8

In Situ Hybridization

A fascinating property of DNA is the complementarity of the nucleotide bases in its two anti-parallel strands, with G always pairing with C and A always pairing with T. This does not involve strong covalent chemical bonds but weak hydrogen bonds. There are three hydrogen bonds between G-C pairs and two between A-T pairs, so strand separation is easier in AT-rich DNA than in GC-rich DNA. Mild heating breaks these hydrogen bonds and is one way to separate the two strands, called *denaturation* or *dissociation*. Reducing the temperature under the right salt conditions leads to *renaturation* (*reassociation* or *reannealing*) of the two strands by reconstitution of the hydrogen bonds. The rate of renaturation depends on the frequency of collision between complementary sequences, which depends on their concentration. The concentration and time required for renaturation determines the *Cot value* (concentration × time). If a high concentration of labeled probe DNA is used, hybridization to

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complementary nucleic acid sequences in the target preparation can be achieved in a reasonably short time. These properties of DNA are extremely important, because they make it possible to detect specific DNA sequences (such as genes) on a nitrocellulose filter (*molecular hybridization*) or in cytological preparations (*in situ hybridization*) by using labeled DNA or RNA probes.

In Situ Hybridization of Repetitive and Unique DNA Sequences

Typically, in situ hybridization involves denaturing a cloned DNA fragment, labeling it with a radioactive, fluorescent, or antigenic tag, and hybridizing it to denatured DNA in fixed metaphase chromosome spreads on a slide. Under highly stringent conditions of temperature and salt concentration, the DNA probe will hybridize only to its complementary strands and not to any of the enormously greater number of strands from other parts of the genome. Under these conditions, bonds formed between imperfectly paired sequences will be short-lived, but bonds between perfectly paired sequences will be stable. Sites of in situ hybridization of radioactive probes can then be detected autoradiographically and those of nonradioactive probes by fluorescence or enzyme-mediated staining.

The strength of signal depends on the length of the cloned DNA probe and the density of label, and determines the *sensitivity*. When ^{3}H-thymidine is used to label probe DNA, the sensitivity is limited, because the half-life of tritium (^{3}H) is 12.8 years, so only a tiny fraction of the atoms will decay in a 3- to 4-week autoradiographic exposure period. Furthermore, the electrons produced by the radioactive decay of tritium may travel some distance before they nucleate silver grains in the photographic emulsion overlying the microscope slide. The grains are spread out within a radius of 1 μm or more around the site of hybridization, which yields rather low *resolution*. However, autoradiographic in situ hybridization does have one advantage: It allows comparisons of the relative sizes of two or more targets. De Capoa et al. (1988) used this approach to determine the relative numbers of ribosomal RNA genes on different acrocentric chromosomes.

Despite the limited sensitivity and resolution of autoradiographic detection of sites of in situ hybridization, this method was successfully used to map tandemly repetitive sequences such as satellite DNAs in heterochromatin and several classes of multicopy genes (40 to 200 copies each). These included

the 18S and 28S ribosomal RNA genes in the NORs of the acrocentric chromosomes, the 5S ribosomal RNA genes at lq42–q43, and the histone genes at 7q32–q36. Autoradiographic detection of single-copy genes was much harder, though achievable (Harper and Saunders, 1984). A major problem was that fewer than 15% of the autoradiographic silver grains were localized at the two specific allelic sites, with the rest of the grains widely scattered and sometimes also concentrated at a few secondary sites. That is, the background was high and the signal-to-noise ratio was low. An obvious reason for the high background was the presence of extremely abundant repetitive sequences throughout the genome. Because of their high number of copies, the repetitive sequences could hybridize more quickly (at a lower Cot value) than unique sequence genes.

A major advance was the discovery that hybridization of labeled probes to repetitive sequences can be blocked by prehybridization to unlabeled genomic DNA or, preferably, Cot1 DNA, the highly repetitive fraction of the genome (Cremer et al., 1988; Pinkel et al., 1988). As a result, nonradioactive (enzyme or fluorescence) labeling techniques have largely replaced autoradiographic labeling because of their greater speed, resolution, and reliability. Almost any gene or other component of the genome can now be mapped.

Fluorescence In Situ Hybridization

Methods involving fluorescent labels are generally preferred over enzyme labels and are the best developed (Trask, 1991; Lichter and Ward, 1990). Bromodeoxyuracil, digoxigenin, dinitrophenol, or other side-groups can be used to label DNA probes, and their sites of hybridization can be detected by highly specific fluorescent or enzyme-linked antibodies (Fig. 8.1). Fluorescent side-groups can be covalently bound to the probe DNA itself, thus eliminating the need to add a fluorescent molecule that binds to the probe. Chromosome-specific probe sets have been prepared that "paint" different chromosomes or parts of chromosomes (Cremer et al., 1988; Pinkel et al., 1988). The power and beauty of these new techniques is reflected by their increasingly wide use, and illustrated in Figs. 8.2, 8.3, 8.4 (see color insert), and 26.1.

