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Efficient Transformation of *Dictyostelium discoideum* Amoebae

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We have transformed *Dictyostelium discoideum* amoebae by using derivatives of a plasmid, pAG60, which was designed for transformation of mammalian cells. The plasmid carries the promoter region of the herpes simplex virus type 1 thymidine kinase gene linked to the bacterial gene *kan*, which codes for the enzyme aminoglycoside 3'-phosphotransferase. *kan* is derived from the Tn*5* transposon. Expression of the phosphotransferase permits direct selection of transformed cells by their resistance to the antibiotic G-418. pAG60 is incapable of transforming *D. discoideum* but is made transformation proficient by cloning *D. discoideum* sequences into the tetracycline resistance gene. The majority of transformed cells grow and develop normally and differentiate to give G-418-resistant spores. These transformants are unstable and rapidly lose their G-418-resistance during growth in the absence of antibiotic selection. Southern blots show that these unstable G-418-resistant transformants carry the pBR322 and *kan* sequences of pAG60. The pAG60-*D. discoideum* recombinant plasmids used for transformation were constructed in a way that might make them mutagenic. We have isolated several developmental mutants after transformation of *D. discoideum* with libraries of pAG60-*D. discoideum* recombinant plasmids. These mutants are G-418 resistant and carry pAG60 in their nuclear DNA. We recovered a pAG60-*D. discoideum* recombinant plasmid from several developmental mutants. This plasmid transforms *D. discoideum* at an elevated frequency and integrates into the nuclear genome. We speculate that integration can result in insertional inactivation of genes that are essential for differentiation but not for growth. Mutagenic transformation occurred only if the transforming plasmid had homology with *D. discoideum* nuclear DNA. A mammalian cell transformation vector, pSV2-*neo*, carried no *D. discoideum* sequences and was able to transform. However, pSV2-*neo* transformation was not mutagenic. These results suggest that direct inactivation and recovery of genes that are essential for differentiation of *D. discoideum* will be possible.

The lower eucaryote *Dictyostelium discoideum* is a useful organism for studying fundamental problems of developmental biology (13). Amoebae multiply in suspension culture with a 3- to 4-h generation time. Differentiation occurs after amino acid starvation, and multicellular development begins about 8 h after starving amoebae are plated on nonnutrient agar. At that time, chemotactically responsive amoebae signal each other by emitting periodic pulses of cyclic AMP and move toward one another by chemotaxis to form multicellular aggregates of ca. 10^5 cells. The multicellular mass forms a single developmental axis and acquires the properties of a morphallactic field (14). During the course of development, only two cell types emerge: spore cells and stalk cells.

We are attempting to identify genes and underlying molecular mechanisms which control the decision between the spore and the stalk cell developmental pathways. Differentiation is controlled at the level of gene expression by regulation of transcription and translation (1, 2). Several laboratories have isolated developmentally regulated genes from *D. discoideum* and begun to characterize their structure and mode of regulation (15, 18, 22). A large number of interesting developmental mutants have been obtained. Functional analysis of cloned genes, as well as identification of essential differentiation genes by in vivo complementation of developmental mutants, awaits the development of an efficient method for DNA-mediated transformation of *D. discoideum* amoebae.

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The antibiotic G-418 inhibits protein synthesis in both procaryotic and eucaryotic cells. The kanamycin resistance genes carried by transposons Tn*601* and Tn*5* encode aminoglycoside 3′-phosphotransferases which inactivate G-418 and other 2-deoxystreptamine antibiotics related to kanamycin (10). Jiminez and Davies (10) showed that transfection of yeasts with a plasmid containing Tn*601* yields a small fraction of cells which are resistant to G-418. Colbère-Garapin et al. (5) and Southern and Berg (20) have constructed pBR322-derived plasmids that express the kanamycin resistance gene of Tn*5* from the thymidine kinase promoter region of the herpes simplex virus or from the simian virus 40 early gene promoter region. Both vectors permit transformation of mouse, monkey, and human cell lines to G-418 resistance. We wanted to develop for *D. discoideum* a similar transformation method which uses a dominant selectable marker such as G-148 resistance.

In this paper we present a method which permits efficient DNA transformation of *D. discoideum* amoebae. While our work was in progress, Hirth et al. reported *D. discoideum* transformation by pBR322 derivatives carrying a probable *D. discoideum* replication origin and a bacterial *kan* gene coupled to a *D. discoideum* actin gene promoter (9). Our method differs from theirs by its much higher transformation frequency and by its reliance on mammalian viral promoters for expression of the *kan* gene. We also report a simple cloning procedure which makes transforming plasmids mutagenic. In the future, these methods should permit us to inactivate and recover genes which are essential for normal differentiation.

MATERIALS AND METHODS

***D. discoideum* strains and culture conditions.** DdB, a subclone of NC-4 (wild type), is our standard type. DdC is a cycloheximide-resistant derivative of DdB. Both were obtained from R. Kessin. Amoebae were grown at 22°C in KPM buffer (3) containing high concentrations of *Escherichia coli* B/r (final absorbancy at 595 nm [A_{595}] = 15). *E. coli* B/r was grown in NZY medium (10 g of NZamine, 5 g of yeast extract, 10 g of NaCl per liter of water) to stationary phase and allowed to settle overnight at 4°C. The cleared supernatant was decanted. The sediment was concentrated by centrifugation, washed in KPM buffer, suspended in KPM buffer at an A_{595} of 50 to 60, and stored at 4°C for up to several weeks. Development on nonnutrient agar and cell cloning were as previously described (19).

Bacterial strains and plasmids. *Escherichia coli* 1106 (803 *hsdR hsdM*) carrying plasmid pAG60 was obtained from Axel Garapin (5). Plasmid pSV2-*neo* was obtained from Paul Berg through R. Kucherlapati. *E. coli* LA102 (C600 *hsdR hsdM supF recBC*), obtained from P. Tiollais (17), was used as a recipient for bacterial transformation.

Enzymes and reagents. Restriction endonucleases and T4 DNA ligase were obtained from Bethesda Research Laboratories. All ligations and endonuclease digestions were in TA buffer (33 mM Tris acetate, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 0.1 mg of bovine serum albumin per ml; pH 7.9) as recommended by O'Farrell et al. (16). [α-^{32}P]dCTP (2,000 Ci/mmol) was from Amersham Corp. Reagents for nick translation were purchased as a kit from New England Nuclear Corp. G-418 (Schering Corp.) contained 0.449 mg of G-418 per mg of solid. Amounts of G-418 referred to in this report were the total solid, not the weight of the active component.

Isolation and analysis of DNA. Nuclear DNA of *D. discoideum* was prepared from cells grown in suspension with *E. coli* B/r. After repeated washes in KPM buffer to remove bacteria, amoebae were suspended in 5% sucrose–0.5 mM EDTA–5 mM magnesium acetate–25 mM Tris-hydrochloride (pH 7.6) and lysed by adding Triton X-100 to a final concentration of 0.4%. Nuclei were isolated and lysed by the method of Firtel (6). Nuclear DNA was purified by two cycles of banding on CsCl-ethidium bromide gradients. Plasmid DNA was obtained from chloramphenicol-induced cultures by the EDTA-Triton X-100 lysis method (11) and was purified by two cycles of equilibrium banding on CsCl-ethidium bromide gradients. Analytical DNA extraction has been described by others (4). DNA electrophoresis was in 0.7% agarose contained in TAE buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA; pH 8.1) or TBE buffer (89 mM Tris borate, 2.5 mM EDTA; pH 8.2).

Cloning of *D. discoideum* sequences into pAG60. Three samples of *D. discoideum* nuclear DNA (10 μg) in TA buffer were digested separately with 10 U each of *Sau*3A, *Hpa*II, and *Taq*I for 1 h at 30, 37, and 65°C, respectively. Endonucleases were inactivated by heating at 65°C, except for *Taq*I, which was inactivated by treatment with diethyl pyrocarbonate (1/10 volume of 1% diethyl pyrocarbonate in ethanol). The products of each digestion were then ligated in their same tubes overnight at 4°C with 10 U of T4 DNA ligase. Each sample of randomized genomic DNA was digested with 10 U each of *Bam*HI and *Hin*dIII for 1 h at 37°C. All of the above reactions were in TA buffer and did not require intervening steps such as phenol extraction, ethanol precipitation, or buffer changes. pAG60 (10 μg) in TA buffer was digested with 10 U each of *Bam*HI and *Hin*dIII for 1 h at 37°C. *D. discoideum* sequences were cloned into pAG60 by mixing 1 μg of *Bam*HI-*Hin*dIII-digested pAG60 with separate 4-μg samples of *Taq*I, *Sau*3A, and *Hpa*II-digested DNA which was randomized and redigested with *Bam*HI and *Hin*dIII as described above. The mixtures were ligated overnight at 4°C in TA buffer with 5 U of T4 DNA ligase. The separate ligation mixtures were used to transform *E. coli* LA102, giving 10^5 to 10^6 transformants per μg of DNA. Ampicillin-resistant transformants were selected on L broth plates containing 100 μg of penicillin per ml, and recombinants (tetracycline sensitive) represented 30 to 50% of the total number of transformants. Analytical preparations of plasmids from several transformants showed *D. discoideum* inserts ranging from 1 to 7 kilobases (kb).

