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The *Drosophila* nervous system as a model for analysing gene expression in complex organisms

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The behavioural complexity of multicellular organisms is derived from their nervous system. The anatomical variety of neuronal types, their functional diversity, and the intricate but specific manner of nerve connection are properties that make the study of the development and function of the brain a difficult task. However, certain simplifying features allow the construction of a systematic approach to the analysis of the complexity of the nervous system: the cellular localization of molecules and their spatial localization to specific parts of the brain often give us clues to their function. A systematic approach requires the ability to analyse rapidly the very large number of low-abundance mRNA species present in the nervous system. These mRNAs presumably encode molecules that eventually generate the functional complexity we see. Some recently developed approaches allow identification and isolation of genes that encode these mRNAs in the fruit fly *Drosophila melanogaster*. This animal has a complex nervous system. Nevertheless the methods of classical and reverse genetics can be combined with modern molecular approaches for a detailed dissection of neuronal development and function. Many of these methods have broad applicability in the study of gene expression in any organism.

FOR those studying development of multicellular organisms, the past few years have seen exciting changes. Technological advances in modern biology have allowed workers to re-examine old questions in new ways. These powerful methods have resulted in the identification and study of developmental events at the molecular and cellular levels. Formal models of how development takes place can be tested in detail and have to contend with a huge amount of experimental information. We are now able to identify molecules involved in many aspects of development and function and study their role in the cell to decipher how they interact to construct an organism.

Our aim in this article is to demonstrate how recent advances in recombinant-DNA technology and genetics are being applied to the study of the *Drosophila* nervous system. We first summarize the importance of the fruit fly *Drosophila melanogaster* for the study of animal development. Secondly, we demonstrate the molecular complexity of the mRNA molecules expressed in the *Drosophila* nervous system, and outline recent genetic and molecular approaches that allow rapid identification and analysis of a large number of mRNA species and their putative protein products and hence studies of their function.

An important fact of research in modern biology is the remarkably small number of organisms being studied. It is clearly necessary to examine more organisms, not only to learn about the diversity of ways in which they develop but also because of the economic and social importance studies on such organisms may have. However, it takes several years, often decades, of work before the groundwork for the study of an organism is ready. In the final part of this article, we examine the applicability of the methods we describe to the study of organisms where genetics is not easily used.

Gains from studies in *Drosophila melanogaster*

The fruit fly *Drosophila melanogaster* has made major contributions to the study of animal development. Many factors continue to ensure the persistent vigour of research in *Drosophila*. Its small genome size is a major advantage (Table 1). Its robustness in the laboratory and the cumulative history of its genetics, together with a short generation span, continue to make genetic studies attractive. The ability to isolate genes and reinsert them into germline chromosomes is a major advantage (see, for example, ref. 1). The organism itself is complex, has a well-developed nervous system, and a wide behavioural repertoire. The facility it provides for genetics and molecular biology thus allows complex questions of development and function to be rapidly addressed.

Table 1. Genome sizes of various organisms.

Organism	Base pairs/ haploid genome
Bacteriophage lambda	48,502
Escherichia coli	4.7×10^6
Saccharomyces cerevisiae (budding yeast)	1.5×10^7
Dictyostelium discoideum (slime mould)	5.4×10^7
Arabidopsis thaliana	7.0×10^7
Caenorhabditis elegans	8.0×10^7
Drosophila melanogaster	1.4×10^8
Gallus domesticus (chicken)	1.2×10^9
Mus musculus (mouse)	2.7×10^9
Rattus norvegeticus (rat)	3.0×10^9
Xenopus laevis	3.1×10^9
Homo sapiens (man)	3.3×10^9
Zea mays (maize)	3.9×10^9
Nicotiana tabacum (tobacco)	4.8×10^9

The developmental genetics of *Drosophila* was given a firm foundation by the pioneering work of E. B. Lewis and D. F. Poulson. Lewis began his work to try and understand the nature of the gene, and his work subsequently opened up the area of how a complex organism is patterned[2,3]. Poulson started the study of the nervous system of *Drosophila* using genetics[4]. His work concentrated on the *Notch* locus and involved the study of embryonic lethal mutations.

