

Localization of β -defensin genes in non human primates

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Defensins are a family of host defence peptides that play an important role in the innate immunity of mammalian and avian species. In humans, four β -defensins have been isolated so far, corresponding to the products of the genes *DEFB1* (h-BD1, GenBank accession number NM_005218); *DEFB4* (h-Bd2, NM_004942.2), *DEFB103* (h-BD3, NM_018661); and *DEFB104* (hBD4, NM_080389) mapping on chromosome 8p23.22. We have localized β -defensin genes on metaphasic chromosomes of great apes and several non-human primate species to determine their physical mapping. Using fluorescent *in situ* hybridization and BAC probes containing the four β -defensin genes, we have mapped the homologous regions to the β -defensin genes on chromosome 8p23-p.22 in non-human primates, while no signals were detected on prosimians chromosomes.

Key words: Innate immunity, molecular evolution, FISH, homology.

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In humans, four β -defensins have been isolated and well characterized so far: i) *DEFB1* (h-BD1, GenBank accession number NM_005218) is constitutively expressed in kidney tubules and at lower levels in the pancreas, lungs and genitourinary tract (Bensch et al. 1995; Schonwetter et al. 1995; Diamond et al. 1996; Zhao et al. 1996; Goldman et al. 1997); ii) *DEFB4* (h-Bd2, NM_004942.2) is primarily induced in skin and other epithelia during inflammation (Harder et al. 1997b; Hiratsuka et al. 1998; Liu et al. 1998; Mathews et al. 1999); iii) *DEFB103* (h-BD3, NM_018661) is secreted by keratinocytes and epithelial cells of the respiratory tract after inflammatory stimuli or after contact with Gram-negative or Gram-positive bacteria (Harder et al. 1997a) and iv) *DEFB104* (hBD4, NM_080389), whose expression seems to be mainly in the testis, stomach, uterus, neutrophils, thyroid, lung and kidney (Garcia et al. 2001).

A lot of studies indicate that these molecules could play an important role in host defence, having both a direct, broad spectrum but salt sensitive antimicrobial activity (Lehrer and Ganz, 2002), and also playing a role in mobilizing other components of innate or adaptive immunity (Yang et al., 2002).

Twenty eight other putative β -defensin genes have been identified in the human genome, using a bioinformatic technique, based primarily on the 6-cysteine motif present in these molecules (Schutte et al., 2002).

Schutte et al. (2002) discovered five conserved β -defensin gene clusters in human and analogous clusters in mouse, using a computational search strategy: they identified the putative second exons of 28 new human and 43 new mouse β -defensin genes organized in five syntenic chromosomal regions. Within each cluster, the similarity of gene sequences and organization suggested each cluster pair arose from a common ancestor and was retained because of conserved functions. Other authors (Semple et

al., 2003) used a strategy that combined a bioinformatic approach and RT-PCR gene expression analysis to identify new β -defensin genes. Using a hidden Markov model constructed from the alignment of over 40 mammalian GenBank β -defensin sequences, they identified four new β -defensin genes in addition to those coding for the four known molecules, in BAC clone sequences localized to the 8p22-p23 region. These genes were confirmed to be functional by RT-PCR amplification.

In our study we have used a FISH approach based on two BAC clones that contain one or more of the four well-characterized human β -defensins (DEFB1, DEFB4, DEFB103 and DEFB104) to localize homologous genes on metaphasic chromosomes of the great apes and several non-human primate species, as well as two prosimians, in order to investigate their genetic mapping and to verify the presence of β -defensin genes in chromosomal regions other than 8p23-p.22 as described by Schutte et al. (2002).

Materials and Methods

Cell lines

Fibroblast cells from *Pan troglodytes* (PTR), *Gorilla gorilla* (GGO) *Pongo pygmaeus* (PPY), *Macaca mulatta* (MMU), *Cercopithecus aethiops* (CAE), *Presbytis cristata* (PCR), *Callithrix jacchus* (CJA), *Callicebus moloch* (CMO), *Lemur catta* (LCA) and *Eulemur macaco macaco* (EMA) were grown in Dulbecco's minimal essential medium (Gibco-BRL Rockville, MD, USA) supplemented with 10% fetal calf serum, 2 mM L-glutamine and penicillin-streptomycin sulfate and cultured at 37°C.

