Identification and characterization of Tdd-1 and related retrotransposons in Dictyostelium.

Gail A. Henderson
IDENTIFICATION AND CHARACTERIZATION OF Tdd-1
AND RELATED RETROTRANSPOSONS IN DICYOSTELIUM

A Thesis
Presented to the
Department of Biology
and the
Faculty of the Graduate College
University of Nebraska

In Partial Fulfillment
of the Requirements for the Degree
Master of Arts
University of Nebraska at Omaha

by
Gail A. Henderson
May, 1993
THESIS ACCEPTANCE

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ABSTRACT

Previous studies in *Dictyostelium discoideum* on the transposable element Tdd-1, have identified two transcription units of opposite polarity. One is a major, developmentally regulated 4.5 kb transcript believed to encode proteins involved in the retrotransposition of this element, and the other, 1.4 kb in size, is a heat-shock inducible RNA. Based on the sequence of the 4.5 kb transcript and the encoded amino acid sequence, it has been proposed that Tdd-1 is a retrotransposon. This implies that the 4.5 kb mRNA is copied by reverse transcriptase, resulting in DNA copies that insert into new sites. The purpose of this study was to identify similar retrotransposons in two other *Dictyostelium* strains, *D. discoideum* V-12 and *D. mucoroides* DM-7, and to estimate retrotransposon copy number.

To identify the Tdd-1 gene in *D. discoideum* strain NC-4, polymerase chain reaction (PCR) primers were designed using the published nucleotide sequence in the known highly conserved amino acid regions of reverse transcriptase. The product of this amplification was a 370-bp fragment, which was then cloned. The identity of the fragment as part of Tdd-1 was confirmed by restriction mapping and DNA sequencing.

To estimate gene copy number, Southern blots were analyzed using a probe made from the cloned fragment. In all Southern blot analysis NC-4 had the highest copy number, followed by *D. discoideum* V-12 and *D. mucoroides* DM-7, respectively. Although the band patterns on Southern blots were not reproducible, *D. discoideum* NC-4 always had more bands than *D. discoideum* V-12, which had more bands than *D. mucoroides* DM-7. This was consistent with PCR results where NC-4 always yielded the greatest concentration of amplified product of the expected size.

A probe was used to identify clones with complementary sequences to the Tdd-1 fragment in a genomic library of *D. mucoroides*. Digoxigenin (dig)-labeling of the
cloned probe was successfully accomplished by employing the PCR. Identification of genomic clones from the lambda library was accomplished using these dig-labeled probes.
ACKNOWLEDGMENTS

My sincere appreciation goes to my major professor, Dr. Tom Weber. Because of his technical expertise, photographic experience, patient critical evaluation of the manuscript, and unfailing optimism, this project was the most valuable learning experience in my academic career. Even during the frustrating times, working in his lab was exciting, challenging, and fun, because that is the kind of atmosphere he has created for his students. He truly knows what it means to be a teacher. Thank you again, Dr. Weber.

Special thanks go to my committee members, Dr. James Van Etten, Dr. Nora Chapman, and Dr. Bruce Chase. Because of their carefully thought-out questions and advice, I came to a better understanding of this project and what it means to be a good scientist. I value this information.

I also owe a debt of gratitude to a host of other people who provided technical assistance as well as emotional support. Annika Weber, John Mullican, Dr. Nancy Hanson, Dr. Bill Tapprich, Dr. Bill deGraw, Dr. Ann Antlfinger, and Dr. Dave Sutherland kindly provided assistance and access to their equipment. Marilynn Larsen, Margaret Esser, Kevin Brewer, and Sarah Lambie also provided valuable assistance and support.

I would like to dedicate this manuscript to my husband Stephen, whose computer literacy was absolutely essential. My undergraduate and graduate education was as much his goal as it was mine. Such unselfish, unfailing support will always be remembered and treasured.
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INTRODUCTION

In the 1940s Barbara McClintock challenged the classical idea of genes being located at fixed loci on the chromosomes. Her pioneering work with maize led to the idea of transposable genetic elements or 'jumping genes.' A skeptical biological community ridiculed her revolutionary concept, but in 1983 she captured the Nobel Prize for her work. Such elements were not actually identified until the 1960s, when it was shown that a substantial proportion of spontaneous mutations in the lac and gal operons of Escherichia coli were the consequence of the insertion of specific fragments of DNA now known as transposable elements. In the early 1970s it was realized that the rapid spread and diversification of multiple antibiotic resistance in bacteria resulted largely from the activity of transposons. It soon became evident that eukaryotic genomes also encoded large numbers of transposable elements and that these were responsible for a sizeable proportion of spontaneous mutations isolated in the laboratory environment (Sherratt, 1991). Whether these transposons are responsible for some or most naturally occurring genetic variation is at present the subject of much debate in the biological community (Travis, 1992).

There are different kinds of transposons found in nature. The simplest is the IS element found in prokaryotes. As shown in Figure 1A, an IS element contains genes for inserting the DNA segment into a chromosome and for mobilizing the element to different locations. They have been detected at many locations in prokaryotic genomes. These IS elements are capable of inserting into a chromosome at locations where there is no homology. This is an example of a transposition event. If the integration of an IS element places it within a gene, then that gene is usually inactivated. An IS element also has the ability to excise itself from the chromosome.
Figure 1. Generalized structure of transposable elements. A: An insertion sequence (IS) element. B: A transposon (Tn).
Inverted repeat sequences
Core area

Direct repeat sequences flanking the IS

Transposon (Tn)
Terminal Modules (repeats)
Core Area

Direct repeat sequences flanking the transposon

Genes for Transposition
Genes for drug resistance, etc.

IS (insertion element)

TGATC ACTAG

IS

(TGATC ACTAG)
If the excision event is perfect, then the gene returns to normal. If, however, excision is imprecise, some or all parts of the gene surrounding the IS element may be deleted and transposed with the element, or the element may transpose to another site on the same or a different chromosome while leaving a copy of itself at the original site. All IS elements that have been sequenced end in a perfect or near perfect inverted repeat segment of between 20 and 40 base pairs.

The second type of transposable element is the transposon (Figure 1B). It is a mobile DNA segment that contains genes for the insertion of the DNA segment into the chromosome and for the mobilization of the element to other locations on the chromosome; but unlike IS elements, it also contains genes of identifiable function, such as drug resistance. Transposons often end in long (800-1500 bp) direct or inverted repeats, which themselves are IS elements.

The most frequent event in a transposon’s life is transposition; that is, the integration of the transposon into new sites in the genome. The site for integration is called the target sequence. Transposition usually results in the repeat of a short base pair segment of the target DNA on either side of the IS element, where only one copy was present before the insertion event. All transposons move precisely, which indicates that an enzyme or enzymes recognize the ends of the elements, excise the elements, and insert them in new locations. This enzyme is transposase, and the transposition event in prokaryotes occurs once in $10^7$ generations, which is considered a rare event. The transposase cleaves the target to give 5' protrusions of characteristic length. This cleavage requires that the enzyme be bound to the element ends. Transposase-mediated cleavage also occurs in the donor, such that the two 3' ends of the element are free to be transferred to the 5' ends of the target. With Moloney Murine Leukemia Virus integrase and phage Mu transposase, strand transfer normally
occurs without cleavage of the other strand of the DNA containing the donor element, whereas Tn10 and Tn7 transposases cleave the other strand, so that the whole element is excised from the donor molecule. After stand transfer, the transposition/integration is completed by DNA replication to fill in the protrusions or, in the case of replicative Mu, to copy the elements (Sherratt, 1991). All elements require some DNA polymerase activity to form the target site duplication, and in some cases this may continue to produce two copies of the element (see Figure 2), one copy that remains at the donor site and another integrated at the target site (Finnegan, 1989).