The work of Lewis and Poulson complemented the work of Klaus Sander[5] in Germany. Sander studied the development of insects using classical surgical methods. The seventies saw the growth of a large group of *Drosophila* developmental biologists, principally in Europe (Spain, Britain and Germany), who used genetics with great success[6-8]. The same period saw the emergence of the recombinant-DNA revolution. This coincidence has resulted in a phenomenal burst of information on the molecular basis of development. Many important *Drosophila* genes have been isolated and studied. Two examples of areas in which we have learnt a lot are early embryonic development and development of the adult eye. It is also remarkable that the studies of both these aspects have proven useful for developmental studies in a wide range of other organisms. Studies in early embryonic development resulted in the identification of the homoeo-box sequence that encodes a DNA-binding domain of a protein[9]. Such motifs have been found in several genes that are important for early embryonic development, such as *fushi-tarazu*, *engrailed* and *Antennapedia*[10]. Using the *Drosophila* homoeo-box genes as probes, homologous genes have been isolated and studied in a variety of organisms. These studies have revealed inherent conserved features of animal development[9]. *Drosophila* eye development has proven to be a facile model system for studying how proto-oncogenes function. The *Drosophila* gene *sevenless* has a tyrosine-kinase domain that shows homology with some vertebrate oncogenes[11,12]. Several genes have been identified that interact with the sevenless gene product[13], thus allowing the genetic dissection of tyrosine kinase ligand–receptor interactions *in vivo*.

Elegant behavioural screens identified many of the first mutants that were found to affect development and function of the eye. Another aspect of the study of the *Drosophila* nervous system that uses behavioural assays is the genetics of olfaction and taste. Several genes that affect the fly's response to odorants or taste stimuli have been identified[14-18]. Mutants in one gene, *gustB*, result in flies that are attracted to salts at concentrations at which the normal fly is repelled[18]. Among the olfactory mutants being studied, the *olfE* gene (ref. 19) has been cloned and the mutant phenotype rescued by P-element-mediated germline transformation[20]. Very little is known about how the chemosensory system develops

and functions and this area is evincing renewed interest.

Molecular studies of genes in the central and peripheral nervous systems have resulted in the analysis of many gene products important for their development[21]. The *achete scute* complex, for example, encodes a family of DNA-binding proteins that have homology with the vertebrate oncogene *myc*[22]. The gene *extramacrochaete* (*emc*) encodes a new family of DNA-binding proteins that show homology with mammalian muscle regulatory factors[23]. All these examples stress the fact that important molecules in one system are often involved in aspects of development of another organism. While their use is often in very different developmental situations the details of their molecular functioning are very similar. Organisms like *Drosophila* thus serve to unravel the molecular steps in a pathway in a rapid manner.

While genetic approaches in *Drosophila*, combined with molecular methods, have yielded significant results, large-scale analysis of the nervous system has inherent complexities, and it is here that the technological changes of the past few years find important applications. Our own current experiments are geared to use of a combination of behavioural, genetic and molecular analyses to study the development and function of the chemosensory and motor pathways of *Drosophila*.

