CHAPTER 4

FISH analysis of the Siamang and Dog using human chromosome 22q bacterial clones

4.1 Introduction

4.1.1 Cross-species FISH

The first FISH analyses in apes were investigations of the fusion event that lead to the generation of human chromosome 2. Detailed comparative cytological banding analysis had indicated that human chromosome 2 arose through the fusion of two ancestral ape chromosomes. Human chromosome regions 2p and 2q are homologous to chromosomes 12 and 13 respectively in the chimpanzee (*Pan troglodytes*) and chromosomes 11 and 12 respectively in the gorilla (*Gorilla gorilla)* and orangutan (*Pongo pygmaeus*) (Yunis and Prakash, 1982). This was confirmed by zoo-FISH of a human chromosome 2 paint to primate metaphase chromosomes (Wienberg, *et al.,* (1990).

By carrying out FISH, using two human chromosome 2-specific cosmids containing the vertebrate telomeric repeat, the nature of the evolutionary rearrangement was confirmed as a telomere-telomere fusion by Ijdo *et al.* (1991). The two clones were FISH-mapped to human chromosome 2q12 as well as to the ends of other chromosomes. It was concluded that the human chromosome 2 locus cloned was the relic of the telomere-telomere fusion and marks the point at which two ancestral ape chromosomes fused to give rise to human chromosome 2 (Ijdo *et al.,* 1991).

Subsequent studies involved the use of a human chromosome 21 alpha-satellite plasmid clone for FISH on great ape and human metaphase chromosomes, (Baldini *et al.,* 1993). The plasmid containing the alphoid sequence hybridised to the centromeric region of all the human, chimpanzee, gorilla and orangutan chromosomes. The clone also identified sequences on human chromosome 2p21. As that region is not centromeric, it was suggested that those sequences might have been derived from an ancestral centromere, which was subsequently inactivated after the evolutionary rearrangement event in order to maintain chromosome stability.

To further extend the study, a panel of human chromosome 2 YACs was used for FISH to investigate the fragmented human chromosome 2 homologues in four chromosomes of the lesser ape *Hylobates lar (H.lar)* (Arnold *et al*., 1996). Previous chromosome painting studies had revealed that human chromosome 2 is homologous to five distinct regions on *H. lar* chromosomes 1, 10, 12 and two parts of 16. But it had not been established which segment of human chromosome 2 was homologous to which *H. lar* chromosome. As well as hybridising *H. lar* chromosome specific paints back on to human chromosome 2, Arnold and colleagues also hybridised YACs specific for the major bands on human chromosome 2 to *H. lar* metaphase chromosomes. From its hybridisation pattern, a YAC was found that identified the rearrangement point between human chromosome 2-homologous material present on *H. lar* chromosomes 10 and 16. This study demonstrated that a combination of reciprocal heterologous chromosome painting and FISH of specific probes, such as YACs, could be used to identify homologies between closely related species, to construct detailed comparative chromosome maps rapidly and to identify evolutionary rearrangement points.

YACs have also been used to identify chromosome rearrangements within the genomes of higher primates. High-resolution G-banding analyses revealed the high degree of morphological conservation of great ape chromosomes (Nickerson and Nelson, 1998). The distribution of heterochromatin and the occurrence of pericentric inversions were the most notable differences. Pericentric inversions may have played an important role in the establishment of reproductive isolation and speciation of the hominoids as they diverged from a common ancestor (Nickerson and Nelson, 1998). Human YAC clones were used for FISH to identify pericentric inversions when comparing the human karyotype to that of the chimpanzee (Nickerson and Nelson, 1998). Five evolutionary pericentric inversion points were identified on the chimpanzee chromosomes homologous to human chromosomes 4, 9 and 12. The YACs spanning an inversion point showed hybridisation signals on both the p and q arms of the corresponding chimpanzee chromosome.

