Starvation Phase of *Physarum polycephalum*: Characterization of Transfer Ribonucleic Acid

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We have begun a series of studies designed to characterize gene expression during differentiation in the slime mold *Physarum polycephalum*. This work concerns the starvation phase of the sporulation sequence and describes some of the quantitative changes which occur in plasmodial constituents during the 3-day starvation period and also describes alterations in the transfer ribonucleic acid (tRNA) population. The results show that whereas the plasmodial tRNA content decreased by 75% during starvation, concurrent de novo synthesis of tRNA also occurred, and they also show that overall amino acid acceptor activity of the starvation-phase tRNA population did not differ significantly from that found in the growth phase. Of the 19 starvation-phase tRNA families assayed, however, 6 were found to have consistently lower acceptor activities than did their growth-phase counterparts. Reverse-phase (RPC-5) chromatographic analysis of five of those families failed to reveal any major differences between growth- and starvation-phase isoacceptors. The data suggest that the depletion and resynthesis of tRNA during the starvation phase results in a quantitative alteration in the composition of the tRNA population and that the alteration is tRNA family and not tRNA isoacceptor specific.

For some time we have been interested in defining changes in gene expression during the life cycle of the myxomycete *Physarum polycephalum*. Initially, we used this biologically well-defined simple eucaryote to identify possible quantitative and qualitative changes in transfer ribonucleic acid (tRNA) during the naturally synchronous growth-phase mitotic cycle (14, 15). More recently, however, we have begun to study the starvation and differentiation phases of the organism. In the work presented here we have (i) partially redefined some of the quantitative changes which occur in plasmodial constituents as the organism proceeds through its starvation phase, (ii) determined that de novo synthesis of tRNA occurs during the starvation phase, and (iii) compared the abilities of growth-phase tRNA and starvation-phase tRNA to be aminoacylated.

MATERIALS AND METHODS

Maintenance of cultures. Surface cultures of *P. polycephalum* subline M₃CVIIG3 were grown as described previously (15, 16), except that glass beads were used as supports, and pans were replaced by large 15-cm glass petri dishes containing a top layer of Schleicher & Schuell 507 filter paper with an underlayer of Whatman 576 filter paper. Three milliliters of a 1:1 dilution of mid-log-phase microplasmodia was used as the inoculum, and all cultures were maintained at 26 ± 0.5°C.

The M₃CVIIG3 subline was chosen for these studies because it had been derived by sporulation of the original M₃CVII line (with which all of our previous data had been obtained) and because under our conditions it was found to be a consistently good sporulator, having an efficiency [i.e., (number of plasmodia sporulated/number of plasmodia starved) × 100] of greater than 95%. Spherules of M₃CVIIG2 were obtained from the McArdle Laboratory, Madison, Wis., germinated on agar plates, and established in shaker culture. We plated these microplasmodia, sporulated the resulting surface cultures, and isolated new plasmodia formed by fusion of the amoebae obtained from the germinated spores. These plasmodia (i.e., subline M₃CVIIG3) were established in shaker culture and used as the experimental material for these studies.

Sporulation procedure. Since relatively large amounts of starvation-phase material were required, the usual sporulation procedure, which called for plating microplasmodia on filter paper, allowing them to fuse, and then feeding them with sporulation medium (5), was abandoned. Instead, microplasmodia were plated on paper, allowed to fuse, and then fed with growth medium. After 24 h, the resulting surface plasmodia were transferred to plates containing sporulation medium. In this manner large amounts of material were obtained, since a 3-ml inoculum increased in mass approximately threefold in 24 h. Furthermore, we had the option, not used for this study, of beginning the starvation phase at known mitotic cycle points.

