Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 8540–8544, December 1985
Developmental Biology

Genes selectively expressed in proliferating *Dictyostelium* amoebae

(cell proliferation/cloned cDNA/coordinated gene expression/slime mold)

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Communicated by J. T. Bonner, July 29, 1985

ABSTRACT **Few eukaryotic genes are expressed only during cell growth and division. We found that the slime mold *Dictyostelium discoideum* is unusual in that it expresses many genes only during proliferation. Thirty-two percent (304/950) of the sequences in a cDNA library made from vegetative mRNA were homologous to RNAs that are present at high levels during growth but at low or undetectable levels during differentiation when no cell growth occurs. *In vitro* translation assays confirmed that one-third of the vegetative cell mRNAs decreased in steady-state levels during differentiation. These vegetative cell-specific transcripts identified a diverse coordinately regulated class of genes: (*i*) 9 of the 10 cDNAs tested hybridized to unique small transcripts ranging from 400 to 620 bases long; (*ii*) the sequences showed various degrees of homology to related species; (*iii*) transcript levels synchronously fell by a factor of >20 during development and synchronously increased during germination. This class of genes may play important roles in normal cell proliferation.**

Although cell proliferation is undoubtedly a complex process requiring many gene products, relatively few of the corresponding genes have been identified. Homology to the RNA tumor virus oncogenes, which alter growth control, has led to the identification of ≈20 mammalian and avian genes that are believed to play a role in cell proliferation (1, 2).

Complementation of temperature-sensitive mutations that block cell division at specific cell cycle stages has been used in yeasts to identify proliferation-specific genes (3, 4).

These approaches using mutant viral or cellular genes and functional assays for their gene products permit only slow and, possibly, limited identification of more genes important for normal cell proliferation. Other cell proliferation-specific genes could perhaps be identified more easily by analyzing the genes differentially expressed in exponentially growing versus growth-arrested cells. However, in synchronized mammalian or yeast cells, few changes in gene expression have been correlated with progress through the cell-division cycle (5–9). These results might be different if the growing cells could naturally be induced into a long period of differentiation in the absence of growth so that levels of growth-related transcripts would diminish.

An interesting and useful feature of *Dictyostelium discoideum* is that the amoebae grow and divide only at the vegetative stage of the life cycle. While logarithmic growth occurs in the presence of sufficient food, starvation both suppresses the rate of cell proliferation and induces development. Developing amoebae aggregate to form a multicellular cell mass, and after 15 hr of starvation a migrating slug forms, within which only a few residual mitoses occur (10–13). Although mitotic activity ceases by 30 hr, these slugs are capable of migrating for several days before completing differentiation and forming dormant spores. Once activated, these spores will germinate and release amoebae that resume vegetative growth.

We assumed that nonproliferating amoebae in older slugs would lack many transcripts required solely for growth and division. cDNA clones corresponding to these vegetative-specific transcripts could be identified by using a cDNA clone bank prepared from vegetative mRNA and differential screening with vegetative and slug cDNA probes. This paper describes our use of a cDNA library from vegetative transcripts to identify a subset of mRNAs selectively expressed at high levels in these actively proliferating amoebae. Additional studies on the temporal regulation of specific mRNA levels and of homologous transcripts in related species support our view that this set of transcripts may be of general importance for cell proliferation.

MATERIALS AND METHODS

Growth and Development of Cells. Amoebae of *D. discoideum* strains DdB and V12M2, *Dictyostelium purpureum*, *Dictyostelium mucoroides*, and *Polysphondylium violaceum* were grown on *Aerobacter aerogenes* and harvested for development as described (14). *D. discoideum* strain AX-3 (15) was grown axenically in broth as described (16). Developmental conditions were according to Kopachik *et al.* (17) for collection of migrating slugs uncontaminated by amoebae of earlier developmental stages and according to Devine *et al.* (18) for harvesting spores washed free of contaminating amoebae and stalk cells.

Nucleic Acid Isolation. Total cellular RNA from amoebae was prepared according to Chirgwin *et al.* (19) by lysis in guanidinium thiocyanate (Fluka) and centrifugation through a cesium chloride cushion; for effective lysis, spore cells required, in addition, mechanical disruption in a French pressure cell. After ethanol precipitation, this RNA was suitable for RNA gel blotting analysis, but a further phenol/chloroform extraction, ethanol precipitation, and wash with 2 M LiCl was performed before poly(A)$^+$ RNA was selected or probe cDNA was prepared (20). The concentration of poly(A)$^+$ mRNA was estimated from quantitative hybridization to ^{3}H-labeled poly(U) (21) and corrected for an average transcript length of 1200, of which 60 bases are polyadenylic acid (22, 23).