A FISH study of chromosome homologies between more distantly related mammalian species has also been reported (Cole *et al*., 1998). Physical mapping using contiguous human YAC and PAC clones was carried out between the human and laboratory mouse, *Mus musculus*. A human YAC from chromosome 22 was identified which spans the evolutionary rearrangement point defining the boundary between material homologous to regions of human chromosome 21 and human chromosome 22 on mouse chromosome 10.

It was noted by the authors that detailed analysis of sequences across evolutionary rearrangement points on chromosomes would provide insight into the processes involved both in chromosome evolution and maintaining regions of conserved synteny.

4.1.2 Aim of this chapter

The aim of the work described in this chapter was to further refine the analysis of the regions corresponding to evolutionary chromosome rearrangement points in material homologous to human chromosome 22q on Siamang chromosome 18 and on dog chromosomes 10 and 26. In order to carry out the analysis, a high-resolution cross-species FISH study was to be carried out by hybridising human chromosome 22q bacterial clones onto Siamang and dog metaphases.

The high-resolution map of human chromosome 22 (Collins *et al.,* 1995) provided a framework for the sequencing effort (Dunham *et al.,* 1999). To identify genomic clones for sequencing, extensive clone maps of the chromosome were constructed using cosmids, fosmids, bacterial artificial chromosomes (BACs) and P1-derived artificial chromosomes (PACs). Clones were identified by screening BAC and PAC libraries using chromosome 22-specific STS markers, or by using cosmid and fosmid libraries derived from flow-sorted DNA from chromosome 22. Overlapping clone contigs were assembled by restriction fingerprinting and ordered relative to each other using the established framework map (Collins *et al.,* 1995).

The high quality of the map, which has been verified by sequencing (http://www.sanger.ac.uk/HGP/Chr22, or http://www.genome.ou.edu/Chr22.html), provided a powerful tool for selecting clones evenly distributed along chromosome 22q for the FISH studies described in this chapter.

RESULTS

4.2 FISH Analysis of Siamang Chromosomes with Human Bacterial Clones

4.2.1 Analysis of Siamang 18 with human 22q BACs

Thirteen BAC clones evenly distributed along human chromosome 22q (from 22q11.23 to 22q13.33) were selected from the Sanger Institute chromosome 22 mapping resource. This equates to an average spacing of approximately one BAC per 3.5 to 4 Mb. The DNA from each BAC clone was isolated by plasmid preparation and biotinylated by nick translation, then hybridised to human and Siamang metaphase chromosomes. Ten human and Siamang metaphase spreads were analysed for each BAC probe.

There is no standard banded karyotype for the Siamang, and high-resolution localisations were not possible. Therefore the localisation of the BAC clones to Siamang chromosome 18 were scored as “proximal” or “distal” within the human chromosome 22 homologous regions on HSY 18p or 18q. The BAC clones used, their human chromosome 22q localisations and their corresponding Siamang chromosome 18 arm localisations are tabulated in Table 4.1.

Table 4.1 Human chromosome 22q localisation (HSA22) and corresponding Siamang chromosome 18 arm localisation (HSY18) of thirteen BAC clones. The bK prefix on the clone names relates to the library from which the clones originated. NA = not assessed

