Volume 9 Number 4 1981 Nucleic Acids Research

Selection and analysis of cloned developmentally-regulated Dictyostelium discoideum genes by hybridization-competition

Giorgio Mangiarotti[1], Stephen Chung, Charles Zuker and Harvey F.Lodish

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Received 15 October 1980

SUMMARY

We describe a new technique for selection of cloned gene segments which are expressed preferentially at one developmental stage but at a relatively low level. A nitrocellulose filter replica of plaques of λ phage which contain approximately 8 KB inserts of genomic DNA is prepared; it is hybridized with a small amount of [^{32}P] labeled mRNA prepared from one developmental stage, in the presence of a several-hundred fold excess of competitor RNA from a different stage. We show that clones of Dictyostelium nuclear DNA which form hybrids under these conditions indeed encode developmentally regulated mRNAs. Our previous analysis of Dictyostelium discoideum differentiation indicated that transcripts from about 12% of the genome appear in mRNA at one defined stage of differentiation - the formation of cell-cell aggregates. A number of our new clones are novel, in that they encode multiple discrete mRNA species all of which accumulate only at the cell aggregate stages; others encode one or more mRNAs which appear at the tight aggregate stage and also one or more which are present throughout differentiation. These latter clones, in particular, would be difficult to identify using other selection techniques.

INTRODUCTION

A study of gene regulation during differentiation frequently requires the isolation of cloned DNA segments which encode developmentally regulated mRNAs. Several papers have described cloning of genes which encode mRNAs which are expresssed at a high level, such as globin or ovalbumin mRNAs (1, 2), and those for which specific selection procedures are available, such as dinydrofolate reductase (3). Frequently, however, it is necessary to select clones of developmentally regulated mRNAs which are maximally expressed at a low level, and for which the protein product is not known. Indirect selection techniques must be employed, and here we describe one which has proven especially useful for selection of genomic Dictyostelium discoideum clones which encode mRNAs which are expressed at specific stages of differentiation.

Isolation of these genomic clones is complicated by the fact that

transcripts from a relatively large fraction of the Dictyostelium genome begin to accumulate at a defined developmental stage. The population of cytoplasmic polyadenylated RNA in growing and pre-aggregating cells (average size 1250 bases) represents transcripts of about 4000 to 4800 genes, equivalent to 19% of the single-copy nuclear DNA genome. After aggregation, transcripts of an additional 3000 genes (10 - 12% of the genome) are accumulated. All of these cytoplasmic polyadenylated species are present on polyribosomes (4, 5). About one-third of the mass of mRNA in these cells represents newly-expressed genes. Most of these "late-specific" genes accumulate to 2 copies per cell; transcripts from about 100 of these genes accumulate to about 100-200 copies per cell, or 0.1% of the mRNA population each (4). Assuming a totally random distribution of gene segments along the chromosome, these results imply that a typical 8 KB DNA segment would encode 2 genes, some of which would be developmentally regulated and some expressed throughout the differentiation cycle. Indeed, our new results, detailed here, indicate that gene clusters do exist in Dictyostelium.

Published procedures for selection of regulated genomic clones involve the hybridization of filter replicas of a "clone bank" (using phage or plasmid vector) with two labeled mRNA probes, each prepared from a different developmental or physiological state (6, 7). Because of differences in the amount of DNA transferred to or retained on the filters, we have found this technique quite irreproducible for selection of regulated Dictyostelium segments. In any case, such techniques might not select a DNA clone which encodes both a common and a regulated mRNA, nor a regulated mRNA which is expressed at a very low level. We have found the procedure described here to be free of most of these difficulties.

MATERIALS AND METHODS

Growth and differentiation of Dictyostelium discoideum

Cells of Dictyostelium discoideum strain AX 3 (8) were grown axenically as described by Alton and Lodish (9), and development was initiated as detailed by Blumberg and Lodish (4).

Construction of genomic DNA library.

Our cloning vector λgt WES λB has been described by Leder, et al. (10) and λ DNA "arms" were prepared by the procedure of Maniatis, et al., (11). Dictyostelium DNA fragments were generated by complete and

partial Eco RI digestion: DNA that had been digested to completion, 1/2 completion, 1/4 completion, and 1/10 completion were mixed in equal amounts and electrophoresed in a 0.8% agarose gel. DNA fragments from 5 to 15 Kb in size were eluted from the gel and ligated to λ DNA arms (11). The recombinant DNA molecules were packaged into λ particles as described in reference 12 except that we used derivatives of the two lysogens, NS428 and NS43, which were resistant to λ adsorption.

Isolation and labeling of cytoplasmic polyadenylated RNA.

Cytoplasmic RNA was extracted from vegetative growing cells and from developing cells and chromatographed on columns of oligo (dT), as described by Blumberg and Lodish (5) except for the following modification. After the first phenol-chloroform extraction the aqueous phase was extracted once with an equal volume of chloroform. Poly (A^+) selected RNA were labeled using γ [^{32}P] ATP and polynucleotide kinase as described in reference 13.

