Steven L. Gersen
Martha B. Keagle
Editors

The Principles of Clinical Cytogenetics

Third Edition

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ISBN 978-1-4419-1687-7 ISBN 978-1-4419-1688-4 (e-Book)
DOI 10.1007/978-1-4419-1688-4
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2012947405

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Preface

In April 2011, the website *The DNA Exchange* ran a story about the origin of our convention of referring to the short and long arms of chromosomes as "p" and "q." Several possible explanations for how this usage came into being were presented in a somewhat whimsical manner.

Did we really go with p from the French *petite* and q because it alphabetically follows p? Was there really a "French vs. English" argument? Was it supposed to be p and g (from the French *grande*) but changed due to a typesetting error? Was Hardy-Weinberg equilibrium ($p + q = 1$) invoked?

This prompted a flurry of comments over the Listserv used by cytogeneticists. Ultimately, several participants of the 1966 "Chicago Conference" weighed in, and Dr. Kurt Hirschhorn, who chaired the session at that conference, confirmed that the decision to go with p and q resulted from a combination of (sometimes spirited) debate, compromise (p really is for *petite*), logic, and, yes, agreement that $p + q = 1$.

This is all great fun. But the story in *The DNA Exchange* also spawned other comments.

It opened with:

> Karyotypes are sooooo 20th century. Time was when a ripe crop of G-banded chromosomes promised a fruitful harvest of genetic secrets. But nowadays a Giemsa-stained karyotype seems like a quaint low resolution black and white TV set – those cute little D & G groups even have rabbit-ear antennas – compared with the bright, sexy colors of FISH, the fine oligonucleotide detail of microarrays, and the dense volumes of data of generated by high throughput DNA sequencing.

Some cytogeneticists took offense at this.

People have been predicting the demise of cytogenetics for decades; this tended to happen each time new technology, such as DNA analysis or fluorescence *in situ* hybridization, became available. And yet we are still here.

Interestingly, this idea was significant as the previous edition of this book went to press in 2005 due to the increasingly important role of many FISH assays. In the preface to that edition, we discussed that while some classically trained cytogeneticists were concerned that FISH was going to put them out of work, Dorothy Warburton had predicted, years earlier, that FISH would actually provide the cytogenetics lab with an even more important diagnostic and prognostic role. She was of course correct.

Now we have microarrays. This edition of our book has a chapter dedicated to this technology, and several authors also deal with it in their individual chapters. The term "cytogenomics" (chromosome analysis using molecular techniques) is working its way into our lexicon.

Once again, there is talk, if not concern, that arrays could mean the unemployment line for cytogeneticists and, if not arrays, then perhaps next-generation sequencing. And once again, Dorothy put things into perspective:

> The way I look at it is that cytogenetics is not about a technique, but a field of knowledge. We may change the way we look at chromosomes, but the questions and problems remain the same. A technique is only as good as our ability to interpret what we see in a way that helps families, and having molecular training does not provide the experience necessary to do this. We would never have known about bal-

> anced translocations without looking at chromosomes, but now we have a way to tell if they are really balanced or not. I also believe that we will never be able to stop using chromosome preparations to interpret what we see on arrays. We have many examples where confirming array data has revealed unexpected kinds of rearrangements, as well as mosaicism. These are things that have much more significance for counseling than a simple call of a dup or del. I don't believe sequencing will change this.
> I was first advised to find another field in 1969 (right before banding). So far I still have a job, although what I look at day to day has changed a great deal. "Classical" is pretty much a synonym for "in the past," so yes, classical cytogenetics may no longer be practiced. However, what is here is exciting and challenging and requires every technique in our playbook.

This third edition of *The Principles of Clinical Cytogenetics* was prompted by significant advances in the field since the last edition of this book was published. So while it is true that the way we look at chromosomes will likely continue to evolve, we do not expect to stop looking at them any time soon.

Shelton, CT, USA — Steven L. Gersen, Ph.D.
Storrs, CT, USA — Martha B. Keagle, M.Ed.

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Part I

Basic Concepts and Background

1 History of Clinical Cytogenetics

Steven L. Gersen

The beginning of human cytogenetics is generally attributed to Walther Flemming, an Austrian cytologist and professor of anatomy, who published the first illustrations of human chromosomes in 1882. Flemming also referred to the stainable portion of the nucleus as *chromatin* and first used the term *mitosis* [1]. In 1888, Waldeyer introduced the word *chromosome*, from the Greek words for "colored body," and several prominent scientists of the day began to formulate the idea that determinants of heredity were carried on chromosomes [2]. After the "rediscovery" of Mendelian inheritance in 1900, Sutton (and, independently at around the same time, Boveri) formally developed a "chromosome theory of inheritance" [3, 4]. Sutton combined the disciplines of cytology and genetics when he referred to the study of chromosomes as *cytogenetics*.

Due in part to improvements in optical lenses, stains, and tissue manipulation techniques during the late nineteenth and early twentieth centuries, the study of cytogenetics continued, with an emphasis placed by some on determining the correct number of chromosomes, as well as the sex chromosome configuration, in humans. Several reports appeared, with differing estimates of these. For example, in 1912, von Winiwarter concluded that men have 47 chromosomes and women 48 [5]. Then, in 1923, T. S. Painter studied (meiotic) chromosomes derived from the testicles of several men who had been incarcerated, castrated, and ultimately hanged in the Texas State Insane Asylum. Based on this work, Painter definitively reported the human diploid chromosome number to be 48 (double the 24 bivalents he saw), even though, 2 years earlier, he had preliminarily reported that some of his better samples produced a diploid number of 46 [6]. At this time, Painter also proposed the X and Y sex chromosome mechanism in man. One year later, Levitsky formulated the term *karyotype* to refer to the ordered arrangement of chromosomes [7].

Despite continued technical improvements, there was clearly some difficulty in properly visualizing or discriminating between individual chromosomes. Even though Painter's number of 48 human chromosomes was reported somewhat conservatively, it was increasingly treated as fact with the passage of time and was "confirmed" several times over the next few decades. For example, in 1952, T. C. Hsu reported that, rather than depending upon histologic sections, examination of chromosomes could be facilitated if one studied cells grown with tissue culture techniques published by Fisher [8]. Hsu then demonstrated the value of this method by using it to examine human embryonic cell cultures, from which he produced both mitotic metaphase drawings and an idiogram of all 48 human chromosomes [9]!

As with other significant discoveries, correcting this inaccuracy required an unplanned event—a laboratory error. Its origin can be found in the addendum that appears at the end of Hsu's paper:

> It was found after this article had been sent to press that the well-spread metaphases were the result of an accident. Instead of being washed in isotonic saline, the cultures had been washed in hypotonic solution before fixation [9].

The hypotonic solution caused water to enter the cells via osmosis, which swelled the cell membranes and separated the chromosomes, making them easier to visualize. This accident was the key that unlocked the future of human cytogenetics. Within one year, Hsu, realizing the potential of this fortuitous event, reported a "hypotonic shock" procedure [10]. By 1955, Ford and Hamerton had modified this technique and had also worked out a method for pretreating cells grown in culture with colchicine so as to destroy the mitotic spindle apparatus and thus accumulate dividing cells in metaphase [11]. Joe Hin Tjio, an American-born Indonesian, learned about these procedures and worked with Hamerton and Ford to further improve upon them.

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S.L. Gersen and M.B. Keagle (eds.), *The Principles of Clinical Cytogenetics, Third Edition*,
DOI 10.1007/978-1-4419-1688-4_1, © Springer Science+Business Media New York 2013

In November of 1955, Tjio was invited to Lund, Sweden, to work on human embryonic lung fibroblast cultures in the laboratory of his colleague, Albert Levan, a Spaniard who had learned the colchicine and hypotonic method in Hsu's laboratory at the Sloan-Kettering Institute in New York. Tjio and Levan optimized the colchicine/hypotonic method for these cells and in January of 1956 (after carefully reviewing images from decades of previously reported work) diplomatically reported that the human diploid chromosome number appeared to be 46, not 48 [12]. They referenced anecdotal data from a colleague who had been studying liver mitoses from aborted human embryos in the Spring of 1955 but temporarily abandoned the research "because the workers were unable to find all the 48 human chromosomes in their material; as a matter of fact, the number 46 was repeatedly counted in their slides." Tjio and Levan concluded their paper:

> …we do not wish to generalize our present findings into a statement that the chromosome number of man is $2n=46$, but it is hard to avoid the conclusion that this would be the most natural explanation of our observations [12].

What was dogma for over 30 years had been overturned in one now classic paper. Ford and Hamerton soon confirmed Tjio and Levan's finding [13]. The era of clinical cytogenetics was at hand. It would take three more years to arrive, however, and it would begin with the identification of four chromosomal syndromes.

The concept that an abnormality involving the chromosomes could have a phenotypic effect was not original. In 1932, Waardenburg made the suggestion that Down syndrome could perhaps be the result of a chromosomal aberration, but the science of the time could neither prove nor disprove his idea; this would take almost three decades [14]. In 1958, Lejeune studied the chromosomes of fibroblast cultures from patients with Down syndrome and in 1959, described an extra chromosome in each of these cells [15]. The trisomy was reported to involve one of the smallest pairs of chromosomes and would eventually be referred to as trisomy 21. Lejeune had proved Waardenburg's hypothesis by reporting the first example of a chromosomal syndrome in man, and in December of 1962, he received one of the first Joseph Kennedy Jr. Foundation International Awards for his work (Fig. 1.1).

Three more chromosomal syndromes, all believed to involve the sex chromosomes, were also described in 1959. Ford reported that females with Turner syndrome have 45 chromosomes, apparently with a single X chromosome and no Y, and Jacobs and Strong demonstrated that men with Klinefelter syndrome have 47 chromosomes, with the additional chromosome belonging to the group that contained the X chromosome [16, 17]. A female with sexual dysfunction was also shown by Jacobs to have 47 chromosomes and was believed to have an XXX sex chromosome complement [18].