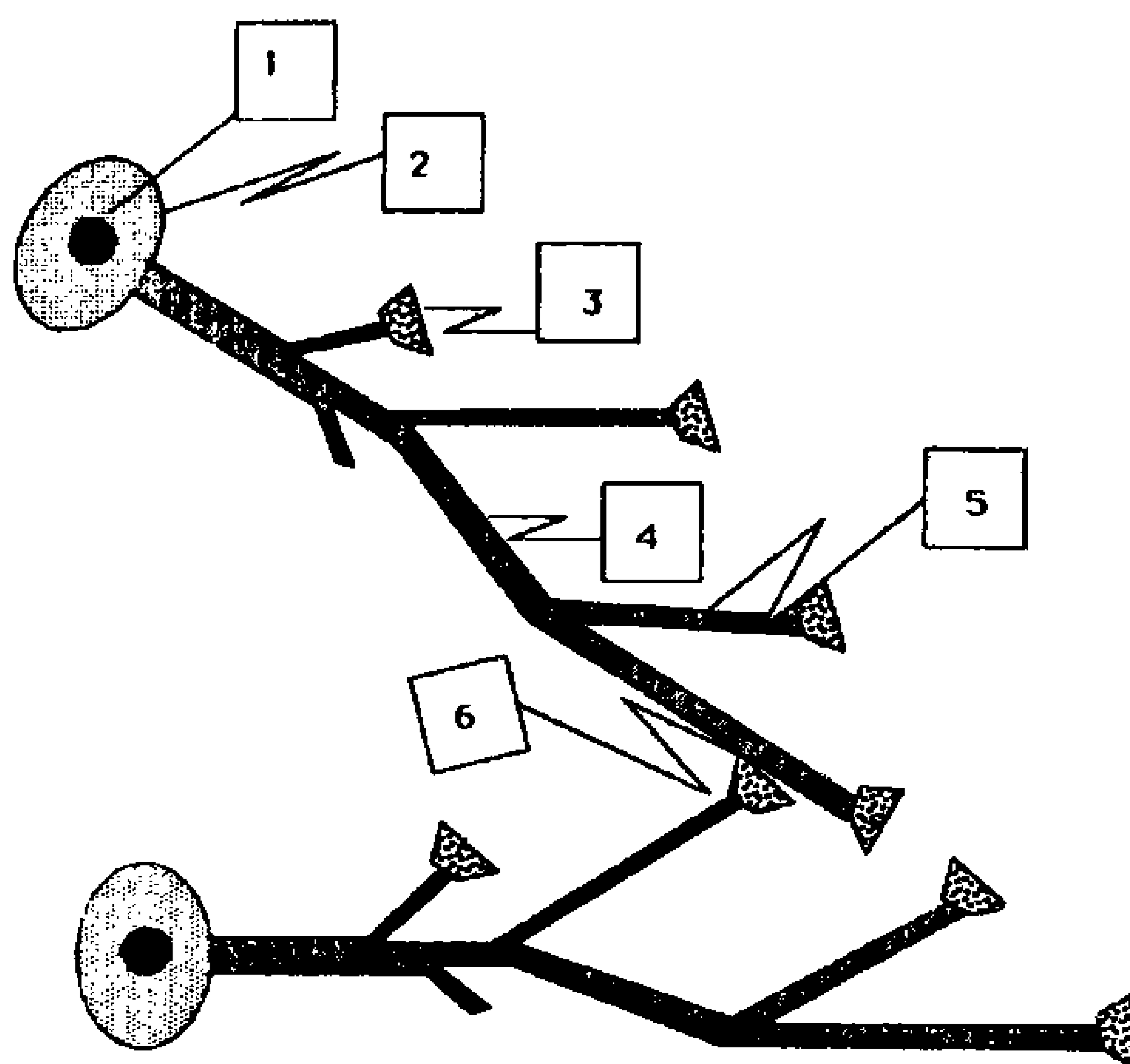

Figure 1. Model neurons, two insect neurons are shown. Some structural features are numbered on one of the neurons: 1, neuronal nucleus; 2, cell body; 3, synapse on dendrite (post-synaptic contact not shown); 4, axon; 5, dendrite; 6, dendrite–axon synapse. (Modified from Kandel, E. R., *Cellular Basis of Behavior*, W. H. Freeman, San Francisco, 1976)

Complexity of the nervous system

In every way we look at it, the nervous system seems complex. Ramon y Cajal used the Golgi silver impregnation method to demonstrate, for the first time, the variety in the anatomy of neurons[24]. Physiologists showed that neurons often have many different kinds of synaptic and conductance properties. Neurochemists have identified and studied many different neurotransmitters and neuromodulators[25]. In addition to the complexity of the mature nervous system, we are also confronted with trying to understand how such a complex tissue develops.

Are there any simplifying features that allow the analysis of this complexity? One of Ramon y Cajal's major contributions was the demonstration that the neuron is the unit of brain architecture[24]. Till then, Golgi's viewpoint that the nervous system was an intricate, net-like structure held sway[24]. Cajal's discovery and the subsequent analysis of the function of single-neuron properties define one level of the problem. One would like to know what gene products are required to construct a particular neuron. Figure 1 shows a model neuron. The localization of gene products to different parts of the neuron can give some idea about their function. For example, a gene product present in the nucleus could be a transcription factor or one involved in RNA splicing, besides other possibilities[25]. Similarly, localization of molecules in the synapse suggests other roles. While we have some idea of the function of most parts of the neuron we have little knowledge of how the neuron is constructed and what the nature of its molecular diversity is.

The functional specialization of animal behaviour has a broad correlation with regional specialization in the brain. Analysis of patients with neurological disorders, for example, has led to identification of domains in the human brain that are important for specific functions[26]. Physiological studies on primates and other mammals have analysed the function of many pathways, in particular the visual system, in some detail[27]. The regional localization of molecules therefore suggests the functional pathway in which they function. As in the case of the neuron, we know little about the molecular constituents and their roles that are responsible for spatial specialization.

Cellular and regional localization of molecules can therefore give clues to their function, help us decide on which molecules to choose to concentrate our study, and ultimately tell us about their roles in the structure, function and development of the nervous system.

Molecular analysis of the nervous system

To understand, in molecular terms, how a structure such as the nervous system is constructed is clearly a

formidable problem. It is now clear that a very large number of molecules go into the construction of this tissue. The mammalian brain, for example, is thought to contain about 40,000 different mRNA molecules specific to it; the actual number may be much higher[28]. This is a complexity much higher than that seen in other tissues such as the liver. Most of the different mRNA molecules in the brain fall into the low-abundance category. Put in another way, many different genes are expressed in the nervous system and they are expressed at low levels[26]. Most methods that allow the isolation of cDNA clones end up isolating molecules in the abundant or moderate category[29]. The study of the molecular complexity of the nervous system therefore requires the ability to prepare cDNA libraries from this tissue and isolate clones that correspond to the low-abundance mRNA species.