Analytical procedures. Dry-weight determinations of whole lyophilized plasmodia were obtained by replicate weighings; protein concentrations were measured by the technique of Lowry et al. (11), as de-

scribed by Mohberg and Rusch (17), and by the Bradford technique (2), and nucleic acid was quantitated by optical density (OD) measurement of total nucleic acid extracts prepared from lyophilized samples, as described by Melera and Rusch (16). The relative amounts of ribosomal RNA (rRNA) and tRNA were obtained by scanning cylindrical polyacrylamide gels at 265 nm after electrophoresis of total nucleic acid samples (16). The amounts of deoxyribonucleic acid (DNA) were also estimated from the polyacrylamide gels (13) and by diphenylamine determinations (3) from the total nucleic acid extracts. Purification of tRNA, partial purification of aminoacyl-tRNA synthetase, aminoacylation reactions, treatment of samples, and calculation of data for both growth- and starvation-phase experiments were all carried out as previously described by Melera and Rusch (15), with the exception that the *Physarum* tRNA^{Gln} used for reverse-phase chromatography (RPC-5) analysis was aminoacylated with rabbit reticulocyte synthetase as described by Hatfield et al. (8). Under these conditions, 10-fold-higher aminoacylation levels were obtained than had been possible with *Physarum* synthetase (D. Hatfield, M. Rice, C. A. Hession, and P. W. Melera, submitted for publication). Base composition analysis of [³H]uridine-labeled tRNA in the presence of 20 μ g of carrier RNA proceeded after digestion of the mixture for 4 h at 37°C with a combination of T₂, T₁, and pancreatic ribonucleases at 5, 1,000, and 200 U/ml, respectively. The resulting nucleotides were spotted on Whatman 3MM filter paper and separated by high-voltage electrophoresis at pH 5.3 as described by Barrell (1). Spots were located by absorption of ultraviolet light, cut out, and counted in Aquasol. Preparation of purified tRNA samples for analysis on reverse-phase chromatography columns was as described by Melera et al. (14), except the RPC-2 matrix was replaced by an RPC-5 matrix as developed by Pearson et al. (19).

Radioactive labeling of surface cultures. Labeling of surface plasmodia was accomplished by direct addition of radioactive precursors to the growth or sporulation medium. Since the starvation phase is carried out in the absence of light, the radioactive precursor(s) was introduced into the petri dishes while the cultures were kept in the dark or illuminated with blue light (Baird Atomic interference filter no. BI-3914), which had previously been shown to be incapable of triggering sporulation (4, 5; W. LeStourgeon, personal communication). Subsequent incubations were carried out in the dark at 26°C. Companion control cultures were routinely carried through these labeling procedures to insure that light induction, as measured by the triggering of sporulation, had not occurred.

RESULTS

The sporulation sequence of *Physarum* occurs in two phases, the starvation phase (defined as that period of time between the initial placement of the growing plasmodium on nonnutrient sporulation medium and the point at which the plasmodium becomes photosensitive, i.e., will form spores after the appropriate illumination

period [4, 5]) and the sporulation phase (defined as the time, approximately 12 to 14 h, between the end of the illumination period and the production of mature spore stalks and heads). Under our conditions, the starvation phase was maintained for a minimum of 72 h, at which time the plasmodia had achieved the photosensitive state.

Figures 1 and 2 show the quantitative changes which occurred on a per-plasmodium basis in plasmodial constituents during the starvation phase. The day 0 values for each component are shown in Table 1. Although results were generally similar to the data previously reported by Daniel (4), some interesting and possibly impor-

FIG. 1. Quantitative changes in plasmodial constituents during starvation. The values plotted represent the mean and standard deviation obtained from two experiments with a total of four to six determinations on each day. Separate experiments were required to obtain the protein, nucleic acid, and dry weight data, since the analytical procedure used for protein (17) was not consistent with the extraction of intact RNA. The techniques which allowed intact RNA to be extracted were also used for the dry weight determinations (16). Starting values in milligrams at day 0 were as follows: protein, 44.4 ± 0.35 ; dry weight, 245 ± 15.56 ; total nucleic acid, 9.17 ± 2.24 .

FIG. 2. Quantitative changes in nucleic acid species during starvation. The values plotted represent the mean and standard deviation obtained from two separate experiments with a total of four to six determinations on each day. The data were obtained as described in the text and by Melera and Rusch (16). Starting values in milligrams at day 0 were as follows: tRNA, 1.02 ± 0.38 ; rRNA, 7.35 ± 1.34 ; DNA, 0.43 ± 0.23 .

