cursors begins and continues for a time, t, at the end of which the replication front is at CD. Two cases must be considered: $t < t_1$ and $t > t_1$ (Figure 8). The radioactivity in fast and slow eluting DNA then corresponds to areas in Figure 8 as shown in Chart I.

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Analysis of Isoaccepting tRNAs during the Growth Phase Mitotic Cycle of *Physarum polycephalum*†

P. W. Melera,*,‡ C. Momeni, and H. P. Rusch

ABSTRACT: A reverse phase chromatography study of *Physarum polycephalum* isoaccepting tRNAs isolated during the growth phase mitotic cycle was undertaken. No significant quantitative or qualitative changes were noted in the 20 tRNA families during the mitotic cycle, although some question remains as to a possible quantitative change in the seryl tRNA population. These data combined with aminoacylation studies reported previously, which showed essentially complete quantitative stability in aminoacylation levels for 20 amino acids throughout the mitotic cycle (Merera, P. W., and Rusch, H. P. (1973), *Biochemistry 12,* 1307), strongly suggest that while under restrictive quantitative and qualitative control itself the possible involvement of tRNA in the active control of growth phase mitotic cycle events or growth phase protein synthesis appears to be minimal. The RPC-2 chromatograms revealed the presence of 44 isoaccepting tRNA species during the growth phase mitotic cycle. Single acceptors were found for seven amino acids, four of which, Asp, His, Ile, and Trp, are coded for by three or less codons, while the remaining three, Ala, Gly, and Val, are coded for by four codons. Only two isoacceptors were found for Leu.

The unique biology of the myxomycete *Physarum polycephalum, i.e.,* a naturally synchronous mitotic cycle during both growth phase and during starvation leading to differentiation, and the ability to maintain all stages of the life cycle in the laboratory (see Rusch, 1970, for a review), coupled with a general lack of information concerning transfer RNA metabolism during the eucaryotic mitotic cycle prompted us to undertake a study of tRNA in this organism.

Recently we reported the results of a quantitative study concerning the *in vitro* aminoacylation levels for 20 amino acids throughout the *Physarum* growth phase mitotic cycle (Melera and Rusch, 1973b). The results showed that during the cycle (1) the amount of tRNA per unit of total nucleic acid remained constant, (2) the level of *in vitro* aminoacylation for the total tRNA population remained constant, (3) the relative levels of *in vitro* aminoacylation for each of the 20 amino acids remained constant, and (4) the synthetase enzymes did not change in their ability to aminoacylate either homologous or heterologous tRNA. From these results it was suggested that a tight quantitative control on the amount of tRNA synthesized and aminoacylated was in effect during the mitotic cycle. It was mentioned, however, that constant *in vitro* aminoacylation levels could conceivably be maintained while considerable variations in isoacceptor profiles might be occurring. Such variations in isoacceptor profiles could be indicative of transcriptional changes in tRNA synthesis or post-transcriptional changes in tRNA maturation and/or modification at various mitotic cycle stages.

This report presents the results of a reverse phase chromatography study designed to characterize the isoaccepting tRNAs of *Physarum* during its growth phase and to compare isoaccepting profiles from early, mid, and late mitotic cycle times.

Materials and Methods

Maintenance of Cultures. Stationary cultures of *Physarum polycephalum* subline M_3C_7 were grown as described previously by Mohberg and Rusch (1969) with the slight modifications of Melera and Rusch (1973b).

Preparation of tRNA and Aminoacyl Synthetase. Purified tRNA shown to be undegraded and uncharged, to possess in-

† From the McArdle Laboratory for Cancer Research, Medical Center University of Wisconsin, Madison, Wisconsin 53706. *Received February 25, 1974.* This work was supported in part by Grants CA-07175 and CA-05002 from the National Cancer Institute.

‡ National Cancer Institute Training Grant Postdoctoral Fellow, 1969–1972. Present address: Sloan-Kettering Institute for Cancer Research, Walker Laboratory, Rye, New York 10580.

tact C-C-A termini, and to be free of detectable DNA, carbohydrate, and protein was prepared as described previously (Melera and Rusch, 1973b) from total nucleic acid preparations (Melera and Rusch, 1973a). Aminoacyl synthetase for 20 amino acids which had been shown to be nuclease free was prepared by Sephadex G-100 chromatography of 105,000*g* supernatants of whole mold homogenates (Melera and Rusch, 1973b).

