Contraction of the type I IFN locus and unusual constitutive expression of IFN-α in bats

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Bats harbor a number of emerging and reemerging viruses, many of which are highly pathogenic in humans and other species, including henipaviruses (Hendra and Nipah), coronavirus (SARS-CoV), rhadoviruses (rabies and lyssaviruses), and filoviruses (Ebola and Marburg), but cause no clinical signs of disease in bats. To determine the role of interferons (IFNs) in the ability of bats to coexist with viruses, we sequenced the type I IFN locus of the Australian black flying fox, Pteropus alecto, providing what is, to our knowledge, the first gene map of the IFN region of any bat species. Our results reveal a highly contracted type I IFN family consisting of only 10 IFN genes, including three functional type IFN-α loci. Furthermore, the three IFN-α genes are constitutively expressed in unstimulated bat tissues and cells and their expression is unaffected by viral infection. Constitutively expressed IFN-α results in the induction of a subset of IFN-stimulated genes associated with antiviral activity and resistance to DNA damage, providing evidence for a unique IFN system that may be linked to the ability of bats to coexist with viruses.


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Data deposition: Nucleotide sequence data have been deposited in the GenBank database [accession nos. KT384445–KT384449 (bat BAC clones 19–21) and KT384440 (cloned 3-kb bat IFN region)]. RNA sequence data have been deposited in the Sequence Read Archive [accession nos. SRP067312 ([uninfected HEK293T cells]) and SRP067371 (PaCKT03 cells)].

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Significance

Here we provide what is, to our knowledge, the first gene map of the type I IFN region of any bat species with the sequence of the type I IFN locus of the Australian black flying fox, Pteropus alecto. The bat IFN locus contains fewer IFN genes compared with any other mammal sequenced to date, including only three IFN-α genes. We also demonstrate that IFN-α genes are constitutively expressed in unstimulated bat tissues and cells and that their expression is unaffected by viral infection. This unusual pattern of IFN-α expression has not been described in any other species to our knowledge and has important implications for the role of innate immunity in the ability of bats to coexist with viruses in the absence of disease.
The U-ISGF3–associated ISGs are driven by distinct IFN-stimulated response elements and include Mx1, ISG15, and STAT1 (20).

Few studies have performed to understand the mechanisms responsible for the ability of bats to coexist with viruses. The sequencing of two bat genomes (Pteropus alecto and Myotis davidii) has revealed several genes involved in the DNA repair and innate immunity pathways that have undergone positive selection in bats compared with other mammals, providing evidence that the evolution of flight could have had inadvertent consequences for the innate immune system of bats (21). Studies have also inferred the existence of seven IFN-α genes in Pteropus vampyrus (22), eight IFN-α subtypes (or alleles) in Dobsonia viridis, and one IFN-α and IFN-ε in Ep特斯icus serotinus (23, 24). However, as only low-coverage bat genomes have been used to identify IFNs for these studies, the exact genome structure of type I IFN family members is yet to be confirmed. Current knowledge on bat type I IFN responses is also very preliminary, with descriptions of type I and III IFN induction following polyinosinic:polycytidylic acid (polyI:C) stimulation of bat cells (25).

Evidence for unique expression patterns of IFN-related genes have also been described in P. alecto, including the constitutive expression of IRF7 and a wider distribution of the type III IFN receptor family members IFN-α and a wider distribution of the type III IFN receptor IFN-ε and ω and a wider distribution of the type III IFN receptor IFN-ε and ω. IFN-ε and IFN-ω expression has been identified in the region 89 kb upstream of IFN-β (proximal 5’ end) or 94 kb downstream of IFN-ε (most 3’ end).

The P. alecto type I IFN locus was compared with the corresponding region from the genomes of 10 other vertebrates. The size of the IFN locus ranged from ∼25 kb in fish to 1 Mb in pig, with a trend toward increasing size through evolution as shown in Fig. 1. The only two exceptions were chicken and bat, both of which have shorter IFN loci of 30 kb and 250 kb, respectively.

