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**Establishment of a transient expression system for *Dictyostelium discoideum***

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**ABSTRACT**

We have established a rapid and sensitive transient expression system for *Dictyostelium discoideum*. We constructed a gene fusion containing the promoter from the *Dictyostelium* Actin 15 gene fused to the firefly luciferase gene. The enzymatic activity of this gene fusion, expressed at very high levels in stable transformants, was measured to determine optimum conditions for transient expression using electroporation to introduce the DNA into cells. With these conditions, we show that a luciferase gene fusion driven by a prestalk, cell-type specific promoter from the *pst*-cathepsin gene expresses luciferase at the appropriate developmental stage. In addition, we present results suggesting that the system will be useful for expressing genes in non-axenic cell lines. Finally, we observe that electroporation is more efficient for obtaining stable transformations than the standard calcium phosphate procedure using extrachromosomally replicating shuttle vectors but less efficient for vectors that integrate into the *Dictyostelium* chromosomes.

**INTRODUCTION**

The study of gene expression in eukaryotes has been greatly advanced using techniques of DNA transfection. In *Dictyostelium*, DNA mediated transformation procedures (1-4) have been established that allow the selection and identification of stable transformants at a moderately high frequency (5), the use of antisense RNA to block the expression of specific gene products from a single gene or a multigene family (6,7; Rubino, Mann, Pinko, Hori and Firtel, manuscript in preparation), and the interruption of an endogenous gene (8,9). DNA mediated transformation has allowed the identification and analysis of *cis*-acting regulatory sequences for a number of genes (10-15). However, these studies require introducing many constructs containing modified regulatory regions into cells to obtain a detailed understanding of the function of specific sequences. In examining *cis*-acting regulatory regions, it would be advantageous to have a more rapid, transient assay system in which constructs could be examined in a day's time, rather than requiring the selection, identification, and growth of stable transformants over 3 weeks.

For mammalian cells, transient assays can be accomplished using a variety of methods to introduce DNA into cells, including calcium phosphate precipitates (16), DEAE dextran-DNA complexes (17), and electroporation (18-20). For many cell types, a number of the methods are equally efficient. In *Dictyostelium*, initial attempts to use calcium phosphate precipitation protocols employed for obtaining stable transformants failed to produce measurable levels of transient gene expression (Howard and Firtel, unpublished observations). In this manuscript, we describe a transient assay system using electroporation for gene transfer and firefly luciferase as a recorder gene (21) driven by a *Dictyostelium* actin promoter. We show that this transient assay system can be effectively used to examine a developmentally regulated promoter for a gene induced by cAMP and preferentially expressed in the prestalk region of the migrating slug. In addition, we find that electroporation provides a

very rapid method for obtaining stable transformants using *Dictyostelium* extrachromosomal shuttle vectors, which contain the putative origin of replication from an endogenous *Dictyostelium* plasmid (22-24; Ahern, Howard and Firtel, submitted for publication). In contrast, electroporation is not as efficient as the calcium phosphate method for obtaining stable transformants with transformation vectors that stably integrate within the genome.

### MATERIALS AND METHODS

CaPO<sub>4</sub>-DNA mediated transformation of *Dictyostelium* cells and selection of stable transformants has been previously described (1,2).

#### Electroporation

We prepared KAx-3 or Ax-2 cells for electroporation by growing them in shaking flasks of HL5 media to a density ranging from  $8 \times 10^5$  to  $2 \times 10^6$  cells/ml. Cells were washed free of media by centrifugation at 500xg, resuspension of the pellet in electroporation buffer (10 mM NaPO<sub>4</sub>, pH 6.1, 50 mM sucrose), a repeat of the centrifugation, followed by a final resuspension of the cells in the same buffer at  $1.3 \times 10^7$  cells/0.8 ml. Supercoiled plasmid DNA [typically 2 mg/ml in 6 mM NaCl, 6 mM Tris HCl, 0.2 mM EDTA, pH 7.5] is added at 20 µg/0.8 ml. We have examined the levels of luciferase expression in transient expression studies as a function of DNA concentration. The level of expression per amount of input DNA was linear up to 60 µg DNA/0.8 ml; however, above 20 µg DNA per 0.8 ml, there was increased cell death with the amount of cell death increasing with DNA concentration. No assays were performed with >60 µg DNA/0.8 ml.

