GENERATION OF cDNA LIBRARIES OF AMOEBA, 8 HOUR, AND 12 HOUR STAGES OF *Dictyostelium discoideum*

by

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(ABSTRACT)

A critical event during the life cycle of *Dictyostelium discoideum* is glycogen turnover. This process is catalyzed by glycogen phosphorylase-2 (*gp-2*). Since *gp-2* expression is first induced during the transition from growth to differentiation, understanding how this gene is controlled may provide some insight into the process of differentiation. In order to identify the trans-acting factors responsible for activating *gp-2* expression, cDNA plasmid libraries of amoebae, and cells at 8 h and 12 h of development were generated. The long-term goals of this project involve screening expression libraries with identified cis-acting elements from the *gp-2* promoter to yield the DNA binding proteins responsible for gene regulation. For this approach to succeed, a high quality cDNA library is essential. The library must contain full-length cDNA that represents the complexity of mRNA present during the developmental stage of interest. Hence, all three libraries were subjected to extensive testing prior to and following cloning. RNA quality and the fidelity of the time points were determined by Northern blot analysis and by RT-PCR for several marker genes. Following cDNA synthesis, the cDNA was assessed for complexity and full-length synthesis by PCR and radioactive primer extension, respectively. Ligation of the cDNA into a vector was performed using several ratios of vector:insert in order to ensure that long cDNA species were included in the plasmid

library. Finally, the presence of the marker genes was confirmed by PCR amplification of plasmid extracted from bacteria transformed with the plasmid library.

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INTRODUCTION

Dictyostelium discoideum is a simple eucaryotic slime mold that feeds on bacteria. When nutrients are abundant, the organism exists as a unicellular amoeba. If nutrients become limited, cells start to differentiate (Darnell, 1990). During differentiation, DNA synthesis and cell division cease. The individual amoebae stream toward aggregation centers in response to cAMP secretion. Mounds of several thousand cells begin to form after around 8 h of starvation, but the cells retain their individual identities and do not fuse. At about 12 h, the "first finger" stage is visible. This structure possesses a discrete tip containing prestalk cells, that will develop into stalk cells at maturation. The finger-like structure then forms a slug, which is a motile, worm-like creature that migrates toward light or warmth. At 20 h, a second finger will form. Finally, true stalk and spore cells develop. The stalk cells elongate and vacuolate, pushing down through the mass of differentiating spore cells and hoisting the mass of spore cells up along the stalk. The stalk contains cells that are destined to die, while spore cells go on to form the next generation. Each spore is surrounded by a rigid spore coat, made of glycoproteins and polysaccharides that are secreted from the spore cells. The spores are metabolically inactive and can tolerate dessication, ultraviolet light, and other harsh treatments. When supplied with water and nutrients, the spores "germinate" to form amoebae. The life cycle is complete in about 24 h.

The enzymes that are involved in glycogen metabolism are important in *Dictyostelium* development (Sucic, *et al.*, 1993). Glycogen phosphorylase-1 (Gp-1) is the dominant isoform of glycogen phosphorylase during the amoeba stage (Sucic *et al.*, 1993). During development, another form of the enzyme, glycogen phosphorylase-2 (*gp-2*), is synthesized. Its expression is first detected at about 8 h of development and the enzyme becomes highly active during the first finger stage. The action of *gp-2* is vital for the

developmental process, as glycogen must be degraded in order to produce the raw materials for the synthesis of the structural end products of differentiated cells. There are various developmental signals that mediate *gp-2* gene expression, *e.g.* cyclic AMP, Differentiation Inducing Factor (DIF), NH_3 and adenosine (Yin *et al.*, 1994; Sucic *et al.*, 1993).

Based on: (1) the temporal expression of *gp-2*, (2) its responsiveness to developmental signals and (3) the requirement of glycogen turnover during differentiation, it is likely that analysis of the control of this gene may uncover basic transcriptional elements involved in the transition from growth to differentiation. Recognition site screening is one approach that can be used to isolate these regulatory factors. This technique involves screening a cDNA expression library with putative promoter elements. Bacteria infected with recombinant phage can be induced to express the cloned cDNA. Radioactively labelled promoter elements will bind only to plaques expressing the corresponding DNA binding proteins. In order for this technique to be successful, it is essential that the cDNA of interest be represented in the library. It is also important that the entire coding sequence of the protein is cloned in order to obtain a funtional protein product. Hence, a complete library must contain one cDNA clone representing each mRNA in the cell and each clone must also possess a full-length cDNA.

The work described below details the construction of three cDNA libraries. At each stage of construction, the products generated were subjected to rigorous testing. The mRNA and cDNA were assessed by RT-PCR and PCR, respectively, for the presence (or absence) of both constitutive and developmentally regulated genes. By using radioactively labelled tracer reactions during cDNA synthesis and PCR primers specific to the 5' end of a marker gene, the presence of full-length cDNA molecules was confirmed. Complexity of the libraries generated was confirmed by PCR amplification of a gene known to be expressed in low quantities. cDNA plasmid libraries were generated for the amoebae stage (when *gp-2* is not expressed), the 8 h stage (when *gp-2* is first expressed) and the 12 h

stage (when *gp-2* is highly expressed). The next step of cloning the cDNA into λgt11 can be performed with the confidence that the libraries consist of full-length messages and are sufficiently complex to recover potential transcription factors.

MATERIALS AND METHODS

Cell Culture and Harvesting

Dictyostelium discoideum strain AX3K was used to inoculate HL5 media (60 mM glucose, 1%(w/v) Oxoid peptone, 0.5% (w/v) Oxoid yeast extract, 2 mM Na_2HPO_4 and 3 mM KH_2PO_4). Stock cultures of amoebae were diluted to a density of 10^5 cells/ml. The medium was supplemented with 25 µg/ml dihydrostreptomycin or streptomycin sulfate to prevent bacterial growth. Cells were shaken at 20°C at 130 rpm until the density reached log phase (5 x 10^6 cells/ml). For differentiated cells, 10^8 cells were washed free of nutrient media, plated on filters saturated with 1X MES LPS (7 mM MES, 20 mM KCl,and 2.5 mM $MgSO_4$, pH 6.5). Plated cells were incubated at 20°C until the desired stage of development was reached.

RNA and mRNA Isolation and Analysis

Total RNA was isolated from *Dictyostelium* cells as described in Current Protocols in Molecular Biology (1991). Briefly, the frozen cell pellet was resuspended in 1 ml denaturing solution per 10^7 cells (2 M guanidinium thiocyanate, 45 mM sodium citrate, pH 7, 1% Sarkosyl and 0.7% (v/v) 2-mercaptoethanol) and mRNA was isolated from total RNA using either the Poly ATract mRNA isolation kit (Promega) or the Poly A+ mRNA isolation kit (Oligotex). mRNA was also directly isolated from *Dictyostelium* cells by the Quick prep micro mRNA purification kit (Pharmacia Biotech). The concentration of RNA and mRNA were assayed spectrophotometrically at 260 nm. Agarose gel electrophoresis was performed using 5 µg of total RNA or 1 µg of mRNA to verify the quality of the RNA. RT-PCR was done using AMV reverse transcriptase and primers directed against actin (5' GGACG GTGAA GATGT TCAAG 3' and 5' CTCCC ACACT GTACC AATCT

3'), glycogen synthase (5' GAAGC ATCTA AACGT GGTAT C 3' and 5' CAGAG TGGAT CAAAG ATGAC AGTTG 3'), glycogen phosphorylase-2 (5' GCAGG TTTAG GTAATGGTGG 3' and 5' TCACC ATGGG AAAGT GAACG 3'), SP60 (5' GTAGG TGCCT TATGT ATGGG 3' and 5' ACTTG CGTTG TCCAA AGGGT 3') and phosphodiesterase inhibitor (PDI) (5' GATAA ATGCA CTAGC CCAGA 3' and 5' GGTGG TTGGC CATGT ATTAA 3'). Northern blots were performed according to standard procedures (Current Protocols in Molecular Biology, 1991) following separation on a formaldehyde gel. A 10 µg sample of total RNA or 3 µg of mRNA was loaded per lane. Hybridizations were performed under stringent conditions (6XSSC, 0.5% SDS, 5X Denhart, 100 µg/ml herring sperm DNA at 65°C) using a glycogen synthase probe lebelled by random priming. Membranes were washed at 65°C in 2XSSC/0.1%SDS, 1XSSC/ 0.1%SDS, and at room temperature in 0.2XSSC/0.2%SDS. Autoradiography was done by exposure to Kodak X-OMAT AR film at -80°C using an intensifying screen.

cDNA Synthesis and Analysis

First strand cDNA was synthesized from 10 µg mRNA or 10 µg total RNA using 150 units AMV reverse transcriptase and 10 µg oligo d(T) primers containing a NotI site. The reaction was performed at 42°C for 1 h in 100 µl final volume containing 125 units RNasin, 4 mM sodium pyrophosphate, and first strand buffer (50 mM Tris HCl pH 8.3 (pHed at 42°C), 50 mM KCl, 0.5 mM spermidine, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1.1 mM each dNTP). DNA polymerase I (115 units) was used to synthesize the second strand from mRNA primers derived from treatment of the DNA/mRNA hybrid with 4 units of RNaseH. Synthesis was performed at 14°C for 4 h using second strand buffer (40 mM

Tris-Cl, pH 7.2, 90 mM KCl, 3 mM $MgCl_2$, 3 mM dithiothreitol and 0.05 mg/ml bovine serum albumin). The cDNA was then treated at 37°C for 10 min with 20 units of T4 DNA polymerase to blunt the ends. For each synthesis reaction, tracer reactions were performed by removing a small aliquot of reaction and adding 5 μCi of alpha-^{32}P-dATP to the reaction. The tracer reactions were used for incorporation assays and gel analyses to assess the quantity and quality of cDNA, as described in the Universal RiboClone cDNA synthesis manual. For gel analysis, a lambda HindIII ladder was labelled with 2 μCi alpha-^{32}P-dATP using a fill-in reaction for use as a DNA marker to estimate the sizes of the first and second strand products. An equal number of counts from each sample was loaded on a 1.4% alkaline agarose gel in alkaline running buffer (30 mM NaOH, 1mM EDTA). The gel was fixed with 7% trichloroacetic acid, dried under a vacuum at 80°C and exposed to X-ray film at -80°C with an intensifying screen. The length of first and second strand cDNA was assessed by comparison to the marker. In cases in which poor sample recovery from precipitation resulted in too few counts for visualization by autoradiography, hot primer extension was used to assess cDNA length. Primer extension was performed using 22.5 ng oligo(dT)-NotI primer, 17 μmoles dNTP without dATP, 10 units Klenow enzyme, 50 μCi alpha-^{32}P-dATP and 75 ng boiled cDNA at 37°C for 1 h. Each sample was loaded on a 1% agarose gel, fixed and exposed as described above for the alkaline agarose gel . The length of cDNA was determined from the autoradiogram by comparing it to the lambda HindIII marker.

The quantity of cDNA synthesized was assessed using DNA Dipstick (Invitrogen) or ethidium dot quantitation assays. For both approaches, cDNA samples were compared to known standards of DNA. The production of double-stranded cDNA was confirmed by S1 nuclease and DNaseI digestion. Briefly, an equal number of counts of each

radiolabelled cDNA from the tracer reactions were treated with S1 nuclease. Similar reactions lacking S1 nuclease were used as controls. The samples were incubated at 37°C for 1 h, then separated on a 1% agarose gel, fixed and exposed to film as described above. For the DNaseI reactions, 20 ng cDNA was incubated at 37°C for 1 h, with or without 10 units DNaseI. A single-stranded actin PCR product (provided by R. Favis) was treated identically. PCR using actin primers was then performed and the products were separated on a 1% agarose gel.

Ligation of cDNA into pBlueScript Vector

To determine the optimal molar ratio for cloning, a 1.5 kb glycogen synthase (*gs*) fragment was used as an insert to simulate cloning of the average expected size of cDNA. The *gs* fragment possessed EcoRI and KpnI ends and was ligated into a pBlueScript vector treated with the same enzymes. The following ratios of vector:insert were tested: 5:1, 1:3, 1:2 and 1:1. To determine the optimal molar ratios for cloning rarer, larger cDNA molecules, a 4.0 kb vector containing a kanamycin resistance cassette was used as an insert. Recombinants were selected by challenging with 100 μg/ml kanamycin and 100 μg/ml ampicillin (pBlueScript contains an ampicillin resistance cassette). Only successful recombinants will be resistant to both antibiotics. Simulation cloning for larger cDNAs is currently in progress. cDNA with EcoRI and NotI ends (5' and 3', respectively) was prepared as follows: 250 ng of cDNA was passed through a Sephacryl S-400 column to remove fragments smaller than 200 bp; 80 pmol EcoRI adaptors were next ligated to the sized cDNA in 10 μl final volume using 800 units T4 DNA ligase at 15°C, overnight. The reaction volumes were increased to 50 μl and the reaction was again passed through a Sephacryl S-400 column to remove unligated EcoRI adaptors. The ligation products were

then digested with NotI at 37°C. cDNA with EcoRI and NotI ends was ligated into pBlueScript that had been previously digested with EcoRI and NotI. Ligations were performed using 1:2 molar ratios of insert to vector, using the same conditions described above. The constructs were then transformed into SURE cells (Strategene) by electroporation (Dower, 1988) (settings: 960 μfaradays of capacitance (extender), 200 ohms of resistance (controller), 25 μfaradays of capacitance (pulser),and 1.8 Volts using a 0.1 cm gapped cuvette). Transformants were selected by incubating the bacteria on LB plates (0.5% (w/v) yeast extract, 86 mM NaCl, 1% (w/v) bactopeptone, 1.5% bacto agar) containing 100 μg/ml ampicillin, 10 μg/ml tetracyclin and plated with 25 μl of 20% IPTG and 50 μl of 2% X-gal. After incubation overnight at 37°C, 10 white colonies were randomly picked for boiling plasmid preps (Sambrook *et al.*, 1989). Extracted plasmid was digested with EcoRI and NotI and subjected to gel electrophoresis. Plasmid libraries were stored in 15% glycerol at -80°C.

Screening cDNA Libraries by PCR

A small amount of a library from the glycerol stock was removed using the tip of sterile needle and was used to inoculate LB media containing 100 μg/ml ampicillin and 10 μg/ml tetracyclin. The bacteria were incubated overnight in a rotary shaker at 220 rpm and 37°C. Plasmids were extracted by the boiling method (Sambrook, 1989) and the amount of DNA determined by the ethidium bromide dot assay. 2 μg of plasmid were linearized by digestion with 10 units SalI at 37°C for 1 h and then analyzed by the ethidium bromide dot

assay. 20 ng of linearized template were PCR amplified using the same conditions as described above.

