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Utilization of FISH in Positional Cloning: An Example on 13q22

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In positional cloning the initial assignment of a gene to a specific chromosomal locus is followed by physical mapping of the critical region. The construction of a high-resolution physical map still involves considerable effort. However, new high-resolution fluorescence in situ hybridization (FISH) techniques have facilitated this process substantially. Here we summarize a strategy that combines a spectrum of FISH techniques [metaphase, interphase, mechanically stretched chromosomes (MSCs), and fiber-FISH on free chromatin] for the construction and characterization of a high-resolution physical map for a positional cloning project. The chromosomal region 13q22, containing the locus of the variant form of the neuronal ceroid lipofuscinosis (vLINCL, CLN5) disease, serves here as an example for this process. We used metaphase FISH to exclude positionally a candidate gene, to refine the locus to 13q22, and to analyze the possible chimerism of the YACs in the region. Both metaphase and interphase FISH techniques were applied to determine the low-resolution distances between the restricting markers. FISH using MSCs confirmed the centromeric–telomeric order of the clones and facilitated the estimation of the size of the gaps between the clones. Finally, fiber-FISH was found to be the method of choice for the construction of an accurate high-resolution map of the contig established over the restricted region. Thus, FISH techniques in combination with genetic mapping data enabled the refinement of the initial 4-cM region to a high-resolution map of only 400 kb in length. Here the FISH strategy replaced the need for many laborious traditional physical mapping methods, e.g., pulsed-field gel electrophoresis.

Positional cloning refers to the identification of a disease gene on the basis of its position on the genomic map (Collins 1992). The entire process is time-consuming and technically demanding. The difficulty of this task is illustrated by the fact that, until now, relatively few of the disease genes originally mapped by linkage analysis have been isolated unless a visible cytogenetic rearrangement has been available, providing a solid landmark for physical mapping (Parrish and Nelson 1993; Collins 1995).

Once a disease gene is genetically assigned to a specific chromosomal region using linkage analysis, the gene "hunter" is typically still left with a distance of several million base pairs. The next step is to close this gap by both genetic and physical means. One of the crucial tasks is to convert the genetic distance to the physical scale and to construct a high-resolution physical map of the region. This step involves the precise localization and high-resolution ordering of clones and markers, with an aim to assemble and characterize a genomic contig over the disease region.

Molecular cytogenetic techniques (for review, see Lichter et al. 1991; Trask 1991; Joos et al. 1994; van Ommen et al. 1995; Palotie et al. 1996) have become the methods of choice for rapid and efficient physical mapping. A special feature of fluorescence in situ hybridization (FISH)-based techniques is the possibility for easy localization, ordering, and visualization of cloned sequences. This makes FISH an efficient tool for producing genome maps over a wide resolution area, from the initial ordering of clones on chromosomes to the assignment of sequences and their accurate chromosomal and genomic localization.

Here we present the use of a panel of FISH techniques as an integral tool of positional clon-

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ing in the characterization of a specific chromosomal region carrying a disease gene.

RESULTS AND DISCUSSION

Based on linkage analysis (<70 individuals), the locus of vLINCL disease (CLN5, MIM 256731) was positioned on 13q21-32 between microsatellite markers D13S162 and D13S160, leaving a gap of 4 cM (Savukoski et al. 1994; Fig. 1A). In patients no visible cytogenetic rearrangements were observed that would have provided a solid landmark for the high-resolution mapping process. However, a tentatively ordered YAC contig from this region was available from CEPH/GENETHON and from the Physical Mapping Group at the Columbia University Human Genome Center.

In the high-resolution mapping of the critical region the strategy relied largely on physical mapping by modern FISH-based molecular cytogenetic techniques. Figure 2 depicts the strategies for FISH in combination with other techniques applied while building the physical map over the CLN5 region.

Metaphase-FISH: Linking the Genetic and Physical Maps

Exclusion of a Candidate Gene TPP2

With a constantly improving map of the human genome, an obvious step is to search for possible candidate genes assigned previously to the region. Traditionally, the available candidate genes are analyzed using STS-content mapping and the localization is refined on the genetic map. We chose for this task two-color metaphase FISH, which does not require the availability of a high-density STS map of the region and gives information about the localization of the gene under analysis in a single experiment.