We incubated this mixture on ice for 10 minutes before adding aliquots of 0.8 ml to 1 ml cuvettes for electroporation. Three capacitance discharge machines were tested: a modified Promega Biotec X-Cell 2000 (exchanging a 14 µF capacitor with a 2 µF capacitor) delivered optimum voltages between 4.25 KV and 4.75 KV; the Promega Biotec X-Cell 450, performed at maximum efficiency with a setting of 2.8 KV/cm, 100 µF of capacitance and a clock setting of 2 ms to cut off the discharge (see Results); and the BioRad Gene Pulsar set with 3 µF capacitor supplied voltages between 2.5 KV/cm and 1.7 KV/cm, yielding  $\tau$  values of 0.6 msec (see Results). In each case, the disposable cuvettes have internal dimensions of 0.4 cm by 1 cm. Cuvettes used with the BioRad machine (BioRad #165-2085) contain an internal, thin metal electrode glued to two sides of the cuvette. In contrast, cuvettes used in the Promega machines (Fisher Scientific #14-385-942) were plain disposable plastic cuvettes. The electrode is supplied as part of the electroporation machine consisting of two metal plates that are inserted into the cuvette and lie against the cuvette walls. In both cases, the electrodes are 1 cm wide and the path length between the electrodes was nominally 0.4 cm. We electroporated the cells at room temperature, waited ten minutes, transferred the cells to Optilux Petri plates (Falcon) and added 8 µl of 0.1 M CaCl<sub>2</sub> and 8 µl of 0.1 M MgCl<sub>2</sub> to a final concentration of 1 mM each. Fifteen minutes later, we added 12 ml of HL5 growth medium and allowed the cells to recover and express the luciferase constructs for varying periods of time (see Results). Cells were harvested for luciferase assays at the average point of maximum expression or 24 hours (see Table 1). During harvesting, medium is first removed to eliminate most cell debris as live cells adhere to the Petri plates within 30 minutes of addition of HL5 medium. When excessive cell death was observed during electroporation, the cells adhered to the plate were rinsed with growth medium or buffer, with remaining cell debris eliminated in subsequent centrifugation steps (see below).

To isolate stable transformants, cells electroporated in the presence of vector DNA were allowed to recover for 5-12 hours in HL5 medium. Then the medium was removed and replaced with HL5 medium containing G418 at 10 µg/ml. Selection and isolation of stable transformants were done according to published procedures (1,2,4).

### Development of electroporated cells

To electroporate cells to be assayed for transient expression in development, we used a cell density of  $3 \times 10^7$  cells/0.8 ml and a DNA concentration of 60  $\mu\text{g}/0.8$  ml. After electroporation, these cells were allowed to recover in HL5 medium for 12 hours. During this time, the live cells adhere to the Petri plates. Cells were freed from the plate by pipetting up and down, and then harvested by centrifugation at 500xg, washed with deionized water, repelleted, and resuspended in 150  $\mu\text{l}$  of 10 mM  $\text{NaPO}_4$  pH 6.1 before plating on 12 mM  $\text{NaPO}_4$ , pH 6.1 agar plates. At the desired morphological stage, cells were scraped off the agar and resuspended in luciferase assay buffer, lysed, and assayed.

### Luciferase assays

We assayed luciferase activity using a modification of a previously described procedure (25). Cells ( $0.5-20 \times 10^6$ ) were harvested and washed as described above, and then resuspended in 50  $\mu\text{l}$  of 100 mM glycylglycine, pH 7.8, 0.2 mM PMSF, 1  $\mu\text{M}$  leupeptin, and 2  $\mu\text{M}$  pepstatin. They were lysed with 3 cycles of freeze-thawing using a dry ice-EtOH bath and a  $\text{H}_2\text{O}$  bath at 24°C. One hundred and fifty microliters of assay buffer (2.7 mM  $\text{MgCl}_2$ , 1.4 mM ATP, 0.36 M glycylglycine, pH 7.8) was put in Monolight luminometer tubes. Various amounts of extract [depending on the light units (LU) produced] were placed in the tubes immediately before assaying in a Monolight 2001 luminometer which automatically injects 100  $\mu\text{l}$  of 1 mM luciferin (suspended in 30 mM glycylglycine pH 7.8) into the tube to initiate the assay. Machine settings for the assay were: automatic mode, integration for 10 seconds, and a 25°C reaction chamber.

