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Stable, selectable, integrative DNA transformation in *Physarum*

(Electroporation; hygromycin; *Physarum polycephalum*; promoter; protist; slime mold; yeast)

Timothy G. Burland[a], Juliet Bailey[a]*, Dominick Pallotta[b] and William F. Dove[a]

[a]*McArdle Laboratory for Cancer Research, Madison, WI 53706, USA; and* [b]*Department of Biology, Faculty of Science and Engineering, Laval University, Quebec G1K 7P4, Canada. Tel. (1-418) 656-3189*

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SUMMARY

The *Physarum polycephalum* actin promoter, *PardC*, can drive transient expression of heterologous genes in *Physarum* amoebae. The *hph* gene, encoding hygromycin (Hy) phosphotransferase, can confer resistance to Hy on a broad spectrum of organisms. When *PardC* is translationally fused to *hph* and transformed into yeasts on high-copy-number vectors, the yeasts become Hy resistant (Hy^R), showing that *PardC-hph* is a functional, selectable genetic element. To establish a stable transformation system for *Physarum*, we electroporated plasmids bearing *PardC-hph* into *Physarum* amoebae and then selected for Hy^R transformants. We show that Hy^R amoebae arise upon the stable integration of *PardC-hph* into the nuclear genome in single copy. These results establish a transformation system that can be used to add plasmid-borne genetic information to *Physarum*.

INTRODUCTION

Stable DNA transformation methods can be used to engineer the genome of an organism, dramatically broadening the spectrum of definitive experimental analyses that can be applied to problems in cell biology. The protist *Physarum polycephalum* provides abundant opportunities for studying areas of broad cell biological significance in the simple context of the unicellular state (reviewed in Burland et al., 1993). Methods for stable DNA transformation of *Physarum* would allow these opportunities to be more fully exploited.

We have developed methods to introduce genes into *Physarum* amoebae wherein expression of the introduced genes is substantial, though transient (Burland et al., 1992; 1993). Expression of the introduced genes was driven by *PardC*, the promoter for the abundantly expressed *ardC* gene of *Physarum* (Hamelin et al., 1988). We have also shown that the *PardC-hph* element, consisting of *PardC* translationally fused to the bacterial *hph* gene, confers Hy^R on yeasts when introduced on high-copy-number vectors (Burland et al., 1991). Previously, however, it was not possible to express exogenous genes stably or integrate them into the genome in *Physarum*, key techniques needed for genetic engineering experiments. The goal of this work was to develop methods and vectors for the stable transformation of *Physarum* amoebae to Hy^R and to characterize the molecular events that give rise to stable transformation in this protist.

Correspondence to: Dr. T.G. Burland, McArdle Laboratory for Cancer Research, 1400 University Avenue, Madison, WI 53706, USA. Tel. (1-608) 262-0982; Fax (1-608) 262-2824; e-mail: burland@oncology.wisc.edu

* Present address: Genetics Department, University of Leicester, Leicester LE1 7RH, UK. Tel. (44-533) 523414.

Abbreviations: *ardC*, gene encoding actin; *axe*, gene conferring ability of amoebae to grow axenically; bp, base pair(s); *cat*, gene encoding chloramphenicol acetyltransferase; DSDM, dilute SDM; *fusA*, gene controlling plasmodial fusion; *gadA*, gene linked to *matA* controlling asexual differentiation; *hph*, gene encoding Hy phosphotransferase; Hy, hygromycin B; kb, kilobase(s) or 1000 bp; *matA*, principal *Physarum* mating type gene; *matB*, gene controlling amoebal cell fusion; MCS, multiple cloning site; *npfC*, gene linked to *matA* controlling asexual differentiation; *P.*, *Physarum*; *PardC*, promoter for *ardC*; ^{R}, resistant/resistance; SDM, semi-defined medium; *TardC*, transcription terminator for *ardC*; wt, wild type.

