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Analysis of gene function in *Dictyostelium*

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Abstract. Over the past ten years, powerful molecular genetic techniques have been developed to analyze gene function in *Dictyostelium*. DNA-mediated transformation using a variety of selections and vectors has allowed the introduction of wild-type or modified genes that are under various forms of transcriptional control. Homologous recombination is efficient and can be used to modify the genome in precise ways. In addition, it is now possible to clone genes based on their mutant phenotype alone, either by insertional mutagenesis, or by screening antisense expression cDNA libraries. Finally, a nearly complete physical map of the genome is available and so genes are easily mapped by physical techniques. We discuss many of these advances within the context of major research problems presently under study.

Key words. Antisense RNA; gene expression; insertional mutagenesis; physical mapping; reporter genes.

Introduction

As described in the other review articles in this issue, *Dictyostelium* has proven to be a useful model for the study of many problems in cell and developmental biology. These studies are only limited by the imaginations of the researchers and the tools at their disposal. In the past 60 years, since the discovery of *Dictyostelium*, advances have been made in the determination of cell fates, the development of assays for chemotaxis and cell adhesion, the definition of conditions for synchronous development, the development of defined growth media, chemical mutagenesis, and parasexual genetics (reviewed in refs 40, 67). Molecular cloning techniques have placed powerful new methods in the arsenal of the *Dictyostelium* biologist. Thus, in the past ten years techniques have been developed for DNA-mediated transformation[54], homologous recombination[6,7,76], antisense-mediated gene inactivation[5], analysis of cell-specific gene expression using reporter genes, high-resolution gene mapping[36,37], and insertional mutagenesis[34]. In this article we will describe these recent advances in the molecular techniques as they pertain to the analysis of gene function in *Dictyostelium*. Detailed protocols describing these techniques are reviewed elsewhere[35,47].

Identification of genes by their function

Cloning *Dictyostelium* genes by methods which exploit the similarity of homologous genes from diverse organisms has been a successful way of cloning genes of known function. It has been much more difficult to clone novel genes by beginning with a cellular function of interest. In the past fifteen years this has been done primarily by purifying a protein using a functional assay, as was done for the cell adhesion protein contact sites A (gp80)[51], and then by using antibodies directed against that protein to screen a cDNA expression library to clone the gene (e.g. ref 56). This process can take longer than a year, and successful cloning cannot be assured. In order to circumvent these difficulties, two new methods for isolating genes based on phenotypic assays have recently been devised.

Insertional mutagenesis

A method for generating insertional mutants by restriction enzyme mediated integration (REMI) has been devised[34]. The integrating plasmid is linearized with a restriction enzyme and introduced by electroporation into cells along with a restriction enzyme. The restriction enzyme stimulates the rate of vector integration 20 to 60-fold in *Dictyostelium*, as was previously observed in yeast[63], such that transformants can be recovered at a frequency of 4×10^{-5} or more. The enzyme also determines the sites of integration by stimulating integration into its cognate recognition sites in the genome.

Successful REMI requires that the single-stranded DNA overhang generated by the restriction enzyme used matches the overhang on the linearized vector. Thus, *Dpn*II (which recognizes GATC and generates GATC overhangs) will stimulate integration of vector DNA linearized with *Bam*HI (which recognizes GGATCC and generates GATC overhangs), but *Eco*RI (which recognizes GAATTC) will not. The majority of

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the integration sites in *Dpn*II REMI transformants are *Dpn*II sites even when the plasmid is linearized with *Bam*HI. Thus, the mediating enzyme apparently determines the genomic restriction site used for integration. Of the enzymes that have been tested to date, *Bam*HI, *Sau*3AI, *Dpn*II, *Eco*RI, *Aha*III, *Bgl*II, *Cla*I, *Not*I, *Xba*I, and *Pst*I have been shown to stimulate integration. These should provide the distribution of sites necessary for the inactivation of every non-essential gene in *Dictyostelium*. For unknown reasons, the enzyme *Hind*III has not been observed to stimulate plasmid integration. *Dpn*II has become the enzyme of choice since its sites are present in coding regions about every 200 bp, on average.