Subtracted-cDNA libraries

The mRNA molecules present in the nervous system may be broadly divided into those that are specific to the nervous system and those that are also present in other tissues. It can be argued that the molecules that are important for the development of the nervous system are likely to be exclusive to it. Another view is that molecules that are important for the nervous system could also be used for the development and function of other tissues. We have many examples of both situations (e.g. the product of the gene *fushi-tarazu* (*ftz*) is required for the correct metamerization of the embryo and is also used in the development of the nervous system[30]; the product of the gene *chaoptin*, required for eye development, is expressed exclusively in the photoreceptor cells[31]). In working terms the problem reduces to whether one should make a subtracted-cDNA library and, if so, what tissue should be used for subtraction. The major advantage of using a subtracted library is that it allows one to deplete the cDNA pool of DNAs that encode 'housekeeping' or basic cellular functions common to all cells. While this assumption has simplistic features it is a useful starting point. There are several ways in which one can construct a subtracted-cDNA library[32], and one way is outlined in Figure 2, *a*. It is not necessarily the most convenient way but it has novel features and is of current interest. The major advantage of the method is that it uses a small amount of starting material. Once a subtracted library is constructed the problem is one of isolating cDNA clones from the library that correspond to rare mRNA species. As mentioned earlier most commonly used methods do not readily identify rare cDNA clones. Palazzolo *et al.*[33] have systematically attempted to address this problem. The outline of their approach is depicted schematically in Figure 2,*b*. The scheme is