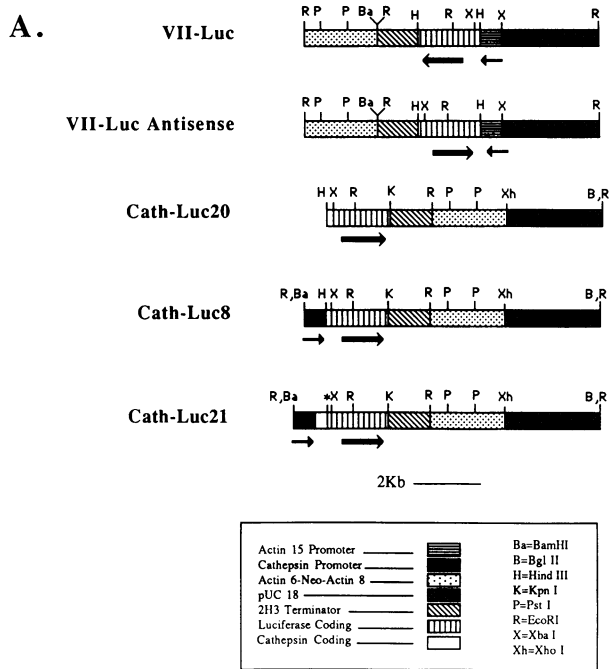
Protein concentration in the lysates was determined using the method of Bradford (26).

## RESULTS

### Establishment of optimum conditions for transient expression

Initially, we tried to obtain transient expression of a recorder gene using calcium phosphate precipitation to introduce DNA into *Dictyostelium* cells with the same protocol that we developed for obtaining stable transformants (1). We attempted to assay the accumulation of RNAs complementary to recorder genes expressed from a number of strong *Dictyostelium* promoters (Actin 6, Actin 15, and Discoidin I- $\alpha$ ) that are transcribed at high levels in vegetative cells and early development (1,7,27-31). After addition of the DNA precipitates to cells followed by a glycerol shock, cells were plated in growth medium and then harvested from 5-36 hours later. All attempts to detect any RNA complementary to the recorder genes using either RNA blot assays or solution hybridization with nick-translated DNA probes or single-stranded high specific activity RNA probes were negative (Howard and Firtel, unpublished observation). Because of the possibility that our assay was not sufficiently sensitive and because it required a substantial amount of time for each assay, we chose to use the firefly luciferase gene as the recorder gene and to assay luciferase activity. Firefly luciferase has several advantages over the conventional enzymatic reporter chloramphenicol acetyltransferase (32) when it is assayed with sensitive detectors. Luciferase detection is 1000 times more sensitive, more rapid, and linear over 5 orders of magnitude using available instrumentation (21,33). Additionally, luciferin can diffuse across the cell membrane of mammalian and *Dictyostelium* cells to allow detection of gene activity in live cells (see 21; Firtel and Howard, unpublished observation).

A construct was made fusing the *Dictyostelium* Actin 15 promoter to the 5' end of the luciferase gene and a 3' transcription terminator/poly(A) addition signal from a *Dictyostelium* gene was added (see Figure 1). The construct was inserted into a vector carrying a gene construct that confers G418 resistance to *Dictyostelium* cells, enabling the selection of stable transformants (1,2). This vector, VII-Luc, was transformed into *Dictyostelium*



B. ATG GAT GGT GAA GAT GTT CAA GCTT GG CAT TCC GGT ACT GTT GGT AAA ATG

Figure 1. Luciferase expression vectors.

A. Shown are the structure of the vectors used for luciferase gene expression. VII-Luc carries the *Dictyostelium* Actin 15 promoter from an *Xba*I site 255 bp upstream from the Cap site, to a *Hind*III site 7 codons into the coding region (ref. 13; see Figure 1B) fused in-frame to the luciferase gene construct lacking introns (clone JD206, refs. 33,34) plus a transcription terminator/poly(A) site from the prespore specific gene 2-H3 (36,37; Fir-tel, unpublished observation). The vector also carries the gene fusion (Act6 promoter-neo<sup>R</sup> gene-Act8 terminator region) conferring G418 resistance to *Dictyostelium* cells used to select stable transformants (1,2). VII-Luc antisense contains the luciferase gene in an antisense polarity.