A third class of transposable elements known as retrotransposons transpose via an RNA intermediate in a way similar to retroviruses. Rather than transposing DNA to DNA it is thought that these transpose by making an RNA copy of the integrated DNA sequence and then by creating a new element. The new element then integrates at a new chromosome location. These transposons, like the proviruses of retroviruses, (1) are generally integrated into host DNA with short reiterations of cellular DNA at either end, (2) have long terminal repeated regions at either end, (3) have binding sites for a tRNA primer near the left LTR, (4) have long open reading frames (ORFs), one of which encodes a putative reverse transcriptase, and (5) have an ORF that can only be expressed by avoidance of a termination codon (Baltimore, 1985).

There is in some classification schemes a second type of retrotransposon regarded as a nonviral element. These usually have two open reading frames, the first being similar to a gag gene and the second encoding a potential reverse transcriptase. They have no terminal repeats, and have a characteristic A-rich sequence at the 3' end of one strand. They make up families comprised of a mixture of complete and incomplete elements, many of them truncated by varying amounts at the 5' end of the
Figure 2. The transposition event. Single-strand breakage occurs in both the target sequence and at the ends of the transposon. The transposon attaches to the target, followed by filling in and sealing, sometimes with duplicated target sequences resulting.
Target sequence

Single-strand breakage at ends of transposon

Attachment of transposon

Filling in and sealing

Duplicated target sequences
strand that has the A-rich sequence. The structure and coding capacity of these elements suggest that they transpose via an RNA intermediate, although this has yet to be proven. Truncated elements probably are incapable of transposing, even in the presence of complete elements, because they have lost sequences necessary for transcription of transposition intermediates (Finnegan, 1989).

Transposition by reverse transcription would be a replicative event as the donor element would not have to excise from the genome. As a result, the number of elements in the genome should increase after each transposition event. A question that arises concerns the nature of the intermediates between the synthesis of the transcript and its appearance as DNA at a new site of chromosome integration. It could be assumed that the RNA-DNA hybrid molecules would look like those involved in retrovirus reverse transcription. Would the homology extend to the formation of retrovirus-like particles during retrotransposon transposition? There is no evidence that infectious particles are released from cells, but there could be intracellular particles. Shiba and Saigo (1983), working with the copia system of Drosophila, found that intracellular reverse transcriptase was associated with a particulate fraction. Retrotransposons could be retroviruses that lack an extracellular phase. Another question concerns how reverse transcriptase is prevented from reverse transcribing other cellular DNAs. Compartmentalization of an inactive precursor into a particle, which apparently occurs in retroviruses (Witte and Baltimore, 1978), would be one way to inhibit random reverse transcription. Another would be the requirement for a tRNA, or other complementary RNA, as a primer (Baltimore, 1985).

In the course of cloning segments of Dictyostelium discoideum genomic DNA that are expressed preferentially during early differentiation, two independent labs isolated transposable elements. Zuker and Lodish in 1981 named the element DIRS-1,
and Rosen, Sivertsen, and Firtel in 1983 termed it Tdd-1. For simplicity it shall be referred to as Tdd-1 in this paper. This element is 4.7 kb in length and consists of 4.1 kb of internal unique sequence flanked by 330 bp of inverted terminal repeats (ITRs) (Zuker et al., 1984). Elements that are inserted at different locations have similar terminal repeats and internal 4.1 kb segments (Capello et al., 1985). It appears to be a retrotransposon containing two transcription units of opposite polarity (Firtel, 1989). One is a major developmentally regulated 4.5 kb transcript believed to encode a protein involved in the transposition of this element, and the other, 1.4 kb in size, is a heat-shock-inducible RNA (Firtel, 1989). This second transcript presumably does not encode a functional protein, since it does not contain an extended open reading frame (ORF) (Capello et al., 1984).

Two other elements, Tdd-2 and Tdd-3, are considered mobile genetic elements because they are present at different chromosomal locations in different laboratory strains and wild-type isolates of *D. discoideum*, but do not appear to be retrotransposons. Unlike Tdd-1, they lack long terminal repeats (LTRs) and are not known to be transcribed during either vegetative growth or multicellular differentiation. An unusual aspect of Tdd-2 and Tdd-3 is that both contain a short (22-base-pair) homologous sequence and structural analysis suggests that one transposon preferentially inserts into the other (Poole and Firtel, 1984).

There are about 50 copies of the intact Tdd-1 element per haploid *D. discoideum* genome and from 50 to 100 copies of partial or truncated elements, according to Firtel (1989). Tdd-1 appears to be present in all strains and is mobile, because it also is present at different chromosomal locations in different laboratory strains and wild-type isolates. Analysis of about 20 genomic clones containing whole or partial elements indicates that the flanking sequences surrounding Tdd-1 consist of
partial Tdd-1 elements, suggesting that Tdd-1 preferentially inserts into other Tdd-1 sequences (Capello et al., Rosen et al., 1984), although it is possible that insertion of Tdd-1 causes rearrangements that result in extensive duplications of the Tdd-1 element (Firtel, 1989). In five out of six genomic examples that were examined by Capello (1984), Tdd-1 had been inserted within a preexisting Tdd-1-related sequence. The exact nucleotides within the target sequence at which the insertions occur were more or less random, except that they were all localized within the right half of the element. The orientation of the incoming element was independent of the orientation of the target sequence, and no target site duplication occurred upon insertion. The simplest explanation given by Capello involved the recognition of integrated Tdd-1 elements as insertional "hot spots", which would require mediation by a Tdd-1-encoded protein.

Tdd-1 is an unusual eukaryotic transposon because it has long terminal repeats that are inverted. Both the left and right repeats flanking a single element frequently contain nucleotide sequence alterations that are found in no other cloned copies of the Inverted Terminal Repeats (ITRs) that have been sequenced. However, the left and right ITRs of individual elements are not identical. At three specific locations in the ITR sequence consistant sequence differences occur between left and right repeats (Capello et al., 1985). The most obvious is 27 bases present only at the distal terminus of all right ITRs. The mechanism that allows an exchange of information between ITRs of the same element must also provide for the maintenance of the sequence differences between them. Tdd-1 encodes three open reading frames longer than 100 codons. All three are present on the same strand and would be encoded by the dominant 4.5 kb RNA that is transcribed rightward from the left ITR. As shown in Figure 3, ORF3 spans more than 2000 bases and encodes a putative polypeptide of 76,000 daltons (Capello et al., 1985).
FIGURE 3. STRUCTURE AND TRANSCRIPTION OF Tdd-1

