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A DNA-mediated transformation system for *Dictyostelium discoideum*

(aminoglycoside G418 resistance)

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ABSTRACT **We have established a transformation system for *Dictyostelium discoideum*. The transformation vector contains the protein coding region of the Tn5 neomycin resistance gene fused to the proposed promoter of the *Dictyostelium* actin 8 gene; the vector also contains a sequence that acts as an autonomously replicating sequence (*ars*) in yeast. Using this vector, we can transform *Dictyostelium* vegetative amoebae to be resistant to aminoglycoside G418 at a frequency of between 10^{-6} and 10^{-4} of the input cells. The transformed cell lines are stable and contain vector sequences integrated within chromosomal DNA.**

Over the past several years the structure and transcriptional properties of many developmentally regulated genes from the cellular slime mold *Dictyostelium discoideum* have been examined (see ref. 1 for review). These include the genes encoding actin (2–4), the carbohydrate binding protein discoidin I (5–7), and those whose expressions are modulated by cyclic AMP (refs. 8 and 9; unpublished data; see refs. 1 and 10 for review). To examine potential regulatory sequences, it will be useful to reinsert these genes into *Dictyostelium* by means of a DNA-mediated transformation system. Ratner *et al.* (11) have previously reported the transformation of mutant *Dictyostelium* amoebae that cannot grow on *Bacillus subtilis* by using plasmids containing wild-type *Dictyostelium* genomic DNA. They obtained *Dictyostelium* transformants that grew on *B. subtilis* and that carried sequences complementary to the transformation vector, but because the gene conferring the ability to grow on *B. subtilis* could not be isolated, a *Dictyostelium* transformation vector could not be established.

Using resistance to the aminoglycoside G418 as the selectable marker, we have established a DNA-mediated transformation system for *Dictyostelium*. Several groups have shown that the neomycin resistance (*neo*^{R}) genes from transposable elements Tn*903* (12) and Tn5 (13, 14) confer resistance to G418 in yeast and mammalian cells, respectively. We have used the Tn5 *neo*^{R} gene to transform *Dictyostelium* cells. In stably transformed cell lines, the *neo*^{R} gene and vector DNA appear to be integrated within the *Dictyostelium* genome. In addition, when DNA is isolated from newly selected colonies, some of the vector DNA is extrachromosomal. Transformed *Dictyostelium* amoebae are developmentally competent and retain G418 resistance throughout their life cycle. Resistant cells grown in the absence of the drug for many generations are still resistant to G418 and carry vector DNA.

MATERIALS AND METHODS

Construction of pCERF·Drp14. A 3-kilobase (kb) *Eco*RI/*Bgl* II fragment containing the actin gene from plasmid pDd actin 8 (3) was inserted into the *Eco*RI/*Bam*HI restriction sites of plasmid pBR322, producing plasmid pCE8. pCE8 was linearized at the *Hin*dIII restriction site within codon 8 of the actin gene, digested with *Bal* 31 exonuclease, and ligated with *Bam*HI linkers. A *Bgl* II/*Bam*HI restriction fragment from pJZ112 (13) carrying the protein-coding region of the *neo*^{R} gene was ligated into the vector and used to transform *Escherichia coli* to penicillin resistance. Analysis of the only transformant, pCEN1, indicated that all of the actin 8 5′ sequences had been removed, and the *Bam*HI linker was immediately adjacent to the *Eco*RI site in pBR322. A 130-base-pair (bp) *Eco*RI/*Eco*RI* fragment carrying the proposed actin 8 promoter was then purified and ligated into the *Eco*RI site of pCEN1. Restriction endonuclease digestion and DNA sequence analysis were used to identify a clone in which the *Eco*RI/*Eco*RI* restriction fragment was inserted in the proper orientation.

After the vector was constructed, the *Eco*RI/*Sal* I restriction fragment carrying the actin 8–Tn5 *neo*^{R} gene fusion was transferred to plasmid pXF3 (obtained from D. Hanahan, Cold Spring Harbor Laboratories). [pXF3 is a pBR322 derivative that lacks sequences thought to inhibit the replication of extrachromosomal DNA in mammalian cells (15).] The resultant vector was called pCERF.