RESULTS

Condition of Extracted RNA

In order to obtain a high yield of full-length cDNA, the RNA template must be undegraded. The condition of the extracted RNA was assessed by agarose gel electrophoresis and Northern blot analysis of the purified RNA as described in Materials and Methods. Figure 1 shows a typical agarose gel. Total RNA from amoebae, 8 h and 12 h of development is shown. The 17S and 26S ribosomal RNA bands (1900 and 4100 bp, respectively) are clearly visible, indicating that the RNA is not degraded. Figure 2 shows a Northern blot of total RNA from amoeba to 28 hrs of development. Radiolabelled glycogen synthase was used as a probe, since glycogen synthase is expressed at low levels throughout the life cycle of *Dictyostelium* cells (Williamson, 1995). When I compared the molecular weight of the bands appearing on the autoradiograph to the map of the glycogen synthase gene (Williamson, 1995), I found that the bands at approximately 2,300 bp corresponded to the predicted length of this gene without any introns. I also determined whether any degradation occurred during separation by agarose gel analysis of the high salt washes used to remove tRNA and rRNA following mRNA isolation. Figure 3 shows the two ribosomal bands were present and therefore not degraded by contaminating RNases, hence the mRNA fraction should also be undegraded.

Furthermore, RT-PCR was carried out using extracted total RNA as template to detect a developmentally regulated gene (gp-2) and a constitutively expressed gene (gs), in order to establish that the time points were accurate (Figure 4). Both of these genes are expressed at a low level. Detection of these genes from genomic DNA serves a positive control for the PCR portion of the reaction. For both genes, PCR primers were designed to surround an intron, so that DNA and RNA could be distinguished. Additionally,

identical reactions lacking reverse transcriptase (RT) were performed in order to show that the RNA-derived band is reverse transcriptase-dependent. Thus, based on this RT-dependence and on the size of the band, it can be established that the band is the result of amplifying mRNA. Figure 4 shows the result of the RT-PCR analysis for amoebae, 8 h and 12 h time points. When using primers for *gs* and *gp-2*, smaller PCR products (400 bp and 200 bp, respectively) can be seen in the lanes containing AMV reverse transcriptase, but the larger DNA-derived PCR products (500 bp and 300 bp, respectively) are seen in the lanes lacking reverse transcriptase. The lack of a 200 bp product for *gp-2* at 0 h was expected because this gene is not expressed in amoebae. The larger PCR products in the lanes containing RT are the result of contaminating genomic DNA. Although the 300 bp genomic product can be seen faintly in the +RT lane for amoeba RNA (See figure 4b, lane 1), it is far fainter than the -RT control (figure 4b, lane2) for this sample. It is possible that this result can be attrituted to promiscuous binding of the *gp-2* 3' primer to non-*gp-2* sequence. Several smaller PCR products can be seen faintly in the +RT lane and these smaller products may be substracting primer, nucleotides, etc. from the reaction amplifying the genomic contaminant, thus reducing the intensity of the 300 bp genomic band. The results show that the quality of mRNA is acceptable because the gs 400 bp PCR product can be detected, even though this gene is expressed only in low quantities. In addition, the RNA-derived PCR product for *gp-2* demonstrates the expected expression pattern of the gene, that is, no expression during amoeba stage and detectable expression at 8 hr. of development.

Gel Analysis and Hot Primer Extension of cDNA

The length of cDNA produced was determined by gel analysis of radiolabelled cDNA from the tracer reactions. Figure 5 shows the result for first and second strand synthesis for the amoebae and 8 h time points and the second strand synthesis for the 12 h

time point. From these experiments, it can be seen that the average size of cDNA generated is around 1 kb, although some species of cDNA greater than 2 kb were generated. As mentioned previously, technical difficulties in recovering and/or loading some radiolabelled tracer reactions into the gels required an alternative approach to determine the length of the cDNA produced. Figure 6 shows the results of a hot primer extension reaction for amoeba cDNA. The average size of cDNA for this sample is about 1 kb, with a smaller fraction of cDNA (as evidenced by the fainter high molecular weight smear). To determine whether double-stranded cDNA was synthesized, DNase I and S1 nuclease were used to digest the cDNA. DNase I digests double-stranded DNA while S1 nuclease digests single-stranded DNA. Figure 7 shows that single-stranded DNA derived from a PCR product (lane 2a) was not digested by DNase I, while double-stranded DNA derived from a PCR product (lane2b) was digested by the enzyme, since PCR can only amplify the single-straded product following digestion. Amoeba, 8 h and 12 h cDNA, however, were digested in the reaction, as PCR cannot produce a product (or significantly less product compared to cDNA lacking DNase I). This indicates that the amoeba, 8 h and 12 h cDNA must be double stranded. Figure 8 shows the result of S1 nuclease digestion on the first and second strand tracer reactions. The first strand reaction is digested by S1 nuclease (compare lanes 2 and 3), while the second strand reaction shows a retarded complex between the enzyme and the double-stranded DNA (lanes 4). The result means that the enzyme is unable to degrade the double-stranded DNA. As shown previously in Figure 6, the results from hot primer extension also indicated that double-stranded cDNA was produced. Because klenow enzyme synthesizes a DNA strand by extending from an oligo(dT) primer, the only way that a product of the expected size for cDNA can be made is if the second strand (containing the series of adenines present in the original 3' end of the message) is available for hybridization with this primer.

Analysis of cDNA by PCR Amplification of Marker Genes

Identical PCR reactions as those described for RT-PCR were used to assay the success of cDNA synthesis. Primers designed to surround introns and against *gp-2*, *PDI* and *SP60* were used to demonstrate that cDNA synthesis resulted in replicating the expression patterns (200 bp product from *gp-2* primers, 529 bp product from PDI, and 418 bp product from SP-60) present in the mRNA. In addition, amplification using actin primers demonstrated that the entire coding sequence of the message was likely to be found in the cDNA generated, because these primers were directed against the extreme 5' end of this gene and includes the start codon. The purpose of amplifying *gs* was to show that even rare messages were converted into cDNA. Figure 9 shows the results for each time point. In all cases except actin amplification, the lower band (as compared to the band generated from genomic DNA) represents the size of that particular region of DNA, minus the intron. Because a PCR product corresponding to the correct size of the RT-PCR product can be generated for the primers surrounding an intron with gs and gp-2, it is reasonable to assume that the PCR product generated for actin should also correspond to amplification from cDNA and not genomic contamination. From this experiment, we can conclude that the cDNA synthesized is of sufficient quality to proceed with cloning, because: 1) the developmental expression patterns present in the mRNA are maintained; 2) rare messages have been converted into cDNA; and 3) the cDNA contains full-length copies of the mRNA.

Preparation and Cloning of cDNA into pBlueScript

In order to decrease the background of non-translatable clones in the λgt11 system, directional cloning is required. By controlling the orientation of the insert, cDNAs will be forced to clone into the vector such that translation will produce a functional protein product. To achieve directional cloning, EcoRI adaptors were ligated on to the ends at the cDNA. By the digesting with Not I, the Not I site in the oligo(dT) primer used for reverse transcription was cleaved. This resulted in an EcoRI "sticky" end on the 5' end and a Not I "sticky" end at the 3' end.

To test whether ligation of the EcoRI adaptors occured, a portion of the radiolabelled second strand tracer reaction was subjected to the same ligation conditions as the cDNA and then self-ligated (Figure 10). To control for self-ligation of the tracer cDNA by the blunt ends, tracer cDNA without EcoRI adaptors was ligated. By comparing the original tracer reaction with the ligated tracer reactions, it can be seen that cDNA lacking adaptors self-ligated very inefficiently, as evidenced by only a slight shift upward of the radioactive smear (lane 3). In contrast, cDNA bearing the EcoRI adaptors self-ligated very efficiently (lane 4): the smear appears as a higher molecular weight complex. This experiment shows that adaptor ligation has occured under the conditions used.

Figure 11 shows the result of cloning amoeba cDNA that has been adaptor-ligated and restricted with Not I into pBlueScript. Of the ten white colonies selected for plasmid extraction, four yielded vector of the correct size and inserts. It is likely that the inserts represent different cDNAs, because each insert is a different molecular weight.

To test whether larger cDNAs were cloned into the vector, PCR was again used to detect a marker gene. Glycogen synthase was chosen because it is known to be expressed in amoebae, and DNA and RNA can be readily distinguished due to an intron present between the primer binding sites. Figure 12 shows the results of this PCR reaction. In

this case, no cDNA was detected. Since the inserts present in Figure 11 appear to be between 0.5 and 1.0 kb in length, it is possible that larger cDNAs cannot clone into the vector under the conditions used. Conditions for cloning were optimized as described in materials and methods (Ligation of cDNA into pBlueScript vector) using a 1.5 kb DNA fragment. Figure 13 shows the results of this simulated cloning effort: the conditions used can very effectively clone fragments up to 1.5 kb. Experiments are currently in progress to optimize the conditions for cloning the larger fragments.

DISCUSSION

To achieve our long range goal of isolating transcription factors by recognition site screening of a λgt11 expression library, a high quality cDNA library must first be produced. We have diligently assayed the results at each step of library production in order to ensure that the end product will contain the identical complement of messages present in the living cells.

During library production, we relied heavily on PCR assays for various marker genes. With the exception of actin 8, which contains no introns, all other marker genes were amplified using primers that surrounded an intron. The presence or absence of this intron indicates that the PCR product was derived from amplification of DNA or RNA/cDNA, respectively. Actin 8 is constitutively expressed at constant, high levels throughout the life cycle of *Dictyostelium* (Romans *et al.*, 1985); hence, it is useful as a positive control for PCR of RNA-derived products. Although it lacks an intron, it is still very useful in determining whether full-length synthesis of cDNA occurred, since the primers are designed to encompass the start codon. Glycogen synthase is included as an additional constitutively expressed gene; however, it is expressed in low levels throughout the life cycle (Williams *et al.*, 1996). This makes it a good marker gene for the presence of rare messages during library construction. Gp2 is inactive during vegetative growth, but becomes transcriptionally active very early in development during aggregation (Rutherford *et al.*, 1992). In addition to its use in determining the precision of the time points collected, gp-2 also serves as a good marker gene for the presence of long messages in the library, because the expected size of gp2 cDNA is around 3.2 kb. SP60 yields a smaller message of 1.8 kb and it is expressed only late in development during the first finger stage (Hong

and Loomis, 1988). SP60 serves as a marker for the presence of intermediate-size cDNAs and confirms the precision of the 12 hr. time points. PDI generates the smallest message (0.95 kb) and is expressed from the onset of starvation to about 8 h of development (Franke *et al.*, 1991). This gene serves as a temporal marker for the precision of the time points, as well as a marker for the presence of smaller messages.

Due to the unusual base composition of *Dictyostelium* (78% A/T overall, but 90% A/T in noncoding regions), there is an increased rate of instability for some cloned fragments (Nellen, 1987). For this reason, library construction was carefully assayed at each step for both the correct expression of the marker genes, as well as the quality of the product generated. RNA extraction and mRNA isolation were shown to result in undegraded products by RT-PCR (Figures 1 and 3) and Northern analysis (Figure 2). The results of cDNA synthesis were assayed for length by radiolabelled tracer reactions (Figure 5), hot primer extension (Figure 6) and PCR using primers directed against the extreme 5' end of the actin marker gene (Figure 9b). These experiments showed that the average size of cDNA generated was about 1 kb, although higher molecular weight species were also present. cDNA synthesis also appears to be full-length, since the 5' end of actin can be amplified from the library. PCR also demonstrated that the diversity of message present in the RNA starting material was converted into cDNA. By using primers surrounding introns that were directed against developmentally regulated and constitutively expressed genes, it was shown that that RNA for this diverse group of marker genes had been converted into cDNA (Figure 9). The cDNA generated was also shown to be correctly modified for directional cloning: EcoRI adaptors efficiently ligated (Figure 10) to the double-stranded cDNA (Figures 7 and 8). Finally, we have succeeded in cloning a subset of the cDNA population (Figure 11).

According to the *Dictyostelium* subset of the SWISS-PROT Protein Sequence Data Bank, Release 34.0 (http://expasy.hcuge.ch/cgi-bin/lists?dicty.txt), the vast majority of

known *Dictyostelium* genes are within the range of sizes that were cloned. Approximately 84% of known *Dictyostelium* genes are less that 2 kb in length. Figure 13 demonstrates that the conditions used can successfully clone a 1.5 kb DNA fragment, hence it is likely that a significant portion of the cDNA population has been cloned. However, it should be kept in mind that the estimated size of *Dictyostelium* genes by other researchers are the result of cDNA and genomic libraries constructed in other laboratories. If such libraries were not subjected to rigorous examination as presented here, it is possible that these libraries may not be complete and may lack certain longer cDNAs. This implies that the database of known genes may be biased towards what can be easily cloned. For this reason, we are continuing our efforts to produce libraries with greater size heterogeneity.

Figure1. Examination of RNA quality.

Lane 1 contains DNA marker IV (1a), lambda HindIII marker (1b), and lambda Sty I marker (1c). Lane 2 contains total RNA from amoeba (1a), 8 h (1b), and 12 h (1c) of development separated on a 1% agarose gel.

