Homologous Gene Replacement in *Physarum*

Timothy G. Burland* and Dominick Pallotta†

*McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706, and †Department of Biology, Faculty of Science and Engineering, Laval University, Quebec G1K 7P4, Canada

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ABSTRACT

The protist *Physarum polycephalum* is useful for analysis of several aspects of cellular and developmental biology. To expand the opportunities for experimental analysis of this organism, we have developed a method for gene replacement. We transformed *Physarum* amoebae with plasmid DNA carrying a mutant allele, *ardDΔ1*, of the *ardD* actin gene; *ardDΔ1* mutates the critical carboxy-terminal region of the gene product. Because *ardD* is not expressed in the amoeba, replacement of *ardD*⁺ with *ardDΔ1* should not be lethal for this cell type. Transformants were obtained only when linear plasmid DNA was used. Most transformants carried one copy of *ardDΔ1* in addition to *ardD*⁺, but in two (5%), *ardD*⁺ was replaced by a single copy of *ardDΔ1*. This is the first example of homologous gene replacement in *Physarum*. *ardDΔ1* was stably maintained in the genome through growth, development and meiosis. We found no effect of *ardDΔ1* on viability, growth, or development of any of the various cell types of *Physarum*. Thus, the carboxy-terminal region of the *ardD* product appears not to perform a unique essential role in growth or development. Nevertheless, this method for homologous gene replacement can be applied to analyze the function of any cloned gene.

THE protist *Physarum polycephalum* displays a variety of strikingly different cell types in its life cycle, including uninucleate amoebae, multinucleate plasmodia, flagellates, spores, and cysts. The different cell types exhibit a wealth of biological phenomena, including two distinct mechanisms of mitosis (e.g., SOLNICKA-KREZEL *et al.* 1991), several mechanisms of motility, and distinct patterns of genetic control of somatic compatibility (reviewed in BURLAND *et al.* 1993b). Biochemical-scale analysis of the unperturbed mitotic cycle is possible in the plasmodium, which grows to large mass while maintaining a perfectly synchronous mitotic cycle (e.g., SCHEDL *et al.* 1984). The ability to culture the vegetative amoebae and plasmodia in haploid or diploid form, together with the option for asexual development, permits isolation of mutants and complementation testing. The occurrence of mating and meiosis in the life cycle of *Physarum* permits Mendelian genetic analysis of mutants (reviewed in BURLAND *et al.* 1993b). To expand the opportunities for experimental analysis of this wealth of biological phenomena found in a single organism, we are developing methods for engineering the genome.

Heterologous genes can be introduced into *Physarum* when they are carried on plasmid DNA and electroporated into amoebae (BURLAND *et al.* 1992, 1993a). A *Physarum* promoter is required for expression of the heterologous genes, and stable transformants have

been obtained by selecting for hygromycin resistance after electroporation of amoebae with DNA carrying the *hph* (hygromycin phosphotransferase) gene under control of the actin gene promoter *PardC* (BURLAND *et al.* 1993a). Stable transformants typically carry a single copy of *PardC-hph* integrated into the nuclear genome at nonhomologous sites.

To develop a method for homologous gene replacement, we chose the *Physarum ardD* actin gene as a test case. The major actin in *Physarum* is encoded by the isocoding *ardA*, *ardB*, and *ardC* genes, with the *ardB* and *ardC* genes accounting for most of the actin expressed in amoebal and plasmodial cell types (HAMELIN *et al.* 1988). ADAM *et al.* (1991) discovered that the *ardD* actin gene encodes a polypeptide with only 83% identity to the major actin found in this protist. Although not as divergent as the very divergent yeast *ACT2* and *act2* actin-like genes (LEES-MILLER *et al.* 1992; SCHWOB and MARTIN 1992), the *ardD* product is significantly different from the other *Physarum* cytoplasmic actins in view of their 100% sequence conservation. This simplifies analysis of specific DNA and RNA sequences in transformants. Of particular convenience for these studies, although *ardD* is expressed in the plasmodium and during development of spherules, which are the dormant encysted form of the plasmodial stage, it is not expressed in the amoeba (ADAM *et al.* 1991). Thus, a recessive lethal phenotype due to mutation of *ardD* is not expected for the amoeba, so haploid rather than diploid amoebae can be used for the attempted gene replacements. Furthermore, mutation of *ardD*

Corresponding author: T. G. Burland, McArdle Laboratory for Cancer Research, 1400 University Ave., Madison, WI 53706.
E-mail: burland@oncology.wisc.edu

should not compromise viability of the amoeba even for alleles that are dominant lethal in cell types where the gene is expressed, and hence it should be possible to recover any amoebal transformant in which *ardD*⁺ is replaced by a mutant allele.

The two most highly conserved regions of actin polypeptides are around positions $\approx 25-75$ and the carboxy-terminal ≈ 60 residues. The carboxy-terminal conserved region interacts with several proteins, including tropomyosin, profilin, depactin, and myosin, as well as with actin itself (HAMBLY *et al.* 1986). Deletion of the carboxy-terminal 20 codons of a flight-muscle actin gene in *Drosophila* destroys muscle function (OKAMOTO *et al.* 1986). For the budding yeast conserved Act1 actin, missense mutations affecting the carboxy-terminal region indicate that several of these residues are important for function (WERTMAN *et al.* 1992), and a mutation that removes the terminal three residues (Lys-Cys-Phe) of Act1 is lethal (JOHANNES and GALLWITZ 1991). The carboxy-terminal region of actins is thus critical for function. In an effort to generate a mutation with a potentially informative phenotype, we sought to replace *ardD*⁺ with *ardD* Δ 1, a mutant allele carrying a deletion spanning intron 5 and exon 6, that mutates the 3'-terminal 54 codons.

