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Expression of Recombinant Glycoproteins in the Simple Eukaryote *Dictyostelium discoideum*

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Introduction

The use of the simple eukaryote *Dictyostelium discoideum* to express recombinant proteins brings a new perspective to this organism, which is well known to developmental biologists. Just as *D. discoideum* occupies a niche in the soil ecosystem, so does the use of the organism in biotechnology revolve around specific areas of protein expression where other eukaryotic systems fail or have deficencies. The aim of this review is twofold. Firstly, we consider various studies which impact on the basic expression system and which have not recently been reviewed. Specifically, we consider promoters, vectors and glycosylation. Secondly, we give our views on the areas of protein expression where *D. discoideum* is likely to have most impact.

Molecular approaches to fundamental biological questions have provided the basis for use of *D. discoideum* in biotechnology. *D. discoideum* is the major experimental model for research into the structure and function of the cytoskeleton. This work commenced with molecular characterisation of cytoskeletal genes, notably actin, myosin and actin binding proteins. There followed simple gene knockout experiments (De Lozanne and Spudich, 1987) and more recently replacement of sections of genes to see how function is affected (Anson *et al.*, 1996). There is also an extensive research base on cell adhesion molecules and G-protein signalling (Parent and Devreotes, 1996; Kim *et al.*, 1996) ; this is a legacy of the organism's status as a model for development of multicellular interactions. The expression and function of these molecules can readily be manipulated *in vivo* in *D. discoideum* and this may be adapted for engineering heterologous proteins. For example, human G-protein linked

Abbreviations: CA1, common antigen; cAMP, cyclic AMP; CAT, chloramphenicol acetyl transferase; CHO cells, Chinese hampster ovary cells; CMF, conditioned medium factor; csA, contact sites A; CSP, circumsporozoite protein; DMSO, dimethyl sulphoxide; Fuc, fucose; GlcNAc, N-acetylglucosamine; Kb, Kilobase pairs; Man, mannose; Man6-P, mannose-6-phosphate; Man6-S, mannose-6-sulphate; NMR, nuclear magnetic resonance; PsA, prespore specific antigen; PSF, prestarvation factor.

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receptors (e.g., muscarinic receptor) can be expressed in *D. discoideum* to form functional receptors that bind pharmaceutically important ligands (Voith and Dingermann, 1995a,b). The fusion of the binding domain of such a receptor to the intracellular region of a *D. discoideum* receptor to form a functional G-protein signalling pathway has the potential to become an important research tool for screening drugs that modify the function of pharmaceutically important receptors.

There is a clear dichotomy emerging between two current uses for expression of heterologous recombinant proteins in *D. discoideum*. On the one hand are studies requiring a functional biological response (e.g., a functional receptor) where the physical amount of protein is relatively unimportant. Indeed, for functional studies 'normal' levels of protein are desired and overproduction may have deleterious effects on the cell. On the other hand are projects where a substantial quantity of correctly folded recombinant protein is needed (e.g., structural studies, antigens for vaccines). In such cases the success of the project depends on relatively high levels of protein expression. Hence, these two aspects demand somewhat different approaches and although many of the basic techniques will be the same, the expression vectors are different.

The *Dictyostelium discoideum* cell

D. discoideum is a simple amoeboid eukaryote with a fast growth rate, very robust cells (5–10 μm in diameter) and its growth requires either bacteria or cheap media and simple equipment. *D. discoideum* also has no requirement for animal proteins and has no known adventitious agents (e.g., viruses). Thus, among eukaryotic expression systems, *D. discoideum* is reminiscent of the *Saccharomyces* expression system. However, notable disadvantages of the yeast system are not present in *D. discoideum*. In particular *D. discoideum* does not hyperglycosylate N- and O- linked glycoforms, it lacks a cell wall and the secretion of proteins is facile. The methods used to culture *D. discoideum* cells and to control gene expression are very different from *Saccharomyces*, being based on a fundamentally different life cycle.

Many key features of the *D. discoideum* expression system relate to its unusual life style. This organism grows as a single-celled amoeba by phagocytosing bacteria and dividing by binary fision. Its natural habitat is in leaf litter, primarily in the world's temperate zones (Raper, 1984; Loomis 1975, 1982). This environment accounts for the cell's natural resistance to osmotic shock (e.g., distilled water) and a variety of chemical insults. To persist in such an unpredictable environment, the cells produce spores that are highly resistant to desiccation, oxidising agents and solvents; this is a particularly useful property when preparing and storing inocula. In contrast, both amoeba and spores of *D. discoideum* are easily killed by heat, e.g., 30 min at 55° (Cotter and Raper, 1968) or simple flaming in 100% alcohol.

When *D. discoideum* amoebae finish consuming their bacterial food source, they undergo a dramatic series of developmental changes. Starving cells migrate into mounds, differentiate to produce a multicellular 'slug' which migrates towards a light source and finally produces a spore bearing structure (Loomis, 1975, 1982). Such dramatic changes in cell function are used as a basic research model for the multicellular interactions required to form differentiated tissues. This research has documented a large series of coordinated changes in gene expression levels following the onset of

starvation, providing well characterised, inducible promoters and selectable markers. Most of the promoters used for biotechnology are induced soon after the onset of starvation. This allows the expression of recombinant proteins in simple buffers.

It should be appreciated that *D. discoideum* DNA is very A+T rich (Kimmel and Firtel, 1982) with a highly biased codon usage (Sharp and Devine, 1989). Codons with G in the third position are rarely used. Whilst the correlation between codon usage and expression level is unclear, no genes with more than 20% rare codons have been expressed in appreciable amounts in *D. discoideum*. The A+T rich expression plasmids are sometimes unstable in *Escherichia coli*, particularly if the gene to be expressed is also A+T rich. Any instability problems usually appear immediately after transformation into *E. coli*; plasmids are stable once established. We have found plasmids are usually stable in *E. coli strain* Dh5a, particularly if incubated at 21° rather than 37°. We have experienced problems with stability in the 'Sure' strain.

Informatics

Advice regarding all aspects of *Dictyostelium* is readily available via the internet. Rex Chisholm (r-chisholm@nwu.edu) maintains a computer mailing list of *Dictyostelium* researchers (dd-email-list@worms.cmb.nwu.edu), a weekly electronic newsletter of abstracts of papers accepted for publication and world wide web pages containing information about vectors, promoters, gene sequences, codon usage tables, gene knockout mutants, references and much more (http://worms.cmb.nwu.edu/dicty.html). YAC libraries and high resolution maps of six of the seven *D. discoideum* chromosomes are available (Loomis *et al.*, 1995; Kuspa and Loomis, 1996). There is also an annual/biannnual international *Dictyostelium* conference.

Maintaining strains: the nuts and bolts

A major advantage of the *D. discoideum* expression system is the relative simplicity of its growth conditions compared to insect or animal tissue culture cells. *D. discoideum* can easily be grown in up to 2 l shaker cultures whilst maintaining accurate control over gene expression. Thus, most microbiology laboratories would have little difficulty in growing 20 litre quantities (i.e., 10 × 2 l cultures) of *D. discoideum* on a weekly basis if required. More simply, *D. discoideum* can be grown in large quantities on bacterial lawns on trays of solid media. As a rough guide, 1 l of either axenic or bacterial growth medium will produce 10^{10} *D. discoideum* cells, roughly 10 g wet weight, 2 g dry weight and 1 g cell protein. The composition of cells varies according to growth conditions (Ashworth and Watts, 1970). For example, stationary phase cells in axenic medium increase by 25% in volume and protein content and by 37 % in dry weight (Soll *et al.*, 1976), whilst during starvation, cells may lose 50% of cell mass.

Most strains of *D. discoideum* are grown at 21° and many laboratory strains contain temperature-sensitive mutations that prevent growth at 26°. Such mutations are used in parasexual genetics to select heterozygous diploid cells from haploid cultures by complementation. *D. discoideum* strains will not grow above 28°, so they are completely nonpathogenic to mammals.

Most non-transformed strains of *D. discoideum* are maintained on lawns of *Klebsiella aerogenes* or *E. coli* on a nutrient agar (Sussman, 1987). Note that particular

strains of *K. aerogenes* or *E. coli* are used which do not produce mucoid substances when grown at 21°. Petri dishes containing 30 ml of SM agar (glucose 10 g, bactopeptone (Oxoid) 10 g, yeast extract 1 g, $MgSO_4.7H_2O$ 1g, K_2HPO_4 1g and KH_2PO_4 2.2 g per litre) are spread with a suspension of bacteria and streaked with a loop of a suspension of *D. discoideum* spores. Isolated colonies of *D. discoideum* become visible as plaques in the lawn of bacteria in 3–4 days. One week after inoculation, individual spore heads can be picked with a loop and suspended in Bonner's salt solution (NaCl 0.6g, KCl 0.75g and $CaCl_2.2H_2O$ 0.4 g per litre) and used as inoculum for further cultures. It is good technique to subculture weekly using as inoculum spore heads selected from near the centre of isolated plaques with a 'non-spready' morphology and lacking sectors with different colonial morphologies. This minimises the chance of selecting variant clones, particularly non-sporing variants. If plates have overgrown, select inoculum from the densest part of the streak, as the zone that has extended growth across the plate may contain fast growing revertants. This is particularly important for transformants where fast growing cells may have lost protein production.

