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Mutagenesis and gene identification in *Dictyostelium* by shotgun antisense

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ABSTRACT **We have developed a mutagenesis technique that uses antisense cDNA to identify genes required for development in *Dictyostelium discoideum*. We transformed *Dictyostelium* cells with a cDNA library made from the mRNA of vegetative and developing cells. The cDNA was cloned in an antisense orientation immediately downstream of a vegetative promoter, so that in transformed cells the promoter will drive the synthesis of an antisense RNA transcript. We find that individual transformants typically contain one or occasionally two antisense cDNAs. Using this mutagenesis technique, we have generated mutants that fail to aggregate, aggregate but fail to form fruiting bodies, or aggregate but form abnormal fruiting bodies. The individual cDNA molecules from the mutants were identified and cloned using PCR. Initial sequence analysis of the PCR products from 35 mutants has identified six novel *Dictyostelium* genes, each from a transformant with one antisense cDNA. When the PCR-isolated antisense cDNAs were ligated into the antisense vector and the resulting constructs transformed into cells, the phenotypes of the transformed cells matched those of the original mutants from which each cDNA was obtained. We made homologous recombinant gene disruption transformants for three of the novel genes, in each case generating mutants with phenotypes indistinguishable from those of the original antisense transformants. Shotgun antisense thus is a rapid way to identify genes in *Dictyostelium* and possibly other organisms.**

The simple eukaryote *Dictyostelium discoideum* is an excellent system for the study of fundamental aspects of cell biology and developmental biology. *Dictyostelium* is an amoeba that lives on soil surfaces and eats bacteria. The cells are haploid and increase in number by fission. When the cells overgrow their food supply and starve, they aggregate using relayed pulses of cAMP as a chemoattractant. An aggregate typically consists of 10^5 cells that differentiate into two main cell types: prestalk and prespore. These cells form a migrating slug that then becomes a fruiting body consisting of a mass of spore cells supported by a thin column of stalk cells (for reviews, see refs. 1 and 2).

There is no general way to map or rescue chemically induced mutations. However, both extrachromosomal and integrating transformation vectors exist for *Dictyostelium* (3–8), and gene expression can be repressed by both antisense RNA and homologous recombination-mediated gene disruption (9–16). Mutants can also be generated by random insertion of transformation plasmids into the genome (17, 18). A portion of the disrupted gene can then be isolated by digesting the DNA of the mutant and recircularizing the DNA so that the resulting plasmid contains genomic DNA flanking the insertion site. In the Kuspa and Loomis technique (restriction enzyme-mediated insertion; REMI), the excised plasmid can then be relinearized and used for homologous recombination to verify that disruption of the gene has caused the mutant phenotype. A limitation of knock-out strategies is that the disruption of many genes is lethal to the cell.

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In contrast, antisense repression can permit a substantial reduction but not a complete block in the synthesis of the corresponding gene products. This has allowed the investigation of the effect of decreased amounts of proteins such as calmodulin, where complete repression is lethal (19). Another advantage of antisense repression is that transformation with a single construct will repress expression of proteins from multiple genes that express essentially identical transcripts, such as the three-member Discoidin I gene family (9). Similar repression by homologous recombination would require three separate rounds of transformation. In this report, we describe a mutagenesis scheme for *Dictyostelium* based on antisense transformation. This method allows the sequence of the repressed mRNA to be examined a few days after isolation of an interesting phenotype and also allows the identification of genes that would not be detected in other mutagenesis strategies.