Replica of a random set of clones

The wells of a microtiter tray (0.6 cm diameter) were washed in 80% ethanol and then sterilized under a hood by ultraviolet light. To each well 50 µl of LB medium (10 gm tryptone, 5 gm yeast extract, 5 gm NaCl and 1 ml 1 N NaOH per liter) containing 1% agarose was added. After the agar had solidified, 50 µl of λ broth (12 gm tryptone, 5 gm NaCl per liter) containing 0.7% agarose and 2 µl of a growing Escherichia coli culture (in λ broth containing 0.2% maltose and 0.01% yeast extract) at an A_{540} of 1.0, were added. Single plaques from the λ-Dictyostelium library were picked into wells with a toothpick. Following an overnight incubation at 37°C, each set of plaques could be replicated into a series of new microtiter trays prepared in the same way by using a metal replicator, constructed in such a way that each tooth corresponds to one well. Each tray could be conserved in a sealed plastic bag at 4°C for at least three months before being replicated. With this procedure, a given set of clones, ordered in a defined way, and therefore identifiable by the two coordinates (see Fig. 1), could be replicated whenever and as many times as needed.

From the tray, the set of plaques could be replicated onto 15 cm diameter petri dishes, each containing 40 ml of LB-agar medium and a layer of 0.7% agarose with E. coli. The replication was usually done directly with the metal replicator. However, occasionally in touching the soft agar layer with the teeth of the replicator, the soft agar

layer was broken. This led to the transfer of small discs of soft agar onto the nicrocellulose paper when the plaques were subsequently blotted. The agarose appeared to trap some labeled RNA probe, non-specifically generating false positive signals when the nitrocellulse paper was autoradiographed following hybridization (see below). To avoid this problem, the transfer of phage from the tray to the replica plate was later done through an intermediate set of wells, each of which contained 50 µl of TM buffer (10 ml TRIS-HCl, pH 7.4; 10 mM $MgSO_4$). Using the metal replicator again, a tiny amount of liquid on each tooth was then deposited on the surface of the replica plate, being careful not to touch the agar to the metal. This procedure reproducibly generated larger plaques than did the direct transfer technique (compare Figs. 1 and 2) and also minimized the background.

DNA blotting

Following overnight incubation at 37°C, each agar plate was left open in the same incubator for at least two hours in order to dry the surface. A 10 cm diameter sheet of nitrocellulose paper (Schleicher and Schuell, BA85) was placed onto its surface for 1 min. The paper was then carefully removed and dropped for 45 sec into a solution of 0.2 N NaOH and 1.5 M NaCl. It was then transferred for 30 sec into a solution of 20 mM TRIS-HCl, pH 7.4, and finally for 30 sec into a solution of 2 X SSC. The filter was wiped for a few seconds and then dried under vacuum for 2 hours at 80°C.

Hybridization reaction

Hybridization buffer contains 50% formamide, 4 X SSC, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.1% SDS, 25 mM EDTA, 20 mM sodium phosphate buffer, pH 7.0. Each dry filter was sealed into a plastic bag of a slightly larger size, containing 20 ml of hybridization buffer and incubated overnight at 37°C. Following this prehybridization the filter was transferred into a second bag containing 5 ml of hybridization buffer, and labeled and competitor RNAs at the concentrations indicated in the individual figure legends. The RNA preparations had been heated in water at 65°C for 5 min and then rapidly chilled in ice water before being mixed with the hybridization buffer. The hybridization reaction was carried out for 3 to 4 days at 37°C in a sealed bag.

Each filter was then washed separately in 100 ml of 50% formamide containing 4 X SSC, twice for 30 min at 37°C; then with 100 ml of 2 X

SSC, twice for 30 min at 50°C; and finally with 200 ml 0.1 X SSC containing 0.1% SDS, twice for 30 min at 50°C. Sometimes, an RNAse step was then added: The washed filters were rewashed thoroughly with 2 X SSC, and they were then incubated in a solution of 2 X SSC, containing 10 µg pancreatic RNase/ml, at room temperature (22 - 24°C) for 10 minutes, and then rewashed in 2 X SSC. The RNAse treatment was found to lower the background, but with some of the clones it also lowered significantly the intensity of the hybrid signal.

Following washing, each filter was dried for a few seconds on a layer of tissues and then covered with saran-wrap and exposed to a Kodak XR-5 film with an intensifying screen, at -70°C. Exposure was usually for two to four days.

Preparation of [^{32}P]-labeled DNA

DNA was isolated from the λ-Dictyostelium clones as described in reference 11. Cloned DNAs were labeled by nick translation (14) except for the following modifications. 0.1 - 1.0 µg of DNA was incubated at 15°C for 3 hours in 0.1 ml of a solution containing 50-100 µCi [^{32}P]-dTTP (600 ci/mmol; Amersham), 30 nmoles each dCTP, dGTP and dATP, 0.1 mg gelatin, 0.05 M Tris-HCl (pH 7.8), 5 mM MgCl, and 0.01 M 2-mercaptoethanol. The reaction was intiated by the addition of 2 units of DNA polymerase I (P-L Biochemicals). Following incubation at 15°C for 5 minutes, 10^{-6} to 10^{-7} mg of DNAase I was added. After 3 hrs at 15°C, 300 µl of a solution containing 0.2 M NaCl, 10 mM Tris-HCl (pH 7.5) and 0.5% SDS was added and the reaction mixture was extracted once with phenol and once with chloroform. Carrier RNA was added and the labeled DNA was precipitated with ethanol.

The DNA was resuspended in 100 µl of 0.3 M NaOH, heated at 100°C for 3 min and desalted on a 1 ml Sephadex G-25 (fine) column preequilibrated in 10 mM Tris-HCl (7.4) and 1 mM EDTA. The final specific activity ranged from 0.2 - 1 x 10^8 cpm/µg DNA.

Gel analysis and transfer of RNA to nitrocellulose paper.