A total of 10 genes with intact ORFs, including three IFN-α genes, one IFN-β, one IFN-ε gene, and five IFN-ω genes, were identified in the assembled bat IFN scaffold. In addition, a single copy of IFN-κ was found on a separate scaffold in the P. alecto genome (scaffold 14). The pattern of a single copy of IFN-κ and IFN-ε is conserved across all species (8). Consistent with the expansion in the genomic size of the IFN locus, gene duplication has occurred in the vertebrate type I IFN family in a step-wise manner, from only four type I IFNs at the basal branch such as in fish to 42 in pig. However, bats do not follow this trend and have only 10 type I IFN loci, three of which are IFN-α genes. Of the species that contain IFN-α genes, bats have the fewest IFN-α family members compared with any other mammalian genome studied (Fig. 1).

The deduced amino acid sequences of the three bat IFN-α genes share a number of features with IFN-α genes from humans.

![Fig. 1. Vertebrate type I IFN gene family among species. Type I IFN loci in selected vertebrate species (loci drawn to scale). IFN genes are annotated and labeled (not drawn to scale). The blocked arrows represent IFN ORFs, and directions indicate strand of the genes. IFN-ω (red), IFN-ε, and IFN-β (green), other intron-less type I IFNs (blue), and the non-IFN gene, KLHL9 (yellow), are shown. The intron-containing fish and frog IFNs are shown in large blue blocked arrows, with white columns to indicate the exon/intron boundaries. The unplaced IFN containing fragments outside the major IFN locus for some species are also shown. The numbers of type I IFNs (including IFN-ω) and IFN-α counts for each species are shown on the right. The phylogenetic tree on the left was drawn according to TimeTree, and the approximate divergence times are labeled (M, million years) (38).](image-url)
and other mammals, including predicted signal peptides and conserved binding domains for IFN-α1 and IFN-α2 for activation of downstream signaling (Fig. S2A). They share 93–96% similarity to each other and 79–85% similarity to human IFN-α genes at the amino acid level.

The bat IFN locus contains an additional eight IFN-α loci that appear to be pseudogenes (IFN-αP). This number is larger compared with humans or mice, which each have five IFN-αPs. Nucleotide alignment of the bat IFN-αP sequences with the three presumably functional IFN-α genes show that many of the IFN-αPs contain conserved partial IFN-αR binding domains, consistent with the likelihood that they once encoded functional IFN-α proteins (Fig. S2B).

**Evolution of Bat IFN-α Families.** IFN-α and IFN-α/β shared a common ancestor ~130 Mya and are interspersed with each other on the mammalian IFN locus (28) (Fig. 1). To determine the evolutionary pressures responsible for the diversification of type I IFN genes, we performed an evolutionary analysis of IFN-α and IFN-α/β families across eight mammalian species. The ratio of nonsynonymous (dN) to synonymous (dS) changes (dN/dS ratio) was measured to examine the selection pressures on the bat IFN-α and IFN-α/β genes. For the bat ancestor, the dN/dS ratio was 0.54, which is similar to the selection pressures on ancestral IFN-α genes from other species including pig (0.58), horse (0.34), humans (0.24), and mice (0.85). The purifying selection of bat IFN-α genes indicates its functional conservation and importance. Interestingly, positive selection for IFN-α was observed at the ancestor of bats (dN/dS ratio = 1.07), and its selection pressure was higher than any nonbat mammalian type I IFN genes.

**IFN-α Maintains a Constitutive and Ubiquitous Expression Pattern in Bat Tissues and Cells.** We then examined the IFN-α mRNA expression in comparison with IFN-β in tissues from three apparently healthy wild-caught *P. alecto* bats. As shown in Fig. 2A, *P. alecto* IFN-α was undetectable across all tissues tested with the exception of testes, in contrast, primers capable of detecting all three bat IFN-α genes demonstrated significant expression of IFN-α in all bat organs tested, with lung and brain the highest and wing the lowest (Fig. 2A). To determine whether the constitutive expression of IFN-α is *P. alecto*-specific, a second bat species, the lesser short nosed fruit bat (*Cynopterus brachyotis*) was also tested. Similar to the *P. alecto* tissues, IFN-α was expressed constitutively in tissues from *C. brachyotis* in contrast to undetectable levels of IFN-β across all tissues tested (Fig. S3A).