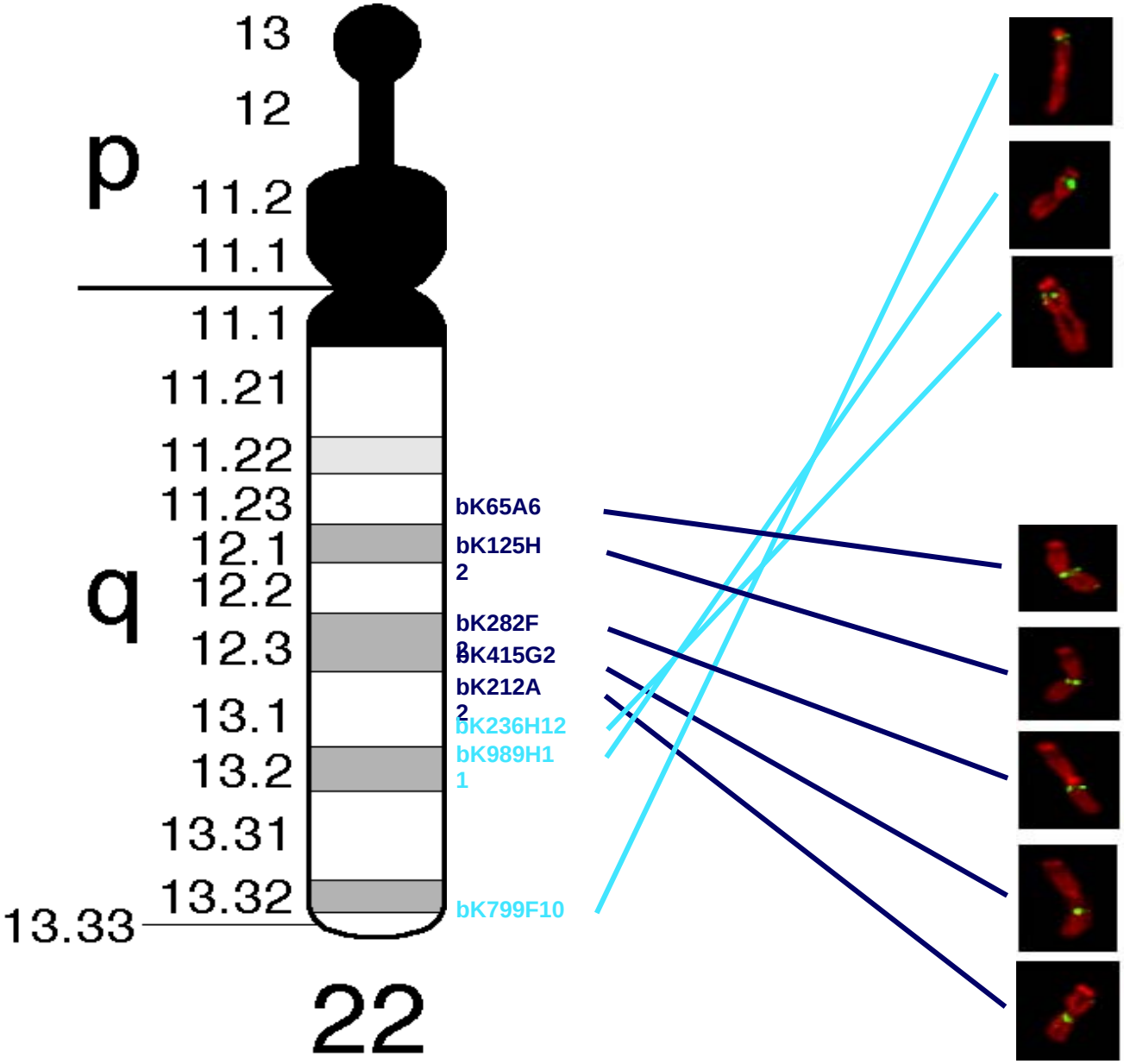
BAC clone	HSA22 localisation	HSY18 localisation
bK65A6	22q11.23	Proximal 18q
bK125H2	22q12.1	Proximal 18q
bK282F2	22q12.3	Proximal 18q
bK415G2	22q12.3	Distal 18q
bK221H1	22q12.3	Distal 18q
bK212A2	22q12	Distal 18q
bK236H12	22q13.1	Proximal 18p
bK206C7	22q13.1	NA
bK229A8	22q13.1	NA
bK216E10	22q13.2	NA
bK989H11	22q13	Proximal 18p
bK1109B5	22q13.31-13.32	NA
bK799F10	22q13.33	Distal 18p

All of the BAC probes gave strong, clean and informative hybridisation signals on human chromosome 22q. Nine of the BAC probes (bK65A6, bK125H2, bK282F2, bK415G2, bK221H1, bK212A2, bK236H12, bK989H11 and bK799F10) gave strong, clean and informative hybridisation signals on Siamang chromosome 18. Four of the BAC probes (bK206C7, bK229A8, bK216E10 and bK1109B5) gave dissipated signals with high background so that it proved difficult to assess confidently signal localisation (NA in table).

