ART TK

chapter 23

Experimental Systems

ART TK

Addition of Expression Profile of Diffuse Large B-cell Lymphoma.

Experimental systems of various types are used to unravel the complex cellular interactions of the immune response. In vivo systems, which involve the whole animal, provide the most natural experimental conditions. However, in vivo systems have a myriad of unknown and uncontrollable cellular interactions that add ambiguity to the interpretation of data. At the other extreme are in vitro systems, in which defined populations of lymphocytes are studied under controlled and consequently repeatable conditions; in vitro systems can be simplified to the extent that individual cellular interactions can be studied effectively. Yet they have their own limitations, the most notable of which is their artificiality. For example, providing antigen to purified B cells in vitro does not stimulate maximal antibody production unless T cells are present. Therefore a study of antibody production in an artificial in vitro system that lacks T cells could lead to the incorrect conclusion that B cells do not synthesize high levels of antibodies. One must ask whether a cellular response observed in vitro reflects reality or is a product of the unique conditions of the in vitro system itself.

This chapter describes some of the experimental systems routinely used to study the immune system. It also covers some recombinant DNA techniques that have revolutionized the study of the immune system in the past decade or so. Other chapters also cover experimental systems and techniques in detail. Table 23-1 lists them and directs the reader to the appropriate location for a description.

Experimental Animal Models

The study of the immune system in vertebrates requires suitable animal models. The choice of an animal depends on its suitability for attaining a particular research goal. If large amounts of antiserum are sought, a rabbit, goat, sheep, or horse might be an appropriate experimental animal. If the goal is development of a protective vaccine, the animal chosen must be susceptible to the infectious agent so that the efficacy of the vaccine can be assessed. Mice or rabbits can be used for vaccine development if they are susceptible to the pathogen. But if growth of the infectious agent is limited to humans and primates, vaccine development may require the use of monkeys, chimpanzees, or baboons.

For most basic research in immunology, mice have been the experimental animal of choice. They are easy to handle, are genetically well characterized, and have a rapid breeding cycle. The immune system of the mouse has been characterized more extensively than that of any other species. The value of basic research in the mouse system is highlighted by the enormous impact this research has had on clinical intervention in human disease.

Inbred Strains Can Reduce Experimental Variation

To control experimental variation caused by differences in the genetic backgrounds of experimental animals, immunologists often work with inbred strains—that is, genetically identical animals produced by inbreeding. The rapid breeding cycle of mice makes them particularly well suited for the production of inbred strains, which are developed by repeated inbreeding between brother and sister littermates. In this way the heterozygosity of alleles that is normally found in randomly outbred mice is replaced by homozygosity at all

TABLE 23-1 Immunological methods described in other chapters

Method	Location
Bone-marrow transplantation	Ch. 2 Clinical Focus
Preparation of immunotoxins	Fig. 4-22
Genetic engineering of chimeric mouse-human monoclonal antibodies	Fig. 5-20 and Ch 5 Clinical Focus
Determination of antibody affinity by equilibrium dialysis	Fig. 6.2
Precipitation reactions	Fig. 6.4
Immunodiffusion and immunoelectrophoresis	Figs. 6.5 and 6.6
Hemagglutination	Fig. 6.7
Radioimmunoassay (RIA)	Fig. 6.9
ELISA assays	Fig. 6.10
ELISPOT assay	Fig. 6.11
Western blotting	Fig. 6.12
Immunoprecipitation	Fig. 6.13
Immunofluorescence	Fig. 6.14
Flow cytometry	Fig. 6.15
Production of congenic mice	Fig. 7-3
Mixed lymphocyte reaction (MLR)	Fig. 14-16
Cell-mediated lympholysis (CML)	Fig. 14-17
Production of vaccinia vector vaccine	Fig. 18-5
Production of multivalent subunit vaccines	Fig. 18-7
HLA typing	Fig. 21-4

loci. Repeated inbreeding for 20 generations usually yields an inbred strain whose progeny are homozygous at more than 98% of all loci. More than 150 different inbred strains of mice are available, each designated by a series of letters and/or numbers (Table 23-2). Most strains can be purchased by immunologists from such suppliers as the Jackson Laboratory in Bar Harbor, Maine. Inbred strains have also been produced in rats, guinea pigs, hamsters, rabbits, and domestic fowl. Because inbred strains of animals are genetically identical **(syngeneic)** within that strain, their immune responses can be studied in the absence of variables introduced by individual genetic differences—an invaluable property. With inbred strains, lymphocyte subpopulations isolated from one animal can be injected into another animal of the same strain without eliciting a rejection reaction. This type of experimental system permitted immunologists to first demonstrate that lymphocytes from an antigen-primed animal could transfer immunity to an unprimed syngeneic recipient.

Adoptive-Transfer Systems Permit the in Vivo Examination of Isolated Cell Populations

In some experiments, it is important to eliminate the immune responsiveness of the syngeneic host so that the response of only the transferred lymphocytes can be studied in isolation. This can be accomplished by a technique called **adoptive transfer:** first, the syngeneic host is exposed to x-rays that kill its lymphocytes; then the donor immune cells are introduced. Subjecting a mouse to high doses of x-rays (650–750 rads) can kill 99.99% of its lymphocytes, after which the activities of lymphocytes transplanted from the spleen of a syngeneic donor can be studied without interference from host lymphocytes. If the host's hematopoietic cells might influence an adoptive-transfer experiment, then higher x-ray levels (900–1000 rads) are used to eliminate the entire hematopoietic system. Mice irradiated with such doses will die unless reconstituted with bone marrow from a syngeneic donor.

The adoptive-transfer system has enabled immunologists to study the development of injected lymphoid stem cells in various organs of the recipient, and have facilitated the study of various populations of lymphocytes and of the cellular interactions required to generate an immune response. Such experiments, for instance, first enabled immunologists to show that a T helper cell is necessary for B-cell activation in the humoral response. In these experiments, adoptive transfer of purified B cells or purified T cells did not produce antibody in the irradiated host. Only when both cell populations were transferred was antibody produced in response to antigen.

SCID Mice and SCID-Human Mice Are a Valuable Animal Model for Immunodeficiency

An autosomal recessive mutation resulting in **severe combined immunodeficiency disease (SCID)** developed spontaneously in a strain of mice called CB-17. These CB-17 SCID mice fail to develop mature T and B cells and consequently are severely compromised immunologically. This defect is due to a failure in V(D)J recombination. SCID mice must be housed in a sterile (germ-free) environment, because they cannot fight off microorganisms of even low pathogenicity. The absence of functional T and B cells enables these mice to accept foreign cells and grafts from other strains of mice or even from other species.

Apart from their lack of functional T and B cells, SCID mice appear to be normal in all respects. When normal bone-marrow cells are injected into SCID mice, normal T and B cells develop, and the mice are cured of their immunodeficiency. This finding has made SCID mice a valuable model system for the study of immunodeficiency and the process of differentiation of bone-marrow stem cells into mature T or B cells.

Interest in SCID mice mushroomed when it was found that they could be used to study the human immune system. In this system, portions of human fetal liver, adult thymus,

TABLE **23-2** Some inbred mouse strains commonly used in immunology

Strain	Common substrains	Characteristics
A	A/He	High incidence of mammary tumors in some substrains
	A/J	
	A/WySn	
AKR	AKR/J	High incidence of leukemia
	AKR/N	
	AKR/Cum	*Thy 1.2* allele in AKR/Cum, and *Thy 1.1* allele in other substrains (*Thy* gene encodes a T-cell surface protein)
BALB/c	BALB/c*j*	Sensitivity to radiation
	BALB/c AnN	Used in hybridoma technology
	BALB/cBy	Many myeloma cell lines were generated in these mice
CBA	CBA/J	Gene (*rd*) causing retinal degeneration in CBA/J
	CBA/H	
	CBA/N	Gene (*xid*) causing X-linked immunodeficiency in CBA/N
C3H	C3H/He	Gene (*rd*) causing retinal degeneration
	C3H/HeJ	High incidence of mammary tumors in many substrains (these carry a mammary-tumor virus that is passed via maternal milk to offspring)
	C3H/HeN	
C57BL/6	C57BL/6J	High incidence of hepatomas after irradiation
	C57BL/6By	High complement activity
	C57BL/6N	
C57BL/10	C57BL/10J	Very close relationship to C57BL/6 but differences in at least two loci
	C57BL/10ScSn	
	C57BL/10N	Frequent partner in preparation of congenic mice
C57BR	C57BR/cd*j*	High frequency of pituitary and liver tumors Very resistant to x-irradiation
C57L	C57L/J	Susceptibility to experimental autoimmune encephalomyelitis (EAE)
	C57L/N	High frequency of pituitary and reticular cell tumors
C58	C58/J	High incidence of leukemia
	C58/LwN	
DBA/1	DBA/1J	High incidence of mammary tumors
	DBA/1N	
DBA/2	DBA/2J	Low immune response to some antigens
	DBA/2N	Low response to pneumococcal polysaccharide type III
HRS	HRS/J	Hairless (*hr*) gene, usually in heterozygous state
NZB	NZB/BINJ	High incidence of autoimmune hemolytic anemia and lupus-like nephritis
	NZB/N	Autoimmune disease similar to systemic lupus erythematosus (SLE) in F_1 progeny from crosses with NZW
NZW	NZW/N	SLE-type autoimmune disease in F_1 progeny from crosses with NZB
P	P/J	High incidence of leukemia
SJL	SJL/J	High level of aggression and severe fighting to the point of death, especially in males Tendency to develop certain autoimmune diseases, most susceptible to EAE
SWR	SWR/J	Tendency to develop several autoimmune diseases, especially EAE
129	129/J	High incidence of spontaneous teratocarcinoma
	129/SvJ	

SOURCE: Adapted from Federation of American Societies for Experimental Biology, 1979, *Biological Handbooks,* Vol. III: Inbred and Genetically Defined Strains of Laboratory Animals.

and adult lymph nodes are implanted into SCID mice (Figure 23-1). Because the mice lack mature T and B cells of their own, they do not reject the transplanted human tissue. The implanted human fetal liver contains immature lymphocytes (stem cells), which migrate to the implanted human tissues, where they mature into T and B cells, producing a **SCID-human mouse.** Because the human lymphocytes are exposed to mouse antigens while they are still immature, they later recognize mouse cells as self and do not mount an immunologic response against the mouse host.

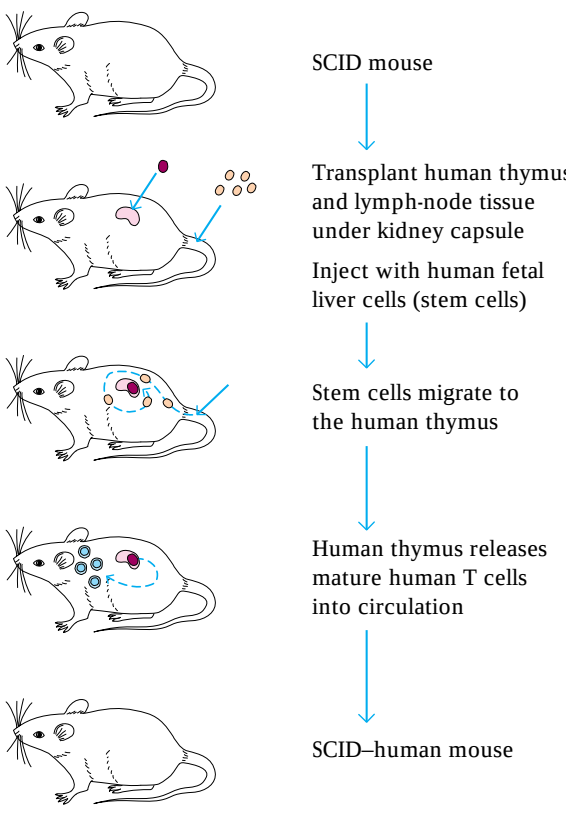


FIGURE 23-1 Production of SCID-human mouse. This system permits study of human lymphocytes within an animal model. In this example, human T-cells are transferred to SCID mouse, but B-cells also can be transferred by the use of bone-marrow precursors.

The beauty of the SCID-human mouse is that it enables one to study human lymphocytes within an animal model. This valuable system has proved useful in research on the development of various lymphoid cells and also as an important animal model in AIDS research, since mouse lymphocytes cannot be infected with HIV, whereas the lymphocytes of a SCID-human mouse are readily infected.

Cell-Culture Systems

The complexity of the cellular interactions that generate an immune response has led immunologists to rely heavily on various types of in vitro cell-culture systems. A variety of cells can be cultured, including primary lymphoid cells, cloned lymphoid cell lines, and hybrid cells.

Primary Lymphoid Cell Cultures

Primary lymphoid cell cultures can be obtained by isolating lymphocytes directly from blood or lymph or from various lymphoid organs by tissue dispersion. The lymphocytes can then be grown in a chemically defined basal medium (containing saline, sugars, amino acids, vitamins, trace elements, and other nutrients) to which various serum supplements are added. For some experiments, serum-free culture conditions are employed. Because in vitro culture techniques require from 10- to 100-fold fewer lymphocytes than do typical in vivo techniques, they have enabled immunologists to assess the functional properties of minor subpopulations of lymphocytes. It was by means of cell-culture techniques, for example, that immunologists were first able to define the functional differences between $CD4^+$ T helper cells and $CD8^+$ T cytotoxic cells.

Cell-culture techniques have also been used to identify numerous cytokines involved in the activation, growth, and differentiation of various cells involved in the immune response. Early experiments showed that media conditioned, or modified, by the growth of various lymphocytes or antigen-presenting cells would support the growth of other lymphoid cells. Conditioned media contain the secreted products from actively growing cells. Many of the individual cytokines that characterized various conditioned media have subsequently been identified and purified, and in many cases the genes that encode them have been cloned. These cytokines, which play a central role in the activation and regulation of the immune response, are described in Chapter 12 and elsewhere.

Cloned Lymphoid Cell Lines

A primary lymphoid cell culture comprises a heterogeneous group of cells that can be propagated only for a limited time. This heterogeneity can complicate the interpretation of experimental results. To avoid these problems, immunologists use cloned lymphoid cell lines and hybrid cells.

Normal mammalian cells generally have a finite life span in culture; that is, after a number of population doublings characteristic of the species and cell type, the cells stop dividing. In contrast, tumor cells or normal cells that have undergone **transformation** induced by chemical carcinogens or viruses can be propagated indefinitely in tissue culture; thus, they are said to be immortal. Such cells are referred to as **cell lines.**

The first cell line—the mouse fibroblast L cell—was derived in the 1940s from cultured mouse subcutaneous connective tissue by exposing the cultured cells to a chemical carcinogen, methylcholanthrene, over a 4-month period. In the 1950s, another important cell line, the HeLa cell, was derived by culturing human cervical cancer cells. Since these early studies, hundreds of cell lines have been established, each consisting of a population of genetically identical (syngeneic) cells that can be grown indefinitely in culture.

Table 23-3 lists some of the cell lines used in immunologic research and briefly describes their properties. Some were derived from spontaneously occurring tumors of lymphocytes, macrophages, or other accessory cells involved in the immune response. In other cases, the cell line was induced by transformation of normal lymphoid cells with viruses such as Abelson's murine leukemia virus (A-MLV), simian virus 40

TABLE 23-3 Cell lines commonly used in immunologic research

Cell line	Description
L-929	Mouse fibroblast cell line; often used in DNA transfection studies and to assay tumor necrosis factor (TNF)
SP2/0	Nonsecreting mouse myeloma; often used as a fusion partner for hybridoma secretion
P3X63-Ag8.653	Nonsecreting mouse myeloma; often used as a fusion partner for hybridoma secretion
MPC 11	Mouse IgG2b-secreting myeloma
P3X63-Ag8	Mouse IgG1-secreting myeloma
MOPC 315	Mouse IgA-secreting myeloma
J558	Mouse IgA-secreting myeloma
7OZ/3	Mouse pre–B-cell lymphoma; used to study early events in B-cell differentiation
BCL 1	Mouse B-cell leukemia lymphoma that expresses membrane IgM and IgD and can be activated with mitogen to secrete IgM
CTLL-2	Mouse T-cell line whose growth is dependent on IL-2; often used to assay IL-2 production
Jurkat	Human T-cell leukemia that secretes IL-2
DO11.10	Mouse T-cell hybridoma with specificity for ovalbumin
PU 5-1.8	Mouse monocyte-macrophage line
P338 D1	Mouse monocyte-macrophage line that secretes high levels of IL-1
WEHI 265.1	Mouse monocyte line
P815	Mouse mastocytoma cells; often used as target to assess killing by cytotoxic T lymphocytes (CTLs)
YAC-1	Mouse lymphoma cells; often used as target for NK cells
HL-60	Human myeloid-leukemia cell line
COS-1	African green monkey kidney cells transformed by SV40; often used in DNA transfection studies

(SV40), Epstein-Barr virus (EBV), or human T-cell leukemia virus type 1(HTLV-1).

Lymphoid cell lines differ from primary lymphoid cell cultures in several important ways: They survive indefinitely in tissue culture, show various abnormal growth properties, and often have an abnormal number of chromosomes. Cells with more or less than the normal diploid number of chromosomes for a species are said to be aneuploid. The big advantage of cloned lymphoid cell lines is that they can be grown for extended periods in tissue culture, enabling immunologists to obtain large numbers of homogeneous cells in culture.