Plasmid DNA was isolated by the EDTA/Triton X-100 method (24).

Construction and Screening of cDNA Library. The procedure of Okayama and Berg (25, 26) was followed in constructing plasmid primer cDNA recombinants using 1.8 μg of pcDV1 and 0.4 μg of poly(A)$^+$ mRNA from *D. discoideum* strain DdB as starting material. Cloning of plasmids in *Escherichia coli* strain DH1 followed Hanahan's (27) protocol. After establishing the clones in microtiter tray wells, the differential colony hybridization method of Williams and Lloyd (28) was used to identify cDNA plasmids exhibiting preferential hybridization to the vegetative cDNA probe.

To distinguish individual plasmids, the DNA was digested with the endonuclease *Taq* I (1 unit/μg) in a buffer (10 mM

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$MgCl_2$/50 mM NaCl/50 mM Tris·HCl, pH 8.0) at 65°C for 1 hr. The fragments were electrophoretically separated on a 1.2% agarose gel.

RNA Gel Blotting and Hybridization. Total denatured RNA was electrophoresed on 1.3% formaldehyde/agarose gels (20) and electroblotted onto GeneScreen Plus hybridization membranes (New England Nuclear) using the manufacturer's recommendations. After baking for 2 hr at 80°C, blots were incubated in hybridization buffer [50% (vol/vol) formamide/0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.9 mM NaCl/50 mM NaH_2PO_4, pH 7.4/5 mM EDTA/0.1% $NaDodSO_4$/sheared denatured herring sperm DNA (100 μg/ml)] at 42°C overnight. Nick-translated (29) plasmid probes (≈1 × 10^7 cpm/μg) were hybridized to the RNA blots in the hybridization buffer at a probe concentration of 100 ng/ml for 2 days at 42°C. Blots were washed three times in 2× SSC (0.3 M NaCl/0.03 M trisodium citrate) with 0.1% $NaDodSO_4$ for 10 min at room temperature and then twice in 1× SSC with 0.1% $NaDodSO_4$ for 1 hr at 65°C. Washed blots were visualized by autoradiography at −80°C with Kodak X-Omat film and Cronex intensifying screens.

***In Vitro* Translation and Two-Dimensional Gel Electrophoresis.** Total cellular RNA (10 μg) was translated in a rabbit reticulocyte cell-free system (Promega Biotec, Madison, WI) with [^{35}S]methionine (30). Samples were lyophilized and dissolved in 9.95 M urea/4% (vol/vol) Nonidet P-40/2% (vol/vol) LKB (pH 5–7) ampholytes/10 mM dithiothreitol. Two-dimensional gel electrophoresis was performed according to Garrels (31) and modified as described (17).

Enzymes and Reagents. Enzymes and reagents for cloning and analysis of plasmids were obtained from P-L Biochemicals. [α-^{32}P]dCTP (>3000 Ci/mmol; 1 Ci = 37 GBq) and [^{35}S]methionine (>600 Ci/mmol) were from Amersham. A nick-translation kit (8160BB, Bethesda Research Laboratories) was used to radioactively label plasmids. Avian myeloblastosis virus reverse transcriptase was the generous gift of J. Ross.

RESULTS

Cloning and Selection of Transcripts from Proliferating Amoebae. A cDNA library in the vector pcDV1 (26) was constructed from poly(A)$^+$-enriched RNA isolated from vegetative amoebae as described. An aliquot of the library was used to transform *E. coli* strain DH1 cells, and 950 ampicillin-resistant colonies were screened by differential colony hybridization (28). One set of filters was hybridized with a cDNA probe prepared from 25 μg of oligo(dT)-primed whole-cell RNA of vegetative amoebae and the other was hybridized with a cDNA probe prepared from whole-cell RNA of the nonproliferating slug cells harvested 24 hr after starvation. The autoradiographs of the filters were scored visually. We found that of the colonies with a positive hybridization signal 32% had a stronger hybridization to the cDNA prepared from vegetative amoebae. Of the remaining, 65% had an approximately equal hybridization, whereas only 3% showed a slightly stronger hybridization to the slug cell cDNA.