Once a mutant of interest is obtained, the next step is to recover the genomic DNA flanking the insertion by plasmid rescue, which is made possible by the bacterial origin of replication and the ampicillin gene in the REMI plasmid. However, this has proven to be the most unpredictable step in the entire procedure. The success of plasmid rescue appears to depend on the use of cesium chloride purified genomic DNA and on performing the monomolecular ligation reactions at a DNA concentration of 1 nanogram per microliter. A significant fraction of REMI insertions (10–20%) appear to be 'impossible' to clone. This is most likely due to deletions of parts of the REMI plasmid that are essential for plasmid selection or maintenance in *E. coli*. Still, the cloning of genomic DNA flanking these rearranged REMI inserts should be possible by using some variation of the polymerase chain reaction.

To prove that the original mutant phenotype is a direct consequence of the insertion event the plasmid DNA cloned from an insertion strain can be used to generate the original disruption in a wild-type strain. Homologous recombination re-establishes the vector at the original insertion site in 20–100% of the transformants, depending on the amount of flanking DNA in the particular clone. In this way the linearized clone can be used to disrupt the gene in a variety of genetic backgrounds for the purpose of generating complex genotypes. Deletions of genomic DNA of 100 bp to 2–3 kb have occasionally been observed to occur near REMI insertion sites. Therefore, the final proof that the gene recovered is the only gene that was mutated should come from phenotypic rescue of the mutant by expressing the cloned gene in the original mutant background.

The REMI technique has been used to identify several mutants with morphological alterations in the development of *Dictyostelium*[14,31,42,64]. It should, however, be pointed out that any mutation for which a selection or screen is available can be recovered. Mutations affecting the expression of a particular gene can easily be detected by protein blots[71] or RNA colony blots[45]. We have recently started a protein colony blot screen for REMI mutants affecting discoidin expression (W.N., unpublished results) and readily found both of the previously described phenotypes of overexpression and strongly reduced expression[1,74]. An azide assay is being used to select mutants defective in myosin function: due to a failure of the cells to round up in response to azide, mutant cells will not detach from a plastic surface when respiration is impaired (J. A. Spudich, personal commun.). Thus, several rounds of azide treatment will result in an enrichment for mutants with defects in the organization of the cytoskeleton. When developed in the presence of the non-hydrolyzable cAMP analog cAMPS, cells are compromized in chemotaxis and completely impaired in the formation of differentiating aggregates. cAMPS-resistant mutants which can bypass the cAMP requirement for development have previously been selected by their ability to form fruiting bodies in the presence of cAMPS[22]. REMI will allow the selection of gene-tagged mutants with this phenotype.

Since it appears as if REMI insertions into restriction sites occur at random, it should be possible to isolate any mutant as a tagged REMI cell line. However, there is one important difference between REMI and chemical mutagenesis in the frequency of obtaining mutants. With nitrosoguanidine mutagenesis about seven mutational events per genome can be achieved, and about 1 in 40 of the survivors displays an obvious developmental phenotype[41]. Developmental mutants occur at a frequency of about 1 in 300 REMI-generated strains, each of which suffered a single mutational event[34]. To have a 95% chance of recovering an insertion in any particular gene about 5×10^5 transformants would have to be screened. Thus, routine screening for a mutation in a single gene will not be a reasonable goal for a single investigator. However, if a multicomponent system is targeted, such as a signal transduction pathway consisting of approximately 20 genes, the examination of the 1,000 or 2,000 clones required to detect a mutation in one of these genes becomes feasible for an individual investigator.

