bo}/W^{bo}$ mice. An apparent protected band was observed in the skin tissue of 18-day pc $W^{bo}/W^{bo}$ embryos, whereas the protected band was barely detectable in the skin tissue of 5-day-old $W^{bo}/W^{bo}$ mice (Fig 3).

In situ hybridization was performed to demonstrate directly that the disappearance of the c-kit signal was earlier than that of mast cells themselves. Three serial sections were cut from the skin of 5-day-old +/+ and $W^{bo}/W^{bo}$ mice; the first section was used for the in situ hybridization of c-kit mRNA, the second section was stained with alcin blue and nuclear fast red to demonstrate mast cells, and the third section was used for the in situ hybridization of MC-CPA mRNA. As shown in Fig 4, some alcin blue-positive mast cells in the skin of +/+ mice expressed mRNA of c-kit or MC-CPA. As a matter of course, some mast cells may express both c-kit and MC-CPA mRNAs, but mast cells are not so large to be observed in three serial sections. Some alcin blue-positive mast cells in the skin of $W^{bo}/W^{bo}$ mice expressed MC-CPA mRNA but rarely expressed c-kit mRNA. Since alcin blue-positive mast cells observed in a section are not always present in the adjacent sections, the number of alcin blue-positive, c-kit expressing, and MC-CPA expressing cells were counted in the skin sections of +/+ and $W^{bo}/W^{bo}$ mice of various ages (embryos of 18 days pc, neonatal mice, and 5-day-old mice). Approximately 80% of alcin blue-positive mast cells appeared to express MC-CPA mRNA in the skin of both +/+ and $W^{bo}/W^{bo}$ mice of all ages examined (Table 1). Approximately 60% (day 18 pc) to 50% (day 5) of alcin blue-positive cells appeared to express c-kit mRNA in the skin of +/+ mice but only 6% (day 18 pc) to 1% (day 5) of alcin blue-positive cells expressed c-kit mRNA in the skin of $W^{bo}/W^{bo}$ mice (Table 1). The very low proportion of c-kit expressing cells in the skin of 5-day-old $W^{bo}/W^{bo}$ mice indicated that the result shown in Fig 4 was not an exceptional case.

Next we stained the skin of +/+ and $W^{bo}/W^{bo}$ mice with ACK2 MoAb to detect c-kit protein-containing cells. Since frozen sections are mandatory for staining with ACK2 MoAb and since cutting good serial sections from frozen materials was very difficult, we identified mast cells by staining with alcin blue and since cutting good serial sections from frozen materials was very difficult, we identified mast cells by staining with alcin blue and nuclear fast red to demonstrate mast cells, and the third section was used for the in situ hybridization of c-kit mRNA. Since alcin blue-positive mast cells observed in a section are not always present in the adjacent sections, the number of alcin blue-positive, c-kit expressing, and MC-CPA expressing cells were counted in the skin sections of +/+ and $W^{bo}/W^{bo}$ mice of various ages (embryos of 18 days pc, neonatal mice, and 5-day-old mice). Approximately 80% of alcin blue-positive mast cells appeared to express MC-CPA mRNA in the skin of both +/+ and $W^{bo}/W^{bo}$ mice of all ages examined (Table 1). Approximately 60% (day 18 pc) to 50% (day 5) of alcin blue-positive cells appeared to express c-kit mRNA in the skin of +/+ mice but only 6% (day 18 pc) to 1% (day 5) of alcin blue-positive cells expressed c-kit mRNA in the skin of $W^{bo}/W^{bo}$ mice (Table 1). The very low proportion of c-kit expressing cells in the skin of 5-day-old $W^{bo}/W^{bo}$ mice indicated that the result shown in Fig 4 was not an exceptional case.

Next we stained the skin of +/+ and $W^{bo}/W^{bo}$ mice with ACK2 MoAb to detect c-kit protein-containing cells. Since
however, the number of c-kit protein-containing cells in the skin of Wsh/Wsh mice dropped from 10% to 2% that of +/+ mice of the same age (Table 1).

DISCUSSION

Despite the deficiency of mast cells in the skin of adult Wsh/Wsh mice, appreciable numbers of mast cells were observed in the skin of Wsh/Wsh embryos and neonatal Wsh/Wsh mice. There are at least three possibilities to explain the result: 1) the c-kit receptor was expressed by embryonal mast cells but not by adult mast cells; 2) the number of c-kit receptors necessary for development of mast cells in embryos is smaller than that of c-kit receptors necessary for survival of mast cells after weaning; 3) embryonal but not adult mast cells may be stimulated through receptors other than c-kit. These three possibilities are not mutually exclusive, but the present result may support the first possibility. Although the in situ hybridization showed few mast cells that expressed c-kit mRNA in the skin of Wsh/Wsh embryos, Southern blotting of RT-PCR products, RNase protection analysis and immunohistochemistry of c-kit protein indicated