This procedure was introduced to us by Tom Maniatis (personal communication). It is nearly a hundred times more sensitive for detection of RNA sequences than the method of Alwine, et al. (15), and it is also more reproducible than a similar method described by Patricia Thomas (16).

RNA species were separated by electrophoresis in a formaldehyde gel as described by Rave, et al. (17). We used 1.5% agarose and electro-

phoresis at 100 V for 6 hrs in a horizontal gel box with circulating buffer. After electrophoresis, the gel was soaked in 10 X SSC for 20 min. It was then blotted onto a nitrocellulose filter which had been soaked in distilled water and equilibrated in 10 X SSC. The mechanism of blotting was the same as described by Southern (18) except that the transfer buffer was 10 X SSC. After blotting the filter was throughly dried under a lamp and baked *in vacuo* at 80°C for 2 hrs. Presoaking, hybridization and washing were the same as described by Alwine, *et al.* (15) except that no glycine was used in the presoaking buffer.

RESULTS

Selection of developmentally-regulated Dictyostelium clones by hybridization competition.

Our studies utilized a library of *Dictyostelium* nuclear DNA, partially digested with EcoRI, and cloned in a λgt *wes* vector. The average DNA insert was 8 KB. Our strategy for selection of clones which encode developmentally regulated mRNAs is to hybridize a filter replica of a set of plaques with [^{32}P] cytoplasmic polyadenylated RNA, isolated from aggregated cells and labeled *in vitro* with polynucleotide kinase, in the presence of a large (several hundred-fold) excess of RNA prepared from growing or pre-aggregating cells. The competitor RNA generally was not preselected for polyadenylated species. The desired clones should generate a [^{32}P] hybrid, detected by radioautography, even in the presence of unlabeled competitor RNA from growing cells.

Figure 1 illustrates an example of this selection procedure, together with a number of essential controls. A random set of *Dictyostelium* clones, each derived from a single recombinant phage, were placed in wells of a microtiter tray and replicated onto four plates, generating a set of replica plaques (see Methods). From each plate DNA was blotted onto a nitrocellulose filter. Panel A shows that about three-fourths of these clones formed a hybrid with [^{32}P] cytoplasmic polyadenylated RNA isolated from growing cells. With other sets of clones the fraction which formed a hybrid with the same probe was lower, around 50%. However, since no unlabeled RNA was present in these hybridization reactions, we do not know whether all of the positive signals represent true RNA-DNA hybrids. In any case, this result is not surprising when it is recalled that 18% of the single-copy genome is represented as cytoplasmic polyadenylated mRNA transcripts in these cells, and that all of

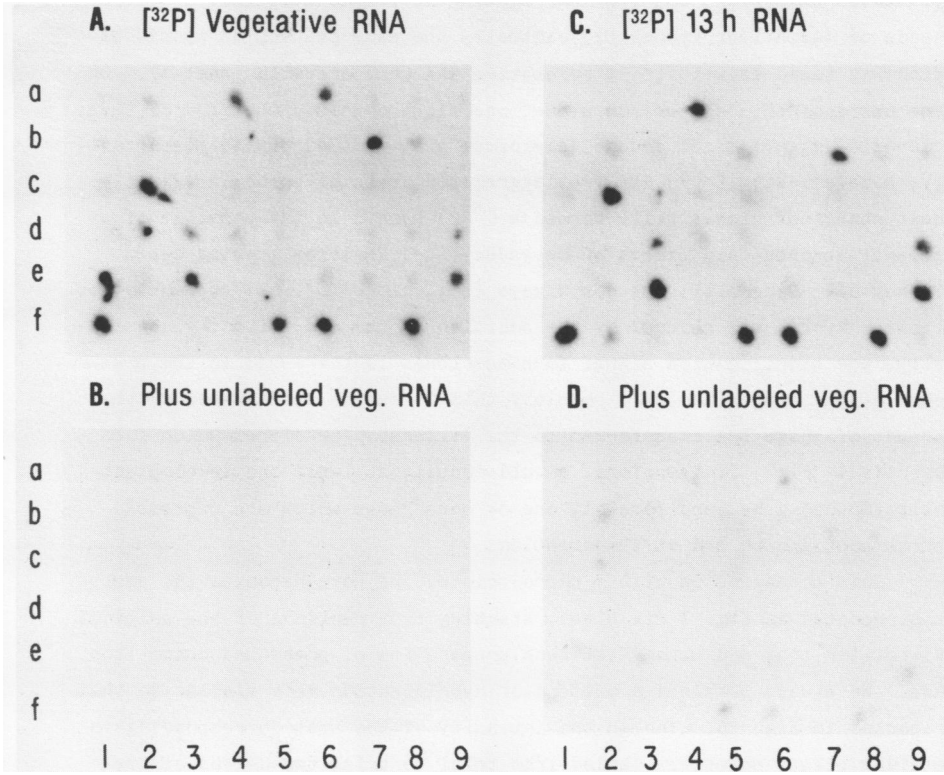

Figure 1. Screening a random sample of λ-Dictyostelium clones by hybridization competition. A random sample of plaques, each derived from a single recombinant pahge, were picked into wells of a microtiter tray and replicated directly onto four agar plates as described in Methods. DNA from the plaques was blotted onto a nitrocellulose filter and hybridized as described in Methods. In panel a) 0.2 μg (1,000,000 cpm) in vitro labeled cytoplasmic poly (A^+) RNA extracted from vegetative cells was added to the hybridization mixture; b) as in a, except 1.0 mg total cytoplasmic RNA extracted from vegetative cells was added; c) the same amount of poly (A^+) RNA from 13 hr cells, with comparable specific radioactivity, was added; d) as in c except 1.0 mg RNA from vegetative cells was added.

