JOURNAL OF BACTERIOLOGY, Nov. 1974, p. 872-879
Vol. 120, No. 2

Printed in U.S.A.

Nature of Ribonucleic Acid Synthesis During Early Sporulation in *Saccharomyces cerevisiae*

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Received for publication 22 August 1974

Phosphate uptake in sporulating cultures of *Saccharomyces cerevisiae* has been found to occur approximately 2 h after the transfer to sporulation medium. Early ribonucleic acid synthesis begins at approximately 4 h and continues to 8 h. Incorporation of phosphate into acid-extractable precursor pools parallels phosphate uptake. In triple-labeling experiments it was observed that the breakdown of vegetatively synthesized ribonucleic acid is not a significant source of precursors for ribonucleic acid synthesis during sporulation. The majority of the ribonucleic acid made in a 10-min period during sporulation does not migrate on gels with precursor or mature ribosomal ribonucleic acid.

Meiosis in the yeast *Saccharomyces cerevisiae* is characterized by an ordered sequence of biochemical and structural events. These have recently been reviewed by Tingle et al. (16). The study of events in the developmental process of sporulation in yeast is facilitated by the ability of yeast to undergo meiosis under nongrowth conditions. The synthesis of ribonucleic acid (RNA) as well as other macromolecules during ascospore formation has been investigated by Esposito et al. (3). A new stable 20S RNA species reported by Kadowaki and Halvorson (6, 7) was found to be made only during sporulation and subsequently identified as a ribosomal RNA (rRNA) precursor (14). It was shown that during sporulation the rate of processing of rRNA is markedly slower than during vegetative growth (14). The experiments reported here with [^{32}P]phosphate were conducted to examine in greater detail the appearance of RNA during meiosis and to analyze the specific RNA products. The results presented in this paper provide more direct evidence that: (i) RNA can be synthesized entirely from internal reserves; (ii) turnover (breakdown and resynthesis) of pre-existing RNA molecules is not a significant factor in providing precursors; (iii) early RNA synthesis is largely restricted to 4 to 8 h after the cells are placed in sporulation medium; (iv) the majority of RNA made is apparently nonribosomal in nature; and (v) phosphate uptake may provide a readily available assayable physiological marker for meiosis in yeast.

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MATERIALS AND METHODS

Organism and cultivation. A diploid strain of *S. cerevisiae*, D649, was used in these experiments. D649 has the genotype *a* MAL2 *trp1 pet6 ade2 lys2/α his4 leu2 thr4 mal adel*. Cells were grown in YEP (yeast extract-peptone medium) containing 2% dextrose as carbon source and transferred to sporulation medium at the end of the first period of growth on glucose (3). Sporulation medium contained 1% potassium acetate (KAc), pH 7 (11). Haploid strains of opposite mating types a *ade2*, *α trp4* were obtained by dissection after sporulation of D649 and were used for comparison purposes to measure [^{32}P]phosphate uptake in nonsporulating cells incubated in 1% KAc. Approximately 80% of the cells of strain D649 sporulate with the first asci appearing at 14 h. Sporulation is essentially complete by 48 h.

Labeling procedures. Vegetative cells were grown in YEP medium for several generations to 1 to 2 units at absorbancy of 260 nm (A_{260}) containing either [^{14}C]adenine or [^{3}H]adenine to label stable RNA species. Adenine pools were diluted by the addition of 100 μg of unlabeled adenine per ml approximately three generations before transfer to KAc medium. In some experiments, [^{32}P]phosphate was added at the end of vegetative growth before transfer to sporulation medium. Approximately 50% of the [^{32}P]phosphate was removed from the medium during this time. In other experiments, sporulating cells were briefly labeled for 10 min with 0.1 to 0.15 mCi of carrier free [^{32}P]phosphate per ml.

Phosphate uptake. Uptake of phosphate during sporulation was determined by addition of [^{32}P]phosphate to the medium. At intervals, samples were taken and the cells were removed by filtration. A sample of the filtrate was used to determine the [^{32}P]phosphate remaining in the culture.