Six of the BAC probes (bK65A6, bK125H2, bK282F2, bK415G2, bK221H1 and bK212A2) hybridised to the long arm of Siamang chromosome 18 and three of the BAC probes

(bK236H12, bK989H11 and bK799F10) hybridised to the short arm (see table 4.1). These two groups of clones are located proximally and distally to the rearrangement breakpoint, respectively, on human chromosome 22. Some of the results of the first round FISH analysis are summarised in Figure 4.1.

Figure 4.1 (next page) Summary of the BAC clone analysis of Siamang metaphase chromosomes. On the left, the location on human chromosome 22 of eight of the tested BAC clones is indicated by their position adjacent to the chromosome 22 ideogram. On the right, eight corresponding Siamang chromosome 18 images show the FISH signal location from each BAC clone. The images are ordered according to the location of the BAC signal. The BACs in dark blue are proximal and the BACs in light blue are distal to the breakpoint on chromosome 22.


13
12
p
11.2
11.1
11.1
11.21
11.22
11.23
12.1
12.2
q
12.3
13.1
13.2
13.31
13.32
13.33
22
bK65A6
bK125H2
bK282F8
bK415G2
bK212A2
bK236H12
bK989H11
bK799F10

It was possible to discern that the BAC clones hybridised to a similar position with respect to centromere, telomere and each other within the regions on Siamang chromosome 18 as they did to human chromosome 22. For example, the clones bK415G2 and bK221H2 are located on human 22q distal to clones bK65A6 and bK125H2. On Siamang 18q, clones bK415G2 and bK221H2 are clearly distal to clones bK65A6 and bK125H2 within the human 22q homologous region. Similarly, the clone bK236H12 is located on human 22q proximal to clone bK799F10. On Siamang 18p, bK236H12 is proximal to bK799F10 within the human 22q homologous region.

The BAC probes bK212A2 and bK236H12 are located in 22q12 and 22q13.1, respectively. The hybridisation of BAC probe bK212A2 to Siamang 18q and bK236H12 to Siamang 18p indicated that the rearrangement breakpoint is located in the region (approximately 3-4 Mb) of 22q between those two clones. The BAC probes proximal to bK212A2 on human 22q all hybridised to Siamang 18q and the BAC probes distal to bK236H12 all hybridised to Siamang 18p.

4.2.2 Analysis of Siamang 18 with clones from human 22q12-13.1

In order to narrow down the position of the human 22q homologous block rearrangement point in Siamang chromosome 18, eleven new bacterial clones were selected for a second round of FISH screening. The one BAC, three PAC, four fosmid and three cosmid clones were selected because they are located on human 22q evenly distributed between the BAC clones bK212A2 and bK236H12. These clones sampled the entire interval and the distance between them was approximately 300 to 400 kb. The DNA from each clone was isolated, biotinylated by nick translation then hybridised to human and Siamang metaphase chromosomes as described earlier (4.2.1). Ten human and Siamang metaphase spreads were analysed for each probe. All of the probes gave informative hybridisation signals on human chromosome 22 and Siamang 18.

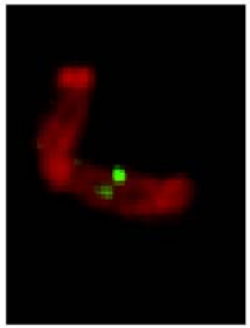
The probes used and the corresponding Siamang chromosome 18 localisation, are tabulated in Table 4.2.

Table 4.2 Human bacterial clone probes from 22q12-q13.1 and their corresponding Siamang chromosome 18 localisation. Clones are listed according to their order from centromere to telomere on chromosome 22.

