

MOLECULAR DIAGNOSTICS

Fundamentals, Methods, & Clinical Applications

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Chromosomal Structure and Chromosomal Mutations

OUTLINE

CHROMOSOMAL STRUCTURE AND ANALYSIS

Chromosomal Compaction and Histones

*Chromosomal Morphology
Visualizing Chromosomes*

DETECTION OF GENOME AND CHROMOSOMAL MUTATIONS

Karyotyping

Fluorescence In Situ Hybridization

OBJECTIVES

- Define mutations and polymorphisms.
- Distinguish the three types of DNA mutations: genome, chromosomal, and gene.
- Describe chromosomal compaction and the proteins involved in chromatin structure.
- Diagram a human chromosome, and label the centromere, q arm, p arm, and telomere.
- Illustrate the different types of structural mutations that occur in chromosomes.
- State the karyotype of a normal male and female.
- Identify the chromosomal abnormality in a given karyotype.
- Compare and contrast interphase and metaphase FISH analyses.
- Distinguish between the effects of balanced and unbalanced translocations on an individual and the individual's offspring.

The **human genome** is all of the genes found in a single individual. The human genome consists of 2.9 billion nucleotide base pairs of DNA organized into 23 chromosomes. As **diploid** organisms, humans inherit a **haploid** set of all their genes (23 chromosomes) from each parent, so that humans have two copies of every gene (except for some on the X and Y chromosomes). Each chromosome is a double helix of DNA, ranging from 246 million nucleotide base pairs in length in chromosome 1 (the largest) to 47 million nucleotide base pairs in chromosome 22 (the smallest; Table 8.1). Genetic information is carried on the chromosomes in the form of the order, or **sequence**, of nucleotides in the DNA helix. A **phenotype** is a trait or group of traits resulting from transcription and translation of these genes. The **genotype** is the DNA nucleotide sequence responsible for a phenotype.

Genotypic analysis is performed to confirm or predict phenotype. In the laboratory, some changes in chromosome structure and changes in chromosome number can

be observed microscopically. Mutations at the nucleotide sequence level are detected using biochemical or molecular methods. Alterations of the DNA sequence may affect not only the phenotype of an individual but the progeny of that individual as well. The latter, **heritable changes**, are the basis for prediction of the phenotype in the next generation. The probability of inheritance of a phenotypic trait can be estimated using logical methods of mendelian genetics and statistics.

A transmissible (inheritable) change in the DNA sequence is a mutation or polymorphism. Although these terms are sometimes used interchangeably, they do have slightly different meanings based on population genetics. A DNA sequence change that is present in a relatively small proportion of a population is a **mutation**. The term, **variant**, may also be used, particularly to describe inherited sequence alterations, thus reserving the term mutation for somatic changes; for example, changes found only in tumor tissue. A change in the DNA sequence that is present in at least 1%–2% of a population is a **polymorphism**. Both mutations and polymorphisms may or may not produce phenotypic differences. Polymorphisms are casually considered mutations that do not severely affect phenotype; this is generally true, as any negative effect on survival and reproduction limits the persistence of a genotype in a population. Some polymorphisms are maintained in a population through a balance of positive and negative phenotype. The classic example is sickle cell anemia, a condition caused by a single-base substitution in the gene that codes for hemoglobin. The alteration is regarded as a mutation, but it is really a **balanced polymorphism**. In addition to causing abnormal red blood cells, the genetic alteration results in resistance to infection by *Plasmodium falciparum*; that is, resistance to malaria. The beneficial trait provides a survival and reproductive advantage that maintains the polymorphism in a relatively large proportion of the population. Examples of benign polymorphisms, that is, those with no selective advantage, are the ABO blood groups and the major histocompatibility complex (see Chapter 15). Polymorphisms used for human identification and paternity testing are discussed in Chapter 10.

DNA mutations can affect a single nucleotide or millions of nucleotides, even whole chromosomes, and thus can be classified into three categories. **Gene mutations** affect single genes and are often, but not always, small changes in the DNA sequence. **Chromosome mutations** affect the structures of entire chromosomes. These

Table 8.1 Sizes of Human Chromosomes in Base Pairs

| Chromosome | Millions of Base Pairs |
|------------|------------------------|
| 1 | 246 |
| 2 | 244 |
| 3 | 199 |
| 4 | 192 |
| 5 | 181 |
| 6 | 171 |
| 7 | 158 |
| 8 | 146 |
| 9 | 136 |
| 10 | 135 |
| 11 | 134 |
| 12 | 132 |
| 13 | 113 |
| 14 | 100 |
| 15 | 90 |
| 16 | 82 |
| 17 | 76 |
| 18 | 64 |
| 19 | 64 |
| 20 | 47 |
| 21 | 47 |
| 22 | 49 |
| X | 154 |
| Y | 57 |

changes require movement of large chromosomal regions either within the same chromosome or to another chromosome. **Genome mutations** are changes in the number of chromosomes. A cell or cell population with a normal complement of chromosomes is **euploid**. Genome mutations result in cells that are **aneuploid**. Aneuploidy is usually (but not always) observed as increased numbers of chromosomes, because the loss of whole chromosomes is not compatible with survival. A single copy of each chromosome (23 in humans) is a **haploid** complement. Humans are normally **diploid**, with two copies of each chromosome. Aneuploidy can result when there are more than two copies of a single chromosome or when there are multiple copies of one or more chromosomes. Down's syndrome is an example of a disease resulting from aneuploidy, where there are three copies, or **triploidy**, of chromosome 21.

Detection of mutations in the laboratory ranges from direct visualization of genome and chromosomal mutations under the microscope to indirect molecular methods to detect single-base changes. Methods used for detection of genome and chromosomal mutations are discussed in this chapter. Methods to detect gene mutations are described in Chapter 9.

Chromosomal Structure And Analysis

Chromosomal Compaction and Histones

An important concept in the understanding of chromosomes is that chromosome behavior is dependent on chromosome structure as well as DNA sequence.¹ Genes with identical DNA sequences will behave differently, depending on their chromosomal location or the surrounding nucleotide sequence. For example, certain functional features, such as the **centromere** (where the chromosome attaches to the spindle apparatus for proper segregation during cell division), are not defined by specific DNA sequences.² It is a well-known phenomenon that a gene inserted or moved into a different chromosomal location may be expressed (transcribed and translated) differently than it was in its original position. This is called **position effect**.

A eukaryotic chromosome is a double helix of DNA. A cell nucleus contains 4 cm of double helix. This DNA must be compacted, both to fit into the cell nucleus and

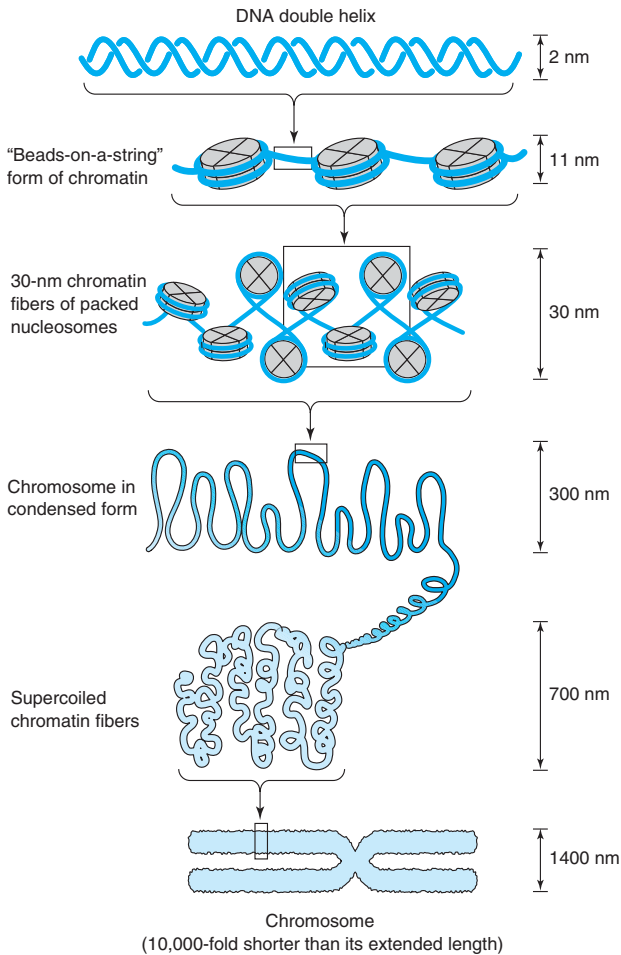
Advanced Concepts

The structure of metaphase chromosomes is maintained by more than just histones. Metaphase chromatin is one-third DNA, one-third histones, and one-third nonhistone proteins. Nonhistone protein complexes, termed condensin I and condensin II, are apparently required for maintenance of mitotic chromosome structure.²⁸