Until the late 1970s, immunologists did not succeed in maintaining normal T cells in tissue culture for extended periods. In 1978, a serendipitous finding led to the observation that conditioned medium containing a T-cell growth factor was required. The essential component of the conditioned medium turned out to be interleukin 2 (IL-2). By culturing normal T lymphocytes with antigen in the presence of IL-2, clones of antigen-specific T lymphocytes could be isolated. These individual clones could be propagated and studied in culture and even frozen for storage. After thawing, the clones continued to grow and express their original antigen-specific functions.

Development of cloned lymphoid cell lines has enabled immunologists to study a number of events that previously could not be examined. For example, research on the molecular events involved in activation of naive lymphocytes by antigen was hampered by the low frequency of naive B and T cells specific for a particular antigen; in a heterogeneous population of lymphocytes, the molecular changes occurring in one responding cell could not be detected against a background of 10^3–10^6 nonresponding cells. Cloned T- and B-cell lines with known antigenic specificity have provided immunologists with large homogeneous cell populations in which to study the events involved in antigen recognition. Similarly, the genetic changes corresponding to different maturational stages can be studied in cell lines that appear to be "frozen" at different stages of differentiation. Cell lines have also been useful in studying the soluble factors produced by lymphoid cells. Some cell lines secrete large quantities of various cytokines; other lines express membrane receptors for particular cytokines. These cell lines have been used by immunologists to purify various cytokines and their receptors and eventually to clone their genes.

With the advantages of lymphoid cell lines come a number of limitations. Variants arise spontaneously in the course of prolonged culture, necessitating frequent subcloning to limit the cellular heterogeneity that can develop. If variants are selected in subcloning, it is possible that two subclones derived from the same parent clone may represent different subpopulations. Moreover, any cell line derived from tumor cells or transformed cells may have unknown genetic contributions characteristic of the tumor or of the transformed state; thus, researchers must be cautious when extrapolating results obtained with cell lines to the normal situation in vivo. Nevertheless, transformed cell lines have made a major contribution to the study of the immune response, and many molecular events discovered in experiments with transformed cell lines have been shown to take place in normal lymphocytes.

Hybrid Lymphoid Cell Lines

In somatic-cell hybridization, immunologists fuse normal B or T lymphocytes with tumor cells, obtaining hybrid cells, or

heterokaryons, containing nuclei from both parent cells. Random loss of some chromosomes and subsequent cell proliferation yield a clone of cells that contain a single nucleus with chromosomes from each of the fused cells; such a clone is called a **hybridoma.**

Historically, cell fusion was promoted with Sendai virus, but now it is generally done with polyethylene glycol. Normal antigen-primed B cells can be fused with cancerous plasma cells, called **myeloma cells** (Figure 23-2). The hybridoma thus formed continues to express the antibody genes of the normal B lymphocyte but is capable of unlimited growth, a characteristic of the myeloma cell. B-cell hybridomas that secrete antibody with a single antigenic specificity, called monoclonal antibody, in reference to its derivation from a single clone, have revolutionized not only immunology but biomedical research as well as the clinical laboratory. Chapter 4 describes the production and uses of monoclonal antibodies in detail (see Figures 4-21).

T-cell hybridomas can also be obtained by fusing T lymphocytes with cancerous T-cell lymphomas. Again, the resulting hybridoma continues to express the genes of the normal T cell but acquires the immortal-growth properties of the cancerous T lymphoma cell. Immunologists have generated a number of stable hybridoma cell lines representing T-helper and T-cytotoxic lineages.

Protein Biochemistry

The structures and functions of many important molecules of the immune system have been determined with the techniques of protein biochemistry, and many of these techniques are in constant service in experimental immunology. For example, fluorescent and radioactive labels allow immunologists to localize and visualize molecular activities, and the ability to determine such biochemical characteristics of a protein as its size, shape, and three-dimensional structure has provided essential information for understanding the functions of immunologically important molecules.

Radiolabeling Techniques Allow Sensitive Detection of Antigens or Antibodies

Radioactive labels on antigen or antibody are extremely sensitive markers for detection and quantification. There are a number of ways to introduce radioactive isotopes into proteins or peptides. For example, tyrosine residues may be labeled with radioiodine by chemical or enzymatic procedures. These reactions attach an iodine atom to the phenol ring of the tyrosine molecule. One of the enzymatic iodination techniques, which uses lactoperoxidase, can label proteins on the plasma membrane of a live cell without labeling proteins in the cytoplasm, allowing the study of cell-surface proteins without isolating them from other cell constituents.

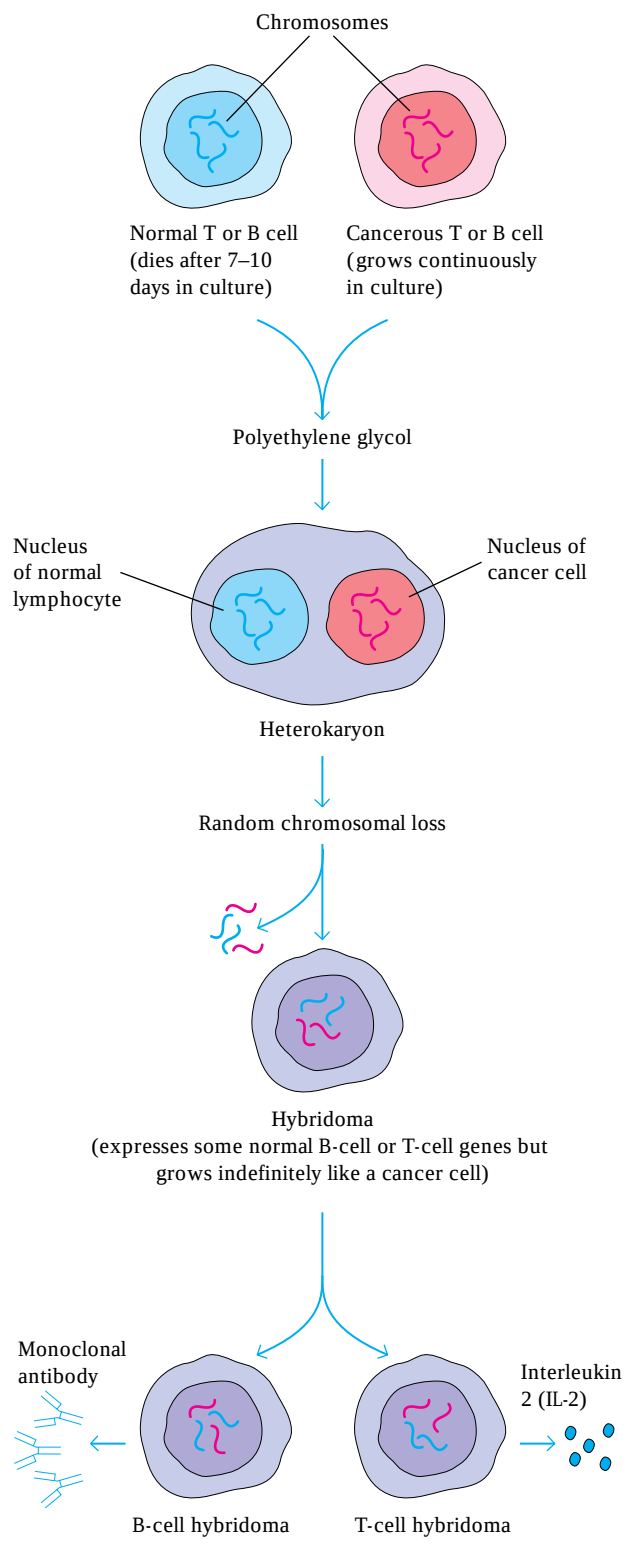


FIGURE **23-2** Production of B-cell and T-cell hybridomas by somatic-cell hybridization. The resulting hybridomas express some of the genes of the original normal B or T cell but also exhibit the immortal-growth properties of the tumor cell. This procedure is used to produce B-cell hybridomas that secrete monoclonal antibody and T-cell hybridomas that secrete various growth factors.

TABLE 23-4 Radioisotopes commonly used in immunology laboratories

Isotope	Half-life	Radiation type*	Autoradiography†
^{125}I	60.0 da	γ	+
^{131}I	6.8 da	γ	+
^{51}Cr	27.8 da	γ	–
^{32}P	14.3 da	β	+
^{35}S	87.4 da	β	+
^{14}C	57.30 yrs	β	+
^{3}H	12.35 yrs	β	–

* γ (gamma) radiation may be detected in a solid scintillation counter. β (beta) radiation is detected in a liquid scintillation counter by its ability to convert energy to photons of light in a solution containing phosphorescent compounds.

† Radiation may also be detected by exposure to x-ray film. ^{35}S and ^{14}C must be placed in direct contact with film for detection. ^{3}H cannot be detected by normal autoradiographic techniques.

A general radiolabeling of cell proteins may be carried out by growing the cells in a medium that contains one or more radiolabeled amino acids. The amino acids selected for this application are those most resistant to metabolic modification during cell growth so that the radioactive label will appear in the cell protein rather than in all cell constituents. Leucine marked with ^{14}C or ^{3}H, and cysteine or methionine labeled with ^{35}S, are the most commonly used amino acids for metabolic labeling of proteins. Table 23-4 lists some properties of the radioisotopes used in immunologic research.

Biotin Labels Facilitate Detection of Small Amounts of Proteins

In some instances direct labeling of proteins, especially with enzymes or other large molecules, as described in Chapter 6, may cause denaturation and loss of activity. A convenient labeling system has been developed which may be used in conjunction with the ELISA and ELISPOT assays described in Chapter 6. This labeling technique exploits the high affinity of the reaction between the vitamin biotin and avidin, a large molecule that may be labeled with radioactive isotopes, with fluorescent molecules, or with enzymes. Biotin is a small molecule (mol. wt. 244) that can be coupled to an antibody (or to any protein molecule) by a gentle chemical reaction that causes no loss of antibody activity. After the biotin-coupled antibody has reacted in the assay system, the labeled avidin is introduced and binding is measured by detecting the label on the avidin molecule (Figure 23-3). The reaction between biotin and avidin is highly specific and of such high affinity that the bond between the two molecules under most assay conditions is virtually irreversible.

Gel Electrophoresis Separates Proteins by Size and Charge

When subjected to an electric field in an electrophoresis chamber, a charged molecule will move toward the oppositely charged electrode. The rate at which a charged molecule moves in a stable field (its electrophoretic mobility) depends upon two factors specific to the molecule: one is the sign and magnitude of its net electrical charge, and the other is its size and shape. All other factors being equal, if molecules are of equal size the one with higher net charge will move faster in an applied electrical field due to the molecular seiving properties of the solid medium. It also follows that small molecules will move faster than large ones of the same net charge. Although there are exceptions in which the shape of a molecule may increase or decrease its frictional drag and cause atypical migration behavior, these general principles underlie all electrophoretic separations.

Most electrophoretic separations are not conducted in free solution but rather in a stable supporting medium, such as a gel. The most popular in reseach laboratories is a polymerized and crosslinked form of acrylamide. Separation on polyacrylamide gels, commonly referred to as *poly*acrylamide

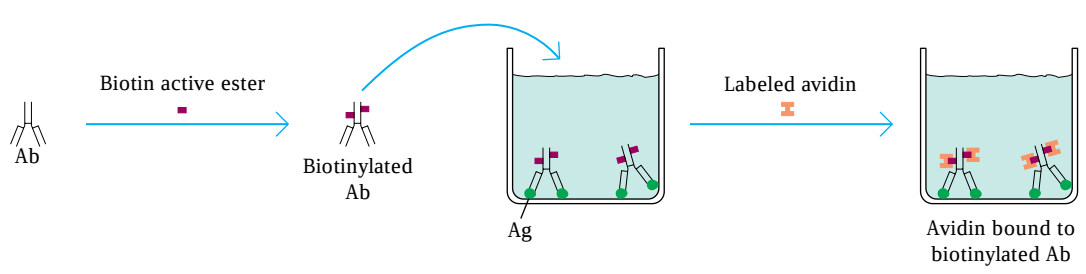


FIGURE 23-3 Labeling of antibody with biotin. An antibody preparation is mixed with a biotin ester, which reacts with the antibody. The biotin-labeled antibody can be used to detect antigens on a solid substrate such as the well of a microtiter plate. After washing away unbound antibody, the bound antibody can be detected with labeled avidin. The avidin can be radioactively labeled or linked to an enzyme that catalyzes a color reaction, as in ELISA procedures (see Figure 6-10).

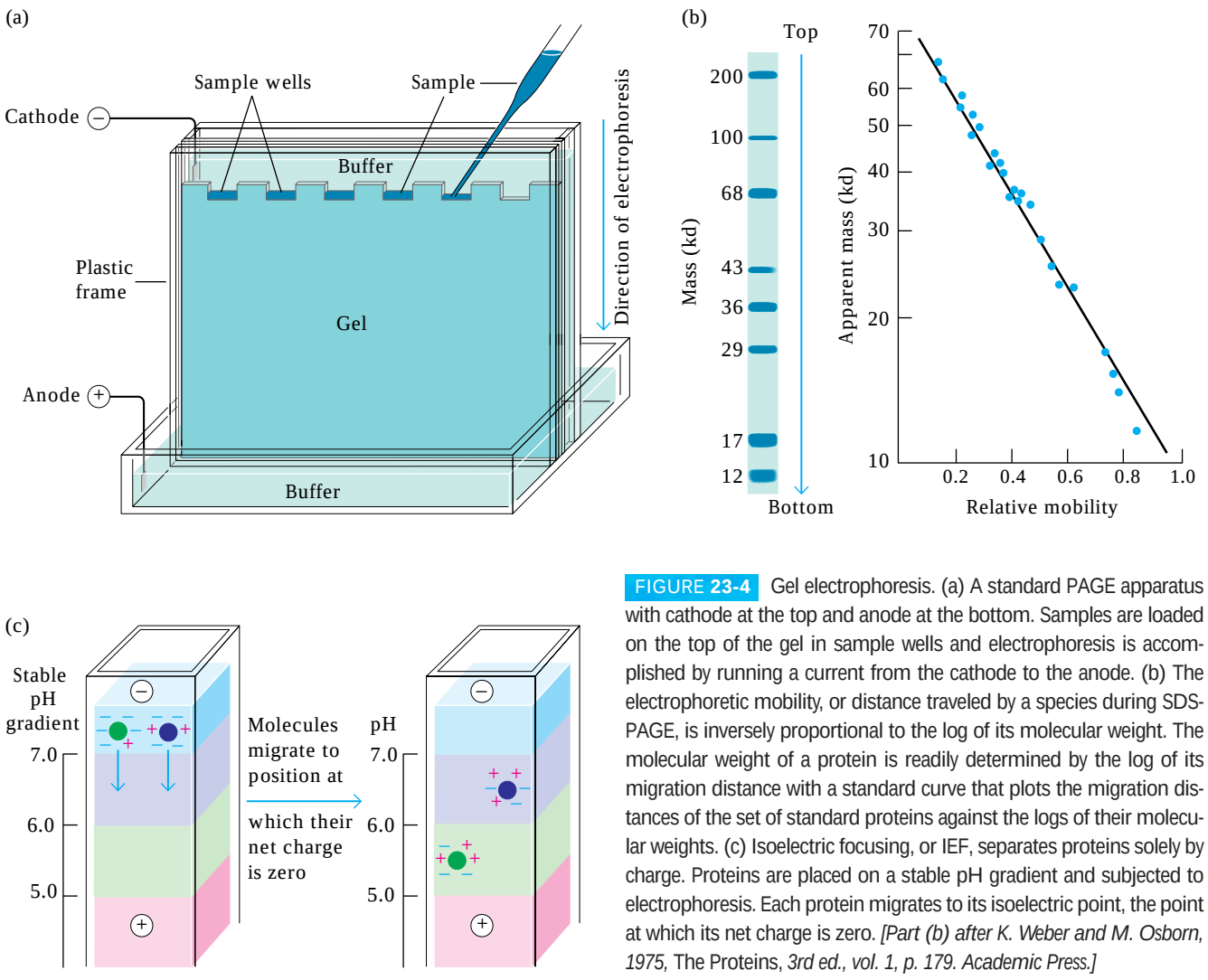


FIGURE 23-4 Gel electrophoresis. (a) A standard PAGE apparatus with cathode at the top and anode at the bottom. Samples are loaded on the top of the gel in sample wells and electrophoresis is accomplished by running a current from the cathode to the anode. (b) The electrophoretic mobility, or distance traveled by a species during SDS-PAGE, is inversely proportional to the log of its molecular weight. The molecular weight of a protein is readily determined by the log of its migration distance with a standard curve that plots the migration distances of the set of standard proteins against the logs of their molecular weights. (c) Isoelectric focusing, or IEF, separates proteins solely by charge. Proteins are placed on a stable pH gradient and subjected to electrophoresis. Each protein migrates to its isoelectric point, the point at which its net charge is zero. *[Part (b) after K. Weber and M. Osborn, 1975,* The Proteins, *3rd ed., vol. 1, p. 179. Academic Press.]*

*g*el *e*lectrophoresis (PAGE), may be used for analysis of proteins or nucleic acids (Figure 23-4a).