Because the transformation frequency of yeast is markedly increased by the presence of an autonomously replicating sequence (*ars*) (16, 17), we chose to isolate a *Dictyostelium* sequence that might function as an *ars*. It had previously been shown that certain *Dictyostelium* genomic DNA sequences could act as *ars*s in yeast (18). *Eco*RI-digested *Dictyostelium* DNA was ligated with *Eco*RI-digested, alkaline phosphatase-treated YIp5 DNA (18). The ligation mix was used to transform *E. coli* to tetracycline resistance. Colonies were picked and each was grown overnight in 2 ml of L broth containing tetracycline at 5 μg/ml. The cells were mixed in groups of 10 cultures each, collected by centrifugation, and then washed once with Tris/sucrose (25% sucrose/50 mM Tris·HCl, pH 7.4). The cells were resuspended in 1 ml of Tris/sucrose containing lysozyme at 1 mg/ml, incubated on ice for 10 min, then collected by centrifugation. These *E. coli* spheroplasts were used to transform the yeast strain SHY2 [*ste-VC9 ura3-52 trp1-289 leu2-3 leu2-112 his3Δ1 can1-100* (29)] according to the protocol of Kingsman *et al.* (19). Some of the resulting transformants were grown in yeast minimal medium containing tryptophan, leucine, and histidine, each at 0.001%, for 4 days at 30°C. DNA was prepared from these cultures according to the procedure of Struhl *et al.* (16).

DNA from the transformed yeast was used to transform *E. coli*. Plasmid DNA was prepared from the *E. coli* transformants and tested for the ability to transform yeast at a high frequency. The yeast strains, SHY2 and 1-7F (*a gal10 trp1 ade8 his3 ura3*), were transformed at a very high frequency with DNAs from two plasmids, pDRp14 and pDRp26. The 2.7-kb DRp14 *Eco*RI restriction fragment was chosen for the vectors used in these experiments because it is considerably smaller than DRp26.

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Abbreviations: kb, kilobase(s); bp, base pair(s).

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DRp14 permitted a 5- to 10-fold higher frequency of transformation in yeast than was obtained by using yeast vectors such as YRp7 or YEp6 (16).

The DRp14 element was transferred into pCERF at the *Eco*RI site to produce plasmid pCERF·DRp14 (Fig. 1).

Transformation Protocol. Five micrograms of DNA from *Dictyostelium* Ax-3 cells (21) and 25 μg of plasmid DNA were placed in 200 μl of 6 mM NaCl/6 mM Tris·HCl/0.2 mM EDTA, pH 7.5, in a small silane-treated tube. Three drops of chloroform was added and then removed under reduced pressure to sterilize the DNA. Two milliliters of 10 mM sodium phosphate buffer, pH 6.2–6.4, and 1 μg of poly(L-ornithine) per μg of DNA were added. [Poly(L-ornithine) (average 110,000 daltons, Sigma) was stored as a 10 mg/ml stock in phosphate buffer over chloroform at 4°C.] The DNA solution was placed in a 125-ml sterile screwcap flask containing 6 ml of phosphate buffer and shaken on an orbital shaker at 50–100 rpm for 20 min.

Ax-3 cells (harvested at 2×10^6 cells per ml) were collected and washed twice with sterile PDF solution (22). The cells were resuspended at a concentration of 1.5×10^7 per ml in phosphate buffer. Two milliliters of cells was added to the DNA/poly(L-ornithine) mixture and shaken as described above for 20 min. One milliliter of 50 mM $CaCl_2$ was added. The cells clumped at this stage. After 10 min of shaking, the cells were washed twice with HL5 medium (23, 24) and then transferred to 300 ml of HL5 medium containing tetracycline at 25 μg/ml, chloramphenicol at 25 μg/ml, and penicillin at 100 μg/ml in a 1-liter screwcap flask. After shaking for 10–20 min, a small aliquot was removed and used to titer survivors by plating on SM plates with *Klebsiella aerogenes* (22). The survival rate was >80%; centrifugation accounts for most of the loss.

