4

General Features of Mitotic Chromosomes

Metaphase Chromosomes

Chromosomes are most often studied at mitotic metaphase, when the chromosomes are shortest and thickest, or most condensed. To obtain suitable metaphases, cells are grown in culture, treated with colcemid to destroy the highly viscous spindle (preventing anaphase), and treated with a hypotonic saline solution to swell the cells. The cell suspension is fixed with methanol-acetic acid, air-dried on a glass microscope slide to achieve optimal spreading and flattening of the chromosomes, and stained with a dye that binds to DNA. At metaphase the duplicated chromosomes each consist of two *sister chromatids*, which become daughter chromosomes after their separation at anaphase (Chapter 2). Each chromatid has two arms separated by a *primary constriction*, or unstained gap (Fig. 4.1). This marks the location of the *centromere*, the site of *spindle*

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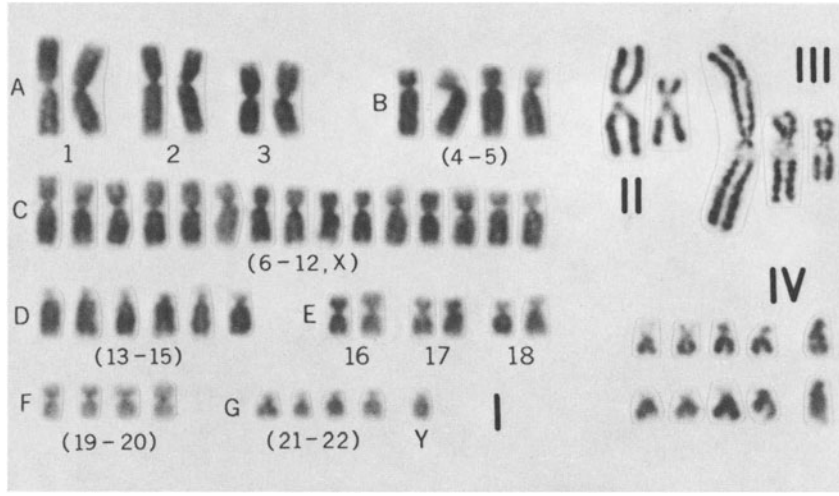

Figure 4.1. (I) Unbanded normal male karyotype from a lymphocyte. (II) Chromosomes 1 and 9 have fuzzy regions of heterochromatin (III) Chromosomes 1, 9, and 16, with fuzzy regions. (IV) G group and Y chromosomes from a father (above) and son (orcein staining) (courtesy of E. Therman).

microtubule attachment, which is essential for the normal movements of the chromosomes during mitotic and meiotic cell divisions. A chromosome without a centromere is *acentric* and either is lost or drifts passively towards a pole of the spindle. Some chromosomes have a *secondary constriction*, called a *nucleolus organizer*. A segment of variable size, a cytological *satellite*, is sometimes visible distal to a secondary constriction.

Each chromosome has a characteristic length (1–10 μm or so) and position of the centromere, which divides the chromosome into a short arm, designated p (petite) at the Paris Conference: 1971 (1972), and a long arm, designated q (since all geneticists know that p + q = 1!). That is, if a gene exists in only two forms (alleles), the fraction of each type, specified by "p" and "q," must together equal 1 (100%). A *metacentric* chromosome has its centromere near the middle. A *telocentric* chromosome has its centromere at the very end and is found only as a result of a structural change. *Submetacentric* chromosomes are intermediate between these two types. *Acrocentric* chromosomes have markedly unequal arms. The *arm ratio* (q/p) is the length of the long arm divided by that of the short arm. The *centromere index* is the length of the short arm divided by the total chromosome length. The length and arm ratio or centromeric index are rarely sufficient

to permit unambiguous identification of a chromosome, whether it is normal or abnormal. Chromosome identification is therefore almost always based on the use of chromosome banding or in situ hybridization with chromosome-specific DNA probes (Chapters 6 and 8).