Aminoacylation Reaction Conditions. All aminoacylation reactions were carried out with standard procedures in 0.5-ml reaction mixtures containing between 0.20 and 0.50 A_{260} unit of purified tRNA (Melera and Rusch, 1973b). The kinetics of each reaction were followed to ensure that the plateau level had been reached in the reaction time of 40 min.

Reverse Phase Chromatography. Aminoacyl tRNAs were isolated from 0.5-ml reaction mixtures by DEAE chromatography on minicolumns as described by Waters and Novelli (1971) and applied to a 1 × 240 cm jacketed glass column containing the RPC-2 freon packing of Weiss and Kelmers (1967). General procedures for RPC-2 chromatography were as outlined by Yang and Novelli (1971). Specific packing and column preparatory techniques were as described by Waters and Novelli (1971). Linear 2-l. gradients (0.35–0.80 M NaCl in freon buffer (0.1 M sodium acetate (pH 4.5), 0.01 M $MgCl_2$, 0.001 M Na_2EDTA, and 0.005 M β-mercaptoethanol)) were generated with an Isco Dialagrad at a flow rate of 80 ml/hr. The column temperature was maintained at 20°. Ten-milliliter samples were collected and, after adding 2 A_{260} units of DNA (Yang and Novelli, 1971), were precipitated by addition of 2 ml of cold 50% Cl_3CCOOH. After vortexing, the samples were held in ice for at least 30 min and the precipitate was then collected on 24-mm Millipore type MA filters, washed three times with 70% ethanol, dried, and counted in 10 ml of toluene scintillation fluid containing 8 g/l. of butyl PBD and 0.5 g/l. of PBBO.[1] Radioactivity was measured in a Packard scintillation spectrometer with appropriate settings for ^3H and ^{14}C discrimination. Recoveries from the RPC-2 columns varied with the aminoacyl tRNA in question and the particular column and packing batch used, but were always between 75 and 100%.

Of obvious importance in a study such as this is the resolving power and reliability of the RPC-2 column which is known to be dependent upon such things as the Chromosorb W batch used, the correct proportion of freon to tertiary amine, column temperature, column age, slope of the elution gradient, heavy metal contamination, SH group integrity of the tRNA molecules, wall effects of the glass column (which are overcome by silanization of the column before use), and, of course, the quality of the aminoacyl tRNA analyzed. These factors either wholly or in part have been described by others (Waters and Novelli, 1971; Yang and Novelli, 1971; Gallo and Pestka, 1970; Merrick and Dure, 1972; and Twardzik *et al.*, 1971), and suffice it here to say that we have considered each of these in the preparation of the data presented here.

In an attempt to verify isoacceptor profiles two procedures were carried out: (1) purified tRNA was heated to 85° for 5 min in low salt buffer, quickly cooled in ice, aminoacylated with the amino acid of choice, and then chromatographed against nonheated control tRNA; (2) the elution gradients were made more shallow, 0.35 M NaCl–0.60 M NaCl. These controls were not carried out for each of the 20 tRNA families, but in no cases tested were there any variations from the profiles of the tRNAs as presented here, except for some slight peak broadening when the shallow gradients were run. We feel that these results in light of our other precautions indicate that artifacts due to tRNA preparation or column technique were at a minimum in this study. The presence of artifactual peaks produced by degradation of tRNA during aminoacylation as described by Merrick and Dure (1972) although not tested for directly would have lead to excessive heterogeneity in the isoacceptor profiles, a problem not experienced here.

As a control on the performance of our columns we used *Escherichia coli* ^3H-labeled leucyl tRNA which was generously supplied by Dr. Larry Waters. Before use with *Physarum* aminoacyl tRNA, each RPC 2 column was first used to chromatograph the *E. coli* tRNA. If the five leucyl tRNAs could not be properly resolved, as shown in Waters and Novelli (1971), or if recoveries were low or broad peaks produced, the column was dismantled and repoured with new packing. This control was repeated after every fifth *Physarum* chromatogram.

Results

Changes in Isoacceptors during the Mitotic Cycle. Early cycle II tRNA (1 hr postmitosis) was aminoacylated with ^{14}C-labeled amino acid and homologous (early) synthetase while late cycle II tRNA (1–2 hr premitosis) was aminoacylated with ^3H-labeled amino acid and homologous (late) synthetase. Eighteen tRNA families were analyzed and the results of ten of these experiments are shown in Figure 1.