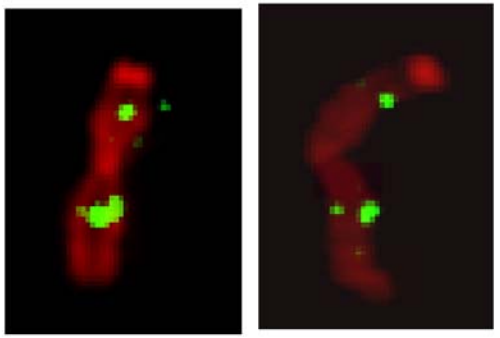
Human 22q clone	Siamang 18 localisation
BAC bK212A2	18q
Fosmid fF24E5	18q
Fosmid fF126G10	18q
Cosmid cE132D12	18q
PAC dJ293L6	18q
Fosmid fF4G12	18q
Fosmid fF45C1	18q + 18p
Cosmid cE81G9	18p
Cosmid cE146D10	18p
PAC dJ151B14	18p

Six of the clones (bK212A2, fF24E5, fF126G10, cE132D12, dJ293L6 and fF4G12) hybridised entirely to Siamang 18q and three (cE81G9, cE146D10 and dJ151B14) hybridised entirely to Siamang 18p. Those clones lie proximal and distal to the human 22q homologous block rearrangement breakpoint, respectively. The fosmid clone fF45C1, which lies distal to fF4G12 and partially overlaps cE81G9 on human 22q13.1, hybridised to both the long arm and the short arm of Siamang 18 (see Figure 4.2).

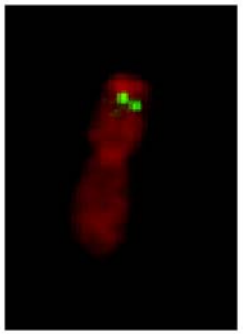
*Figure 4.2 (*next page) Summary of the second round FISH analysis of Siamang metaphase chromosomes. At the bottom of the figure coloured bars indicate the location of fF4G12, fF45C1 and cE81G9 on human chromosome 22 and the overlap between fF45C1 and cE81G9. The dark blue and light blue bar colours indicate the regions proximal and distal to the breakpoint on chromosome 22, respectively. At the top of the figure four corresponding Siamang chromosome 18 images show the FISH signal location from each clone. The chromosome 18 images are ordered from left to right according to the location of the clone on human chromosome 22.



fF4G12 hybridises
to
HSY 18q



fF45C1 hybridises
to
HSY 18p and 18q



cE81G9 hybridises
to
HSY 18p

In order to produce hybridisation signals on both Siamang chromosome 18 arms, fF45C1 must contain sequences homologous to sections of both 18q and 18p. It seemed possible that fF45C1 contains the sequence in human 22q13.1 that spans the homologous block rearrangement point in Siamang chromosome 18. On further careful analysis of the Siamang gibbon metaphase spreads after hybridisation with fF45C1, it was apparent by eye that the FISH signal in 80% of metaphases analysed was weaker on 18p than on 18q. In the other cases, the signals appeared to be of equal intensities.

4.3 FISH Analysis of Dog Chromosomes with Human 22q13.1 Bacterial Clones

4.3.1 FISH analysis of Dog chromosomes with individual bacterial clones

In Chapter 3, the reciprocal zoo-FISH analysis between human chromosome 22 and dog chromosomes 10 and 26 indicated that the junction between material homologous to dog chromosomes 10 and 26 was located in 22q13.1. In the previous section of this chapter, human fosmid clone fF45C1 was identified as containing sequences spanning the evolutionary homologous block rearrangement point in Siamang chromosome 18. Fosmid fF45C1 was localised to human chromosome 22q13.1. Based on those results, clones from the same region of human 22q13.1 were selected for FISH analysis of dog metaphase chromosomes to investigate the possibility that the rearrangement in Siamang chromosome 18 had reverted the human chromosome 22-specific material back to it's ancestral state, represented in the dog.