The sex chromosome designation of these syndromes was supported by (and helped explain) a phenomenon that had been observed 10 years earlier. In 1949, Murray Barr was studying fatigue in repeatedly stimulated neural cells of the cat [19]. Barr observed a small stained body on the periphery of some interphase nuclei, and his records were detailed enough for him to realize that this was present only in the nuclei of female cats. This object, referred to as sex chromatin (now known as X chromatin or the Barr body), is actually the inactivated X chromosome present in nucleated cells of all normal female mammals but absent in normal males. The observation that the Turner syndrome, Klinefelter syndrome, and putative XXX patients had zero, one, and two Barr bodies, respectively, elucidated the mechanism of sex determination in humans, confirming for the first time that it is the presence or absence of the Y chromosome that determines maleness, not merely the number of X chromosomes present, as in *Drosophila.* In 1961, the single active X

Fig. 1.1 Jérôme Lejeune receives a Joseph P. Kennedy Jr. Foundation International Award for demonstrating that Down syndrome results from an extra chromosome (Photo courtesy of the John F. Kennedy Library, Boston, MA)

chromosome mechanism of X-dosage compensation in mammals was developed by Mary Lyon and has been since known as the Lyon hypothesis [20].

It was not long after Lejeune's report of the chromosomal basis of Down syndrome that other autosomal abnormalities were discovered. In the April 9, 1960, edition of *The Lancet*, Patau et al. described two similar infants with an extra "D-group" chromosome who had multiple anomalies quite different from those seen in Down syndrome [21]. In the same journal, Edwards et al. described "a new trisomic syndrome" in an infant girl with yet another constellation of phenotypic abnormalities and a different autosomal trisomy [22]. The former became known as Patau syndrome or "D trisomy" and the latter as Edwards syndrome or "E trisomy." Patau paper incredibly contains a typographical error and announces that the extra chromosome "belongs to the E group," and Edwards reported that "the patient was … trisomic for the no. 17 chromosome," but we now know these syndromes to be trisomies 13 and 18, respectively.

Also in 1960, Nowell and Hungerford reported the presence of a small chromosome in patients with chronic myelogenous leukemia. Using the proposed nomenclature method at the time, this was designated Philadelphia chromosome 1 (Ph^1), and it demonstrated, for the first time, an association between chromosomes and cancer [23–25] (Fig. 1.2). Still referred to as the "Philadelphia chromosome" for historical purposes, this phenomenon was eventually relegated to nothing more than a curiosity during the 1960s, as the concept of a clinical association between chromosomes and cancer fell out of favor.

In 1963 and 1964, Lejeune et al. reported that three infants with the *cri du chat* ("cat cry") syndrome of phenotypic anomalies, which includes severe mental retardation and a characteristic kitten-like mewing cry, had a deletion of the short arm of a B-group chromosome, designated as chromosome 5 [26, 27]. Within two years, Jacobs et al. described "aggressive behavior, mental subnormality and the XYY male," and the chromosomal instabilities associated with Bloom syndrome and Fanconi anemia were reported [28–30].

Additional technical advancements had facilitated the routine study of patient karyotypes. In 1960, Peter Nowell observed that the kidney bean extract phytohemagglutinin, used to separate red and white blood cells, stimulated lymphocytes to divide. He introduced its use as a mitogen, permitting a peripheral blood sample to be used for chromosome analysis [31]. This eliminated the need for bone marrow aspiration, which had previously been the best way to obtain a sufficient number of spontaneously dividing cells. It was now feasible to produce mitotic cells suitable for chromosome analysis from virtually any patient.

Yet, within nine years of the discovery of the number of chromosomes in humans, only three autosomal trisomies, four sex chromosome aneuploidies, a structural abnormality (a deletion), an acquired chromosomal abnormality associated with cancer, and two chromosome breakage disorders had been described as recognizable "chromosomal syndromes." A new clinical laboratory discipline had been created; was it destined to be restricted to the diagnosis of a few abnormalities?

This seemed likely. Even though certain pairs were distinguishable by size and centromere position, individual chromosomes could not be identified, and as a result, patient-specific chromosome abnormalities could be observed but not defined. Furthermore, the existence of certain abnormalities, such as inversions involving a single chromosome arm (so-called *para*centric inversions) could be hypothesized, but not proven, because they could not be visualized. Indeed, it seemed that without a way to definitively identify each chromosome (and more importantly, regions of each chromosome), this new field of medicine would be limited in scope to the study of a few disorders.

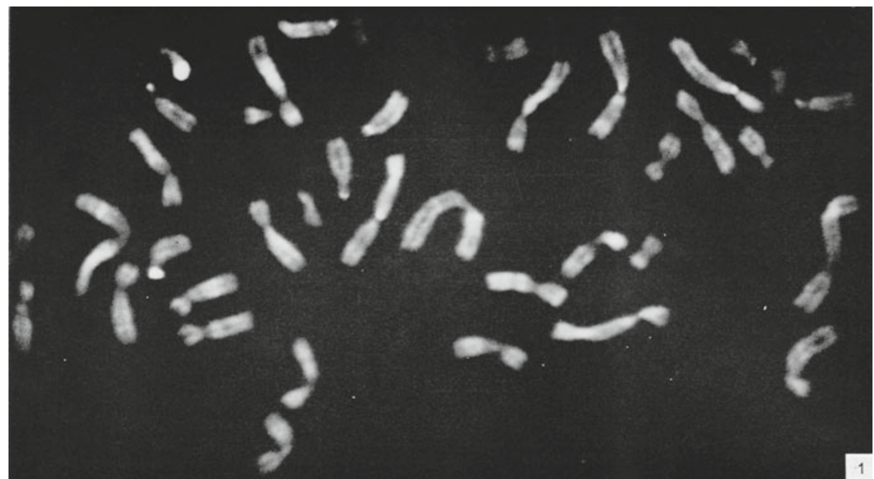

Fig. 1.2 The first photograph of a Q-banded cell published by Caspersson in 1970. The figure was originally labeled "Quinacrine mustard treated human metaphase chromosomes (male) from leukocyte culture. Fluorescence microscope × 2,000" (Reprinted with permission from Caspersson et al. [33], Elsevier)

For three more years, clinical cytogenetics was so relegated. Then, in 1968, Torbjörn Caspersson observed that when plant chromosomes were stained with fluorescent quinacrine compounds, they did not fluoresce uniformly but rather produced a series of bright and dull areas across the length of each chromosome. Furthermore, each pair fluoresced with a different pattern, so that previously indistinguishable chromosomes could now be recognized [32].

Caspersson then turned his attention from plants to the study of human chromosomes. He hypothesized that the quinacrine derivative quinacrine mustard (QM) would preferentially bind to guanine residues and that C-G rich regions of chromosomes should therefore produce brighter "striations," as he initially referred to them, while A-T rich regions would be dull. Although it ultimately turned out that it is the A-T rich regions that fluoresce brightly and that ordinary quinacrine dihydrochloride works as well as QM, by 1971, Caspersson had successfully produced and reported a unique "banding" pattern for each human chromosome pair [33, 34]. See Fig. 1.3.

For the first time, each human chromosome could be positively identified. The method, however, was cumbersome. It required a relatively expensive fluorescence microscope and a room that could be darkened, and the fluorescence tended to fade or "quench" after a few minutes, making real-time microscopic analysis difficult.

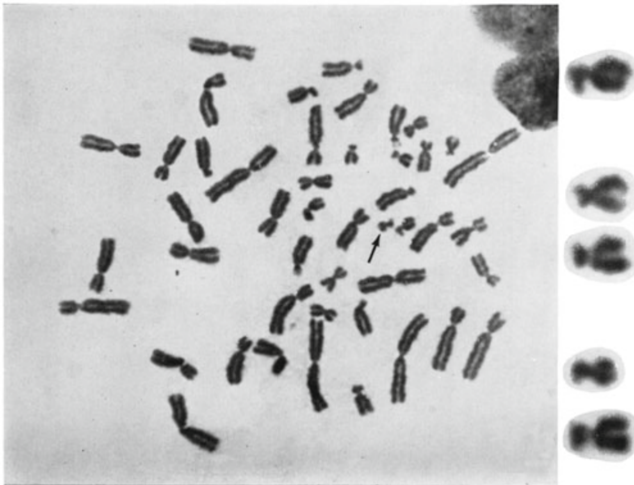

Fig. 1.3 One of the first photomicrographs of a metaphase spread from a patient with chronic myelogenous leukemia, indicating the Philadelphia chromosome. Reported a decade before routine chromosome banding, the authors (correctly) interpreted the abnormal chromosome to represent the next-to-smallest human chromosome and reported it as being a chromosome 21: "*Note* the Ph^1 chromosome (*arrow*). To *right* are shown, from *bottom to top*, 21, Ph^1, 22, 22, and Y. The Ph^1 chromosome is apparently a 21 which has lost approximately one half of its long arm." However, although chromosome banding demonstrated that the chromosome involved in Down syndrome is actually the smallest human chromosome, the term "trisomy 21" was already too common to be changed, and so the numbering of the two smallest human chromosomes was reversed. The Philadelphia chromosome is therefore described as being derived from chromosome 22 (Figure courtesy of Alice Hungerford and reprinted with permission from Nowell and Hungerford [25])

These difficulties were overcome a year later, when Drets and Shaw described a method of producing similar chromosomal banding patterns using an alkali and saline pretreatment followed by staining with Giemsa, a compound developed for identification, in blood smears, of the protozoan that causes malaria [35]. Even though some of the chromosome designations proposed by Drets and Shaw have been changed (essentially in favor of those advocated by Caspersson), this method, and successive variations of it, facilitated widespread application of clinical cytogenetic techniques. While the availability of individuals with the appropriate training and expertise limited the number and capacity of laboratories that could perform these procedures (in some ways still true today), the technology itself was now within the grasp of any facility.

