Arch Microbiol (1999) 172 : 364–376

ORIGINAL PAPER

Juliet Bailey · Lynnette J. Cook
Richard Kilmer-Barber · Emma Swanston
Lilianna Solnica-Krezel · Karin Lohman
William F. Dove · Jennifer Dee · Roger W. Anderson

Identification of three genes expressed primarily during development in *Physarum polycephalum*

Received: 21 June 1999 / Accepted: 17 August 1999

Abstract During the life cycle of *Physarum polycephalum*, uninucleate amoebae develop into multinucleate syncytial plasmodia. These two cell types differ greatly in cellular organisation, behaviour and gene expression. Classical genetic analysis has identified the mating-type gene, *matA*, as the key gene controlling the initiation of plasmodium development, but nothing is known about the molecular events controlled by *matA*. In order to identify genes involved in regulating plasmodium formation, we constructed a subtracted cDNA library from cells undergoing development. Three genes that have their highest levels of expression during plasmodium development were identified: *redA*, *redB* (regulated in development) and *mynD* (myosin). Both *redA* and *redB* are single-copy genes and are not members of gene families. Although *redA* has no significant sequence similarities to known genes, *redB* has sequence similarity to invertebrate sarcoplasmic calcium-binding proteins. The *mynD* gene is closely related to type II myosin heavy-chain genes from many organisms and is one of a family of type II myosin genes in *P. polycephalum*. Our results indicate that many more *red* genes remain to be identified, some of which may play key roles in controlling plasmodium formation.

Key words Slime moulds · *Physarum polycephalum* · Plasmodium development · Differential gene expression · Myosin · Calcium-binding protein

Abbreviations *LIA* Liver infusion agar · *SDM* Semi-defined medium · *DSDM* Dilute SDM · *DSPB* DSDM with phosphate buffer · *SCP* Sarcoplasmic calcium-binding protein

Introduction

The development of uninucleate, haploid amoebae into multinucleate, syncytial plasmodia in the acellular slime mould (Myxomycete) *Physarum polycephalum* provides a useful system in which to study changes in gene expression during the development of individual cells. As a result of differences in gene expression, amoebae and plasmodia differ greatly in many aspects of behaviour and cellular organisation, including locomotion and mitosis. Comparisons of stage-specific cDNA libraries have demonstrated that approximately 5% of genes are expressed in amoebae but not in plasmodia, and a further 5% show plasmodium-specific expression (Pallotta et al. 1986; Sweeney et al. 1987). As a first step towards understanding the control of development and identifying potential regulatory genes, we sought genes expressed primarily during the development of amoebae into plasmodia and showing little or no expression in the vegetative cell types.

In natural populations, development of amoebae into plasmodia is sexual and under the control of three polymorphic mating-type loci. Two loci, *matB* and *matC*, influence the frequency of fusion between haploid amoebae but have no effect on the events that follow (Youngman et al. 1979, 1981; Kawano et al. 1987). The third mating-type locus, *matA*, has no influence over amoebal fusion,

J. Bailey · L. Solnica-Krezel[1] · W. F. Dove
McArdle Laboratory for Cancer Research,
1400 University Avenue, Madison, WI 53706, USA

J. Bailey (✉) · L. J. Cook · R. Kilmer-Barber · E. Swanson · J. Dee
Department of Genetics, University of Leicester,
University Road, Leicester, LE1 7RH, UK
e-mail: jab9@le.ac.uk,
Tel.: +44-116-2523414; Fax: +44-116-2525101

K. Lohman[2]
Department of Biochemistry, University of Wisconsin,
Madison, WI 53706, USA

R. W. Anderson
Department of Molecular Biology and Biotechnology,
University of Sheffield, Sheffield, S10 2TN, UK

Present addresses:
[1] Department of Molecular Biology,
Vanderbilt University, Box 1820 Station B,
Nashville, TN 37235, USA

[2] DuPont Agricultural Products,
Delaware Technology Park, One Innovation Way,
P.O. Box 6104, Newark, DE 19713, USA

but it controls the development of fusion cells into diploid plasmodia and therefore is the key gene controlling development (Dee 1987). If the fusing amoebae carry different alleles of *matA*, cell fusion is followed by nuclear fusion and the resulting diploid zygote undergoes rounds of synchronous mitoses without cytokinesis to form a diploid multinucleate plasmodium (Dee 1987; Bailey et al. 1990). When plasmodia starve in the light, they form sporangia; meiosis occurs inside the spores that hatch to release amoebae, thus completing the life cycle. Strains carrying *gadA* mutations, which are genetically inseparable from *matA*, are able to develop apogamically; single haploid amoebae develop directly into haploid plasmodia (Bailey et al. 1987, 1992; Anderson et al. 1989).

Time-lapse cinematographic analyses show that in apogamic development, the developing haploid amoeba enters an extended cell cycle more than twice as long as a vegetative cell cycle, during which it continues to grow and becomes irreversibly committed to plasmodium formation (Bailey et al. 1987). The *P. polycephalum* cell cycle does not normally possess a G1 phase, and it has been demonstrated that the extension of the cell cycle is due to a lengthening of the G2 phase (Bailey et al. 1987). During the extended cell cycle, the developing cell acquires some aspects of plasmodial behaviour (e.g. the ability to ingest amoebae and fuse with genetically identical plasmodia) and loses amoebal characteristics such as the ability to transform into a flagellated cell. In addition, some of the alterations in gene expression that accompany development are initiated during this cell cycle (Sweeney et al. 1987; T'Jampens et al. 1997). At the end of the extended cell cycle, the large uninucleate cell becomes binucleate by mitosis without cytokinesis. A haploid multinucleate plasmodium arises by further rounds of synchronous mitoses unaccompanied by cytokinesis (Bailey et al. 1987). Similar cinematographic analyses of sexual development indicate that following cell and nuclear fusion, all zygotes undergo an extended period of growth before becoming binucleate by mitosis without cytokinesis (Bailey et al. 1990). Commitment to plasmodium development coincides with the time of cell and nuclear fusion. In other respects, the changes in cell cycle lengths and cellular organisation follow a similar pattern in both types of development. These analyses demonstrate that an extended cell cycle is a characteristic feature of plasmodium formation.

Studies of cellular organisation and gene expression have confirmed the importance of the extended cell cycle in plasmodium development. The alterations in microtubule organisation and tubulin gene expression have been well-studied [reviewed in Burland et al. (1993)]. Amoebae possess an extensive microtubule network, and mitosis is accompanied by breakdown of the nuclear envelope (Havercroft et al. 1981; Havercroft and Gull 1983). Plasmodia, in contrast, have few microtubules in interphase (Salles-Passador et al. 1991), and mitosis occurs inside the intact nuclear membrane (Havercroft and Gull 1983). The reorganisation of the microtubule network begins during the extended cell cycle, and in the majority of cells the plasmodium-specific β2-tubulin isotype is first detected at this time (Solnica-Krezel et al. 1990, 1991). However, some uninucleate developing cells do not express β2-tubulin even though changes in microtubule organisation are seen (Solnica-Krezel et al. 1990, 1991). These observations suggest that changes in tubulin isotype composition alone do not bring about the reorganisation of the microtubular structures during development.

Since the cytological evidence indicates that the activation of plasmodium-specific genes is not sufficient to bring about the observed changes in cellular organisation and behaviour, other genes must be involved in regulating and controlling development. It has been suggested that these genes would probably have peak levels of expression during plasmodium development (Bailey 1995, 1997). The cDNA libraries previously constructed from amoebae and plasmodia (Pallotta et al. 1986; Sweeney et al. 1987) were not designed to isolate such genes. We therefore constructed a cDNA library from cultures of apogamically developing cells. From this library, we identified clones for genes showing peak levels of expression during development. In addition, we developed methods to overcome the asynchrony of development in mating cultures so that gene expression during sexual development can also be investigated.

Materials and methods

Cells and culture conditions for apogamic development

Apogamic development is temperature-sensitive; plasmodia form in cultures at 26 °C, but development is inhibited at 30 °C. Amoebal stocks of the apogamic strain CL (Cooke and Dee 1974) were maintained at 30 °C. To obtain proliferating amoebae, LIA plates (liver infusion agar; Blindt et al. 1986) were inoculated with 3×10^5 amoebal cysts plus 0.1 ml of standard bacterial suspension (Blindt et al. 1986) and were incubated at 26 °C for 24 h to allow excystment, followed by 48 h at 30 °C. To obtain cultures of developing cells, DSDM plates (Dilute Semi-Defined Medium; Dee et al. 1997) were inoculated with 5×10^5 amoebal cysts plus 0.1 ml of standard bacterial suspension and were incubated at 26 °C. Microplasmodia were cultured in axenic shaken culture at 30 °C in SDM (Semi-Defined Medium; Dee et al. 1997). To produce macroplasmodia, 100 μl of dense microplasmodial suspension was dropped onto Oxoid Nuflow filters (placed on metal grids over SDM in petri dishes), and the cells were allowed to fuse together and grow prior to harvesting 16–30 h later.