Essentially no differences in isoacceptors could be detected between the early and late cycle tRNAs (Figure 1). Indeed, in only one instance was any quantitative change indicated, and that was in the seryl tRNA profile. The initial observation was that $tRNA_{2.4}^{Ser}$ (isoacceptor peaks are numbers from left to right and do not necessarily correspond with others in the literature) apparently constituted a larger percentage of the late seryl tRNA population than it did of the early seryl tRNA population. Since the specific acceptor activity for serine remains constant over the mitotic cycle (Melera and Rusch, 1973b) the relative change in $tRNA_{2.4}^{Ser}$ was probably not due to a general decrease in the remainder of the seryl tRNA isoacceptors, but apparently represented a quantitative increase in $tRNA_{2.4}^{Ser}$. We have had problems, however, reproducing the overall serine profile and we have not investigated early and late tRNAs from other *Physarum* cycles. We feel, therefore, more evidence is required before the observed serine shift can be accurately interpreted.

In no other instances, including recently obtained data for the two remaining tRNA families, cystine and glutamine, were any significant quantitative changes in isoacceptors detected and in no case was there any indication of a qualitative change in isoacceptors (*i.e.*, the appearance or disappearance of a given isoacceptor at a specific time in the cycle) during the growth phase mitotic cycle. In addition, several mid-cycle tRNAs charged with mid-cycle enzyme gave isoacceptor profiles essentially identical with tRNA isolated from early and late cycle (data not shown). Heterologous charging, *i.e.*, late tRNA aminoacylated with early enzyme, early tRNA aminoacylated with late enzyme, etc., caused no changes in the isoacceptor profiles.

These data confirm and extend our original observations (Melera and Rusch, 1973b) on the stability of *Physarum* tRNA during the growth phase mitotic cycle. They also indicate that no changes in tRNA transcription, maturation, or modification, as these processes might effect the total number or relative amounts of isoacceptors per tRNA family, occur

[1] Abbreviations used are: PBD, 2-(4′-*tert*-butylphenyl)-5-(4′-biphenyl)-1,3,4-oxidiazole; PBBO, 2-(4′-biphenyl)-6-phenylbenzoxazole.

