

## EXPERIMENTAL PATHWAYS

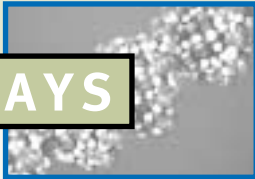
### Coupling Oxidation to Phosphorylation

A link between oxidation and phosphorylation was first proposed by the Russian biochemist Vladimir Engelhardt in 1932. Engelhardt found that cells placed under anaerobic conditions rapidly depleted their store of ATP. When aerobic conditions were restored, ATP was rapidly resynthesized.<sup>1</sup> The manner in which two basic biological processes—the oxidation of substrates and the phosphorylation of ADP—are coupled to one another became one of the most important and controversial areas of research in cell biology. Attention turned to a basic question: How is the energy released by the movement of electrons along the respiratory chain made available for use in ATP synthesis?

The first major hypothesis to explain the coupling of phosphorylation to oxidation was based on experience gained from research on other energy-coupled reactions. In 1953, E. C. Slater of Cambridge University proposed that the energy released by electron transport was trapped in a high-energy chemical intermediate that was subsequently used in a reaction forming ATP.<sup>2</sup> Note in Figure 3.27 that numerous biological compounds have a higher phosphate transfer potential than ATP. Any compound of this type could act as a high-energy phosphate donor in the phosphorylation of ADP. The search for the high-energy chemical intermediate in oxidative phosphorylation occupied the activities of numerous laboratories for at least a decade, but no such compound was ever discovered.

In 1961, Peter Mitchell of the University of Edinburgh proposed a radically different mechanism, which he called **chemiosmosis**, to explain the coupling of oxidation to phosphorylation. Most of the earlier researchers in the field of bioenergetics were biochemists who were accustomed to studying reactions as they occur in a soluble medium, whether in a test tube or the fluid phase of the cell. But oxidative phosphorylation had been shown to be intimately associated with cellular membranes—either the inner membrane of mitochondria or the plasma membrane of aerobic bacteria. Mitchell realized that membrane structure would have to be a key factor in any hypothesis to explain oxidative phosphorylation. Mitchell's earlier research on membrane transport led him to appreciate the fact that substances (whether electrons, ions, or sugars) were moved in a specific direction (*vectorially*) from one side of a membrane to the other side. Some of the earlier work that led Mitchell to consider vectorial movements as a key to oxidative phosphorylation is reviewed by R. N. Robertson.<sup>3</sup>

In his chemiosmosis theory, Mitchell proposed that the movement of electrons down the respiratory chain results in the translocation of protons from one side of the inner mitochondrial membrane to the other.<sup>4,5</sup> Mitchell came to this conclusion after considering the nature of electron carriers (page 195). There are two types of carriers that make up the respiratory chain: ones that accept both protons and electrons and ones that can accept only electrons. In the chemiosmotic hypothesis, Mitchell stressed the importance of the spatial arrangement of the various carriers within the energy-transducing membrane. He suggested that those carriers that



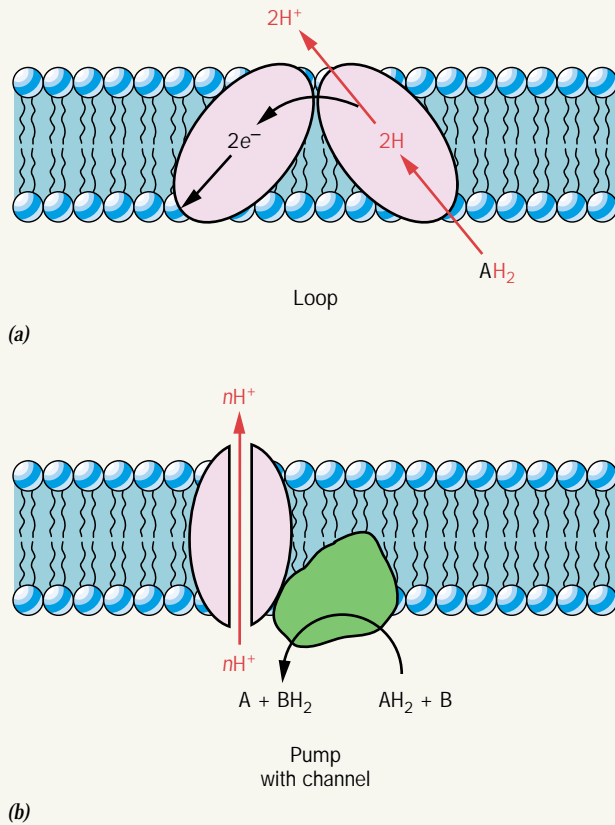
bind both protons and electrons (such as flavoproteins) would be present on the inner side of the mitochondrial membrane so as to remove protons from the matrix. For these protons to be released on the other side, the electrons must be passed to carriers on the outer side of the membrane that would not accept the protons (such as cytochromes). Given the proper organization of carriers within the membrane, electrons could be passed along the entire chain to oxygen, while protons would simply be shuttled across the membrane in one direction. Each passage of an electron from a carrier on the inner side to one on the outer side and back to the inner side again would constitute a redox loop (see Figure 2a). Initially, the chemiosmotic hypothesis predicted the existence of three such loops, as shown in Figure 1. In the first loop, for example, a pair of hydrogen atoms is carried by FMN; the electrons are transferred to an iron-sulfur protein, while the protons are discarded on the outer (cytosolic) side of the membrane.

In the chemiosmotic hypothesis, the movement of protons across the membrane leads to the establishment of an electrochemical gradient that represents a high-energy intermediate linking substrate oxidation to ADP phosphorylation. Mitchell proposed that, for the gradient to be maintained, the inner mitochondrial membrane was highly impermeable to protons as well as other ionic species. At the same time, he recognized that, depending on the types of transport systems within the membrane, the translocation of protons could lead to either a pH gradient or an electrical gradient, or a combination of both (page 202).

A good hypothesis makes specific predictions that can be tested experimentally. The chemiosmotic hypothesis made a number of predictions that stimulated a huge research effort in the field of bioenergetics, much of it aimed at disproving the radical new hypothesis. Included among the predictions of the chemiosmotic hypothesis were the following:

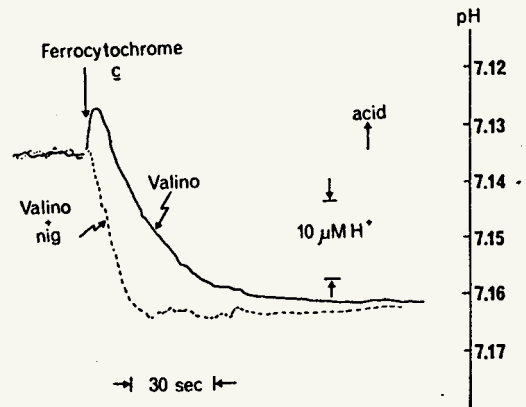
1. The oxidation of substrate and subsequent transport of electrons should be accompanied by the vectorial movement of protons.
2. Electron carriers should be arranged within the inner membrane to form redox loops by which protons could be picked up on one side of the membrane and released on the other side.
3. The inner mitochondrial membrane should be highly impermeable to protons. Furthermore, uncoupling agents, such as dinitrophenol, might act by making the membrane permeable to protons, thus abolishing the electrochemical gradient and destroying the organelle's ability to generate ATP.
4. Actively respiring mitochondria should exhibit a measurable proton gradient and/or an electric potential across their inner membrane.
5. The presence of a proton electrochemical gradient across the inner mitochondrial membrane should provide the energy required to drive the phosphorylation of ADP.





**Figure 2** The loop (a) and pump (b) models of proton translocation. (REPRINTED FROM Y. KAGAWA, *BIOCHIM. BIOPHYS. ACTA* 505:76, 1978. RECONSTITUTION OF THE ENERGY TRANSFORMER, GATE AND CHANNEL SUBUNIT REASSEMBLY, CRYSTALLINE ATPASE AND ATP SYNTHESIS. COPYRIGHT 1978, WITH PERMISSION OF ELSEVIER SCIENCE.)

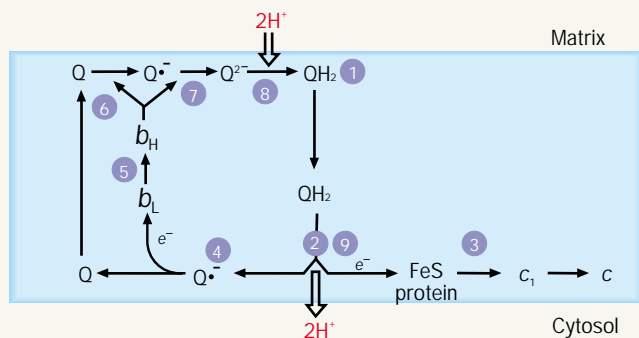
The most direct evidence for the existence of proton pumps within the respiratory chain has come from research in the laboratory of Mårten Wikström of the University of Helsinki beginning in the early 1970s. Wikström focused his attention on cytochrome oxidase, which, according to the original chemiosmotic hypothesis, constitutes the electron-carrying limb of the third redox loop (Figure 1). Because cytochrome oxidase consists of redox centers (hemes and copper ions) that carry only electrons, it should be impossible according to the original chemiosmotic hypothesis for this component of the respiratory chain to translocate protons. Yet Wikström and his colleagues demonstrated that cytochrome oxidase does indeed move protons across a membrane, both within the mitochondrial membrane and when incorporated into artificial vesicles.<sup>11,12</sup> To incorporate the enzyme into vesicles, purified cytochrome oxidase was dispersed into a medium containing detergent-solubilized phospholipids. When the detergent molecules were removed by dialysis, vesicles formed that contained cytochrome oxidase embedded within a phospholipid bilayer. When reduced cytochrome *c* was added to a preparation of these vesicles, electrons were transferred from the cytochrome *c* molecules to  $O_2$ . If cytochrome oxidase acted only to transfer electrons, one would expect the surrounding medium to become alkaline



**Figure 3** Proton ejection from membrane vesicles containing cytochrome *c* oxidase. The vesicles were suspended in unbuffered 0.1 M KCl. After 5-minute preincubation, 10  $\mu$ l of a suspension of the electron donor ferrocyclochrome *c* (i.e., reduced cytochrome *c*) was added to a final concentration of 2  $\mu$ M. Addition of ferrocyclochrome led to an initial ejection of protons, making the medium more acidic. Ejection was counteracted by uncoupling agents. The number of protons ejected initially exceeded the concentration of cytochrome oxidase by almost two orders of magnitude, indicating that the ejected protons derive from the inner aqueous space of the vesicles and that the proton-transport function of cytochrome oxidase is most probably an intrinsic property of the complex. (Valinomycin (valino) and nigericin (nig) are ionophores, compounds that move ions across membranes. Valinomycin is added to allow  $K^+$  ions to move across the membrane in response to the membrane potential formed by proton translocation. Nigericin, which acts as an uncoupler by moving  $H^+$  ions across the membrane, blocks the initial acidification of the medium.) (REPRINTED FROM M. K. F. WIKSTRÖM AND H. T. SAARI, *BIOCHIM. BIOPHYS. ACTA* 462:354, 1977. THE MECHANISM OF ENERGY CONSERVATION AND TRANSDUCTION BY MITOCHONDRIAL CYTOCHROME *C* OXIDASE. COPYRIGHT 1977, WITH PERMISSION OF ELSEVIER SCIENCE.)

due to the consumption of protons as  $O_2$  was reduced to  $H_2O$ . Instead, the medium became acidic (Figure 3), indicating that protons were being expelled from the vesicles. The rate of acidification paralleled the rate of electron flow from cytochrome *c* to  $O_2$ , and acidification was abolished by uncouplers, such as dinitrophenol. These results provided strong evidence of the inherent capacity of cytochrome oxidase to translocate protons across a membrane.

Mitchell did not accept the conclusion that cytochrome oxidase was a proton pump. As it became increasingly evident that the respiratory chain contained only two carriers (FMN and UQ of Figure 1) capable of combining with both protons and electrons and, therefore, could only contain two traditional redox loops, Mitchell modified the chemiosmotic model. He proposed that the additional pair of protons, which were originally thought to be translocated by the third redox loop, were instead shuttled across the membrane by means of a "Q cycle" that operated in the cytochrome *bc\_1* complex (Figure 4). In the Q cycle, the two

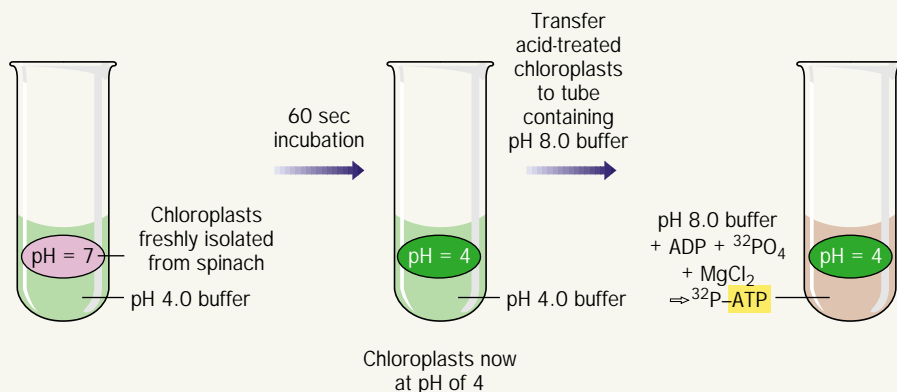


**Figure 4** The Q cycle. In the late 1960s, Mitchell introduced the idea of a Q cycle that is able to translocate an additional pair of protons to make up for the absence of a third loop involving cytochrome oxidase. Shown here in its current form, the Q cycle has been widely adopted to explain the passage of protons through the cytochrome  $bc_1$  (complex III) portion of the respiratory chain. A reduced ubiquinol ( $QH_2$ ) enters the complex (step 1) from the quinone pool and is oxidized at the cytosolic side of the membrane (step 2), releasing its pair of  $H^+$  ions. The two electrons carried by the  $QH_2$  travel along very different pathways. One of the electrons is transferred to an iron-sulfur protein, then to cytochrome  $c_1$  (step 3), and eventually to cytochrome  $c$ . The other electron, which remains associated with a ubisemiquinone, ( $Q\cdot^-$ , step 4), is transferred through the two heme groups of cytochrome  $b$  (step 5) back to the matrix side of the membrane. The electron carried by the heme is used to partially reduce a ubiquinone (step 6), forming a ubisemiquinone molecule at the matrix side of the membrane. Steps 1–6 are then repeated by the oxidation of another ubiquinol, which releases two more  $H^+$  ions into the cytosol and delivers a second electron to both cytochrome  $c$  and cytochrome  $b$  (this repeating series of reactions is not shown). The second electron passed through cytochrome  $b$  is used to reduce ubisemiquinone ( $Q\cdot^-$ ) to the ubiquinol state ( $Q^{2-}$ ) (step 7). The  $Q^{2-}$  picks up a pair of  $H^+$  ions from the matrix (step 8) to form another  $QH_2$ . The newly formed  $QH_2$  diffuses back through the membrane, where it is oxidized, releasing another pair of  $H^+$  ions from the cytosolic side of the membrane (step 9). Thus, as a result of the Q cycle, an extra proton is released into the cytosol for each electron transferred to cytochrome  $c$ .

electrons carried by ubiquinol follow separate pathways in which each electron ultimately leads to the translocation of two protons. According to Mitchell's revised proposal, cytochrome oxidase remained as an electron-carrying limb of the last redox loop. Mitchell's proposal of a Q cycle proved to be another farsighted suggestion, because a large body of evidence now indicates that cytochrome  $bc_1$  (complex III) does indeed translocate protons by means of this type of cycle. A current version of the Q cycle is shown in Figure 4. At the same time, however, the operation of the Q cycle has no relationship to cytochrome oxidase, which has been clearly shown to operate as a proton pump.

