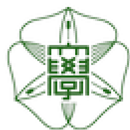
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Studies on karyotype evolution in higher primates by comparative chromosome mapping of immunoglobulin $C\varepsilon$ genes with fluorescence in situ hybridization

Hideyuki Tanabe

A thesis presented to the Graduate School of Science, Hokkaido University, in partial fulfillment of the requirements for the degree of Doctor of Science

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Introduction

The understanding of evolutionary processes in mammals has been greatly facilitated by the development of cytogenetic, cellular and molecular procedures in the last two decades. The advent of differential staining techniques of Q-, G-, R-, and C-bands has permitted the unequivocal identification of human chromosomes as well as other primate chromosomes (Paris Conference, 1971, Supplement, 1975; ISCN 1978; Pearson *et al.*, 1979; ISCN 1985). The application of chromosome banding to cytogenetic studies of mammalian chromosomes has made it possible to monitor more accurately the divergence of chromosome structure in mammalian evolution (Dutrillaux, 1979; Dutrillaux *et al.*,1982a; Seuánez, 1984; O'Brien *et al.*, 1988).

Cytogenetic analyses of G-banding pattern have carried out in more than 80 primate species, and these results have led to several general conclusions. Perhaps most striking is the extensive conservation of chromosome banding patterns among various primate species (Grouchy et al., 1978; Dutrillaux, 1979; Seuánez, 1979, 1984). Comparative chromosome analysis has showed that extensive chromosome banding homology exists not only between closely related primates, such as man and the great apes (chimpanzee, pygmy chimpanzee, gorilla, and orangutan) (Dutrillaux, 1979; Seuánez, 1979; Dutrillaux et al., 1982a; Yunis and Prakash, 1982), but also to a lesser extent between distantly related primates such as man and the wooly monkey (Dutrillaux *et al.*, 1980; Clemente *et al.*, 1987). Only a few chromosome rearrangements between man and the great apes have been observed, and all the human chromosomes have tentative homologues in these species (Dutrillaux, 1979). Yunis and Prakash (1982) have used high-resolution banding of the chromosomes of man and the great apes to reconstruct the chromosomal rearrangements that have presumably

occurred and become fixed during hominoid evolution. Their report confirmed previous observations (Turleau *et al*., 1972; Dutrillaux *et al*., 1975; Miller, 1977; Seuánez, 1979; Stanyon and Chiarelli, 1982) that most of the postulated rearrangements were pericentric inversions, although other rearrangements, such as Robertsonian translocations, paracentric and pericentric inversions, fusions, and fissions, have also occurred in primate species. For example, comparative karyotype analysis between human and the great apes showed the difference in the diploid number of man (2n=46) and the great apes (2n=48). Human chromosome 2 has no single counterpart in the great apes. Instead, there is an acrocentric homologue pair for 2p, and another acrocentric homologue pair for 2q. This chromosome comparison between species suggested that human chromosome 2 emerged as a result of a fusion between two nonhomologous ancestral chromosomes similar to these arm counterparts. This fusion event explains the difference in diploid number between man and the great apes (Grouchy *et al*., 1972; Turleau *et al*., 1972; ISCN 1978; Dutrillaux, 1979; Seuánez, 1979). The high extent of chromosome conservation in primates has allowed the construction of chromosome phylogenies, which demonstrate tentative chromosomal changes that have occurred during the simian radiation (Dutrillaux, 1979; Couturier and Dutrillaux, 1981; Dutrillaux *et al*., 1982b).

Modern primates include 175 species, and a representative phylogenetic tree of hominoid based on several morphological characters (dentition, skeletal traits, cephalic arterial system, larynges, and others) is presented in Fig. 1. Although several methodologies of molecular evolution have been extensively applied to the hominoids (man and apes), relatively less molecular data are available for primate species outside this group (King and Wilson, 1975; Koop *et al*., 1986; Sibley and Ahlquist, 1987).

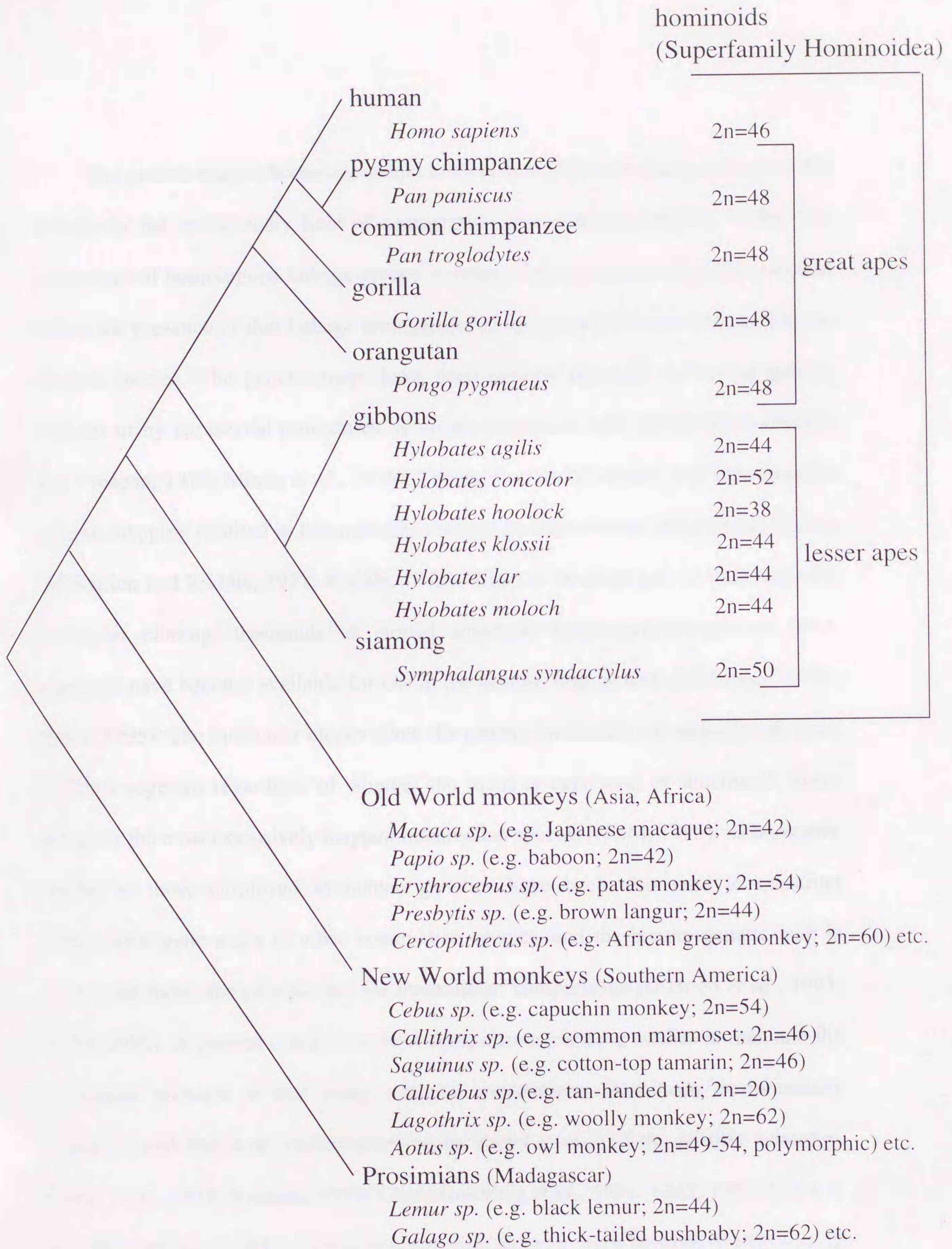


Fig. 1. Consensus phylogenetic tree of primates

Classification of the Order Primates following Simpson (1945) and Goodman (1975). The tree shows phylogenetic relationships. Diploid chromosome number of each species is also described.

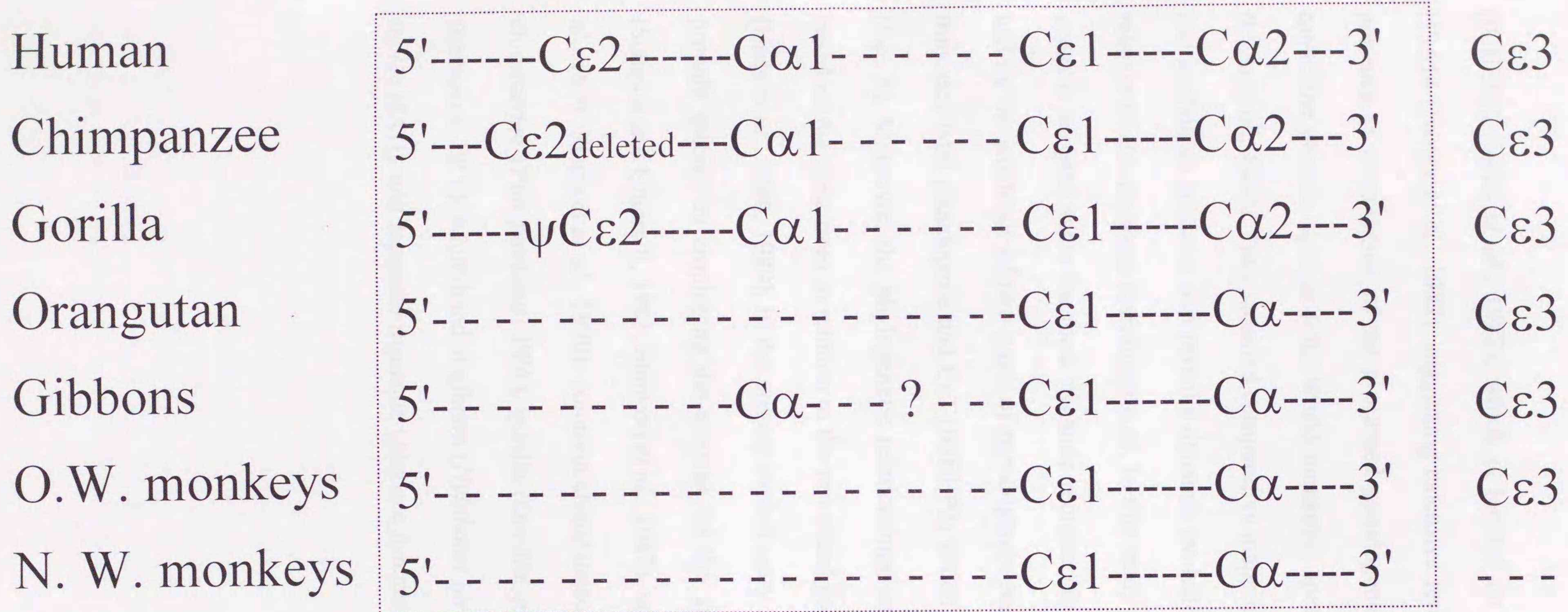
The genetic map of homologous loci in a number of mammalian species provides a basis for the evolutionary field of comparative gene mapping (HGM3, 1976). The occurrence of homologous linkage groups in related species is generally interpreted to reflect the presence of that linkage arrangement in species which were ancestral to the modern species. The genetic maps have been derived primarily by sexual genetic analysis or by parasexual procedures by means of somatic cell hybridization (Ruddle and Creagan, 1975; Minna *et al.*, 1976). The application of somatic cell hybridization to gene mapping resulted in a remarkable increase in chromosome assignments in man (McKusick and Ruddle, 1977; Ruddle, 1978). With the development of techniques for molecular cloning, thousands of cloned structural genes and anonymous DNA segments have become available for use in the genetic map as well (McKusick, 1991; HGM, 1995). The molecular clones allow the genetic localization of virtually any gene or DNA segment regardless of whether the locus is expressed or functional. Since human is the most extensively mapped mammalian species (HGM, 1995), technologies similar to those employed in human genetics have been also used to construct comparative gene maps in other mammalian species, and the human genetic map is used as an index for primate and for mammalian comparisons (O'Brien *et al.*, 1993; HGM, 1995). A general conclusion from comparative mapping studies in man and the nonhuman primates is that many syntenic associations have been evolutionarily conserved and that such conservation can be traced to each of the primate suborders (Estop *et al.*, 1979; Seuánez, 1979; Creau-Goldberg *et al.*, 1981, 1982, 1983; Estop *et al.*, 1983; Seuánez, 1984). A positive correlation seems to exist between morphological and syntenic changes at the chromosome level. This makes morphological attributes, such as banding patterns useful indicators of syntenic homologies in the primate order.

Furthermore, an important advance in human gene mapping has been the development of in situ hybridization of cloned probes to metaphase chromosomes (Pardue and Gall, 1970). This method involves the molecular hybridization of radiolabelled cloned probes to homologous DNA segments on a metaphase chromosome, followed by an autoradiography and chromosome banding procedure. Regional localization of genes can be directly visualized on individual chromosomes. More recently, nonisotopic hybridization techniques, particularly fluorescence in situ hybridization (FISH), have become an important tool in studies of genome mapping and chromosome structure and function (Pinkel *et al*., 1986; Lichter *et al*., 1988; Trask, 1991). Currently composite DNA probe sets for delineating whole chromosomes, chromosomal regions, or gene-specific loci are available (Morrison, 1993). In particular whole chromosomes or large chromosomal regions in a metaphase are painted by FISH using an entire chromosomes as probe DNA. This procedure called "chromosome painting" (Cremer *et al*., 1988; Lichter *et al*., 1988; Pinkle *et al*., 1988) has become a valuable tool to elucidate karyotype rearrangements in primate evolution, since FISH with human chromosome-specific probes has enabled interspecific chromosome homologies to be much precise. This new approach is called as comparative chromosome painting (Wienberg *et al*., 1990, 1992), or ZOO-FISH (Scherthan *et al*., 1994). For example, the well-known fusion-origin of human chromosome 2 was confirmed by the human chromosome 2 library which painted two chromosome pairs in all great apes (Jauch *et al*., 1992; Wienberg *et al*., 1994). In contrast to previous comparative gene mapping experiments that have been restricted to single-copy seqeunces, the chromosome painting approach provides an overall comparison of DNA sequence homologies for complete chromosomes.

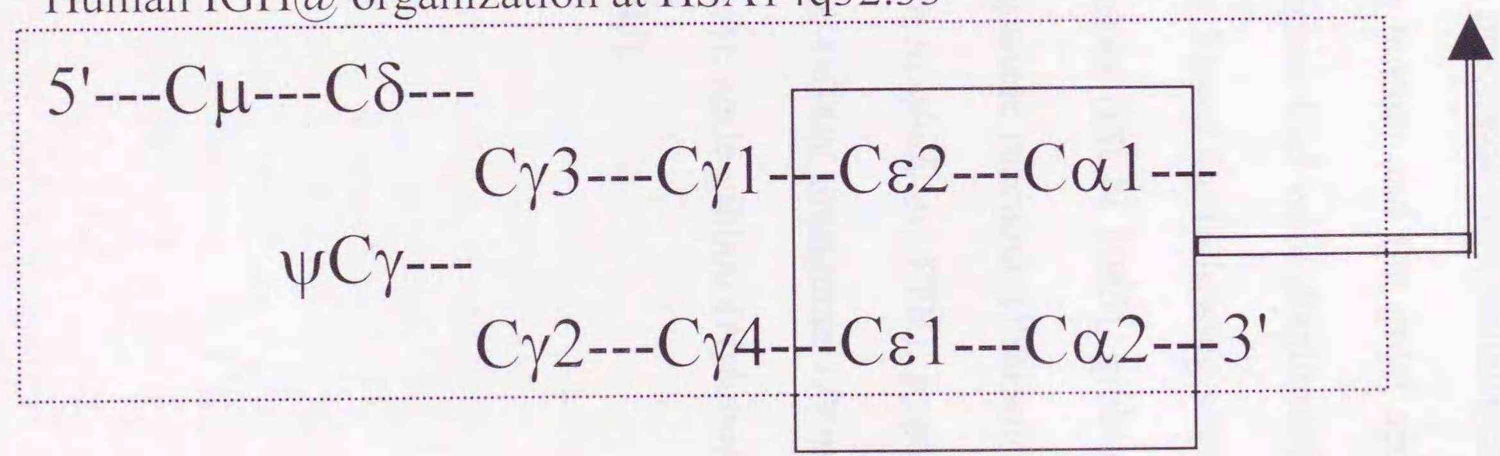
Although FISH data clearly show that the human genome is closely related to the great apes by the presence of similar DNA sequences providing another phylogenetic parameter for interspecific comparison, whole chromosome painting does not allow the demonstration of homology among subchromosomal regions or intrachromosomal rearrangements, which are supposed to be prominent in the evolution of human and great ape karyotypes. However, these subchromosomal regions or intrachromosomal rearrangements can be readily detected by defined human probes that span subregions of chromosomes, especially if one of the assumed breakpoints is included (Ried *et al*., 1993; Toder *et al*., 1993; Arnold *et al*., 1995). As the human genome analysis is remarkably progressing, an increasing number of DNA probes will become available for comparative gene mapping studies of any region of interest. Therefore, in this thesis I used both procedures of ZOO-FISH and comparative mapping for detailed analysis of genome alignment and also precise recognition of chromosome rearrangements in homologous segments.

In the present study, I analyzed localization of genes for immunoglobulin heavy chain in order to obtain additional information about homologies as well as chromosome rearrangements in karyotypes of primate species at the molecular cytogenetic level for better understanding of their karyotype evolution. Immunoglobulins are the effector molecules of the immune system and they are composed of two identical light (L) and heavy (H) chains, each of which consists of variable (V) and constant (C) regions, and have a Y-shaped structure connected by the hinge region (Honjo *et al*., 1989). The antigen-binding sites that are formed by a complex of their V regions including three small hypervariable regions of both L and H chains could bind numerous different antigens. There are five classes of H chains, α,

δ, ε, γ, and μ, which form the different classes of antibodies, IgA, IgD, IgE, IgG, and IgM, respectively, that determine the each effector function. The human H chain genes, which are divided into the ~300 variable (V_H), ~20 diversity (D), 6 joining (J_H), and 11 constant (C_H) gene segments, are arrayed in a direction from telomere to centromere to build up a huge gene cluster at the distal region of chromosome 14 (HSA14q32.33) and the total length is estimated to encompass 2.5 to 3 megabases DNA, making it one of the largest gene clusters in the mammalian genome (Croce *et al.*, 1979; Flanagan and Rabbitts, 1982; Shimizu *et al.*, 1982; Blackwell and Alt, 1989; Cox *et al.*, 1991). In this gene cluster the combinatorial somatic recombination of the VDJ-segments greatly increases the diversity of antibody that occurs in the B lymphocyte in which the antibody is expressed and the process called class switching allows the antibodies with varied biological properties (Davis *et al.*, 1980; Gellert, 1992). During the switch recombination, DNA deletion occurs by cutting and rejoining between the assembled VDJ sequence and the upstream of the particular C_H gene segment. From the evolutionary view point, likewise, the dynamic genetic rearrangements such as deletions, duplications, insertions, and gene conversions as well as point mutations have occurred in the genesis of C_H genes (defined as to IGH@; Cox and Donlon, 1989) during the course of primate evolution (Honjo *et al.*, 1981; Max *et al.*, 1982; Flanagan *et al.*, 1984; Migone *et al.*, 1984; Ueda *et al.*, 1985, 1988; Kawamura *et al.*, 1992; see Fig. 2). The human IGH@ contains 9 functional C_H genes and 2 pseudogenes with the order of 5'-Cμ-Cδ-Cγ3-Cγ1-Cε2(ψ)-Cα1-ψCγ-Cγ2-Cγ4-Cε1-Cα2-3'. It seems that a Cγ gene must have been duplicated to give the subcluster of Cγ-Cγ-Cε-Cα, after which the entire group was then duplicated (Flanagan and Rabbitts, 1982; Kawamura and Ueda, 1992). IGH@ contains another pseudogene, the processed pseudogene Cε3



Human IGH@ organization at HSA14q32.33



IGH@ has evoluved by the gene duplication involving the Cγ-Cγ-Cε-Cα region after Old World monkeys branched out. Organization of the Cε and Cα genes is described in the upper dotted rectangle with Cε3 gene at each side from human to New World monkeys (Kawamura and Ueda, 1992).

Fig. 2. Organization of IGH@ in higher primate genomes

(IGHEP2) (Ueda *et al*., 1982), which is located on a different chromosome from HSA14 (Battey *et al*., 1982), suggesting extensive reorganization of IGH@ loci. The presence of homologues of the processed pseudogene Cε3 has been demonstrated in catarrhine primate species (Old World monkeys, apes, and human) so far examined (Ueda *et al*., 1986). Thus, detailed comparative mapping of IGH@ loci between human and nonhuman primates will reveal a dynamic evolutionary trail of this gene family in relation to chromosome rearrangements. In this study among IGH@ a class of the Cε genes is adopted since the most dynamic reorganization might be occurred in the Cε loci by the existence of two types of pseudogenes, namely, Cε2 (IGHEP1) which is a truncated-type pseudogene and Cε3 (IGHEP2) which is a processed-type pseudogene (Fig. 3). Moreover, the phylogenetic relationships among human and the great apes based on the gene trees in relation to the processed pseudogene Cε3 were documented (Ueda *et al*., 1986, 1989). In the present evolutionary study I used the following seven primate species by employing the systems for the great apes (ISCN 1985), gibbons (Stanyon and Chiarelli, 1983; Stanyon *et al*., 1987), and Japanese macaque (Pearson *et al*., 1979; Stanyon *et al*., 1990): common chimpanzee (*Pan troglodytes*; PTR), pygmy chimpanzee (*Pan paniscus*; PPA), gorilla (*Gorilla gorilla*; GGO), orangutan (*Pongo pygmaeus*; PPY), white-handed gibbon (*Hylobates lar*; HLA), agile gibbon (*Hylobates agilis*; HAG), and Japanese macaque (*Macaca fuscata*; MFU).