Antisense mutagenesis

Those genes that are present in multiple copies and those that are necessary for the growth would likely escape insertional tagging by REMI. However, a complementary approach to insertional mutagenesis has been developed by Richard Gomer and his colleagues which should allow the isolation of genes that are inaccessible by REMI (R. Gomer, personal commun.). The technique might be termed random antisense cDNA expression mutagenesis. It involves constructing a copy-number normalized cDNA library in an extrachromosomal expression vector such that antisense RNAs of the corresponding cDNAs are expressed in *Dictyostelium*. Mutants can be isolated by the same criteria as used for REMI, and the cDNA responsible

for the mutant phenotype can be easily cloned by plasmid rescue into *E. coli*. The timing of expression of the antisense RNA can be controlled by choosing the appropriate promoter. Thus, it should be possible to select for genes that cause an 'antisense phenotype' when expressed in particular cell types or at different times of development. Such variations may be useful for isolating genes required for late developmental events without biasing against genes also required for an earlier step, or for growth. In addition, the expression libraries themselves could be constructed from mRNA expressed at particular times or in different cell types.

Since phenotypes are often caused by mutations in genes directly required for the particular process that is disrupted, insertional and antisense mutagenesis techniques are powerful ways to isolate novel genes of interest. Whether the cloned gene is directly required for a particular function depends critically on how well the phenotype can be characterized with respect to the function under study. Since many of the phenotypes of *Dictyostelium* developmental mutants are broadly defined, many genes are now being isolated for which no specific biochemical or cellular assays exist. Often a clue to developing such an assay comes from the predicted amino acid sequence of the genes. When the primary sequence fails to provide such a hint, more creative routes to understanding gene function must be used.

Functional analysis of genes

Gene targeting techniques

The introduction of specific alterations in the genome by homologous recombination has proven to be an effective approach for examining the function of genes. Examples are targeted disruption of the *Dictyostelium* myosin heavy chain gene[7], disruption of the ABP-120 gene (*abpC*) which in certain genetic backgrounds leads to cells with altered motility[4], isolation of gene disruption mutants with cDNA coding for annexin-VII (*nxnA*)[12], the selective elimination of the contact sites A protein by gene disruption of *csaA*[25], targeted gene disruption of *carC* which encodes the cAMP receptor subtype cAR3 expressed during multicellular stages of *Dictyostelium* development[32], disruption of the sporulation-specific gene *spiA*[58], and disruption of the major vault protein-alpha gene (*mvpA*) in *Dictyostelium*[70], to mention a few. Targeted gene disruption has become much more efficient since low copy selection systems like *thyA* and *pyr5-6* have become available. For simple gene disruptions or gene replacements homologous recombinants are obtained at high frequencies in most cases when the prototrophic *thyA* or *pyr5-6* selections are used[13,33]. The *thyA* gene is essential for growth in the absence of thymidine and so *thyA* constructs can be selected in *thyA*$^-$ cells. Similarly, the *pyr5-6* gene can be selected for in *pyr5-6*$^-$ mutants by demanding growth in the absence of uracil.

No firm 'rules' for targeting vector construction have been established. In general, 0.1 to 1.0 kb of DNA homologous to the target sequence should flank the selectable marker on each side (reviewed in ref. 35). Linear fragments work better than circular plasmids. In addition, fragments with ends that correspond to the the genomic site to be targeted (as opposed to vector sequences) appear to increase the probability of homologous recombination at the desired site.

Antisense mediated gene inactivation

In order to define functions of gene products, antisense-mediated gene inactivation has become an attractive alternative to gene disruption by homologous recombination. Antisense gene inactivation is accomplished by introducing stable plasmid constructs that express antisense RNA corresponding to the gene or gene family one wants to disable functionally. Antisense effects are dominant in diploid organisms. In haploid cells like *Dictyostelium* they provide the advantage that gene families may be silenced in a single step (e.g. ref 5). Especially when the gene of interest is essential for growth, antisense transformants, in contrast to gene knock-outs, may be viable because cells with reduced but not completely abolished expression can be selected. Using an inducible promoter[2] to drive antisense transcription, it has been possible to isolate strains with reduced amounts of small G-proteins (G. Weeks, personal commun.), and of calmodulin[39], which could not be obtained by gene disruption. A compilation of successful antisense experiments in *Dictyostelium* is presented by Hildebrandt[28]. It should be possible to down-regulate gene expression selectively in a particular cell type by using cell-type specific promoters. This may, however be hampered by a regulatory mechanism in *Dictyostelium* which reduces or abolishes antisense effects in later development (see below).