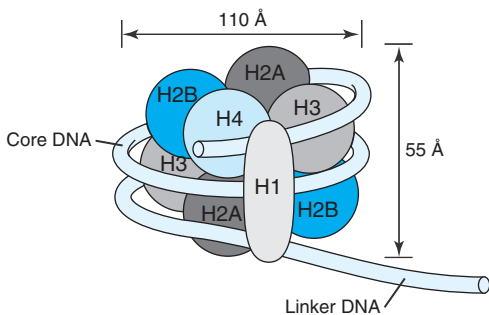
for accurate segregation in mitosis. There is an 8000-fold compaction of an extended DNA double helix to make a metaphase chromosome (Fig. 8-1).³ Winding of DNA onto **histones** is the first step. Histones are the most abundant proteins in cells. There are five histones: H1, H2a, H2b, H3, and H4. Approximately 160–180 bp of DNA is wrapped around a set of 8 histone proteins (2 each of H2a, H2b, H3 and H4) to form a **nucleosome**. Nucleosomes can be seen by electron microscopy as 100-Å beadlike structures that are separated by short strands of free double helix (Fig. 8-2). DNA wrapped around histones forms a “bead-on-a-string” arrangement that comprises the 10-nm or **10-micron fiber**. The 10-micron fiber is further coiled around histone H1 into a thicker and shorter 30-nm or **30-micron fiber**. The 30-nm interphase fibers represent the “resting state” of DNA. The fibers are locally relaxed into 10-nm fibers for DNA metabolism as required during the cell cycle. These fibers are looped onto protein scaffolds to form 300-nm fibers; before entry into the M phase of the cell cycle (mitosis), the looped fibers are wound into 700-nm solenoid coils.⁴ The 700-nm coils are compacted into the 1400-nm fibers that can be seen in metaphase nuclei and in karyotypes.

Historical Highlights

Before 1943, histones were thought to contain genetic information. Their function was later thought to be structural. It is now known that modification of histones, through acetylation, methylation, phosphorylation, or ubiquitination, plays a role in other cellular functions such as recombination, replication, and gene expression.³²



■ **Figure 8-1** DNA compaction into metaphase chromosomes. (From, Alberts, *Molecular Biology of the Cell*, 4th edition, Garland Science, New York, 2002.)



■ **Figure 8-2** DNA wrapped around eight histone proteins (2 each of histone 2A, 2B, 3, and 4) forms a nucleosome. A further association with histone H1 coils the nucleosomal DNA into a 30-nm fiber.

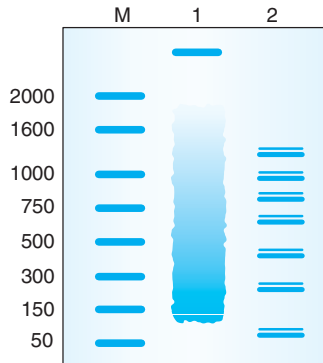
When the DNA is relaxed into 10-micron fibers for transcription or replication, the placement of nucleosomes along the double helix can be detected using nucleases (e.g., Mung bean nuclease, or DNase I). These enzymes cut the double helix in the **linker region**, the part of the double helix that is exposed between the histones.

To make 30-nm chromatin fibers, the internucleosomal DNA is associated with histone H1, and the beaded structure is wound into a solenoid coil. Loss of this level of organization is the first classic indicator of **apoptosis**, or programmed cell death. The 30-nm fibers are uncoiled, and the exposed linker DNA between the nucleosomes becomes susceptible to digestion by intracellular nucleases. The DNA wrapped into the nucleosomes remains intact so that DNA isolated from apoptotic cells contains "ladders," or multiples of discrete multiples of ~180 bp. These ladders can be resolved by simple agarose electrophoresis (Fig. 8-3). The remainder of the proteins involved in DNA compaction are the **nonhistone proteins**.

Chromosome topology (state of compaction of the DNA double helix) affects gene activity; for instance in chromosome X inactivation in females. More highly compacted DNA is less available for RNA transcription. Maintenance of the more highly compacted state of DNA in closed chromatin, or **heterochromatin** (in contrast to open chromatin, or **euchromatin**), throughout interphase may require special proteins called condensin proteins or condensin-like protein complexes.

Advanced Concepts

Members of a family of proteins called SMC proteins control chromosome condensation in eukaryotes and other aspects of chromosome behavior, including chromosome segregation in prokaryotes. Two of the SMC proteins, XCAP-C and XCAP-E, first isolated from frog eggs,²⁹ are integral parts of the **condensin complex**, a protein scaffold structure that can be isolated from both mitotic and interphase cells. This complex in the presence of topoisomerase can wrap DNA around itself in an ATP-driven reaction. Although the exact role of this complex in condensation and decondensation is not yet completely defined, this ability to change chromosome architecture is a significant feature of DNA metabolism.



■ **Figure 8-3** Apoptotic DNA (lane 2) is characterized by the ladder seen on gel electrophoresis. This is in contrast to degraded DNA from necrotic cells (lane 1). Lane M contains molecular weight markers.

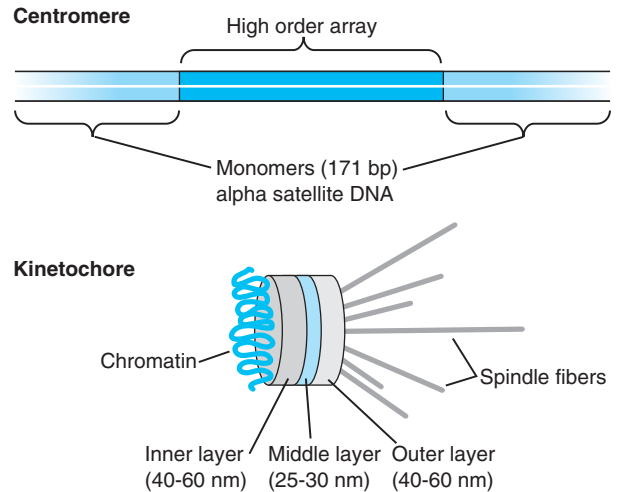
Chromosome Morphology

Mitotic chromosomes have been distinguished historically by their relative size and centromere placement. As previously stated, the **centromere** is the site of attachment of the chromosome to the spindle apparatus. The connection is made between microtubules of the spindle and a protein complex, called the **kinetochore**, that assembles at the centromere sequences (Fig. 8-4). At the nucleotide level, the centromere is composed of a set of highly repetitive **alpha satellite** sequences.⁵ Microscopically, the centromere appears as a constriction in each compacted metaphase chromosome. Chromosomes are **metacentric**, **submetacentric**, **acrocentric**, or **telocentric**, depending on the placement of the centromere (Fig. 8-5). The placement of the centromere divides the chromosome into arms.

There are no telocentric human chromosomes. Human chromosomes are acrocentric or submetacentric and so have long and short arms (Table 8.2). The long arm of a chromosome is designated **q**, and the short arm is designated **p**. Acrocentric chromosomes have a long arm length:short arm length ratio of from 3:1 to 10:1. Chromosomes 13 to 15, 21, and 22 are acrocentric.