Pool extraction. Ten milliliters of cells (5 A_{600} units) in KAc medium were centrifuged at 5000 × *g* at room temperature. The residual medium was re-

moved from the cell pellet with a Pasteur pipette. The cells were then suspended in 2 ml of 1 M formic acid and extracted for 60 min at 0 C. Cell debris was removed by centrifugation and the formic acid was removed by lyophilization. The resulting material was resuspended in water for further analysis. Chromatography of this fraction was accomplished by a two-dimensional electrophoretic technique (S. J. Sogin, B. L. A. Carter, and H. O. Halvorson, Exp. Cell Res., in press).

Isolation of RNA. The cells were broken in the French press as described by Bhargava and Halvorson (1) except that the homogenizing buffer contained 0.004 M tris(hydroxymethyl)aminomethane (pH 7.8), 0.5 M NaCl, 0.0015 M ethylenediaminetetraacetic acid, 1 M sorbitol, and 20% glycerin. RNA was isolated as previously described (14). Base ratios were determined on samples which had been treated with 1 μg of deoxyribonuclease I per ml in 0.1 M sodium acetate, pH 5.0, and 0.005 M $MgCl_2$ for 30 min at 4 C.

Polyacrylamide gel electrophoresis. Gels (3 or 4.5%; 9 mm diameter) were prepared by the method of Loening (8) and run at 10 mA per gel for 4.5 h (4.5% gel) or 12 h (3% gels) for analytical procedures. The gels were sliced into 1-mm sections with a Joyce Loebl gel slicer. For analytical runs, the slices were dried onto paper strips and counted by using toluene-based scintillation cocktail containing 4 g of 2,5-bis-[2-(5-*tert*-butylbenoxazoy)]-biophene per liter in a Beckman LS250 liquid scintillation spectrometer. Where ^3H was used, slices were dissolved with 0.5 ml of H_2O_2 at 80 C for 8 h. The dissolved slices were then counted in 10 ml of Bray's solution (2). In preparative procedures, the gel slices were placed in scintillation vials containing 1 ml of a buffer, 0.005 M ethylenediaminetetraacetic acid, pH 7.2, 0.1 M NaCl, and counted directly using Cerenkov emissions. Peak slices were pooled and an additional 1 ml of buffer were added (0.005 M ethylenediaminetetraacetic acid, pH 7.2, and 0.1 M NaCl). The slices were allowed to elute overnight in the cold with gentle agitation. Eighty-five to ninety percent of the ^{32}P counts were eluted in this fashion.

It is well known that in yeast [^{32}P]phosphate is incorporated into polyphosphate which is a common contaminent of isolated RNA (13). The use of polyacrylamide gel electrophoresis overcomes this difficulty since fractionation of large molecular weight RNA by this technique separates RNA from polyphosphate. Polyphosphate migrates at *S* values below 7*S* and none of the fractions in the 3% gel contained polyphosphate.

Determination of RNA base ratios. RNA was hydrolyzed in 0.2 N NaOH at 37 C for 18 h. The hydrolysate was spotted on Whatman 3MM chromatography paper and the paper was saturated with a buffer containing 0.5% pyridine and 5% acetic acid, pH 3.5 (12). The individual bases were separated by a Savant electrophoresis system at 5,000 V for 2 to 3 h. The radioactive areas were localized by autoradiography on the electrophoretogram, excised, and counted to determine the relative amount of radioactivity in each base.

Isolation and chromatography of tRNA. Transfer RNA (tRNA) was isolated by extraction of cells with phenol (5). The crude tRNA was purified by chromatography on DEAE cellulose (diethylaminoethyl-cellulose). The fractions eluting between 0.3 M and 1.0 M KCl were collected and precipitated with ethanol. The purified unfractionated tRNA was separated into isoacceptor species by chromatography on BD-cellulose (benzoylated-DEAE) as described by Gillam et al. (4).

Materials. Materials were obtained from the following: carrier free [^{32}P]phosphate and [^{32}P]phosphate, [^3H]adenine (15Ci/mmol), and [^{14}C]adenine (52.5 mCi/mmol) from New England Nuclear; unlabeled adenine from Aldrich Chemicals; 3MM paper from Whatman Paper Co.; BD-cellulose from Schwartz/Mann; and deoxyribonuclease from Worthington Biochemical Corporation.