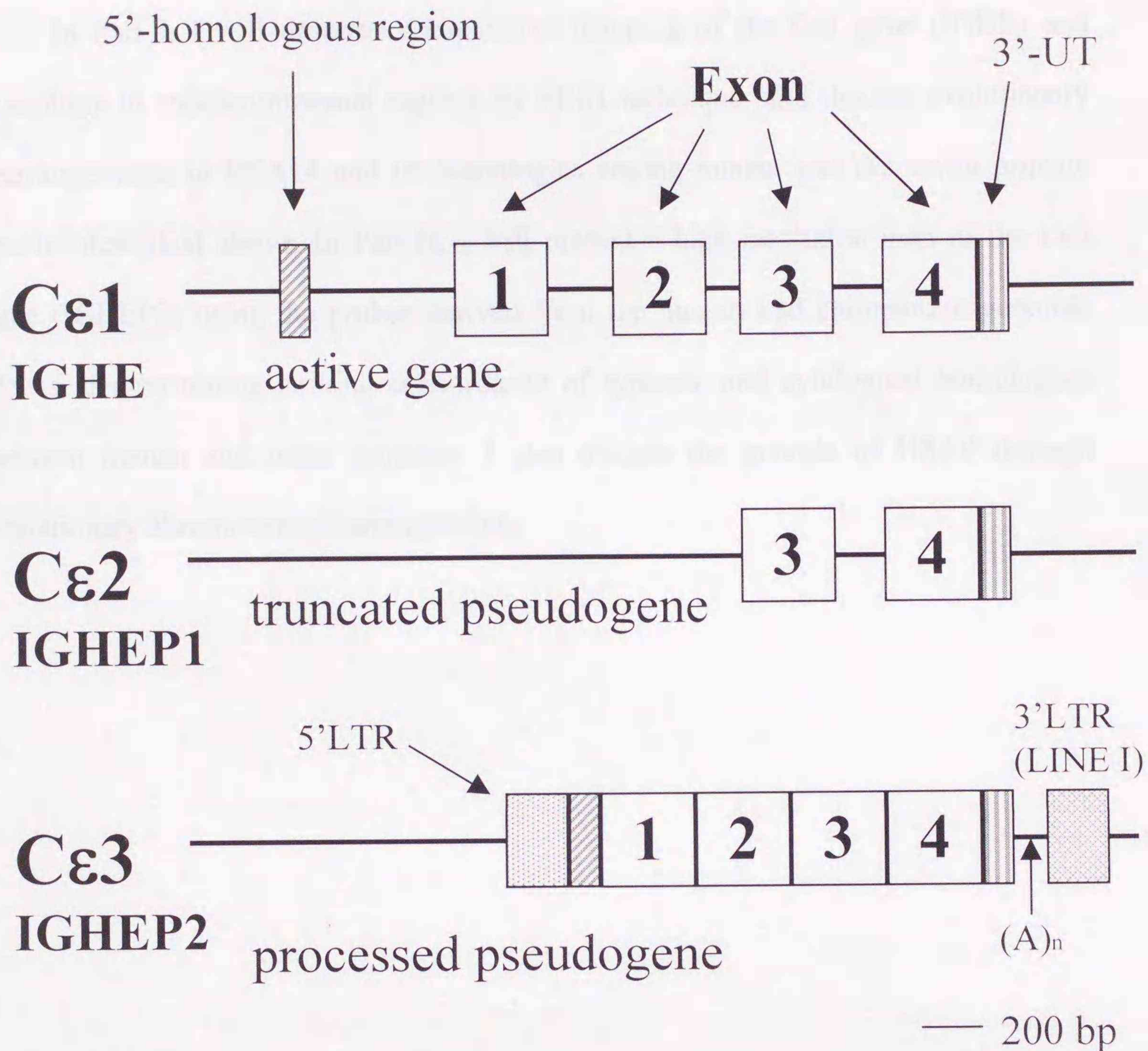


Fig. 3. Schematic representation of the structures of the Cε1, Cε2, and Cε3 genes

Schematic representation of the structures of the human Cε genes. The Cε1 gene (IGHE) is only active gene in the human genome. The Cε2 gene (IGHEP1) is categorized as a truncated pseudogene that lacks the 5' upstream region including exon 2. The Cε3 gene (IGHEP2) lacks the three introns entirely and has an A-rich sequence followed by the 3'-untranslated region so far indicating that the Cε3 gene is categorized as a processed pseudogene. The Cε3 gene has also the 5' homologous segment (100bp) with the Cε1 gene and long terminal repeat (LTR)-like sequences in both 5'- and 3'-flanking regions which contain terminal inverted repeats. The open boxes indicate the exons. The hatched boxes indicate the homologous segment in the 5'-flanking region and the 3'-untranslated region, respectively. The dotted boxes indicate the 5' and 3' LTR-like sequences. The 3' LTR-like sequence is homologous to the LINE I DNA.

In Part I, I will describe comparative mapping of the Cε1 gene (IGHE) and homology in subchromosomal regions by FISH technique, and discuss evolutionary rearrangements in HSA14 and its homologies among human and the seven primate species described above. In Part II, I will present a high-resolution map of the Cε3 gene (IGHEP2) using the probes derived from the human and chimpanzee genomic DNAs, demonstrating striking concordance of syntenic and cytological homologues between human and other primates. I also discuss the genesis of HSA9 through evolutionary chromosome rearrangements.

Part I:

Comparative mapping of the Cε1 gene (IGHE) in higher primates by FISH: Syntenic organization of HSA14 with its primate homologues

Materials and methods

Cells and metaphase spreads

Chromosome preparations from the common chimpanzee (*Pan troglodytes*, PTR), white-handed gibbon (*Hylobates lar*, HLA), agile gibbon (*Hylobates agilis*, HAG), and Japanese macaque (*Macaca fuscata*, MFU) were obtained from cultured lymphocytes of male individual blood samples of each species (courtesy of Primate Research Institute, Kyoto University). Pygmy chimpanzee (*Pan paniscus*, PPA) and orangutan (*Pongo pygmaeus*, PPY) chromosomes were prepared from Epstein-Barr virus immortalized lymphoblastoid cell lines PapE2 (established from a male individual) and PopE1 (established from a male individual of F1 hybrid) that were kindly provided by Dr. Ishida, The University of Tokyo (Ishida, 1991; Ishida and Yamamoto, 1987). Gorilla (*Gorilla gorilla*, GGO) chromosomes of a female individual were prepared from skin fibroblasts that were also kindly provided by Dr. Ishida. Human metaphase spreads were also obtained from cultured blood lymphocytes of healthy human males and females. Blood lymphocytes were cultured in RPMI1640 medium (Nissui) with 10% FBS (JRScientific; heat inactivated fetal bovine serum), mitogen, and 7.5% total blood at 37 ℃ for 70 hours. Phytohemagglutinin-M (PHA-M; Difco, final 2.5%) or concanavalin A (Con-A; Sigma, final 25μg/ml; only for Japanese macaque) was used as mitogen. Colcemid solution (GibcoBRL) was applied at final concentration of 0.04μg/ml for last 2 hours incubation and harvested cells were suspended in the hypotonic solution (0.075M KCl) for 25 min and fixed with methanol-acetic acid (3:1) fixative. Cell suspension was dropped on the slide and air-dried. The slides were stored in the freezer until using.

QFQ technique

McIlvaine's buffer (0.05 M citric acid/0.15 M Na_2HPO_4/pH 7.0) and Quinacrine/Hoechst staining solution (Quinacrine musturd (Sigma, 50μg/ml)/Hoechst 33258 (Sigma, 0.5μg/ml) in McIlvaine's buffer) were prepared and QFQ technique was performed by the methods of Caspersson *et al*. (1970), Yoshida *et al*. (1975), and Verma and Babu (1989) with slightly modifications. The slides were stained in Quinacrine/Hoechst staining solution for 30 min in dark, rinsed in distilled water and McIlvaine's buffer, and sealed with the cover glass after mounting the McIlvaine's buffer. The slides were observed under a fluorescence microscope and the metaphases were photographed. The photographed metaphases were superimposed on the same images with fluorescent signals after FISH procedure. Banded slides were destained in methanol-acetic acid (3:1) mixture and dried through a series of ethanol (70% and then absolute) prior to FISH procedure.

DNA Probe

A phage clone of the human genomic Cε1 gene, Ch4A-H-Igε-12 (Nishida *et al*., 1982), that was kindly provided by Dr. Ueda, The University of Tokyo, was used as the DNA probe for IGHE. The specific hybridization of this probe with orthologous Cε1 gene, IGHE, of nonhuman primates has been established (Ueda *et al*., 1985). This probe was labelled with biotin-11-dUTP (Enzo) by nick translation (Nick Translation Kit; Boehringer) and purified through a Sephadex G-50 spin column (Boehringer). The Cy3 labelled human chromosome 14 specific DNA (Amersham) and SpectrumGreen labeled chromosome 9 specific DNA (Vysis) were also used as the whole chromosome painting probes. The labelled probe DNA (100ng) was precipitated by ethanol with human Cot-1 DNA (GibcoBRL; 10μg) and sonicated salmon sperm DNA as a carrier (Sigma; 2.5μg) and disolved in 100% formamide (BRL; Specialty Reagent).

FISH procedure

FISH was carried out as described in detail elsewhere (Lichter *et al.*, 1988; Pinkel *et al.*, 1986; Takahashi *et al.*, 1990; Viegas-Péquignot *et al.*, 1989) with some modifications (Fig. 4). The chromosome spreads were heat-denatured, dehydrated through a series of ethanol (70% and then absolute), and mounted by the hybridization mixture including the DNA probe that was heat-denatured in advance. After hybridization (incubation in a humid chamber at 37 ℃ for 20 hours or more), the slides were washed by a series of incubation in 50% formamide/2xSSC and 2xSSC at 42℃ (1xSSC: 0.15M NaCl/0.015M sodium citrate ($Na_3C_6H_5O_7$)/pH7.0). The detection system of flurescent signals after washing was according to the protocol of Cambio supplied, namely, the biotinylated DNA probe was visualized by FITC-conjugated avidin and biotinylated anti-avidin antibody combination system (Chromosome Painting Kit; Cambio). The metaphases were counterstained with 0.5μg/ml 4'6'-diamidino-2-phenylindole (DAPI; Sigma) and 1μg/ml propidium iodide (PI; Sigma) by mounting the antifade medium (90% (v/v) glycerol (Merck), 1% (v/v) Evans Blue (Enzo), and 1% (w/v) 1,4 diazabicyclo-(2.2.2)octane (DABCO; Sigma) in 2xSSC). QFQ-banded metaphases were photographed beforehand and superimposed after FISH procedure with flurescent signals on the same metaphases. To localize the Cε1 gene (IGHE) on these primate chromosomes, approximately 40 metaphases per species were scored and one or two fluorescent signals detected on the specific positions of one pair of chromosomes were counted. Chromosome banded regions in the primate chromosomes of IGHE were assigned by using standard or proposed karyotypes of these species as references (Pearson *et al.*, 1979; Stanyon and Chiarelli, 1983; ISCN 1985; Stanyon *et al.*,1987, 1990; Jauch *et al.*, 1992; see Fig. 5).

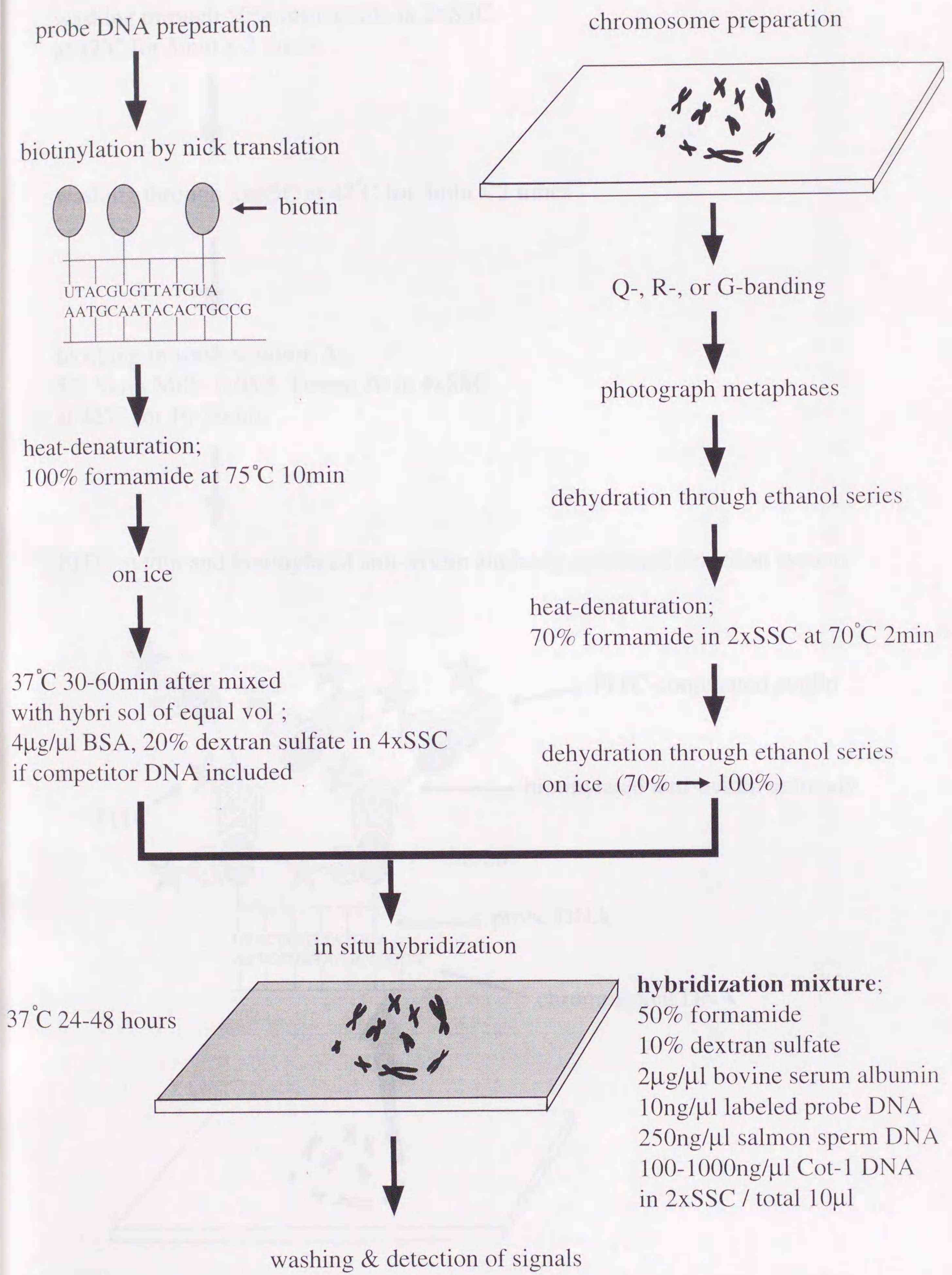


Fig. 4. Schematic representation of FISH procedure:
(a) hybridization procedure

washing through 50% formamide in 2xSSC
at 42°C for 5min x 2 times

washing through 2xSSC at 42°C for 5min x 2 times

blocking in wash solution A;
5% Skim Milk, 0.05% Tween 20 in 4xSSC
at 42°C for 10-30min

FITC-avidin and biotinylated anti-avidin antibody combined detection system

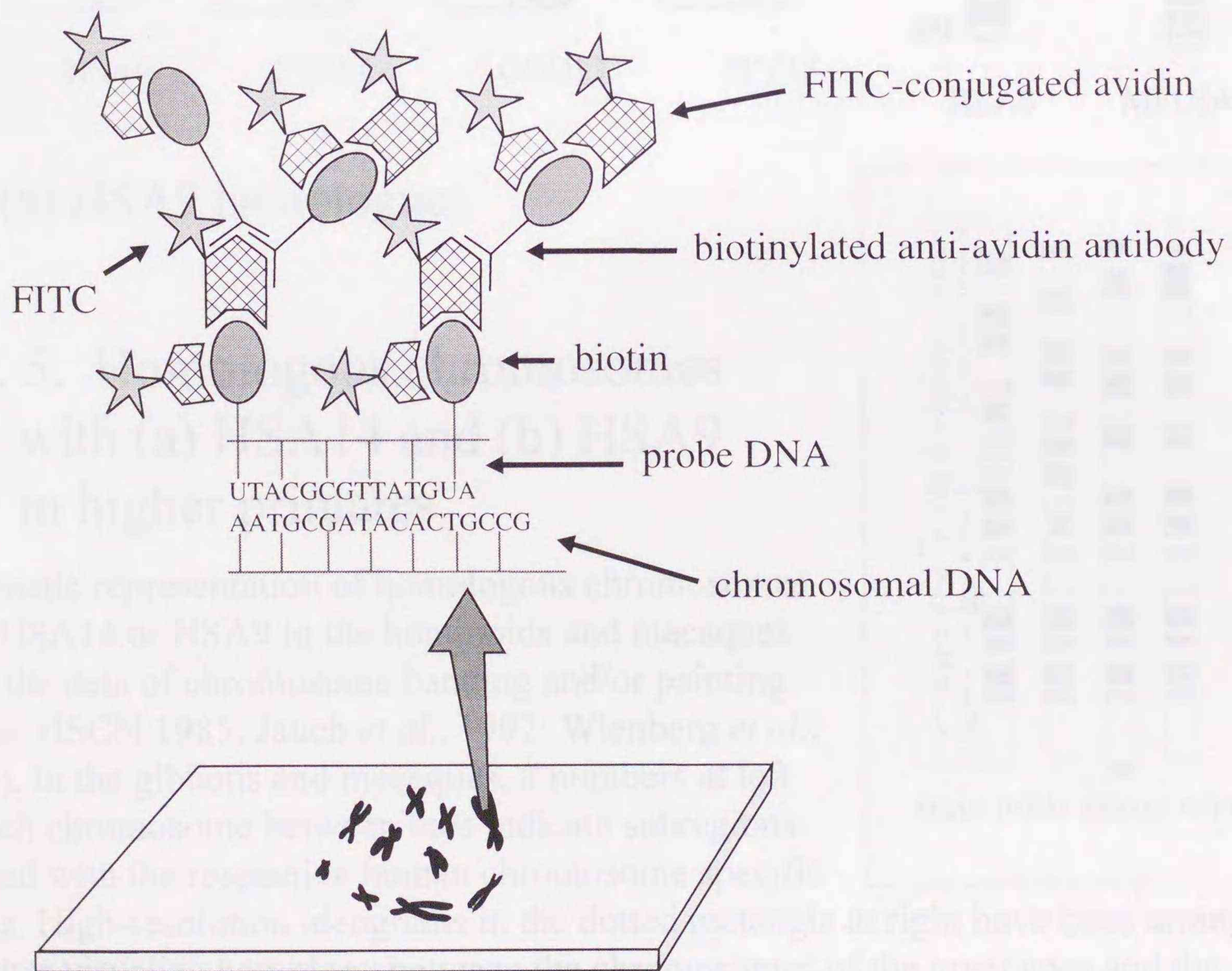
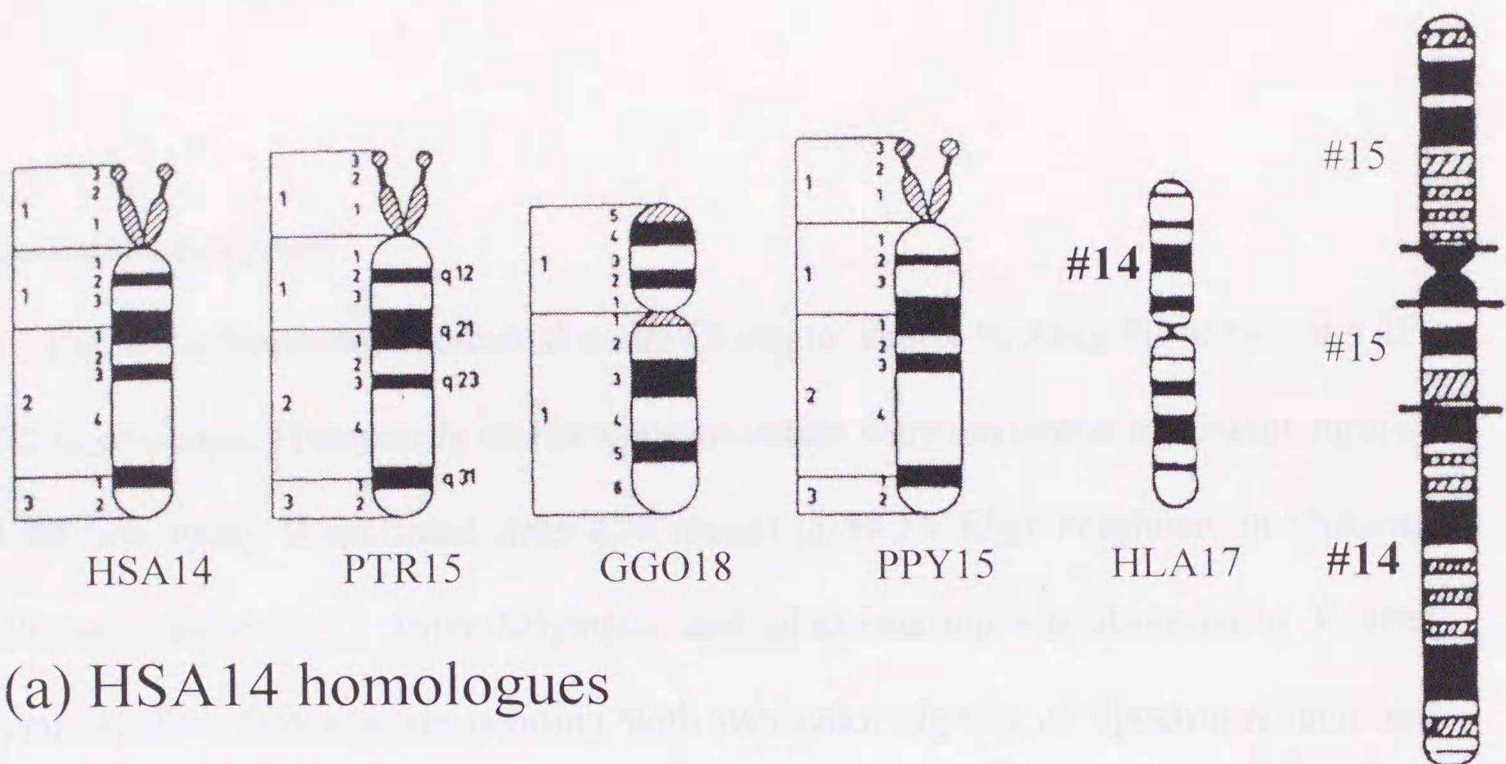
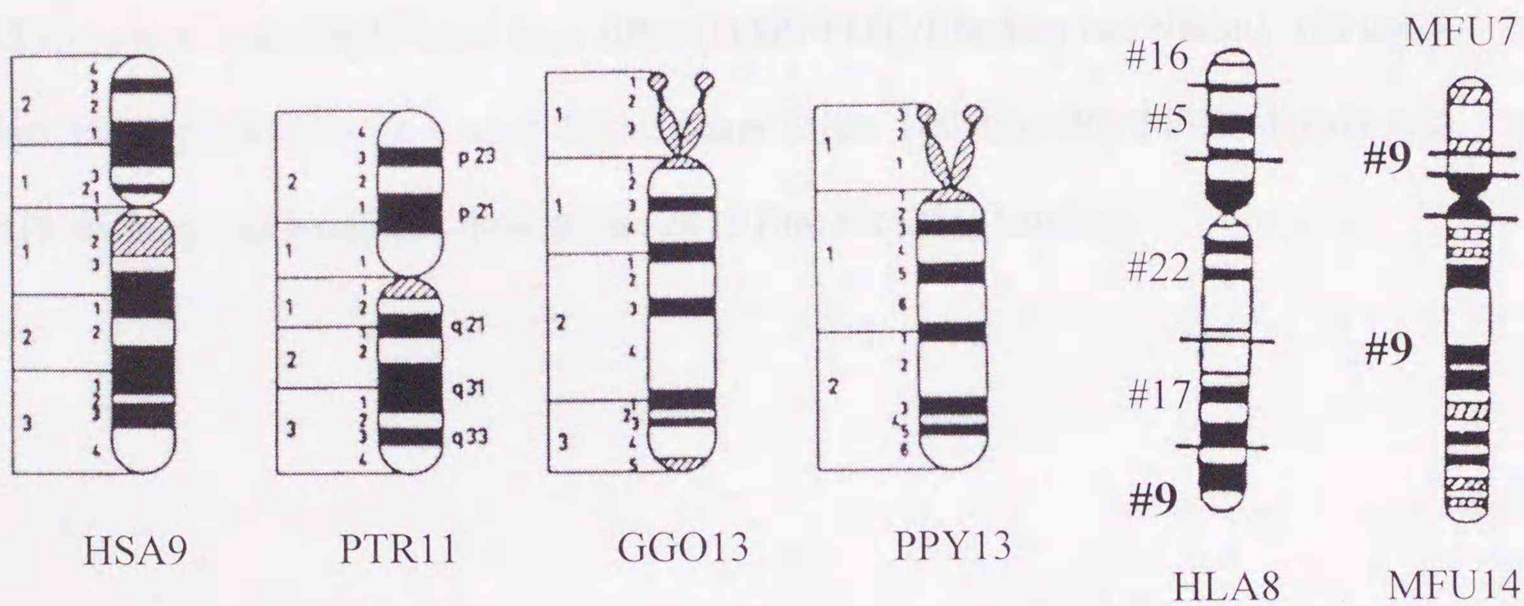


Fig. 4. Schematic representation of FISH procedure:
(b) fluorescence detection





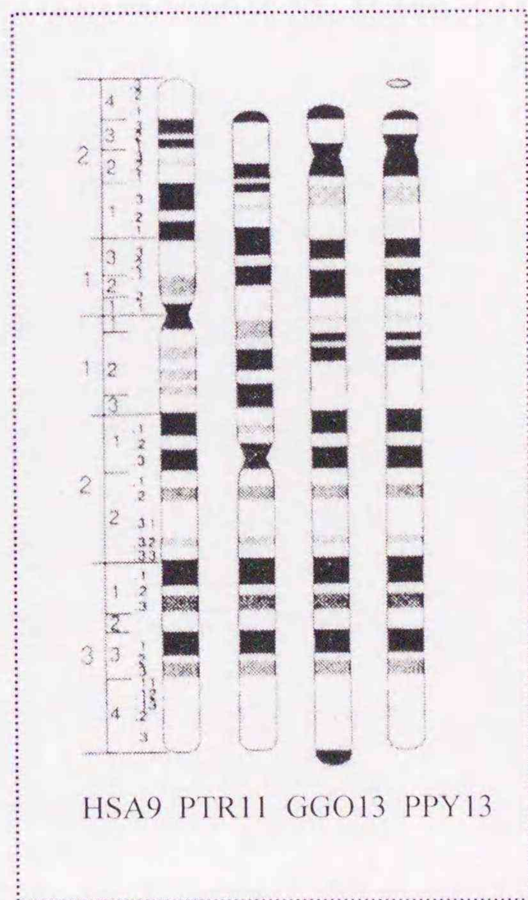


Fig. 5. Homologous chromosomes with (a) HSA14 and (b) HSA9 in higher primates

Schematic representation of homologous chromosomes with HSA14 or HSA9 in the hominoids and macaques from the data of chromosome banding and/or painting studies (ISCN 1985; Jauch *et al.*, 1992; Wienberg *et al.*, 1992). In the gibbons and macaques, # numbers at left of each chromosome between bars indicate subregions painted with the respective human chromosome specific paints. High-resolution ideograms in the dotted rectangle at right have been arranged to better visualize homology between the chromosomes of the great apes and the human complement that implying the evolutionary breakpoints involving pericentric inversions (Yunis and Prakash, 1982).