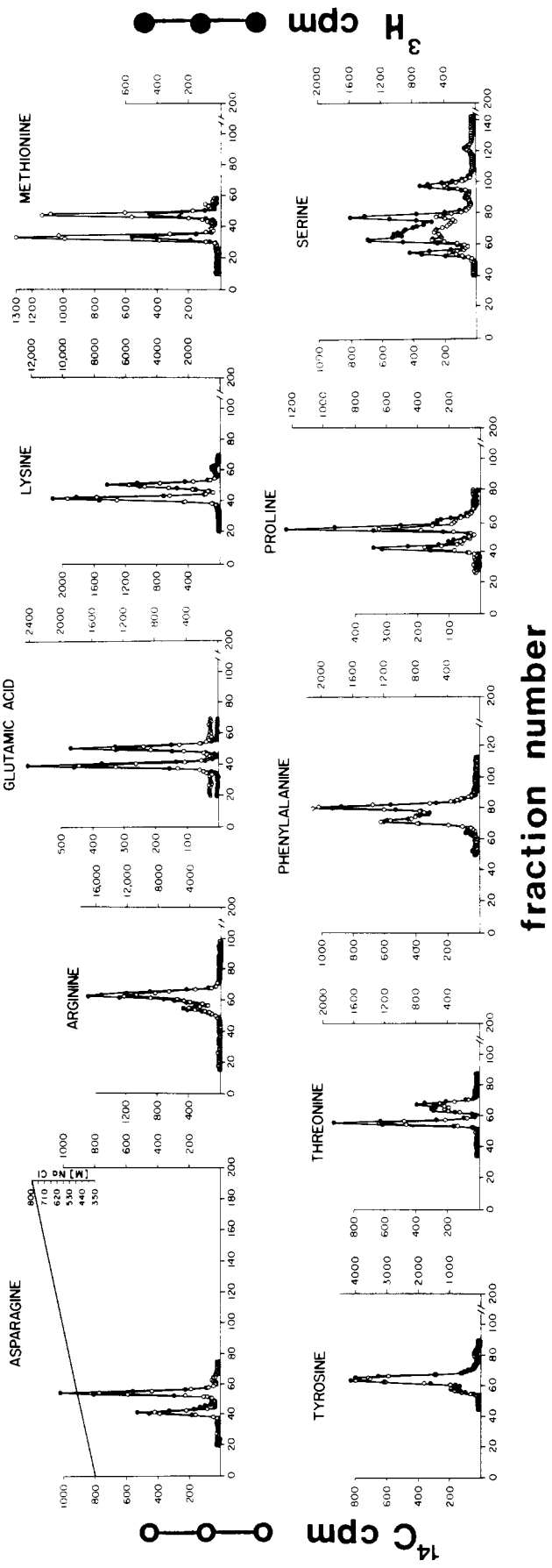

FIGURE 1: RPC-2 chromatograms of *Physarum* aminoacyl tRNAs. All columns were run at 20°. Linear 2-l. gradients (0.35 M NaCl–0.80 M NaCl) were run at a flow rate of 80 ml/hr. Further details are described under Materials and Methods: (O) early cycle tRNA; (●) late cycle tRNA.

during this phase of the *Physarum* life cycle. The data further suggest that the progression of *Physarum* through its growth phase mitotic cycle is not accompanied by the production of translationally functional mRNAs containing unique codewords requiring unique tRNAs for translation, or by the production of mRNAs with synonym codewords in varying proportions sufficient to warrant quantitative shifts in tRNA isoacceptors.

Characterization of Physarum Isoaccepting tRNAs. Under the conditions used in this study, we were able to identify isoacceptor tRNAs for 18 amino acids and recently (P. W. Melera, unpublished observations) for two more, cystine and glutamine. Considering all shoulders and small peaks to represent individual species, and allowing for 7 seryl tRNAs, the total number of isoacceptors for the 20 amino acids was 44; cystine and glutamine had 2 isoacceptors each. This number is considerably lower than the 56 isoacceptors reported for mouse plasma cell tumors (Yang and Novelli, 1971), normal and leukemic lymphocytes (Gallo and Pestka, 1970), and *E. coli* (Muench and Safille, 1968), but is in agreement with the recent results of Clarkson *et al.* (1973) who showed that *Xenopus* contains a minimum of 43 tRNAs as detected by molecular hybridization.

Under our conditions of preparation and chromatography, seven amino acids showed single acceptor profiles: Ala, Asp, Gly, His, Ile, Trp, and Val. Four of them, Asp, His, Ile, amd Trp, although of interest because of the isoacceptor heterogeneity found for these amino acids in other systems, were not suspect since there are no more than three codons for any one of them (Asp-2, His-2, Ile-3, and Trp-1) and the single acceptors, assuming wobble pairing (Crick, 1966), could conceivably translate all the assigned codons. The other three, however, Ala, Gly, and Val, were the basis of some question. Ala, Gly, and Val form a group of amino acids, each of which has four codons, all of which begin with G

$$\begin{matrix} \text{U} & & \text{U} & & \text{U} \\ \text{C} & & \text{C} & & \text{C} \\ \text{Ala-GC A,} & \text{Gly-GG A,} & & \text{and Val-GU A} \\ \text{G} & & \text{G} & & \text{G} \end{matrix}$$

This arrangement is suited for wobble pairing, wherein the single acceptor could translate three of the four possible codons. However, in strict accordance with the theory, one codon for each amino acid would remain untranslatable by the single acceptors (GCG, GGG, and GUG for Ala, Gly, and Val, respectively). A similar situation exists in *Physarum* with the amino acid Leu for which there are six codons

$$\begin{matrix} \text{U} \\ \text{C} \\ \text{CU A and UU} \begin{matrix} \text{A} \\ \text{G} \end{matrix} \\ \text{G} \end{matrix}$$

but only 2 isoacceptors. In this case, assuming wobble pairing, one of the isoacceptors could translate three of the four possible CU codons

$$\begin{matrix} \text{U} \\ \text{CUC} \\ \text{A} \end{matrix}$$

leaving CUG untranslatable, while the other isoacceptor could translate

$$\text{UU} \begin{matrix} \text{A} \\ \text{G} \end{matrix}$$

Although attempts thus far to resolve multiple isoacceptors for Ala, Gly, Leu, and Val by heating, or by shallow gradient elution (see Materials and Methods), have been negative, it is reasonable to assume that we have either failed to resolve all of