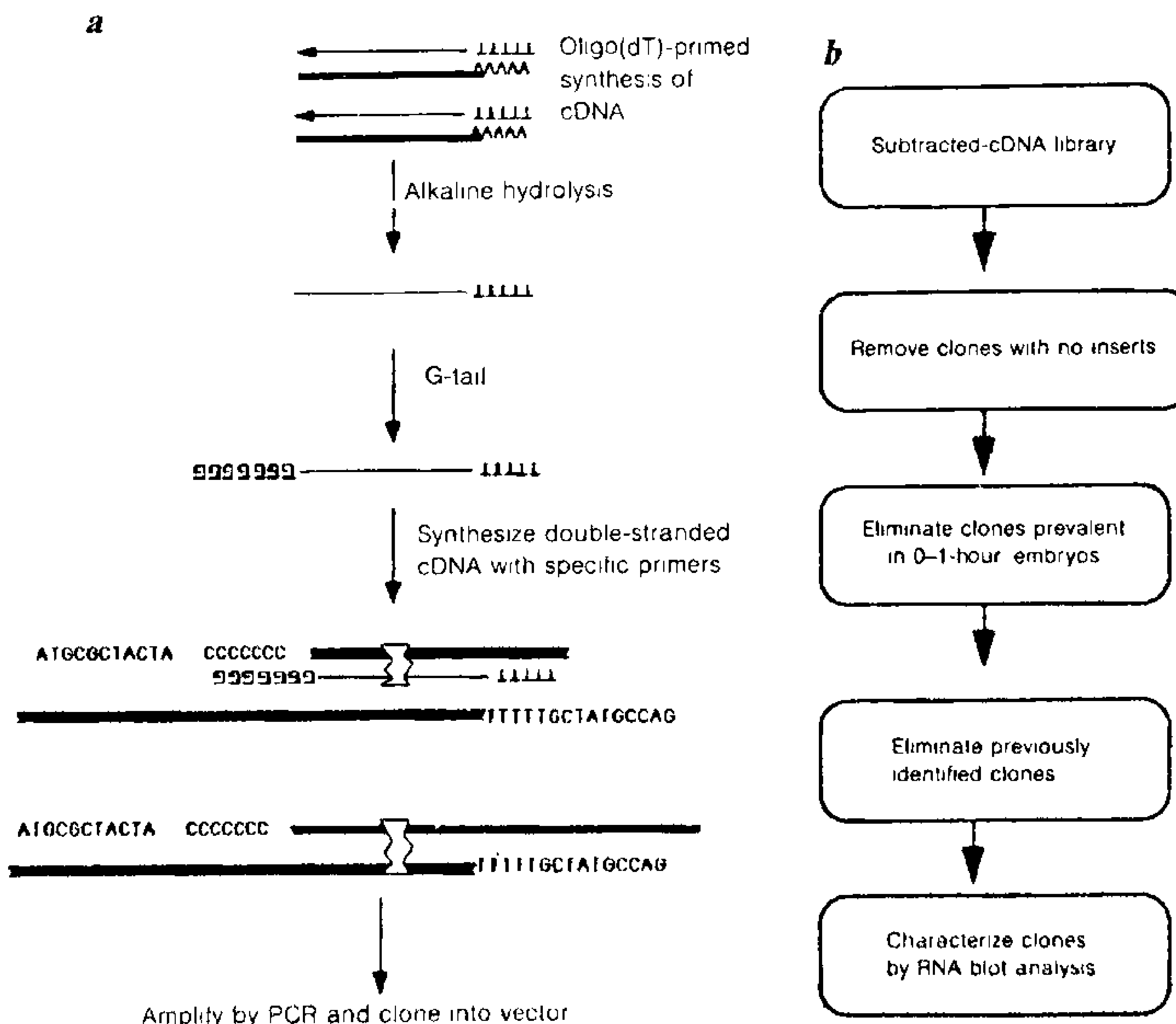

Figure 2. **a**, A scheme for construction of a cDNA library using the polymerase chain reaction. The library can be constructed with a small amount of starting material. The vectors in which such libraries are constructed can be chosen to allow the synthesis of antisense RNA in large amounts. This can be hybridized with RNA from the subtractive tissue, and the unhybridized RNA selected by fractionation and used to construct a subtracted library. See ref. 33 for details. **b**, Sorting a subtracted-cDNA library for clones that correspond to low-abundance mRNA species (see ref. 33)

based on a step-by-step sorting of a subtracted-cDNA library. Phage plaques that do not contain insert are identified and removed from the clones being analysed. Next, plaques that correspond to highly prevalent cDNAs from the subtractive tissue, but are still present in the library, are identified and removed. A set of plaques that contain insert are analysed individually, using labelled inserts from them as probes on RNA blots. Antisense RNA, labelled with ^{32}P, is used as a probe on an RNA blot that contains polyA$^+$ RNA from the adult head and, in another lane, polyA$^+$ RNA from the 0–1-h embryo. Thus, by a direct screen on an RNA blot, cDNA clones are isolated that are expressed in the adult head but not in the 0–1-h embryo. Once the first set of clones is analysed in this manner, the next set is picked for analysis. Before the second set is analysed on RNA blots, the clones that are common with the previously analysed set are removed. This procedure is continued with subsequent sets of clones, analysing individual clones in each set on RNA blots after clones cross-hybridizing with the previous sets have been removed. In this manner 436 cDNA clones were isolated and characterized. Quantitative estimates of the abundance of the mRNAs corresponding to these clones show that some of these clones represent mRNAs that are about 0.002% of the total polyA$^+$ RNA present in the adult head. Many clones fell into the class that could not detect RNAs from both head and 0–1-h embryos on RNA blots. These could, presumably, fall into an abundance category even lower than 0.002%. The pattern of expression of these clones during development was analysed. The important result from this study is that genes that are expressed in the head and not in 0–1-h embryos are expressed in other parts of the adult and stages of development in many possible combinations. For example, some clones correspond to RNAs that are exclusive to the head, others to head and body, yet others to head and 24-h embryos, and so on. These results indicate that molecules used in the adult head have roles in other parts of the animal or in other stages. The detailed analysis of such cDNA clones is the next step in the process, and this is outlined after another method for the identification of tissue-specific gene products is described.

The 'enhancer-trap' method

A genetic method of identifying genes based on their pattern of expression will obviously have major use in *Drosophila*. While classical *Drosophila* genetics has been widely applied to identifying genes on the basis of mutant phenotype, there are some important limitations to a mutational approach. Genes important for late developmental events will be missed if their products also have vital early roles: lesions in such a gene will lead to early lethality that will obscure detection of the later function. Many gene products, especially in the nervous system, that are likely to be expressed in a small number of cells or at very low levels serve important cellular functions, but may mutate to give phenotypes whose meaning may not be clear. For example, the only readily discernible phenotype of many embryonic mutants is death. The cause may be loss of the gene product in a very small number of cells during development of the nervous system, but this is not readily seen from the phenotype. The method devised by O'Kane and Gehring[34] solves many of these problems in an ingenious way (Figure 3,*a*). The method uses the properties of the *Drosophila* P transposable elements and eukaryotic enhancers. A reporter gene, the *lacZ* gene of *E. coli*, which encodes the enzyme β-galactosidase, is put under the control of a weak, constitutive promoter. This construct can be genetically mobilized by P-element transposition to different sites on the *Drosophila* chromosomes. Appropriate host strains and genetic markers allow its course to be controlled and mapped. Lines of flies can be set up, each bearing one such P-element insert at a particular chromosomal position. The expression of the reporter gene is strongly influenced by genomic