G418 was added to the cells at a concentration of 10 μg of active drug per ml 10–20 min after dilution into HL5 medium (see above). (G418 stock was prepared in 10 mM Hepes, pH 7.0, at a concentration of 2 mg/ml. The actual activity of the drug varies from lot to lot.) The cells were shaken for 4.5–5 days and then harvested.

K. aerogenes powder was prepared by collecting stationary-phase cells from SM medium (22), washing twice with 10 mM Tris·HCl at pH 7.4, lyophilizing, and then grinding to a fine powder. The powder was suspended in HL5 medium (0.1 g/ml) and autoclaved. Then 0.2 ml of the bacterial suspension was added to each *Dictyostelium* transformation culture and the cells were collected by centrifugation (2,800 rpm, 15 min, Beckman J54.2 rotor in a Beckman JC-6 centrifuge), washed with 20 ml of HL5 medium (3,000 rpm for 10 min, Sorvall HB-4 rotor), and resuspended in 2 ml of the *Klebsiella* suspension with tetracycline, chloramphenicol, penicillin (as above), and active G418 at 30 μg/ml. It should be noted that *Dictyostelium* cells are less sensitive to G418 when plated on lyophilized bacteria than when shaken in axenic medium.

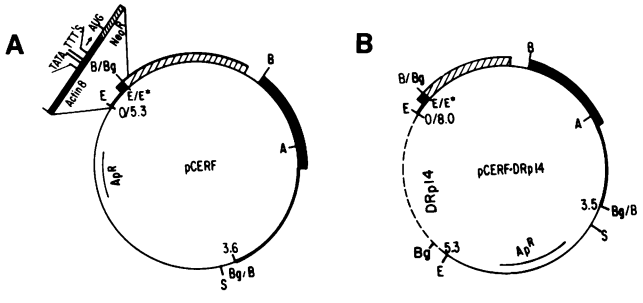

FIG. 1. Restriction map of pCERF (*A*) and pCERF·DRp14 (*B*). The regions shown as thick black lines are derived from pDd actin 8. The black bars contain the regions encoding actin 8 mRNA. The thin solid line denotes sequences derived from pXF3. The hatched bar denotes the Tn*5* *neo*^{R} gene. The thin solid line 3′ to this represents DNA that contains 3′ flanking sequences from Tn*5*. The broken line denotes the DRp14 element. Also shown in *A* are the relative location of the TATA box and oligo(dT). [Oligo(dT) regions are found between the TATA box and the 5′ end of all *Dictyostelium* polymerase II genes and have been proposed to be part of the actin 8 promoter (refs. 1 and 20; unpublished data).] The numbers inside the plasmid circle represent lengths in kb from the *Eco*RI site, which is labeled O. Ap^R, ampicillin resistance. Restriction site abbreviations are A, *Ava* II; B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; S, *Sal* I. The B/Bg sites identify the position of the joining of *Bam*HI and *Bgl* II restriction sites. The unique restriction sites that could be used for inserting a DNA molecule into this vector are the *Bgl* II, *Bam*HI, and *Sal* I sites.

The cells were then plated. Two Whatman 3 MM filters (12.5 cm) were placed in a 15 × 150 mm Petri dish and saturated with 15–20 ml of HL5 medium containing G418 at 30 μg/ml plus the other three antibiotics. A Millipore cellulose filter pad and then a 47-mm black Millipore HABP filter were placed on top and allowed to absorb liquid but not to become wet on the top surface. The cells were then spread evenly on three filters and the plates were sealed with Parafilm (see Fig. 2*A*). Transformants appeared in 3–5 days. After transformants were visible, they were picked and transferred onto 0.5-cm circles of autoclaved, lyophilized bacteria on black Millipore filters (see Fig. 3*B*). The filters were placed on top of Whatman 3 MM filter paper saturated with antibiotic solution plus active G418 at 25–35 μg/ml. Approximately 14 circles of bacteria were placed on each 47-mm HABP membrane.

