

Molecular complementation of a genetic marker in *Dictyostelium* using a genomic DNA library

(genetic selection)

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ABSTRACT We constructed a partial *Sau3A* *Dictyostelium* genomic DNA library in a shuttle vector that replicates extrachromosomally in *Dictyostelium* cells. This library was used to complement *Dictyostelium* strain HPS400, which lacks thymidylate synthase activity and requires exogenous thymidine for growth. We have used a modified high-frequency transformation protocol that allows the introduction of the library into a sufficient number of *Dictyostelium* cells to select complementing plasmids. Using this approach, we have isolated a gene (Thy1) that complements the thymidine growth requirement of HPS400. The gene encodes a 1.2-kilobase RNA and the derived amino acid sequence shows no homology to thymidylate synthase, a protein highly conserved throughout evolution, or any other protein sequence in the data base examined. Thy1 provides an important selectable marker for transforming *Dictyostelium* cells. In addition, this work suggests that it will be possible to isolate genes that are essential for developmental processes in *Dictyostelium* by complementation.

The cellular slime mold *Dictyostelium discoideum* is an excellent system for the study of eukaryotic cell and developmental biology. The cells grow as solitary vegetative amoebae, which develop when nutrient starved. Up to 10^5 cells will aggregate, form slugs, and finally form mature fruiting bodies consisting of at least two different cell types: stalk cells and spore cells (1, 2). Mutations have been isolated for many developmental steps and have been instrumental for understanding the various processes controlling signal transduction and cellular differentiation (3–12). One important class of mutants is defective in the signal transduction pathways operating during aggregation (3–6, 8, 12–15). In another class, cells do not make differentiation-inducing factor (DIF) but can respond to DIF, a morphogen essential for stalk and spore differentiation (16). Mutations have also been isolated that affect other cellular functions (8, 10, 17).

To study many of these processes on a molecular level, it is essential to isolate the mutant gene. In most eukaryotes, this is difficult. A systematic method to isolate mutant genes in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, in the mold *Aspergillus nidulans*, and in mammalian tissue culture cells employs genomic library complementation (18–22). This technique operates through rescuing a recessive mutant phenotype by introducing a cloned copy of the wild-type gene. The mutant strain is transformed with the pooled DNA from a genomic library cloned into an extrachromosomal shuttle vector. The transformants are then screened for the appearance of the wild-type phenotype. These rescued transformants putatively contain a wild-type copy of the mutant gene or, possibly, a suppressor. The plasmid containing the wild-type gene is isolated by introducing the DNA from the rescued mutant

into *Escherichia coli*. The complementing ability of the isolated plasmid is confirmed by the high frequency at which it rescues the mutant phenotype.

Recently, a series of extrachromosomal shuttle vectors for *D. discoideum* has been constructed in this laboratory (23, 24). They contain a *Dictyostelium* actin promoter–bacterial neomycin gene (*neo*) fusion for selection in *Dictyostelium* with G418, a bacterial plasmid backbone for selection and propagation in *E. coli*, and a region from the endogenous plasmid Ddp1 that confers extrachromosomal replication in *Dictyostelium*. In this paper, we report the successful complementation of the thymidine-requiring mutant HPS400, which has no detectable thymidylate synthase activity in cell lysates (17). For this, we used a modified shuttle vector to construct a genomic library for complementation. The pooled DNA from this library, introduced into *Dictyostelium* by electroporation, transforms at a frequency high enough to screen the entire genome. This work demonstrates the potential for isolating genes essential in a variety of developmental processes.†

MATERIALS AND METHODS

Materials. Restriction enzymes were obtained from Promega Biotec, except for *Cla* I, which was from Boehringer Mannheim. Alkaline phosphatase was from Sigma. DNA polymerase I, Klenow fragment, and T4 DNA ligase were from Promega Biotec. Hybridization membranes were from Micron Separations (Westboro, MA), and modified T7 DNA polymerase (Sequenase) was from United States Biochemical. *Bam*HI linkers were from Promega Biotec. The plasmid vector pGEM7Zf(+) was from Promega Biotec.

Growth of *Dictyostelium* Strains. Standard laboratory strain KAx-3 was grown axenically in HL5 medium as described (25). Thymidine-requiring mutant HPS400 was a gift from R. Deering (Pennsylvania State University) and was grown axenically in HL5 supplemented with thymidine (100 μ g/ml) (17).