MATERIALS AND METHODS

Construction of pV18neo Antisense Vector. The *Dictyostelium* actin 8 terminator contained in the *Hin*dIII-*Eco*RI fragment of pDneoII (12) was ligated into *Hin*dIII/*Eco*RI-digested pBluescript SK$^+$ (Stratagene). After digestion with *Pst*I and *Hin*dIII, the fragment containing the terminator was ligated into *Pst*I/*Hin*dIII-digested pSP72. The *Xba*I site of the resulting plasmid was destroyed by digesting with *Bam*HI and *Sal*I and ligation with a mix of the oligos GATCCGCCCGCGACG and TCGACGTCGCGGGCG to create pSP72A8, preserving the *Bam*HI and *Sal*I sites into which the cDNA library was later ligated. The V18 promotor fragment was isolated as a *Bam*HI/*Hin*dIII fragment from pV18p3–90 (20) and was ligated into the *Bam*HI and *Hin*dIII sites of pBluescript SK$^+$. A *Cla*I/*Bam*HI fragment containing the promotor was isolated from the resulting plasmid and was ligated with the 3-kb fragment from a *Bam*HI/partial *Cla*I digest of pSP72A8 to produce pHC. The *Bam*HI and *Sal*I sites of pUC 19 were destroyed by digesting with *Bam*HI and *Sal*I and ligation with a mixture of oligonucleotides GATCAGGCTCTAGACCT and TCGAAAGGTCTAGAGCCT to produce pUC19noBS, containing an intact *Xba*I site between the two destroyed sites. The 1.2-kb *Hin*dIII fragment of pHC containing the V18 promoter and actin 8 terminator was ligated into the *Hin*dIII site of pUC19noBS, and then the *Xba*I fragment of pA15T containing the neomycin resistance cassette (5) was ligated into the *Xba*I site of the resultant plasmid to make pV18neo.

Construction of the Antisense cDNA Library *Dictyostelium*. RNA was isolated from vegetative cells and from cells starved on filter pads for 2.5, 5.0, 7.5, and 10.0 hr, following Jain *et al.* (21). Equal amounts of the RNA were pooled and poly(A)$^+$

Abbreviations: REMI, restriction enzyme-mediated insertion; Ka, Klebsiella aerogenes.

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RNA was prepared using the Fast Track kit (Invitrogen). A directional cDNA library was constructed from 1.2 μg of poly(A)$^+$ RNA using the SuperScript Plasmid System (GIBCO/BRL). We followed the manufacturer's protocol precisely with the exception of substituting the oligo CGGGATCCGGAATCCCTTTTTTTTTTTTTTT for the supplied oligo in the first strand reaction. The resultant cDNA has a *Bam*HI site on the end corresponding to the 3′ end of the mRNA and a *Sal*I site on the other. The cDNA was next digested with *Bam*HI and ligated into the *Bam*HI and *Sal*I sites of pBluescript. Transformation of *Escherichia coli* Sure cells (Stratagene) with known amounts of circular DNA and with an aliquot of the ligation indicated that the library size was 9 × 10^5 independent clones.

The pool of cloned cDNA was then amplified using 30 cycles of PCR (1 min at 94°C, 1 min at 44°C, and 2 min at 72°C) with T7 and T3 as primers. The reaction contained ≈0.03% of the original ligation as template. The PCR products were electrophoresed on an agarose gel and all the products from 200 bp to 10 kb were pooled and isolated using the GeneClean Kit (Bio 101). The pooled product (10 μg) was then digested with 200 units of *Bam*HI and then with 280 units of *Sal*I in 100-μl reactions. We next isolated fragments ranging in size from 200 bp to 10 kb as described above. *Bam*HI/*Sal*I-digested pV18neo (100 ng) was ligated to ≈1 μg of cDNA fragments, and the ligation was used to transform Sure cells. The library from each ligation contained ≈15,000 independent clones. The cDNA inserts from 24 randomly selected clones ranged in size from 250 to 1500 bp, with an average size of 500 bp. The transformed cells were grown in a 500-ml culture overnight and the plasmid DNA prepared from this culture was then used to transform *Dictyostelium* cells.