Spore stocks on silica gel are prepared from spread plates on SM agar lawns. After one week's growth, the spore heads are collected by sharply rapping the inverted Petri dishes on the bench so the spore heads fall into the lid. A glass vial of sterile (dry heat), pure (no blue cobalt chloride), fine mesh silica gel is cooled in a freezer. The spores are resuspended in 200 µl sterile horse serum, added to the cold vial of silica gel and well mixed by vigorous shaking. Silica stocks that are well sealed and stored at 4° in a dark container over blue silica gel last up to ten years. Cultures are revived by simply sprinkling a few grains of the silica stock onto a fresh bacterial lawn. Longer term storage is possible as lyophilised spores sealed under vacuum in glass vials. Amoeba or spores can also be stored in liquid nitrogen (Williams and Newell, 1976).

The two methods used to transform *D. discoideum* are electroporation (Knecht *et al.*, 1990; Mann *et al.*, 1994) and calcium phosphate precipitation (Nellen *et al.*, 1984a,b). After transformation, we maintain the cells in axenic media under non-selective conditions overnight, add 10µg G418/ ml for a further 24 h and then plate on bacteria in the presence of G418.

Transformed *D. discoideum* cultures are routinely maintained under G418 (Geneticin) selection. The strains of *K. aerogenes* or *E. coli* used to maintain non-transformed cultures will not grow with G418, although the bacteria can be grown separately and then spread on to selective agar. The best approach is to maintain the cells on a culture of *Micrococcus luteus* PRF3 which is able to grow slowly in the presence of G418 (Wilczynska and Fisher, 1994). Alternatively, *E. coli* can be transformed with a plasmid (e.g., pUC4K) conferring neomycin resistance (and hence G418 resistance) (Hughes *et al.*, 1992). However, this has the disadvantage that the bacteria degrade the G418 and so higher initial amounts of G418 have to be used and selection may decrease during growth. *D. discoideum* transformants are streaked onto lawns of *M. luteus* grown on SM/5 agar (glucose 2 g, Bactopeptone (Oxoid) 2 g, yeast extract 0.2 g, $MgSO_4.7H_2O$ 1 g, K_2HPO_4 1 g and KH_2PO_4 2.2 g per litre) containing 10 µg G418/ml added immediately before pouring the agar and any plates not used within 1 week (stored at 4°) discarded. Stock solutions of 10 mg active G418 /ml are filter sterilised and stored frozen. Note that many G418 preparations are only 60%–70% active. Stocks maintained on *M. luteus* grow more slowly, needing to be

subcultured fortnightly, and have been shown stably to maintain the ability to produce recombinant proteins after subculture for at least 2 years.

Spore stocks for the production of recombinant proteins are prepared on *K. aerogenes* spread plates using an inoculum from cultures maintained on *M. luteus* under selective conditions. The harvested spores are resuspended in axenic broth (below), counted and cooled on ice. 10% DMSO is added and the spores dispensed at 10^7 per aliquot before being snap frozen and stored at –80°.

A defined medium for the growth of *D. discoideum* is available (Franke and Kessin, 1977; Mann *et al.*, 1994). This has not been utilised for the production of recombinant proteins, but has been used more often for selection against uracil or thymidine requiring strains (Mann *et al.*, 1994). *D. discoideum* requires the growth factors biotin, cyanocobalamin, folic acid, lipoic acid, riboflavin and thiamine (Watts, 1977). In addition, the majority of amino acids are essential, with omission of only alanine, aspartic acid, glutamine, serine and tyrosine having no effect on growth, while growth is increased if cysteine, proline, asparagine and glutamic acid are supplied (Franke and Kessin, 1977). The requirement for a large number of essential amino acids is a reflection of *D. discoideum* naturally eating a perfectly balanced source of nutrients – whole bacterial cells.

D. discoideum will grow on a wide range of Gram negative organisms, but does not like most *Pseudomonas* spp, some of which produce substances toxic to *D. discoideum* (Depraitere and Darmon, 1978). Growth on *Bacillus subtilis* is slower than on *E. coli*, but all laboratory strains will grow on *B. subtilis* as mutations in *bsgA*, *bsgB* and *bsgC* loci are used as genetic markers.

Vectors

INTEGRATING VECTORS

D. discoideum shuttle vectors which do not have a *D. discoideum* origin of replication integrate into the chromosomal DNA and hence are called integrating vectors.

The promoter used to drive the selective marker and/ or the nature of the gene in the selective marker determines the number of copies of the vector found in the chromosomes, usually as a tandem array. In a key study, Knecht *et al.* (1986) showed that two integrating vectors gave very different copy numbers. The two vectors contain two different promoters driving different neomycin phosphotransferase (*neo*) genes conferring resistance to G418. Cells transformed with a vector containing an actin 15 promoter controlling the *neo* gene from Tn903 carried less than five copies, while cells transformed with a vector containing an actin 6 promoter controlling the *neo* gene from Tn5 carried more than 200 copies. Despite the difference in copy number, the two sets of transformants produced a similar amount of RNA from the *neo* genes. It appears that survival under selection is related to the RNA levels produced by the cassette; fewer copies of an effective selection cassette are required compared with a cassette producing low levels of RNA. The copy number of integrating vectors can also be increased by raising the G418 concentration from 10 μg/ml to 100 μg/ml to increase selective pressure.

Whereas low copy number vectors are suitable for functional studies, high copy numbers of integrating vectors are preferred for most work on expression of

recombinant proteins, as cloned promoters usually give less expression than the native promoter driving its single copy chromosomal gene. This consideration eliminates for protein production a number of very effective selectable markers which give single copy integration, e.g., selection for uracil auxotrophy using the *D. discoideum* UMP-synthase (DdPYR4-5) gene (Kalpaxis *et al.*, 1990). This selectable marker is remarkable as it can be used in repeated cycles of transformation to select both ura$^+$ (in the absence of uracil) and ura$^-$ cells (in the presence of uracil and 5-fluoro orotic acid). The blastocidin selective marker is also very effective, but gives a low copy number more suitable for gene disruption studies than recombinant protein production (Sutoh, 1993). A range of high copy number selective markers other than G418 have been published, but are not widely used. High copy number selective markers for bleomycin resistance (Chang *et al.*, 1991) and resistance to the related antibiotic phleomycin (Leiting and Noegel, 1991) are considerably more expensive for routine use. A hygromycin selection marker gave erratic transformation results in integrating vectors, but worked in extrachromosomal plasmids (Egelhoff *et al.*, 1989; Knecht and Kessin, 1990). Finally, the thymidylate synthase selective marker, which gives 40 copies of vector per cell (Chang *et al.*, 1989), has not been widely adopted because it is only effective in one cell line (HPS400) with a requirement for thymidine. Some care has to be taken to exclude thymidine from the selective media.

Despite the alternatives listed above, G418 selective makers have been used in all studies producting high levels of recombinant proteins. The reason is that G418 is cheap and effective at the levels (>5μg/ml) used in *D. discoideum*. The low copy number actin 15 promoter/Tn903 *neo* gene selection marker is the most commonly used G418 selective marker, compared to the actin 6/Tn5 *neo* gene. Although direct comparisons of the transformation frequency of high and low copy number vectors are not available, it may be easier to integrate a low number of copies of a vector early in selection, effectively obtaining a higher frequency of transformants.

The choice of selection cassette influences the promoter used for expression, as any vector containing two copies of the same promoter sequence is likely to be unstable in *E. coli*. Thus, vectors that use the actin 15 promoter in the selection cassette have to use an alternative promoter for expression, usually either the strong discoidin gamma promoter (e.g., pVEII (Blusch *et al.*, 1992), pVEII dATG lacking a start codon (Rebstein *et al.*, 1993), or pVEII MCS containing a longer multiple cloning site), or the actin 6 promoter (e.g., pDNeoII (Witke *et al.*, 1987), pDNeo67 (Da Silva and Klein, 1990)). Vectors that use the strong actin 15 promoter for expression use the G418 cassette containing the actin 6 promoter (e.g., pDEX H; Faix *et al.*, 1992). Integration vectors are also available for expression during development using the csA promoter (Faix *et al.*, 1992, 1995).

EXTRACHROMOSOMAL VECTORS

A notable feature of the *Dictyostelium* expression system is the availability of extrachromosomal plasmid vectors, which are relatively unusual in eukaryotes. At least 13 different circular, extrachromosomal plasmids have been descibed in *Dictyoselium* sp. differing in size (1.3–27 kb) and copy number (50–300 copies per cell) (Metz *et al.*, 1983; Orii *et al.*, 1987; Hughes *et al.*, 1988). Only Ddp1 and Ddp2 have been used to construct recombinant vectors. The plasmids are nuclear, as

evidenced by their purification from the nucleus and organisation in nucleosomes (Ashktorab and Welker, 1988). They bear no structural or sequence similarity to the well known 2micron plasmids of *Saccharomyces* (Farrar and Williams, 1988). Plasmids Ddp1, Ddp2, and Ddp5 can coexist at high copy number, but as might be expected, Ddp2 based plasmids are mutually incompatible (Hughes and Welker, 1989).

The original Ddp2 based shuttle vectors were rather large and unwieldy, incorporating much of the 5.8 kb Ddp2 plasmid and a selective marker into an *E.coli* plasmid (Leiting and Noegel, 1988). The addition of further sequences to control gene expression frequently produced vectors that were unstable in *E. coli*. The most recent use of a plasmid incorporating a large section of Ddp2, resulted in a 13 Kb extrachromosomal vector with a copy number of 10–50 per *D. discoideum* cell (Yin *et al.*, 1994).