RESULTS AND DISCUSSION

(a) Sensitivity of *Physarum* to hygromycin

Growth of *Physarum* amoebae plated on lawns of killed bacteria is completely inhibited by 50 μg Hy/ml (Hy was obtained from CalBiochem Inc., Davis, CA, catalogue No. 400050). However, at this concentration, the spontaneous frequency of Hy^R mutants is about 10^{-5}. We therefore increased the Hy to 100 μg/ml for the selection of transformants. At the higher concentration, no Hy^R mutants were observed among over 10^{10} wt amoebae plated.

(b) Generation of Hy^R transformants

This work was initiated in the course of optimizing transformation conditions for the transient expression of heterologous genes. We applied improvements in transient expression techniques to our stable transformation attempts as they were made, and different transformants described here were obtained under slightly different experimental conditions. The conditions for preparing transformation-competent cells and electroporation we now favor are as described in Burland et al. (1992) except that (*i*) cells are washed and electroporated in 40 mM sucrose/10 mM Hepes pH 8.2 instead of 30 mM sucrose/10 mM Hepes pH 8.2; (*ii*) washed cells are not transferred immediately to ice but rather transferred to a refrigerator at 0–4°C for 3 h, then placed on ice immediately before electroporation; and (*iii*) cells are electroporated at 0.85 kV and 25 μF, with 1000 Ω resistance in parallel. The haploid amoebal strain used for transformation was LU352 (Dee et al., 1989), genotype *matA2 gadAh npfC5 matB3 fusA1 axe*. Cells were cultured at 26°C in the growth media described (Dee et al., 1989).

Amoebae were grown axenically in SDM broth prior to transformation. Following transformation and outgrowth in SDM, cells were cultured on DSDM plates with 0.2 ml undiluted formalin-killed bacteria per plate. Amoebal cells were electroporated with either of two plasmids. Plasmid TB38 (Fig. 1) carries the selectable *PardC-hph* element and the actin gene terminator *TardC* (Burland et al., 1991). In addition, this plasmid bears the *ars1* and *ura4*$^+$ genes from *Schizosaccharomyces pombe*, which permit selection and multicopy replication of the molecule in fission yeast and budding yeast. Like pTB33 (Burland et al., 1991), which lacks *TardC*, pTB38 can transform fission yeast and budding yeast to Hy resistance, showing that the *PardC-hph* element in this vector remains functional. Plasmid TB41 (Fig. 1) carries no yeast sequences but, like pTB38, carries *PardC-hph* and *TardC*. In addition, pTB41 carries a 2.1-kb *Xba*I fragment of the *Physarum* nuclear genome. Hybridization to Southern blots of *Physarum* nuclear DNA indicates that this 2.1-kb fragment includes sequences that are highly repeated in the genome (not shown). We added this fragment of repeated DNA to the plasmid to test whether the presence of additional homologous DNA would markedly increase the transformation efficiency compared with pTB38; such an increase would be expected if transformation occurs predominantly by homologous integration, as the repeated DNA sequence would greatly increase the opportunities for homologous recombination between plasmid and genome.

*Hin*dIII cuts once in both pTB38 and pTB41, immediately 5′ to *PardC* (Fig. 1). For each plasmid, about 7.5 μg *Hin*dIII-digested and 20 μg uncut plasmid DNA were mixed and electroporated into 5×10^7 competent amoebae, in duplicate. Amoebal cells from duplicate electroporations were pooled, counted to determine survival, grown out for two days (1–2 generation times), and inoculated on lawns of killed bacteria on plates containing 100 μg/ml Hy. Plates were examined for colonies 1–3 weeks after inoculation, and one Hy^R colony, named 41T1, was found from the pTB41-treated cells, but none from pTB38-treated cells.