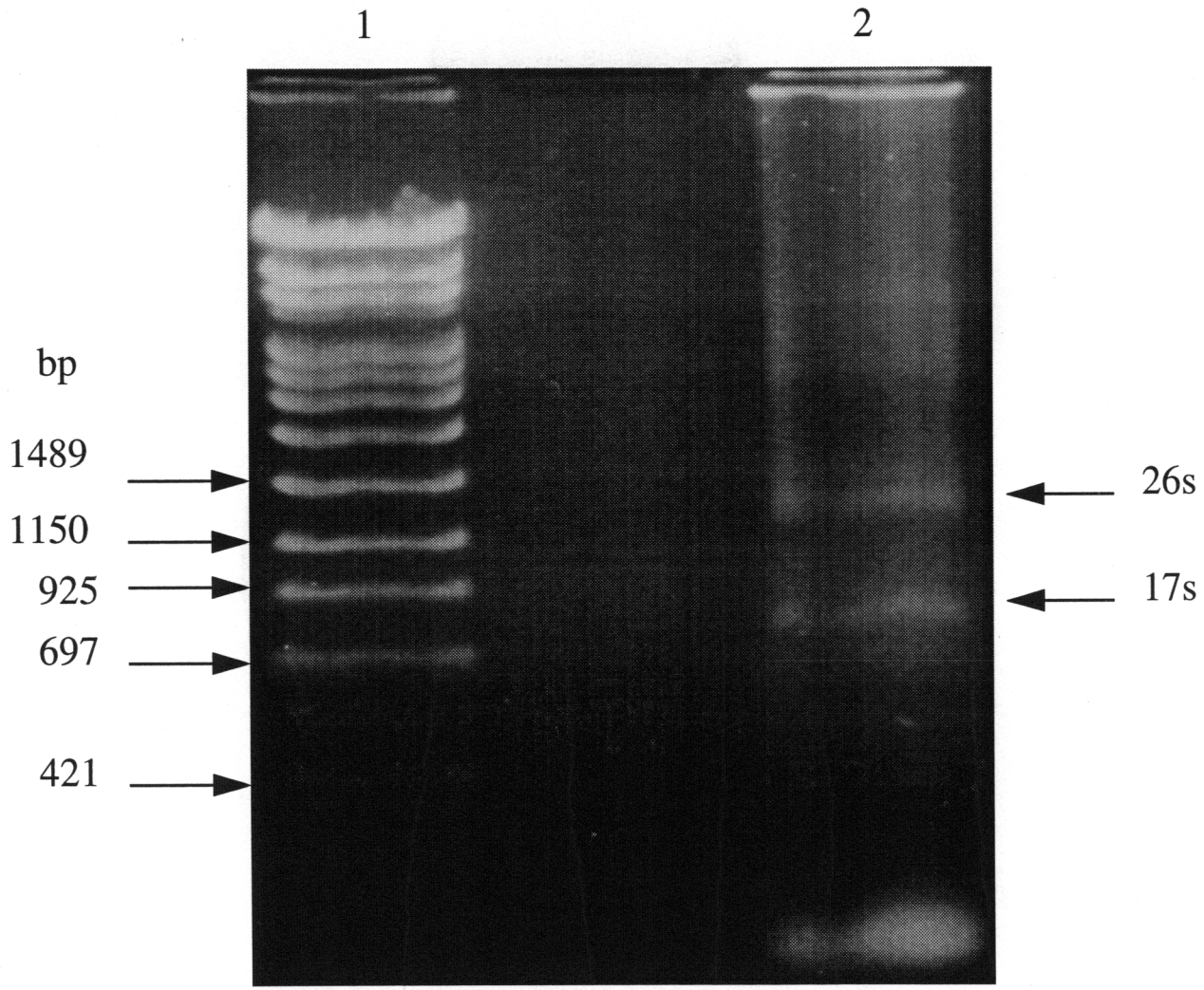


Figure 1a. Examination of amoeba RNA quality

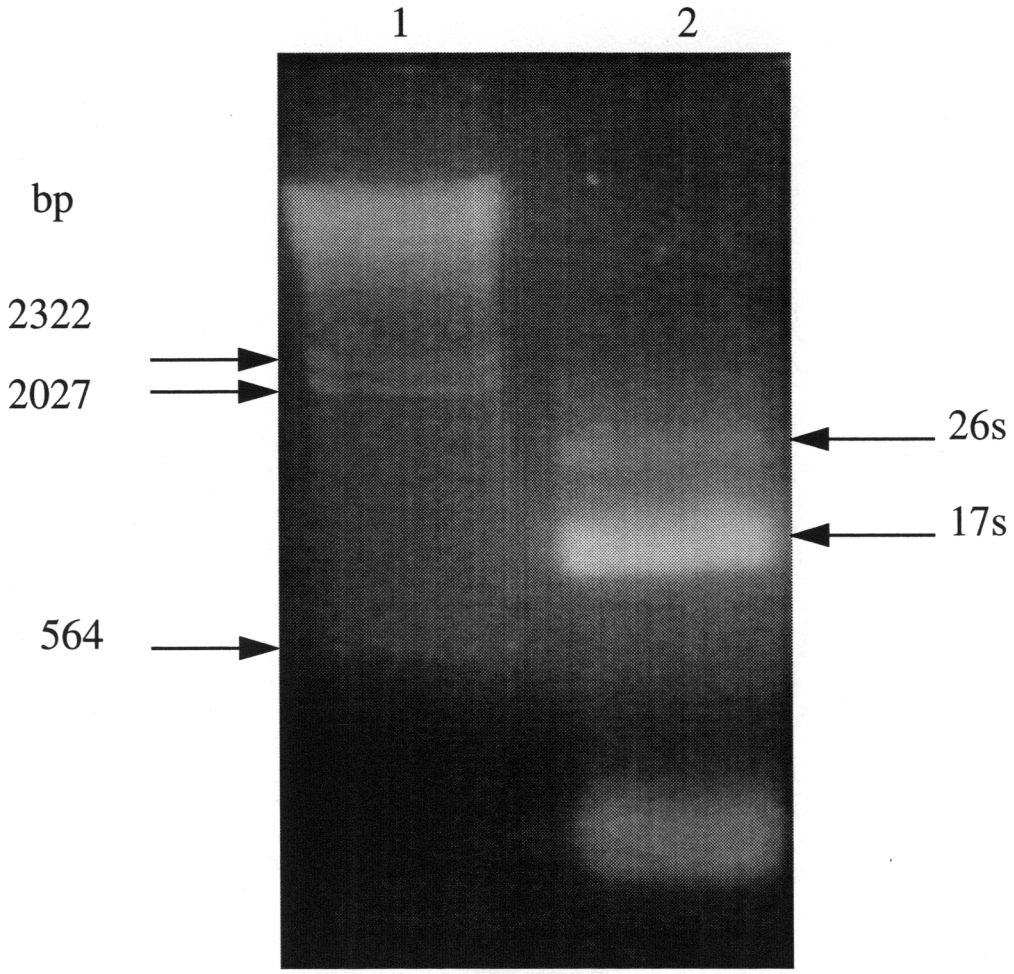


Figure 1b. Examination of 8 hours RNA quality.

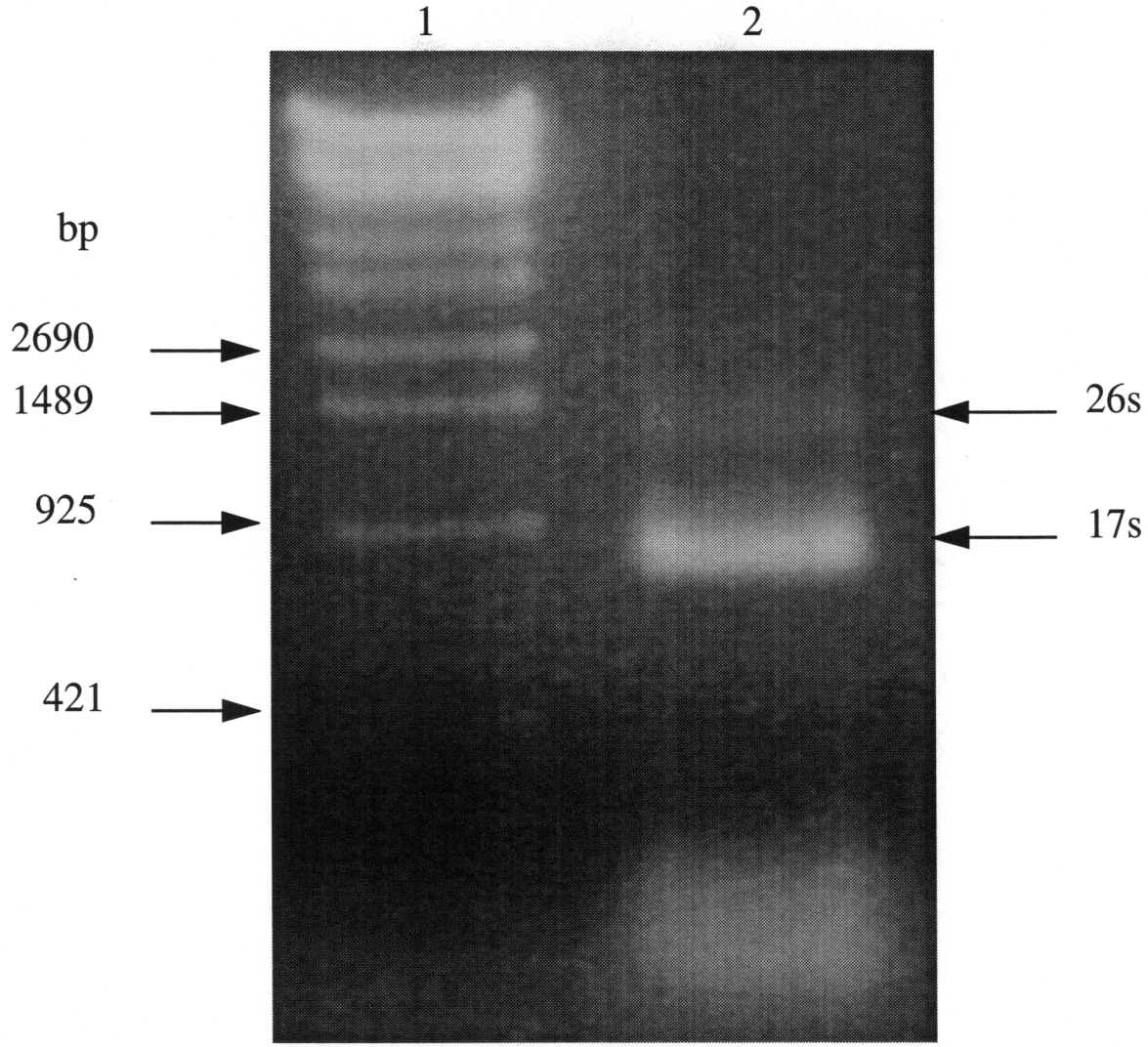


Figure 1c. Examination of 12 hours RNA quality.

Figure 2. Analysis of mRNA from amoeba to 28 hours of development.

2A: Ethidium bromide stained formaldehyde gel of total RNA with RNA ladder as a marker in lane 1; 2B: Northern blot of 2A using radiolabeled glycogen synthase as a probe.

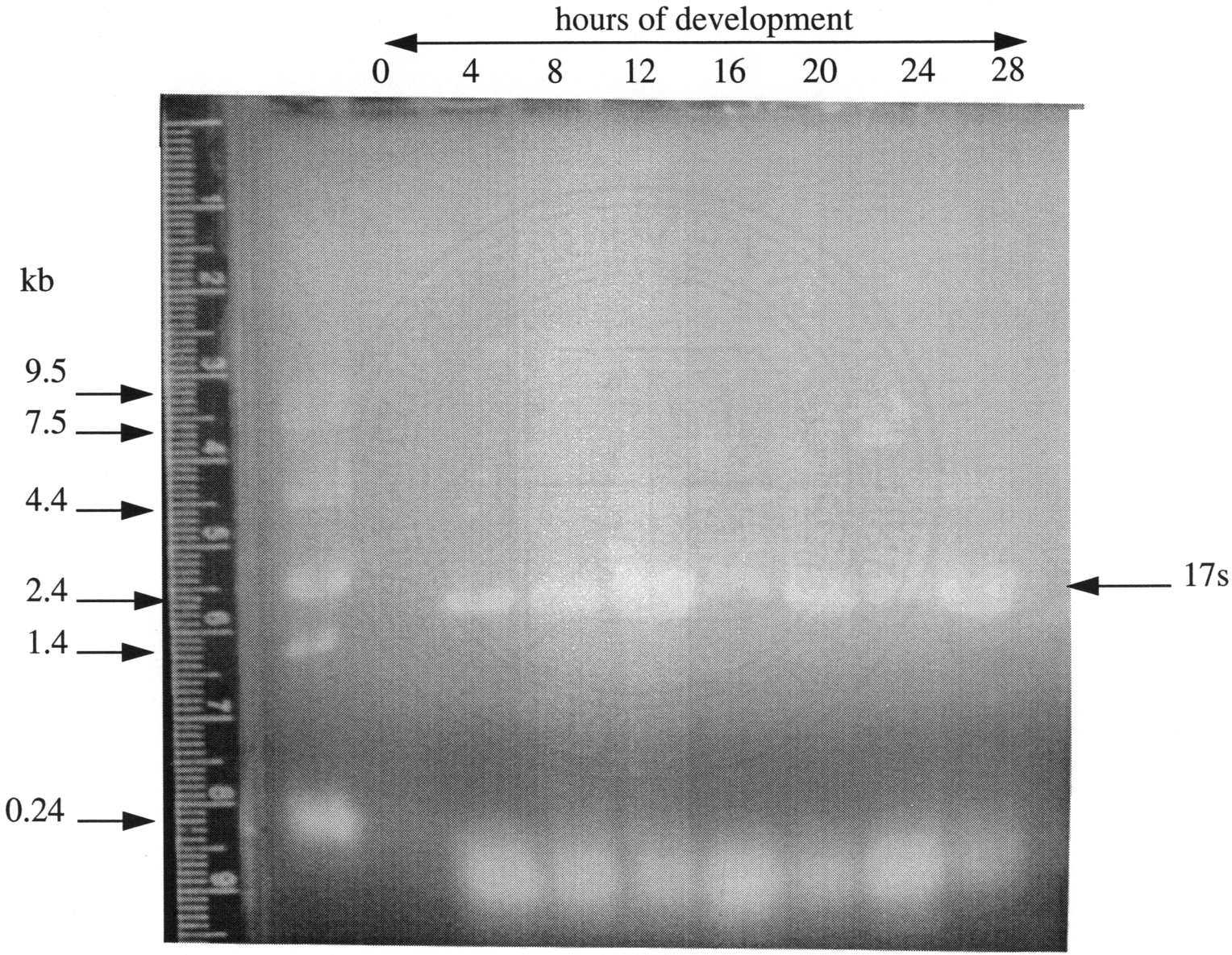


Figure 2a. Formaldehyde gel of total RNA.