In *Dictyostelium* and in other organisms, attempts to establish general rules for the most effective regions of a gene in antisense experiments were unsuccessful[24,62]. Taking the data together, no rules could be defined. This is not surprising since the sequence and the secondary structure of an RNA determine rates and efficiency of interaction with the corresponding antisense transcript, and these properties are certain to be different for each gene fragment used in an antisense experiment. For several *E. coli* antisense systems it has been shown that minor changes in the 'kissing complex' (secondary structures required for RNA annealing) can have dramatic effects on RNA-RNA interactions (for review see ref 57). Since it is at present not possible to determine RNA secondary structures in vivo which could interfere with sense/antisense interactions, it is difficult to predict the most effective antisense gene

construct. Thus, one should test gene fragments of different sizes and from different locations within the transcript produced by the gene.

In many cases antisense transcripts are unstable. That is, even when the construct is strongly transcribed, little or no RNA accumulation is observed. This may be due to a lack of polyadenylation. In experiments with plants it was found that the addition of a polyadenylation signal at the 3′ end of the antisense gene markedly increases the antisense effect[24].

In addition to reducing or abolishing gene expression, there are other applications of the antisense approach in *Dictyostelium*. Maniak and Nellen[46] used antisense constructs to investigate a transcriptional feedback mechanism where a 'back-up' promoter is activated when the level of the mRNA transcribed from the regular promoter decreases. Introduction of an antisense construct directed against the endogenous mRNA could activate the feedback mechanism and enhance expression of a reporter gene driven by the feedback-regulated back-up promoter.

Another antisense approach has been suggested to design new regulatory features. A gene of interest driven by a constitutive promoter is fused with a small tag sequence, for convenience, a sequence encoding an inert epitope is used. In a second vector, the antisense configuration of this sequence is fused to a developmentally regulated promoter. Cotransformation of these two constructs will result in a downregulation of the gene of interest when the developmentally induced promoter is up-regulated[53].

Holt and colleagues used the antisense replacement technique in mammalian cells to investigate mutations in essential genes which could not be deleted by homologous recombination[30]. These authors first transformed cells with a *c-fos* gene which lacked part of the 5′ untranslated region and contained mutations of interest. Subsequently, an antisense construct directed against the 5′ untranslated region which was part of the endogenous wild type transcript, but not of RNA derived from the mutated gene construct, was introduced into the cells. The endogenous mRNA could thus be substantially downregulated and the gene product was essentially replaced by the mutated form. Similar experiments in *Dictyostelium* could provide a useful alternative to the gene replacement method.