To confirm and further characterize the apparent specific and strong hybridization of some colonies to cDNA prepared from vegetative amoebae, we nick-translated nine plasmids (now termed pcD-D1 to pcD-D9) from 9 of the 304 vegetative-specific colonies. These were used as probes for hybridization to gel blots of RNA from vegetative or slug cells. As shown in Fig. 1, probes from pcD-D1, -D4, and -D7 each hybridized to a single species of RNA that was at least 20-fold more abundant in vegetative than in slug cells. The corresponding mRNAs were undetectable in an RNA blot of 10 μg of total slug RNA. We also examined about 5 times more slug mRNA by fractionating 2 μg of poly(A)$^+$-enriched mRNA on gel blots. The transcripts, as illustrated here for pcD-D6, were still undetectable in slug cells.

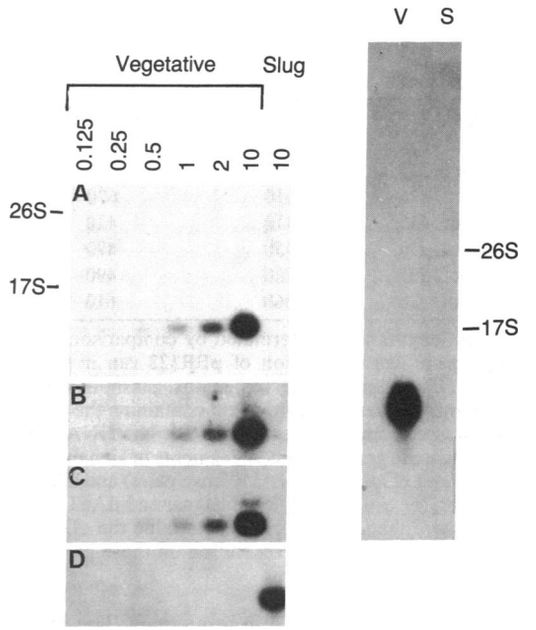

FIG. 1. RNA blot hybridization to plasmids from a vegetative-cell cDNA library. (*Left*) Total cellular RNA of bacterial-grown strain V12M2 amoebae (0.125, 0.25, 0.5, 1, 2, and 10 μg) and RNA from developing slug stage amoebae harvested after 24 hr of starvation (10 μg) was electrophoretically size-separated on formaldehyde agarose gels (1.3%) and electroblotted onto hybridization transfer membranes (GeneScreen Plus). The membrane was hybridized with nick-translated plasmid DNA, washed, and exposed to x-ray film as described. Hybridization by vegetative cell-specific cDNA plasmids is shown by pcD-D4 (*A*), pcD-D1 (*B*), and pcD-D7 (*C*). Hybridization by a nick-translated plasmid that contains a cDNA insert homologous to an abundant transcript of slug but not vegetative cells is shown in *D*. This probe was used as a control for RNA blot analysis to show that the slug-cell RNA was intact. (*Right*) Polyadenylylated RNA (2 μg) from vegetative (V) and slug (S) cells hybridized with pcD-D6.

cDNA Clone Characterization. We analyzed these plasmids after digestion with *Taq* I (Fig. 2) and found that 90% (9/10) of the cDNA inserts had different sets of fragments, indicat-

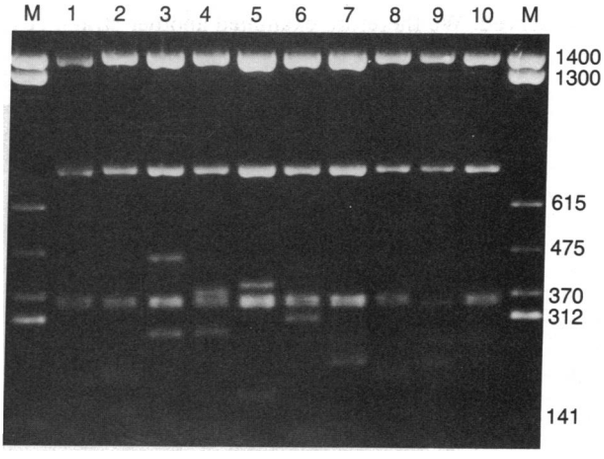

FIG. 2. Restriction enzyme digestion analysis of plasmids pcD-D1 to pcD-D9. Plasmid DNA was digested with *Taq* I and electrophoresed on 1.2% agarose gels. DNA size markers (M) were from *Taq* I-digested pBR322 DNA. Lanes: 1–7, pcD-D1 to -D7; 8, pcD-D2 separate isolate; 9 and 10, pcD-D9 and -D10. Three of the common vector fragments are ≈1400, ≈770, and ≈50 bases long.