Construction of Extrachromosomal Vector pATANB43. The bacterial plasmid backbone of pATANB43 (see Fig. 1) was created by replacing the *Eco*RI/*Sal* I fragment of pAT153 with the *Bgl* II/*Xho* I polylinker sequence from pSP73, destroying the *Eco*RI and *Bgl* II sites (L. Haberstroh and R.A.F., unpublished data). This vector was termed pATSP. The *Eco*RI/*Bam*HI fragment from plasmid B10SX (26), containing the actin promoter–*neo*^R (neomycin resistance) gene fusion, was cloned into the *Eco*RI/*Bam*HI site of pATSP and was named pATAN. The *Sal* I/*Bam*HI fragment from plasmid CI (24), containing sequences required for extrachromosomal replication from the endogenous *Dictyostelium* plasmid Ddp1, was placed into the *Bam*HI/*Sal* I site of pATAN. The *Bam*HI/*Bgl* II fragment of this construct was deleted to

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M27713).

make the shuttle vector pATANBS, which has a unique *Sal*I site. This *Sal*I site was replaced with a *Bam*HI linker to form pATANB43, which has a unique *Bam*HI site.

Construction of Complementation Library. *Dictyostelium* nuclear DNA was prepared from axenically grown KAX-3 cells as described (27). The genomic DNA was partially digested with *Sau*3A and size-fractionated on a sucrose gradient. The fractions containing DNA from 3 to 12 kilobases (kb) were pooled and purified. Four hundred nanograms of insert was ligated to 100 ng of pATANB43 that had been linearized with *Bam*HI and treated with alkaline phosphatase. The ligated DNA was transformed by bacterial electroporation (28, 29) into *E. coli* strain MC1061 [*F*⁻, *araD139*, Δ (*ara*, *leu*) 7679, Δ (*lacI*74, *galU*⁻, *galk*⁻, *hsr*⁻, *hsm*⁺, *strA*], yielding 5×10^5 bacterial colonies from 16 electroporations. These colonies were pooled into eight separate library fractions containing 60,000–70,000 colonies each. They were diluted to 10^8 cells per ml with L broth and shaken for 80 min at 37°C. An equal volume of glycerol was added and the stocks were stored at –85°C. DNA from these pools was made by standard procedures.

Transformation of *Dictyostelium* by Electroporation and Isolation of DNA. The electroporation protocol used was a variation of the method developed by Howard *et al.* (30). The electroporation machine used was the Bio-Rad Gene Pulser with a 5- Ω resistor in series with the sample chamber. Cells were grown logarithmically in HL5 medium supplemented with thymidine (100 μ g/ml), harvested at 1.5 – 3.5×10^6 cells per ml, diluted with 2 vol of electroporation buffer (10 mM sodium phosphate, pH 6.1/50 mM sucrose; 4°C), and pelleted by centrifugation. The cells were gently resuspended at 3×10^7 cells per ml in electroporation buffer, and 0.2 ml of cells was mixed with 15 μ g of DNA and then electroporated once at 1.0–1.4 kV per 0.2-cm cuvette at 3 μ F. After 10 min, cells were removed to an Optilux Petri dish (20 \times 100 mm) and CaCl₂ and MgCl₂ were added to “cure” the cells. After 15 min, 12 ml of HL5 medium with thymidine was added to the plate. The cells were allowed to recover for 20–30 hr, washed off the plate, pelleted, resuspended in selected medium (HL5 medium without thymidine), and used to inoculate 10 plates at a density of 2.0×10^6 cells per plate. By analyzing the results of serial dilutions and determining the greatest dilution that still yielded G418-resistant (G418^R) colonies, we calculated that $\approx 0.5\%$ of the cells were transformed to G418 resistance by using library DNA in these experiments.

Total *Dictyostelium* DNA was isolated as described (27) and transformed into *E. coli* strain MC1061 by bacterial electroporation (28, 29).

Analysis of RNA and DNA from Transformants. RNA was isolated from vegetative cells and sized on denaturing gels, blotted, and probed as described (31, 32). The sequencing was performed on double-stranded plasmid DNA by using modified T7 DNA polymerase (Sequenase) as recommended by the manufacturer.