RESULTS

To determine the optimal period for performing short-term labeling experiments, we examined the utilization of [^{32}P]phosphate from KAc medium (Fig. 1). The amount of phosphate remaining in KAc medium at various stages of sporulation was determined by removing the cells by filtration and measuring the radioactivity remaining in the filtrate. In the sporulating culture (D649) the cells take up phosphate from the medium only after a lag of 2.5 to 3 h (T_2 to T_3) and continue to T_6 to T_7 (Fig. 1). Although the D649 cultures do not completely exhaust the medium of phosphate, they take up approximately 80% of the phosphate added in the absence of carrier. It should be noted in this experiment that the exponential rate of phosphate uptake between T_3 and T_6 is unaffected by the pH fluctuations occurring during this period (3, 9).

For purposes of comparison, two haploid strains of opposite mating types (which do not sporulate) were obtained from the diploid D649 parent. Both haploid strains and the diploid strain were grown vegetatively and placed in sporulation medium containing [^{32}P]phosphate. The haploid strains, unlike the diploid, immediately removed phosphate from the medium and continued until phosphate was nearly exhausted (Fig. 1).

Precursor pools. Accurate measurements on rapidly labeled RNA required that the precursor pools were rapidly labeled. Since there was a delay in [^{32}P]phosphate uptake into RNA during the early phases of sporulation (Fig. 1), we examined the kinetics of labeling of the acid extractable pools in which nucleoside phosphate precursors to nucleic acid synthesis are found. The disappearance of labeled phosphate from the medium coincided with the appearance of label into the acid extractable fraction

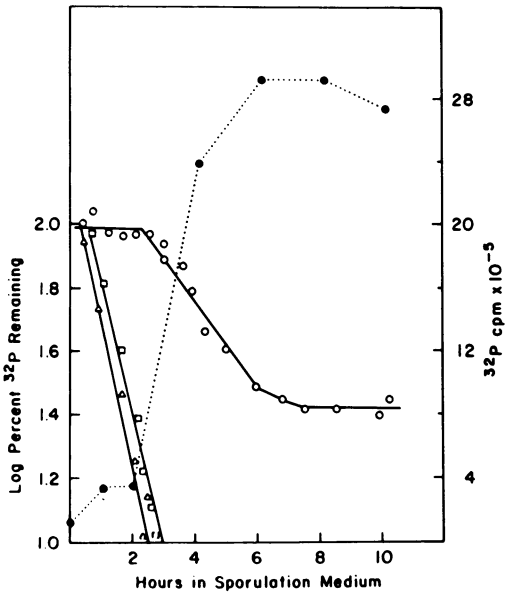

FIG. 1. *Uptake of [^{32}P]phosphate and accumulation into acid-extractable material during sporulation. Uptake of labeled [^{32}P]phosphate was determined as in Materials and Methods. 1 μCi of [^{32}P]phosphate per ml was added to each culture. (—O—) diploid D649 grown in YEP, (—Δ—) a ade$_2$, (—□—) α thr$_4$, (····●····) ^{32}P incorporated into acid extractable material.*

(Fig. 1). Fractionation of this material by a two-dimensional electrophoretic technique indicated that all mono-, di-, and trinucleotide phosphates were radioactive. Thus, if RNA were made during the period of phosphate uptake, then this RNA would be radioactive.