Some of the clones shown in this figure are also shown in the following figures. Among these are GM6 (a1), GM10(b2), GM15(c2), GM19(e2), GM21(e9), GM22(f1), GM23(f5), GM24(f6), GM45(a6) and GM55(f4).

these sequences are found on polysomes (5). As expected (panel B), all hybridization was abolished by the addition of a several hundred-fold excess of unlabeled RNA isolated from the same cells. (Note that the competitor is total cytoplasmic RNA, of which only about 2% is polyaden-

ylated.) When [^{32}P] RNA from aggregating cells was utilized, in the absence of competitor (panel C), virtually the same pattern of hybridization was found as with [^{32}P] vegetative RNA (compare with panel A). On the basis of this comparison alone, one might conclude that few of these clones (e.g. e9) might encode developmentally-induced mRNAs. Importantly, however, with [^{32}P] RNA from aggregated cells as probe, a significant number of clones still formed a [^{32}P] hybrid in the presence of a several hundred-fold excess of polyadenylated RNA from growing cells (panel d). Generally, but not always (e.g. clones b2, b8) the amount of labeled hybrid was reduced by the addition of the competitor. Occasionally, the hybridization signal to some clones is increased in the presence of the competitor RNA; possibly this is due to differences in the amount of phage DNA transferred to the filters. The clones which form hybrids in Fig. 1d, therefore, should contain at least one developmentally-induced gene, and possibly one or more genes which are expressed throughout growth and differentiation.

This procedure is highly reproducible. We have repeated the analysis reported in Fig. 1 six times, starting from replicas of the original microtiter tray and using different preparation of probe and competitor RNA. We always obtained a pattern of hybridization very similar to that reported in Fig. 1D. Though the intensity of the spot corresponding to a given plaque sometimes varied from trial to trial the degree of concordance among the different trials was greater than 90%. As an example (Fig. 2), some of the plaques have been repicked from the original wells and ordered in a new tray. The analysis described in Fig. 1, was repeated using the indirect plaque transfer technique (see Methods); this generated large plaques and thus a more intense hybridization signal. The results obtained were consistent with those obtained in the first screening (see legend).

We have, however, encountered two artifacts. Occasionally a plaque was missing in a replicate plate (see, for example, the legend to Fig. 2). This can be controlled by inspecting each plate before blotting. Sometimes a false positive signal was observed, clearly due not to hybridization to DNA, but to non-specific sticking of the labeled probe to material other than DNA which was transferred from the plate to the nitrocellulose filter. Though this background problem can be greatly reduced by replicating the plaques first into a small amount of buffer, and then to the agar plate (discussed in Methods) it may be advisable to

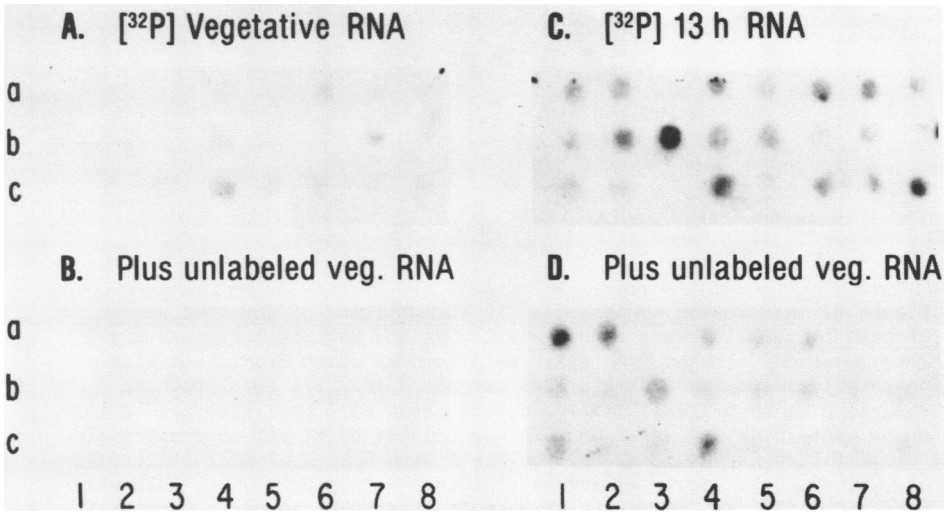

Figure 2. Reproducibility of the competition hybridization procedure. Some of the clones selected from the study in Fig. 1d and from similar experiments were repicked into a new microtitration tray and replicated (using the "liquid transfer procedure" described in Methods) onto four plates. From each plate DNA was blotted and hybridized with the same RNA probes and competitor as described for each corresonding panel of Fig. 1. Some of the clones indicated in Fig. 1 are also present here. These are GM6(a1), GM10(a2), GM19(a4), GM21(a6), GM22(a7), GM23(a8), GM45(c2) and GM55(c3). The plaque corresonding to GM 6 was missing from the plate used for panel A. Also included in Fig. 2 are an additional clone, GM27(b1), and SC253(b3), which was isolated by a different procedure and which has been shown to encode a single mRNA species present in 13 and 22 hr cells, and absent in vegetative or 6 hr cells (Stephen Chung, Charles Zuker, and Harvey F. Lodish, manuscript in preparation).

run the competition test in parallel on a set of two duplicate filters, in order to avoid any chance of error.