What followed was a cascade of defined chromosomal abnormalities and syndromes: aneuploidies, deletions, microdeletions, translocations, inversions (including the paracentric variety), insertions, mosaicisms, and a seemingly infinite number of patient- and family-specific rearrangements.

In 1973, Janet Rowley demonstrated that the "Philadelphia chromosome" was actually the result of a translocation involving chromosomes 9 and 22, and in that same year, she also described an (8;21) translocation in AML [36, 37]. The association between chromosomes and cancer could no longer be ignored. The decades that followed saw an ever-increasing collection of rearrangements and other cytogenetic anomalies associated with neoplasia. These were eventually cataloged by Felix Mitelman in what has become an ongoing project of incredible dedication; the first volume was published in 1983, and the most recent version is an online database with close to 60,000 entries [38, 39].

Thanks to the host of research applications made possible by the precise identification of smaller and smaller regions of the karyotype, genes began to be mapped to chromosomes at a furious pace. The probes that resulted from such research have given rise to the discipline of molecular cytogenetics, which utilizes the techniques of fluorescence *in situ* hybridization (FISH). In recent years, this exciting development and the many innovative procedures derived from it have created even more interest in the human karyotype. A perfect example involves the union of information gleaned from the Human Genome Project with molecular techniques such as comparative genomic hybridization (GCH) or single nucleotide polymorphism (SNP) analysis. Combining these using computer and droplet technologies has given rise to the chromosome microarray, which is already becoming the next step in the evolution of clinical cytogenetics.

In the summer of 2006, geneticists from around the world met in Bethesda, Maryland, to celebrate "50 Years of 46

Fig. 1.4 In July 2006, geneticists from around the world met in Bethesda, Maryland, to celebrate "50 Years of 46 Human Chromosomes: Progress in Cytogenetics"

Human Chromosomes: Progress in Cytogenetics" (Fig. 1.4), and in 2010, we gathered in Philadelphia for a "Philadelphia Chromosome Symposium: Past, Present, and Future—The 50th Anniversary of the Discovery of the Philadelphia Chromosome." This group had the honor of being addressed by Dr. Peter Nowell, Dr. Janet Rowley, Dr. Felix Mitelman, and Mrs. Alice Hungerford, wife of the late Dr. David Hungerford.

More than one million cytogenetic and molecular cytogenetic analyses are now performed annually in more than 400 laboratories worldwide, and this testing is now often the standard of care [40, 41]. Pregnant women over the age of 35, or those with certain serum-screening results, are routinely offered prenatal cytogenetic analysis, and many also have prenatal ploidy analysis via FISH. For children with phenotypic and/or mental difficulties and for couples experiencing reproductive problems, cytogenetics has become a routine part of their clinical workup. FISH has permitted us to visualize changes that are too subtle to be detected with standard chromosome analysis, and chromosome microarrays provide even greater resolution. Cytogenetics and FISH also provide information vital to the diagnosis, prognosis, therapy, and monitoring of treatment for a variety of cancers, and cancer arrays are gaining utility as well.

It was really not so long ago that humans had 48 chromosomes. One has to wonder whether any of the pioneers of this field could have predicted the modern widespread clinical use of chromosome analysis, in all its forms. But perhaps it is even more exciting to wonder what lies ahead.

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DNA, Chromosomes, and Cell Division

2

Martha B. Keagle

Introduction

The molecule deoxyribonucleic acid (DNA) is the raw material of inheritance and ultimately influences all aspects of the structure and functioning of the human body. A single molecule of DNA, along with associated proteins, comprises a chromosome. Chromosomes are located in the nuclei of all human cells (with the exception of mature red blood cells), and each human cell contains 23 different pairs of chromosomes.

Genes are functional units of genetic information that reside on each of the 23 pairs of chromosomes. These units are linear sequences of nitrogenous bases that code for protein molecules necessary for the proper functioning of the body. The genetic information contained within the chromosomes is copied and distributed to newly created cells during cell division. The structure of DNA provides the answer to how it is precisely copied with each cell division and to how proteins are synthesized.

DNA Structure

James Watson and Francis Crick elucidated the molecular structure of DNA in 1953 using X-ray diffraction data collected by Rosalind Franklin and Maurice Wilkins, and model building techniques advocated by Linus Pauling [1, 2]. Watson and Crick proposed the double helix: a twisted, spiral ladder structure consisting of two long chains wound around each other and held together by hydrogen bonds. DNA is composed of repeating units—the nucleotides. Each nucleotide consists of a deoxyribose sugar, a phosphate group, and one of four nitrogen-containing bases: adenine (A), guanine (G), cytosine (C), or thymine (T). Adenine and guanine are purines with a double-ring structure, whereas cytosine and thymine are smaller pyrimidine molecules with a single ring structure. Two nitrogenous bases positioned side by side on the inside of the double helix form one rung of the molecular ladder. The sugar and phosphate groups form the backbone or outer structure of the helix. The fifth (5′) carbon of one deoxyribose molecule and the third (3′) carbon of the next deoxyribose are joined by a covalent phosphate linkage. This gives each strand of the helix a chemical orientation with the two strands running opposite or antiparallel to one another.

Biochemical analyses performed by Erwin Chargaff showed that the nitrogenous bases of DNA were not present in equal proportions and that the proportion of these bases varied from one species to another [3]. Chargaff noted, however, that concentrations of guanine and cytosine were always equal, as were the concentrations of adenine and thymine. This finding became known as Chargaff's rule. Watson and Crick postulated that in order to fulfill Chargaff's rule and to maintain a uniform shape to the DNA molecule, there must be a specific complementary pairing of the bases: adenine must always pair with thymine, and guanine must always pair with cytosine. Each strand of DNA, therefore, contains a nucleotide sequence that is complementary to its partner. The linkage of these complementary nitrogenous base pairs holds the antiparallel strands of DNA together. Two hydrogen bonds link the adenine and thymine pairs, whereas three hydrogen bonds link the guanine and cytosine pairs (Fig. 2.1). The complementarity of DNA strands is what allows the molecule to replicate faithfully. The sequence of bases is critical for DNA function because genetic information is determined by the order of the bases along the DNA molecule.

DNA Synthesis

The synthesis of a new molecule of DNA is called replication. This process requires many enzymes and cofactors. The first step of the process involves breakage of the hydrogen

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S.L. Gersen and M.B. Keagle (eds.), *The Principles of Clinical Cytogenetics, Third Edition*,
DOI 10.1007/978-1-4419-1688-4_2, © Springer Science+Business Media New York 2013

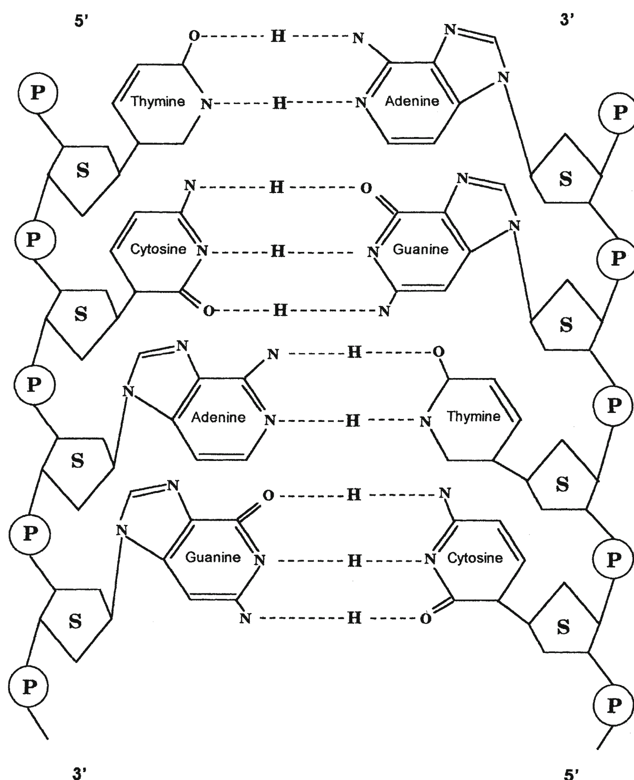

Fig. 2.1 DNA structure. Schematic representation of a DNA double helix unwound to show the complementarity of bases and the antiparallel structure of the phosphate (P) and sugar (S) backbone strands

bonds that hold the DNA strands together. DNA helicases and single-strand binding proteins work to separate the strands and keep the DNA exposed at many points along the length of the helix during replication. The area of DNA at the active region of separation is a Y-shaped structure referred to as a replication fork. These replication forks originate at structures called replication bubbles, which, in turn, are at DNA sequences called replication origins. The molecular sequence of the replication origins has not been completely characterized. Replication takes place on both strands, but nucleotides can only be added to the 3′ end of an existing strand. The separated strands of DNA serve as templates for production of complementary strands of DNA following Chargaff's rules of base pairing.

The process of DNA synthesis differs for the two strands of DNA because of its antiparallel structure. Replication is straightforward on the leading strand. The enzyme DNA polymerase I facilitates the addition of complementary nucleotides to the 3′ end of a newly forming strand of DNA. In order to add further nucleotides, DNA polymerase I requires the 3′-hydroxyl end of a base-paired strand.

DNA synthesis on the lagging strand is accomplished by the formation of small segments of nucleotides called Okazaki fragments [4]. After separation of the strands, the enzyme DNA primase uses ribonucleotides to form a ribonucleic acid primer.