Advanced Concepts

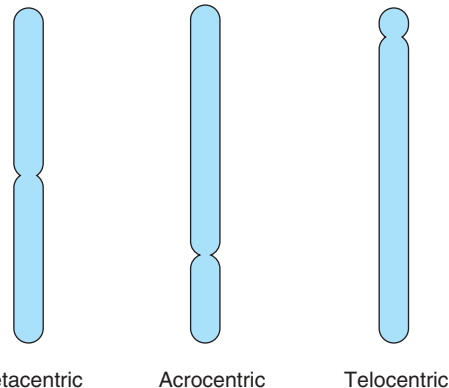
Some plants and insects have **holocentric** chromosomes. During cell division, these chromosomes form kinetochores along their entire length.



■ **Figure 8-4** The centromere (top) consists of tandem repeats of 171 base pair sequences flanking sets of single repeat units, or monomers repeated in groups in a higher order array. The kinetochore (bottom) is a protein structure that connects the centromeres to the spindle apparatus.

Visualizing Chromosomes

Conventional cytological stains, such as Feulgen's, Wright's, and hematoxylin, have been used to visualize chromosomes. An advance in the recognition of individual chromosomes was the demonstration that fluorescent stains and chemical dyes can react with specific chromosome regions. This region-specific staining results in the formation of band patterns where portions of the chro-



■ **Figure 8-5** The arms of metacentric chromosomes (left) are of equal size. Acrocentric chromosomes (center) divide the chromosome into long arms and short arms. Telocentric centromeres (right) are at the ends of the chromosome.

Table 8.2 Classification of Chromosomes by Size and Centromere Position

| Group | Chromosomes | Description |
|-------|-------------|---|
| A | 1, 2 | Large metacentric |
| | 3 | Large submetacentric |
| B | 4, 5 | Large submetacentric |
| C | 6–12, X | Medium-sized submetacentric |
| D | 13–15 | Medium-sized acrocentric with satellites |
| E | 16 | Short metacentric |
| | 17, 18 | Short submetacentric |
| F | 19, 20 | Short metacentric |
| G | 21, 22 | Short acrocentric with satellites |
| | Y | Short acrocentric |

mosome accept or reject the stain. For cytogenetic analysis, this allows unequivocal identification of every chromosome and the direct detection of some chromosomal abnormalities. Underlying the region-specific staining is the implication that the reproducible staining patterns occur as a result of defined regional ultrastructures of the mitotic chromosomes.

When chromosomes are stained with the fluorescent dyes quinacrine and quinacrine mustard, the resulting fluorescence pattern visualized after staining is called **Q banding** (Fig. 8-6). This method was first demonstrated in 1970 by Caspersson, Zech, and Johansson.⁶ The results of this work confirmed that each human chromosome could be identified by its characteristic banding pattern. Q banding gives a particularly intense staining of the human Y chromosome and thus may also be used to distinguish the Y chromosome in interphase nuclei. Because Q banding requires a fluorescent microscope, it is not as widely used as other stains that are detectable by light microscopy.

The chemical dye Giemsa stains in patterns, or **G bands**, similar to those seen in Q banding. The appearance of G banding differs, depending on the treatment of the chromosomes before staining.⁷ Mild treatment (2× standard saline citrate for 60 minutes at 60°C) yields the region-specific banding pattern comparable to that seen with fluorescent dyes. Use of trypsin or other proteolytic agents to extract or denature proteins before Giemsa staining was found to map structural aberrations more clearly and is the most commonly used staining method

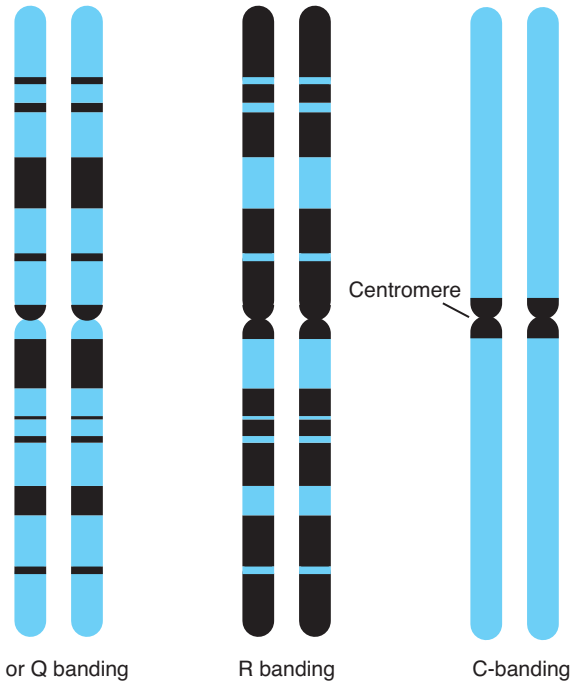


Figure 8-6 Reproducible staining patterns on chromosomes are used for identification and site location. Heterochromatin stains darkly by G or Q banding (left); euchromatin stains darkly by R banding (center); C banding stains centromeres (right).

for analyzing chromosomes.^{8,9} G bands can also be produced by Feulgen staining after treatment with DNase I.¹⁰ The number of visualized bands can be increased from about 300 to about 500 per chromosome by staining chromosomes before they reach maximal metaphase condensation. This is called **high-resolution banding**.

Harsher treatment of chromosomes (87°C for 10 minutes, then cooling to 70°C) before Giemsa staining will produce a pattern opposite to the G banding pattern called **R banding**.¹¹ R bands can also be visualized after staining with acridine orange.¹²

Alkali treatment of chromosomes results in centromere staining, or **C banding**.¹³ Centromere staining is absent in G band patterns. C bands may be associated with heterochromatin, the “quiet,” or poorly, transcribed sequences along the chromosome that are also present around centromeres. In contrast, euchromatin, which is relatively rich in gene activity, may not be stained as much as heterochromatin in C banding.

Advanced Concepts

The correlation between heterochromatin and staining may also hold for noncentromeric G and Q bands. This association is complicated, however, because a variety of procedures and stains produce identical banding patterns. The correlation of staining with heterochromatin is contradicted by observations of the X chromosome. Although one X chromosome is inactive and replicates later than the active X in females, both X chromosomes stain with equal pattern and intensity. Staining differences, therefore, must be due to other factors. Possible explanations for differential interactions with dye include differences in DNA compaction, sequences, and DNA-associated nonhistone proteins.

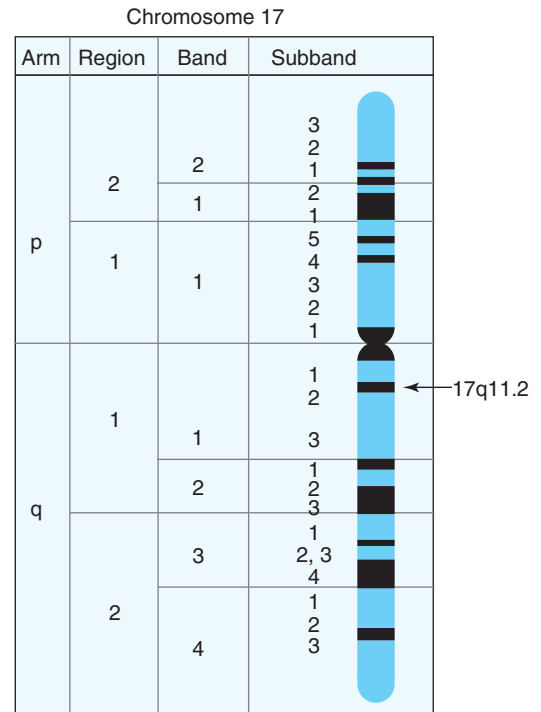
Nucleolar organizing region staining (**NOR staining**) is another region-specific staining approach. Chromosomes treated with silver nitrate will stain specifically at the constricted regions, or stalks, on the acrocentric chromosomes.