Cells and culture conditions for sexual development

Sexual development is not temperature-sensitive, but to ensure comparability between experiments, all matings were carried out at 26 °C. Amoebal stocks were maintained at 26 °C on DSPB plates (DSDM with phosphate buffer; Dee et al. 1997) with standard bacterial suspension and were harvested from these for RNA isolation. For two-strain matings, strains LU648 (Cooke and Dee 1975) and CH508 (Youngman et al. 1979) were used; these strains cross efficiently since they carry different alleles at the mating-type loci (see Table 2). The amoebae were harvested in water and adjusted to 6×10^6 cells ml^{-1}. Equal volumes of the two strains were mixed together and combined with 2 vol. of concentrated bacteria (Youngman et al. 1981). The mixed suspension was inoculated as 10-μl spots (60 per plate) on SM-2 agar plates (Kawano et al. 1987) and were incubated at 26 °C. Microplasmodia and macroplasmodia were cultured in the same way as CL plasmodia.

Table 1 Strains used to generate sexually developing cultures

Strain	Genotype	Reference
CH508	*matA2 matB3*	Youngman et al. (1979)
DP14	*matA7 matB7*	Collins (1975)
DP15	*matA8 matB8*	Collins (1975)
DP74	*matA12 matB5*	Kirouac-Brunet et al. (1981)
DP75	*matA11 matB6*	Kirouac-Brunet et al. (1981)
DP89	*matA15 matB12*	Kirouac-Brunet et al. (1981)
DP90	*matA16 matB13*	Kirouac-Brunet et al. (1981)
LU648	*matA1 matB1*	Cooke and Dee (1975)
PpII1.17–100	*matA5 matB10*	Collins (1975)
PpII1.17–80	*matA6 matB11*	Collins (1975)

For ten-strain matings (Table 1), amoebal cysts were harvested in 5 ml 10 mM phosphate buffer (pH 6; Gorman et al. 1977) and were pelleted by gentle centrifugation at $405 \times g$ for 1.5 min. The cells were resuspended in 2 ml buffer and placed on a reciprocating shaker at 120 strokes per minute at 26 °C until the majority of cysts had hatched into flagellates. The cell density in each suspension was adjusted to 6×10^6 cells ml^{-1}, and an equal volume of each of the ten strains was mixed together. The amoebae were then mixed with an equal volume of concentrated bacteria and plated as described for two-strain matings.

Assays for developing cells in apogamic development

Two assay methods were used to determine the proportions of cells at different stages of development in cultures harvested for RNA isolation:

1. Phase-contrast microscopy was used to determine the proportions of different cell types by counting the number of nuclei per cell in a sample of 500–1,000 cells.

2. Replating assays were used to determine the proportion of cells committed to development. These assays are based on the assumption that cells committed to development at the time of harvesting grow into plasmodia on the assay plates, while noncommitted cells continue to proliferate as amoebae. A sample of the harvested cells was plated on DSDM assay plates (Blindt et al. 1986), and the proportions of plasmodia and amoebal colonies on these assay plates were determined after 4–7 days of incubation.

Assays for developing cells in sexual development

Replating assays (see above) and microscopy analysis were used to determine the proportions of different cell types in cultures undergoing sexual development. Four cell types were distinguished by phase-contrast microscopy: (1) amoebae, (2) fusion cells with two haploid nuclei, (3) zygotes with one diploid nucleus, and (4) multinucleate cells with two or more diploid nuclei. The total number of developing cells was calculated from the replating assays and also by summing the numbers of cells in categories 2–4; the total numbers of developing cells calculated from these two measurements were very similar (data not shown).

RNA isolation

Amoebae and developing cells were harvested from agar plates with ice-cold water, pelleted at $405 \times g$ for 1.5 min, and then washed twice with ice-cold water. Microplasmodia from 50-ml cultures inoculated the previous day with 5 ml of dense microplasmodial suspension were pelleted and washed as above. Macroplasmodia were scraped directly into the lysis medium. Total RNA was isolated using the method of Chomczynski and Sacchi (1987) with modifications by Puissant and Houdebine (1990).

Construction of the ML8 cDNA library

PolyA$^+$ RNA was isolated using an oligo(dT) column (Sambrook et al. 1989). Multiple first-strand synthesis reactions were set up with each containing 5 μg polyA$^+$ RNA in 50 μl. Reverse transcription used RNase H$^-$ Moloney murine leukaemia virus reverse transcriptase with oligo(dT)*Not*I primers (Promega). Second-strand synthesis reactions were carried out in 400-μl volumes with a small amount of ^{32}P-α-dCTP in addition to *Escherichia coli* DNA ligase, DNA polymerase and RNaseH. The cDNAs were phosphorylated by T4 kinase, blunt-ended by T4 DNA polymerase, and then precipitated.

The radioactive cDNA molecules were fractionated over Sepharose CL-4B (Sambrook et al. 1989), and fractions containing molecules over 500 nucleotides were pooled. Next, *Eco*RI adapters (Promega) were attached with T4 DNA ligase. The cDNAs were treated with T4 kinase and digested with *Not*I. Excess *Eco*RI adapters and *Not*I-cut cDNA ends were removed with spin columns (Sambrook et al. 1989). The resulting cDNA molecules were ligated into *Eco*RI/*Not*I-digested and phosphatased pBluescript II KS$^-$ and were transformed into *E. coli* XLI-Blue by electroporation. After outgrowth at 37 °C for 1 h, glycerol was added to 10% and the library was stored at –80 °C. This primary cDNA library was designated ML8.

Amplification and subtraction of the ML8 library

Approximately 30% of ML8 was amplified to give ML8A by growth overnight at 30 °C on LB plates containing tetracycline and ampicillin; the bacteria were harvested in LB plus 10% glycerol and were frozen. To obtain single-stranded phagemid DNA for subtraction, 2 ml of ML8A was shaken at 37 °C in 2 × YT containing ampicillin. After 20 min, helper phage (M13K07; Vieira and Messing 1987) and kanamycin were added. After 6 h, the bacteria were pelleted and the supernatant collected. To each 30 ml of supernatant, 7.5 ml 2.5 M NaCl and 20% PEG solution were added. After 24 h at 4 °C, the phagemids were pelleted, resuspended in 1.5 ml TE per aliquot, and spun at $250{,}000 \times g$ for 18 h in a CsCl gradient. The phagemid band was harvested and dialysed against TE for 1 h. The dialysed phage were broken open by addition of 200 μl CTAB [5 ml 1 M Tris (pH 7.5), 5 ml 0.5 M EDTA (pH 8), and 100 mg cetyltrimethylammoniumbromide]. The released DNA was ethanol-precipitated, cleaned by phenol/chloroform extraction, and resuspended at approximately 0.2 μg μl^{-1}.

To enrich for cDNAs representing genes of interest, the single-stranded phagemid DNA was hybridised against an excess of biotinylated polyA$^+$ RNA from amoebae and plasmodia (Sive and St. John 1988). The nonhybridised phagemids were purified using streptavidin and phenol/chloroform washes (Sive and St. John 1988), resuspended in water, and electroporated into *E. coli* DH5αMCR. This subtracted cDNA library was designated ML8S.

Preparation of subtracted probes for screening ML8S

Three subtracted probes were synthesised, one each using polyA$^+$ RNA isolated from amoebae, plasmodia, and the library source culture. The first cDNA strand of the probes was synthesised as above with the inclusion of 5 μl ^{32}P-β-dCTP. After cDNA synthesis, the RNA was denatured, and unincorporated nucleotides were removed on a Sephadex G-50 column (Sambrook et al. 1989). The probes were precipitated and subtracted against the relevant biotinylated polyA$^+$ RNA (Sive and St. John 1988). Finally, the second strand of the cDNA was synthesised and the whole was used as a probe.

Screening ML8S

Approximately 3×10^4 colonies from ML8S were plated out onto 10 × 9-cm Hybond-N filters placed on LB plates containing ampicillin. Following overnight incubation at 37 °C, three replica filters

were prepared from each master plate (Sambrook et al. 1989). One filter from each set was hybridised with each of the subtracted probes. Colonies that hybridised to only one or two of the three probes were transferred to LB plus ampicillin and grown overnight at 37 °C before storage at –80 °C. To identify individual bacterial colonies containing cDNA inserts, the frozen stocks were plated out at low density and rescreened using subtracted probes. Individual colonies hybridising to only one or two of the probes were picked, and plasmid DNA was isolated using Qiagen kits. The cDNA inserts were digested from the vector, purified, and used as probes.