One of the genes, tripeptidyle peptidase II (TPP2), localized in the vicinity of our critical region to 13q32-33 by radioactive ISH, seemed potentially interesting (Martinsson et al. 1993). TPP2 is a high-molecular-weight serine exopeptidase that at neutral pH removes tripeptides from the amino terminus of longer peptides (Tompkinson and Jonsson 1991). In cohybridization of TPP2 with YACs positive for either of the markers

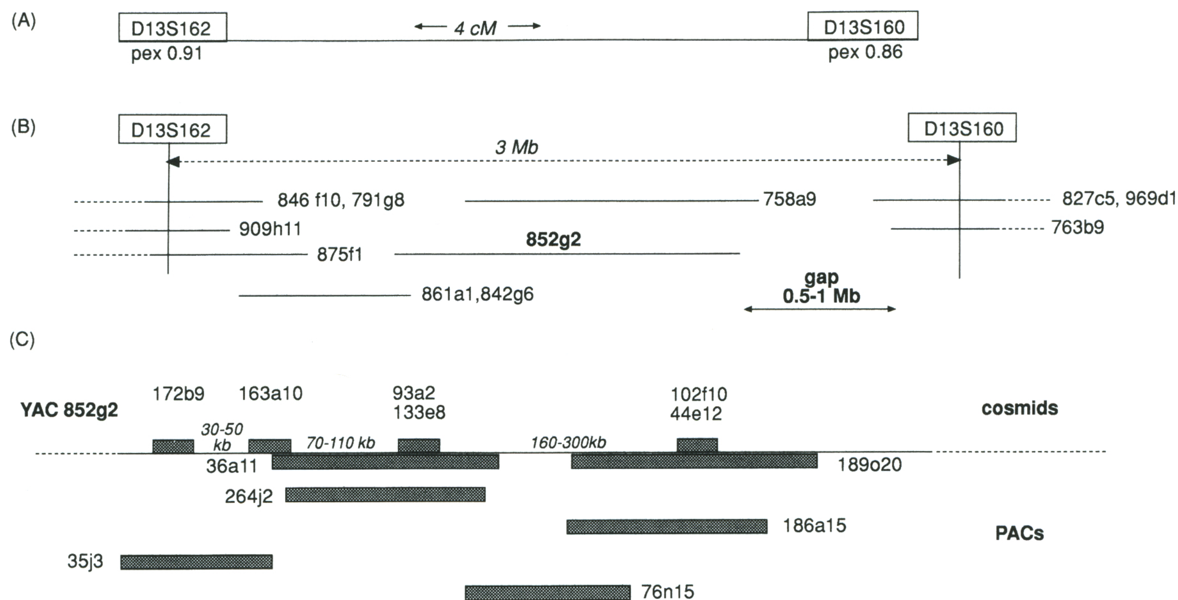

Figure 1 Generating maps over the CLN5 region at 13q22. (*A*) Starting point for physical mapping: CLN5 locus positioned between the polymorphic markers D13S162 and D13S160 by linkage analysis (Savukoski et al. 1994) and the estimated distance in cM. (*B*) Preliminary physical map encompassing the locus at 13q22. Nonchimeric YAC clones important for further study are shown as horizontal lines. The estimation of the physical distance between markers D13S162 and D13S160 was based on metaphase FISH analysis and the size of the gap was analyzed on MSCs. (*C*) High-resolution map of the contig from the CLN5 critical chromosomal region based on fiber-FISH analysis. The bars above the horizontal line represent the cosmid contigs, which here served also as landmarks, and the bars under the line PACs within the YAC 852g2. Calculated distances between the cosmids based on fiber-FISH are indicated.

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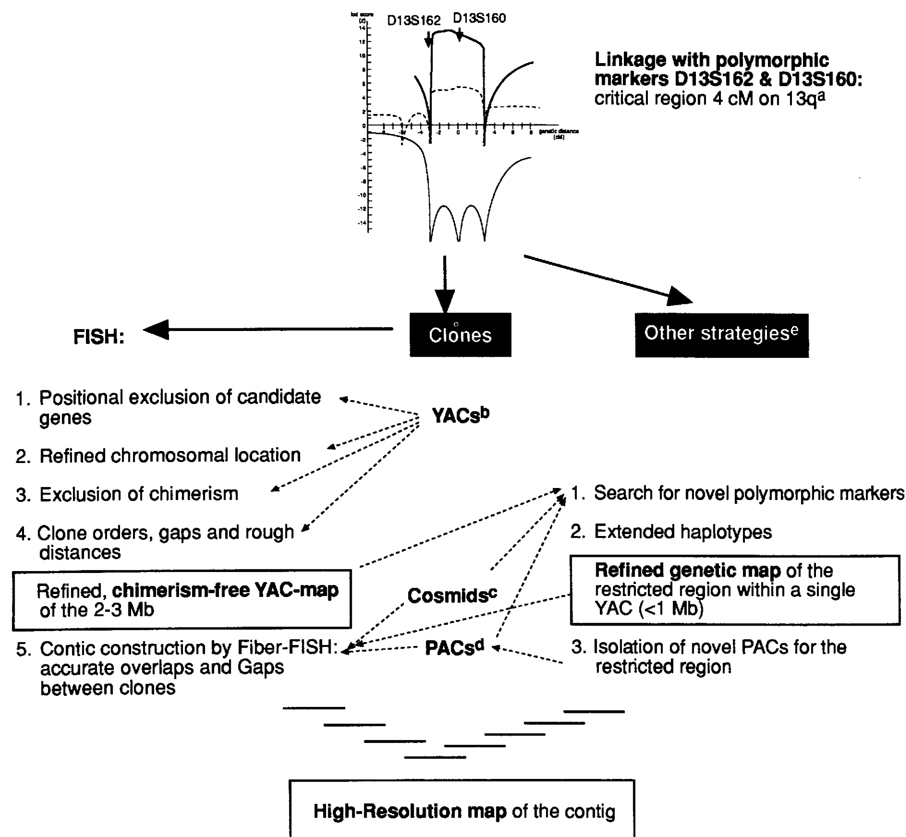