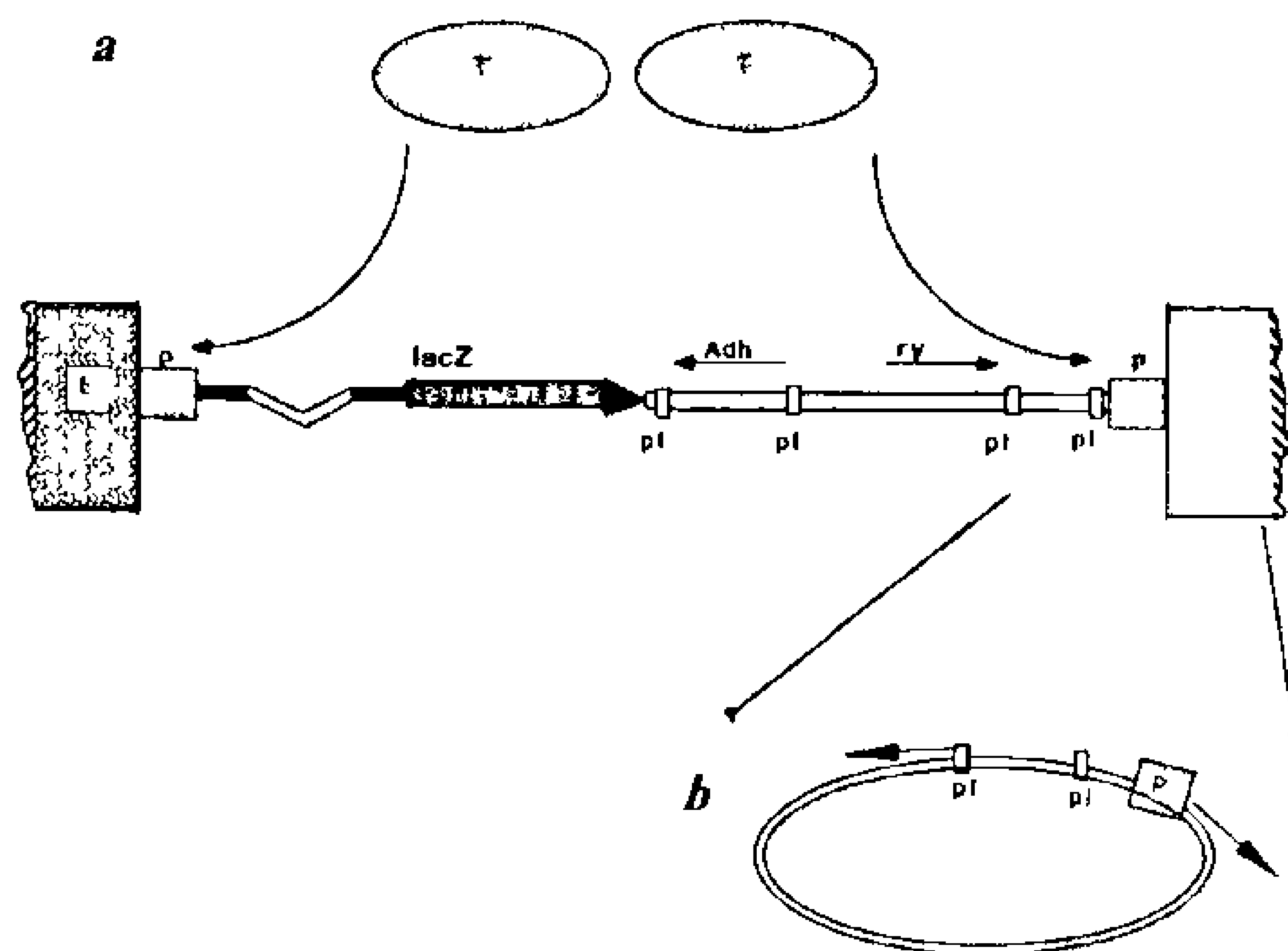

Figure 3. **a**, Outline of scheme for generation of enhancer-trap P-element insert-bearing flies. P, P-element ends, the weak constitutive promoter at the left end drives the reporter gene, pl, polylinkers. *lacZ*, β-galactosidase gene, fused in frame to a truncated (and nonfunctional) transposase gene, E, genomic enhancer that affects the expression of the reporter gene in a tissue-specific manner. *Adh*, alcohol dehydrogenase gene present in the vector for use in selection schemes. *ry*, wild-type gene for xanthine dehydrogenase, confers eye colour phenotype in crosses in which the P element is mobilized. T, transposase from genomic source. Modified from Wilson *et al.*[35] **b**, Plasmid rescue of flanking genomic DNA. The genomic DNA is digested with an enzyme that cuts once in a polylinker, diluted, and recircularized. Bacteria are transformed with this DNA and plated on ampicillin-containing plates. The Amp-resistance gene and the bacterial origin of replication allow plasmids that contain flanking *Drosophila* genomic DNA to replicate and grow on selective drug-containing plates[35].

enhancer elements: the histochemical pattern of expression of the reporter gene seems very often to match the pattern of expression of the gene the now 'trapped' enhancer normally drives. The enhancer-trap method has proved to be remarkably effective in detecting gene expression in a wide variety of tissues, the nervous system in particular. Expression in a very small number of cells is often seen. One major advantage of the method is that it allows the histochemical analysis of gene expression in the heterozygote throughout development even if the homozygote has a lethal phenotype[35].