Northern and Southern blotting

Northern blots were run with 10 μg of total RNA in 1.1% agarose gels using the method of Murray et al. (1994) and Promega RNA size markers. Genomic DNA was isolated from CL microplasmodia using the method of T'Jampens et al. (1997). The DNA was digested to completion with an excess of enzyme and electrophoresed in 0.8% agarose gels with λ-*Hin*dIII and ΦX174-*Hae*III size markers. Transfer to Hybond-N membrane followed the method of Sambrook et al. (1989), and the nucleic acid was fixed to the membranes by exposure to UV irradiation prior to hybridisation. Radio-labelled DNA probes were synthesised by the method of Feinberg and Vogelstein (1983). High-stringency experiments were carried out at 65 °C using the method of Church and Gilbert (1984); post-hybridisation washes were in 0.2–0.04 M Na_2HPO_4 (pH 7.5) with 1% SDS. For low-stringency experiments, the temperature was dropped to 52 °C and washes were in 0.2–0.1 M Na_2HPO_4. All filters were exposed at –80 °C with an intensifying screen.

DNA sequence analysis

Both strands of all clones were sequenced using a Sequenase Version 2.0 Kit (Amersham). All primer design and sequence analysis was carried out with the University of Wisconsin GCG software program (Devereux et al. 1984). Databases were searched using the TFASTA and BLAST programs.

Results

Constructing and screening the subtracted cDNA library

By manipulating culture conditions, apogamic cultures containing a high proportion of uninucleate developing cells were obtained, although these also contained amoebae and multinucleate cells. To identify genes expressed primarily in developing cells, we constructed a cDNA library from a developing culture of the apogamic strain CL (Cooke and Dee 1974) and enriched it for the genes of interest using cDNA subtraction (Sive and St. John 1988). The culture contained 46% uninucleate cells committed to development, 10% multinucleate developing cells, and 44% amoebae (Table 2: 56% developing cells). This primary library, designated ML8, contained approximately 1×10^7 phagemid-carrying bacteria. Analysis of a random sample indicated that 95% of the phagemids contained inserts ranging in size from 500 to 2,000 bp, averaging approximately 900 bp; smaller cDNAs were removed during construction. Part of ML8 was amplified (to give ML8A), and single-stranded phagemids in which the sequence of the inserts was complementary to mRNA were produced.

Table 2 Distribution of cell types in cultures of the apogamic strain CL used for RNA isolation. The values for uninucleate cells were determined from replating assays; the values for multinucleate cells were determined by microscopic analysis. The percentage of uninucleate developing cells equals the total percentage of developing cells minus the total percentage of multinucleate developing cells

Sample	Amoebae (%)	Uninucleate developing cells (%)	Multinucleate developing cells (%)	Total developing cells (%)
1%	98.9	1.1	0	1.1
4%	95.9	3.4	0.7	4.1
10%	90.4	9.3	0.3	9.6
11%	89.3	10.4	0.3	10.7
22%	77.9	21.2	0.9	22.1
23%	77.3	18.8	3.9	22.7
38%	62.3	32.5	5.2	37.7
39%	61.0	34.7	4.3	39.0
56%	44.0	46.1	9.9	56.0

Subtractive hybridisation against polyA$^+$ RNA from amoebae and plasmodia removed plasmids containing amoeba-specific, plasmodium-specific, and constitutively expressed genes. After subtraction, the nonhybridised phagemids were used to transform *E. coli* DH5αMCR to give the ML8S library. Since plasmids without cDNA inserts are not removed by hybridisation against RNA, they form a higher proportion of the subtracted library (ML8S) than of the primary library (85% vs 5%).

Part of the ML8S library was plated out, and after overnight growth three replica plates were made from each original. Each set of three replica plates was screened with the following probes: (1) amoebal probe synthesised using RNA from amoebae and subtracted against RNA from amoebae to reduce the frequency of messages for constitutively expressed and amoeba-specific genes, (2) plasmodial probe synthesised using RNA from microplasmodia and subtracted against RNA from microplasmodia, and (3) developing cell probe synthesised using RNA from the library source culture and subtracted against RNA from amoebae and plasmodia. A first screen yielded approximately 150 colonies that hybridised with only one or two of the probes; colonies that hybridised to all three probes were discarded since they represented constitutively expressed genes. Following a second screening of approximately 20 of the 150 colonies, eight colonies were chosen for analysis. More of the 150 colonies were screened subsequently, and further cDNA clones were analysed (see below).

Gene expression during apogamic development

To examine gene expression by Northern blotting, total RNA was isolated from (1) amoebae from the apogamic strain CL growing on bacteria at 30 °C (the nonpermissive temperature for apogamic development); (2) nonapogamic LU353 amoebae (Dee et al. 1989) growing under the

same conditions; (3) CL microplasmodia from shaken axenic culture, each containing a few hundred nuclei but with no clear vein network; and (4) CL macroplasmodia grown axenically on a surface and containing a network of veins through which protoplasmic streaming was occurring. In addition, total RNA was isolated from several developing cultures of the apogamic strain CL, each containing a different proportion of cells committed to apogamic development (in the range of 1–56%). The proportion of uninucleate committed cells was calculated by subtracting the percent multinucleate cells, determined by microscopic analysis, from the total percent committed developing cells, determined by replating assay (Table 2). In all cases, the majority of committed cells were uninucleate and were therefore traversing the long cell cycle. Although it was possible to obtain cultures with more than 60% committed cells, many of the developing cells in such cultures were multinucleate, making them unsuitable for our purposes.

We planned to identify developmentally regulated genes by their pattern of expression rather than by sequence similarity to known genes. In order to be sure we could clearly distinguish these genes from previously identified classes, we examined expression during apogamic plasmodium development of known constitutive, amoeba-specific, and plasmodium-specific genes. Although the expression patterns of these genes in amoebae and plasmodia were already known, their expression patterns during development had not been examined. All experiments were repeated several times, and the data shown are representative of the results obtained. The constitutive gene studied was actin (Hamelin et al. 1988). This mRNA was used to monitor RNA integrity; all the samples contain easily detected actin signal, and the RNA is not degraded.

The expression patterns of the amoeba-specific *(proA)* and plasmodium-specific *(proP)* isoforms of the actin-binding protein profilin were also examined. The *proA* gene gives rise to two major transcripts that differ only in the length of their 3′-untranslated regions (Binette et al. 1990). The *proA* mRNA was present in all cultures containing amoebae (Fig. 1), but no transcripts from this gene were detected in RNA samples from either microplasmodia or macroplasmodia. The relative abundance of the two *proA* transcripts was the same in all samples, with the larger mRNA being more abundant at all times. The *proP* mRNA was present in trace amounts in cultures containing as few as 4% developing cells, and transcript levels increased as the percent of developing cells increased, with maximum levels in microplasmodia and macroplasmodia (Fig. 1). In agreement with previous results (Binette et al. 1990), we could not detect *proP* mRNA in RNA from amoebae. The expression pattern of *proP* is similar to that previously observed for two other plasmodium-specific genes, β2-tubulin and fragminP (Solnica-Krezel et al. 1988; T'Jampens et al. 1997).

Once the patterns of expression of these three genes had been established, the expression patterns of the eight cDNA inserts identified above was examined. Each hybridised to a different mRNA species, indicating that they represented eight different genes. Six of the mRNA species were present at low levels in all samples, and these constitutively expressed genes were not studied further. The remaining two cDNA clones hybridised to different 800-nucleotide mRNAs with a novel expression pattern; these new genes were designated *redA* and *redB* (regulated in development; Fig. 1). No transcripts from either gene were detected in RNA from amoebae, indicating that these clones did not represent constitutively expressed or amoeba-specific genes. Trace amounts of signal were observed in cultures containing as few as 4% developing cells, and the levels of mRNA increased as the percent of developing cells increased (Fig. 1). The maximum levels of transcripts from both genes were in cultures containing 39% and 56% developing cells, with lower levels of expression in microplasmodia, and still lower levels in macroplasmodia. This expression pattern was clearly different from the patterns described above (Fig. 1), indicating that these cDNAs represented a new group of genes with maximum levels of expression in developing cells.