TABLE 1. Quantitative measurements of plasmodial constituents at day 0^a

Expt	Mean $\pm$ standard deviation (mg)					
	Protein	Dry wt	TNA ^b	rRNA	tRNA	DNA
1	44.2 $\pm$ 2.4	256 $\pm$ 1.41	10.95 $\pm$ 0.21	8.29 $\pm$ 0.63	1.29 $\pm$ 0.16	0.59 $\pm$ 0.07
2	44.7 $\pm$ 3.6	234 $\pm$ 6.66	7.58 $\pm$ 0.07	6.40 $\pm$ 0.17	0.75 $\pm$ 0.18	0.27 $\pm$ 0.04

^a Values were obtained from three separate determinations, as described in the text. Variations in nucleic acid levels are due to differences in mitotic cycle time at day 0.

^b TNA, Total nucleic acid.

tant differences were noted. The rate of depletion of total nucleic acid was rapid (Fig. 1) during the initial 24 h, with about 35% of the total nucleic acid being lost from the plasmodia. This represented a sevenfold-greater loss than that previously reported (4). Paralleling this rapid decrease in total nucleic acid, the dry weight of the organism fell by 40% during the same time period, whereas protein increased about 10% during the initial 24 h and did not begin to fall until after 48 h of starvation. This observation differed considerably from previously reported data (4) which indicated that protein levels decreased by about 20% during the initial 48 h of the starvation phase. Analysis of the amounts of RNA and DNA during this time (Fig. 2) showed that, as expected, the depletion of rRNA (which accounts for 80 to 85% of the total nucleic acid [16]) followed closely the depletion rate of total nucleic acid. Although perhaps decreasing somewhat more rapidly during the initial 24 h of starvation, tRNA diminished over the course of the starvation phase in a manner very similar to that of rRNA. Hence, at day 4 the concentrations of both rRNA and tRNA had decreased to approximately 25% of growth levels. The data concerning DNA depletion were not as clear because of the wide variation encountered due to differences in mitotic cycle time at day 0, but nevertheless indicated a decrease in DNA content to about 30% of growth levels by day 3. The suggestive increase in DNA content between days 3 and 4 was consistent with the putative mitosis occurring during this time (21).

Although the quantitative data of Fig. 2 demonstrated that a dramatic depletion of tRNA from the plasmodia had occurred during the starvation phase, previous results obtained by radiolabeling (7) had indicated that RNA synthesis also occurred during this time. To verify that de novo synthesis of tRNA took place during starvation, day 3 plasmodia were labeled for 4 h with [³H]uridine, and the nucleic acids were extracted and fractionated on polyacrylamide gels (Fig. 3) as described above. To insure that the radioactivity associated with the 4S region of the gel was, in fact, incorporated into tRNA, total nucleic acid labeled for 4 h with [³H]uridine

FIG. 3. Electrophoresis of [³H]uridine-labeled day 3 RNA. Day 3 plasmodia were incubated for 4 h in the dark with 20 μ Ci of [³H]uridine per ml as described in the text. Total nucleic acid was extracted, and the cytoplasmic-enriched "A" fraction was analyzed by electrophoresis on 2.8% polyacrylamide gels as described previously (16).

was prepared from day 3 plasmodia, and tRNA was prepared as described previously (15). The resulting purified tRNA had a specific activity of 1.4×10^4 cpm/ μ g, whereas the rRNA fraction had a specific activity of 2.1×10^3 cpm/ μ g. To demonstrate that the incorporation of radioactivity into the day 3 tRNA had resulted from de novo synthesis and not from CCA 3' end turnover or from modification of existing molecules, etc., [³H]uridine-labeled day 3 tRNA was subjected to base composition analysis. The results of these experiments clearly showed that the bulk of the radioactivity found in day 3 tRNA and originally supplied as [³H]uridine was present in uridine monophosphate, indicating that de novo synthesis of tRNA had taken place at day 3 (Table 2).