The inducibility of bat IFN-α and IFN-β was then compared in primary cell lines derived from nine different *P. alecto* tissues before and after transfection with polyIC for 3 h. The responses of primary cells confirmed our finding from the bat tissues demonstrating that IFN-α maintains a constitutive expression pattern in unstimulated bat primary cells. However, upon polyIC treatment, IFN-α was not significantly induced. This is in clear contrast to IFN-β, which was highly induced in polyIC-treated bat cells (Fig. S3B).

To examine the production patterns of bat IFN-α and IFN-β in response to viral challenge, we used two bat viruses and a mouse paramyxovirus to infect *P. alecto* kidney cell line PaKiT03 cells. Both Hendra virus (HeV) and Pulau virus (PuV) are bat-borne viruses carried by *Pteropus* bats. Sendai virus (SeV, *Cantell strain*) is a mouse paramyxovirus and is used in IFN research because of its ability to induce type I IFN through the production of defective interfering particles (29). Only SeV infection resulted in significant induction of IFN-β (P < 0.05). The absence of IFN-β induction is likely the result of antagonism of the IFN-β response by bat-borne viruses as reported previously for HeV (30). In contrast, IFN-α was significantly induced by SeV (P < 0.05) but to a lesser extent compared with the induction of IFN-β. Infection of bat cells with the two bat-borne viruses, HeV and PuV, caused no change in the constitutive IFN-α expression pattern (Fig. 2B). RNA sequencing (RNAseq) data available from HeV-infected human (HEK293T) and bat (PaKiT03) cells was used to confirm our findings (31). In bat cells, the constitutive IFN-α expression pattern was confirmed by using read depth counts of IFN-α transcripts in uninfected cells and showed little change following HeV infection. In contrast, few IFN-α transcripts were detected in infected or uninfected human cells (Fig. S3C). As a comparison, read mapping of RNAseq data from uninfected human and bat cells failed to detect IFN-β in either cell line. To confirm that the bat cells were not harboring an unrecognized infection, we used BLASTX to query the RNAseq data for the presence of sequences corresponding to known pathogens. Among the 64 million paired end reads in our dataset, no transcripts showed significant homology to known viruses or microbes. Even unknown viruses would be expected to show some sequence similarity to known virus families, as described previously for RNAseq data from bat tissues (32). This further supports our conclusion that the constitutive expression of IFN-α in bats is not associated with active viral infection.

Although the constitutive expression of bat IFN-α at the protein level has not been confirmed as a result of the absence of a bat-specific antibody, a high level of IFN-α protein expression would be expected to lead to the induction of ISGs. In human cells, continuous IFN-β exposure has been shown to lead to steady-state induction of the U-ISGF3–dependent proteins, with no sustained increase in other IFN-β–induced proteins (20). To determine whether the constitutive expression of IFN-α in bat cells resulted in induction of U-ISGF3–associated genes, we compared the expression of ISGF3-dependent and U-ISGF3–dependent transcripts in RNAseq data from uninfected human (HEK293T) and bat (PaKiT03) cells. Previous analyses describing U-ISGF3 and ISGF3– induced ISGs in human cells were used as the basis for distinguishing bat ISGs in the present study (20). Expression was
calculated using normalized read counts based on four replicates of RNAseq data from each cell line. Using a cutoff of 1.5-fold upregulation between cell lines, 61.5% (16 of 26 genes) of U-ISGF3-dependent ISGs were expressed at a higher level in bat compared with human cell lines, compared with only 23.0% (6 of 26) that had higher expression in human cells. Conversely, 48.5% (17 of 42) of ISGF3-dependent ISGs displayed higher expression in human compared with bat cells, and only 33.3% (14 of 42) were higher expression in bat cells (Fig. 2C). The U-ISGF3–associated ISGs with the highest expression in bats included well-known antiviral proteins including bone marrow stromal cell antigen 2 (BST2; also known as tetherin) and Mx1. The expression of a subset of genes that were up-regulated in either bat or human cells was validated by using quantitative RT-PCR (qRT-PCR), confirming the pattern obtained from the RNAseq dataset (Fig. S4).