By screening the amplified library (ML8A), we estimated that approximately 0.1% of the clones in ML8A were *redA* clones, while *redB* was more abundant at

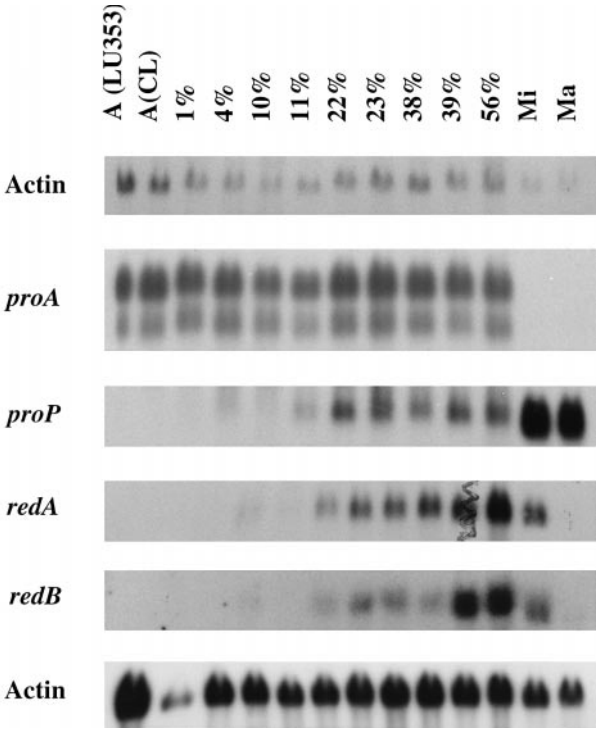

Fig. 1 Gene expression during apogamic development. The results shown come from two separate blots. Blot 1 was probed for the following mRNAs: actin (*upper*; 1,400 nucleotides), *proA* (600 and 500 nucleotides) and *proP* (520 nucleotides). Blot 2 was probed for actin *(lower), redA* (800 nucleotides) and *redB* (800 nucleotides). The RNA samples used were *A(LU353)* RNA from LU353 amoebae, *A(CL)* RNA from CL amoebae, *Mi* RNA from CL microplasmodia, and *Ma* RNA from CL macroplasmodia; *1–56%* RNA samples from developing cultures (see Table 2). The CL amoebal lane in the *lower actin blot* appears to carry less actin mRNA than in the *upper actin blot*, possibly as a result of incomplete transfer to the membrane

0.25%. The *red* mRNAs are, therefore, of low abundance, but since the library source culture contained several different cell types, it is not possible to estimate expression levels in individual cells rather than in the population as a whole. Northern blotting demonstrated that both mRNAs were approximately 800 nucleotides in length, indicating that the cDNA clones for both genes were missing approximately 150 nucleotides since they were both approximately 650 nucleotides in length. Based on data from other *P. polycephalum* genes (Hamelin et al. 1988; Binette et al. 1990), we predicted that the polyA$^+$ tail represented 40–50 of these missing nucleotides and the 5′-untranslated region another 40, leaving approximately 70 bases of the coding region to be identified.

Extensive Southern blot analysis at both high and low stringency indicated that *redA* and *redB* are both single-copy genes (data not shown) with no closely related genes present in the *P. polycephalum* genome.

Table 3 Distribution of cell types in cultures of sexually developing cells used for RNA isolation. The total for developing cells equals the total for fusion cells, zygotes, and multinucleate cells as determined by microscopic analysis

Sample	Amoebae (%)	Fusion cells (%)	Zygotes (%)	Multinucleate developing cells (%)	Total developing cells (%)
2%[a]	97.7	0.8	1.5	0	2.3
4%[a]	95.7	2.1	2.2	0	4.3
17%[a]	82.8	3.7	13.5	0	17.2
24%[a]	76	1.2	14.4	8.4	24.0
38%[b]	62	4.1	25.7	8.2	38
41%[b]	59	4.9	29.1	7.0	41

[a] These samples were isolated from matings involving two strains, LU648 and CH508

[b] These samples were isolated from matings involving ten strains; see Table 1 for list of strains used

Gene expression during sexual development

Although cellular studies have indicated that, following commitment, a similar sequence of events occurs in sexual and apogamic development (Bailey et al. 1990), there have been no studies of gene expression during sexual development. We therefore extended our analysis to include sexual development. RNA was isolated from amoebae of strains LU648 and CH508 (Table 1), from microplasmodia and macroplasmodia formed by crossing these two strains, and from cultures containing different numbers of developing cells. For comparison with uninucleate committed cells in apogamic development, we were particularly interested in fusion cells and zygotes since these are the stages undergoing the extended cell cycle (Bailey et al. 1990). Microscopic analysis was used to determine the distribution of the different cell types in each developing sample (Table 3), and replating assays were used to determine the proportion of committed cells in these cultures (data not shown). The proportion of developing cells calculated from the replating assays was very close to that calculated by totaling the number of fusion cells, zygotes, and multinucleate developing cells (Table 3).

Northern blotting was carried out using the RNA samples described above and the control genes used in our analysis of apogamic development; the data shown in Fig. 2 a are representative of the results obtained. Actin signal was detected in all lanes, and the mRNA was not significantly degraded. Both transcripts from the *proA* gene were present in LU648 and CH508 amoebae, and the amount of *proA* mRNA and the relative levels of the two transcripts remained approximately constant in cultures containing amoebae, just as in apogamic development (compare Figs. 1 and 2 a). No *proA* mRNA was detected in microplasmodia or macroplasmodia. The *proP* mRNA was abundant in microplasmodia and macroplasmodia (Fig. 2 a), and trace amounts were detected in cultures containing 24% developing cells after a long exposure, but we could not detect *proP* mRNA in any of the other developing cultures; this result is in contrast to those from apogamic development, in which *proP* mRNA was detected in cultures containing as few as 4% developing cells. Although low levels of the *red* gene transcripts were detectable in the 24% developing cells and in microplasmodial samples after long exposures (data not shown), it was not possible to determine the levels of the *red* gene expression in the different cultures.

We also wished to examine gene expression in sexually developing cultures containing higher numbers of fusion cells and zygotes. Although we achieved good mixing of the amoebae, mating occurred with poor synchrony, so that in cultures containing more than approximately 25% developing cells, most of the developing cells were multinucleate and were not of interest. The rate-limiting factor in sexual development is the frequency with which compatible amoebae meet and fuse within the cultures. In the case of mixtures of two strains, only half of the possible meetings between pairs of amoebae involve compatible cells. In order to increase the proportion of meetings between compatible amoebae, we increased the number of strains mixed to ten, each of which carried a different allele of *matA* and *matB* (Table 1); in this case, 90% of random pairwise meetings would be between compatible amoebae. Cinematographic analyses indicated that amoebae in ten-strain mixtures normally fused in pairs just as in two-strain mixtures (Barber 1998). Using these ten-strain mixtures, cultures containing a much higher proportion of developing cells were obtained, the majority of which were fusion cells and zygotes (Table 3).

Northern blot analysis (Fig. 2 b) was then carried out using RNA isolated from the ten-strain mixtures (38% and 41% developing cells) together with several of the two-strain samples; the experiments were repeated several times, and the data shown are representative of the results obtained. Although the levels of actin mRNA do vary somewhat, there is clearly a signal in all lanes. The expression pattern of *proA* was as expected; both transcripts were detected in all samples containing amoebae, but not

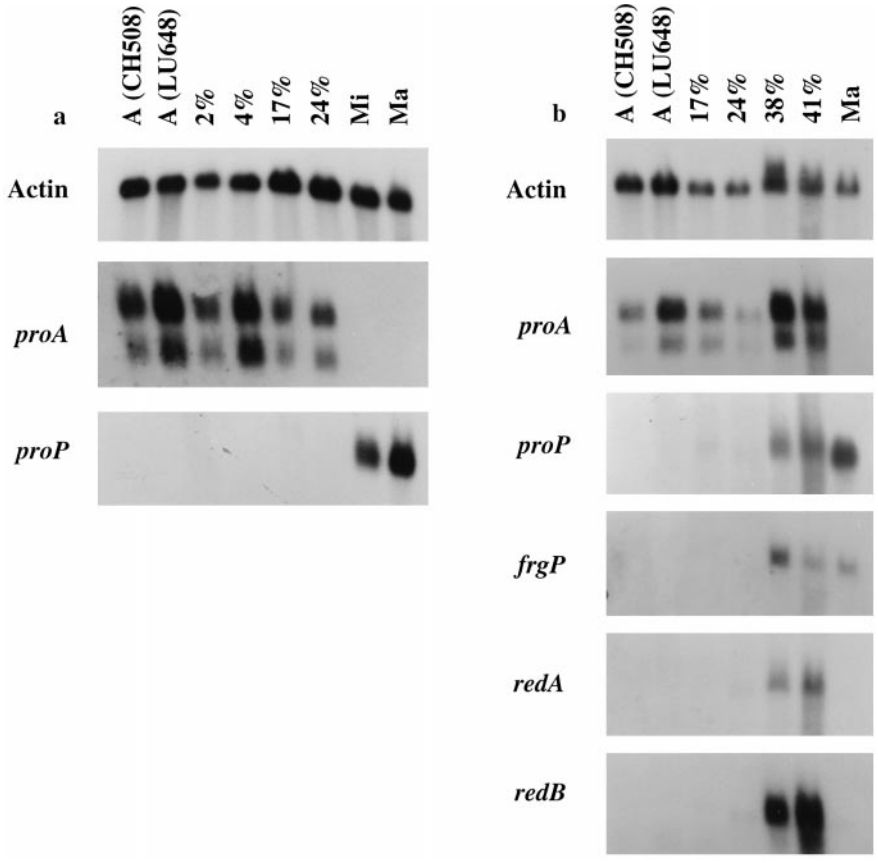

Fig. 2 a, b Gene expression during sexual development. **a** Gene expression in two-strain mixtures. **b** Gene expression in ten-strain mixtures. The following mRNAs were studied: actin, *proA*, *proP*, *frgP* (1,200 nucleotides), *redA*, and *redB*. The RNA samples used were: *A(LU648)* RNA from LU648 amoebae, *A(CH508)* RNA from CH508 amoebae, *Mi* RNA from crossed microplasmodia of genotype CH508 × LU648, and *Ma* RNA from crossed macroplasmodia of the same genotype; *2–24%* RNA samples from developing cultures made by mating CH508 and LU648 (see Table 3); *38–41%* RNA samples from ten-strain mixtures (see Tables 1 and 3)

in the plasmodial sample (Fig. 2 b). Expression of *proP* was maximal in the macroplasmodial samples, but significant levels of expression were observed in the 38% and 41% samples from ten-strain matings. Low levels of signal were also seen in the 17% and 24% two-strain mating cultures, but expression was not detected in the other samples. The expression pattern of the plasmodium-specific fragmin gene, *frgP* (T'Jampens et al. 1997), was the same as that observed for *proP*.