Amino Acid Sequence Homology Search. Protein sequence homology searches were performed against an updated version of the NEWAT data base (33, 34).

RESULTS

Construction of Genomic Complementation Library. Nuclear DNA was partially digested with *Sau*3A and fragments from 3 to 12 kb were pooled and ligated into pATANB43 (Fig. 1). The ligation mixture was introduced into *E. coli* strain MC1061 by bacterial electroporation, generating 5×10^5 primary colonies. Analysis of random transformants showed a variety of insert sizes up to 12 kb, although some plasmids had inserts <3 kb, possibly due to recombination. Because most *Dictyostelium* genes are relatively small, the library should contain inserts capable of expressing most genes.

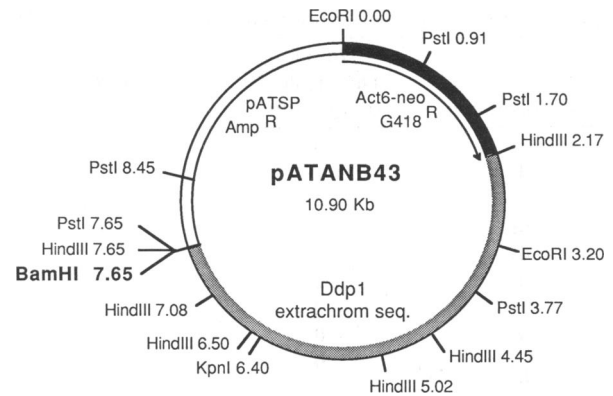


FIG. 1. Partial restriction map of pATANB43. The plasmid was constructed as described from DNA fragments from pATAN (pAT153 bacterial plasmid backbone) (□), B10SX (actin 6 promoter–bacterial *neo* gene fusion) (■), and CI (Ddp1 extrachromosomal sequences) (■). The unique *Bam*HI site was used for inserting the genomic DNA.

Most *Dictyostelium* genes contain one or two introns of 100–150 base pairs (bp), and essential cis-acting regulatory sequences, all of which appear to lie 5' to the coding region, are contained within <1 kb of the cap site. Thus, a gene for a 40-kDa protein would be contained on a DNA fragment of 2–2.5 kb. An 80-kDa protein would require a 3.5-kb DNA fragment. Given that the haploid genome size of *Dictyostelium* is 5×10^7 bp (27), the library contains ≈ 20 genomic equivalents of insert DNA. The colonies were pooled into eight separate library fractions containing 60,000–70,000 transformants each. Library DNA was made from each of these fractions.

Complementation of Thymidine-Requiring Mutant. HPS400 is a thymidine-requiring mutant isolated by Podgorski and Deering (17) that requires ≥ 20 μ g of exogenous thymidine per ml for growth in the axenic medium HL5. Thymidylate synthase is the enzyme that transfers the methylene group of 5,10-methylene tetrahydrofolate to deoxyuridylate to produce thymidylate (TMP) utilized in DNA synthesis. Mutants lacking thymidylate synthase activity require either TMP or thymidine, which is converted to TMP by thymidine kinase. There are three lines of evidence indicating that HPS400 has altered thymidylate synthase activity (17) and suggesting that the genetic lesion in HPS400 may be associated with thymidine metabolism. First, the TMP precursors deoxyuridine and deoxyuridylate do not support growth of the mutant in minimal medium. Second, HPS400 lacks the requirement for adenine, guanine, or serine in minimal medium, as would be predicted if the mutant had altered levels of 5,10-methylene tetrahydrofolate. Finally, cell lysates from the mutant have no thymidylate synthase activity. We have used HPS400 as a model system to demonstrate genomic library complementation in *Dictyostelium*.