In one common application, the electrophoresis of proteins through a polyacrylamide gel is carried out in the presence of the detergent sodium dodecyl sulfate (SDS). This method, known as SDS-PAGE, provides a relatively simple and highly effective means of separating mixtures of proteins on the basis of size. SDS is a negatively charged detergent that binds to protein in amounts proportional to the length of the protein. This binding destroys the characteristic tertiary and secondary structure of the protein, transforming it into a negatively charged rod. A protein binds so many negatively charged SDS molecules that its own intrinsic charge becomes insignificant by comparison with the net charge of the SDS molecules. Therefore, treatment of a mixture of proteins with SDS transforms them into a collection of rods whose electric charges are proportional to their molecular weights. This has two extremely useful consequences. First, it is possible to separate the components of a mixture of proteins according to molecular weight. Second, because the electrophoretic mobility, or distance traveled by a species during SDS-PAGE, is inversely proportional to the logarithm of its molecular weight, that distance is a measure of its molecular weight. The gel is stained with a dye that reacts with protein to visualize the locations of the proteins. The migration distance of a protein in question is then compared with a plot of the distances migrated by a set of standard proteins (Figure 23-4b).

Another electrophoretic technique, isoelectric focusing (IEF), separates proteins solely on the basis of their charge. This method is based on the fact that a molecule will move in an electric field as long as it has a net positive or negative charge; molecules that bear equal numbers of positive and negative charges and therefore have a net charge of zero will not move. At most pH values, proteins (which characteristically bear a number of both positive and negative charges) have either a net negative or a net positive charge. However,

for each protein there is a particular pH, called its isoelectric point (pI), at which that protein has equal numbers of positive and negative charges. Isoelectric focusing makes use of a gel containing substances, called carrier ampholytes, that arrange themselves into a continuous pH gradient when subjected to an electric field. When a mixture of proteins is applied to such a gel and subjected to electrophoresis, each protein moves until it reaches that point in the gradient where the pH of the gel is equal to its isoelectric point. It then stops moving because it has a net charge of zero. Isoelectric focusing is an extremely gentle and effective way of separating different proteins (Figure 23-4c).

A method known as two-dimensional gel electrophoresis (2D gel electrophoresis) combines the advantages of SDS-PAGE and isoelectric focusing in one of the most sensitive and discriminating ways of analyzing a mixture of proteins. In this method, one first subjects the mixture to isoelectric focusing on an IEF tube gel, which separates the molecules on the basis of their isoelectric points without regard to molecular weight. This is the first dimension. In the next step, one places the IEF gel lengthwise across the top of an SDS-polyacrylamide slab (that is, in place of the sample wells in Figure 23-4a) and runs SDS-PAGE. Preparatory to this step, all proteins have been reacted with SDS and therefore migrate out of the IEF gel and through the SDS-PAGE slab according to their molecular weights. This is the second dimension. The position of the proteins in the resulting 2D gel can be visualized in a number of ways. In the least sensitive the gel is stained with a protein-binding dye (such as Coomassie blue). If the proteins have been radiolabeled, the more sensitive method of autoradiography can be used. Alternatively, silver staining is a method of great sensitivity that takes advantage of the capacity of proteins to reduce silver ions to an easily visualized deposit of metallic silver. Finally, immunoblotting—blotting of proteins onto a membrane and detection with antibody (see Figure 6-13)—can be used as a way of locating the position of specific proteins on 2D gels if an appropriate antibody is available. Figure 23-5 shows an autoradiograph of a two-dimensional gel of labeled proteins from murine thymocytes.

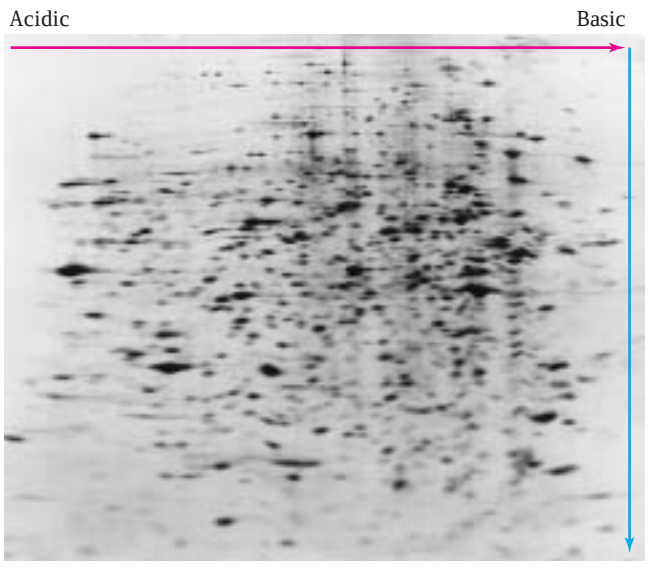


FIGURE **23-5** Two-dimensional gel electrophoresis of ^{35}S-methionine labeled total cell proteins from murine thymocytes. These proteins were first subjected to isoelectric focusing (direction of migration indicated by red arrow) and then the focused proteins were separated by SDS-PAGE (direction of migration indicated by blue arrow). The gel was exposed to x-ray film to detect the labeled proteins. *[Courtesy of B. A. Osborne.]*

X-Ray Crystallography Provides Structural Information

A great deal of information about the structure of cells, parts of cells, and even molecules has been obtained by light microscopy. The microscope uses a lens to focus radiation to form an image after it has passed through a specimen. However, a practical limitation of light microscopy is the limit of resolution. Radiation of a given wavelength cannot resolve structural features less than about 1/2 its wavelength. Since the shortest wavelength of visible light is around 400 nm, even the very best light microscopes have a theoretical limit of resolution of no less than 200 nm.

Because of the much shorter wavelength (0.004 nm) of the electron at the voltages normally used in the electron microscope, the theoretical limit of resolution of the electron microscope is about 0.002 nm. If it were possible to build an instrument that could actually approach this limit, the electron microscope could readily be used to determine the detailed atomic arrangement of biological molecules, since the constituent atoms are separated by distances of 0.1 nm to 0.2 nm. In practice, aberrations inherent in the operation of the magnetic lenses that are used to image the electron beam limit the resolution to about 0.1 nm (1Å). This practical limit can be reached in the examination of certain specimens, particularly metals. Other considerations, however, such as specimen preparation and contrast, limit the resolution for biological materials to about 2 nm (20 Å). To determine the arrangement of a molecule's atoms, then, we must turn to x-rays, a form of electromagnetic radiation that is readily generated in wavelengths on the order of size of interatomic distances. Even though there are no microscopes with lenses that can focus x-rays into images, x-ray crystallography can reveal molecular structure at an extraordinary level of detail.

X-ray crystallography is based on the analysis of the diffraction pattern produced by the scattering of an x-ray beam as it passes though a crystal. The degree to which a particular atom scatters x-rays depends upon its size. Atoms such as carbon, oxygen, or nitrogen, scatter x-rays more than do hydrogen atoms, and larger atoms, such as iron, iodide, or mercury give intense scattering. X-rays are a form of electromagnetic waves;

as the scattered waves overlap, they alternately interfere with and reinforce each other. An appropriately placed detector records a pattern of spots (the diffraction pattern) whose distribution and intensities are determined by the structure of the diffracting crystal. This relationship between crystal structure and diffraction pattern is the basis of x-ray crystallographic analysis. Here is an overview of the procedures used:

OBTAIN CRYSTALS OF THE PROTEIN OF INTEREST. To those who have not experienced the frustrations of crystallizing proteins, this may seem a trivial and incidental step of an otherwise highly sophisticated process. It is not. There is great variation from protein to protein in the conditions required to produce crystals that are of a size and geometrical formation appropriate for x-ray diffraction analysis. For example, myoglobin formed crystals over the course of several days at pH 7 in a 3 M solution of ammonium sulfate, but 1.5 M ammonium sulfate at pH 4 worked well for a human IgG1. There is no set formula that can be applied, and those who are consistently successful are persistent, determined, and, like great chefs, have a knack for making just the right "sauce."

SELECTION AND MOUNTING. Crystal specimens must be at least 0.1 mm in the smallest dimension and rarely exceed a few millimeters in any dimension. Once chosen, a crystal is harvested into a capillary tube along with the solution from which the crystal was grown (the "mother liquor"). This keeps the crystal from drying and maintains its solvent content, an important consideration for maintaining the internal order of the specimen. The capillary is then mounted in the diffraction apparatus.

GENERATING AND RECORDING A DIFFRACTION PATTERN. The precisely positioned crystal is then irradiated with x-rays of a known wavelength produced by accelerating electrons against the copper target of an x-ray tube. When the x-ray beam strikes the crystal, some of it goes straight through and some is scattered; sensitive detectors record the position and intensity of the scattered beam as a pattern of spots (Figure 23-6a,b).

INTERPRETING THE DIFFRACTION PATTERN. The core of diffraction analysis is the mathematical deduction of the detailed structure that would produce the diffraction pattern observed. One must calculate to what extent the waves scattered by each atom have combined to reinforce or cancel each other to produce the net intensity observed for each spot in the array. A difficulty arises in the interpretation of complex diffraction patterns because the waves differ with respect to phase, the timing of the period between maxima and minima. Since the pattern observed is the net result of the interaction of many waves, information about phase is critical to calculating the distribution of electron densities that is responsible. The solution of this "phase problem" looms as a major obstacle to the derivation of a high-resolution structure of any complex molecule.

The problem is solved by derivatizing the protein—modifying it by adding heavy atoms, such as mercury, and then obtaining crystals that have the same geometry as (are iso-

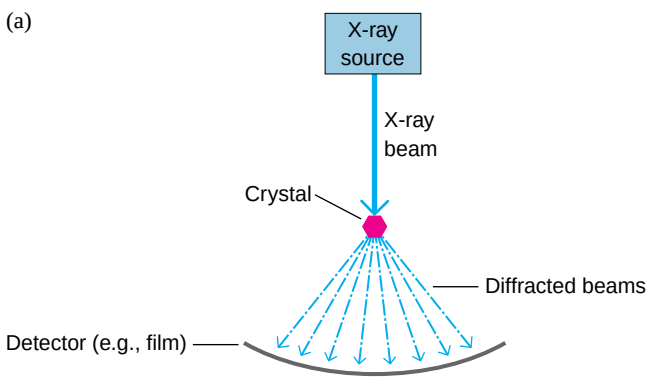


FIGURE **23-6** X-ray crystallography. (a) Schematic diagram of an x-ray crystallographic experiment in which an x-ray beam bombards the crystal and diffracted rays are detected. (b) Section of x-ray diffraction pattern of a crystal of murine IgG2a. (c) Section from the electron-density map of murine IgG2a. *[Part (a) from L. Stryer, 1995,* Biochemistry, *4th ed.; parts (b) and (c) courtesy of A. McPherson.]*

morphous with) those of the underivatized protein. The diffraction pattern of the isomorphous crystal is obtained and compared with that of the native protein. Usually, armed with a knowledge of the diffraction patterns of two or more isomorphous heavy-atom derivatives, the phases for the native protein can be calculated by reference to the characteristic diffraction patterns generated by heavy-atom landmarks. The phases established, it is possible to move on to a calculation of the distribution of electron density. This is accomplished by Fourier synthesis, a mathematical treatment particularly suited to the analysis of periodic phenomena such as those involving waves. In this case, it is used to compute the distribution of electron density along the *x*, *y*, and *z* axes within a unit cell of the crystal. The deduced electron density can then be visualized on a computer (Figure 23-6c).

DERIVATION OF THE STRUCTURE. The resolution of a model depends upon a number of factors. First of all, the ultimate resolution possible is set by the quality of the crystal and the internal order of the crystal. Even the highest-quality crystals have a degree of internal disorder that establishes a limit of resolution of about 2 Å. Second, a factor of paramount importance is the number of intensities fed into the Fourier synthesis. A relatively small number of spots may produce a low-resolution (6 Å) image that traces the course of the polypeptide chain but provides little additional structural information. On the other hand, the processing of data provided by tens of thousands of spots allows the tracing of very detailed electron-density maps. Provided one knows the amino-acid sequence of the protein, such maps can guide the construction of high-resolution, three-dimensional models. Amino-acid sequence data is necessary because it can be difficult, and in some cases impossible, to unambiguously distinguish among some amino-acid side chains on even the most detailed electron-density maps.

Since 1960, when the first detailed structures of proteins were deduced, the structures of many thousands of proteins have been solved. These range from small and (relatively) simple proteins such as lysozyme, consisting of a single polypeptide chain, to poliovirus, an 8,500,000 dalton, stunningly complex nucleoprotein made up of RNA encased by multiple copies of four different polypeptide subunits. Of particular importance to immunologists are the large number of immunologically relevant molecules for which detailed crystal structures are now available. These include many immunoglobulins, most of the major and minor proteins involved in the MHC and T-cell–receptor complexes, and many other important immunological macromolecules, with new structures and structural variants appearing every month.

Recombinant DNA Technology

The various techniques called recombinant DNA technology have had an impact on every area of immunologic research. Genes can be cloned, DNA can be sequenced, and recombinant proteins can be produced, supplying immunologists with defined components for study of the structure and function of the immune system at the molecular level. This section briefly describes some of the recombinant DNA techniques commonly employed in immunologic research; examples of their use have been presented throughout the book.

Restriction Enzymes Cleave DNA at Precise Sequences

A variety of bacteria produce enzymes, called *restriction endonucleases*, that degrade foreign DNA (e.g., bacteriophage DNA) but spare the bacterial-cell DNA, which contains methylated residues. The discovery of these bacterial enzymes in the 1970s opened the way to a major technological advance in the field of molecular biology. Before the discovery of restriction endonucleases, double-stranded DNA (dsDNA) could be cut only with DNases. These enzymes do not recognize defined sites and therefore randomly cleave DNA into a variable series of small fragments, which are impossible to sort by size or sequence. In contrast, restriction endonucleases recognize and cleave DNA at specific sites, called restriction sites, which are short double-stranded segments of specific sequence containing four to eight nucleotides (Table 23-5).

TABLE 23-5 Some restriction enzymes and their recognition sequences

Microorganism source	Abbreviation	Sequence* 5′→3′ / 3′→5′
Bacillus amyloliquefaciens H	*Bam*HI	G|G A T C C C C T A G|G
Escherichia coli RY 13	*Eco*RI	G|A A T T C C T T A A|G
Haemophilus aegyptius	*Hae*III	G G|C C C C|G G
Haemophilus influenzae Rd	*Hin*dIII	A|A G C T T T T C G A|A
Haemophilus parainfluenzae	*Hpa*I	G T T|A A C C A A|T T G
Nocardia otitidis-caviarum	*Not*I	G C|G G C C G C C G C C G|G C G
Providencia stuartii 164	*Pst*I	G T G C A|G G|A C G T C
Staphylococcus aureus 3A	*Sau*3A	|G A T C C T A G|

*Blue lines indicate locations of single-strand cuts within the restriction site. Enzymes that make off-center cuts produce fragments with short single-stranded extensions at their ends.

SOURCE: New England Biolabs, http://www.neb.com.

A restriction endonuclease cuts both DNA strands at a specific point within its restriction site. Some enzymes, such as *Hpa*I, cut on the central axis and thus generate blunt-ended fragments. Other enzymes, such as *Eco*RI, cut the DNA at staggered points in the recognition site. In this case, the end of each cleaved fragment is a short segment of single-stranded DNA, called a *sticky end*. When two different DNA molecules are cut with the same restriction enzyme that makes staggered cuts, the sticky ends of the fragments are complementary; under appropriate conditions, fragments from the two molecules can be joined by base pairing to generate a recombinant DNA molecule. Several hundred different restriction endonucleases have been isolated and many are available commercially, allowing researchers to purchase enzymes that cut DNA at defined restriction sites.

Cloning of DNA Sequences

The development of DNA-cloning technology in the 1970s provided a means of amplifying a given DNA fragment to such an extent that unlimited amounts of identical DNA fragments (cloned DNA) could be produced.

Cloning Vectors Are Useful to Replicate Defined Sequences of DNA

In DNA cloning, a given DNA fragment is inserted into an autonomously replicating DNA molecule, called a cloning vector, so that the inserted DNA is replicated with the vector. A number of different viruses have been used as vectors, including bacterial viruses, insect viruses, and mammalian retroviruses. A common bacterial virus used as a vector is bacteriophage λ. If a gene is inserted into bacteriophage λ and the resulting recombinant λ phage is used to infect *E. coli*, the inserted gene will be expressed by the bacteria.

Retroviruses, which can infect virtually any type of mammalian cell, are a common vector used to clone DNA in mammalian cells. Retroviruses are RNA viruses that contain reverse transcriptase, an enzyme that catalyzes conversion of the viral RNA genome into DNA. The viral DNA then integrates into the host chromosomal DNA, where it is retained as a provirus, replicating along with the host chromosomal DNA at each cell division. When a retrovirus is used as a vector in research, most of the retroviral genes are removed so that the vector cannot produce viral particles; the retroviral genes that are left include a strong promoter region, located at the 5′ end of the viral genome, in a sequence called the *long terminal repeat* (LTR). If a gene is inserted into such a retroviral vector and the vector is then used to infect mammalian cells, expression of the gene will be under the control of the retroviral promoter region.