Figure 2 Schematic representation of strategies for the physical mapping of the CLN5 locus on 13q22. [a]Savukoski et al. (1994). [b]Tentatively ordered YAC-contigs were obtained from CEPH/GENETHON. [c]Initially ordered cosmid clones. [d]PAC library (Ioannou et al. 1994) was provided, and the Isolation of applied PAC-clones is described by Klockars et al. (1996). [e]Other strategies are described in detail in Klockars et al. (1996).

D13S160 or D13S162, the TPP2 gene could be assigned to the most telomeric band of chromosome 13, 13q34. This locus is clearly more distal than any of the clones localized to the vLINCL region (Fig. 3, part 3). Thus in this case, two-color metaphase FISH was sufficient to exclude TPP2 as a candidate gene for the disease.

Refined Chromosomal Localization of the CLN5 Critical Region

The markers D13S162 and D13S160 flanking the CLN5 critical region had been assigned earlier by genetic means (Dib et al. 1992; Bowcock et al. 1993; Buetow et al. 1994), by radiation hybrid mapping (Shaw et al. 1995), and by somatic cell hybrids (Hawthorn and Cowell 1995) to the region ranging from 13q21 to 13q32. The accurate chromosomal localization of this genomic region had to be verified and possibly refined. Here, one-and two-color FISH was applied to metaphase chromosomes. Thus, YAC-clones positive to either of the flanking polymorphic markers D13S160 or D13S162 were hybridized to replication-banding chromosomes. The results refined the location of the CLN5 region 13q22 (Fig. 3, part 1a–c). This is in agreement with the recently published chromosome 13 integrated map data (Kooy et al. 1995).

Analysis of the YAC-Contig: Exclusion of Chimeric YACs

On the first-generation physical map approximately 90% of the genome has been reported to be covered in a contig of YAC clones (Cohen et al. 1993). In many positional cloning projects the construction of a YAC contig and analysis of the isolated clones is the first step in the attempted isolation of a disease gene (Hellsten et al. 1995; Järvelä et al. 1995). In this example also the physical map construction was begun by use of the available YAC clones covering the region in question.

Several YACs from the 13q22 had been isolated, ordered tentatively, and assembled to the contig by CEPH/GENETHON and by the Physical Mapping Group at the Columbia University Human Genome Center. However, because chimerism is a well-known phenomenon in YACs (e.g., Green et al. 1991), examination of each clone before studying it in further positional cloning steps is essential. Metaphase FISH is the most convenient tool for the detection of chimerism in YAC or other clones. Thus, each of the YAC clones initially assigned to the region was analyzed for possible chimerism using conventional FISH to metaphase spreads (Fig. 3, part 4a,b). The only drawback of FISH analysis is the inability to detect minor (<1- to 2-kb) components of chi-

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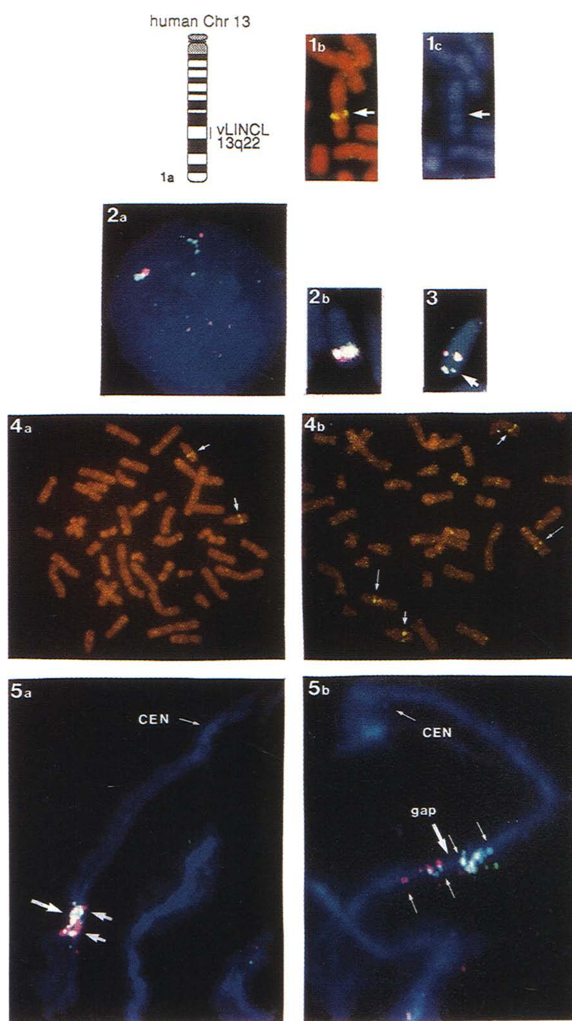