The structure of ribonucleic acid (RNA) is similar to that of DNA, except that each nucleotide in RNA has a ribose sugar instead of deoxyribose and the pyrimidine thymine is replaced by another pyrimidine, uracil (U). RNA also differs from DNA in that it is a single-stranded molecule. This RNA primer is at the beginning of each Okazaki segment to be copied, provides a 3′-hydroxyl group, and is important for the efficiency of the replication process. The ribonucleic acid primer then attracts DNA polymerase I. DNA polymerase I brings in the nucleotides and also removes the RNA primer and any mismatches that occur during the process. Okazaki fragments are later joined by the enzyme DNA ligase. The process of replication is semiconservative because the net result is creation of two identical DNA molecules, each consisting of a parent DNA strand and a newly synthesized DNA strand. The new DNA molecule grows as hydrogen bonds form between the complementary bases (Fig. 2.2).

Protein Synthesis

The genetic information of DNA is stored as a code; a linear sequence of nitrogenous bases in triplets. These triplets code for specific amino acids that are subsequently linked together to form protein molecules. The process of protein synthesis involves several types of ribonucleic acid.

The first step in protein synthesis is transcription. During this process, DNA is copied into a complementary piece of messenger RNA (mRNA). Transcription is controlled by the enzyme RNA polymerase, which functions to link ribonucleotides together in a sequence complementary to the DNA template strand. The attachment of RNA polymerase to a promoter region, a specific sequence of bases that varies from gene to gene, starts transcription. RNA polymerase moves off the template strand at a termination sequence to complete the synthesis of an mRNA molecule (Fig. 2.3).

Messenger RNA is modified at this point by the removal of introns—segments of DNA that do not code for an mRNA product. In addition, some nucleotides are removed from the 3′ end of the molecule, and a string of adenine nucleotides are added. This poly(A) tail helps in the transport of mRNA molecules to the cytoplasm. Another modification is the addition of a cap to the 5′ end of the mRNA, which serves to aid in attachment of the mRNA to the ribosome during translation. These alterations to mRNA are referred to as mRNA processing (Fig. 2.4). At this point, mRNA, carrying the information necessary to synthesize a specific protein, is transferred from the nucleus into the cytoplasm of the cell, where it then associates with ribosomes. Ribosomes, composed of ribosomal RNA (rRNA) and protein, are the site of

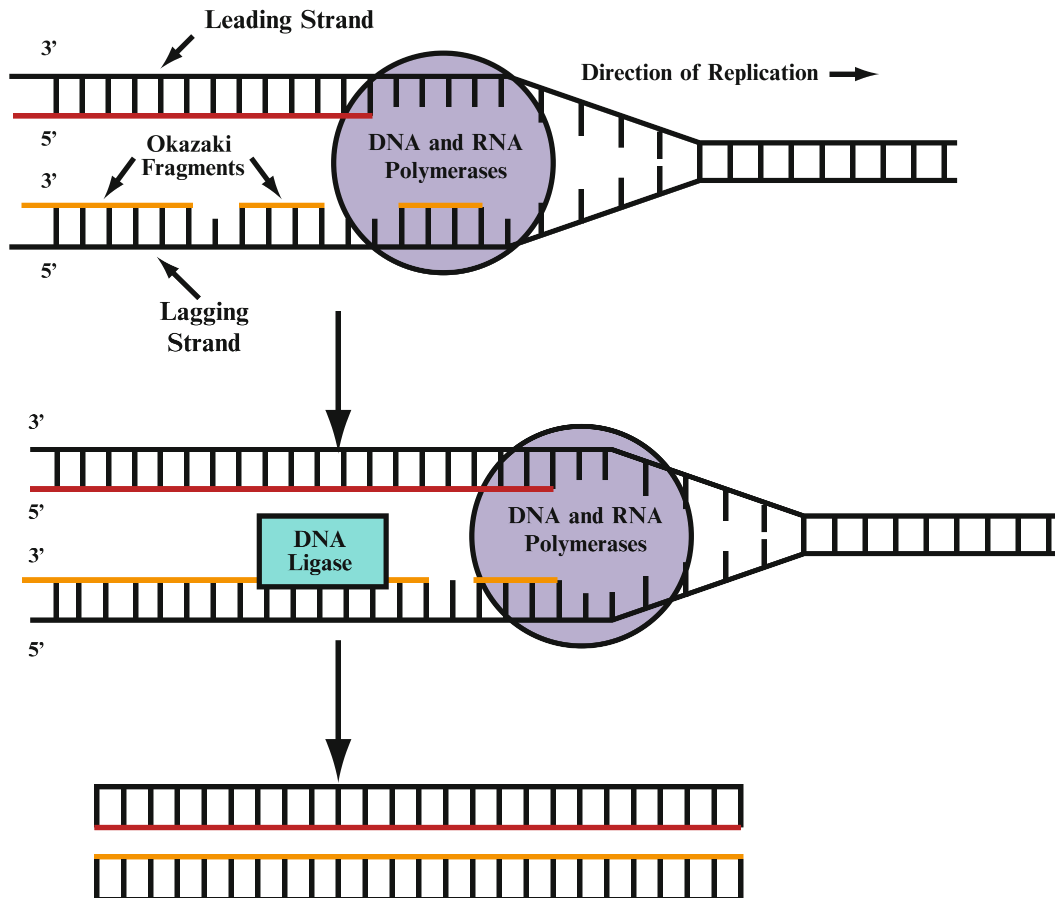

Fig. 2.2 Semiconservative replication. Complementary nucleotides are added directly to the 3′ end of the leading strand, whereas the lagging strand is copied by the formation of Okazaki fragments

protein synthesis. Ribosomes consist of two subunits that come together with mRNA to read the coded instructions on the mRNA molecule.

The next step in protein synthesis is translation. A chain of amino acids is synthesized during translation by using the newly transcribed mRNA molecule as a template, with the help of a third ribonucleic acid, transfer RNA (tRNA). Leder and Nirenberg and Khorana determined that three nitrogen bases on an mRNA molecule constitute a codon [5, 6]. With four nitrogenous bases, there are 64 possible three-base codons. Sixty-one of these code for specific amino acids, and the other three are "stop" codons that signal the termination of protein synthesis. There are only 20 amino acids, but 61 codons. Therefore, most amino acids are coded for by more than one mRNA codon. This redundancy in the genetic code is referred to as degeneracy.

Transfer RNA molecules contain "anticodons"—nucleotide triplets that are complementary to the codons on mRNA. Each tRNA molecule has attached to it the specific amino acid for which it codes.

Ribosomes read mRNA one codon at a time. Transfer RNA molecules transfer the specific amino acids to the synthesizing protein chain (Fig. 2.5). The amino acids are joined to this chain by peptide bonds. This process is continued until a stop codon is reached. The new protein molecule is then released into the cell milieu and the ribosomes split apart (Fig. 2.6).

DNA Organization

Human chromatin consists of a single continuous molecule of DNA complexed with histone and nonhistone proteins. The DNA in a single human diploid cell, if stretched out, would be approximately 2 m in length and therefore must be condensed considerably to fit within the cell nucleus [7]. There are several levels of DNA organization that allow for this.

The DNA helix itself is the first level of condensation. Next, two molecules of each of the histones H2A, H2B, H3, and H4 form a protein core: the octamer. The DNA double

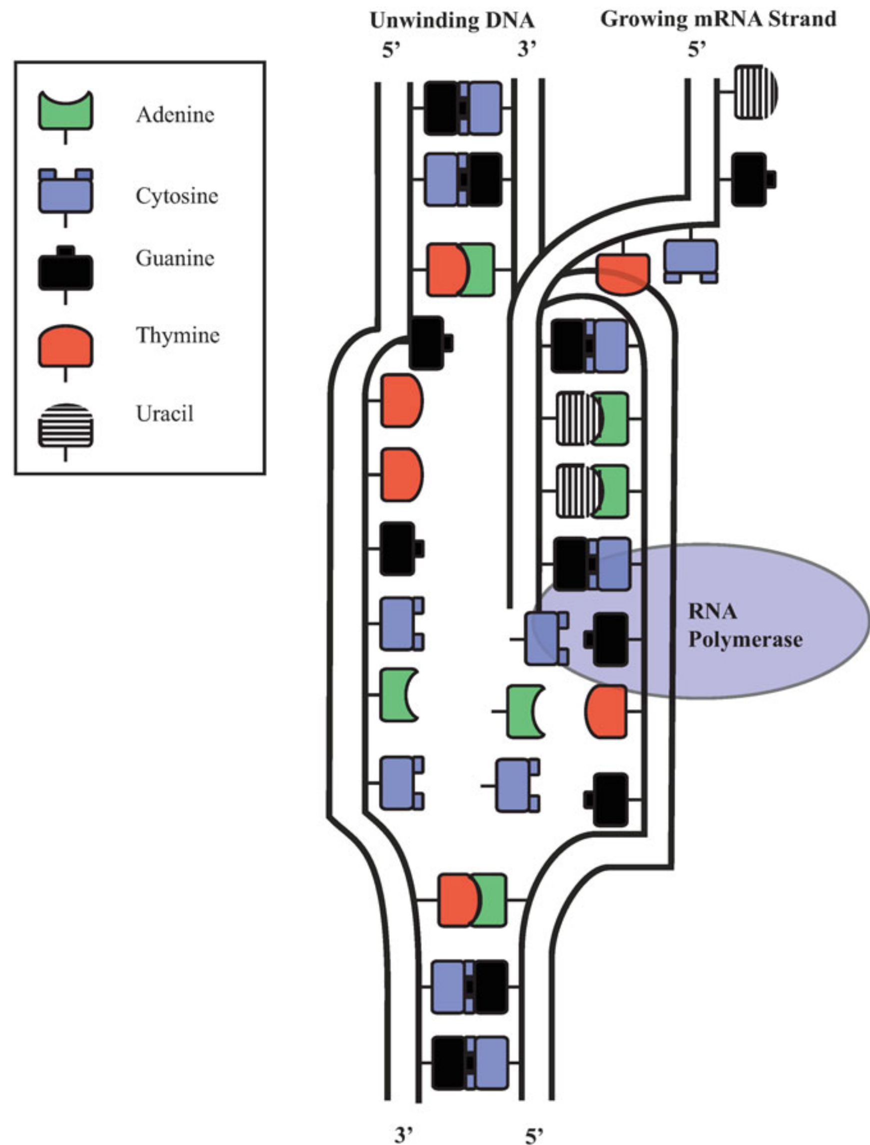

Fig. 2.3 Transcription. A DNA molecule is copied into mRNA with the help of RNA polymerase

helix winds twice around the octamer to form a 10-nm nucleosome, the basic structural unit of chromatin. Adjacent nucleosomes are pulled together by a linker segment of the histone H1. Repeated, this gives the chromatin the appearance of "beads on a string." Nucleosomes are further coiled into a 30-nm solenoid, with each turn of the solenoid containing about six nucleosomes. The solenoids are packed into DNA looped domains attached to a nonhistone protein matrix. Attachment points of each loop are fixed along the DNA. The looped domains coil further to give rise to highly compacted units, the chromosomes, which are visible with the light microscope only during cell division. Chromosomes reach their greatest extent of condensation during mitotic metaphase (Fig. 2.7).