Ddp2 based vectors were revolutionised by the discovery of two functional regions of the Ddp2 plasmid: a 626 bp origin of replication and the 'Rep gene' – a 2.7 Kb *trans*-acting open reading frame which is essential for plasmid replication (Slade *et al.*, 1990, 1995; Chang *et al.*, 1990: Leiting *et al.*, 1990). Thus it is now possible to construct much smaller extrachromosomal vectors which incorporate just the 626 bp origin of replication. Such plasmids are more stable in *E.coli* and convenient to handle. However, it must be stressed that extrachromosomal replication in *D.discoideum* only occurs if the Ddp2 'Rep gene' is supplied in *trans*, usually from a separate plasmid integrated into the chromosomal DNA. This may conveniently be acheived by co-transforming cells with both the integrating and extrachromosomal vector at the same time (Chang *et al.*, 1990; Slade *et al.*, 1991). This two vector system maintains a 100 copies of extrachromosomal plasmid per cell (Chang *et al.*, 1990), which is less than the 190–300 copies per cell of the parent Ddp2 plasmid (Noegel *et al.*, 1985; Hughes and Welker, 1989). In contrast to the results of Chang *et al.* (1990), Manstein *et al.* (1995) report less than 10 copies per cell using a similar Ddp2 based, extrachromosomal vector. This dramatic difference in plasmid copy number is most likely due to a difference in the number of chromosomal copies of the Ddp2 Rep gene in the host: a large number of copies of the 'Rep gene' may increase plasmid replication. The AX3-ORF$^+$ cells used by Manstein *et al.* (1995) were created using the auxotrophic pyr506 selective marker which usually gives single or low copy numbers of integrating plasmids, whilst Chang *et al.* (1990) used a G418 selective marker controlled by the actin 6 promoter which typically gives over 200 copies of an integrated plasmid (Knecht *et al.*, 1986). An important difference between these two Ddp2 vector systems is in the transformation frequency: the two vector system of Chang *et al.* (1990) had a low transformation efficiency of around $1/10^6$ cells, presumably limited by the frequency of integration/amplification of the plasmid containing the 'Rep gene', whilst the single vector system (i.e., with Rep already incorporated into the recipient cell) of Manstein *et al.* (1995) achieved a transformation of up to $1/3 \times 10^4$ cells. The combination of low copy number of extrachromosomal plasmid and higher transformation frequency is much more suitable for the expression of libraries and functional studies. However, it is probable that a low copy number vector may be more likely to be lost by random segregation at cell division under non-selective conditions, as is likely to be required for bulk or commercial production of recombinant proteins.

Under selective conditions, Ddp2 based vectors are highly stable (Dittrich *et al.*,

1994; Manstein *et al.*, 1995). Using non-selective growth conditions, Hughes and Welker (1989) showed that plasmids containing most of Ddp2 were completely stable and maintained in a narrow range of copy number. The copy number was independent of the amount of transforming DNA and genotype of the host, but was doubled in diploid strains. Ddp2 became markedly less stable following the insertion of *E.coli* plasmid sequences (pGEM3Z) and deletion of one copy of an inverted repeat (which causes instability during growth in *E. coli*) so that 50% of cells had lost the plasmids after 160 generations of non-selective growth on bacteria (Hughes *et al.*, 1992). Note that marked strain differences in plasmid stability were observed. Deletion of further non-coding Ddp2 sequences reduced stability under non-selective conditions to less than 1% after 160 generations. Compared to extrachromosomal Ddp2 based vectors, transformants containing integrating vectors exhibited a much greater variation in stability, with some transformants being stable and others very unstable (Hughes *et al.*, 1992). Unfortunately similar stability experiments under non-selective conditions have not been carried out with the more recent Ddp2 based vectors. In our experience with the pMUW series of plasmids, the loss of protein production occurs sporadically at a low frequency in axenic culture in the absence of selection. In some cases of lost production, the control of the actin 15 promoter may have changed since the cells are G418 resistant and it appears that the plasmid is intact.

At present there are two different families of expression vectors that incorporate the Ddp2 origin of replication. The original pMUW family of plasmids (Slade *et al.*, 1995; Dittrich *et al.*, 1994) have a similar structure to the pDXA family of plasmids (Manstein *et al.*, 1995). Both families of plasmids use the actin 15 promoter, a multiple cloning site for insertion of the gene to be expressed, a polyadenylation sequence and the Ddp2 origin of replication. These vectors differ by the pDXA family incorporating a Tn5 based (G418) selective marker operated by the actin 6 promoter; the pMUWfamily relying on co-transformation with an integrating vector which carries a G418 selective marker. Most of the pMUW family are designed to secrete the recombinant protein and so incorporate a *D. discoideum* secretion signal sequence (*psp*A gene) 5' to the polylinker, whilst the pDXA family are designed for the expression of cytoplasmic proteins fused to peptide tags and so lack a secretion signal. The pMUW plasmids are 3.1 kb whilst the pDXA plasmids are 6.1 kb.

The homologous plasmid Ddp1 allows the construction of alternative extrachromosomal plasmid vectors that are completely compatible with the Ddp2 vectors (Hughes and Welker, 1989). Ddp1 has been fully sequenced (Farrar *et al.*, 1994) and used to construct stable extrachromosomal vectors (Hughes *et al.*, 1994). Ddp1 is very different from Ddp2, being a 13.7 kb plasmid with nine different RNA transcripts. All of the plasmid genes expressed during growth are essential for long term maintenance so the minimal Ddp1 sequence required for replication is the 7.4 kb Csp45I fragment (Hughes *et al.*, 1994). This fragment has been cloned into pGEM7Z with a G418 selective marker to form the 13 kb p155dI shuttle vector which transforms *D.discoideum* with a high frequency of $1/3 \times 10^4$ cells. Although, there are no published reports of using Ddp1 vectors for the production of recombinant proteins it is rumoured that expression levels are low. Plasmid p155dI is stable for at least 400 generations in the absence of selection and has a similar copy number (100) to the parent plasmid Ddp1 (Hughes *et al.*, 1994).

Promoters

In *D. discoideum*, gene expression has been studied under conditions similar to the natural life cycle. When attempts are made to increase expression levels of recombinant proteins using conditions different to those used in classical experiments, it becomes obvious that the regulation of gene expression is complex.

Over 20 *D. discoideum* promoters have been isolated and characterised in detail, providing a range of promoters that could be used for the expression of recombinant proteins. The *D. discoideum* life cycle, having distinct phases of growth and development, offers a simple way of controlling the expression of heterologous genes, e.g., by using a promoter, such as discoidin I, that is induced by the onset of starvation (Rosen *et al.*, 1973; Siu *et al.*, 1976; Ma and Firtel, 1978). These promoters provide control over expression that works well using minimal equipment, such as flasks on a simple shaker, that is available in most microbiology laboratories. However, this approach does mean that activation of the promoters is intimately linked with the cell culture conditions.

The promoters used for recombinant protein production are chosen for the ease of manipulation in bulk culture. To avoid stability problems, particularly in scale up, there must be ways of turning the promoter off. Thus, strong promoters that are active during growth (e.g., cytochrome C oxidase promoter; Rizzuto *et al.*, 1993) are not widely used. Promoters that are active in the late stages of *D. discoideum* development (e.g., promoters for spore coat protein SP60, SP70 and SP96) are completely suppressed during growth, but they can only be used in cultures grown on agar as the late stages do not develop properly in liquid culture. Whilst very large scale agar cultures can easily be produced, they are not so simple as shaken cultures, particularly with respect to timing.

The best levels of recombinant protein expression have been obtained using promoters that are induced as the cells enter starvation, when amino acids are likely to be more available than later in starvation. Note that several of these promoters (e.g., discoidins, actin 15) are also active during the later stages of growth in axenic medium, which offers the advantage of a continuous exogenous supply of amino acids during gene expression. Promoters that operate later in starvation (e.g., csA) cannot be induced in the presence of exogenous amino acids (Marin, 1976, 1977). During starvation, the energy metabolism and biosynthetic requirements of *D. discoideum* cells is met by breakdown of cellular proteins, with the cell volume and protein levels decreasing by half in 24 h of starvation.

The earliest starvation-induced promoters, are those involving genes in the 'prestarvation response' (Clarke *et al.*, 1987, 1988). These promoters are controlled by both the cells nutritional status and an extracellular 'prestarvation factor' (PSF). PSF is an autocrine signal secreted by growing cells that accumulates in the medium in proportion to cell density (Rathi *et al.*, 1991; Rathi and Clarke, 1992). Cells grown on bacteria only respond to PSF when the bacteria become depleted, inducing the prestarvation genes about three generations before the cells cease logarithmic growth (Clarke *et al.*, 1987). In axenic cultures the cells are slightly starved, being limited by the volume of medium (0.2–0.8 μl h^{-1}per 10^6 cells) that can be pinocytosed (North, 1983; Thilo and Vogel, 1980; Maeda, 1983). Thus, at best the maximum rate of protein uptake is only sufficient for the cells to replicate in 6–8 h. In practice, axenic

cells double in 8–16 h (depending on conditions and strain), 3–4 times slower than cells growing on bacteria. It is clear that axenic cells, compared to bacterially grown cells, have a whole suite of changes in metabolism (e.g., increased glycogen accumulation) and the regulation of a range of enzymes (e.g., an increased production and secretion of alpha-mannosidase, N-acetylglucosaminidase) (Ashworth and Watts, 1970; Ashworth and Quance, 1972). At low cell densities axenic cells have enzyme compositions similar to bacterially grown cells, but during exponential growth the enzyme composition is similar to the early stages of starving cells (Burns *et al.*, 1981). PSF also induces the production of a range of proteins required for the early stages of multicellular development, e.g., contact sites B, an EDTA sensitive adhesion protein (Rathi and Clarke, 1992). PSF is produced only by growing cells and production decreases in starvation to low levels after 4 h. Thus, PSF will be maximal under the high cell concentrations ($>5 \times 10^6$) used for the production of recombinant proteins unless the starving cells are washed. PSF is a heat labile, non-dialysable glycoprotein protein which binds to the lectin ConA (Rathi and Clarke, 1992). Note that the PSF does not inhibit cell growth (Clarke *et al.* 1988). However, stationary phase cells produce other factors which, if added to log phase cells, will block cell division and depress the rate of transcription, but which do not affect transcription in stationary phase cells (Yarger *et al.*, 1974; Yarger and Soll, 1975; Soll *et al.*, 1976; Ferguson and Soll, 1976). These growth inhibitory factors provide an explanation of why most *D. discoideum* cultures stop growing at around $1–2 \times 10^7$ cells per ml in the presence of excess nutrients, but their effect on the transcription of prestarvation genes is unclear. Axenically grown, stationary phase cells (1×10^7/ml) no longer express the prestarvation gene discoidin I (Devine *et al.*, 1982).