Library DNA was electroporated into $\approx 1 \times 10^8$ HPS400 cells, and transformants were selected for growth in the absence of thymidine (see *Materials and Methods*). Sixteen independent electroporations of 6×10^6 cells were performed, with each of the eight library fractions used twice. Cells were allowed to recover for 24 hr, in which time the cell number increased ≈ 3 -fold. These cells were then washed and diluted into 10 plates in medium without added thymidine. The cells from each electroporation were kept separate throughout the experiment. The results are summarized in Table 1. Thymidine-independent cells were found in 9 of the 16 electroporations. In most cases, more than one plate from a single electroporation was positive. Since the electroporated cells had divided and increased in number 3-fold before the plates were split for selection, positive plates from a

Table 1. Results of genomic library complementation of HPS400

Electroporation	No. of Thy ⁺ plates (n _{total} = 10)	No. of Thy ⁺ G418 ^R plates (n _{total} = 10)
1A	0	0
1B	1	0
2A	0	0
2B	6	0
3A	0	0
3B	2	0
4A	2	2
4B	0	0
5A	0	0
5B	0	0
6A	8	8
6B	8	8
7A	4	4
7B	2	2
8A	1	0
8B	0	0
Total 16	34	24

Two electroporations were done for each of the eight separate library fractions and then split into 10 separate plates for selection. Total number of cells electroporated, $\approx 10^8$; number of cells per electroporation, 6×10^6 . Cells from 16 electroporations with library DNA were split into 10 plates per electroporation upon initiation of selection. Thymidine-independent colonies were seen after 12–30 days of selection. Plates with thymidine-independent cells (Thy⁺) were counted and tested for growth in medium containing G418 (10 $\mu\text{g}/\text{ml}$). A direct determination of the number of independent Thy⁺ transformants was not possible since this strain does not produce discrete clones on plastic dishes. However, a minimum estimate can be obtained. The cells were split and plated onto 10 plates in selection medium 20–30 hr after electroporation. At this point, total cell number had increased ≈ 3 -fold. Electroporations 6A and 6B both had 8 of 10 plates showing Thy⁺ cells. This indicates that there were minimally 8 Thy⁺ cells before splitting the cells and suggests that electroporations 6A and 6B had multiple Thy⁺ transformants. Statistically, it is expected that there would be >1 Thy⁺ cell on some of the plates just after splitting. Since the exact number of Thy⁺ transformation events cannot be determined, the results are displayed as the number of plates containing Thy⁺ cells.

single electroporation may, in some cases, be descended from a single transformation event. In another case (e.g., electroporations 6A and 6B), the number of positive plates (8 of 10) is sufficient to suggest that there were multiple transformation events. Five of the nine thymidine-independent cell lines were G418^R, suggesting that the Thy⁺ phenotype was due to the action of a complementing plasmid. The Thy⁺ G418-sensitive (G418^S) colonies are probably revertants (see Discussion). A similar frequency of reversion to Thy⁺ was seen in cells electroporated without DNA (≈ 1 per 6×10^7 cells) (see Table 1). Parallel experiments indicated that 5×10^5 or 0.5% of the cells were transformed to G418^R. Thus, the frequency of complementing plasmids in the library is $\geq 10^{-5}$. As described in the legend to Table 1, it is not possible to determine the actual number of initial Thy⁺ transformants.

Isolation of Complementing Plasmids and Rescue. Total genomic DNA was isolated from the nine thymidine-independent cell lines, each from a different electroporation, and was used to transform *E. coli*. Fifty nanograms of total DNA from the five G418^R cell lines gave between 1000 and 4000 colonies, while only background levels of colonies were seen with DNA from the revertant and the four thymidine-independent G418^S lines (<50 colonies). The plasmids isolated from the Thy⁺ G418^R lines had either a 3.9- or a 3.0-kb insert. Representative clones of these plasmids were termed Thy1P-1 and Thy1P-42, respectively. Plasmids Thy1P-1 and Thy1P-42 were used to transform HPS400 again and both rescued the mutation at high efficiency ($\approx 0.5\%$ of the cells

transformed to Thy⁺). In addition, these Thy⁺ cells are also G418^R. When these plasmids are used to select for G418^R, the same high frequency (0.5%) of cells is transformed. Moreover, cells transformed and selected for Thy⁺ grow at the same rate as either transformed cells selected for G418^R or untransformed HPS400 (data not shown; see Discussion). Restriction enzyme mapping of the *Dictyostelium* genomic DNA inserts from the two plasmids showed that both had the same 3.0-kb partial *Sau3A* fragment and thus came from the same genetic locus. Thy1P-1 has a 0.9-kb *Sau3A* fragment not present in Thy1P-42, accounting for the size difference between the two inserts. The map of the Thy1P-42 insert is shown in Fig. 2. Thy1P-42 was used for the subsequent analysis since it was smaller and showed identical rescuing ability. The 3.0-kb *Bam*HI/*Hind*III fragment from Thy1P-42 also transforms HPS400 to thymidine independence when subcloned into the plasmid pGEM7Zf(+), which lacks any extrachromosomal DNA sequence and transforms by integrating randomly into the chromosome. The transformation frequency is consistent with that observed for integrating vectors carrying the actin-*neo*^R gene fusion conferring G418^R (data not shown).