Microscopes and films

The metaphases were screened under Olympus Vanox-S, AH2-FL or Nikon Y2F-EFD2 microscope. Fluorescein and propidium iodide were excited at the wavelength of 450-490 nm using B-excitated filter (Olympus) or B-2A filter combination (Nikon). DAPI was excited by UV filter (Olympus) and QFQ-banding was observed by V-filter (Olympus). For chromosome painting with two color signals of SpectrumGreen and Cy3 fluorochromes, triple-band pass filter (DAPI/FITC/Rhodamine; Nikon) was used. Films photographed were Kodak Ektachrome Dyna 100 film for FISH signals and DAPI staining and Kodak Technical Pan 2415 film for QFQ banding.

Results

Comparative chromosome painting with human chromosome 14 DNA probe

I employed FISH with a human chromosome 14 specific composite DNA probe, WCP#14, to demonstrate homology between the human 14 and its counterpart in seven primate species. This probe produced uniform hybridization signals in one pair of whole chromosomes in each species of six primates except for Japanese macaque whose hybridization signals appeared on only a distal part of the long arm of a chromosome pair (Fig. 6). Each painted chromosome was identified by Q-banding as chromosome15 of both common (PTR15) and pygmy (PPA15) chimpanzees, chromosome18 of gorilla (GGO18), chromosome 15 of orangutan (PPY15), chromosome 17 of both white-handed (HLA17) and agile (HAG17) gibbons, and chromosome 7 of Japanese macaque (MFU7), respectively. The present painting results confirmed the homology previous works (ISCN 1985; Jauch *et al*., 1992).

Comparative mapping of IGHE in higher primates

To investigate whether syntenic region is maintained in these homologous chromosomes during evolution, I performed comparative mapping at the single gene level by FISH using a human Ch4A-H-Igε-12 probe for the human Cε1 gene (IGHE). This probe gave hybridization signals on human chromosome 14q32.3 (Fig. 7), confirming the localization of the IGH@ gene cluster. This probe also hybridized to specific loci on metaphase chromosomes of each species, indicating existence of homologous chromosome regions among these species. Hybridization signals were observed in telomeric regions of long arm of acrocentric chromosomes in both common and pygmy chimpanzees and orangutan, whereas in gorilla, both white-handed and agile gibbons, and Japanese macaque, signals were found in telomeric

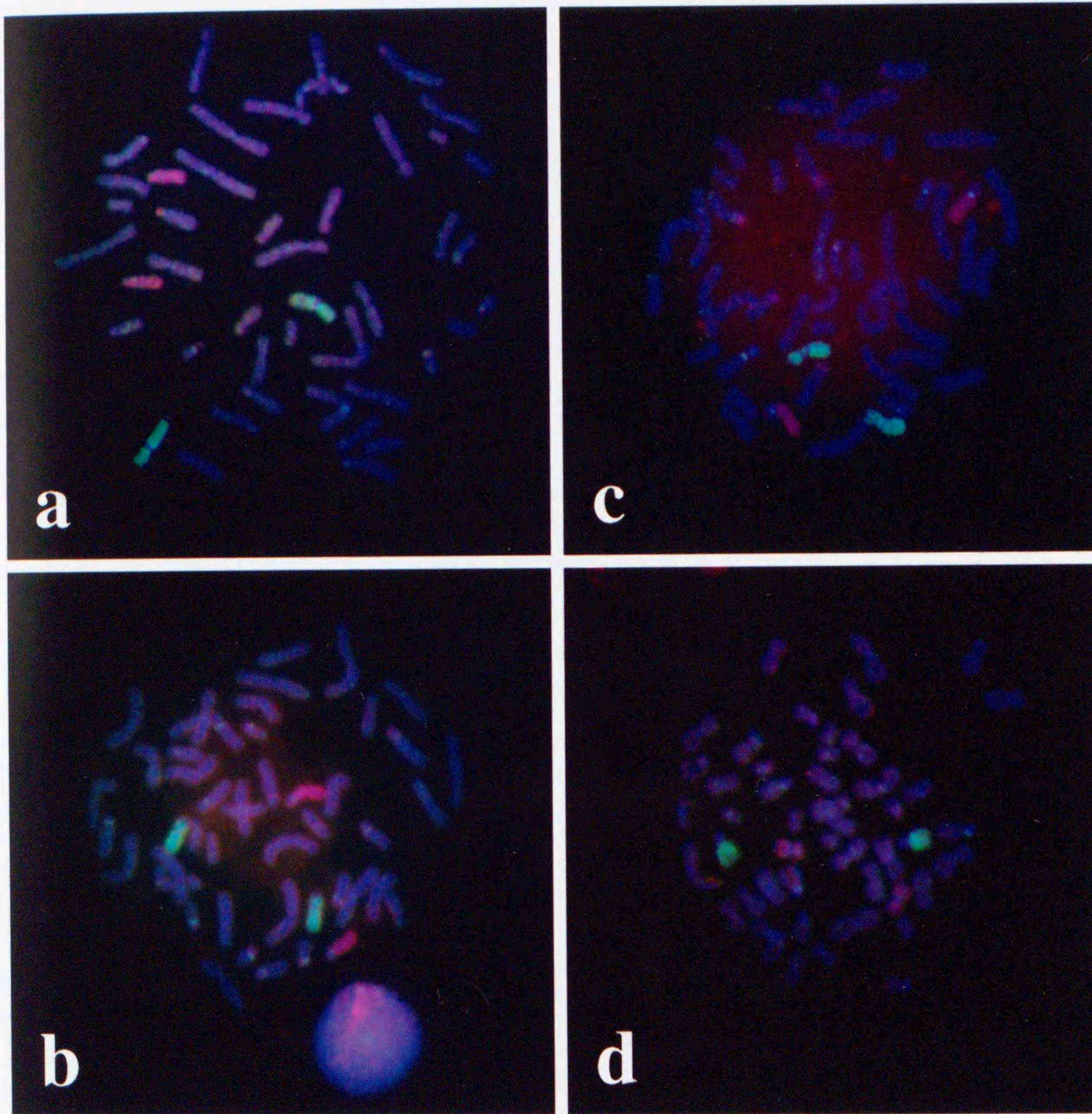


Fig. 6. Human and primate metaphases hybridized with WCP probes (WCP#14 and WCP#9)

Cy3-labelled WCP#14 and SpectrumGreen-labelled WCP#9 probes were hybridized to metaphases of human (**a**), common chimpanzee (**b**), pygmy chimpanzee (**c**), and gorilla (**d**).

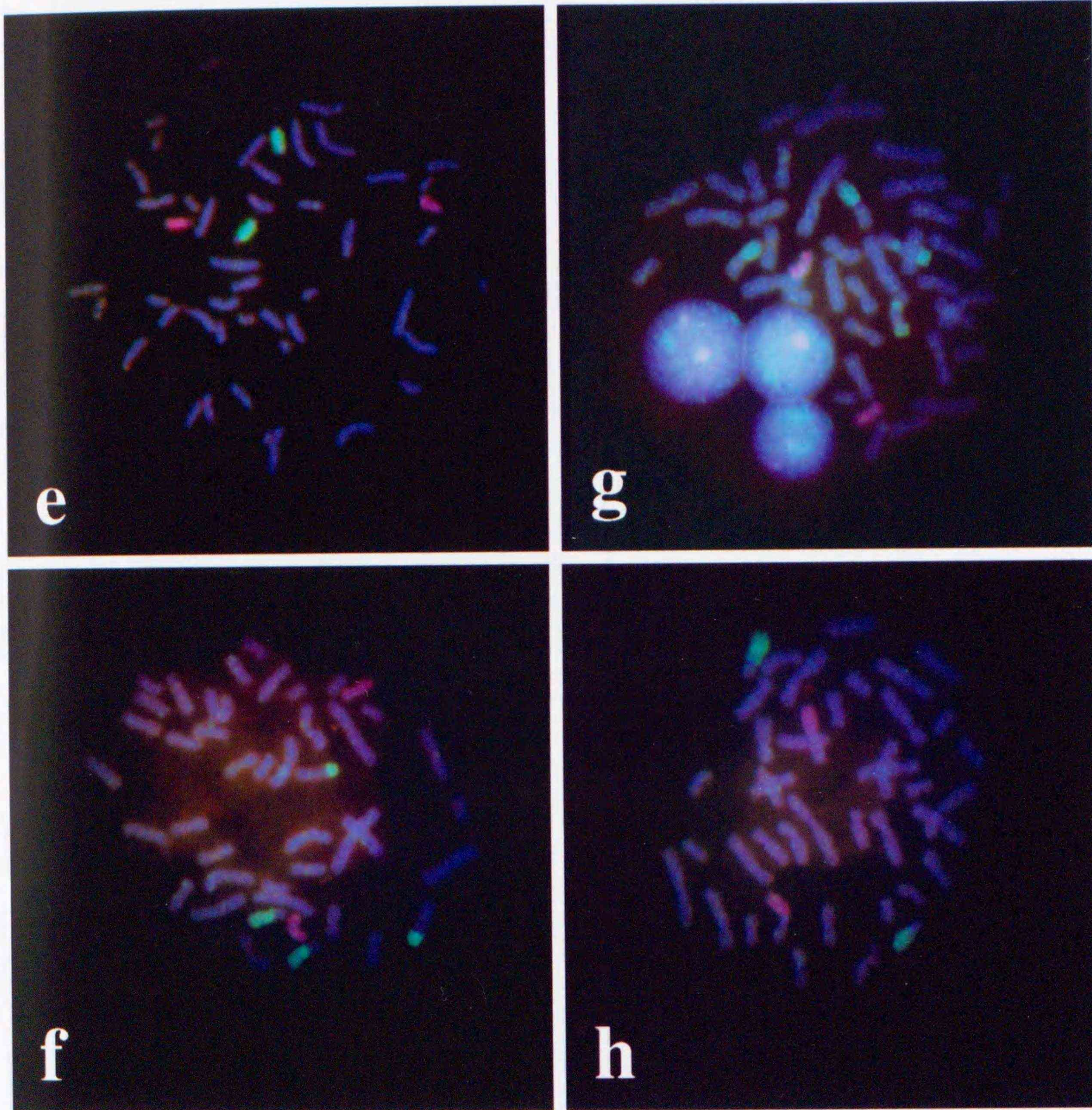


Fig. 6. Human and primate metaphases hybridized with WCP probes (WCP#14 and WCP#9)

Cy3-labelled WCP#14 and SpectrumGreen-labelled WCP#9 probes were hybridized to metaphases of orangutan (**e**), white-handed gibbon (**f**), agile gibbon (**g**), and Japanese macaque (**h**).

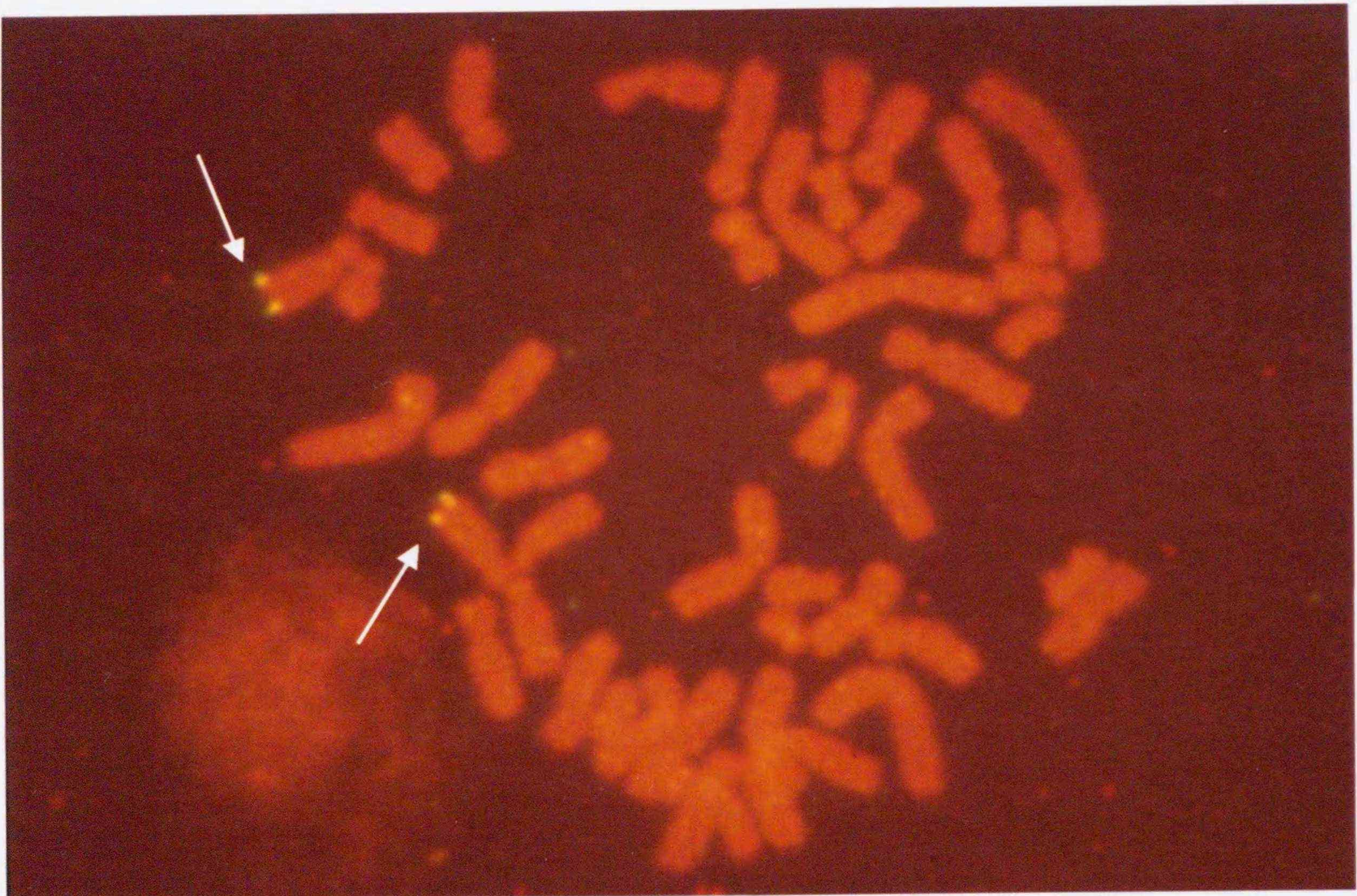


Fig. 7. Human metaphase hybridized with the Cε1 gene

Localization of the human Cε1 gene (IGHE) to the terminus region of D-group chromosomes, namely HSA14q32.3, by FISH. Arrows indicate the twin-spot fluorescent signals.

regions of submetacentric chromosomes. The frequencies of cells showing single and double hybridization signals on each chromosome were 72.2% for common chimpanzee, 66.7% for pygmy chimpanzee, 72.4% for gorilla, 67.7% for orangutan, 60.6% for white-handed gibbon, 67.5% for agile gibbon, and 62.9% for Japanese macaque, respectively. After chromosome identification by Q-banding, IGHE were mapped to bands 15q32 in both chimpanzees (PTR15 and PPA15), 18q16 in gorilla (GGO18), 15q32 in orangutan (PPY15), 17qter in both gibbons (HLA17 and HAG17), and 7q29 in Japanese macaque (MFU7) (Figs. 8-14). Therefore, these results indicate syntenic conservation in homologous chromosome segments among primate species here examined. A summary of FISH mapping data is given in Fig. 15.

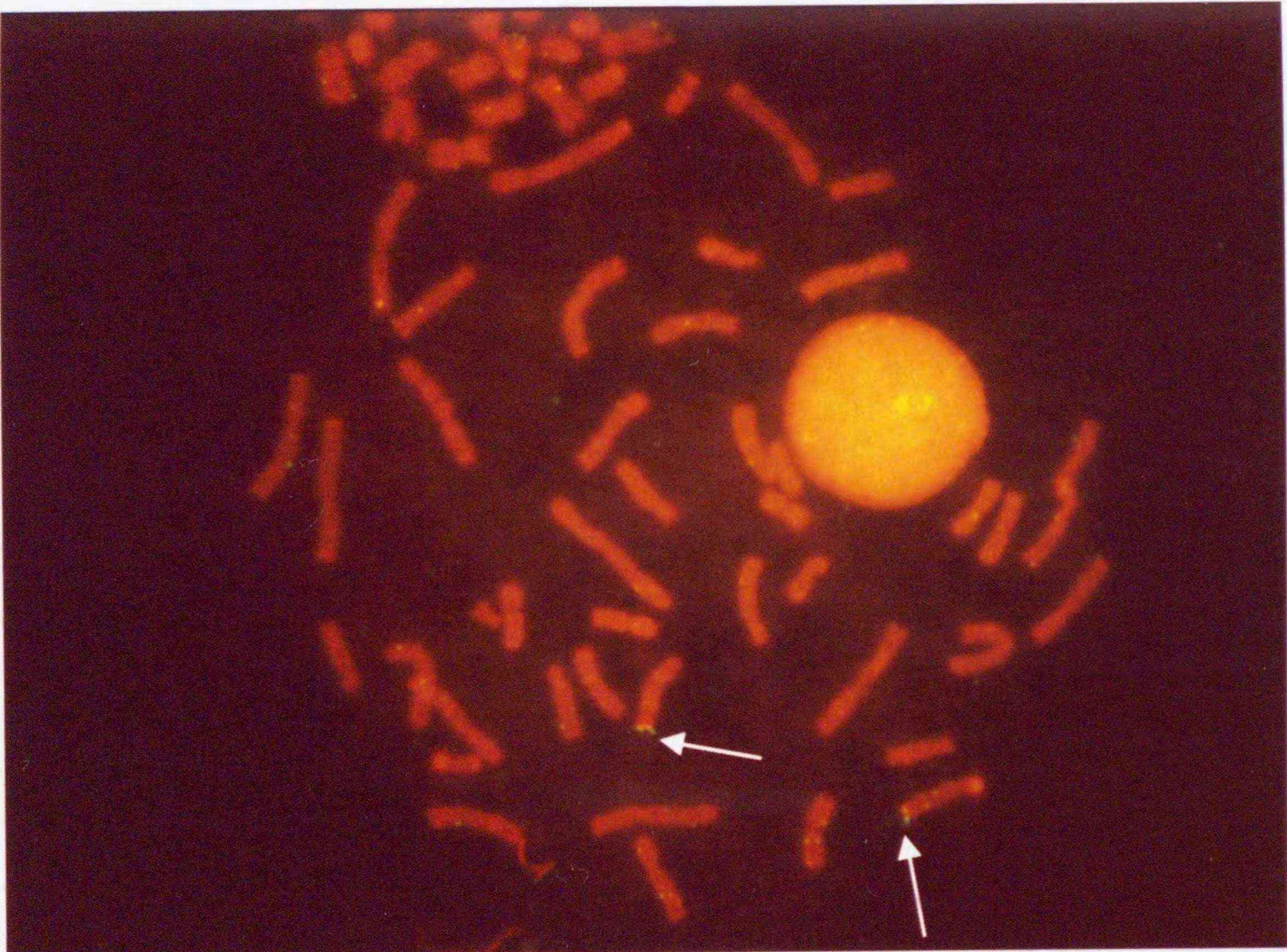


Fig. 8. Common chimpanzee metaphase hybridized with the Cε1 gene

Localization of the common chimpanzee orthologous Cε1 gene (IGHE) to PTR15q32 by FISH. The white arrows indicate the twin-spot fluorescent signals.

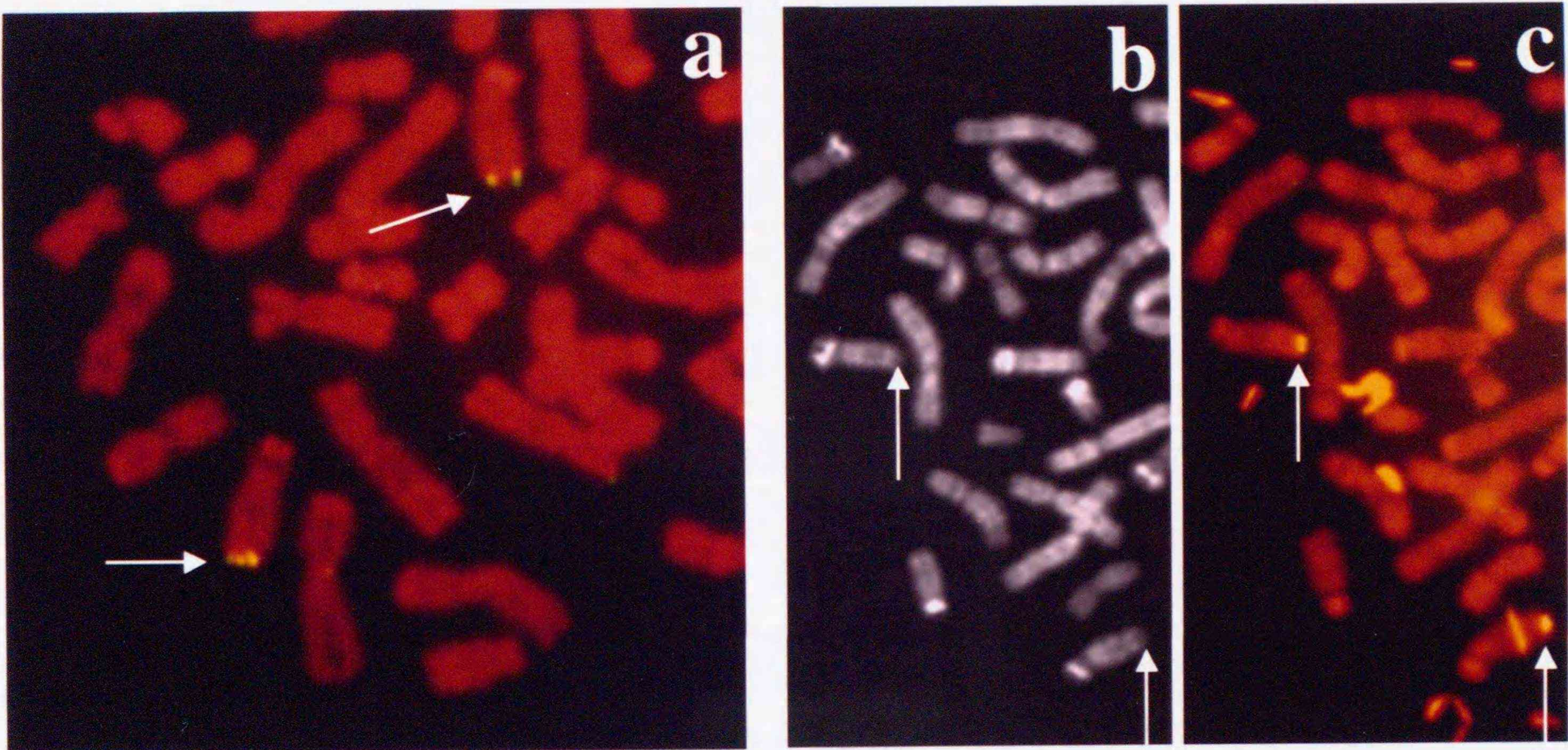


Fig. 9. Pygmy chimpanzee partial metaphases hybridized with the Cε1 gene combined with Q-banding

Localization of the pygmy chimpanzee orthologous Cε1 gene (IGHE) to PPA15q32 by FISH. The Q-banded image (**b**) is the same as that of (**c**). Arrows indicate the fluorescent signals (**a**, **c**) and the banded same positions (**b**).