DNA transformation of *D. discoideum* amoebae. The following were our standard protocols. Vegetative amoebae growing in suspensions of *E. coli* B/r were

washed free of bacteria by four or more low-speed centrifugations in KPM buffer, suspended in 50 mM $CaCl_2$ at a concentration of 5×10^6 cells per ml, and incubated on ice for 20 min. The cells (20 ml) were centrifuged at 1,000 × *g* for 5 min at 4°C. Amoebae (2.5×10^7) were suspended by adding 300 μl of cold 100 mM $CaCl_2$. Plasmid DNA (2 to 25 μg) in 20 to 40 μl was added, and the mixture was maintained on ice for 60 min.

Treated cells were processed in one of two ways. In protocol I, independent clones of G-418-resistant transformants were obtained by plating dilutions of the mixture onto lawns of autoclaved *Enterobacter aerogenes* in the presence of 40 μg of G-418 per ml as described previously (9). Plaques appeared after 3 days at 22°C.

Protocol II was used when the goal of an experiment was to test whether a single recombinant plasmid or a library was capable of transforming *D. discoideum*. Treated amoebae were suspended by adding the entire transformation mix into 100 ml of KPM buffer (22°C) containing sufficient *E. coli* B/r to given a final A_{595} of 15. This culture was shaken on a New Brunswick Gyrotory shaker at 250 rpm for 16 to 18 h at 22°C. After overnight growth in the absence of drug, transformants expressing aminoglycoside 3'-phosphotransferase were selected by diluting the culture to 10^4 to 10^5 amoebae per ml and adding G-418 to a final concentration of 40 μg/ml. The culture was maintained in log phase (1×10^4 to 5×10^6 amoebae per ml) by dilution into KPM buffer containing G-418 (40 μg/ml) and *E. coli* B/r (A_{595} = 15). Growth of amoebae was monitored by directly measuring cell number with a hemacytometer. Transformation was also successful when G-418 was added immediately upon dilution of the transformation mix into KPM buffer containing *E. coli* B/r. Successful transformation required rapid growth of treated amoebae. Transformants were not obtained when growth was slow, and transformants died quickly when cultures entered stationary phase.

We prefered to select G-418-resistant transformants in suspension culture, rather than on plates, because less of this expensive drug was required in suspension cultures. Independent transformants were isolated by separate transformation experiments or by diluting a transformation mixture immediately before growth into separate suspension cultures.

Determining transformation efficiency. The fraction of treated cells that became transformed was determined in two ways. A sample of 2.5×10^7 cells was transformed as described above with either pBM7.6.1 or a *D. discoideum* library in pAG60. Transformed cells were diluted serially into a series of 40-ml cultures of KPM buffer containing G-418 (40 μg/ml) and *E. coli* B/r (A_{595} = 15). The cultures were shaken (250 rpm) at 22°C as above and were monitored for growth for the next 7 to 15 days. The smallest number of cells which yielded a transformed culture was noted. Alternatively, serial dilutions of the transformation mixture were plated clonally onto lawns of autoclaved *E. aerogenes* in the presence of 40 μg of G-418 per ml as outlined above. Controls were treated identically except that either no plasmid DNA was added or pAG60 supercoiled DNA not containing *D. discoideum* inserts was added.

Recovery of plasmids from developmental mutants. Nuclear DNA was obtained from G-418-resistant amoebae by the nuclear isolation and CsCl-ethidium bromide banding methods described above. LA102 (10^8 cells) was transformed with 1 or 2 μg of nuclear DNA by using a common protocol (4). Amp^+ transformants were selected and screened for tetracycline sensitivity as described above. Plasmid DNA was recovered by amplification as described before.

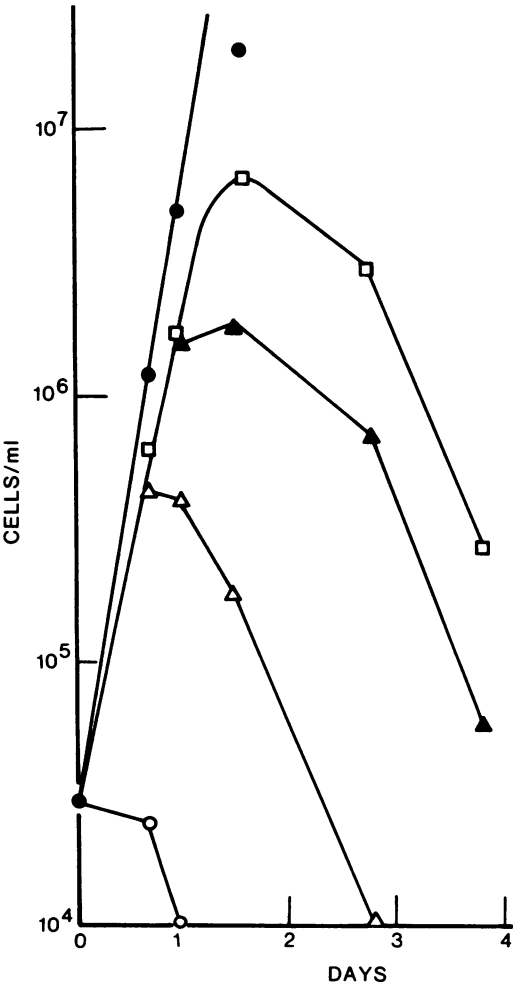

FIG. 1. Sensitivity of *D. discoideum* amoebae to G-418. *D. discoideum* amoebae growing exponentially in suspension cultures of *E. coli* B/r were diluted into 10-ml cultures containing *E. coli* B/r (A_{595} = 15) and various concentrations of G-418: (●) none, (□) 20 μg/ml, (▲) 30 μg/ml, (△) 40 μg/ml, or (○) 50 μg/ml. Cultures were incubated at 22°C in a New Brunswick Gyrotory shaker (250 rpm). Cell number was measured with a hemacytometer. In cultures like these which have large amounts of bacteria, amoebae grow linearly up to 1.5×10^7 to 2.0×10^7 amoebae per ml.

RESULTS

Growing amoebae were sensitive to low levels of G-418 (Fig. 1). Cell death and lysis were rapid in the presence of G-418 at 50 μg/ml. Death was

delayed by 1 to 2 days at slightly lower concentrations and occurred after three to four generations when G-418 was present at 40 μg/ml. We used either 20 or 40 μg/ml for all subsequent experiments. *D. discoideum* amoebae appeared slightly more sensitive than mouse L, simian Vero, or HeLa cells to low concentrations of G-418 (5).

We were unable to detect amoebae which were spontaneously resistant to G-418 at 25, 50, or 100 μg/ml in four attempts to detect such mutants in suspension cultures containing at least 2.5×10^7 amoebae. This was in accord with the failure of others to find spontaneous resistant mutants of mouse L cells (frequency, $<4 \times 10^{-8}$) (5).

Transformation of *D. discoideum* amoebae. Plasmid pAG60 (Fig. 2) efficiently transforms mouse L cells, making them resistant to G-418. We tested its ability to transform *D. discoideum* amoebae by adding 25 μg of supercoiled pAG60 to 2.5×10^7 amoebae, using protocol II outlined above. We anticipated that the heterologous viral *tk* promoter might be inefficient in *D. discoideum*; therefore, we used the lowest concentrations of G-418 which were convenient for selection, 40 and 20 μg/ml. In spite of this precaution, all treated cultures died within 3 to 4 days. G-418-resistant cells failed to appear even after a total of 14 to 21 days of culturing. Thus, pAG60 did not yield detectable transformants of *D. discoideum*.