Expression of *redA* and *redB* was clearly detected in the RNA from the ten-strain cultures. For both *red* genes, the maximum level of expression was observed in the ten-strain samples (38% and 41%), with trace amounts of expression in the 24% sample and no detectable signal in cultures containing fewer developing cells (Fig. 2 b). Only low levels of expression were observed in the macroplasmodial sample after long exposure (data not shown). Although the basic pattern of expression of all the genes examined was similar in apogamic and sexual development, gene expression was detected in apogamic cultures containing as few as 4% developing cells, while in sexual development no signal was detected in cultures containing less than 24% developing cells.

The sequence of *redA*

The longest *redA* cDNA clone identified contained 674 nucleotides from the estimated 800 (EMBL accession no. Y18123). Using *Taq*I-digested CL genomic DNA, a 900-bp genomic fragment (EMBL accession no. Y18122) was amplified by inverse PCR (Ochman et al. 1990) using the following primers: (1) 5′-CAAATGCCACCTTCACTATG-3′ and (2) 5′-TATTGACAAGAAACCAGGC-3′. Comparison of the cDNA and genomic sequences reveals that the first 297 bases of the genomic clone do not overlap with the cDNA clone. This 297-base region contains several ATG start codons, only one of which is in the same reading frame as the cDNA, is in the correct position to give an mRNA of the observed length, and is not followed immediately by a stop codon. Assuming this ATG is the start of the *redA* coding region, the first 237 bases of the genomic clone represent promoter sequences, and the next 60 nucleotides represent the section of *redA* open reading frame not present in the cDNA clone. The complete amino acid sequence of *redA* was deduced from the combined cDNA and genomic sequences. The RedA protein has a predicted molecular weight of approximately 24 kDa and contains 223 amino acids. Sequence analysis did not reveal any conserved domains or motifs, and comparisons with the sequences in the databases revealed no significant similarity to any listed proteins. Therefore, *redA* is a novel gene of currently unknown function.

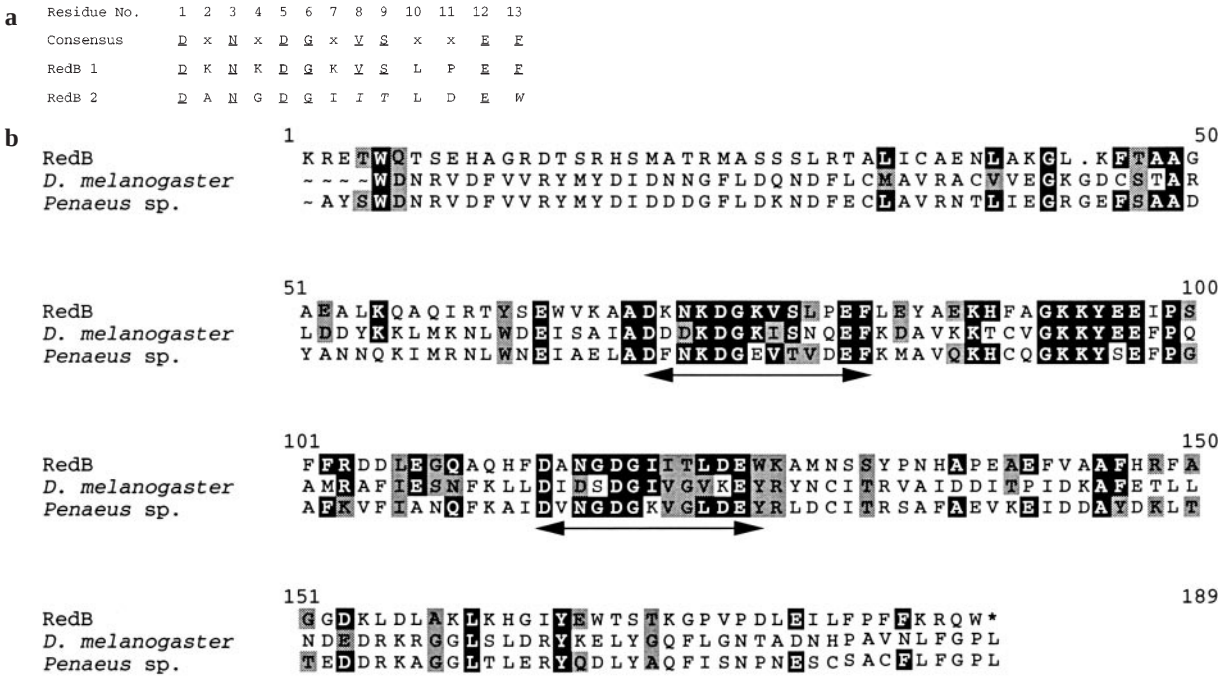

Fig. 3 a, b The structure of RedB. **a** Comparison of the calcium-binding domains in the deduced RedB sequence to the consensus sequence. The *underlined residues* are identical to the consensus sequence, while *italics* designate permitted alternative residues and *X* shows where many different residues can occur; those present are on the permitted list. **b** Comparison of the deduced RedB amino acid sequence with the sequences of the sarcoplasmic calcium-binding proteins from *Penaeus* sp. (shrimp; Takagi and Konishi 1984) and *Drosophila melanogaster* (Kelly et al. 1997). The *black boxes* indicate amino acids in RedB that are shared with at least one of the other proteins (27%), while similar amino acids are *shaded* (12%). The calcium-binding domains are indicated. One gap *(dot)* was inserted for the best fit

The sequence of *redB*

Sequencing of the partial *redB* cDNA clone (EMBL accession no. Y18124) indicated that there is only one possible open reading frame giving rise to 187 amino acids before the stop codon. Sequencing of a partial genomic clone (EMBL accession no. Y18125) indicated that *redB* contains at least three short introns. This pattern of short exons and introns is similar to that seen in other *P. polycephalum* genes including *redA* (Binette et al. 1990). Amino acid sequence analysis indicates that the RedB protein contains two calcium-binding domains. Calcium-binding domains generally occur in pairs, and there is a consensus sequence for 13 amino acids in the most invariant region (Moncrief et al. 1990; Nakayama et al. 1992); these are compared to the RedB domains in Fig. 3 a. Comparisons with sequences in the databases indicated that RedB has significant identity with sarcoplasmic calcium-binding proteins (SCPs) from invertebrates such as shrimp and *Drosophila melanogaster* (Fig. 3 b; Takagi and Konishi 1984; Kelly et al. 1997).

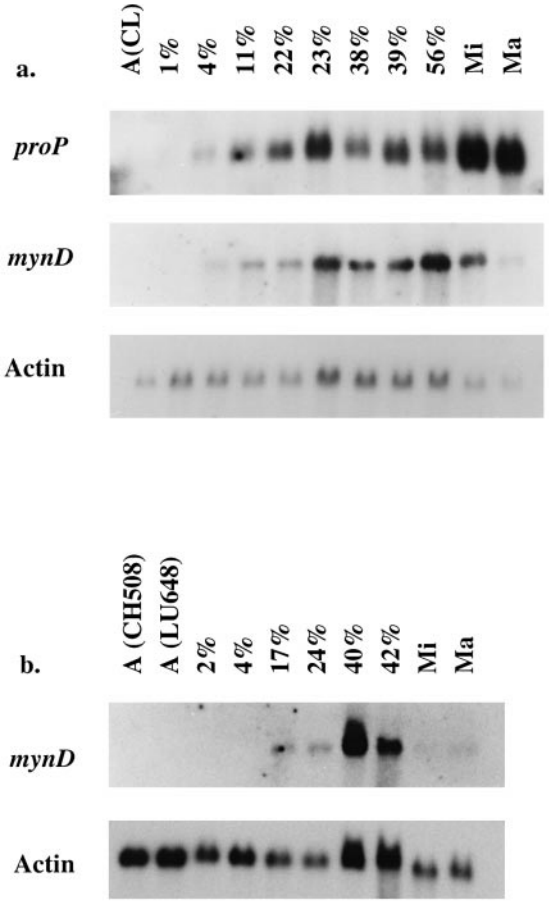

Fig. 4 a, b Expression of *mynD* during plasmodium development. For explanations of the RNA samples used, see legends to Figs. 1 and 2. These experiments were repeated several times, and the results shown are representative of the results obtained. **a** The expression of *mynD*, actin and *proP* during apogamic development. **b** Expression of *mynD* and actin during sexual development