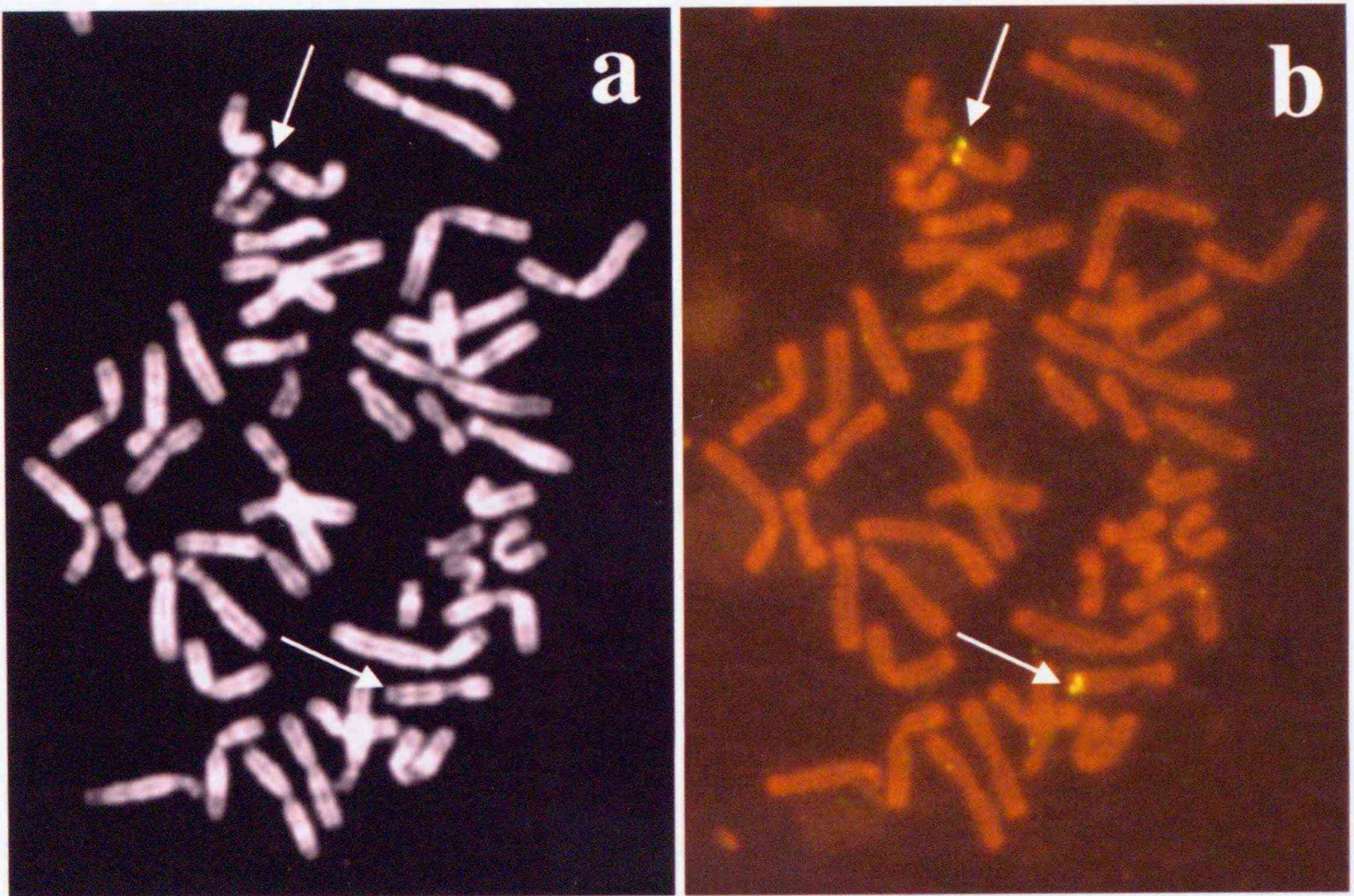


Fig. 10. Gorilla metaphase hybridized with the Cε1 gene combined with Q-banding

Localization of the gorilla orthologous Cε1 gene (IGHE) to GGO18q16 by FISH (**b**) combined with Q-banding (**a**). Arrows indicate the fluorescent signals (**b**) and the same positions (**a**).

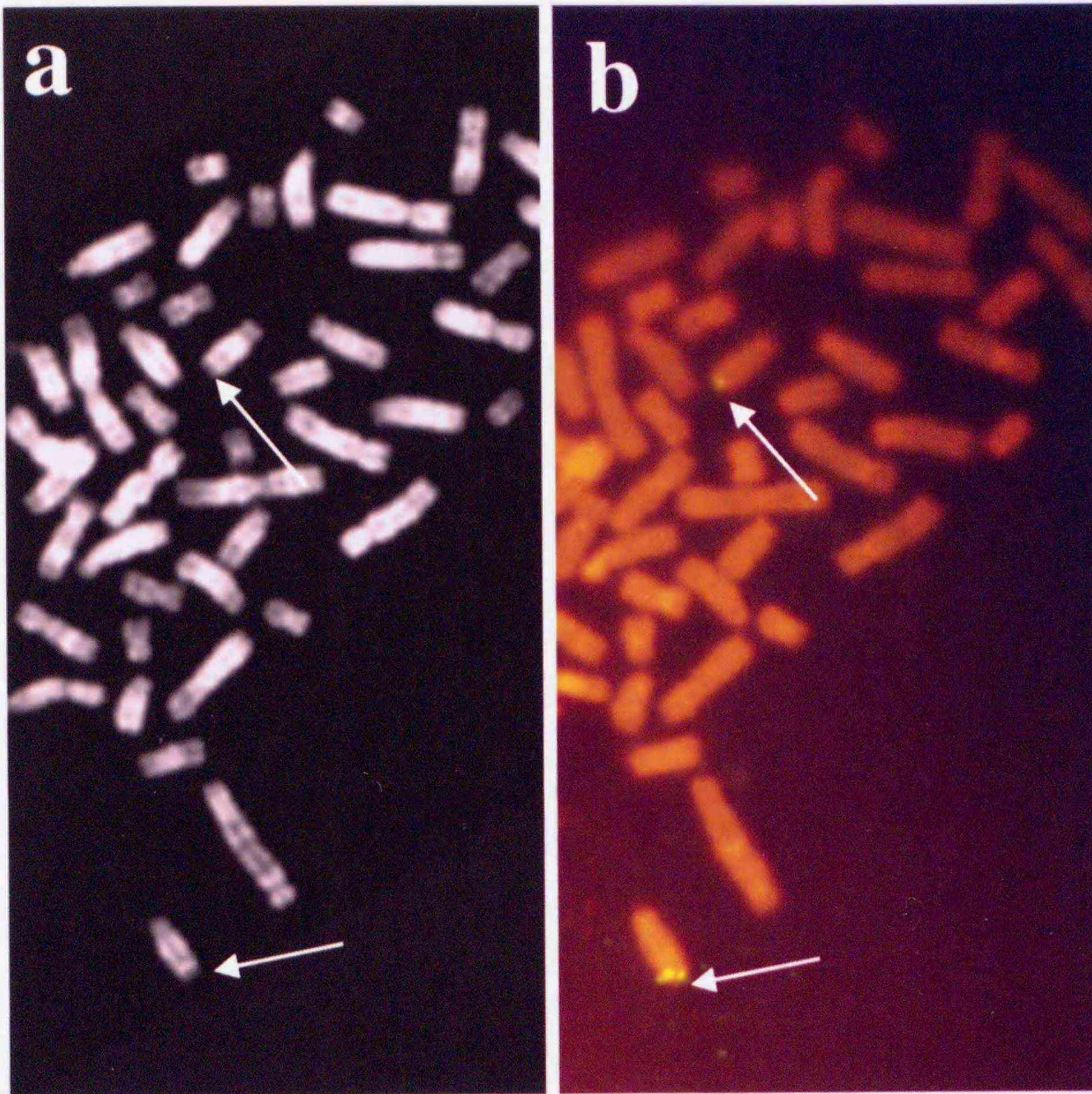


Fig. 11. Orangutan partial metaphase hybridized with the Cε1 gene combined with Q-banding

Localization of the orangutan orthologous Cε1 gene (IGHE) to PPY15q32 by FISH (**b**) combined with Q-banding (**a**). Arrows indicate the fluorescent signals (**b**) and the same positions (**a**).

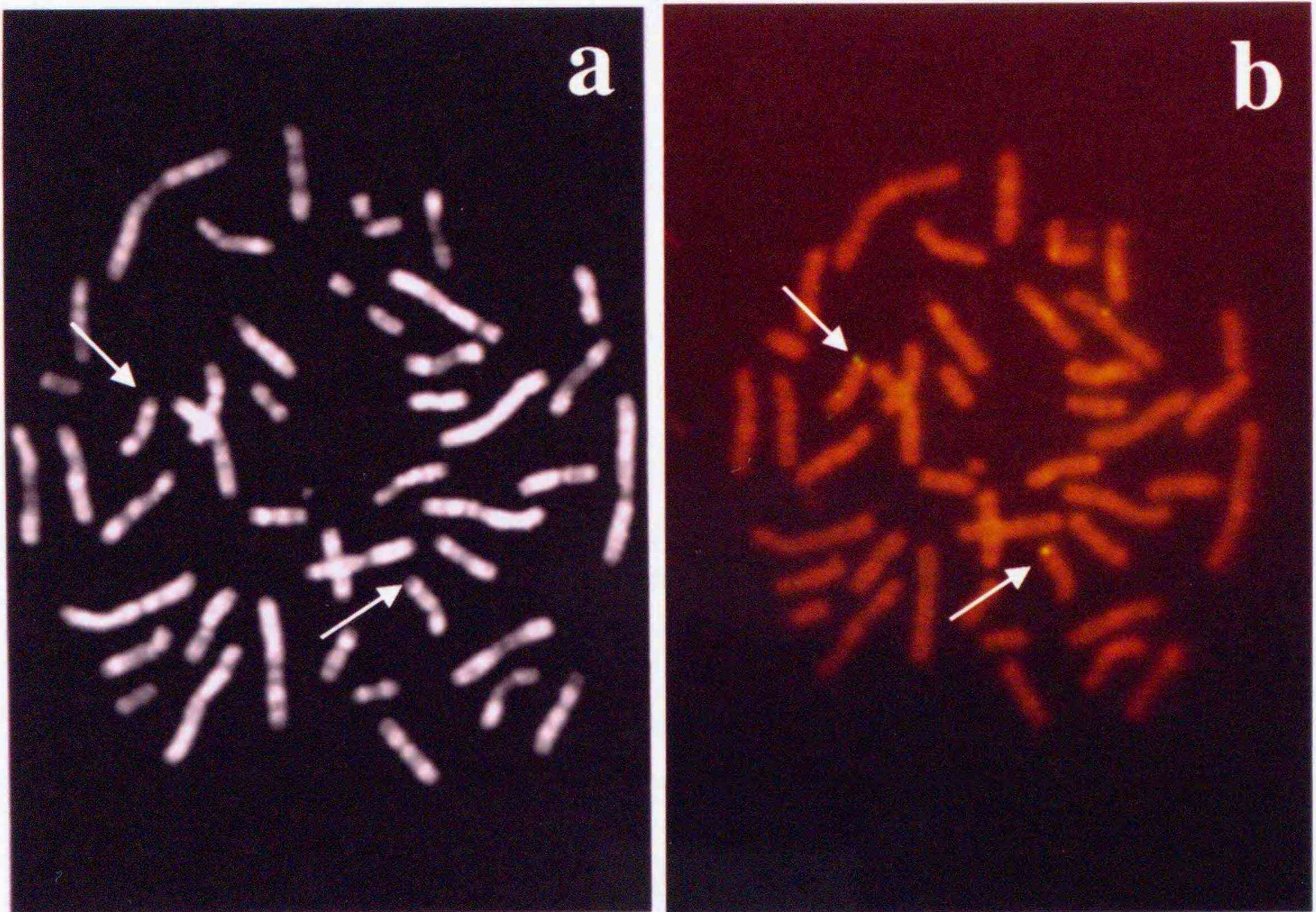


Fig. 12. White-handed gibbon metaphase hybridized with the Cε1 gene combined with Q-banding

Localization of the white-handed gibbon orthologous Cε1 gene (IGHE) to HLA17qter by FISH (**b**) combined with Q-banding (**a**). Arrows indicate the fluorescent signals (**b**) and the same positions (**a**).

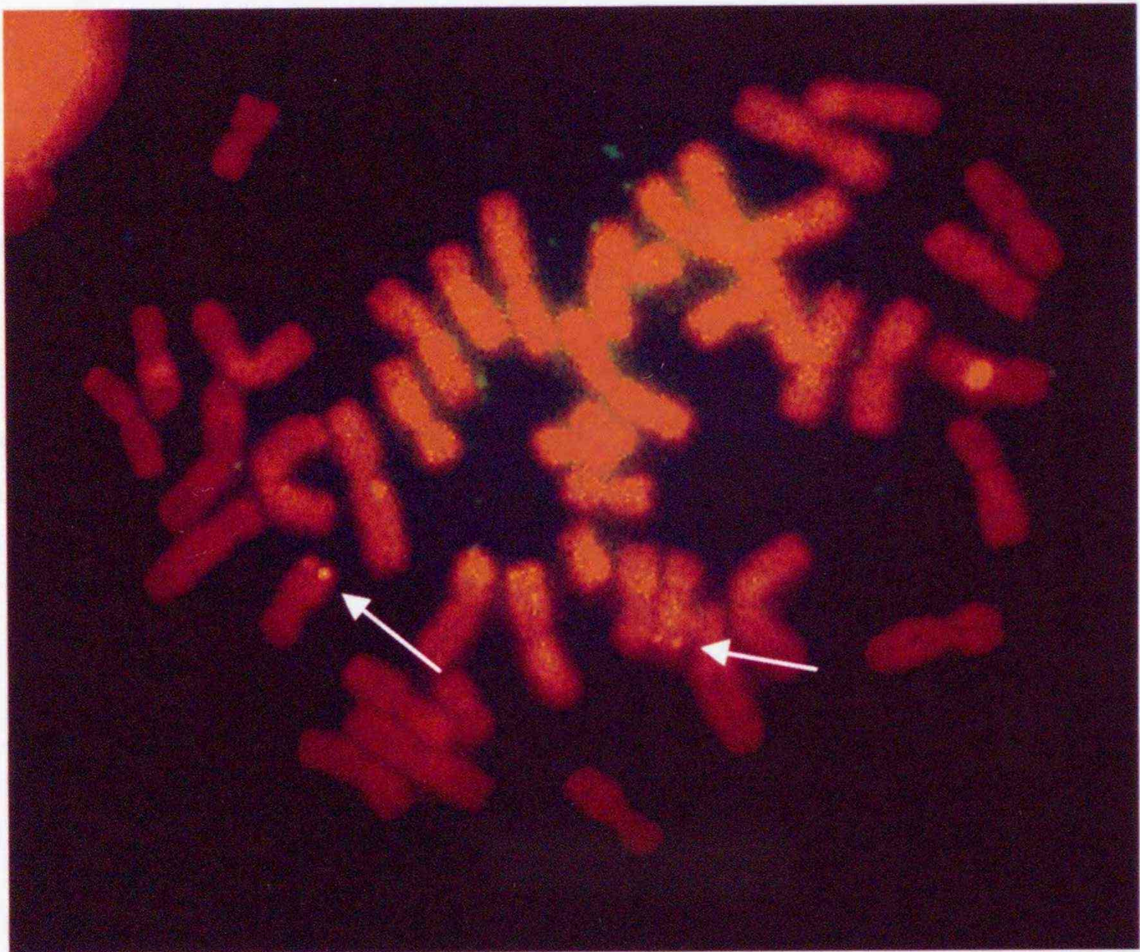


Fig. 13. Agile gibbon metaphase hybridized with the Cε1 gene

Localization of the agile gibbon orthologous Cε1 gene (IGHE) to HAG17qter by FISH. Arrows indicate the twin-spot fluorescent signals.

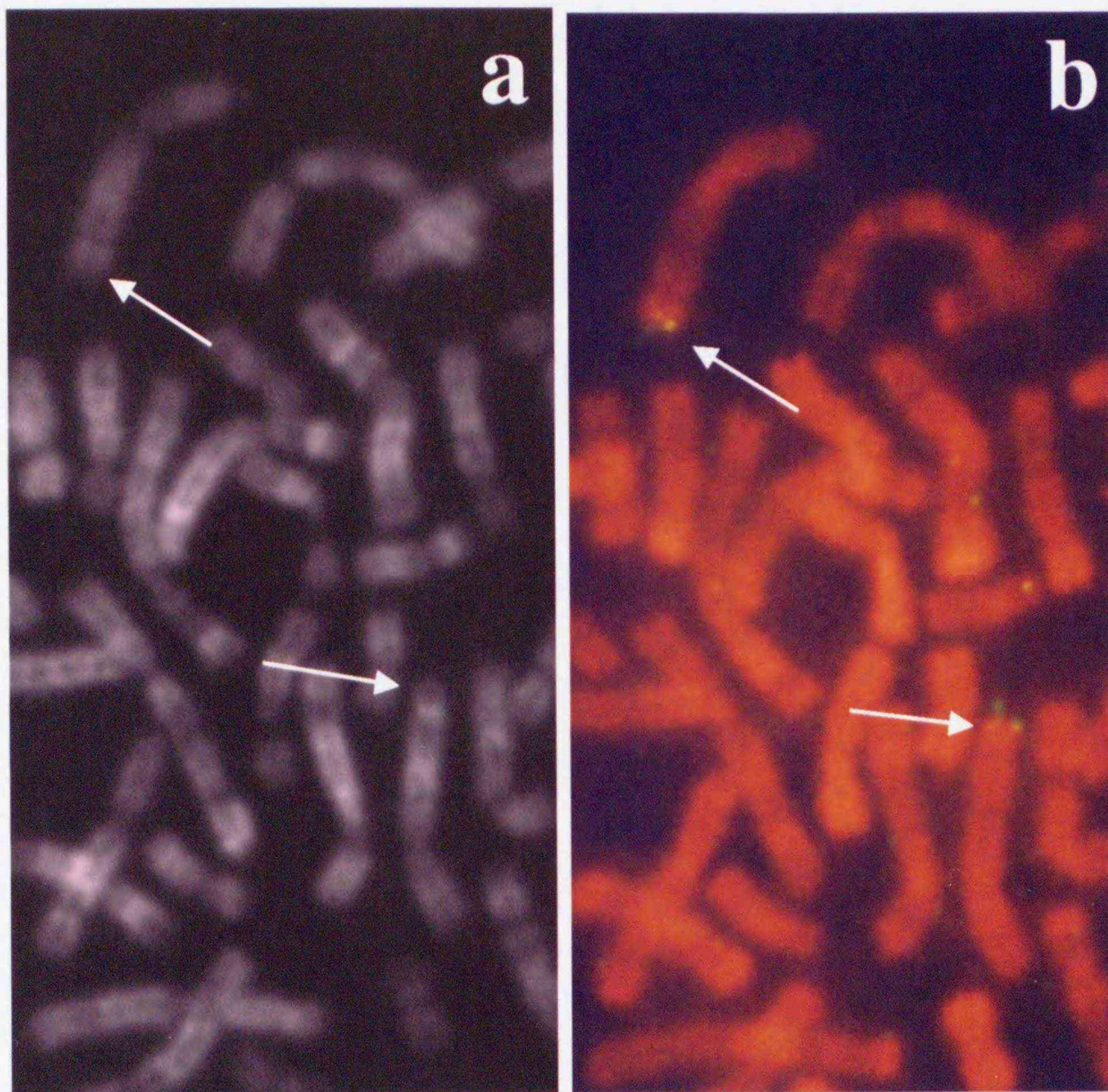


Fig. 14. Japanese macaque partial metaphase hybridized with the Cε1 gene combined with Q-banding

Localization of the Japanese macaque orthologous Cε1 gene (IGHE) to MFU7q29 by FISH (**b**) combined with Q-banding (**a**). Arrows indicate the fluorescent signals (**b**) and the same positions (**a**).

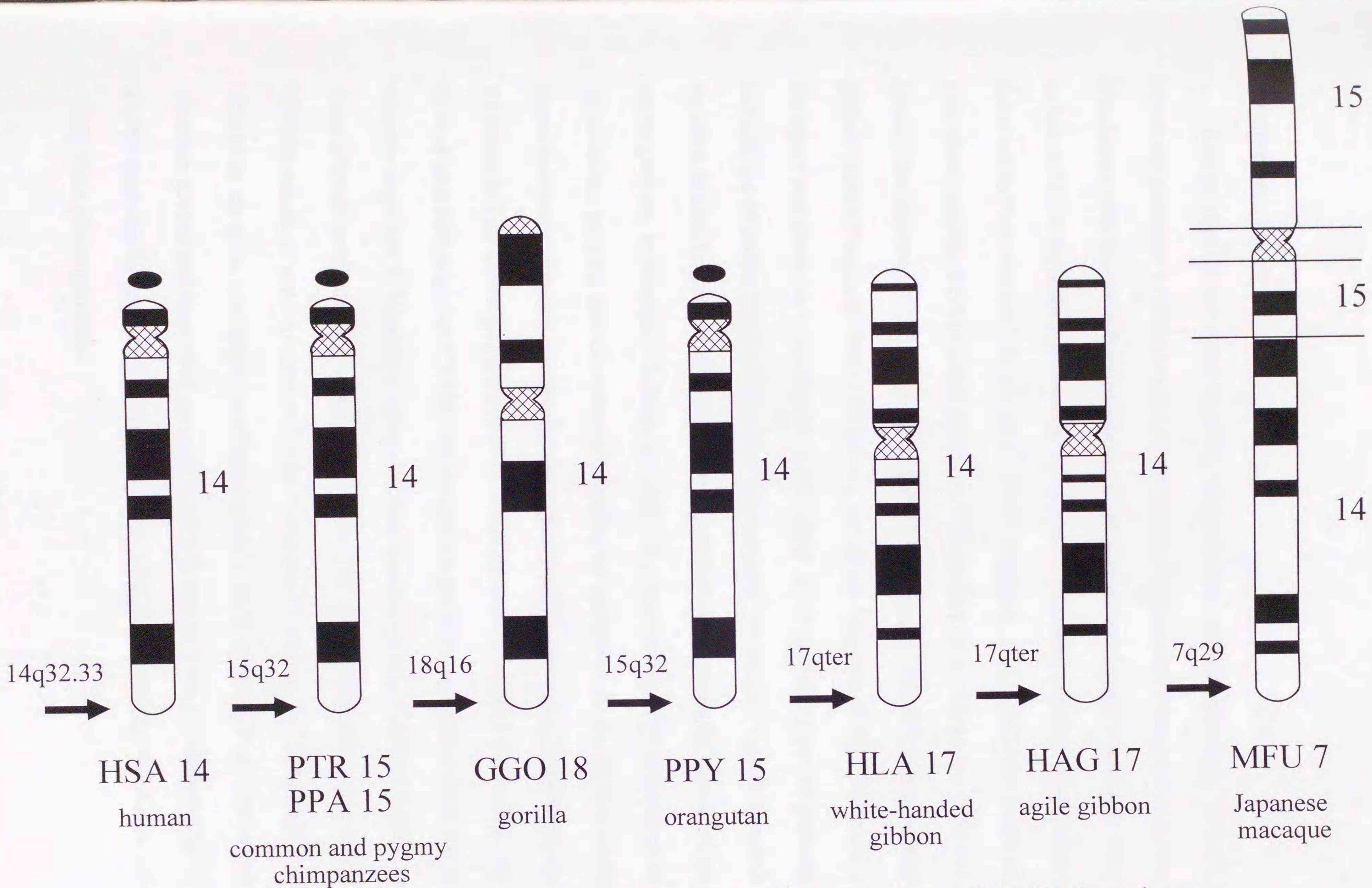


Fig. 15. Ideogramatic representation of comparative mapping of IGHE in primates

Ideogramatic representation of the localization of primate Cε1 genes, IGHE. Arrows indicate the position of IGHE; the chromosome band number and species common name are given below each arrow. Numbers at the right of each chromosome indicate subregions painted with human chromosome 14- or 15-specific library referred to in the references (Jauch *et al.*, 1992; Wienberg *et al.*, 1992).