Cath-Luc20 is similar to the above vector but the luciferase gene is not fused to a *Dictyostelium* promoter.

The cath-Luc8 and cath-Luc20 constructs contain the prestalk cell-specific, developmentally regulated promoter from pst-cathepsin (11,14,15) fused to the luciferase gene. Cath-Luc8 and 21 are in-frame gene fusions that extend from -313 bp upstream of the Cap site to different regions within the coding sequence. Both contain the 5'-cis-acting sequences necessary for proper temporal and cAMP regulation of pst-cath (11,14,15). Pst-cathepsin contains a 28 amino acid leader sequence at the N-terminus that is presumably responsible for the localization of the pst-cathepsin in vesicles in *Dictyostelium* prestalk cells (11,35,36). Cath-Luc8 is a gene fusion adding the first 3 codons of the N-terminus of cathepsin and 33 nucleotides (11 codons) of luciferase 5' DNA in-frame with the full length luciferase coding sequence (clone JD206, ref. 33,34). In cath-Luc21 119 codons of pst-cath and thus the entire presumed leader sequence is fused as a *Bam*HI/*Bgl*III fusion (see \* in Figure 1 in map of cath-Luc 21) with the full length luciferase clone JD207 which is identical to the clone JD206 except a *Bgl*III site replaces the *Hind*III site. A similar construct with a different recorder gene ( $\beta$ -glucuronidase) has been expressed in *Dictyostelium* transformants and the protein product is also localized in vesicles (11).

The arrows indicate polarity of the coding sequence of the luciferase gene (larger, heavier arrow) and the polarity of transcription of the promoter (smaller, thinner arrow).

B. The sequence from the Act15 AUG translation initiation codon through the luciferase initiation codon is given, with actin coding depicted in plain type, and luciferase printed in bold face.

TABLE 1: Comparison of Luciferase Activities with Vector VII-Luc

Transient Expression in Light Units/ $\mu$ g Protein				
CaPO <sub>4</sub> (KAX-3)	3 hrs	7.5 hrs	24 hrs	50 hrs
	4.45	23.5	23.5	18.3
<b>Electroporation</b>				
<i>strain KAX-3</i>	3 hrs	7 hrs	26 hrs	46 hrs
	ND	262	617	383
<i>strain Synag7</i>	12 hrs			
	2.65			
<b>Stable Transformants</b>				
<i>Stable Line (KAX-3)</i>	sense:	5.1x10 <sup>6</sup>		
	antisense:	727		
	5 hour development:	1.1x10 <sup>6</sup>		

The table shows enzyme expression of the Actin 15-luciferase gene. For cells treated with CaPO<sub>4</sub>-precipitated DNA, this shows their transient expression, assayed in the hours indicated, after the glycerol shock step of the calcium-phosphate transformation procedure as described previously (1). For electroporation, the time represents hours after the electrical discharge. We subtracted machine background of 40-45 LU (light units) from the absolute levels before calculating LU/ $\mu$ g protein. The background was independent of amount of extract. The stable transformed cells were transformed with VII-Luc using the calcium phosphate precipitation protocol and subsequently selected for G418 resistance. Luciferase levels were measured in extracts from mid-log phase cells. Several transformed cell populations were examined and all had similar levels of expression. The calcium phosphate and electroporation experiments shown here are representative experiments with the same KAX-3 cell population. The level of expression varied from experiment to experiment (see Figure 2).