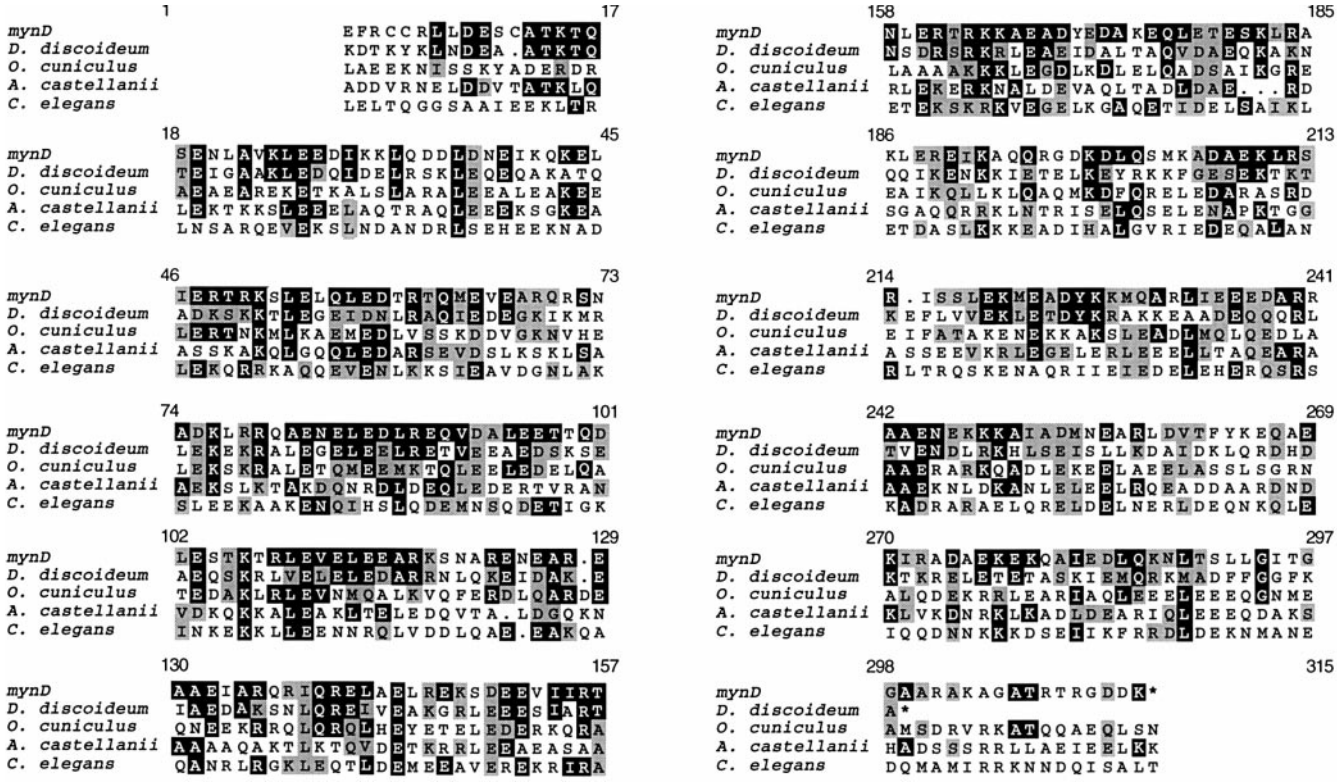

Fig. 5 Amino acid sequence of MynD as compared to that of other myosin heavy chains. Comparison of MynD with *Dictyostelium discoideum* large myosin heavy chain (Warrick et al. 1986), *Oryctolagus cuniculus* (rabbit) smooth-muscle myosin (Nagai et al. 1988), *Acanthamoeba castellanii* myosin II (Hammer et al. 1987) and*Caenorhabditis elegans* myosin II (Dibb et al. 1989). Amino acids are arranged into 28-residue repeats. In a few places, gaps *(dots)* were inserted for the best fit. The *black boxes* indicate amino acids in MynD that are shared with at least one of the proteins shown, while *shaded boxes* indicate similar amino acids

Identification of *mynD*

From the second batch of clones screened, we identified several more candidate *red* genes, one of which is described here. A 1,000-nucleotide cDNA that cross-hybridised to a 6,500-nucleotide mRNA was identified. In apogamic development, the new gene was not expressed in cultures containing less than 4% developing cells, but expression levels increased as the proportion of developing cells in the cultures increased and declined in plasmodia (Fig. 4 a). As expected from our previous results, actin expression was detected in all samples, and *proP* showed maximum levels of expression in plasmodia (Fig. 4 a). Northern blot analysis of sexual development indicated that this new gene is expressed at maximum levels in developing cultures, at very low levels in plasmodia, and not at all in amoebae (Fig. 4 b); actin expression was detected in all samples. Thus, the expression pattern of this new gene is similar to those of *redA* and *redB*, suggesting that it represents another *red* gene.

Results from Southern blot analysis at high stringency (data not shown) showed that for some enzymes that had no restriction sites within the cDNA, two bands were detected, suggesting that there were introns in the section of the gene covered by the cDNA clone; this observation was not surprising given our experiences with *redA* and *redB*. When Southern blotting was carried out at low stringency with the same enzymes, two or three bands were detected in addition to those expected from the high-stringency results (data not shown). This suggests that this new gene is a member of a gene family with three or possibly four members.

The entire 1,000-nucleotide cDNA clone was sequenced (EMBL accession no. AJ133501) and compared to sequences in the databases. A high degree of identity was observed between our clone and the tail region of myosin type II heavy-chain genes from many organisms; therefore, we named this gene *mynD* (myosin Developmental). There is little similarity with the previously identified *P. polycephalum* myosin-like proteinA, MlpA (data not shown). The MlpA protein is encoded by a constitutively expressed 2,400-nucleotide mRNA (Murray et al. 1994). The amino acid sequence of MynD is 32% identical and a further 23% similar to the *Dictyostelium discoideum* large myosin heavy-chain protein (Fig. 5; Warrick et al. 1986). In total, over 50% of the amino acids in MynD are shared with at least one of the myosin proteins shown (Fig. 5).

Secondary structure calculations (data not shown) indicate that MynD will adopt a virtually uninterrupted α-helix coiled-coil formation indicative of the tail region of type II myosins. Although the *mynD* cDNA clone does not contain the full myosin tail sequence, structural analysis indicates that, as in the case of other myosin tails,

mynD contains repeating 28-amino-acid-residue zones, each made of four heptad repeats; these repeats are common in proteins that form a coiled-coil structure. Hydrophobic residues are concentrated at positions "a" and "d" within these heptad repeats, presumably helping to stabilise the coiled-coil structure (data not shown).

Discussion

By screening a subtracted cDNA library made from apogamically developing cells, we identified three genes showing a novel pattern of expression. All had their highest levels of expression in developing cultures, with lower levels of expression in microplasmodia and macroplasmodia; no expression was detected in amoebae. These genes were identified by detailed screening of less than 1% of the subtracted cDNA library ML8S, suggesting that many more *red* genes remain to be identified.

Even at their maximum levels of expression, *redA* and *redB* mRNAs never represented more than 0.5% of the total mRNA present in the cultures, suggesting that we might have had to screen 200 colonies from a nonsubtracted cDNA library to identify just one of these genes. The fact that *redA* and *redB* were identified from only eight colonies indicates that the enrichment technique was successful. Our success rate of one in four was similar to that reported by Wan et al. (1996; 1 in 5.6) in a study comparing commonly used methods of identifying differentially expressed genes.

The RedB protein had significant sequence similarity to sarcoplasmic calcium-binding proteins from invertebrates. The first members of this family of proteins were identified from invertebrate muscle, where it has been proposed that they act as calcium buffers (Wnuk et al. 1982). However, recent findings indicate that SCPs are expressed in some nerve cells (Kelly et al. 1997), indicating that their role is more complex than originally suggested and that their exact functions are unclear. Further studies on RedB function are planned and may help to elucidate the roles of SCPs in invertebrates.