Expression of Isolated Sequence. Hybridization of RNA blots containing vegetative RNA from KAx-3 with the 3.0-kb *Bam*HI/*Hind*III insert fragment from Thy1P-42 showed a single mRNA of 1.2 kb, which we have termed Thy1. This RNA was mapped to the 1.7-kb *Bam*HI/*Cla*I fragment by hybridization to subfragments of the Thy1P-42 insert. This 1.7-kb fragment was hybridized to RNA blots of vegetative RNA from four strains: KAx-3, HPS400, 7B-3 (a Thy⁺ G418^R complemented mutant), and 8A-9 (a Thy⁺ G418^S putative revertant). The results are shown in Fig. 3. KAx-3, HPS400, and the putative revertant all show mRNAs of equal size and abundance, while the complemented mutant had an mRNA of similar size but with a 5-fold greater level of expression.

Sequence Analysis of Thy1. The 1.5-kb *Nsi*I/*Cla*I fragment (see Fig. 1) was sequenced. Analysis of the DNA sequence revealed only a single extended open reading frame in either orientation when the sequence of the 90-bp putative intron is deleted. This putative intron contains consensus splice donor and acceptor sequences and A+T-rich internal sequences similar to other *Dictyostelium* introns (35). The open reading frame of 260 amino acids, with the intron, is shown in Fig. 4. The open reading frame also has a codon usage consistent with that of other *Dictyostelium* genes (36, 37). Thymidylate synthase is a protein highly conserved throughout evolution, showing 45% amino acid identity between *E. coli*, *S. cerevisiae*, and humans. Computer analysis of the Thy1 open reading frame shows no homology to thymidylate synthase from other systems (33, 34). Analysis of any of the short open

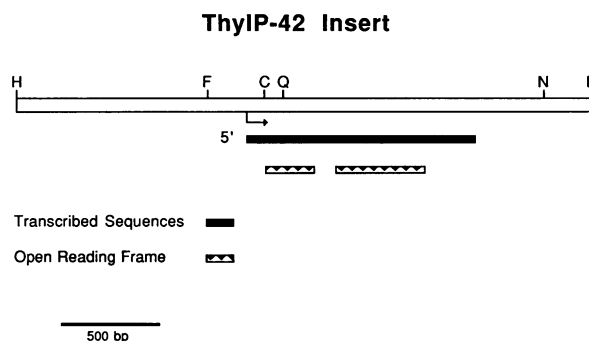


FIG. 2. Partial restriction map of Thy1P-42 insert and location of transcribed sequences. Location of transcribed sequences was determined by hybridization to vegetative RNA from strain KAx-3. Locations of the open reading frame and intron were determined by analysis of the sequence in Fig. 4. B, *Bam*HI; C, *Cla*I; F, *Cfo*I; H, *Hind*III; N, *Nsi*I; Q, *Nco*I.

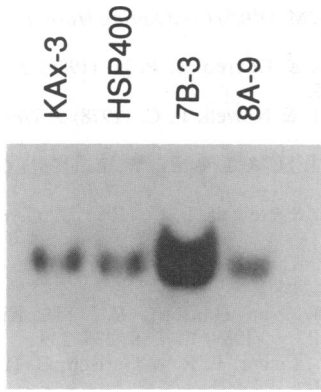


FIG. 3. Expression of Thy1P-42 insert. Thy1P-42 insert was labeled with ^{32}P and hybridized to vegetative RNA blots from wild-type axenic strain KAX-3, thy^- mutant HPS400, positive 7B-3 (Thy^+ G418^R complemented mutant), and positive 8A-9 (Thy^+ G418^R putative revertant).

reading frames in either orientation also shows no sequence homology of thymidylate synthase. Thus, presumably Thy1 does not encode thymidylate synthase. No homology was seen to any other protein sequence in the data base examined.