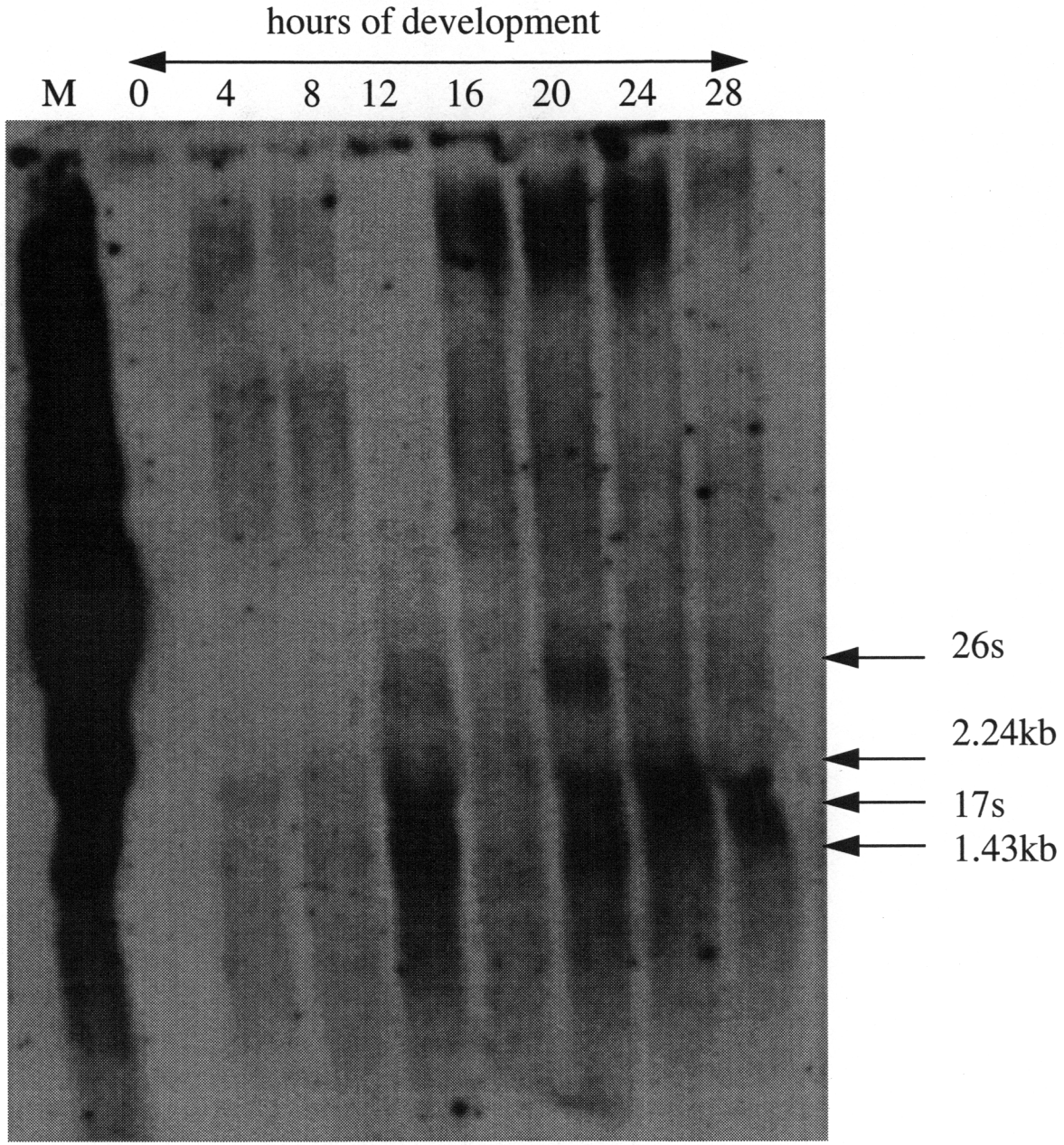


Figure 2b. Northern blot of total RNA.

Figure 3. tRNA and rRNA condition after mRNA isolation.

Lane 1: lambda HindIII as a marker; Lane 2: high salt washes from the mRNA isolation procedure from amoebae (3a), 8 h (3b), and 12 h (3c) of development; Lane 3: mRNA from amoeba (3a), 8 h (3b), and 12 h (3c).

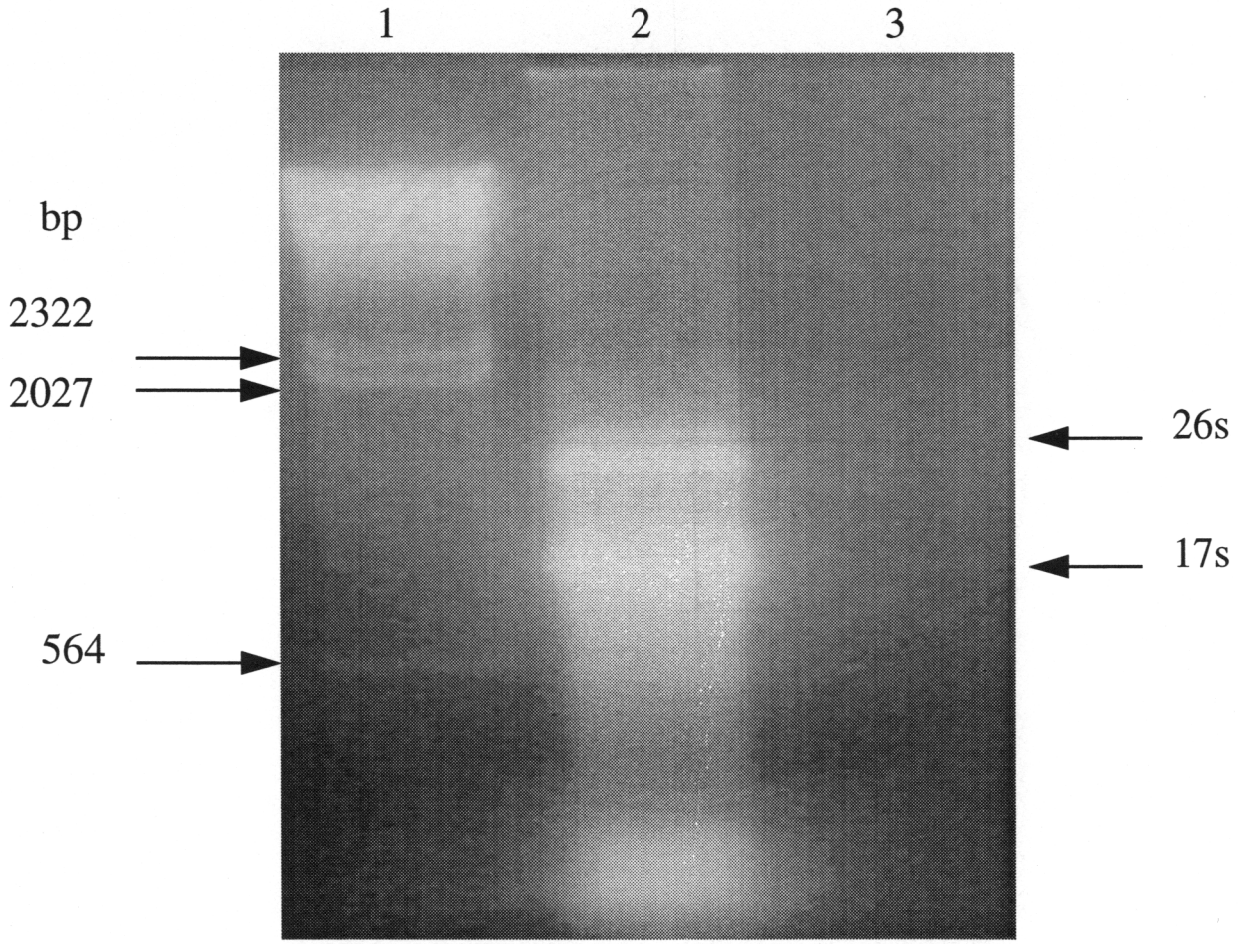


Figure 3a. Amoeba tRNA and rRNA condition after mRNA isolation.

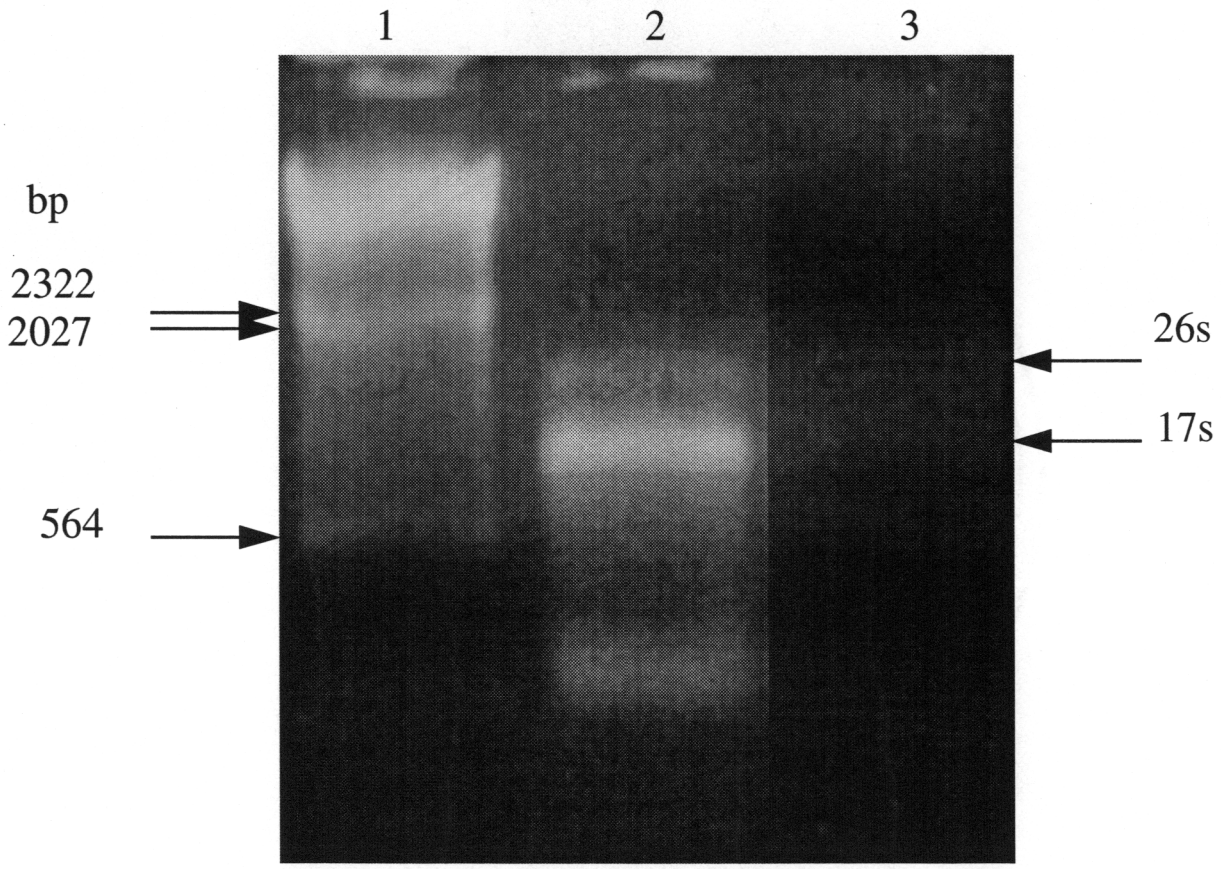


Figure 3b. 8 hours tRNA and rRNA condition after mRNA isolation.

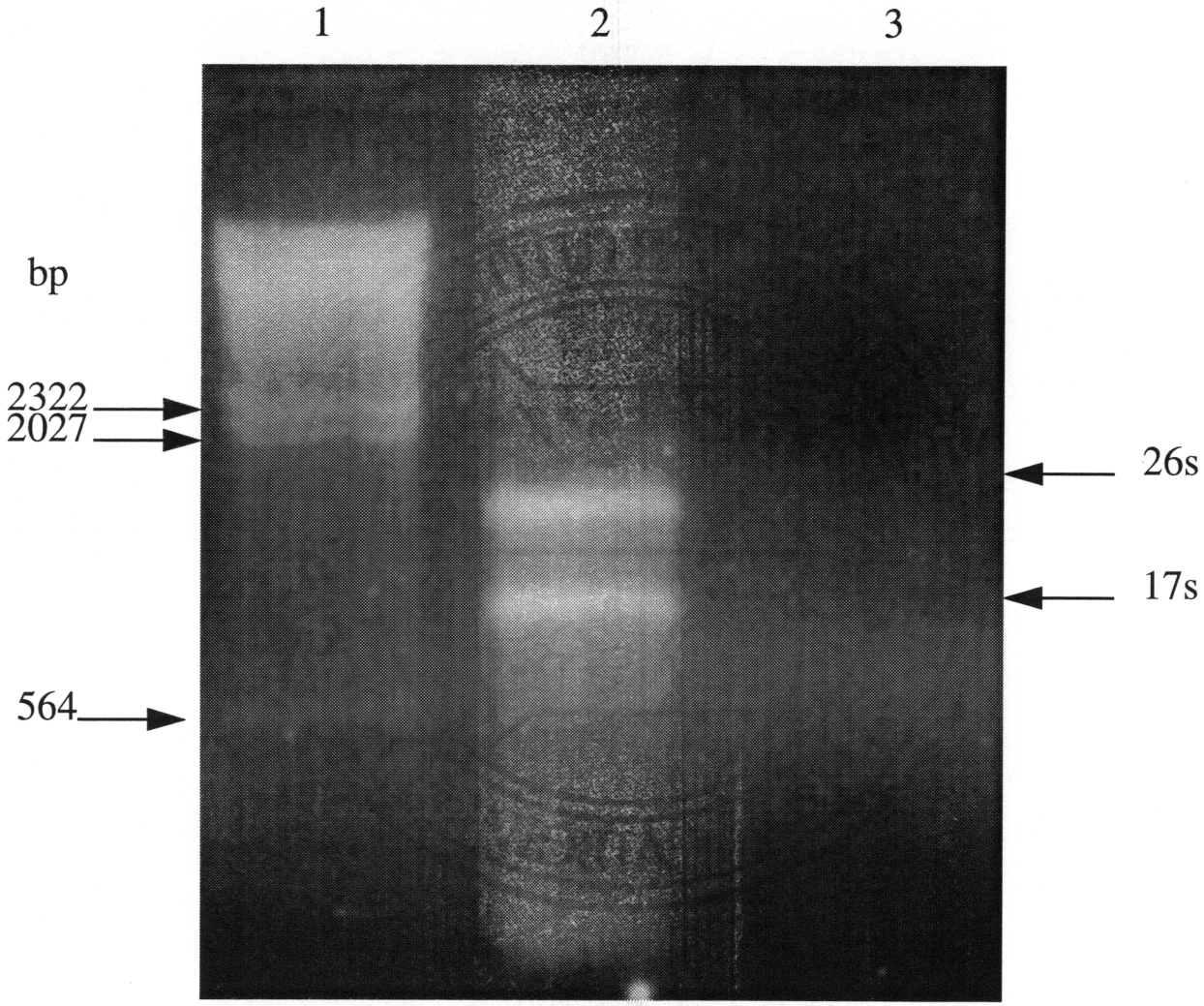
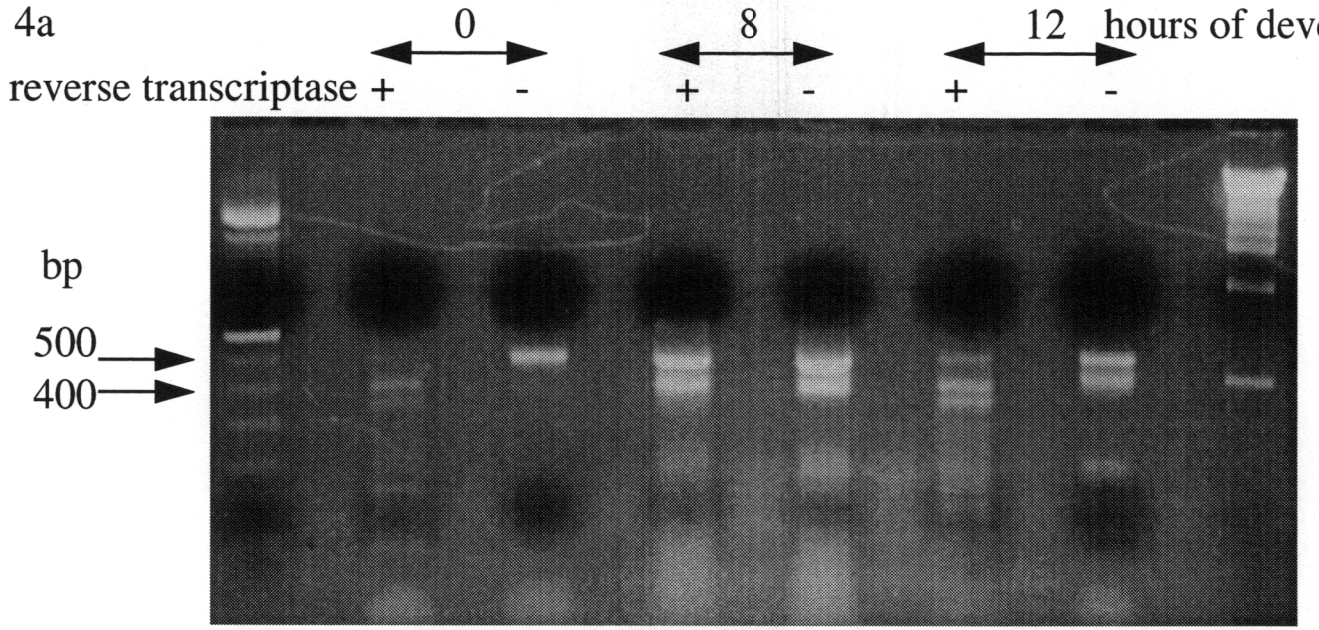


Figure 3c. 12 hours tRNA and rRNA condition after mRNA isolation.