MATERIALS AND METHODS

Vectors: Plasmids were constructed using standard techniques (SAMBROOK *et al.* 1989), and enzymes were obtained from Promega Inc. (Madison, WI) except where noted. pTB44 was constructed in two steps. First, the *Bgl*II-*Bam*HI fragment of the deleted *ardD* allele from pZAP-*ardD* (Figure 1) (ADAM *et al.* 1991) was cloned into the *Bam*HI site of pBluescript KSII- (Stratagene Inc., La Jolla, CA) to produce pTB43. Then the *Hind*III-*Xho*I fragment of the selectable marker *PardC-hph* from pTB37 (BURLAND *et al.* 1993a) was cloned into *Hind*III-*Xho*I-cut pTB43 to produce pTB44 (Figure 1). To construct pTB45, pZAP-*ardD* was digested with *Bgl*II and the ends were made double stranded with Klenow polymerase. The ends of the *Hind*III-*Xho*I fragment of *PardC-hph* from pTB37 were likewise made double stranded, and then the fragment was cloned into the blunted *Bgl*II site of pZAP-*ardD* to produce pTB45 (Figure 1). Plasmid DNA was prepared by alkaline lysis and organic extraction, as described in BURLAND *et al.* (1992), without further purification.

Strains and culture conditions: Amoebal strain LU352, genotype *matA2 gadAh npfC5 matB3 fusA1 axe* (DEE *et al.* 1989), was used as wild-type for transformations. Amoebal strains MA389 (*matA4 gadA⁺ npfC⁺ matB1 fusA2 axe*) (BURLAND *et al.* 1993a) and LU897 (*matA1 matB1 fusA2 whiA1*) (ANDERSON and DEE 1977) were used for crossing to transformed amoebae. Culture temperature was 26° except where noted.

Amoebae were grown in SDM broth before transformation and transformed by electroporation (BURLAND *et al.* 1993a). We modified transformation conditions over the course of these experiments as improvements were developed using transient expression of luciferase as an assay for efficiency (BAILEY *et al.* 1994). We provide here only details of the last and most successful experiment. Cells were washed once in HBS (40 mM sucrose, 10 mM Hepes, pH 8.2, both chemicals ultrapure from GIBCO-BRL, Gaithersburg, MD) at room tem-

perature, resuspended in HBS at 10⁸ cells/ml, and then cooled for 3 hr in a refrigerator. [However, transient gene expression tests indicate that direct transfer of the washed cells to ice rather than to a refrigerator leads to superior transformation efficiency (BURLAND and BAILEY 1994).] Aliquots of 0.5 ml cells were electroporated with 8 μ g DNA each at 0.88 kV and 25 μ F, with 1,000 Ω resistance in parallel, using a Gene Pulser (BioRad, Hercules, CA) and 4-mm cuvettes. Further treatment and selective plating were as described (BURLAND *et al.* 1993a). Once transformants were confirmed to be hygromycin resistant (Hyg^R), further growth for DNA isolation and developmental analyses was performed in the absence of hygromycin.

Asexual and sexual plasmodium development from amoebae was carried out on DSDM plates with live bacteria (DEE *et al.* 1989). Spores were obtained by placing plates of confluent plasmodia in an incubator containing a cool white 20-W fluorescent light 12–24" from the plasmodia, cycling 12 hr on and 12 hr off. Sclerotia were obtained by incubating plasmodia on plates in the dark. Spherules (or "microsclerotia") were obtained by incubation in salts medium (MOHBERG 1982). Sclerotia were germinated by transferring to fresh SDM plates. Spores were germinated, recloned, and analyzed as described (BURLAND *et al.* 1993a).

Flagellates were obtained by first culturing amoebae on liver plates (BURLAND *et al.* 1984) with live bacteria and then harvesting the amoebae by flooding the plates with 10 ml water. These cell suspensions were then agitated at 26°, and samples were observed at intervals by phase-contrast microscopy to determine whether the characteristic long flagellum of the flagellate became visible, whether the typical gross changes in cell shape and motility occurred, and whether the timecourse and extent of development was normal, with $\geq 70\%$ of the population developing into flagellates.

Isolation and analysis of nucleic acids: DNA was isolated from nuclei and analyzed by Southern blotting as described (BURLAND *et al.* 1993a). The DNA probe for *ardD* was the *Bgl*II-*Bam*HI fragment of the cloned gene from pZAP-*ardD*. The *Bgl*II site lies at position 732 of the gene; hence, this probe lacks 732 bp of the 5' end of the gene as well as having the intron 5/exon 6 deletion (Figure 1) (ADAM *et al.* 1991). In Northern blotting experiments, the 402-bp *Dra*I fragment from the 3'-noncoding transcribed region of *ardD* was also used separately as a probe. The DNA probe for *hph* was the 1.3-kb *Bam*HI fragment of *hph* from pLG83 (GRITZ and DAVIES 1983). DNA probes were radiolabeled by random priming using the Prime-a-Gene kit (Promega Inc.) and [α -³²P]dCTP (6,000 Ci/mmol, NEN Research Products, Boston, MA).

RNA was isolated (PUISSANT and HOUEBINE 1990), and 10 μ g/lane was electrophoresed through a 1.2% agarose gel in MOPS buffer (SAMBROOK *et al.* 1989) and blotted to nitrocellulose. As a control for RNA loading, a blot was also probed with Ppc16, a cDNA corresponding to a constitutive 850-nt mRNA (SCHEDL *et al.* 1984). Blots probed with the locus-specific 3' *ardD* fragment and Ppc16 were washed at 65° in 1 $\times$ SSC (SAMBROOK *et al.* 1989), and blots probed with the *Bgl*II-*Bam*HI fragment were washed stringently with 0.2 $\times$ SSC at 65° to prevent cross-hybridization to mRNAs from the *ardB* and *ardC* actin genes.