We expected that stable transformants would result if pAG60 contained *D. discoideum* DNA sequences that permitted integration of the plasmid in the nuclear genome. If the herpes simplex virus *tk* promoter did not act in *D. discoideum*, such transformants would go unnoticed because they would not be selectable by G-418 resistance. However, if the *tk* promoter did function and integration was mutagenic as a result of insertional inactivation of genes or sequences that are essential for differentiation but not for growth, then it should be possible to detect transformants by screening for developmental mutants. Therefore, we constructed pAG60-*D. discoideum* recombinants in a way that should make many of these recombinant plasmids mutagenic.

Figure 3 outlines our strategy. The *D. discoideum* nuclear genome was randomized by digesting nuclear DNA with *Taq*I, *Sau*3A, or *Hpa*II in three separate reactions. After religation, complete double digestion with *Bam*HI and *Hin*dIII was performed. *Bam*HI-*Hin*dIII fragments from the three randomized genomes were cloned into the tetracycline resistance gene of pAG60. Many recombinant plasmids were expected to carry at least one *D. discoideum* gene sequence that was an internal fragment of a nuclear gene. Insertion of the plasmid into this internal region by recombination was expected to inactivate the gene. Having several gene fragments in each plasmid was expected to increase the number of genes targeted by each plasmid molecule.

D. discoideum amoebae treated with a mixture of 35 different pAG60-*D. discoideum* recombinant DNA plasmids were efficiently transformed to a G-418-resistant phenotype. Figure 4 shows the results of two experiments. In the absence of selection with G-418, amoebae treated with the recombinant plasmid preparation grew as well as untreated normal cells (generation time, 3.2 h). Cells untreated with recombinant DNA died quickly in the presence of 40 μg of G-418 per ml. However, cultures of cells treated with the mixture of recombinant plasmid DNA continued to grow when G-418 was added to 40 μg/ml after growth for 16 to 18 h in the absence of selection. Usually cultures of transformed cells grew for two to four generations at a normal rate. This was followed by 6 to 12 h of slower growth or decreasing cell numbers and then by a return to normal growth rates. This pattern was expected if only a subset of all cells are transformed to G-418 resistance. For convenience, we selected transformed amoebae in suspensions of living *E. coli* B/r; however, efficient transformation also occurred if transformed cells were selected in suspensions of autoclaved *E. coli* B/r. Calcium chloride treat-

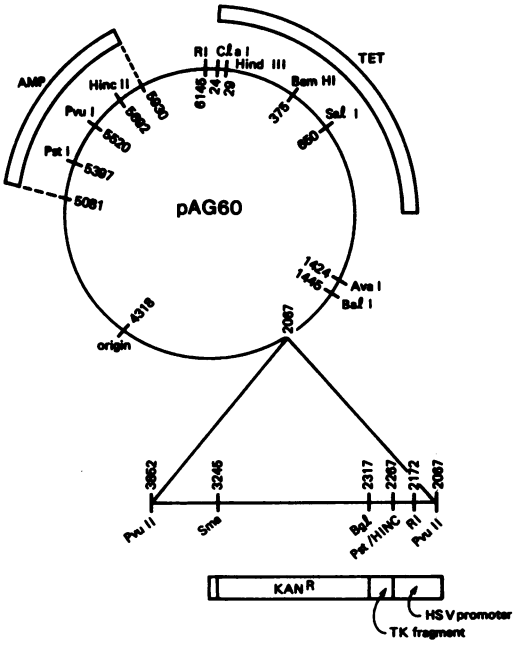

FIG. 2. Structure of plasmid pAG60. TET, tetracycline; HSV, herpes simplex virus.

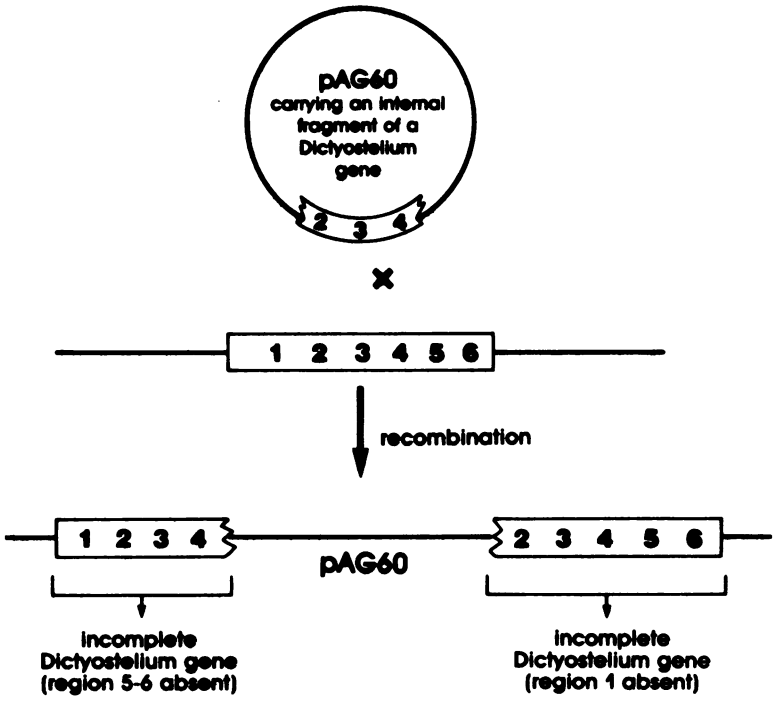

FIG. 3. Insertional inactivation of *D. discoideum* genes. Plasmid pAG60 is shown carrying a fragment of a *D. discoideum* gene. The entire gene is shown to be composed of six regions, 1 through 6. Homologous recombination between the intact nuclear gene and the gene fragment (regions 2 through 4) carried by the plasmid leads to insertion of the plasmid with the formation of two incomplete genes on each side of the plasmid. If the inactivated gene is nonessential for growth, stable, G-418-resistant transformants will grow in the presence of the antibiotic G-418.

ment, per se, was not harmful (50 to 70% survival), and cells remained G-418 sensitive after calcium treatment.

Occasionally (ca. 10% of the time) control suspension cultures of amoebae that received drug (40 μg/ml) but no transforming DNA were not completely killed. Their cell number declined for several days but began to increase after 5 to 7 days in culture. When this occurred, the control cultures were diluted 10-fold into suspension cultures containing fresh drug (40 μg/ml) and were incubated with shaking at 22°C. Invariably, all cells died. Occasional growth of control cultures probably resulted from our need to use the minimal effective concentration of drug which killed normal cells. This was required because transformants did not grow in G-418 concentrations of 75 μg/ml or higher.

The frequency of transformation was determined by serial dilution and direct plating. Both procedures showed that ca. 1% of all amoebae were transformed when 2×10^7 cells were treated with 25 μg of pBM7.6.1, a transformation-proficient pAG60-*D. discoideum* recombinant vector described in detail below, or with a mixture of 10 plasmids which included pBM7.6.1. Thus, ca. 10^4 transformants per μg of plasmid DNA were obtained under these conditions. Similar results were obtained with as little as 2 μg of pBM7.6.1 DNA.

Stability of transformants. Cultures of transformed cell grew for an unlimited number of generations in the presence of 40 μg of G-418 per ml and displayed a normal generation time of ca. 3.5 h. When differentiation was induced within a few days after transformation by plating transformed amoebae onto nonnutrient agar, normal differentiation and morphogenesis occurred in the presence and in the absence of G-418 to produce spores that were resistant to G-418 (40 μg/ml). Because nontransformed cells failed to differentiate in the presence of G-418 (40 μg/ml), the plasmid must have persisted within the transformed amoebae during differentiation. Differentiation was usually abnormal when transformed cultures were grown for more than a week under drug selection before being plated onto nonnutrient agar. We will show below that this was the result of selection during growth for stable transformants, many of which were developmental mutants.