Plasmids are another common type of cloning vector. A plasmid is a small, circular, extrachromosomal DNA molecule that can replicate independently in a host cell; the most common host used in DNA cloning is *E. coli*. In general, the DNA to be cloned is inserted into a plasmid that contains an antibiotic-resistance gene. After the recombinant plasmid is incubated with bacterial cells, the cells containing the recombinant plasmid can be selected by their ability to grow in the presence of the antibiotic.

Another type of vector that is often used for cloning is called a cosmid vector. This type of vector is a plasmid that has been genetically engineered to contain the COS sites of λ-phage DNA, a drug-resistance gene, and a replication origin. COS sites are DNA sequences that allow any DNA up to 50 kb in length to be packaged into the λ-phage head.

Cloning of cDNA and Genomic DNA Allows the Isolation of Defined Sequences

Messenger RNA (mRNA) isolated from cells can be transcribed into complementary DNA (cDNA) with the enzyme reverse transcriptase. The cDNA can be cloned by inserting it into a plasmid vector carrying a gene that confers resistance to an antibiotic, such as ampicillin. The resulting recombinant plasmid DNA is subsequently transferred into specially treated *E. coli* cells by one of several techniques; the transfer process is called **transfection.** If the foreign DNA is incorporated into the host cell and expressed, the cell is said to be **transformed.** When the cells are cultured on agar plates containing ampicillin, only transformed cells containing the ampicillin-resistance gene will survive and grow (Figure 23-7). A collection of DNA sequences within plasmid vectors representing all the mRNA sequences derived from a cell or tissue is called a *cDNA library.* A cDNA library differs from a genomic library (see Figure 23-8) by virtue of the fact that it contains only the sequences derived from mRNA, the sequences that represent expressed genes.

Genomic cloning, cloning of the entire genome of an animal, requires specialized vectors. *E. coli* plasmid vectors are impractical for cloning of all the genomic DNA fragments that constitute a large genome because of the low efficiency of *E. coli* transformation and the small number of transformed colonies that can be detected on a typical petri dish. Instead, cloning vectors derived from bacteriophage λ are used to clone genomic DNA fragments obtained by cleaving chromosomal DNA with restriction enzymes (Figure 23-8). Bacteriophage λ DNA is 48.5 kb long and contains a central section of about 15 kb that is not necessary for λ replication in *E. coli* and can therefore be replaced with foreign genomic DNA. As long as the recombinant DNA does not exceed the length of the original λ-phage DNA by more than 5%, it can be packaged into the λ-phage head and propagated in *E. coli.* This means that somewhat more than 1.5×10^4 base pairs can be cloned in one particle of λ phage. A collection of λ clones that includes all the DNA sequences of a given species is called a *genomic library.* It has been calculated that about 1 million different recombinant λ-phage particles would be needed to form a genomic DNA library representing an entire mammalian genome, which contains about 3×10^9 base pairs.

Often the 20–25 kb stretch of DNA that can be cloned in bacteriophage λ is not long enough to include the regulatory

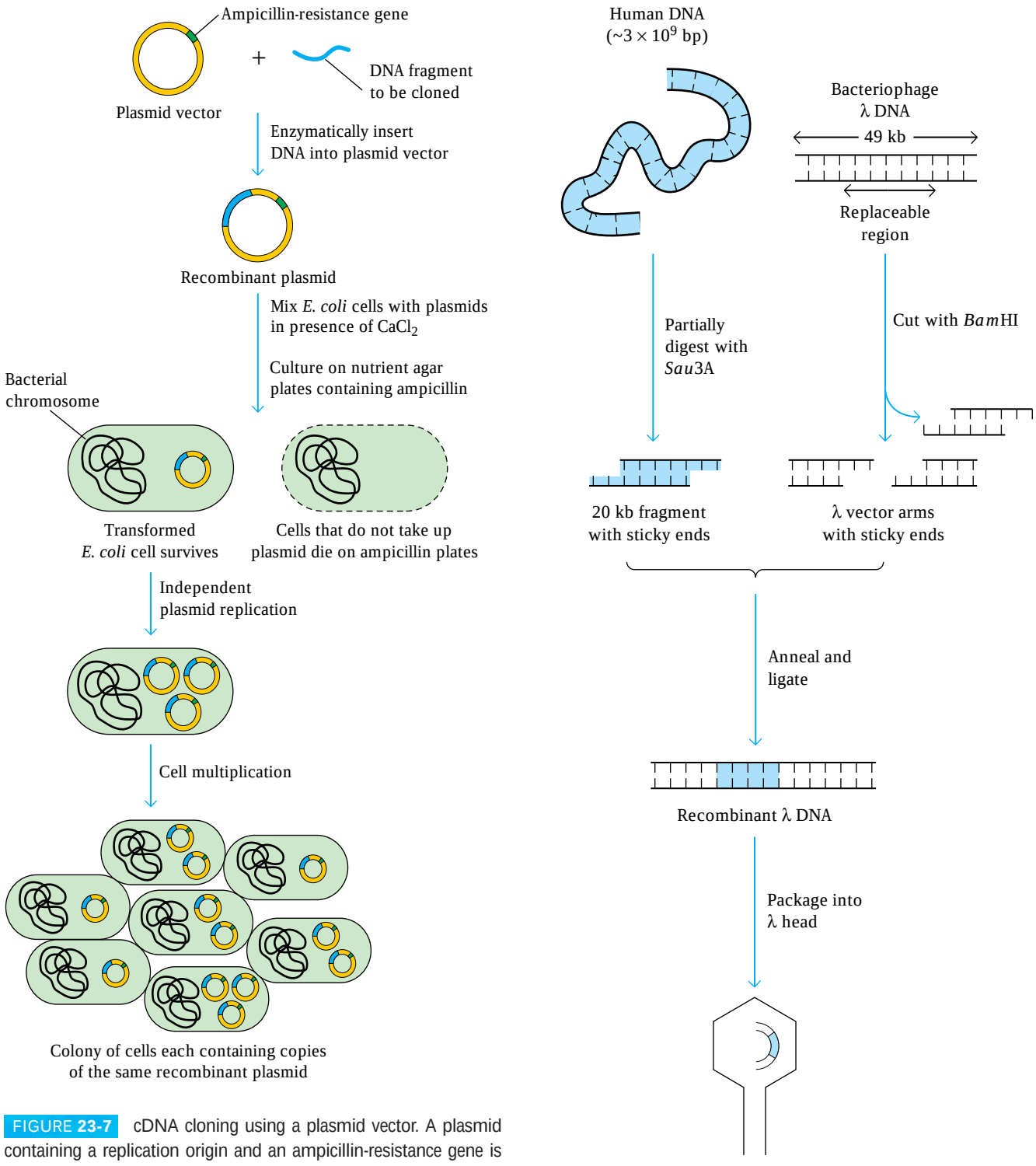


FIGURE **23-7** cDNA cloning using a plasmid vector. A plasmid containing a replication origin and an ampicillin-resistance gene is cut with a restriction endonuclease that produces blunt ends. After addition of a poly-C tail to the 3′ ends of the cDNA and of a complementary poly-G tail to the 3′ ends of the cut plasmid, the two DNAs are mixed, annealed, and joined by DNA ligase, forming the recombinant plasmid. Uptake of the recombinant plasmid into *E. coli* cells is stimulated by high concentrations of $CaCl_2$. Transformation occurs with a low frequency, but the transformed cells can be selected in the presence of ampicillin. *[Adapted from H. Lodish et al., 1995,* Molecular Cell Biology, *3rd ed. Scientific American Books.]*

FIGURE **23-8** Genomic DNA cloning using bacteriophage λ as the vector. Genomic DNA is partly digested with *Sau*3A, producing fragments with sticky ends. The central 15-kb region of the λ-phage DNA is cut out with *Bam*HI and discarded. These two restriction enzymes produce complementary sticky ends, so the genomic and DNA fragments can be annealed and ligated. After the resulting recombinant DNA is packaged into a λ-phage head, it can be propagated in *E. coli.*

TABLE 23-6 Vectors and maximum length of DNA that they can carry

Vector type	Maximum length of cloned DNA (kb)
Plasmid	20
Bacteriophage λ	25
Cosmid	45
Bacteriophage P1	100
Bacterial artificial chromosome (BAC)	100–300
Yeast artificial chromosome (YAC)	>1000

sequences that lie outside the 5′ and 3′ ends of the direct coding sequences of a gene. As noted already, larger genomic DNA fragments—between 30 and 50 kb in length—can be cloned in a cosmid vector. A recombinant cosmid vector, although not a fully functional bacteriophage, can infect *E. coli* and replicate as a plasmid, generating a cosmid library. Recently, a larger *E. coli* virus, called bacteriophage P1, has been used to package DNA fragments up to 100 kb long. Even larger DNA fragments, greater than a megabase (1000 kb) in length, can be cloned in yeast artificial chromosomes (YACs), which are linear DNA segments that can replicate in yeast cells (Table 23-6). The BAC, or bacterial artifical chromosome, is another useful vector. BACs can accept pieces of DNA up to 100–300 kb in length. Although YACs accept larger inserts of foreign DNA, BACs are much easier to propagate and are the vector of choice for many large-scale cloning efforts.

Selection of DNA Clones

Once a cDNA or genomic DNA library has been prepared, it can be screened to identify a particular DNA fragment by a technique called *in situ hybridization*. The cloned bacterial colonies, yeast colonies, or phage plaques containing the recombinant DNA are transferred onto nitrocellulose or nylon filters by replica plating (Figure 23-9). The filter is then treated with NaOH, which both lyses the bacteria and denatures the DNA, allowing single-stranded DNA (ssDNA) to bind to the filter. The filter with bound DNA is then incubated with a radioactive probe specific for the gene of interest. The probe will hybridize with DNA in the colonies or plaques on the filter that contain the sought-after gene, and they can be identified by autoradiography. The position of the positive colonies or plaques on the filter shows where the corresponding clones can be found on the original agar plate.

Various radioactive probes can be used to screen a library. In some cases, radiolabeled mRNA or cDNA serves as the probe. If the protein encoded by the gene of interest has been purified and partly sequenced, it is possible to work backward from the amino-acid sequence to determine the probable nucleotide sequence of the corresponding gene. A known sequence of five or six amino-acid residues is all that is needed to synthesize radiolabeled oligonucleotide probes with which to screen a cDNA or genomic library for a particular gene. To cope with the degeneracy of the genetic code, peptide segments containing amino acids encoded by a limited number of codons are usually chosen. Oligonucleotides representing

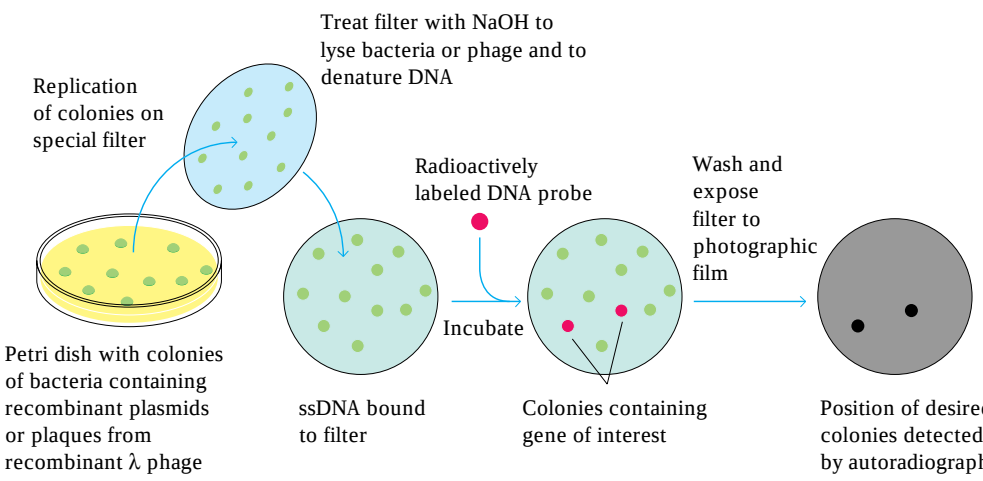


FIGURE 23-9 Selection of specific clones from a cDNA or genomic DNA library by in situ hybridization. A nitrocellulose or nylon filter is placed against the plate to pick up the bacterial colonies or phage plaques containing the cloned genes. After the filter is placed in a NaOH solution and heated, the denatured ssDNA becomes fixed to the filter. A radioactive probe specific for the gene of interest is incubated with the filter. The position of the colonies or plaques containing the desired gene is revealed by autoradiography.

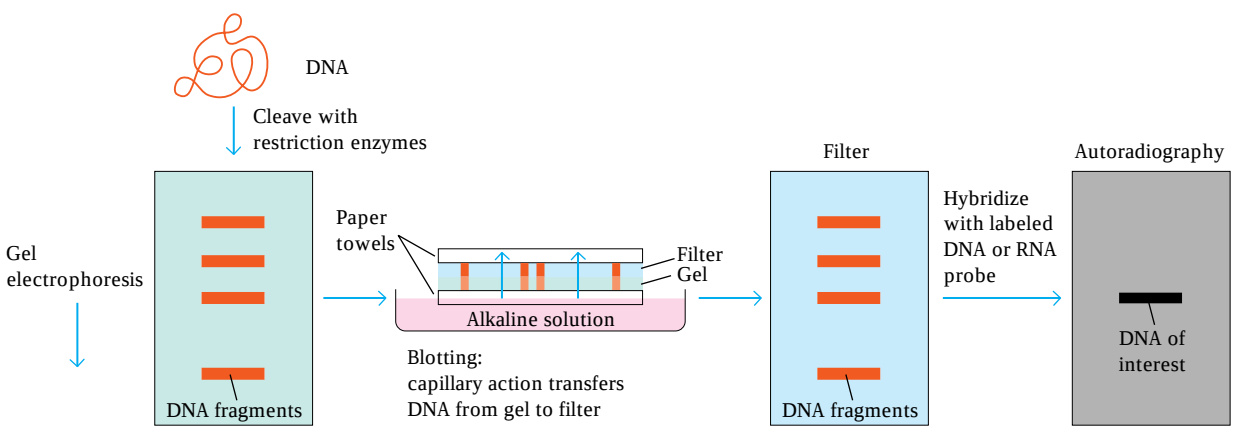


FIGURE **23-10** The Southern-blot technique for detecting specific sequences in DNA fragments. The DNA fragments produced by restriction-enzyme cleavage are separated by size by agarose gel electrophoresis. The agarose gel is overlaid with a nitrocellulose or nylon filter and a thick stack of paper towels. The gel is then placed in an alkaline salt solution, which denatures the DNA. As the paper towels soak up the moisture, the solution is drawn through the gel into the filter, transferring each ssDNA band to the filter. This process is called blotting. After heating, the filter is incubated with a radiolabeled probe specific for the sequence of interest; DNA fragments that hybridize with the probe are detected by autoradiography. *[Adapted from J. Darnell et al., 1990,* Molecular Cell Biology, *2nd ed., Scientific American Books.]*

all possible codons for the peptide are then synthesized and used as probes to screen the DNA library.

Southern Blotting Detects DNA of a Given Sequence

DNA fragments generated by restriction-endonuclease cleavage can be separated on the basis of length by agarose gel electrophoresis. The shorter a fragment is, the faster it moves in the gel. An elegant technique developed by E. M. Southern can be used to identify any band containing fragments with a given gene sequence (Figure 23-10). In this technique, called **Southern blotting,** DNA is cut with restriction enzymes and the fragments are separated according to size by electrophoresis on an agarose gel. Then the gel is soaked in NaOH to denature the dsDNA, and the resulting ssDNA fragments are transferred onto a nitrocellulose or nylon filter by capillary action. After transfer, the filter is incubated with an appropriate radiolabeled probe specific for the gene of interest. The probe hybridizes with the ssDNA fragment containing the gene of interest, and the position of the band containing these hybridized fragments is determined by autoradiography. Southern-blot analysis played a critical role in unraveling the mechanism by which diversity of antibodies and T-cell receptors is generated (see Figures 5-2 and 9-2).

Northern Blotting Detects mRNA

Northern blotting (named for its similarity to Southern blotting) is used to detect the presence of specific mRNA molecules. In this procedure the mRNA is first denatured to ensure that it is in an unfolded, linear form. The mRNA molecules are then separated according to size by electrophoresis and transferred to a nitrocellulose filter, to which the mRNAs will adhere. The filter is then incubated with a labeled DNA probe and subjected to autoradiography. Northern-blot analysis is often used to determine how much of a specific mRNA is expressed in cells under different conditions. Increased levels of mRNA will bind proportionally more of the labeled DNA probe.

Polymerase Chain Reaction Amplifies Small Quantities of DNA

The polymerase chain reaction (PCR) is a powerful technique for amplifying specific DNA sequences even when they are present at extremely low levels in a complex mixture (Figure 23-11). The procedure requires that the DNA sequences that flank the desired DNA sequence be known, so that short oligonucleotide primers can be synthesized. The DNA mixture is denatured into single strands by a brief heat treatment. The DNA is then cooled in the presence of an excess of the oligonucleotide primers, which hybridize with the complementary ssDNA. A temperature-resistant DNA polymerase is then added, together with the four deoxyribonucleotide triphosphates, and each strand is copied. The newly synthesized DNA duplex is separated by heating and the cycle is repeated. In each cycle there is a doubling of the desired DNA sequence; in only 25 cycles the desired DNA sequence can be amplified about a million-fold.