Figure 4. Analysis of mRNA by RT-PCR.

mRNA from amoeba (0), 8 and 12 h of development was subjected to RT-PCR using primers for gs (4A) and gp-2(B). + and - refer to the presence or absence of reverse transcriptase, respectively. For gs, the 500 bp band is DNA-derived, while 400 bp band is RNA-derived. For gp-2, the 300 bp band is DNA-derived, while the 200 bp band is RNA-derived.


4a
0
8
12 hours of development
reverse transcriptase + - + - + -
bp
500
400

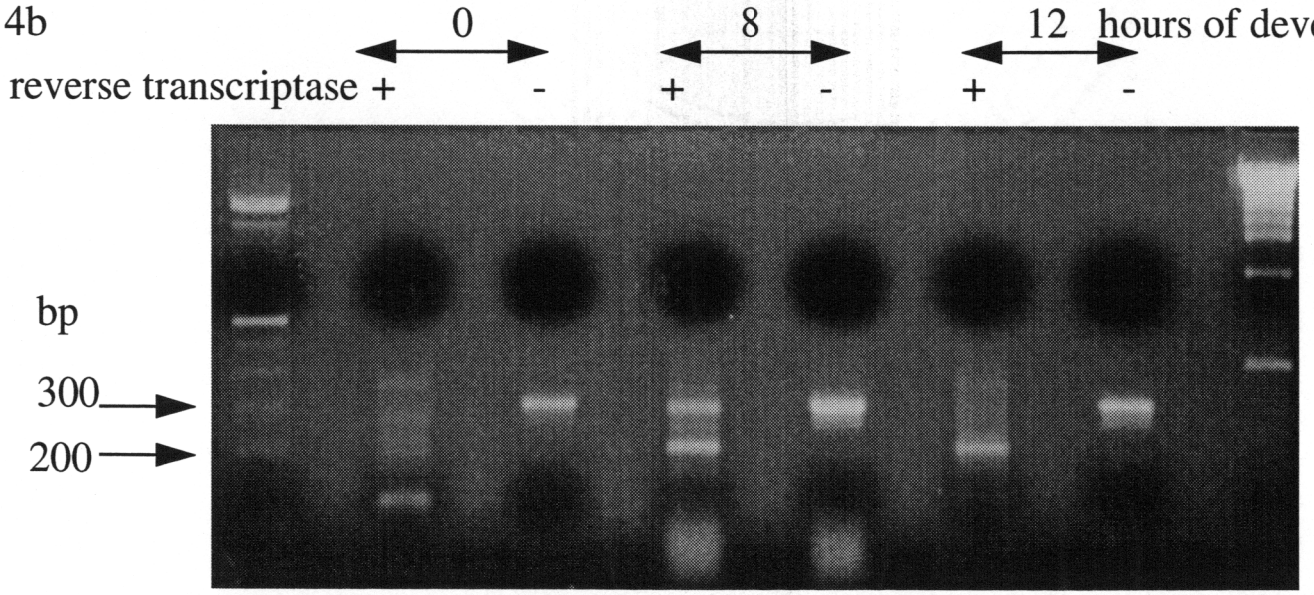

4b
0
8
12 hours of development
reverse transcriptase + - + - + -
bp
300
200

Figure 5. Tracer reactions of first and second strand cDNA synthesis.

Amoeba (5A), 8 h (5B), and 12 h (5C) cDNA synthesis tracer reactions for first (second lane in all figures) and second strand (third lane in all figures) separated on an alkaline agarose gel. Lambda HindIII is lane 1 in all figures.

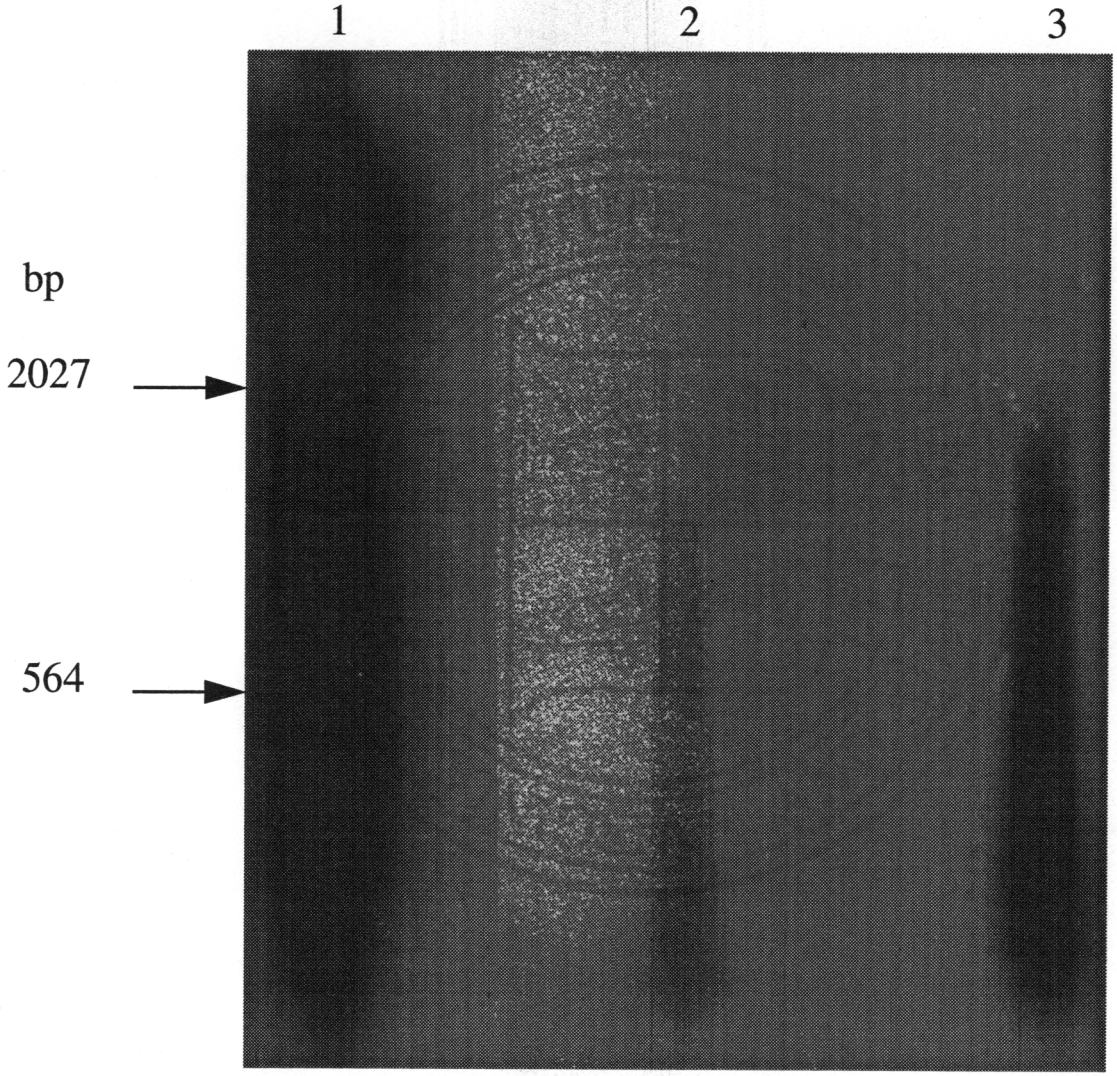


Figure 5a. First and second strand of amoeba cDNA.

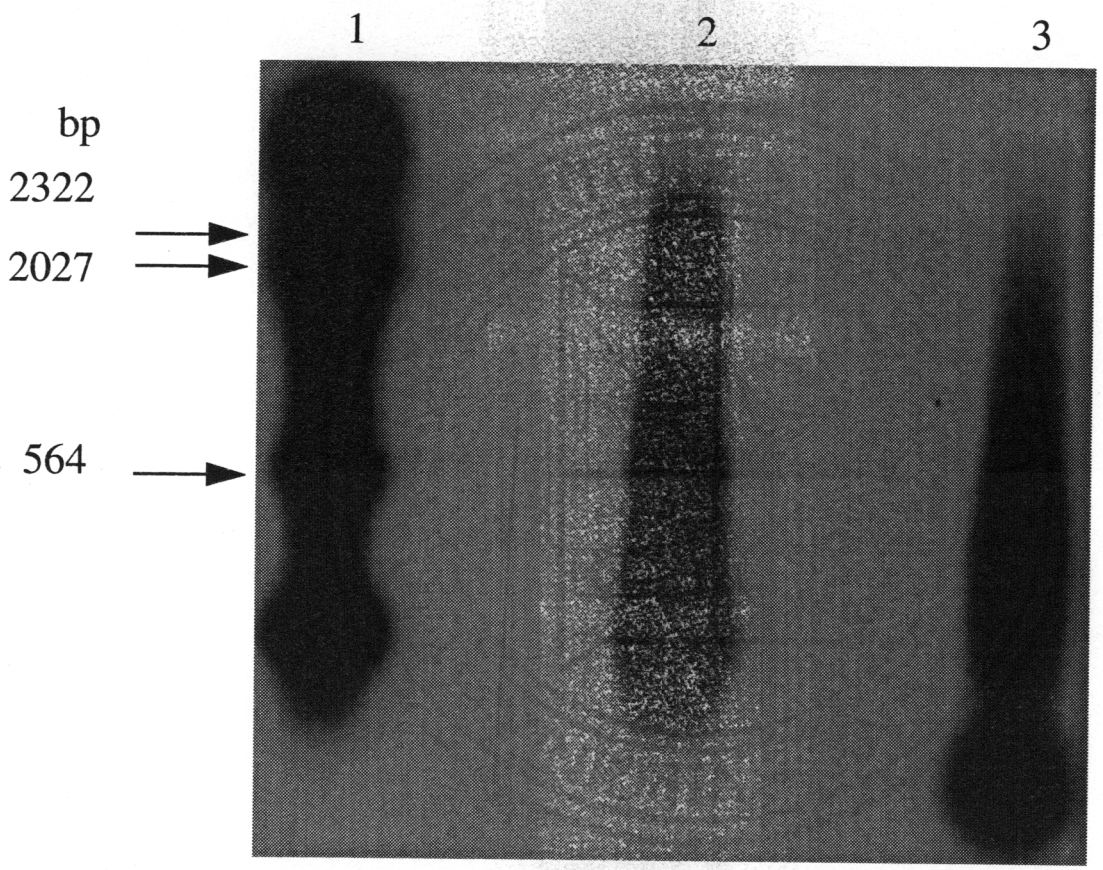


Figure 5b. First and second strand of 8 hours cDNA.

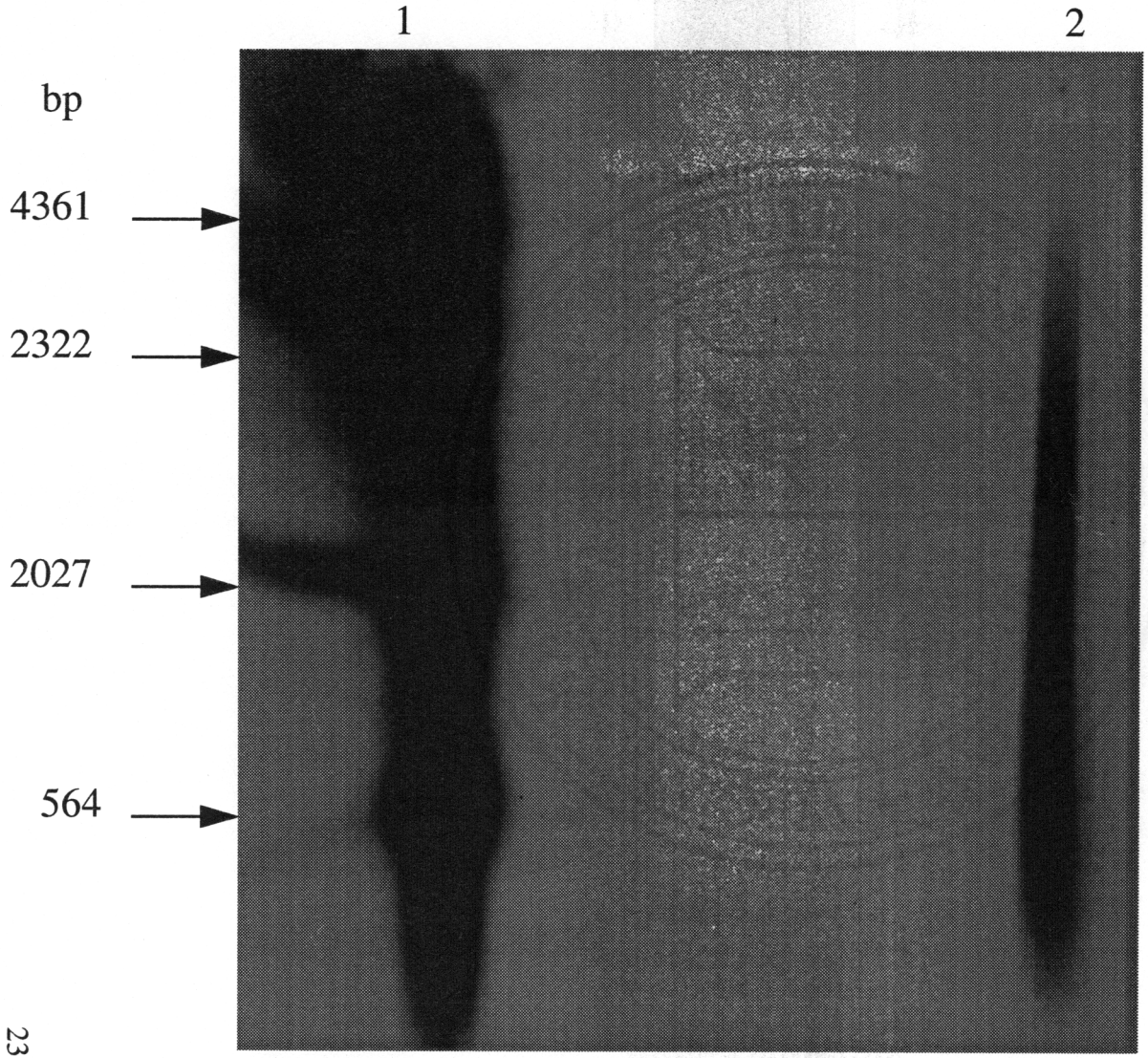


Figure 5c. Second strand of 12 hours cDNA.