Very long DNA fragments can be used as hybridization probes after blocking the hybridization of interspersed repeats, thus markedly reducing the noise level generated by such hybridization. This is useful because long probes yield strong fluorescent signals that are readily detectable by simple fluorescence

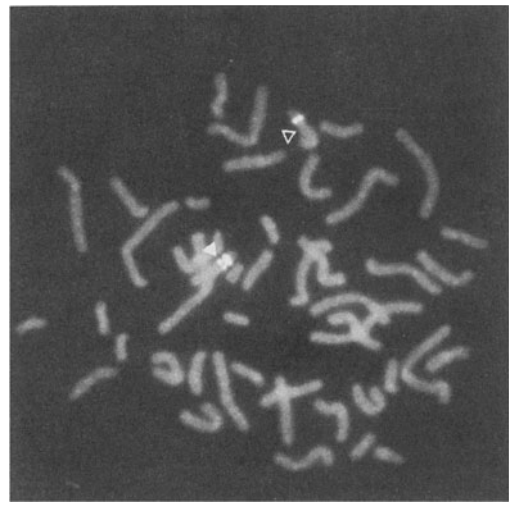


Figure 8.1. Demonstration by FISH of a deletion of the Prader-Willi/Angelman syndrome region, 15q11–q13. Hybridization of a YAC cloned from this region produces a signal on one homologue (filled arrowhead) but none on the other homologue (open arrowhead). The classical satellite probe, D15Z1, produces a large signal in the centromeric region of each chromosome 15 (reproduced from Kuwano et al., Hum Mol Genet 1:417–425, 1992, with permission of Oxford University Press).

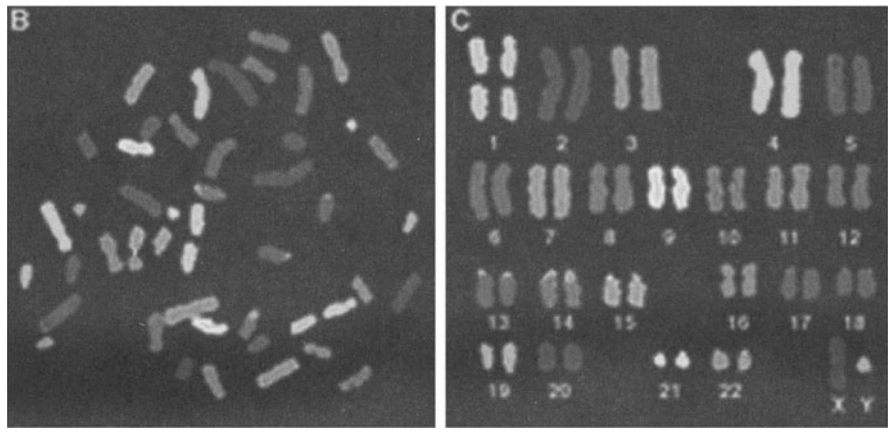


Figure 8.2. Spectral karyotyping after simultaneous hybridization of 24 combinatorially labeled chromosome-specific (painting) probes. B, a metaphase spread; C, the karyotype of this metaphase. Regions rich in repetitive sequences, such as the short arms of numbers 13, 14, 15, 21, and 22, show color variations that are expected after suppression hybridization (reprinted with permission from Schröck et al., Multicolor spectral karyotyping of human chromosomes. Science 273:494–497, copyright 1996, American Association for the Advancement of Science) (See color insert).

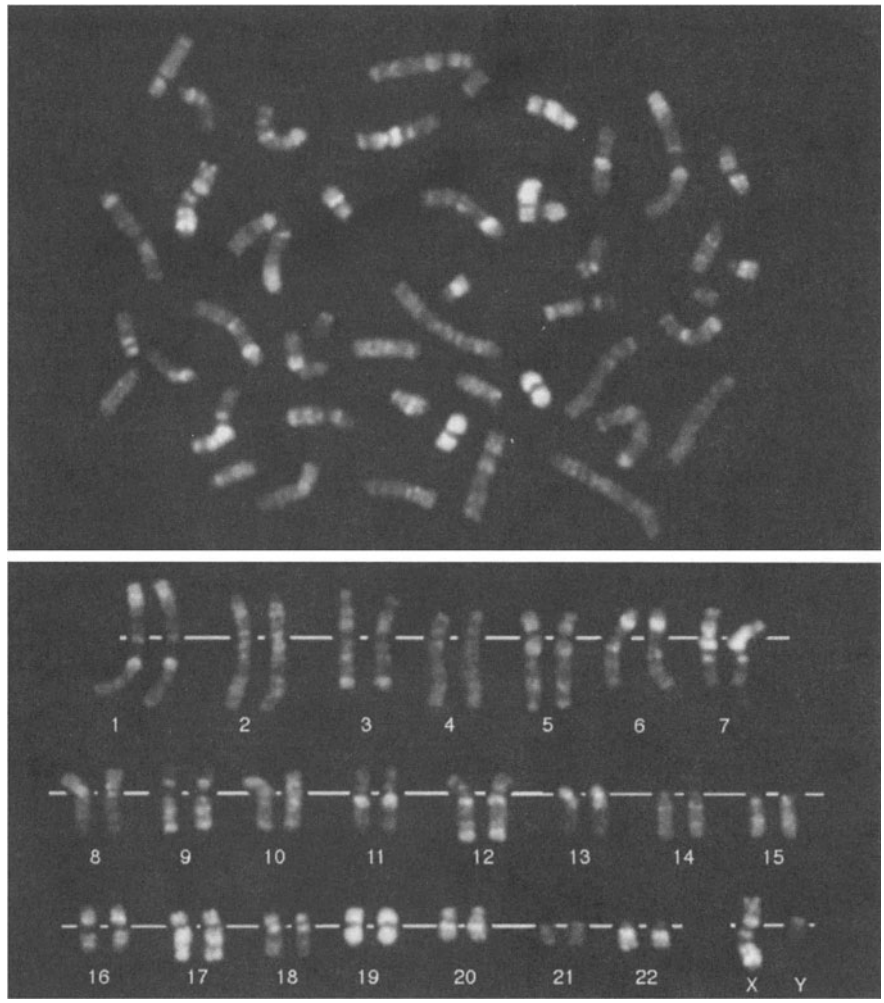


Figure 8.3. A metaphase spread (above) and a karyotype (below) after in situ hybridization and detection of multiplex probes. Red and green bars represent regions that hybridized to fragments in only one probe pool, and yellow bars represent regions that hybridized equally to fragments in both probe pools. Mixed colors are due to overrepresentation of fragments from one pool. Regions that fail to hybridize to fragments in either pool show background DAPI blue fluorescence (reproduced from Müller et al., Toward a multicolor chromosome bar code for the entire human karyotype by fluorescence in situ hybridization, Hum Genet 100, fig. 3, p 273, copyright 1997, Springer-Verlag) (See color insert).