In a second experiment, duplicate 800-μl suspensions of amoebae were electroporated with (*i*) 7.5 μg linear pTB38; (*ii*) 10 μg circular pTB38; (*iii*) 7.5 μg linear pTB41; and (*iv*) 10 μg circular pTB41, as described above. A second Hy^R colony, 41T2, was recovered from the cells treated with linear pTB41 DNA. The second experiment was then repeated except that only one electroporation of amoebae was performed for each DNA sample. From this experiment, two Hy^R colonies, 38T1 and 38T2, were recovered from cells treated with uncut pTB38 DNA. Overall, the frequency of transformants in these initial experiments was approximately 10^{-8} per cell.

(c) Stability of the Hy^R phenotype

To assess the stability of the Hy^R phenotype, we wanted to determine whether transformants would still be Hy^R after amoebal growth followed by plasmodium development and plasmodial growth, in the absence of selection. In strain LU352, the *gadAh* mutation at the complex *matA* locus (Anderson et al., 1989) permits the normally heterothallic *matA2* amoebae to develop asexually ('self') to form plasmodia. The *npfC5* mutation at *matA* (Anderson et al., 1989) prevents this selfing and allows indefinite growth of pure amoebal cultures. However, *npfC5* reverts to wt at low frequency, and plasmodia can be obtained by selfing of the revertants under appropriate conditions when needed (Dee et al., 1989). To obtain plasmodia from LU352 transformants, we inoculated clones of amoebae onto DSDM plates with live bacteria and grew them until plasmodia developed. Plasmodia were then transferred to SDM plates for further outgrowth to