Although G-418 resistance was stable during differentiation, drug resistance was unstable during growth in the absence of selection. G-

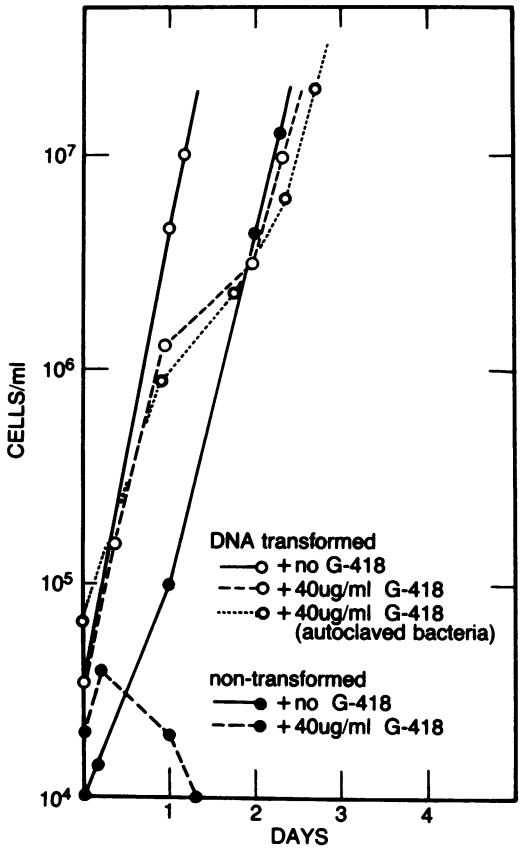

FIG. 4. Effect of G-418 on the growth of DNA-transformed and nontransformed cells. Amoebae pretreated with 50 mM and 100 mM $CaCl_2$ were divided into two portions. One portion (nontransformed) was diluted into suspension cultures containing *E. coli* B/r at A_{595} = 15. The other portion (DNA transformed) was treated as indicated in the text with supercoiled pAG60-*D. discoideum* recombinant DNA. Treated cells were diluted into suspension cultures containing *E. coli* B/r as above. After overnight growth (four generations) at 22°C with shaking at 250 rpm, each culture was divided into two portions and diluted into *E. coli* B/r (A_{595} = 15) containing either no drug or 40 μg of G-418 per ml. Cell number was monitored with a hemacytometer during subsequent growth at 22°C (250 rpm).

418-resistant spores or amoebae were plated clonally on lawns of bacteria, and after ca. 40 generations, cells from the edges of more than 20 plaques were used to inoculate separate suspension cultures containing 40 μg of G-418 per ml. None produced a G-418-resistant culture.

Presence of plasmid sequences in G-418-resistant cells. The above results indicated that a library of pAG60-*D. discoideum* recombinant plasmids was able to transform *D. discoideum*, although pAG60 itself could not. We sought individual pAG60-*D. discoideum* recombinant plasmids that were transformation proficient by testing the transformation capacity of a smaller mixture of plasmids. A mixture of pAG60 and 35 plasmids prepared by randomization of the nuclear genome with *Taq*I (10 plasmids), *Hpa*II (5 plasmids), and *Sau*3A (20 plasmids) as described above was used to transform *D. discoideum* amoebae by protocol II. pAG60 was included originally in the mixture as the carrier. Later experiments showed it to be nonessential for transformation with the transformation-proficient plasmids described later. A population of G-418-resistant amoebae was readily obtained with this mixture. Nuclear DNA from the uncloned population was digested with *Eco*RI, probed with nick-translated pBR322, and subsequently reprobed with a *Sma*I-*Bgl*II fragment corresponding to the kanamycin resistance gene of pAG60. The nuclear DNA of this mixture of G-418-resistant cells contained major bands of 2.2, 2.8, 4.0, and ca. 11.2 kb which had homology with pBR322 (Fig. 5, lane 3). We cannot be certain of the origin of some of these fragments because we used an uncharacterized, complex mixture of plasmids. We interpret them as follows. The 4.0-kb fragment was the large *Eco*RI fragment of pAG60 (and its *D. discoideum* recombinant plasmids), because it carried the *kan* gene. The 2.2-kb fragment was the smaller *Eco*RI fragment of pAG60. This fragment must be absent in pAG60-*D. discoideum* recombinant plasmids, because *D. discoideum* DNA has been inserted between the *Bam*HI and *Hin*dIII sites. Drawing on the results of others, we speculate that the 2.2-kb fragment persisted in these cells as a result of pAG60 undergoing intermolecular recombination with a replication-proficient pAG60-*D. discoideum* recombinant plasmid to form a dimer. Alternatively, this fragment might have contained a 0.35-kb *D. discoideum* sequence that replaced the *Hin*dIII-*Bam*HI fragment of pAG60. The 2.8-kb fragment probably came from a pAG60 recombinant in which the 0.35-kb *Hin*dIII-*Bam*HI fragment within the small *Eco*RI fragment was replaced by a 0.95-kb *D. discoideum* sequence. The 11- to 12-kb *Eco*RI fragment also contained the *kan* gene (cf. lane 3 with lane 6 in Fig. 5). We presume that this fragment resulted from the loss of one *Eco*RI site in a pAG60-*D. discoideum* recombinant plasmid by rearrangement of cloned *D. discoideum* genomic segments in *E. coli* as previously reported (9). If this was the case, then the 11- to 12-kb fragment resulted either from *Eco*RI linearization of a freely replicating pAG60-*D. discoideum* recombinant plasmid deleted for one *Eco*RI site or by *Eco*RI excision of a fragment of a pAG60-*D. discoideum* recombinant plasmid integrated into high-molecular-weight nuclear DNA. We cannot distinguish

these alternatives in this instance; however, we show later that plasmid intergration can occur.

Detection of plasmid sequences in developmental mutants obtained without prior selection. Transformation might occasionally be mutagenic if a plasmid carrying only an internal fragment of a gene, i.e., a sequence devoid of the 5′ and 3′ flanking sequences, inserted via homologous recombination into a nuclear gene (Fig. 3). Transformants of this kind should be relatively stable and should have a developmental defect if the gene is essential for differentiation but not for growth. To test for mutagenic transformation, *D. discoideum* amoebae were transformed with the above mixture of 35 plasmids and grown for 5 days without selection for G-418 resistance. Amoebae were plated clonally, again in the absence of G-418, and clones were observed for developmental defects. Aggregation-defective (Agg) mutants were observed (frequency, ca. 10^{-3}). In contrast, we never observed spontaneous aggregation mutants in nontransformed cultures (frequency, $<10^{-4}$).

Nuclear DNA from one of these mutants contained 2.1-, 2.8-, 4.0-, 5.8-, 7.0-, and 14.2-kb *Eco*RI fragments that are derived from pAG60, as shown by their homology with pBR322 (Fig. 5, lane b, left panel). Only the 4.0-, 5.8-, and 7.0-kb fragments include the *kan* gene (Fig. 5, lane b, right panel). The 4.0-kb fragment can be carried by both integrated and extrachromosomal plasmids. We do not know the origin of the 5.8- and 7.0-kb fragments. However, we think they are likely to result from (i) partial digestion of one or several integrated plasmids, (ii) complete digestion of integrated plasmids that have lost an *Eco*RI site during the integration event by, for example, gene conversion, or (iii) complete digestion of extrachromosomal plasmids that have lost an *Eco*RI site by plasmid rearrangement during propagation. We have not attempted to resolve these possibilities. The 14.2-kb fragment contains plasmid sequences homologous to pBR322 (Fig. 5, lane b, left side), but it did not contain the *kan* gene (Fig. 5, lane b, right side). The 14.2-kb fragment could be a 2.2-kb *Eco*RI fragment of pAG60 with an additional 12.3-kb *D. discoideum* DNA insert that replaced the 0.32-kb *Hin*dIII-*Bam*HI fragment; alternatively, it could be derived from a samller pAG60-*D. discoideum* recombinant plasmid that inserted into the nuclear genome. Although the mutant was obtained without drug selection, it was G-418 resistant even after more than 50 generations of growth in the absence of drug. Spontaneous revertants grew and developed normally and were G-418 sensitive.

Developmental mutants obtained by transformation and drug selection. Because we observed both drug resistance and mutation induction, we concluded that efficient transformation occurs with our procedures. These results were obtained with a mixture of pAG60 and 35 uncharacterized plasmids. We sought transformation-proficient plasmids by testing the transforming ability of a subset of plasmids in this mixture. Transformation would be detected by both drug resistance and mutation induction.