For unknown reasons, antisense experiments do not always work as desired. In some cases no reduction of the targeted gene product has been observed and sometimes, the (sense) mRNA is present at wild type levels despite high antisense expression. It has also been reported that antisense cell lines become unstable: mostly, the expression of the antisense construct is unaltered but the effect on the corresponding mRNA or the respective gene product is lost. Although there is no explanation for this, recent experiments highlight some of the problems with antisense mediated gene inactivation. Three lines of evidence suggest a developmental regulation of antisense effects in *Dictyostelium*. In one case it has been observed that mRNA accumulation is efficiently suppressed in early development, but in later development sense and antisense RNA co-exist in the cells and downregulation is abolished. The two other cases refer to the *psvA* gene which is regulated by an endogenous antisense mechanism[27]. Under normal developmental conditions, the *psvA* antisense transcript inhibits *psvA* mRNA accumulation during growth and after mechanical disaggregation of slugs. However, when slugs are disaggregated in the presence of cAMP, both the sense and the antisense transcripts are accumulated and there is only a minor decrease in the production of the *psvA* gene product. This is probably due to an inhibition of translation by antisense transcripts (Hildebrandt, Sadiq and Nellen, unpublished). Most importantly, the interaction between the complementary transcripts (or their degradation) is abolished. A similar situation is observed in the mutant HM28 which, in the presence of cAMP, can develop mature spores from single cells in the monolayer assay. Under these conditions both sense and antisense RNA are accumulated and apparently do not interact. These results suggest that regulated compounds of the antisense machinery exist which control either hybridization of the complementary RNAs or degradation of the hybrids[60].

Nothing is known about the regulation of antisense effects in *Dictyostelium* or any other eukaryotic organism. The involvement of hybrid promoting proteins, RNA helicases and dsRNase has recently been proposed[52]. Candidates for all three classes of proteins have been identified in *Dictyostelium* (ref 44, Oberosler, Schirmacher and Nellen, unpublished). Interestingly, a developmentally regulated dsRNase activity has been detected in plants[48] which coincides with resistance against dsRNA viruses (J. Matousek, personal commun.).

In some rare cases, sense inhibition (also known as co-suppression in plants) has been observed in *Dictyostelium*[61]. As in the plant studies, this phenomenon is not at all understood. In some experiments with plants, co-suppression could be attributed to methylation of the endogenous gene[49,72]. In *Dictyostelium* this seems unlikely since CpG methylation does not occur or is at least very rare[66].

In summary, antisense experiments in *Dictyostelium* are successful in most cases. A better understanding of the mechanisms of antisense mediated gene inactivation will help to improve application of the technique, and allow one to foresee situations where this approach is likely to fail due to endogenous regulatory pathways.

Analysis of gene expression

LacZ

Gene and operon fusions to the *E. coli lacZ* gene encoding β-galactosidase have proven to be powerful tools in prokaryotic molecular genetics. Analogous constructs, producing functional β-galactosidase under transcriptional control of a eukaryotic promoter, are similarly useful in studying gene expression in eukaryotes[23,38,59,68]. Transformation with *lacZ* gene fusions followed by in situ staining of β-galactosidase expressing cells has been adopted for *Dictyostelium* as well. Such constructs allow one to assay developmentally regulated promoters by staining whole developing organisms. The detection of β-galactosidase activity in situ is as sensitive as immunohistochemistry but technically less demanding. Several vectors are available which allow convenient insertion of any promoter under study[26].

Reporter genes are being used to study expression levels of promoter mutations, to analyze cell type specific gene expression and to follow the behavior of single cells in the multicellular stages of development[15]. Until recently, this was confined to 'snapshots' because cells had to be fixed for staining. With the establishment of the green fluorescent protein as a fluorescent in vivo marker, it will now be possible to observe cell movement in real time[3].

Another recent development has been the use of reporter genes (β-galactosidase and β-glucosidase) that produce unstable proteins. By comparing results with unstable and stable reporters, it was shown for the first time that during slug migration there is a continous de-differentiation and re-differentiation between cell types[8].

In order to measure expression levels quantitatively, CAT and luciferase are most frequently used as reporters. Both activities are easy to measure and there are no endogenous interfering activities in *Dictyostelium*. Determination of CAT activity by the scintillation assay[50,55] can in part compensate for the lower sensitivity of this reporter in comparison to luciferase. The availability of two reliable reporters may be used to compare relative expression levels of two promoters.

In addition to reporters that produce markers with enzymatic activity, immunological markers have also proven useful in *Dictyostelium*. A notable example is a myc epitope to which antibodies are commercially available. The advantage of this technique lies in the fact that the marker sequence is very small and can be integrated at various sites of a gene under examination.