Many clones encode multiple species of mRNA.

Two types of studies showed that many of our selected clones indeed encode multiple species of mRNA, some (or all) of which are developmentally regulated. In the first, constant amounts (0.1 to 0.2 µg) of purified phage DNA are denatured and directly applied to nitrocellular filters. (The amounts of DNA are, we estimate, about 1000 times more than in the plaque hybridization system (Fig. 1, 2)). These are hybridized with [^{32}P] mRNA from 22 hr cells, in the presence of various amounts of competitor RNAs. A key control for these studies is illustrated in Fig. 3. Here two DNA clones are used, one (GM5) hybridizes to an equal ex-

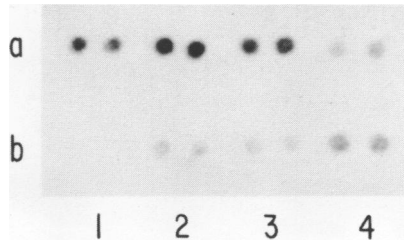

Figure 3. Resolution by hybridization-competition of the mRNA sequenced encoded by two different cloned DNAs. DNA was extracted (see Methods) from clone GM5 (chosen from a series of clones which hybridized with comparable intensity to RNA probes extracted from all developmental stages; G.M. unpublished data) and from clone GM27 (see Fig. 2). A solution containing 0.2 µg of GM5 DNA was spotted by itself on a nitrocellulose filter (spots a1 and b1) or mixed with 0.1 µg of GM27 DNA (spots a2, b2, a3, b3). Spots a4 and b4 contained 0.2 µg GM27 DNA. Following fixation of DNA, as described in Methods, the sheet of paper was cut into two strips (a and b). Strip a was hybridized with 0.1 µg poly (A^+) 22 hr RNA (in vitro labeled to a specific activity of 1.2 X 10^7 cpm/µg); strip b was hybridized with the same RNA probe, in the presence of 0.6 mg unfractionated cytoplasmic RNA extracted from vegetative cells.

tent with RNA from growing and differentiating cells (data not shown); the other, GM27, hybridizes only to RNA from postaggregating cells. DNA from these clones individually (spots a1 and a4) or mixed together (lanes a2 and a3) all hybridize, as expected, to [^{32}P] RNA from 22 hr cells. Hybridization to GM5 DNA was totally blocked by competitor RNA from growing cells (spot b1) whilst hybridization to GM27 RNA was unaffected (spot b4). Hybridization in the presence of competitor to the artificial mixture of the two clones, was proportional to the amount of GM27 DNA spotted on the sheet (spots b2, b3). Thus, hybrids formed by the two DNAs can easily be resolved by competition.

A quantitative competition experiment, utilizing 10 new clones, is depicted in Figs. 4 and 5. It is based on the "annealing with mixed competitors" technique employed by Bolle, et al. (19) in a study of phage T4 gene expression. In our experiment, DNA purified from each recombinant plage lysate was spotted in 22 equal aliquots in an horizontal line on a sheet of nitrocellulose paper. The paper was cut in vertical strips, each containing 2 spots corresponding to each of the clones and the strips were hybridized separately with labeled RNA from 22 hr cells. The strip presented in line 1 was hybridized in the absence of competitor RNA. In lines 2 to 5 increasing amounts of un-