Once an insertion strain of interest has been identified, the next step is to identify a cDNA clone whose pattern of expression is similar to that of the reporter gene. The initial steps in this, outlined in Figure 3*b*, involve rapid isolation of chromosomal DNA flanking the insertion. This DNA is labelled and used as a probe to screen a wild-type genomic library to isolate DNA from the region of the insert. The genomic DNA can be used to screen a cDNA library made from the appropriate developmental stage or tissue to isolate cDNA clones from the region. The position of the cDNA clones on the genomic clone can be mapped readily. Usually the clone closest to the site of the P-element insertion is the best candidate. RNA *in situ* hybridization to tissue whole mounts or sections using this clone allows a test of the validity of the method. A recent RNA *in situ* method using non-radioactive probes provides a rapid and moderately sensitive way of doing this[36].

At the present time the enhancer-trap approach and the cDNA-cloning approach outlined earlier have both resulted in identification of cDNAs that are to be analysed further.

Rapid analysis of cDNA clones

For rapid isolation of full-length cDNA clones using information from shorter clones, conventional methods, which require screening of cDNA libraries, isolation of more clones, and analysis of their sizes by electrophoresis, are time-consuming when a large number of clones are to be isolated. In cases where very-large-molecular-weight RNA molecules are to be isolated additional problems are encountered. This is especially so if the RNA species are low in abundance. One method to circumvent this problem is by using DNA sequence information from already isolated shorter-length cDNA clones to synthesize sequence-specific primers to be used for reverse transcription. A known sequence can be tailed to the synthesized cDNA and thus a template for the polymerase chain reaction (PCR) is generated, the gene-specific primer and the tailed sequence serving as primers. (This method is sensitive, though the error in fidelity when PCR is used must be kept in mind when interpreting sequence data from such clones. Analysis of independent multiple clones will help in this matter.)

Methods that address the rapid sequencing of cDNA clones must contend with handling a large number of clones simultaneously. The cDNA libraries can be constructed in vectors that readily allow the conversion of phage to expression-vector plasmid constructs that also allow rapid production of single-stranded phage for sequencing (e.g. ref. 37). Several approaches are being taken to generate sequence information rapidly. It is possible to generate transposable-element insertions at random positions in a cloned insert and use the known sequence of the insert ends to prime sequencing reactions. Methods for identification of clones bearing inserts at random positions have recently been described[38].

Another aspect of the analysis of cDNA clones is the generation of fusion proteins in expression systems. Proteins thus synthesized can be used to raise antibodies specific to the cellular protein that results from translation of the mRNA corresponding to the cDNA clone. Many parameters affect the efficient production of protein in bacterial expression vectors. If the sole purpose of generation of fusion protein is its use as an immunogen, efficient expression vectors, for example those that generate fusions with the phage T7 gene 10 protein whose production can be induced, are available. Once a protein has been synthesized, it can be used as an immunogen to raise monoclonal antibodies or polyclonal antibodies. The antibodies must be analysed to ensure that they are against domains encoded by the cDNA clones. This step is currently slow compared to steps that require analysis of nucleic acids. Recent reports have described generation of libraries of immunoglobulin molecules in bacteria. These expression libraries can be screened with an antigen for clones that produce antibodies specific to it. If this method is tested and shown to have wide applicability, it will greatly enhance the speed of generating antibodies and at the same time reduce costs.

Reverse genetics

One of the principal advantages with *Drosophila* is the ability to examine the *in vivo* function of a gene product through genetics. When a large number of cDNA clones have been isolated, it is important to be able to study the function of the gene products that correspond to these cDNAs by mutating the respective genes. One requirement for systems of reverse genetics are strong selection schemes that allow isolation of mutants in the genes of interest. In the case where sequence information is available for the gene in question there exist methods to select for P-element insertions in or

adjacent to the gene[39,40]. The method uses features of PCR. A large number of flies with random P-element insertions are generated These flies are propagated stably by individual or group matings. Parents from these lines are pooled, DNA extracted, and these pools of DNA used as template in PCR with one primer being a gene-specific primer and the primer on the opposite strand being an oligonucleotide homologous to P-element termini. Exponential amplification of DNA is obtained only when the P-element insertion is close to the gene-specific primer. This method can be applied to other organisms as well[41]. One disadvantage of the method is that sequence information for the gene is required, preventing the use of this method in mass screens in which many cDNA clones are simultaneously analysed. Another disadvantage arises from the extreme sensitivity of the method, which gives rise to false positives. In spite of these problems the method is powerful, and this and other methods of reverse genetics are likely to see more application.