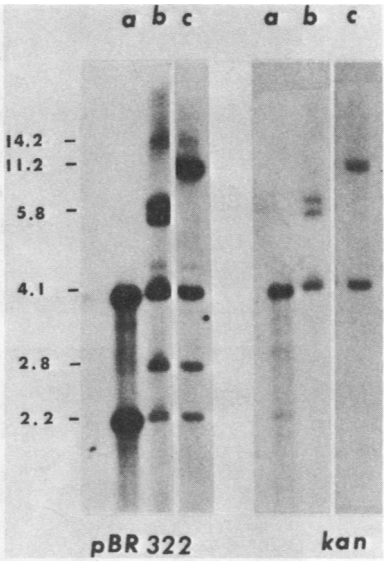

FIG. 5. Southern blot hybridization analysis of transformed *D. discoideum*. Lanes of a horizontal 0.7% agarose gel were loaded with 25 pg of *Eco*RI-digested pAG60 DNA (a) or with 5 μg of *Eco*RI-digested nuclear DNA from Agg⁻ G-418-resistant cells (b) or from G-418-resistant cells (c). After electrophoresis the gel was transferred by the method of Southern onto nitrocellulose paper and hybridized with 10^6 cpm of nick-translated pBR322 per ml (1.6×10^7 cpm/μg) (left lanes). Hybridization was for 48 h at 42°C in 50% formamide–0.75 M NaCl–0.075 M sodium citrate–0.2% bovine serum albumin–0.2% polyvinylpyrolidone–0.2% Ficoll–0.1% sodium dodecyl sulfate–1.21 mM sodium PP_i. Blots were washed and visualized by autoradiography at −80°C for 17 to18 h with Kodak X-Omat film and Cronex intensifying screens. Molecular weight markers (not shown) were from a mixture of *Eco*RI and *Hin*dIII digests of lambda DNA. After the pBR322 hybridization pattern was visualized the nitrocellulose filter was washed twice in 0.015 M NaCl and 0.0015 M sodium citrate at 95°C for 15 min. The clean nitrocellulose filter was hybridized as described above with 5×10^5 cpm of nick-translated kanamycin gene (*Sma*I-*Bgl*II fragment from pAG60) per ml(10^7 cpm/μg) (right lanes).

Vegetative amoebae were transformed by treating 2.5×10^7 cells with 25 μg of a mixture of 10 individual *Taq*I-scrambled recombinants. Transformants were selected for growth in suspension cultures containing 40 μg of G-418 per ml (protocol II). After 12 days under selection, amoebae were cloned on solid medium in associ-

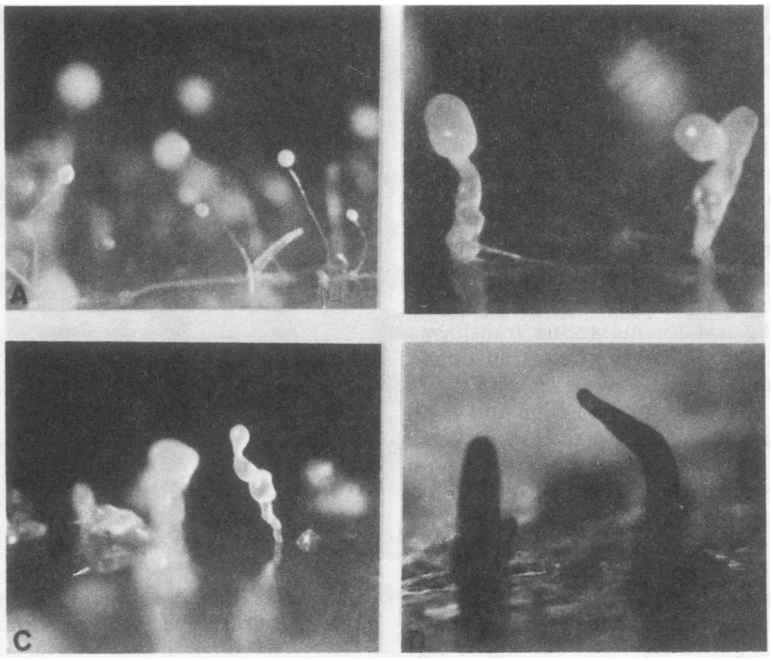

FIG. 6. G-418-resistant transformants. (a) Resistant clone which develops normally; (b) Pmd-3; (c) Pmd-4; (d) Pmd-5.

ation with *E. aerogenes*. Developmental mutants appeared at a frequency of 3.6%. Mutants were either Agg or postmound defective (Pmd). The morphologies of a G-418-resistant clone which develops normally (Fig. 6A) and representative Pmd mutants (Fig. 6B, C, and D) were compared. Phase-contrast microscopy showed that terminal differentiation of both spore and stalk cells was absent in all Pmd mutants. All Pmd mutants were G-418 resistant. Their mutant phenotype was unstable, but it could be maintained by recloning. All Pmd mutants yielded wild-type revertants, which may be G-418 resistant or G-418 sensitive. All Pmd mutants also formed Agg sectors. Agg mutants and Agg segregants of Pmd mutants did not revert or yield sectors with other phenotypes. Agg mutants were G-418 resistant. Two independent G-418-resistant transformants which were wild type for development contained vector sequences in high-molecular-weight DNA (*Xba*I digest) (Fig. 7d and e). These transformants also contained a single *Hin*dIII fragment (*Hin*dIII digest) that was homologous to the pBR322 sequences carried by pAG60 (Fig. 7b and c). This simple pattern suggests that only 1 or a few of the 10 recombinant plasmids used in this experiment were transformation proficient. Two independent Pmd mutants also contained plasmid sequences, but in different high-molecular-weight forms. These plasmid sequences appeared to be inte-

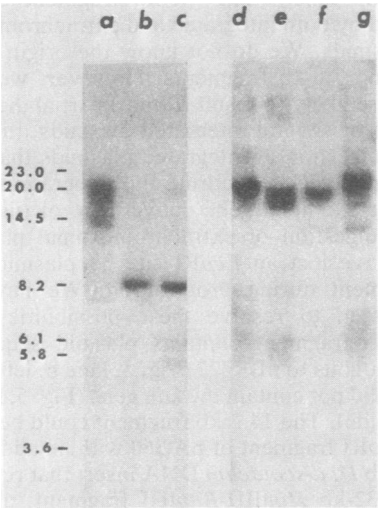

FIG. 7. Southern blot hybridization of *D. discoideum* nuclear DNA. Lanes of a horizontal 0.7% agarose gel were loaded with 5 μg of *Hin*dIII-digested nuclear DNA from (a) a Pmd-4 mutant, (b) wild-type, developing G-418-resistant clone 5 (wt5), (c) wild-type, developing G-418-resistant clone 3 (wt3), or nuclear DNA digested with *Xba*I from (d) wt3, (e) wt5, (f) Pmd-5, or (g) Pmd-4. After electrophoresis, DNA was transferred onto a nitrocellulose filter and hybridized with 2×10^6 cpm of nick-translated pBR322 per ml (1.2×10^8 cpm/μg). Hybridization conditions were as described for Fig. 5.

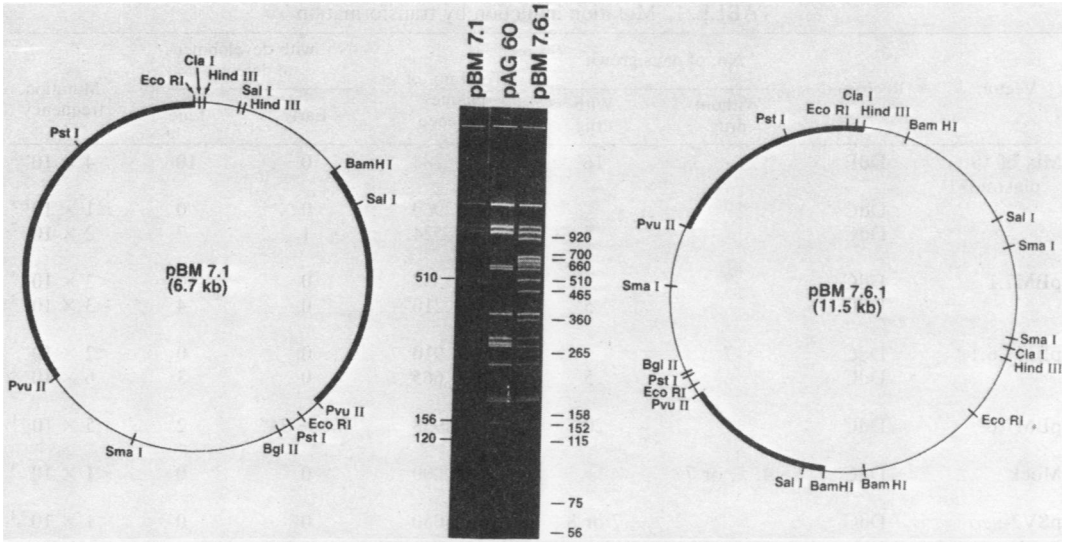

FIG. 8. Structures of plasmids pBM7.1 and pBM7.6.1 and the 5% acrylamide gel electrophoresis pattern of *Taq*I-digested pBM7.1, pAG60, and pBM7.6.1 DNA stained with etidium bromide. Thick lines represent regions of pAG60 that are homologous to pBR322. The narrow line terminated by *Pvu*II sites is the region of pAG60 that has the *tk* promoter and the bacterial *kan* gene. The narrow line terminated by *Hin*dIII and *Bam*HI denotes the *D. discoideum* DNA insert.

grated in nuclear DNA because Southern transfers of uncut Pmd mutant DNA electrophoresed on 0.7% agarose gels showed the sequences to be present in fragments larger than 23 kb (data not shown). We conclude that this mixture of 10 recombinant plasmids contains at least 1 transformation-proficient plasmid.