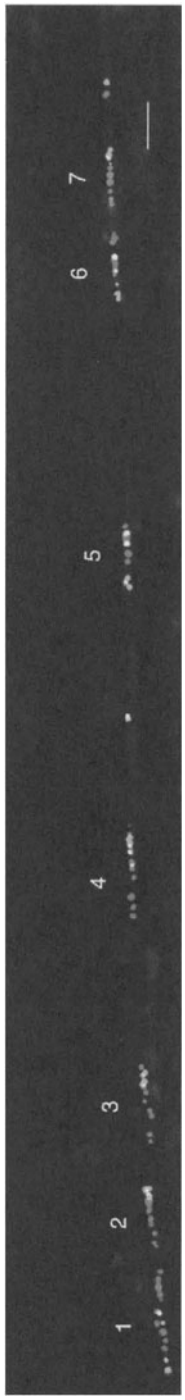


Figure 8.4. High-resolution fiber-FISH using the DAZ G5 probe from the 5′ end of the DAZ gene (green fluorescence) and the DAZ G21 probe from the 3′ end of the gene (red fluorescence) to label extended S phase Y chromatin. Seven DAZ red-green signals (genes or pseudogenes) are seen in a linear array, with at least one gene inverted with respect to the others (Gläser et al., Chromosome Research 6: figure 4, p 484, 1998, with kind permission from Kluwer Academic Publishers) (See color insert).

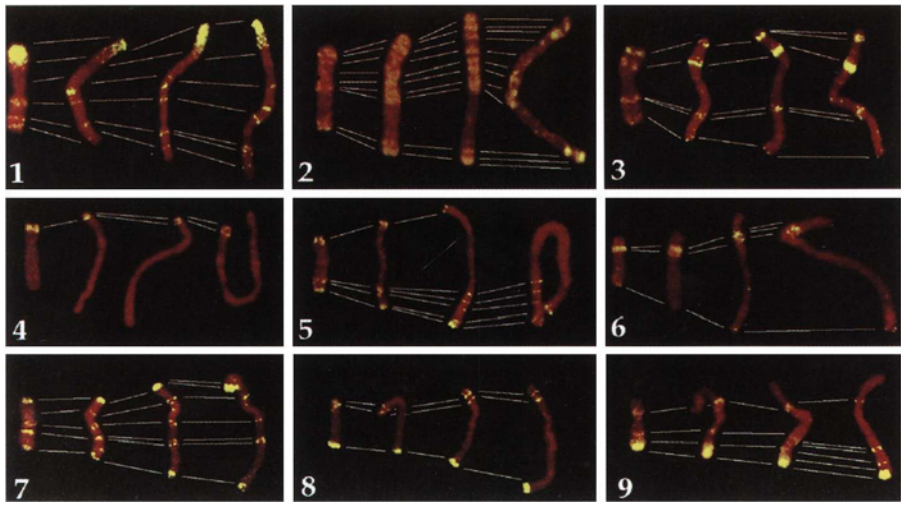
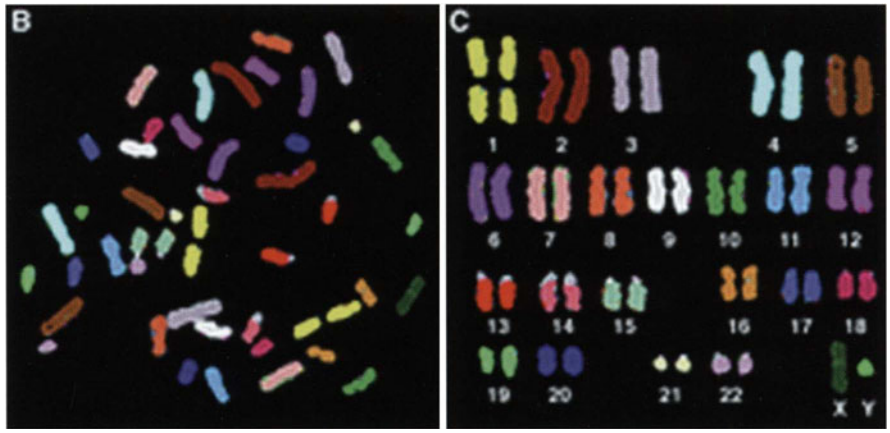


Figure 7.2. Hybridization of GC-rich H3 isochore DNA to chromosomes 1–9 at different stages of contraction. Biotinylated H3 isochore DNA was detected with avidin-FITC (yellow) on propidium iodide-stained chromosomes (red) (Saccone et al., Identification of the gene-richest bands in human prometaphase chromosomes, Chrom Res 7: figure 1, p 382, copyright 1999, with kind permission from Kluwer Academic Publishers).