Other Methods. *Dictyostelium* genomic DNA and plasmid DNA were isolated as described (25). Plasmid DNA was isolated from stationary-phase cultures grown in L broth containing the appropriate antibiotic. DNA labeling, blot hybridization, and sequence analysis were done as described (2, 3, 7).

In the experiments in which DNA from transformed *Dictyostelium* cells was used to transform *E. coli*, a 10- to 100-pg range of total cell DNA was used. Within this range, the number of transformants was linear with DNA concentration. Control experiments in which *Dictyostelium* genomic DNA was added to supercoiled plasmid DNA showed that the genomic DNA did not affect the transformation frequencies. Supercoiled pCERF·DRp14 gave 1–2 × 10^6 transformants per μg.

Dictyostelium axenic cell line Ax-3, obtained from R. Kessin (Harvard University), was utilized in all experiments. It was grown in HL5 medium containing phosphate buffer (24). G418 was a gift of Peter Daniels (Anti-Infectives Chemical Research, Schering, Bloomfield, NJ).

RESULTS

Vector Construction. We chose to construct a vector containing a dominant drug resistance gene fused to a *Dictyostelium* promoter to allow its expression in *Dictyostelium*. Because the actin 8 gene is expressed at high levels throughout *Dictyostelium* development (3), we linked the proposed promoter for this gene to the coding region of the *neo*^{R} gene from Tn5 in the pBR322 derivative pXF3. The resultant vector is called pCERF.

The frequency of transformation in yeast is markedly increased by sequences that allow the plasmid DNA to replicate autonomously (*ars*) (16, 17). Stinchcomb *et al.* (18) showed that DNA sequences from many eukaryotes, including *Dictyostelium*, act as *ars* in yeast. Because it was possible that such a sequence would also function in *Dictyostelium*, potential *Dictyostelium* replicons (DRps) were isolated. One of the sequences that conferred a very high frequency of transformation in yeast, DRp14, was then inserted into the *Eco*RI site of pCERF, yielding the vector pCERF·DRp14 (see Fig. 1*B*).

Transformation of *Dictyostelium* Vegetative Amoebae. *Dictyostelium* vegetative amoebae were transformed as described in *Materials and Methods* and incubated in axenic medium with

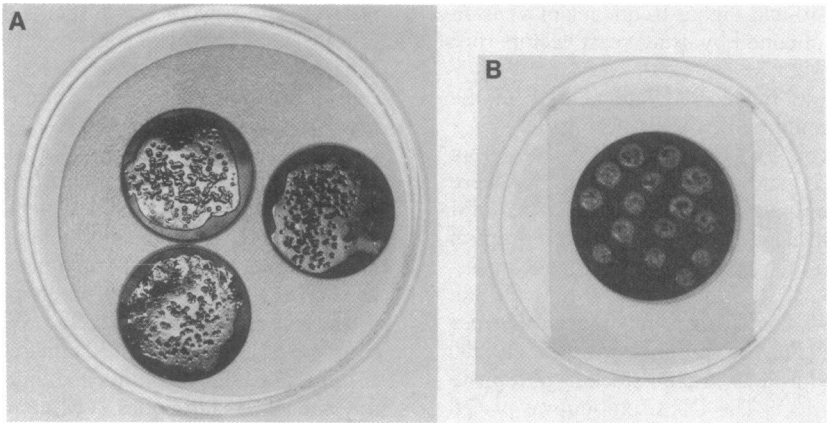

FIG. 2. Clonal isolation of transformed *Dictyostelium* cells. (*A*) Three Millipore filters (47-mm HABP) covered with autoclaved, lyophilized bacteria on black Millipore filters to facilitate seeing the colonies. "Plaques" in the lawn are colonies derived from individual *Dictyostelium* transformed cells. Colonies shown were allowed to grow for several days longer than usual to make them easily visible in the photograph. (*B*) Individual colonies from transformants shown in *A* were used to inoculate small (0.5-cm diameter) circles of lyophilized bacteria on 47-mm Millipore filters. Usually 14 or 15 individual transformed colonies can be picked with a toothpick and grown on each filter. The dark areas in the midst of the bacteria represent regions that have been eaten by transformed *Dictyostelium* cells.