Points of initiation of the major Tdd-1 RNAs; the 4.5 kb RNA transcribes from left to right, and E1, from right to left (Cohen et al., 1984). The positions and lengths of the three Tdd-1 open reading frames (ORFs) are also shown.
Consensus
Tdd-1

4.5 kb RNA

500 bp

HSP

ORF1

ORF2

ORF3
The analysis of nucleotide sequences from the vertebrate retroviruses, animal DNA viruses, and from some plant viruses has shown that many of these encode an RNA-dependent polymerase involved in their replication (Kamer and Argos, 1984). A comparison of these pol gene products has shown that a region of about 200 amino acids in these polypeptides can be aligned to display a significant homology. (Toh et al., 1983). At the core of this homologous region are ten invariant amino acid positions in almost all known reverse transcriptase genes. A comparison of the Tdd-1-encoded polypeptides with the amino acid sequence of the pol genes of Rous sarcoma virus (RSV) (Schwartz et al., 1983) and Moloney murine leukemia virus (MoMLV) (Schinnick et al., 1981) showed that ORF3 is homologous to this conserved reverse transcriptase region. In Figure 4, the 200 amino acid regions of homology between ORF3 and MoMLV are aligned with the sequence of the RNA-dependent polymerase of Cauliflower Mosaic Virus (CaMV, Gardner, et al., 1981). Amino acid positions identical with the Tdd-1 ORF3 sequence are boxed. The ten invariant amino acid positions present in the reverse transcriptase sequences described by Toh et al., (1983) are denoted by ♦ above the sequence (Capello et al., 1985). The Tdd-1 ORF3 product is identical at 53 out of 200 amino acid positions (26%) with MoMLV and at 47 out of 200 (24%) with the CaMV pol proteins, and it contains all ten of the invariant reverse transcriptase residues.

The purpose of this thesis was to identify and clone Tdd-1, or related elements, from two strains of D. discoideum and from D. mucoroides DM-7. Putative elements were amplified from genomic DNA using primers designed based on the conserved regions of Tdd-1 using the polymerase chain reaction. A Tdd-1 fragment was cloned from a PCR product of D. discoideum NC-4. The identity of this fragment was confirmed by restriction mapping and DNA sequencing. The clone was then used to
Figure 4. The 200 amino acid regions of homology between ORF 3 of Tdd-1 and MoMLV are aligned with the sequence of the RNA-dependent polymerase of Cauliflower mosaic virus (CaMV, Gardner, et al., 1981). Amino acid positions identical with the Tdd-1 ORF3 sequence are boxed. The known reverse transcriptase ten invariant amino acid positions are denoted by "♦" above the sequence.
prepare a probe for analysis of Southern blots to estimate gene copy number. In addition, the probe was used to identify clones with complementary sequences to the Tdd-1 fragment in a genomic library of *D. mucoroides*. Ultimately the cloned transposons will be used for mutagenesis and molecular tagging of disrupted genes.
MATERIALS AND METHODS

ORGANISM AND CULTURE CONDITIONS

*D. discoideum* strains NC-4 and V-12 along with *D. mucoroides* strain DM-7 were grown in association with *E. coli* B/r as a nutrient source. Stock cultures were maintained on 0.2% lactose-peptone agar plates (0.2% lactose, 0.2% peptone, 1.5% agar). To obtain amoebae for DNA isolation, \(1.0 \times 10^5\) spores and 0.1 ml of a turbid suspension of *E. coli* B/r were inoculated into 250 ml flasks containing 40 ml glucose-yeast-peptone broth (Weber and Raper, 1971). Flasks were incubated at 23° C in covered gyratory shaking water baths. Amoebae in early stationary phase were harvested after 56 hours. The cells were harvested by centrifugation at 500 xg (2000 rpm) in a Sorvall GSA rotor for 15 minutes at 4° C. The amoebae were washed twice with sterile deionized water to remove residual bacteria, centrifuging as above.

DNA ISOLATION

Nuclear DNA was isolated essentially as described by Richardson et al. (1990). Washed cells were resuspended in ice-cold nuclei buffer (40 mM Tris-HCl, pH 7.6, 15% sucrose, 0.1 mM EDTA, 6 mM MgCl\(_2\), 40 mM KCl, 5 mM DTT, 0.4% NP-40) to \(1-5\times 10^8\) cells/ml. After 5 minutes on ice, the nuclei were pelleted by centrifugation at 10,000 xg for 10 minutes in a Sorvall SS-34 rotor and the supernatant was discarded. This lysis step was repeated once. Nuclei were resuspended in 0.3 vol proteinase K buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 0.5 % SDS, 100 \(\mu\)g/ml proteinase K) and then digested 60 minutes at 60° C. After cooling to room temperature, the DNA was extracted sequentially with phenol, phenol-chloroform, and chloroform and then
ethanol precipitated. After air-drying the DNA was resuspended in sterile deionized water. Quantitative and qualitative assessment of the DNA was determined by ultraviolet absorption spectrophotometry at 260 and 280 nanometers.

**PCR AMPLIFICATION OF Tdd-1**

Oligonucleotide primers were constructed based on highly conserved amino acid sequences of the reverse transcriptase gene. Toh et al (1983) reported that all known reverse transcriptase sequences contained invariant amino acids at ten positions. Two groups consisting of eight conserved amino acids 130 residues apart were chosen to design primers. Designed into the forward primer and the reverse primer were restriction enzyme sites (Kpn I and Bam HI respectively) to facilitate cloning. The primers were designed as follows:

**Dictyostelium discoideum 5’**

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<th>Forward Primer</th>
<th>TTA CAT GGT AAA ACT CGA TAT CAA GAA ....</th>
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<tr>
<td></td>
<td>TA CAT GGT Ac c ACT CGA TAT CAA</td>
</tr>
<tr>
<td></td>
<td>Kpn I</td>
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**Dictyostelium discoideum 5’**

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<th>Reverse Primer</th>
<th>CC GgA TCG ATT TGT AAT CCG AGA AAA GTA ...</th>
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<tr>
<td></td>
<td>CC GgA  TCc ATT TGT AAT CCG AGA A</td>
</tr>
<tr>
<td></td>
<td>Bam HI</td>
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The primers were synthesized by Operon, Inc. of California.