Chromosome Structure

A chromosome consists of two sister chromatids, each of which is comprised of a contracted and compacted double helix of DNA. The centromere, telomere, and nucleolar organizer regions are functionally differentiated areas of the chromosomes (Fig. 2.8).

The Centromere

The centromere is a constriction visible on metaphase chromosomes where the two sister chromatids are joined together. The centromere is essential to the survival of a chromosome

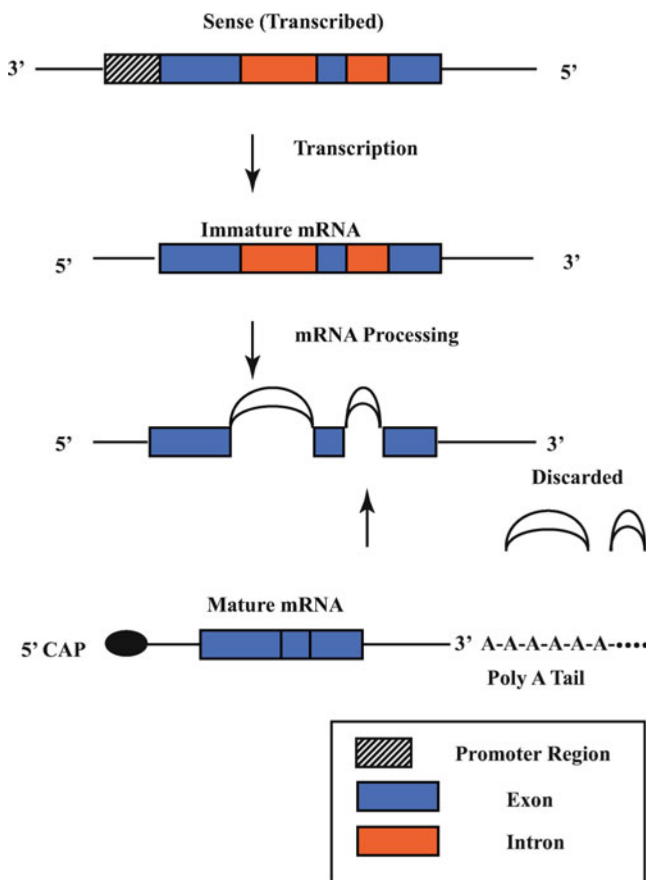

Fig. 2.4 Messenger RNA processing. The transcribed strand of DNA is modified to produce a mature mRNA transcript

during cell division. Interaction with the mitotic spindle during cell division occurs at the centromeric region. Mitotic spindle fibers are the functional elements that separate the sister chromatids during cell division.

Human chromosomes are classified based on the position of the centromere on the chromosome. The centromere is located near the middle in metacentric chromosomes, near one end in acrocentric chromosomes, and between the middle and end in submetacentric chromosomes. The kinetochore apparatus is a complex structure consisting of proteins that function at the molecular level to attach the chromosomes to the spindle fibers during cell division. Although the kinetochore is located in the region of the centromere, it should not be confused with the centromere. The latter is the DNA at the site of the spindle-fiber attachment.

The Nucleolar Organizer Regions

The satellite stalks of human acrocentric chromosomes contain the nucleolar organizer regions (NORs), so-called because this is where nucleoli form in interphase cells. NORs are also the site of ribosomal RNA genes and production of rRNA. In humans, there are theoretically ten nucleolar organizer regions, although all may not be active during any given cell cycle.

The Telomeres

The telomeres are the physical ends of chromosomes. Telomeres act as protective caps to chromosome ends, preventing end-to-end fusion of chromosomes and DNA degradation resulting after chromosome breakage. Nonhistone proteins complex with telomeric DNA to protect the ends of chromosomes from nucleases located within the cell [9]. The telomeric region also plays a role in synapsis during meiosis. Chromosome pairing appears to be initiated in the subtelomeric regions [10].

Telomeres contain tandem repeats of the nitrogenous base sequence TTAGGG over 3–20 kb at the chromosome ends [11]. At the very tip of the chromosome, the two strands do not end at the same point, resulting in a short G-rich tail that is single stranded. Because of this, DNA synthesis breaks down at the telomeres and telomeres replicate differently than other types of linear DNA. The enzyme telomerase synthesizes new copies of the telomere TTAGGG repeat using an RNA template that is a component of the telomerase enzyme. Telomerase also counteracts the progressive shortening of chromosomes that results from many cycles of normal DNA replication. Telomere length gradually decreases with the aging process and with increased numbers of cell divisions in culture. The progressive shortening of human telomeres appears to be a tumor-suppressor mechanism [12]. The maintenance of telomeric DNA permits the binding of telomeric proteins that form the protective cap at chromosome ends and regulate telomere length [12]. Cells that have defective or unstable telomerase will exhibit shortening of chromosomes, leading to chromosome instability and cell death.

Types of DNA

DNA is classified into three general categories: unique sequence, highly repetitive sequence DNA (>105 copies), and middle repetitive sequence DNA (102–104 copies). Unique sequence or single-copy DNA is the most common class of DNA, comprising about 75% of the human genome [13]. This DNA consists of nucleotide sequences that are represented only once in a haploid set. Genes that code for proteins are single-copy DNA. Repetitive or repeated sequence DNA makes up the remaining 25% of the genome and is classified according to the number of repeats and whether the repeats are tandem or interspersed among unique sequence DNA [13].

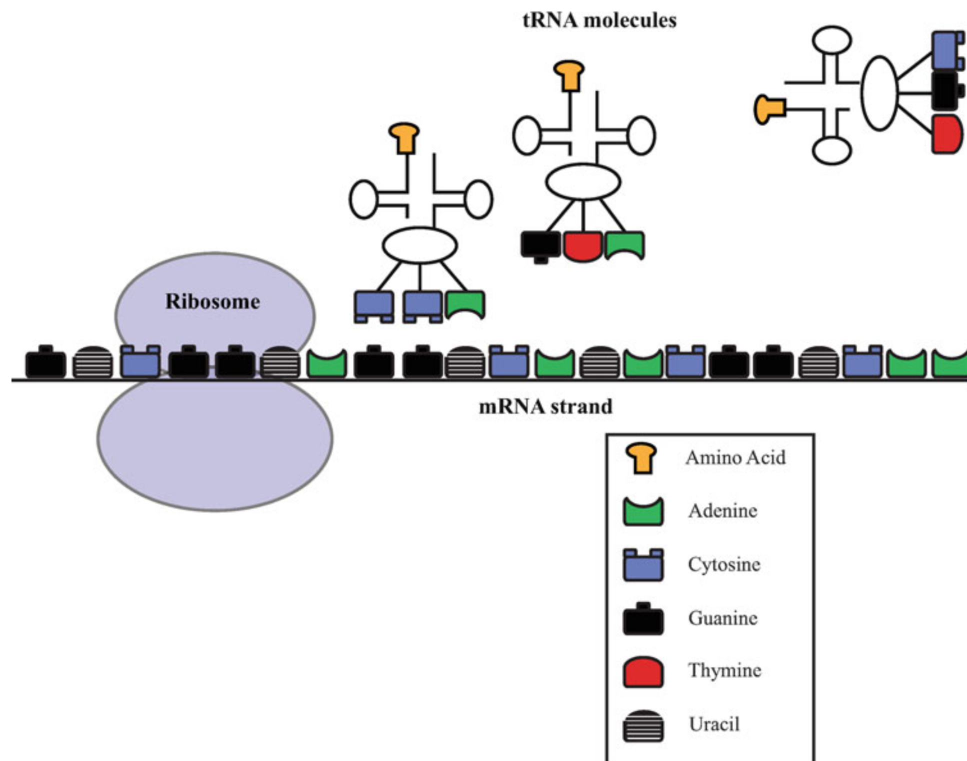

Fig. 2.5 Translation. Transfer RNA molecules bring in specific amino acids according to the triplet codon instructions of mRNA that are read at the ribosomes

Repetitive, tandemly arranged DNA was first discovered with a cesium chloride density gradient. Repetitive, tandem sequences were visualized as separate bands in the gradient. This DNA was termed satellite DNA [14]. Satellite DNA is categorized, based on the length of sequences that make up the tandem array and the total length of the array, as α (alpha)-satellite, minisatellite, and microsatellite DNA.