G418 to kill nontransformed cells. In axenic medium transformed cells are viable but do not divide (see below). To isolate clones of single transformants, the cells were then plated in association with lyophilized, autoclaved *K. aerogenes* and G418 (Fig. 2*A*). Transformed cells grow in the presence of G418 when lyophilized *K. aerogenes* is the food source. Table 1 gives the results of a series of representative experiments. Using the plasmid pCERF·DRp14, we consistently obtained transformants at frequencies of 10^{-6} to 10^{-4} of the input cells. Upon replating (Fig. 2*B*), approximately 70% of the clones transformed with pCERF·DRp14 grew under selection. pCERF, which lacks the DRp14 sequence, gave a very low frequency of transformation ($<10^{-6}$ of the input cells). pXF3 and pML5, which carries the *neo*^{R} gene without the actin promoter, gave few if any survivors on the initial screening. Colonies that did appear from these plasmids in the initial screen did not grow upon rescreening. Because the frequency is variable from experiment to experiment, it has been difficult to determine more accurately the efficiency of transformation.

In yeast, if vector DNA is linearized within sequences homologous to chromosomal DNA, the transformation frequency is much greater than if the vector is circular (26). Linearization may increase the frequency at which the vector recombines within the chromosome. To test the effect in *Dictyostelium*, pCERF DRp14 was linearized at several restriction sites. Linearization at the *Bgl* II site in the DRp14 element increases in the efficiency of transformation by 5- to 10-fold, whereas linearization at the *Bam*HI or *Sal* I restriction sites has little if any effect on the frequency. Cleavage at the *Eco*RI sites excises the DRp14 sequence and results in a low frequency of transformation comparable to that of pCERF.

State of Vector DNA in Transformed Cell Lines. G418-resistant clones were examined for the presence of pCERF·DRp14 DNA sequences. DNA was isolated from colonies grown on lyophilized bacteria in the presence of G418 and analyzed by DNA blot hybridization to nick-translated pML5 DNA [Tn5 *neo*^{R} gene inserted into pML2, a pBR322 derivative (15)] (see Fig. 3*A*). Several bands are observed in the lanes containing DNA from clones resistant to G418 (lanes D, E, and F) whereas no hybridization is observed with control DNA (lane C). In the case of all three transformed cell lines three bands are observed. Each lane shows a band (c) that corresponds in size to the smaller *Pst* I/*Eco*RI fragment from pXF3 and the *Eco*RI/*Pst* I fragment from the 5′ end of the actin–Tn5 gene fusion, which comigrate. The rest of the bands from the independently derived cell lines differ in size, although the patterns in lanes D and F are similar. There are no bands that correspond to the size of the largest *Pst* I/*Eco*RI fragment of pCERF·DRp14 (see lane A).

Fig. 3*B* shows hybridization of nuclear DNA from another series of transformed lines with a purified Tn5 *neo*^{R} gene probe. Lanes B–H (with DNA from transformants) show a band that is the same size as the Tn5 *neo*^{R} gene *Eco*RI/*Hin*cII fragment from pCERF·DRp14. No hybridization is seen in the two lanes with DNA from Ax-3 untransformed cells. Strain 4 (see lane E) has an additional lower molecular weight fragment that hybridizes, indicating the presence of two *neo*^{R} genes, one with a deletion.

Two types of experiments were done to examine the physical

Table 1. Frequency of transformation of *Dictyostelium*

Vector	Fraction of cells surviving: First screening*	Fraction of cells surviving: Second screening†
	Experiment 1	
pXF3	1.3×10^{-7}	0
pCERF	0	0
pCERF·DRp14	2.9×10^{-5}	0.72
	Experiment 2	
pXF3	8×10^{-7}	0
pCERF	1×10^{-6}	0
pCERF·DRp14	$7\text{–}10 \times 10^{-5}$	0.68
	Experiment 3	
pXF3	0	0
pCERF·DRp14	6×10^{-6}	0.75
	Experiment 4	
pXF3	5×10^{-7}	0
pCERF·DRp14	2.9×10^{-6}	0.80
pCERF·DRp14 linearized‡	1.8×10^{-5}	0.67

Four different but representative experiments were chosen. Note that the number of cells surviving the first screening ranged between 10^{-6} and 10^{-4} of the input cells over all experiments done. In approximately 10% of the experiments there were no surviving colonies.