To optimize amplification of the desired fragment, different concentrations of genomic DNA were added to the reaction mix. Amounts tested were 10 μg, 1 μg, 100 ng, 10 ng, and 1 ng. A negative control was also run with sterile TE replacing the DNA.
The initial reaction mix consisted of:

- 30 µl sterile deionized water
- 10 µl 10x amplification buffer including MgCl₂ (Promega)
- 4 µl dATP: 1.25 mM, pH 7.0
- 4 µl dTTP: 1.25 mM, pH 7.0
- 4 µl dCTP: 1.25 mM, pH 7.0
- 4 µl dGTP: 1.25 mM, pH 7.0
- 5 µl Forward Primer: 20 µmol/µl
- 5 µl Reverse Primer: 20 µmol/µl
- Template DNA
- Sterile deionized water to 100 µl
- 0.5 µl Taq Polymerase (Promega)

A second reaction mixture was tested in an attempt to obtain greater yield. This was from PCR Protocols (Innis et al., 1990) and consisted of:

- 10 µl 10x amplification buffer
- 10 µg genomic DNA
- 6 µl MgCl₂ from Taq polymerase kit
- 64 µl dNTPs (1.25 mM, pH 7.0)
- 5 µl Forward Primer
- 5 µl Reverse Primer
- 0.5 µl Taq Polymerase

Initial polymerase chain reactions followed conditions set forth by Sambrook, et al. (1989). The expected end product was a 370-bp fragment identical to that contained in ORF 3 of Tdd-1. Conditions for denaturation, annealing, and polymerization were as follows:

- **First Cycle**: 5 minutes at 94°C, 2 minutes at 50°C, 3 minutes at 72°C
- **Subsequent Cycles (28)**: 1 minute at 94°C, 2 minutes at 50°C, 3 minutes at 72°C
- **Last Cycle**: 1 minute at 94°C, 2 minutes at 50°C, 10 minutes at 72°C
- **Final Step**: Hold at 4°C
A second set of conditions for the reactions was also tested. This protocol was suggested by John Mullican (personal communication):

<table>
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<tr>
<th>Cycle</th>
<th>2.5 minutes at 94°C</th>
<th>1 minute at 50°C</th>
<th>1.5 minutes at 72°C</th>
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<tr>
<td>First Cycle</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Subsequent Cycles (38)</td>
<td>2.5 minutes at 94°C</td>
<td>1 minute at 50°C</td>
<td>1.5 minutes at 72°C</td>
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<tr>
<td>Last Cycle</td>
<td>1 minute at 94°C</td>
<td>1 minute at 50°C</td>
<td>5 minutes at 72°C</td>
</tr>
<tr>
<td>Final Step</td>
<td>Hold at 4°C</td>
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After amplification gel electrophoresis was used to confirm the presence of the appropriate size fragment. Ten microliters of DNA from the amplification was loaded into the wells of a 1% agarose gel using an IBI MPH electrophoresis chamber and run at 100 volts for 1 hour. One microliter (1 mg/ml) of a 100-bp DNA ladder (BRL) was run as a size standard.

**CLONING**

The amplified Tdd-1 fragment was purified by ammonium acetate and ethanol precipitation. Three ligation procedures were used in attempts to clone the fragment. One procedure was a staggered-end ligation, and the other two were blunt-end ligations with different ligation reactants. The initial ligation involved digesting the selected vector, pUC 19, and the amplified product with Kpn I and Bam HI for 1 hour at 37°C and then following the ligation procedure as found in Maniatis et al., 1982. The plasmid was first diluted to a final concentration of 0.4 μg/μl. The DNA mixture consisted of 1 μl of insert (0.2 μg/μl), 1 μl of diluted pUC 19, and 8 μl H2O. Dilute T4 Ligase was prepared by adding 8μl of water, 1 μl of 10X buffer, and 1 μl of T4 ligase (30 units/μl). The DNA mixture was heated to 45°C for 5 minutes and then placed on ice. One microliter of ligation buffer and one microliter of dilute ligase was then added. This solution was maintained at 16°C for 4 hours, and then transferred to
refrigerator for overnight incubation. This was followed by transformation into *E. coli* DH5α by the Standard Transformation Procedure (Hanahan, 1985). Five ml of SOB medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, pH 6.9) was inoculated with *E. coli* DH5α and incubated overnight at 37°C. The overnight culture was diluted 1:25 in SOB medium and incubated at 37°C with agitation to an A₆₀₀ of 0.5. The cells were transferred to a 50 ml polypropylene centrifuge tube and incubated on ice for 15 minutes. The cells were collected by centrifugation at 500g (3000 rpm) at 4°C with a Sorvall GLC-2 type SS-34 rotor. The pellet was resuspended in 8 ml cold TFB (10 mM K-MES, pH 6.3, 100 mM RbCl, 45 mM MgCl₂·4 H₂O, 10 mM CaCl₂·2H₂O, 3 mM HaCoCl₃) by gentle mixing and incubated for 15 minutes on ice. The cells were again collected by centrifugation as before and resuspended in 2 ml cold TFB. Seven µl cold DnD (1.53 gram dithiothreitol, 9 ml DMSO, 100 µl 1M KOAc, pH 7.5) was added by gentle swirling. This suspension was placed on ice for 10 minutes, then an additional 7 µl of cold dithiothreitol and dimethyl sulfoxide (DnD) was added and the suspension was again swirled and iced for 10 minutes. Aliquots of 210 µl were pipetted into chilled polypropylene tubes. Twenty microliters of the DNA solution was added with swirling, and the resulting mixture was incubated on ice for 30 minutes. The cells were then heat-shocked in a 42°C water bath for 90 seconds and placed on ice for 2 minutes. Volumes of 10, 50 and 100 µl were aliquoted into 200 µl SOC medium (SOB medium plus 20 mM glucose) and incubated at 37°C for 60 minutes with occasional gentle mixing. Various dilutions of the cells were plated in duplicate on LB plates containing ampicillin (50 µg/µl), X-gal (40 µl/plate), and IPTG (10µl/plate) using an inoculum of 10, 50, or 100 µl per plate. As a control, pUC 19 without an insert was transformed into similar host cells and plated.
The second ligation procedure used a blunt-end cut of the plasmid using the enzyme Hinc II and a fill-in of the insert as published in the Promega Technical Bulletin No. 57. The standard reaction is as follows:

- 26 µl of partially digested genomic DNA (4µl pUC 19, 1 µg/µl + 22 µl of insert, 0.2 µg/µl)
- 5 µl 10x fill-in buffer containing dGTP and dATP
- 5 µl 10 mM dCTP and dTTP
- 2 µl Klenow (5 u/µl, final 1 u Klenow/ µg DNA)
- 12 µl sterile water

This mixture was incubated at 37°C for 30 minutes, then extracted with phenol-chloroform and chloroform, and precipitated with ammonium acetate and ethanol.

After this ligation, transformation followed Hanahan's Standard Transformation Procedure, again using DH5α cells as the recipients. Platings were done as before, and the resulting white colonies were picked and screened using the Quick Plasmid Size Screen (N. Hanson, personal communication). The gel for the electrophoresis chamber was prepared by melting 0.48 g agarose in 12 ml 5X TBE and 47 ml deionized water. After the solution was cooled to 50°C, 300 µl of 10% SDS was added and the gel was poured. The running buffer was comprised of 0.05% SDS in 1X TBE. The working solution for the protoplasting buffer was freshly prepared by mixing together 1 µl of DNase-free RNase (10 mg/ml) and 4 µl of lysozyme (5 mg/ml) with 200 µl protoplasting buffer solution (30 mM Tris-HCl, pH 8.0, 5 mM Na₂EDTA, 50 mM NaCl, 20% sucrose). Selected colonies were placed into 5 µl of protoplasting buffer and allowed to sit for 30-40 minutes at room temperature. Two microliters of lysis buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM Na₂EDTA, 2% SDS, 5% sucrose, 0.04% bromphenol blue) were added to each of the wells. The protoplast suspensions were then added, and the gel was run at 120 volts for 1 to 1½ hours. The gel was then washed for 2 hours in water and stained in ethidium bromide (0.5 µg/ml).
After destaining, the approximate size of the plasmids was determined by comparison with known standards.