<table>
<thead>
<tr>
<th>Mice</th>
<th>Genotype</th>
<th>Alcian Blue Positive Cells</th>
<th>MC-CPA mRNA Expressing Cells</th>
<th>c-kit mRNA Expressing Cells</th>
<th>c-kit Protein Positive Cells</th>
</tr>
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<tbody>
<tr>
<td>Embryos of 18 days pc</td>
<td>+/+</td>
<td>378 ± 25 (9)</td>
<td>305 ± 14 (6)</td>
<td>232 ± 27 (6)</td>
<td>355 ± 33 (6)</td>
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<tr>
<td></td>
<td>Wsh/Wsh</td>
<td>146 ± 7* (6)</td>
<td>115 ± 10* (6)</td>
<td>9 ± 1* (6)</td>
<td>36 ± 5* (6)</td>
</tr>
<tr>
<td>Neonatal mice</td>
<td>+/+</td>
<td>364 ± 13 (6)</td>
<td>290 ± 8 (6)</td>
<td>220 ± 16 (6)</td>
<td>335 ± 7 (6)</td>
</tr>
<tr>
<td></td>
<td>Wsh/Wsh</td>
<td>134 ± 8* (6)</td>
<td>108 ± 5* (6)</td>
<td>7 ± 1* (6)</td>
<td>19 ± 4* (6)</td>
</tr>
<tr>
<td>5-day-old mice</td>
<td>+/+</td>
<td>332 ± 10 (10)</td>
<td>255 ± 20 (6)</td>
<td>165 ± 8 (6)</td>
<td>301 ± 17 (6)</td>
</tr>
<tr>
<td></td>
<td>Wsh/Wsh</td>
<td>97 ± 5*† (6)</td>
<td>85 ± 4* (6)</td>
<td>1 ± 1*† (6)</td>
<td>7 ± 1*† (6)</td>
</tr>
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Number of mice is shown in parentheses.

* P < .01 when compared with the values of +/+ mice of the same age by t-test.

† P < .01 when compared with the values of 18-day pc embryos of the same genotype.
that at least some mast cells in the skin of \(W^b/W^b\) embryos produced c-kit protein. On day 5 after birth, however, c-kit mRNA was barely detectable in the skin of \(W^b/W^b\) mice even by the RNase protection analysis. Moreover the number of c-kit protein-containing cells was significantly smaller in the skin of 5-day-old \(W^b/W^b\) mice than in the skin of 18-day pc \(W^b/W^b\) embryos. The abolishment of c-kit mRNA expression appeared to be specific, because the expression of MC-CPA mRNA was detectable by in situ hybridization in the skin mast cells of 5-day-old \(W^b/W^b\) mice. Taken together, the expression of c-kit mRNA was abolished first, then the content of c-kit protein dropped to undetectable levels, and then the disappearance of \(W^b/W^b\) mast cells themselves followed.

The present result is consistent with the speculation of Galli et al.\(^8\) that the number of mast cells in different anatomical sites is determined with the frequency of effective interaction between SCF and c-kit receptors. Galli et al.\(^8\) injected recombinant human SCF (rhSCF) to baboons or cynomolgus monkeys. The number of mast cells remarkably increased in various anatomical sites, but the number of mast cells dropped to preinjection levels after discontinuation of rhSCF injection. In the case of Galli et al., the limiting factor is the concentration of SCF, but in the present experiment the limiting factor is the number of c-kit receptors on the surface of mast cells.

The c-kit mRNA is expressed in the cerebellum, testis, and spleen of adult \(W^b/W^b\) mice.\(^25\) The c-kit cDNA was cloned from the cerebellum of \(W^b/W^b\) mice with RT-PCR, and the nucleotide sequence was determined. No significant change was detectable in the nucleotide sequence.\(^25\) In the previous study, we demonstrated that the c-kit gene was not transcribed in CMC derived from the spleen of \(W^b/W^b\) mice.\(^25\) This indicated that the expression of the c-kit gene in \(W^b/W^b\) mice was influenced by cell types. The present result confirmed the previous study and suggested that the expression of c-kit gene by \(W^b/W^b\) mast cells was also influenced by the age of the animals. In the skin of \(W^b/W^b\)
embryos of 18 days pc, melanoblasts were not detectable, whereas appreciable numbers of mast cells were detectable in the skin. Duttlinger et al. recently reported the presence of c-kit protein-containing melanoblasts in the skin of W/Wb embryos of 11 days pc, and they attributed the absence of melanoblasts at later stages to ectopic c-kit expression in the dermatoine. We speculate simply that the expression of c-kit mRNA was abolished earlier in melanoblasts than in mast cells and that melanocytes disappeared faster than mast cells of W/Wb mice due to the lack of c-kit receptors. Yasuda et al. reported the sequence of 244 bases in the marine c-kit promoter region, and Yamamoto et al. that of 1215 bases in the human c-kit promoter region. Moreover, Duttlinger et al. obtained the results suggesting that the Wb mutant allele arose as a result of a deletion or a rearrangement in the vicinity of the c-kit gene. There is a possibility that the deletion or the rearrangement suggested by Duttlinger et al. may contain a regulatory region of the c-kit that is specific for melanocytes and mast cells. The precise molecular mechanism that may explain the abnormal c-kit gene transcription in W/Wb mice remains to be investigated.

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