Figure 3 Physical mapping by FISH: Examples from experiments with metaphase spreads, interphase nuclei and mechanically stretched chromosomes as targets. (*1*) Refined assignment of the CLN5 locus. (*a*) Idiogram of human chr 13 with the indicated refined locus; (*b*) fluorescent hybridization signals (arrow) of YAC 909f11 (D13S162) on chr 13 counterstained with propidium iodide; (*c*) assignment of the locus (arrow) using intensive DAPI-staining of the replication-banded chromosome. (*2*) Estimation of the physical distance between polymorphic markers D13S160 and D123S162. (*a*) An image of the interphase mapping of YACs 827c5 (XRITC) and 875f7 (FITC). Note the difficulty in determining the center of the fluorescent hybridization spots and thus the distance between them. (*b*) An image of a metaphase chromosome cohybridized with YACs 791g8 (FITC) (D13S162) and 827c5 (XRITC) (D13S160). Despite the partly overlapping signals of the large probes, the orientation of the clones is resolvable. (*3*) Exclusion of the candidate gene TPP2: An image of a cohybridization of YAC 846f10 (XRITC) and TPP2 cDNA (FITC) (arrow). (*4*) Analysis of the possible chimerism of YAC-clones on metaphase spreads. (*a*) Nonchimeric YAC 909h11. Note the intensive hybridization signals on chromosome 13 only (arrows). (*b*) Chimeric YAC 935e7. Despite strong nonspecific Alu background, specific fluorescent hybridization spots can be detected on both chr 13 (short arrows) and chr 7 (long arrows). (*5*) Estimation of the size of the gap between contigs established from D13S162 and D13S160 by use of FISH on MSCs. (*a*) Cohybridization of YAC 846f10 (XRITC) (D13S162), 758a9 (FITC), and 763b9 (XRITC) (D13S160). The green signal of 758a9 (long arrow) was detected in the center and overlapped with the red signals from both of the flanking clones (short arrows) equally. (*b*) Cohybridization of YACs 758a9 (FITC) and 763b9 (XRITC), flanking the gap region, to an extensively stretched chromosome. The gap (large arrow) between the hybridization loci can be estimated roughly as being shorter than the size of the corresponding YACs (small arrows), i.e., <1 Mb.

meric clones. Of the 21 clones, only 13 proved to be not chimeric and thus suitable for the creation of the chimera-free YAC map and for further analysis (Fig. 2).

Preliminary Physical Distance between Polymorphic Markers

FISH analysis was performed to convert the 4-cM genetic distance between the flanking markers to the physical scale and to confirm the centromere-telomere orientation of the clones. Preliminary estimates of the physical distance between the markers was carried out by cohybridizing corresponding, differently labeled YAC clones pairwise to metaphase spreads (minimum resolution 1–2 Mb) (Lawrence et al. 1990; Trask et al. 1993) and interphase nuclei (informative resolution range 0.1–1 Mb) (Lawrence et al. 1990; Trask et al. 1991; Senger et al. 1993). Analysis on metaphase chromosomes demonstrated partially overlapping large signals from YACs positive to region-restricting markers. The centromere–telomere orientation of the clones was detectable in each of the 40–60 chromosomes analyzed (Fig. 3, part 2b). This indicated that the interval between the

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bordering markers of the critical region should be at least 1–2 Mb (Lawrence et al. 1990; Trask et al. 1993) and that the region is orientated TEL-D13S160-D13S162-CEN.

As metaphase preparations include a large number of interphase nuclei, these can be utilized as an additional source of data for distance estimation. In this case the variation in distance measurements between the signals of different YAC clones in interphase nuclei proved to be large [coefficient of variation (CV) = 50–70%], which made the interpretation of individual results problematic (Fig. 3, part 2a). Earlier studies have shown that the mean measured interphase distance between the probes' midpoints (in μm) correlates with the real genomic distance (in kb) of <1 Mb (Lawrence et al. 1990; Trask et al. 1991; Senger et al. 1993). Furthermore, according to a random walk model at genomic distances <2 Mb, the square of the mean interphase distance is linearily related to the known genomic distance (van den Engh et al. 1992). Thus, here, the great variation in the measured distances between probes in interphase nuclei suggested indirectly that the distances between the hybridized YACs exceeded 1–2 Mb. In addition, as the hybridization signal of the large YAC inserts varied in size, the determination of the midpoint for the signal was complicated (Fig. 3, part 2a). This increased the possibility of unreliable gap measurements. Furthermore, as interphase nuclei in metaphase preparations were used, these cells include all stages from G1 to G2, thus adding inaccuracy to interphase FISH distance determination.