TABLE 2. Distribution of [^3H]uridine in day 3 tRNA^a

Nucleotide ^b	^3H cpm	% of total label
CMP	5,320	20.1
AMP	93	0.4
GMP	589	2.2
UMP	20,494	77.3

^a [^3H]uridine-labeled day 3 tRNA was purified and subjected to base composition analysis as described in the text. Approximately 5×10^4 cpm of tRNA was enzymatically hydrolyzed in the presence of carrier RNA and spotted on Whatman 3MM filter paper, and the nucleotides were separated by high-voltage electrophoresis. Only that radioactivity associated with the four major nucleotide spots which had been located by ultraviolet adsorption was measured.

^b CMP, Cytidine 3'-monophosphate; AMP, adenosine 3'-monophosphate; GMP, guanosine 3'-monophosphate; UMP, uridine 3'-monophosphate.

Further analysis of the OD and radioactivity profiles of Fig. 3 indicated that differences existed in the ratios of mature rRNA to tRNA. During growth, a 4-h labeling period similar to that shown in the data of Fig. 3 generated a radioactivity profile which was essentially identical to the OD profile (data not shown), with mature rRNA accounting for about 82% of the total counts and tRNA accounting for about 11% (16). When the relative amount of radioactivity incorporated into mature rRNA and tRNA at day 3 was determined, however, it was found that 61% was rRNA and 39% was tRNA, whereas the OD profile was essentially identical to that found in growth, i.e., 82% rRNA and 11% tRNA. Therefore, approximately three times more [^3H]uridine had been accumulated into day 3 tRNA during the 4-h labeling period than would have been expected from the growth data or from the OD profile of day 3 RNA. Although we do not have information concerning the absolute rates of synthesis and degradation, i.e., the turnover of rRNA and tRNA at day 3, the data of Fig. 2 and the OD profile of Fig. 3 show that the overall rates of depletion of rRNA and tRNA were similar during the starvation phase since the relative amounts of these two RNA species, i.e., 80% rRNA and 10% tRNA, did not change appreciably over the 3-day time course. Since the depletion of both RNA species was so extensive during the starvation period, it seems reasonable to suggest that the rates of degradation of both rRNA and tRNA are probably quite similar during this time and that the radioactivity profile of Fig. 3 indicates a relative enhancement of de novo tRNA synthesis, as compared with rRNA synthesis, at day 3. That this enhancement did not alter the OD profile of day 3 RNA indicates that the amount, i.e., mass, of

tRNA synthesized was not sufficient to alter the endogenous levels of tRNA present; i.e., little or no net synthesis of tRNA occurred.

The effects of the depletion and resynthesis of tRNA during the starvation phase were analyzed by a comparison of the amino acid acceptor activity of the day 4 tRNA population with that of the day 0 tRNA population. With the conversion factor 1 mg of tRNA = 22 units of absorbance at 260 nm and with the assumption that tRNA has an average molecular weight of 26,000, it was calculated (Table 3) that day 0 enzyme was able to acylate day 0 tRNA to 83% of its expected capacity, whereas the same enzyme acylated day 4 tRNA to 77% of its capacity. In both cases, these charging levels represented minimal values since cysteine levels had not been determined, and when compared with our previously reported values (15), the observed leucine charging levels were extraordinarily low. These results indicated that the total amino acid acceptance per OD unit of the tRNA population had been maintained during the starvation phase. However, of the 19 day 4 tRNA families assayed, 6, including arginine, glutamine, glycine, histidine, proline, and threonine, displayed acylation levels consistently lower than those of their day 0 controls (Table 3).