THE DISCOIDIN Iγ PROMOTER

The best characterised examples of prestarvation regulated genes are discoidin I, alpha-mannosidase and N-acetylglucosaminidase. The conditions of growth dramatically influence the patterns of discoidin I expression (Alexander *et al.*, 1990). During growth on a lawn of *Klebsiella*, the RNA from the three discoidin I genes is virtually undetectable (Rowekamp *et al.*, 1980; Ma and Firtel, 1978), but when grown in a suspension of bacteria, the discoidin I genes are induced three generations before stationary phase (Clarke *et al.*, 1987; Alexander *et al.*, 1990; Wetterauer *et al.*, 1993b). When cells grown on bacterial lawns are starved, discoidin I RNA increases one thousand fold, reaching a peak of around 1% of total RNA at 7-9 h starvation (Ma and Firtel, 1978; Rowekamp *et al.*, 1980; Devine *et al.*, 1982). The discoidin I RNA is selectively degraded after cell aggregation, an effect mediated by extracellular cAMP. Protein production lags behind RNA being about 0.1%, 0.6% and 1% of total cell protein at 4, 8 and 10 h respectively after starvation (Siu *et al.*, 1976; Ma and Firtel, 1978). Cells grown in axenic broth contain about 10% of the discoidin I RNA found in starving cells (Devine *et al.*, 1982).

Most of expression is controlled at the level of transcription, but it is important to appreciate that protein expression is also controlled at the translation level. Alexander *et al.* (1990, 1991) showed that cells with discoidin I expression induced during growth in bacterial suspentions may not produce any more discoidin I protein when starved.. Despite the cells containing RNA for discoidin I, synthesis

of discoidin is blocked at the translation stage. Similarly, starving axenically grown cells may not produce CAT protein when the CAT gene is driven by the discoidin I γ promoter (Blusch *et al.*, 1992). These observations have profound implications for the production of recombinant proteins under similar experimental conditions. The gene specific mechanisms selectively controlling translation levels are unknown. In the case of a ribosomal protein rp1024, the 5' untranslated region of the mRNA controls a rapid drop in translation at the start of starvation (Steel and Jacobson, 1991). Strain VI41 appears selectively to block the translation of discoidin RNA (Wetterauer *et al.*, 1993a). Unfortunately, most work on the *D. discoideum* promoters concentrates on RNA levels rather than protein production. This reflects a focus by molecular biologists on gene regulation and not on the effective output.

The discoidin Iγ promoter (previously known as discoidin I-B, I-C or Ic) is the best characterised promoter used for the expression of recombinant proteins (Poole and Firtel, 1984a). The most commonly used construct contains 411 bp of discoidin γ promoter (5' to the start codon). Although the 411 bp discoidin promoter has the correct temporal pattern of regulation, it actually produces less than 25% of reporter enzyme (CAT), compared to a longer 1.2 kb promoter (Vauti *et al.*, 1990; Blusch *et al.*, 1992). However, as the basal level of CAT expression was also reduced using the shorter promoter, both constructs had a similar 20 fold induction between growth at 10^6/ml in bacterial suspension and starvation for 11 h. The discoidin γ promoter contains an integrated Tdd-2 mobile genetic element (from –810 bp to at least –4 Kb) which is associated with some chromosomal instability (Poole and Firtel, 1984a,b), but its effect, if any, on expression is unknown.

In bacterial suspension culture, using the 411 bp discoidin Iγ promoter to control the luciferase gene, the recombinant protein per unit cell protein rises logarithmically between 1×10^6 cells/ml and stationary phase, increasing tenfold between 8×10^6 and 1.5×10^7 cells per ml (Wetterauer *et al.*, 1993b). This expression can be increased by using strain VI88 which constitutively over-expresses discoidin promoters 10 to 100 fold, giving a 30 fold increase in recombinant protein production (Wetterauer *et al.*, 1993b). However, this lack of gene regulation could lead to strain stability problems.

A notable feature of the discoidin Iγ promoter is that below 10^6 cells per ml in axenic medium it can be repressed by the addition of 1mM folic acid to axenic medium (Blusch *et al.* 1992). 1 mM folate can inhibit the growth rate (Slade, personal observation) and is much higher than the 0.25 μM commonly added to axenic media to stimulate growth as an essential vitamin (Watts, 1977). Repression with folate is useful for the expression of growth inhibitory proteins. For example, to produce a truncated cyclin B protein, the discoidin I γ promoter was repressed with folate to allow selection of transformants in axenic medium and during growth transformants were maintained below 2×10^6 cells per ml in the presence of folate to minimise cyclin B production (Luo *et al.*, 1994).

THE ACTIN 6 AND 15 PROMOTERS

D. discoideum cells are actively motile and phagocytic requiring a very active cytoskeleton. Actin represents about 1% of protein synthesis in vegetative cells (Tuchman *et al.*, 1974) and then increases three fold during the first 3 h of starvation

(Alton and Lodish, 1977). There is an unusually large family of 17–20 actin genes (Romans and Firtel, 1985a,b), of which the best studied are actin 6, 8, and 15. Actin 8, the most abundantly transcribed gene, is expressed at 14%–27% of total actin RNA throughout growth and development, while actin 6 RNA is expressed at about 10% of that level during growth on bacterial lawns and is induced two fold on starvation (Romans *et al.*, 1985). Actin 6 was originally used in G418 expression cassettes because early reports suggested that it comprised 23% of total actin RNA in axenic medium and in early starvation (McKeown and Firtel, 1981: Nellen *et al.*, 1984b), but it is now recognised that the actin 6 promoter is rather weak (Da Silva and Klein, 1990). RNA transcripts from the actin 6 promoter were present at very low levels in growing cells and induced after 2–6 h of starvation (Knecht *et al.*, 1986). During axenic growth, 10 to 20 copies of the csA gene controlled by the actin 6 promoter produced only 20%–25 % of the RNA of the authentic single copy csA gene active during development (Da Silva and Klein, 1990). Full expression of the actin 6 promoter requires 599 bp 5 to the start codon (Nellen *et al.*, 1986), although most vectors use 719 bp from a convenient EcoRI site. The actin 6 promoter has been used for the production of secreted homologous proteins in axenic medium (Blume and Ennis, 1991; Souza *et al.*, 1995).

While the actin 15 promoter is used in several expression vectors, the conditions used for its control are not well characterised. The actin 15 promoter has the same induction profile as the actin 6 promoter (Nellen *et al.*, 1986) and is similar to the prestarvation promoters discoidin I, α-mannosidase and N-acetylglucosaminidase. However, the addition of folate to low density axenic cultures had no effect on the protein production from the actin 15 promoter (Slade, unpublished); a notable difference from the discoidin γ promoter. One similarity between the actin 15 and discoidin γ promoters is that starving axenically grown cells produces no extra recombinant protein, unlike bacterially grown cells. The actin 15 promoter is only weakly active during growth on bacteria, but we have observed that RNA levels start to rise before the onset of starvation. When starvation is induced by removing bacteria, RNA levels increase tenfold reaching peak levels at 2–6 h (Cohen *et al.*, 1986; I. Wilson, personal communication). Typically, maximum protein expression levels are observed at around 6 h starvation. In axenic cultures, some activity of actin 15 promoter is observed throughout the growth curve, but protein production is maximal in stationary phase (Slade, unpublished).

All the sequences for the expression of the actin 15 promoter are contained in 297 bp of DNA from a 5' XbaI site to the start codon. However, while Cohen *et al.* (1986) report that deletion to –182 bp allows normal levels of induction by starvation, Hori and Firtel (1994) show that a curved, A+T rich sequence from –275 bp to –244 bp acts as an enhancer in logarithmically growing, axenic cells, with deletion of this region reducing expression of recombinant luciferase 3–6 fold. It should be noted that the pMUW series of plasmids lack most of this enhancer sequence, being truncated at –247 bp. The effect of this deletion is unclear since, for most purposes, expression of recombinant protein during growth is undesirable and there appears to be no effect on the induction of expression by starvation (Cohen *et al.*, 1986). There is evidence that the strength of the actin 15 promoter is due to a remarkable poly (dT) sequence containing 45 dT residues adjacent to the TATA box (Hori and Firtel, 1994).