The third ligation/ transformation procedure was similar to the second except the E. coli SURE strain was used as the host. After plating on LB/Amp/Tet/X-gal/IPTG plates and growing overnight at 37°C, resulting white colonies were picked as possible transformants. Eighteen colonies were chosen for further testing by PCR analysis (J. Mullican, personal communication). These colonies were replated, grown overnight as before, and transferred to 100 μl sterile deionized water. They were then heated to 95°C in the thermal cycler to cause cell lysis. Two microliters of this lysate were then added to:

- 2 μl 10x reaction buffer
- 2 μl 1.25 mM dATP
- 2 μl 1.25 mM dCTP
- 2 μl 1.25 mM dTTP
- 2 μl 1.25 mM DGTP
- 0.5 μl forward primer
- 0.5 μl reverse primer
- 1.08 μl MgCl2
- 10.92 μl sterile deionized water
- 1 μl Taq Polymerase, diluted 1:10

PCR profiles used were as follows:
- First Cycle: 2.5 minutes at 94°C
- Subsequent Cycles (40): 10 seconds at 50°C, 1 minute at 72°C, 5 seconds at 94°C
- Third Cycle: 1 minute at 50°C, 2 minutes at 72°C, Hold at 10°C

The resulting products were run in a 1.2% agarose gel along with a 100 bp ladder to test for production of a fragment 370 bp in length. Fifteen μl of sample and 2μl of loading buffer were added per well. Based on these results, two colonies were chosen for further testing.
**PLASMID ISOLATION**

Two clones, 1-1 and 1-4 were selected and isolated using the Rapid Isolation of Plasmid DNA Procedure from Promega. A single colony was picked from a plate and inoculated into 250 ml of TB (3.0 grams bacto-tryptone, 6 grams bacto-yeast extract, 0.1 ml glycerol, 5.785 grams dibasic potassium phosphate, and 3.135 grams monobasic potassium phosphate) medium containing 50 μg/ml ampicillin. The cells were grown overnight in a shaker at 37°C. The cells were then centrifuged at 5000g at 4°C for 15 minutes using the Sorvall GSA rotor. The next step was to resuspend the cells in 6 ml of ice-cold freshly prepared lysis buffer (25mM Tris-HCl, pH 8.0, 10mM EDTA, and 50mM glucose) and incubate in ice water for 15 minutes. Twelve ml of freshly prepared 0.2N NaOH containing 1% SDS was added, mixed by inversion, and incubated in ice water for 10 minutes. Next, 7.5 ml of 3M sodium acetate, pH 4.6, was added and mixed by gentle vortexing and iced for 20 minutes. After centrifuging at 12,000g for 15 minutes, the supernatant was transferred to another tube and the pellet discarded. A DNase-free RNase solution with a final concentration of 20 μg/ml was added and the mixture incubated at 37°C for 1 hour. Following extractions with phenol-chloroform and chloroform:isoamyl alcohol (24:1), an ethanol-NaCl precipitation followed by centrifugation at 12,000xg for 10 minutes pelleted the desired plasmid DNA. This pellet was then washed with 70% ethanol and centrifuged at 12,000xg for 5 minutes. The pellet was then dried under vacuum and dissolved in sterile deionized water. The concentration of the DNA was determined by measuring absorbance at 260 nanometers with a Varian model 634 spectrophotometer. Purity of the samples was assessed by determining the 260 nm/280 nm absorption ratios (Berger, 1987). The size of the insert was determined by digesting the plasmids with the
restriction endonuclease Bam HI to linearize the plasmid, followed by fractionation of the digested DNA on a 1% agarose gel. The plasmid pUC 19 was used as a control and also linearized with Bam HI to show size differences between the plasmids containing the insert and the control plasmid with no insert. Four micrograms of DNA were loaded in each well of the gel containing EtBr (0.5 ug/ml) and electrophoresis was carried out for approximately 1.5 hours at 100 volts utilizing an IBI MPH electrophoresis chamber.

After determining which plasmids contained DNA inserts, based on size differentiation, four micrograms of each vector was digested with the enzyme Eco RI to linearize them for treatment in PCR reactions. The presence of linearized template was confirmed by agarose gel electrophoresis, as previously described. The linearized plasmid was then added to the PCR reaction mix described earlier for production of the 370 bp insert. The resulting product was tested for correct size and approximate concentration by gel electrophoresis. It was then purified by NH₄Ac and ethanol precipitation and centrifuged at 30,000 rpm for 30 minutes in the ultracentrifuge at 4°C. This was followed by a 70% EtOH rinse and another spin for 15 minutes at 30,000 rpm. After air drying and resuspension in sterile deionized water, the product was digested with the two restriction endonucleases Alu I and Mbo II. This was done to confirm the identity of the fragment as the partial Tdd-1 fragment. Ten micrograms of each sample were digested with Alu I, Mbo II, and a combination of Alu I and Mbo II for approximately 3 hours at 37°C. The digested samples were then run on a 3% Nusieve gel at 50 volts for 2 hours.
SEQUENCING

Further confirmation of the identity of the cloned fragment was obtained by determining its nucleotide sequence. The sequencing was carried out by Margaret Esser using the Sequenase 2.0 protocol from U.S. Biochemicals. Two primers were used for this sequencing: the M13 universal primer for the forward direction and the reverse primer used for PCR was used for sequencing in the reverse direction.

PROBE SYNTHESIS

Eight micrograms of plasmid 1-1 was linearized with Bam HI to prepare the sample for PCR treatment. The digest was carried out at 37° C for 1.5 hours. This digested DNA was then added to the standard PCR reaction mix substituting a mixture of 9.75μl diluted (1:10) digoxigenin-labelled dUTP and 5.25μl dTTP for dTTP only. This method was adapted from Celeda et al (1992). The probe was purified by NH₄Ac/ethanol precipitation and centrifugation at 30,000 rpm for 30 minutes, followed by a 70% EtOH wash and 15 minute spin at 30,000 rpm. Quantification of the probe followed procedures recommended by Boehringer Mannheim (1991) for digoxigenin (dig)-labeled DNA. Dig-labeled control DNA was diluted to final concentrations of 100 pg/μl, 10pg/μl, 1 pg/μl, and 0.1 pg/μl. The labeled DNA probe was diluted in the same manner. One microliter samples of the dilutions were spotted directly onto a dry Hybond N+ membrane (Amersham). The DNA was then UV-linked to the membrane. Chemiluminescent detection of the probe followed procedures in the DIG Luminescent Detection Kit No. 1 from Boehringer Mannheim Biochemicals. The membrane was washed briefly in washing buffer (Tris-HCl, 100 mM/l; NaCl, 150 mM/l; pH 7.5). It was then incubated for 30 minutes with buffer 2
(1% blocking reagent in Buffer 1). The antibody conjugate supplied in the kit was
diluted 1:5000 in Buffer 2, and 20 ml/100 cm² membrane was added to a heat-sealable
bag and incubated 30 minutes at room temperature. The unbound conjugate was
removed by washing twice for 15 minutes with 100 ml of Buffer 1 for each 100 cm² of
membrane. Next the membrane was equilibrated for 2 minutes with 20 ml of Buffer 3
(Tris-HCl, 100 mM/l; NaCl, 100 mM/l; MgCl₂, 50 mM/l; pH 9.5) added per 100 cm²
of membrane. The freshly prepared color solution consisted of 45 μl NBT-solution, 35
μl X-phosphate-solution, and 10 ml of Buffer 3. The membrane, along with this color
solution, was heat-sealed, wrapped in foil and incubated in the dark to allow color
development to occur. Probe concentration was determined by comparing the intensity
of the sample spots to those of the control DNA.