Bac clones

The BAC clones RP11-791N7, RP11-287p18, and RP11-351d16 were obtained from UCSC database, April 2003. The former two are in the 8p22-p23 region (<http://genome.ucsc.edu/>, April 2003), the latter clone is a test probe containing the conserved RET gene.

FISH experiments

Chromosome preparations were obtained following standard protocols. For stretched chromosomes, cells were resuspended in hypotonic solution (HCM: hepes, 100 mM; glycerol, 1M; CaCl₂, 100mM; MgCl₂, 0.5M, taken to 10 ml in H₂O) and left for 15 minutes. The resuspension was centrifuged using a cytospin (800-1200 rpm for 5-15 minutes) in order to produce stretched chromosomes (Laan et al., 1995). Slides were hybridized *in situ* basically as described by Lichter et al. (1990), with minor modifications. BAC probes (200 ng) were labelled by nick translation using nucleotides modified with fluorochrome cy3; hybridization was performed at 37°C in 2XSSC, 50% (v/v) formamide, 10% (v/v) dextran sulfate, 5 μ g human Cot1 DNA (Gibco-BRL), and 3 μ g sonicated salmon sperm DNA, in a volume of 10 ml. Post-hybridization washings were at 60°C in 0.1XSSC (three times, high stringency). Washes of FISH experiments using human probes on primates were performed at lower stringency: 37°C in 2XSSC-50% formamide ($\times 3$), followed by three washes at 42°C in 2XSSC ($\times 3$). Digital images were obtained using a Leica DMRXA epifluorescence microscope equipped with a cooled CCD camera (Princeton Instruments, NJ, USA). DAPI was used to counterstain the chromosomes in order to recognize them on the basis of the DAPI banding pattern. Cy3 and DAPI fluorescence signals, detected with specific filters, were recorded separately as grey scale images. Pseudocoloring and merging of images were performed using Adobe Photoshop™ software.

Results and Discussion

We have performed a query on the UCSC database (<http://genome.ucsc.edu/> April 2003) using the mRNA sequences of four known β -defensins, corresponding to the genes: *DEFB1* (NM_005218); *DEFB4* (formerly *DEFB2*, NM_004942.2); *DEFB103* (formerly *DEFB3*, NM_018661) and *DEFB104* (formerly *DEFB4*, NM_080389) and found the corresponding BAC clones, which are listed in Table 1. We then used the RP11-287p18 clone, containing the DEFB4-DEFB103-DEFB104 cluster

Table 1. Bac clone localization of defensin genes (position is referred to UCSC database April 2003).

Bac clone	Defensin gene	Mapping	Position(kb)
RP11-791n7	<i>DEFB1</i>	Chr 8	6736-6925
RP11-287p18	<i>DEFB4-DEFB103-DEFB104 cluster 1</i>	Chr 8	7330-7501