In conclusion, metaphase FISH refined the chromosomal localization of the CLN5 critical region with positional exclusion of the candidate gene TTP2. It allowed the creation of the chimera-free YAC map over the critical chromosomal region and the estimation of the preliminary physical distances between the CLN5 region flanking polymorphic markers.

FISH on Mechanically Stretched Chromosomes

Analysis of the Size of the Gap in the YAC–Contig

The narrower the refined critical region, the greater the demand for higher resolution in physical mapping. Metaphase FISH provides mapping with a resolution of 1–2 Mb. Thus, on the submegabase level, the resolution of metaphase FISH chromosomes is no longer sufficient for the successful ordering of probes. Further analyses require more decondensed chromosomal structures.

While establishing the YAC map of the region, there was no information available about the size of the unfilled gap between the contig from D13S162 and YACs positive to D13S160. For this task we used mechanically stretched chromosomes (MSCs), providing a mapping resolution ~10–20 times higher than conventional metaphase chromosomes (highest resolution ~100 kb) (Laan et al. 1995b). Long stretched chromosomes are achieved by extending them after hypotonic treatment during cytocentrifugation (Haaf and Ward 1994). As compared with interphase nuclei, which provide a similar mapping resolution, one of the benefits of the extended chromosomes is that they provide information on the centromere–telomere orientation of the clones.

YAC clones flanking the gap region, 763b9 and 758a9, were cohybridized on stretched chromosomes with a YAC positive to marker D13S162, 846f10 (Fig. 1b). The hybridization showed that 758c9 detected with green overlapped simultaneously with 763b9 and 846f10, both detected with red fluorescence (Fig. 3, part 5a). This means that the unfilled gap between 758a9 and 763b9 is about the same size as the contig-covered region between 758b9 and 846f10. However, MSC preparations are characterized by a high variability in target stretching and thus cannot be used for direct measurements of probe distances. The gaps can be estimated only indirectly by comparison with known flanking distances (Laan et al. 1995b). Thus, as on maximally stretched chromosomes where the specific hybridizations are more fiber-like, the gap between clones 763b9 and 758a9 did not exceed the size of the hybridization signals of the corresponding YAC clones and could thus be interpretated as being <1 Mb (Fig. 3, part 5b). In addition, the hybridization confirmed the orientation of the YACs flanking the gap: CEN-758a9-763b9-TEL.

Fiber-FISH and High-resolution Mapping of the Restricted Region

The final construction of the physical map over the restricted DNA region requires a high-resolution approach, allowing precise map construction at the 1-kb level. Traditionally, in most

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of the physical mapping studies, construction of a genomic map at the 1-Mb level has been carried out by use of pulsed-field gel electrophoresis (PFGE) or restriction mapping, followed by Southern analysis of the fragments, and characteristically involves tedious isolation of the ends of large clones. In our study, FISH on extended DNA fibers (Heiskanen et al. 1994) was the method of choice for establishing the precise physical map over the CLN5 region. The advantages of fiber-FISH techniques are high resolution within the range of 1–500 kb and physical mapping accuracy (Parra and Windle 1993; Heiskanen et al. 1995; Florijn et al. 1995; Weier et al. 1995). Visual mapping enables easy ordering and orientation of the clones, as well as measurements of the overlaps and gaps between the isolated clones. The unique feature of fiber-FISH is the possibility to order also clones that do not overlap with each other. This is not possible by restriction mapping, for example.

Our contig construction was initiated with the available YACs. However, in construction of the high-resolution map, medium-sized insert clones, PACs, and cosmids were used. Initially ordered partial cosmid contigs between D13S162 and D13S160 were assembled by Columbia University Human Genome Center, and a P1-derived artificial chromosome (PAC) library was used. The recently introduced PAC cloning system has numerous advantages when compared with YACs or cosmids (Ioannou et al. 1994).

As a result, a high-resolution map of a continuous contig over the putative CLN5 region was established solely on the basis of fiber-FISH mapping in combination with STS content mapping data (Fig. 2). The details of this PAC contig covering the vLINCL critical region is described elsewhere (Klockars et al. 1996). Figure 4 demonstrates the process of contig construction. Usually a conventional fluorescent microscope is sufficient for visualization of FISH signals. However, a digital multicolor image-analysis system based on high-quality microscope and CCD-camera applications enabled rapid and efficient analysis of fiber-FISH preparations and the physical map construction.