**SOUTHERN BLOT ANALYSIS**

Single digests of genomic DNA from *D. discoideum* NC-4 and V-12 and *D.
mucoroides* DM-7 were carried out with Eco RI, Bam HI, and Hind III. In each
instance 40 μg DNA was incubated with 20 units of restriction enzyme for 5 hours at
37°C. After digestion the samples were heated to 55º C for 5 minutes and then placed
on ice for 5 minutes. Samples were then loaded into wells of a 0.7% agarose gel
along along with 3 μl of Ficoll loading buffer. The gel was run at 25 volts for
approximately 18 hours. The DNA was transferred to a Hybond membrane
(Amersham) by capillary action in an ascending 0.4 M NaOH buffer for 4.5 hours as
described by Boehringer Mannheim (1992). The DNA was immobilized on the
membrane by baking for one half hour at 120º C.

Prehybridization and hybridization was accomplished according to procedures
set forth in the Boehringer Mannheim Technical Bulletin supplied with the DIG
Luminescent Detection Kit. The membrane was placed in polyethylene heat-sealable bags with 20 ml of prehybridization buffer (formamide, 50%; 5x SSC; blocking reagent, 2%; N-laurylsarcosine, 0.1%; SDS, 0.02%) for each 100 cm² of membrane and incubated for 1-2 hours at 42°C. After removal of the prehybridization buffer, denatured probe (from 100 to 200 µg/ml) and 2.5 ml of hybridization buffer for each 100 cm² membrane was added. Hybridization was carried out in a 37°C shaking water bath for approximately 24 hours.

After hybridization, the membranes were washed twice for five minutes each at room temperature with 50 ml of 2x SSC; SDS, 0.1%, per 100 square cm of membrane and twice for 15 minutes at 68°C with 0.5x SSC; SDS, 0.1%. Chemiluminescent detection followed procedures from the Boehringer Mannheim DIG Luminescent Detection Kit. Chemiluminescent images were obtained by exposing Kodak XAR-2 film to the filters at room temperature for 4.5 hours.

**SCREENING OF GENOMIC LIBRARY WITH GENOMIC T-dd 1 PROBE**

A dig-labelled probe from NC-4 genomic DNA was prepared in a manner similar to production of the cloned probe. This was then used to screen a genomic library of *D. mucoroides* in lambda gt 11 previously prepared by Dr. Weber. Fifty ml flasks of LB medium with tetracycline (1 µl/ml) and 0.2% maltose were inoculated with *E. coli* KW 251 and incubated overnight at 37°C. Magnesium sulfate was then added to a final concentration of 10 mM.

The phage containing the genomic DNA were then diluted to concentrations of 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, and 10⁻⁶ in phage buffer (20 mM Tris-HCl, pH 7.4; 100 mM NaCl; and 10 mM MgSO₄). To 250 µl of each dilution was added 250 µl of the KW 251 bacteria. The phage were allowed to adsorb for 30 minutes at 37 °C. This
mixture was then added to 3 ml of molten top agar, and 200 μl/plate was plated in
duplicate. These plates were incubated overnight at 37 °C. Plaque lifts were
performed using Hybond nylon filters. Chemiluminescent detection of positive
plaques followed the same procedures used in Southern blot analysis.

Three plaques were picked from the 10^-4 dilution plate and each put into 1 ml
SM buffer (phage buffer + 0.1% gelatin) with 1 drop of chloroform. These were
incubated at room temperature for 1 hour and stored at 4°C. Plaque lifts were
repeated two successive times on dilutions of phage from the original positive plaques
to ensure isolation of pure clones containing sequences hybridizing to the Tdd-1 probe.
These clones were stored at 4°C in SM buffer.
RESULTS

GROWTH OF AMOEBAE AND DNA ISOLATION

Vegetative amoebae from *D. mucoroides* strain DM-7 and *D. discoideum* strains NC-4 and V-12 were harvested when the amoebae reached stationary phase. This occurred after approximately 56 hours of growth in GYP broth, at which time there were approximately $5.0 \times 10^6$ amoebae/ml. Growth periods of up to 60 hours caused negligible changes in total DNA recovered. Slight modifications were made in the published DNA isolation protocol in an effort to increase yield. The protease K treatment was carried out for 1 hour instead of 15 minutes, and the incubation temperature for this digest was decreased to 55-60° instead of the recommended 65°C. Average yields increased from a low of 0.5 μg/μl to a high of 4.0 μg/μl. The purity of the DNA as measured by 260/280 absorption ratios also increased from 1.7 to 1.9.

PCR AMPLIFICATION OF Tdd-1 FRAGMENT

The optimum amount of DNA for amplification was determined by varying the amount of genomic NC-4 DNA added to the mixture (Figure 5). Amounts tested were 0, 0.001, 0.01, 0.1, 1, and 10 μg. The greatest yield was obtained using 10 μg of DNA, although 1μg also produced a visible product.

Total genomic DNA from each of the three strains of *Dictyostelium* was used as the template DNA to determine if PCR would produce the expected 370-bp fragment. Ten micrograms of DNA from each strain was added to the reaction mix. Care was taken to add the same amount of DNA, based on absorbance readings, to each reaction. Although it is very difficult to quantify PCR reactions, genomic DNA from *D. discoideum* strains NC-4 and V-12 produced a greater yield from the primers than did
Figure 5. Agarose gel electrophoresis of PCR products obtained from different amounts of template DNA. *D. discoideum* genomic DNA was used in these reaction mixtures. Each lane of a 1% agarose gel was loaded with 10 µl of the PCR product. DNA template: Lane 1, 0.001 µg; Lane 2, 0.01 µg; Lane 3, 0.1 µg; Lane 4, 1 µg; Lane 5, 10 µg; Lane 6, Control: TE; and Lane 7, Hind III cut lambda DNA.
DM-7 (Fig. 6). Identical results were obtained in three separate experiments, with NC-4 always producing more than the other two strains.

**CLONING**

In order to proceed with cloning it was necessary to isolate the amplified 370-bp fragment from NC-4 genomic DNA. The PCR products were separated on a 1.5% agarose gel with 10 μl added per well (Figure 7). These specific fragments were then eluted from the gel using an IBI electroelution chamber. The eluate was diluted 50% with sterile deionized water and then ethanol precipitated and centrifuged at 30,000 rpm at 4°C for 1 hour. The resulting pellet was suspended in sterile deionized water.

Ligation of this fragment was hampered because very little product was recovered from the elution. However, the initial cloning appeared to be successful based on the size of the cloned fragment as determined by gel electrophoresis and by fragments obtained from restriction digests of the insert with Alu I and Mbo II. However, the nucleotide sequence obtained for the cloned fragment bore no resemblance to the sequence expected for the 370-bp fragment.