Staining of chromosomes with 4',6-diamidino-2-phenylindole (**DAPI**) was first described in 1976 as a way to detect mycoplasma contamination in cell cultures.¹⁴ DAPI binds to the surface grooves of double-stranded DNA and fluoresces blue under ultraviolet light (353-nm wavelength). DAPI can be used to visualize chromosomes as well as whole nuclei.

Chromosome banding facilitates detection of small deletions, insertions, inversions, and other abnormalities and the identification of distinct chromosomal locations.

Advanced Concepts

Chromosomes can be prestained with the DNA-binding oligopeptide distamycin A to enhance chromosomal distinctions.^{30,31} DAPI/distamycin A staining is useful in identifying pericentromeric breakpoints in chromosomal rearrangements and other rearrangements or chromosomes that are too small for standard banding techniques.



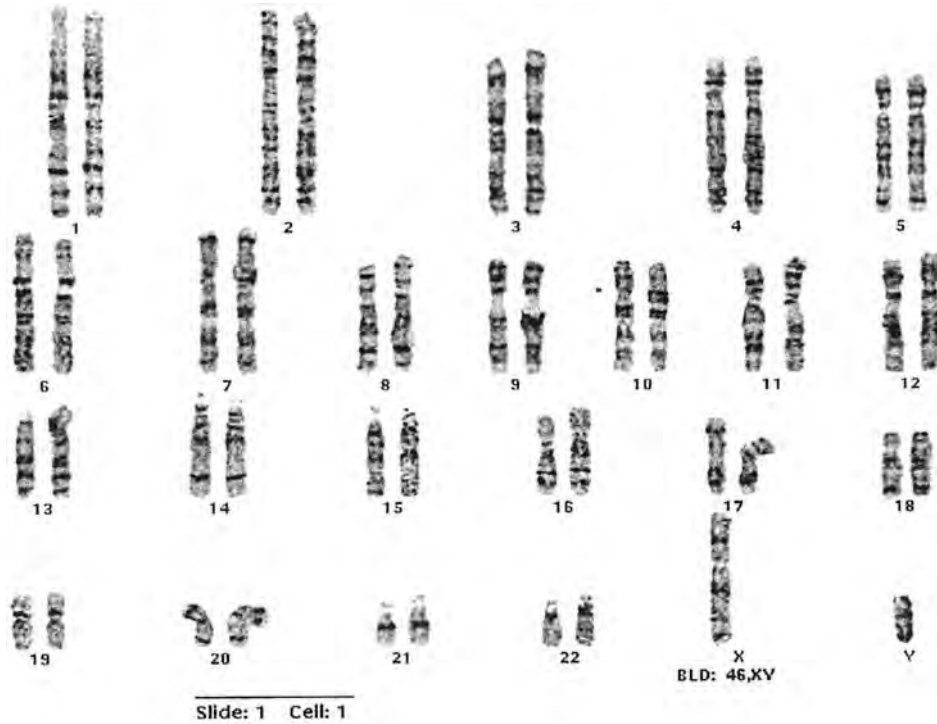
■ **Figure 8-7** Identification of chromosomal location by G-band patterns. Locations are designated by the chromosome number 17 in this example, the arm q, the region 1, the band 1 and the sub-band 2.

For this purpose, the reproducible G-banding pattern has been ordered into regions, comprising bands and subbands. For example, in Figure 8-7 a site on the long arm (q) of chromosome 17 is located in region 1, band 1, sub-band 2, or 17q11.2.

Detection Of Genome And Chromosomal Mutations

Karyotyping

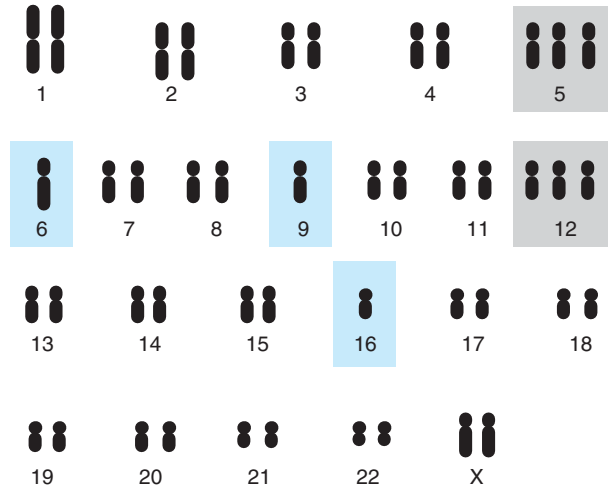
Genome mutations, or aneuploidy, can be detected by indirect methods, such as flow cytometry and more directly by karyotyping. A **karyotype** is the complete set of chromosomes in a cell. Karyotyping is the direct observation of metaphase chromosome structure by arranging metaphase chromosomes according to size. Karyotyping requires collecting living cells and growing them in culture in the laboratory for 48–72 hours. Cell division is stimu-



■ **Figure 8-8** A normal male karyotype. There are 22 sets of autosomes, one inherited from each parent, and one pair of sex chromosomes, XY. This karyotype is designated 46, XY.

lated by addition of a **mitogen**, usually phytohemagglutinin. Dividing cells are then arrested in metaphase with colcemid, an inhibitor of microtubule (mitotic spindle) formation. The chromosomes in dividing cells that arrest in metaphase will yield a **chromosome spread** when the cell nuclei are disrupted with hypotonic saline. The 23 pairs of chromosomes can then be assembled into an organized display, or **karyotype**, according to their size and centromere placement (Fig. 8-8). Aneuploidy can be observed affecting several chromosomes¹⁵ (Fig. 8-9) or a single chromosome (Fig. 8-10).

Karyotyping can also detect chromosomal mutations such as **translocations**, which are the exchange of genetic material between chromosomes. Translocations can be of several types. In **reciprocal** translocations, parts of two chromosomes exchange, i.e., each chromosome breaks, and the broken chromosomes reassociate or recombine with one another. When this type of translocation does not result in gain or loss of chromosomal material, it is **balanced** (Fig. 8-11, Fig. 8-12). Balanced translocations can



■ **Figure 8-9** Aneuploidy involving multiple chromosomes. Chromosomes 5 and 12 are triploid; chromosomes 6, 9, and 16 are monoploid.



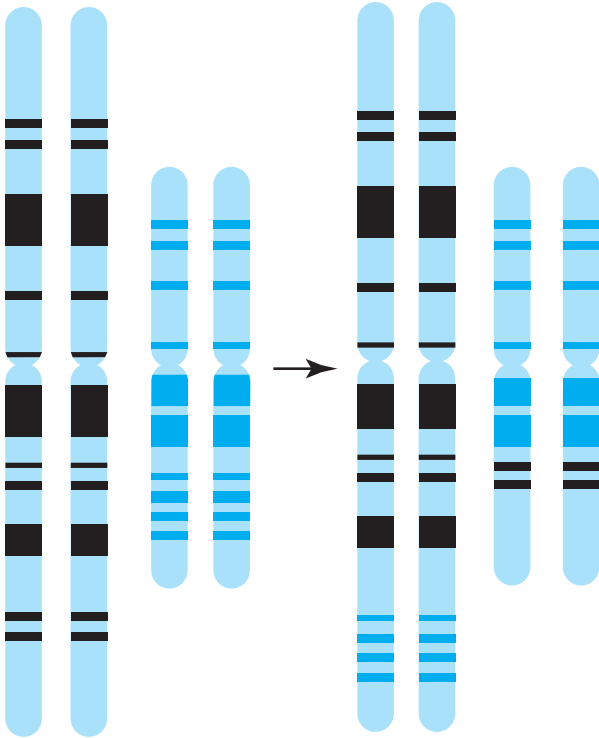
■ **Figure 8-10** Aneuploidy involving the Y chromosome (XYY syndrome). This is designated 47,XYY.

occur, therefore, without phenotypic effects. Balanced translocations in germ cells (cells that give rise to eggs or sperm) can, however, become **unbalanced** by not assorting properly during meiosis; as a result, they affect the phenotype of offspring. A **robertsonian** translocation involves the movement of most of one entire chromosome to the centromere of another chromosome (Fig. 8-13). This type of translocation can also become unbalanced during reproduction, resulting in a net gain or loss of chromosomal material in the offspring.