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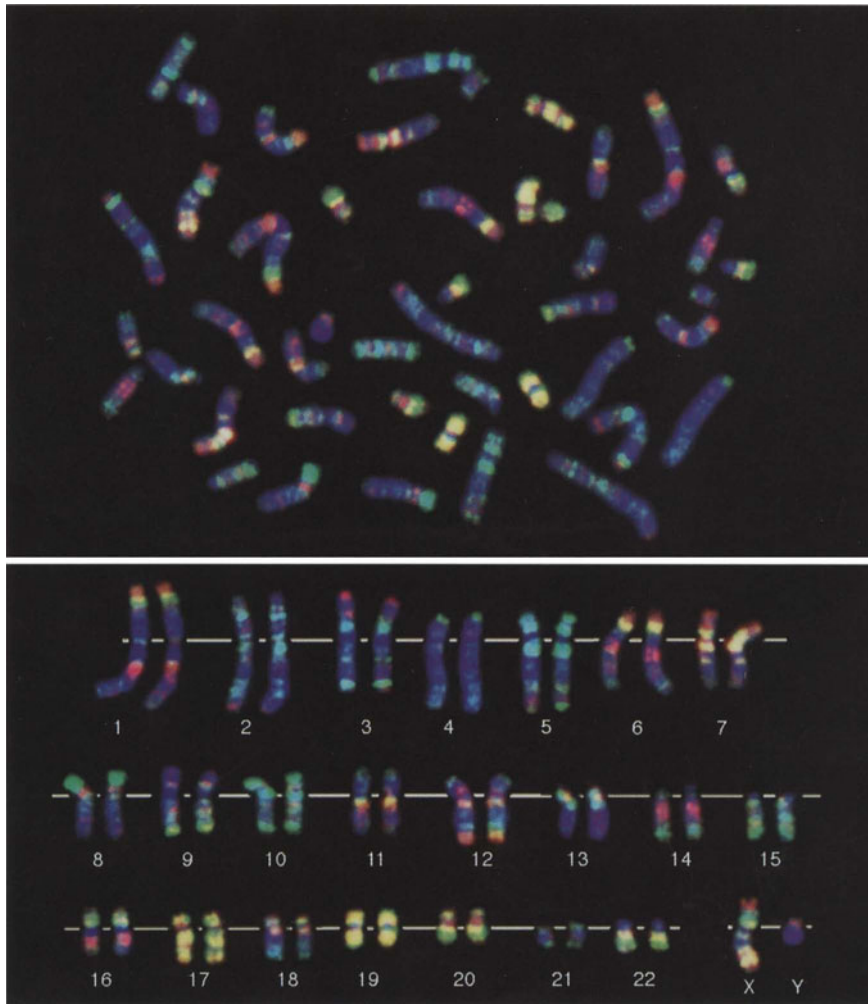
Color Plate II



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Color Plate III

Figure 8.4. High resolution fiber-FISH using the DAZ G5 probe from the 5′ end of the DAZ gene (green fluorescence) and the DAZ G21 probe from the 3′ end of the gene (red fluorescence) to label extended S phase Y chromatin. Seven DAZ red-green signals (genes or pseudogenes) are seen in a linear array, with at least one gene inverted with respect to the others (Gläser et al., Chromosome Research 6: figure 4, p 484, 1998, with kind permission from Kluwer Academic Publishers).

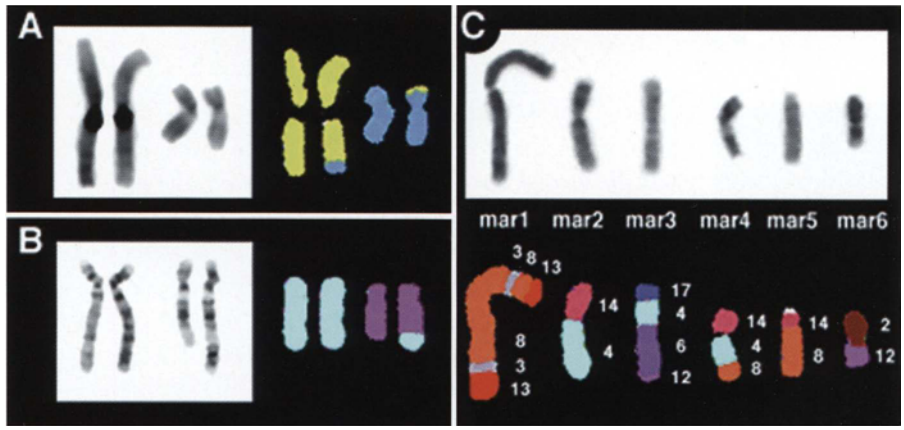


Figure 8.6. (A) Partial C-band and spectral (painting) karyotypes of the father of a mentally retarded child, showing an otherwise cryptic t(1;11) translocation. (B) Partial G-band karyotype of an ataxic patient; note extra material on a chromosome 11; the spectral karyotype shows its origin from a chromosome 4. (C) Six marker chromosomes from a breast cancer cell line; spectral karyotyping shows the chromosomes involved in these complex rearrangements (reprinted with permission from Schröck et al., Multicolor spectral karyotyping of human chromosomes. Science 273:494–497, copyright 1996, American Association for the Advancement of Science).