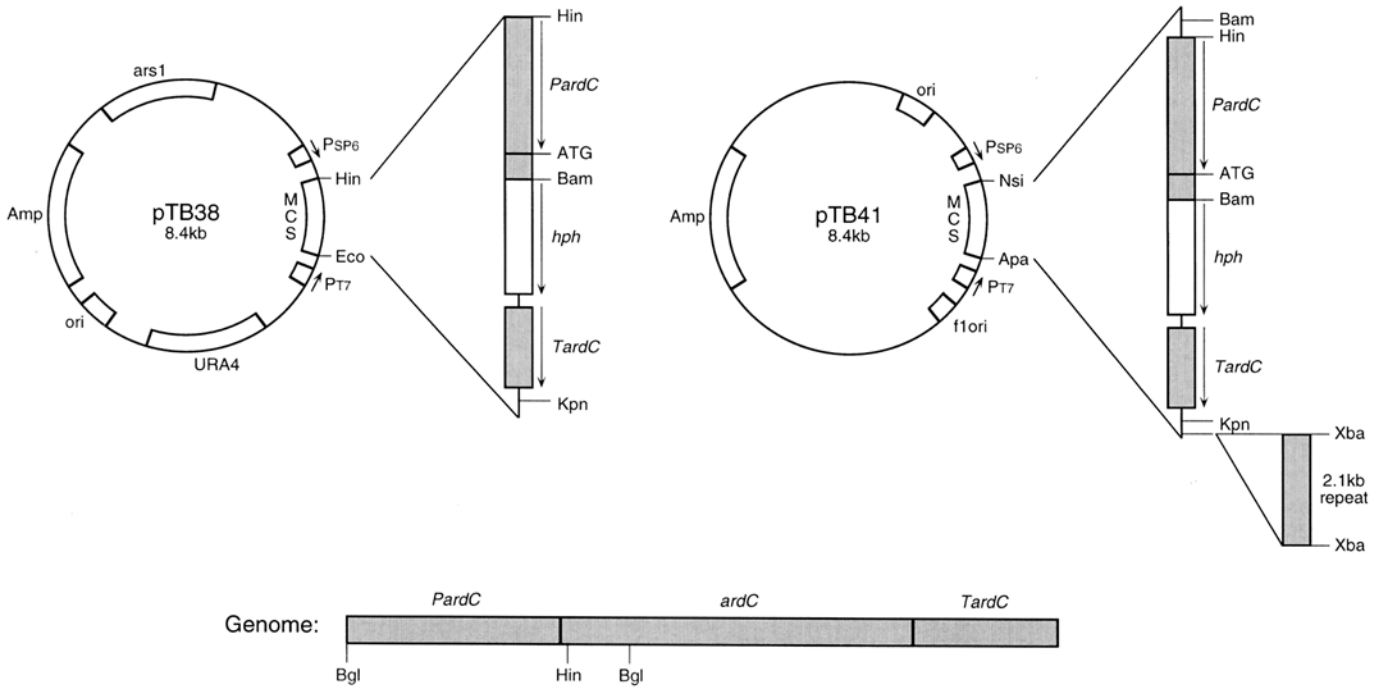

Fig. 1. Plasmid maps. Plasmid pTB38 is based on vector pGEM3Z (Promega, Madison, WI, USA) and contains yeast genes *ars1* and *ura4*$^+$. Plasmid TB41 is based on vector pGEM7Zf(+) (Promega) and contains no yeast genes. Shaded boxes represent *Physarum* sequences. Amp, β-lactamase-encoding gene; *ardC*, actin-encoding gene; ATG, start codon; *ori*, bacterial origin of DNA replication; MCS, multiple cloning site; P_{SP6}, P_{T7}, bacteriophage promoters; *PardC*, *ardC* promoter; *TardC*, *ardC* terminator; Apa, *Apa*I site; Bam, *Bam*HI site; Bgl, *Bgl*II site; Eco, *Eco*RI site; Hin, *Hin*dIII site; Kpn, *Kpn*I site; Nsi, *Nsi*I site; Xba, *Xba*I site. **Methods:** Plasmids were constructed using standard techniques (Sambrook et al., 1989), and enzymes were obtained from Promega. To construct pTB38, the *Pst*I fragment of *TardC* from pLAV-TerC (Burland et al., 1992) was blunt-ended with Klenow polymerase and ligated into the *Sma*I site of pTB33 (Burland et al., 1991). To construct pTB41, the *Hin*dIII-*Kpn*I fragment of *PardC-hph* from pTB33 (Burland et al., 1991) was ligated into *Hin*dIII + *Kpn*I-digested pGEM7Zf(+) (Promega) to create pTB37 (not shown). Next, the blunt-ended *Pst*I fragment of *TardC* (see above) was ligated into the *Sma*I site of pTB37 to create pTB40 (not shown). Separately, gel-purified *Xba*I-digested fragments of *Physarum* CLdxLU215 plasmodial nuclear DNA in the range 1–5 kb were ligated into the *Xba*I site of pTB20 (not shown), a plasmid consisting of a *Bam*HI-*Bgl*II fragment of *hph* from pLG83 (Gritz and Davies, 1983) ligated into the *Bam*HI site of pGEM7Zf(+), to create a small plasmid library of *Physarum Xba*I fragments. One of these *Xba*I fragments, 2.1 kb long and present in pTB21, was identified as containing repeated sequences from the *Physarum* genome, and this *Xba*I fragment was purified and ligated into the *Xba*I site of pTB40 to create pTB41. Plasmid DNA was prepared either in crude form or resin-purified (Burland et al., 1992), though transformant amoebae have been obtained from both crude and resin-purified preparations, and the plasmid DNA quality has not yet shown any overt difference in stable transformation efficiency.

at least 9 cm diameter. This strategy of unselected growth and development represents at least 20 mean generation times for the amoebae and at least a further 20 mean plasmodial cell cycle times since selection was removed. Selfed plasmodia were then tested for growth on 200 μg Hy/ml, a concentration at which untransformed plasmodia barely grow. All transformant plasmodia were resistant to Hy. This indicates that the Hy^R phenotype is stable through amoebal growth, plasmodium development and plasmodial growth. Assuming the resistance determinant to be stable, plasmodia were expected to be Hy^R if the resistance is due to the presence of *PardC-hph*, as *ardC*, whose promoter drives *hph* in the transforming plasmids, is expressed in both amoebae and plasmodia (Hamelin et al., 1988).