Transformation with plasmids recovered from developmental mutants. We recovered two transformation-proficient plasmids from the nuclear DNA of several developmental mutants. Nuclear DNA prepared from four G-418-resistant, normally developing cells and from five Pmd mutants was used without further treatment, *i.e.*, no restriction digestion or religation, to transform *E. coli* LA102. amp$^+$ transformants were obtained from the nuclear DNA of Pmd-3, -4, -5, and -11. Plasmid pBM7.6.1 was recovered from each of these mutants as an Amp$^+$ bacterial transformant (Fig. 8). It contains the restriction sites of pAG60 and includes 12 additional *Taq*I fragments within the *D. discoideum* DNA insert. This plasmid has a restriction pattern which is identical to that of pBM7.6, 1 of the 10 recombinant plasmids used in the transformation experiment that gave us the developmental mutants Pmd-3, -4, -5, and -11. We conclude that pBM7.6 and pBM7.6.1 are the same plasmid. A second plasmid was recovered only from Pmd-4. It contains a small insert, ca. 0.5 kb. Because this second plasmid may be a degradation product of pBM7.6.1, it has not been studied further, except to show that it is transformation proficient. We sought additional transforming plasmids by testing the transformation ability of all of the 10 *Taq*I-scrambled recombinant plasmids. Only two, pBM7.1 and pBM7.6, were able to transform *D. discoideum* amoebae (protocol II). pBM7.1 also contains the restriction sites of pAG60 and includes three additional *Taq*I fragments within the *D. discoideum* insert (Fig. 8).

Mutagenic transformation by recombinant plasmids. The presence of pBM7.6.1 in several developmental mutants led us to ask whether this plasmid is mutagenic. Two different strains of *D. discoideum* were transformed (protocol II) and grown with or without selection for G-418 resistance. If pBM7.6.1 or other plasmids were mutagenic, we expected that morphological developmental mutations would appear with increased frequency. This frequency might be quite low because only a few genes can be represented in the 12 *Taq*I fragments carried by pBM7.6.1; however, coselection for G-418 resistance should increase this frequency.

Table 1 summarizes several transformation experiments. Treatment of amoebae with calcium chloride but not with plasmid DNA did not induce mutations. In contrast, pBM7.1, pBM7.6, and the plasmid recovered from developmental mutants, pBM7.6.1, did induce developmental mutations. Selection for drug resistance should have increased the frequency of mutations by eliminating untransformed cells or unstable transformants that had lost the plasmid. Cultures transformed with pBM7.6.1 and main-

TABLE 1. Mutation induction by transformation

Vector	Recipient	No. of days grown		Total no. of plaques screened	No. with developmental defect		Mutation frequency
		Without drug	With drug		Early	Late	
Mix of 10 plasmids	DdB		16	284	0	10	4×10^{-2}
	DdC	7		960	0	0	$\leq 1 \times 10^{-3}$
	DdC		5	524	1	7	2×10^{-2}
pBM7.1	DdC	7		557	0	0	$<2 \times 10^{-3}$
	DdC		5	1,210	0	4	3×10^{-3}
pBM7.6.1	DdC	7		910	0	0	$<2 \times 10^{-3}$
	DdC		5	665	0	3	6×10^{-3}
pBM7.6	DdC		20	938	404 + 34[a]	2	5×10^{-1}
Mock	DdC	4, 5, or 7		>10,000	0	0	$<1 \times 10^{-4}$
pSV2-*neo*	DdC		7 or 8	1,050	0	0	$<1 \times 10^{-3}$

[a] A total of 404 mutants are "flat" Agg and 34 mutants are "rippled" Agg.

tained under G-418 selection for 5 days contained Pmd mutants (0.6%) but no Agg mutants (0 of/665). When drug selection was maintained for 20 days, pBM7.6 (equivalent of pBM7.6.1) gave many Agg mutants (43%). In contrast, pBM7.1 generated late-development mutants, but none was defective for aggregation. Mutagenesis was host strain independent. Another plasmid, pSV2-*neo*, did not induce mutations, although it did transform (see below). Finally pAG60, which does not transform, did not induce mutations (data not shown). We selected mutants in suspension culture, rather than on plates, to conserve drug. Independent mutants were obtained by diluting a transformation mix into separate suspension cultures.

Mutagenic transformation by plasmid pBM7.6.1 was associated with insertion of the plasmid into *D. discoideum* nuclear DNA (Fig. 9). A population of amoebae transformed by this plasmid contained *Hin*dIII fragments, larger than or equal to 8.2 kb, which were homologous with the plasmid. Several repetitions of this experiment utilizing several exposure periods show many discrete fragments larger than 8.2 kb. Fragments larger than 8.2 kb could have arisen only from plasmids that had integrated into nuclear DNA by recombination in region I. Integration via this route predicts that a small *Hin*dIII fragment (3.3 kb) will be present. Probes homologous to region II of pBM7.6.1 detected this *Hin*dIII fragment in transformants, but it was much less abundant than the large 8.2-kb *Hin*dIII fragment (data not shown). The 8.2-kb *Hin*dIII fragment could have originated from freely replicating plasmids or from plasmids inserted by recombination in region II (Fig. 9). The underrepresentation of the 3.3-kb *Hin*dIII fragment in transformants (relative to the 8.2-kb *Hin*dIII) suggests that most insertion events are in region II, as expected from the larger size of this region compared with region I.

The majority of transformants obtained with pBM7.6.1 were unstable. A culture was transformed (protocol II) with pBM7.6.1 and grown under G-418 selection for 8 days before being divided into two portions. One portion was grown for an additional 9 days in drug; the other was grown for 9 days in the absence of drug. Cells grown continuously in drug contained *Hin*dIII fragments (>8.2 kb) which were homologous to pBR322 sequences in the plasmid (Fig. 10, lane c, left side). In contrast, the very small amount of hybridization obtained with DNA of cells from which selection had been removed (Fig. 10, lane d, left side) was probably due to rearranged plasmid sequences. A cloned, stable, G-418-resistant transformant contained several high-molecular-weight *Hin*dIII fragments (Fig. 10, lane b, left side).

Figure 10 also provides preliminary evidence for plasmid insertion by homologous recombination. The 6.6- and 1.7-kb *Hin*dIII fragments from nontransformed *D. discoideum* nuclear DNA were homologous to pBM7.6.1 (Fig. 10, lane e, right side). These *Hin*dIII fragments would be absent in transformed cells if the plasmid had inserted into them by homologous recombination. The 6.6- and 1.7-kb *Hin*dIII fragments were present in cloned G-418-resistant transformants that had either normal development (Fig. 10, lane b, right side) or a late developmental defect (Pmd-4; (Fig. 10, lane a, right side). However, in a population of transformed cells that had been