For strain *Synag7*, peak activity was observed at 1.75 KV/cm and 3  $\mu$ F using the Gene Pulsar. ND, no detectable activity over background.

cells and stable transformants were selected. The vector was integrated into the genome with an average copy number of ~100 copies of vector per haploid genome (data not shown). Luciferase activity was assayed in cell extracts as described in Materials and Methods. As shown in Table 1, stable transformants produce ~5x10<sup>6</sup> light units (LU) per  $\mu$ g protein or 500-1000 LU per cell with a machine background of 40-50 LU per assay. In addition, an antisense construct of VII-Luc in which the luciferase gene was inserted in an antisense polarity downstream from the Act15 promoter was used. This vector gave a low level of expression (see Table 1), presumably from a cryptic promoter in the vector or from read-through transcription from the Act6-neo<sup>R</sup> gene fusion. These results indicated that we had a sensitive and rapid assay for enumerating the optimal conditions for electroporation.

When construct VII-Luc was introduced into *Dictyostelium* cells using calcium phosphate precipitates, we detected low levels of luciferase activity (~24 LU/ $\mu$ g protein) in transient assays, whereas the resulting stable transformants expressed extremely high levels of enzyme activity (~5x10<sup>6</sup> LU/ $\mu$ g protein). These results suggested that while calcium phosphate precipitates are functional in yielding stable transformants, the introduced DNA is either is not rapidly expressed under the transient assay conditions or does not enter a sufficient fraction of the cells to yield high levels of luciferase activity. In an attempt to obtain higher levels of expression, we

TABLE 2: Examination of Conditions for Transient Expression

capacitance ( $\mu\text{F}$ )	field strength (KV/cm)	$\tau$ (ms)	cell death (%)	LU/ $\mu\text{g}$ protein
1.0	0.75	0.3	0	0.9
	1.0	0.3	0	8.0
	1.5	0.3	0	35.0
	2.0	0.3	0	32.0
	3.75	0.2	0	45.0
3.0	0.75	0.7	0	16.3
	1.0	0.7	0	35.0
	2.0	0.7	0	152
	2.5	0.7	10	262
	3.75	0.6	20	271
	5.0	0.6	90	78.0
25.0	0.75	5.5	0	9.2
	1.0	5.0	10	39.0
	1.25	4.8	20	28.0
	1.5	5.1	50	27.3
	2.0	4.6	60	0.40
100	1.125	20.0	90	0.09
100	1.125	2.0	20	159
600	1.125	2.0	90	41.0
1500	0.875	2.0	70	84

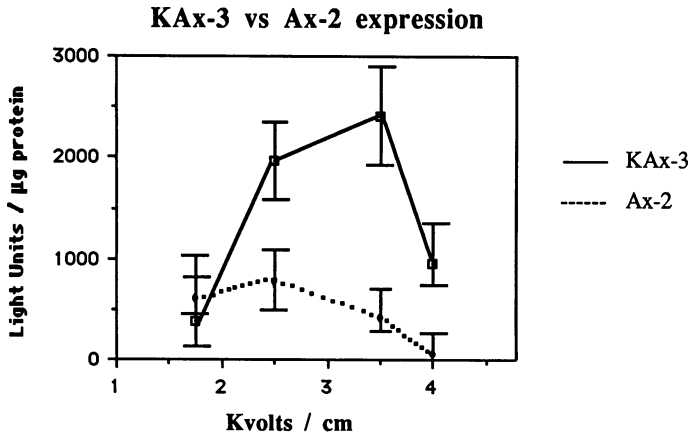
Luciferase expression in transient assays using vector VII-Luc as a consequence of capacitance, electric field strength, and pulse length. For capacitance values 1-25 $\mu\text{F}$ , the pulse time was fixed as a direct consequence of  $\tau\text{RC}$  where R is resistance and C is capacitance. We used the Gene Pulsar for capacitance from 1 to 25  $\mu\text{F}$ . For values 100-150 $\mu\text{F}$ , the Promega Biotec X-Cell 450 was used with the exponential discharge being abruptly halted by a circuit clock with variable stop time indicated. The percent of the cells that died as a result of the electrical discharge is shown. Cells were assayed (see Materials and Methods for the electroporation and assay protocols) 7 hours following electroporation. For most experiments, the LU/ $\mu\text{g}$  protein values continued to increase up to ~25 hours after electroporation before slowly declining (see Table 1). Absolute light unit values were recorded, background subtracted, and normalized to lysate protein concentrations.

assayed transient expression of the Act15-luciferase gene construct using electroporation since the method has been successful in other systems.