To assess the inheritance of the Hy^R phenotype, a cross of each transformant with MA389 was analyzed. MA389, genotype *matA4 gadA*$^+$ *npfC*$^+$ *matB1 fusA2 axe*, is a heterothallic progeny clone of a cross between BEN210 (Burland et al., 1984) and LU381 (Dee et al., 1989). Genetic analysis was as described in Burland et al. (1984). MA389 is imperfectly inbred with the strain used for transformation, and growth rates among the progeny of such crosses can be variable. In order to avoid scoring as sensitive progeny that were resistant but slow growing, we tested progeny amoebae for resistance to 50 μg Hy/ml rather than 100 μg Hy/ml as used for selection. Except for testing of recloned progeny, crosses were performed entirely in the absence of Hy.

All four crossed heterozygous plasmodia were Hy^R, demonstrating that Hy^R is dominant. Hy^R segregated approximately 1:1 among the progeny of crosses to 38T1, 38T2 and 41T2 (14/24, 12/23 and 8/23 progeny amoebae were Hy^R, respectively), as expected for normal meiotic segregation of a single nuclear determinant. However, there was a deficit of Hy^R progeny for the cross of MA389 with transformant 41T1 (4/24 Hy^R progeny; $\chi^2 = 10.7$, $P < 0.005$). The reason for this deficit is unclear. For all

four crosses, recombination was observed between Hy^R and both *matA* and *fusA*. These results show that in each case the transforming element can be inherited through meiosis in the absence of selection.

(d) Southern blot analysis of transformants

To test for the presence of the *PardC-hph* element in the genome of the transformants, DNA was isolated for Southern blotting. Amoebae of transformants were subcultured from the original selective plate and grown with and without Hy. For unselected cultures, at least 20 mean cell generation times elapsed from the initial transfer off drug to the time of isolation of the DNA. To purify DNA from the plasmodial stage, microplasmodial cultures growing in SDM broth were used to set up small (ca. 8 cm diameter) surface cultures of synchronous plasmodia, from which DNA was isolated in G2 phase of the mitotic cycle. DNA, isolated from whole cells or from isolated nuclei, was then digested with one of three restriction enzymes: *(i)* *Bam*HI, which cuts between *PardC* and *hph* in both pTB38 and pTB41 and also cuts 5′ to *PardC* in pTB41 (Fig. 1); *(ii)* *Bgl*II, which cuts neither plasmid but which generates a 1.5-kb fragment of *PardC* from genomic DNA (Fig. 1); and *(iii)* *Hin*dIII, which cuts each plasmid once immediately 5′ to *PardC* (Fig. 1).

When Southern blots were probed with *hph*, no signal was observed in wt, as expected, but each of the four transformants showed one restriction fragment hybridizing to this probe, consistent with each transformant containing a copy of *hph* (Fig. 2a). The sizes of *Physarum* genomic fragments hybridizing to *hph* are distinct for 41T1, 41T2 and 38T1 (Fig. 2a), indicating distinct integration events in these three transformants. The *hph* bands are the same for all three restriction digests for 38T1 and 38T2, which were obtained from the same transformed cell mixture, and we interpret this result as a single transformation event followed by cell replication during outgrowth, before selective plating. All of the *Hin*dIII-generated *hph* fragments identified in the transformants are distinct from the size of the linear plasmid (Fig. 2a), indicating that *hph* is not maintained extrachromosomally on molecules the size of the input plasmids.

Although transforming plasmid DNAs are commonly integrated in multiple copies after the transformation of other protists (e.g., Knecht and Loomis, 1987; Kindle et al., 1989), this is clearly not the case for the three *Physarum* transformants. Integration at multiple separate sites would have led to multiple restriction fragments hybridizing to *hph*, and integration of multiple copies of the 8.4-kb plasmids in tandem would have produced an 8.4-kb *Hin*dIII fragment that would hybridize intensely to *hph*, along with less intensely hybridizing 'junction fragment(s)'. Hybridization intensity for *hph* in the transformant DNAs is close to the equivalent of one copy of *hph* per nucleus (Fig. 2a), confirming that *hph* is present in single copy. No differences were observed in hybridization intensity or band pattern between amoebal and plasmodial DNA (not shown), nor between DNA from cells grown with or without Hy selection (e.g., Fig. 3a), nor between whole cell and nuclear DNA (e.g., Fig. 3b). We conclude that *hph* is integrated in single copy in the nuclear genome, rather than in mitochondrial DNA that might be contaminating the nuclear preparations, and that this association is stable in the absence of selection and through asexual and sexual development.