Table 1. Characterization of cDNA plasmids and transcripts

Plasmid	cDNA size, base pairs	mRNA size, bases
pcD-D1	380	510
pcD-D2	410	450
pcD-D3	390	400
pcD-D4	610	620
pcD-D5	410	420
pcD-D6	450	470
pcD-D7	280	490
pcD-D8	360	610

Transcript lengths were determined by comparison to DNA size markers from a *Taq* I digestion of pBR322 run in formaldehyde agarose gels. To determine cDNA lengths, plasmids were digested with *Hpa* I and the size of the fragment containing the cDNA linkers and insert was determined in comparison to the DNA size markers run in 1.2% agarose gels. The linker segments of simian virus 40 early region DNA and dGdC bridge (≈150 base pairs) and simian virus 40 DNA carrying the polyadenylylation signal and dAdT bridge (≈200 base pairs) were then subtracted to determine the cDNA length to within ≈20 bases (the limit of resolution on these gels).

ing that the inserts were unique sequences. The cDNA insert and homologous transcript sizes were estimated (Table 1). All transcripts were smaller than the mean of 1200 bases for *Dictyostelium* (23). Since the Okayama and Berg vectors selectively clone full-length copies, small genes might be preferentially cloned. Alternatively, the vegetative-specific transcripts and corresponding translation products might be small.

***In Vitro* Translation Analysis.** We examined the products of *in vitro* translated vegetative and slug total cell RNA to estimate independently the number and sizes of vegetative-specific transcripts (Fig. 3). Among the most abundant proteins detected by two-dimensional polyacrylamide gel electrophoresis, 21 are vegetative-specific, 56 are shared, and 24 are slug-specific proteins. The percentage of vegetative-specific transcripts in vegetative RNA is 27% (21/77). This estimate compares favorably with our other estimate above, which was derived from screening a vegetative cDNA library. However, many high molecular weight vegetative-specific proteins were found, and the corresponding transcripts would not be small.

Homology of Sequences in Related Species. If these mRNA transcripts are required for an essential function during proliferation, then we might expect that the sequences would be conserved. We therefore examined another strain, AX3, and several other species of cellular slime molds for homologous sequences. RNA was isolated from both *D. discoideum* of bacterial grown (V12M2) and axenically grown cultures (AX3), *D. purpureum, D. mucoroides*, and *P. violaceum*. RNA blot analysis (Fig. 4) indicated that some sequences are more conserved than others at this moderate level of stringency (see *Materials and Methods*). For instance, plasmids pcD-D6 and D10 hybridize to a single band in all species, whereas pcD-D5 does not hybridize to mRNA of *D. mucoroides* or *P. violaceum*. As noted by Raper (32), there is evidence that among the species examined here *P. violaceum* is least related to *D. discoideum*. Thus, it would not be surprising to find sequence divergence and reduced hybridization to this species.

Decline of Transcript Levels in Developing Cells. Our aim was to correlate the levels of specific mRNAs with the occurrence of cell-proliferation transitions in the life cycle. As previously mentioned, cell proliferation is not immediately arrested upon starvation. In one study, Zada-Hames and Ashworth (12) found an 82% increase in total cell number from 0 hr to the first sign of aggregation at 8–9 hr. For this reason, we did not expect the levels of vegetative cell-specific mRNAs that are essential for proliferation to decline precipitously until 8–10 hr of development had passed. When we prepared RNA from developing cells at intervals after starvation and analyzed it on blots, we found that transcript levels were high at 0, 1, 2, and 4 hr. In contrast, they fell drastically by 8 hr and continued to decline to the undetectable levels characteristic of the slug-stage cells, which do not divide (Fig. 5).

Reappearance of Transcripts After Germination. A slug continues through development to form a fruiting body composed of inviable stalk cells and viable spores. These spores can be effectively germinated after a heat shock at 45°C (33) to give a highly synchronous population of emergent, feeding, and dividing amoebae. Germination begins within 1 hr after the 0.5-hr heat shock, and the release of amoebae occurs 3 hr after heat shock. We examined RNA from spores and germinating cells for the appearance of the vegetative cell-specific transcripts (Fig. 6). No pcD-D homologous transcripts were detected in spores or 1 hr after heat shock. However, by 2.5 hr after heat shock, all of the transcripts were present. The coincident appearance of these transcripts during the initial growth and emergence reinforces our belief that these transcripts are important for proliferation.