Reintroduction of cloned genes into germline chromosomes

In 1982 Rubin and Spradling revolutionized *Drosophila* genetics by discovering a method to reintroduce cloned genes into *Drosophila* germline chromosomes[42,43]. One obvious advantage of this method is that it allows the rescue of mutant phenotypes by germline transformation. This allows clear definition of the limits of the gene in terms of function. But the application of the method has far exceeded just this advantage Regulatory regions of genes and functional domains of gene products can be studied readily because the genomic DNA can be manipulated *in vitro* and stably reintroduced into the fly. Genes can be induced to be expressed at inappropriate times or places and the effects studied[44].

Recently a library of *Drosophila* DNA has been constructed in yeast artificial chromosomes (YAC)[45] The insert size here is in the range of a few hundreds of kilobase pairs. Such large inserts can be manipulated readily in yeast. The YAC library allows rapid mapping of cDNAs to specific clones and therefore to specific chromosomal regions. One of the major advantages of yeast is the ease with which homologous-recombination events can be selected. While it is possible thus to manipulate *Drosophila* DNA in yeast, the DNA size is too large for handling and reintroduction into *Drosophila*. A recent paper described transfer of yeast DNA into human chromosomes by cell fusion[46]. The development of such methods in *Drosophila*, in combination with recent genetic methods for generating deficiencies[47], will greatly increase our ability to handle large regions of DNA for analysis.

Application to other complex genomes

The methods we have described address important cell-biological questions. In many plants and animals we need similar methods that allow rapid identification, isolation and study of molecules that play crucial roles in cellular and organismal development. *Drosophila* has advantages because of the small size of its genome and the facility it allows for genetic manipulation. But many of the methods outlined above can be applied to more complex genomes.

The polymerase chain reaction, using the thermostable *Taq* polymerase, allows a stringency of selection that facilitates isolation of genes by homology. Oligonucleotide primers are synthesized by conceptually translating conserved regions of a protein to generate degenerate probes that take into account codon redundancy. These primers can be used on complex genomes to amplify homologous genes rapidly, using PCR, without having to screen large genomic libraries at low stringency, a procedure that has to contend with false positives. This method allows rapid cloning of homologues of important genes that have been identified in other systems[48]. YAC technology has been applied to generate genomic libraries in plants and mammals, and allows rapid mapping of cloned DNA. As mentioned earlier, it is now possible to construct cDNA libraries from a small amount of tissue using PCR. The function of many important genes can be studied by introducing them into animals where manipulation is straightforward. For example, the genes involved in cell division are functionally conserved; the human *cdc28* gene rescues a yeast-mutant defect, and its functional domains can be studied in yeast. In general, organisms with strong genetics, like yeast, *Drosophila*, the nematode *Caenorhabditis elegans*, mouse and *Arabidopsis*, are more and more likely to serve as useful reaction systems for analysis of the function of genes cloned from other organisms.

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Molecular basis of immune response against parasites

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Parasites, both unicellular and multicellular, have evolved many strategies to evade or overcome host immune response. The molecular basis of parasite adaptations and immune-system mechanisms of killing of parasites are subjects of intense study. The best known of parasite adaptations for evading host immune response is surface-antigen variation, the genetic basis of which has been well studied in at least a few parasites. The immune response to parasites has various components, broadly the B-cell-derived antibody and the T-cell responses, and distinct populations of lymphoid cells are involved. The role of proteins coded by the major histocompatibility complex is also important. Recent understanding of the mechanism of antigen recognition has opened up the promising area of the molecular basis of vaccine development.

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A wide range of organisms, some unicellular, others complex multicellular, invade humans and animals and are dependent on the host for their survival. These organisms (parasites) very often cause disease leading to much morbidity and mortality. Many parasites need vectors for transmission, e.g. snails and the tsetse fly transmit schistosomes and *Trypanosoma brucei* respectively. Often development of the parasite proceeds partly in the vector and is completed in the host. Some parasites develop in the host and multiply intracellularly: promastigotes of *Leishmania* transform into amastigotes in macrophages, sporozoites of *Plasmodium* become merozoites in hepatocytes.

For over a century biomedical science has tried to understand host parasite relationships for the major human parasites. While several aspects of this complex phenomenon are becoming clear, important questions