Discussion

Comparative chromosome banding analysis has shown homology between human chromosome 14 (HSA14) and its equivalent chromosome of the great apes, but homologies with the white-handed gibbon, agile gibbon, and Japanese macaque were unclear until the application of recently developed chromosome painting techniques as described in "Introduction" (Jauch *et al.*, 1992; Wienberg *et al.*, 1992). As proposed after these studies, HSA14 corresponds to PTR15 (common chimpanzee), PPA15 (pygmy chimpanzee), GGO18 (gorilla), PPY15 (orangutan), HLA17 (white-handed gibbon), HAG17 (agile gibbon), and a distal part of the long arm of MFU7 (Japanese macaque) and these were confirmed in this study. Although chromosome painting methods are effective for identification of chromosome homologies, further approach for more detailed analysis to identify syntenic chromosome segments for chromosomal rearrangements is necessary. Indeed, a combined procedure of FISH techniques of chromosome painting and comparative mapping as performed in the present study demonstrated conservation of syntenic segments of IGHE locus among primate species. As shown in Fig. 15, the Cε1 gene of IGHE localized at the terminal region of the long arms of each homologue of HSA14. This assignment provides us a new information of syntenic segments at telomeric region and that human genome is closely similar to these primate species at the single gene level. The present data also suggest that GGO18 which is only a submetacentric chromosome among the homologues of HSA14 in the great apes might have been derived from in addition to one pericentric inversion at least one more changes at the telomeric regions during evolution (Fig. 5). Further analyses by comparative mapping using other DNA markers in HSA14 will clarify these rearrangements.

In contrast to the great apes which have diploid chromosome numbers of 48, the lesser apes show karyotypic variation: *Hylobates agilis* (2n=44), *Hylobates concolor* (2n=52), *Hylobates hoolock* (2n=38), *Hylobates klossii* (2n=44), *Hylobates lar* (2n=44), *Hylobates moloch* (2n=44), and *Symphalangus syndactylus* (2n=50). Moreover, these lesser apes show little similarity of chromosome banding patterns to human chromosomes (Chiarelli, 1972; Tantravahi *et al*., 1975; Warburton *et al*., 1975; Stanyon and Chiarelli, 1982, 1983; Prouty *et al*., 1983; Van Tuinen and Ledbetter, 1983; Stanyon *et al*., 1987). Painting with 22 human autosome libraries to these hylobatid metaphases showed considerably complicated chromosomal rearrangements including numerous translocations in hylobatid chromosomes (Jauch *et al*., 1992; Koehler *et al*., 1995a, 1995b). These results demonstrated that the 22 autosomes have been divided into 51 elements to composing the 21 gibbon autosomes (2n=44) (Jauch *et al*., 1992), while at least 33 translocations have occurred in the siamong (*Symphalangus syndactylus*, recently changed to *Hylobates syndactylus*; 2n=50) (Koehler *et al*., 1995a). In spite of many rearrangements between human and gibbon chromosomes, the present painting studies with the HSA14 DNA probe to white-handed gibbon (HLA) and agile gibbon (HAG) gibbon metaphases showed that only each chromosome 17 of white-handed gibbon (HLA17) and agile gibbon (HAG17) was painted, although HSA14 and these gibbon chromosome 17s are morphologically different, indicating no interchromosomal rearrangements. However, the present comparative mapping of IGHE and the morphological difference in HSA14 and gibbon chromosome 17s indicate the occurrence of complicated intrachromosomal rearrangements including at least one pericentric inversion event, since HSA14 is acrocentric and both HLA17 and HAG17 are submetacentric, and no banding

homology is found between HSA14 and HLA17 or HAG17, although further comparative mapping of many markers is necessary for details of intrachromosomal rearrangements. It is interesting to know why HSA14 homologues in gibbon karyotypes are highly conserved without interchromosomal rearrangements, because extremely high evolutionary rate and interchromosomal rearrangements have been noted in gibbon genomes (Marks 1982; Stanyon and Chiarelli, 1983; Stanyon *et al*., 1987; Jauch *et al*., 1992; Arnold *et al*., 1996).

In Japanese macaque, HSA14 corresponded to the distal part of the long arm of the macaque chromosome 7 (MFU7) by the painting studies (Wienberg *et al*., 1992; Stanyon *et al*., 1995) and by the chromosome banding studies (Dutrillaux *et al*., 1978; Soares *et al*., 1982; Creau-Goldberg *et al*., 1983; Small *et al*., 1985; Seuánez, 1987; Stanyon *et al*., 1990). The present comparative mapping of IGHE confirmed syntenic segment between HSA14 and MFU7. This study also indicated that the human genome is closely similar to that of the macaque at the single gene level.

The present comparative mapping of IGHE in higher primates supports the previous chromosome banding and painting studies and suggests that HSA14 has a high degree of syntenic organization with its homologues of the great apes, white-handed and agile gibbons, and Japanese macaque.

The combined procedure of ZOO-FISH and comparative mapping here used directly indicates regions of interspecies chromosome homology as well as syntenic segments at the DNA level. This means that chromosome rearrangements that occurred during primate evolution can be defined by these FISH techniques for better understanding of phylogenetic relationships.

Summary

To understand the karyotype evolution in human and higher primates, comparative mapping by FISH was performed using human Cε1 gene (IGHE) as a DNA probe. This gene is well investigated at the DNA level and will be a suitable probe for the analysis of evolutionary rearrangements due to that the multiple recombinational events have occurred in the IGH@ family during the course of primate evolution. All members of human IGH@ except for the Cε3 gene (IGHEP2) have been assigned onto HSA14q32.33. Therefore, molecular organization and evolution of HSA14 in relation to the region of IGH@ can be traced through comparative mapping of IGHE in seven species of nonhuman primates. The IGHE of the common chimpanzee (*Pan troglodytes*; PTR), pygmy chimpanzee (*Pan paniscus*; PPA), gorilla (*Gorilla gorilla*; GGO), orangutan (*Pongo pygmaeus*; PPY), white-handed gibbon (*Hylobates lar*; HLA), agile gibbon (*Hylobates agilis*; HAG), and Japanese macaque (*Macaca fuscata*; MFU) was assigned to the telomeric region of HSA14 homologues in each species, namely, to PTRl5q32 (coromon chimpanzee), PPAl5q32 (pygmy chimpanzee), GGO18ql6 (gorilla), PPY15q32 (orangutan), HLA17qter (white-handed gibbon), HAG17qter (agile gibbon), and MFU7q29 (Japanese macaque), respectively. These results indicated that IGHE provides a new telomeric DNA marker for nonhuman primates and supported the results from the previous chromosome banding and painting studies showing that HSA14 has high degree of syntenic organization with its primate homologues.

Part II:

Comparative mapping of the Cε3 gene (IGHEP2) in higher primates by FISH: Genealogical consideration for the evolutionary genesis of HSA9 and its primate homologues

Materials and methods

Metaphase spreads

Metaphase spreads of human and seven species of nonhuman primates (common chimpanzee (*Pan troglodytes*), pygmy chimpanzee (*Pan paniscus*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), white-handed gibbon (*Hylobates lar*), agile gibbon (*Hylobates agilis*), and Japanese macaque (*Macaca fuscata*)) were obtained in the same manner as described in the section of "Materials and methods" in Part I. For high-resolution banding patterns in human chromosomes, lymphocyte cultures were treated with ethidium bromide (5-10μg/ml) and colcemid (0.02μg/ml) for last 2 hours before harvesting as described (Ikeuchi and Sasaki, 1979; Ikeuchi, 1984).

QFQ technique

QFQ technique was performed by the same manner as described in the section of "Materials and methods" in Part I. Approximately 30-40 metaphases from each species were photographed and then superimposed on the same images with fluorescent signals after FISH procedure. Banded slides were destained in methanol-acetic acid (3:1) mixture and dehydrated through a series of ethanol (70% and absolute) prior to FISH procedure.

RBG technique

Lymphocytes synchronized with excess thymidine were allowed to incorporate BrdU as described (Latt,1973; Dutrillaux *et al*.,1976; Verma and Babu,1989; Takahashi *et al*., 1990) with a minor modification. Briefly, peripheral blood lymphocytes were cultured in TC199 medium (Nissui) with 10% heat-inactivated fetal bovine serum (FBS; JRScientific), and 2.5% PHA-M (Difco) at 37 ℃. Thymidine (Sigma; final 300μg/ml) was added to the cultures, and 15.5 hr later cells were washed and BrdU

(Sigma; final 25μg/ml) was added. Colcemid solution (GibcoBRL) was applied at final concentration of 0.04μg/ml for the last 30 min incubation. Chromosome preparations made in the same manner as described before in Part I were stained with Hoechst 33258 solution (lμg/ml in 1/15M Sorensen's buffer pH6.4 (Iatron)), exposed under the UV transilluminator (BioRad) for 10 to 20 min, and rinsed in l/15M Sorensen's buffer (pH6.4). Then the slides were stained with 3% Giemsa solution (Merck) in 1/15M Sorensen's buffer (pH6.4) for 10 min and the metaphases were photographed. Banded slides were destained in methanol-acetic acid (3:1) mixture and dehydrated through an ethanol series (70% and absolute) prior to FISH procedure.

GTG technique

GTG technique was performed by the method of Seabright (1971) with modifications. The slides were placed in 30% H_20_2 solution for 10 min to age the air-dried preparations and washed in distilled water. Then the slides were treated in trypsin solution (0.25%, Gibco) for 5 to 30 seconds on ice and promptly washed in cold 70% ethanol. The slides were stained in 4% Giemsa solution (Merck) for 10 min in l/15M Sorensen's buffer (pH6.8) and the metaphases were photographed. Banded slides were destained in methanol-acetic acid (3:1) mixture, refixed in 3.7% formaldehyde/PBS(-), and dehydrated through an ethanol series (70% and absolute) prior to FISH procedure (Klever et al., 1991).

DNA Probes

The genomic clone of the human Cε3 gene, WES-H-Igε-31 (Nishida *et al*., 1982), and a phage clone of the chimpanzee genomic Cε3 gene, Ch28-PTR-Igε-301 (Ueda *et al*., 1989), were kindly provided by Dr. Ueda, The University of Tokyo. WES-H-Igε-31 was used for only human chromosomes because of its repetitive sequences of

LINE1 that reduced the specific signals. LINE1 DNAs of 4 clones (pUK19A, pUK20A, pUK30A, and pUK3 IA; Hattori *et al*. (1985); courtesy of JCRB Gene Bank, NlH, Tokyo) were used as the competitor as well as human Cot-1 DNAs (GibcoBRL). Ch28-PTR-Igε-301 was used for comparative mapping in nonhuman primates after excluding 3' LINE1sequences since a flanking region of this clone was longer than that of the human genomic clone WES-H-Igε-31. A BamHI-BglII fragment (6.4kb) of Ch28-PTR-Igε-301 was subcloned into a plasmid vector pUCI 8 and used as the DNA probe. Three cosmid marker DNAS on human chromosome 9 (HSA9), cCI9-37, cCI9-135, and cCI9-208, whose map positions were at 9q22.1→q22.2, 9q22.32→q22.33, and 9p13.3→p13.2, respectively (Takahashi *et al*., 1994), were also used (courtesy of JCPB Gene Bank, NlH, Tokyo). These probes were labeled with biotin-11-dUTP (Enzo) by nick translation and purified through a Sephadex G-50 spin column (Boehringer) as described in Part I. The SpectrumGreen labelled HSA9 specific DNA probe (WCP#9; Vysis) was also used as the whole chromosome painting probe.

FISH procedure

FISH was carried out as described in the same manner in Part I. To localize the human Cε3 gene (IGHEP2) a total of 163 metaphases from were examined. For nonhuman primates, approximately 40 metaphases per species were scored. Three cosmid markers on HSA9 were applied only to the great ape species.

Microscopes and films

Observations were performed using microscopes and photographs were taken using films as described in Part I. For RBG and GTG banding, Fuji Minicopy HRII film was used.

Results

Comparative chromosome painting with human chromosome 9 DNA probe

FISH of the human chromosome 9 DNA probe (WCP#9) to metaphases of each species resulted as expected in a specific delineation of chromosome homology. WCP#9 painted one pair of chromosomes in the great apes and Japanese macaque, and two chromosome pairs in the white-handed gibbon and in the agile gibbon. These painted chromosomes were PTR11 (common chimpanzee), PPA11 (pygmy chimpanzee), GGO13 (gorilla), PPY13 (orangutan), distal portion of HLA8q and proximal portion of HLA13q (white-handed gibbon), centromeric and distal regions of HAG8q (agile gibbon) and proximal portion of HAG13q , and MFU14q (Japanese macaque), respectively (Fig. 6) in accordance with the painting data reported by Jauch *et al*. (1992). According to Stanyon *et al*. (1987), balanced inversion and translocation polymorphisms have been reported for different gibbon species, and three forms of inversion of gibbon chromosome 8 are noted as 8a, 8b, and 8c. Our data also indicated polymorphism to form 8b and 8b in white-handed gibbon and 8c and 8c in agile gibbons, respectively (Figs. 23a and 24a). WCP#9 probe indicated that the form 8b had one painted part of distal q subregion whereas the form 8c showed two painted parts of centromeric and distal q subregions (Fig. 6).

Regional assignment of the human Cε3 gene (IGHEP2)

FISH using WES-H-Igε-301 probe for the Cε3 gene (IGHEP2) to human metaphases resulted in assignment of the gene to the short arm of chromosome 9. The IGHEP2 region has been localized to band 9p24 (Fig. 16). The frequency of fluorescent signals of one or two-spots in 9p24 was 58.9% (96/163) (Figs.16 and 17), and no twin-spots on other chromosomes were observed. The locus for IGHEP2 was

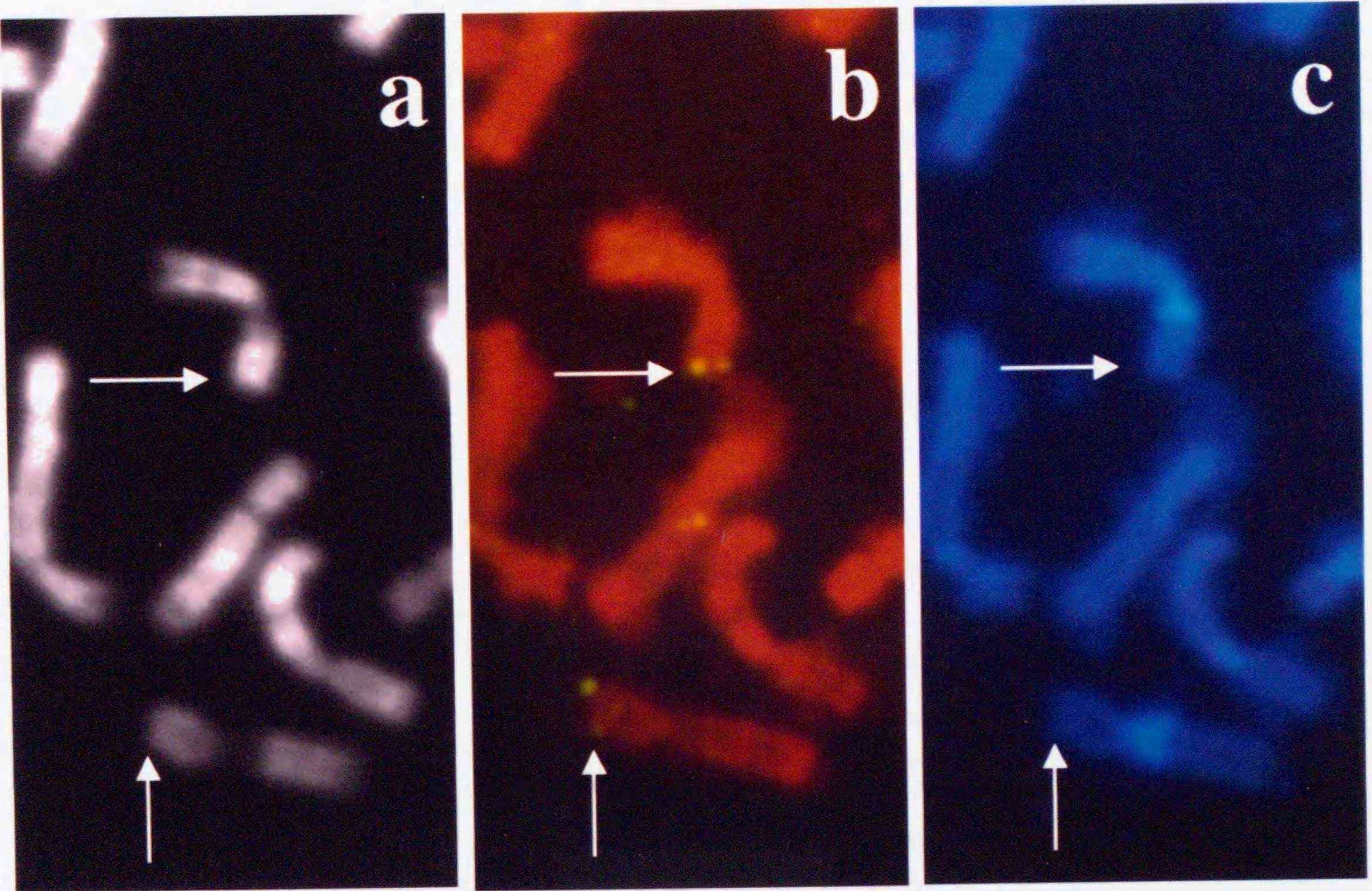


Fig. 16. Human partial metaphase hybridized with the Cε3 gene combined with Q-banding and DAPI-staining

Localization of the human Cε3 gene (IGHEP2) to the terminus region of human chromosome 9 (HSA9), namely HSA9p24, by FISH (**b**) combined with Q-banding (**a**) and with DAPI-staining (**c**). Arrows indicate the twin-spot fluorescent signals (**b**) and the same positions (**a**, **c**).

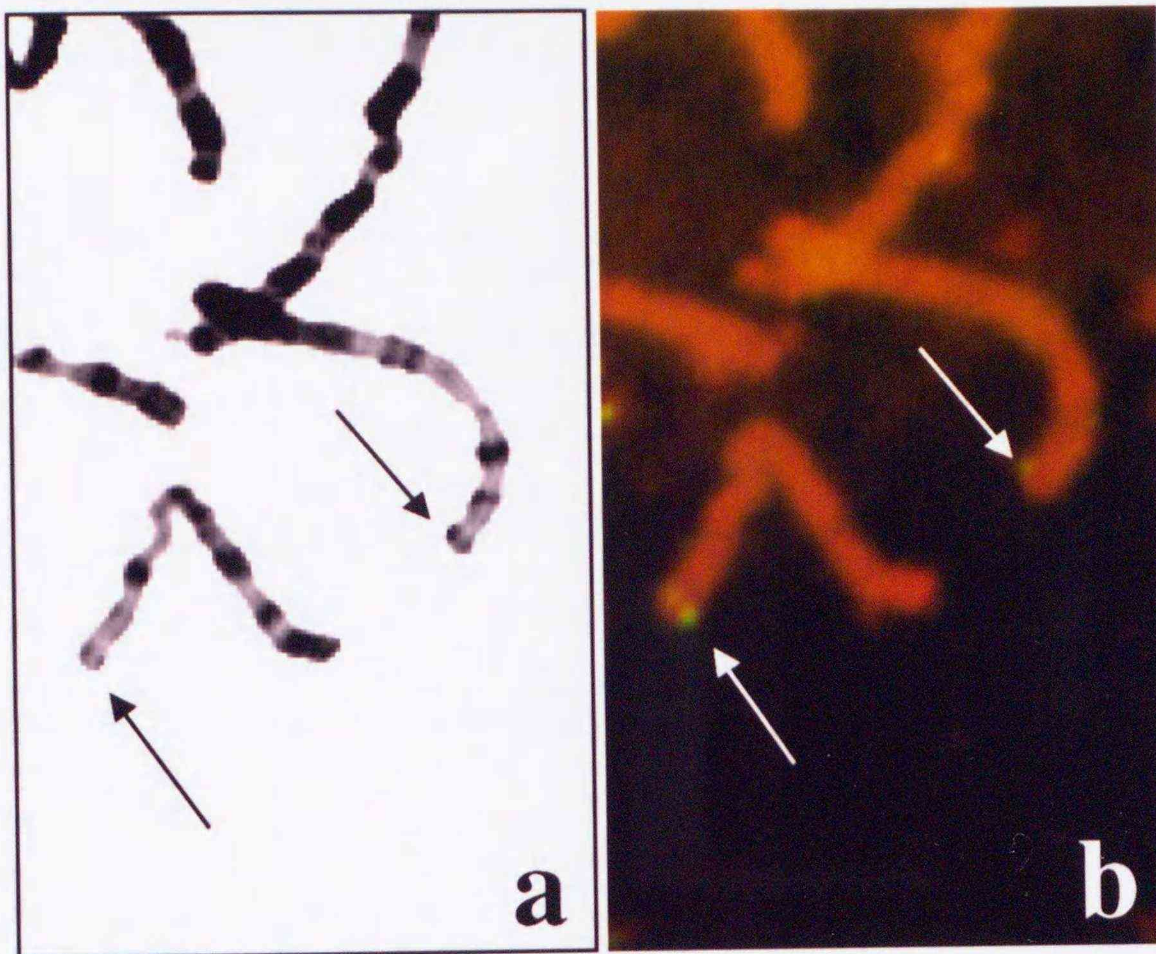


Fig. 17. Human partial metaphase hybridized with the Cε3 gene combined with R-banding

Localization of the human Cε3 gene (IGHEP2) to HSA9p24 by FISH (**b**) combined with R-banding (**a**). Arrows indicate the fluorescent signals (**b**) and the same positions (**a**).

further examined by high-resolution banding at approximately 600 or more band-stage, and assigned to 9p24.2→p24.1 (Fig. 18).

Comparative mapping of the Cε3 gene (IGHEP2) in higher primates

The DNA probe for the chimpanzee genomic Cε3 gene used to comparative mapping in higher primates hybridized to specific chromosomes of each species. The IGHEP2 region has been localized to PTR11q34 (common chimpanzee), PPA11q34 (pygmy chimpanzee), GGO13q22 (gorilla), PPY13q16 (orangutan), HLA8qter (white-handed gibbon), HAG8qter (agile gibbon), and MFU14q22 (Japanese macaque), respectively (Figs. 19-25). The frequencies of cells showing specific single and double hybridization signals on each chromosome were 75.3% for common chimpanzee, 72.8% for pygmy chimpanzee, 70.4% for gorilla, 64.2% for orangutan, 58.7% for white-handed gibbon, 54.8% for agile gibbon, and 53.2% for Japanese macaque, respectively.