Alpha-satellite DNA is a repeat of a 171-base pair sequence organized in a tandem array of up to a million base pairs or more in total length. Alpha-satellite DNA is generally not transcribed and is located in the heterochromatin associated with the centromeres of chromosomes (see later). The size and number of repeats of satellite DNA is chromosome specific [15]. Although α-satellite DNA is associated with centromeres, its role in centromere function has not been determined. A centromeric protein, CENP-B, has been shown to bind to a 17-base pair portion of some α-satellite DNA, but the functional significance of this has not been determined [16].

Minisatellites have repeats that are 20–70 base pairs in length, with a total length of a few thousand base pairs. Microsatellites have repeat units of two, three, or four base pairs, and the total length is usually less than a few hundred base pairs. Minisatellites and microsatellites vary in length among individuals and, as such, are useful markers for gene mapping and identity testing.

The genes for 18S and 28S ribosomal RNAs are middle repetitive sequences. Several hundred copies of these genes are tandemly arranged on the short arms of the acrocentric chromosomes.

Dispersed repetitive DNA is classified as either short or long. The terms SINEs (short interspersed elements) and LINEs (long interspersed elements) were introduced by Singer [17]. SINEs range in size from 90 to 500 base pairs. One class of SINEs is the Alu sequence. Many Alu sequences are transcribed and are present in nuclear pre-mRNA and in some noncoding regions of mRNA. Alu sequences have high G-C content and are found predominantly in the Giemsa-light bands of chromosomes [18]. LINEs can be as large as 7,000 bases. The predominant member of the LINE family is a sequence called L1. L1 sequences have high A-T content and are predominantly found in the Giemsa-dark bands of chromosomes [17]. See Chaps. 3 and 4.

Chromatin

There are two fundamental types of chromatin in eukaryotic cells: euchromatin and heterochromatin. Euchromatin is loosely organized, extended, and uncoiled. This chromatin contains active, early replicating genes, and stains lightly with GTG-banding techniques (see Chap. 4).

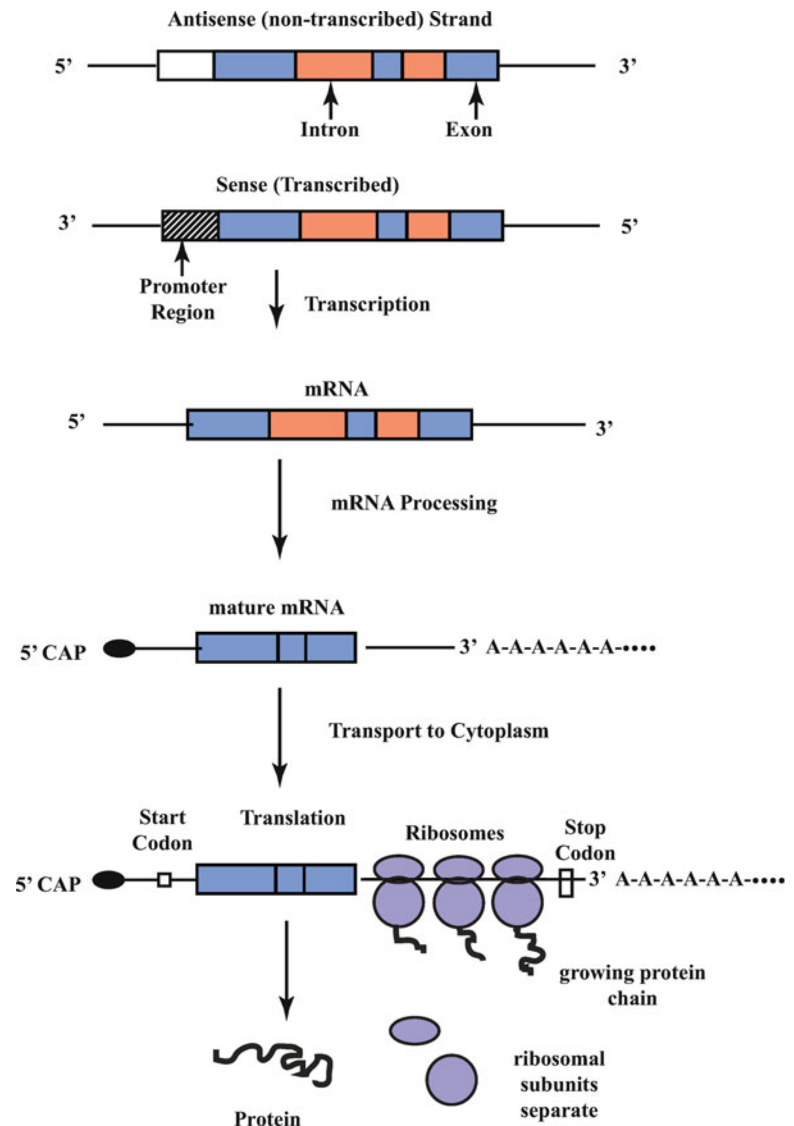

Fig. 2.6 Overview of protein synthesis. DNA is transcribed to mRNA, which is modified to mature transcript and then transferred to the cytoplasm of the cell. The codons are read at the ribosomes and translated with the help of tRNA. The chain of amino acids produced during translation is joined by peptide bonds to form a protein molecule

There are two special types of heterochromatin that warrant special mention: facultative heterochromatin and constitutive heterochromatin. Both are genetically inactive, late replicating during the synthesis (S) phase of mitosis, and are highly contracted.

Constitutive Heterochromatin

Constitutive heterochromatin consists of simple repeats of nitrogenous bases that are generally located around the centromeres of all chromosomes and at the distal end of the Y chromosome. There are no transcribed genes located in constitutive heterochromatin, which explains the fact that variations in constitutive heterochromatic chromosome regions apparently have no effect on the phenotype. Chromosomes 1, 9, 16, and Y have variably sized constitutive heterochromatic regions. The heterochromatic regions of these chromosomes stain differentially with various special staining techniques, revealing that the DNA structure of these regions is not the same as the structure of the euchromatic regions on the same chromosomes. The only established function of constitutive heterochromatin is the regulation of crossing-over—the exchange of genes from one sister chromatid to the other during cell division [19].

Facultative Heterochromatin

One X chromosome of every female cell is randomly inactivated. The inactivated X is condensed during interphase and replicates late during the synthesis stage of the cell cycle. It is termed facultative heterochromatin. Because these regions are inactivated, it has been proposed that facultative heterochromatin regulates gene function [20].

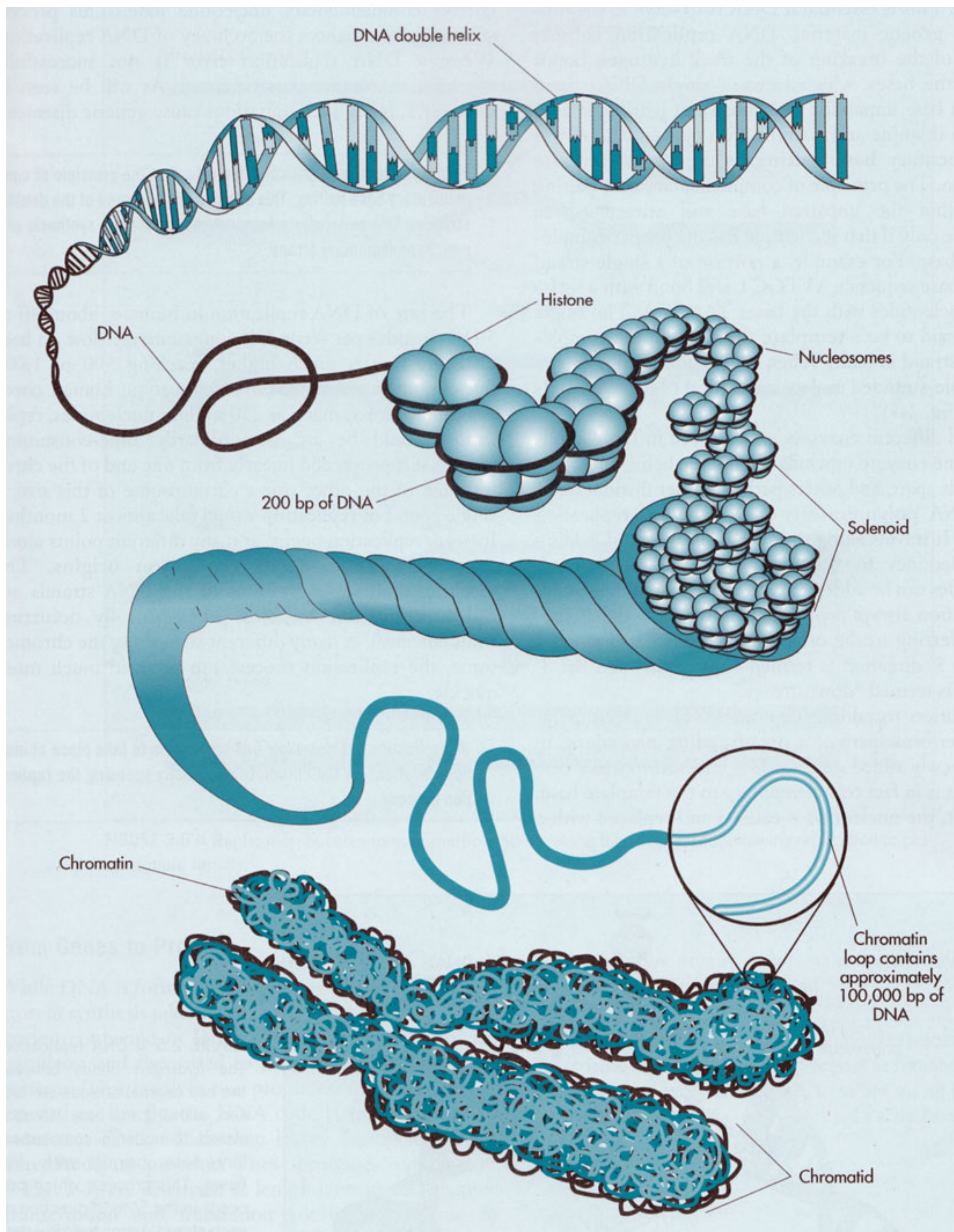

Fig. 2.7 The levels of DNA organization (Reprinted with permission from Jorde et al. [8])

Cell Division

An understanding of cell division is basic to an understanding of cytogenetics. Dividing cells are needed in order to study chromosomes using traditional cytogenetic techniques, and many cytogenetic abnormalities result from errors in cell division.