PCR amplification and cycle sequencing: Reverse-transcription-PCR amplification (RT-PCR) was based on the method of KAWASAKI (1990). Whole cell RNA (1 μ g) was used to template reverse transcription reactions catalyzed by 200 U H⁻ MMLV reverse transcriptase, in 10 μ l of solution containing 50 mM KCl, 20 mM Tris, pH 8.4, 2.5 mM MgCl₂, 100 μ g/ml bovine serum albumen, 1 mM dithiothreitol, 500 nM of each primer, and 500 μ M each of dATP, dCTP, dGTP,

FIGURE 1.—Plasmid and genomic maps. Physarum coding sequences are shown as the larger open boxes, with hatched boxes for introns, smaller boxes for noncoding flanking regions, and a black box for the bacterial *hph* gene. Vector sequences are shown as solid horizontal lines. The numbers beneath the genomic region indicate the size in base pair. Restriction sites derived from vector sequences are in parentheses, whereas sites from genomic sequences are not. Dotted lines connect identical restriction sites. “Δ” following a restriction site indicates deletion of the site during cloning. Genomic sequences are drawn to scale, but vector sequences are expanded to show restriction site detail. The genomic *Hind*III fragment for *ardD* is 4.2 kb (SCHEDL and DOVE 1982), whereas the cloned allele is deleted (Δ1) for a region of ~1 kb spanning most of intron 5 and part of exon 6 (ADAM *et al.*, 1991; this work).

and dTTP. RNA, primers, and water were mixed and heated to 80° and then allowed to cool to room temperature before adding the rest of the reagents. Reactions were then incubated at 37° for 90 min. Next, reactions were made to 50 μ l by adding 36 μ l water, 1 U *Taq* polymerase (Promega), and 4 μ l of a solution containing 500 mM KCl, 200 mM Tris, pH 8.4, 25 mM MgCl₂, 1 mg/ml BSA, and 10 mM dithiothreitol. Reactions were then covered with mineral oil. This mixture was transferred from ice to a thermal cycling machine (Coy Inc., Grass Lake, MI), heated to 94°, and incubated for 2 min. Then, 30 cycles of amplification were performed on the schedule 94° for 1 min, 60° for 2 min, and 72° for 2 min. Finally, reactions were incubated for another 10 min at 72°. PCR amplification of DNA was performed as for amplification of reverse-transcribed products but in 25- μ l volumes, using 10–20 ng DNA as substrate.

RT-PCR and PCR products were analyzed on 2.5% LE agarose gels (FMC Corp., Rockland, ME) in TBE buffer (SAMBROOK *et al.* 1989), and products were visualized by staining with ethidium bromide. Gels of amplified product were blotted and probed as described above for gels of genomic DNAs.

RT-PCR products were sequenced using the dsDNA Cycle Sequencing System supplied by GIBCO-BRL and [γ -³²P]-labeled ATP (3,000 Ci/mmol, NEN Research Products) to end-label oligonucleotide sequencing primers. Sequencing reac-

tions were analyzed by electrophoresis through 6% acrylamide/8 M urea gels, followed by autoradiography.

For PCR and RT-PCR amplifications of *ardD* sequences, the forward primer used was 5'-TTCTTCCCTC↓AACTATGAACTT (nucleotides 1834–1855 of the genomic allele of *ardD* as described in Genbank entry M59234), which straddles a 5-codon deletion (↓) present naturally in the *ardD* exon 5 sequence compared with the *ardA*, *B* and *C* actin genes (ADAM *et al.* 1991). This is designed to amplify *ardD* sequences preferentially over the other actin sequences. The reverse primer used was 5'-TGATGCAAGTTACAACAACG (nucleotides 2425–2406), derived from the 3'-noncoding transcribed region of *ardD*, which bears no homology to the corresponding region of the other actin genes (ADAM *et al.* 1991). We also used oligonucleotides III and IV (ADAM *et al.* 1991) to confirm the change in amplicon sizes obtained from wild-type and disruptant RNA.

For sequencing, the “reverse” primer used was 5'-GAG-ACGATGCGAGG (nucleotides 2375–2358), which is derived from the 3'-noncoding region of *ardD*, several nucleotides internal to the PCR amplicon. To confirm two nucleotide differences we found in the transcript sequence from strain LU352 compared with the genomic DNA sequence reported for strain M₃CV (ADAM *et al.* 1991), we sequenced the opposite strand of RT-PCR amplicon using the forward

TABLE 1
Recovery of hygromycin-resistant transformants

Plasmid	Restriction digest	Number of experiments	Aggregate mass of DNA used (μ g)	Aggregate number of survivors of electroporation	Aggregate number of transformants	Overall frequency of transformants (per cell)
pTB44	None	16	590	3.7×10^8	0	
pTB44	<i>AgeI</i>	5	100	2.9×10^8	0	
pTB44	<i>BstEII</i>	3	40	8.5×10^7	0	
pTB44	<i>SphI</i>	8	150	9.0×10^7	0	
pTB44	<i>NotI</i>	8	129	3.3×10^8	21	6.4×10^{-8}
pTB44	<i>NotI</i> + <i>XhoI</i>	17	357	5.8×10^8	105	1.8×10^{-7}
pTB45	None	16	507	3.7×10^8	0	
pTB45	<i>AgeI</i>	5	77	1.5×10^8	0	
pTB45	<i>BstEII</i>	3	14	6.2×10^7	0	
pTB45	<i>SphI</i>	6	123	5.5×10^7	0	
pTB45	<i>NotI</i>	8	81	1.8×10^8	0	
pTB45	<i>NotI</i> + <i>BglII</i>	13	243	5.1×10^8	0	

sequencing primer 5'-GTCCCAGGAATTGCTGAT (nucleotides 2045–2061). This region of *ardD* had been entered erroneously in Genbank as 5'-GTCCAGGAATTGCTGAT, omitting the C residue (underlined above) between nucleotides 2047 and 2048.

RESULTS

Vectors for gene replacement: The cloned genomic allele of the *ardD* actin gene, *ardD* Δ 1, carries a deletion spanning most of intron 5 and the first 128 bp of exon 6 (Figure 1) (ADAM *et al.* 1991), mutating codons from position 314 onward of the 367-codon gene. We sought to replace the *ardD*⁺ allele in the genome with *ardD* Δ 1 and constructed two vectors for this purpose. pTB45 carries *ardD* Δ 1 interrupted by the selectable element *PardC-hph* (Figure 1). This element can confer resistance to hygromycin on Physarum cells that integrate the DNA into the nucleus (BURLAND *et al.* 1993a). pTB44 carries a fragment of *ardD* Δ 1, which is deleted from the 5' end to the *BglII* site at position 732 in the coding region (Figure 1), and in this case *PardC-hph* is downstream of the mutant *ardD* allele.