The Chromosome Complement and Karyotype

Somatic cells have two complete sets of chromosomes, so the chromosome number, 46, is referred to as *diploid*, or 2n. The gametes have one complete set of 23 chromosomes, with the *haploid* number, n. The chromosome complement consists of 22 pairs of *autosomes* (non-sex chromosomes) and one pair of sex chromosomes (XX in females, XY in males). Their analysis is aided by constructing a *karyotype*, a display in which the chromosomes of a single metaphase spread are aligned in pairs, generally from longest to shortest, with the short arm of each chromosome at the top. The chromosome constitution of an individual is referred to as his or her karyotype. A diagrammatic karyotype, or *ideogram*, is usually based on the analysis of multiple cells. Figure 4.1 shows a karyotype prepared from an unbanded metaphase spread from a normal male. Chromosomes that can sometimes be individually distinguished by their length and arm ratio are so listed in the figure. Chromosome 9 can sometimes be identified by the presence of a less-stained region on the long arm near the centromere. The rest of the chromosomes can be classified only as belonging to one or another group. The X chromosome belongs to the large C group of eight chromosome pairs. Chromosomes 1 and 3 are metacentric; chromosome 2 and the chromosomes in the B, C, and E groups are submetacentric. The D and G group chromosomes are acrocentric and all have a nucleolus organizer (NOR) on the short arm.

Human chromosome banding was discovered by Caspersson et al. (1970). Banding techniques (Chapter 6) enable each chromosome to be distinguished by its pattern of darker and lighter bands. Consequently, an enormous number of structural changes undetectable by the earlier methods became identifiable. A G-banded karyotype is presented in Fig. 4.2. The chromosomes are arranged numerically according to length, with one exception; chromosome 22 is actually longer than 21. Since the chromosome that in the trisomic state causes Down syndrome had long been called 21, this number has been retained. The overwhelming importance of chromosome banding in human cytogenetics is amply demonstrated in many chapters of this book.