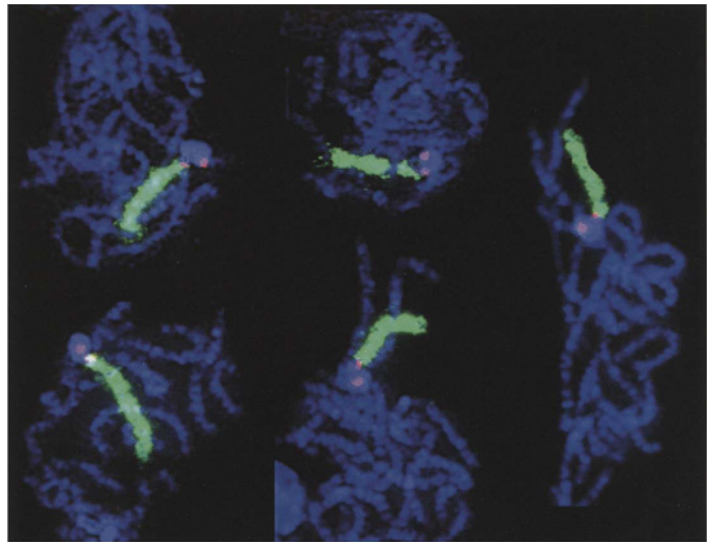


Figure 9.6. Association of bivalent 15 with the sex vesicle in five normal pachytene nuclei stained blue with DAPI. Bivalent 15 is made visible with a 15-specific paint (green) and its centromeric region with a 15-specific α-satellite probe (red). The sex vesicle (blue clump) is marked by an X-specific α-satellite probe (red) (Metzler-Guillemain et al., Chrom Res 7:369, Bivalent 15 regularly associates with the sex vesicle in normal male meiosis, fig. 1, copyright 1999, with kind permission from Kluwer Academic Publishers).

microscopy. The nick translation reaction used to label the probes also yields the short fragments best suited for hybridization. The fluorescence does not spread from the site of hybridization nearly as far as autoradiographic silver grains do, so the resolution, or precision, of mapping is much higher with fluorescence in situ hybridization (FISH) than with autoradiography. The results do not require a statistical analysis of the distribution of signals throughout the entire genome, because the background is so low that virtually every fluorescent signal counts (see Figs. 3.5, 8.4, and 9.6).

Highly sensitive CCD (charge-coupled device) digital cameras and computer-assisted data acquisition and processing methods permit routine detection of even faint fluorescent signals. With them, FISH can detect hybridization to sequences as short as 1 kb. It is even possible to distinguish single RNA transcripts within a cell and to determine the average number of transcripts of a particular gene within a cell. By using three different fluorochromes to label three probes, each complementary to a different segment of a gene, one can determine the rate of elongation of a growing (nascent) RNA transcript and the distance separating the RNA polymerase II transcriptional complexes. These correspond well to the results determined by electron microscopy (Femino et al., 1998).

Probes for highly repetitive (TTAGGG)n telomeric sequences are widely used, and for a variety of purposes. They have been used to show that apparently terminal deletions have been stabilized by the capture of TTAGGG repeats (Figs. 8.1 and 15.3). Pericentric inversions (Chapter 13) can be detected by a fascinating method called Chromosome Orientation and Direction FISH, or COD-FISH (Bailey et al., 1996). This takes advantage of the fact that telomeric repeats (TTAGGG in the G-rich strand, CCCTAA in the C-rich strand) all have the same orientation with respect to the end of the chromosome, just as centromeric α-satellite sequences do. Cells grown for one cycle in the presence of BrdU incorporate BrdU in place of T into each newly replicated strand of DNA. UV light selectively breaks down BrdU-substituted DNA, and exonuclease III can then destroy the entire damaged strand, leaving a single strand of DNA in each sister chromatid. A single-stranded probe complementary to one strand of telomeric or centromeric DNA will thus find a hybridizable (complementary) sequence in only one of the two sister chromatids. The single-stranded telomeric probe will hybridize to the short-arm telomere of one chromatid and the long-arm telomere of the other chromatid; the centromeric probe will hybridize to only one of these chromatids. However, if a pericentric inversion

has occurred, the centromeric probe will hybridize to the other chromatid (Fig. 13.6).

Replication Timing by FISH

The time of replication of individual genes can be determined in various ways. The standard method involves incorporating BrdU during part of the S phase in synchronized cell cultures and detecting the BrdU-containing strand with antibodies to BrdU. Alternatively, the BrdU-containing strand can be destroyed by photolysis and its complementary strand destroyed with single-strand-specific nuclease. This procedure eliminates only the DNA segments or genes that were replicated while BrdU was present. Specific gene probes can then be hybridized to the remaining DNA in solution or on Southern blots (Chapter 3). Selig et al. (1992) devised an ingenious cytological method to determine the time at which a particular gene is replicated. DNA probes for the genes in question are hybridized to interphase nuclei in synchronized cultures and the sites of hybridization detected by FISH. Each allele appears as a single dot before replication and as paired dots after replication has occurred and a sufficient degree of separation of sister chromatids has taken place in that region (Fig. 3.5). Torchia et al. (1994) and Boggs and Chinault (1994) applied this technique to several X-linked loci. Those known to undergo X-inactivation, such as *HPRT* and *FRAXA*, showed a high degree of asynchrony in the time of replication of the two alleles, whereas the two alleles of genes that escape X-inactivation, such as *ZFX* and *RPS4X*, replicated in synchrony. The *XIST* gene, which is active only on the inactive X chromosome, showed marked asynchrony, and both research groups suggested that the active XIST allele replicated early and the inactive allele late. However, Gartler et al. (1999), using both the standard method and that of Selig et al. (1992), concluded that the inactive XIST allele replicated early and the active allele late (Fig. 8.5).