Transformations with *ardD* Δ 1 vectors: Plasmids pTB44 and pTB45 were electroporated into amoebae either as circular molecules or as molecules linearized with various restriction enzymes (Table 1). Circular plasmids, and plasmids linearized within the *ardD* coding region using either the *SphI*, *AgeI*, or *BstEII* sites, were designed to act as insertion vectors. Plasmids linearized with *NotI* or *NotI* plus a second enzyme were designed to act as replacement vectors. Cells were then plated on hygromycin, and Hyg^R colonies were sought. No Hyg^R transformants were obtained with any of the preparations of pTB45 (Table 1). Previous experience transforming Physarum to hygromycin resistance using plasmids carrying the *PardC-hph* element suggested we

should have obtained $\geq 10^{-8}$ transformants per cell treated and that both linear and circular DNA molecules could yield transformants (BURLAND *et al.* 1993a). Further, judging by transient expression assays using firefly luciferase (BAILEY *et al.* 1994), the methods we used for electroporation are more efficient than those used previously. Thus, >13 pTB45 transformants were expected from the number of cells treated. We do not know why transformants were not obtained with pTB45, but note that *PardC-hph* lies downstream of the start of *ardD*, in the same orientation, and that *ardD* is not expressed in the amoeba. This raises the possibility that *PardC-hph* expression would be diminished by an *ardD* regulatory element when introduced into amoebae in this configuration.

No Hyg^R transformants were obtained with circular pTB44 either, even though 3.7×10^8 survivors of electroporation were plated in all (Table 1). Nor were any transformants obtained with pTB44 digested with *AgeI*, *BstEII*, and *SphI*, which all cut within the region of homology between *ardD* Δ 1 and *ardD*⁺ (Figure 1). However, many transformants were obtained when cells were electroporated with pTB44 linearized with either *NotI* or *NotI* + *XhoI* (Table 1). Both the *NotI* and *XhoI* sites are in vector rather than *ardD* sequences (Figure 1). The readily measurable frequency of recovery of transformants for pTB44-*NotI* and pTB44-*NotI*-*XhoI* DNAs (Table 1) suggests that the absence of transformants among pTB45-treated cells and among cells treated with the circular preparations of pTB44 DNA reflects some characteristic of the input DNA rather than a chance failure to recover transformants. We do not believe that this characteristic is DNA purity, however, because three independent preparations of both pTB44 and pTB45, made by the same procedure, were used in different experiments, and all three prepara-

tions of pTB44 yielded transformants when the DNA was digested with *NotI* + *XhoI*. This makes remote the possibility that impurities account for failure of linear DNA preparations to transform *Physarum*.

Site of integration of *ardDΔ1*: We isolated nuclear DNA from 23 transformants obtained with pTB44-*NotI*-*XhoI*-digested DNA and from 15 transformants obtained with pTB44-*NotI*-digested DNA. These nuclear DNAs were analyzed by Southern blotting. Most transformants contained the normal copy plus a second copy of *ardD*, and in these *hph* was located on the same *KpnI* fragment as the second copy of *ardD* but on a different *HindIII* fragment, as expected if the *ardDΔ1-PardC-hph* DNA fragment integrates as one piece. Examples are shown in Figure 2. The patterns of hybridization are consistent with nonhomologous integration of *ardDΔ1-PardC-hph* at separate sites in the different transformants. Absence of an extra *HindIII* fragment of *ardD* in transformant 44T42 may reflect coincidental generation of a fragment the same size as the wild-type allele; the *KpnI* digests clearly show an additional *ardD* fragment for these transformants. The intensity of hybridization of the *HindIII* fragments of the ectopic *ardD* sequences is similar to that of the endogenous one, indicating that only a single extra copy of *ardD* has integrated in each transformant.

From the 38 independent transformants analyzed, two, 44T5 (from *NotI*-*XhoI*-digested pTB44) and 44T19 (from *NotI*-digested pTB44) did not carry the wild-type 4.2-kb *HindIII* fragment of *ardD*. Instead, each carried a smaller fragment of ≈ 3.2 kb (Figure 2), as expected for replacement of *ardD*⁺ with *ardDΔ1* (Figure 1).

Hybridization of the *hph* probe to the *HindIII* digest of 44T5 DNA appears less than expected for one copy of *hph* per genome, but this is probably due to an artefact. A low intensity of hybridization of 44T5 DNA to *hph* is also seen in Figure 4. However, hybridization of *hph* to the *KpnI* digest of 44T5 is consistent with a complete copy of *hph* in every nucleus, and the intensity of hybridization of the *HindIII* fragment of *hph* in progeny of crosses involving 44T5 is typical for a single copy gene (see Figure 4). As with nonhomologous transformants, *hph* is carried on the same *KpnI* fragment as the introduced *ardDΔ1* allele in 44T5 and 44T19, indicating that the integration event did not separate *PardC-hph* from *ardDΔ1*. For 44T5, this main *KpnI* fragment that hybridizes to both *hph* and *ardDΔ1* is ≈ 5 kb (Figure 2), as expected if most or all of the *NotI*-*XhoI* fragment of pTB44 integrated into the genome in one piece (Figure 1). For 44T19, however, the corresponding *KpnI* fragment is ≈ 14.5 kb (Figure 2), consistent with integration of a slightly smaller fragment of DNA, not including the *KpnI* site downstream of *hph*. These are the first examples of homologous gene replacement in *Physarum*.

Recovery of two independent replacement events shows that gene replacement is reproducible.