DIFFERENT SIGNALS FOR DEVELOPMENTAL PROMOTERS

Expression of developmentally regulated genes requires a second cell density sensing signal 'conditioned medium factor' (CMF) which is different to PSF described above (Clarke *et al.*, 1992), although CMF also causes elevated expression of discoidin I in starving cells (Gomer *et al.*, 1991). CMF's function is to ensure that multicellular development only occurs above a threshold cell density. CMF is slowly secreted by starving cells and the extracellular level signals the cell density. Although CMF does not appear to be secreted by growing cells (Gomer *et al.*, 1991), it is present in internal vesicles (Yuen *et al.*, 1991). The receptor for CMF is virtually absent from growing cells and is maximally expressed in cells starved for 6–8 h (Jain and Gomer, 1994). CMF is an 80 kDa protease sensitive glycoprotein (Gomer *et al.*, 1991; Jain *et al.*, 1992), but after 10 h of starvation, proteolytic break down of CMF produces 0.5–6.5 kDa glycopeptides which are 100 fold more active than the intact molecule (Yuen *et al.*, 1991). A threshold concentration of CMF is required for activation of the cAMP receptor cAR1 (Van Haastert *et al.*, 1996), a seven transmembrane domain G-protein coupled receptor that is essential for aggregation (Klein *et al.*, 1988).

The promoter from the contact sites A (csA) gene seems particularly suited to the expression of proteins that are inhibitory to growing cells (Faix *et al.*, 1992, 1995). This promoter has the advantage of being very tightly regulated, expression being virtually absent during growth and it is induced 300 fold by starvation (Faix *et al.*, 1995). Expression starts after 3 h of shaking in 17mM phosphate buffer and is maximal between 6 and 9 h of starvation (Faix *et al.*, 1992, 1995). One quirk of this promoter is that it is fully induced in starving cells from logarithmic cultures (5×10^6 cells/ml axenic broth), but stationary phase cells (1.2×10^7 cells/ml) need both starvation and pulsing with cAMP for full expression (Faix *et al.*, 1992). It should also be noted that the expression of the csA promoter can be influenced by the degree of cell-cell adhesion (Desbarats *et al.*, 1994). The csA promoter in an integrating vector produced 5–10mg of a soluble fragment of csA protein per litre (Faix, pers. comm.), similar to the yield of soluble PsA (a smaller homologous gene) produced using the actin 15 promoter in an extrachromosomal vector (Dittrich *et al.*, 1994).

In nature, *D. discoideum* maintains contact with a solid substrate, which allows it to respond to starvation by moving towards other cells emitting chemotactic signals of cAMP and then aggregate, forming stable cell-cell contacts. These processes are very limited when cells are shaken in buffer, as used for the production of most recombinant proteins. The pulses of cAMP are initially detected by cAR1 (Klein et *al.*, 1988) which couples the extracellular cAMP pulses to gene expression, repressing genes expressed in growth and inducing the expression of genes characteristic of the multicellular stages of the life cycle (Gerisch *et al.*, 1976; Klein and Darmon, 1977; Kimmel and Carlisle, 1986; Mann *et al.*, 1987; Peters *et al.*, 1991). This process has important effects on the use of promoters from the multicellular stages of the life cycle. Such promoters (e.g., Ras) will function normally if the cells are allowed to aggregate on solid media (e.g., agar), but will only function in shaken suspensions of cells while cAMP pulses are added to the culture. This process is practicable on a small scale, but would be difficult in bulk cultures. In practice, Ras is the only promoter that absolutely requires cAMP that has been used for the production of recombinant proteins, being used for membrane proteins to avoid possible toxicity problems (Voith and

Dingermann, 1995a; Reymond *et al.*, 1995). The mRNA from promoters induced in multicellular stages is rapidly degraded in the absence of cAMP (Mangiarotti *et al.*, 1989).

The regulation of *D. discoideum* promoters appears to be independent of copy number, suggesting that expression is controlled by *trans*-acting activating proteins rather than repressing proteins which might be titrated out. Thus, introducing 100 or more copies of any promoter into a cell does not appear to make gene suppression 'leaky'. However, there is evidence that gene activating proteins do become limiting. Thus 100–150 copies of the csA promoter produced about 20 fold more RNA and 10–15 fold more protein than the single copy native gene (Faix *et al.*, 1992, 1995). Similarly, variations in the copy number of the discoidin Iγ promoter had little effect on the level of CAT reporter activity, with independent transformations of the same construct always having similar relative activity (Vauti *et al.*, 1990).

Signal sequences

D. discoideum genes usually start with the sequence AAAATG, which is similar to the consensus translation initiation signals for other protozoa (Yamauchi, 1991). As this initiation signal is very different from the vertebrates consensus sequence of CC(A/G)CCATG (Kozak, 1986, 1991), the start of most mammalian genes expressed in *D. discoideum* have been modified to AAAATG. Although there seems to be little evidence as to whether or not this alteration is required, most workers consider the change to be on the safe side. For secreted proteins, it is common to replace both the initiation signal and the entire secretion signal with an authentic *D. discoideum* secretion signal. This is easier than just modifying the start of the gene as the 5' non-coding, A+T rich sequence has few unique restriction sites. Replacing the secretion signal effectively removes rare codons from the start of the gene and allows confidence that the signal will be cleaved correctly. The m2 muscarinic acid receptor and *Plasmodium* CSP are examples where replacing the secretion signals have increased protein production (Voith and Dingermann, 1995a; Reymond *et al.*, 1995). The two secretion signals commonly used are from csA and PsA genes (Wong and Siu, 1986; Noegel *et al.*, 1986; Early *et al.*, 1988). Plasmid pMUW1630 encodes a PsA secretion signal, but the DNA sequence is modified to move NsiI and NdeI restriction sites to the signal cleavage site to allow easy insertion of genes (Dittrich *et al.*, 1994), while in plasmid pMUW2442 these sites are moved to lie entirely within the signal to avoid any changes in the gene sequence of the mature protein. This changes the cleavage site from the authentic PsA amino acid sequence of ANA/ in pMUW1630 to ALA/ in pMUW2442, as occurs in the SP96 gene (Fosnaugh and Loomis, 1989). Both sequences are correctly cleaved from recombinant proteins (Dittrich *et al.*, 1994; I. Wilson, personal communication).

A transcription termination signal is essential for high level expression in *D. discoideum*. Constructs provided with an actin 8 terminator produced 10–20 times more β-galactosidase activity compared with constructs having no terminator (Dingermann *et al.*, 1989). *D. discoideum* will recognise a single AATAAA polyadenylation site as a 3' processing signal provided it is embedded in a A+T rich sequence, but apparently will not use the sequence in a G+C rich environment as may occur in mammalian signals (Maniak and Nellen, 1991).

Production of recombinant proteins

SECRETION

The conditions for production of secreted recombinant proteins are best divided into growth of the cells and then induction of expression. There are two alternative methods for the bulk growth of cells. Many laboratories prefer growth in an axenic medium as this is traditional for most molecular biology groups. However, using the actin 15 promoter, we have found that growth on bacteria is preferable for the production of proteins which are toxic to cells and hence inhibit growth in axenic medium where the actin 15 promoter is active. Some transformants simply will not grow at all in axenic medium, particularly if they are producing membrane proteins or proteins that are retained in a compartment of the secretory pathway, e.g., rotavirus VP7. Growth on bacteria is much faster than in axenic medium, usually a 10 fold increase in 24 h in cells grown on bacteria compared to a doubling in axenic medium, which translates into a 1-week production cycle using bacteria, but at least 2 weeks using axenic medium. The growth conditions are also important factors in the purification of a secreted protein. We have experienced difficulties in purifying proteins away from collagen and other peptides from the 14.3g/l peptone in axenic medium, whilst a simple buffer can be used for expression from bacterially grown cells. Under all growth conditions, inoculum concentrations lower than 10^5 /ml may result in a prolonged and erratic lag phase before growth commences.

Axenic broth contains 15.4 g glucose, 14.3 g Oxoid bactopeptone L37, 7.1 g yeast extract, 0.25 g dihydrostreptomycin, 0.48 g KH_2PO_4 and 20 mM MES buffer adjusted to pH 6.1 (modified from Watts and Ashworth, 1970). In recent years, growth in axenic media has been highly dependent on the batch and source of peptone used, so it is wise to consult widely as to what peptone currently works best. Other peptones frequently used are Oxoid Neutralised peptone L34 and Difco Proteose peptone. To avoid animal protein sources, a peptone made from soya can be used, but growth is not as good. The effect of a poor peptone is lack of growth at low dilutions and non-transformed strains entering stationary phase at $3–5 \times 10^6$ rather than $8–10 \times 10^6$ cells per ml. A culture entering stationary phase at a low cell density initially appears to be producing more recombinant product, but the final production is higher in cultures entering stationary phase at a high cell density. Problems with the peptone (particularly at low cell density) can be partially overcome by routine supplementation with 5 µg/ml vitamin B12 and 2 µg/ml folic acid immediately before use, using 1000 fold concentrated stock solutions filter sterilised and stored frozen. Folic acid is soluble in 0.5M $NaHCO_3$.