Cloned, PCR-Generated, and In Situ-Generated Probes

The first probes for in situ hybridization were fragments of DNA that had been inserted into bacterial viruses (circular plasmids or linear bacteriophages) that

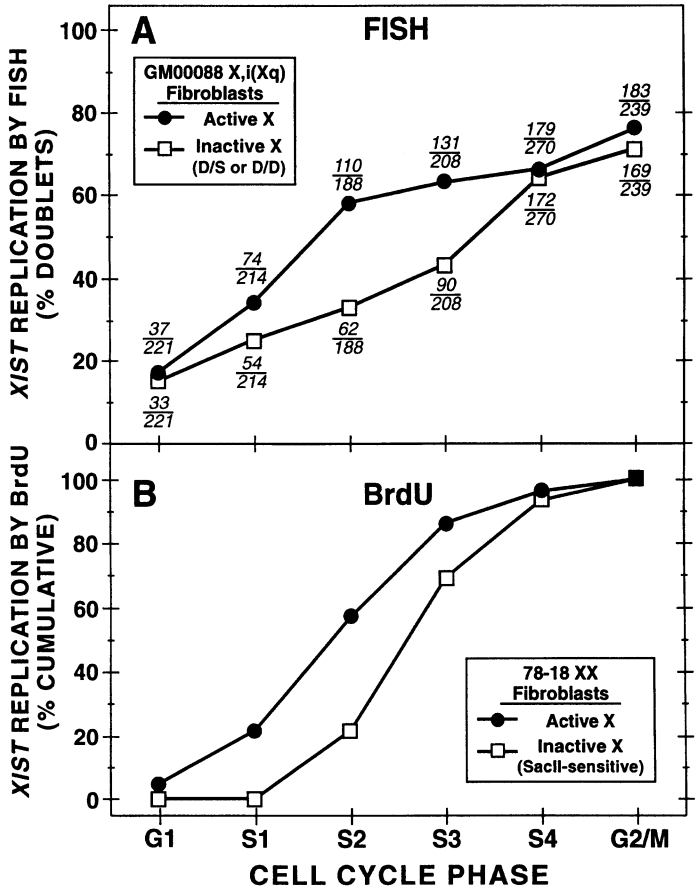


Figure 8.5. FISH and BrdU analysis of the replication time of the *XIST* alleles in X,i(Xq) fibroblasts. (A) Percentage of cells with doublets of the active X (closed circles) and the inactive i(Xq) (open squares) in each fraction of the cell cycle. The inactive i(Xq) was scored as replicated if doublets were present at either or both *XIST* loci. (B) Cumulative replication values for the active X (closed circles) and inactive i(Xq) (open squares) in each fraction of the cycle (reproduced from Gartler et al., Hum Mol Genet 8:1085–1089, 1999, with permission of Oxford University Press).

were then grown in bacteria to develop clones of identical fragments. Larger fragments have been successfully cloned in cosmids (35–55 kb) and in bacterial artificial chromosomes (BACs, 100–250 kb; Shizuya et al., 1992) or yeast artificial chromosomes (YACs, 300–1500 kb; Burke et al., 1987). An example of their use is the creation of a complete set of probes specific for each

chromosome end, capable of detecting by FISH deletions, translocations, or other rearrangements (Knight et al., 1997). The polymerase chain reaction (PCR) has been used to generate unique probes or libraries of probes from a whole genome, a whole chromosome, or a chromosome segment (Lüdecke et al., 1989). Some of the most useful probes have been produced using an oligonucleotide complementary to part of an *Alu* sequence as one primer and an arbitrary oligonucleotide sequence for the other. This generates a library of fragments, most of them from the gene-rich R-bands, which are rich in *Alu* sequences. This technique can be used with a variety of chromosome sources: flow-sorted chromosome-specific (or enriched) pools, rodent-human hybrids that contain a specific human chromosome or chromosome fragment, and microdissected fragments from a specific chromosome region. Microdissection (a scrape from a single or several chromosomes on a slide) and formation of a PCR-amplified pool of DNA fragments from a tiny region can be used to yield a chromosome-band-specific painting probe (Lüdecke et al., 1989; Chen et al., 1997).

A method has been developed for the in situ generation of label, called *primed in situ* (PRINS) *labeling*. A labeled oligonucleotide primer is annealed to its complementary sequences in the genome and used to initiate replication in situ. This works quite well with repetitive DNA sequences, such as centromeric α-satellite DNA, and takes no more than an hour. PRINS gives good results with even fairly low copy repeats if the labeled product is at least 1 kb in length. For lower copy repeats, a modification involving PCR in situ sometimes works (Gosden and Lawson, 1994). By using either end of the consensus *Alu* sequence as the oligonucleotide primer (Alu-PRINS), one can selectively label euchromatin (T- and R-bands, mainly) and detect small regions of euchromatin in an aberrant location (Cullen et al., 1997).

Chromosome-, Region-, and Band-Specific Painting Probes

In 1988, two groups reported the successful use of chromosome-specific libraries for the recognition of numerical and structural chromosome abnormalities (Cremer et al., 1988; Pinkel et al., 1988). Flow sorting was used to prepare fractions highly enriched for each chromosome, and the DNA from each of

these was fragmented and the pool of fragments ligated into bacterial virus DNA. Bacteria were infected with the mixture of recombinant virus DNA to yield a library of human DNA fragments derived almost entirely from a single chromosome: a *chromosome-specific library*. Although repetitive human sequences common to many chromosomes are abundant in such libraries, their hybridization can be blocked by prehybridization with an excess of unlabeled DNA or the highly repetitive Cot1 fraction of DNA. The nonrepetitive sequences in the labeled library hybridize, producing a fluorescent signal along the length of the chromosome (Fig. 8.2). The number of chromosomes labeled with such a probe is easily identifiable, in both metaphase spreads and interphase nuclei. Translocations involving the painted chromosome are also readily apparent, even when they are very tiny (Popp et al., 1993). Chromosome painting probes can be used in conjunction with locus-specific probes (Fig. 9.6).