Three human chromosome 22 clones (BAC bk256C5, fosmid fF45C1 and cosmid cE81G9) were selected for hybridisation individually to dog metaphases. The BAC clone bk256C5 contains sequences, which span those cloned in fF45C1 and cE81G9. The reason for selecting the larger-insert BAC clone was to provide a longer stretch of sequence for hybridisation to the dog chromosomes, which might prove to be more successful than hybridising the shorter fosmid and cosmid clones.

Up to 250 ng of biotinylated DNA from each clone were hybridised to dog metaphase chromosomes, but no clear FISH signals were visualised for any of the individual clones.

4.3.2 FISH analysis of Dog chromosomes with contiguous bacterial clones from human chromosome 22q13.1

As the hybridisation of individual human chromosome 22q13.1 clones to dog metaphases was unsuccessful, an alternative approach was attempted by hybridising several contiguous bacterial clones from the region simultaneously.

A mixture of DNA from three bacterial clones contiguous on human 22q13.1 and overlapping fF45C1 was used for FISH analysis of dog metaphase chromosomes. The three contiguous clones used were BAC bK833B7, PAC dJ394H8 and PAC dJ1170K4. The hybridisation of these three clones together provided the equivalent of a probe 450 kb in length. The sequence of clone fF45C1 is located near the centre of the three contiguous clones.

If the human 22q homologous block rearrangement breakpoint identified in fF45C1 is also present in the canine karyotype the mixed clones could generate hybridisation signals on dog chromosomes 10 and 26. Each signal would be generated by DNA at least 200 kb in length, which is of sufficient size to visualise by fluorescence microscopy after cross-species FISH (Matthew Breen, personal communication).

Biotinylated DNA from the three clones was mixed and tested by hybridisation to human and Siamang metaphase spreads. After detection, ten metaphase spreads were analysed each. A single bright hybridisation signal on HSA 22q13.1 and two bright hybridisation signals on Siamang 18p and 18q were revealed. The contiguous clone signals appeared in the same location on Siamang 18 as the two signals generated by fF45C1.

Biotinylated DNA from the contiguous clones was mixed and hybridised to canine metaphase spreads. After detection, one bright specific hybridisation signal was revealed, only on dog chromosome 26. (Figure 4.3)