TABLE 3. Aminoacylation of growth-phase and starvation-phase *Physarum* tRNA

Amino acid	Mean $\pm$ standard deviation (pmol/OD unit) ^a		
	Growth tRNA + growth enzyme	Starvation tRNA + growth enzyme	Difference (%) ^b
Ala	83.4 $\pm$ 6.3	82.3 $\pm$ 5.4	-1.4
Arg	107.1 $\pm$ 7.5	86.4 $\pm$ 4.3	-19.3
Asn	63.8 $\pm$ 5.3	60.1 $\pm$ 8.4	-5.8
Asp	61.9 $\pm$ 7.3	68.5 $\pm$ 3.8	+10.6
Glu	78.4 $\pm$ 6.4	72.8 $\pm$ 6.8	-7.1
Gln	25.6 $\pm$ 4.7	12.3 $\pm$ 4.4	-52.0
Gly	113.7 $\pm$ 19.8	92.7 $\pm$ 12.7	-18.5
His	51.3 $\pm$ 7.8	44.1 $\pm$ 3.5	-14.0
Ile	86.0 $\pm$ 9.9	80.1 $\pm$ 2.9	-6.9
Leu	29.0 $\pm$ 1.0	27.9 $\pm$ 0.4	-3.8
Lys	115.7 $\pm$ 3.5	104.8 $\pm$ 3.7	-9.4
Met	96.1 $\pm$ 10.2	99.4 $\pm$ 8.2	+3.4
Phe	60.5 $\pm$ 4.0	64.4 $\pm$ 2.8	+6.4
Pro	73.3 $\pm$ 3.0	63.2 $\pm$ 1.0	-13.8
Ser	119.8 $\pm$ 8.2	129.8 $\pm$ 7.2	+8.3
Thr	88.9 $\pm$ 2.4	75.8 $\pm$ 0.9	-14.9
Trp	33.3 $\pm$ 4.2	33.2 $\pm$ 3.1	0
Tyr	25.7 $\pm$ 8.1	24.8 $\pm$ 8.3	-3.5
Val	130.8 $\pm$ 8.1	116.0 $\pm$ 4.5	-11.3
Total	1,444.3 $\pm$ 127.7	1,338.6 $\pm$ 92.3	

^a Values are for three separate determinations with two different enzyme and three different tRNA preparations. Data were obtained as described in the text.

^b Percent difference indicates the relative increase (+) or decrease (-) in the acylation levels for the 19 tRNA families analyzed from growth- and starvation-phase tRNA populations as indicated in footnote a.

To determine whether the quantitative alterations in the tRNA families noted above had been accompanied by similar quantitative or perhaps qualitative changes in the isoaccepting tRNA's of those families, cochromatography of

day 4 tRNA with day 0 tRNA was performed on RPC-5 columns. In four of the five families analyzed, day 4 tRNA was indistinguishable from day 0 tRNA (Fig. 4), indicating that changes to specific isoacceptors within a family could not

FIG. 4. RPC-5 chromatographic analysis of starvation-phase $tRNA^{Gly}$, $tRNA^{Pro}$, $tRNA^{Thr}$, and $tRNA^{Arg}$. Day 0 and 4 tRNA's were aminoacylated with [^{14}C]- and [3H]-labeled amino acids, respectively, with day 0 synthetase. After charging, appropriate amounts of each were mixed and prepared for reverse-phase chromatography as described previously (14). RPC-5 columns were developed with 0.4 to 0.8 M linear NaCl gradients at pH 4.5 (A) $tRNA^{Gly}$; (B) $tRNA^{Pro}$; (C) $tRNA^{Thr}$; (D) $tRNA^{Arg}$. Codon assignments where shown were taken from the data of Hatfield et al. (submitted for publication). $tRNA^{His}$ is not shown since only one histidine isoacceptor has been resolved (unpublished data). Day 0 tRNA, ^{14}C counts per minute; day 4 tRNA, 3H counts per minute.

explain the quantitative alterations noted from the acylation experiments (Table 3). Indeed, even in the case of the glutamine tRNA family, which did undergo some distributional changes during starvation (Fig. 5), the isoacceptor lost only accounted for 10% of the total glutamine tRNA and could not therefore have accounted for the observed 50% decrease in glutamine acceptor activity at day 4 (Table 3).

Taken together, the data presented in Table 3 and in Fig. 4 and 5 suggest that a quantitative alteration in the relative composition of the *Physarum* tRNA population occurs during the starvation phase but that the alteration takes place primarily at the level of tRNA families as a whole and not at the level of individual tRNA isoacceptors.

Since codon recognition properties have recently been assigned to several *Physarum* tRNA's (Hatfield et al., submitted for publication) as shown in Fig. 4 and 5, the point can also be made, at least for the tRNA families analyzed here, that the frequency of codon usage for a particular amino acid, as reflected by the presence of a specific tRNA isoacceptor, does not change during starvation. In fact, the glutamine isoacceptor which was apparently lost during starvation (Fig. 5) displayed the same codon recognition properties as did those of the major glutamine isoacceptor, suggesting that no diminution in the ability to decode glutamine code words had resulted from its loss.