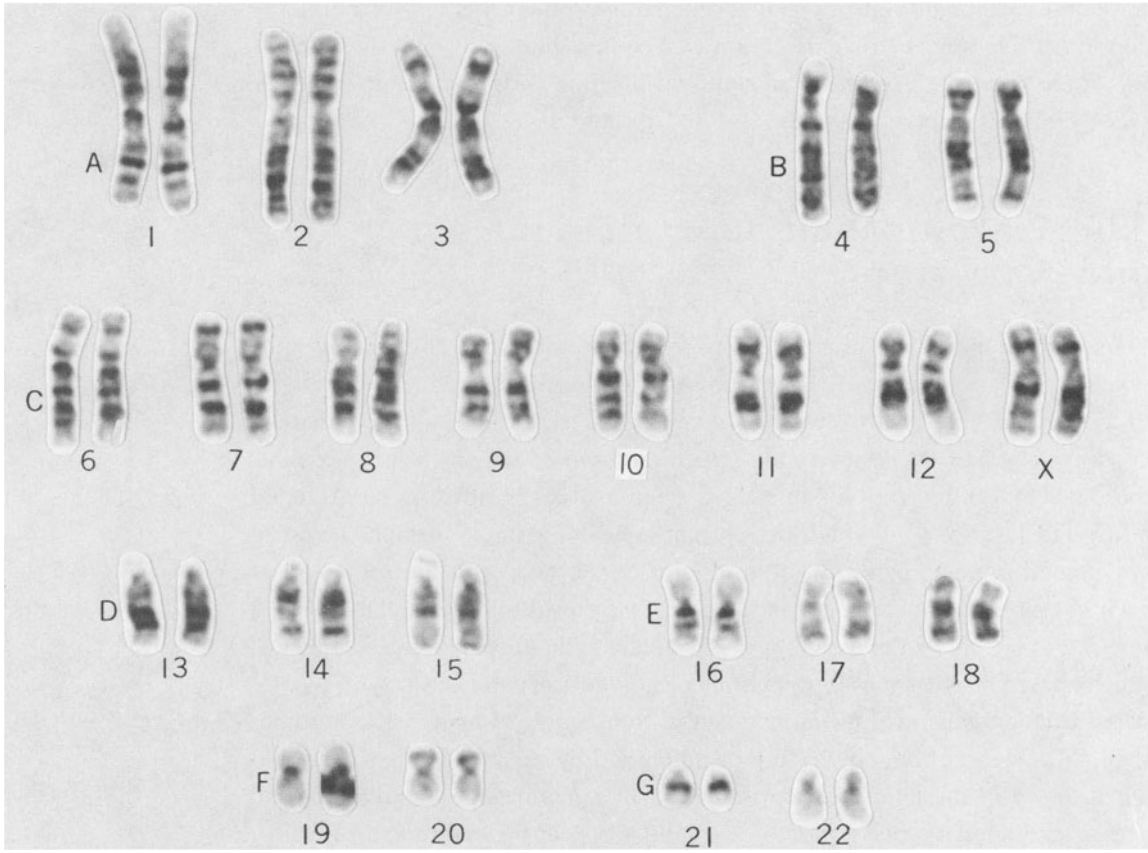

Figure 4.2. Normal female karyotype by G-banding.

DNA Content and DNA-Based Flow Cytometric Karyotypes

The DNA content of Feulgen-stained nuclei or fluorochrome-stained chromosomes (Mayall et al., 1984) and chromosome bands (Caspersson et al., 1970) can be determined by cytophotometry; automated scanning methods facilitate such measurements. However, for many purposes flow cytometry has replaced cytophotometry. The flow cytometer is an instrument that can be used to measure the DNA content of single chromosomes at a rate of several thousand per minute. The flow cytometer converts a suspension of chromosomes that have

been doubly stained with two fluorochromes into microdrops and shoots these through a detector that measures the intensity of fluorescence of each microdrop at two wavelengths and prepares a statistical profile of all the measurements. The suspension is made so dilute that virtually no droplet contains more than one chromosome. The thousands of individual measurements of the DNA content of each droplet then fall into rather sharply defined clusters, forming what is called a flow karyotype (Fig. 4.3). A fluorescence-activated cell sorter is able to isolate one class of microdrops, based on DNA content, and this technique has been used to prepare chromosome fractions so enriched for a single chromosome that quite workable PCR-amplified libraries can be constructed for use as chromosome-specific painting probes (Chapter 8). These techniques have exciting phylogenetic and clinical diagnostic applications (Ferguson-Smith, 1997).

Centromeres and Kinetochores

Centromeres, or kinetochores, are essential to attach chromosomes to the spindle so they can be distributed correctly to the daughter cells in mitosis and meiosis. Centromere malfunction leads to nondisjunction or other maldistribution of chromosomes. The terms centromere and kinetochore are sometimes used interchangeably. To avoid ambiguity, we shall use *centromere* to refer to the chromatin core (DNA plus histones) at the primary constriction and *kinetochore* to refer to the complex proteinaceous structure at the centromere that mediates attachment of spindle microtubules and chromosome movement in metaphase and anaphase. Choo (1997) and Lee et al. (1997) have reviewed the kinds of DNA sequences found at human centromeres, which contain large amounts of simple-sequence DNA, just like other constitutive heterochromatin. The most abundant, and the only one found on every chromosome, is called α-satellite DNA. It is made up of long tandem repeats of a basic monomeric sequence that is approximately 170 bp long. Its total length varies from chromosome to chromosome, but the amounts at all centromeres are very large, ranging from about 300 to 5000 kb. Thus, the 170-bp sequence is repeated over and over some 1700 to 29,000 times at a centromere. This satellite can serve as a molecular probe for centromeric sequences in intact chromosomes. Most cloned α-satellite fragments hybridize preferentially to the centromeric region of a specific chromosome. In fact, chromosome-specific α-satellite probes for every chromosome except 13 and 21 are now in common use for rapid analysis, by fluorescence in