Transformation of *Dictyostelium discoideum*. Ax-4 axenic *Dictyostelium* cells were transformed and grown in soft agar containing 30 μg/ml of G418 following Knecht *et al.* (8). Four to six weeks after transformation, colonies of transformants were transferred with a sterile toothpick onto an agar plate spread with *Klebsiella aerogenes* (Ka) bacteria following Sussman (22). Transformants with interesting phenotypes were transferred to a 3-ml shaking culture of HL5 containing 10 μg/ml of G418. Cells were grown to ≈3 × 10^6 per ml and were then diluted to 1 × 10^6 per ml. Cells (10–20) of transformants that grew in the presence of G418 were mixed with Ka bacteria and spread on an 85-mm agar plate to isolate clones following Sussman (22). Clones repeating the original phenotype were put back in shaking culture as described.

Isolation and Cloning of Antisense cDNAs. To isolate the antisense cDNA contained in the pV18neo vector, 2 × 10^4 logarithmically growing transformed cells were collected by centrifugation for 1 min at 1000 × *g*. The cell pellet was resuspended in 40 μl of 1× PCR reaction buffer containing gelatin (Perkin–Elmer) and treated with 4 μl of PreTaq (GIBCO/BRL) for 5 min at 75°C. The reaction was stopped by the addition of 4 μl of 20 mM EGTA and incubated at 75°C for 10 min, followed by 6 min at 100°C. A 100-μl quick start PCR reaction (Perkin–Elmer) contained 2 μl of the treated cells (template) and 200 ng each of V18 (CCTATCACCCTCCTTATATTTCAC), an oligonucleotide complimentary to the V18 promoter, and PL19 (CCTTCGACCTGCAGGCATGC), an oligonucleotide complimentary to the altered pUC polylinker. Products were obtained after 30 cycles of (1 min at 94°C, 1 min at 37°C, 1 min at 72°C). Following a 7 min 72°C extension step and phenol/chloroform extraction, the PCR product was isolated by a 5-min centrifugation at 2000 × *g* over an UltraFree-MC 30,000 kDa cutoff spin filter (Millipore) and washed with additional TE. The retained product was digested with *Sal*I and *Bam*HI. After phenol/chloroform extraction and ethanol precipitation, 15% of the digested product was used in a ligation reaction with 50 ng of *Bam*HI/*Sal*I-digested pV18neo. One-fifth of the ligation was used to transform Sure cells. Plasmid DNA from individual bacterial colonies was prepared by minipreps and analyzed by *Bam*HI/*Sal*I digestion. Plasmids that contained an insert were further analyzed by sequencing the insert using the V18 primer and PC20 (AAGCCTGCATGCCTGCAG), which allows sequencing from the actin 8 terminator side. These results were then used to search the EMBL and GenBank data bases.

Generation of Gene Disruption Mutants. To generate a knock-out mutant, we used the DIV2 plasmid of the REMI system (18). The DIV2 plasmid was altered by ligating oligonucleotides Stf1 (CGTCGACGCTGCAGGATCGATCGTGCA) and Stf2 (CGATCAATCCTGCAGCGTCGACGTGCA) into the *Pst*I site of the plasmid. This ligation separates the *Sph*I and *Pst*I sites by an additional 22 nucleotides. We used PCR to amplify portions of three of the novel cDNAs. The PCR primers were designed so that the PCR products would contain *Sph*I and *Pst*I restriction sites on the 5′ fragment and *Ava*I and *Eco*RI sites on the 3′ fragment. These pairs of sites lie at either end of the *pyr5–6* gene of the DIV2 plasmid. For transformation, the resulting plasmid was cleaved with *Sph*I and *Eco*RI. This generated cDNA fragments with part of the coding region replaced by the *pyr5–6* gene; for one of the genes, *smlA*, 95 bp was replaced. A portion of this fragment (20 μg) was gel purified as described above and used to transform *Dictyostelium* DH1 cells (a gift of Peter Devreotes, Johns Hopkins University, Baltimore). Transformation and selection of homologous recombination gene disruption transformants followed the protocol of Kuspa and Loomis (18). DNA and RNA isolation, gel electrophoresis, and Southern and Northern blot analyses were done as described (21).