Comparative mapping of three cosmid markers on HSA9 in the great apes

Three cosmid markers of cCI9-37, cCI9-135 and cCI9-208 on HSA9 provided further mapping data in the common and pygmy chimpanzees and orangutan. The two closely linked markers of cCI9-37 (on HSA9q22.1→q22.2) and cCI9-135 (on 9q22.32 →q22.33) were both regionally assigned to PTR11p11 (common chimpanzee), PPA11p11 (pygmy chimpanzee), and PPY13q22 (orangutan) (Figs. 19c, 20d, and 22c), whereas another marker, cCI9-208 (on HSA9p13.3→p13.2) was regionally assigned to PTR11q22, PPA11q22, and PPY13q12 (Figs. 19d, 20f, and 22d), as summarized in the ideogram in Fig. 26.

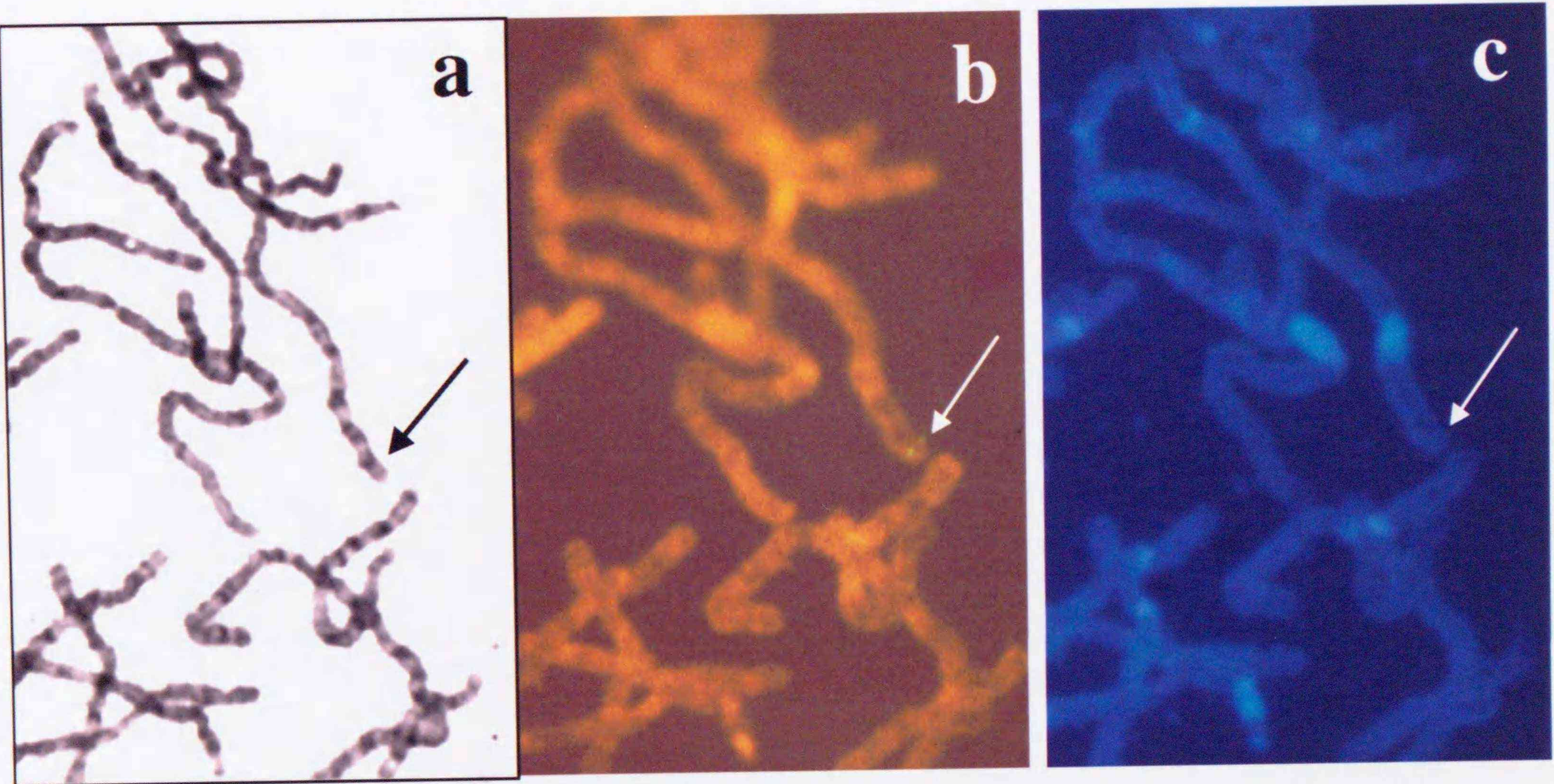


Fig. 18. Human partial metaphase hybridized with the Cε3 gene combined with high resolution G-banding and DAPI-staining

Localization of the human Cε3 gene (IGHEP2) to HSA9p24.2-p24.1 by FISH (**b**) combined with high resolution G-banding (**a**) and DAPI-staining (**c**). DAPI-staining reveals the centromeric heterochromatin on HSA9. The white arrows indicate the twin-spot fluorescent signals (**b**) and the same position (**c**) and the black arrow indicates the same position on the banded region (**a**).

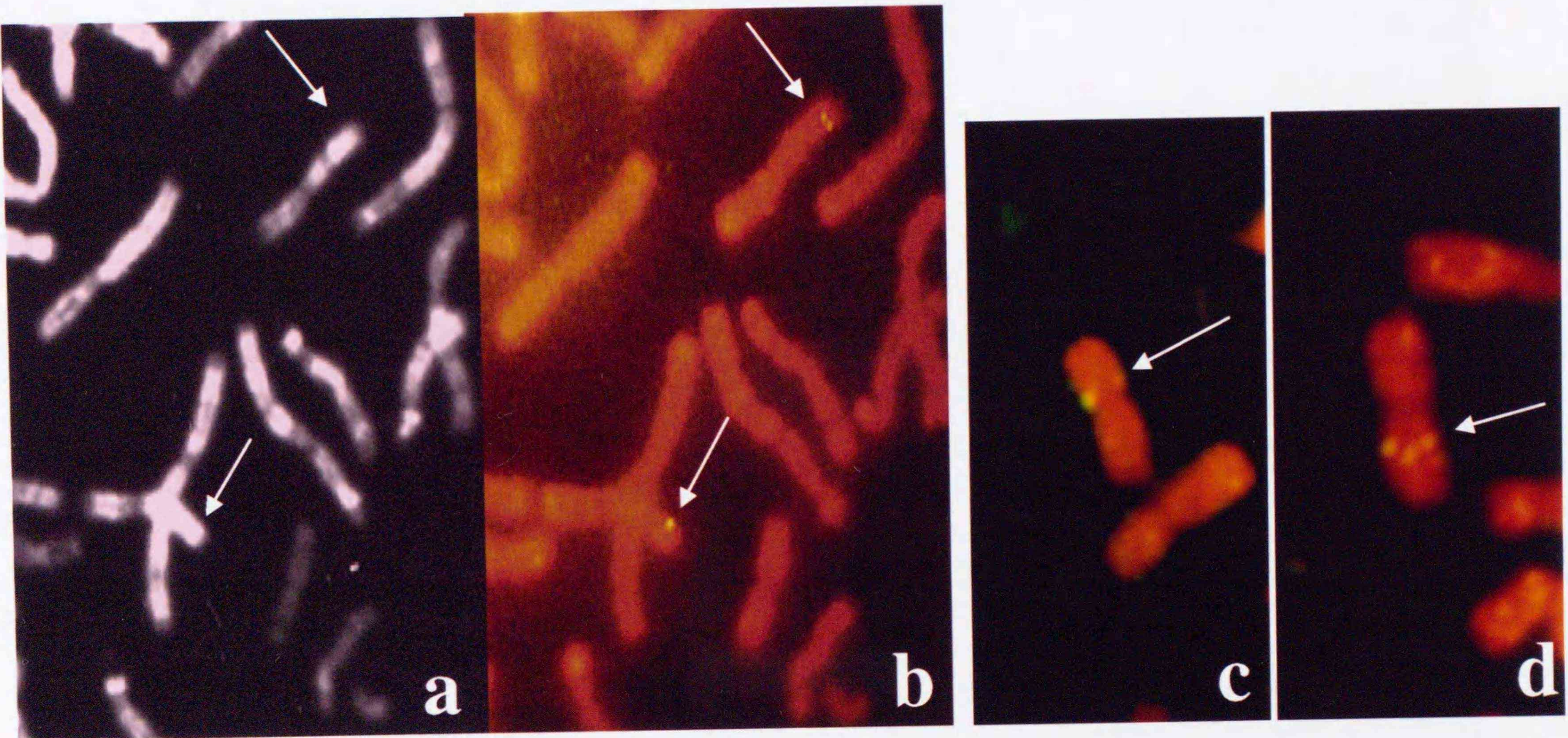
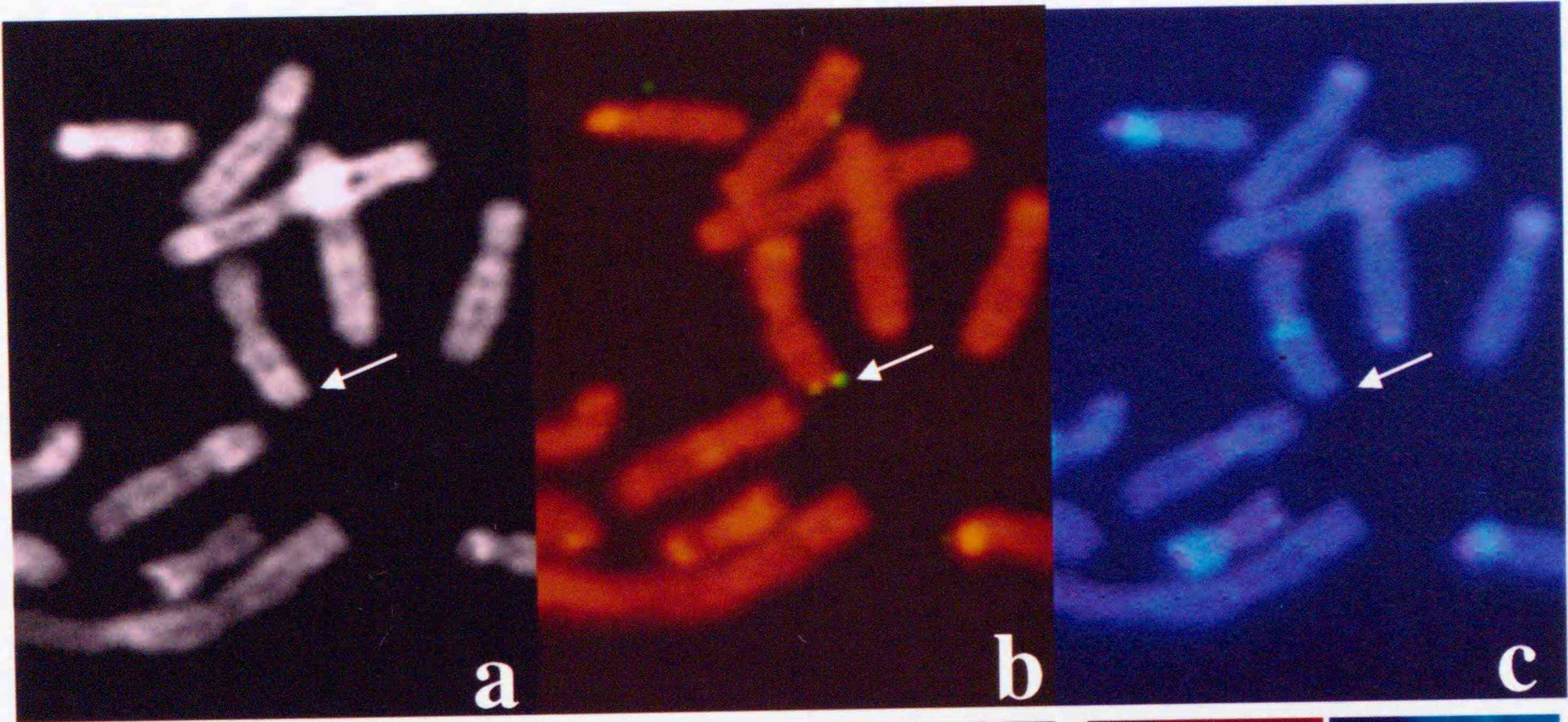


Fig. 19. Common chimpanzee partial metaphases hybridized with the Cε3 gene combined with Q-banding and hybridized with cosmid markers, cCI9-135 and cCI9-208.

Common chimpanzee partial metaphases: (**a**), (**b**) arrows indicate localization of Cε3 gene (IGHEP2) to PTR11q34 by FISH combined with Q-banding; (**c**) an arrow indicates localization of cCI9-135 to PTR11p11 by FISH; (**d**) an arrow indicates localization of cCI9-208 to PTR11q22 by FISH.



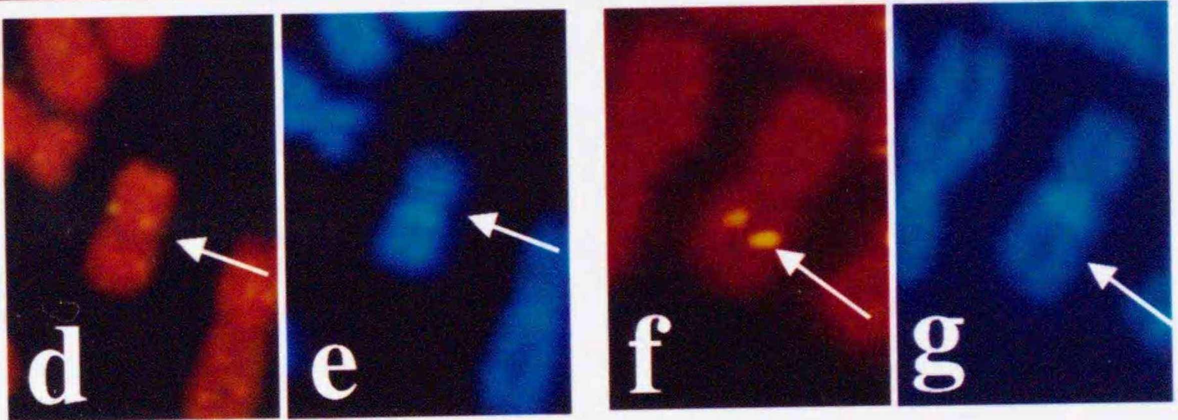


Fig. 20. Pygmy chimpanzee partial metaphases hybridized with the Cε3 gene combined with Q-banding and cosmid markers, cCI9-37 and cCI9-208.

Pygmy chimpanzee partial metaphases: (**a**), (**b**), (**c**) arrows indicate localization of Cε3 gene (IGHEP2) to PPA11q34 by FISH combined with Q-banding and DAPI-staining; (**d**), (**e**) arrows indicate localization of cCI9-37 to PPA11p11 by FISH; (**f**), (**g**) arrows indicate localization of cCI9-208 to PPA11q22 by FISH. The DAPI images of (**c**), (**e**), (**g**) reveal positions of the centromeric heterochromatin.

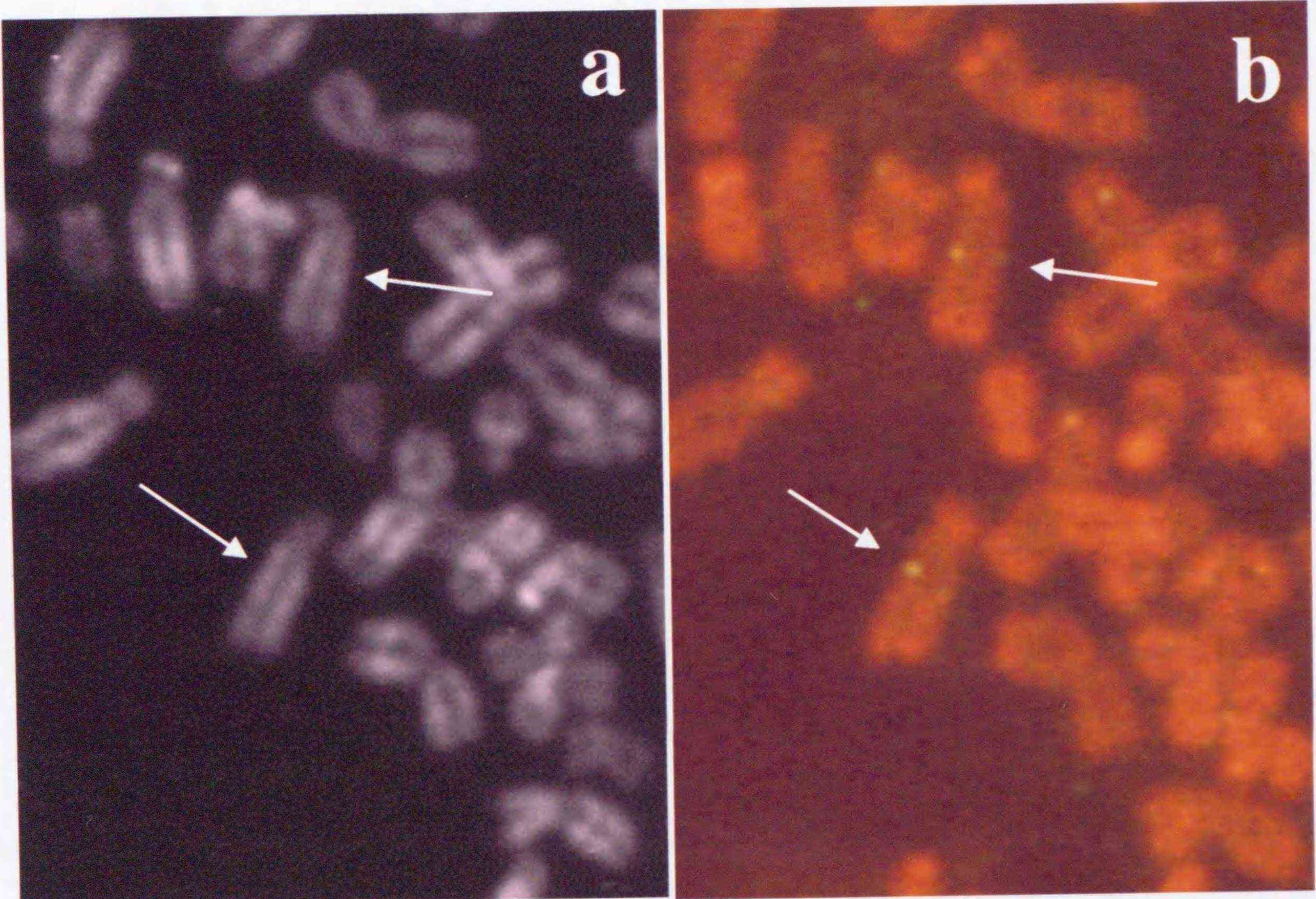


Fig. 21. Gorilla partial metaphase hybridized with the Cε3 gene combined with Q-banding

Localization of the gorilla orthologous Cε3 gene (IGHEP2) to GGO13q22 by FISH (**b**) combined with Q-banding (**a**). Arrows indicate the fluorescent signals (**b**) and the same positions (**a**).

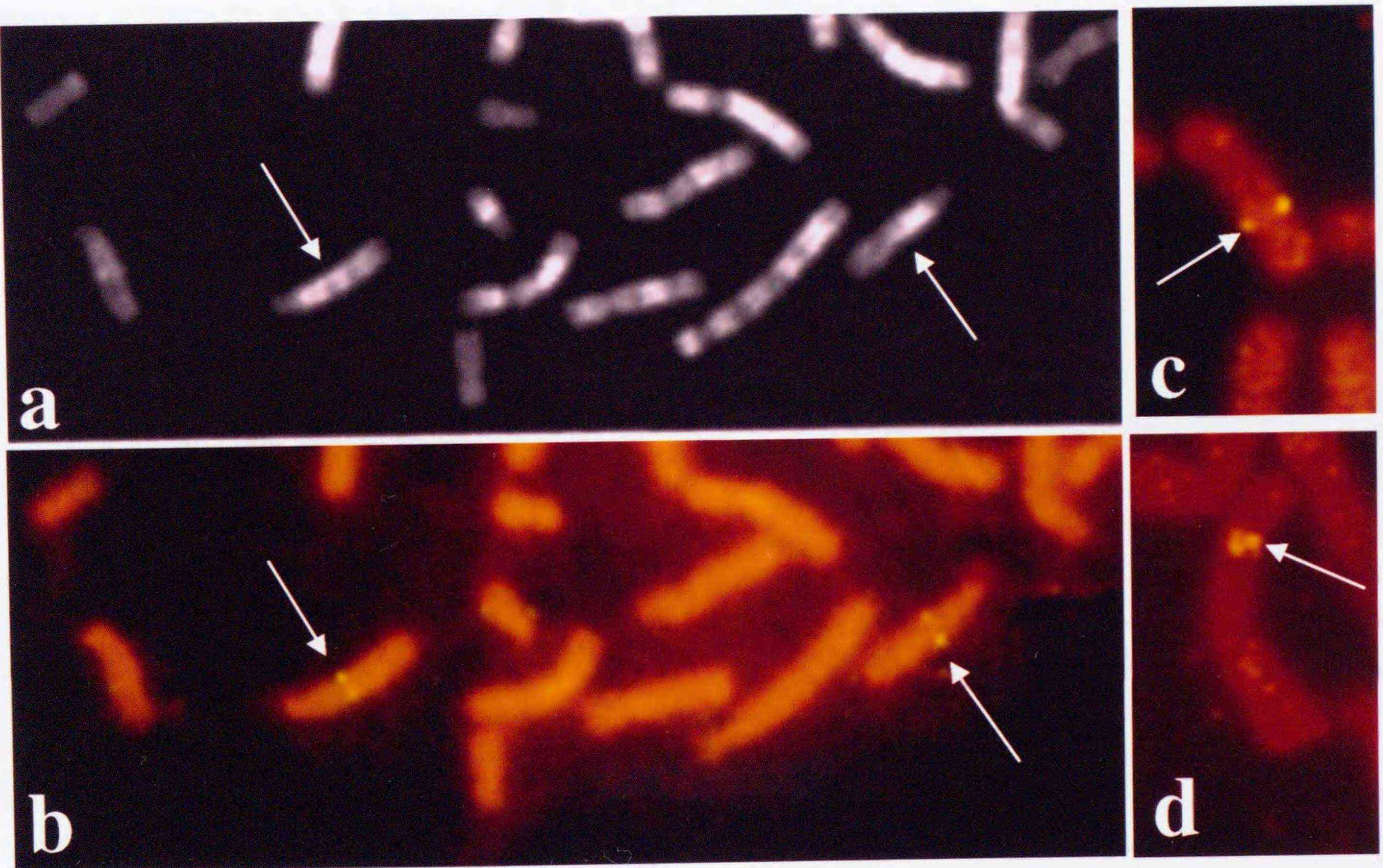


Fig. 22. Orangutan partial metaphases hybridized with the Cε3 gene combined with Q-banding and hybridized with cosmid markers, cCI9-37 and cCI9-208.

Orangutan partial metaphases: (**a**), (**b**) arrows indicate localization of Cε3 gene (IGHEP2) to PPY13q16 by FISH combined with Q-banding; (**c**) an arrow indicates localization of cCI9-37 to PPY13q22 by FISH; (**d**) an arrow indicates localization of cCI9-208 to PPY13q12 by FISH.