The cloned genomic *ardDΔ1* sequence contains a central ≈ 1.2 -kb *DraI* fragment that spans the deletion (Figure 1). Southern blotting of *DraI*-digested DNAs reveals a single 1.2-kb *ardD* fragment in the two replacement strains 44T5 and 44T19, as expected, whereas a 2.2-kb fragment is present in wild-type. In two nonhomologous transformants, both 1.2-kb and 2.2-kb *DraI* fragments of *ardD* are detected (Figure 3). This confirms that *ardD*⁺ was replaced by the deleted *ardDΔ1* allele in 44T5 and 44T19. We have not been able to recover DNA for this deleted region either by molecular cloning (ADAM *et al.* 1991) or PCR amplification of wild-type genomic DNA (*e.g.*, Figure 5). The *DraI* Southern blots show that there is ~ 1 kb of DNA deleted from intron 5/exon 6 in the *ardDΔ1* genomic clone, only 128 bp of which is from exon 6. Thus, intron 5 appears to be larger than most introns so far found in *Physarum*.

Segregation of the replaced allele through meiosis: It is important to determine whether gene replacements of the sort we have constructed are heritable through meiosis. Where a particular replacement and/or disruption event could be lethal, the initial replacement would need to be made in heterozygous form in a diploid amoebal strain. Diploid amoebae carrying appropriate mutations at the *matA* locus can be selfed to form plasmodia (ANDERSON and YOUNGMAN 1985), and these diploid plasmodia can undergo normal meiosis to yield recombinant haploid amoebal progeny, among which the replacement event can be sought to determine whether it is viable. Because we used a haploid amoebal strain in this work, to test this general feasibility we simply crossed the *ardDΔ1* mutant amoeba to an *ardD*⁺ haploid amoebal strain, MA389, to make a diploid plasmodium. We then analyzed haploid meiotic progeny of this crossed plasmodium.

The hygromycin resistance associated with *PardC-hph* typically segregates 1:1 among haploid meiotic progeny of crosses of transformants with a hygromycin-sensitive (*Hyg*^S) strain such as MA389, but occasionally a deficit of *Hyg*^R progeny is found (BURLAND *et al.* 1993a). When *ardDΔ1* strain 44T5 was crossed with MA389, a deficit of *Hyg*^R progeny was also observed, with only 7 of 24 progeny *Hyg*^R, significantly less than a 1:1 ratio of *Hyg*^R:*Hyg*^S progeny ($\chi^2 = 4.16$, $P < 0.05$) but consistent with a 1:3 ratio. Genomic DNA from three *Hyg*^S and three *Hyg*^R progeny of this cross was analyzed by Southern blotting (Figure 4). All six progeny carried both *ardDΔ1* and *hph*, and in all six *ardDΔ1* and *hph* were on the same *KpnI* fragment as they are in 44T5. This argues against major rearrangement of *ardDΔ1-PardC-hph* through meiosis, which might have led to a *Hyg*^S phenotype of progeny carrying *hph*. There remained a possibility that the *Hyg*^R phenotype of 44T5

FIGURE 2.—Presence of novel *ardD* and *hph* fragments in nuclear DNA. Nuclear DNA from wild-type (C) and from hygromycin-resistant transformants of pTB44 (numbers) was digested with *Hind*III (top) or *Kpn*I (bottom) and analyzed by Southern blotting. Replicate blots were probed with either the *Bgl*III-*Bam*HI fragment of *ardD* (left) or with *hph* (right). W, position of the wild-type *ardD* allele; Δ, position of the *Hind*III fragment of the integrated *ardD*Δ*I* allele where it has replaced the wild-type allele in transformants 44T5 and 44T19; Δ5, positions of the two *Kpn*I fragments of the replaced *ardD* allele in 44T5; Δ19, position of the *Kpn*I fragment of the replaced *ardD* allele in 44T19. Numbers at the side indicate positions of molecular weight markers in kilobase.

was not caused by *PardC-hph*, though given that no Hyg^R colonies have been observed in over 10^{10} non-transformed cells plated (BURLAND *et al.* 1993a), this is highly unlikely. We considered more likely the possibility that an unlinked suppressor of Hyg^R is present in MA389. In this case, one expects that on average two thirds of the Hyg^S progeny of this cross would carry *ardD*⁺ and not *hph*; we attribute to chance the fact that all three Hyg^S progeny tested carried *ardD*Δ*I* and *hph*.

To test for the presence of a suppressor, one of the

Hyg^S progeny that carried *ardD*Δ*I* and *hph* was crossed to Hyg^S strain LU897, which is more closely related to LU352, the strain used for transformation, than is MA389. From this cross of two Hyg^S strains, 14 of 46 progeny were Hyg^R , consistent with the 1:3 ratio of Hyg^R : Hyg^S progeny expected if the hygromycin sensitivity of *hph*-carrying progeny of the first cross were due to the presence of an unlinked suppressor carried by MA389. Southern blots confirmed that four Hyg^S progeny tested from this cross carried *ardD*⁺ and not *hph*,

FIGURE 3.—Presence of a 1-kb internal deletion from the cloned *ardD* allele in transformants. Southern blot of *DraI* digests of nuclear DNA probed with *ardD*. C, wild-type; 5, 19, 27, 28, pTB44 transformants 44T5, 44T19, 44T27 and 44T28, respectively. Numbers at the side indicate positions of molecular weight markers in kilobase.

whereas two Hyg^R progeny tested carried *ardDΔ1* and *hph* (Figure 4). Again, there is no evidence from the Southern blots of genetic rearrangements among the progeny of crosses involving *ardDΔ1-PardC-hph*. Thus, the gene replacement construction is stable through meiosis.

Analysis of *ardD* transcripts: If engineered alleles are to be useful for analysis of gene function, they must be expressed in a predictable way. To test the expression of *ardDΔ1*, RNA was isolated from plasmodia and spherules and analyzed by Northern blotting, using a 3'-noncoding probe specific for the *ardD* gene (ADAM *et al.* 1991) as well as the deleted *ardD* probe used for Southern blotting. There appeared to be no major change in size or abundance of *ardD* mRNA compared with wild-type in strains carrying *ardDΔ1* (data not shown), whether the mutant allele replaced *ardD*⁺ or was present in addition. Thus, *ardDΔ1* is expressed in a similar pattern to *ardD*⁺. However, there remained a possibility that a minor change in transcript size could have been overlooked.