RESULTS

In an attempt to generate developmental mutants, we transformed *Dictyostelium* cells with a cDNA library produced from the mRNA of vegetative and developing cells. The cDNA was directionally cloned in an antisense orientation in an expression cassette (Fig. 1) consisting of the *Dictyostelium* V18 promoter, a polylinker, and the terminator of actin 8, on a vector containing a neomycin-resistance cassette. The promoter from the V18 gene has been shown to be active in vegetative cells and in starving cells up to 8–10 hours after the onset of starvation (20, 23). The antisense vector was designed so that transformed cells will become resistant to neomycin and express antisense RNA complementary to the mRNA from which the cDNA was derived. The vector does not contain the sequences required to be maintained episomally, and therefore only integrated copies are maintained.

Dictyostelium cells were transformed with the antisense library, and the development of individual clones of neomycin-resistant cells was assessed by growth on a lawn of Ka bacteria (22). As *D. discoideum* cells grow, they consume the Ka lawn and create a clearing. Cells at the center of this clearing starve, and, as a consequence, begin development. From an initial screen of 1426 transformants, 149 did not form normal fruiting bodies. The majority of these mutants (129) formed smaller than normal fruiting bodies. Of the 20 others, one failed to aggregate, eight aggregated but had greatly delayed fruiting body formation, five formed bunches of fruiting bodies projecting from irregular aggregates, four formed fruiting bodies with a large spore mass and a thick stalk, and two formed fruiting bodies with elongated stalks and small spore masses (Fig. 2). To ensure that the putative mutants were drug-resistant and clonal, samples of each mutant were inoculated into liquid media containing G418. After two passages, the phenotypes of the cells were reassessed on Ka bacterial plates. Southern blot analyses of DNA from the transformants indicated that there were 8 to 20 copies of the antisense vector per cell.

We used PCR to isolate the antisense cDNA fragment(s) from 15 of the small fruiting body mutants and the 20 mutants

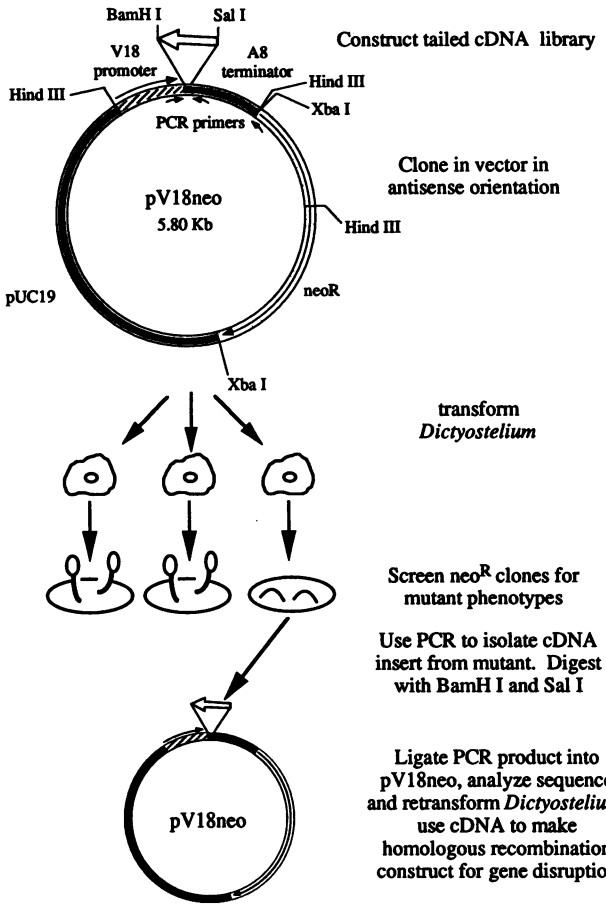

FIG. 1. Diagram of the pV18neo antisense vector and outline of the shotgun antisense mutagenesis method. In the pV18neo diagram, the PCR primers are, from left, V18, PC20, and PL19.