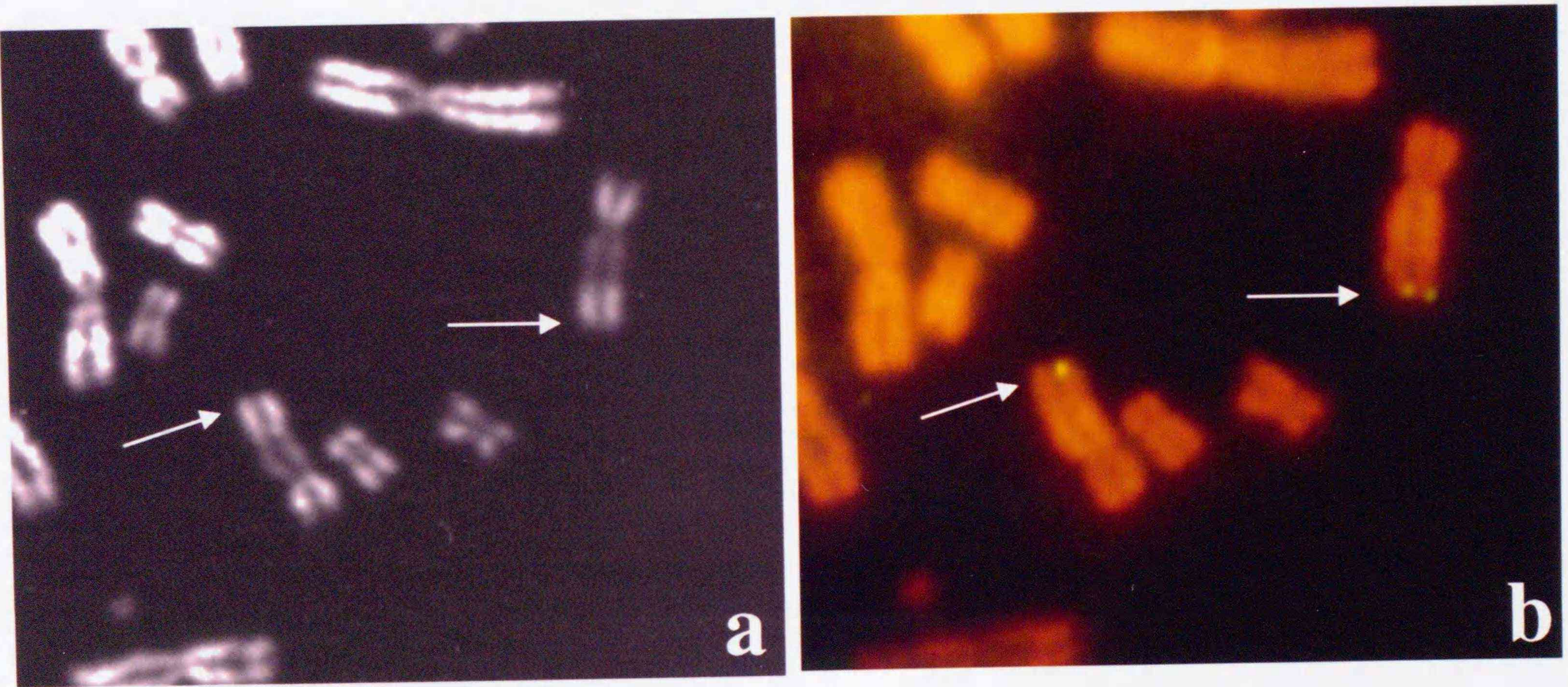


Fig. 23. White-handed gibbon partial metaphase hybridized with the Cε3 gene combined with Q-banding

Localization of the white-handed gibbon orthologous Cε3 gene (IGHEP2) to HLA8qter by FISH (**b**) combined with Q-banding (**a**). Arrows indicate the fluorescent signals (**b**) and the same positions (**a**).

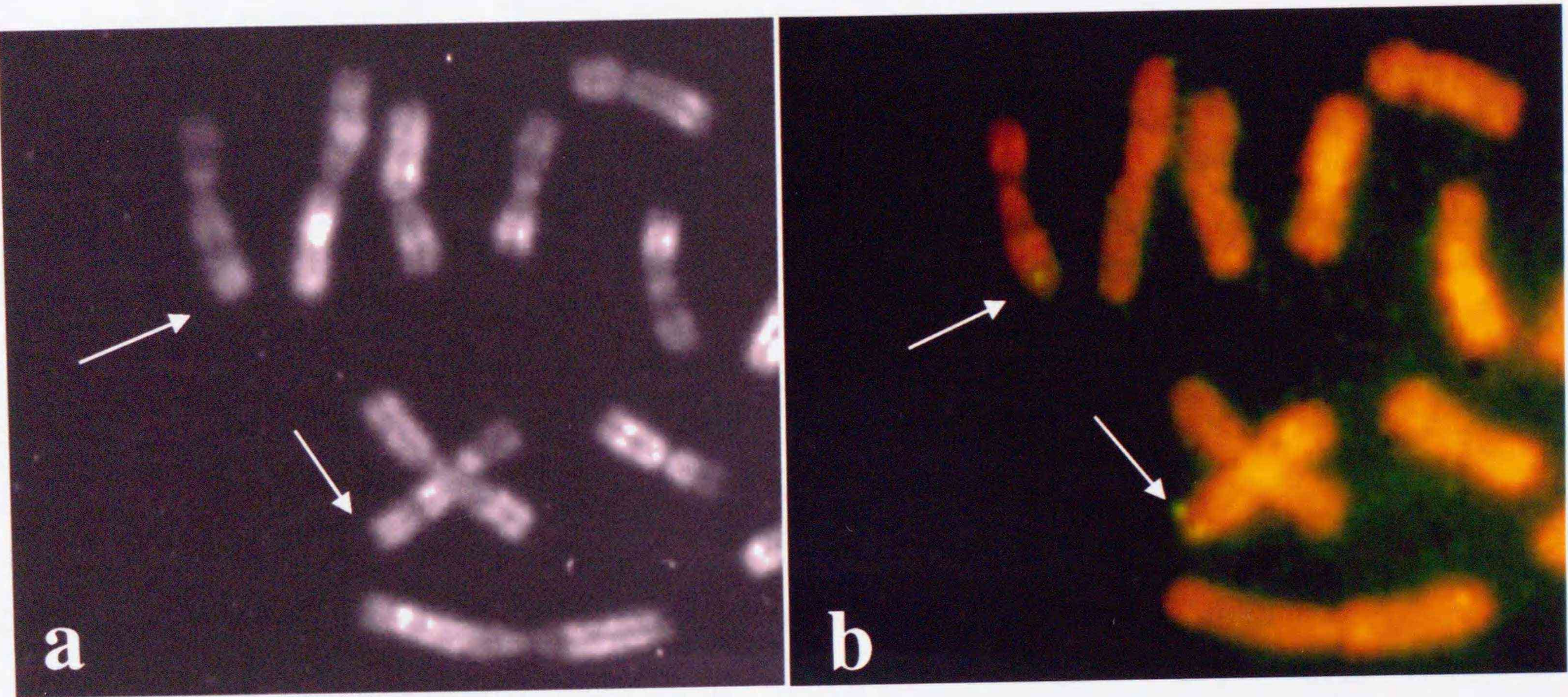


Fig. 24. Agile gibbon partial metaphase hybridized with the Cε3 gene combined with Q-banding

Localization of the agile gibbon orthologous Cε3 gene (IGHEP2) to HAG8qter by FISH (**b**) combined with Q-banding (**a**). Arrows indicate the fluorescent signals (**b**) and the same positions (**a**).

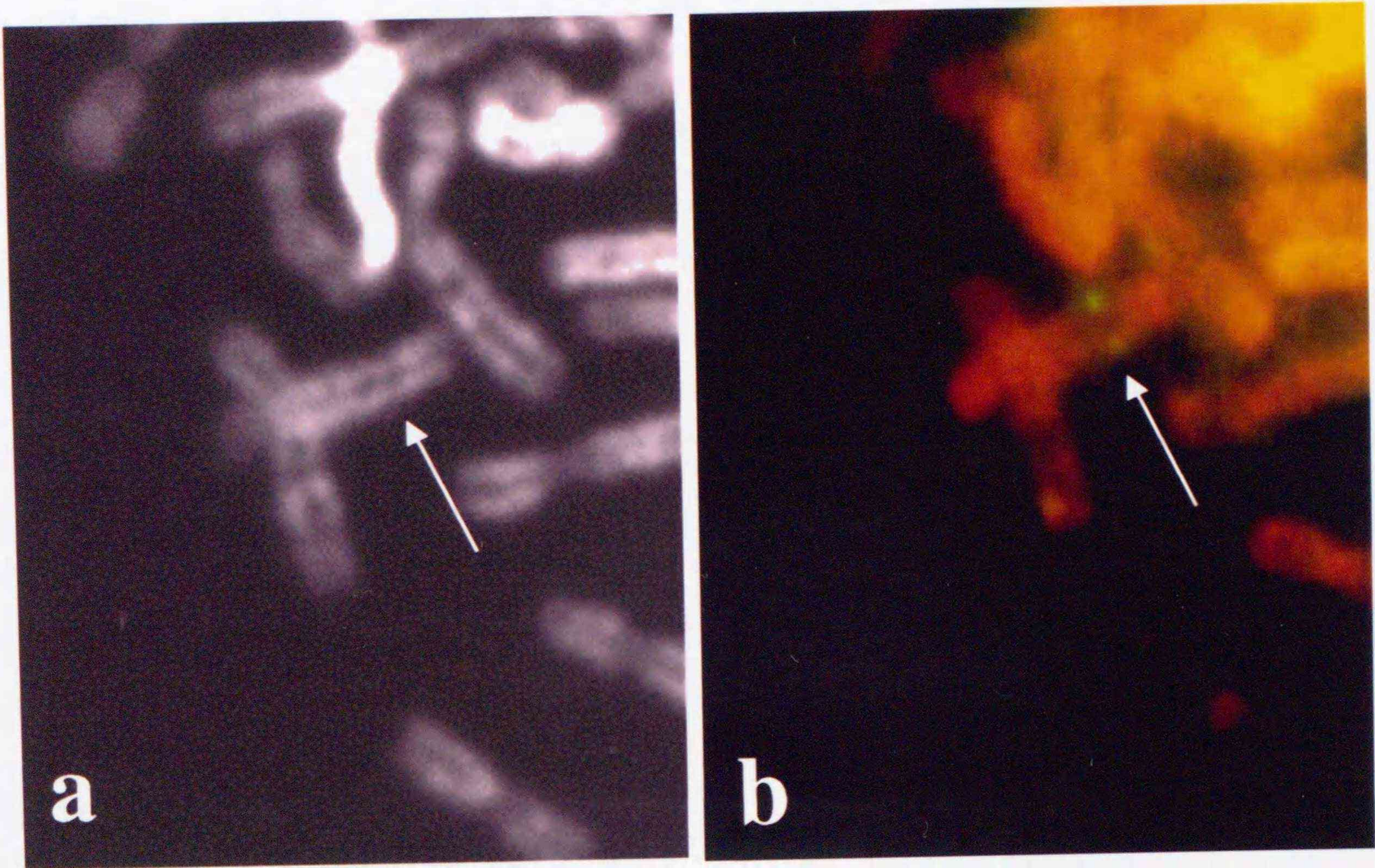
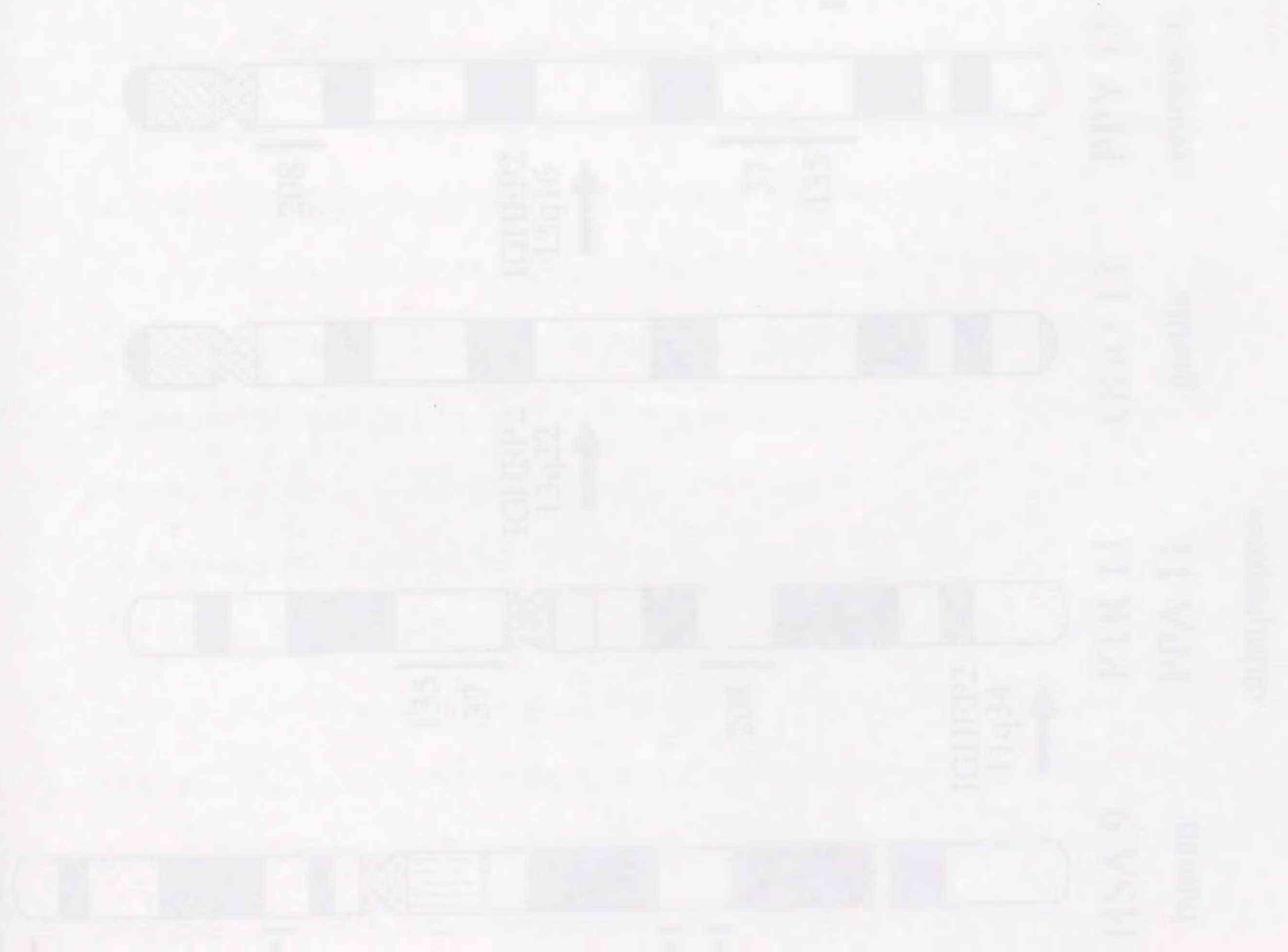


Fig. 25. Japanese macaque partial metaphase hybridized with the Cε3 gene combined with Q-banding

Localization of the Japanese macaque orthologous Cε3 gene (IGHEP2) to MFU14q22 by FISH (**b**) combined with Q-banding (**a**). Arrows indicate the fluorescent signals (**b**) and the same position (**a**).

Legend to Figure 26

Figure 26. Ideogramatic representation of the localization of primate Cε3 genes (IGHEP2) and three cosmid markers. Arrows indicate the positions of IGHEP2. Arrows and/or vertical bars indicate the positions of cosmid markers (only the clone numbers are shown). The chromosome number and species common name are given below each chromosome. The ideograms are referred to in ISCN (1985), Pearson *et al*. (1979), and Stanyon *et al*. (1987).



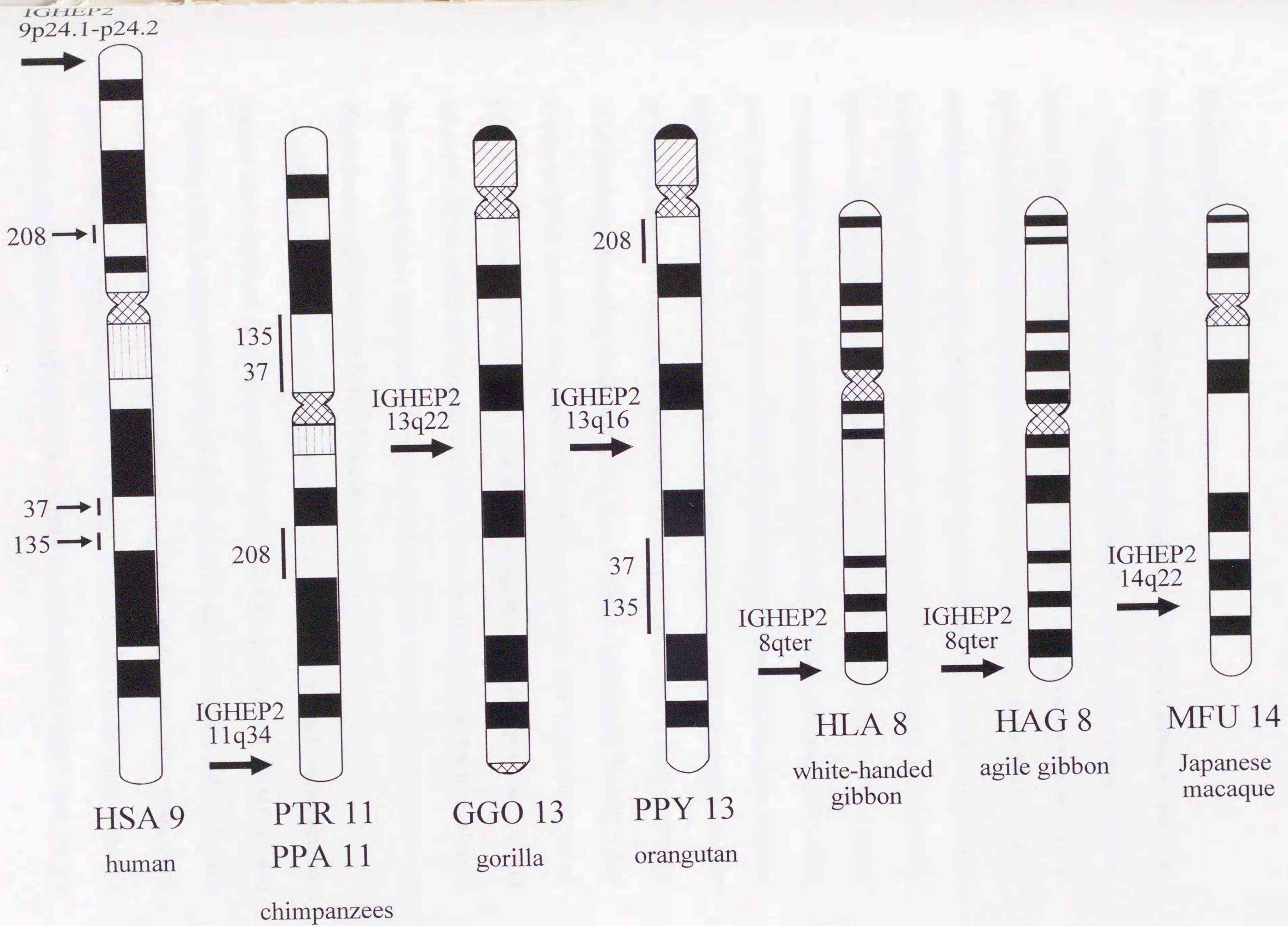


Fig. 26. Ideogramatic representation of comparative mapping of IGHEP2 in primates

Discussion

The locus of IGHEP2 mapped to HSA9p24.2→p24.1 as a valuable telomeric marker

Application of high resolution banding technique provided a fine mapping of the human Cε3 gene (IGHEP2) to 9p24.2→p24.1 (Fig. 18). This new locus will be a useful marker at the telomeric region of the short arm of chromosome 9. DNA markers near telomeric regions have been provided for analysis of cryptic translocations such as Cri-du-Chat syndrome (Overhauser *et al*., 1989) and Miller-Dieker lissencephaly syndromes (Alvarado *et al*., 1993; Kohler *et al*., 1994; Kuwano *et al*., 1991). Cryptic translocations are hardly detected by conventional cytogenetic methods, but rather easily detected by application of FISH methods using chromosome-specific markers including telomeric markers (Lamb *et al*., 1989; Ledbetter, 1992). Recently Kohler *et al*. (1994) detected a cryptic translocation t(9;17)(p24.2;p13.3) in a large family by FISH methods. According to the list of the current genetic markers on 9p, only a few telomeric DNA markers have been obtained (Povey *et al*., 1997) and Povey *et al*. (1997) commented on the shortage of markers on 9p. IGHEP2 thus provides a useful telomeric DNA marker on 9p for investigating chromosomal rearrangements in clinical disorders and also in comparative chromosome mapping.

Evolutionary aspects of the IGHEP2 locus

During the course of primate evolution, it is stated that multiple recombination events have occurred in the immunoglobulin (IGH@) gene cluster (Flanagan and Rabbitts, 1982; Lefranc *et al*., 1982; Max *et al*., 1982; Takahashi *et al*., 1982; Hisajima *et al*., 1983; Flanagan *et al*., 1984; Migone *et al*., 1984; Ueda *et al*., 1985, 1986, 1988; Kawamura and Ueda, 1992; Kawamura *et al*., 1992; Fig. 2). In the present study I assigned the IGHE to HSA14q32.33 and IGHEP2 to HSA9p24.2→p24.1, indicating a

dynamic chromosome rearrangement in the IGH@ gene cluster. Dynamic evolutionary changes in the organization of the Cε genes in primates were investigated by Southern hybridization analysis (Ueda *et al*., 1985, 1986; Fig. 2). Every catarrhine species (15 species of hominoids and Old World monkeys including orangutan, gibbons, macaques, baboons, gelada, patas monkey, and green monkey) possesses the processed Cε pseudogene (IGHEP2), suggesting that an ancient origin of the Cε pseudogene had occurred before branching into Old World monkeys and hominoids (Ueda *et al*., 1985). On the other hand, the Cε2 gene (IGHEP1) seems to be evolutionarily unstable because the IGHEP1 exists in only human and gorilla but not in chimpanzee and the other species. However, two Cε genes, IGHE and IGHEP2 are conserved in all species (Fig. 2). New World monkeys and prosimians possess only IGHE and no IGHEP1 and IGHEP2 (Ueda *et al*., 1986). Thus, IGHEP2 can provide a suitable probe for comparative gene mapping and for study in further genetic rearrangements of IGH@ in catarrhine primates.

IGHEP2 is categorized as a processed type pseudogene because of a likely product of IGHE mRNA and of the different locus from IGH@ cluster (Battey *et al*., 1982; Nishida *et al*., 1982; Ueda *et al*., 1982). The evolutionary rate of IGHEP2 based on the analysis of DNA sequences has been higher than that of IGHE during hominoid evolution (Ueda *et al*., 1986, 1989). However, the probe for the chimpanzee Cε3 gene (IGHEP2) hybridized to other hominoid and macaque chromosomes, suggesting conservation of a region homologous with the chimpanzee pseudogene of IGHEP2. Therefore, the region for the pseudogene can serve as a useful marker for study in evolutional chromosomal rearrangements. This may extend to other pseudogenes of IGH@, although it needs to be examined. Interestingly, IGHEP2 located closely to

breakpoints of chromosome inversions as found in PPY13q16 and GGO13q22, although both regions for IGHE and IGHEP2 did not generate themselves as chromosome breakpoints.