Figure 6. Hot primer extension.

The length of amoeba cDNA (lane 2) compared to lambda HindIII marker (lane 1).

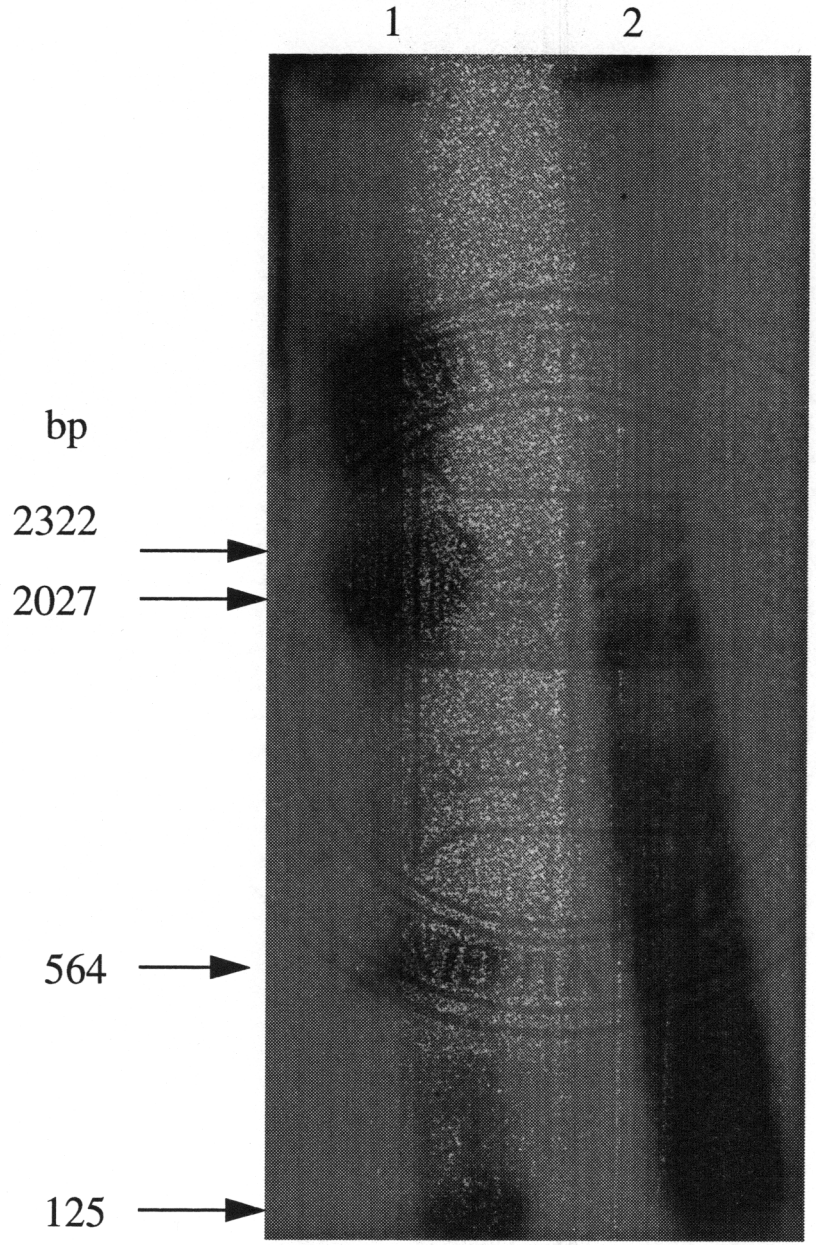


Figure 6. Hot primer extension.

Figure7. Double-stranded cDNA determination by DNase I.

Figure 7a shows digested and undigested amoeba cDNA (lane 4 and 5, respectively), and 8 h cDNA of development (lane 6 and 7, respectively). Figure 7b shows digested and undigested 12 h cDNA of development (lane 4 and 5, respectively). Digested and undigested single-stranded actin PCR product (Figure 7a) and digested and undigested double-stranded actin PCR product (Figure 7b) is in lane2 and 3, respectively. Lambda HindIII marker is in lane 1 in both figures.

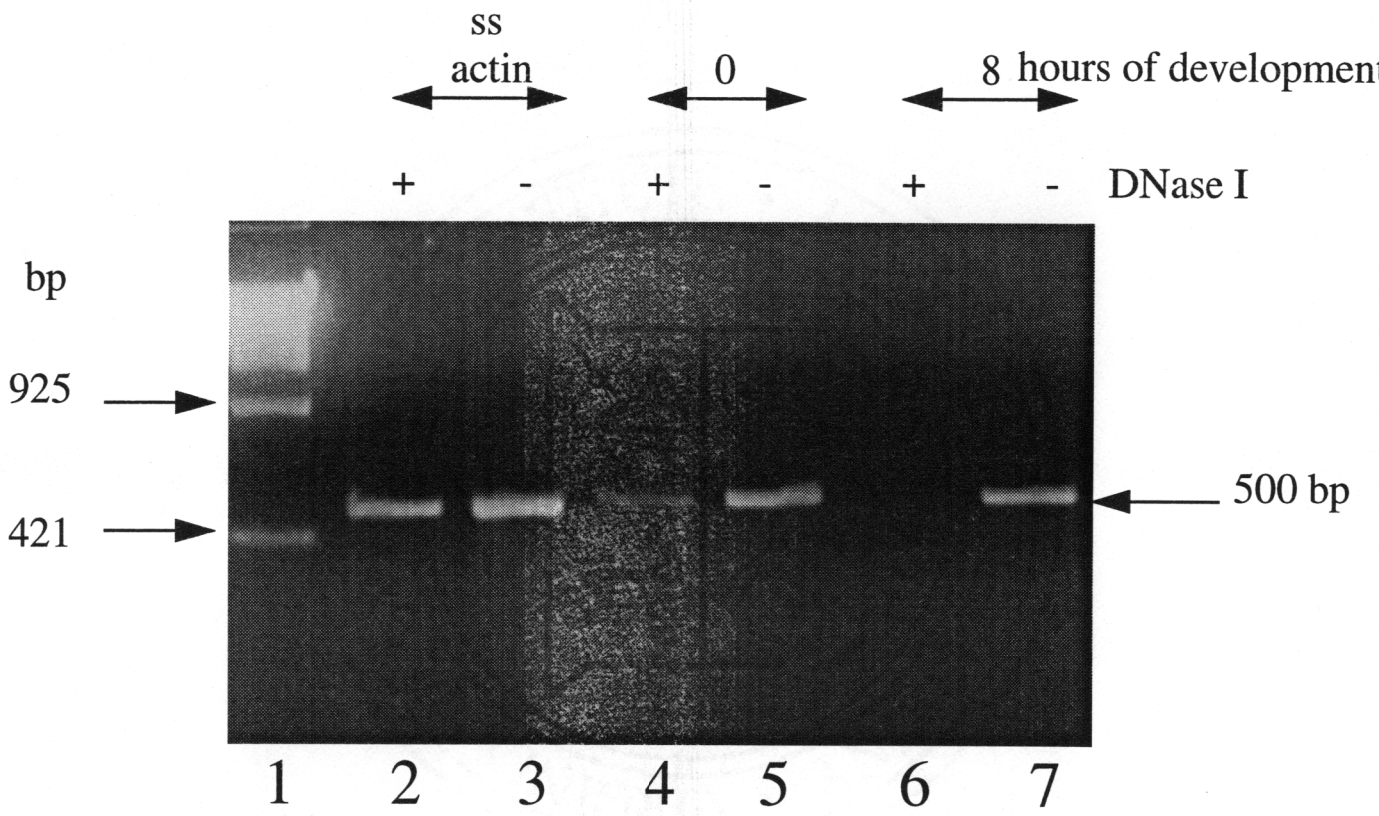


Figure 7a. Double-stranded cDNA determination by DNase I.

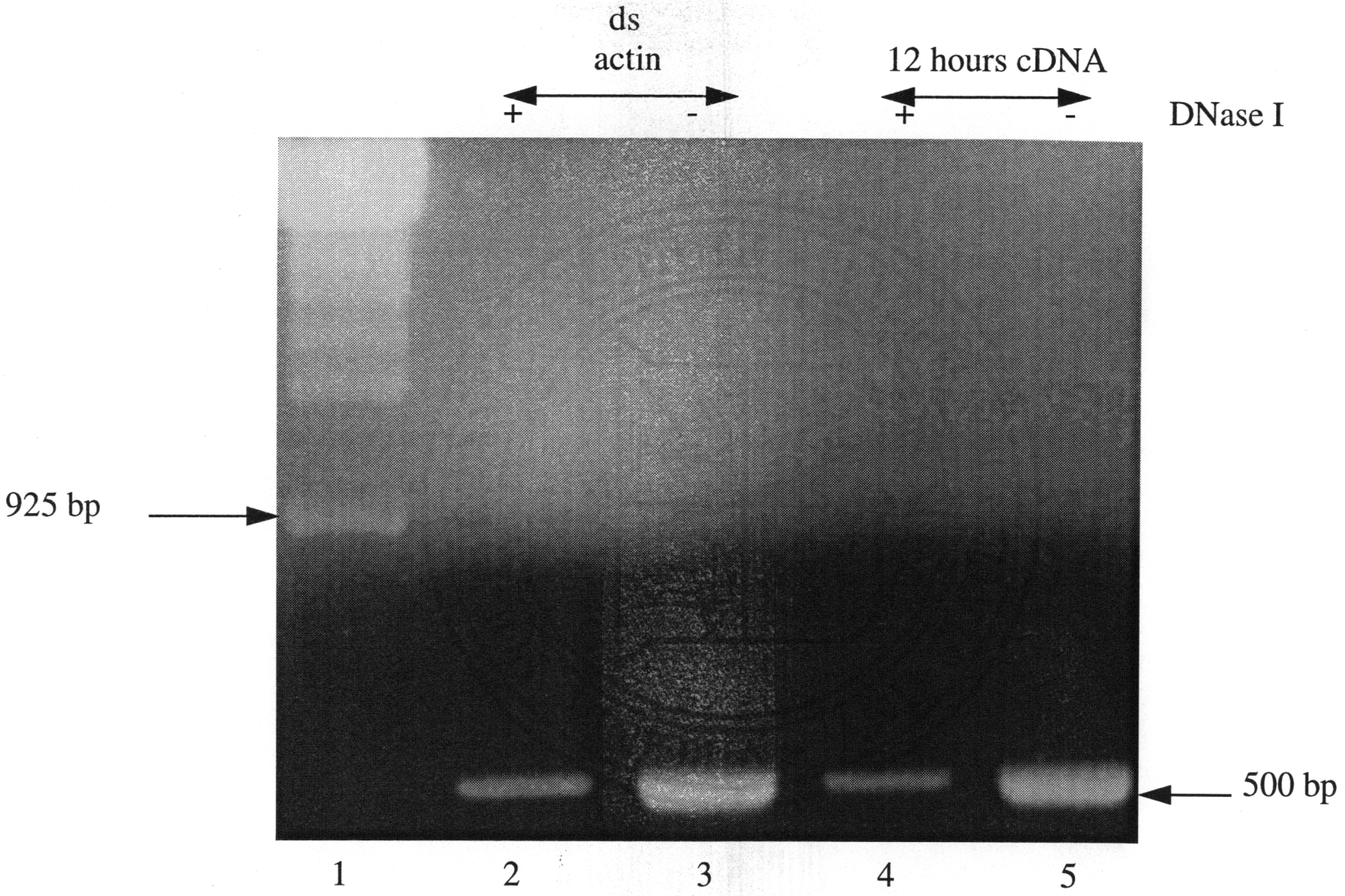


Figure 7b. Double-stranded cDNA determination by DNase I.

Figure 8. Double-stranded cDNA determination using S1 nuclease.

An equal number of counts from the first and second strand cDNA tracer reactions from 12 h of development were incubated with (+) and without (-) S1 nuclease.

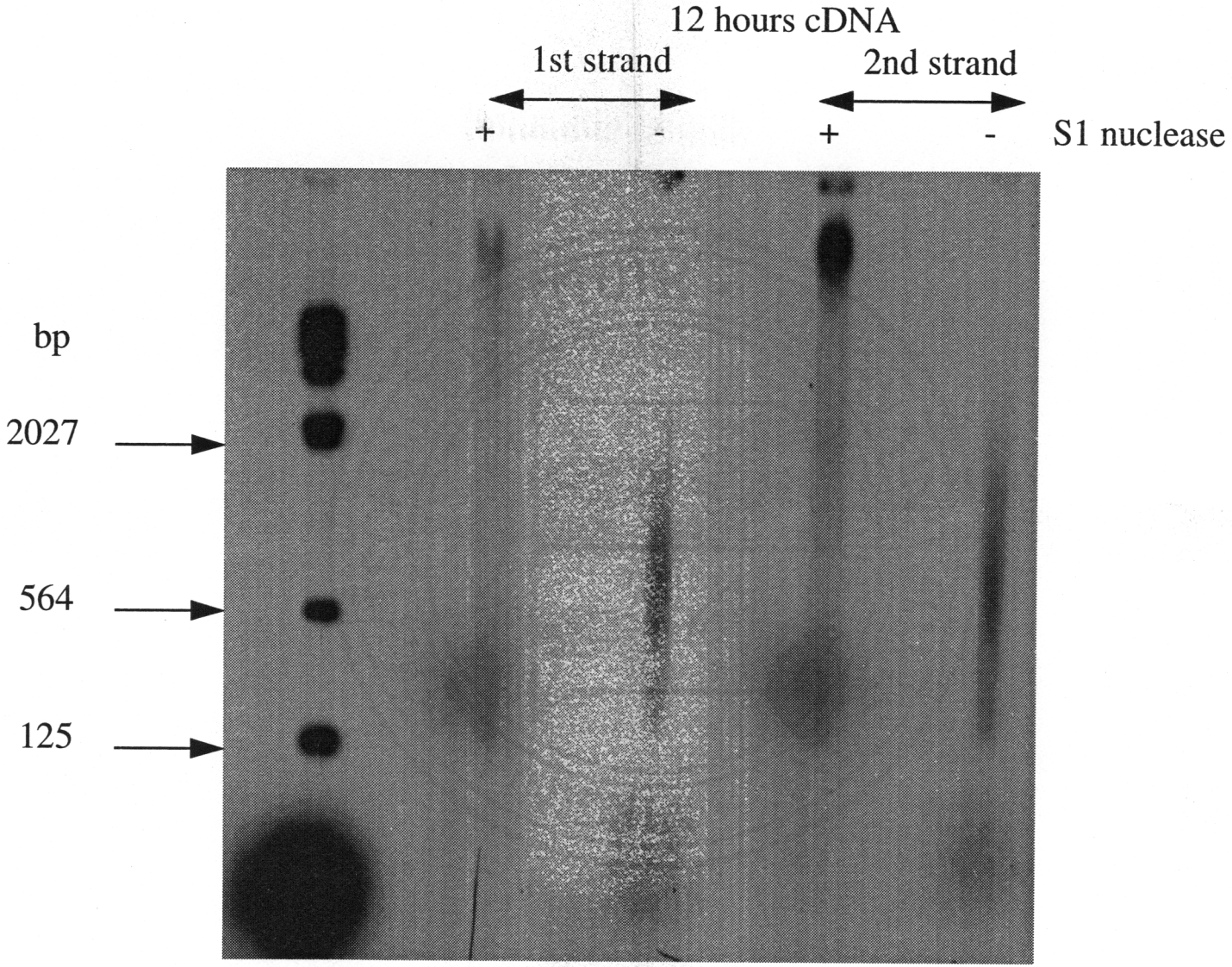


Figure 8. Double-stranded cDNA determination by S1 nuclease.