with other abnormal phenotypes. Of these 35 transformants, 31 contained one PCR product, two contained two PCR products, and two did not yield a PCR product (Fig. 3). The cDNA fragments ranged in size from ≈200 to 2000 bp. Once isolated, the cDNAs were cloned into the antisense expression vector and were sequenced. Sequence analysis showed that the PCR fragments from 6 of the 35 transformants were novel cDNAs. The derived amino acid sequence from the open reading frame of one of these six had 50% identity to cathepsin D and other cysteine proteases, whereas the other five showed no significant sequence similarity to any sequence either in the EMBO or GenBank data bases. Fifteen products were identical to rRNA genes. Eight products were fragments of the cDNA encoding V14, a ribosomal protein (24). One product was a fragment of *Dictyostelium* actin cDNA (25) and one product was a fragment of the *Dictyostelium* cyclophilin cDNA (26). Similar transformations with antisense expression controlled by an actin promoter, which is inactive in bacterially grown cells and turns on only after cells starve, yielded a much higher percentage of transformants in which antisense expression of rRNA fragments caused abnormal development (data not shown).

To determine if the cDNA(s) in the antisense expression vector caused the phenotype of interest, we transformed *Dictyostelium* with the antisense vector containing each of the PCR-isolated cDNA's. All six of the novel PCR products and the cyclophilin fragment regenerated the phenotype of the corresponding original mutant. In each case, 30–80% of the transformants had the original phenotype, with the exception of *rtoA* (Fig. 2), which had the original phenotype in only 5% of transformants. These results indicated that the mutant

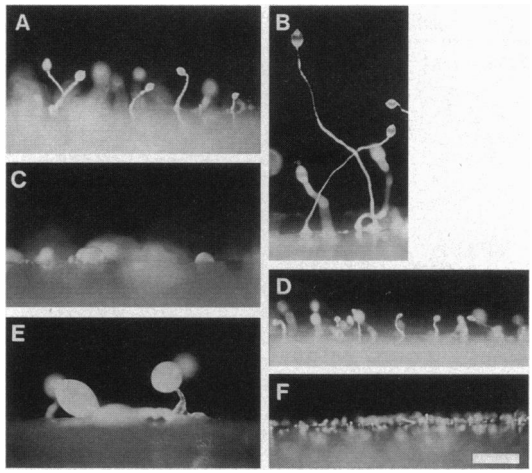

FIG. 2. Mutant phenotypes generated by shotgun antisense transformation. Cells were grown on agar plates spread with Ka bacteria. Side views of the aggregates or fruiting bodies that formed in the center of clearings are shown. (*A*) Wild-type Ax-4 fruiting bodies. (*B*) Cyclophilin antisense produces tall fruiting bodies. (*C*) *rtoA* antisense produces mounds that form fruiting bodies after many days (*D*) A cysteine protease-homologue antisense produces small fruiting bodies. (*E*) *ftbA* antisense produces large spore masses. (*F*) *smlA* antisense produces very small fruiting bodies. The cDNAs from the latter four mutants represent novel genes. Antisense repression of two other novel genes generated small fruiting bodies. (Bar = 0.5 mm.)

phenotypes resulted from transformation by the respective antisense constructs. In contrast, when constructs containing rRNA or V14 antisense cDNAs were transformed into cells, each construct generated a wide variety of phenotypes. Southern blot analyses of DNA isolated from mutants generated with the novel cDNAs or cyclophilin indicated that the endogenous genes were intact and therefore the mutants were not caused by integration or recombination events (data not shown).