An alternative to electroelution was chosen in order to increase the amount of purified, amplified DNA available for ligation. After amplification of the desired fragment by PCR, the product was purified by ammonium acetate and ethanol precipitation, followed by centrifugation at 30,000 rpm for 15 minutes. This was followed with a rinse in 70% ethanol and another 15 minute spin at 30,000 rpm 4°C. The dried pellet was resuspended in sterile deionized water. This procedure
Figure 6. Agarose gel electrophoresis of PCR products obtained from amplification of three strains of *Dictyostelium*. Each lane of a 1% agarose gel was loaded with 10 μl of PCR product. Lane 1, *D. discoideum* strain V-12; Lane 2, *D. mucoroides* strain DM-7; Lane 3, *D. discoideum* strain NC-4; and Lane 4, Hind III cut lambda DNA.
Figure 7. Agarose gel electrophoresis for separation of amplified DNA to be used in cloning. Each well of a 1% agarose gel was loaded with 10 μl of amplified *D. discoideum* NC-4 DNA from one PCR reaction. Marker at far right is Hind III-cut lambda.
substantially increased the concentration of the fragment to be used in the ligation procedure.

Results from the second attempt indicated a successful cloning based on the size of the fragment and the results of restriction digests. However, subsequent testing by comparing the linearized sample to known standards indicated that the desired fragment was no longer present in the vector.

The most successful cloning procedure involved blunt-cutting the pUC 19 vector with Sma I and filling in the ends of the PCR fragment using the Klenow enzyme. The ligated vector-insert was transformed into the \textit{E. coli} SURE strain. This host was used in place of \textit{E. coli} DH5α in hopes of establishing a stable clone. Eighteen white colonies were picked from the agar plates as possible transformants. These were PCR-tested to determine which colonies contained the desired clone. After running 15 μl of the resulting lysates on a 1.2% agarose gel, three possible positive transformants were apparent. These results are shown in Figure 8. The two wells showing the most intensely stained bands were chosen for further study.

Plasmid isolations were performed on these two transformants. The resulting plasmids were linearized with Eco RI enzyme as was the pUC 19 control plasmid. They were then run on a 1% agarose gel. It is apparent in Figure 9 that the two test plasmids ran behind the control, indicating a slightly larger size. Following these positive results, the linearized plasmids were again subjected to PCR amplification to produce the 370-bp fragment in quantities large enough to perform restriction digests with Alu I and Mbo II.

After digestion, the DNA was separated on a 3% Nusieve gel for 2 hours at 50 volts producing the bands shown in Figure 10. These bands correlate with those reported in the Genbank sequence for \textit{D. discoideum}. The Alu I digest gave two bands:
Figure 8. Electrophoretic analysis of transformed cells. Transformed cells were lysed and their DNA amplified by PCR using designed Tdd-1 primers. Then 15 μl of each product was loaded into the wells of a 1.2% agarose gel. Marker in the far right lane is a 100-bp DNA ladder (1 μg/μl).
Figure 9. Agarose gel electrophoresis of plasmids isolated from transformed cells. Plasmids were first linearized with Eco RI, and 10 µl of each digest was then loaded onto a 1% agarose gel. Lane 1, Plasmid 1-1; Lane 2, Plasmid 1-4; Lane 3, pUC 19 control; and Lane 4, 100-bp DNA ladder (1µg/µl).
Figure 10. Nusieve gel electrophoresis of digests of cloned 370-bp fragment. The composition of the gel was 2.5% Nusieve, 0.5% SeaPlaque Agarose. Ten µl of each digest was loaded into the wells. Lane 1, Fragment digested with Mbo II; Lane 2, Alu I/ Mbo II cut; Lane 3, Alu I cut; Lane 4, Marker DNA prepared by digesting pSPORT with Pvu II.
132 and 237 bp long, an Mbo II digest gave two bands: 255 and 115 bp long. A double digest with the two enzymes together resulted in three bands 132, 123, and 115 bp long. Although the fragments from the double digest are not clearly separated on the gel, the pattern obtained is consistent with these results.

**SEQUENCING**

DNA sequencing was performed on one clone to confirm the identity of the fragment as part of the Tdd-1 sequence of NC-4. As seen in Figure 11, the sequence matched all but 8 bases of the published nucleotide sequence of the *D. discoideum* strain NC-4 genomic region of the Tdd-1 fragment. Further testing by linearizing the plasmid and comparing it to the linearized pUC control confirmed the stable maintenance of the clone in *E. coli* SURE.

**PROBE PRODUCTION AND SOUTHERN BLOT ANALYSIS**

The linearized plasmid carrying the Tdd-1 fragment was next used to produce a labelled probe for analysis of Southern blots to estimate gene copy number. Initially the product of one PCR amplification procedure was used as the probe, with the final concentration estimated at 100 μg/μl (See Figure 12). To increase the concentration of the probe, four amplification products, each starting with about 10 μg of plasmid DNA, were pooled and purified by sodium acetate and ethanol precipitation. According to the detection protocol this should have produced a probe strong enough to successfully screen the *Dictyostelium* genomic DNA. Figure 13 shows electrophoresis of the DNA from the three strains after being digested separately with three different
Figure 11. Published Tdd-1 sequence compared to sequence of clone 1-1. The top line gives the Tdd-1 sequence from Genbank, and the second line gives the results of sequencing clone 1-1. Nucleotides in bold type are mismatches, and "n" refers to undiscernible nucleotides. The "n's" at the beginning represent the primer region.
5`
gaatcgattt tgtaatccga gaaaaatgaat tgattgagtt ggttgcagaa cactccttttc
nnnnnnnnnn nnnnnnnnn nnnnnnataat tgattgagtt ggttgcagaa cactccttttc
(primer region)

tagatttaac ttgaaaccta gtttgacaaag taagttccatt gttttttttaa ggtgggataaa
tagatttaac ttgaaaccta gtttgacaaag taagttccatt gttttttttaa ggtgggataaa

acattctttct tttgttgaac cgacgattaa tagatcgttcc aagtatgcga tgacgggataac
acattctttct tttgttgaac ctacgattaa tagatcgttcc aagtaagcga tgacgggataac

gttgatatct ctcaacatttc gaagtacagg tcttaacaac attgtaagag tacgaggagc
gttgatatct ctcaacatttc gaagtacagg tcttaacagg attgtaagag tacgaggagc

tgtcgataaac ccgaacggca ttgtttttcca acggtagtgc gaacctttccc acacgaagcg
tgtcgataagc ccgaacggca ttgtttttcca acggtagtgc gaacctttccc acacgaagcg

gaataagtct ctgtattgccg gatctactaa aacgtgggaga taggctttct tgaatatcggag
gaataagtct ctgtattgccg gatctactaa tacgtgggagg taggctttct tgaatatcggag