The most important feature of the chemiosmotic hypothesis originally proposed by Mitchell was the idea that the energy released by electron transport was stored as an electrochemical proton-based gradient across the inner membrane. If this proposition is correct, then establishing a gradient by artificial means that is unrelated to electron transport should also drive ATP formation. This prediction was tested in 1966 in an ingenious experiment conducted by Andre Jagendorf and Ernest Uribe of Johns Hopkins University (Figure 5).<sup>13</sup>

As discussed in the following chapter, chloroplasts generate ATP by a process, termed photophosphorylation, that uses the same basic mechanism that powers oxidative phosphorylation in mitochondria. Thus the chemiosmotic hypothesis applies just as well to chloroplasts (and to the plasma membranes of aerobic bacteria) as it does to mitochondria. Jagendorf had demonstrated earlier that illumination of isolated chloroplasts generates a proton gradient across the chloroplast membranes such that the inside is acidic relative to the outside. To artificially impose a gradient across the membranes of chloroplasts, Jagendorf and Uribe prepared isolated chloroplasts from spinach cells and suspended them *in the dark* in a tube containing a pH 4 buffer for about 60 seconds (Figure 5), the time required for the protons of the medium to cross the chloroplast membrane so as to lower the pH within that compartment. After the internal compartments of the chloroplasts had dropped to approximately pH 4, the acidified chloroplasts were injected in the dark into a second tube containing a medium buffered at pH 8, along with the substances necessary to make radioactively labeled ATP. Transfer of the chloroplasts to the alkaline buffer created a transitory, 10,000-fold  $H^+$  gradient ( $10^{-8}$  M  $[H^+]$  vs.  $10^{-4}$  M  $[H^+]$ ) across the internal chloroplast membranes. Within a few seconds of the



**Figure 5** The production of ATP in isolated chloroplasts in response to an artificial pH gradient.

Table 1

Kinetics of Alkaline Decay  
and of Phosphorylation of ADP

Reaction	—Yield of ATP at Time (in sec)—					
	0	2	4	6	30	60
pH 8 Decay	41	7	1.7	1.1	0.4	—
ADP + P → ATP	3	27	48	47	42	47

Source: A. Jagendorf and E. Uribe, *Proc. Natl Acad. Sci. U.S.A.* 55:173, 1966.

**Table 1** ATP is given in nmol/mg chlorophyll. The alkaline decay (line 1) was measured by injecting the acid chloroplasts into a medium buffered at pH 8, then waiting the indicated number of seconds before adding ADP,  $Mg^{2+}$ , and  $^{32}P$ -labeled phosphate, followed by a standard 15 seconds with these reagents before stopping the reaction by adding trichloroacetic acid (TCA). The second line showing the phosphorylation time course was determined by injecting acidified chloroplasts into the complete phosphorylation reaction mixture and waiting the indicated number of seconds before stopping the reaction with TCA. In the top line, it is evident that the longer the wait before adding the chloroplasts, the less ATP was synthesized because the pH gradient was being dissipated. In the second line, the amount of ATP synthesized increased as the time of incubation in the labeled reaction mixture increased, up to a point at about 4 seconds, when no further increase was evident because, once again, the pH gradient had been dissipated during the period of ATP synthesis.

time of transfer, newly synthesized, labeled ATP could be detected (line 2, Table 1). The results indicated that a pH gradient, by itself, was capable of driving the phosphorylation of ADP.

A second ingenious experiment that illustrated the powers of a proton gradient in the formation of ATP was carried out by Efraim Racker and Walter Stoekenius in 1974.<sup>14</sup> Recall from page 161 that purple bacteria of the genus *Halobacterium* possess a protein called bacteriorhodopsin that acts as a light-driven proton pump. When bacteriorhodopsin is incorporated into artificial phospholipid vesicles (liposomes), the protein establishes a proton gradient across the vesicle membrane following illumination. When artificial vesicles were prepared that contained both bacteriorhodopsin and the purified mitochondrial ATP synthase, illumination of the vesicles in the presence of ADP and  $P_i$  was accompanied by the formation of ATP. Clearly, the proton gradient generated by a light-driven bacterial pump was driving the phosphorylation of ADP using the enzymatic machinery of the mitochondrion.

The results of these last two experiments are important in a number of ways. Not only do they demonstrate that an electrochemical gradient can provide the free energy for ATP

formation, but they also indicate that electron transport and ATP formation need not be *directly* coupled. In these experiments, no electron transport is taking place; therefore, phosphorylation can occur independently from electron transport. Electron transport normally generates a high-energy state that is used to drive phosphorylation, but there are no particular conditions of that high-energy state that cannot be met by a gradient formed in other ways.

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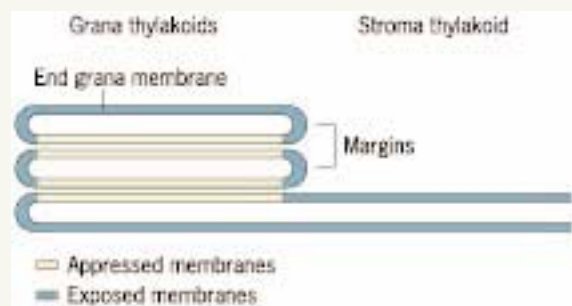
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## EXPERIMENTAL PATHWAYS

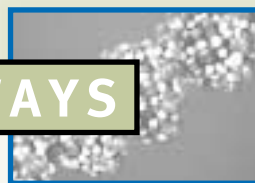
### Organization of the Thylakoid Membrane

With the discovery that photosynthesis required the cooperation of two distinct photosystems connected by an electron transport chain, it was assumed that all of the components involved in electron transport resided in very close proximity to one another within the thylakoid membrane. As indicated in Figure 6.3 and discussed in the accompanying text, the thylakoids of higher plants normally occur as part of interconnected stacks (or grana). The membrane of the stroma thylakoids (the lamellae that interconnect the grana) of higher plants is referred to as *nonappressed membrane* because it resides in contact with the stroma rather than being pressed against another membrane. In contrast, most of the grana thylakoid membrane is referred to as *appressed membrane* because its outer surface is pressed against the outer surface of an adjacent thylakoid. Exceptions are the lateral margins and upper and lower surfaces of the grana thylakoids, which are also in contact with the stroma (Figure 1).

During the 1960s and 1970s, techniques were developed whereby appressed and nonappressed membranes could be separated from one another and their protein composition determined.<sup>1</sup> The results of these, and other studies, led to a model proposed by James Barber of Imperial College in London,<sup>2</sup> and by Bertil Andersson and Jan Anderson at a research institute in Australia<sup>3</sup> that stressed the lack of uniformity of the various components of the thylakoid membranes (Figure 2). In this model, PSI and ATP synthase are located almost exclusively in nonappressed membrane, whereas PSII is segregated primarily in the appressed regions. The only major protein complex distributed evenly between the two types of membrane regions is the cytochrome *b<sub>6</sub>f* complex, which lies in the electron transport chain connecting PSII with PSI (see Figure 6.13).<sup>4</sup>

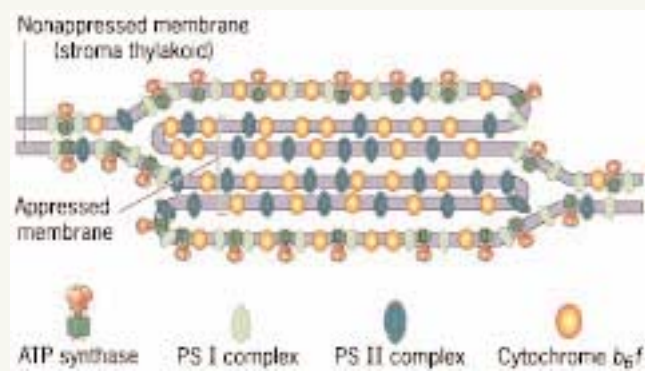


**Figure 1** The outer membrane surface of the thylakoids can be divided into two structurally different regions: the appressed membranes of the grana thylakoids whose surface is in contact with other membranes, and the nonappressed (exposed) membranes of the stroma thylakoids and the ends and margins of the grana thylakoids that are also exposed to the stroma. (FROM J. M. ANDERSON, FEBS LETT., 124:1, 1981.)

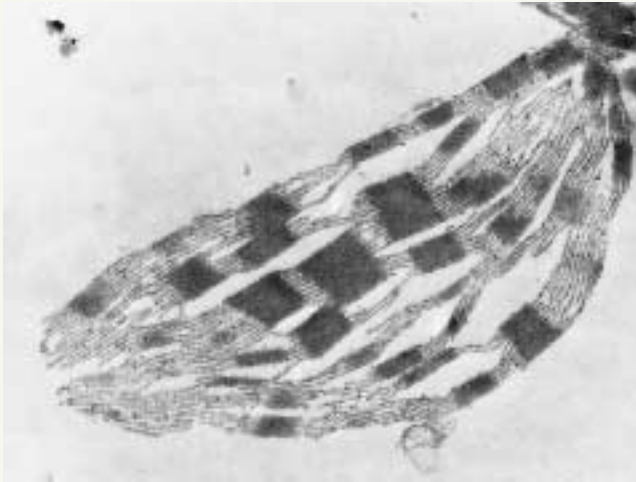


Reliance on purified membrane fractions as the basis for determining lateral heterogeneity led to concerns that the disruption and purification procedure was introducing artifacts. These objections were overcome when the localization of various components was demonstrated visually with the use of gold-labeled antibodies.<sup>5,6</sup> Using this nondisruptive technique, researchers showed that PSII proteins were excluded from the nonappressed thylakoid membranes, whereas PSI proteins were excluded from the appressed regions (Figure 3). In contrast, the cytochrome *b<sub>6</sub>f* complex was distributed evenly along the entire membrane system, as shown in the model in Figure 2.

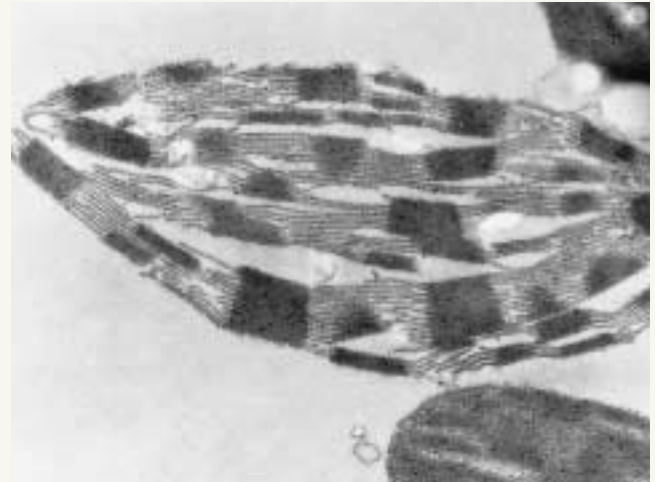
The demonstration that the two photosystems are spatially separated from one another within the thylakoid membrane has led to our current view of the dynamic nature of the photosynthetic membrane in which mobile carriers, such as plastoquinone and plastocyanin, are able to diffuse within the membrane, carrying electrons from one protein complex to another. The high degree of fluidity of the thylakoid membrane, which would be needed to facilitate such lateral movements, has been demonstrated by measurements of diffusion coefficients of fluorescently labeled plastoquinone molecules within the lipid bilayer using the technique of FRAP (page 145).<sup>7</sup> Coefficients for PQ in thylakoid membranes have been measured as high as  $10^{-6}$  cm<sup>2</sup>/sec (compared to  $10^{-8}$  cm<sup>2</sup>/sec for lipids in most membranes), suggesting that these photosynthetic membranes may be the most fluid of any major biological membrane. It was calculated in this study that the average distance between PSII and the nearest cytochrome *b<sub>6</sub>f*



**Figure 2** A model of the organization of the thylakoid membrane proposed in 1981 in which the two photosystems are spatially segregated: PSII is located in the appressed membrane regions and PSI (along with ATP synthase) is located in the nonappressed membrane regions. Cytochrome *b<sub>6</sub>f* is evenly distributed. (AFTER J. M. ANDERSON AND B. ANDERSSON, TRENDS BIOCHEM. SCI. 7:291, 1982. THE ARCHITECTURE OF PHOTOSYNTHETIC MEMBRANES: LATERAL AND TRANSVERSE ORGANIZATION. COPYRIGHT 1982, WITH PERMISSION FROM ELSEVIER SCIENCE.)



(a)



(b)

**Figure 3** Electron micrographs of thin sections of maize mesophyll cell thylakoids labeled with antibodies to (a) the D subunit of the PSI reaction center and (b) cytochrome *b*-559 of the PSII reaction center. PSI reaction centers are localized

almost exclusively on stroma thylakoids and end membranes of grana thylakoids, whereas PSII reaction centers are localized almost exclusively on appressed membranes of the grana thylakoids. (COURTESY OF O. VALLON.)

complex is about 73 nm. Based on the diffusion coefficient above, it was calculated that a PQ molecule should be able to diffuse about 2800 nm in 20 msec, which is the calculated half-time for cytochrome *b*<sub>6</sub>*f* reduction after a series of saturating light flashes. Thus, even if a PQ molecule were to take a highly devious path in diffusing between the two protein complexes, it ought to be able to make the connection in the required time.

In 1977, John Bennett of the University of Warwick in England made a discovery that would prove to have important implications in the study of thylakoid membrane structure of higher plants. Bennett incubated illuminated leaf chloroplasts in a medium containing radioactively labeled phosphate; then he extracted the proteins from the chloroplasts and fractionated the protein mixture using SDS-polyacrylamide gel electrophoresis.<sup>8</sup> Several proteins became rapidly labeled during the incubation, one of which was identified as the major light-harvesting chlorophyll *a/b* binding protein (referred to as LHCII in the text, page 227). Subsequent studies revealed the presence in the thylakoid membrane of a protein kinase that was responsible for phosphorylating LHCII.<sup>9</sup> Attention then turned to the mechanism by which the kinase was regulated and the importance of phosphorylation of light-harvesting proteins in higher plants.

Normally, LHCII does not become phosphorylated when chloroplasts are incubated in the dark. However, the activity of the protein kinase is not strictly light dependent, because it can be activated by incubation of chloroplasts in the dark in a medium containing a strong reducing agent, such as reduced ferredoxin.<sup>10</sup> These results suggested that one of the electron carriers of the photosynthetic chain must be reduced before the protein kinase is activated. A number of pieces of evidence suggested that the key component was plastoquinone.<sup>11</sup> Plastoquinone is the terminal electron acceptor of the PSII reaction center (see Figure 6.10). The pool of plastoquinone molecules in the membrane would be ex-

pected to accumulate in a reduced state (PQH<sub>2</sub>) as electrons are transferred to PQ but are not passed on to subsequent carriers. This condition would be expected to occur at times when PSII is operating at a higher level than PSI, that is, when the activity of the two photosystems is out of balance.

Based on various data, it was proposed that the phosphorylation of LHCII by the activated protein kinase served to correct a temporary imbalance resulting from the overexcitation of PSII relative to PSI. It was shown, for example, that LHCII phosphorylation leads to an increase in the transfer of energy to PSI at the expense of PSII.<sup>12</sup> This suggested that phosphorylation leads to the dissociation of LHCII from PSII, where it normally resides in the appressed regions of the thylakoid membrane. This phosphorylated LHCII complex would then migrate into the nonappressed regions of the membrane, where it would become closely associated with PSI. This association should allow the transfer of energy from LHCII to the PSI reaction center.