FIG. 5. RPC-5 analysis of tRNA^{Gln}. Day 0 and 4 tRNA's were aminoacylated with [³H]glutamine (30 Ci/mmol) with rabbit reticulocyte synthetase. Each sample was chromatographed on a separate RPC-5 column and developed with a 0.475 to 0.625 M linear NaCl gradient at pH 4.5. (A) Day 0 tRNA^{Gln}; (B) day 4 tRNA^{Gln}. [³H]glutamine counts per minute.

DISCUSSION

Although described previously by Daniel (4) and Sauer et al. (21), our somewhat different conditions for initiating the *Physarum* sporulation sequence prompted us to redefine some of the more critical parameters pertinent to our interests. That the initial large losses in nucleic acid content and dry weight were accompanied by a net synthesis of protein (Fig. 1) during the first 24 h of starvation, instead of the previously reported decrease (4), may simply have reflected the possibility that a log-phase-growth surface plasmodium has more energy reserve, i.e., stored glycogen, lipid, etc., than does a similar mass of log-phase-growth microplasmodia. However, the net increase in protein indicates that protein synthesis persisted during the initiation of the starvation phase. Since it is reasonable to assume that some of the proteins necessary to initiate and maintain the starvation phase are synthesized at this time, the data obtained from the sporulation protocol presented here suggest that phase-specific protein synthesis may occur during the initial 24 h of starvation. Preliminary sodium dodecyl sulfate-polyacrylamide slab gel analysis of cytosol proteins taken after 24 h of starvation has indicated that both quantitative and qualitative alterations in protein synthesis do occur at this time (P. W. Melera and J. P. Davide, unpublished data).

When compared with the OD profile, the pulse-label profile of day 3 RNA (Fig. 3) suggests that the synthesis of tRNA might be enhanced over that of rRNA. This suggestion is based upon two observations: (i) the specific activity of tRNA synthesized at day 3 during a 4-h labeling period with [³H]uridine was seven times greater than the rRNA synthesized at that time, and (ii) the rates of depletion of both rRNA and tRNA (Fig. 2) were similar during this time. Although no direct information is available concerning the rates of RNA synthesis at day 3, the observations are consistent with a recent report which suggests that the activity of RNA polymerase I, the enzyme responsible in eucaryotes for the synthesis of rRNA (20), decreases during starvation (9). Presumably due to the accumulation of a specific inhibitor, the decrease in polymerase I activity could account for the observed relative decrease in [³H]uridine incorporation into rRNA at day 3. A relative increase in RNA polymerase III activity (the enzyme in eucaryotes responsible for the synthesis of tRNA [12, 25]) could also account for the data of Fig. 3; however, no measurements of its activity during the starvation phase have been made.

Fig. 2 and 3 and Table 2 show that, even

though a massive depletion of tRNA occurred during starvation, de novo synthesis of tRNA also took place. The consequence of this turnover was indicated by the data given in Table 3, which showed that a quantitative alteration in the tRNA population had resulted; i.e., six starvation-phase tRNA families showed consistently lower aminoacylation levels than did their growth-phase controls. Since RPC-5 chromatographic analysis of five of these families had shown that starvation-phase tRNA, with the exception of one minor glutamine isoacceptor, was indistinguishable from growth-phase tRNA, we conclude that the lowered acylation levels of the tRNA families in question resulted from their being present in lower amounts in the starvation-phase tRNA population.

The results described here are considerably different from those of Palatnik et al. (18), who reported that during starvation in *Dictyostelium* no quantitative changes in tRNA families occurred but that alterations in RPC-5 profiles did occur, presumably resulting from specific tRNA modification. Our results are, however, consistent with several recent reports (10, 22-24, 26) which have associated specific protein synthetic efficiency with the overall quantitative distribution of tRNA populations and not with the presence or absence of specific isoacceptors. Whether evidence can be obtained to suggest that the starvation-phase tRNA population of *Physarum* has undergone a "functional or quantitative adaption" (6) to specifically accommodate the synthesis of starvation-phase specific proteins remains to be determined.

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