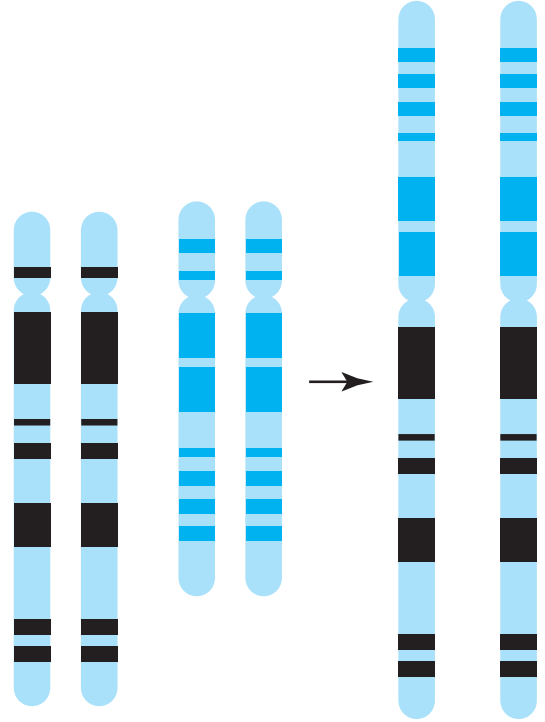
Other types of chromosome mutations that are sometimes visible by karyotyping are shown in Figure 8-14. A **deletion** is a loss of chromosomal material. Large deletions covering millions of base pairs can be detected using karyotyping; smaller **microdeletions** are not always easily seen using this technique. An **insertion** is a gain of chromosomal material. The inserted sequences can arise from duplication of particular regions within the affected chromosome or from fragments of other chromosomes. As with deletions, altered banding patterns and a change in the size of the chromosomes can indicate the occurrence of this event. **Inversions** result from excision, flipping, and reconnecting chromosomal material within

Historical Highlights

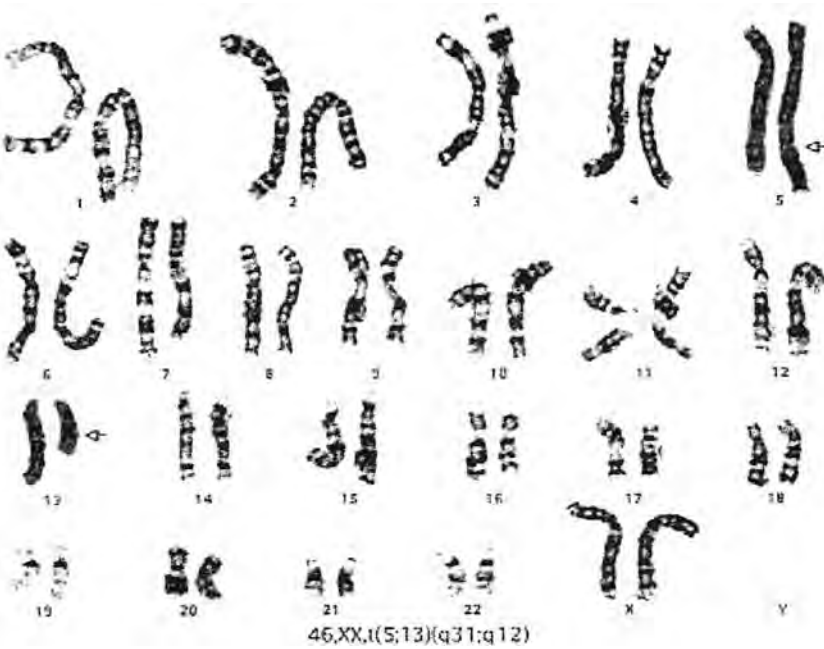
The first chromosome mutations were visualized in the 1960s in leukemia cells. Peter Nowell and colleague David Hungerford observed an abnormally small chromosome 22 in leukemia cells, which they labeled the “Philadelphia” chromosome. A few years later, Janet Rowley, using chromosome banding, noted that tumor cells not only lost genetic material, they exchanged it. In 1972 she first described the translocation between chromosomes 8 and 21, t(8;21) in patients with acute myeloblastic leukemia. In that same year, she demonstrated that the Philadelphia chromosome was the result of a reciprocal exchange between chromosome 9 and chromosome 22. She went on to identify additional reciprocal translocations in other diseases, the t(14;18) translocation in follicular lymphoma and the t(15;17) translocation in acute promyelocytic leukemia. This was the first evidence that cancer had a genetic basis.



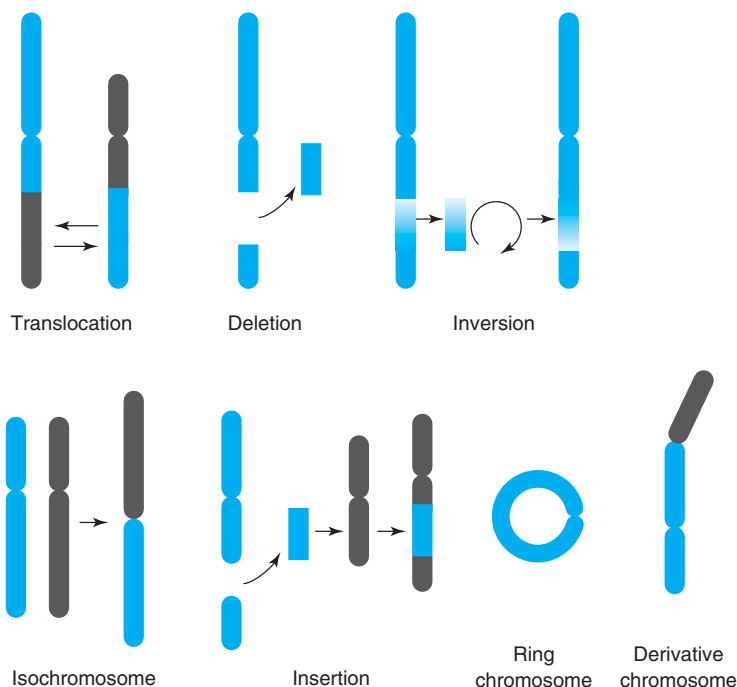
■ Figure 8-11 A balanced reciprocal translocation.



■ Figure 8-13 A robertsonian translocation.



■ Figure 8-12 A karyotype showing a balanced reciprocal translocation between chromosomes 5 and 13. This is designated 46, XX,t(5;13).



■ **Figure 8-14** Chromosome mutations involving alterations in chromosome structure.

the same chromosome. **Pericentric inversions** include the centromere in the inverted region, whereas **paracentric inversions** involve sequences within one arm of the chromosome. An **isochromosome** is a metacentric chromosome that results from transverse splitting of the centromere during cell division. Transverse splitting causes two long arms or two short arms to separate into daughter cells instead of normal chromosomes with one long arm and one short arm. The arms of an isochromosome are, therefore, equal in length and genetically identical. A **ring chromosome** results from deletion of genetic regions from both ends of the chromosome and a joining of the ends to form a ring. A **derivative chromosome** is an abnormal chromosome consisting of translocated or otherwise rearranged parts from two or more unidentified chromosomes joined to a normal chromosome.