To confirm that the antisense RNA was blocking expression of the corresponding gene, we used homologous recombination to disrupt the genomic sequence. The gene disruption experiment was done by cloning cDNA fragments into sites flanking the *pyr5–6* gene (a gene that allows cells to grow in uracil-minus media). A DNA fragment with a portion of the *smlA* cDNA on each end of the *pyr5–6* gene was purified and used to transform DH1 *pyr5–6*-minus cells. Southern blot analyses of a transformant that grew in uracil-minus medium indicated that they contained a disrupted *smlA* gene (Fig. 4). A Northern blot showed that the *smlA* knock-out cells contained no *smlA* mRNA (Fig. 4). The *smlA* knock-out cells had a small-fruiting-body phenotype, similar to that of the original and repeated *smlA* antisense transformants (Fig. 5). These results indicated that antisense repression of *smlA* with the

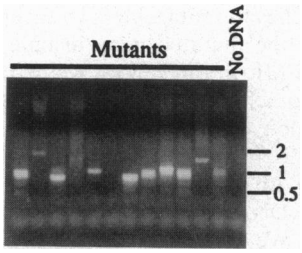

FIG. 3. Isolation of antisense cDNAs by PCR from transformants. An ethidium bromide-stained agarose gel shows PCR products from mutants with abnormal fruiting bodies from a pV18neo shotgun antisense transformation. The "No DNA" lane shows the results of PCR with no template DNA. Tick marks at right indicate 2.0-, 1.0-, and 0.5-kb markers.

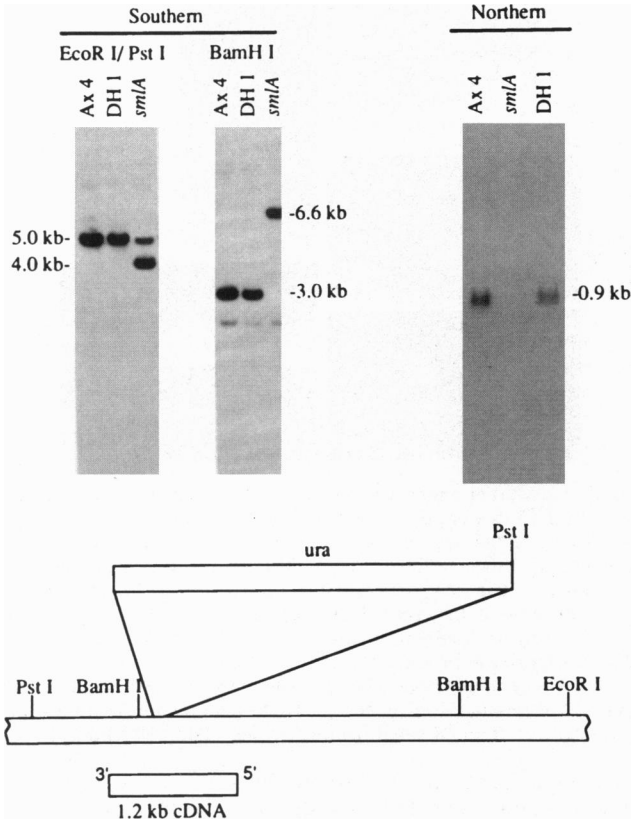

FIG. 4. Disruption of a gene identified by shotgun antisense. Map at bottom shows a region of the *smlA* genomic DNA indicating the insertion site of the ura (*pyr5–6*) cassette; 1.2 kb cDNA indicates the cDNA fragment used to construct the homologous recombination gene disruption DNA and also used to probe the Southern and Northern blots. At top left of the figure, the Southern blot autoradiogram shows DNA from Ax-4 wild-type and DH1 ura$^-$ parental strains, as well as the *smlA* knockout strain after digestion with both *Eco*RI and *Pst*I, or digestion with *Bam*HI. The Northern blot autoradiogram shows total vegetative cell RNA from the three strains. 10 μg of RNA was used for each lane and the blot was stained with methylene blue to verify that equal amounts of RNA were transferred and that the rRNA bands were not significantly degraded.