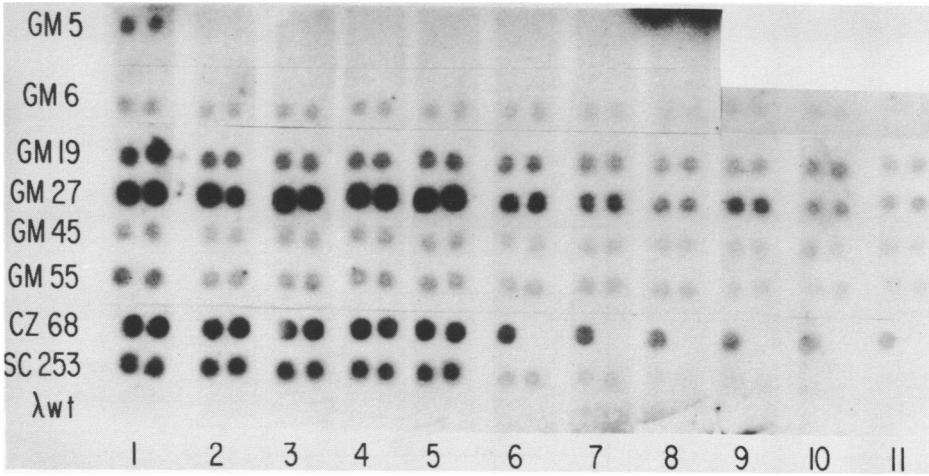

Figure 4. Analysis of cloned gene segments by hybridization-competition. DNA was extracted from some of the clones shown in Figs. 1-3, and from clone CZ68, isolated by a different procedure and shown to encode two different RNA's, one "constitutive" and the other specific for late development (S.C., C.Z. and H.L. manuscript in preparation). λ wild type DNA ($_{\eta}$wt) was a commercial preparation from Biolabs, Inc. Three μl aliquots of a solution (about 0.2 mg DNA per ml) were spotted on a nitrocellulose sheet. After fixation, the sheet of paper was cut in eleven vertical strips (labeled here with numbers 1 - 11), each containing duplicates of DNA from each clone. In the case of CZ68, only one spot of DNA was used for samples 6 - 11. Each strip was hybridized in a separate bag. All solutions contained 0.1 μg poly (A^+) RNA from 22 hr cells, labeled with γ [^{32}P] ATP and polynucleotide kinase (sp. act. 1.5 X 10^7 cmp/μg). To strip 1, no competitor RNA was added; to strip 2, 0.6 mg of total cytoplasmic RNA extracted from vegetative cells; to strips 3, 4, and 5, 1.2, 1.8 and 3.0 mg of the same unlabeled RNA were added, respectively. To strip 6, 7, and 8, was added 0.6 mg total cytoplasmic RNA from growing cells plus 0.6, 1.2 and 2.4 mg of total cytoplasmic RNA extracted from 13 hr cells, respectively. To strips 9, 10, and 11, 0.6 mg total cytoplasmic RNA from growing cells were added plus 0.6, 1.2 and 2.4 mg of total cytoplasmic RNA extracted from 22 hr cells.

labeled vegetative RNA were added. In lines 6 to 8, and in lines 9 to 11, the same amount of unlabeled vegetative RNA was added as in line 2, plus amounts of unlabeled RNA derived from cells at 13 hr of development (lines 6 to 8) or at 22 hr (lines 9 to 11) sufficient to bring the amount of total unlabeled RNA to the same levels as in lines 3 to 5, respectively.

The amount of label found in the hybrid formed in each set of conditions was determined both by scanning the film (Fig. 5) and by cutting

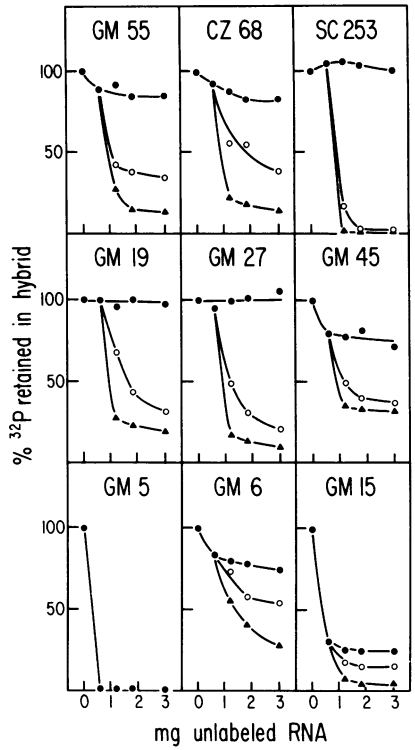

Figure 5. Quanitation of the autoradiograph shown in Fig. 4. The optical denisty of each spot is expressed as a percent of the intensity of the spot generated in the absence of competitor RNA. Different exposures of the radioautogram were scanned in a Joyce-Lobel microdensitometer using a full-scale pen diflection of 1.16 optical density units, a value within the linear range of the film.

Each strip was scanned twice. Two peaks were thus obtained for each spot, and four peaks for each doublet of spots (see Fig. 4). The height of the four peaks for each doublet was averaged. Normally the difference between the two values obtained for a given spot in the two scanning trials or between the two spots of a given doublet was less than 15%. Clone GM19 shows, in the absence of competitor RNA (lane 1) two spots which clearly differ in intensity. In previous trials hybridization to GM19 DNA showed little or no competition by unlabeled total vegetative mRNA, thus we have scanned only the least intense spot on the left side. The intensity of the background (poorly recognizable in the photographic reproduction in Fig. 4) surrounding this spot made the quantitation of its intensity difficult. Thus the 100% value for clone GM19 in this particular trial may be in error.

● plus various amounts of total cytoplasmic RNA from vegetative cells

o plus 0.6 mg of RNA from vegetative cells and various amounts of RNA from 13 hr cells (see legend to Fig. 4).

▲ plus 0.6 mg of RNA from vegetative cells and various amounts of RNA from 22 hr cells (see legend to Fig. 4).

and counting each doublet of spots in a scintillation spectrometer. The latter results are not shown, since they were very similar to those obtained by scanning.

The analysis of Figure 4 and 5 indicate that our selected clones fall into three distinct classes. Some (GM27, SC253; and perhaps GM19, see legend to Fig. 5)) form with the developmental RNA probe a hybrid which is fully resistant to competition by unlabeled vegetative RNA, but is sensitive to competition by RNA derived from 13 hr developing cells and even more by RNA derived from 22 hr cells. The simplest explanation is that clones of this class encode one or more mRNAs all of which are induced during development. Other clones, such as GM15, GM55, and CZ68 form hybrids with [^{32}P] developmental RNA in which two distinct components can be resolved. One is sensitive to competition by unlabeled vegetative RNA and the other is fully resistant, though sensitive to competition by unlabeled developmental RNA. These clones must encode at least two gene segments one of which is expressed only at a late stage of differentiation, and the other which is expressed at all stages. Finally, some cloned DNAs, such as GM6, and GM45, formed a hybrid which is more sensitive to competition by developmental RNA than by vegetative RNA. However the extent of sensitivity of these hybrids to competition by vegetative RNA could not be precisely determined, since no plateau was reached with the amounts of competitor RNA used. The simplest explanation is that clones of this class contain genes which encode mRNA molecules which are present in vegetative cells, but which accumulate to a much higher extent during development.