Evolutionary genesis of HSA9 and its primate homologues

Comparative banding analysis between human and primates has been used for a number of years as a basis for studying primate phylogenies (Turleau *et al.*, 1972; Dutrillaux, 1979; Yunis and Prakash, 1982). Yunis and Prakash (1982) have used high resolution banding techniques to chromosomes of human and the great apes to reconstruct chromosome rearrangements that have presumably occurred and become fixed during hominoid evolution. They reported that the orangutan lineage was first branched off after 6 karyotypic changes and subsequently each lineage of the gorilla after 14 karyotypic changes, chimpanzee after 15 karyotypic changes, and human after 11 karyotypic changes has occurred. Most of the postulated chromosomal rearrangements were pericentric inversions, although other rearrangements of paracentric inversions, fusions, etc., have also occurred. The presumed common ancestors of human and the great apes shared a substantially identical genetic set, which might have possessed a more-or-less orang-gorilla type of chromosomes equivalent to human chromosomes. For HSA9 as examined in this thesis successive pericentric inversions and accumulation of heterochromatin from the orang-gorilla type ancestral HSA9 might have generated the present HSA9. However, the details of this evolutionary scenario of HSA9 were still unclear, even with high-resolution banding techniques. The present comparative mapping of the IGHEP2, the other three anonymous markers and painting with the chromosome specific probe supports the hypothesis of Yunis and Prakash (1982) for the origin of HSA9. My results confirmed

HSA9 homologues previously suggested by chromosome banding in the great apes (Yunis and Prakash,1982). Moreover, I defined the breakpoints of the pericentric inversions that occurred in the human-chimp ancestor of the present orangutan chromosome 13 (PPY13p13 and PPY13q16) and gorilla chromosome 13 (GGO13p13 and GGO13q22), and in the chimp ancestor of the present chimpanzees chromosome 11 (the proximal region between PTR/PPA11p11 and PTR/PPA11q22). Putting these data together with other comparative mapping data involving the ABL1 proto-oncogene (Verma and Luke, 1994), it is hypothesized that HSA9 genesis took place as follows; the human-chimp-gorilla-orang ancestral HSA9 chromosome was an orang-gorilla type acrocentric chromosome (PPY13/GGO13), and nucleolar organizer regions appeared on PPY13p only after branching from a human-chimp-gorilla common ancestor. Then, after the gorilla branched off, the first pericentric inversion occurred in a human-chimp ancestor with breakpoints at the regions of the present PPY13q16 and PPY13p13, and centromeric heterochromatin subsequently accumulated in the human-chimp ancestral chromosome. The second pericentric inversion took place in the chimp branch, with breakpoints in the middle region of the present PTR/PPA11p11 and the proximal portion of the PTR/PPA11q22. Further accumulation of centromeric heterochromatin and G-positive band on the short arm occurred in the human branch, generating the present HSA9. The inversion breakpoints that occurred in the human-chimp ancestral chromosome were proximal to the markers cCI9-208 and cCI9-37, corresponding to the present HSA9 proximal region between HSA9p13.3 and HSA9q22.2 (Fig. 27). This model will be refined and confirmed by the analysis of further comparative mapping of other DNA markers of HSA9 to the great apes.

Legend to Figure 27

Figure 27. Schematic representation of an evolutionary scenario on HSA9 genesis in relation to the pericentric inversions took place in hominoid speciation. The small arrows combined with the vertical bar indicate the region of the pericentric inversions (see text for detailed scenario on the genesis of HSA9). Arrows or vertical bars indicate the positions of assigned genes. Present HSA9 shows the accumulation of centromeric heterochromatin and an additional G-positive band on the short arm. The nucleolar organizer regions (NORs) appeared on the short arm of PPY13 (Seuánez, 1979). Comparative mapping data of the gene for ABL1 (v-abl oncogene homolog 1) is referred to in Verma and Luke (1994).

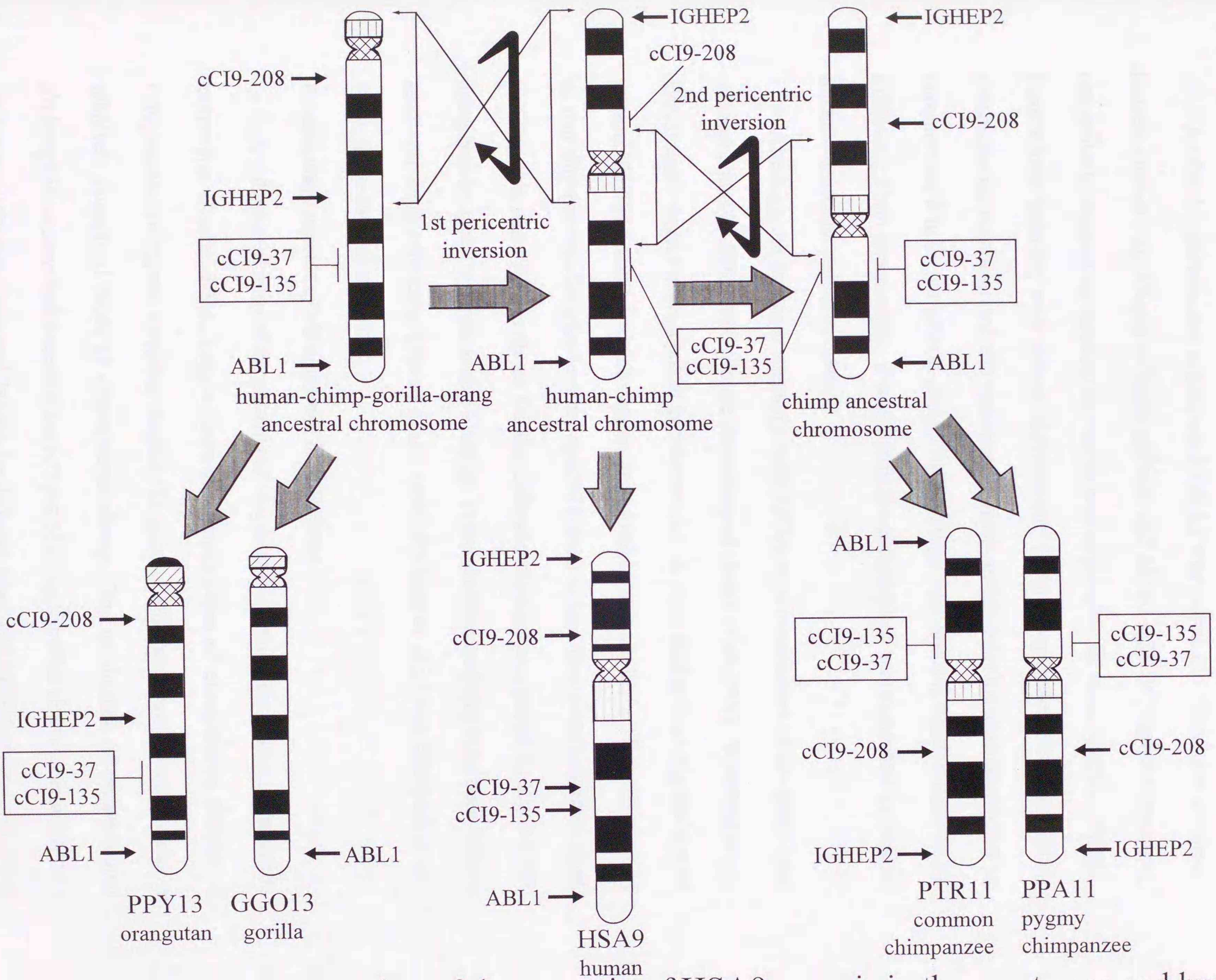


Fig. 27. Schematic representation of the scenario of HSA9 genesis in the great apes and human

As for the gibbons and macaques, IGHEP2 was mapped to HLA8qter of white-handed gibbon , HAG8qter of agile gibbon, and MFU14q22 of Japanese macaque, respectively, suggesting interchromosomal rearrangements in these species. Indeed, chromosome painting with human chromosome 5- and 9-specific probes to gibbon chromosomes demonstrated the presence of inter- or intraspecific rearrangements in chromosome 8 in the lar gibbon group (2n=44) (Figs. 5, 6, and 26). Further analysis by FISH on gibbon chromosome 8 will clarify the chromosome rearrangement as well as intrachromosomal polymorphism.

Although the syntenic segments with HSA9 to chromosomes of the great apes and macaques by FISH methods are demonstrated (Jauch *et al.*, 1992; Wienberg *et al.*, 1992), there were some technical problems such as high background signals due to nonspecific hybridization or no signals by probably an inability of hybridization of human chromosome libraries to other species chromosomes. However, Scherthan *et al.* (1994) improved FISH methods for the different mammalian species as ZOO-FISH using human specific chromosome libraries. Presently their methods have been widely used with high efficiency (Apiou *et al.*, 1996; Richard *et al.*, 1996; Sherlock *et al.*, 1996; in the present thesis).

Evolutionary aspects of chromosome breakpoints

A cohesive picture of the patterns of chromosomal evolution among mammals is beginning to take shape, largely from the combination of comparative studies of cytogenetics and gene mapping studies. In general, the standard observation is a relatively conserved mode of chromosome change. The occurrence of chromosomal exchange is so slow that ancestral karyotypes of living families or even certain orders (primates, carnivores, marsupials) can be deduced from ZOO-FISH analysis of living

species (Scherthan *et al.*, 1994). The syntenic predictions of these cytological conclusions usually, but not always, have been affirmed.

The rule of chromosomal conservation has a number of exceptional species in every group thus far studied. In primates, the gibbons and owl monkeys have highly rearranged karyotypes relatively to the ancestral forms. Stanyon and Chiarelli (1983) hypothesized that changes in the hylobatid karyotype are characterized by an extremely high evolutionary rate compared with other primates. Recently this was confirmed by chromosome painting studies (Jauch *et al.*, 1992; Koehler *et al.*, 1995a, 1995b). The mechanism of this rapid rate of chromosomal evolution in gibbons remains still unanswered, but the molecular data suggested that the evolutionary rate of genes or whole genome in gibbons is within the range of other primates (Sibley and Ahlquist, 1987; Kawamura *et al.*, 1990, 1991; Kawamura and Ueda, 1992).

Relatively few reports have been published on the breakpoints in evolutionary chromosome changes in primates. Previously, fragile site expression was studied in human and the great apes in relationships to a potential involvement of the site in chromosome rearrangements in evolutionary changes. However, no apparent correlation was found between the expression of a certain fragile site and the sites of chromosome rearrangement in evolutionary changes (Miro *et al.*, 1987; Smeets and Klundert, 1990), although there was a report of the breakpoint of pericentric inversion between Bornean and Sumatran orangutans at 2q14 (PPY2q14) which corresponds to a conserved fragile site in human (FRA3B of HSA3p14) and primate species (Seuánez et al. 1979).

Summary

Karyotypic homology in relation to human chromosome 9 (HSA9) was studied through comparative mapping of the Cε3 gene (IGHEP2) in primates. First of all, IGHEP2 was regionally assigned onto the terminal region of HSA9p, at band 9p24.2→p24.1 by FISH. This result suggests that IGHEP2 is a useful novel telomeric DNA marker not only for investigating the HSA9 genesis through comparative gene mapping but also for cytogenetic analyses of cryptic translocations. The primate IGHEP2 was assigned to the region of HSA9 homologues in each species, namely, to PTR11q34 (common chimpanzee), PPA11q34 (pygmy chimpanzee), GGO13q22 (gorilla), PPY13q16 (orangutan), HLA8qter (white-handed gibbon), HAG8qter (agile gibbon), and MFU14q22 (Japanese macaque), respectively. Besides, whole chromosome paints of HSA9 demonstrated that PTR11, PPA11, GGO13, PPY13, HLA8q, HAG8q, and MFU14q were homologous with HSA9. These results supported the data from the previous chromosome banding and painting studies and indicated that the pericentric inversions occurred at least twice during the course of hominoid evolution. To verify the breakpoints of presumed pericentric inversions on the ancestral great ape chromosomes, three DNA markers on HSA9, cCI9-37 (9q22.1→q22.2), cCI9-135 (9q22.32→q22.33), and cCI9-208 (9p13.3→p13.2), were also assigned to PTR/PPA11p11 (cCI9-37 and 135), PTR/PPA11q22 (cCI9-208), PPY13q22 (cCI9-37 and 135), and PPY13q12 (cCI9-208). Putting these data together with other comparative mapping data including ABL1, it is hypothesized that HSA9 genesis took place as follows (cf. Fig. 27); the human-chimp-gorilla-orang ancestral HSA9 chromosome was an orang-gorilla type acrocentric chromosome (PPY13/GGO13), and nucleolar organizer regions appeared on PPY13p only after branching from a human-

chimp-gorilla common ancestor. Then, after the gorilla branched off, the first pericentric inversion occurred in a human-chimp ancestor with breakpoints at the site of the present PPY13q16 and PPY13p13 regions, and centromeric heterochromatin subsequently accumulated in the human-chimp ancestral chromosome. The second pericentric inversion took place in the chimp branch, with breakpoints in the middle of the present PTR/PPA11p11 region and the proximal part of the PTR/PPA11q22 region. Further accumulation of centromeric heterochromatin occurred in the human branch, generating the present HSA9 with an additional slight G-positive band on the short arm. The inversion breakpoints that occurred in the human-chimp ancestral chromosome were proximal to the markers cCI9-208 and cCI9-37, corresponding to the present HSA9 proximal region between HSA9p13.3 and HSA9q22.2, demonstrating more detailed breakpoints than those of G-banding pattern.

The present FISH pocedures of comparative mapping and painting can clarify the intrachromosomal rearrangements which could not be detected so far by chromosome banding and/or painting alone and provide more information on the genesis of HSA9 supported by the previous chromosome banding and painting studies.

Concluding remarks

In the present thesis, karyotype evolution in human and higher primates was studied by comparative chromosome mapping of immunoglobulin Cε genes combined with chromosome painting by FISH techniques. Although recently developed FISH techniques for chromosome painting are powerful for identifying chromosome homologues in mammalian species, it is still impossible to detect the evolutionary breakpoints of intrachromosomal rearrangements. Thus, I have used a combined method for comparative mapping and chromosome painting to identify chromosome breakpoints in evolutionary changes. Here, I analyzed loci of immunoglobulin Cε genes, namely Cε1 gene (IGHE) and Cε3 gene (IGHEP2), because these genes are well investigated at the DNA level and the multiple recombinational events have occurred in the IGH@ family during the course of primate evolution. IGHE is a functional gene that can express active heavy chain proteins of IgE molecule but IGHEP2 is categorized as a processed-type pseudogene. The presence of homologous DNA sequences of IGHE and those of IGHEP2 has been demonstrated by Southern analysis in the genomes of hominoids and Old World monkeys. The human IGHE has been mapped to human chromosome 14 (HSA14) at band 14q32.33, whereas the locus of the IGHEP2 differs in human genome, suggesting recent extensive reorganization of the immunoglobulin Cε loci.

The present studies confirmed that each locus of IGHE and IGHEP2 in primates mapped to different chromosomes but to homologous regions of each HSA14 and HSA9, respectively. This demonstrates the similar syntenic association in loci of the Cε genes among nonhuman primates here examined, indicating that despite the multiple recombinational events found in the Cε loci during evolution, the syntenic segments have remained intact. These results also suggest that evolutionary changes of

the Cε genes in the DNA sequences may be different from evolutionary chromosomal changes.

Moreover, the present combined FISH procedures offered the genesis of HSA9 during evolutionary rearrangements. The comparison of syntenic groups and painting results has revealed that the pericentric inversions occurred at least twice during the course of hominoid evolution. The first pericentric inversion occurred in a human-chimp ancestor after the gorilla branched off and the second pericentric inversion took place in the chimp branch. An orang-gorilla type acrocentric chromosome (PPY13/GGO13) seemed to be ancestral, and nucleolar organizer regions appeared on PPY13p only after branching away from a human-chimp-gorilla common ancestor. Then, after the gorilla branched off, the first pericentric inversion occurred in a human-chimp ancestor with breakpoints at the site of the present PPY13q16 and PPY13p13 regions, and centromeric heterochromatin subsequently accumulated in the human-chimp ancestral chromosome. The second pericentric inversion took place in the chimp branch, with breakpoints in the middle of the present PTR/PPA11p11 region and the proximal part of the PTR/PPA11q22 region. Further accumulation of centromeric heterochromatin and addition of G-positive band on the short arm occurred in the human branch, generating the present HSA9. Thus, the inversion breakpoints have been subregionally determined by the present procedures.

It is noteworthy that recently developed novel molecular cytogenetic approches such as comparative genomic hybridization (CGH) procedure first reported by Kallioniemi *et al*. (1992) and spectral karyotyping (SKY) method demonstrated by Schröck *et al*. (1996). CGH procedure provides information on gains or losses of DNA sequences in targeted cells by measuring FISH signals on each chromosome and

utilized to various tumor cells. Analysis of interspecies genomes by CGH may be possible to show species specific chromosomal subregions with amplifications or deletions in several megabases. On the other hand, SKY method allows that all human chromosomes have been visualized simultaneously in 24 different colors by combinatorial chromosome painting probes labelled with 5 kinds of fluorchromes utilizing Fourier spectroscopy, which can identify the specific emission spectral patterns of each fluorochrome combination corresponding to each human chromosome. This new technique will shed light on the molecular cytogenetic analysis of highly rearranged karyotypes with numerous marker chromosomes such as in tumor cells and facilitate the comparative cytogenetic studies among interspecies since so far only two or three different colored painting probes can be applied at a time.

No matter how excellent these novel technologies are in the application to interspecies chromosome painting (ZOO-FISH) that will allow us to see the patterns of genome exchange by direct observation, there are still intriguing questions unanswered in the evolutionary breakpoints of chromosomes at the fine structure level. Are they random or do they represent evolutionary "fragile sites" that tend to divide an ancestral mammalian genome into discrete conserved units? Is there a relationship between chromosomal breakpoints in primate evolution and the specific breakpoints observed in human tumorigenesis? Are there interesting genes or sequences at these breakpoints? These questions, of course, require maps of much greater density around the evolutionary junctions in order to determine their relative similarities in different mammalian taxa. Eventually, of course, they must be defined at the nucleotide level - now a realistic expectation from the human genome sequencing initiative, especially if it is accompanied by concentrated gene mapping of prototype species from other

mammalian families. Rapid technological advances driven by human genome mapping have clearly facilitated progress in comparative gene mapping in other species. As the human genome project provides nearly 6000 genes and 16000 expressed sequence tags in linear order on 24 chromosomes, making high resolution gene-dense map, comparative mapping will be more and rapidly extended by use of these human genome data for phylogenetic description of the genomes of mammalian ancestors.

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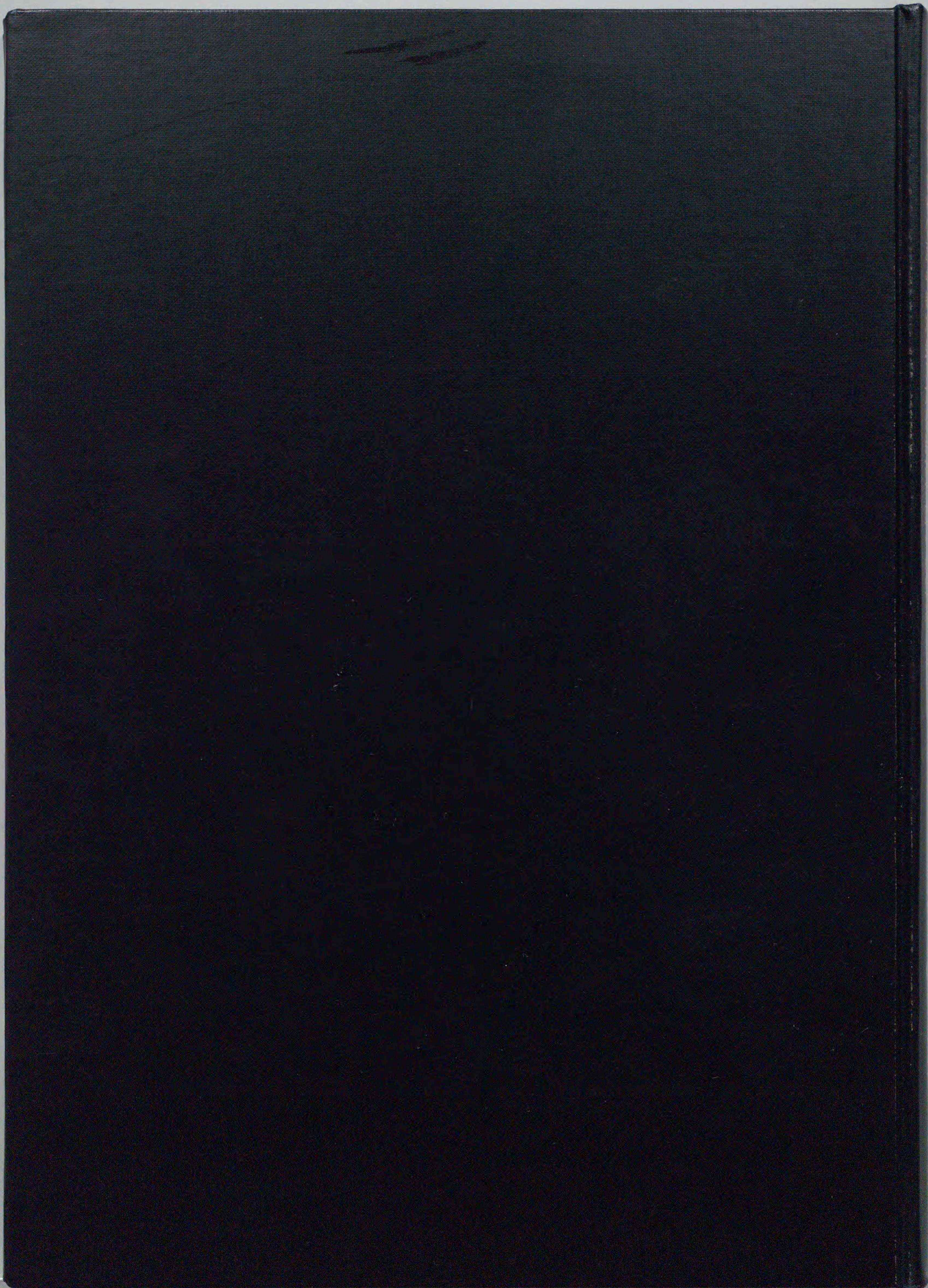

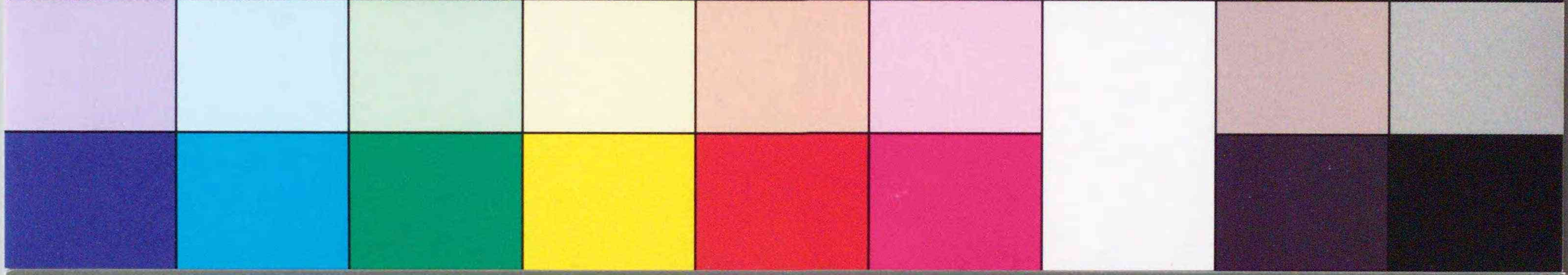

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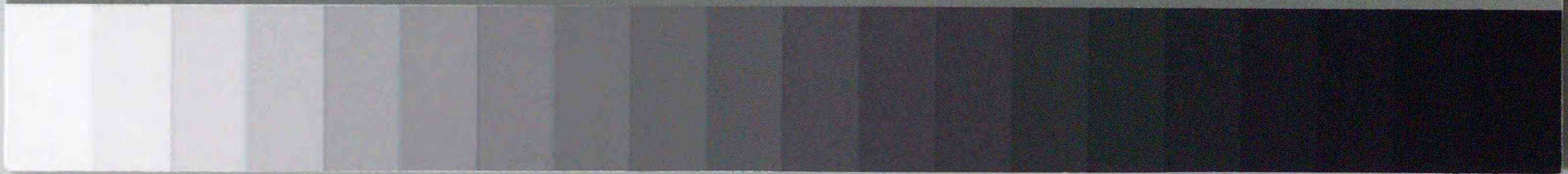

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