There are two types of cell division: mitosis and meiosis. Mitosis is the division of somatic cells, whereas meiosis is a special type of division that occurs only in gametic cells.

The Cell Cycle

The average mammalian cell cycle lasts about 17–18 h and is the transition of a cell from one interphase through cell division and back to interphase [21]. The cell cycle is divided into four major stages. The first three stages, gap 1 (G1), synthesis (S), and gap 2 (G2), comprise interphase. The fourth and final stage of the cell cycle is mitosis (M) (Fig. 2.9).

The first stage, G1, is the longest and typically lasts about 9 h [21]. Chromosomes exist as single chromatids during this

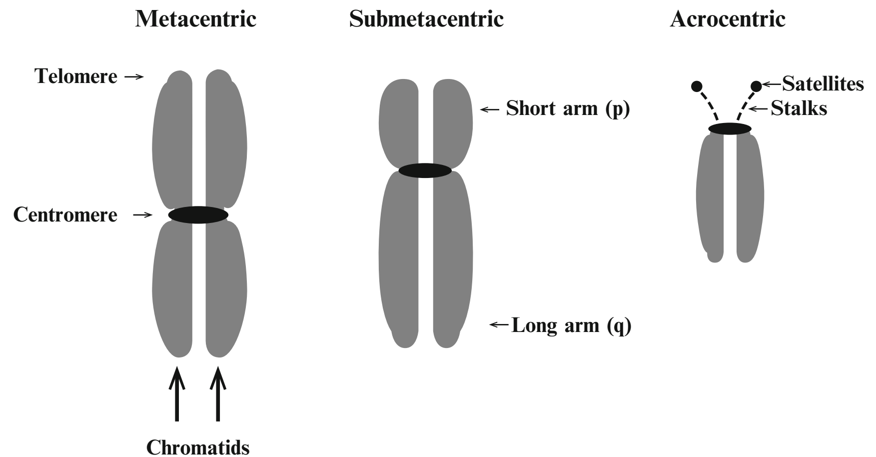

Fig. 2.8 The functional and structural components of metaphase chromosomes

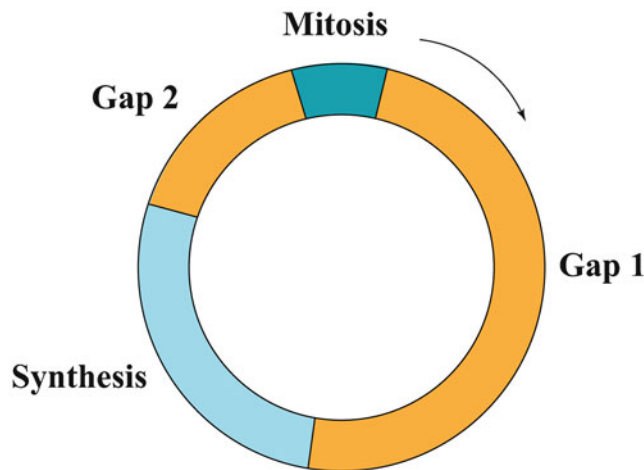

Fig. 2.9 The cell cycle: gap 1, synthesis, gap 2, and mitosis

stage. Cells are metabolically active during G1, and this is when protein synthesis takes place. A cell might be permanently arrested at this stage if it does not undergo further division. This arrested phase is referred to as gap zero (G0).

Gap 1 is followed by the synthesis phase, which lasts about 5 h in mammalian cells [21]. This is when DNA synthesis occurs. The DNA replicates itself, and the chromosomes then consist of two identical sister chromatids.

Some DNA replicates early in S phase, and some replicates later. Early replicating DNA contains a higher portion of active genes than late-replicating DNA. By standard G-banding techniques, the light-staining bands usually replicate early, whereas the dark-staining bands and the inactive X chromosome in females replicate late in the S phase.

Gap 2 lasts about 3 h [21]. During this phase, the cell prepares to undergo cell division. The completion of G2 represents the end of interphase.

The final step in the cell cycle is mitosis. This stage lasts only 1–2 h in most mammalian cells. Mitosis is the process by which cells reproduce themselves, creating two daughter cells that are genetically identical to one another and to the original parent cell. Mitosis is itself divided into stages (Fig. 2.10).

Mitosis

Prophase

Chromosomes are at their greatest elongation and are not visible as discrete structures under the light microscope during interphase. During prophase, chromosomes begin to coil, become more condensed, and begin to become visible as discrete structures. Nucleoli are visible early in prophase but disappear as the stage progresses.

Prometaphase

Prometaphase is a short period between prophase and metaphase during which the nuclear membrane disappears and the spindle fibers begin to appear. Chromosomes attach to the spindle fibers at their kinetochores.

Metaphase

During metaphase, the mitotic spindle is completed, the centrioles divide and move to opposite poles, and the chromosomes line up on the equatorial plate. Chromosomes reach their maximum state of contraction during this phase. It is metaphase chromosomes that are traditionally studied in cytogenetics.

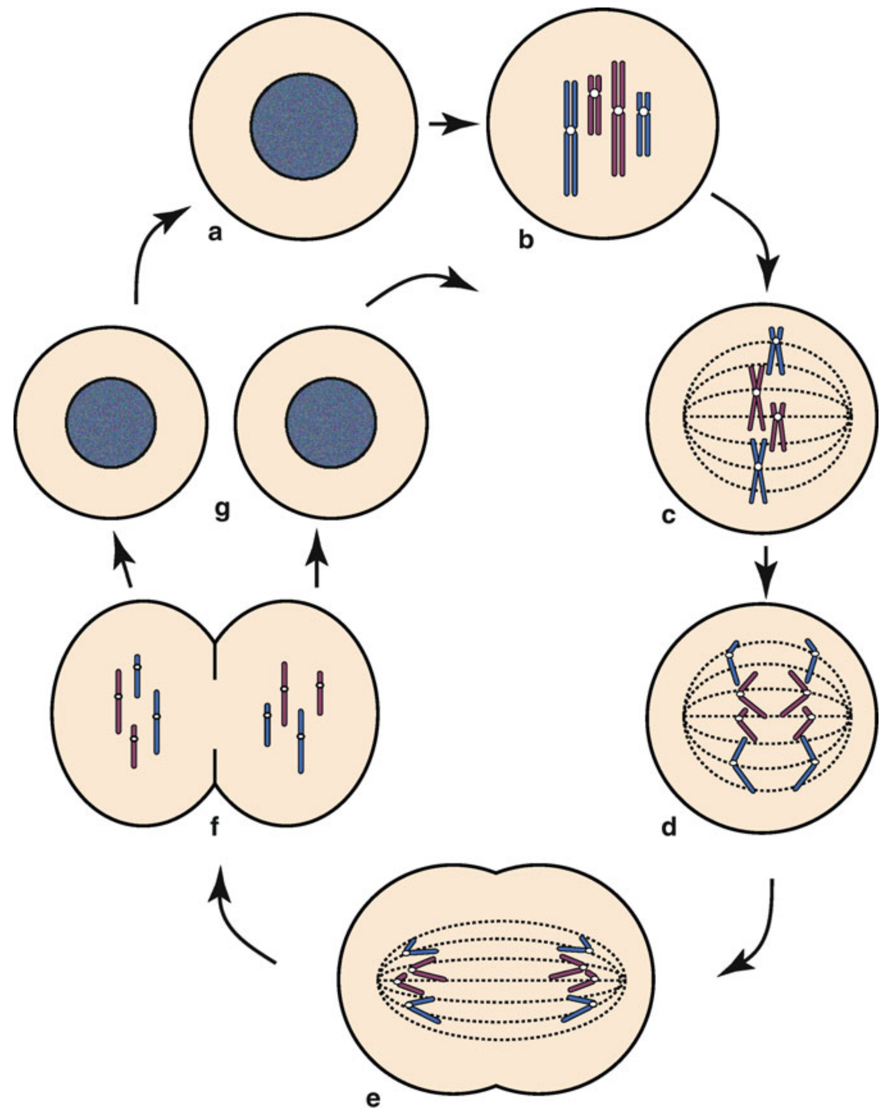

Fig. 2.10 Mitosis. Schematic representation of two pairs of chromosomes undergoing cell division: (**a**) interphase, (**b**) prophase, (**c**) metaphase, (**d**) anaphase, (**e**) telophase, (**f**) cytokinesis, and (**g**) interphase of the next cell cycle

Anaphase

Centromeres divide longitudinally and the chromatids separate during this stage. Sister chromatids migrate to opposite poles as anaphase progresses.

Telophase

The final stage of mitosis is telophase. The chromosomes uncoil and become indistinguishable again, the nucleoli reform, and the nuclear membrane is reconstructed. Telophase is usually followed by cytokinesis, or cytoplasmic division. Barring errors in DNA synthesis or cell division, the products of mitosis are two genetically identical daughter cells, each of which contains the complete set of genetic material that was present in the parent cell. The two daughter cells enter interphase, and the cycle is repeated.