Figure 9. Examination of cDNA by PCR amplification of marker genes.

cDNA from amoeba (Am), 8 h and 12 h of development were PCR amplified using primers directed against glycogen synthase (gs), glycogen phosphorylase-2 (gp-2), phosphodiesterse inhibitor (PDI), SP-60, and actin. G: genomic DNA, M: lambda HindIII marker (Figure 9a and 9c), lambda Sty I marker (Figure 9b).

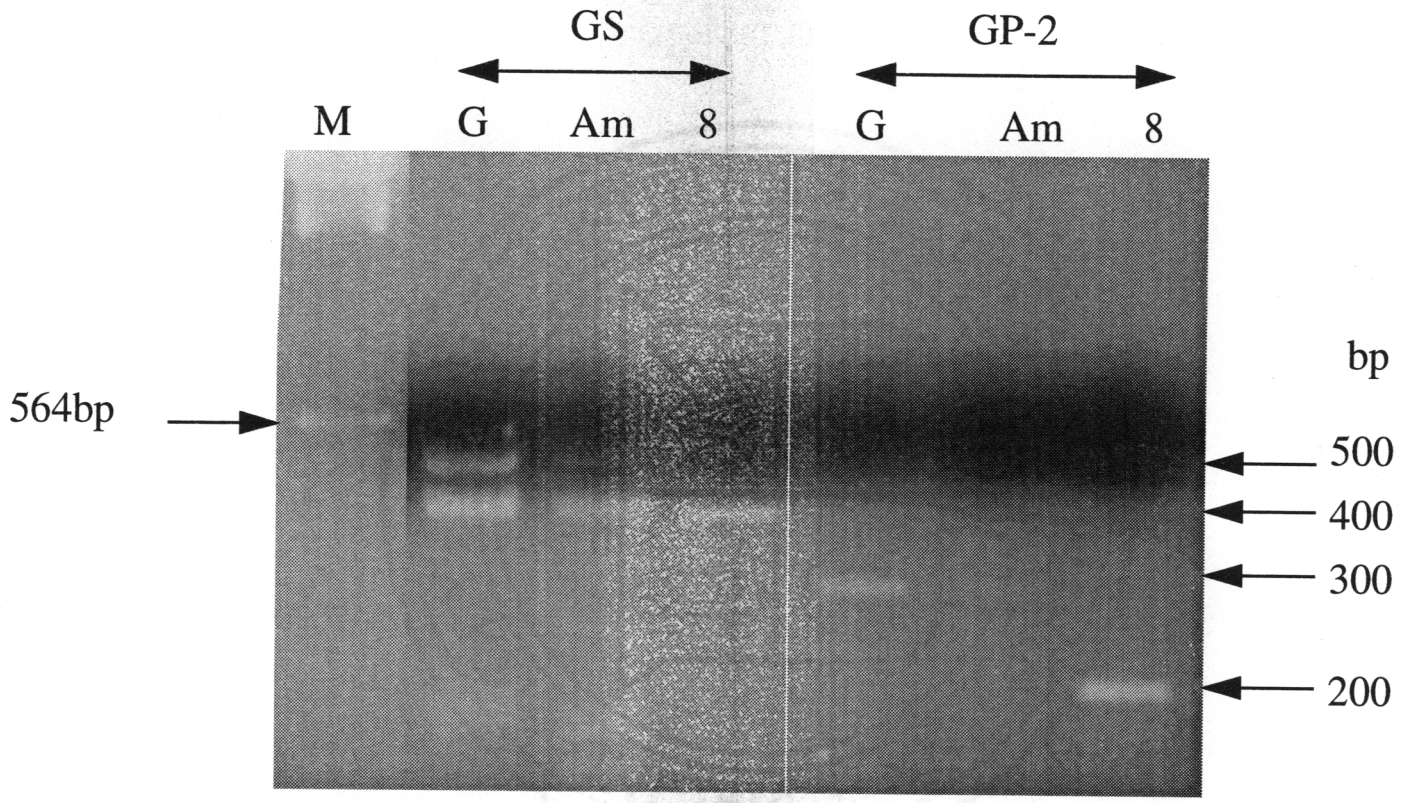


Figure 9a. Examination of cDNA by PCR amplification of GS and GP-2.

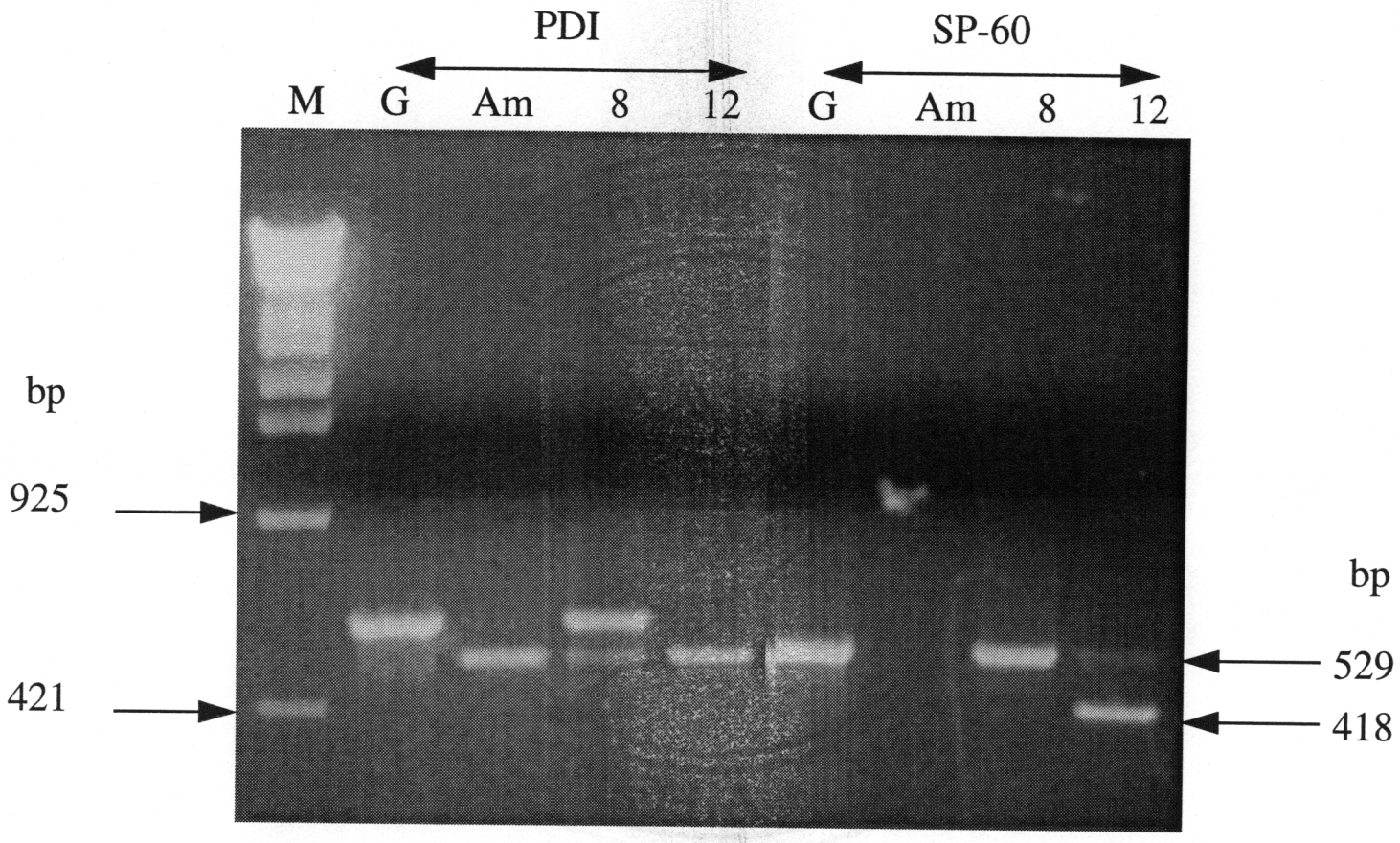


Figure 9b. Examination of cDNA by PCR amplification of PDI and SP-60.

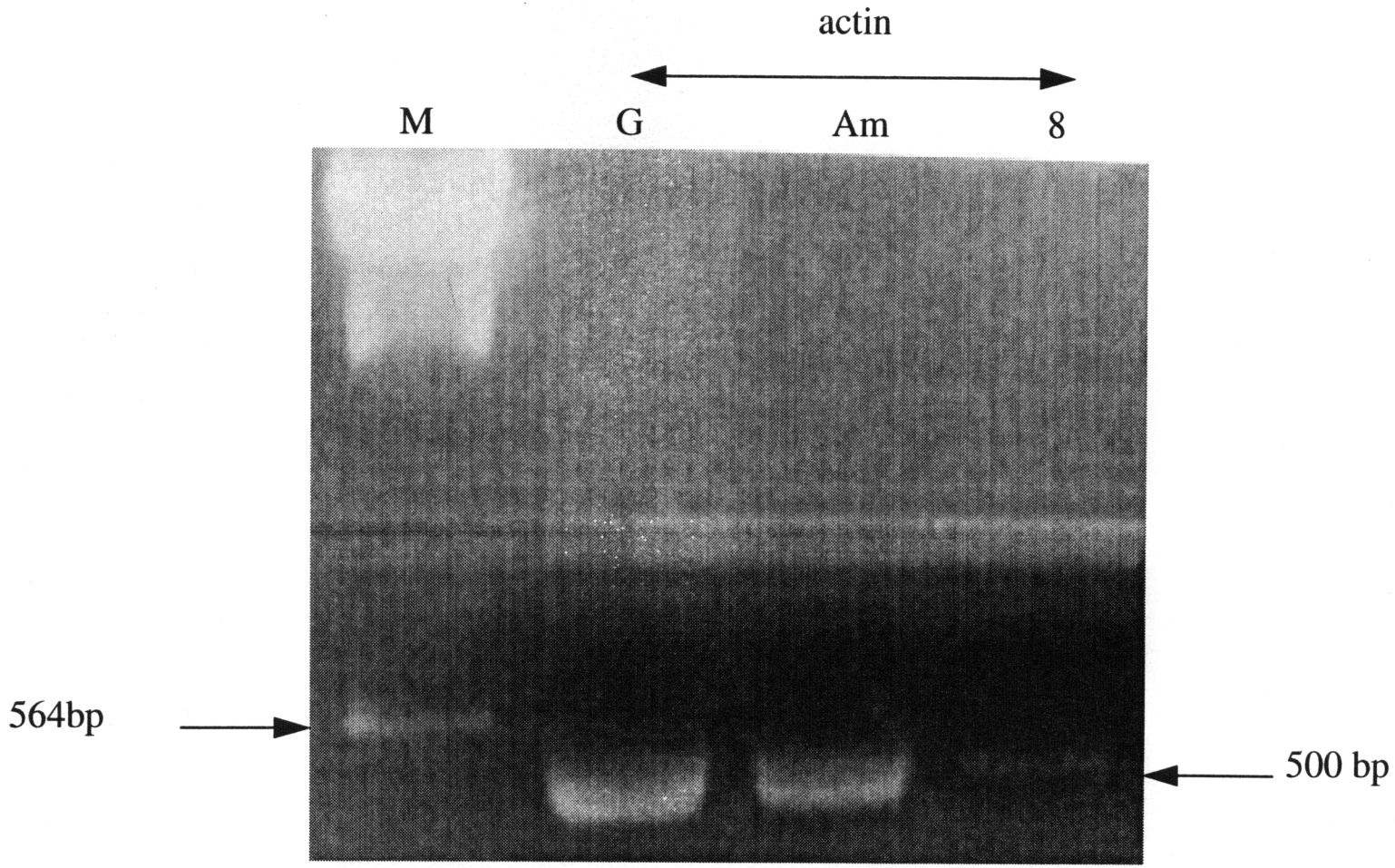


Figure 9c. Examination of cDNA by PCR amplification of actin.

Figure 10. Ligation of EcoRI adaptors to cDNA.

Tracer reactions from the second strand synthesis of cDNA from 8 h of development were ligated to EcoRI adaptors and then subjected to self-ligation. Lane 1: lambda HindIII marker; Lane 2: cDNA without EcoRI adaptors and T4 DNA ligase; Lane 3: self-ligated cDNA without EcoRI adaptors, but with T4 DNA ligase; Lane 4: cDNA bearing EcoRI adaptors self-ligated.