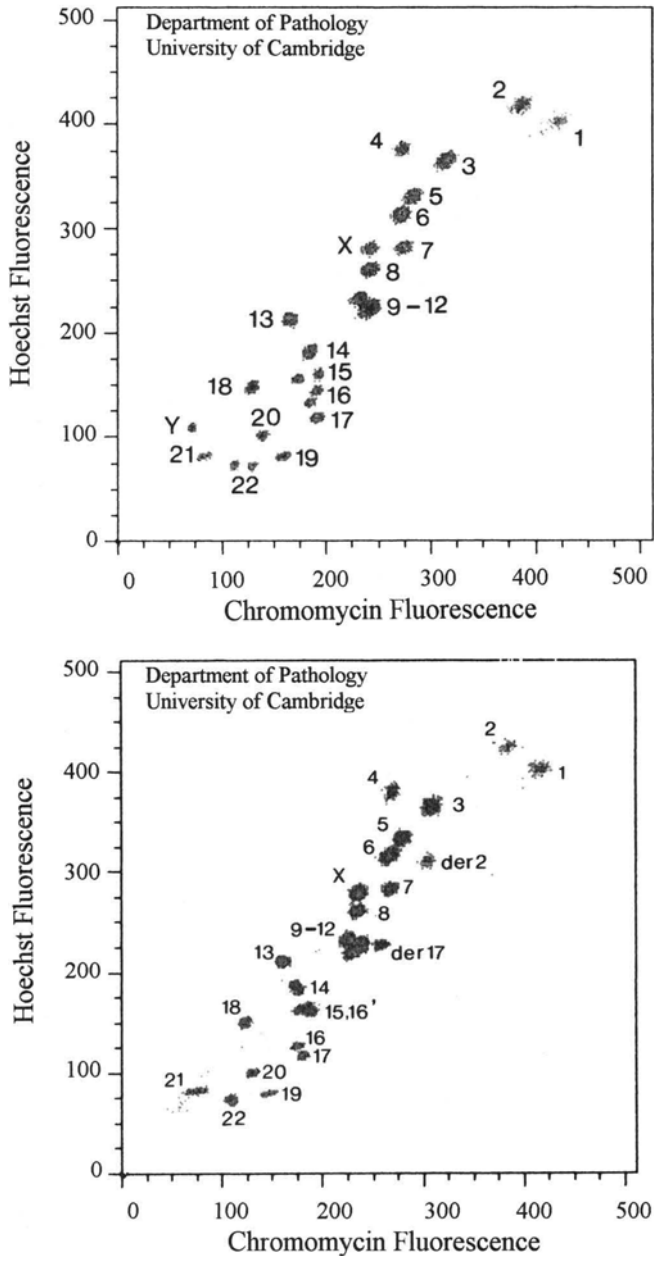

Figure 4.3. Two-fluorochrome bivariate flow cytometric karyotypes. (top) In this individual, all the chromosomes except numbers 9–12 differ in their DNA content or GC richness, and so do the homologues of chromosomes 15 and 16. (bottom) In this individual, a reciprocal 2; 17 translocation has produced derivative der 2 and der 17 chromosomes of novel sizes (Ferguson-Smith, 1997, reproduced with permission of S. Karger AG, Basel).

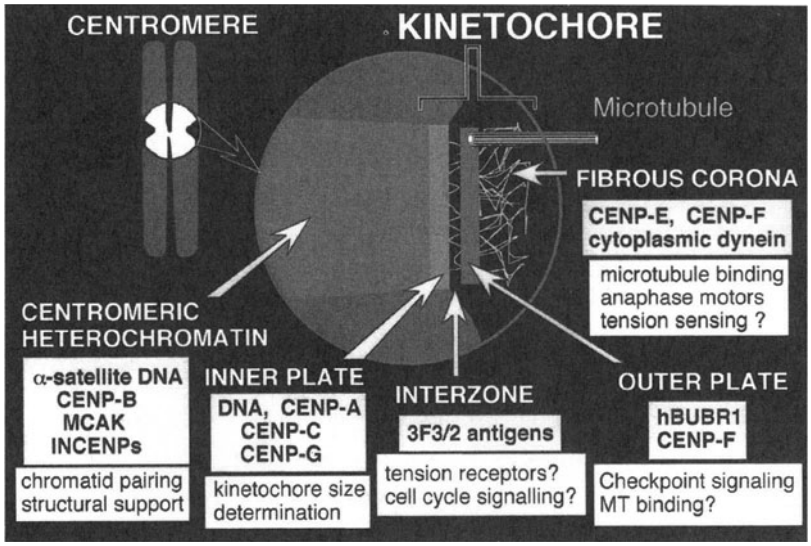

Figure 4.4. Model of an active centromere, or kinetochore; see text for details (courtesy of William Earnshaw).

situ hybridization (FISH), of the chromosome content of cells (Choo, 1997; see also Chapter 8). For examples, see Figures 8.1 and 9.6.