Meiosis

Meiosis takes place only in the ovaries and testes. A process involving one duplication of the DNA and two cell divisions (meiosis I and meiosis II) reduces the number of chromosomes from the diploid number ($2n=46$) to the haploid number ($n=23$). Each gamete produced contains only one copy of each chromosome. Fertilization restores the diploid number in the zygote.

Meiosis I

Meiosis I is comprised of several substages: prophase I, metaphase I, anaphase I, and telophase I (Fig. 2.11).

Prophase I

Prophase I is a complex stage that is further subdivided as follows.

Leptotene

In leptotene, there are 46 chromosomes, each comprised of two chromatids. The chromosomes begin to condense but are not yet visible by light microscopy. Once leptotene takes place, the cell is committed to meiosis.

Zygotene

Zygotene follows leptotene. Homologous chromosomes, which in zygotene appear as long thread-like structures, pair locus for locus. This pairing is called synapsis. A tripartite structure, the synaptonemal complex, can be seen with electron microscopy. The synaptonemal complex is necessary for the phenomenon of crossing-over that will take place later in prophase I.

Synapsis of the X and Y chromosomes in males occurs only at the pseudoautosomal regions. These regions are located at the distal short arms and are the only segments of the X and Y chromosomes containing homologous loci. The nonhomologous portions of these chromosomes condense to form the sex vesicle.

Pachytene

Synapsis is complete during pachytene. Chromosomes continue to condense and now appear as thicker threads. The paired homologs form structures called bivalents, sometimes referred to as tetrads because they are composed of four chromatids.

The phenomenon of crossing over takes place during pachytene. Homologous or like segments of DNA are exchanged between nonsister chromatids of the bivalents. The result of crossing over is a reshuffling or recombination of genetic material between homologs, creating new combinations of genes in the daughter cells.

Diplotene

In diplotene, chromosomes continue to shorten and thicken, and the homologous chromosomes begin to repel each other. This repulsion continues until the homologous chromosomes are held together only at points where crossing-over took place. These points are referred to as chiasmata. In males, the sex vesicle disappears, and the X and Y chromosomes associate end to end.

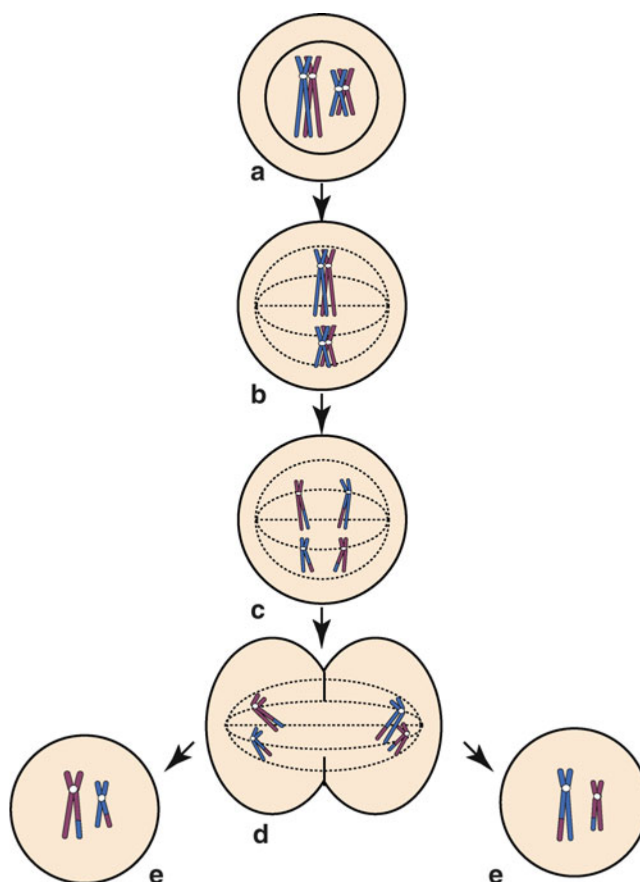

Fig. 2.11 Schematic representation of two chromosome pairs undergoing meiosis I: (**a**) prophase I, (**b**) metaphase I, (**c**) anaphase I, (**d**) telophase I, and (**e**) products of meiosis I

Diakinesis

Chromosomes reach their greatest contraction during this last stage of prophase.

Metaphase I

Metaphase I is characterized by disappearance of the nuclear membrane and formation of the meiotic spindle. The bivalents line up on the equatorial plate with their centromeres randomly oriented toward opposite poles.

Anaphase I

During anaphase I, the centromeres of each bivalent separate and migrate to opposite poles.

Telophase I

In telophase, the two haploid sets of chromosomes reach opposite poles, and the cytoplasm divides. The result is two cells containing 23 chromosomes, each comprised of two chromatids.

Meiosis II

The cells move directly from telophase I to metaphase II with no intervening interphase or prophase. Meiosis II proceeds much like mitotic cell division except that each cell contains only 23 chromosomes (Fig. 2.12).

The 23 chromosomes line up on the equatorial plate in metaphase II, the chromatids separate and move to opposite poles in anaphase II, and cytokinesis occurs in telophase II. The net result is four cells, each of which contains 23 chromosomes, each consisting of a single chromatid. Owing to the effects crossing-over and random assortment of homologs, each of the new cells differs genetically from one another and from the original cell.

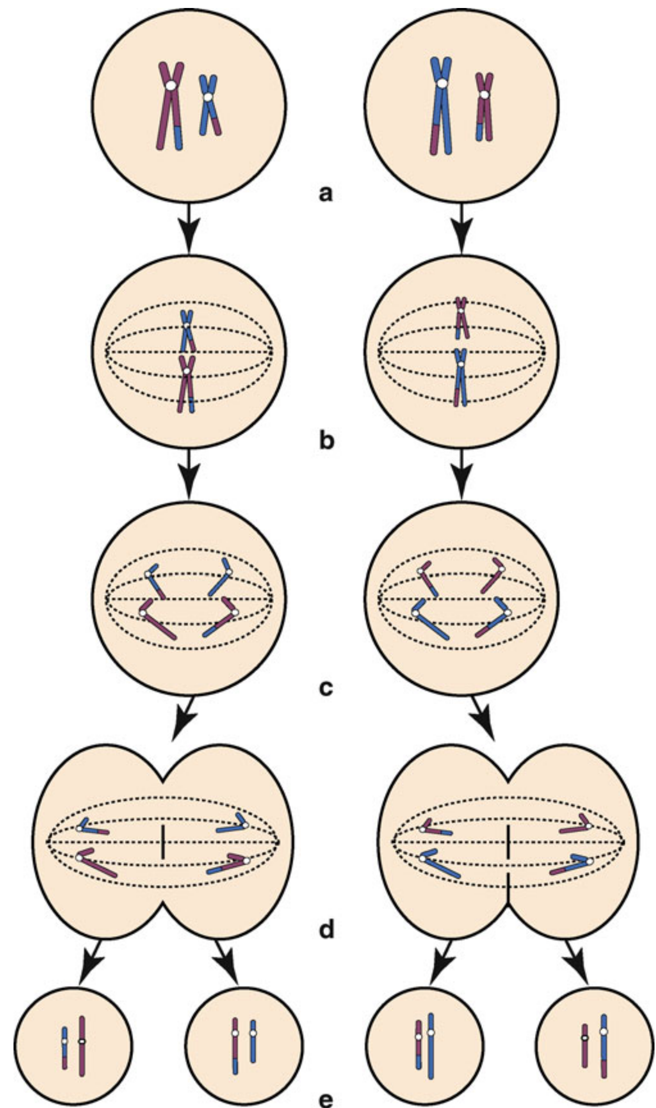

Fig. 2.12 Schematic representation of two chromosome pairs undergoing meiosis II: (**a**) products of meiosis I, (**b**) metaphase II, (**c**) anaphase II, (**d**) telophase II, and (**e**) products of meiosis

Spermatogenesis and Oögenesis

The steps of spermatogenesis and oögenesis are the same in human males and females; however, the timing is very different (Fig. 2.13).

Spermatogenesis

Spermatogenesis takes place in the seminiferous tubules of the male testes. The process is continuous and each meiotic cycle of a primary spermatocyte results in the formation of four nonidentical spermatozoa. Spermatogenesis begins with sexual maturity and occurs throughout the postpubertal life of a man.

The spermatogonia contain 46 chromosomes. Through mitotic cell division, they give rise to primary spermatocytes. The primary spermatocytes enter meiosis I and give rise to the secondary spermatocytes, which contain 23 chromosomes, each consisting of two chromatids. The secondary spermatocytes undergo meiosis II and give rise to spermatids. Spermatids contain 23 chromosomes, each consisting of a single chromatid. The spermatids differentiate to become spermatozoa, or mature sperm.

Oögenesis

Oögenesis in human females begins in prenatal life. Ova develop from oögonia within the follicles in the ovarian cortex. At about the third month of fetal development, the oögonia, through mitotic cell division, begin to develop into diploid primary oöcytes. Meiosis I continues to diplotene, where it is arrested until sometime in the postpubertal reproductive life of a woman. This suspended diplotene is referred to as dictyotene.

Subsequent to puberty, several follicles begin to mature with each menstrual cycle. Meiosis I rapidly proceeds with an uneven distribution of the cytoplasm in cytokinesis of meiosis I, resulting in a secondary oöcyte containing most of the cytoplasm, and a first polar body. The secondary oöcyte, which has been ovulated, begins meiosis II. Meiosis II continues only if fertilization takes place. The completion of meiosis II results in a haploid ovum and a second polar body. The first polar body might undergo meiosis II, or it might degenerate. Only one of the potential four gametes produced each menstrual cycle is theoretically viable.

Fertilization

The chromosomes of the egg and sperm produced in meiosis II are each surrounded by a nuclear membrane within the cytoplasm of the ovum and are referred to as pronuclei. The male and female pronuclei fuse to form the diploid nucleus of the zygote, and the first mitotic division begins.