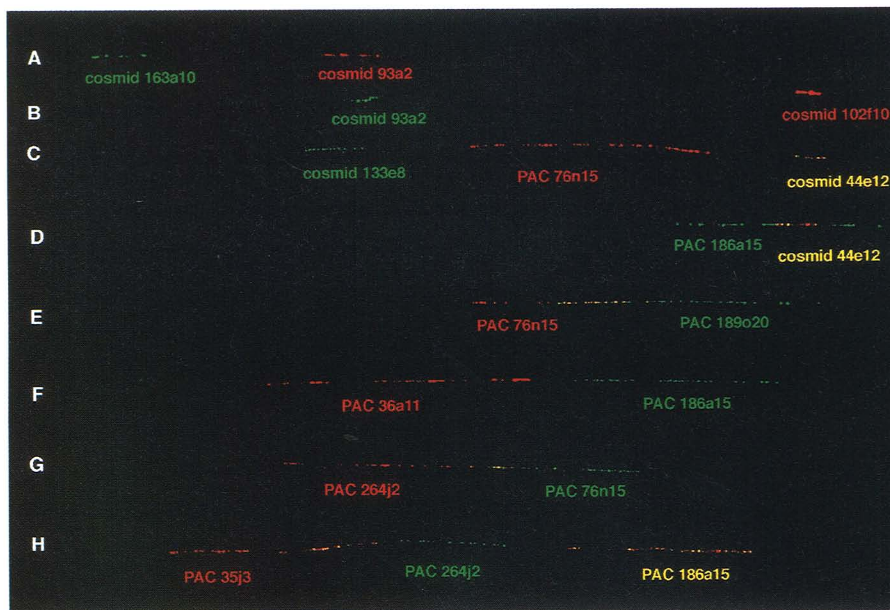

Figure 4 Physical mapping by FISH: Construction of a high-resolution map of a contig on the CLN5 region. Examples shown represent only a selection of experiments performed by fiber-FISH in the process of physical map construction. Each of the mapping results was confirmed by hybridizations of different probe combinations and STS-content mapping. Cohybridizations on DNA fibers of (*A*) cosmids 163a10 (FITC, green) and 93a2 (XRITC, red) separated by ~70–110 kb; (*B*) cosmids 93a2 (green) and 102f10 (red) separated by ~160–300 kb; (*C*) cosmids 133e8 (green), 44e12 (FITC + XRITC = yellow) and PAC 76n15 (red). PAC is located between the cosmids and not overlapping with them. (*D*) PAC 186a15 (green) covering the cosmid 44e12 (yellow); (*E*) PAC 76n15 (red) overlapping with PAC 189o20 (green); (*F*) PAC 36a11 (red) and PAC 186a15 (green) with a clear visible gap; (*G*) PAC 264j2 (red) overlapping with PAC 76n15 (green); and (*H*) PAC 35j3 (red) overlapping with PAC 264j2 (green) and the orientation of the latter toward PAC 186a15 (yellow).

Assignment of the Cosmid Contigs

The mapping was initiated by the precise localization of six cosmid contigs, localized and ordered tentatively between the markers D13S160 and D13S162. Representative clones of two cosmid contigs, 173f6 and 159b11, proved to be chimeric with two hybridization loci, 13q22 and 13q13-14, and thus could not be included in the map. The order and physical dis-

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Table 1. Gaps between Cosmid Contigs within YAC 852g2

Cosmid pair	No. of analyzed images	Mean length of gap (kb)	Standard deviation (kb)
172b9/163a10	11	40	10
163a10/93a2	8	90	20
93a2/102f10	6	230	70

The gap calculation was based on the known insert sizes of cosmid clones (~30 kb).

tances between the remaining four cosmid contigs, represented by clones 172b9, 163a10, 93a2, and 102f10 were determined by fiber-FISH. The gaps were measured in kbs based on the known lengths of the inserts of the flanking cosmids (~30 kb) (Table 1). Clones 172b9 and 163a10 resided only ~30–50 kb apart (data not shown), whereas the distance between 163a10 and 93a2 was estimated to be ~70–110 kb (Fig. 4A), and the gap between 93a2 and 102f10 ~160–300 kb (Fig. 4B).

For each probe combination, the analysis of only one or two hybridization preparations was sufficient. Usually 10–30 images were scanned to ensure the probe order, the number of image analyses required depending on the distances between the probes. From the photographed images, approximately 10 were applied for the distance measurements.

Filling the Gaps with Isolated PAC Clones

There were distinct gaps between the mapped cosmid clones representing four separate contigs (Figs. 1C, 4A–C). More clones were needed to fill in the gaps to generate a contig over the key area thought to contain the CLN5 gene. PAC clones containing inserts of up to 200 kb were isolated from the region (Klockars et al. 1996), having an advantage over cosmids whose inserts are only up to 40 kb (Ioannou et al. 1994). PACs were used to cover the gaps and to complete the construction of a high-resolution physical map over the region.