The kinetochore can be visualized by electron microscopy as a trilaminar structure resting on the surface of the heterochromatin at the primary constriction. Heterochromatin protein 1 (HP1) binds firmly to centromeric heterochromatin and serves to bind other specific proteins to the site. For example, the inner centromeric protein, INCENP, has a 13-amino-acid sequence that targets it to HP1 (Ainsztein et al., 1998). The molecular structure of the centromere/kinetochore is beginning to be understood in considerable detail (reviewed by Pluta et al., 1995). Figure 4.4 is a model showing the location of the major functional components. Most of these are described in the following paragraphs. The kinetochore has dense outer and inner plates separated by a less dense plate (*interzone*). Spindle microtubules insert into the outer plate or the fibrous corona surrounding it, which contains the motor protein dynein. Almost half the microtubules insert into the outer plate, the rest into the surrounding region or the inner plate. The inner plate contains chromatin, as shown by its disruption by DNAse and its abundant phosphate, detectable by electron

spectroscopic imaging (Rattner and Bazell-Jones, 1989). Kinetochores form attachments to the spindle fibers and contain the cytoplasmic motor protein dynein, which is involved in propelling kinetochores and their attached chromosomes to the spindle poles.

Kinetochores can be divided into subunits that retain the essential functions of the intact structure (Zinkowski et al., 1991). When DNA replication is blocked with hydroxyurea, cells blocked at the transition from the G1 to the S phase accumulate. When caffeine, an inhibitor of the DNA damage checkpoint discussed in Chapters 2 and 26, is added to these cells, they undergo mitosis even though the chromosomes have not replicated, and the chromosomes fall to pieces. The kinetochores break up into fragments, but these are still able to function as kinetochores. They line up on the metaphase plate, form attachments to the spindle fibers, and migrate to the spindle poles despite the absence of attached chromosomes. Kinetochores vary in size, with large sizes more common. Chromosomes with small kinetochores are more likely to be involved in nondisjunction than chromosomes with large kinetochores; that is, there appears to be a lower size limit for effective kinetochore function (Cherry and Johnston, 1987).

Antibodies in the serum of patients with the CREST form of an autoimmune disease called scleroderma bind specifically to the centromere and kinetochore. Consequently, fluorescent CREST antibodies will "light up" the centromeric regions of both metaphase and interphase chromosomes. These antibodies identify at least seven centromeric proteins (CENPs). In addition, there are inner centromeric proteins (INCENPs) that may play a role in holding sister chromatids together until the beginning of anaphase, and other proteins that may just be passengers. The known CENPs include the following:

1. CENP-A, a homologue of histone H3, has been immunolocalized to the inner kinetochore plate. CENP-A is present in centromeric nucleosomes in place of histone H3 and alters the chromatin structure. How is this achieved despite the high affinity of histone H3 for DNA? The answer appears to be that histone H3 synthesis is limited to the S phase and none is available when centromeric heterochromatin is replicated in very late S, whereas CENP-A is available even into early G2 (Shelby et al., 1997).
2. CENP-B binds to a conserved 17-bp DNA sequence, the "CENP-B box," that is present in many α-satellite repeats (Masumoto et al., 1989).
3. CENP-C binds to DNA and localizes to the inner kinetochore plate (Yang et al., 1996). In a dicentric chromosome in which one centromere has been

inactivated, immunostaining shows that CENP-B is present at both the active and the inactive centromeres, whereas CENP-C is present only at the active centromere and is thus diagnostic of active centromeres (Page et al., 1995). Microinjection of antibodies to CENP-C during interphase causes the trilaminar kinetochore at the next metaphase to be smaller and unable to bind microtubules. CENP-C is thus necessary for kinetochore function (Tomkiel et al., 1994). Cells from some scleroderma patients who produce antibodies to CENP-C appear to have a somewhat increased incidence of aneuploid cells (Jabs et al., 1993).