To grow axenic cultures of strains producing recombinant proteins, we inoculate an aliquot of 10^7 spores into 20 ml axenic broth containing 100 µg ampicillin per ml and 5 µg G418 per ml in a 100 ml Erylenmeyer flask which is rotated on an orbital shaker at 150 rpm. Ampicillin is needed only in the initial culture to avoid growth of any of the streptomycin resistant *Micrococcus* in the spore inoculum. Cell numbers double daily (depending on the strain and protein expressed) and are usually diluted 10 fold towards the end of logarithmic growth, roughly $3–5 \times 10^6$ cells per ml. Cultures are diluted twice weekly, building up to 2-l volumes in 5-l litre flasks. When sufficient volumes of cultures have been produced, cultures are allowed to enter stationary phase at $8–10 \times 10^6$ cells per ml where the bulk of the recombinant protein is produced. The

health of stationary phase cultures can be monitored by pH, as cultures containing MES buffer usually start to die after rising above pH 7. Using the actin 15 promoter, the maximum yield of secreted proteins is achieved by maintaining the cells in stationary phase. Several harvests can be made from one flask. This is achieved by placing stationary phase cultures on ice for 4 h, decanting the supernatant from the settled cells and replacing with fresh medium. Not all cells settle so the density of cells drops from 1×10^7/ml to 0.6×10^7/ml during decanting and then increases in a few days. This process can be repeated indefinitely every 3–4 days, depending on aseptic technique, and producing about twice the concentration of recombinant proteins found in the primary cultures. If the recombinant protein is attacked by proteolysis, 3 μM E64 should be added to cultures to irreversibly inhibit cysteine proteases. It should be noted that only particular strains of *D. discoideum* (e.g., AX3, NP2) possessing three recessive mutations are able to grow well in axenic medium (Williams *et al.*, 1974).

Bulk cultures of *D. discoideum* can also be produced on bacteria using the methods similar to Zhou-Chou *et al.* (1990). We have found *E. coli* B/r to be preferable to *K. aerogenes* as less polysaccharide is produced. The *D. discoideum* cells are grown on a bacterial lawn in 38x38x2.5 cm stainless steel trays with close fitting lids (designed by John Wheldrake, Flinders University, South Australia). Each tray containing 1.5 l SM agar is inoculated on a Friday with a suspension of *E. coli* B/r and *D. discoideum* spores and allowed to grow at 21° for 3 days. The growth on two trays is resuspended in 2-l 20 mM MES buffer containing 0.1 mM $CaCl_2$ and shaken in a 5-l flask (Emslie *et al.*, 1995). This synchronises the onset of starvation, which is patchy in the trays. For the production of recombinant proteins, it is preferred to harvest the *D. discoideum* at 6–8 × 10^6 cells per ml before the onset of starvation. The *D. discoideum* cells are resuspended at 1×10^7/ml in fresh buffer to starve. This procedure allows precise timing of the starvation phase and produces a higher quality product for purification, largely free from bacterial cell components. A variation on this procedure is to grow the *D. discoideum* cells entirely in MES buffer on suspension of *E. coli* cells with an absorbance of 5 at 420 nm. For this method, the *E. coli* are grown separately in trays incubated at 37° for 1–2 days.

It is interesting that *D. discoideum* is able completely to degrade the murein-lipoprotein complex in bacterial cells walls (Braun *et al.*, 1972) and also deacylates the lipid A of the lipopolysaccharide endotoxin rendering it non-toxic (Verret, 1984; Malchow *et al.*, 1969; Rosner *et al.*, 1979; Verret *et al.*, 1982a, b). The polysaccharide portion of the endotoxin is secreted essentially intact except for dephosphorylation and the complete removal of the fatty acid residues (Malchow *et al.*, 1969). The kinetics of growth on live bacterial cultures (Sussman, 1961) or heat sterilised bacteria (Gezelius, 1962) are of interest for some expression work.

Examples of proteins secreted from *D. discoideum* include the homologous glycoproteins PsA (Dittrich *et al.*, 1994; Zhou-Chou *et al.*, 1995), cellulase (Blume and Ennis, 1991), cysteine protease (Souza *et al.*, 1995). The secretion of glutathione S-transferase provides a convenient means of purifying fusion proteins (Dittrich *et al.*, 1994; Jung *et al.*, 1996). Additional heterologous glycoproteins include human antithrombin III (Dingermann *et al.*, 1991), rotavirus VP7 (Emslie *et al.*, 1995, 1996a) and a soluble form of the human mast cell IgE receptor (Wilson, Slade and Williams, unpublished).

CELLULAR RETENTION IN THE ENDOPLASMIC RETICULUM

During a rotavirus infection of mammalian cells, single shelled rotavirus particles assemble in the cytoplasm and then bud through into the endoplasmic reticulum, where the outer capsid protein VP7 is located (Bellamy and Both, 1990). In *D. discoideum*, recombinant VP7 is retained in the endoplasmic reticulum, even when a secretion signal is used (Emslie *et al.*, 1995). The recombinant VP7 contains both N-glycosylated and non-glycosylated forms with the same apparent molecular weights as viral VP7. Crucially, the recombinant VP7 possesses conformation-dependent neutralising epitopes (Emslie *et al.*, 1995).

The transformants producing VP7 die in axenic medium, presumably because the accumulation of the protein in the endoplasmic reticulum is toxic to the cells. Not surprisingly, the protein secretion pathway is essential for growth. The VP7 transformants have to be maintained on bacteria where expression of the VP7 under the actin 15 promoter is minimal. Expression is obtained by starving bacterially fed cultures, as described above. The presence of calcium ions in the starvation buffer significantly increased expression, with the intracellular VP7 being rapidly degraded if calcium was depleted (Emslie *et al.*, 1996a). The level of VP7 in the cells appeared to be relatively constant from 4–8 h of starvation, perhaps indicating a steady state between synthesis and degradation/secretion.

SURFACE PRESENTATION OF ANTIGENS

Three antigens (*D. discoideum* csA, PsA and *Plasmodium* CSP) have been displayed on the surface of *D. discoideum* cells by attachment to GPI anchors. The most effective display of GPI-linked antigens has been in non-growing cells, because GPI-anchored proteins expressed during growth can cause cell culture problems. The most successful promoters were the contact sites A (csA) promoter and the *ras* promoter, both of which are induced after 6 h of starvation and stimulated by cAMP.

The most interesting example is the malarial circumsporozoite antigen (CSP) which is difficult to obtain in the native conformation present on the surface of sporozoites. CSP from *Plasmodium faliciparum* has been expressed on the surface of developmental *D. discoideum* cells using the *ras* promoter (Reymond *et al.*, 1995; Fasel *et al.*, 1992). In order for the CSP protein to be transported to the cell surface, both the CSP secretion signal and the signal sequence for the attachment of a GPI anchor on to CSP needed to be replaced with the corresponding signals from the csA gene (Reymond *et al.*, 1995). Cells were grown in axenic medium, resuspended at 5×10^6 cells /ml in 20 mM phosphate buffer containing magnesium and starved for 6 h before the promoter was induced by the addition of 200 uM cAMP over 1 h. Immunofluorescent staining demonstrated punctate patches of CSP on the surface of *D. discoideum* cells similar to those observed in sporozoites. The GPI-linked, cell surface CSP adopted a conformation which did not react with antibodies against the N-terminal peptide, unlike denatured CSP in western blots. Antisera of mice immunised with the CSP bearing *D. discoideum* reacted with the sporozoite surface antigen. Surface expression of CSP was observed in only 20%–40% of cells (as expected using the *ras* promoter) and the yield was estimated as between 0.03% – 0.3% of total cell protein, roughly 0.15–1.5 mg CSP /litre. The CSP could be extracted from the cells by Triton X-114 and it partitioned into the detergent phase, as expected of a GPI-anchored protein.

The conditions used for the expression of GPI-linked antigens may be quite critical. Two homologous proteins csA (Da Silva and Klein, 1990; Faix *et al.*, 1990, 1992) and pre-spore antigen (PsA) (Slade, unpublished) have their own signals for the addition of the GPI anchor. However, expression of these antigens under growth conditions using the actin 15 promoter interfered with cell growth. In the case of csA transformants (selected in axenic medium) the cells grew three times slower (20 h doubling) than normal and were highly unstable (Faix *et al.*, 1992), whilst PsA transformants (selected on bacteria) were unable to grow in axenic medium. This reduced growth rate was not reported for csA expression controlled by the weaker actin 6 promoter (Da Silva and Klein, 1990). The effect on the cell's growth could be due to interference with nutrient uptake due to the putative cell adhesion properties of these molecules, although the PsA was not detected on the cell surface during growth. Alternatively, the cells may not add GPI anchors efficiently under growth conditions leading to accumulation of the proteins in the secretory pathway as observed in mutants of the GPI pathway in mammalian cells (Fasel *et al.*, 1991; Morgan and Caras, 1992; Delahunty *et al.*, 1993), although it has been shown that csA expressed in growing *D. discoideum* can be radiolabelled with components of the GPI anchor (Da Silva and Klein, 1990). CsA was efficiently and stably overexpressed using its own promoter (Faix *et al.*, 1992), but multicellular development was impaired, as has been observed with the overexpression of a number of other membrane proteins such as gp70 from the feline leukemia virus and gpD from equine herpes virus (Slade, personal observation).

RECOMBINANT HUMAN M2 MUSCARINIC RECEPTOR.

The G-protein coupled receptors, such as the human muscarinic receptors, are of intense interest for the design and testing of pharmaceutical drugs. The *D. discoideum* cAMP receptors (cAR1-4) belong to the same family so *D. discoideum* should have all the machinery necessary to express functional human receptors and it is conceivable that such receptors could be linked to the *D. discoideum* G-protein signalling system to form functional signalling pathways.