DISCUSSION

Genetically, we have demonstrated genomic library complementation in the cellular slime mold *D. discoideum*. All five Thy^+ G418^R transformants contain sequences from the same locus. This locus complements the mutant HPS400 repeatedly and efficiently, in both extrachromosomal and integrat-

ing vectors. The transformation protocol used has a sufficiently high frequency that the entire library can be introduced in 1 day. Complementing plasmids are easily recovered from *Dictyostelium* by transforming *E. coli* with *Dictyostelium* total cell DNA. The library appears to be representative of the entire *Dictyostelium* genome, since two different complementing plasmids were isolated from three different library fractions.

One use for the isolated sequence is as a selectable marker. Recombinant plasmids containing the Thy1P-42 insert could be selected for in HPS400. These transformed cells would then be available for the introduction of a second plasmid containing the bacterial *neo*^R gene and selecting for G418^R by using either integrating or extrachromosomal vectors. Another dominant drug selection for *Dictyostelium* has recently been established by using the bacterial hygromycin-resistance gene driven by a *Dictyostelium* actin promoter (38). While potentially very useful, this selection only works on a high-copy-number extrachromosomal vector that is already very large, making it difficult to clone large inserts.

The nature of the mutation and the function of the complementing clone are unknown. Although HPS400 appears to be altered at the level of thymidylate synthase activity, the exact step mutated in this process is not clear and it is not proven that the phenotype results from the absence of thymidylate synthase activity. The isolated gene, Thy1, is either the wild-type copy of the gene mutated in HPS400 that confers the requirement for thymidine or a suppressor of that mutation. It is not possible for us to distinguish between these possibilities since the molecular defect of the original mutation is not known. That we obtain DNA from only a single genetic locus may be significant and could mean that we have isolated the wild-type counterpart of the mutated gene in