Because the 3' splice acceptor site for intron 5 is absent from the *ardDΔ1* allele, incorrect splicing of *ardDΔ1* transcripts is expected. To test this prediction, we examined RNA by RT-PCR, using oligonucleotides that flank the intron 5/exon 6 region to prime polymerization by reverse transcriptase and *Taq* polymerase. If none of the shortened intron 5 were spliced from the *ardDΔ1* transcript, the PCR should amplify a 446-

bp fragment from the *ardDΔ1* mRNA template and a 502-bp DNA fragment from the *ardD*⁺ mRNA template. If splicing occurs via a 3' acceptor site in exon 6, the *ardDΔ1* transcript would be smaller than 446 bp. The RT-PCR analysis confirms the presence of a ≈500-bp fragment in wild-type RNA and indicates that the fragment amplified from 44T5 RNA is only ≈370 bp (Figure 5). In the nonhomologous *ardDΔ1* transformant, both 500-bp and 370-bp fragments are amplified. Because the amplified fragments from 44T5 RNA are smaller than the corresponding fragment amplified from pTB44 and 44T5 DNA, they could not have been produced from cellular DNA contaminating the RNA samples. Thus, intron 5 is spliced out in the 44T5 replacement strain, presumably using an alternative 3' splice acceptor site in exon 6. In addition, because the smaller 370-bp RT-PCR product is found in nonhomologous *ardDΔ1* transformant 44T20, it appears that the *ardDΔ1* allele is expressed in this strain, despite the absence of the 5'-flanking region and a significant portion of the 5'-coding sequence (Figure 1).

To determine the position of the novel splice site for *ardDΔ1* and thereby confirm that the coding potential of the mRNA had been altered, RT-PCR products were analyzed by cycle sequencing. The cycle sequencing of wild-type RT-PCR products largely confirmed the coding potential of exon 6 and part of exon 5 of *ardD*⁺ previously reported (ADAM *et al.* 1991), with two minor exceptions. We found a cytosine at position 2284 (of the Genbank *ardD*⁺ sequence) instead of an adenosine residue, implying histidine instead of glutamine at position 346 of the polypeptide, and at position 2223 we found a cytosine instead of a guanosine residue, implying an alanine instead of a glycine at position 326. Both differences were confirmed by cycle sequencing the opposite strand of DNA from a separately amplified RT-PCR product. It is possible that these differences reflect a difference between the strains used. Neither of these differences affects our conclusions.

For 44T5, the transcript carries an out-of-frame deletion compared with the wild-type mRNA, from codon 314 onward (Figure 6). Thus, in the strain carrying the *ardDΔ1* allele, an alternative 3' splice site, 145 nt into exon 6, is used to remove intron 5. The region around this site would be 5'-AUAG↓UUCA-3', where the arrow indicates the splice site. This maintains a consensus AG dinucleotide immediately upstream of the splice. The frame change in 44T5 mRNA implies translational read-through beyond the wild-type stop codon and a polypeptide of 356 residues, all beyond residue 313 being mutant (Figure 6).

In addition to the major novel splice site used for 44T5 mRNA, we found evidence for a minor additional site. This is most readily observed by examining the autoradiogram of the "top" position of the sequencing ladder (Figure 5). For wild-type, the ladder termina-

FIGURE 4.—Segregation of integrated transforming DNA through meiosis. Nuclear DNAs from wild-type (C), gene replacement strain 44T5 (5) and segregants from crosses that are either resistant (R) or sensitive (S) to hygromycin were analyzed by Southern blotting after digestion with either *Hind*III (top) or *Kpn*I (bottom) and probed with either *ardD* (left) or *hph* (right). The progeny clone of 44T5xMA389 marked S' was used as the parent in the cross S'xLU897. W, wild-type alleles of *ardD*; Δ , deleted alleles of *ardD*. Numbers at the side indicate positions of molecular weight markers in kilobase.

tion is evident from the intense band in all four lanes corresponding to the end of the RT-PCR amplicon, where all chain elongation must terminate. For 44T5, instead of a single intense termination band, there are two (Figure 5), both shorter than that of wild-type. The major of these two bands is slightly smaller than the minor one. Close examination of the sequencing ladder indicated the presence of "shadow" bands in the lanes corresponding to 44T5 cycle sequencing products, that is, bands of lower intensity but of the same mobility as the major band in a second of the four lanes on the sequencing ladder. In the early-terminated portion of the sequencing ladder, these shadow bands could be read as a separate sequence, on the assumption that absence of a shadow band at a specific position means that the major and minor sequences are the same. This shadow sequence indicated that a second transcript from *ardD Δ 1* is generated using a secondary 3' splice site (Figure 6), 129 nt into exon 6, generating an in-frame deletion of codons 314–356. Assuming that both *ardD Δ 1* transcripts are amplified with equal efficiency, this second transcript would be minor. Thus, for either of two alternative transcripts, the homologous gene replacement mutates the carboxy-terminal region of the gene product, as it was designed to do.

Cellular phenotype of the *ardD Δ 1* strain 44T5: We investigated whether the *ardD Δ 1* mutation in transformant 44T5 would affect any of the cell types and developmental transitions found in *Physarum*. Recovery of amoebae carrying the *ardD Δ 1* replacement indicates as expected that there is no effect on viability of the amoeba. The crosses involving 44T5 demonstrate that sexual development is also unaffected, at least in crosses between *ardD $^{+}$* and *ardD Δ 1* strains, and *ardD Δ 1* / *ardD $^{+}$* heterozygous plasmodia grew normally. We also found no effect on meiosis, spore development, or spore germination from such heterozygous plasmodia.