The human muscarinic m2 receptor (fused with a csA secretion signal peptide) has been expressed under three promoters allowing different conditions of expression (Voith and Dingermann, 1995a, b). The best receptor activity was achieved in axenic cultures (2×10^6 cells/ml) using the discoidin γ promoter. Under these expression conditions, the recombinant m2 receptors in whole cells or in isolated membrane preparations showed binding characteristics similar to authentic receptors. Under the actin 6 promoter (which is expressed during growth in axenic culture) little functional receptor was observed, perhaps due to some interference with cell growth (Voith and Dingermann, 1995b). Under the control of the *ras* promoter (which functions at a late stage in starvation), the receptors displayed a Hill coefficient lower than one, indicating a decrease in receptor function (Voith and Dingermann, 1995a). Possible reasons for poor receptor function include interference with cAR receptors which are produced at this time (and are needed for activation of the *ras* promoter) or perhaps differences in post-translational modifications.

INTRACELLULAR PRODUCTION

Manstein *et al.* (1995) produced mutant catalytic domains of myosin using a Ddp2

based vector containing the actin 15 promoter. Cells were grown in axenic medium to a density of 8×10^6 cells per ml, with 15 l of medium yielding about 15 g (wet weight) of cells. Cells were lysed with 300 ml buffer containing 0.5% Triton-X100, 15 μg/ml RNaseA and protease inhibitors. The recombinant protein was collected in a pellet (230.000g, 1 h), solubilised and fractionated by chelation chromatography using a histidine tag. The final yield was 5 mg recombinant protein per 10 gram cells or roughly 0.5% of cell protein.

Manstein *et al.* (1995) reported good results for the affinity purification of intracellular recombinant proteins using a C-terminal histidine tag by nickel chelation chromatography. In this example, most of the soluble cytoplasmic proteins were removed prior to chromatography by centrifugation and selective solubilisation of the recombinant myosin fragment. It should be noted that histidine tags are probably not suited to the production of secreted proteins from axenically grown cells where peptone constituents are likely to interfere with chromatography as reported in *Saccharomyces* (Kaslow and Shiloach, 1994) and the tag may be attacked by extracellular proteases. Manstein*et al.* (1995) also reported a vector that allows the production of proteins with a N-terminal histidine tag and/or a C-terminal human c-myc epitope (Mab 9E10). A further epitope YL1/2 (Glu-Glu-Phe) from a-tubulin was found to cross react with a homologous 120 kDa protein.

Glycosylation of proteins in *Dictyostelium discoideum*

One of the most fundamental differences between eukaryotic expression hosts is the nature of the glycosylation structures. Part of the interest in expressing glycoproteins in *D. discoideum* is in the ability to produce a range of glycosylation structures. In some instances it may be desirable to express proteins with typical protozoan glycosylation, which can be an important part of antigenic determinants. Other applications seek to change the form of glycosylation to see the effect on protein function or simply to test the evolutionary conservation of glycosylation site recognition. Glycoproteins produced for the analysis of protein structure using NMR techniques generally require more compact, less variable glycoforms than produced in mammalian cells.

The glycosylation structures in *D. discoideum* can be manipulated, as the haploid genome makes it easy to select mutants or produce gene disruptions and replacements. Thus it should be possible to tailor *D. discoideum* glycosylation for particular purposes using a range of mutants that produce modified glycosylation structures. Our initial approach to this concept has been to do a detailed structural analysis of the glycoforms added to peptides predicted to become O-glycosylated. These peptides are fused to the carboxyl terminus of the easily purified glutathione S-transferase (Jung*et al.*, 1997).

Whilst all eukaryotes use the same peptide motif for N-glycosylation, the picture is less clear for O-linked glycosylation. *D. discoideum* appears to use very similar O-linked glycosylation motifs to mammals (Gooley *et al.*, 1991). Although as yet the glycosylation sites of few recombinant mammalian proteins produced in *D. discoideum* have been characterised in detail, no incorrect glycosylation sites have been observed. Compared with products expressed in mammalian cells, most glycoproteins expressed in *D. discoideum* have a smaller apparent molecular weight on SDS gels and

form more compact bands, suggesting smaller, less variable glycosylation structures. The basic N-linked glycosylation structure in *D. discoideum* glycoproteins is similar to the mammalian high mannose structure, but can be manipulated according to the time of expression. A major difference from mammalian glycosylation is that *D. discoideum* do not appear to contain galactose, N-acetylgalactosamine or sialic acids.

Digests of whole cells in early stages of starvation contain predominantly $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ high mannose glycosylation structures, while after 18–24 h of starvation the glycosylation structures range in size from $Man_9GlucNAc_2$ to $Man_3GlucNAc_2$ (Sharkey and Kornfeld, 1991b). These changes in size correlated with the appearance of mannosidase MI and MII activities which are low in growing cells, but increase following starvation. MI activity (which shortens $Man_9GlucNAc_2$ to $Man_5GlucNAc_2$) rose sharply from 6 to 12 h of starvation and MII activity (which acts only on $Man_5GlucNAc_2$ structures) rose from 12–18 h of starvation (Sharkey and Kornfeld, 1991a). MI and MII are membrane bound and have similar activities to golgi enzymes in rat liver (Tabas and Kornfeld, 1979). Neither processing mannosidase should be confused with a soluble lysosomal α-mannosidase which has an acid pH optimum and is produced in large quantities during the growth phase. There is no evidence for hypermannosylation of either N- or O-linked glycoforms in *D. discoideum*. This is in marked contrast to yeasts, where both N- and O-glycoforms may have hundreds of mannose residues added.

An unusual modification found only in *D. discoideum* is an 'intersecting' N-acetylglucosamine residue linked β1–4 to the mannose residue linked α1–6 to the β-linked core mannose. On the basis of ConA binding, this modification appears to be present on 42% of the N-linked structures in growing cells (Sharkey and Kornfeld, 1991b). The regulation of the 'intersecting' N-acetylglucosaminyltransferase is different from the mannosidases, being present in growing cells, maximally expressed at 6 h starvation and dropping to low levels at 12 h starvation, paralleling a decrease in the proportion of N-linked structures containing the 'intersecting' N-acetylglucosamine (Sharkey and Kornfeld, 1989, 1991a,b). The 'intersecting' N-acetylglucosamine may be part of the common antigen 2 (CA2) epitope as antibody recognition of CA2 is inhibited by N-acetylglucosamine. However, this epitope is only exposed after boiling the proteins (Freeze, 1986). Mammalian glycosylation may also contain 'bisecting' N-acetylglucosamine, but this is in a different position, being linked β1–4 to the inner mannose residue which in turn is linked β1–4 to the GlcNAc core (Hard *et al.*, 1992).

Some *D. discoideum* N-linked glycosylation also contains fucose. In growing *D. discoideum* cells about 50% of structures are endo-H resistant and are radio-labelled with fucose, unlike the Endo-H sensitive structures (Ivatt *et al.*, 1981, 1984). This has been interpreted as fucosylation on the core N-acetylglucosamine residues (Henderson, 1984). This conclusion is supported by the detection of fucose in neutral Endo-H resistant oligosaccharides, but fucosylation does not appear to account for all the Endo-H resistance as charged oligosaccharides contained little fucose (Freeze and Wolgast, 1986). The Endo-H resistant structures parallel fucose labelling until about 13 h of starvation when the proportion of Endo-H resistant glycosylation decreases and fucosylated endo-H sensitive oligosaccharides are synthesised carrying fucose in a terminal position that protects against α-mannosidase digestion (Ivatt *et al.*, 1981, 1984). However, this 'late processing pathway' is severely suppressed in shaken cultures (Ivatt *et al.*, 1984) which are used for the production of recombinant proteins.

The modification of N-linked oligosaccharides on lysosomal enzymes has been extensively studied, partly because the enzymes are easy to produce, being readily secreted (Ashworth and Quance, 1972; Dimond *et al.*, 1981; Freeze, 1986). *D. discoideum* lysosomal enzymes bind to the mannose-6-phosphate receptor of mammalian cells faster than mammalian lysosomal enzymes and are then transported to the lysosomes (Freeze *et al.*, 1980). The greater binding to the mammalian man-6-P receptor is apparently due to a high proportion of high mannose glycosylation structures carrying two phosphate residues on the *D. discoideum* enzymes (Freeze, 1985). It appears that *D. discoideum* would be a very good host for the expression of human lysosomal enzymes for the treatment of lysosomal storage diseases. The phosphate residues are actually present in an unusual methyl phosphodiester linkage to the 6 position of mannose (Gabel *et al.*, 1984; Freeze and Wolgast, 1986b; Lang *et al.*, 1986), unlike the mammalian man-6-phosphate monoester. A point of interest is that attempts have failed to demonstrate a man-6-P receptor in *D. discoideum* (Meirendorf *et al.*, 1985; Cardelli *et al.*, 1986).

D. discoideum lysosomal enzymes and some plasma membrane proteins also contain unusual mannose-6-sulphate groups which are highly immunogenic and known as the 'common antigen' CA1 (Knecht and Dimond, 1981; Freeze *et al.*, 1984). A range of mutants have been isolated that do not produce the CA1 epitope (Knecht *et al.*, 1984). Mutant HL244 completely lacks sulphation, yet it appears to produce normal glycosylation structures and have normal targeting to the lysosome (Freeze *et al.*, 1990; Cardelli *et al.*, 1990). Temperature-sensitive mutations that produce low levels of sulphation at restrictive temperatures have also been reported by Boose and Henderson (1986), but the nature of the defects are unknown. More interestingly, selection for the absence of sulphation led to a range of mutants that produced truncated N-linked oligosaccharides. Mutants HL241 and HL243 synthesise a truncated lipid-linked precursor $Man_6GlcNAc_2$, lacking three terminal sugars on the a1–6 mannose branches (Freeze *et al.*, 1989). The levels of N-linked glycosylation is nearly normal in HL241, unlike HL243 (Freeze *et al.*, 1990a,b). On lysosomal enzymes, these oligosaccharides are still sulphated (and phosphorylated), but lack the CA1 epitope indicating the requirement for a specific arrangement of Man-6S residues on the α1,6 branch (Freeze *et al.*, 1989, 1990b). Additional evidence on the location of the CA1 epitope is its presence in the *modA* strain M31 which lacks the α1,3 glucosidase involved in trimming the inner two glucose residues in the $Glc_3Man_9GlcNAc_2$ precursor of N-linked oligosaccharides, so protecting the α1,4 mannose branch from further modification (Freer *et al.*, 1978; Freeze and Miller, 1980; Freeze *et al.*, 1983; 1990a). High mannose N-linked oligosaccharides can carry up to six sulphate residues, so sulphation may occur in most of the 7 outer mannose residues that do not have a glycosidic linkage in the 6 position (Freeze and Wolgast, 1986a).