To establish conditions for electroporation, we varied capacitance, length of pulse, and electric field strength using two different, commonly employed axenic strains of *D. discoideum*, KAx-3, and Ax-2. Preliminary results suggested concentrating on field strength and capacitance. Pairwise combinations of capacitance and electric field strength were then tried on log phase vegetative cells. The level of luciferase activity was used to evaluate the efficiency of our experiments. Table 2 depicts the salient portion of these studies for strain KAx-3. As can be seen, the voltage optimums for the two strains were broad and different and strain Ax-2 showed a reproducibly higher level of transient expression than KAx-3 (see Figure 2). Once the optimal parameters were identified as assayed by maximum luciferase expression, a variety of buffers, cell densities, DNA concentrations, and pre- and post-electroporation treatments were tried, yielding the protocol used in subsequent experiments and described in the Materials and Methods.

#### Transient expression with a developmentally regulated cell-type specific promoter

To determine if this system could be extended to measure the activity of developmentally induced genes, we made a series of constructs by fusing the luciferase gene in-frame to the promoter from the *pst*-cathepsin



**Figure 2.** Comparison of transient expression of axenic strains KAx-3 and Ax-2. Shown are a comparison of VII-Luc transient expression in two axenic strains over a range of field strengths at the optimum capacitance of 3  $\mu$ F. The points represent the average of four experiments. The bars indicate the range of values for each voltage. This value is shown to give the reader a better feel of the reproducibility since the ranges are low for these types of experiments.

gene, a cAMP inducible gene preferentially expressed in anterior, prestalk cells during the early formation of the multicellular aggregate. The endogenous gene, pst-cathepsin, is not expressed in vegetative cells, is induced during the loose aggregate stage (~10 hours of development), and is maximally expressed at the finger stage (~15 hours of development). Gene fusions with the pst-cath promoter have been previously shown to have proper temporal and spatial expression in developing aggregates (11,14,15).

Cath-Luc20 (no promoter) and cath-Luc8 and 21 (cathepsin promoter) (see Figure 1), were transformed into *Dictyostelium discoideum* and stable transformants selected. Vegetative levels of expression are compared to induced levels during development in Table 3. There is a >1000 fold increase in the level of expression with constructs carrying the pst-cath promoter when the two developmental stages were compared.

It is interesting to note that the construct lacking the promoter showed very low levels of luciferase activity in vegetative cells, as did the antisense construct of VII-Luc. Presumably, this is due to non-specific or cryptic initiation within the vector or in the 5' flanking sequences. RNA blot analysis was performed on RNA isolated from vegetative and developing cells transformed with the two vectors carrying the pst-cath promoter. No RNA band was detectable in RNA from vegetative cells, while abundant RNA of appropriate size was seen on blots using RNA from finger stage cells probing with either a luciferase gene probe to measure the transformed gene or a pst-cath gene probe to assay endogenous gene expression (data not shown). No transcript was seen at either stage in cells transformed with cath-Luc20. Because of the higher level of expression in stable transformation, we assayed the transient expression of construct cath-Luc8. Table 3 shows the results of a representative experiment. In all such experiments, parallel control experiments are carried out with VII-Luc to ensure that the electroporation worked efficiently (see legend to Table 3). As can be seen from the results in Table 3, the gene is induced in transient assays, as we have previously shown for the endogenous gene and the luciferase gene expressed from this promoter in stable transformants. The luciferase gene shows a very low level of expression in vegetative cells and a 40-90-fold induction of expression in finger stage cells over vegetative

TABLE 3: Expression of pst-cath/luciferase Gene Fusions

LU/ $\mu$ g protein		
Electroporation	12 hrs axenic (HL5)	Finger stage of development
cath-Luc8(a)	1.4 (151 LU/assay)	77 (31,464 LU/assay)
cath-Luc8(b)	1.1 (107 LU/assay)	148 (51,564 LU/assay)
Stable Line	vegetative	Finger stage of development
cath-Luc8(a)	50.4	$1.75 \times 10^5$
cath-Luc8(b)	57.5	$0.95 \times 10^5$
cath-Luc20	1.5	1.1
cath-Luc21	10	$2.8 \times 10^4$

This table shows enzyme expression driven by the developmentally induced promoter pst-cath in strain Ax-2. For some of the samples, the absolute number of LU is given to allow the reader a better comprehension of the level of luciferase activity above background. There are 3 constructs. Cath-Luc20 lacks the pst-cath promoter. Cath-Luc8 and 21 constructs are in-frame gene fusions that extend from -313 bp upstream of the Cap site to different regions within the coding sequence (see Figure 1).