**IFNα2 and IFNα3 Are the Main Constitutively Expressed Bat IFNs.** To test which bat IFN-α gene is constitutively expressed, TaqMan quantitative PCR (qPCR) assays were used to distinguish the three bat IFN-α genes in *P. alecto* tissues. IFN-α distribution among bat organs from three individual bats demonstrates that IFN-α2 and IFN-α3 are constitutively expressed in all organs tested, whereas IFN-α1 is expressed to a lesser extent and only in a subset of tissues. IFN-α2 and IFN-α3 displayed a similar expression pattern across most organs with the exception of the thymus, in which IFN-α3 was higher (Fig. 3A). These data confirm that *P. alecto* has three expressed IFN-α genes, of which IFN-α2 and IFN-α3 contribute to the majority of the constitutive expression of IFN-α.

For human IFN-α1, the unique promoter structure, and the simultaneous recruitment of IRF3 with the transcriptional coactivators CBP and p300, leads to a weak expression of endogenous IFN-α1 (18). To explore whether constitutively expressed bat IFN-α genes also have unique promoters, we analyzed the proximal promoters of bat IFN-α genes. A region 200 bp upstream of the putative translation start which contains the three IRF binding modules in human and mouse IFN-α genes was chosen for this analysis (19, 33). The three modules were identified in bat IFN-α1 and IFN-α3, and modules I and III were conserved with those of functional human IFN-α genes whereas module II was identical to that of human IFN-α2, which is nonfunctional (19). In contrast, the bat IFN-α2 promoter contains mutations within module I and nucleotide insertions within modules II and III, which would prevent it from binding to IRFs (Fig. 3B). Promoter assays demonstrated that only IFN-α1 and IFN-α3 responded to IRF3 and IRF7, whereas IFN-α2 failed to respond even in the presence of mitochondrial antiviral-signaling protein (MAVS), which is known to stimulate IRF activation (19) (Fig. S5). These findings are consistent with bat IFN-α2 being regulated by factors other than IRF3 and IRF7 to maintain its constitutive expression pattern.

**P. alecto IFN-α Proteins Are Functional.** To assess the functionality of *P. alecto* IFN-α proteins, plasmids encoding the three individual IFN-α ORFs were transiently transfected into human HEK293T cells. We chose HEK293T cells because of their high transfection efficiency, and also because the human IFN-αR cannot respond to bat IFN- and trigger downstream signaling (Fig. S6). Cell supernatant was collected as IFN-α and IFN-α3, and modules I and III were conserved with those of functional human IFN-α genes whereas module II was identical to that of human IFN-α2, which is nonfunctional (19). In contrast, the bat IFN-α2 promoter contains mutations within module I and nucleotide insertions within modules II and III, which would prevent it from binding to IRFs (Fig. 3B). Promoter assays demonstrated that only IFN-α1 and IFN-α3 responded to IRF3 and IRF7, whereas IFN-α2 failed to respond even in the presence of mitochondrial antiviral-signaling protein (MAVS), which is known to stimulate IRF activation (19) (Fig. S5). These findings are consistent with bat IFN-α2 being regulated by factors other than IRF3 and IRF7 to maintain its constitutive expression pattern.

**Discussion**

Type I IFNs provide the first line of defense against viral infection and are typically expressed only at low levels in unstimulated cells but are rapidly induced following infection. An increase in the size of the IFN locus has been accompanied by the evolution of a family of IFN-α genes that display distinct roles in the antiviral immune response of most mammals. Pathologically, bats, which are important reservoirs for a variety of viruses, have a contracted IFN locus and have only three functional IFN-α loci that are expressed constitutively in the absence of viral infection. The constitutive expression of bat IFN-α results in the up-regulation of a distinct subset of ISGs that may have implications for the ability of bats to coexist with viruses and resist DNA damage associated with flight.

The bat type I IFN locus was remarkably contracted in the bat genome at ~250 kb compared with other eutherian mammals that range from 350 kb (mouse) to 1,000 kb (pig). The smaller genome size of flying mammals has been speculated to be related to the evolution of flight (36), with bats and birds having smaller genomes compared with other species (37). However, the contraction of the IFN locus is striking, with only three functional IFN-α genes in the bat genome compared with 7–18 IFN-α loci in other mammals. Contraction of the bat IFN locus appears to have occurred after the divergence of bats from ungulates ~80 Mya (38). The presence of eight IFN-α pseudogenes provides further evidence for the contraction of the bat IFN locus from a large IFN-α family in the ancestral bat genome. The dN/dS ratio, indicative of purifying selection pressures shaping ancestral bat IFN-α emphasizes that the three functional bat IFN-α genes are conserved and functionally important to the host. In comparison, bat IFN-α genes experienced positive selection at the
ancestral branch, suggesting host–pathogen antagonism has continued through the long coevolutionary history of bats and viruses.