the isoacceptors for these amino acids or that the single acceptors we have found are capable of translating four codons instead of three as predicted by the "wobble hypothesis." Both possibilities must be given careful consideration since RPC-2 chromatography has been reported to give poor resolution for particular aminoacyl tRNAs (Yang and Novelli, 1971), while both valyl $tRNA_{Ia}$ and valyl $tRNA_{Ib}$ from rat liver have each been shown to recognize the four valine codons (Nishimura and Weinstein, 1969) when assayed by the ribosome–trinucleotide binding technique of Nirenberg and Leder (1964).

Discussion

We have attempted to establish the growth phase of *Physarum polycephalum* as a biological and biochemical base line against which changes in tRNA metabolism induced by starvation, differentiation, aging, etc. can be measured. We have shown that the amount of tRNA per milligram dry weight of tissue or per unit of total nucleic acid does not vary during growth and throughout the mitotic cycle, *i.e.*, the growth phase mitotic cycle (Melera and Rusch, 1973a,b). We have also established that the *Physarum* tRNA population as measured by total and relative *in vitro* aminoacylation levels for the 20 amino acids (Melera and Rusch, 1973b), as well as the isoacceptor tRNA profiles for the 20 tRNA families, remain essentially constant during the growth phase mitotic cycle. With the possible exception of the seryl tRNA profiles noted in this report and the elevated valine acceptor activity of late cycle tRNA (Melera and Rusch, 1973b) no indication of any fluctuation in the tRNA population has been detected and the probability for the involvement of tRNA as a controlling element in mitotic cycle events or general growth phase protein synthesis appears minimal. Since the overall tRNA population does remain so stable, it is suggested that the synthesis, maturation, and modification of tRNA remain constant throughout the cycle insofar as these parameters affect the amount of tRNA present, the level of *in vitro* aminoacylation, and the chromatographic elution profiles of the tRNAs themselves. Changes in the ability of tRNA to bind to ribosomes as a result of a specific modification (Gefter and Russell, 1969), which might not affect aminoacylation level or chromatographic profile but which could possibly affect the rate of protein synthesis or some similar modifications affecting translation, would not have been detected here, and obviously cannot be ruled out as control systems through which tRNA may act. It must also be pointed out that all our determinations are confined to the time frame of 1 hr in Melera and Rusch (1973b) and 3 hr in the present report. No data have been obtained for mitosis itself.

In terms of gene number Newlon *et al.* (1973) have reported that 0.004% of the growth phase *Physarum* genome is complimentary to *Physarum* tRNA. This corresponds to approximately 1000 tRNA genes per diploid *Physarum* genome and an average redundancy of 23 for each of the 44 isoaccepting *Physarum* tRNAs. Newlon *et al.*, (1973) also report that *Physarum* has 300 rRNA genes per diploid genome. These data compared with those of Hatlen and Attardi (1971) indicate that *Physarum* contains tRNA genes and rRNA genes in similar numbers and proportion (3.3:1) to yeast and *Drosophila*.

The detection of 44 isoaccepting tRNAs in *Physarum* combined with the recent data of Clarkson *et al.* (1973), which indicated the presence of 43 isoaccepting tRNAs in *Xenopus*, is of interest since both bacteria (Muench and Safille, 1968) and mammals (Yang and Novelli, 1971; Gallo and Pestka, 1970) have been shown to contain 56 isoacceptors. Unfortunately, a more complete list of the numbers of isoacceptors found in various organisms is not available, and attempts at retrieving comparable data from the literature have been unsuccessful due to considerable differences in preparation techniques, chromatographic systems, and controls performed. However, the data at hand raise some interesting questions concerning how many tRNAs are actually required for mRNA translation in both pro- and eucaryotes (Jukes, 1973).

Certainly, the participation of tRNA in processes other than translation is clear (Littauer and Inouye, 1973). Perhaps this participation is on a scale greater than generally thought suggesting the possibility that large numbers of tRNAs may not be required for *in vivo* protein synthesis and that many of the observed changes in isoacceptor profiles which occur under so many diverse biological conditions may have little to do with the translational control of protein synthesis.

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