To test for a recessive phenotype of *ardD Δ 1*, we examined whether the haploid 44T5 transformant could complete the selfing cycle. LU352, the *matA2* parent strain for the transformants, carries mutations at *matA* that facilitate experimental control of the selfing cycle. The *gadAh* mutation allows the normally heterothallic *matA2* strain to self by apogamy to form haploid plasmodia (ANDERSON *et al.* 1989). The *npfC5* mutation at *matA* prevents this selfing, but plasmodia can be obtained by rare but reliable reversion of *npfC5* to *npfC $^{+}$* under specific culture conditions. Such selfed plasmodia grow normally and can complete meiosis to yield viable haploid spores by virtue of rare diploid nuclei in

FIGURE 5.—A difference between *ardD* transcripts from wild-type and transformants. (Top) Southern blot of RT-PCR amplicons of RNA, and PCR amplicons of DNA, resolved on 2.5% agarose gels and probed with the *Bgl*II-*Bam*HI fragment of *ardDΔ1*. W, wild-type nucleic acid template; T5, T20, nucleic acid templates from transformants 44T5 and 44T20; 0, no nucleic acid added; 44, 20 ng pTB44 DNA template; P, plasmodial RNA; S, spherule RNA. Numbers at the side indicate size markers in base pair. (Bottom) The top of the cycle sequencing ladder for RT-PCR-generated amplicons of *ardD*⁺ and *ardDΔ1* RNA. A, C, G, and T indicate lanes for reactions carrying the indicated dideoxynucleotide. W, the position of the strong termination point of the amplicon from wild-type; Δ, the termination points for major and minor amplicons from *ardDΔ1* replacement strain 44T5.

the otherwise haploid multinucleate plasmodium (LAFFLER and DOVE 1977). We found that 44T5 selfed to form normal plasmodia, which could sporulate apparently normally to yield viable spores. We also found that a 44T5 plasmodium could form a sclerotium (the name given to plasmodial cysts when they develop on solid surfaces) and that the sclerotium was capable of developing back into a plasmodium.

We also tested the developmental transitions of the amoebal stage of the life cycle. Haploid 44T5 amoebae were capable of developing into morphologically normal flagellates, as determined by phase-contrast microscopy, over a similar timecourse as found for LU352 (e.g., PAUL *et al.* 1992). Similarly, 44T5 amoebae could form morphologically normal cysts *en masse*, which in turn could germinate back into amoebae. Finally, we

grew plasmodia and amoebae at 20° and 31° to test for a possible cold-sensitive or thermosensitive phenotype, but no difference in growth of 44T5 and wild-type was observed. Thus, there is no evidence that the *ardDΔ1* mutation has any effect on growth or development in *Physarum*.

DISCUSSION

We have demonstrated that homologous gene replacement can be reproducibly achieved in *Physarum*. This capability presents broad opportunities for analysis of questions in cellular and developmental biology in this protist (BURLAND *et al.* 1993b).

The frequency of transformation to hygromycin resistance in previous experiments was around 10^{-8} per cell (BURLAND *et al.* 1993a). We improved this frequency during the course of the experiments reported here, with the last experiment yielding 14 transformants at a frequency of 8.5×10^{-8} per cell for pTB44-*NotI* DNA and 84 transformants at a frequency of 5.1×10^{-7} per cell for pTB44-*NotI*-*XhoI* DNA. This improvement probably reflects superior electroporation conditions that were developed using transient expression of a luciferase reporter gene (BAILEY *et al.* 1994). It is also evident that digestion of pTB44 with *NotI* + *XhoI* yields significantly more transformants than does digestion with *NotI* alone. The single digest produces a linear DNA molecule of 8.4 kb, whereas the double digest cuts the DNA molecule carrying the selectable marker down to 5.4 kb. This suggests that smaller DNA fragments transform *Physarum* amoebae with higher efficiency.

Neither circular plasmid yielded transformants in these experiments. It appears that linear DNA may be necessary for transformation in *Physarum*, as appears to be the case for the protist *Trypanosoma* (LEE and VAN DER PLOEG 1990) but not for the protists *Chlamydomonas* (KINDLE *et al.* 1989) or *Tetrahymena* (KAHN *et al.* 1993). Our earlier observation of a stable *Physarum* transformant arising from circular plasmid DNA (BURLAND *et al.* 1993a) may reflect linearization of the supposedly circular DNA either by damage *in vitro* or enzymatically *in vivo*.

Transformation with circular DNA or with DNA linearized within a region of homology readily generates transformants carrying homologous integrations in yeasts. However, our abundant trials show that use of circular DNA may not be the ideal strategy for homologous replacement in *Physarum*. The data rather suggest that linearizing the DNA at sites that flank the region of homology will produce transformants among which can be found clones carrying homologous gene replacements. This is analogous to integration of transforming DNA in *Tetrahymena*, where linear DNA fragments undergo homologous integration. However, in the case of this ciliate, most or all integration events are at the

FIGURE 6.—Sequences of transcripts for the exon 5/exon 6 region of *ardD* and implied polypeptide products. For both mutant and wild-type products, sequences were read at least from nucleotides corresponding to positions 2056 to 2338 of the genomic sequence of *ardD* reported in Genbank entry M59234. The coding potential of the 3' region of the transcripts was then deduced by combining this information with the known *ardD*⁺ sequence. The upper region shows nucleotide sequences deduced for *ardD* transcripts from RT-PCR amplimers of RNA from plasmodia carrying wild-type (*ardD*⁺) and deleted (*ardDΔ1*) alleles. Nucleotide residues in bold were read directly from cycle sequencing gels of RT-PCR products. Other nucleotides were added from published data (ADAM *et al.* 1991). Lowercase indicates the nucleotides from the 3'-noncoding region of *ardD* that are expected to be translated from the major *ardDΔ1* transcript due to a frameshift caused by the novel splice. Nucleotide numbering starts at position 1 for the beginning of exon 6, while negative numbers refer to positions in exon 5. Asterisks indicate sequence differences detected compared with the original report (ADAM *et al.* 1991). The lower region shows inferred amino acid residues for positions 303–367 of the *ArdD* polypeptide. Uppercase letters indicate residues found in wild-type; lowercase letters indicate mutant residues. Dashes indicate absence of a residue.

homologous site (KAHN *et al.* 1993; GAERTIG *et al.* 1994). Furthermore, circular plasmid DNA carrying a ribosomal DNA origin can integrate into homologous sites in either the chromosomes or the extrachromosomal ribosomal DNA of *Tetrahymena* (KAHN *et al.* 1993).