Have the plasmid DNAs integrated at homologous or nonhomologous sites? In the *Physarum* genome, there is no *Hin*dIII site immediately 5′ to *PardC*, but there is one 3′ to *PardC* near the beginning of the *ardC* coding region (Fig. 1) that was removed from the plasmids during construction. Homologous integration of either plasmid at *PardC* via a simple recombination event would thus produce a unique, diagnostic 1.1-kb *Hin*dIII fragment of *PardC*. None of the *Hin*dIII fragments recognized by the *PardC* probe (Fig. 2b) is 1.1 kb, ruling out the possibility of a simple recombination event giving rise to homologous integration at *PardC*. Further, the *PardC* probe shows that the wt *PardC* fragment in each transformant is unchanged, and that each transformant contains a second *PardC* element (Fig. 2b), as expected if integration does not occur at *PardC*, regardless of the nature of the recombination event between genome and plasmid. (The wt *Bam*HI fragment of *PardC* is faint for 41T2 because the purified DNA was partially degraded, which disporportionately reduces the relative amount of larger fragments.) Thus, we can rule out homologous integration at *PardC*. We cannot be sure whether homologous integration has occurred at *TardC* or at a site related to the 2.1-kb *Xba*I repeat sequence. However, the most likely explanation is that integration is at nonhomologous sites. Since the length of sequence homology is only 700 bp for *TardC* compared with 1100 bp for *PardC*, it would seem more likely that homologous integration would have occurred at *PardC*, assuming the integration event would not be lethal. Further, if homologous recombination were the favored mechanism for integration, transformation with pTB41, which carries the *Physarum* genomic repeated DNA sequence, should have been dramatically more efficient than for pTB38.

For *Bgl*II and *Hin*dIII digests of DNA, the additional *PardC* sequence resides on the same restriction fragment as *hph*, consistent with the absence of sites for these endonucleases between the two elements in the input plasmids. *Bam*HI, which cuts between *PardC* and *hph* in the plasmids (Fig. 1), separates *PardC* and *hph* onto different restriction fragments for 41T1 and 41T2 but did not