This conclusion is consistent with a number of findings:

- Freeze-fracture analysis shows that 8.0-nm particles thought to represent light-harvesting complexes become redistributed in the thylakoid membranes of pea chloroplasts from the appressed to the nonappressed regions upon phosphorylation.<sup>13</sup>
- Light-harvesting proteins in membrane fractions isolated from nonappressed regions are 10 times more heavily phosphorylated than the same proteins in appressed regions.<sup>14</sup>
- Phosphorylation leads to a decrease in the light-absorption capacity of PSII and a corresponding increase in that of PSI.<sup>15</sup>

The appressed regions of the membrane are sites where proteins from adjacent membranes come into close contact with one another. Phosphorylation of light-harvesting proteins

would add negative charges to the protein surface, which might cause the proteins to experience an electrostatic repulsion from neighboring proteins. This type of repulsion has been suggested as the major force that drives the phosphorylated LHCII out of the appressed portions of the thylakoid.<sup>16</sup>

The migration of LHCII complexes from one part of the thylakoid to another may serve a variety of photosynthetic functions in addition to correcting temporary imbalances between the two photosystems. It has been shown, for example, that overexcitation resulting from high light intensities can damage the photosynthetic machinery. Because of its high oxidizing potential, PSII is particularly susceptible to damage due to the formation of highly toxic oxygen radicals. Because exposure to bright light leads to the phosphorylation of LHCII and its dissociation from PSII, this phenomenon might limit the damage to PSII from overexcitation. The LHCII migration may also serve to regulate the balance between cyclic and noncyclic photophosphorylation. Some of the early studies indicated that the thylakoid protein kinase was also sensitive to the NADP<sup>+</sup>/NADPH ratio in the chloroplast. As this ratio decreases, and the relative level of NADPH increases, the need for noncyclic electron transport diminishes. Phosphorylation of LHCII would be expected to funnel more energy into PSI, which could be used to stimulate cyclic photophosphorylation, a process that allows the chloroplast to continue to produce ATP without the formation of additional NADPH.<sup>17</sup>

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## EXPERIMENTAL PATHWAYS

### The Role of Gap Junctions in Intercellular Communication

**B**ased on the information presented in Chapter 4, you might presume that synaptic transmission always occurs by movement of neurotransmitter molecules from the presynaptic neuron to the postsynaptic cell. This was the prevailing view until the 1950s, when Edwin Furshpan and David Potter of University College in London found a notable exception. Furshpan and Potter were studying synaptic transmission between giant neurons in the nerve cord of the crayfish. They noted that a small, *subthreshold* depolarization induced in the presynaptic nerve cell produced a very rapid (0.1 msec) depolarization in the postsynaptic cell.<sup>1,2</sup> If the nerve cells had been connected by a chemical synapse, a subthreshold change in membrane potential should not have been propagated to the postsynaptic cell, because it would not be sufficient to stimulate the release of neurotransmitter molecules. Even if neurotransmitter molecules were released, they could not possibly induce such a rapid change in the postsynaptic cell. Furshpan and Potter concluded that the two nerve cells were connected by a different type of synapse, an *electrotonic synapse*, in which ionic currents in the presynaptic cell could flow directly into the postsynaptic cell on the other side of the synapse. It was presumed that this type of cell-cell connection, which allows for the flow of ions between cells, was peculiar to excitable cells, such as neurons, which are specialized for cell-cell communication.

During the early 1960s, Yoshinobu Kanno and Werner Loewenstein of Columbia University were studying the permeability properties of the nuclear envelope, the membranous complex that bounds the nucleus. To determine whether ions were capable of flowing across the nuclear envelope, they had turned to the very large cells that make up epithelial tissues of the larval fruit fly (cells that contain the giant chromosomes that had proved so useful to geneticists). These cells were large enough to allow penetration of microelectrodes capable of inducing and recording ionic currents (Figure 1). To their surprise, Kanno and Loewenstein found that when ions were injected into the nucleus of



**Figure 1** Micrograph showing recording microelectrodes being inserted into living cells of the Malpighian tubule of an insect cell. (COURTESY OF WERNER R. LOEWENSTEIN.)

one cell, not only did the ion flux (measured as an electrical current) spread into the cytoplasm of that cell, but it flowed directly into the cytoplasm of an adjacent cell. In fact, the potential recorded in the adjacent cell was almost as great as that in the cell in which the current was originally induced. Kanno and Loewenstein concluded that the epithelial cells that make up the salivary gland are *electrically coupled* to one another, meaning that ions are able to flow freely from cell to cell through low-resistance cell junctions.<sup>3</sup> If small inorganic ions could pass through these junctions between neighboring cells, what about larger substances? When a small volume of the fluorescent ion fluorescein (molecular weight of 376 daltons) was injected into the cytoplasm of one cell with a micropipette, the fluorescence rapidly spread into adjacent cells until the entire epithelial layer glowed from the presence of the tracer (as in Figure 7.33). In contrast, none of the fluorescent dye leaked out of the cells into the external medium, indicating that fluorescein molecules were diffusing directly from the cytoplasm of one cell into the cytoplasm of adjacent cells by means of permeable cell-cell contacts.<sup>4</sup> Similar observations were soon made on a variety of different types of epithelial and mesenchymal cells, including those of various mammals, indicating that these communicating junctions are widespread.

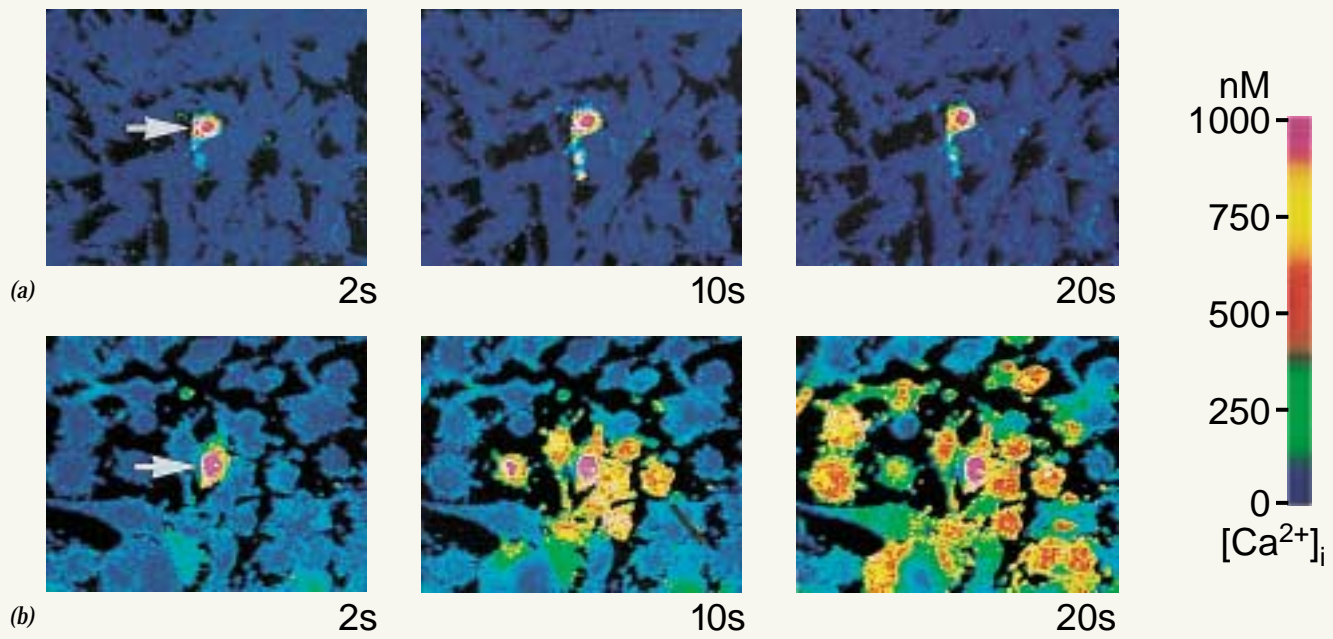
Electron microscopic studies had shown that animal cells are bounded by a continuous plasma membrane. This new data suggested that the structure of this membrane had to be modified at sites where cells made contact with other cells. Otherwise, it would be impossible for substances to move directly from the cytoplasm of one cell into another. The discovery of cell junctions containing channels between closely applied cells was made in 1967 by Jean Paul Revel and M. J. Karnovsky.<sup>5</sup> Electron micrographs of these junctions showed a distinct cleft between adjoining cells, which led the researchers to name them “gap junctions” to distinguish them from tight junctions, where adjoining cells make direct contact.

Studies were carried out to learn more about the size of the channels connecting the cytoplasms of adjacent cells. Loewenstein’s laboratory tested fluorescent probes that were linked to peptides of varying size. They found that molecules up to about 1200 daltons were able to diffuse between insect larval salivary gland cells.<sup>6</sup> Based on estimates of the dimensions of these molecules, they concluded that the effective diameter of the channel was approximately 10 to 15 Å (1.0 to 1.5 nm), a value that matched closely with that estimated from high-resolution micrographs of gap junctions taken with the electron microscope.<sup>7</sup>

### The Role of Gap Junctions in Cancer

One of the first questions considered by Kanno and Loewenstein following their discovery of permeable intercellular contacts was whether these same types of contacts





**Figure 2** Calcium waves induced by mechanical stimulation in (a) a control culture of rat C6 glioma cells and (b) a clone of the same cells shown in a, but transfected with connexin43 DNA. The injected DNA is transcribed and translated in the transfected cells, and the connexin protein molecules are incorporated into the plasma membrane. When one of the nontransfected cells is mechanically stimulated, there is very little passage of  $Ca^{2+}$  into its neighbors. In contrast, when cells that express the connexin gene are mechanically stimulated, a wave of  $Ca^{2+}$  passes from cell to cell through the gap junctions formed by the connexin43 protein. (FROM ANDREW C. CHARLES ET AL., J. CELL BIOL. 118:197, 1992; BY COPYRIGHT PERMISSION OF THE ROCKEFELLER UNIVERSITY PRESS.)

were present in cancer cells. The growth rates of normal cells were known to be influenced by stimuli from their environment. It was possible that one of the factors that allowed cancer cells to escape from the types of growth control mechanisms that prevail in normal cells was a loss in

their ability to receive regulatory molecules from neighboring cells. Kanno and Loewenstein investigated this possibility by measuring the flow of ionic current in normal liver tissue as compared to that in a variety of liver tumors. Whereas ionic current readily flowed between normal cells of a rat



**Figure 3** (a) When a small mass of C6 glioma cells is implanted into the brain of a rat, the cells develop into a large tumor mass after two weeks growth. (b) The same cells that have been transfected with connexin43 DNA and are

presumed to engage in gap junction intercellular communication have developed into a much smaller tumor during the same time period. (FROM CHRISTIAN C. G. NAUS ET AL., CANCER RES. 52:4210, 1992.)

liver, no passage of current could be detected between the cells of any of the liver tumors that were investigated.<sup>8</sup>

Since these initial studies, hundreds of different types of cancer cells have been analyzed for their ability to carry out gap-junction intercellular communication (GJIC). The initial results of Loewenstein and Kanno have been found to hold true for most, but not all, cancer cells that have been investigated.<sup>Reviewed in 9,10</sup> It is not surprising that not all cancer cells exhibit the same properties with regard to GJIC. The conversion of a normal cell into a malignant cell is a multistage phenomenon that can occur as the result of changes in a wide variety of different genes (Chapter 16). There are different mechanisms by which a cell can lose growth control. Some of these mechanisms do seem to involve the loss of the cells' ability to transmit signals across gap junctions. Among those tumors where this does occur, there is often a progressive loss in GJIC as the cells become more and more malignant.<sup>11</sup> In addition, there may be a correlation between the loss of GJIC and an increase in the metastatic potential of a population of cells.<sup>12</sup> Metastatic potential is a property of cancer cells that enables them to ignore adjacent cells and move off on their own. It would be expected that such behavior could occur only if a cell severed its communication links with neighboring cells.

Evidence obtained by correlations between one condition (e.g., loss of GJIC) and another condition (e.g., malignancy) is circumstantial. The best evidence of a direct, causal relationship between the two conditions has been obtained by Christian Naus and colleagues at the University of Western Ontario in studies in which cancer cells are forced to express gap junction proteins (connexins). When C6 glioma cells from a rat brain tumor are transfected with DNA that encodes the protein connexin43, there is a dramatic increase in GJIC between the tumor cells (Figure 2) and a corresponding decrease in the growth rate of the cells.<sup>13,14</sup> Similarly, when C6 glioma cells that have been transfected with connexin43-coding DNA are implanted into the brains of adult rats, they generate much smaller tumors than the nontransfected glioma cells (Figure 3).<sup>15</sup> Similar findings have been reported on human cancer cells.<sup>16–18</sup> For example, when human mammary carcinoma cells are transfected with genes encoding connexins that are expressed in normal mammary cells, the capability of the cells to form tumors in susceptible mice is greatly reduced. One of the transfected cell lines exhibited suppressed growth for several weeks and then displayed a sudden increase in malignancy. When analyzed, these cells no longer expressed the connexin protein, suggesting that the transfected gene had been lost or inactivated during tumor growth.<sup>17</sup>

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## EXPERIMENTAL PATHWAYS

## The Molecular Motor That Drives Fast Axonal Transport

Most compound light microscopes have a pair of ocular lenses through which the observer views the field, and a third “ocular lens” where a camera is placed to photograph the field. With the development of video technology, it became possible to replace the film camera with a video camera and watch events under the microscope on a television screen. Not only did the video camera provide a new way to use a light microscope, it provided researchers with a means to observe structures considered to be below the limit of resolution of the instrument ( $0.02\ \mu\text{m}$ ).<sup>1,2</sup> There are several reasons for this phenomenon, the most notable one being that video cameras greatly enhance the contrast of objects in the field, which has a magnifying effect. When video observation is combined with differential interference contrast (DIC) microscopy (Section 18.1), objects as small as intermediate filaments (10 nm diameter) and microtubules (25 nm diameter) can be resolved. The technique is known as *video-enhanced contrast-differential interference contrast microscopy*, or *VEC-DIC*.

Robert Allen of Dartmouth College was one of the pioneers of videomicroscopy. Allen had long been interested in cytoplasmic movement, and one of the first projects he undertook using VEC-DIC microscopy was the examination of living nerve cells. It had been known for a number of years that synaptic vesicles moved by fast axonal transport from the cell body down the length of the axon at rates up to  $5\ \mu\text{m}/\text{sec}$ . In 1982, Allen and his colleagues reported on the movement of very small vesicles (30–50 nm) down the length of squid giant axons by VEC-DIC. The vesicles moved continuously in one direction, either toward the terminus (anterograde) or toward the cell body (retrograde), at velocities averaging  $2.5\ \mu\text{m}/\text{sec}$  and as fast as  $5\ \mu\text{m}/\text{sec}$ .<sup>3</sup> The vesicles moved along linear elements, presumably either IFs or microtubules, which could not be distinguished.

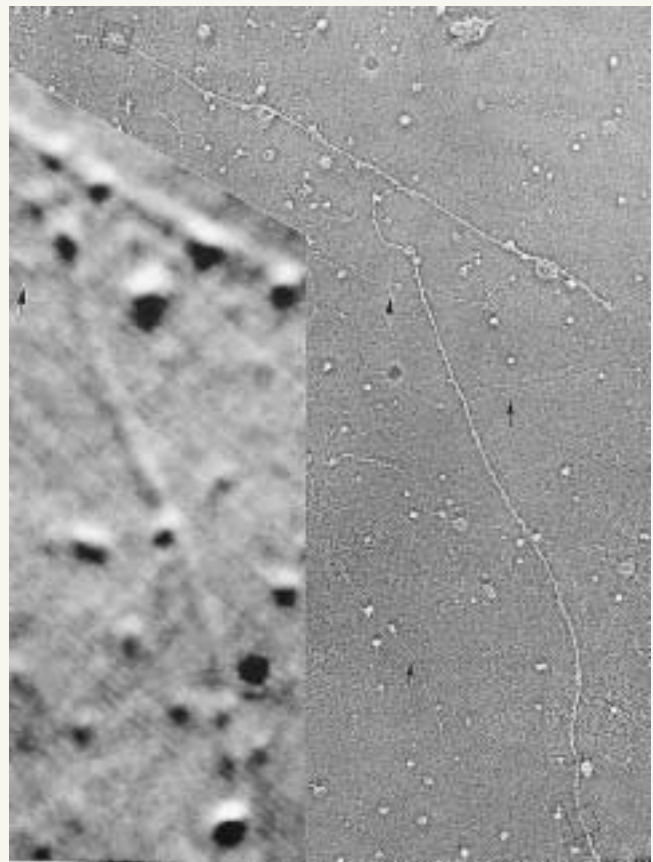
One of the advantages of working with giant axons is that the cytoplasm (or *axoplasm*) can be squeezed out of the cell not unlike the way toothpaste can be squeezed out of its container. The movement of vesicles continued at about the same rate in extruded axoplasm as in the whole cell.<sup>4</sup> The linear elements that defined the pathways of movement could be seen with VEC-DIC even more clearly in the extruded axoplasm than in the intact axon. Most important, the demonstration that fast axonal transport could continue outside the confines of an intact cell opened the door to understanding the molecules that were responsible for organelle transport.