Short-term labeling. To examine RNA species which do not accumulate or which are not made continuously during sporulation, the following double-label experiment was performed. First, the cells were grown in medium containing [^{14}C]adenine, followed by the addition of an excess of unlabeled adenine (see Materials and Methods) to insure that the majority of the [^{14}C]adenine was incorporated into stable RNA, and then transferred to KAc sporulation medium. The sporulating cells were briefly labeled at various times by removing a sample of cells and adding [^{32}P]phosphate to the medium for 10 min. The RNA was then extracted from the labeled cells and the ^{32}P/^{14}C ratio was determined. The ^{14}C label provided an internal standard reference for the stable RNA made before sporulation. For this purpose the ^{14}C label was functionally equivalent to the optical density of the RNA synthesized vegetatively, whereas the ^{32}P-labeled RNA was a measure of the RNA made during sporulation and to a lesser extent of polyphosphate. We found that the polyphosphate component of the short-term label material remained at 15 to 20% of the total phosphate incorporation throughout the experimental period. This estimate was derived from the amount of inorganic phosphate appearing on the electrophoretogram of the material analyzed for base ratios. The ^{32}P/^{14}C ratio increased to a maximum at T_6 and then declined (Fig. 2). This change in ratio was in agreement with the restriction of RNA synthesis to a small portion (T_4 to T_8) of the early sporulation period as shown below and elsewhere (for review see 16). It should be noted that the rate of uptake of phosphate was identical at T_4, T_5, and T_6 and that the results at T_8 and T_{10} may be ameliorated by the decline in the rate of uptake of phosphate. An alternative interpretation of the ratio data would be a constant rate of RNA synthesis, accompanied by a breakdown of stable RNA. However, the total amount of RNA extracted from these cells increased during the course of the experiments, which is consistent with previously published results (3). Activity may have been lowered during the period in which no ^{32}P was incorporated into acid-extractable material by turnover (breakdown

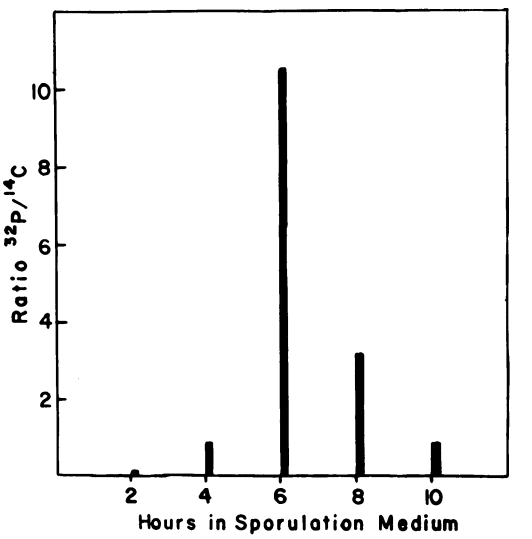

FIG. 2. *Timing of RNA synthesis. Fifty milliliters of vegetative cells were prelabeled with 15 μCi of [^{14}C]adenine (52.5 mCi/mmol) and then grown in the presence of excess unlabeled adenine to the end of glucose growth. The culture was then diluted 1:5 in KAc medium and 30-ml volumes of the sporulating culture were removed and labeled for 10 min with 5 mCi of [^{32}P]phosphate. The cells were poured over ice and RNA was isolated as described.*

and resynthesis). This question was treated in the manner described below.

Preferential use of internal reserves during early stages of sporulation. The possibility must be considered that in the early stages of sporulation (T_0 to $T_{2.5}$) internal phosphate and/or stable RNA species are utilized as precursors for de novo RNA synthesis since we did not detect incorporation of label into acid-extractable material during this time. To test this possibility, the following triple-labeled experiment was undertaken: cells were first grown in YEP medium containing [^{3}H]adenine to label vegetative RNA, and at an A_{600} of 1 unit, an excess of unlabeled adenine was added for three generations to eliminate ^{3}H from the precursor pool. After three generations of growth, [^{32}P]phosphate was added just before the stationary phase of growth to label the precursor pools as well as RNA made during this time. The culture was then transferred to the KAc sporulation medium. Finally, [^{32}P]phosphate was added at T_0 in KAc sporulation medium to follow the course of incorporation of precursor (labeled during sporulation) into RNA. RNA was extracted at various intervals and analyzed by polyacrylamide gel electrophoresis. Since this procedure separates polyphosphate from RNA, isotope incorporation can be used as a measure of RNA synthesis.