Results of karyotyping analyses are expressed as the number of chromosomes/nucleus (normal is 46), the sex chromosomes (normal is XX or XY), followed by any genetic abnormalities observed. A normal karyotype is 46, XX in a female or 46, XY in a male. 46,XX,del(7)(q13) denotes a deletion in the long arm q of chromosome 7 at region 13. 46,XY,t(5;17)(p13.3;p13) denotes a translocation between the short arms of chromosomes 5

and 17 and region 13, band 3, and region 13, respectively. 47,XX+21 is the karyotype of a female with Down's syndrome resulting from an extra chromosome 21. Klinefelter's syndrome is caused by an extra X chromosome in males; for example, 47,XXY. Table 8.3 shows a list of some of the terms used in expressing karyotypes.

Table 8.3 A List of Descriptive Abbreviations

| Abbreviation | Indication |
|--------------|----------------------------------|
| + | gain |
| - | loss |
| del | deletion |
| der | derivative chromosome |
| dup | duplication |
| ins | insertion |
| inv | inversion |
| I, iso | isochromosome |
| mat | maternal origin |
| pat | paternal origin |
| r | ring chromosome |
| t | translocation |
| tel | telomere (end of chromosome arm) |

Fluorescence In Situ Hybridization

Interphase FISH

Fluorescence in situ hybridization (FISH) is a widely used method to detect protein, RNA as well as DNA structures in place in the cell or in situ. For cytogenetic analysis, fixed cells are exposed to a probe. The probe is a 60–200-kb fragment of DNA attached covalently to a fluorescent molecule. The probe will hybridize or bind to its complementary sequences in the cellular DNA. In interphase FISH, the bound probe can be visualized under a fluorescent microscope in the nucleus of the cell. Probes are designed to be specific to a particular chromosome or chromosomal regions so that the image under the microscope will correlate with the state of that chromosome or region. For example, a probe to any unique region on chromosome 22 should yield an image of two signals per nucleus, reflecting the two copies of chromosome 22 in the somatic cell nucleus (Fig. 8-15). A deletion or duplication of the region that is hybridized to the probe will result in a nucleus with only one signal or more than two signals, respectively. Multiple probes spanning large regions are used to detect regional deletions.^{16,17} One advantage of interphase FISH is that growth of cells in culture is not required. FISH methods are, therefore, used commonly to study prenatal samples, tumors, and hematological malignancies, not all of which are conveniently brought into metaphase in culture.

Translocations or other rearrangements can be detected using probes of different colors complementary to regions on each chromosome taking part in the translocation (Fig. 8-16). A translocated chromosome will combine the two probe colors with a loss of one of each signal. Analysis of

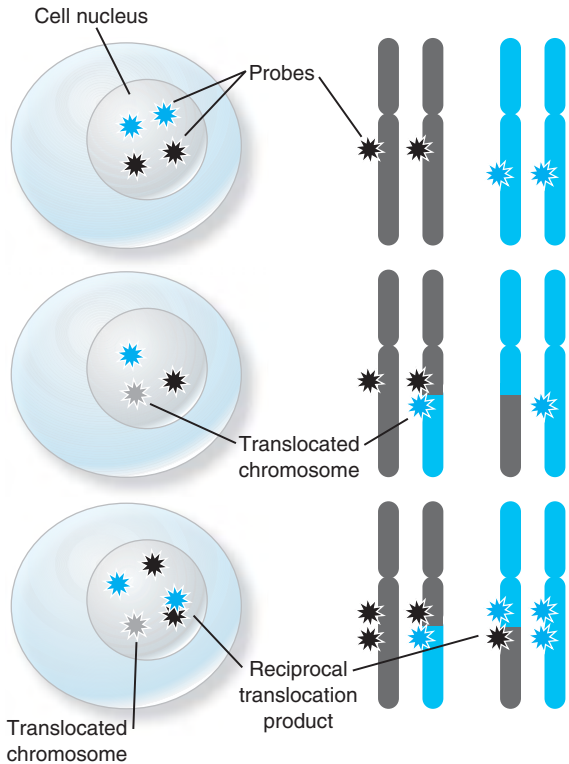


Figure 8-16 FISH analysis using distinct probes to detect a translocation. A normal nucleus has two signals from each probe (top). A translocation involving the two chromosomes combines the two probe colors (middle). Dual-fusion probes confirm the presence of the translocation by also giving a signal from the reciprocal breakpoint (bottom).

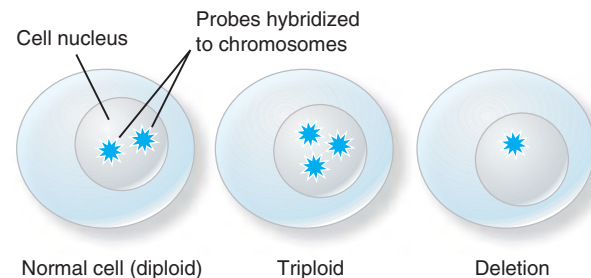
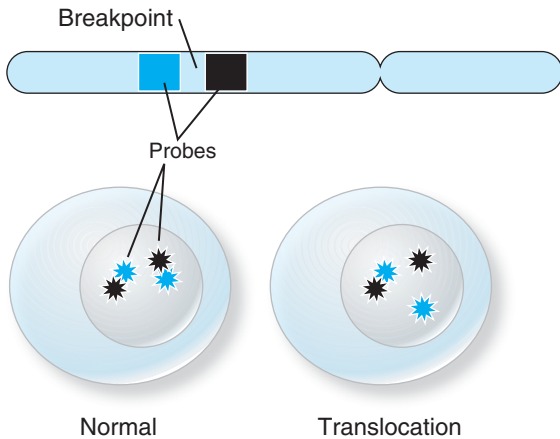


Figure 8-15 FISH analysis for a normal diploid cell (left), triploidy (center), and deletion (right).

translocation signals is sometimes complicated by false signals that result from two chromosomes landing close to one another in the nucleus, such that the bound probes give a signal similar to that exhibited by a translocation. These false signals can often be distinguished from true translocations by the size of the fluorescent image, but this distinction requires a trained eye. Accounting for false-positive signals as background noise limits the sensitivity of this assay.

The sensitivity of interphase FISH analysis can be increased using dual color probes, or **dual fusion probes**. These probes, 0.8–1.5 Mb in size, are designed to bind to regions spanning the breakpoint of both translocation



■ **Figure 8-17** Break-apart probes bind to the chromosome flanking the translocation breakpoint region. Normal cells will display the combination signal (bottom left), and a translocation will separate the probe signals (bottom right).

partners. A translocation will be observed as a signal from both the translocation junction and the reciprocal of the translocation junction; e.g., $t(9;22)$ and $t(22;9)$; see Fig. 8-16. Dual color **break-apart probes**, 0.6–1.5 Mb, are another approach to lower background as well as to identify translocation events where one chromosome can recombine with multiple potential partners. These probes are designed to bind to the intact chromosome flanking the translocation breakpoint. When a translocation occurs, the two probes separate (Fig. 8-17). Sometimes called tri-FISH, break-apart probes are not the same as tricolor probes (see below).

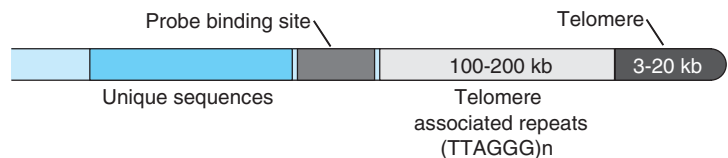
Centromeric probes (CEPs) are designed to hybridize to highly repetitive alpha satellite sequences surrounding centromeres. These probes detect aneuploidy of any chromosome. Combinations of centromeric probes and region-specific probes are often used to confirm deletions or amplifications in specific chromosomes. Addition of a CEP to dual color probes serves as a control for amplification or loss of one of the chromosomes involved in the

translocation. This combination of CEP and dual color probes comprises a **tricolor probe**. For example, the IGH/MYC CEP 8 Tri-color Dual Fusion Translocation Probe (Vysis) is a mixture of a 1.5-Mb-labeled probe, complementary to the immunoglobulin heavy chain region (IGH) of chromosome 14, an approximately 750-kb distinctly labeled probe complementary to the *myc* gene on chromosome 8 and a CEP to chromosome 8. The IGH probe contains sequences homologous to the entire IGH locus as well as sequences extending about 300 kb beyond the 3' end of the IGH locus. The *myc* probe extends approximately 400 kb upstream and about 350 kb 3' beyond the *myc* gene. CEP 8 targets chromosome 8 alpha satellite sequences and serves as a control to detect amplification of *myc* or loss of the chromosome 8 derivative resulting from the translocation.