In 1985, a series of papers appeared out of the collaborative efforts of Ronald Vale, Bruce Schnapp, Thomas Reese, and Michael Sheetz that shed considerable light on the mechanism of axonal transport. To begin their studies, they diluted extruded axoplasm by addition of an appropriate salt solution, which had the effect of scattering the contents of the axoplasm so that individual filaments could be observed more readily with VEC-DIC microscopy.<sup>5</sup> Under these conditions, vesicles of widely differing size moved continuously along



linear filaments at a rate of about  $2.2\ \mu\text{m}/\text{sec}$ . Organelle movement was also shown to require ATP. In the absence of ATP, organelles became immobile but remained bound to the filaments.

Vale and his colleagues developed a method to observe organelle movement in extruded axoplasm and then fix the preparation so that the same filament and organelle seen on video could be examined in the electron microscope (Figure 1).

1.5  $\mu\text{m}$ 

**Figure 1** Corresponding video (left) and electron micrograph (right) of the same two filaments that had been seen by video to participate in vesicle transport. Both images are shown at the same magnification; the difference in size of the filaments illustrates the manner in which video microscopy inflates the apparent size of small objects. (Arrows point to smaller filaments that are not relevant to the discussion.) (REPRINTED FROM B. J. SCHNAPP ET AL., *CELL* 40:458, 1985. SINGLE MICROTUBULES FROM SQUID AXOPLASM SUPPORT BIDIRECTIONAL MOVEMENT OF ORGANELLES. COPYRIGHT 1985 BY PERMISSION OF CELL PRESS AND ELSEVIER SCIENCE.)

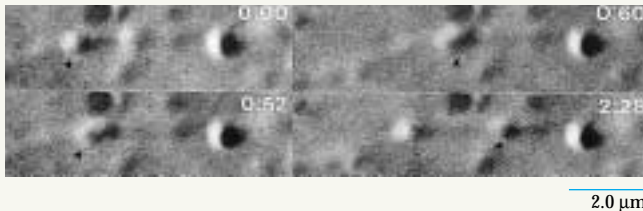
Close examination revealed that two organelles could move along the same filament in opposite directions and, in fact, could pass each other without colliding (Figure 2).<sup>6</sup> This observation suggested that each filament has several tracks along which organelles can move. Under the electron microscope, the filaments had a size and structure that suggested they were microtubules. In this case, the “tracks” were likely to be individual protofilaments.

Use of immunofluorescent antibodies confirmed that the filaments were made of tubulin. Because all the protofilaments of a microtubule are oriented in the same direction, and organelles are capable of moving in either direction along a microtubule, some factor other than the microtubule itself must determine the direction of movement. Attention turned to the motor protein that might move along a microtubule. By 1985, dynein had been isolated and characterized as the motor protein responsible for ciliary movement (page 358), and there were indications from studies on sea urchin eggs that there might be a cytoplasmic form of dynein. But it was unlikely, even if dynein were responsible for organelle movement, that it would be able to transport materials in both directions along the same microtubule.

In a subsequent paper, Vale and his colleagues successfully reconstituted a system of axonal transport from separate ingredients.<sup>7</sup> The microtubules to be used as the “tracks” for movement were prepared from soluble tubulin that was polymerized *in vitro* in the presence of taxol, a drug that promotes assembly and stabilizes the polymer (page 350). The assembled microtubules were then washed with 1 M NaCl to remove any nontubulin proteins that might be associated with the structures. Subsequent analysis of the protein from these microtubules showed them to consist solely of tubulin.

Axoplasm prepared from squid brain was separated into several fractions by centrifugation through a sucrose density gradient (Section 18.6). One of the fractions contained vesicles in the size range (0.05–0.5  $\mu\text{m}$ ) that were known to be transported *in vivo*. When this organelle fraction was added to the purified microtubule fraction in the presence of ATP, virtually no movement of the organelles was seen.

A second fraction from the axoplasm was obtained from the supernatant that was left at the top of the tube following centrifugation through the sucrose gradient. This supernatant (designated S2) contained a complex mixture of polypeptides, as shown by polyacrylamide gel electrophoresis (PAGE), but it was free of organelles, microtubules, and smaller filaments.



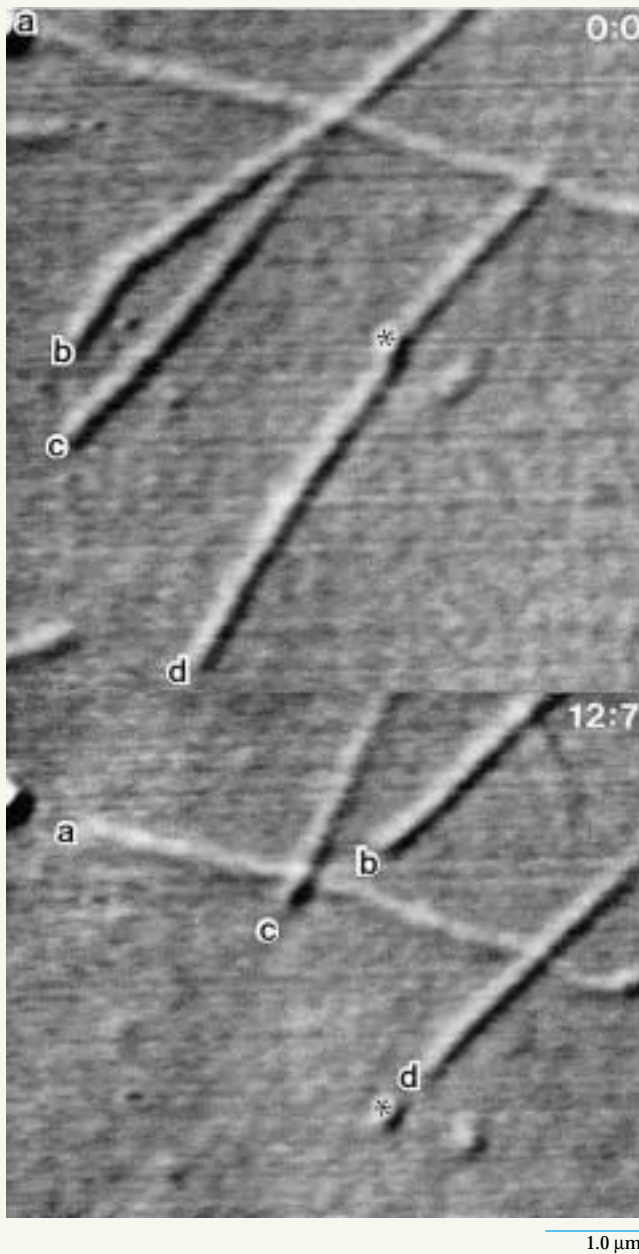
**Figure 2** Sequence of video micrographs showing bidirectional movement of organelles (triangles) on the vertically oriented filament shown in Figure 1. (REPRINTED FROM B. J. SCHNAPP ET AL., *CELL* 40:458, 1985; BY PERMISSION OF CELL PRESS AND ELSEVIER SCIENCE.)

When the S2 supernatant was mixed with the organelle fraction and the purified microtubules in the presence of ATP, organelles became attached to the microtubules and moved along them. Unlike the results with extruded axoplasm, the movement of organelles promoted by the S2 supernatant occurred in only one direction along a single microtubule.

Not only did the organelles move along the microtubules, but the microtubules themselves moved along the surface of the glass coverslip (Figure 3). Movement of the microtubules required the addition of the S2 supernatant but not the organelles. When a small drop of supernatant was placed on a coverslip and allowed to remain a few minutes, and then the coverslip was gently washed and blotted before adding the microtubules, the microtubules still moved over the coverslip in the presence of ATP. This indicated that the factor in the supernatant that promoted movement could adhere to glass and still retain its ability to act as a microtubule motor. Subsequent experiments indicated that when latex beads were incubated in the S2 supernatant and then added to microtubules stuck to a coverslip, the beads would move in a single direction along a stationary microtubule. Translocation of the microtubules over the glass surface, or the beads over the microtubules, required the addition of ATP. If adenylyl imidodiphosphate (AMP-PNP), an ATP analogue that could not be hydrolyzed, was added instead of ATP, all of these movements were blocked. These investigators concluded, “All of these movements may be driven by a single, soluble ATPase that binds reversibly to organelles, beads, or glass, and generates a translocating force on a microtubule.”

The soluble component promoting organelle movement was sensitive to trypsin and heat denaturation, indicating that it was a protein, one that presumably acted in a manner similar to myosin or dynein. The development of an *in vitro* system capable of generating movement provided the necessary assay to allow the purification of the motor protein in the S2 supernatant responsible for microtubule-based organelle translocation. A model of how the motor protein in the S2 supernatant might be operating in the movement of beads, vesicles, and microtubules is shown in Figure 4.

By the end of 1985, Vale and his coworkers had reported on the purification of the motor protein.<sup>8</sup> The purification was based on the earlier demonstration that particles from squid axoplasm would attach tightly to microtubules in the presence of the nonhydrolyzable ATP analogue, AMP-PNP.<sup>9</sup> One explanation for these results was that the motor protein associated with these organelles was binding to a microtubule and then becoming stuck there because it was unable to hydrolyze the AMP-PNP bound to its ATPase site. If this was the case, then it might be possible to purify the motor protein by allowing the S2 supernatant to interact with microtubules in the presence of AMP-PNP. Following this protocol, it was found that a protein of approximately 600 kDa would bind to the microtubules and that this protein was released by addition of ATP. When the released protein was applied to a preparation of purified microtubules, the microtubules were translocated across the coverslip. Further analysis of the polypeptide components of the protein indicated that it was distinct from both myosin and dynein and thus belonged to a novel class of force-generating molecules. They named the protein kinesin (from the Greek *kinein*, to move).



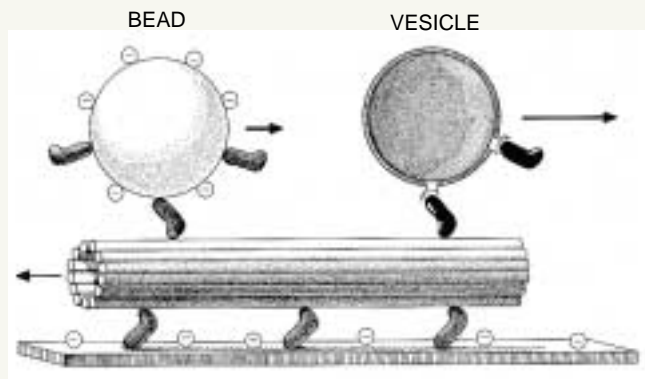
**Figure 3** The movement of purified microtubules along a glass surface in the presence of S2 supernatant. Elapsed times are indicated at the upper right in seconds: tenths of seconds. Three microtubules, designated b, c, and d, are seen to move diagonally from the lower left to the upper right, while another microtubule (a) moves horizontally. An organelle (asterisk) attached to the coverslip provides a stationary reference point. (REPRINTED FROM R. D. VALE ET AL., *CELL* 40:564, 1985. ORGANELLE, BEAD, AND MICROTUBULE TRANSLOCATIONS PROMOTED BY SOLUBLE FACTORS FROM THE SQUID GIANT AXON. COPYRIGHT 1985 BY PERMISSION OF CELL PRESS AND ELSEVIER SCIENCE.)

In the last paper of the 1985 series, Vale and his colleagues reported on additional properties of kinesin.<sup>10</sup> As noted above, they had shown that the S2 supernatant (which contains the soluble kinesin molecules) promoted movement

of organelles along single microtubules in only one direction. To determine the direction of translocation, it was necessary to utilize microtubules whose plus and minus ends could be identified. This was accomplished by allowing purified tubulin to polymerize onto centrosomes *in vitro* and using these microtubules of defined polarity as substrates for particle movement. As discussed on page 000, microtubules grow with their plus ends away from the centrosome. When latex beads with bound kinesin molecules were added to these microtubules, the beads always moved along the microtubular track toward its plus end. It was evident that kinesin was a plus end-directed microtubule-based motor. Because anterograde movement within the axon is also plus end-directed, the researchers concluded that kinesin was the motor responsible for anterograde vesicle transport in the nerve cell.

But movement of organelles in intact axons can occur in either anterograde or retrograde directions. Recall that extruded axoplasm promotes the movement of particles in both directions along the same microtubule. When latex beads were incubated in a crude solubilized fraction (designated S1a) from brain tissue, these beads also were capable of bidirectional movement along single microtubules. Based on these findings, it appeared that the crude soluble fraction contained both the anterograde motor (kinesin) and the retrograde motor (an unknown protein), and that further purification steps between S1a and S2 had removed the retrograde motor. Attention then turned to attempting to distinguish the two molecular motors.

In one experiment an antibody was prepared against purified kinesin.<sup>10</sup> The antibody molecules were then covalently linked to Sepharose beads that were packed in a column. The crude S1a fraction was passed through the column to remove kinesin molecules from the fraction. When latex beads were incubated with the fluid that had passed through the column, they moved along microtubules solely in the retrograde direction, providing further evidence that anterograde and



**Figure 4** A schematic model showing how a motor protein in the S2 supernatant could support the translocation of organelles, beads, and microtubules. The direction of organelle and bead movement along microtubules is opposite to the direction of microtubule movement on glass, as indicated by the arrows. (REPRINTED FROM R. D. VALE ET AL., *CELL* 40:567, 1985; BY PERMISSION OF CELL PRESS AND ELSEVIER SCIENCE.)

retrograde movements required different motor proteins and that kinesin was not the retrograde motor.

In another experiment, the crude S1a fraction was treated with N-ethylmaleimide (NEM) and vanadate prior to incubation with the latex beads. Beads incubated with this fraction no longer moved bidirectionally, but moved only toward the plus end of the microtubules, that is, in the anterograde direction. These results indicated that the anterograde and retrograde motor proteins had different sensitivities to drugs, providing further evidence that they were different proteins. Because NEM and vanadate are agents known to inactivate dyneinlike motor proteins, the possibility was raised that the retrograde motor was a dyneinlike protein.

In the past decade, considerable advances have been made in the study of the structure and function of kinesin. Kinesin has been implicated in the movement of different types of organelles in diverse cell types. Because kinesin is a motor protein that causes organelles to move along microtubules, translocated organelles must be able to bind kinesin molecules. A protein thought to act as the kinesin receptor has been isolated from the membranous vesicles of embryonic brain cells.<sup>11</sup> This membrane protein has been named *kinectin* and may be part of a family of proteins involved in the type of vesicle trafficking described in Chapter 8. Antibodies against kinectin inhibit the binding of kinesin to vesicles and halt their movement *in vitro*, suggesting that kinectin is a component of the vesicle membrane that anchors the motor protein.<sup>12</sup> The molecular basis of kinesin action is being actively investigated using *in vitro* motility assays. Taken together, these studies have provided considerable insight into the mechanism by which membranous organelles are moved in a directed manner from one part of a cell to another.