Type I IFN mRNA and proteins have been detected in tissues of healthy mice maintained in pathogen-free environments, but only in extremely low quantities (17). In humans, IFN-α1 mRNA is detectable in healthy spleen, liver, and kidney, but not in other organs (13). Bats are unusual in that IFN-α mRNA is detectible across all organs from apparently healthy individuals of at least two bat species. In contrast, IFN-β is barely detectable. Furthermore, stimulation of bat cells with dsRNA ligand polyIC results in the up-regulation of IFN-β while the expression of IFN-α mRNA remained similar to that of unstimulated cells 3 h following stimulation. Our previous work also showed extremely low induction of IFN-α in response to polyIC, up to a maximum of only approximately threefold at 9 h post transfection of bat lung cells (25). These findings suggest that IFN-α is not significantly up-regulated in response to cytoplasmic dsRNA sensing in bats. Nevertheless, the high baseline levels of IFN-α mean that substantial quantities of IFN-α mRNA can be detected even in the absence of immune stimulation. Similarly, Rousettus aegyptiacus lung cells had an apparent low level of constitutive IFN-α expression even before stimulation, and significant levels of IFN-α mRNA were not present until 8 h after polyIC treatment (25). Thus, IFN-α in bats forms two layers of protection: the constitutive and the induced IFN-α. The human IFN-α response peaks at 2, 8, and 12 h following SeV infection (18), whereas, in bats, the IFN-α response is constitutively activated and further increased 8–9 h post infection (25). The two layers of response are anticipated to provide bats with immediate protection but also allow them to react with a higher response when stimulated. Whether the two layers of the bat IFN-α response are dependent on each other or have different antiviral functions remains to be determined.

Very low levels of constitutively expressed IFN-α/IFN-β in humans and mice are believed to play a role in priming downstream responses, rather than having a direct role in antiviral immunity (16, 17). However, the ability of recombinant bat IFN-α to inhibit viral replication is consistent with constitutive IFN-α having a direct role in antiviral immunity in vivo. HeV has been demonstrated to block IFN production and signaling in bat cells (30), but does not affect the basal expression of IFN-α. RNAseq analysis supported our conclusion that bat IFN-α is constitutively expressed at a much higher level than human IFN-α, and is almost unaffected by HeV infection. Thus, the basal expression of bat IFN-α appears to be capable of avoiding the consequences of viral antagonism (at least by HeV and PulV) that leads to inhibition of the IFN-β response (30).

Constitutive expression of IFN-α would be expected to result in the corresponding induction of ISGs. To test this hypothesis, we used the available RNAseq dataset to compare expression of ISGs in human and bat cells (31). Human cells continually exposed to IFN-β express a distinct subset of ISGs that are driven by U-ISGF3, which leads to extended resistance to virus infection and DNA damage (20). The ISG response of bat cells appears to be enriched in ISGs associated with U-ISGF3. These ISGs were previously annotated, thus confirming they are counterparts to the corresponding human genes (21). Among the ISGs are well-characterized intracellular antiviral factors BST-2 (tetherin), which has been reported to restrict replication of HIV, Ebola, and Marburg viruses, and Mx1, which is recognized as having broad-spectrum antiviral activity against many RNA viruses (including influenza virus) and some DNA viruses (40, 41). Such ISGs may provide a “switched-on” defense mechanism to blunt virus replication and potentially viral pathogenesis in bats. The ISGs driven by U-ISGF3 do not appear to mediate the acute inflammatory responses often associated with IFN responses and may therefore contribute to the ability of bats to tolerate high levels of IFN-α with no pathological consequences. Furthermore, this subset of ISGs has been linked to resistance to DNA damage in human cells (20). The evolution of a prolonged ISG response in bats may be yet another adaptation caused by the evolution of flight that has had inadvertent consequences for antiviral immunity (20).