Each chromosome arm has a unique set of repeat sequences located just before the end of the chromosome, called the **telomere** (Fig. 8-18). These sequences have been studied for the development of a set of DNA probes specific to the telomeres of all human chromosomes. **Telomeric probes** are useful for the detection of chromosome structural abnormalities such as cryptic translocations or small deletions that are not easily visualized by standard karyotyping.

Because interphase cells for FISH do not require culturing of the cells and stimulating division to get metaphase spreads, as is required for standard karyotyping, interphase FISH is faster than methods using metaphase cells and is valuable for analysis of cells that do not divide well in culture, including fixed cells.^{18,19} Furthermore, as 200–500 cells can be analyzed microscopically using FISH, the sensitivity of detection is higher than that of metaphase procedures, which commonly examine 20 spreads. A limitation of FISH, however, is the inability to identify chromosomal changes other than those at the specific binding region of the probe. In contrast, karyotyping is a more generic method that can detect any chromosomal change that causes

■ **Figure 8-18** The binding sites for telomeric probes are unique sequences just next to the telomeric associated repeats and telomeric repeat sequences at the ends of chromosomes.



changes in chromosomal size, number, or banding pattern within the sensitivity limits of the procedure.

Preparation of the sample is critical in interphase FISH analysis, both to permeabilize the cells for optimal probe-target interaction and to maintain cell morphology.²⁰ Optimal results are obtained if fresh interphase cells are incubated overnight (aging) after deposition on slides. After aging overnight, cells are treated with protease to minimize interference from cytoplasmic proteins and fixed with 1% formaldehyde to stabilize the nuclear morphology. Before DNA denaturation, the cells are dehydrated in graded concentrations of ethanol. Paraffin-embedded tissues must be dewaxed in xylene before protease and formaldehyde treatment.

The quality of the probe should also be checked and its performance validated before use. Fluorescent probes (DNA with covalently attached fluorescent dyes) are usually purchased from vendors, which may also supply compatible hybridization reagents and controls. Nevertheless, it is recommended that the probe performance be observed on control tissue before use on patient samples. Under a fluorescent microscope with the appropriate color distinction filters, the signal from the probe should be bright, specific to the target in the cell nuclei, and free of high background. Probes differ in their signal characteristics and intensities; the technologist should become familiar with what to expect from a given probe on different types of tissues.

Similar to Southern and Northern blotting procedures, both probe and target must be denatured prior to hybridization. The amount of time taken to hybridize and use Cot-1 DNA (to reduce nonspecific binding) or facilitators such as dextran sulfate (to increase the effective probe concentration) depend on the sequence complexity of the probe (see Chapter 6). A probe 10 ng–1 µg may be used in a hybridization volume of 3–10 µL. The hybridization of the probe on the target cells should be performed at 37–42°C in a humidified chamber. The slides are cover-slipped and sealed to optimize the hybridization conditions.

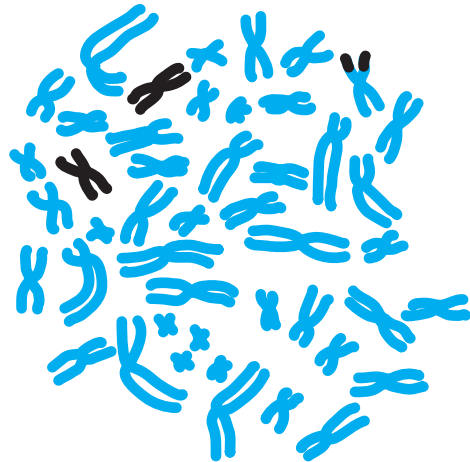
Following hybridization and the removal of unbound probe by rinsing in Coplin jars, the sample is observed microscopically. The probe signals should be visible from entire intact nuclei. Although adequate numbers of cells

must be visible, crowded cells where the nuclei and signals overlap do not yield accurate results. Furthermore, different tissue types have different image qualities and characteristics that must also be taken into account when assessing the FISH image.

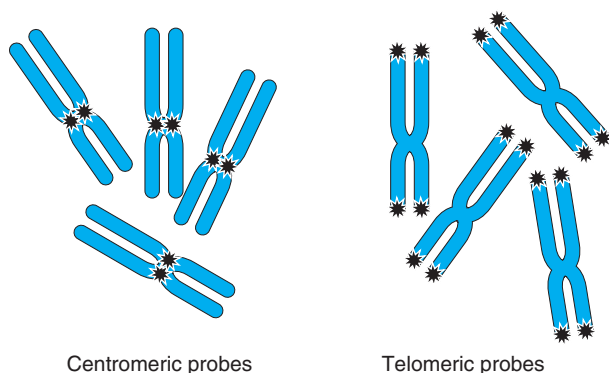
Metaphase FISH

Metaphase analysis has been enhanced by the development of fluorescent probes that bind to metaphase chromosomal regions or to whole chromosomes. Probes that cover the entire chromosome, or **whole chromosome paints**, are valuable for detecting small rearrangements that are not apparent by regular chromosome banding (Fig. 8-19). By mixing combinations of five fluorochromes and using special imaging software, **spectral karyotyping** can distinguish all 23 chromosomes by chromosome-specific colors.²¹ This type of analysis can be used to detect abnormalities that affect multiple chromosomes as is sometimes found in cancer cells or immortalized cell lines.^{22–24} Telomeric and centromeric probes are also applied to metaphase chromosomes (Fig. 8-20) to detect aneuploidy and structural abnormalities.

Preparation of chromosomes for metaphase FISH procedures begins with the culture of cells for 72 hours. About 45 minutes before harvesting, colcemid is added



■ **Figure 8-19** Chromosome painting showing a derivative chromosome formed by movement of a fragment of chromosome 12 (black) to an unidentified chromosome.

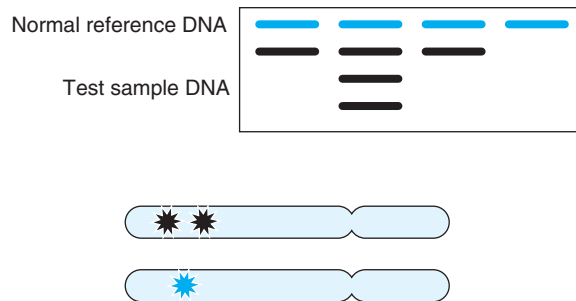


■ **Figure 8-20** Centromeric (left) and telomeric (right) probes on metaphase chromosomes.

to the cultures to arrest cells in metaphase. The cells are then suspended in a hypotonic medium (0.075 M KCl) and fixed with methanol/acetic acid (3:1). The fixed-cell suspension is applied to an inclined slide and allowed to dry briefly. A second treatment with 70% acetic acid may improve the chromosome spreading and decrease background. Condensed chromosome spreads, especially those from cultured metaphases, may be affected by temperature and humidity. Under a phase contrast microscope, the chromosomes should appear well separated with sharp borders. Cytoplasm should not be visible. Once the slide is dried, hybridization proceeds as discussed above for interphase FISH.

Intrachromosomal amplifications or deletions can be detected by **comparative genome hybridization (CGH)**.^{25,26} In this method, DNA from test and reference samples is labeled and used as a probe on a normal metaphase chromosome spread (Fig. 8-21). CGH has the advantage of being able to identify the location of deletions or amplifications throughout the genome.²⁷ The resolution (precise identification of the amplified or deleted region), however, is not as high as can be achieved with **array CGH** (see Chapter 6).