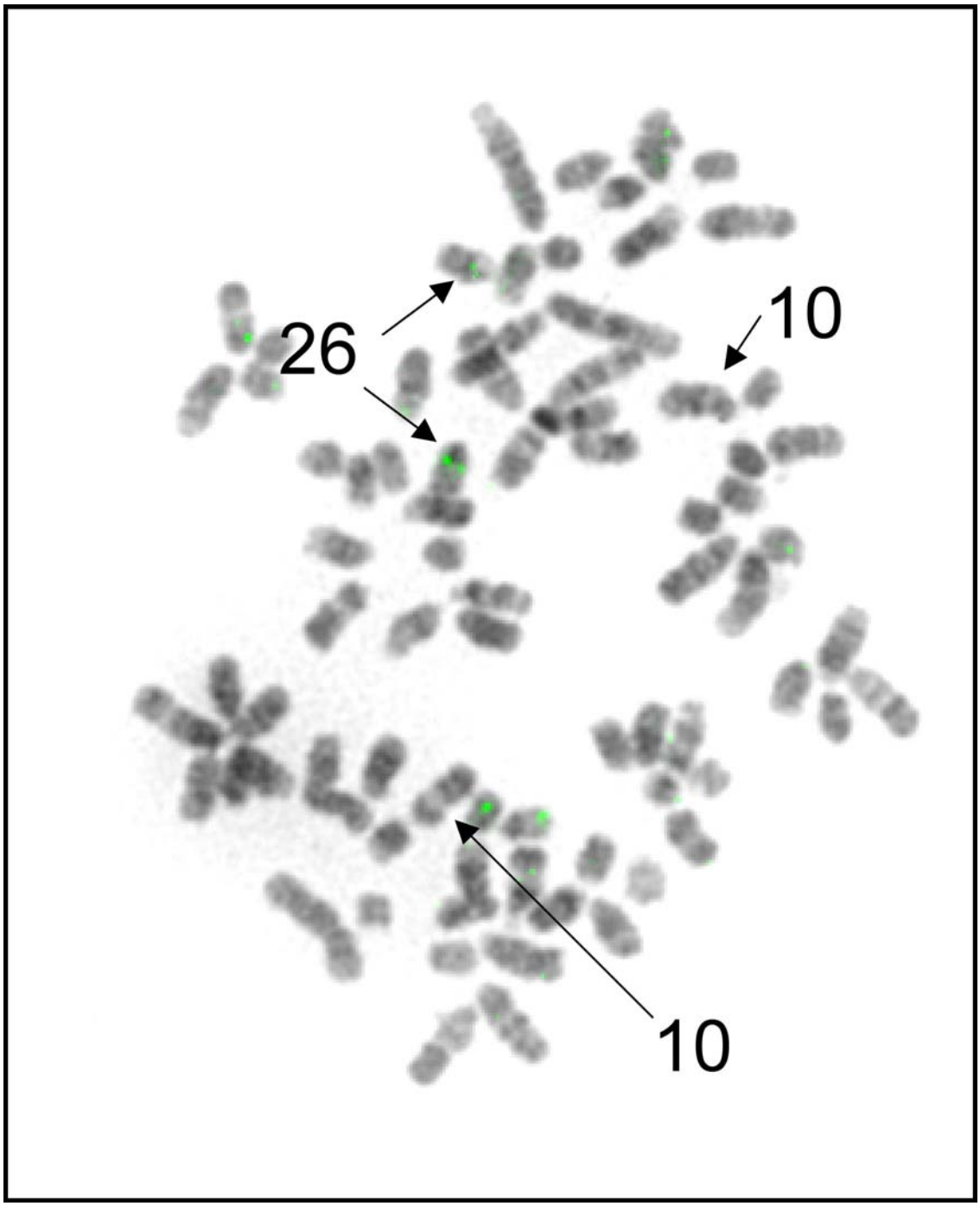


Figure 4.3 Hybridisation signal (pseudocoloured green) of three contiguous human chromosome 22 BAC clones onto a canine metaphase spread. Canine chromosomes 10 and 26 are indicated (arrows).

Thirty metaphase spreads were analysed and although there were several non-specific signals on other dog chromosomes (see Figure 4.3), no specific signal was detected reproducibly on dog chromosome 10.

One possible explanation for this observation might be that the sequence on dog chromosome 10 has diverged sufficiently so that the probe did not hybridise well at the stringency used for the study. Alternatively, it could be explained if the rearrangement breakpoint identified in human chromosome 22q13.1 was not present in the dog karyotype.

4.3 Discussion

In Chapter 3, low-resolution cytogenetic analysis by reciprocal zoo-FISH indicated that the rearrangement points between human chromosome 22 and its two syntenic blocks in the dog lie within 22q13.1. In this chapter, a higher resolution FISH analysis has been carried out on Siamang chromosome 18 using bacterial clones from human chromosome 22q. This resulted in the identification of a fosmid clone from human 22q13.1, which hybridised to both Siamang chromosome 18 arms, which therefore is likely to contain sequences, which span the homologous block rearrangement point.

The evolutionary rearrangements that gave rise to the situation in the three species are most likely to have occurred as independent events. Nevertheless, the similar location of the ends of the gibbon and dog syntenic blocks on human chromosome 22q13.1 meant that a higher resolution analysis of the dog was needed to provide more information. In fact, the hybridisation of clones containing sequences spanning the rearrangement point in the gibbon identified only a single location in the dog genome.

The majority of previous cross-species FISH studies have relied on the use of YAC clones due to their availability and the size of their inserts. Distantly related species are likely to possess more genome sequence divergence than closely related species.