4. CENP-D may be the RCC1 protein, a regulator of chromosome condensation (Bischoff et al., 1990).
5. CENP-E is also found at active but not inactive centromeres (Page et al., 1995). Like dynein, it is close to the surface of the outer kinetochore plate (Wordeman and Mitchison, 1995). Its role is to cross-link microtubules (Liao et al., 1994).
6. CENP-F (mitosin) has a poorly understood role in microtubule binding to the outer kinetochore plate.
7. CENP-G binds to the same α-1 satellite family as CENP-B (He et al., 1998).

Additional proteins that are present in kinetochores include tubulin (the monomer from which microtubules are assembled) and cytoplasmic dynein (an ATPase that provides the motive force for moving materials along microtubules in cells). Another motor protein, mitotic centromere-associated kinesin (MCAK), is found throughout the centromeric region and between the kinetochore plates from prophase through telophase (Wordeman and Mitchison, 1995). The 3F3/2 antigen is in the interzone between the two kinetochore plates. It may function in sensing tension and in the cell cycle signaling that initiates anaphase (Chapter 2).

The assembly of the kinetochore proceeds by unknown mechanisms. It usually occurs at sites of large arrays of tandemly repetitive α-satellite DNA. The formation of a kinetochore at only one of the two α-satellite arrays on dicentric chromosomes with one inactive centromere (Chapter 22) indicates that this repetitive centromeric DNA is not sufficient for kinetochore assembly. Is it even necessary? The α-satellite sequences vary from chromosome to chromosome, although the short 17-bp CENP-B box, which binds the CENP-B protein, does seem to be conserved in at least some of the 170-bp repeats of this satellite on each chromosome. The strongest evidence that α-satellite is not essential for centromeric function is the occurrence of neocentromeres that contain *no* α-

satellite (Tyler-Smith et al., 1999). CENP-C and CENP-E, which are present at active but not inactive centromeres, are also present at these neocentromeres (Depinet et al., 1997). What is unclear is whether potential neocentromeres are restricted to sequences that share some higher-order structure with the normal centromeric DNA that enables it to bind HP1 protein so that CENP-C and INCENP can bind and thus anchor the inner kinetochore plate. Much more work is needed to understand kinetochore assembly and function.

Another function of centromeres is to carry passenger proteins into the daughter nuclei. Some proteins that are centromeric during mitotic prophase are later found attached to spindle fibers. Other proteins move from the chromosome and become concentrated under the cell membrane in the region where the cleavage furrow (for cytokinesis) will appear. Some of the passengers are nucleolar proteins, which are involved in ribosomal RNA synthesis and processing in the nucleolus. The storage of nucleolar proteins on the chromosomes may be necessary for the very rapid reconstitution of nucleoli and reinitiation of ribosomal RNA synthesis in late anaphase. Ribosomal RNA synthesis is still active in prophase and is absent only from prometaphase to late anaphase. Some of the ribosomal RNA genes remain decondensed throughout mitosis and are still associated with the nucleolar transcription factor, upstream binding factor (UBF) (Gebrane-Younes et al., 1997). This accounts for the presence of secondary constrictions and the silver staining capability of nucleolus organizer regions, described below.

Telomeres

Both ends of every chromosome are capped by special structures called telomeres. The tandemly repetitive telomeric unit, TTAGGG in one strand and CCCTAA in the other, is repeated several thousand times to give a stretch of DNA about 10 kb long at each end of each chromosome (Moyzis et al., 1988). Telomeres are critical for the complete replication of chromosome ends, as described in Chapter 3. They also play an essential role in the pairing of homologous chromosomes in prophase of meiosis (Chapter 10). Telomeres are protected from exonucleases that attack free ends of DNA by a protein that binds specifically to the single-stranded tail. Other telomere-specific proteins may play a role during meiosis in the association of telomeres with the nuclear envelope, and with each other, and may have additional functions (Broccoli et al., 1997).