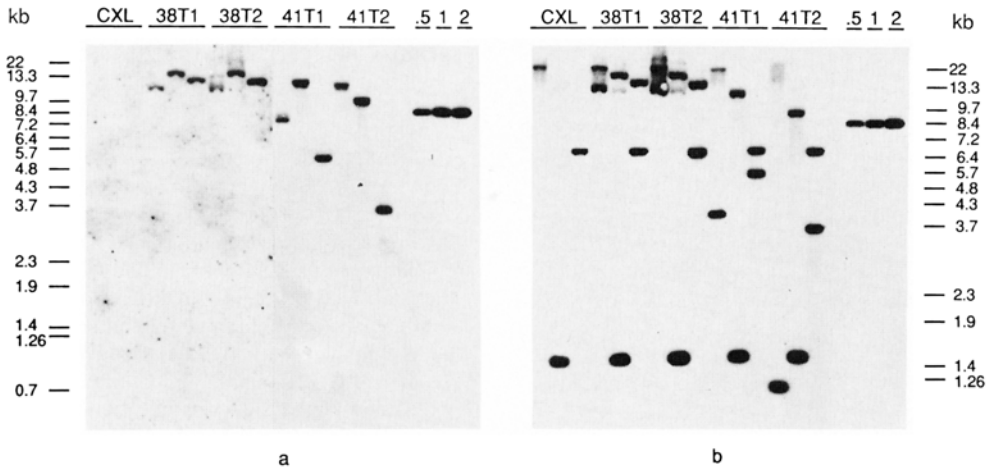

Fig. 2. Presence of *hph* and an additional copy of *PardC* in transformant DNA. Each set of three lanes contains DNA from the same strain, digested with *Bam*HI, *Bgl*II or *Hin*dIII (left to right, respectively), except for the rightmost set, which is a copy number control consisting of *Hin*dIII-digested pTB41 DNA, diluted and mixed with sonicated herring testis DNA, equivalent to 0.5, 1 and 2 copies of pTB41 per genome. *Hin*dIII digestion produces a linear plasmid of 8.4 kb. CXL is the inbred wt plasmodium CLdxLU215, which has the same genetic background as LU352. CXL, 38T1 and 38T2 DNAs are from nuclei isolated from selfed plasmodia grown without selection, 41T1 DNA is from nuclei isolated from amoebae grown on Hy, and 41T2 DNA is from nuclei isolated from amoebae grown without selection. Numbers at the side indicate the positions of molecular size markers in kb. (Panel **a**) Probed with *hph*. The gel for 38T1 and 38T2 was run separately from the gel for 41T1 and 41T2. (Panel **b**) Probed with *PardC*. All DNAs were resolved on the same gel. **Methods:** *Physarum* genomic DNA was purified and blotted as described (Schedl et al., 1984). The *PardC* probe was a 1.1-kb *Hin*dIII-*Bam*HI fragment gel-purified from pTB37. The *hph* probe was a 1.3-kb *Bam*HI fragment of *hph* from pLG83 (Gritz and Davies, 1983). The *hph* fragment was purified from pLG83 to ensure that no *Physarum* DNA fragments could contaminate the probe. Probes were denatured and then radiolabelled using random hexamers to prime Klenow polymerase replication in the presence of [^{32}P]dCTP (using the 'Prime-a-Gene' kit from Promega). Radiolabel was obtained from New England Nuclear (catalogue No. NEG-013H). Genomic DNA (3 μg) was digested overnight in 500-μl reactions with a 50-fold excess of restriction enzyme to ensure complete digestion, then precipitated with ethanol and resuspended in 20 μl TE before electrophoresis on 15-cm-long 0.7% agarose gels at $\leq$0.8 V/cm. Gels were then blotted to nitrocellulose, hybridized with DNA probes and washed in 0.2 × SSC at 62°C five times for 15 min each. SSC is 0.15 M NaCl/0.015 M Na_3-citrate pH 7.0, and TE is 10 mM Tris/1 mM EDTA pH 7.6.

appear to separate *PardC* and *hph* for 38T1/2. This raises the possibility that the *PardC-hph* element in the pTB38 transformants underwent mutation. However, since the *Bam*HI site is in the first few codons of *hph* (Burland et al., 1991), any mutation is unlikely to be a major rearrangement. It is more likely that *PardC* and *hph* are on separate *Bam*HI fragments that are too similar in size to be resolved on the gels of DNA from this transformant; the wt *Bam*HI fragment of *PardC* is $\approx$20 kb, and resolution is poor in this region of the gel.

Probing similar Southern blots with linearized pGEM7Zf(+) vector DNA failed to detect related sequences in transformants 38T1/2 and 41T1 (not shown), suggesting that little if any of the vector DNA survived the recombination events that gave rise to the transformants. A single crossover between circular plasmid and the genome would not produce this result, suggesting that the integration events are more complex. By contrast, the pGEM7Zf(+) probe hybridized strongly to 41T2 DNA digests, consistent with the presence of most or all of the vector sequences at one copy per genome in this transformant (Fig. 3c). Again, the fact that the restriction fragments are distinct from the 8.4-kb size of pTB41 argues against the extrachromosomal maintenance of monomer plasmid DNA. Transformant 41T2 is the only one we transformed using linear rather than circular DNA or a mixture of linear and circular DNA, but with so few transformants we cannot say whether linear DNA is for some reason superior to circular DNA for the integrative transformation of larger fragments of DNA into *Physarum* chromosomes.