A second type of analysis has confirmed our interpretation of each of the three classes of cloned DNA segments (fig. 6). Here poly (A^+)-containing RNA isolated at five times of differentiation - growing cells and at 6 (preaggregation), 9 (early aggregation), 13 (tight aggregates) and 22 hours (culmination) of differentiation - were run in parallel lanes of a formaldehyde-agarose gel. The RNA was blotted to and immobilized on a sheet of nitrocellulose, and then hybridized to [^{32}P] labeled cloned DNA. (Note that the amount of RNA from growing cells is twice that from the other stages).

Clone GM27 hybridizes to one major mRNA species (3500 bases) and 2 or 3 minor ones. All of these are absent from vegetative and 6 hr cells, present in 13 hr cells, and present at a higher level in 22 hr cells. This is consistent with the results of Fig. 5, in which hybridi-

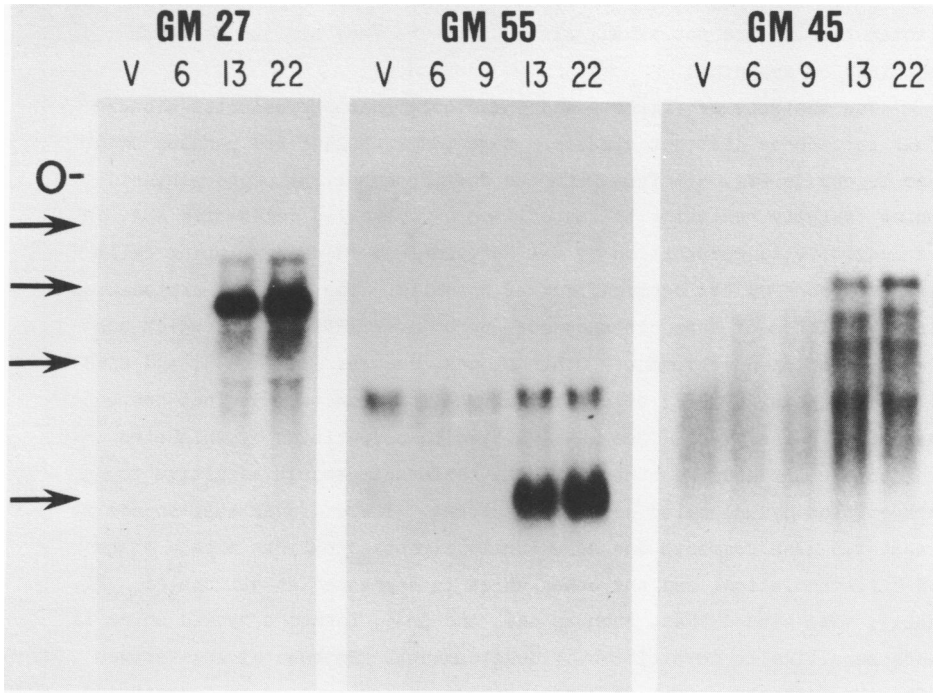

Figure 6. Accumulation during development of mRNAs complementary to some of the selected clones. Poly (A^+) RNA species extracted from growing cells (4 µg) and from cells at the indicated stages of development (2 µg) were separated by electrophoresis and blotted onto nitrocellulose paper as described in Methods. DNA was extracted from the indicated clones was labeled by nick-translation and hybridized to the nitrocellulose filter as described in Methods. The arrows correspondes to marker DNA (from top to bottom) of 9500, 4300, 2100 and 1300 bases. "O" indicates the origin.

zation of [^{32}P] 22 hr mRNA to GM27 DNA was not competed by unlabeled RNA from growing or 6 hr cells, and the ability of 13 hr RNA to compete was about half that of RNA from 22 hr cells. Clearly GM27 encodes several mRNAs which appear in cells only after aggregation. Similar results were obtained with clones GM19 and SC253 (data not shown).

Clone GM55, by contrast, encodes two discrete mRNA species. One (1800 bases) is present in more-or-less equal amounts throughout differentiation. The other (1200 bases) appears only after aggregation, and accumulates to a level about 10-fold that of the constitutive species. Clearly, these two mRNAs correspond to the two discrete components ob-

servered in the hybridization-competition study (Fig. 5). An analysis of clones CZ68 and GM15 yielded similar results (data not shown).

Clone GM45, a representative of the third class, encodes at least 4 discrete mRNA species. One of these (1800 bases) appears to be present throughout growth and differentiation, although its amount increases markedly between 9 and 13 hours. Three other, (2400, 3000 and 4200 bases) appear absent in growing cells, present at a low level in 6 and 9 hour cells, and at a higher level in post-aggregation cells. These results apppear consistent with the hybridization-competition curves in Fig. 5, but we have not attempted any quantitative comparison of the data for GM45 from Figs. 5 and 6.

DICUSSION

We have shown that plaque competition hybridization can be used to screen a library of Dictyostelium discoideum genomic DNA for genes expressed only or mainly during development. The same technique could be used to screen for groups of genes expressed specifically at any given stage of development, or differentially in different types of cells, such as spores or stalks (20), or sequentially during spore germination (21). Experiments along these lines are in progress.