In principle, a probe specific for any region or band can be generated by microdissection followed by PCR amplification of sequences from the region or band, and a number of such probes have been developed. Using a more global approach, Rocchi and his collaborators have prepared a panel of subchromosomal painting libraries (paints) representing over 300 regions of the human genome (Antonacci et al., 1995). The presence of ribosomal RNA gene clusters and several repetitive sequence families on all the acrocentric chromosomes reduces the specificity of chromosome painting probes prepared from these chromosomes. A novel way around this problem is to prepare painting probes from gorilla or chimpanzee acrocentric chromosomes, which lack all these repetitive elements but are otherwise highly homologous to the human acrocentrics (Müller et al., 1997a).

Multicolor FISH, Spectral Karyotyping, and Bar Codes

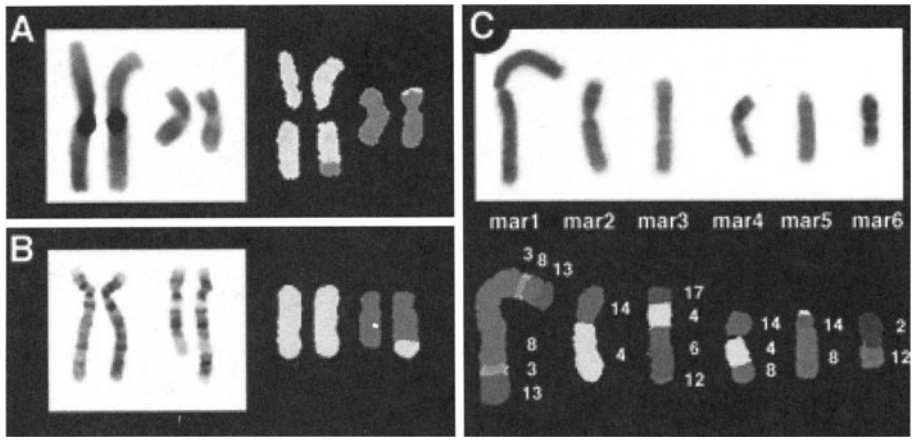
Simultaneous detection of each chromosome in a metaphase spread is now routine, using either multicolor FISH (M-FISH; Speicher et al., 1996) or spectral karyotyping (SKY; Schröck et al., 1996). These methods use 24 chromosome painting probes and five fluorochromes in a combinatorial labeling scheme that labels each of the 22 autosomes and the X and Y differently. M-FISH

requires a series of image acquisitions with a change of optical filters, while SKY uses Fourier spectroscopy and an interferometer to evaluate the fluorescence emission patterns in a single, longer image acquisition (Fig. 8.2). Neither method detects intrachromosomal rearrangements such as most deletions, duplications, or inversions, but they are very useful for detecting other structural as well as numerical changes (Fig. 8.6; see color insert). Even minute chromosome rearrangements may be detectable using multicolor FISH with chromosome-specific painting probes (Popp et al., 1993).

Alu-PCR from human-mouse hybrids that contain a limited number of human chromosomes has been used to generate two different human-specific probe sets. These sets were derived pretty much at random from the human genome, and for use one is labeled with red and one with green fluorescence. Chromosome regions that hybridize to probes from both pools appear yellow, while regions that hybridize to probes from only one pool appear either red or green. The 110 distinct signals per haploid set produce a unique multicolor pattern of variously colored dots along each chromosome, a "bar code" that allows chromosome identification and recognition of rearrangements (Fig. 8.3). Extension of this approach using additional signals or additional colors should make this technique increasingly useful (Müller et al., 1997b).

High-Resolution (Interphase and Fiber) FISH

Techniques have been developed for multicolor fluorescence labeling of chromosomes, which allows two or more specific probes to be identified in a single nucleus. Interphase chromatin in hypotonically swollen nuclei can be stained with these fluorescent probes; the distance between the bands in such fluorescence-labeled chromosomes is directly proportional to the molecular distance (up to about 1 million base pairs) separating the two binding sites (Lichter and Ward, 1990; Trask, 1991). These new techniques will improve the diagnostic power of cytogenetics and even allow chromosome changes to be detected in nondividing differentiated cells. The use of multiple probes and multicolor fluorescence labeling permits much more accurate analysis and detection of subtle changes in structure and copy number (Trask, 1991).



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Chromatin can be released from interphase nuclei and the extended chro matin fibers spread out on lysine-coated slides. These are used for fiber FISH, to determine the order of three or more genes that are close to one another, or to analyze chromosome abnormalities (Mann et al., 1997). Pelizaeus-Merzbacher disease of the central nervous system is frequently caused by a duplication of the *PLP* (proteolipid protein) gene at Xq22. Fiber FISH detected a tiny duplication in three different families. In a fourth family, the two *PLP* signals were widely separated even in metaphase chromosomes, indicating there had been a major structural rearrangement (Woodward et al., 1998). Using differentially fluorescent probes from the 5′ and 3′ ends of the *DAZ* gene, Gläser et al. (1998) demonstrated a linear array of seven *DAZ* genes in Y chromatin fibers, with at least one of them inverted with respect to the others (Fig. 8.4). Shiels et al. (1997) used fiber FISH to estimate the length of ribosomal RNA genes (rDNA) and α-satellite arrays on human chromosome 22 from a human-rodent cell hybrid. There were up to 10 repeats of the 43-kb rRNA gene in a 155-μm cluster, although the full