Of the three functional IFN-α loci in the bat genome, all three show some level of expression in bat tissues and cells, but IFN-α2 and IFN-α3 account for the majority of the constitutive expression pattern. IRF3 and IRF7 drive IFN expression in humans and other species by binding to unique IRF modules in the promoter regions (18). Curiously, despite the high expression of IFN-α2, no intact IRF binding elements were present in the promoter region of this gene. Furthermore, only module I and III appear to be potentially functional in the promoter regions of IFN-α3 and IFN-α1. Similar observations have been made in mouse IFN-α13 and IFN-ε, in which the IFN promoters have lost modules for IRF binding but maintain a constitutive expression pattern in certain organs (thymus, spleen, and spinal cord for IFN-α13; female reproductive tract for IFN-ε) (42, 43). In both cases, IFN gene expression is independent of viral infection. It is possible that the constitutively expressed IRF7 of bats contributes to the constitutive expression of bat IFN-α3 and IFN-α1 (27). However, the absence of IFR binding modules in IFN-α2 and the failure of IRF3 or IRF7 to induce IFN-α2 is consistent with the possibility that other transcription factors drive expression of bat IFN-α genes. Differences in the induction of IFNs may provide the opportunity to avoid antagonism by viruses that target IFN production pathways (44).

IFN genes evolve by gene duplication and deletion, and the loss of genes indicates their functions have become redundant, as observed in birds, which have a highly contracted IFN family (45, 46). Natural selection can result in mutations that favor less than the complete repertoire of functional genes, often with favorable consequences. This has been termed the “less-is-more” hypothesis (47). The contraction of the type I IFN family in bats with corresponding changes in their expression patterns is consistent with this hypothesis. Bats use fewer IFN-α genes to efficiently perform the functions of as many as 13 IFN-αs in other species and have a system that is constitutively ready to respond to infection.

In summary, we present an evolutionarily unique bat type I IFN locus with the discovery of only three functional bat IFN-α genes. Although bats have fewer IFN-α family members, the constitutive and ubiquitous expression pattern of IFN-α in bats may provide bats with a highly effective system for controlling viral replication.

**Methods**

**Bat Tissues and Cells.** Tissues were collected from *P. alecto* and *C. brachyotis* bats as described in **SI Methods**. Details of the *P. alecto* primary and
immortalized cell lines and culture conditions are described in SI Methods. All animal experiments were conducted following guidelines approved by the Australian Animal Health Laboratory (AAHL) ethics committee (AEC1389 and AEC1557) or Singapore animal ethics committee [B01/12 (AA) 12].

**Viral Infection.** PaKTI03 cells were mock-infected or infected with HeV, Sendai virus, or PulV as described in SI Methods. IFN-α viral protection assays were performed in PaKTI03 cells as described in SI Methods.

**IFN Locus Sequencing and Annotation.** Detailed descriptions of the sequencing, annotation, and comparative analysis of the bat type I IFN locus with other species are provided in SI Methods.

**Comparative and Evolutionary Analysis of the Mammalian Type I IFN Locus and IFN Genes.** Comparative analysis of the bat IFN locus was performed with the corresponding genomic region from other vertebrates and is described in SI Methods and Tables S1–S9. Evolutionary analyses were performed by using sequence alignments of IFN-α and IFN-ω genes across a variety of vertebrates and is described in SI Methods.

**Analysis of IFN and ISG Transcription Abundance.** RNAseq datasets from *P. alecto* PaKTI03 and human HEK293T cells obtained from mock and HeV infection are described in SI Methods (31). Analyses to determine changes in transcript abundance of IFNs and ISGs are described in SI Methods. qRT-PCR validation of gene expression was performed on total RNA from tissues or cells as described previously (25) and described in SI Methods. Primers are listed in Table S10.

**ISG Induction and Antiviral Activity of Bat IFN-α.** Details of the cloning and expression of recombinant *P. alecto* IFN-α (IFN-α1–3) and IFN-ω are described in SI Methods. The activity of the recombinant IFN-α proteins was determined by their ability to induce the production of IFGs and inhibit virus-mediated cytolysis as described in SI Methods.

**Luciferase Promoter Assays.** Details of the luciferase promoter assays used to test the ability of the three bat IFN-α genes to respond to IRF3 and IRF7 are described in SI Methods.

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