Analysis of a larger number of transformants will be needed before conclusions can be made as to the relative frequencies of homologous versus nonhomologous integration. The availability of luciferase expression vectors for *Physarum* (Burland et al., 1993), together with the simplicity and reliability of the luciferase assay (DeWet et al., 1987), is leading to rapid improvements in transformation methods, which in turn will lead to more efficient generation of stable *Physarum* transformants for further analysis.

(e) Conclusions

Our results establish a routine method for DNA transformation of *Physarum*. The success of DNA transformation in the analysis of biological problems involving, for example, myosin function and signal transduction in *Dictyostelium* (e.g., DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Howard et al., 1992) shows how powerful this technology can be in protists. The

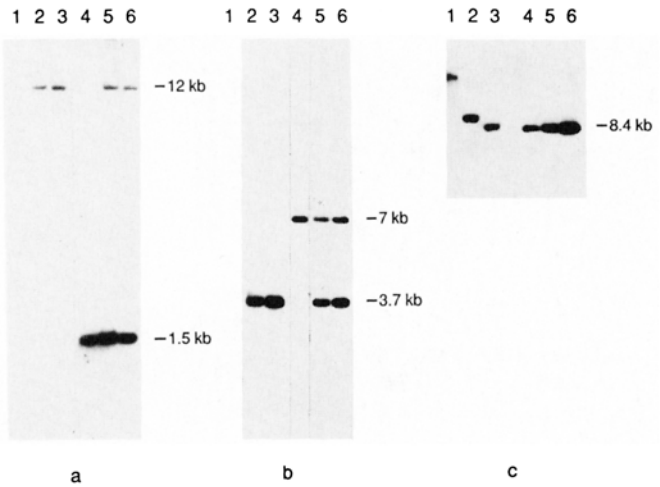

Fig. 3. Nuclear location and stability of *hph* in transformants, and presence of vector. (Panel **a**) Southern blot of nuclear versus whole cell transformant DNA. *Bgl*II digests of 41T1 DNA (lanes 2, 3, 5, 6) isolated from amoebal nuclei (lanes 2, 5) or whole cells (lanes 3, 6). Lanes 1 and 4 are *Bgl*II digests of control wt DNA. Lanes 1–3 probed with *hph*, lanes 4–6 probed with *PardC-hph*. Note similar band intensities for the transforming *PardC-hph* fragment at ≈12 kb in both whole cell and nuclear DNA preparations. The *Bgl*II fragment at ≈1.5 kb is the endogenous *PardC* sequence. (Panel **b**) Southern blot of selected versus unselected transformant DNAs. *Hin*dIII digests of wt (lanes 1, 4) and 41T2 (lanes 2, 3, 5, 6) DNA, probed with *hph* (lanes 1–3) or *PardC-hph* (lanes 4–6). The 41T2 nuclear DNA was isolated from amoebae grown with (lanes 2, 5) or without (lanes 3, 6) Hy selection. Note similar band intensities for the transforming *PardC-hph* fragment at ≈3.7 kb whether cells were grown with or without selection. The fragment at ≈6.5 kb is the endogenous *PardC* sequence. (Panel **c**) Southern blot probed with pGEM7Z(f)+, showing presence of vector sequences in 41T2. Lanes 1–3, 41T2 nuclear DNA from unselected amoebae, digested with *Bam*HI, *Bgl*II and *Hin*dIII, respectively. Lanes 4–6, copy number control, same as Fig. 2. **Methods:** The *hph* probe was as described in the legend to Fig. 2. The *PardC-hph* probe was a 2.2-kb *Hin*dIII-*Xho*I fragment gel-purified from pTB37, and the pGEM7Zf(+) probe was digested with *Hin*dIII prior to radiolabelling.

DNA transformation technology for *Physarum*, together with the variety of cell types and the meiotic sexual cycle of this protist, promises even greater power for the analysis of questions in cell biology.

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