In principle, this technique can be used to screen any library of cloned cDNAs on genomic DNAs for genes expressed only in a given condition during the life cycle of any cell or organism. Whenever a complex population of mRNAs is to be used as a probe to screen such a library, rather than a single isolated mRNA, the use of competition may offer several advantages over the conventional colony or plaque hybridization techniques introduced by Grunstein and Hogness (6) and Benton and Davis (7). The latter techniques are based on the difference in the intensity of the hybrids observed on replicate filters tested with different labeled probes. The introduction of competition (a) simplifies the screening procedure, since it can be done with a single filter hybridization test (see Fig. 1d and 2d); and thus (b) avoids the problem of unequal transfer of DNA onto the replicate filters, which may by itself influence the intensity of the hybridization signals; (c) reduces the problems of background hybridization, since the presence of a large excess of unlabeled RNA minimizes the non-specific sticking of the labeled probe to the filters; and (d) automatically eliminates hybridization to ribosomal DNA clones, which may give such intense spots after radioauto-

graphy that they obscure the analysis of other clones. Finally, with genomic DNA libraries, where each cloned fragment may contain more than one gene, the use of competition avoids the possibility that the appearance of an mRNA specific for one stage is compensated by the disappearance of another mRNA, and therefore that the clone goes undetected. It is significant to note that while about one-third of the random genomic DNA clones tested by plaque competition hybridization have been found to carry genes specific for development, only a few clones specific for late development, out of several thousand tested, could be detected by the conventional plaque hybridization technique using two [^{32}P] RNA probes (data not shown).

It should be noted, however, that the competition-hybridization technique would not allow detection of genes whose expression is modulated over a small range. These genes would be difficult to detect with any technique.

We have also shown (Figs 4 and 5) that hybridization-competition can be used to analyze complex gene segments which encode more than one mRNA molecule. In particular, it can determine the minimum number of gene segments encoded by the DNA, and the relative abundance of the transcripts from the different segments at various times during differentiation. Results obtained with this technique are confirmed by a different type of analysis, in which mRNA from different stages is separated by gel electrophoresis and hybridized *in situ* to labeled, cloned DNA (Fig. 6 and data not shown).

Our data have several implications for gene regulation in *Dictyostelium*. First, as mentioned above, a large fraction of the randomly chosen 3 KB genomic clones carry genes expressed only or mainly during development. This is in line with the great increase in the mRNA complexity detected during development of *Dictyostelium discoideum* by Blumberg and Lodish (4). We shall extend this analysis to a larger sample of clones to obtain a statistically reliable estimate of the number of genes expressed specifically during development.

Second, a significant fraction of cloned DNA fragments contain both "constitutive" genes and genes specific for development. This may suggest that the two kinds of genes are interspersed in *Dictyostelium* genome, rather than being grouped according to their specificity of expression. We are currently analysing additional clones to clarify this point.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Science Foundation and from the National Institutes of Health. G.M. held an EMBO Senior Fellowship; SC held a postdoctoral fellowship from the Damon Runyon-Walter Winchel Foundation; and CZ held a predoctoral fellowship from the Whitaker Health Sciences Fund. We thank Naomi Cohen for expert technical assistance.

[1]Permanent address: Cattedra di Biologia Generale per Medicina, University of Turin, Italy

REFERENCES

1. Maniatis, R., Kee, S.G., Efsratiadis, A. and Kafatos, F.G. (1976) Cell 8, 163-182.
2. McReynolds, L.A., Monahan, J.J., Bendure, D.W., Woo, S.L.C., Paddock, G.V., Salser, W., Dorson, J., Moses R.E. and O'Malley, B.W. (1977) J. Biol. Chem. 252, 1840-1843.
3. Chang, A.C.Y., Nunberg, J.H., Kaufman, R.J., Erlich, H.A., Schimke R.T. and Cohen, S.N. (1978) Nature 275, 617-624.
4. Blumberg, D.D. and Lodish, H.F. (1980) Devel. Biol., 78, 285-300.
5. Blumberg, D.D. and Lodish, H.F. (1980) Devel. Biol., 78, 268-284.
6. Grunstein, M. and Hogness, D.S. (1975) Proc. Natl. Acad. Sci. USA 72, 3961-3965.
7. Benton, N.D. and Davis, R.W. (1977) Science 196, 180-182.
8. Loomis Jr., W.F. (1971) Exp. Cell Res. 60, 285-289.
9. Alton, T.H. and Lodish H.F. (1977) Devel. Biol. 60, 180-260.
10. Leder, P., Tiemier, D. and Enquist, L. (1977) Science, 196, 175-177.
11. Maniatis, T., Hardison, R.C., Lacy, E., Laver, J., O'Connell, C., Quon, D., Sim, G.K. and Efstratiadis, A. (1978) Cell 15, 687-701.
12. Sternberg, N., Tiemeier, D. and Enquist, L. (1977) Gene 1, 255-280.
13. Williams, J.G. and Lloyd, M.M. (1979) J. Mol. Biol. 129, 19-35.
14. Rigby, P.W.J., Deckmann, M., Rhodes, C. and Berg, P. (1977) J. Mol. Biol. 113, 237-251.
15. Alwine, J.D., Kemp, D.J., and G.R. Stark (1977) Proc. Natl. Acad. Sci. USA 74, 5350-5354.
16. Thomas, P. (1980) Proc. Natl. Acad. Sci. USA. In the press.
17. Rave, N., Crkvenjakov, R. and Boedtker, J. (1979) Nucleic Acids Res. 6, 3559-3567.
18. Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.
19. Bolle, A., Epstein, R.H., Salser, W. and Geiduschek, E.P. (1968) J. Mol. Biol. 31, 325-338.
20. Colona, A. and Lodish, H.F. (1980) Devel. Biol., in press.
21. Dowbenko, D.J. and Ennis, H.L. (1980) Proc. Natl. Acad. Sci. USA 77, 1791-1795.