Engineering protein expression

Several attempts to construct promoters which can be induced by an extracellular signal failed in *Dictyostelium*. The introduction of the *tet* repressor and its corresponding binding site was unsuccessful, as was a promoter engineered for regulation by steroid hormones. Similarly, the use of heterologous heatshock promoters or multi-drug resistance promoters did not give the desired results. A system for gene induction by an extracellular signal is based on a modified *pol*III promoter which can be regulated by tetracycline. In combination with a suppressor tRNA and the respective stop codon in a gene of interest this can be used as a rather complicated but functional inducible system[10]. Critical to the operation of this system is the fact that amber suppression in *Dictyostelium* does not interfere with growth or differentiation, since *Dictyostelium* uses almost exclusively UAA (opal) codons as translation terminators[65,73].

Much more convenient but also rather limited in application is the use of endogenous promoters which are regulated in development or can be manipulated by exogenous signals. To date, the most commonly used, regulated expression constructs are based on the csA promoter which is strictly off in growing cells but can be efficiently induced in development by pulses of cAMP[17]. An alternative is the discoidin promoter which is off in cells growing on a bacterial food source and strongly induced with the onset of development. In addition, it can be down-regulated during axenic growth by folate, and re-induced by diluting out the signal molecule[2]. Another possibility is to grow cells at very low densities and then induce the discoidin promoter by prestarvation factor, PSF[39] (see chapter by Clarke and Gomer). Expression levels can be further manipulated by the use of truncated promoters which display decreased transcription but are still regulated by folate[2]. The discoidin promoter offers the additional advantage that, in mutants which overexpress discoidin, reporter constructs are also overexpressed under control of the discoidin promoter[75].

There are also cell-type specific promoters available which are only active in later development, during the multicellular stages. Thus for production of heterologous proteins, these promoters are of limited interest. However, some developmentally regulated promoters may allow for the expression of cytotoxic proteins. The *rasD* promoter has been successfully used to drive the expression of the circumsporozoite protein from *Plasmodium falciparum*[18]. Activity of this promoter can be induced by external cAMP addition. Since this promoter is very tightly regulated, toxic proteins could be expressed upon addition of cAMP after large-scale cell culture. This promoter in combination with the *csA* leader peptide has recently been successfully used to express human muscarinic receptor subtypes in *Dictyostelium*. After induction of such cells, membrane preparations can be used for receptor ligand-binding assays[77].

Dictyostelium discoideum naturally secretes a wide variety of proteins. Homologous signal sequences can be

used to construct genes whose products should be secreted. A tested signal sequence is that of *pspA*, a gene coding for a surface associated protein in *Dictyostelium*[11]. Some heterologous signal sequences also function in *Dictyostelium*. For example, human antithrombin III[9], VP7 protein from SA11 rotavirus[16], and human Alpha 1 antitrypsin (Zündorf and Dingermann, in preparation) have been expressed and secreted from *Dictyostelium*.

Expression of heterologous genes in *Dictyostelium* is complicated by the uncommon codon bias where in accordance with the extremely high A/T content of the *Dictyostelium* genome As or Ts are preferred in the third codon position. This has led to a corresponding adaptation of tRNAs[29]. Therefore, expression of heterologous genes is frequently not very efficient in *Dictyostelium* , most likely because the required tRNAs are not available at the appropriate levels. In several examples, however, it has been shown that adapting the first eight codons to the *Dictyostelium* codon bias significantly improves expression levels (ref 9; C. Reymond, personal commun.). Apparently, efficient initiation is most important for reasonable translation efficiency. The issue of whether the protein expression from a heterologous gene is improved when its codons are optimized towards *Dictyostelium* codon usage is currently under examination (Dingermann et al., work in progress).