The DNA amplified by the PCR can be further characterized by Southern blotting, restriction-enzyme mapping, and

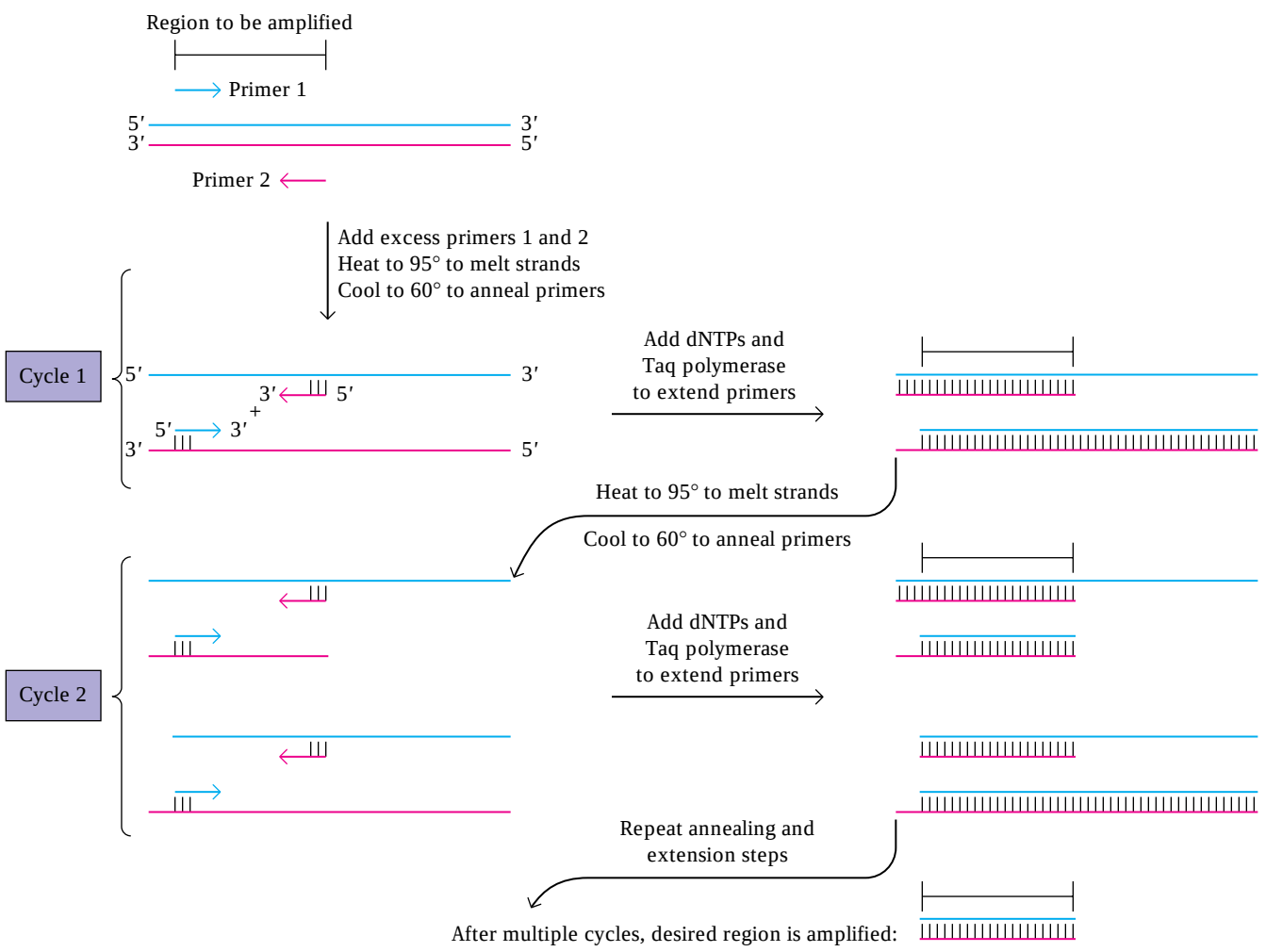


FIGURE **23-11** The polymerase chain reaction (PCR). DNA is denatured into single strands by a brief heat treatment and is then cooled in the presence of an excess of oligonucleotide primers complementary to the DNA sequences flanking the desired DNA segment. A heat-resistant DNA polymerase is used to copy the DNA from the 3′ ends of the primers. Because all of the reaction components are heat stable, the heating and cooling cycle can be repeated many times, resulting in alternate DNA melting and synthesis, and rapid amplification of a given sequence. *[Adapted from H. Lodish et al., 1995,* Molecular Cell Biology, *3rd ed., Scientific American Books.]*

direct DNA sequencing. The PCR technique has enabled immunologists to amplify genes encoding proteins that are important in the immune response, such as MHC molecules, the T-cell receptor, and immunoglobulins.

Analysis of DNA Regulatory Sequences

The transcriptional activity of genes is regulated by promoter and enhancer sequences. These sequences are *cis-acting,* meaning that they regulate only genes on the same DNA molecule. The promoter sequence lies upstream from the gene it regulates and includes a TATA box, where the general transcription machinery, including RNA polymerase II, binds and begins transcription. The enhancer sequence confers a high rate of transcription on the promoter. Unlike the promoter, which always lies upstream from the gene it controls, the enhancer element can be located anywhere with respect to the gene (5′ of the promoter, 3′ of the gene, or even in an intron of the gene).

The activity of enhancer and promoter sequences is controlled by transcription factors, which are DNA-binding proteins. These proteins bind to specific nucleotide sequences within promoters and enhancers and act either to enhance or suppress their activity. Enhancer and promoter sequences and their respective DNA-binding proteins have been identified by a variety of techniques, including DNA footprinting, gel-shift analysis, and the CAT assay.

DNA Footprinting Identifies the Sites Where Proteins Bind DNA

The binding sites for DNA-binding proteins on enhancers and promoters can be identified by a technique called DNA footprinting (Figure 23-12a). In this technique, a cloned DNA fragment containing a putative enhancer or promoter sequence is first radiolabeled at the 5′ end with ^{32}P. The labeled DNA is then divided into two fractions: one fraction is incubated with a nuclear extract containing a DNA-binding protein; the other DNA fraction is not incubated with the extract. Both DNA samples are then digested with a nuclease or a chemical that makes random cuts in the phosphodiester bonds of the DNA, and the strands are separated. The resulting DNA fragments are run on a gel to separate fragments of different sizes. In the absence of DNA-binding proteins, a complete ladder of bands is obtained on the electrophoretic gel. When a protein that binds to a site on the DNA fragment is present, it covers some of the nucleotides, protecting that stretch of the DNA from digestion. The electrophoretic pattern of such protected DNA will contain blank regions (or footprints). Each footprint represents the site within an enhancer or promoter that binds a particular DNA-binding protein.

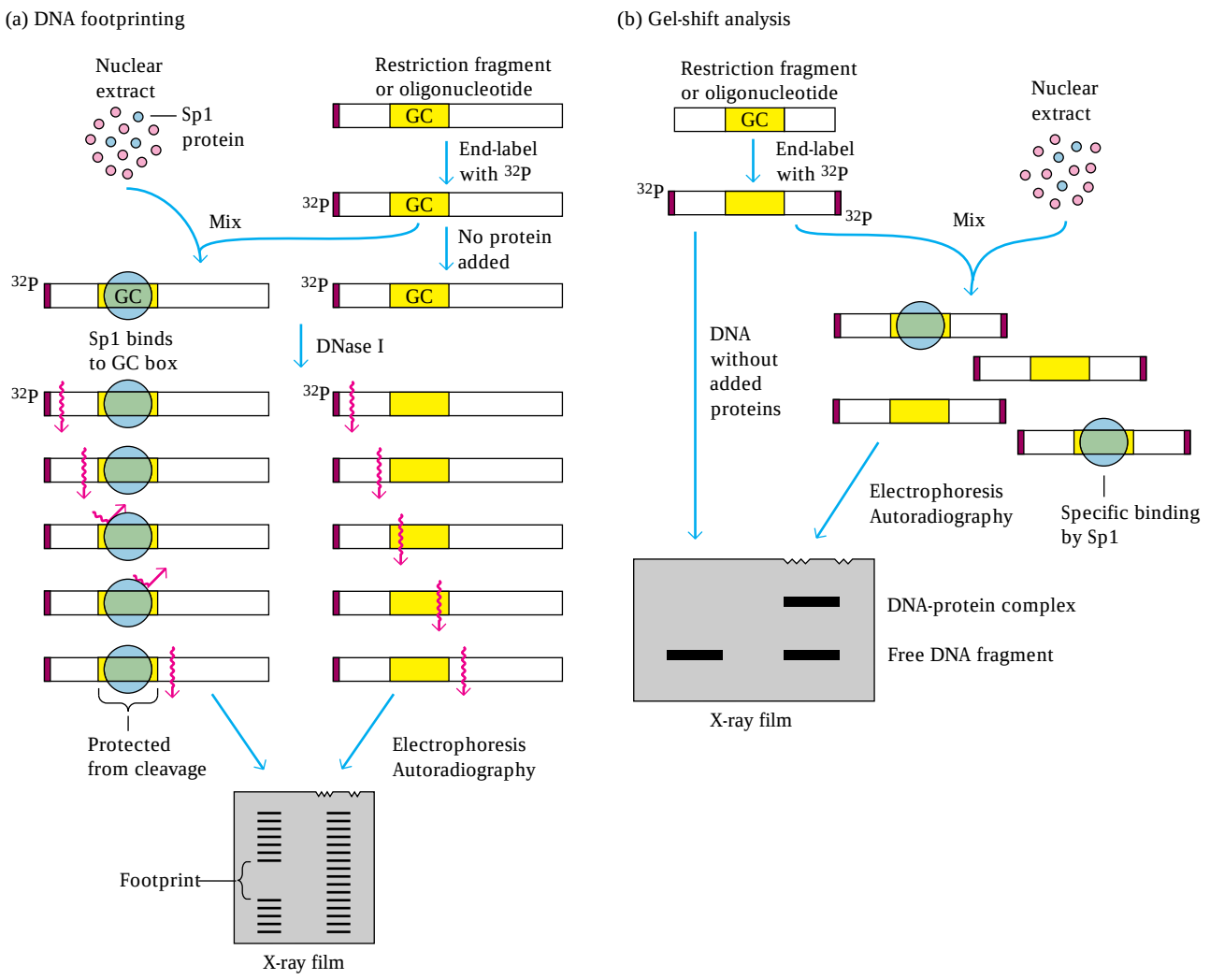


FIGURE **23-12** Identification of DNA sequences that bind protein by DNA-footprinting and gel-shift analysis. (a) In the footprinting technique, labeled DNA fragments containing a putative promoter or enhancer sequence are incubated in the presence and absence of a DNA-binding protein (e.g., Sp1 protein, which binds to a "GC box," a GC-rich region of DNA). After the samples are treated with DNase and the strands separated, the resulting fragments are electrophoresed; the gel then is subjected to autoradiography. A blank region (footprint) in the gel pattern indicates that protein has bound to the DNA. (b) In gel-shift analysis, a labeled DNA fragment is incubated with a cellular extract containing transcription factors. The electrophoretic mobility of the DNA-protein complex is slower than that of free DNA fragments. *[Adapted from J. D. Watson et al., 1992,* Recombinant DNA, *2nd ed., W. H. Freeman and Company.]*

Gel-Shift Analysis Identifies DNA-Protein Complexes

When a protein binds to a DNA fragment, forming a DNA-protein complex, the electrophoretic mobility of the DNA fragment in a gel is reduced, producing a shift in the position of the band containing that fragment. This phenomenon is the basis of gel-shift analysis. In this technique, radioactively labeled cloned DNA containing an enhancer or a promoter sequence is incubated with a nuclear extract containing a DNA-binding protein (Figure 23-12b). The DNA-protein complex is then electrophoresed and its electrophoretic mobility is compared with that of the cloned DNA alone. A shift in the mobility indicates that a protein is bound to the DNA, retarding its migration on the electrophoretic gel.

CAT Assays Measure Transcriptional Activity

One way to assess promoter activity is to engineer and clone a DNA sequence containing a *reporter gene* attached to the promoter that is being assessed. When this sequence, or construct, is introduced into eukaryotic cells, transcription will be initiated from the promoter if it is active, and the reporter gene will be transcribed and its protein product synthesized. Measuring the amount of this protein produced is thus a way to determine the activity of the promoter.

Most reporter genes are chosen because they encode proteins that can be easily measured, such as the enzyme chloramphenicol acetyltransferase (CAT), which transfers the acetyl group from acetyl-CoA to the antibiotic chloramphenicol (Figure 23-13). The more active the promoter, the more CAT will be produced within the transfected cell. By introducing mutations into promoter sequences and then assaying for promoter activity with the corresponding reporter gene, conserved sequence motifs have been identified within promoters. Another reporter gene, the firefly luciferase gene, is also convenient and easy to use. Luciferase activity is analyzed by the emission of light, which is detected by a luminometer.

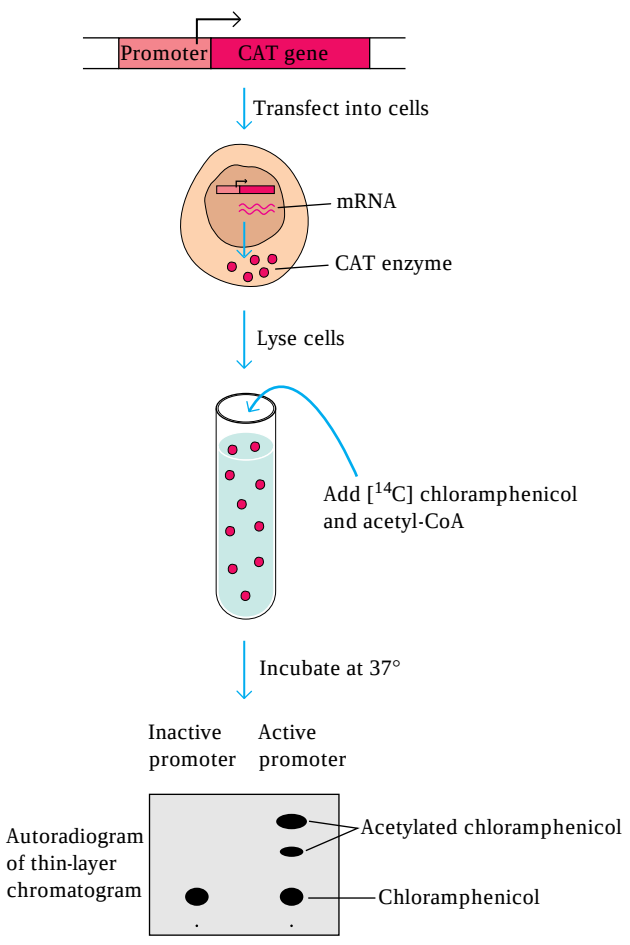


FIGURE **23-13** CAT assay for assessing functional activity of a promoter sequence. In this assay, a DNA construct consisting of the promoter of interest and the reporter gene encoding chloramphenicol acetyltransferase (CAT) is introduced (transfected) into eukaryotic cells. If the promoter is active, the CAT gene will be transcribed and the CAT enzyme will be produced within the transfected cell. The presence of the enzyme can easily be detected by lysing the cell and incubating the cell lysate with [^{14}C] chloramphenicol and acetyl-CoA. If present, the CAT enzyme will transfer the acetyl group from acetyl-CoA to the chloramphenicol, forming acetylated chloramphenicol, which can be easily detected by thin-layer chromatography. *[Adapted from J. D. Watson et al., 1992,* Recombinant DNA, *2nd ed., W. H. Freeman and Company.]*

Gene Transfer into Mammalian Cells

A variety of genes involved in the immune response have been isolated and cloned by use of recombinant DNA techniques. The expression and regulation of these genes has been studied by introducing them into cultured mammalian cells and, more recently, into the **germ line** of animals.