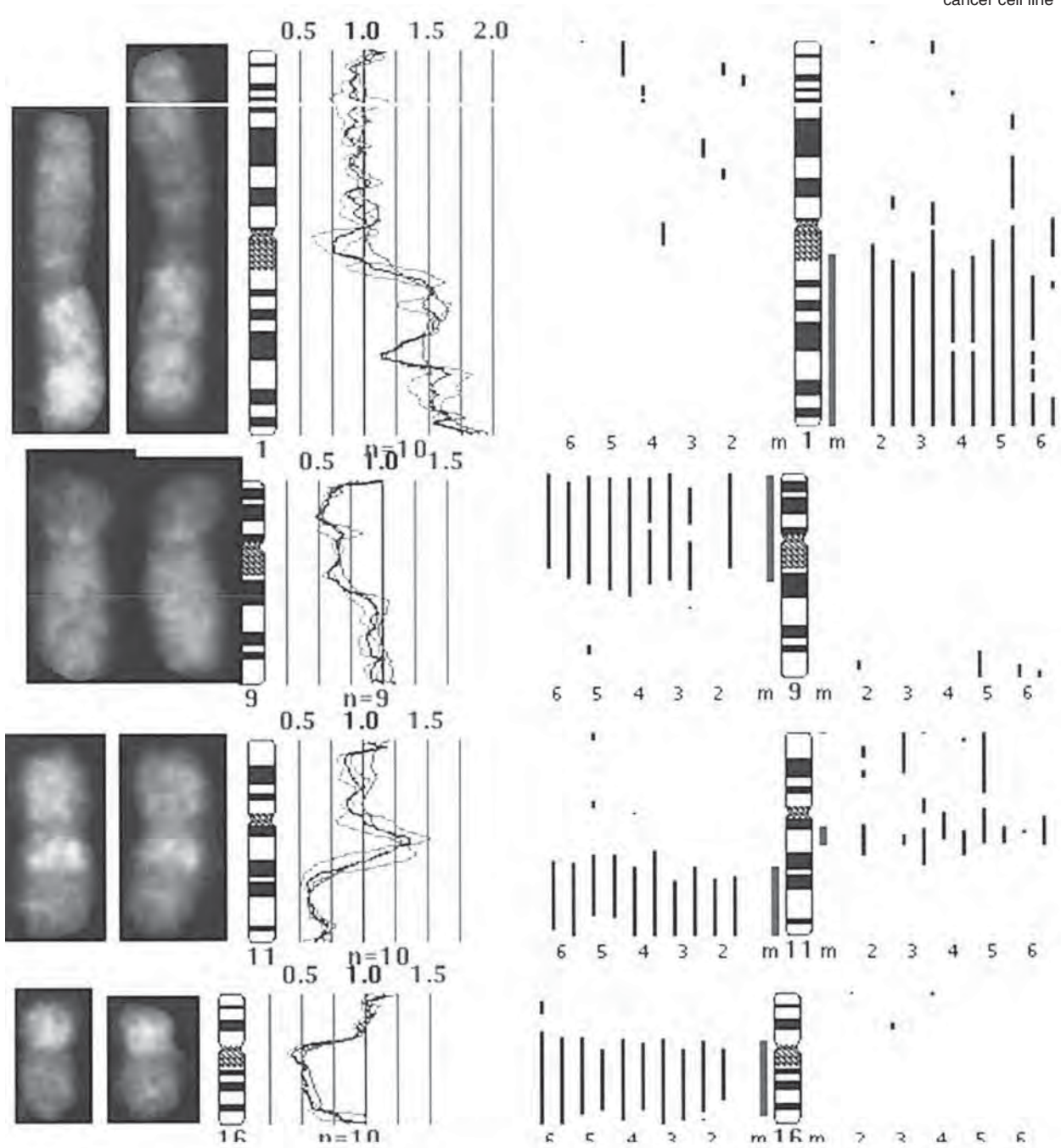
For CGH, the test DNA is isolated and labeled along with a reference DNA. Cyanine dyes are used as fluorescent labels for test and reference DNA for CGH. The two colorimetrically distinct dyes, Cy3 and Cy5, are commonly used for this purpose. Cy3, which fluoresces at a



■ **Figure 8-21** In CGH, the test sample is compared with a normal reference sample on a metaphase spread. Normally, test and reference signals are equal. A higher test signal denotes an amplification, and a higher reference signal denotes a deletion.

wavelength of 550 nm, is often represented as “green,” and Cy5, which fluoresces in the far-red region of the spectrum (650–667 nm), is represented as “red.” Derivatives of these dyes, such as Cy3.5, which fluoresces in the red-orange region, are also available. Because these dyes fluoresce brightly and are water-soluble, they have been used extensively for CGH using imaging equipment.

Labeling (attachment of Cy3 or Cy5 dye to the test and reference DNA) is achieved by nick translation or primer extension in which nucleotides covalently attached to the dye molecules are incorporated into the DNA sequences. Dye-nucleotides commonly used for this type of labeling are 5-amino-propargyl-2'-deoxycytidine 5'-triphosphate coupled to the Cy3 or Cy5 fluorescent dye (Cy3-AP3-dCTP, Cy5-AP3-dCTP) or 5-amino-propargyl-2'-deoxyuridine 5'-triphosphate coupled to the Cy3 or Cy5 fluorescent dye (Cy3-AP3-dUTP, Cy5-AP3-dUTP). DNA to be tested is partially digested with DNase to produce fragments that will bind efficiently to the denatured DNA in a metaphase chromosome spread. Separate aliquots of test and reference DNA are labeled with different Cy3 and Cy5 dyes, respectively, before application to a normal metaphase spread. An example of results from a CGH analysis is shown in Figure 8-22. Despite its utility and versatility in detecting chromosomal abnormalities, CGH does require advanced technical expertise. Array CGH is less comprehensive, but more specific, for detection of particular abnormalities.



■ **Figure 8-22** CGH analysis of four chromosomes from a cancer cell line. Amplified or deleted areas can be observed where the test and reference signals are not equal. The vertical lines on the diagram at right represent results from six different chromosomal spreads analyzed for excess reference signal (left of ideogram) or test signal (right of ideogram).

• STUDY QUESTIONS •

- During interphase FISH analysis of a normal specimen for the t(9;22) translocation, one nucleus was observed with two normal signals (one red for chromosome 22 and one green for chromosome 9) and one composite red/green signal. Five hundred other nuclei were normal. What is one explanation for this observation?
- Is 47; XYY a normal karyotype?
- What are the genetic abnormalities of the following genotypes?
47, XY, +18
46, XY, del(16)p(14)
iso(X)(q10)
46,XX del(22)q(11.2)
45, X
- A chromosome with a centromere not located in the middle of the chromosome but not completely at the end, where one arm of the chromosome is longer than the other arm, is called:
 - metacentric
 - acrocentric
 - paracentric
 - telocentric
- A small portion of chromosome 2 has been found on the end of chromosome 15, and a small portion of chromosome 15 has been found on the end of chromosome 2. This mutation is called a:
 - reciprocal translocation
 - inversion
 - deletion
 - robertsonian translocation
- Phytohemagglutinin is added to a cell culture when preparing cells for karyotyping. The function of the phytohemagglutinin is to:
 - arrest the cell in metaphase
 - spread out the chromosomes
 - fix the chromosomes on the slide
 - stimulate mitosis in the cells
- A CEP probe is use to visualize chromosome 21. Three fluorescent signals are observed in the patient's cells when stained with this probe. These results would be interpreted as consistent with:
 - a normal karyotype
 - Down's syndrome
 - Klinefelter's syndrome
 - technical error
- Cells were harvested from a patient's blood, cultured to obtain chromosomes in metaphase, fixed onto a slide, treated with trypsin, and then stained with Giemsa. The resulting banding pattern is called:
 - G banding
 - Q banding
 - R banding
 - C banding

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