The levels of phosphorylation and sulphation of lysosomal enzymes decreases between 6 and 10 h of starvation (Knecht *et al.*, 1985; Bennett and Dimond, 1986; Moore *et al.*, 1987; Levi *et al.*, 1987), although the membrane-bound csA protein has a range of sulphated isoforms after 8 and 14 h of starvation (Stadler *et al.*, 1983) with the sulphation occurring in the golgi (Hohmann *et al.*, 1985). Sulphation appears to continue for longer in shaked cultures (as used for expression) than in cells allowed to aggregate (Davis and Wheldrake, 1986).

Initial mass spectrometry on a secreted soluble human receptor produced in *D.*

discoideum in the first 6 h of starvation indicates that individual N-linked structures have masses in the range 2180–2320 Daltons (Wilson, personal communication) compared to a calculated mass of 2209 D for a $Man_9GlucNAc_3Fuc_1$ structure or 2289 D including one sulphate group. Although in the right ball park, this clearly needs to be repeated using purified N-linked structures. Two dimensional gel electrophoresis showed this protein to consist of multiple isoelectric forms, but whether or not the protein is sulphated is unclear. An important practical implication of these multiple isoelectric forms is the poor resolution of this molecule on ion exchange chromatography.

The comparatively recent development of techniques for the characterisation of O-linked glycosylation means that most of the O-linked structures are being determined using recombinant proteins, rather than the homologous proteins used to characterise N-linked glycosylation. Easily purified recombinant proteins (e.g., GST fusion proteins, Jung *et al.*, 1997) provide convenient molecules for a full battery of modern analytical techniques to analyse individual glycosylation sites. Hitherto, it has been difficult to tie glycoforms recognised by antibodies to particular structures and glycosylation sites, despite a large literature about the distribution of antigenic glycoforms in both wild type and mutant strains producing modified structures (Champion *et al.*, 1995).

The best general classification of O-linked glycoforms comes from the analysis of mutants lacking sets of glycoforms. The *mod*B mutation (Murray *et al.*, 1984; Loomis *et al.*, 1985) is particularly important as *mod*B strains lack the ability to add the first sugar (N-acetylglucosamine) to the peptide acceptor and so lose epitopes on a wide range of proteins. For example, in *mod*B strains the 'contact sites A' protein (csA) lacks any reaction with antibody E28D8, while 'prespore specific antigen' (PsA) lacks any reaction with antibody MUD50. In recombinant PsA the MUD 50 epitope has been shown to be single N-acetylglucosamine residues with a B linkage to the threonine in a repeated amino acid sequence of $(PTVT)_n$ (Zhou-Chou *et al.*, 1995; Zachara *et al.*, 1996). The same epitope also occurs on the recombinant sequence $(PSVS)_n$ (Jung *et al.*, 1997) and $(PTET)_n$ from the spacer in the *D. discoideum Cel*A gene (Slade, unpublished observation). However, there is evidence that in the authentic PsA (produced later in starvation) this glycosylation contains 2 moles of N-acetylglucosamine and one of phosphate (Haynes *et al.*, 1993) although it is still recognised by the MUD 50 antibody. The *mod*B glycosylation in csA must also have a different structure as MUD50 and E28D8 antibodies do not cross react, but should contain the same core N-acetylglucosamine.

A particularly interesting aspect of *mod*B glycosylation is that over 50 cytoplasmic mammalian proteins such as neurofilament proteins and nuclear pore proteins contain single N-acetylglucosamine residues (Hart *et al.*, 1989; Haltiwanger *et al.*, 1992; Dong *et al.*, 1993; Dong and Hart, 1994), although in most cases the sites of glycosylation are not identified. Recombinant proteins with this modification have been produced in eukaryotic expression systems, but the proteins are retained with in the cell and hence are only available in small quantities (Starr *et al.*, 1990; Reason *et al.*, 1992; Greis *et al.*, 1994; Ku and Omary, 1994). It may prove possible to secrete larger amounts from *D. discoideum*. O-linked N-acetylglucosamine residues are also important in secreted antigens of *Entamoeba histolytica* (Stanley *et al.*, 1995), *Plasmodium falciparum* (Nasir-ud-Din *et al.*, 1992; Dieckmann-Schuppert *et al.*, 1993) and *Schistosoma mansoni* (Nyame *et al.*, 1987).

D. discoideum produces another group of highly immunogenic O-linked glycosylation structures found on lysosomal cysteine proteases, cell surface proteins and spore coat proteins (Gustafson and Milner, 1980; Gustafson and Gander, 1984; Mehta *et al.*, 1996; Emslie *et al.*, 1996b). These epitopes (recognised by monoclonal antibodies 83.5, MUD62 and MUD166) are frequently referred to as 'type 3' glycosylation and all appear to be linked by an unusual phosphodiester linkage. As yet, the core O-P-GlcNAc structure has only been detected on serine residues in *D. discoideum* and the structure may also contain fucose residues. In a recombinant cysteine protease, the serine -O-P-GlcNAc structure appears to be on an extended serine-rich loop containing poly-S, SGSQ and SGSG motifs, but the precise sites of glycosylation are unknown (Souza *et al.*, 1995). However, Freeze and Ichikawa (1995) have demonstrated a membrane fraction that exhibits *in vivo* GlcNAc-α-1-P transferase activity on a synthetic peptide substrate containing a SGSG motif. A range of mutants (*mod*C, *mod*D, *mod*E) are available which produce type 3 glycosylation with reduced fucosylation (Champion *et al.*, 1995; Griffiths *et al.*, 1996). Phosphoglycosylation has yet to be reported in mammals, but is important in parasitic protozoa, frequently as highly immunogenic antigens. For example, *Leishmania mexicana* secretes an acid phosphatase containing serine-P-mannose linkages (Ilg *et al.*, 1994) and the cell surface of *Trypanosoma cruzi* contains threonine-P-Xylose linkages (Haynes *et al.*, 1996). Thus, one possible use of expression in *D. discoideum* is in the analysis and production of parasite glycoproteins containing phosphodiester oligosaccharides.

The most intensively studied forms of *D. discoideum* O-linked glycosylation are those that are highly immunogenic in mammals. In contrast, we know comparatively little about less immunogenic structures that are also present in mammalian proteins. For example, it appears that *D. discoideum* produces a single O-linked fucose, but without specific antibodies to map its distribution, its presence is only known from detailed biochemical analysis on individual proteins, e.g., the spore coat protein SP96 (Mreyen, unpublished results). O-linked fucose is reported on mammalian epidermal growth factor domains (Harris *et al.*, 1992) and a range of CHO cell surface proteins (Stults and Cummings, 1993).

Conclusions

There is a general view that no one expression system fulfils all requirements for recombinant protein production. Accordingly, one should choose the most appropriate expression system for a particular protein or application. *D. discoideum* has the basic advantage of being among the cheapest of the eukaryotic systems, but it has had little development compared to other, more established expression systems. Nonetheless the large background of basic research in *D. discoideum* means that rapid progress has been made in a very wide range of different types of protein expression. With such an adapable, basic cell, it is difficult to predict the limits of what this expression system may achieve.

To date, the notable successes of the *D. discoideum* expression system are with proteins that cannot easily be produced in other expression systems. However, as the technology for the *D. discoideum* expression becomes more established, we can expect to tackle proteins that can be expressed in other organisms. What would be the

advantages of using *D. discoideum*? One obvious advantage, among eukaryotic expression systems, is the absence of any requirement for serum or other animal proteins, combined with low costs and simple technology. This seems ideal for low cost animal and human vaccines. Some of the most suitable proteins for expression in *D. discoideum* are antigens of various parasites which are a serious problem in less developed countries. Thus, it is possible that the initial large scale adoption of *D. discoideum* expression system for bulk proteins will be in the third world.

The first application for *D. discoideum* expression is the small scale production of biologically active proteins for structural studies. Examples include fragments of cytoskeletal proteins for functional and X-ray crystallography studies, small glycoproteins for structural characterisation by NMR, and, perhaps most excitingly, hybrid receptors as reporter systems for drug discovery. Thus, cells equipped with hybrid receptors linking extracellular segments of mammalian receptors to intracellular signalling pathways could be used to screen for drug antagonists and agonists. Techniques are becoming available to clone libraries in *Dictyostelium* cells for the surface presentation of adhesion molecules, antigens or antibodies. Such applications require a basic, easy-to-manipulate cell with an exposed outer membrane, properties not often present in other simple eukaryotic systems which have cell walls (e.g., filamentous fungi, yeast) or lyse during during protein production (e.g., bacculovirus infected insect cells) or are substrate-dependent as in many tissue culture systems.

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