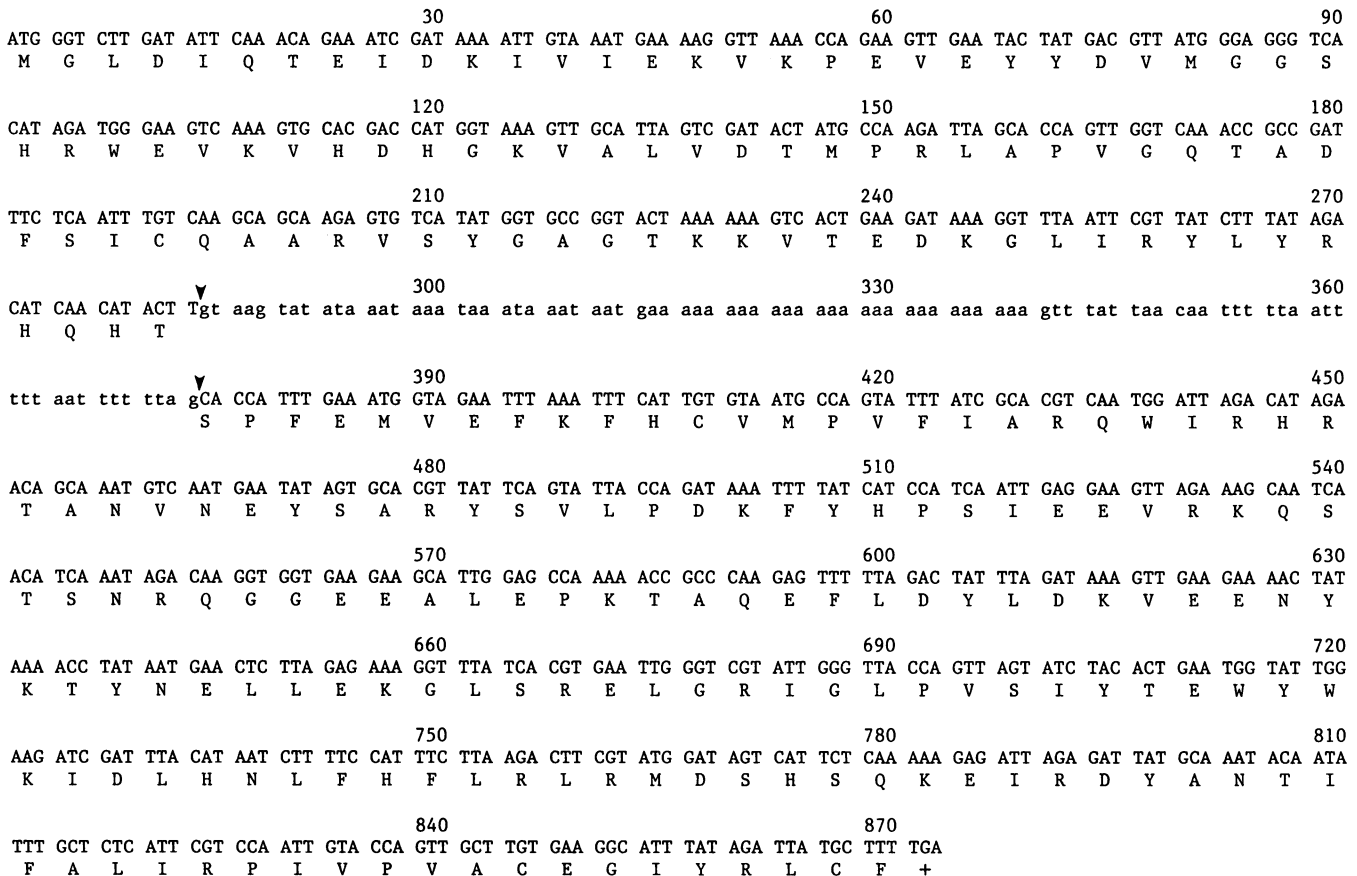


FIG. 4. DNA and derived amino acid sequence (single-letter code) of open reading frame from Thy1P-42 insert. Arrowheads indicate the putative mRNA splice sites as deduced from the DNA sequence.

HPS400. If we have isolated a suppressor, our library or our method may not be as universally applicable as our experiments suggest. We see an ≈ 5 -fold overexpression of the Thy⁺ mRNA in our transformants, but this is a function of the *Dictyostelium* transformation system in which extrachromosomal vectors are present in multiple copies in the cell (23, 24). Moreover, integrating vectors produce multiple-copy tandem arrays (26). Since we see the same high frequency of transformation of HPS400 cells to Thy⁺ or G418^R and since Thy⁺ cells are also G418^R, we do not feel that this is a suppressor. A suppressor gene expressed at a level only 5-fold higher than the endogenous gene might be expected to have a lower frequency of transformation and a slower growth rate than cells selected for G418^R and grown in the presence of thymidine. This was not observed.

A spontaneous revertant of HPS400 able to grow in HL5 medium in the absence of added thymidine, termed HPS401, was isolated by Podgorski and Deering (17) and was characterized along with HPS400. The single revertant was found in 1.8×10^8 cells at a similar frequency to the one revertant in 6×10^7 cells that we have observed. The two mutations, HPS400 and HPS401, map to different chromosomes (39). Unlike HPS400, which requires 20 μg of thymidine per ml to grow in minimal medium, HPS401 requires only 1 μg of thymidine per ml. Further analysis indicates that the transport of thymidine is increased in this mutant, enabling it to utilize the estimated 6 $\mu\text{g}/\text{ml}$ found in the standard rich HL5 axenic medium (40). It is possible that if Thy1 is a suppressor gene, it may be of the same type present in HPS401.

We have demonstrated genomic library complementation in *Dictyostelium* and used it to isolate a selectable marker that provides the ability to transform cells previously transformed with another selectable marker. Complementation will now allow the cloning of genes for which phenotypically distinct mutations exist, given appropriate screening procedures. Many developmental mutations, especially those affecting signal transduction processes (see Introduction), have been characterized in *Dictyostelium*, and their complementation will lead to the isolation of genes that control these various developmental processes. This approach has been especially successful for the sterile mutations in *S. cerevisiae*, resulting in many genes controlling the yeast mating-type response (41). Because of the availability in *Dictyostelium* of powerful techniques, such as antisense inactivation and gene disruption (42, 43), for the analysis of gene function, *Dictyostelium* genes isolated by complementation can be further manipulated to understand their biological role.

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