Amoebae and plasmodia differ greatly in actin organisation (Uyeda and Furuya 1985; Stockem and Brix 1994) but express the same actin genes (Hamelin et al. 1988). These two cell types express different isoforms of several actin-binding proteins including profilin and fragmin (Binette et al. 1990; T'Jampens et al. 1997), suggesting that changes in actin-binding proteins may be responsible for the differences in actin organisation. Prior to this study, nothing was known about the timing of the switch from one profilin isotype to the other during plasmodium formation. Our results showed that in apogamic development, the *proP* mRNA was first detected in cultures containing as few as 4% developing cells, most of which were uninucleate, and gradually increased to its maximum levels in plasmodia. The expression pattern of *proP* is very similar to that previously observed for the plasmodium-specific β2-tubulin gene (Solnica-Krezel et al. 1988). Since cytological studies indicate that β2-tubulin protein is present in the microtubules of uninucleate committed cells (Solnica-Krezel et al. 1990, 1991), given the similarity in expression pattern of β2-tubulin and *proP*, it seems likely that the ProP protein will also be present in uninucleate committed cells.

The type II myosin found in amoebae and plasmodia has many functional and structural similarities to skeletal muscle myosin and colocalises with actin in a microfilament network (Taniguchi et al. 1978; Ohl and Stockem 1995). In amoebae, microfilaments are concentrated within the pseudopodia, although there also appears to be a cortical network under the cell membrane (Uyeda and Furuya 1985). In axenically cultured microplasmodia (the cell type labelled "Mi" in our RNA samples) there is a cortical microfilament system under the cell membrane and, in some cases, a few cytoplasmic fibrils. In macroplasmodia (the cell type labelled "Ma" in our RNA samples), in addition to the cortical layer there is an extensive network of cytoplasmic fibrils in frontal regions and bundles of filaments surrounding the veins (Ohl and Stockem 1995).

Kohama and colleagues (Kohama et al. 1986; Uyeda and Kohama 1987) have purified myosin from amoebae and plasmodia and have demonstrated that antibodies raised against amoebal myosin do not detect myosin from plasmodia in Western blots and vice versa. Immunofluorescence microscopy on cells from developing apogamic cultures indicate that amoebal myosin is present in uninucleate committed cells and amoebae, whereas plasmodial myosin is detected in uninucleate developing cells and plasmodia but not in amoebae (Uyeda and Kohama 1987). It has been suggested, therefore, that amoebae and plasmodia express different myosin heavy-chain genes and that the plasmodial gene is activated during the extended cell cycle, while the amoebal gene is switched off at a similar stage of development (Uyeda and Kohama 1987).

Although amoeba-specific and plasmodium-specific myosins have been identified biochemically, *mynD* is the first *P. polycephalum* type II myosin heavy-chain gene to be cloned. Since it is not expressed in amoebae, the *mynD* gene cannot code for the amoebal myosin. Plasmodial myosin is normally isolated from macroplasmodia since it is more abundant in these cells than in microplasmodia (Ohl and Stockem 1995). We predict, therefore, that the gene for plasmodial myosin will have an expression pattern similar to that of other plasmodium-specific, actin-binding proteins such as profilinP (this work) and fragminP (T'Jampens et al. 1997). Although *mynD* is expressed in macroplasmodia, its expression level is low in these cells as compared to that of *proP* (Fig. 4), suggesting that it does not code for the major plasmodial myosin but instead represents a third *P. polycephalum* myosin isoform that is most abundant during plasmodium formation.

Our data from low-stringency Southern blotting suggest that the *P. polycephalum* type II myosin heavy-chain gene family has three or four members. These would presumably include the biochemically identified amoeba-specific and plasmodium-specific myosins in addition to *mynD*. However, the *mynD* clone covers a region of the

gene that is not well-conserved since as long as the α-helical coiled-coil structure is maintained, there is great flexibility in sequence. It is possible, therefore, that Southern blotting with our partial clone did not identify all members of this gene family. In order to determine conclusively how many type II myosin heavy-chain genes are present in the *P. polycephalum* genome and when they are expressed, we plan to clone the remainder of the *mynD* gene and use this to probe Southern and Northern blots.

Our studies suggest there may be a difference between apogamic and sexual development in the timing of activation of the *red* and the plasmodium-specific genes. In apogamic development, these classes of genes were expressed in cultures containing less than 10% committed cells, whereas in sexual development, expression was undetected until 17–24% cells committed to development were present. Commitment is defined operationally by the ability of a developing cell to grow into a plasmodium when washed off the culture plate and replated onto an assay plate (Blindt et al. 1986). In sexual plasmodium formation, commitment is acquired close to the time of amoebal fusion (Shipley and Holt 1982). In apogamic development, uninucleate cells become committed to development approximately halfway through the extended cell cycle (Bailey et al. 1987). The relationship between an uninucleate committed cell in apogamic development and a fusion cell in sexual development is unknown at present, and differences in the state of the cells at the time of commitment may account for any differences in gene expression.

All three genes were expressed at higher levels in microplasmodia than in macroplasmodia. Microplasmodia are used by many laboratories as the major cell type studied, but they are not thought to represent a naturally occurring cell-type. They are grown in shaken axenic culture and, due to the action of the shaker, fragment into pieces, each containing a few hundred nuclei. Our results suggest that the difference in expression levels is not due to the induction of gene expression in microplasmodia by growth in shaken culture since the *red* genes are not expressed in CLd-AXE amoebae growing in shaken culture under identical conditions (data not shown). As noted earlier, macroplasmodia and microplasmodia differ in their cytoskeletal organisation, and it is possible that these may be related to differences in *red* gene expression, particularly in the case of *mynD*.

The ultimate aim of this project is to identify genes involved in the regulation of plasmodium development in *P. polycephalum* and to elucidate their functions. Previously, genes required for plasmodium formation have been identified by isolating mutants blocked in development. Genetic analysis has indicated that these *npf* mutants (no plasmodium formation) fall into two groups: those linked to *matA* and those unlinked to *matA* (Anderson et al. 1989; Solnica-Krezel et al. 1995). Analyses of strains carrying *npf* mutations unlinked to *matA* have mapped the mutations to many different genes; each mutant shows a different terminal phenotype (Bailey et al. 1992; Solnica-Krezel et al. 1995). In most cases, developmental abnormalities have been first observed during the extended cell cycle, confirming that this is an important period in plasmodium development. Although it is not possible to clone the genes identified, it seems likely that some of the *npf* genes will also be shown to be *red* genes.

It has been postulated that *matA* acts as a transcription factor and activates a cascade of gene action ending with the activation of plasmodium-specific genes and the repression of amoeba-specific genes (Bailey 1995, 1997). If this is the case, then some of the *red* genes may be a part of such a cascade. Although all three genes show similar patterns of expression during development, this does not imply that they play similar roles in the amoebal-plasmodial transition. In order to understand their precise functions during development, we are undertaking further cellular and molecular studies including immunofluorescence microscopy using specific antibodies and gene disruption using methods similar to those of Burland and Pallotta (1995). By combining the results from several different techniques, we hope to elucidate the functions of the *red* genes during plasmodium development and to learn more about the control and regulation of plasmodium development.

Acknowledgements The authors would like to thank Dr. T. Burland for expert technical help and patient guidance during the early part of this project, and J. Foxon, L. King and A. Kobayashi for technical assistance. L. Solnica-Krezel was supported by Core Grant no. CA-07175, Program Project Grant in Tumor Biology no. CA-23076 from the National Cancer Institute, and a Wisconsin Power and Light Foundation Fellowship in Cancer Research. J. Bailey was supported by the University of Wisconsin Graduate School. J. Bailey and L. J. Cook are supported by The Wellcome Trust (grant nos. 034789 and 042524). R. Kilmer-Barber received a Biotechnology and Biological Sciences Research Council PhD studentship. E. Swanston is supported by a University of Leicester PhD studentship. K. Lohman was supported by National Institutes of Health predoctoral training grant no. T32 GM07215.