Physical gene mapping

The physical mapping of cloned genes provides a powerful way of organizing genetic information. Newly discovered genes can be distinguished from known genes, and closely related members of gene families can be defined. When a genomic map includes an ordered clone library, physical mapping also provides direct access to genomic clones. To improve the resolution of gene mapping in *Dictyostelium*, a physical mapping project has been initiated, and a first generation map has been completed[43]. It consists of a restriction map of *Apa*I, *Bgl*I, *Not*I, *Sma*I, and *Sst*II endonucleases which cut *Dictyostelium* genomic DNA infrequently, 109 plasmid insertion sites, and over 150 mapped genes.

Restriction fragment length polymorphisms (RFLPs) generated by REMI were instrumental in constructing the physical map of the *Dictyostelium* genome. Random insertions of the plasmid DIV6 can be used to generate RFLPs in the range of 10 to 1500 kb since DIV6 carries restriction sites for *Apa*I, *Bgl*I, *Not*I, *Sma*I, and *Sst*II endonucleases[36]. When high molecular weight DNA from a DIV6 insertion strain is digested with one of these rare-cutting enzymes, a unique polymorphism occurs because the large restriction fragment bearing the plasmid insertion is fragmented at the insertion site. Thus, each DIV6 insertion strain bears a single large restriction fragment which is marked at a unique site, and this can be revealed by hybridization with any gene probe that is contained in that fragment. Using a population of such marked strains, new genes can be mapped to most of the large restriction fragments. The contiguity of unmarked small fragments with a nearby large restriction fragment can be established by using different restriction enzymes, and can be confirmed by genomic clones that span the shared restriction sites[36,43].

Ordered clone libraries provide a higher degree of mapping resolution than RFLPs induced by insertion. *Dictyostelium* genomic clones carried as yeast artificial chromosomes (YACs) have been assembled into ordered arrays that represent the genome with a high degree of fidelity (ref 37; A. K. and W. F. Loomis, work in progress). YACs also provide a way to analyze gene families or repetitive elements. Beginning with the DNA from one member of the family used as a probe, one low-stringency hybridization of the probe to the ordered YACs will map all genomic loci that contain at least one member of the family. Fine structure restriction mapping of the YAC clones from each locus by Southern analysis allows one to determine the number of genes at each locus. This approach has been used to map 13 myosin genes[69]. The YACs from each locus may also be used to subclone genome fragments of any newly discovered gene. Purified YACs can be used as substrates for amplification of a gene by the polymerase chain reaction (PCR). In addition, direct subcloning from YACs has also proven to be extremely useful for isolating genomic clones[34].

Prospects

Advances in the molecular genetic techniques for *Dictyostelium* have allowed detailed analyses of its growth and development. Signal transduction pathways in *Dictyostelium* are very similar to those in higher eukaryotes but are much more accessible to investigation and manipulation. Cell motility and cytoskeletal dynamics are of general interest to cell biologists. The advances in molecular biology have made *Dictyostelium* a widely used model system to study the signals and the structures involved in cell locomotion (see chapter by Luna and Noegel). These studies should benefit tremendously in the future from an ability to isolate new genes directly by insertional and antisense mutagenesis. For the future, isolating genes through protein interaction screens using the yeast two-hybrid system[19] should provide a powerful way to connect the functions of genes isolated by mutational analyses.

Finally, there is increasing focus on using *Dictyostelium* as an alternative eukaryotic system for the production of biologically active substances. With the accumulated knowledge, the available techniques, and the efficient and inexpensive cultivation of *Dictyostelium*, the system

offers itself as an attractive alternative expression system for the production of heterologous proteins. Several promoters that operate at various stages of the life cycle have been characterised which allow expression of highly toxic proteins. *Dictyostelium* is also known to have N-linked sugars of the high mannose type, and it can phosphorylate and glycosylate serine and threonine residues as well as produce proteins with GPI tails[21]. With the availability of powerful molecular tools, applied research is likely to become an active area of *Dictyostelium* work in the future.

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