Therefore, the use of large-insert clones for cross-species FISH studies probably increases the chances of successful probe hybridisation and subsequent specific FISH signals. Although there are a considerable number of YACs available, which form part of the human chromosome 22 framework map (Dunham, *et al.,* 1999), BACs were selected to carry out the first cross-species FISH analyses of Siamang chromosomes. It was a well-established fact that at least 30% of YAC clones are chimaeric. The aim of the work for this chapter was to map the human 22q homologous block evolutionary rearrangement point in Siamang chromosome 18. One of the key indicators that a clone contains sequences, which span a breakpoint would be the occurrence of two FISH signals on Siamang 18. Without a guarantee that the YACs were not chimaeric, it was felt that their use could have reduced the reliability of the cross-species FISH results. The occurrence of chimaerism in BAC clones had not been reported.

The size of insert in a BAC clone can range from 150 to 500 kb. PAC inserts range from 30 to 300 kb, whilst cosmid and fosmid clone inserts range from 35 to 45 kb. The clones used for this study harbour inserts only a fraction of the size of the average CEPH megaYAC clone. However, as long as reliable signals were generated by FISH on to Siamang metaphase chromosomes, there was an advantage to using smaller insert clones. The fact that one clone was identified, which generated signals on both arms of Siamang 18, and, therefore contained sequences spanning the homologous block rearrangement point, meant that the location of breakpoint in human chromosome 22 was narrowed down to within a smaller interval than would have been possible with a larger-insert clone.

For the FISH studies, clones were selected which had been used successfully before as part of the mapping process of human 22. For the first round of screening, the thirteen BAC clones were selected on the basis of their mapped location on human 22q and the quality of the FISH signal. Although all the BACs gave good strong signals on human metaphases, after hybridisation to Siamang metaphase chromosomes, four of the BAC clones gave dissipated FISH signals with high background. Therefore, it was concluded

that the hybridisation problems for the four clones was probably due to sequence differences which might have arisen since the ancestors to the lesser apes and great apes diverged.

At the time of these studies, there was no standard karyotype for the Siamang gibbon. However, for the purposes of these studies, it was sufficient to map the BAC clones relative to the centromere and each other within the human 22q homologous blocks on Siamang 18p or 18q. All of the BAC clones appeared to have retained their orientation with respect to the centromere, telomere and each other in the 22q homologous regions on Siamang 18. These results are consistent with there not having been any intrachromosomal rearrangements within the two blocks after the gross rearrangement.

By mapping the ordered BAC clones to Siamang 18p or 18q, it was possible to narrow down the location of the homologous block rearrangement breakpoint to between two BACs (bK212A2 and bK236H12) located in 22q12 and 22q13.1, respectively. In order to narrow down further the position of the breakpoint, eleven new bacterial clones, distributed between bK212A2 and bK236H12, were selected for the second round of FISH screening. Seven of those clones were fosmids or cosmids with relatively small insert sizes of 35 to 45 kb each. There was concern that those clones would be too small to generate meaningful FISH signals. However, all of the clones in the second round of screening gave informative hybridisation signals.

The fosmid clone fF45C1 (mapped to human chromosome 22q13.1) generated signals on Siamang 18p and 18q. The signals on each arm were adjacent to the junction between the human 22q- and human 16p-homologous segments. Fosmid fF45C1 was thus identified as likely to contain sequences spanning the 22q-homologous block breakpoint. From the observations that the FISH signal in 80% of the Siamang metaphases was weaker on 18p than on 18q, it could be speculated that the sequence spanning the homologous block breakpoint was positioned towards the distal end of the fosmid.

Having identified a clone containing sequences spanning an evolutionary chromosome rearrangement point in the Siamang, the next stage of the work towards analysing the nature of the syntenic block boundaries was to identify and analyse the Siamang sequences, which span the homologous block junctions. This work is described in the next two chapters.