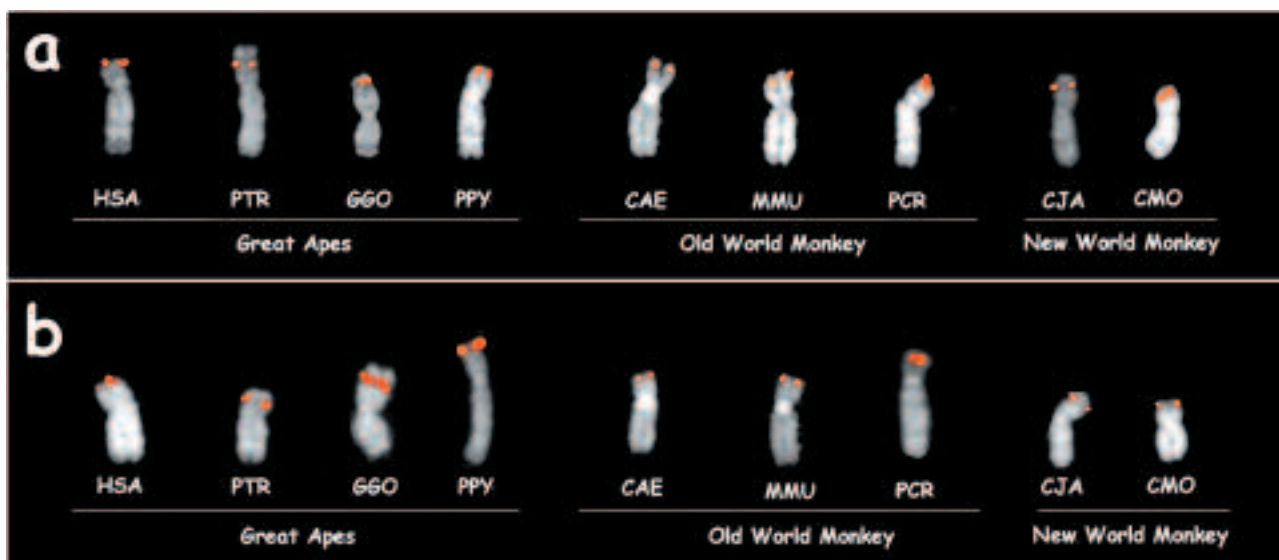


Figure 1. Comparative FISH analysis of the β -defensin genes. *In situ* hybridization of the BAC RP11-791n7 probe, containing the *DEFB1* gene (a) and of the BAC RP11-287p18 probe, containing the *DEFB4-DEFB103-DEFB104* cluster (b) onto metaphasic chromosome preparations from *Homo sapiens* (HSA), the *Great Apes* *Pan troglodytes* (PTR), *Gorilla gorilla* (GGO) and *Pongo pygmaeus* (PPY), the Old World Monkeys *Macaca mulatta* (MMU), *Cercopithecus aethiops* (CAE) and *Presbytis cristata* (PCR) and the New World Monkeys: *Callithrix jacchus* (CJA) and *Callicebus moloch* (CMO). (a) chromosome 8 for HSA, 8 for PTR GGO, PPY, CAE and MMU; 7 for PCR; 13 for CJA and 9 for CMO (b) from left to right, chromosome 8 HSA, 8 PTR GGO and PPY; 8, for CAE; 8 for MMU; 7, for PCR; 13 for CJA and 9 for CMO. Human chromosome 8 is the homologous of the reported non-human primate chromosomes.

and the RP11-791n7 clone, containing only *DEFB1* (Table 1) for the comparative mapping of these genes in primates.

Metaphasic chromosomal preparations of ten non-human primate species, including *Pan troglodytes* (PTR), *Gorilla gorilla* (GGO), *Pongo pygmaeus* (PPY) (Great Apes group), *Macaca mulatta* (MMU), *Cercopithecus aethiops* (CAE), *Presbytis cristata* (PCR) (Old World Monkeys group), *Callithrix jacchus* (CJA), *Callicebus moloch* (CMO) (New World Monkeys group), *Lemur catta* (LCA) and *Eulemur macaco macaco* (EMA) (prosimians group) were hybridized with the two BACs. The RP11-791n7 clone (containing *DEFB1*) showed signals in correspondence to 8p23-p22 in human preparations and in homologous bands in chromosomes from the non-human primate species (Figure 1A), but not in prosimian chromosomes (LCA and EMA, see Figure 2A). In the latter case co-hybridization experiments, using the RP11-351D16 BAC clone, which contains the evolutionarily conserved *RET* gene, as test probe (Carbone et al. 2002), were performed to confirm the negative FISH results obtained on LCA and EMA chromosomes. A positive hybridization signal was detected only for the test probe (Figure 2B).

The RP11-287p18 BAC clone, containing the

DEFB4, *DEFB103*, *DEFB104* cluster, detected a signal on human chromosome 8, as well as in homologous regions for the Great Apes. Homologous regions to chromosome 8 were detected in the Old and New world Monkeys (see Figure 1B and Table 2). This clone, like RP11-791n7, did not result in a FISH signal with prosimian metaphasic chromosome preparations (which were positive for the test probe in co-hybridization experiment).

In humans, four β -defensins have been isolated and well-characterised so far, and their genes (*DEFB1*, *DEFB4*, *DEFB103* and *DEFB104*) map on chromosome 8 (8p22-p23). We have identified BAC probes containing these genes and localized homologous regions on different non-human primate chromosomes by using fluorescence *in situ* hybridization (FISH). The localization of *DEFB1* (BAC clone RP11-791n7) on this chromosome in the different primate species indicated a strong conservation of the homology ranging from humans to New World Monkeys. The localization of the *DEFB4*, *DEFB103* and *DEFB104* gene cluster (BAC clone RP11-287p18) indicated the presence of these genes on chromosome 8 (or homolog chromosomes) in the Great apes, in the Old World Monkeys and in the New World Monkeys.