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## EXPERIMENTAL PATHWAYS

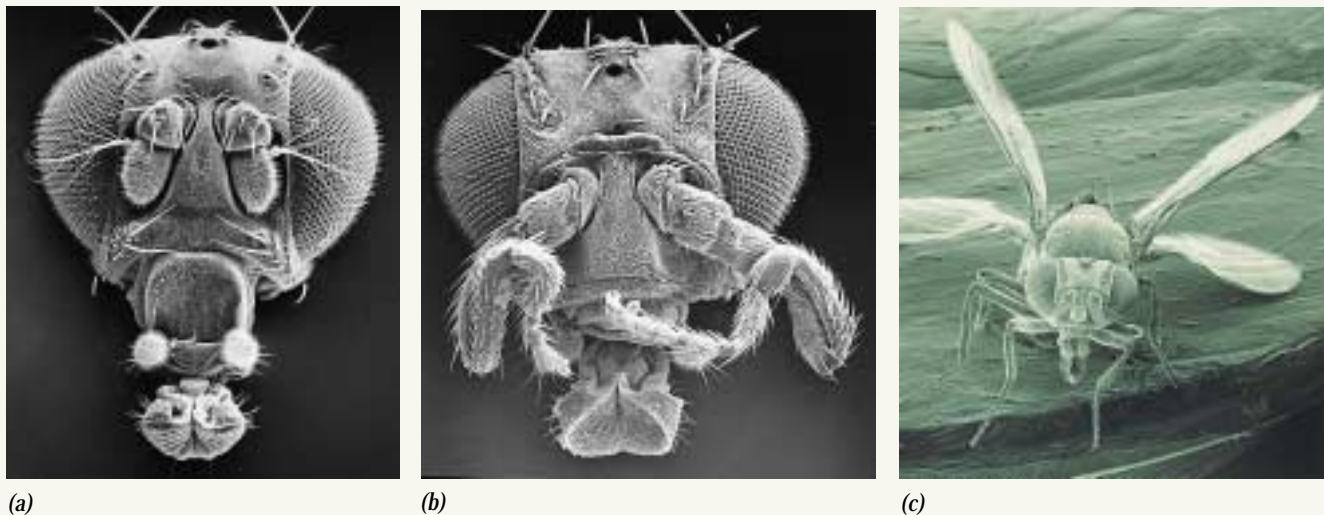
## Genes That Control Embryonic Development

**D**arwin's theory of evolution by natural selection rested heavily on his observations that members of a population possess inheritable characteristics that vary from one individual to another. One of Darwin's most fervent supporters was William Bateson, who published a treatise in 1894 describing the variation that existed in natural populations of various types of organisms.<sup>1</sup> Bateson's observation of human skeletons revealed rare examples in which one type of vertebra was replaced by another type. He coined the term "homeosis" to describe variations in which one part of the body seemed to be changed into a structure that is normally located somewhere else.

With the rediscovery in 1900 of Mendel's work on pea plants, and with Morgan's establishment of the fruit fly as a laboratory organism for genetic study, biologists were in a better position to appreciate the importance of a mutation that could cause a dramatic change in body form. The first homeotic mutant in fruit flies was described in 1915 by Calvin Bridges, one of Morgan's colleagues.<sup>2</sup> Like other types of flies, the wild-type fruit fly has only one pair of wings, which are located on the second thoracic segment. The third (and last) thoracic segment bears a pair of balancing organs (halteres). In contrast, the third thoracic segment of the mutant discovered by Bridges had a pair of small wings in place of the halteres. Bridges named the mutant *bithorax* (*bx*). Further studies indicated that *bx* was part of a cluster of similar genes that controlled the directions in which both the thoracic and abdominal segments developed. The cluster of genes, which was located on the third chromosome, became known as the bithorax complex (BX-C). In 1926, a second type

of homeotic mutant was described in *Drosophila*, in which the antennae that normally develop on an anterior segment of the head are replaced by a pair of legs.<sup>3</sup> This gene was named *antennapedia* (*Antp*) and was shown to be part of a different cluster of genes, named the antennapedia complex (ANT-C), on the third chromosome. Scanning electron micrographs of a normal fruit fly and two homeotic mutants are shown in Figure 1.

Much of the work that identified the genes that make up the two homeotic gene complexes was carried out by Edward B. Lewis of the California Institute of Technology.<sup>4</sup> The fact that the genes that make up the bithorax and antennapedia complexes (1) are present in tandem array and (2) have similar functions in controlling the developmental pathway of various segments prompted Lewis to suggest that these genes arose as the result of duplication and divergence of a single ancestral gene. One way to test evolutionary relationships among genes is to determine whether or not they possess homologous DNA sequences. The first indications that homeotic genes did indeed share a region of similar sequence came from studies carried out in Walter Gehring's laboratory at the University of Basel in Switzerland, which were published as a series of papers in 1984. In one of these reports, DNA was extracted from adult flies, digested with the restriction enzyme *EcoR*I, and subjected to electrophoresis through an agarose gel to separate the resulting fragments according to their size.<sup>5</sup> The DNA fragments in the gel were denatured by soaking the gel in a solution of NaOH, and the gel was then transferred to a nitrocellulose membrane. In this technique, which is called Southern blotting after its developer



**Figure 1** Scanning electron micrographs of (a) a normal fruit fly, (b) an *Antp* mutant in which the antennae are replaced by a pair of legs, and (c) a fly bearing a mutation in

the *bx* cluster in which two of the three thoracic segments bear a pair of wings. (A,B: COURTESY OF F. R. TURNER; C: FROM DAVID SCHARF/PETER ARNOLD.)

Edwin Southern, the single-stranded DNA fragments become bound to the membrane in the same position they occupied within the gel (see Figure 18.35). The DNA is permanently fixed to the membrane by drying it under vacuum at 80°C. The membrane is then incubated in solutions containing radioactively labeled, single-stranded DNA probes, which bind (hybridize) to complementary DNA fragments on the filter. The unbound, labeled DNA can be washed away, and the locations of the bound, labeled probe can be determined autoradiographically by pressing the membrane against a sheet of X-ray film.

In these experiments, the labeled probes were prepared from short fragments of the BX-C or ANT-C complexes that had been previously cloned. Using the Southern blots prepared from total DNA, as described above, it was found that certain of the labeled probes hybridized to a number of different-sized fragments on the nitrocellulose membrane. The DNA therefore contained a number of different sites that were complementary to a single labeled probe representing either a small part of the ANT-C or BX-C complex.

The next step was to see if this same labeled probe would bind to restriction fragments prepared from specific genes. It was found that each of three different genes from the homeotic complexes (*antennapedia*, *ultrabithorax* [*Ubx*], and *fushi tarazu* [*ftz*]) contained a sequence that hybridized to the labeled fragment, indicating that all three genes shared a region having a similar nucleotide sequence. This region of sequence similarity was termed the *homeobox*.<sup>5</sup>

The homeobox is not part of a regulatory region of the DNA but resides within the coding portion of the genes. Subsequent analysis of the regions of the three homeotic genes that bound to the same probe indicated that the region of homology was limited to a stretch of 180 base pairs that codes for a 60 amino acid portion of the polypeptide called the *homeodomain*. Sequence analysis of the three genes indicated that the homeobox in *Antp* shows 77 percent identity with the homeobox of *ftz* (i.e., 138 nucleotides of the 180 were perfect matches). The homeoboxes of *ftz* and *Ubx* showed 75 percent identity, while those of *Antp* and *Ubx* showed 79 percent identity. Moreover, many of the nucleotide substitutions that distinguished the homeobox of the three genes caused one codon for an amino acid to be replaced by another codon that specified the same amino acid. Of the 60 amino acids encoded by the homeobox, 45 of the amino acids are identical in all three genes.<sup>6</sup>

The role of the protein encoded by these genes was also considered. The fact that the homeodomain was rich in basic amino acids (approximately 19 of the 60 amino acids were either lysine, arginine, or histidine) raised the possibility that the protein functioned by binding to DNA. Although the amino acid sequence of the homeodomain did not show extensive homology to that of any other known protein, it did show a weak homology to a pair of yeast proteins that were known to function as DNA-binding transcription factors. Thus based on the basic nature of the homeodomain and the weak homology to a DNA-binding protein, it was proposed that homeotic genes might encode proteins that act as DNA-binding transcription factors. This proposal was consistent with the idea that homeotic genes acted like “master control” genes whose product controlled the expression of a variety of subordinate genes.

Once it was established that the homeobox sequence was a consistent feature of the homeotic genes of *Drosophila*, the question arose as to whether a similar sequence was present in the genes of other organisms. To test this possibility, William McGinnis and his coworkers prepared Southern blots using fragmented, total genomic DNA from a number of organisms and tested the ability of the separated fragments to bind a labeled DNA probe containing the *Drosophila* homeobox sequence.<sup>6</sup> When the DNA tested in the Southern blot was isolated from *Drosophila*, six bands on the nitrocellulose blots were labeled (with three corresponding to *ftz*, *Ubx*, and *Antp* and three to other genes). DNA from beetles, earthworms, chickens, mice, and humans also produced a number of labeled bands, indicating the presence in each of these organisms of a variety of genes containing the homeobox sequence. Approximately six to eight restriction fragments from both mouse and human DNA were found to bind to the *Drosophila* homeobox-containing DNA probe in these early studies. In contrast, DNA from bacteria failed to show the presence of homeobox sequences. Not only did genes of mice and humans possess a homeobox, the sequence was soon shown to be remarkably conserved between flies and mammals, members of two widely divergent phyla. It was shown, for example, that one of the restriction fragments from the mouse genome encoded a polypeptide in which 44 of the 60 amino acids of the homeobox were identical to those encoded by the homeobox of the *Antp* gene of *Drosophila* (Figure 2).<sup>7</sup>

	1	10	20																	
Mouse10	Ser	Lys	Arg	Gly	Arg	Thr	Ala	Tyr	Thr	Arg	Pro	Gln	Leu	Val	Glu	Leu	Glu	Lys	Glu	Phe
<i>Antp</i>	Arg	Lys	Arg	Gly	Arg	Gln	Thr	Tyr	Thr	Arg	Tyr	Gln	Thr	Leu	Glu	Leu	Glu	Lys	Glu	Phe
<i>ftz</i>	Ser	Lys	Arg	Thr	Arg	Gln	Thr	Tyr	Thr	Arg	Tyr	Gln	Thr	Leu	Glu	Leu	Glu	Lys	Glu	Phe
<i>Ubx</i>	Arg	Arg	Arg	Gly	Arg	Gln	Thr	Tyr	Thr	Arg	Tyr	Gln	Thr	Leu	Glu	Leu	Glu	Lys	Glu	Phe
	21	30	40																	
Mouse10	His	Phe	Asn	Arg	Tyr	Leu	Met	Arg	Pro	Arg	Arg	Val	Glu	Met	Ala	Asn	Ala	Leu	Asn	Leu
<i>Antp</i>	His	Phe	Asn	Arg	Tyr	Leu	Thr	Arg	Arg	Arg	Arg	Ile	Glu	Ile	Ala	His	Leu	Leu	Cys	Leu
<i>ftz</i>	His	Phe	Asn	Arg	Tyr	Ile	Thr	Arg	Arg	Arg	Arg	Ile	Asp	Ile	Ala	Asn	Ala	Leu	Ser	Leu
<i>Ubx</i>	His	Thr	Asn	His	Tyr	Leu	Thr	Arg	Arg	Arg	Arg	Ile	Glu	Met	Ala	Tyr	Ala	Leu	Cys	Leu
	41	50	60																	
Mouse10	Thr	Glu	Arg	Gln	Ile	Lys	Ile	Trp	Phe	Gln	Asn	Arg	Arg	Met	Lys	Tyr	Lys	Lys	Asp	Gln
<i>Antp</i>	Thr	Glu	Arg	Gln	Ile	Lys	Ile	Trp	Phe	Gln	Asn	Arg	Arg	Met	Lys	Trp	Lys	Lys	Glu	Asn
<i>ftz</i>	Ser	Glu	Arg	Gln	Ile	Lys	Ile	Trp	Phe	Gln	Asn	Arg	Arg	Met	Lys	Ser	Lys	Lys	Asp	Arg
<i>Ubx</i>	Thr	Glu	Arg	Gln	Ile	Glu	Ile	Trp	Phe	Gln	Asn	Arg	Arg	Met	Lys	Leu	Lys	Lys	Glu	Ile

**Figure 2** A comparison of the amino acid sequences that make up the homeodomain of one mouse gene and three fruit fly genes (*antp*, *ftz*, and *Ubx*). The underlined amino acids are identical between the mouse homeodomain and fly *Antp* gene products. (REPRINTED FROM W. MCGINNIS, ET AL., CELL 38:677, 1984. MOLECULAR CLONING AND CHROMOSOME MAPPING OF A MOUSE DNA SEQUENCE HOMOLOGOUS TO HOMEOTIC GENES OF *DROSOPHILA*, BY PERMISSION OF CELL PRESS AND ELSEVIER SCIENCE.)

The 1984 papers on the homeobox created considerable excitement among both developmental biologists, who were searching for clues about the genes that control development, and molecular biologists, who were searching for proteins that control transcription. The finding that the mammalian genome contains genes with sequences that were homologous to the homeotic genes that play such an important role in the development of *Drosophila* was particularly compelling. The development of fruit flies and mammals could not be more different, yet the genetic mechanisms that control the development of the two types of organisms may be remarkably similar. Attention turned to the role of homeotic genes during mammalian development. Over the next few years, investigators sought to answer three major types of questions:

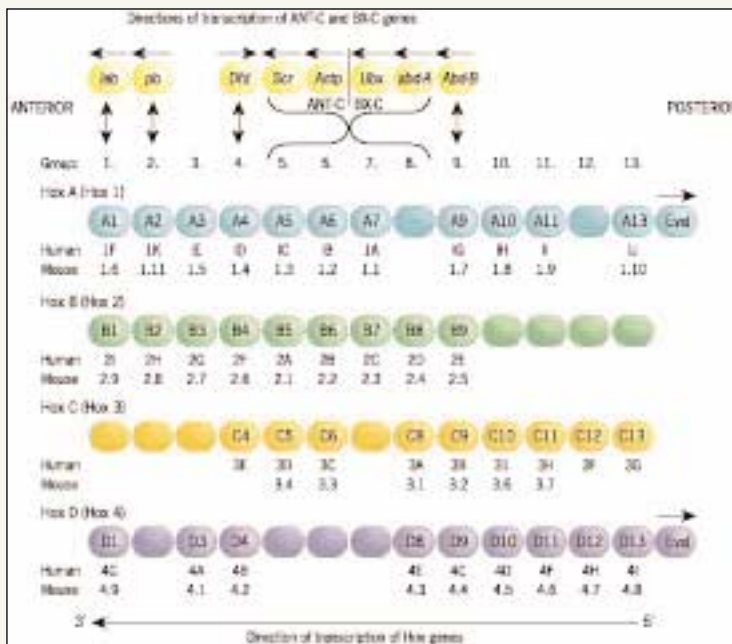
1. How many homeobox-containing genes are present in the genomes of vertebrates, and how are the genes organized within the chromosomes?
2. At what stages of embryonic development and in which embryonic tissues are homeobox-containing genes transcribed?
3. What is the role of these genes during development?

The first of these questions was probed by a variety of genetic and molecular techniques. Studies on the mouse indicated that there were four distinct clusters of homeobox-containing genes located on four different chromosomes.<sup>8</sup> The four clusters in the mammalian genome have been named HoxA through HoxD.<sup>9</sup> Each gene cluster contains a series of genes that are very similar to genes occupying comparable positions in clusters on other chromosomes (Figure 3). It is striking that several of the genes of the two *Drosophila* homeotic-gene clusters (ANT-C and BX-C) have homologues within the mouse (and human) genomes, and the order of the homologues within the gene clusters is identical in both the insects and mammals. This observation suggests that the an-

cestor of both arthropods and vertebrates, which are thought to have diverged approximately 700 million years ago, must have possessed a similar cluster of genes that determined its body plan during embryonic development. According to one evolutionary scenario, the ancestral cluster was split in two during the evolution of insects (Figure 3), whereas during the evolution of vertebrates, the ancestral cluster underwent duplication and divergence to generate four separate complexes of homeotic genes.

The second of these questions was studied by in situ hybridization, in which sections of embryos were prepared and incubated with labeled, single-stranded DNA probes containing the homeobox sequences. Those cells of the embryo that are actively transcribing a gene containing the homeobox sequence have RNA transcripts that are complementary to the labeled DNA probe. During the incubation, the labeled DNA binds to complementary transcripts, forming DNA-RNA hybrids whose location can be determined autoradiographically. Using this approach, it was found that specific parts of the mouse embryo transcribed particular homeobox genes at particular stages of development.<sup>10</sup> Moreover, the linear order of the *Hox* genes along a chromosome parallels the expression of that gene along the anterior-posterior axis of the embryo. Genes having lower numbers within each cluster of Figure 3 (said to be more 3', as indicated at the bottom of Figure 3) are expressed earlier in development and in more anterior regions of the embryo.<sup>11,12</sup> A similar observation had been made much earlier by E. B. Lewis on the homeotic genes of the *Drosophila* clusters.