Cloned Genes Transferred into Cultured Cells Allow in Vitro Analysis of Gene Function

Diverse techniques have been developed for transfecting genes into cells. A common technique involves the use of a retrovirus in which a viral structural gene has been replaced with the cloned gene to be transfected. The altered retrovirus is then used as a vector for introducing the cloned gene into cultured cells. Because of the properties of retroviruses, the recombinant DNA integrates into the cellular genome with a high frequency. In an alternative method, the cloned gene of interest is complexed with calcium phosphate. The calcium-phosphate–DNA complex is slowly precipitated onto the cells and the DNA is taken up by a small percentage of them. In another transfection method, called electroporation, an electric current creates pores in cell membranes through which the cloned DNA is taken up. In both of these latter methods, the trans-

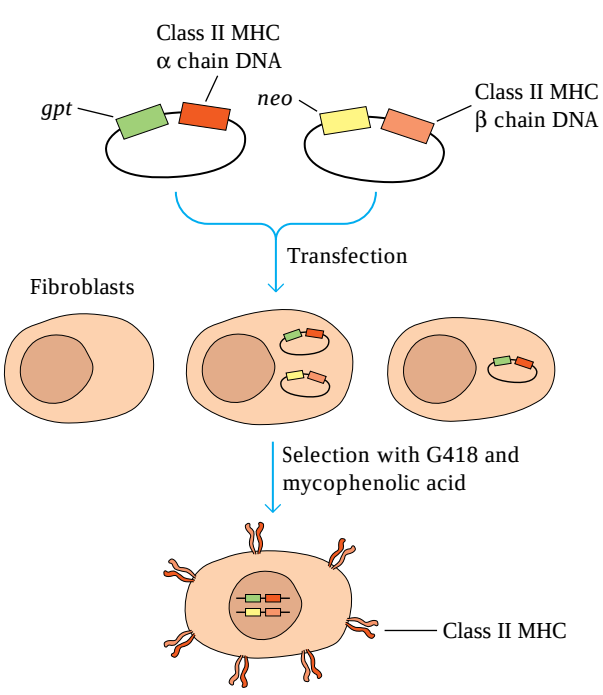


FIGURE 23-14 Transfection of the genes encoding the class II MHC α chain and β chain into mouse fibroblast L cells, which do not normally produce these proteins. Two constructs containing one of the MHC genes and a selectable gene were engineered: the α-chain gene with the guanine phosphoribosyl transferase gene (*gpt*), which confers resistance to the drug G418, and the β-chain gene with a neomycin gene (*neo*), which confers resistance to mycophenolic acid. After transfection, the cells are placed in medium containing both G418 and mycophenolic acid. Only those fibroblasts containing both the *neo* and *gpt* genes (and consequently the genes encoding the class II MHC α and β chains) will survive this selection. These fibroblasts will express both class II MHC chains on their membranes.

fected DNA integrates, apparently at random sites, into the DNA of a small percentage of treated cells.

Generally, the cloned DNA being transfected is engineered to contain a selectable marker gene, such as one that confers resistance to neomycin. After transfection, the cells are cultured in the presence of neomycin. Because only the transfected cells are able to grow, the small number of transfected cells in the total cell population can be identified and selected.

Transfection of cloned genes into cells has proved to be highly effective in immunologic research. By transfecting genes involved with the immune response into cells that lack those genes, the product of a specific gene can be studied apart from interacting proteins encoded by other genes. For example, transfection of MHC genes, under the control of appropriate promoters, into a mouse fibroblast cell line (L929, or simply L, cells) has enabled immunologists to study the role of MHC molecules in antigen presentation to T cells (Figure 23-14). Transfection of the gene that encodes the T-cell receptor has provided information about the antigen-MHC specificity of the T-cell receptor.

Cloned Genes Tranferred into Mouse Embryos Allow in Vivo Analysis of Gene Function

Development of techniques to introduce cloned foreign genes (called **transgenes**) into mouse embryos has permitted immunologists to study the effects of immune-system genes in vivo. If the introduced gene integrates stably into the germ-line cells, it will be transmitted to the offspring. Two techniques for producing transgenic mice are described in this section; one of these has been used to produce **knockout mice,** which cannot express a particular gene product (Table 23-7).

Transgenic Mice Aid in the Analysis of Gene Function

The first step in producing transgenic mice is injection of foreign cloned DNA into a fertilized egg. In this technically demanding process, fertilized mouse eggs are held under suction at the end of a pipet and the transgene is microinjected into one of the pronuclei with a fine needle. The transgene integrates into the chromosomal DNA of the pronucleus and is passed on to the daughter cells of eggs that survive the process. The eggs then are implanted in the oviduct of "pseudopregnant" females, and transgenic pups are born after 19 or

TABLE 23-7 Comparison of transgenic and knockout mice

Characteristic	Transgenic mice	Knockout mice
Cells receiving DNA	Zygote	Embryonic stem (ES) cells
DNA constructs used	Natural gene or cDNA	Mutated gene
Means of delivery	Microinjeciton into zygote and implantation into foster mother	Transfer of ES cells to blastocyst and implantation into foster mother
Outcome	Gain of a gene	Loss of gene

20 days of gestation (Figure 23-15). In general the efficiency of this procedure is low, with only one or two transgenic mice produced for every 100 fertilized egg collected.

With transgenic mice, immunologists have been able to study the expression of a given gene in a living animal. Although all the cells in a transgenic animal contain the transgene, differences in the expression of the transgene in different tissues has shed light on mechanisms of tissue-specific gene expression. By constructing a transgene with a particular promoter, researchers can control the expression of a given transgene. For example, the metallothionein promoter is activated by zinc. Transgenic mice carrying a transgene linked to a metallothionein promoter express the transgene only if zinc is added to their water supply. Other promoters are functional only in certain tissues; the insulin promoter, for instance, promotes transcription only in pancreatic cells. Transgenic mice carrying a transgene linked to the insulin promoter, therefore, will express the transgene in the pancreas but not in other tissues.

Because a transgene is integrated into the chromosomal DNA within the one-celled mouse embryo, it will be integrated into both somatic cells and germ-line cells. The resulting transgenic mice thus can transmit the transgene to their offspring as a Mendelian trait. In this way, it has been possible to produce lines of transgenic mice in which every member of a line contains the same transgene. A variety of such transgenic lines are currently available and are widely used in immunologic research. Included among these are lines carrying transgenes that encode immunoglobulin, T-cell receptor, class I and class II MHC molecules, various foreign antigens, and a number of cytokines. Several lines carrying oncogenes as transgenes also have been produced.

Gene-Targeted Knockout Mice Assess the Contribution of a Particular Gene

One of the limitations with transgenic mice is that the transgene is integrated randomly within the genome. This means that some transgenes insert in regions of DNA that are not transcriptionally active, and hence the gene is not expressed. To circumvent this limitation, researchers have developed a technique in which a desired gene is targeted to specific sites within the germ line of a mouse. The primary use of this technique has been to replace a normal gene with a mutant allele or a disrupted form of the gene, thus knocking out the gene's function. Transgenic mice that carry such a disrupted gene, called *knockout mice*, have been extremely helpful to immunologists trying to understand how the removal of a particular gene product affects the immune system. Various knockout mice are being used in immunologic research, including mice that lack particular cytokines or MHC molecules.

Production of gene-targeted knockout mice involves the following steps:

- Isolation and culturing of embryonic stem **(ES) cells** from the inner cell mass of a mouse blastocyst

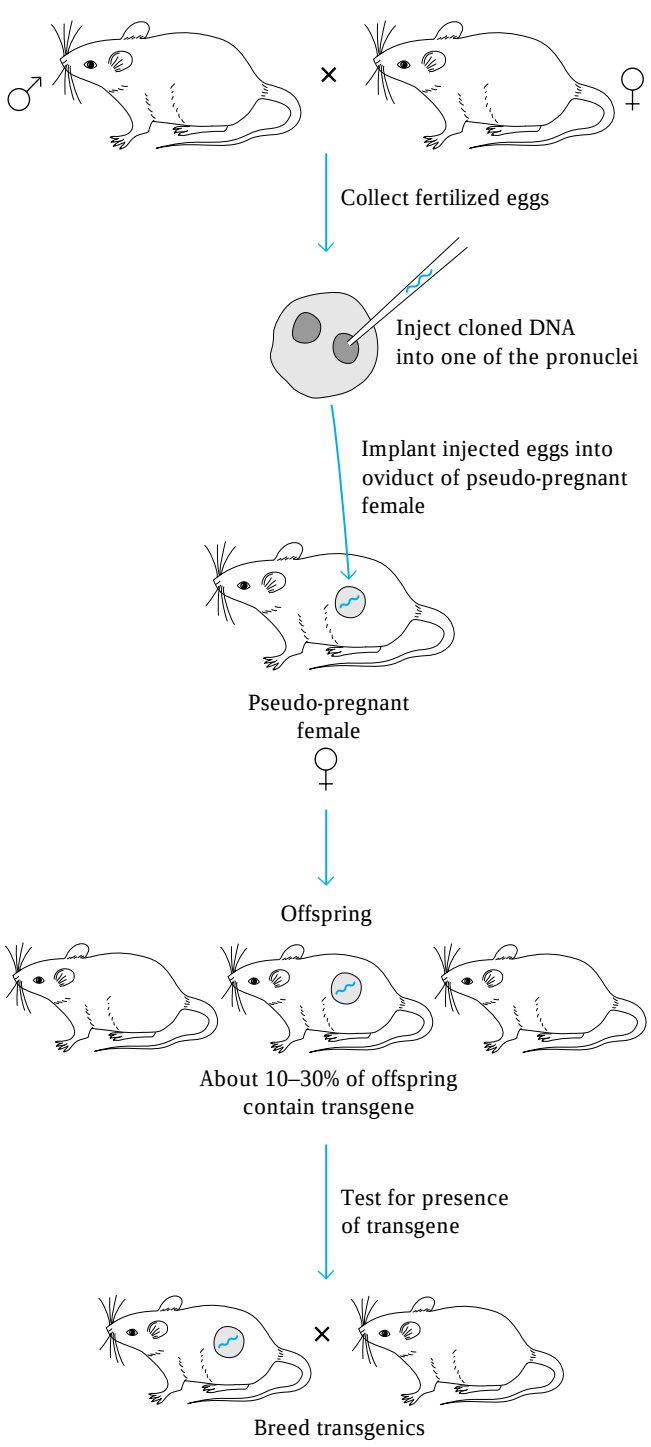


FIGURE **23-15** General procedure for producing transgenic mice. Fertilized eggs are collected from a pregnant female mouse. Cloned DNA (referred to as the transgene) is microinjected into one of the pronuclei of a fertilized egg. The eggs are then implanted into the oviduct of pseudopregnant foster mothers (obtained by mating normal females with a sterile male). The transgene will be incorporated into the chromosomal DNA of about 10%–30% of the offspring and will be expressed in all of their somatic cells. If a tissue-specific promoter is linked to a transgene, then tissue-specific expression of the transgene will result.

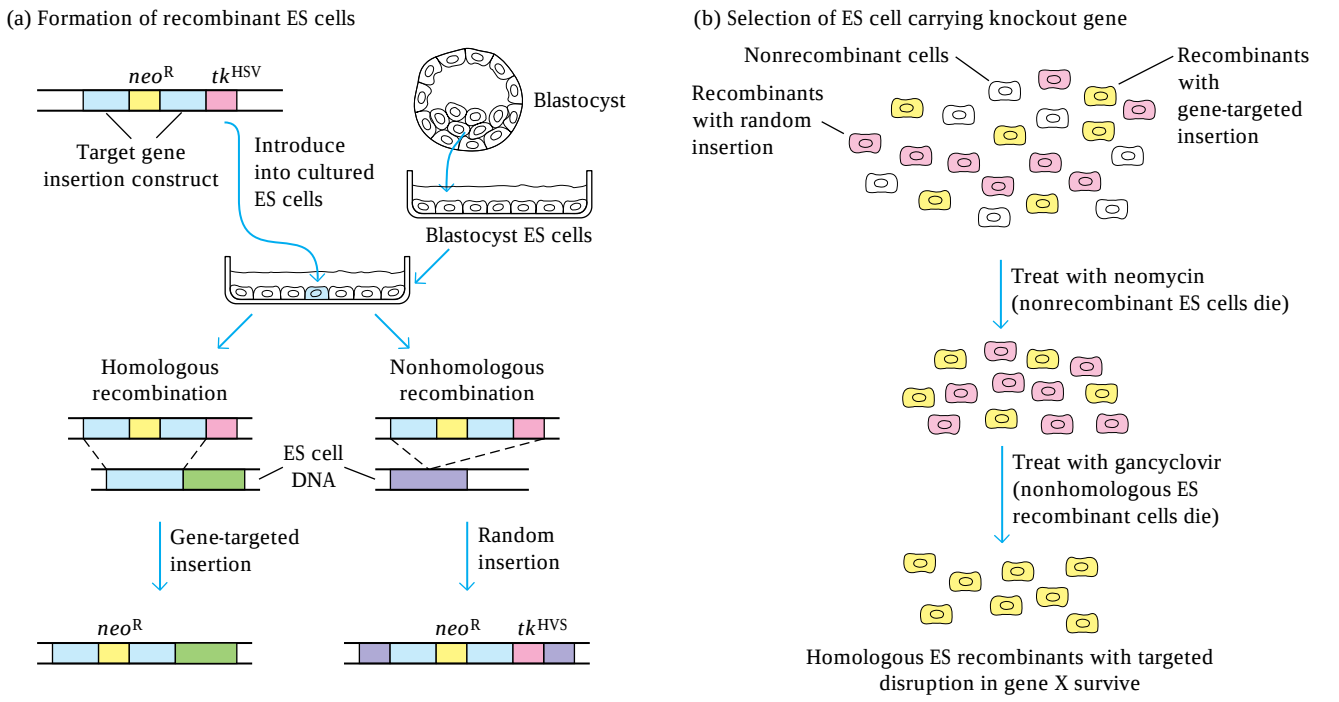


FIGURE **23-16** Formation and selection of mouse recombinant ES cells in which a particular target gene is disrupted. (a) In the engineered insertion construct, the target gene is disrupted with the *neo*^{R} gene, and the thymidine kinase *tk*^{HSV} gene is located outside the target gene. The construct is transfected into cultured ES cells. If homologous recombination occurs, only the target gene and the *neo*^{R} gene will be inserted into the chromosomal DNA of the ES cells. If nonhomologous recombination occurs, all three genes will be inserted. Recombination occurs in only about 1% of the cells, with nonhomologous recombination much more frequent than homologous recombination. (b) Selection with the neomycin-like drug G418 will kill any nonrecombinant ES cells because they lack the *neo*^{R} gene. Selection with gancyclovir will kill the nonhomologous recombinants carrying the *tk*^{HSV} gene, which confers sensitivity to gancyclovir. Only the homologous ES recombinants will survive this selection scheme. *[Adapted from H. Lodish et al., 1995,* Molecular Cell Biology, *3rd ed., Scientific American Books.]*

- Introduction of a mutant or disrupted gene into the cultured ES cells and selection of homologous recombinant cells in which the gene of interest has been knocked out (i.e., replaced by a nonfunctional form of the gene)
- Injection of homologous recombinant ES cells into a recipient mouse blastocyst and surgical implantation of the blastocyst into a pseudo-pregnant mouse
- Mating of chimeric offspring heterozygous for the disrupted gene to produce homozygous knockout mice

The ES cells used in this procedure are obtained by culturing the inner cell mass of a mouse blastocyst on a feeder layer of fibroblasts or in the presence of leukemia-inhibitory factor. Under these conditions, the stem cells grow but remain pluripotent and capable of later differentiating in a variety of directions, generating distinct cellular lineages (e.g., germ cells, myocardium, blood vessels, myoblasts, nerve cells). One of the advantages of ES cells is the ease with which they can be genetically manipulated. Cloned DNA containing a desired gene can be introduced into ES cells in culture by various transfection techniques. The introduced DNA will be inserted by recombination into the chromosomal DNA of a small number of ES cells.

The insertion constructs introduced into ES cells contain three genes: the target gene of interest and two selection genes, such as *neo*^{R}, which confers neomycin resistance, and the thymidine kinase gene from herpes simplex virus (*tk*^{HSV}), which confers sensitivity to gancyclovir, a cytotoxic nucleotide analog (Figure 23-16a). The construct often is engineered with the target-gene sequence disrupted by the *neo*^{R} gene and with the *tk*^{HSV} gene at one end, beyond the sequence of the target gene. Most constructs will insert at random by nonhomologous recombination rather than by gene-targeted insertion through homologous recombination. As illustrated in Figure 23-16b, a two-step selection scheme is used to obtain those ES cells that have undergone homologous recombination, whereby the disrupted gene replaces the target gene.