ttttacc -Tdd-1
tggtaccatg taggg -pUC 19 with insert
3`
Figure 12. Quantification of dig-labelled probe by chemiluminescent detection. Top row is dig-labelled control DNA, and bottom row is dig-labelled Tdd-1 probe. Concentrations are, from left to right: (1) 1 ng/µl, (2) 100 pg/µl, (3) 10 pg/µl, (4) 1 pg/µl, and (5) 0.1 pg/µl.
Figure 13. Agarose gel electrophoresis of genomic DNA after digestion with three restriction enzymes. Forty μg of genomic DNA from *D. discoideum* strains NC-4 and V-12 and *D. mucoroides* strain DM-7 were each treated to separate digests of Eco RI, Bam HI, and Hind III (4 μl per digest). Digests were carried out for 4.5 hours at 37°C, with 2 additional μl of enzyme being added after 2 hours. A 0.7% gel was loaded with the digested DNA and allowed to run overnight at 20 volts. Lane 1, NC-4, Eco-cut; Lane 2, NC-4, Bam-cut; Lane 3, NC-4, Hind III-cut; Lane 4, DM-7, Eco-cut; Lane 5, DM-7, Bam-cut; Lane 6, DM-7, Hind III-cut; Lane 7, V-12, Eco-cut; Lane 8, V-12, Bam-cut; Lane 9, V-12, Hind III-cut.
enzymes to prepare for Southern Blot analysis. Specific bands appear at different locations on the gel for each of the three strains, indicating that the digests were performed adequately. After transfer to the Hybond membrane, these fragments were probed with the labeled cloned fragment. Results were disappointing, with only faint bands appearing from the blot analysis. Decreased stringency of washings slightly improved the results, but the bands were still faint and the patterns were not reproducible. Detection by dig-labelled probes did not appear to be sensitive enough for this experiment. Figure 14 shows the result of one representative experiment with faint bands on the membrane for the *D. discoideum* strains NC-4 and V-12, but no visible bands for *D. mucoroides* DM-7, even when 40 μg of genomic DNA was loaded per well.

**GENOMIC LIBRARY SCREENING WITH Tdd-1 PROBES**

Screening a genomic library of *D. mucoroides* in lambda gt 11 with a probe made from PCR amplification of genomic NC-4 DNA proved very effective. After 4 rounds of plaque lifts several clones were isolated from the genomic library of *D. mucoroides* that contained nucleotide sequences complementary to the Tdd-1 sequence of NC-4.
Figure 14. Southern Blot Analysis of *D. discoideum* strains NC-4 and V-12 and *D. mucoroides* strain DM-7 genomic DNA after restriction digests. Hybridization lasted 19.5 hours at 37°C. Exposure time was three hours and took place at room temperature. Lanes 1-3: NC-4 DNA cut with Eco RI, Bam HI, and Hind III, respectively; Lanes 4-6: DM-7 DNA cut with Eco RI, Bam HI, and Hind III respectively, and Lanes 7-9: V-12 DNA cut with Eco RI, Bam HI, and Hind III respectively. Each digest used 30 units of restriction enzyme.
DISCUSSION

Using primers designed specifically for the Tdd-1 element of \textit{D. discoideum} based on the published sequence (Capello et al., 1985), PCR analysis of \textit{D. discoideum} NC-4 genomic DNA resulted in the amplification of a single fragment of DNA 370 bp in length. Optimal conditions for the PCR included using 10 \( \mu \text{g} \) of genomic DNA per application and using the protocol given by John Mullican (personal communication). The DNA from these PCR analyses, assumed to be a portion of Tdd-1, was cloned into pUC 19. The cloned fragment was then confirmed as part of Tdd-1 by restriction mapping and DNA sequencing. By using the clone as a probe for Southern blot analysis of genomic DNA, an attempt was made to estimate gene copy numbers in \textit{D. discoideum} strains NC-4 and V-12 and \textit{D. mucoroides} strain DM-7. In all Southern blot analysis \textit{D. discoideum} NC-4 had the highest copy number, followed by \textit{D. discoideum} V-12 and \textit{D. mucoroides} DM-7, respectively. Although the band patterns on Southern blots were not reproducible, \textit{D. discoideum} NC-4 always had more bands than \textit{D. discoideum} V-12, which had more bands than \textit{D. mucoroides} DM-7. This was consistent with PCR results where \textit{D. discoideum} NC-4 always yielded the greatest concentration of amplified product of the expected size. The same probe was also used successfully to screen a genomic library of \textit{D. mucoroides} to identify clones with sequences complementary to Tdd-1.

Dig-labeling of the cloned probe was successfully accomplished by employing the PCR, as seen by the quantification results. Probing the Southern blots for the estimated copy number gave inconclusive results. It was very difficult to achieve enough sensitivity to accurately determine copy number. However, successful identification of genomic clones from the lambda library was accomplished using dig-labeled probes.
From the sequence of the element and the amino acid sequence homology, it has been proposed that Tdd-1 is a retrotransposon (Capello et al., 1985). This implies that the 4.5-kb mRNA is copied by a reverse transcriptase, resulting in DNA copies that insert into new sites (H. Varmus and P. Brown, 1989). Analysis of the proteins expressed by adjacent and slightly overlapping ORFs in retrotransposons suggests that, at least in some cases, a shift in the reading frame occurs during translation so that the protein is encoded by two reading frames (Clare and Farbaugh, 1985). Since ORF-3 has homology to retrovirus reverse transcriptase, it is assumed that the ORF-3 product serves in an equivalent manner for Tdd-1, although the presence of reverse transcriptase in *D. discoideum* has not yet been documented. There is no direct evidence that any of the ORFs actually specify proteins *in vivo* (Firtel, 1989). It is known that a portion of Tdd-1 is preferentially transcribed during development of *D. discoideum* into sorocarps (Firtel, 1989), but it is not known if Tdd-1 is also transcribed during the formation of macrocysts, whether Tdd-1 encodes proteins that are essential for *Dictyostelium* differentiation, or whether transcription during this time of development is causally related to the mobility of the element *in vivo* (Firtel, 1989).

There does not appear to be a direct relationship among any of the three mobile genetic elements from *D. discoideum* and mutations affecting growth or development (Firtel, 1989). This is possibly because of the preferential insertion of Tdd-1 into partial Tdd-1 sequences and of Tdd-3 into Tdd-2. Thus, while the elements are quite mobile within the genome, mutagenesis by insertion into genes is an unlikely event. However, Soll and collaborators (1987) identified high frequency mutating strains which could lead to a genetically functional system for the isolation of mutants in which the genes of interest are tagged by a mobile element. The use of such mutants
would allow rapid cloning of the particular gene, aid in its analysis, and help to dissect regulatory pathways that regulate cellular differentiation (Firtel, 1989).

Possible further analysis would include sequencing of the Tdd-1 retrotransposon including the surrounding DNA, and also identifying possible movement of these elements during sorocarp versus macrocyst development. Another area of related research would be to determine if the concentration of Tdd-1 transcripts increased during macrocyst development as it does during sorocarp development. It should be possible to subclone Tdd-1-like elements from the genomic library to be used for insertional mutagenesis. Even though Firtel states that he was unsuccessful in creating mutagenesis within the NC-4 strain, it might be possible to do so between species, such as from NC-4 to DM-7, because there might be enough differences in the nucleotide sequences between the two to override the preferential insertion spots. This could ultimately be used to screen for developmental genes important in Dictyostelium pathways.
REFERENCES