Attempts to understand the role of *Hox* genes in mammalian development have only just begun. Several different approaches have been taken, including injection into embryos of antibodies directed against homeobox proteins, production of transgenic mice containing extra copies of the *Hox* genes that are overexpressed during embryonic development, and the formation of knockout mice that are lacking both copies of one of the *Hox* genes. Taken together, these



**Figure 3** The organization of the genes that make up the four mammalian *Hox* complexes. Each complex contains 13 genetic loci, but not all loci have functional genes. The 39 functional *Hox* genes of mammals are named according to a nomenclature proposed in the 1992 paper from which this is taken. Alignment with *Drosophila* genes is shown at the top. The direction of transcription of the genes is indicated by the single-headed arrows. (FROM M. P. SCOTT, CELL 71:551, 1992. VERTEBRATE HOMEBOX GENE NOMENCLATURE. BY PERMISSION OF CELL PRESS AND ELSEVIER SCIENCE.)

studies suggest that, as in the fruit fly, the homeobox genes direct each part of the embryo to develop along a pathway that is consistent with its position along the anterior-posterior axis of the embryo.

The products of the *Hox* genes are transcription factors that either activate or repress the expression of other genes involved in the development of embryonic structures. Because there are only a handful of homeotic proteins, it is presumed that each of them regulates (both directly and indirectly) the transcription of a large number of subordinate genes. The first studies of the DNA-binding activity of the homeodomain were carried out using a protein encoded by the *engrailed* gene, one of the homeobox-containing genes of the fruit fly.<sup>13</sup> To better define the DNA-binding activity of that protein, various parts of the *engrailed* coding sequence were fused to the bacterial  $\beta$ -galactosidase gene. The fusion proteins that resulted from expression of the fused gene were allowed to bind to DNA fragments prepared from the genome. The binding of a fusion protein to the DNA was monitored by precipitating DNA-protein complexes with an antibody against  $\beta$ -galactosidase.

Using this procedure, it was determined that DNA-binding activity was localized to a region of the engrailed protein consisting of the homeodomain and an additional 12 amino acids on its N-terminal flank. The DNA sequence that bound the homeodomain was determined by allowing the engrailed fusion protein to bind to genomic DNA fragments, then treating the DNA-protein complexes with an enzyme (DNase I) that digests all of the DNA that is not protected by a bound protein. The enzyme was then removed and the undigested DNA purified and sequenced. This type of experiment provided a “footprint” of the DNA sequence bound by the protein.

Results of these experiments indicated that the engrailed protein bound DNA fragments bearing a sequence equal to or very similar to TCAATTAAAT. In most cases, this sequence was repeated several times within the protected region of the DNA. Identification of a DNA sequence to which the homeodomain can bind provided the tool needed to search for such sites in the regulatory regions upstream from genes that are known to play a role in embryonic development. Identification of a homeodomain recognition site also made it possible to describe the three-dimensional structure of the complex formed by the homeodomain and the DNA to which it binds.<sup>14</sup> The homeodomain was determined to contain a DNA-binding motif, called a helix-turn-helix (HTH), similar to that identified previously in bacterial repressors that bound to operator sites in bacterial operons (page 518). The key aspects of the interaction between the DNA and a homeotic gene product are shown in Figure 4.

One of the most important questions concerning the activity of homeotic genes is how the product of one homeotic gene carries out functions distinct from those of other genes. As noted above, the amino acid sequences of the homeodomains of different homeotic proteins are very similar to one another. As a result, it was initially expected that differences in DNA-binding activity of different homeotic gene products would be determined by those parts of the proteins residing outside of the homeodomain; these are the regions of the proteins that are very different from one another. One way to determine which part or parts of related proteins de-



**Figure 4** Schematic diagram showing the relationship of the N-terminal arm of the homeodomain and the DNA double helix. The position of the  $\alpha$  helices is indicated by the cylinders. (FROM C. R. KISSINGER ET AL., CELL 63:581, 1990. CRYSTAL STRUCTURE OF AN ENGRAILED HOMEODOMAIN-DNA AT 2.8 Å RESOLUTION: A FRAMEWORK FOR UNDERSTANDING HOMEODOMAIN-DNA INTERACTIONS. BY PERMISSION OF CELL PRESS AND ELSEVIER SCIENCE.)

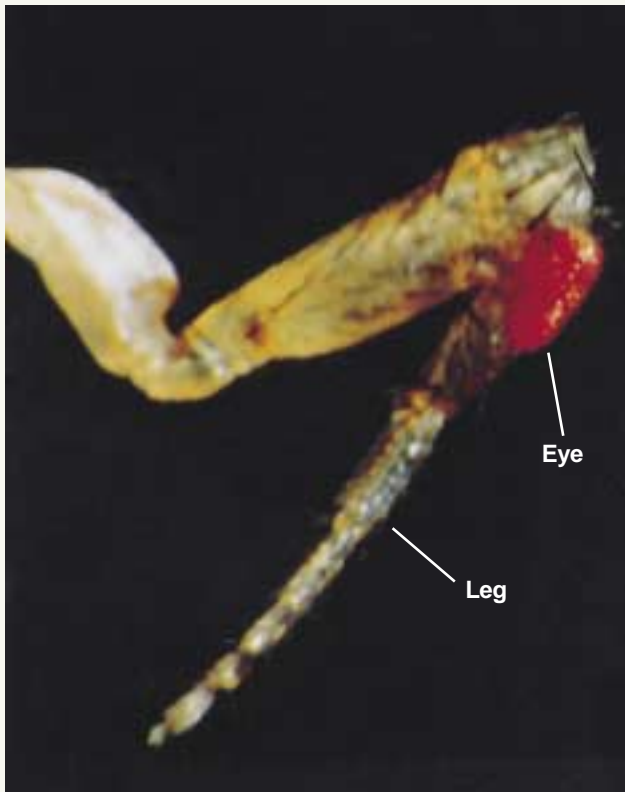
termine their functional specificity is to construct genes that encode hybrid proteins containing parts of more than one member of the group. These hybrid genes can then be introduced into a fruit fly or mouse embryo, and their effects on development can be followed. Experiments in which parts of homeotic proteins are swapped with one another have been carried out in a number of laboratories, and all of the results indicate that functional specificity is present within the amino acid sequence of the homeodomain itself. Thus if a hybrid gene encodes a protein having its homeodomain from protein A and the remainder from protein B, the hybrid protein functions as if it were protein A.<sup>15,16</sup>

Results of these experiments raised important questions about how proteins with such similar DNA-binding domains—in some cases differing by only a few amino acids—can activate different batteries of genes that cause parts of the embryo to develop into different types of structures. Like other transcription factors discussed in this chapter, homeotic gene products also bind to the DNA as dimers. A dimeric transcription factor must recognize two different sites on the DNA, which greatly increases the specificity of the interaction between the protein and DNA. Specificity is also increased through cooperative binding in which one protein can only bind to the DNA in conjunction with other proteins.<sup>Reviewed in 17, 18</sup> In fact, differences in amino acid sequence among different homeotic proteins may determine differences in protein-protein interactions. Ultimately, the signals that cause a particular segment of a fly to develop into an antenna rather than a leg may depend on subtle differences in affinity of homeotic transcription factors for particular nucleotide and/or amino acid sequences.

The importance of transcription factors in triggering the formation of a particular organ is evident from studies on a gene that controls eye development. An insect eye is a complex organ, requiring the concerted activity of an estimated 2500 different gene products. *Eyeless* (*ey*) is a *Drosophila* mu-

tant that fails to develop eyes, indicating that the product of the *ey* gene plays a role in eye formation. But the importance of the protein was not realized until 1995 when Walter Gehring and his coworkers demonstrated that the product of the *ey* gene single-handedly triggers the development of an eye in some of the most unlikely parts of a fly's body, such as a wing or a leg (Figure 5).<sup>19</sup>

To carry out these experiments, researchers prepared two groups of genetically engineered fruit flies. One group carried extra copies of the *eyeless* gene located downstream from a regulatory region that could be activated by a yeast transcription factor called GAL4. The second group carried copies of the yeast *GAL4* gene whose expression was controlled by a nearby enhancer (page 528) that responded to tissue-specific transcription factors. Consequently, the *GAL4* gene was only expressed in specific tissues of these animals. When members of these two groups of flies were mated, the offspring contained both types of foreign gene constructs and consequently expressed the *ey* gene in those cells that produced GAL4. If GAL4 was expressed in wing cells, so too was the product of the *ey* gene, which caused the cells in the wing to give rise to an eye. Some of the offspring of these matings produced as many as 14 different eyes located in a variety of abnormal (i.e., *ectopic*) sites around the body. In addition to providing headlines for newspapers around the world, the



**Figure 5** The leg of this fruit fly bears a fully formed eye that has formed as the result of the forced expression of the *eyeless* gene within cells of the leg primordium during the development of the adult. (COURTESY OF U. KLOTER, G. HALDER, AND W. J. GEHRING.)

research suggested that the *ey* gene was a true master control gene for eye formation. The product of the gene can presumably activate other genes, triggering a chain reaction that leads to the ordered construction of the parts of an eye.

Because the eyes of arthropods and vertebrates are so differently constructed, it has always been assumed that the two types of eyes evolved independently. Yet experiments have shown that mammals, including both mice and humans, contain a gene (called *Pax6*) that is homologous to *eyeless* that has also been shown to be involved in eye formation. In fact, the mammalian gene can substitute for the fly gene in causing the formation of ectopic eyes in fruit flies. Thus, even though flies and mammals develop very different types of eyes, the evidence suggests that the development of eyes is controlled by a similar type of “master gene” and that the two eyes are actually evolutionarily related.

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## EXPERIMENTAL PATHWAYS

### The Role of Human NER Deficiencies in DNA Repair Research

In 1968, James Cleaver of the University of California made a discovery that started a new field of molecular biology and human genetics. It was known that persons suffering from the rare inherited disorder xeroderma pigmentosum (XP) were extremely sensitive to exposure to the sun (discussed previously in the Human Perspective). Cleaver had been studying the capability of cultured mammalian cells to carry out DNA repair. It had previously been demonstrated that bacteria exhibiting an increased sensitivity to ultraviolet radiation carried mutations in one of the genes required for DNA repair. Cleaver wondered whether xeroderma pigmentosum might be caused by similar types of mutations in the human genome. To test this possibility, skin biopsies were taken from three persons with XP and one normal subject; the tissues were placed in culture, and fibroblasts from each of the skin samples were isolated. The ability of fibroblasts from each of these sources to repair DNA damage resulting from ultraviolet irradiation was then determined.<sup>1</sup>

As discussed on page 573, removal of pyrimidine dimers from a DNA strand requires endonucleases to cut the strand and a polymerase to fill in the gap. This latter process is called repair replication and can be assayed by measuring the incorporation of radioactively labeled thymidine into cells that are not engaged in S-phase DNA replication. In one series of experiments, Cleaver irradiated cultures of fibroblasts with various doses of ultraviolet light, labeled the cells with [<sup>3</sup>H]thymidine for three hours, and then fixed and processed the cells for autoradiography. The percentage of cells labeled in each of four cultures is shown in Table 1. Whereas all of the normal cells subjected to UV irradiation were engaged in repair replication (as indicated by the presence of large numbers of silver grains over the cell nuclei in the autoradi-

ographs), only about one-quarter of cells from persons with XP showed evidence of this activity. These results indicated that repair replication in fibroblasts from persons with XP is either completely absent or occurs at a much reduced level compared with that of cells from healthy individuals.

Studies in bacteria had revealed that repair of UV-damaged DNA required the activity of different enzymes to excise the lesion, repair the DNA strand, and ligate the replacement DNA with the unrepaired portion of the strand. In the wake of Cleaver's initial studies on XP cells, a number of laboratories began to focus on the specific deficiencies at the molecular level that generated the XP phenotype. One of the first studies was conducted by R. B. Setlow and his colleagues at Oak Ridge National Laboratory.<sup>2</sup> In one series of experiments, these researchers compared the ability of normal and XP cells to excise pyrimidine dimers, which is the first step in the repair pathway (see Figure 13.27). The two types of cells were incubated with [<sup>3</sup>H]thymidine for 24 hours. The cells were then washed free of isotope, unlabeled medium was added to the cultures, and the cells were irradiated through the bottoms of the Petri dishes with ultraviolet light. Then at various time periods following irradiation, DNA was extracted from samples of the two groups of cells, and the proportion of labeled thymine present as pyrimidine dimers was determined by acid hydrolysis of the DNA followed by paper chromatography. The results of the experiments are shown in Figure 1. Whereas normal cells removed 50–70 percent of the dimers in a 24-hour period, XP cells exhibited little or no excision. The average initial excision rate in XP cells was at least tenfold lower than that observed in control cells.

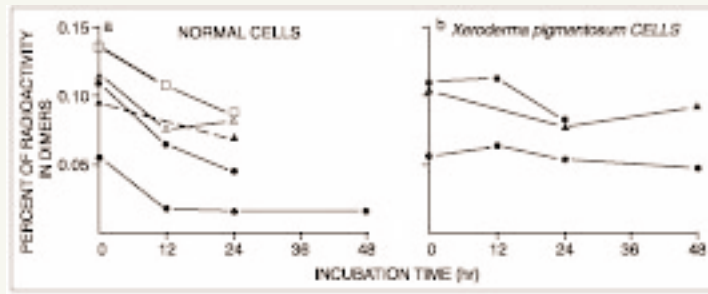
In a second part of the study, these researchers sought to determine the relative numbers of single-strand breaks in the DNA of normal and XP cells that had been subjected to UV irradiation. The presence of single-strand breaks is evidence of endonuclease activity, which is required for the first step of nucleotide excision repair (see Figure 13.27). To assay for single-strand breaks, the DNA of irradiated cells was extracted, layered on the top of an alkaline sucrose gradient, and subjected to ultracentrifugation for 90 minutes at 30,000 rpm. Larger DNA fragments (which have larger sedimentation coefficients) move more rapidly during centrifugation than smaller fragments and, as a result, are located closer to the bottom of the tube at the end of the sedimentation period. The alkalinity of the gradients causes the two strands of each DNA duplex to separate, and thus those strands that have breaks along their length behave as smaller molecules in the gradients than strands that lack such breaks.

The results of this type of experiment conducted on normal cells are shown in Figure 2. Ten hours after irradiation, the sedimentation coefficient of the DNA of irradiated cells was considerably smaller than that of unirradiated cells, indicating the presence of single-strand breaks. By 24 hours, the DNA from irradiated and nonirradiated cells had similar sedimentation profiles, indicating that the single-strand

**Table 1** Labeling Indices of Human Skin Fibroblasts Incubated for 3 hr in [<sup>3</sup>H]thymidine After Various Doses of Ultraviolet Light

	Control	100 erg/mm <sup>2</sup>	1,000 erg/mm <sup>2</sup>
Normal	41.2%	100%	100%
Xeroderma pigmentosum (5-yr-old male)	24.2%	25.3%	27.4%
Xeroderma pigmentosum (9-yr-old female)	19.0%	23.2%	15.0%
Xeroderma pigmentosum (29-yr-old male)	22.2%	28.9%	26.0%

Source: Reprinted with permission from J. E. Cleaver, *Nature* 218:655, 1968, © copyright 1968, Macmillan Magazines Ltd.



**Figure 1** When normal cells are subjected to UV irradiation (left curves), thymine-containing dimers form in the DNA, but they are removed over time as indicated by the drops in each of the curves, which represent separate experiments with different UV exposures. In contrast, the thymine-

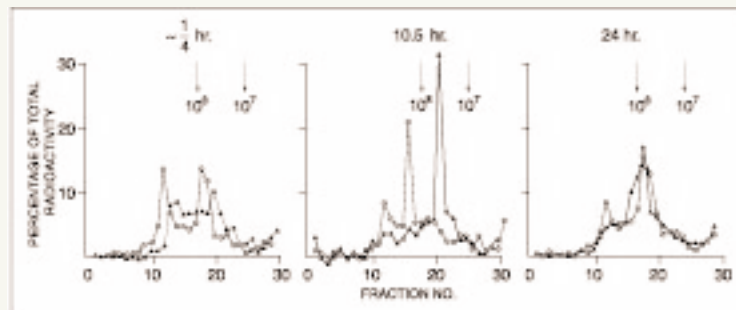
containing dimers that form in the DNA of XP cells after similar UV exposure are not removed over time (right curves). (FROM R. B. SETLOW ET AL., PROC. NAT'L ACAD. SCI. U. S. A. 64:1038, 1969.)

breaks had been repaired. In contrast, the sedimentation profile of the DNA extracted from irradiated XP cells remained similar at all times following irradiation, indicating the absence of single-strand breaks in the DNA. These results suggested that these XP cells lack an endonuclease capable of making the incision in the DNA strand containing a pyrimidine dimer.