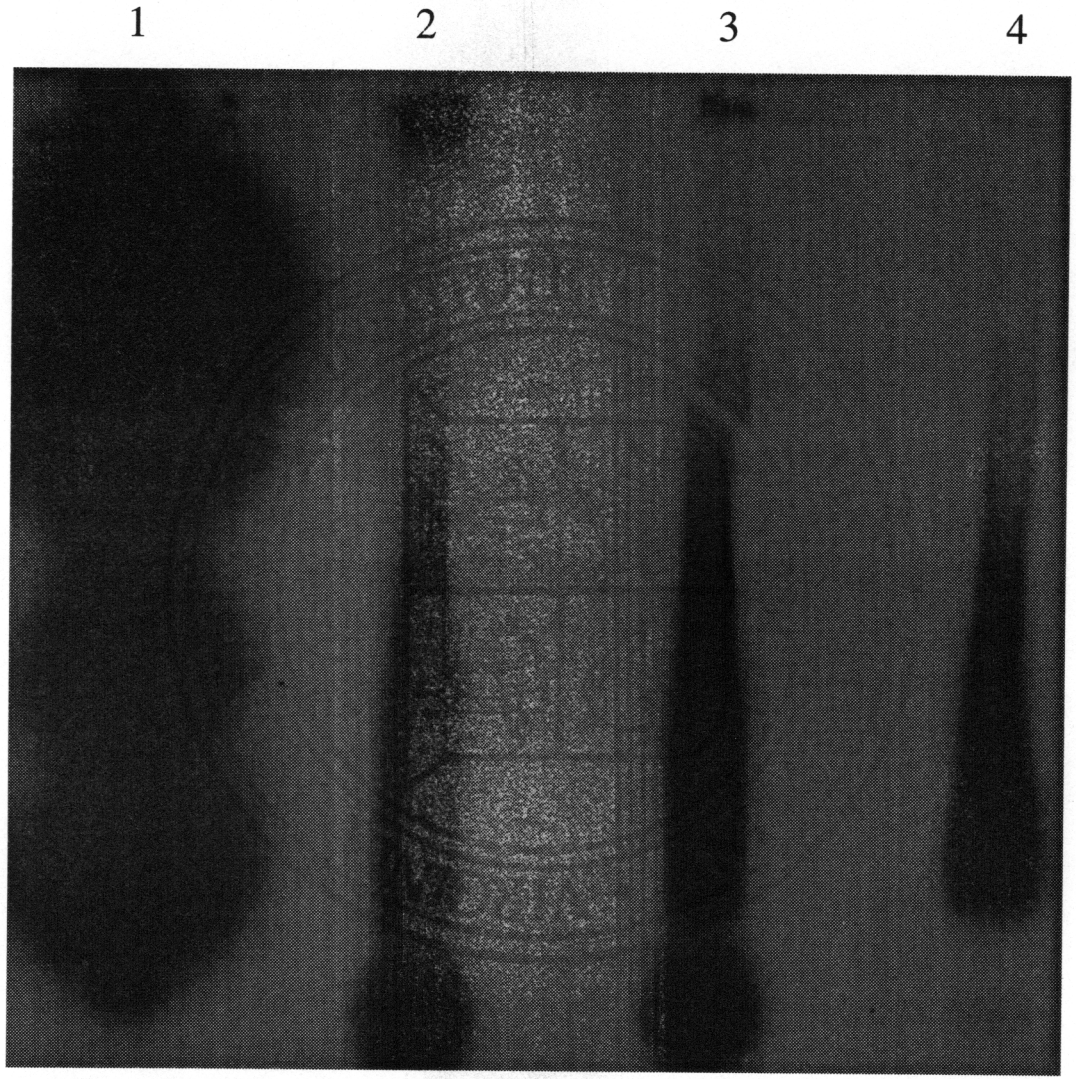


Figure 10. Ligation of EcoRI adaptors to cDNA.

Figure 11. Representative inserts from amoeba cDNA plasmid library.

Plasmid extracted from 10 white colonies (lane 3-12) were digested to determine the size of the insert. Lane 1 and 2 contain digested and undigested vector, respectively. M signifies lambda HindIII marker.

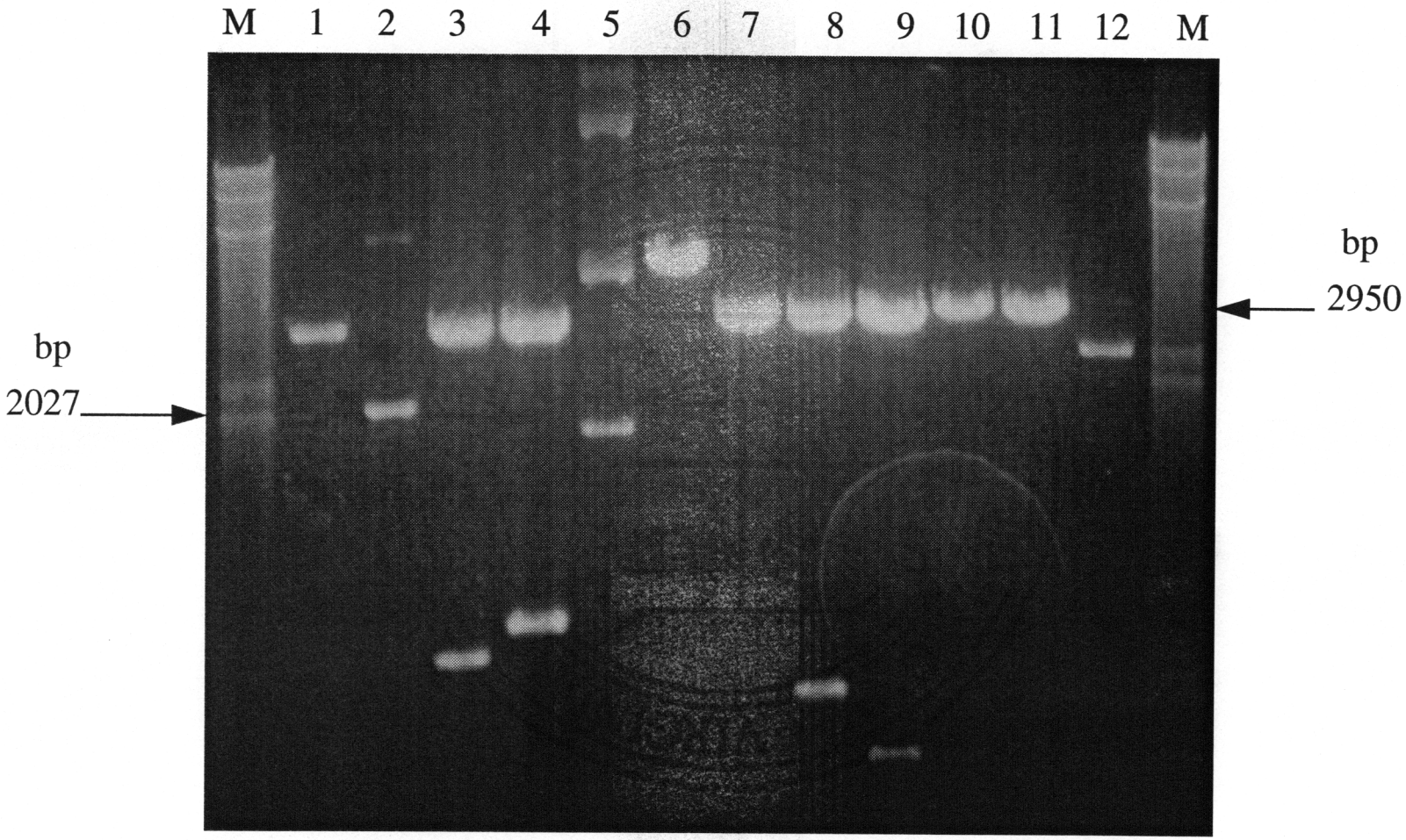


Figure 11. Representative inserts from amoeba cDNA plasmid library.

Figure 12. PCR screening of cloned cDNA for gs.

Plasmid extracted from a random population of bacteria transformed with either amoeba or 12 h cDNA plasmid libraries were PCR amplified using gs primers.

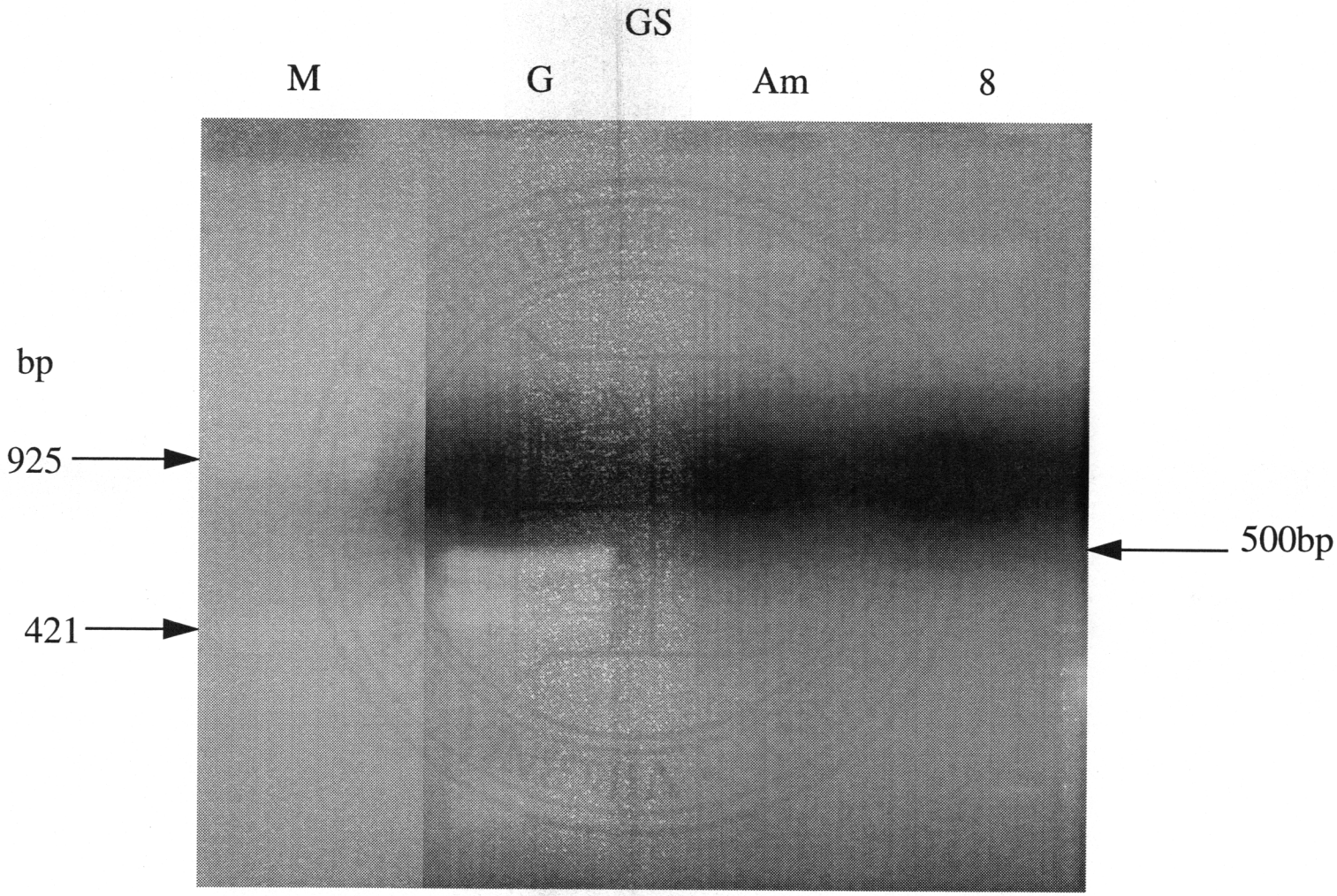


Figure 12. PCR screening of cloned cDNA for GS.

Figure 13. Cloning of a 1.5 kb insert into pBlueScript.

Lane 1 (undigested) and lane 2 (digested) contain the original cloned insert. Lane 3-6 contain digested plasmid from white colonies transformed with the new construct. M: lambda Sty I marker.

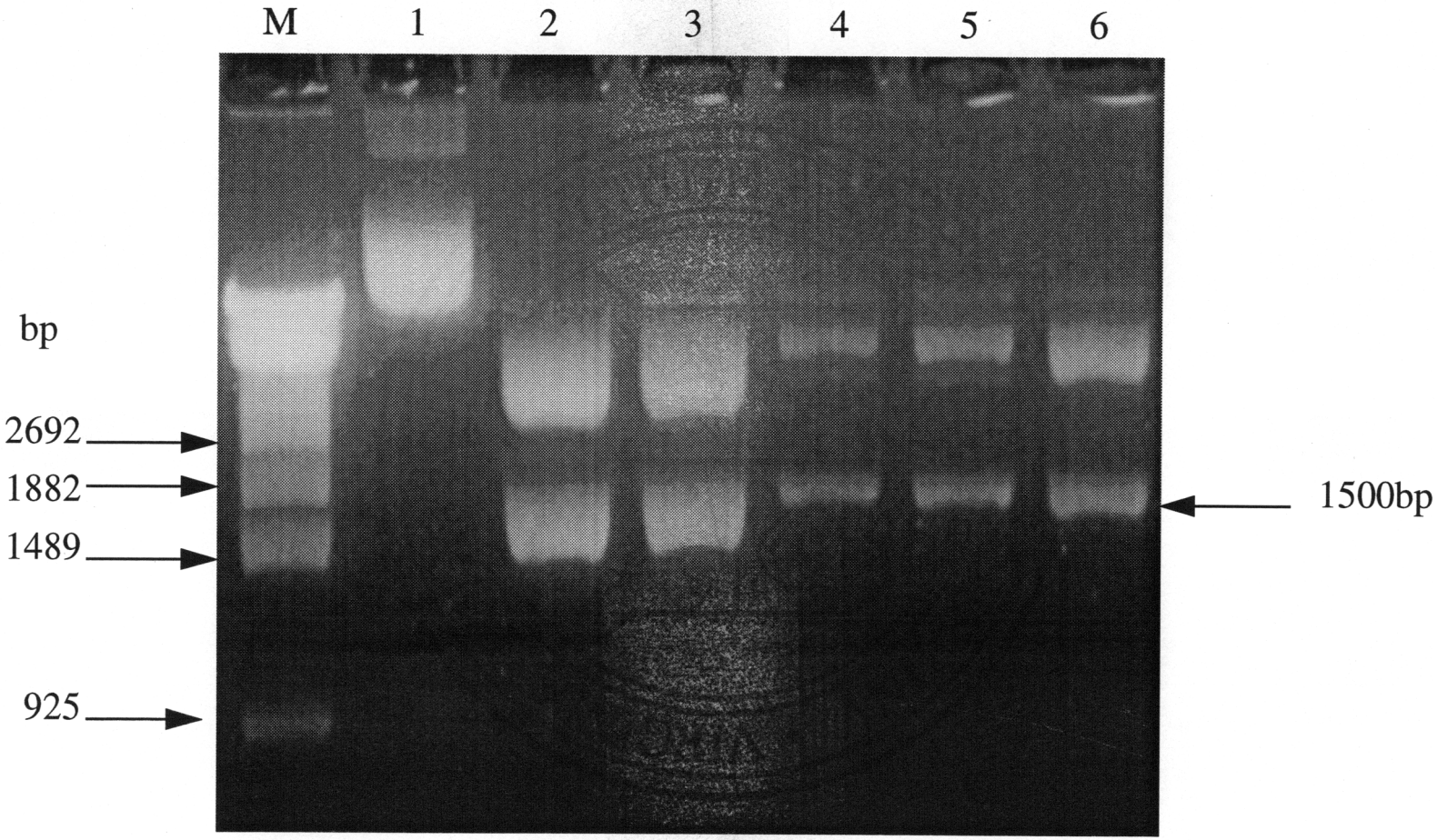


Figure 13. Cloning of a 1.5 kb insert into pBlueScript.

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