Nucleolus Organizers and Ribosomal RNA Genes

The nucleolus is a nuclear organelle that is not bounded by a membrane. It is the site of transcription and processing of the 45S ribosomal RNA (rRNA) precursor into 28S, 18S, and 5.8S rRNAs and the site of the assembly of these rRNAs and over 80 different proteins into the two ribosomal subunits. These join to form the cytoplasmic ribosomes that play a key role in translating mRNAs into proteins. Nucleoli, which disappear during mitosis, are formed at telophase at specific chromosome sites called nucleolus organizer regions (NORs). Telophase is the time that rRNA synthesis starts up again after having been shut down from prometaphase to late anaphase (Gebrane-Younes et al., 1997). NORs can be strongly stained with a silver nitrate solution (Goodpasture and Bloom, 1975), because a specific nucleolar protein quickly reduces the silver ions to native silver. NORs are located on the short arms of the acrocentric chromosomes of the D and G groups, numbers 13, 14, 15, 21, and 22 (Fig. 20.3).

Immunofluorescence studies have shown that the proteins of the rRNA transcriptional machinery remain associated with active NORs, but not inactive NORs, throughout mitosis (Roussel et al., 1996). As interphase progresses, the number of nucleoli decreases, because nucleoli tend to fuse together into larger nucleoli, by an unknown mechanism and for unknown reasons. Even at metaphase there is a residual trace of this fusion, called *satellite association*, in which the short arms of 2–10 acrocentric chromosomes are touching or very close to one another. The tandemly repetitive ribosomal RNA gene clusters (rDNA) are heteromorphic, showing variations in the length of the secondary constrictions, the amount of silver staining of the NORs, and the amount of rDNA. The frequency with which a chromosome is involved in satellite association is strongly correlated with the size of its silver-stained NOR and less strongly with its number of rRNA genes, reflecting the importance of rRNA gene activity for nucleolar fusion (Miller et al., 1977).

NORs are usually visible either as secondary constrictions or as faintly stained regions, depending upon the amount of chromatin distal to the NOR. However, any rRNA gene clusters that have been inactivated, either as a result of the absence of a species-specific transcription factor or by intense methylation of the rRNA genes, do not form secondary constrictions or stain with silver nitrate (Miller et al., 1976; Tantravahi et al., 1981). The number of rRNA genes in the cluster on each acrocentric chromosome is variable, usually ranging from

perhaps 10 to 100 copies, although accurate estimates are few. Srivastava et al. (1993) isolated DNA from a human-rodent somatic cell hybrid (Chapter 23) that contained a single chromosome 22 as its only human acrocentric chromosome. They digested the DNA with the restriction enzyme *Eco*RV, which does not cut rDNA, and size-separated the resulting DNA fragments by pulsed-field gel electrophoresis. The human rDNA fragment was 1.6 Mb in size. Since each rRNA gene is 43 kb long, the cluster contained, at most, 39 contiguous rRNA genes. The chromatin distal to an NOR may be visible as a cytological satellite. This appears to consist solely of short, tandemly repetitive sequences called satellite DNA, or constitutive heterochromatin, which contains no genes and is highly variable in amount. Much of it appears to consist of long runs of (GACA)n (Fig. 20.3; Guttenbach et al., 1998).

Constitutive and Facultative Heterochromatin

In any type of cell, only a fraction of the genes are transcribed. The rest of the genome is maintained in an inactive configuration, called *heterochromatin*, in which transcription cannot take place (Hennig, 1999). Heterochromatin tends to be clumped in interphase nuclei and is replicated late in the S phase (Chapter 3). Its generally out-of-phase behavior throughout the cell cycle, or *allocycly*, has long been known. One type, *constitutive heterochromatin*, can be visualized by C-banding techniques (Chapter 6) and consists of satellite DNA. The other type of transcriptionally silent chromatin is called *facultative heterochromatin*. It contains potentially transcribable sequences that are specifically inactivated in certain cell types or at certain phases of development. The best-known example of facultative heterochromatin is the inactive X chromosome (Chapter 18).

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