Nucleotide excision repair is a complex, multistep process, and there is no reason to expect that all persons with XP have the same defective gene. In 1969, a relatively simple way to test this premise by means of cell fusion was developed.<sup>3</sup> Cell fusion is a technique, illustrated in Figure 4.28, in which two cells are fused to produce a hybrid cell that contains the combined chromosomes of both participating cells. Consider what might be expected if one were to fuse two cells containing *different* genetic defects. Suppose one cell has two defective alleles of gene *a*, but two normal alleles of gene *b*, whereas the other cell has the opposite genetic composition. The hybrid cell will contain normal alleles for both genetic

functions and thus possess a normal phenotype. In other words, the chromosomes from the two cells complement one another as long as the defects are located in separate genes. In contrast, if the two participating cells have defects in the same gene, then the hybrid lacks a normal gene for this trait and exhibits the same genetic defect as both of the participating cells. The first test of this premise was carried out using two human male cell strains, each carrying a different X-linked mutation. The resulting hybrid cells were shown to synthesize both normal gene products, confirming the ability of the X chromosomes from the two cells to complement one another.<sup>3</sup>

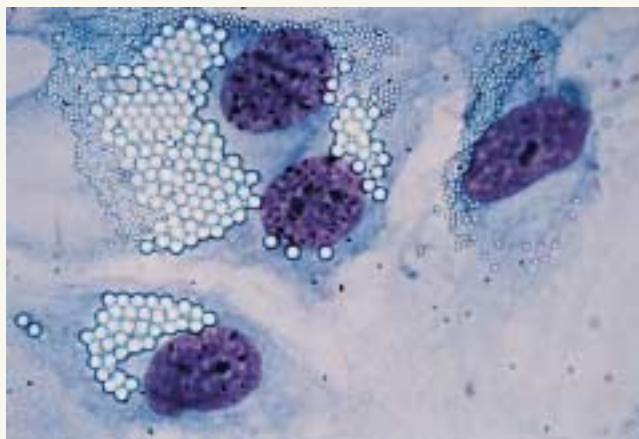
The first use of cell hybridization for testing intergenic complementation in xeroderma pigmentosum was conducted in 1972 in Dirk Bootsma's laboratory at Erasmus University in The Netherlands. It had been suspected that XP might result from deficiencies at different genetic loci because not all persons with the disease exhibited the same symptoms or severity of the disease. If different persons



**Figure 2** Sedimentation patterns of DNA extracted from normal cells that were either unirradiated controls (open circles) or irradiated at zero time with  $150 \text{ ergs/mm}^2$  of UV light (closed triangles). Cells were grown for the indicated times before the DNA was sedimented through an alkaline sucrose density gradient (the alkalinity ensures that the DNA sediments as single strands). The bottom of each centrifuge tube (which would contain the largest strands) is on the left side of each curve. The presence of DNA repair activity is indicated by the peak of smaller DNA strands at

10.5 hours, which reflects the presence of single-strand breaks that accompany the repair process. The disappearance of this peak at 24 hr corresponds to the ligation of the repaired DNA to unaltered strands. The arrows indicate the expected positions of single-stranded DNA of molecular weights of  $10^7$  and  $10^8$  as estimated from separate sedimentation experiments of [ $^3\text{H}$ ]thymidine-labeled phage DNA. (FROM R. B. SETLOW ET AL., PROC. NAT'L ACAD. SCI. U. S. A. 64:1039, 1969.)

with XP have mutations in different genetic loci, then one might expect some of the hybrids resulting from the fusion of cells from different patients to possess a full complement of normal genes and thus to regain their DNA repair capability. This was exactly what was found.<sup>4</sup> When cells from a number of XP patients were fused in various combinations, some of the hybrids retained their UV sensitivity, indicating that both original cells contained defects in the same gene. Other hybrids showed genetic complementation, as evidenced by the incorporation of a low level of [<sup>3</sup>H]thymidine, which was measured autoradiographically. An example of this type of experiment is shown in Figure 3. It is evident in these experiments that thymidine was incorporated into DNA as part of a repair process rather than as part of normal replication that occurs during S phase because the level of incorporation is orders of magnitude less than that observed in an S-phase cell. In addition, this low-level, repair-type incorporation is never observed in hybrids formed between two cells from the same XP strain (i.e.,



**Figure 3** The use of cell fusion to demonstrate that XP can be caused by defects in different genes in different persons. Fibroblasts from two different XP patients were marked by inclusion of latex beads of different diameters, and the cells were then fused with one another by addition of inactivated Sendai virus. The culture was then subjected to UV irradiation and subsequently incubated in the presence of [<sup>3</sup>H]thymidine. The incorporation of the labeled precursor into DNA as the result of nucleotide excision repair was monitored autoradiographically. The level of DNA repair in a nucleus is reflected in the number of silver grains over that nucleus. The micrograph shows three cells: one hybrid cell and two unfused cells. The unfused cell from the XP-C group contains the large-sized beads, and the unfused cell from the XP-G group contains the small-sized beads. One of these cells exhibits no DNA repair capacity, and the other a very limited activity. The hybrid cell situated near the center of the micrograph contains two nuclei, one from each patient (as indicated by the presence of both types of beads in the cytoplasm). Both nuclei exhibit silver grains, which demonstrates the ability of the genes of one nucleus to complement the defect in the genes of the other nucleus.

(FROM JAN H. J. HOEIJMAKERS, *TRENDS GENET.* 9:211, 1993.)

from the same person), but occurs only when the fused cells are from different strains.

Over the next several years, numerous studies using the cell fusion assay were conducted to test the ability of XP cells from different patients to complement one another. These studies led to the division of cells from XP patients into seven different complementation groups, referred to as XP-A to XP-G.<sup>5</sup> As long as two cells from different complementation groups were fused, the hybrids regained their ability to carry out DNA repair. These results suggested that at least seven different genes encoded products required for the repair of UV-damaged DNA, which reflects the complexity of the process.

As the studies on humans were proceeding, other investigators were isolating rodent cells that were sensitive to UV light and presumed to have DNA repair deficiencies similar to those found in cells isolated from persons with XP.<sup>6</sup> These rodent cell lines have proven valuable as a means of isolating human genes involved in nucleotide excision repair. Suppose a rodent cell has the same deficiency as a person that falls into one of the XP groups. If this cell were to take up a human DNA fragment that contained the normal gene at that locus, then this transfected cell would regain its DNA repair capacity and would possess normal resistance to UV light. Those few cells in the population that have regained their normal DNA repair ability can then be selected by exposure of the cell culture to UV light, a treatment that kills those cells that are still repair deficient. (Selection can also be accomplished by incorporating genes for drug resistance into the human DNA fragments and using the drug to select for transfected cells.) Once a culture of UV-resistant transfected cells is obtained, the DNA can be extracted, and the human DNA fragment responsible for correcting the DNA repair deficiency can be isolated and its encoded product identified. The human DNA fragments can be selectively purified from the rodent DNA because they carry marker DNA sequences used as targets for DNA probes used in the isolation procedure.

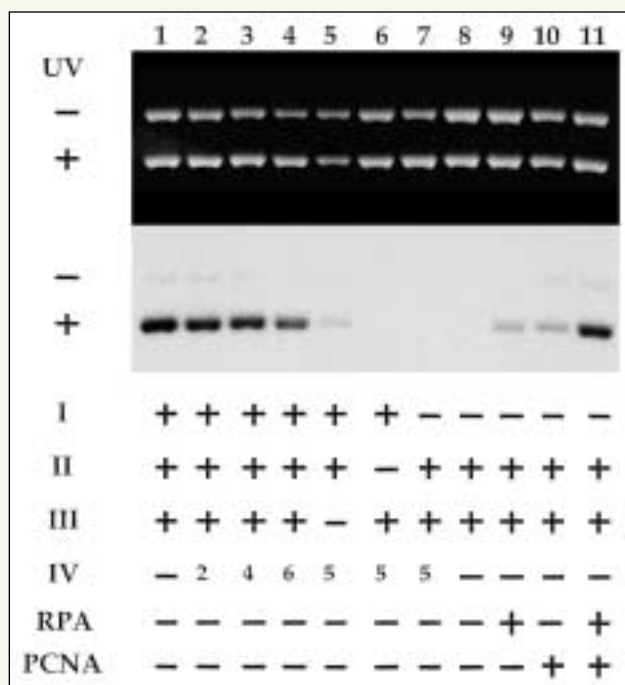
Using this transfection-correction technique, researchers have isolated several genes from human DNA that code for DNA repair proteins. Because they were isolated due to their ability to complement defects in rodent cells, these human genes were called *ERCC* (excision repair cross complementing) genes. The first such gene to be isolated was called *ERCC1*, and the encoded amino acid sequence showed significant homology to a protein called *RAD10* that had been isolated from yeast cells and was known to be involved in nucleotide excision repair.<sup>7</sup> These experiments provided the first indication that DNA repair systems were highly conserved in eukaryotic organisms from yeast to humans.

Over the next several years, a number of other correlations were made between human *ERCC* genes and yeast *RAD* genes. For example, a second human gene capable of correcting a DNA repair deficiency in UV-sensitive rodent cells was discovered and designated *ERCC2*. Analysis of the nucleotide sequence of *ERCC2* indicated that it encoded a polypeptide homologous to that encoded by *RAD3*, a yeast gene whose product acted as an ATP-dependent 5' → 3' DNA helicase.<sup>8</sup> Helicases are enzymes involved in unwinding the DNA duplex during transcription, replication, repair, and recombination (e.g., page 559). A third human gene, *ERCC3*, was also discovered that encodes a protein with

DNA helicase activity. Just as important, *ERCC3* was able to correct the UV sensitivity of cells from one of the human XP complementation groups, specifically XP-B.<sup>9</sup> Over the next few years, a number of genes were isolated from human DNA that corrected DNA repair deficiencies in rodent cell lines and in XP-derived skin fibroblasts and were homologous to yeast *RAD* genes.<sup>reviewed in 10</sup>

A surprising discovery was made in 1993 in Jean-Marc Egly's laboratory in Strasbourg, France, when it was shown that the product of the *ERCC3* gene was a DNA helicase that was also a subunit of the general transcription factor TFIIH (page 455).<sup>11</sup> Until this discovery it was assumed that transcription was the only process that required TFIIH. This finding suggested that the *ERCC3* gene product has a dual role in unwinding DNA in preparation for both transcription and DNA repair. This finding may also explain how the template strand of transcriptionally active genes can be preferentially repaired. If an RNA polymerase is moving along the template strand and becomes stalled by a lesion in its path, then TFIIH may disembark from the transcription machinery and unwind the DNA duplex, creating single-stranded regions that are accessible to other repair proteins.

A major advance in the study of nucleotide excision repair was reported in 1995 by Richard Wood and his colleagues, who reconstituted the entire process in vitro using purified protein components.<sup>12</sup> The steps taken in this study illustrate how reconstitution of a complex biochemical process can be achieved. To begin the study, an extract of human HeLa cells was fractionated by chromatography through a phosphocellulose column into four crude fractions. A mixture of fractions I, II, and III was found to repair DNA damage caused by UV irradiation, indicating that all of the components necessary for nucleotide excision repair were present in these combined fractions (Figure 4). It had been shown previously that fraction I contained RPA (a single-stranded DNA-binding protein) and PCNA (the sliding clamp for DNA polymerase  $\delta$  and  $\epsilon$ ). When purified RPA and PCNA were combined with fractions II and III, the mixture repaired damaged DNA, indicating that fraction I could be replaced by these two purified proteins (Figure 4). Fraction II was then subdivided by chromatography through hydroxyapatite into four fractions (IIa, IIb, IIc, and IId), which were mixed in various combinations to test for activity. Of the various combinations, a mixture of fractions IIa, IIb, and IId was fully active in DNA repair. Fraction IId contained the protein XPG, and addition of purified XPG substituted for fraction IId in the reconstitution reaction. Analysis of the other fractions (IIa, IIc, and III) led to the identification of numerous other human proteins required in the various steps of nucleotide excision repair. In the final step of the study, all of the repair proteins purified from the various fractions were combined in the same reaction mixture, and this mixture performed the entire repair process. Altogether, 12 distinct proteins, consisting of about 30 different polypeptide subunits, were required in the reaction mixture. Omission of any of these components prevented damage-dependent DNA repair. More recently, the entire process of NER has been achieved in the test tube using a combination of recombinant human repair proteins synthesized from cloned DNA (including TFIIH) and replication proteins (e.g., DNA polymerase  $\delta$  and RFC) purified from human cells.<sup>13</sup>



**Figure 4** UV-irradiated and nonirradiated DNA were incubated with various HeLa cell fractions, and their ability to carry out DNA repair was determined. In this figure, the top part of the photograph (blackened background) shows the position of the nonirradiated (-) and irradiated DNA (+) after electrophoresis through an agarose gel. The lower part of the photograph (lighter background) shows an autoradiograph of the same gels. A dark band in the row for the irradiated DNA (+ row) indicates that DNA repair has occurred. The lower part of the figure indicates which fractions were added to the mixture. The numbers in the row for fraction IV indicate the micrograms of this fraction that were added. The data indicates that fraction IV is not required for nucleotide excision repair and that purified RPA and PCNA can substitute for fraction I. (FROM A. ABOUSSEKHRA ET AL., *CELL* 80:860, 1995, COURTESY OF RICHARD D. WOOD; BY PERMISSION OF CELL PRESS.)

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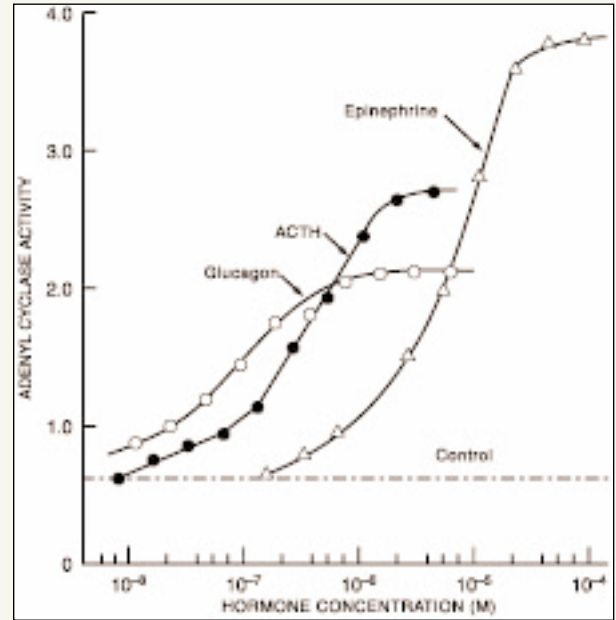
## EXPERIMENTAL PATHWAYS

### The Discovery and Characterization of GTP-Binding Proteins

During the late 1950s and 1960s, Earl Sutherland and his colleagues at Case Western Reserve University discovered that certain hormones, such as epinephrine, act by binding to a specific receptor at the cell surface, which activates the enzyme adenylyl cyclase on the inner side of the membrane. Activation of adenylyl cyclase leads to the production of a second messenger, cyclic AMP, which diffuses into the cytoplasm and initiates the cell's response.<sup>reviewed in 1</sup> By the end of the 1960s, the concept of the second messenger was well established, but little was known about the precise molecular mechanism that allowed a hormone (or other ligand) bound at the outside of the membrane to activate adenylyl cyclase activity. One possibility considered likely was that the active site of the adenylyl cyclase was part of the hormone receptor itself.