The ES cells obtained by this procedure are heterozygous for the knockout mutation in the target gene. These cells are clonally expanded in cell culture and then injected into a

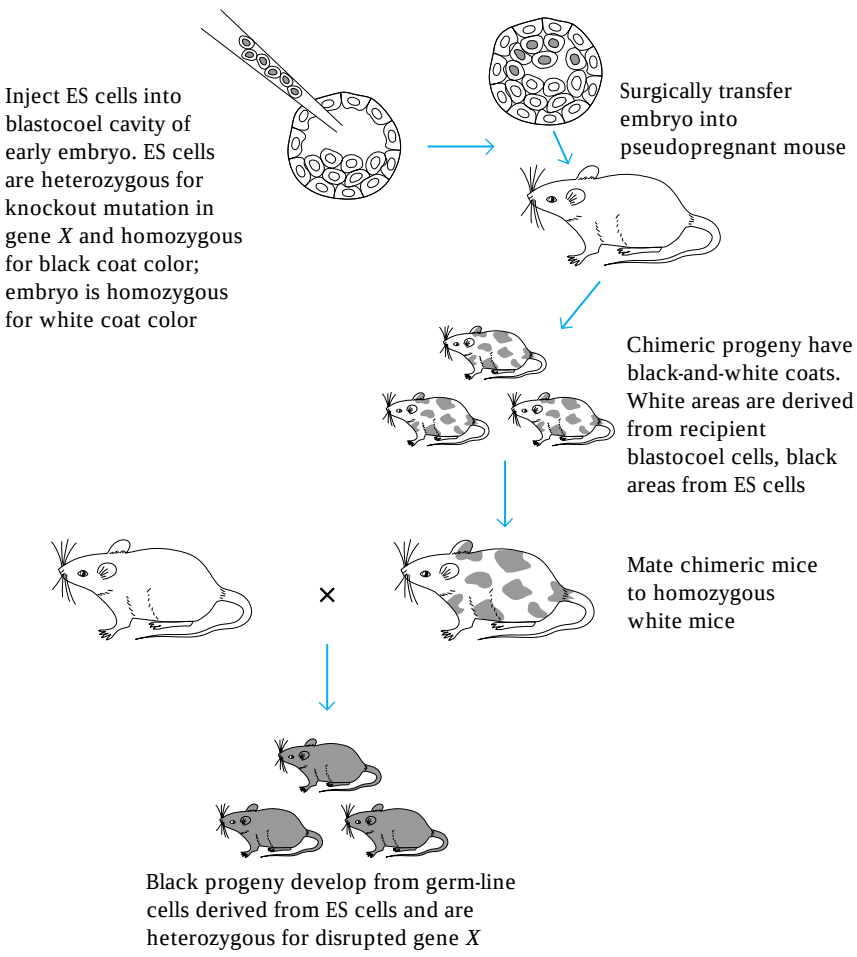


FIGURE 23-17 General procedure for producing homozygous knockout mice. ES cells homozygous for a marker gene (e.g., black coat color) and heterozygous for a disrupted target gene (see Figure 23-18) are injected into an early embryo homozygous for an alternate marker (e.g., white coat color). The chimeric transgenic offspring, which have black-and-white coats, then are mated with homozygous white mice. The all-black progeny from this mating have ES-derived cells in their germ line, which are heterozygous for the disrupted target gene. Mating of these mice with each other produces animals homozygous for the disrupted target gene, that is, knockout mice. *[Adapted from M. R. Capecchi, 1989,* Trends Genet. ***5:****70.]*

mouse blastocyst, which subsequently is implanted into a pseudo-pregnant female. The transgenic offspring that develop are chimeric, composed of cells derived from the genetically altered ES cells and cells derived from normal cells of the host blastocyst. When the germ-line cells are derived from the genetically altered ES cells, the genetic alteration can be passed on to the offspring. If the recombinant ES cells are homozygous for black coat color (or other visible marker) and they are injected into a blastocyst homozygous for white coat color, then the chimeric progeny that carry the heterozygous knockout mutation in their germ line can be easily identified (Figure 23-17). When these are mated with each other, some of the offspring will be homozygous for the knockout mutation.

"Knock-In" Technology Allows the Replacement of an Endogenous Gene

In addition to deleting a gene of choice, it also is possible to replace the endogenous gene with a mutated form of that gene. As in the strategy for knocking out a gene, DNA constructs that carry mutations in a particular gene can be exchanged for the endogenous gene. It also is possible to replace all of an endogenous gene with a DNA sequence of choice. In a recent report, for example, the CD4 gene was replaced with the one for β-galactosidase. In these experiments, the CD4 promoter was left intact to drive the expression of β-galactosidase, which catalyzes the color change of certain reporter chemicals to blue. Because the CD4 promoter drove the expression of β-galactosidase, only those thymic cells destined to express CD4 turned blue in the presence of the reporter chemicals. Data from these experiments were useful in tracing CD4/CD8 lineage commitment in developing T cells.

Inducible Gene Targeting, the Cre/*Lox* System, Targets Gene Deletion

In addition to the deletion of genes by gene targeting, recent experimental strategies have been developed that allow the specific deletion of a gene of interest in precisely the tissue of choice. These technologies rely on the use of site-specific recombinases from bacteria or yeast. The most commonly used recombinase is Cre, isolated from bacteriophage P1. Cre recognizes a specific 34-bp site in DNA known as *loxP* and, upon recognition, catalyzes a recombination. Therefore, DNA sequences that are flanked by *loxP* are recognized by Cre and

the recombinational event results in the deletion of the intervening DNA sequences. In other words, animals that ubiquitously express Cre recombinase will delete all *loxP*-flanked sequences. The real innovation of this technique is that expression of the Cre recombinase gene can be controlled by the use of a tissue specific promoter. This allows tissue-specific expression of the recombinase protein and thus tissue-specific deletion of DNA flanked by *loxP.* For example, one could express Cre in B cells using the immunoglobulin promoter, and this would result in the targeted deletion of *loxP*-flanked DNA sequences only in B cells.

This technology is particularly useful when the targeted deletion of a particular gene is lethal. For example the DNA polymerase β gene is required for embryonic development. In experiments designed to test the Cre/*lox* system, scientists flanked the mouse DNA polymerase β gene with *loxP* and mated these mice with mice carrying a Cre transgene under the control of a T-cell promoter (Figure 23-18a). The results of this mating are offspring that express the Cre recombinase specifically in T cells. Using such mice, the scientists were able to examine the effects of deleting the enzyme DNA polymerase β specifically in T cells. The effects of the deletion of this gene could not be examined in a conventional gene-targeting experiment, because deletion of DNA polymerase β throughout the animal would be lethal. However, with the Cre/*lox* system, it now is possible to examine the effects of the deletion of this gene in a specific tissue of the immune system.

The Cre/*lox* system also can be used to turn on gene expression in a particular tissue. Just as the lack of a particular gene may be lethal during embryonic development, the expression of a gene can be toxic. To examine tissue-specific expression of such a gene, it is possible to insert a translational stop sequence flanked by *loxP* into an intron at the beginning of the gene (Figure 23-18b). Using a tissue-specific promoter driving Cre expression, the stop sequence may be deleted in the tissue of choice and the expression of the potentially toxic gene examined in this tissue. These modifications of gene-targeting technology have been very useful in determining the effects of particular genes in cells and tissues of the immune system.

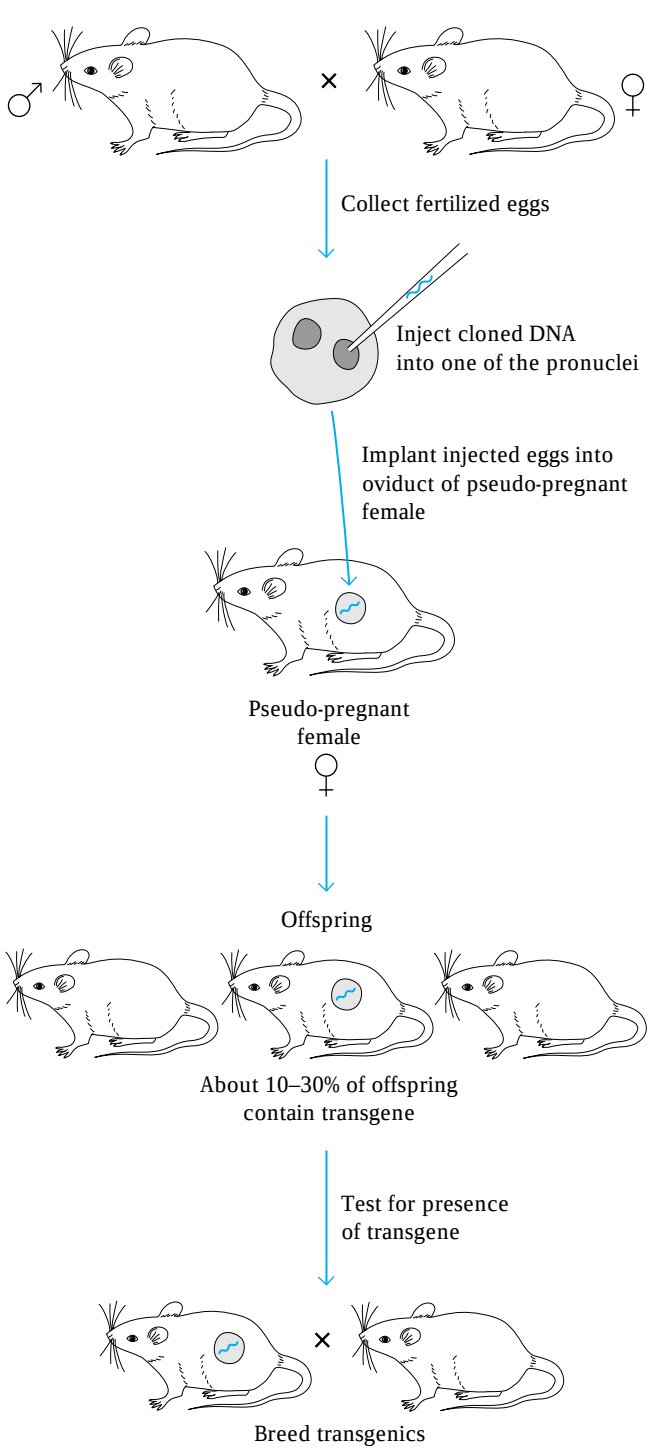


FIGURE **23-18** Gene targeting with Cre/*loxP* (a) Conditional deletion by Cre recombinase. The targeted DNA polymerase β gene is modified by flanking the gene with *loxP* sites (for simplicity, only one allele is shown). Mice are generated from ES cells by standard procedures. Mating of the *loxP*-modified-mice with a Cre transgenic will generate double transgenic mice in which the *loxP*-flanked DNA polymerase β gene will be deleted in the tissue where Cre is expressed. In this example, Cre is expressed in thymus tissue (striped) so that deletion of the *loxP*-flanked gene occurs only in the thymus (white) of the double transgenic. Other tissues and organs still express the *loxP*-flanked gene (orange). (b) Activation of gene expression using Cre/*lox.* A *loxP*-flanked translational STOP cassette is inserted between the promoter and the potentially toxic gene, and mice are generated from ES cells using standard procedures. These mice are mated to a transgenic line carrying the Cre gene driven by a tissue-specific promoter. In this example, Cre is expressed in the thymus, so that mating results in expression of the toxic gene (blue) solely in the thymus. Using this strategy, it is possible to determine the effects of expression of the potentially toxic gene in a tissue-specific fashion. *[Adapted from B. Sauer, 1998,* Methods ***14:****381.]*

Microarrays—An Approach for Analyzing Patterns of Gene Expression

In the past few years, a new approach has emerged designed to assess differences in gene expression between various cell types or the same cells treated in different fashions. This technology, referred to as microarray technology or *gene profiling*, has the ability to rapidly and reliably scan large numbers of different mRNAs. The principle is simple and is derived from what we already know about RNA and DNA hybridization. mRNA is isolated from a given sample. Then, when cDNA synthesis is initiated the first strand of the cDNA is labeled with the tag. This forms the pool of target sequences.

The next step is to hybridize the labeled cDNA to a microarray. There are many microarrays commercially available, which fall mainly into two classes; those composed of cDNA, and those composed of oligonucleotides. Microarrays of cDNAs are, as the name suggests, a collection of cDNA that have been arranged, or arrayed, on a solid substrate in defined locations. The substrate varies but usually is a nylon membrane or a glass slide. If a very small amount of cDNA is used, the spots of cDNA arrayed on the substrate can be as small as 100–300 μm in size; it is relatively simple to array as many as 30,000 cDNAs on a single microscope slide (Figure 23-19a). The actual process of arraying the cDNA is usually accomplished using robotics. The cDNAs are most frequently obtained from available cDNA libraries and, in some cases, are PCR products amplified from the cDNA library using primers specific for certain known genes.

The oligonucleotide arrays are usually a collection of oligos 20–25 nucleotides long (Figure 23-19b). The advantage of this type of array is that one only needs sequences of genes of interest. No cDNA library is needed. However, the cost of assembling such an array is high, since the oligos have to be made and then spotted onto the filter or glass slide. Another problem with this approach is, depending upon the length of the oligo, there can be a degree of non-specific hybridization that hinders the final analysis of the data. This problem can be avoided by making longer oligos—which further increases the cost. For these reasons, oligo arrays are used most often by large pharmaceutical or biotechnology companies.

Although the source of the targets used for both cDNA and oligo arrays are cDNA, the preparation of the target differs depending upon the microarray. The target preparation for cDNA arrays involves labeling the cDNA with different fluorescent dyes such as Cy3 and Cy5 (Figure 28-19a). Cy3 and Cy5 are cyanine-based dyes that are easily conjugated to nucleic acids and are highly stable and emit less background fluorescence than conventional fluorescent dyes. Suppose you wish to compare two different cell types, or one cell type in two different states of activation. cDNA from one population is prepared using mRNA as a template. First strand synthesis of the mRNA is performed using one nucleotide conjugated to Cy3. Then, using mRNA from the second cell population, cDNA is prepared using a nucleotide conjugated to Cy5. These two populations of cDNA, one marked with Cy3 and the other with Cy5, are hybridized to the microarray. If one of the targets hybridizes to a cDNA on the array, a green (Cy3) or red (Cy5) fluorescence emission is detected. If both hybridize to the cDNA, yellow fluorescence is detected (the combination of the red and green emissions from both dyes). The arrays are analyzed by scanning the array at two different wavelengths to distinguish between the Cy3 and Cy5 signals. Once scanned at two wavelengths, the signals are compared and the signal intensity of each dye is determined and compared. The results are presented as a ratio between the two samples.

In the case of oligo-based microarrays, the usual approach is to label the target cDNA with a biotin-labeled nucleotide during first-strand synthesis of the mRNA. The biotin-labeled cDNA is hybridized to the oligo array and detected by the use of the fluorescent strepavidin (Figure 28-19b). The procedure is then repeated with cDNA from the other cell type and another microarray is used. The resultant microarrays are analyzed by either phosphoimaging or fluorescent-based scanning. This is most commonly accomplished using specialized scanners developed for scanning microarrays.

The difference between this procedure and the cDNA-based array described above is that two microarrays are used. This is possible since the method for producing the oligo-based microarrays is more precise and it is possible to ensure that the same oligo will be present in precisely the same position on two separate microarrays. This is not possible with the technology used to prepare cDNA microarrays. Therefore, both targets must be hybridized to the same array to derive an accurate comparison. There is an advantage to using two microarrays. Quantitation of expression levels is easier when using one labeled target per microarray. When two targets are hybridized to the same array, it is always necessary to "subtract" the fluorescence of one target from the other before it is possible to obtain quantitative data. Since only one target is hybridized to a single oligo microarray, subtraction is not necessary.

The application of microarray technology to immunology is apparent. One could easily ask what is the difference between T cells and B cells. Or what is the difference between an activated T cell and a resting T cell? The list of possible comparisons is immense. To begin to answer some of the interesting immunology questions, Louis Staudt and co-workers at the NIH have developed an array they term "Lymphochip." The Lymphochip is an array that consists of more than 10,000 human genes and is enriched in genes expressed in lymphoid cells. It also includes genes from normal as well as transformed lymphocytes. This particular microarray has provided a great deal of useful information, including a profile of T cells compared to B cells, plasma cells compared to germinal center B cells, and gene expression pattens induced by various signaling pathways. The Lymphochip and other clinical applications of microarrays are described in the Clinical Focus box.

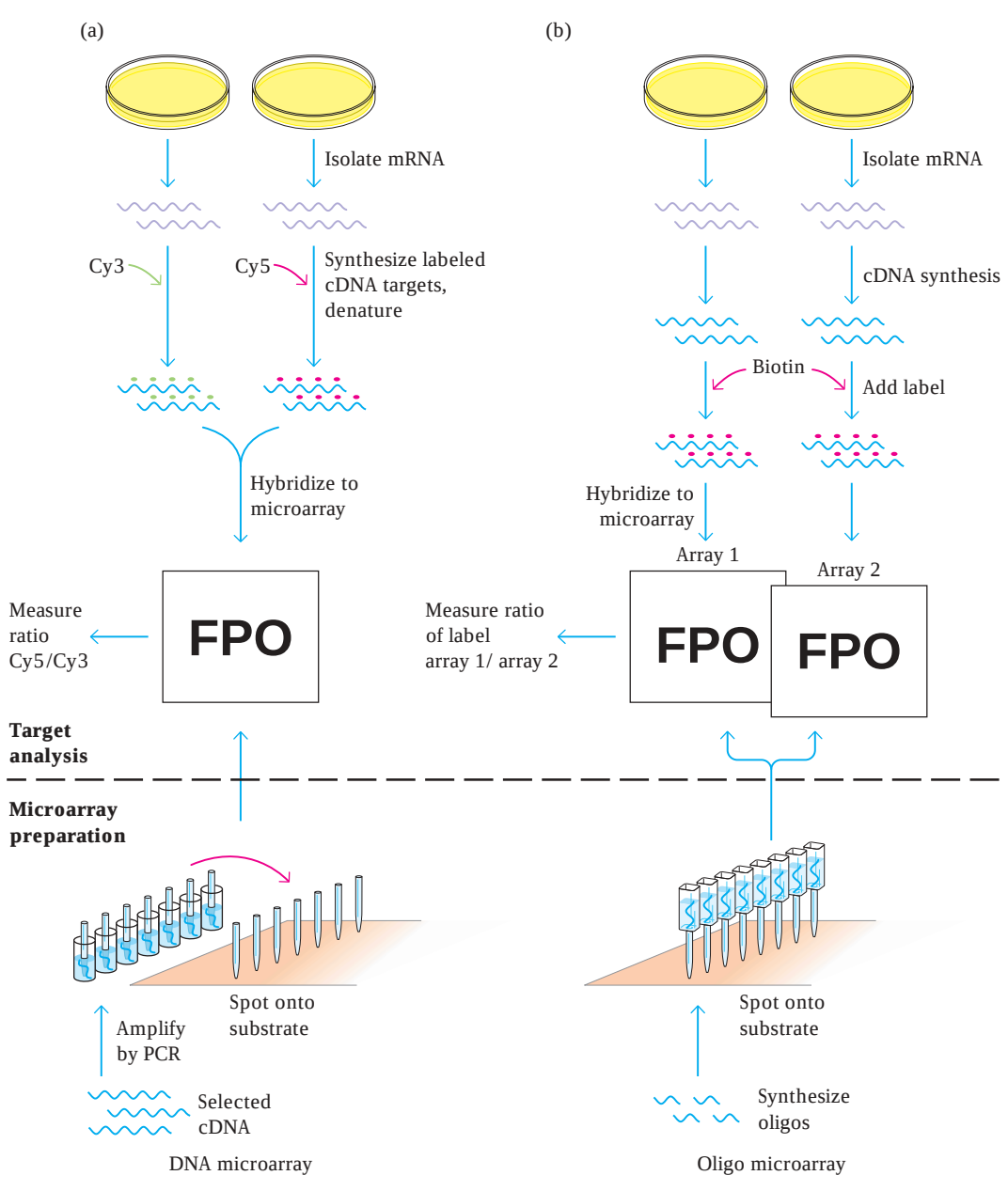


FIGURE 23-19 DNA microarray analysis using cDNA microarrays (a) or high-density oligonucleotide microarrays (b). As described in the text, microarray analysis relies on the isolation of RNA from the tissues or cells to be analyzed, the conversion of RNA into cDNA, and the subsequent labeling of DNA during target preparation. The labeled target sequences are hybridized to either a cDNA microarray (a) or an oligo microarray (b).