If there is a significant RNA synthesis in the period T_0 to $T_{2.5}$, then the ^{33}P/^{3}H ratio should increase. This prediction is based on the rationale that if [^{3}H]adenine-containing RNA is broken down, then the radioactive contribution to the adenine pool will be substantially lowered by the excess of unlabeled adenine which was added during vegetative growth. Therefore, any RNA made would have a lower ^{3}H specific activity by virtue of the fact that the pool dilution would be sufficient to make it unlikely that this RNA would contain a significant amount of radioactivity. In other words, in a situation in which an excess of unlabeled material is added, the specific activity of a stable synthesized component decreases as a function of the amount of synthesis of this component. This is true regardless of whether turnover is a factor in this synthesis. This situation is not the case for the [^{32}P]phosphate added during stationary phase. The ^{32}P isotope was not diluted by an excess of unlabeled material. If the [^{33}P]phosphate has equilibrated between the pools and the RNA then the specific activity of the [^{33}P]RNA would remain constant. If there is a disproportionation between the ^{33}P in the pool and the ^{33}P in stable RNA, then the ^{33}P specific activity would increase with de novo synthesis of RNA regardless of whether or not previously synthesized material was used for precursor. Under these conditions RNA synthesis would utilize pools chased in ^{3}H and unchased for ^{33}P, so that new RNA incorporates relative greater amounts of ^{33}P than ^{3}H such that in the event of synthesis the ^{33}P/^{3}H ratio must increase. This prediction is independent of the presence or absence of [^{32}P]phosphate in the KAc medium. If, however, the ^{32}P incorporated into RNA is, indeed, representative of the RNA synthesis occurring, then the ^{32}P/^{3}H ratio would increase at the same time as the ^{33}P/^{3}H ratio shows a significant increase.

This was the case as demonstrated in Fig. 3. As can be seen, little RNA synthesis occurs during T_0 to T_4 from either ^{33}P or ^{32}P. The increase in the ^{33}P/^{3}H ratio indicates that internal pools of phosphorylated precursors were present early in sporulation but not utilized until T_5.

Analysis of short-term labeled material. A more detailed insight into RNA synthesis during sporulation is obtained by examination by polyacrylamide gel electrophoresis of the RNA synthesized during short-term labeling. Vegetative cells were first grown in a medium containing [^{14}C]adenine, then grown for several generations in the presence of excess unlabeled adenine, washed, and then transferred to KAc

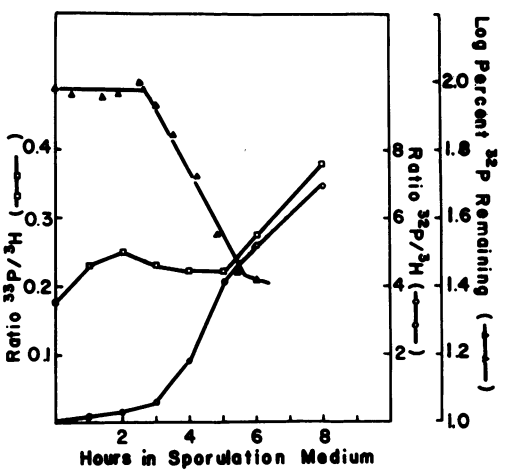

FIG. 3. *Incorporation of RNA precursors into RNA during sporulation. Vegetative cells (50 ml) were labeled with 400 μCi of [^{3}H]adenine (15 Ci/mmol) and then grown in the presence of unlabeled adenine. [^{32}P]phosphate (100 μCi) was added to stationary phase cells and uptake was followed to 50% depletion of isotope. The cells were transferred to sporulation medium and approximately 1 μCi of [^{32}P]phosphate per ml was added. The cells were harvested and RNA was extracted as described.*

sporulation medium. At intervals samples were exposed to [^{32}P]phosphate for 10 min and the RNA was extracted and separated on polyacrylamide gels. Three-percent gels were used to separate rRNAs and 4.5% to separate the smaller RNA species, 5.8S, 5S, and tRNA. The gels were sliced and measured for ^{14}C and ^{32}P radioactivity. The results for 3% gels are shown in Fig. 4 and 4.5% gels are shown in Fig. 5. As can be seen, the radioactivity from RNA made during vegetative growth is exclusively in 26S and 18S RNA species, the smaller 5.8S and 5S rRNAs and in tRNA.