* This column gives the fraction of cells that formed colonies after the first screening (see Fig. 2*A*).

† Colonies were randomly picked with toothpicks and placed on a grid of bacteria with G418 at 25 μg/ml as shown in Fig. 2*B*. The number of colonies picked and checked for growth in the presence of G418 ranged between 30 and 60 for pCERF·DRp14. The number in this column indicates the fraction of the clones that grew.

‡ DNA was linearized at the *Bgl* II site in the DRp14 sequence.

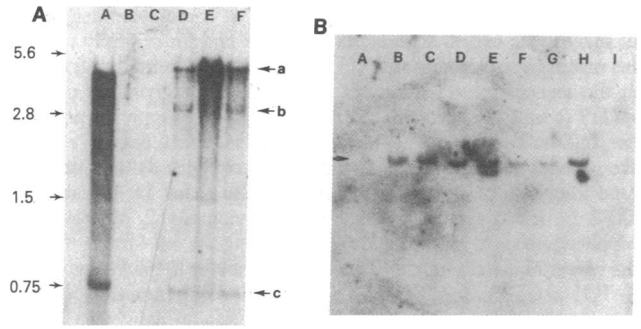

FIG. 3. Hybridization of DNA from transformed cell lines. (*A*) DNA from three different cell lines transformed with supercoiled pCERF·DRp14 was digested with *Pst* I and *Eco*RI, size-fractionated on agarose gels, and blotted onto nitrocellulose. In addition, DNA from Ax-3 nontransformed cells and ≈5 haploid genome equivalents of pCERF·DRp14 were treated in the same manner. The DNA blot filter was hybridized to pML5 (Tn*5* *neo*^{R} gene inserted into pML2) nick-translated DNA, washed, and autoradiographed. Because of the extremely small amounts of DNA isolated from some of the transformed cell lines grown in association with autoclaved bacteria in the presence of G418, the concentration of DNA from several strains was estimated from the intensity of the ribosomal DNA bands relative to known amounts of wild-type axenic DNA in lanes A and I. It should be noted that, in *Dictyostelium*, the 88-kb extrachromosomal ribosomal DNA represents approximately 1/6th of the total genomic DNA, and therefore restriction fragments of the ribosomal DNA are visible on restriction digests (see refs. 25 and 27). Lane A, pCERF·DRp14 DNA; lane G, no DNA; lane C, 2 μg of Ax-3 DNA; lane D, 1 μg of DNA from strain A; lane E, 3 μg of DNA from strain G; lane F, 1 μg of DNA from strain C. Numbers on the left are sizes of molecular weight markers in kb. (*B*) *Dictyostelium* DNA was isolated from several clones transformed with pCERF·DRp14 and several secondary cell lines derived from one of these original transformants. DNAs from these cell lines and from untransformed wild-type *Dictyostelium* axenic cells were digested with *Hin*cII and *Eco*RI, size-fractionated on agarose gels, blotted onto nitrocellulose, and hybridized to a nick-translated purified Tn*5* *neo*^{R} gene probe. An arrow indicates the molecular weight of the *Eco*RI/*Hin*cII restriction fragment from pCERF·DRp14 carrying the actin 8–Tn*5* *neo*^{R} gene fusion. The DNA concentration was estimated as described for *A*. Lane A, 2 μg of DNA from nontransformed Ax-3 cells; lane B, 1 μg of DNA from transformed cell line 1; lane C, 1 μg of DNA from transformed cell line 2; lane D, 1 μg of DNA from transformed cell line 3; lane E, 2 μg of DNA from transformed cell line 4; lane F, 1 μg of DNA from a secondary derivative (2a) of cell line 2 grown on live bacteria; lane G, 1 μg of DNA from a secondary cell line (2b) derived from strain 2 grown in axenic medium; lane H, 1 μg of DNA of a secondary cell line (2c) derived from strain 2 grown in axenic medium; lane I, 2 μg of Ax-3 DNA.