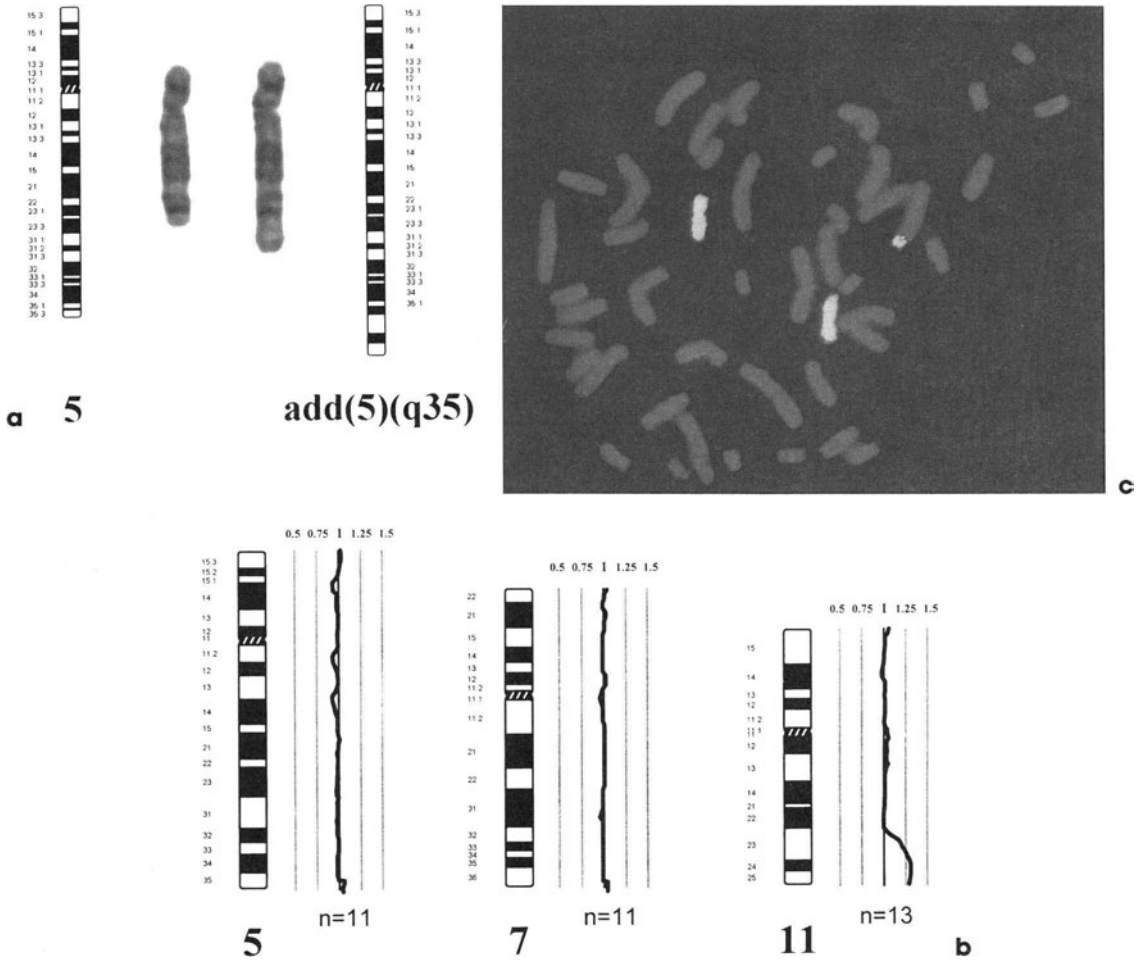

Figure 8.7. (a) Partial karyotype and ideogram of two chromosomes 5, one with additional material on it. (b) Ideograms and CGH (comparative genomic hybridization) profiles indicate that the extra material is from 11q23–qter. (c) Confirmation by FISH using a chromosome 11–specific painting probe (Levy et al., 1997, with permission of S. Karger AG, Basel).

rDNA array on this specific chromosome has nearly 40 copies (Srivastava et al., 1993). The fiber length of the centromeric α-satellite array was 930 μm, providing a better minimal estimate of the size of this array: 2600 kb (2.6 Mb). A modification of this technique uses linearized cosmid DNA fibers attached to a glass slide and aligned in parallel by a process called molecular combing (Weier et al., 1995).

Comparative Genomic Hybridization

Comparative genomic hybridization (CGH) is a molecular cytogenetic approach for genome-wide screening for differences in copy number of any DNA sequence from an individual, and it requires only a few nanograms of DNA as FISH probes. Equal amounts of differently labeled DNA from the test individual (green) and reference DNA (red) are cohybridized to normal metaphase spreads. Copy number differences show up as altered ratios of green-to-red fluorescence intensities (Fig. 26.1). This ratio is calculated along the length of each chromosome by a digital image analysis system. Increased copy number gives an increased ratio, while losses or deletions give a decreased ratio. Levy et al. (1997) used CGH to determine the origin of a de novo translocation in a newborn with multiple congenital anomalies and an unbalanced karyotype (Fig. 8.7). This method has been applied to more than 1500 tumor DNA samples and revealed six previously unknown gene amplifications (Forozan et al., 1997). For a list of publications on CGH, see http://nhgri.nih.gov/dir/ccg/cgh.

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