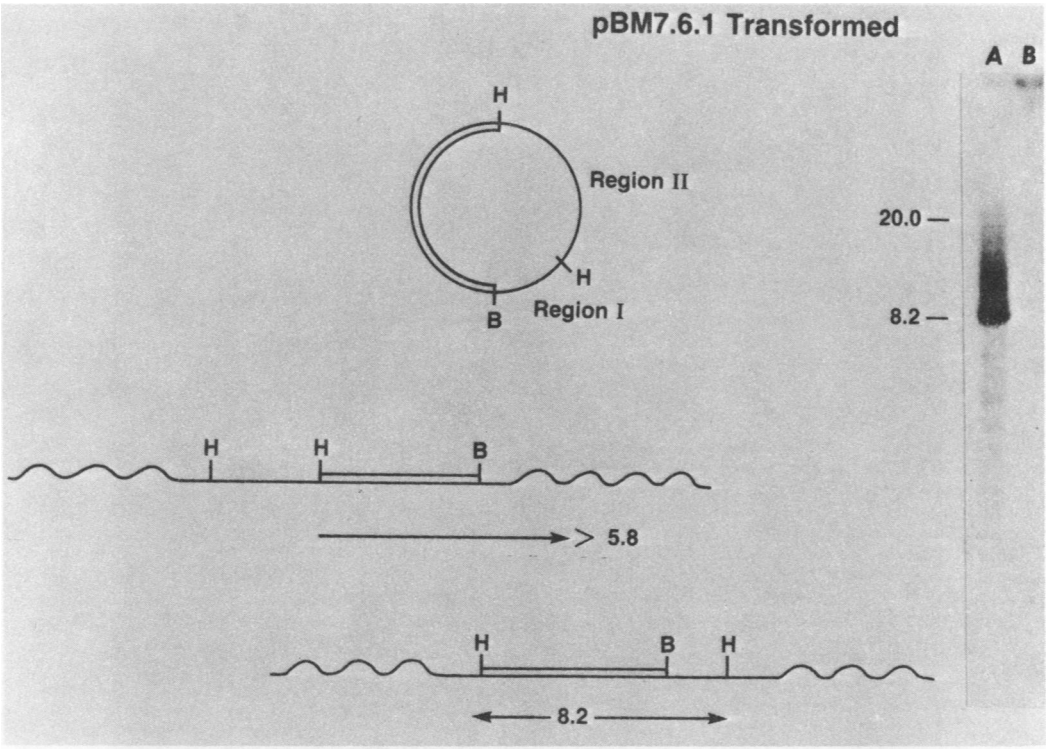

FIG. 9. Insertion of plasmid into nuclear DNA. (a) Nuclear DNA (5 μg) from amoebae transformed with pBM7.6.1 and selected for G-418 resistance (protocol II) for 17 days; (b) nuclear DNA (5 μg of untransformed amoebae. A southern transfer was hybridized with pBR322 (1.2×10^8 cpm/ml) by the method noted for Fig. 5. Double lines represent pAG60 sequences, single straight lines are cloned *D. discoideum* sequences, and wavy lines are *D. discoideum* nuclear sequences that are not in the recombinant plasmid.

under drug selection for 17 days, the 1.7-kb fragment was almost completely absent, the amount of 6.6-kb fragment was diminished, and new high-molecular-weight fragments appeared (Fig. 10, lane c, right side).

Transformation by pSV2-*neo*. The preceding results show that some pAG60-*D. discoideum* recombinants efficiently transform *D. discoideum*, as judged by drug resistance and mutagenesis. A nonmutagenic vector is required for transformation-mediated genetic complementation of developmental mutants. Instead of constructing a nonmutagenic, replication-proficient pAG60-*D. discoideum* rcombinant vector (by cloning large inserts), we tested the transforming ability of pSV2-*neo*. pSV2-*neo* transformed *D. discoideum* amoebae with an efficiency comparable to that of pBM7.6.1, but it did not induce mutations (Table 1). pSV2-*neo* had two intracellular forms, replicative form (RF) III (6 kb) and RFII (9.2 kb). Supercoils (RFI) were not observed (Fig. 11). Hirth and his colleagues also failed to see RFI forms in transformed *D. discoideum* amoebae even though transforming plasmids were recovered (9).

DISCUSSION

Our first goal was to establish an efficient method for DNA-mediated transformation of *D. discoideum*. It should be possible to develop a transformation system based on complementation of several auxotrophs which have been isolated in an axenic strain by Franke and Kessin (7). However, a large number of important developmental mutants which ought to be analyzed by transformation methods have been isolated in the wild-type strain NC-4 or its derivatives. Crossing developmental mutations into an appropriate auxotrophic background before transformation studies begin would be difficult in many cases. However, a transformation method based upon a dominant selectable marker, such as G-418 resistance, permits analysis of developmental mutants in both axenic and nonaxenic strains. Hirth et al. (9) recently described a transformation procedure based on selection of G-418-resistant *D. discoideum* amoebae. Our method differs from theirs by being simpler and more efficient.

Our transformation method gives transform-

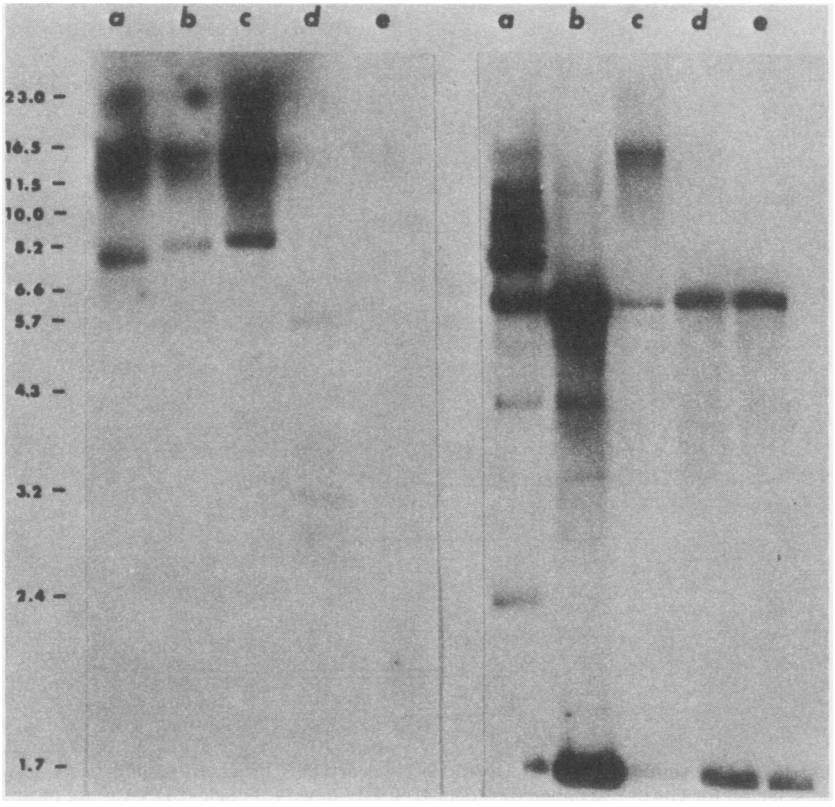

FIG. 10. Southern blot hybridization of nuclear DNA from normal and mutant G-418-resistant cells and wild-type nuclear DNA. Each lane of a horizontal 0.7% agarose gel was loaded with 5 μg of *Hin*dIII-digested *D. discoideum* nuclear DNA from (a) G-418-resistant Pmd-4 mutant cells, (b) wild-type, G-418-resistant cells, (c) cells transformed with pBM7.6.1 and selected for 17 days in G-418, (d) cells transformed with pBM7.6.1 and selected for 8 days in G-418 followed by 9 days without selection, and (e) wild-type, nontransformed cells. After electrophoresis, DNA was transferred onto nitrocellulose paper and hybridized with pBR322 (2×10^6 cpm/ml; 1.4×10^8 cpm/μg). After the pBR322 hybridization pattern was visualized, the nitrocellulose paper was washed as described above and rehybridized with pBM7.6.1 (2×10^6 cpm/ml; 1.5×10^8 cpm/μg). Hybridization conditions were the same as those described for Fig. 5.

ants with a high efficiency (ca. 1% of treated cells and 10^4 transformants per μg of DNA). We have not tested the calcium phosphate precipitation method of transformation (21) for we expect that DNA precipitates will be efficiently destroyed in the lysosomes after ingestion by amoebae, such as *D. discoideum*, which are adapted for feeding by endocytosis. The commonly employed method of transforming yeasts in the presence of polyethylene glycol (8) and our variations of this method were unsuccessful, probably as a result of delayed polyethylene glycol toxicity. Neither were we able to transform amoebae by fusion with bacterial spheroplasts (19).

Plasmid pAG60 is able to transform mouse, simian, and human cultured cells by integration in the nuclear genome to give ca. 100 stable transformants per μg of DNA (5). We should easily detect *D. discoideum* transformation at this level; however, we never obtained transformants when amoebae were treated with pAG60 (frequency, $< 2 \times 10^{-7}$). pAG60 becomes transformation proficient only after *D. discoideum* sequences are cloned into the *tet* gene. These sequences might have roles in integration, replication, or other functions, but in any case they permit efficient transformation. The ability of pSV2-*neo* to transform *D. discoideum* amoebae suggests that viral replication origins may provide one functional requirement for plasmid propagation in *D. discoideum*.

The high efficiency of this transformation method should make complementation of developmental mutants possible. Even though most transformants are unstable during growth, G-418-resistant amoebae differentiate normally in the absence of drug to yield G-418-resistant spores. Thus, the plasmid persists during the 24-h interval during which complete differentiation

occurs. Therefore, we expect that complementation of many developmental mutants will be possible and that the complementing recombinant plasmid can be recovered after the resulting spores are germinated in growth medium containing G-418. pSV2-*neo* is an appropriate cloning vector for complementation studies because it is nonmutagenic.