state of vector DNA in transformants. If any of the vector sequences were extrachromosomal in *Dictyostelium*, they might be expected to transform *E. coli*. Approximately 50% of the newly transformed cell lines (15–20 generations after selection) contain DNA that transforms *E. coli*. The frequency of transformation, compared to supercoiled plasmid DNA, indicated that if the extrachromosomal DNA in the *Dictyostelium* cells were supercoiled, there would be 0.1–2 copies per cell (see *Materials and Methods*). When the DNA isolated from these *E. coli* transformants was analyzed, it contained an intact actin 8/*neo*^{R} gene fusion, the ampicillin resistance gene, and the origin of replication from pXF3, and some, but not all, sequences from the 2.7-kb DRp14 element. There were also deletions in the sequences derived from pXF3. When DNAs from the different transformants were compared, some of the deleted regions were found to vary among DNAs isolated from different strains (unpublished observations). When DNA isolated from cells that were grown for many generations in axenic medium was tested, no *E. coli* transformants were observed. This suggests that either there was no extrachromosomal DNA in these cells or that the sequences required for selection or growth in *E. coli* were modified.

When DNA from transformed cells grown on bacteria with G418 or in axenic medium without G418 was cleaved with *Hin*dIII, blotted, and probed with purified Tn5 DNA, a single restriction fragment of differing size was observed in each of the transformed cell lines (data not shown). Because there are no *Hin*dIII sites on pCERF·DRp14, it is likely that the vector sequences are integrated. This result is supported by the following observations: DNA from several transformants, which was not digested with restriction enzymes, was sized on agarose gels, blotted, and hybridized to a Tn5 *neo*^{R} gene probe. The only hybridization observed was to the very high molecular weight chromosomal DNA, suggesting that at least some of the DNA was integrated (data not shown). This hybridization pattern was also observed for DNA isolated from strains that carry extrachromosomal DNA. We have been unable to detect the extrachromosomal DNA on blots, possibly because of size heterogeneity or the failure of DNA of a given size to run as a homogeneous band, possibly because of variability in the number of superhelical turns. We have no evidence for or against linear extrachromosomal DNA.

The transformed phenotype is maintained through development or growth on nonselective medium. Ten different transformed lines were allowed to develop. All developed normally when plated on paper filters or on SM plates with *K. aerogenes* (22, 24). Individual sorocarps (each containing ≈7×10^4 spores) were plated in the presence of G418. At least 50% of the spores from each sorocarp formed a colony. On average, the plating efficiency of the spores grown on filters with lyophilized *Klebsiella* and G418 was 80–90% of that on live *Klebsiella*. One sorocarp from each cell line was also inoculated in axenic medium. Cells were grown in the absence of G418 for at least 20 generations and aliquots were plated on lyophilized *Klebsiella* with G418 and on live bacteria in the absence of the drug. Again, cells plated on G418 had an 80–90% colony-forming efficiency relative to the cells plated without drug.

The DNAs from substrains derived from strain 2 have been examined. *Eco*RI/*Hin*cII digests of DNA from these substrains was analyzed by DNA blot hybridizations. As shown in Fig. 3*B* (lanes F, G, and H), one band with a molecular weight identical to that of the parental strain (lane C) is observed in all substrains. In a similar experiment, using a different parental transformant, *Hin*dIII digests were probed (data not shown). In this case the *neo*^{R} fragment from some substrains had a different molecular weight than that of the parental strain. The vector or adjacent sequences must have been altered.