In the electroporation assay, cells were plated on Petri plates in HL5 axenic medium to "recover" for 12 hours. One half of the cells were harvested at this point and one half were plated for development and then harvested at the finger stage. The actual numbers of light units measured over machine background are given. See Materials and Methods for details.

In all such experiments, a parallel control experiment using vector VII-Luc and the same culture of cells was used to ensure that the electroporation worked efficiently. The level of luciferase activity in the VII-Luc control had to be within the deviation for those cells and electroporation conditions as shown in Table 2 and Figure 2. The control cells were also harvested at 12 hours. In these experiments, the internal control, VII-Luc, gave 1500 LU/ $\mu$ g protein after 12 hours recovery and 350 LU/ $\mu$ g protein at the finger stage.

Results with two stable cell lines (a and b) carrying cath-Luc8 are given. In addition, the results of two separate electroporation experiments (a and b) with cath-Luc8 are given.

The machine background was subtracted from all values shown (either extracts from cells lacking a luciferase gene construct or no extract). Machine background is independent of amount of extract from cells not carrying a luciferase gene construct.

cells. Since there was no detectable hybridizing band on blots of RNA from vegetative cells, even on very long exposures, we did not determine if the low level of expression in vegetative cells in the transient assays was due to read-through transcription from a cryptic promoter or due to "leaky" expression of the pst-cath promoter during growth.

#### Transient expression in non-axenic strains

Wild-type strains of *Dictyostelium* use bacteria as a food source and cannot be grown in axenic medium. A large number of mutants have been isolated from various wild-type strains and the phenotypes of a number of these have been analyzed in some detail at the biochemical and molecular level. Such mutants provide an important means for probing the mechanisms controlling cellular differentiation in this organism. Therefore, the ability to examine the expression of modified, exogenous gene constructs in such strains is desirable. At present, the standard transformation protocol fails to yield stable transformants using these strains, probably due, in part, to the need to continually grow the cells on bacteria as a food source at various steps in the protocol.

We have used the electroporation procedure to examine transient expression of the VII-Luc construct in strain *Synag7*, a mutant altered in cAMP activation of adenylate cyclase (P. Devreotes, personal communication) and thus of interest for studies of cAMP regulation of gene expression. Cells grown in a shaking culture containing autoclaved *Klebsiella aerogenes* were harvested and separated from bacteria by differential centrifugation



and then electroporated at a range of field strengths from 1.25 to 3.5 KV/cm. The results shown in Table 1 indicate the level of luciferase activity in lysates examined 12 hours after electroporation. While the value is ~100-fold lower than that obtained for axenically grown KAx-3, the luciferase activity remains substantially above background and in a range where it can be quantitatively used to examine gene expression. Part of the reason for the low activity may be due to the fact that this (Act15) and other actin promoters are expressed at lower levels in vegetative cells growing on bacteria than in axenic medium (3,27,28).

#### Isolation of stable transformants using electroporation

The convenience of electroporation led us to try to use electroporation to obtain stable transformants. We compared the frequency of formation of stable transformants using electroporation and calcium phosphate precipitation with either vectors that integrate within the chromosome or vectors that replicate extrachromosomally. The extrachromosomally replicating shuttle vectors carry the Act6-neo<sup>R</sup> fusion gene that confers G418 resistance to *Dictyostelium* cells and the presumed origin of replication from the *Dictyostelium* endogenous plasmid Ddp-1 (23; Ahern, Howard and Firtel, manuscript in preparation). These DNAs replicate extrachromosomally and are stably maintained for extended periods in the absence of selection. A number of shuttle vectors were tested, including Ddp1-20, which is ~19 kb (23). After the selection of stable transformants, DNA was isolated from populations and tested to determine if the DNA was integrated or replicating extrachromosomally (data not shown, see ref. 23 for methods). Using the two transformation protocols, we observed that that electroporation gave a >10 fold reduced number of stable transformants using integrating vectors such as VII-Luc relative to the number of transformants obtained using calcium phosphate precipitates in parallel experiments (data not shown). In contrast, electroporation gave a several fold higher frequency of transformation using extrachromosomally replicating vectors in parallel experiments (data not shown). Moreover, with extrachromosomal vectors and electroporation, confluent plates of stable transformants could be obtained in <1/4 the time as with calcium phosphate precipitates. In all cases tested, the integrating vectors were present as tandem linear domains within the chromosomes while shuttle vector DNA replicated extrachromosomally whether the DNA was introduced by calcium phosphate precipitates or electroporation (data not shown). The rapidity of the procedure makes it the method of choice for transformation using such vectors.