To learn more about the relationship between hormone receptors and adenylyl cyclase, Martin Rodbell and Lutz Birnbaumer at the National Institutes of Health began a series of experiments on the isolated plasma membranes of fat cells and liver cells.<sup>2</sup> One of the first questions they sought to answer was whether these cells, which respond to several different hormones by producing cAMP, possess a single adenylyl cyclase that is activated by all the various hormones or have separate adenylyl cyclases for each of the hormones to which they respond. Fat cells were chosen for these initial studies because they are readily isolated free of other cell types, they respond to several hormones that cause a rapid increase in intracellular levels of cAMP (stimulating enzymes involved in lipid degradation), and their plasma membranes can be isolated as "ghosts" by osmotic lysis. Six different hormones (ACTH, epinephrine, glucagon, TSH, LH, and prolactin) were found to stimulate adenylyl cyclase activity in isolated fat-cell ghosts. The dose response curves of the first three of these hormones acting separately are shown in Figure 1. As shown in Table 1, when these hormones were combined in sets of two or three, their effects on adenylyl cyclase activity were not additive, suggesting that each of these hormones stimulates the same population of adenylyl cyclase molecules. It was shown, however, that the different hormones interact with spatially distinct receptors. Together, these findings led Rodbell and Birnbaumer to propose that, even though the various hormone receptors stimulate a common population of adenylyl cyclase molecules, the receptors and the adenylyl cyclase effectors exist separately from one another in the plasma membranes of fat cells.

In 1971, Rodbell and his colleagues published a series of papers on the stimulation of adenylyl cyclase in isolated plasma membranes of liver cells in response to glucagon and epinephrine. First, they measured the binding of radioactively labeled glucagon molecules to receptors on isolated plasma membranes. Because ATP is a substrate of the adenylyl cyclase reaction, the effects of ATP on the binding of labeled glucagon were monitored, as were the effects of the three other common nucleoside triphosphates, UTP, CTP,



**Figure 1** The effect of varying concentrations of ACTH, epinephrine, and glucagon on adenylyl cyclase activity of fat cell ghosts. The dashed line represents the activity in the absence of added hormones. (Adenylyl cyclase was initially named adenylyl cyclase and later called adenylyl cyclase.) (FROM L. BIRNBAUMER AND M. RODBELL, *J. BIOL. CHEM.* 244:3478, 1969.)

and GTP. It was during these investigations that the special properties of GTP were first noted.<sup>3</sup>

One of the observed effects of the nucleoside triphosphates was to cause the dissociation of labeled glucagon that was bound to the isolated plasma membranes. Whereas ATP, UTP, and CTP did not affect dissociation of glucagon from the membranes at concentrations less than 100 mM, GTP stimulated dissociation at concentrations as low as 0.05 mM. A comparison of the effects of ATP and GTP in causing glucagon dissociation from the membrane is shown in Figure 2. (Note that the concentration is plotted on a log scale, indicating the very large differences in effectiveness of the two triphosphates.) These results suggested to the authors that guanyl nucleotides induce a change in the state or properties of the glucagon receptor that decreases its affinity for glucagon. In addition, they found that liver membranes can hydrolyze GTP.

In the last paper of the series, Rodbell and his colleagues addressed the question as to whether the effects of the guanyl nucleotides on the binding of glucagon bear any relationship to the actions of glucagon on the adenylyl cyclase system of the liver membranes.<sup>4</sup> As shown in Figure 3, GTP stimulated the basal activity of adenylyl cyclase and did so at concentrations as low as 0.01 mM. None of the other nucleotides pos-

Table 1

Effects of Combinations of Hormones, at Supramaximal Concentrations, on Adenylyl Cyclase Activity in Fat Cell Ghosts\*

Additions	Change in adenylyl cyclase activity due to hormones			
	Experiment 1 (37°)		Experiment 2 (30°)	
	Found	Calculated if additive	Found	Calculated if additive
ACTH	0.57 ± 0.02		1.19 ± 0.07	
Epinephrine	1.00 ± 0.06		1.79 ± 0.11	
Glucagon	0.32 ± 0.01		0.57 ± 0.04	
ACTH + epinephrine	0.80 ± 0.04	1.57	2.04 ± 0.12	2.98
Epinephrine + glucagon	0.99 ± 0.05	1.26	2.13 ± 0.10	2.36
ACTH + glucagon	0.64 ± 0.02	0.89	1.33 ± 0.06	1.76
ACTH + epinephrine + glucagon	0.85 ± 0.04	1.88	2.30 ± 0.10	3.55

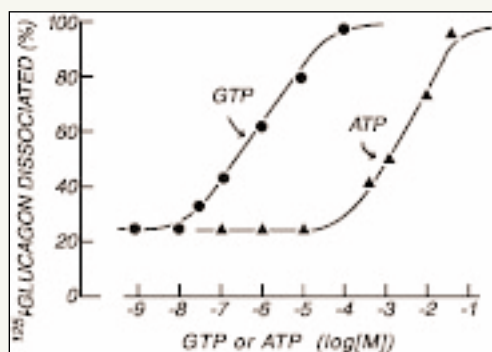
\*Adenylyl cyclase activity was measured at either 37° in absence of, or at 30° in presence of, ATP-regenerating system. The following concentrations of the hormones used, either individually or when combine: ACTH, 400 µg per ml; epinephrine, 400 µg per ml; glucagon, 60 µg per ml. Values are the mean of triplicate determination ± standard deviation.

SOURCE: L. Birnbaumer and M. Rodbell, *J. Biol. Chem.* 244:3479, 1969.

sessed this activity. The authors concluded that guanyl nucleotides are required for the activation of adenylyl cyclase by glucagon. Subsequent studies in other laboratories showed that GTP enhances the response of adenylyl cyclase systems to other hormones, including peptide hormones, catecholamines (e.g., epinephrine), and prostaglandins.

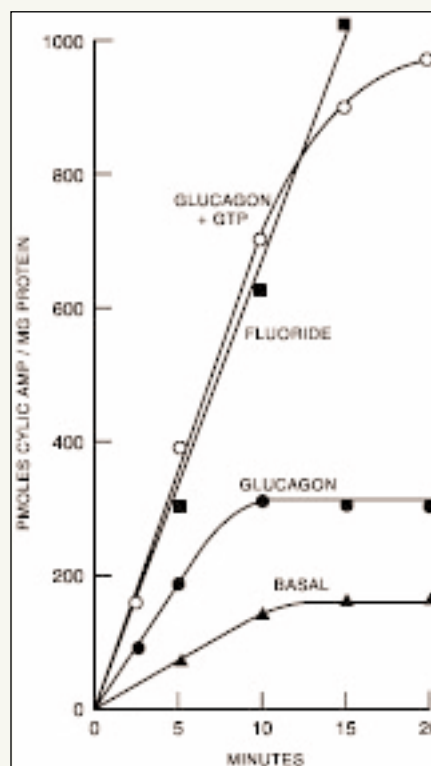
Nonhydrolyzable analogues of nucleoside triphosphates are useful because they allow researchers to distinguish between the effects of nucleotide binding from those of nucleotide hydrolysis. When a nonhydrolyzable analogue of GTP, 5'-guanylylimododiphosphate, Gpp(NH)p, was incubated with liver plasma membranes, glucagon, ATP, and

other ingredients necessary for adenylyl cyclase activation, Gpp(NH)p mimicked the stimulatory effects of GTP. In fact, the nonhydrolyzable GTP analogue activated adenylyl cyclase even in the absence of hormones.<sup>5</sup> These results indicated



**Figure 2** Effects of varying concentrations of GTP and ATP on dissociation of [<sup>125</sup>I]glucagon from plasma membranes of liver cells. In this experiment, the membranes were preincubated for 15 minutes in a medium containing labeled glucagon. Then, either GTP or ATP was added in the presence of a large excess of unlabeled glucagon. After 15 minutes of incubation in one or the other nucleoside triphosphate, samples were taken to measure the labeled glucagon remaining bound. The percentage of bound, labeled glucagon that dissociated during the incubation with ATP or GTP was calculated by comparing the bound radioactivity present at the beginning and at the end of the 15-minute incubation with the nucleoside triphosphate.

(FROM M. RODBELL ET AL., *J. BIOL. CHEM.* 246:1873, 1971.)

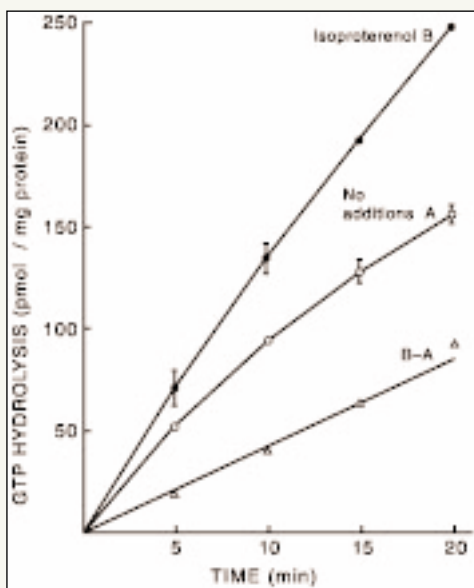


**Figure 3** The effect of fluoride ion, glucagon, and a combination of glucagon and GTP on the formation of cyclic AMP by a preparation of isolated liver cell plasma membranes. The marked stimulation by GTP shown here was achieved with a GTP concentration of 0.01 mM. Fluoride ion was also found to be an effective activator of adenylyl cyclase and was used in numerous subsequent assays. (FROM M. RODBELL ET AL., *J. BIOL. CHEM.* 246:1879, 1971.)

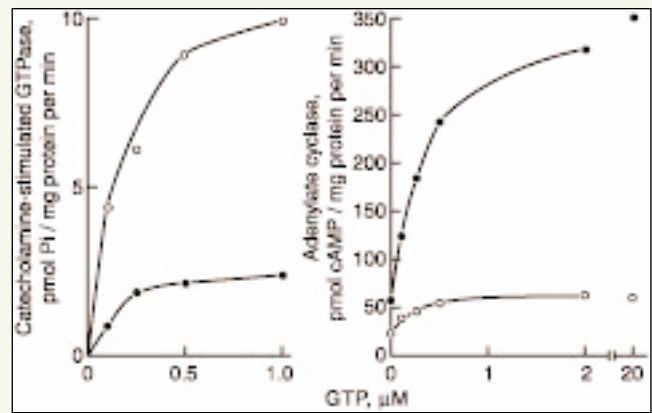
that the binding of GTP, rather than its hydrolysis, played an important role in activating adenylyl cyclase. The primary difference between the effects of GTP and a nonhydrolyzable GTP analogue is that the former stimulates adenylyl cyclase only transiently, whereas the latter stimulates the enzyme for a prolonged period. These findings led investigators to focus on the mechanism by which GTP is normally hydrolyzed by the adenylyl cyclase system.

In the mid-1970s, a number of laboratories, including Rodbell's, identified a GTPase located within the plasma membranes of various cells. In one key study, Don Cassel and Zvi Selinger of the Hebrew University of Jerusalem found that addition of isoproterenol (a catecholamine hormone similar to epinephrine) to a plasma membrane GTPase assay system caused a 30–70 percent increase in the hydrolysis of [ $^{32}$ P]GTP as measured by the release of  $^{32}$ P<sub>i</sub> (Figure 4).<sup>6</sup> Cassel and Selinger proposed that the hydrolysis of GTP by the GTPase serves to turn off the activated adenylyl cyclase, returning the enzyme to the basal inactive state.

In a subsequent study, these same researchers made an important connection between the GTP-binding protein, as it came to be called, and the infamous cholera toxin. Cholera is a disease caused by a bacterial toxin that produces excessive diarrhea and accompanying water loss in infected individuals. During the early 1970s, a number of laboratories found that cholera toxin acts by stimulating adenylyl cyclase in the cells of the intestinal epithelium. It became apparent that the cholera toxin mimics the action of hormones by acting on one of the components commonly present in the adenylyl cyclase



**Figure 4** The time course of GTP hydrolysis in the presence and absence of the catecholamine isoproterenol by isolated turkey erythrocyte membranes. The effect of the hormone is seen in the lower curve, which plots the difference in GTP hydrolysis between the hormone-treated and control membrane preparations. (REPRINTED FROM D. CASSEL AND Z. SELINGER, *BIOCHIM. BIOPHYS. ACTA* 452:543, 1976. CATECHOLAMINE-STIMULATED GTPASE ACTIVITY IN TURKEY ERYTHROCYTE MEMBRANES. COPYRIGHT 1976, WITH PERMISSION FROM ELSEVIER SCIENCE.)



**Figure 5** Effect of the cholera toxin on the catecholamine-stimulated GTPase (left) and adenylyl cyclase (right) activities at various GTP concentrations. Membranes pretreated in the absence (open circles) or presence (closed circles) of toxin were assayed for GTPase and adenylyl cyclase activities at the indicated GTP concentrations. In the presence of the toxin, the lower GTPase activity (left) leads to an elevated level of adenylyl cyclase activity (right). (FROM D. CASSEL AND Z. SELINGER, *PROC. NAT'L ACAD. SCI. U. S.A.* 74:3309, 1977.)

system. In 1977, Cassel and Selinger demonstrated that the toxin acted by inhibiting the GTPase activity of the membrane, which had the effect of maintaining the adenylyl cyclase in a prolonged stimulated state (Figure 5).<sup>7</sup>

The results of these and other experiments demonstrated the existence of a regulatory component of the hormone-adenylyl cyclase system that was activated by GTP binding and deactivated by GTP hydrolysis. Attention turned to several other questions. Is the GTP-binding protein an integral part of the adenylyl cyclase or a separate component? If the GTP-binding protein is a separate component, what is its structure and how does it interact with the other major components of the system, the receptor and the adenylyl cyclase?

The purification of the GTP-binding protein was accomplished in 1980 by Alfred Gilman and his co-workers at the University of Virginia. Purification was achieved by extracting liver plasma membrane proteins in detergent and subjecting the mixture to six successive chromatographic procedures (Table 2).<sup>8</sup> As indicated in the table, the specific activity of the detergent extract increased approximately 2,000-fold during the purification procedure, which corresponds to a purification of 5,000- to 10,000-fold from plasma membranes, or nearly 100,000-fold from total cellular protein.

When the purified GTP-binding protein was subjected to SDS-PAGE, three distinct polypeptides were found to be present (relative molecular masses of 52, 45, and 35 kDa), indicating that the regulatory protein was a multisubunit complex. Of the three polypeptides identified in the initial study, the 52 kDa polypeptide was present in much smaller and more variable amounts and ultimately was found not to be a member of the GTP-binding protein. The 45 kDa polypeptide became known as the  $\alpha$  subunit and the 35 kDa polypeptide as the  $\beta$ -subunit. A third polypeptide of 9 kDa (the  $\gamma$  subunit) was

Table 2

## Purification of the Regulatory Component of Adenylyl Cyclase from Rabbit Liver\*

Step	Protein (mg)	Total units (nmol/min)	Recovery (%)	Specific activity (nmol/min · mg)
Chelate extract of membranes	2020	4380	100	2.2
DEAE-Sephacel	114	2930	67	26
Ultrogel AcA 34	9.8	1820	42	190
Heptylamine-Sephacel	0.70	740	17	1060
Hydroxyapatite	0.03	350	8.0	1200
GTP-Sephacel	0.18	290	6.6	1600
DEAE-Sephacel	0.039	150	3.5	3800

\*Rabbit liver plasma membranes (8.6 g of protein) were extracted and purified. Activity was measured by the reconstitution of the fluoride-activated adenylyl cyclase activity. Specific activity is defined as the amount of reconstituted activity per amount of protein added to the reconstitution. Recoveries are cumulative through the preparation.

SOURCE: J. K. Northup et al., *Proc. Natl. Acad. Sci. U.S.A.* 77, 6518, 1980.

subsequently discovered. Of the three subunits, the 45 kDa  $\alpha$  subunit was shown to be the polypeptide that contains the GTP-binding site.

The purification of the GTP-binding protein set the stage for the analysis of the molecular and biochemical properties of the protein. As a result of these studies, Gilman proposed that the activation of adenylyl cyclase follows the dissociation of the GTP-binding protein into its subunits, and that the dissociated  $\alpha$  subunit with a bound GTP is responsible for activating the cAMP-producing enzyme.<sup>9,10</sup>

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