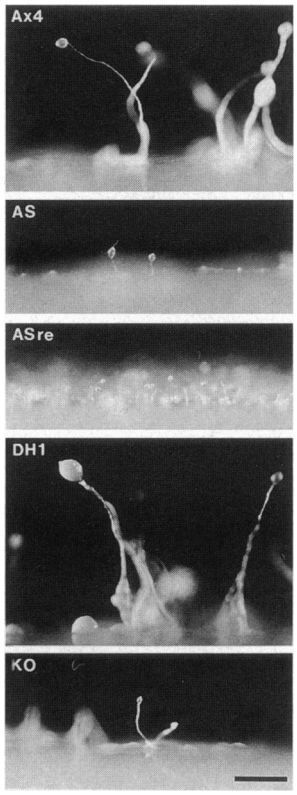

FIG. 5. Phenotypes of the *smlA* antisense and gene disruption transformants. Ax-4 wild-type cells were used for antisense; AS shows the *smlA* clone isolated from a shotgun antisense transformation; ASre shows Ax-4 cells transformed with the pV18neo vector containing the antisense cDNA isolated from the original *smlA* antisense transformant cells. DH1 cells are the ura$^-$ cells used for homologous recombination and KO shows the smlA homologous recombination gene disruption cell line. (Bar = 0.5 mm.)

pV18neo vector and disruption of *smlA* produce cells with the same phenotypes. The disruption of two other genes identified in the original screen, *ftbA* and *rtoA* (Fig. 2), also caused the loss of the corresponding mRNA transcripts, and phenotypes which matched those of the antisense mutants (data not shown).

DISCUSSION

Previous approaches to mutagenesis by techniques that allow rapid cloning of the mutated gene include transposon mutagenesis and REMI. In this report, we describe a successful new technique to achieve the same aims. We demonstrated that transformation of *Dictyostelium* with a cDNA library cloned in an antisense orientation in an expression vector produced cells with developmental defects. PCR allowed isolation of the cDNA fragments that encode the antisense RNA fragments. We demonstrated that in each of the seven mutants that were further analyzed, the PCR fragment, when cloned into the expression cassette in an antisense orientation, regenerated the phenotype of the cells from which the PCR fragment was obtained. This technique has allowed the identification of six novel genes that appear to play roles in development. For three of the genes, the causal role of the antisense constructs in generating the mutant phenotypes was confirmed by gene disruption.

The *Dictyostelium* cells were transformed with a calcium phosphate precipitate of DNA. The DNA used in the transformation was a library containing thousands of different cDNA inserts. Thus, a DNA precipitate would contain many plasmids and many antisense cDNAs. Because there were 8 to 20 copies per cell of the antisense vector, an initial concern was that a putative mutant cell would stably integrate many different cDNAs. However, PCR from putative mutants generally yielded only one fragment, indicating that transformed cells integrated and amplified a single molecule from the DNA precipitate. This made isolation of the antisense cDNA straightforward. The low efficiency in reobtaining the *rtoA* phenotype when transforming *Dictyostelium* cells with the *rtoA* antisense construct may be due to the fact that some constructs integrate into the genome in a complex manner so that during integration they become rearranged (27).

Dictyostelium cells transformed with antisense cDNA derived from rRNA or the V14 gene (a ribosomal protein) gave rise to a number of different phenotypes. These phenotypes may be due to a general inhibition of protein synthesis, and thus the different phenotypes might be caused by a variability in the exact timing or extent of the blocks in synthesis. We have recently begun doing shotgun antisense with an autosubtracted library (28) and find that this greatly reduces the prevalence of rRNA and V14 cDNAs.

The shotgun antisense mutagenesis approach described in this report has proven valuable in the identification of genes in *Dictyostelium*. Because antisense can repress expression of proteins encoded by multigene families (9) and can be used to

partially repress expression of genes where a complete repression is lethal (19), this shotgun antisense technique is complementary to the REMI technique of Kuspa and Loomis (18). An additional important advantage of shotgun antisense mutagenesis is that by allowing selection of the antisense promoter and of the mRNA pool used for the antisense cDNA, this mutagenesis can be targeted, in *Dictyostelium* or other systems, toward genes expressed in specific tissues or at specific developmental stages.

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