SUMMARY

- Inbred mouse strains allow immunologists to work routinely with syngeneic, or genetically identical, animals. With these strains, aspects of the immune response can be studied uncomplicated by unknown variables that could be introduced by genetic differences between animals.
- In adoptive-transfer experiments, lymphocytes are transferred from one mouse to a syngeneic recipient mouse that has been exposed to a sublethal (or potentially lethal) dose of x-rays. The irradiation inactivates the immune cells of the recipient, so that one can study the response of only the transferred cells.
- With in vitro cell-culture systems, populations of lymphocytes can be studied under precisely defined conditions. Such systems include primary cultures of lymphoid cells, cloned lymphoid cell lines, and hybrid lymphoid cell lines. Unlike primary cultures, cell lines are immortal and homogeneous.

CLINICAL FOCUS

Microarray Analysis as a Diagnostic Tool for Human Diseases

It is almost impossible to distinguish visually between B and T cells without molecular analysis. Similarly, it can be quite difficult to distinguish one tumor from another. Two of the best-known acute leukemias are AML, which arises from a myeloid precursor (hence the name, *a*cute *m*yeloid *l*eukemia) and ALL (acute *l*ymphoid *l*eukemia), which arises from lymphoid precursors. Both leukemias are derived from hematopoietic stem cells, but the prognosis and treatment for the two diseases are quite different. Until recently, the two diseases could be diagnosed with some degree of confidence using a combination of surface phenotyping, karotypic analysis, and histochemical analysis, but no single test was conclusive; reliable diagnosis depended upon the expertise of the clinician.

The difference between an ALL diagnosis and an AML diagnosis can mean the difference between life and death. ALL responds best to corticosteroids and chemotherapeutics such as vincristine and methotrexate. AML is usually treated with daunorubicin and cytarabine. The cure rates are dramatically diminished if the less appropriate treatment is delivered due to misdiagnosis.

In 1999, a breakthrough in diagnosis of these two leukemias was achieved using microarray technology. Todd Golub, Eric Lander, and their colleagues isolated RNA from 38 samples of acute leukemia, labeled the RNA with biotin, and hybridized the biotinylated RNA to commercial high-density microarrays that contained oligonucleotides corresponding to some 6817 human genes. Whenever the biotin-labeled RNA recognized a homologous oligonucleotide, hybridization occurred. Analysis revealed a group of 50 genes that were highly associated with either AML or ALL when compared with control samples. These 50 genes were then used to sample nucleic acid from 34 independent leukemias as well as samples from 24 presumed-normal human bone-marrow or blood samples. The result? A set of markers that clearly classified a tumor as ALL or AML.

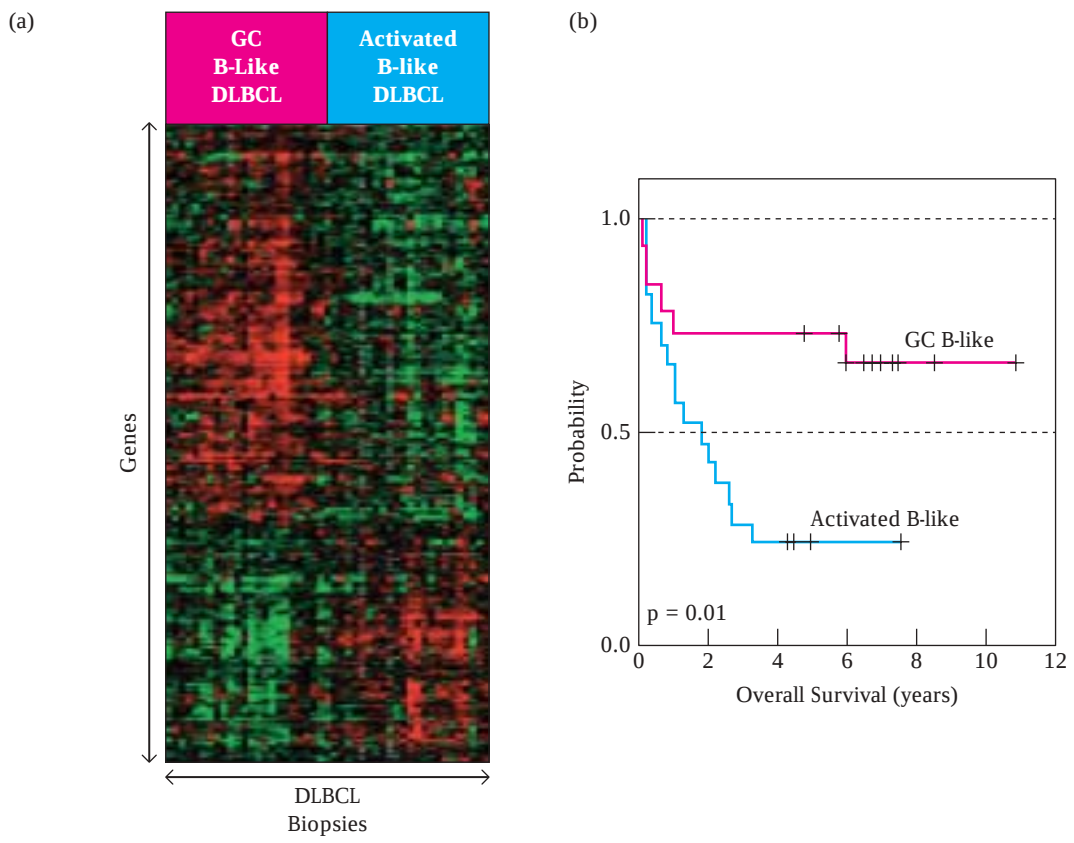
The results of the microarray analysis further suggested that the treatments for AML and ALL can be targeted more precisely. For example, an AML expressing genes *x, y,* and *z* might respond to one treatment modality better than an AML that expresses *a, b,* and *c.* Several pharmaceutical companies have established research groups to evaluate different treatments for tumors based on the tumor's microarray profile. This designer-approach to oncology is expected to produce much more effective treatments of individual tumors, and ultimately, enhanced survival rates.

Microarray analysis is likely to be very useful in the diagnosis of tumors of the immune system. Most notably, a laboratory at the National Institutes of Health (NIH) has developed a specialized DNA microarray containing more than 10,000 human cDNAs that are enriched for genes expressed in lymphocytes. Some of these cDNAs are from genes of known function, others are unknown cDNAs derived from normal or malignantly transformed lymphocyte cDNA libraries. This specialized array is called the "Lymphochip" because the lymphocyte cDNAs are arrayed on a silicon wafer. The group at NIH asked whether they could use the Lymphochip to divide the B-cell leukemia known as diffuse large B-cell lymphoma (DLBCL) into subgroups, an important question because this type of lymphoma has a highly variable clinical course, with some patients responding well to treatment while others respond poorly. Earlier attempts to define subgroups within this group had been unsuccessful. A definition of subgroups within DLBCL could be useful in designing more effective treatments. Using the Lymphochip, the group at NCI identified two genotypically distinct subgroups of DLBCL. One group was comprised of tumors expressing genes characteristic of germinal-center B cells and was called "germinal-center–B-like DLBCL (see Figure). The other group more resembled activated B cells and was termed "activated B-like DLBCL." Significantly, patients with germinal-center–B-like DLBCL had a higher survival rate than those with activated B-like DLBCL. Normally all patients with DLBCL receive multi-agent chemotherapy. Patients who do not respond well to chemotherapy are then considered for bone-marrow transplantation. The data obtained from this study suggests that patients with activated B-like DLBCL will not respond as well to chemotherapy and may be better served

- Biochemical techniques provide tools for labeling important proteins of the immune system. Labeling antibodies with molecules such as biotin and avidin allows accurate determination of the level of antibody response. Gel electrophoresis is a convenient tool for separating and determining the molecular weight of a protein.
- The ability to identify, clone, and sequence immune-system genes using recombinant DNA techniques has revolutionized the study of all aspects of the immune response. Both cDNA, which is prepared by transcribing mRNA with reverse transcriptase, and genomic DNA can be cloned. Generally, cDNA is cloned using a plasmid vector; the re-

by bone-marrow transplantation shortly after diagnosis. As a direct result of this work, ongoing clinical trials are evaluating how best to treat patients with activated B-like DLBCL.

Gene profiling is not restricted to diagnosis of cancer. This technology provides us with a unique opportunity to examine differences between any distinct populations of cells. One can compare which genes are expressed in common or differentially in a naïve T cell and a memory T cell. What is the difference between a normal T cell and a T cell dying by apoptosis? Comparisons like these will be a rich source of insight into differences in cell populations. The key to using this valuable information will be the development of tools to analyze the vast quantities of data that can be obtained from this new approach.



Diffuse large B-cell lymphoma (DLBCL) is at least two distinct diseases. (a) Shown are differences in gene expression between samples taken from patients with either germinal center B-like DLBCL (left, orange) or activated B-like DLBCL (right, blue). Relative expression of the 100 genes (*y* axis) that discriminate most significantly between the two DLBCL types is depicted over a 16-fold range using the graded color scale at bottom. Note the strikingly different gene expression profiles of the two diseases. (b) Plot of overall DLBCL patient survival following chemotherapy. Gene expression profiles of tumor-biopsy samples allow the assignment of patients to the correct prognostic categories and may aid in the treatment of this complex disease. *[Adapted from L. M. Staudt, 2002. Gene expression profiling of lymphoid malignancies.* Annu. Rev. Med. ***53****:303-318.]*

combinant DNA containing the gene to be cloned is propagated in *E. coli* cells. Genomic DNA can be cloned within a bacteriophage vector or a cosmid vector, both of which are propagated in *E. coli.* Even larger genomic DNA fragments can be cloned within bacteriophage P1 vectors, which can replicate in *E. coli*, or yeast artificial chromosomes, which can replicate in yeast cells. Polymerase chain reaction (PCR) is a convenient tool for amplifying small quantities of DNA.

- Transcription of genes is regulated by promoter and enhancer sequences; the activity of these sequences is

controlled by DNA-binding proteins. Footprinting and gel-shift analysis can be used to identify DNA-binding proteins and their binding sites within the promoter or enhancer sequence. Promoter activity can be assessed by the CAT assay.

- Cloned genes can be transfected (transferred) into cultured cells by several methods. Commonly, immune-system genes are transfected into cells that do not normally express the gene of interest. Cloned genes also can be incorporated into the germ-line cells of mouse embryos, yielding transgenic mice, which can transmit the incorporated transgene to their offspring. Expression of a chosen gene can then be studied in a living animal. Knockout mice are transgenics in which a particular target gene has been replaced by a nonfunctional form of the gene, so the gene product is not expressed. The Cre/*lox* system provides a mechanism that allows tissue-specific expression or deletion of a particular gene.
- Microarrays are a powerful approach for the examination of tissue-specific gene expression and comparison of gene expression in different cells. It has already begun to revolutionize the study of gene regulation and gene expression.

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USEFUL WEB SITES

http://www.biomednet.com/db/mkmd

Access to all known knockouts in mice, updated regularly.

http://www.jax.org/

Home page for The Jackson Laboratory, the major repository of inbred mice in the world.

http://www.neb.com/

Home page for New England Biolabs, a molecular biology company. Useful information concerning restriction enzymes is found at this site, under Technical Resources.

http://www.public.iastate.edu/~pedro/research_tools.html

A very useful site for molecular biology, containing links to many informative sites. Updated regularly.

Study Questions

CLINICAL FOCUS QUESTION How has microarray technology changed disease diagnosis and how is it likely to influence treatment of diseases in the future?

1. Explain why the following statements are false.
   a. The amino-acid sequence of a protein can be determined from the nucleotide sequence of a genomic clone encoding the protein.
   b. Transgenic mice can be prepared by microinjection of DNA into a somatic-cell nucleus.
   c. Primary lymphoid cultures can be propagated indefinitely and are useful in studies of specific subpopulations of lymphocytes.

2. Fill in the blanks in the following statements with the most appropriate terms:
   a. In inbred mouse strains, all or nearly all genetic loci are _____ ; such strains are said to be _____ .

b. SCID mice have a genetic defect that prevents development of functional _____ and _____ cells.

c. B-cell hybridomas are formed by fusion of _____ with _____. They are capable of _____ growth and are used to produce _____.

d. A normal lymphoid cell that undergoes _____ can give rise to a cell line, which has an _____ life span.

3. The gene diagrammed below contains one leader (L), three exons (E), and three introns (I). Illustrate the primary transcript, mRNA, and the protein product that could be generated from such a gene.

4. The term *transfection* refers to which of the following?

a. Synthesis of mRNA from a DNA template

b. Synthesis of protein based on an mRNA sequence

c. Introduction of foreign DNA into a cell

d. The process by which a normal cell becomes malignant

e. Transfer of a signal from outside a cell to inside a cell

5. Which of the following are required to carry out the PCR?

a. Short oligonucleotide primers

b. Thermostable DNA polymerase

c. Antibodies directed against the encoded protein

d. A method for heating and cooling the reaction mixture periodically

e. All of the above

6. Why is it necessary to include a selectable marker gene in transfection experiments?

7. What would be the result if a transgene were injected into one cell of a four-cell mouse zygote rather than into a fertilized mouse egg before it divides?

8. A circular plasmid was cleaved with *Eco*RI, producing a 5.4-kb band on a gel. A 5.4-kb band was also observed when the plasmid was cleaved with *Hind*III. Cleaving the plasmid with both enzymes simultaneously resulted in a single band 2.7 kb in size. Draw a diagram of this plasmid showing the relative location of its restriction sites. Explain your reasoning.

9. DNA footprinting is a suitable technique for identifying which of the following?

a. Particular mRNAs in a mixture

b. Particular tRNAs in a mixture

c. Introns within a gene

d. Protein-binding sites within DNA

e. Specific DNA sites at which restriction endonucleases cleave the nucleotide chain

10. Explain briefly how you might go about cloning a gene for interleukin 2 (IL-2). Assume that you have available a monoclonal antibody specific for IL-2.

11. You have a sample of a mouse DNA-binding protein and of the mRNA that encodes it. Assuming you have a mouse genomic library available, briefly describe how you could select a clone carrying a DNA fragment that contains the gene that encodes the binding protein.

12. What are the major differences between transgenic mice and knockout mice and in the procedures for producing them?

13. How does a knock-in mouse differ from a knockout mouse?

14. How does the Cre/*lox* technology enhance knockout and knock-in strategies?

15. For each term related to recombinant DNA technology (a–i), select the most appropriate description (1–10) listed below. Each description may be used once, more than once, or not at all.

Terms

a. _____ Yeast artificial chromosome

b. _____ Restriction endonuclease

c. _____ cDNA

d. _____ COS sites

e. _____ Retrovirus

f. _____ Plasmid

g. _____ cDNA library

h. _____ Sticky ends

i. _____ Genomic library

Descriptions

(1) Cleaves mRNA at specific sites.

(2) Cleaves double-stranded DNA at specific sites.

(3) Circular genetic element that can replicate in *E. coli* cells.

(4) Used to clone DNA in mammalian cells.

(5) Formed from action of reverse transcriptase.

(6) Collection of DNA sequences within plasmid vectors representing all of the mRNA sequences derived from a cell.

(7) Produced by action of certain DNA-cleaving enzymes.

(8) Used to clone very large DNA sequences.

(9) Used to introduce larger-than-normal DNA fragments in λ-phage vectors.

(10) Collection of λ clones that includes all the DNA sequences of a given species.