We have estimated the copy number of *neo*^{R} sequences by DNA dot hybridization (data not shown). Comparison of the intensity of hybridization to the quantitation standards indicates that each cell line has between 0.5 and 2 copies of complementary DNA per haploid genome. Two substrains, 2a and 2b, derived from transformed cell line 2 show a lighter hybridization to DNA blots (see Fig. 3*B*) and to the dot blots than cell line 2 or substrain 2c. The *neo*^{R} sequences may have been lost by some of the cells in these two substrains. DNA from cell line 2 shows 2–3 copies of vector sequences per haploid gene, but it should be noted that this cell line contains ≈1 copy of extrachromosomal DNA per haploid genome as well as integrated DNA copy (copies). After extended growth, extrachromosomal DNA is not detectable in strain 2 by the transformation assay.

DISCUSSION

We have established a transformation system for *D. discoideum* using a *Dictyostelium* actin–Tn5 gene fusion, which confers resistance to the drug G418. Because the frequencies of transformation are variable (see Table 1), it has not been possible to

determine the absolute efficiencies of various vectors. If the G418 concentration is altered by as little as 30% in the initial selection, the number of transformants is changed significantly. The variability in transformation frequency may be the result of the narrow range in sensitivity to G418 of transformed and nontransformed cells. There may be other factors that change the sensitivity of the cells and cause variability in the transformation frequency. Dot-blot hybridizations indicate that most cell lines contain 1 or 2 copies of vector sequences. We have examined the vector copy number in some of transformed lines that are resistant to high concentrations of G418. DNA from cell lines grown on lyophilized bacteria with G418 at 50 μg/ml appears to have the same copy number as DNA from strains that can grow with G418 at only 35 μg/ml. The increased resistance is therefore probably not due to an increase in the number of resistance genes.

We have constructed other vectors in an attempt to obtain more reproducible or higher frequencies of transformation. One vector carrying the Tn*903* *neo*^{R} gene without a *Dictyostelium* promoter gave a frequency of transformation that is lower and even more variable than that obtained with pCERF·DRp14. The transformants were also less stable. When the Tn*903* *neo*^{R} gene was inserted into the pCERF·DRp14 vector, the transformation frequencies were similar to the frequency of pCERF·DRp14. These transformants were stable and contained both *neo*^{R} genes.

The results suggest that a sequence that acts as an *ars* in yeast is needed to transform *Dictyostelium* with a high frequency. At the present time, we do not know if the DRp14 sequence acts as an origin of replication in *Dictyostelium*. When DNA is linearized by *Bgl* II within the DRp14 element, a higher frequency of transformation than either closed circular DNA or DNA linearized at other restriction sites in the vector results. Orr-Weaver *et al.* (26) have shown recently that linearization of yeast vector DNA within a homologous yeast region promotes integration and increases the transformation frequency. It is possible that linearization of the vector within the DRp14 sequence promotes integration and therefore transformation. Another vector has been tested that contains part of the central 3bp *Sal* I restriction fragment of the extrachromosomal palindromic ribosomal DNA from *Dictyostelium* (27) but lacks the DRp14 element. In *Tetrahymena*, the central fragment of the extrachromosomal palindromic ribosomal DNA contains an origin of replication (28) that can also act as an *ars* in yeast (29). Although we do not yet know if the *Sal* I fragment from *Dictyostelium* can function as an *ars* in yeast, this vector transforms *Dictyostelium* with the same frequency as does pCERF·DRp14 (unpublished observation).

When there are sufficient cells to first isolate and analyze the transforming DNA, at least some of the vector DNA is extrachromosomal. The copy number of the extrachromosomal DNA is 0.1–2 copies per cell. If the DNA has been modified to inhibit its ability to transform *E. coli*, this value would be an underestimation. In mammalian cells, transformed DNA appears to integrate before cell division takes place (30). Whether the extrachromosomal DNA is replicating autonomously or represents copies derived from an integrated copy is not known. After extended growth of transformed cell lines, no extrachromosomal DNA can be recovered by transformation of *E. coli*.

A high frequency of transformation in *Dictyostelium* (potentially 10^5 transformed cells with an input of 10^9 amoebae) will prove useful in isolating potentially interesting developmental genes by complementation. The availability of this transformation system will also allow us to study the function of specific nucleotide sequences in regulating gene activity by inserting homologous and heterologous genes that have been modified *in vitro*.

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