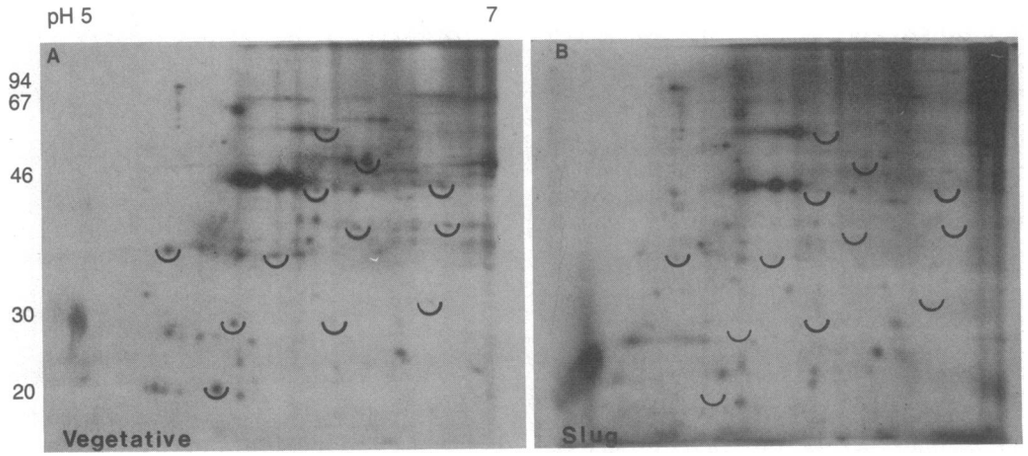

FIG. 3. Cell-free translation of vegetative- and slug-cell RNA. Total cellular RNA (10 μg) was translated in a reticulocyte lysate. The translated products that incorporated [^{35}S]methionine were resolved by two-dimensional gel electrophoresis and visualized by fluorography. Twelve of the 21 proteins preferentially translated from vegetative cell RNA are identified by a semicircle.

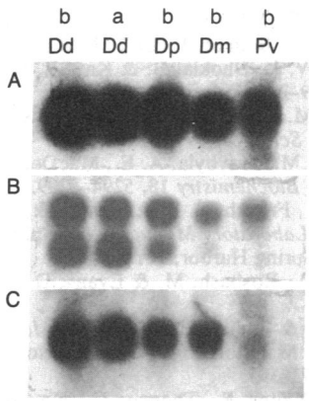

FIG. 4. Homologous transcripts in related slime mold species. RNA blot hybridizations were conducted as described in the legend to Fig. 1 by using 10 μg of total cellular RNA prepared from logarithmic-phase cells. Cells were grown axenically (a) or with bacteria (b). Species listed in increasing phylogenetic distance from *D. discoideum* (Dd) are *D. purpureum* (Dp), *D. mucoroides* (Dm), and *P. violaceum* (Pv). Probes are pcD-D6 (*A*), pcD-D5 (bottom) and pcD-D10 (top) (*B*), and pcD-D7 (*C*).

DISCUSSION

By taking advantage of the natural transition of *Dictyostelium* amoebae from proliferative to nonproliferative states, we identified mRNAs that are present only in proliferating cells. At present, we do not know if these genes are homologous to the few known proliferation-related genes of mammals and yeasts, or if they represent a novel set. This relationship can be elucidated by sequence analysis. The function of these genes, either in the cell cycle *per se* or for ancillary activities, such as phagocytosis, can be revealed by using mutagenic transformation to insert thermosensitive mutations into these genes (34, 35). These combined methods will permit more rapid analysis of the genetic requirements for proliferation.

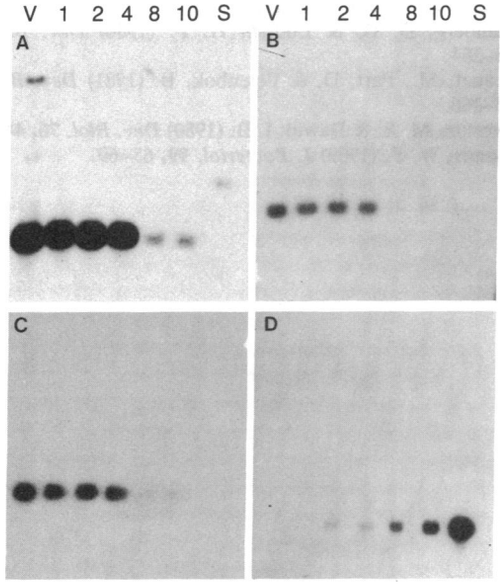

FIG. 5. Decline of transcript levels in developing cells. Amoebae of V12M2 were grown in association with bacteria, washed, and allowed to develop on buffered agar. At intervals, cells were collected and total cellular RNA was extracted. The procedures for RNA blot hybridization are described in the legend to Fig. 1. (*A–C*) Autoradiographs of identical RNA blots hybridized to nick-translated plasmids pcD-D6, pcD-D1, and pcD-D7, respectively. (*D*) Control hybridization (see Fig. 1) to show that some transcripts increase in abundance during development.