Our second goal was to create mutagenic vectors that yield clearly defined developmental mutants. Plasmids that carry randomized *D. discoideum* sequences are mutagenic. Mutagenesis is probably due to insertion of the plasmid into *D. discoideum* genes, rather than to induction of error-prone DNA repair by introduction of exogenous DNA into cells. Support for this conclusion is provided by (i) pBM7.6.1, which gives rise to developmental mutants like the ones from which it was derived, (ii) pBM7.1, which yields a different set of developmental mutants, and (iii) plasmids like pAG60 and pSV2-*neo* that have no homology with *D. discoideum* and are not mutagenic. Preliminary evidence suggests that insertion is by homologous recombination.

The developmental phenotype of G-418-resistant transformants generated by pBM7.6.1 is unstable. G-418-resistant, normally developing clones sector to give Agg and Pmd mutants. Most Pmd mutants decay to an Agg phenotype. In addition to an inserted plasmid, many Pmd mutants apparently contain extrachromosomal copies of pBM7.6.1 because this plasmid can be recovered without our attempting to form circular plasmids by restriction and ligation of mutant nuclear DNA. We speculate that Pmd mutants result from integration of one copy of this plasmid into a gene that is essential for postmound differentiation. Subsequent integration of another copy of this plasmid into a gene required for aggregation converts Pmd mutants to the Agg phenotype. In support of this interpretation, Northern transfers show that pBM7.6.1 is homologous to several developmentally regulated polyadenylated RNAs, and the most abundant of these is maximally expressed at the aggregation stage (E. Meller and S. L. Barclay, unpublished data). pBM7.6.1 has no apparent homology to vegetative polyadenylated RNA. Since G-418 resistance is much more stable in Pmd and Agg mutants than in normally developing, G-418-resistant transformants, cultures initiated by transformation with mutagenic plasmids are expected to accumulate developmental mutants.

These results suggest two applications of our method. First, a library of small randomized *D. discoideum* genomic sequences in pSV2-*neo* might serve as a general mutagen. One could screen or select a desired developmental mutant and from it recover a plasmid that carrries a portion of the targeted gene. Second, many cDNA probes may be mutagenic because most are incomplete at both the 5′ and 3′ ends. Thus, one could determine the functional role of differentiation genes which currently are identified only as cDNA or genomic clones.

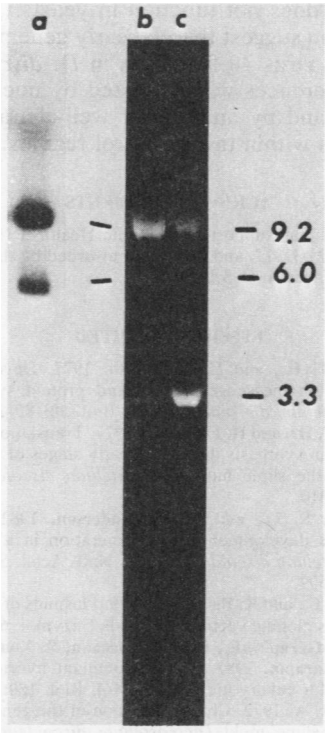

FIG. 11. Intracellular forms of pSV2-*neo*. (a) Nuclear DNA from a population of pSV2-*neo D. discoideum* transformants was Southern blot hybridized with nick-translated pBR322. Electrophoresis was on a gel separate from (b) and (c), but conditions were otherwise the same. Hybridization was as described in the legend to Fig. 5. (b) Undigested pSV2-*neo* DNA (1 μg) obtained from bacteria was electrophoresed in 0.7% agarose and stained with ethidium bromide. (c) pSV2-*neo* DNA obtained from bacteria and purified on a CsCl-ethidium bromide gradient was *Hin*dIII digested and electrophoresed on the same gel as DNA in (b). The single *Hin*dIII site in pSV2-*neo* is cut inefficiently. Nicked circles (RFII) have an apparent size of 9.2 kb, linear molecules (RFIII) are 6.0 kb, and supercoils (RFI) obtained from bacteria have an apparent size of 3.3 kb. Lane c is a control to show that supercoils are poorly cut by *Hin*dIII to give open circles (apparently size, 9.2 kb). Prolonged digestion with *Hin*dIII or brief digestion with other enzymes yields linear molecules (6.0 kb).

Finally, our results indicate that two mammalian promoter regions have at least partial activity in *D. discoideum* amoebae. *D. discoideum* transformants apparently use the herpes simplex virus *tk* promoter region of pAG60 for expression of the kanamycin resistance gene. The *tk*

promoter does not function in yeasts (12). Our results also suggest that the early gene promoter of simian virus 40 functions in *D. discoideum*. These inferences will be tested by nuclease S1 mapping and by analysis of well-characterized delections within these control regions.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Alton, T. H., and H. F. Lodish.** 1977. Developmental changes in messenger RNAs and protein synthesis in *Dictyostelium discoideum*. Dev. Biol. **60**:180–206.
2. **Alton, T. H., and H. F. Lodish.** 1977. Translational control of protein synthesis during the early stages of differentiation of the slime mold *Dictyostelium discoideum*. Cell **12**:301–310.
3. **Barclay, S. L., and E. J. Henderson.** 1982. Thermosensitive development and regeneration in a mutant of *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. U.S.A. **79**:505–509.
4. **Bolivar, F., and K. Backman.** 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol. **68**:245–268.
5. **Colbère-Garapin, F., F. Horodniceanu, P. Kourilsky, and A.-C. Garapin.** 1981. A new dominant hybrid selective marker for eukaryotic cells. J. Mol. Biol. **150**:1–14.
6. **Firtel, R. A.** 1972. Characterization of the genome of the cellular slime mold *Dictyostelium discoideum*. J. Mol. Biol. **66**:363–377.
7. **Franke, J., and R. Kessin.** 1978. A defined minimal medium for axenic strains of *Dictyostelium discoideum*. Nature (London) **272**:537–538.
8. **Hinnen, A., J. B. Hicks, and G. R. Fink.** 1978. Transformation of yeast. Proc. Natl. Acad. Sci. U.S.A. **75**:1929–1933.
9. **Hirth, P.-K., C. A. Edwards, and R. A. Firtel.** 1982. A DNA-mediated transformation system for *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. U.S.A. **79**:7356–7360.
10. **Jiminez, A., and J. Davies.** 1980. Expression of a transposable antibiotic resistance element in *Saccharomyces*. Nature (London) **287**:869–871.
11. **Kahn, M., R. Kalter, C. Thomas, D. Figurski, R. Meyer, E. Remant, and D. R. Helinski.** 1979. Plasmid cloning vehicles derived from plasmids ColEI, F, R6K, and RK2. Methods Enzymol. **68**:268–280.
12. **Kiss, G. B., R. E. Pearlman, K. V. Cornish, J. D. Friesen, and V. L. Chan.** 1982. The herpes simplex virus thymidine kinase gene is not transcribed in *Saccharomyces cerevisiae*. J. Bacteriol. **149**:542–547.
13. **Loomis, W. F.** 1983. The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
14. **MacWilliams, H. K., and J. T. Bonner.** 1979. The prestalk-prespore pattern in cellular slime molds. Differentiation **14**:1–22.
15. **Mangiarotti, G., S. Chung, C. Zuker, and H. F. Lodish.** 1981. Selection and analysis of cloned developmentally-regulated *Dictyostelium* genes by hybridization-competition. Nucleic Acids Res. **9**:947–963.
16. **O'Farrell, P. H., E. Kutter, and M. Nakanishi.** 1980. A restriction map of the bacteriophage T4 genome. Mol. Gen. Genet. **179**:421–435.
17. **Purel, C., C. Marchal, A. Louise, A. Fritsch, and P. Tiollais.** 1979. Bacteriophage lambda-*E. coli* K12 vector-host system for gene cloning and expression under lactose promoter control. Mol. Gen. Genet. **170**:161–169.
18. **Rowekamp, W., and R. A. Firtel.** 1980. Isolation of developmentally regulated genes from *Dictyostelium*. Dev. Biol. 79:409–418.
19. **Schaffner, W.** 1980. Direct transfer of cloned genes from bacteria to mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **77**:2163–2167.
20. **Southern, P., and P. Berg.** 1982. Mammalian cell transformation with SV40 hybrid plasmid vectors, p. 41–45. *In* Y. Gluzman (ed.), Eukaryotic viral vectors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. U.S.A. **76**:1373–1376.
22. **Williams, J. G., and M. M. Lloyd.** 1979. Changes in the abundance of polyadenylated RNA during slime mold development measured using cloned molecular hybridization probes. J. Mol. Biol. **129**:19–35.