In Figure 4 the establishment of the continuous contig is demonstrated from cosmids 102f10/44e12 toward cosmid 163a10 (Figure 4A–H). Cosmid contig represented by clone 172b9 was omitted from the map because it does not belong to the restricted key area. Other nonchimeric cosmid contigs were used as landmarks for the region. The gaps left by cosmid contigs were filled in by PACs isolated by use of novel markers (STSs and polymorphic markers) positive to the cosmid contigs and PACs assigned previously to the region.

The localization of cosmid contigs inside PAC clones was refined (Fig. 4D). The orientation, gaps, and overlaps between the isolated PACs were detected using fiber-FISH (Fig. 4C,E–H). Each of the mapping results was confirmed by hybridizations of different probe combinations and was supported by the STS content mapping. Altogether 10 probe combinations in fiber-FISH were needed to establish the cosmid-PAC contig map. On the other hand, for each of the 10 probe combinations one or two hybridization slides were sufficient to provide reliable results. This is because each fiber-FISH slide contains tens or even hundreds of hybridization signals, providing confidence in the analysis. In total, the size of the overlapping PAC and cosmid contig containing the CLN5 locus was about 400 kb (Fig. 1C).

CONCLUSION

FISH provides an alternative to traditional physical mapping strategies. A large spectrum of FISH techniques with different levels of sensitivity, resolution, and accuracy, combined with speed and easy interpretation, offers a system that can be implemented in many positional cloning studies. Furthermore, in many cases visual FISH mapping is quicker (e.g., fiber-FISH vs. PFGE), less laborious (e.g., centromere–telomere direction), and more accurate (e.g., chromosomal localization) than conventional mapping techniques. In detection of YAC chimerism FISH is indispensable.

Metaphase FISH offers an easy alternative for the chromosomal localization of probes and

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gives information about the initial genomic distribution of probe sequences after a single experiment. However, the condensation level of DNA in chromosomes is high, so the mutual ordering of probes is possible only when clones are >1–2 Mb apart. Interphase FISH allows mapping at a much higher resolution (100–1000 kb), but the disadvantage of the technique is the indirect interpretation of the quantitative data, influenced by the type of probes, hybridization background, and three-dimensional structure of nuclei. MSCs are a suitable target for high-resolution ordering of probes (>100 kb apart) with regard to their telomeric/centromeric orientation, but do not provide accurate information on the distances between them. However, the size of the gap or overlaps can be estimated indirectly by comparison with the known neighboring distances or from quantitative data. Finally, fiber-FISH is the method of choice when accurate distance and overlap measurements are necessary from a range of a few kb up to 500 kb. Thus, a refined map of the established contig can be constructed. It is also seen that, moving from low- to high-resolution analysis, the map estimations at different resolution levels correspond well.

Here we demonstrated how the physical mapping information was collected using mainly visual mapping by FISH techniques. PFGE or even conventional restriction mapping, which are by now the most widespread approaches for large-scale physical mapping and contig description, were not necessary throughout the physical mapping process from the initial 4-cM regional assignment to the construction of a high-resolution map of the CLN5 locus. Compared with traditional techniques, the applied FISH approach proved to be advantageous, first, by reducing the time necessary for the high-resolution map construction, and, second, by giving information on clones not overlapping with each other.

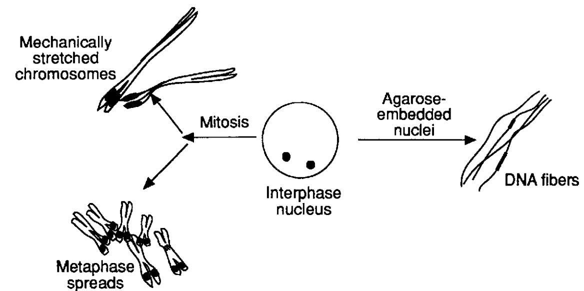

Figure 5 Schematic representation of the hybridization targets for FISH.

METHODS

The original references for the FISH techniques applied in the study, and their physical mapping resolution spectrum, are summarized in Table 2. A panel of different FISH targets is depicted in Figure 5.