The e-cloning results obtained by Schutte et al

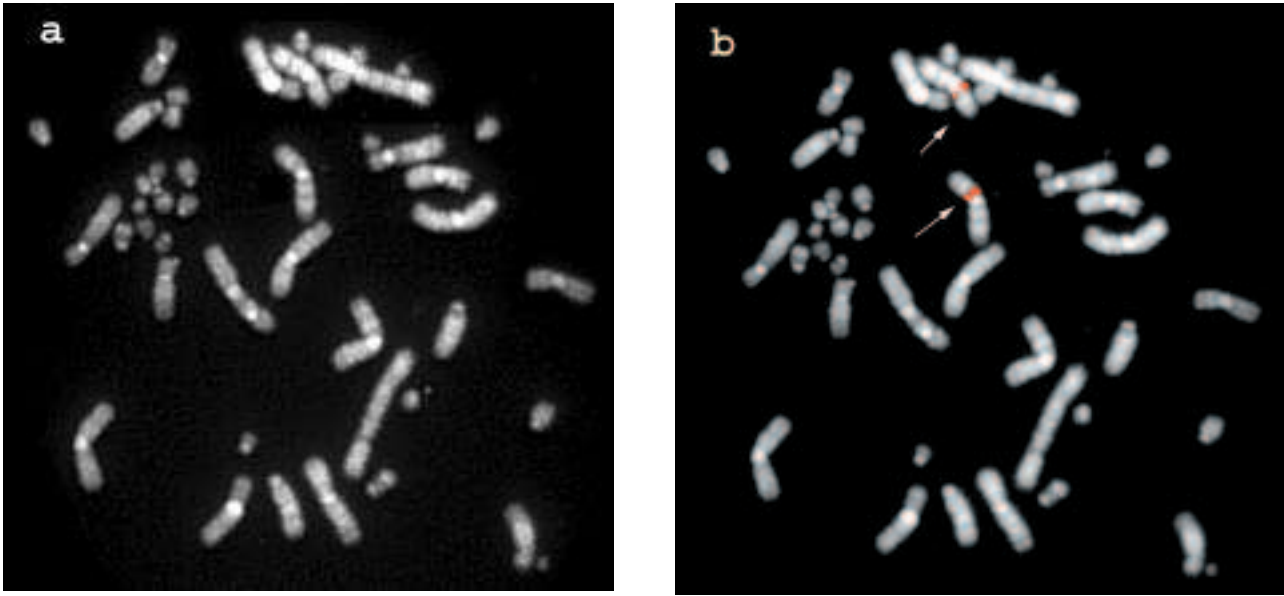


Figure 2. *In situ* hybridization of the BAC RP11-791n7 probe, containing the DEFB1 gene and of the BAC RP11-351d16, containing the conserved RET gene, onto metaphasic chromosome preparations from *E. macaco* (EMA). No hybridization was detected for both BAC RP11-791n7 (a). A positive signal of hybridization is visible (arrows) only for BAC RP11-351d16, the control probe (b).

Table 2. Comparative FISH mapping. Mapping on non-human primates was performed following Yunis (1982) nomenclature for Great Apes, Finelli *et al.* (1999) for *Cercopithecus aethiops* (CAE), Wienberg *et al.* (1992) for *Macaca mulatta* (MMU), Bigoni *et al.* (1997) for *Presbytis cristata* (PCR), Sherlock *et al.* (1996) for *Callitrix jacchus* (CJA) and Stanyon *et al.* (2000) for *Callicebus moloch* (CMO). In each species signals are localized on homologues region of human chromosome 8.

BAC	HSA	PTR	GGO	PPY	CAE	MMU	PCR	CJA	CMO
RP11-791n7	8	8	8	8	8	8	7	13	9
RP11-287p18	8	8	8	8	8	8	7	13	9

(2002) using a bioinformatic approach, indicate the existence of three other β -defensin gene clusters localized in humans on chromosomes 6p12, 20q11.1 and 20p1. In our study no other putative β -defensin genes were found by FISH in these regions in the human or monkey genomes.

This discrepancy could derive from the different approaches used to study the localization and syntax of β -defensin. Schutte *et al.* (2002) method to detect defensin genes in different chromosomal regions was based on the hypothesis that all β -defensins should share the characteristic six-cysteine motif, and thus concerned only exon 2. However, their data are not supported by experimental verification of gene expression and functionality. Our FISH approach has led us to identify chromosomal regions with β -defensin genes, in both human and non-human primate chromosomes, that shared similarity at the level of their whole nucleotide sequence.

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