#### DISCUSSION

Our transient expression protocol for *Dictyostelium* makes use of vectors containing the firefly luciferase gene driven by a *Dictyostelium* actin promoter (vector VII-Luc), and electroporation to introduce DNA into cells. After using this system to optimize transient expression in vegetative cells, we determined that a promoter from a developmentally regulated gene, *pst-cathepsin*, shows proper developmental regulation in a transient expression system. The timing and level of expression of *pst-cath/luciferase* gene constructs (*cath-Luc*) in transient assays suggest that this system will be useful in examining promoter mutations. We also examined the expression of VII-Luc in a non-axenic mutant cell line. While the level of expression was substantially lower than that obtained with axenic cells, it still should represent a quantitative tool for examining gene expression in non-axenic cells. The combination of electroporation as a method for introducing DNA into *Dictyostelium* cells and luciferase as a recorder gene should allow a sensitive, rapid assay for promoter function in *Dictyostelium*. In addition, production of luciferase enzyme does not seem to be deleterious to the cell; VII-Luc containing cell lines has been passaged for many months under moderate G418 selection (5µg/ml) without diminution of levels of luciferase produced or an altered rate of cell division.

We found that electroporation is a more efficient method of producing stable transformants using extrachromosomal vectors, but less efficient using integrating vectors. The disparity of results in comparison to using

the calcium phosphate protocol for these vectors is puzzling. Perhaps the agglomeration of DNA in a calcium phosphate precipitate in *Dictyostelium* allows a larger number of DNA molecules to enter an individual cell. Possibly this is necessary for producing stable transformants, although the number of cells that it enters may be relatively low, or possibly the calcium phosphate precipitate itself may somehow enhance the ability to form stable integrants. The reduced number of stable transformants, obtained with integrating vectors using electroporation, may be due to a lower number of DNA copies entering each cell. This may reduce the frequency at which the vector integrates into a chromosome. In contrast, one would expect that a single copy of an extrachromosomal vector would be sufficient to establish stable transformation. Ddp1 based extrachromosomal vectors are stably maintained in *Dictyostelium* cells with the copy number ranging between 20-100 copies per cell (23; Ahern *et al.*, submitted for publication). Presumably, one or only a few copies of an extrachromosomal vector entering the cell amplify to the normal copy number. The ability to obtain good transient expression using electroporation may result from DNA entering a large fraction of the cells or from the DNA's accessibility to transcription in the time frame of the assay relative to DNA introduced by calcium phosphate precipitates.

Some points are worthy of note in the experiments to optimize transient expression. Transient luciferase activity can be detected as early as 5.5 hours following electroporation (data not shown). This transient activity reaches a peak between 25 and 30 hours and slowly declines as cell division continues. The successful introduction of an ~19 kb extrachromosomal vector, Ddp1-20 (23), indicates that large plasmid molecules are not barred from entering cells through this route (Ahern *et al.*, submitted for publication). Extrachromosomal vectors carrying the Act15-luciferase gene fusion have also been introduced into *Dictyostelium* cells and express high levels of luciferase activity.

The establishment of the transient assay system should substantially aid the rapidity at which promoters can be analyzed in *Dictyostelium*. In addition, the availability of electroporation as an additional method of transformation in this organism should aid a number of other studies aimed at understanding mechanisms controlling cellular differentiation in this organism. The protocols and approaches established for *Dictyostelium* should be helpful in establishing similar approaches for a number of other organisms in which the ability to transform cells has been limited.

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