Hybridization Targets

Metaphase spreads from human peripherial blood lymphocyte cultures were prepared by standard protocols. Nuclei from the same preparations were used as targets for the interphase mapping. To obtain G-banded chromosomes, lymphocyte cultures were synchronized with 200 μg/ml

Table 2. FISH Techniques Applied in the Study

Technique	Mapping resolution	Original methodological reference
Single-color metaphase FISH on BrdU-banded chromosomes	1–3 Mb	Lichter et al. 1990; Takahashi et al. 1990; Lemieux et al. 1992
Two-color metaphase FISH	1–2 Mb	Lichter et al. 1990; Hopman et al. 1986; Tkachuk et al. 1990
Interphase FISH	>50–100 kb (upper limit of utility range 1 Mb)	Lawrence et al. 1990; Trask et al. 1991; Senger et al. 1993
FISH on mechanically stretched chromosomes	>100 kb	Haaf and Ward 1994; Laan et al. 1995b
Fiber-FISH	>1–5 kb (upper limit of utility range 500 kb)	Heiskanen et al. 1994

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5-bromodeoxyuridine (BrdU) overnight, washed with phosphate buffered saline (PBS), and reincubated with 0.3 μg/ml thymidine for an additional 6–7 hr (Lemieux et al. 1992; Tenhunen et al. 1995). Colcemid was added 20 min before harvesting. Prepared chromosomal slides were stained with 1 μg/ml Hoechst 33258 for 5 min and exposed for 30 min to a 302 nm UV lamp at a distance of 10 cm.

MSCs were prepared essentially as described by Haaf and Ward (1994) and Laan et al. (1995b). Briefly, 2000–4500 cells from phytohemagglutinin-stimulated lymphocyte cultures were used for one slide preparation. First, cells were washed in PBS and treated with 10 mM Hepes buffer for 5–15 min. Next, 0.5 ml aliquots of the hypotonic cell suspension were cytocentrifuged (Cytospin 2, Shandon) to an ethanol-cleaned glass slide at 800–1200 rpm for 4–15 min. Preparations were fixed in methanol at −20°C for 15–45 min.

Preparation of DNA fibers is described in detail by Heiskanen et al. (1994, 1995). Briefly, a 1/6–1/10 of 100 μl agarose-embeddeed PFGE block containing about 5 μg human or YAC DNA was placed on a microscopic slide precoated with 0.15% gelatin and 0.2% Poly-L-Lysine. Agarose was melted with 20 μl of deionized water and the DNA extended mechanically on a slide.

Genomic YAC, PAC, and Cosmid Clones

The initially assembled YAC contigs were obtained from CEPH/GENETHON and the Physical Mapping Group at the Columbia University Human Genome Center. Human sequences in the isolated YAC clones were enriched with Alu-specific primers Tc65 and 278 (Nelson et al. 1989). Partial cosmid contigs were assembled and ordered by the same group and the data provided by S.G. Fisher (Human Genome Center, Columbia University, New York, NY.) A genomic PAC library (Ioannou et al. 1994) was provided by P.J. de Jong (Roswell Park Cancer Institute, Buffalo, NY) and the isolation of PAC clones for the CLN5 region is described by Klockars et al. (1996). The tripeptidyle peptidase II (TPP2) cDNA plasmid clone (3.9kb) was a kind gift from B. Tompkinson (Biomedical Centre, Uppsala University, Sweden).

FISH

FISH was performed using probes labeled by nick translation with either biotin-11-dUTP (Sigma Chemical) or digoxigenin-11-dUTP (Boehriger Mannheim) using standard protocols (Lichter et al. 1988; Laan et al. 1995a,b). 40–200 ng of each of the labeled probes with 10- to 30-fold excess of unlabeled Cot-1 DNA (Life Technologies, Gaithersburg, MD) was applied per slide. In single-color experiments formed hybrids were detected using avidin-FITC (Vector Laboratories; Pinkel et al. 1986; Laan et al. 1995a). For double-color hybridizations, biotinylated and digoxigenin-labeled probes were detected as described earlier (Heiskanen et al. 1994; Laan et al. 1995b).

Chromosomal DNA was stained with 0.025 μg/ml DAPI (4′-6′ diamino-2-phenylindole) (Sigma Chemical) and/or 2 μg/ml propidium iodide. The slides were mounted in antifade medium (Vector Laboratories).

Fluorescence Microscopy

Single-color experiments on standard metaphase spreads were examined with a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 100× objective (NA 1.25) and photographed using Ektachrome ASA 400 color film. For double-color hybridizations a digital multicolor image analysis system was used. This is based on the Olympus BX50 microscope and Photometrics PXL camera (Photometrics, Tucson, AZ) attached to a PowerMac 7100/Av workstation. IPLab software (Signal Analytics Corp., Vienna, VA) controls for the camera operation, image acquisition, and Ludl Filter wheel (Ludl Electronics, Hawthorne, NY) were equipped with Chromatechnology multiband-pass filters. Alternatively, another system was used composed of the Nikon SA microscope (Tokyo, Japan) and the Xillix CCD camera (Vancouver, BC, Canada) interfaced to a Sun LX workstation (Sun Microsystems, Mountain View, CA) and running the Scil-image image processing software (TNO Delf, Netherlands).

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