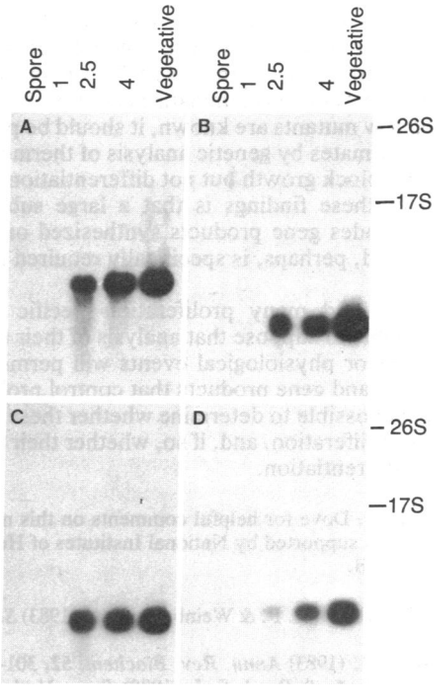

FIG. 6. Increase of transcript levels after germination of spores. Spores were heat-shocked to induce synchronous germination, and RNA was isolated at intervals after heat shock. The procedures for RNA blot hybridization are described in the legend to Fig. 1. Hybridization was to nick-translated plasmids of pcD-D4 (*A*), pcD-D3 (*B*), pcD-D2 (*C*), and pcD-D6 (*D*).

The alternative, collecting growth mutants to be analyzed by complementation with genomic libraries, is much slower.

Transcription of vegetative-specific genes appears to be coordinately regulated because the accumulated levels of all decrease during development and increase during germination with identical time courses. What controls the selective expression of the genes? A comparative structural sequence analysis will be necessary to determine whether there are common, possibly regulatory, sequences surrounding these coordinately expressed transcripts. Kimmel and Firtel (36, 37) have identified such a genomic sequence (M4-Band 4) repeated 50–100 times in the *Dictyostelium* genome that hybridizes to ≈1% of total vegetative poly(A)$^+$ RNA. However, the percentage of mRNA hybridization to M4-Band 4 dramatically increases instead of decreases during development; it seems unlikely, therefore, that M4-Band 4 plays any role in regulation of transcription of these proliferation-specific transcripts.

What proportion of the genes expressed during vegetative growth are selectively expressed only during this stage? There are consistent estimates from solution hybridization experiments that vegetative amoebae have a total of 4000–5000 discrete species of mRNA and 300–800 of these transcripts are present at >100 copies per cell (38–40). It is mainly these relatively abundant transcripts that are detected by the differential hybridization methods used here (7, 41). The percentage of colonies showing a higher hybridization to vegetative probe cDNA was 32%. Since we tend not to isolate a sequence repeatedly (Fig. 2), we estimate that 100–300 genes (1/3 of abundant transcripts) form a proliferative class of genes. The other genes common for both vegetative and developing cells we call "vital" because they are transcribed in all metabolically active cells.

Our estimate of the frequency of vegetative-specific genes was independently confirmed by *in vitro* translation assays in which the vegetative-specific gene products numbered 27%.

It should be noted that RNA excess solution hybridization studies capable of measuring even low abundance transcripts enabled Firtel to estimate that ≈10.5% of all vegetative sequences are not found in culminating cells (38). Finally, and although too few mutants are known, it should be possible to verify these estimates by genetic analysis of thermosensitive mutations that block growth but not differentiation (42). The implication of these findings is that a large subset of all transcripts encodes gene products synthesized only during proliferation and, perhaps, is specifically required for proliferation.

Having identified many proliferation-specific genes, it seems reasonable to suppose that analysis of their regulation by other genes or physiological events will permit identification of genes and gene products that control proliferation. It may also be possible to determine whether these genes are essential for proliferation, and, if so, whether their shut off is needed for differentiation.

We thank W. F. Dove for helpful comments on this manuscript. This research was supported by National Institutes of Health Grant GM33584 to S.L.B.

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