Although we observed no transformants when plasmid DNA was linearized inside the region of *ardD* homology, we doubt that there is a barrier to transformation when transforming DNA has *Physarum* sequences at the ends. Rather, the single digests used to linearize plasmids by cutting in *ardD* produced 8.4-kb molecules, which would have transformed less efficiently than did the 5.4-kb doubly digested plasmids. Further, a majority of the experiments using plasmid DNAs with single digestions in *ardD* were done in the earlier stages of this work, when the transformation efficiency was around

10^{-8} per cell. We attribute failure to recover even non-homologous transformants from DNAs cut within *ardD* to chance. Absence of transformants from transformations with circular DNAs are, however, more significant, as circular plasmid DNAs were tested in more experiments under the most efficient conditions we developed, and we have failed to obtain transformants with other circular plasmids that yield transformants when linearized (data not shown).

The improved transformation frequency of $\sim 10^{-6}$ – 10^{-7} per cell, together with a frequency of homologous replacement events $\sim 5\%$ of transformants, allowed us to obtain quite readily cells carrying engineered *ardD* alleles. Using these same methods, it will be possible to mutate any gene for which a cloned copy can be obtained. However, it is doubtful that our experiments represent the highest possible frequency of homo-

gous integration that can be obtained among a set of Physarum transformants. The region of homology between the mutant *ardDΔI* allele in pTB44 and *ardD*⁺ is 1,455 bp 5' to the 1-kb deletion and 628 bp 3' to the deletion. Whereas this would be ample homology for efficient homologous integration in budding yeast (ROTHSTEIN 1983) and trypanosomes (LEE and VAN DER PLOEG 1990), it may not be so for mammalian cells. Further, in mouse embryonal stem (ES) cells, for example, it can be difficult to obtain homologous integration of added DNA in nonexpressed genes (CAPECCHI 1989), though for some expressed genes, homologous integration events can be as common as we have found for the nonexpressed *ardD* gene in Physarum. If expressed genes are similarly more readily targeted in Physarum, there would be no difficulty in replacing them by transformation with engineered alleles, because we have already replaced *ardD* in amoebae, where the gene is not expressed. Higher efficiency of homologous integration may also be possible in Physarum if longer stretches of homology are present on the transforming DNA, as is found for ES cells (CAPECCHI 1989).

As found for transformation of Physarum with other vectors carrying *PardC-hph* (BURLAND *et al.* 1993a), the present results indicate that only a single copy of the added DNA has integrated into the genome of each transformant. This contrasts with multicopy integration of transforming DNA commonly found in other protists, such as Trypanosoma (LEE and VAN DER PLOEG 1990), Dictyostelium (NELLEN and FIRTEL 1985), and Chlamydomonas (KINDLE *et al.* 1989; KINDLE 1990). The level of transient expression of heterologous genes in Physarum is a function of promoter strength (BURLAND *et al.* 1992; BAILEY *et al.* 1994). If this holds true for integrated genes, it will be extremely convenient experimentally, as it would allow precise control of the level of gene expression.

The molecular events that gave rise to the gene replacements in Physarum presumably involved gene conversion. This sort of homologous integration is typically observed when vectors similar to pTB44 are linearized such that the free ends flank the region of homology. In the case of pTB44, neither end of the linearized DNA molecule is homologous to the *ardD* genomic region. Linearizing at the *NotI* site 5' to *ardD* leaves 20 bp of nonhomologous vector polylinker flanking the region of homology, while the 3' end of the fragment is downstream of the bacterial *hph* gene and is thus over 1 kb from homologous sequences on the vector. Nevertheless, virtually the whole of the *ardDΔI* fragment and the selectable marker must have integrated in the *ardDΔI* replacement strain 44T5. This is reminiscent of DNA integration in ES cells where placing significant tracts of nonhomologous sequence at either end of a linear

targeting vector appears not to hinder homologous recombination (CAPECCHI 1989).

The replaced *ardDΔI* allele can be recovered among meiotic progeny of crosses, a key feasibility for future disruption of essential genes in diploids. Initial concern regarding the apparently non-Mendelian segregation of the Hyg^R phenotype was allayed by evidence that a suppressor is responsible for the abnormal segregation. Genetic analysis shows that this problem can be avoided in future by crossing transformants with congenic strains rather than with less related strains that may carry many genetic differences. However, given that suppressors of Hyg^R appear to exist naturally in Physarum populations, it may be helpful to screen a variety of different strains to seek any that might be more susceptible than LU352 to transformation by *PardC-hph* to Hyg^R.

Our observations of cellular phenotypes, combined with analysis of *ardDΔI* transcripts, suggest that the carboxy-terminal 54 residues of ArdD are not required for growth or development in Physarum. No morphological abnormalities were detected by electron microscopic analysis of the plasmodial stage either (T. SHERWIN and K. GULL, personal communication), though the carboxyl terminus of ArdD still may have a significant role that is yet to be detected. It remains possible that elimination of the entire *ardD* gene would be overtly detrimental or lethal if a unique role is played by the N-terminal 304 residues of the polypeptide. However, this seems unlikely in view of the critical function of the carboxy-terminal domain of conserved actins. The *ardD* gene is more likely functionally redundant, perhaps diverging in sequence through neutral drift, as seems to have occurred for the divergent *betC* β -tubulin gene of Physarum, which is also expressed predominantly in the plasmodial stage (BURLAND *et al.* 1988).

The set of tests of cellular phenotype we describe can be performed at low cost and with little technical sophistication. They thus form a useful core of tests that can be used to screen for phenotypes of transformants on a routine basis. This will be useful not only for testing effects of specific gene replacement events but also for screening nonhomologous integrants for developmental effects, where the integration events may have inactivated important genes (*e.g.*, TAM and LEFEBVRE 1993). The integrated transforming DNA would then serve as a tag with which the gene affected could be cloned.

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