References

Anderson RW, Hutchins G, Gray A, Price J, Anderson SE (1989) Regulation of development by the *matA* complex locus in *Physarum polycephalum*. J Gen Microbiol 135:1347–1359

Bailey J (1995) Plasmodium development in the myxomycete *Physarum polycephalum*: genetic control and cellular events. Microbiology 141:2355–2365

Bailey J (1997) Building a plasmodium: development in the acellular slime mould *Physarum polycephalum*. Bioessays 19:985–992

Bailey J, Anderson RW, Dee J (1987) Growth and development in relation to the cell cycle in *Physarum polycephalum*. Protoplasma 141:101–111

Bailey J, Anderson RW, Dee J (1990) Cellular events during sexual development from amoeba to plasmodium in the slime mould *Physarum polycephalum*. J Gen Microbiol 136:739–751

Bailey J, Solnica-Krezel L, Anderson RW, Dee J (1992) A developmental mutation *(npfL1)* resulting in cell death in *Physarum polycephalum*. J Gen Microbiol 138:2575–2588

Barber R (1998) Changes in cell fusion behaviour and gene expression during sexual development of *Physarum polycephalum*. PhD Thesis, University of Leicester, Leicester, UK

Binette F, Benard M, Laroche A, Pierron G, Lemieux G, Pallotta D (1990) Cell-specific expression of a profilin gene family. DNA Cell Biol 9:323–334

Blindt AB, Chainey AM, Dee J, Gull K (1986) Events in the amoebal-plasmodial transition of *Physarum polycephalum* studied by enrichment for committed cells. Protoplasma 132:149–159

Burland TG, Pallotta D (1995) Homologous gene replacement in *Physarum*. Genetics 139:147–158

Burland TG, Solnica-Krezel L, Bailey J, Cunningham DB, Dove WF (1993) Patterns of inheritance, development and the mitotic cycle in the protist *Physarum polycephalum*. Adv Microb Physiol 35:1–69

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995

Collins OR (1975) Mating types in five isolates of *Physarum polycephalum*. Mycologia 67:98–107

Cooke DJ, Dee J (1974) Plasmodium formation without change in nuclear DNA content in *Physarum polycephalum*. Genet Res 23:307–317

Cooke DJ, Dee J (1975) Methods for the isolation and analysis of plasmodial mutants in *Physarum polycephalum*. Genet Res 24: 175–187

Dee J (1987) Genes and development in *Physarum*. Trends Genet 3:208–213

Dee J, Foxon JL, Anderson RW (1989) Growth, development and genetic characteristics of *Physarum polycephalum* amoebae able to grow in liquid axenic medium. J Gen Microbiol 135: 1567–1588

Dee J, Foxon JL, Hill W, Roberts EM, Walker MH (1997) Contact with a solid substratum induces cysts in axenic cultures of *Physarum polycephalum* amoebae: mannitol induced detergent-resistant cells are not true cysts. Microbiology 143:1059–1969

Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programmes for the VAX. Nucleic Acids Res 12:387–395

Dibb NJ, Maruyama IN, Krause M, Karn J (1989) Sequence analysis of the complete *Caenorhabditis elegans* myosin heavy chain gene family. J Mol Biol 205:603–613

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Gorman JA, Dove WF, Shiabe E (1977) Anisomycin sensitive mutants of *Physarum polycephalum* isolated by cyst selection. Mol Gen Genet 151:253–259

Hamelin M, Adam L, Lemieux G, Pallotta D (1988) Expression of three unlinked isocoding actin genes of *Physarum polycephalum*. DNA 7:317–328

Hammer JA, Bowers B, Paterson BM, Korn ED (1987) Complete nucleotide sequence and deduced polypepetide sequence of a nonmuscle myosin heavy chain gene from *Acanthamoeba*: evidence of a hinge in the rodlike tail. J Cell Biol 105:913–925

Havercroft JC, Gull K (1983) Demonstration of different patterns of microtubule organisation in *Physarum polycephalum* myxamoebae and plasmodia using immunofluorescence microscopy. Eur J Cell Biol 32:67–74

Havercroft JC, Quinlan RA, Gull K (1981) Characterisation of a microtubule organising centre from *Physarum polycephalum* myxamoebae. J Ultrastruct Res 74:313–321

Kawano S, Kuroiwa T, Anderson RW (1987) A third multiallelic mating-type locus in *Physarum polycephalum*. J Gen Microbiol 133:2539–2546

Kelly LE, Phillips AM, Delbridge M, Stewart R (1997) Identification of a gene family from *Drosophila melanogaster* encoding proteins with homology to invertebrate sarcoplasmic calcium-binding proteins (SCPs). Insect Biochem Mol Biol 27: 783–792

Kirouac-Brunet J, Masson S, Pallotta D (1981) Multiple allelism at the *matB* locus in *Physarum polycephalum*. Can J Genet Cytol 23:9–16

Kohama K, Takano-Ohmuro H, Tanaka T, Yamaguchi Y, Kohama T (1986) Isolation and characterization of myosin from amoebae of *Physarum polycephalum*. J Biol Chem 261:8022–8027

Moncrief ND, Kretsinger RH, Goodman M (1990) Evolution of EF-hand calcium-modulated proteins. 1. Relationships based on amino acid sequences. J Mol Evol 30:522–562

Murray M, Foxon JL, Sweeney F, Orr E (1994) Identification, partial sequence and genetic analysis of *mlpA*, a novel gene encoding a myosin-related protein in *Physarum polycephalum*. Curr Genet 25:114–121

Nagai R, Larson DM, Periasamy M (1988) Characterization of a mammalian smooth muscle myosin heavy chain cDNA clone and its expression in various smooth muscle types. Proc Natl Acad Sci USA 85:1047–1051

Nakayama S, Moncrief ND, Kretsinger RH (1992) Evolution of EF-hand calcium-modulated proteins. 2. Domains of several subfamilies have diverse evolutionary histories. J Mol Evol 34:416–448

Ochman H, Medhora MM, Garza D, Hartl DL (1990) Amplification of flanking sequences by inverse PCR. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 219–227

Ohl C, Stockem W (1995) Distribution and function of myosin II as a main constituent of the microfilament system in *Physarum polycephalum*. Eur J Protistol 31:208–222

Pallotta D, Laroche A, Tessier A, Shinnick T, Lemieux G (1986) Molecular cloning of stage specific mRNAs from amoebae and plasmodia of *Physarum polycephalum*. Biochem Cell Biol 64: 1294–1302

Puissant C, Houdebine L-M (1990) An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Biotechniques 8:148–149

Salles-Passador I, Moisand A, Planques V, Wright M (1991) *Physarum* plasmodia do contain microtubules! J Cell Sci 100: 509–520

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Shipley GL, Holt CE (1982) Cell fusion competence and its induction in *Physarum polycephalum* and *Didymium iridis*. Dev Biol 90:110–117

Sive HL, St John T (1988) A simple subtractive hybridization technique employing photoactivable biotin and phenol extraction. Nucleic Acids Res 16:10937

Solnica-Krezel L, Dove WF, Burland TG (1988) Activation of a β2-tubulin gene during early development of the plasmodium in *Physarum polycephalum*. J Gen Microbiol 134:1323–1331

Solnica-Krezel L, Diggins-Gilicinski M, Burland TG, Dove WF (1990) Variable pathways for developmental changes in composition and organization of microtubules in *Physarum polycephalum*. J Cell Sci 96:383–393

Solnica-Krezel L, Burland TG, Dove WF (1991) Variable pathways for developmental changes in mitosis and cytokinesis in *Physarum polycephalum*. J Cell Biol 113:591–604

Solnica-Krezel L, et al (1995) Characterisation of *npf* mutants identifying developmental genes in *Physarum*. Microbiology 141:799–816

Stockem W, Brix K (1994) Analysis of microfilament organisation and contractile activities in *Physarum*. Int Rev Cytol 149: 145–215

Sweeney GE, Watts DI, Turnock G (1987) Differential gene expression during the amoebal-plasmodial transition in *Physarum*. Nucleic Acids Res 15:933–945

Takagi T, Konishi K (1984) Amino acid sequence of the a chain of sarcoplasmic calcium binding protein obtained from shrimp tail muscle. J Biochem 95:1603–1615

Taniguchi M, Yamazaki K, Ohta J (1978) Extraction of contractile protein from myxamoebae of *Physarum polycephalum*. Cell Struct Funct 3:181–190

T'Jampens D, et al (1997) Molecular cloning, overexpression, developmental regulation and immunolocalization of fragminP, a gelsolin-related actin binding protein from *Physarum polycephalum* plasmodia. J Cell Sci 110 : 1215–1226

Uyeda TQP, Furuya M (1985) Cytoskeletal changes visualised by fluorescence microscopy during amoeba-to-flagellate and flagellate-to-amoeba transformations in *Physarum polycephalum*. Protoplasma 126 : 221–232

Uyeda TQP, Kohama K (1987) Myosin switching during amoeboplasmodial differentiation of slime mold, *Physarum polycephalum*. Exp Cell Res 169 : 74–84

Vieira J, Messing J (1987) Production of single-stranded plasmid DNA. Methods Enzymol 153 : 3–11

Wan JS, et al (1996) Cloning differentially expressed mRNAs. Nature Biotech 14 : 1685–1691

Warrick HM, De Lozanne A, Leinwand LA, Spudich JA (1986) Conserved protein domains in a myosin heavy chain gene from *Dictyostelium discoideum*. Proc Natl Acad Sci USA 83 : 9433–9437

Wnuk W, Cox JA, Stein EA (1982) Parvalbumins and other soluble high-affinity calcium-binding proteins from muscle. In: Cheung WY (ed) Calcium and cell function, vol 2. Academic Press, New York, pp 243–278

Youngman PJ, Pallotta DJ, Hosler B, Struhl G, Holt CE (1979) A new mating compatibility locus in *Physarum polycephalum*. Genetics 91 : 683–693

Youngman PJ, Anderson RW, Holt CE (1981) Two multiallelic mating compatibility loci separately affect zygote formation and zygote differentiation in the myxomycete *Physarum polycephalum*. Genetics 97 : 513–530