The radioactivity profile for 10-min ^{32}P-labeled RNA differs significantly from that observed for vegetative cells (17). With the exception of the T_5 sample analyzed on 3% gels, no significant amount of rRNA precursor could be detected during a 10-min labeling during sporulation, whereas a significant amount of precursor rRNA was observed in vegetative cells in the same interval (13, 17). During sporulation, short-term labeling leads to a diffuse RNA pattern, particularly on the 4.5 gels. The majority of the ^{32}P migrates as RNA between 18S and 8S size species.

We have previously shown the rate of rRNA synthesis is markedly slower during the meiotic process. The observation that apparently no rRNA precursor accumulates after a 10-min period with the exception of T_5 may be that the rate of rRNA synthesis is sufficiently depressed that the heterogeneous RNA that we see masks the precursor synthesis.

The RNA products were further characterized by examining the base compositions of the various fractions. In Table 1, the base ratios for 10-min ^{32}P-labeled RNA are tabulated. The compositions are consistent with the exception of the sample taken at T_2 which is unusually high in uridine and low in cytosine. This may simply reflect the comparatively low rate of uptake at this time and indicate differences of either pool size or pool utilization during periods of minimal amounts of RNA synthesis.

RNA species synthesized during sporulation. The RNA profiles, demonstrated on the 4.5% acrylamide gels (Fig. 5), indicated that some tRNA was being synthesized during sporulation. To investigate if this represented synthesis of a single or limited class of tRNA isoacceptors or a general synthesis of tRNA species, tRNA was chromatographed on BD-cellulose columns. As described by Gillam et al. (4), chromatography of tRNA on BD-cellulose resolves tRNA isoacceptors although not achieving complete purification. For this experiment tRNA was labeled with ^{32}P for the first 12 h of sporulation. Unlabeled vegetative tRNA was added as an ultraviolet marker and the sample was chromatographed on BD-cellulose (Fig. 6). Comparison of the ultraviolet absorbence and ^{32}P profiles indicate that the tRNA

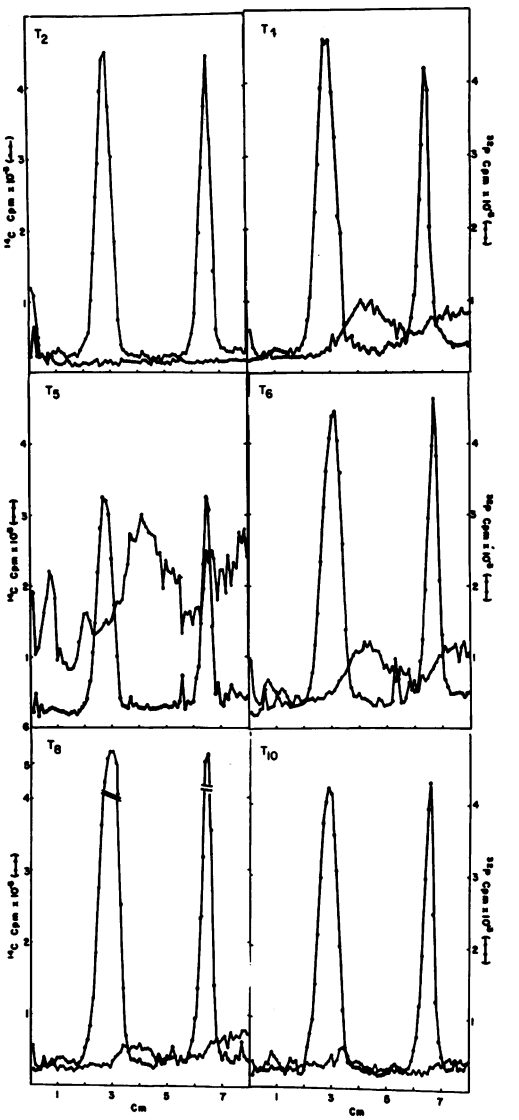

FIG. 4. *Analysis of sporulation RNA products on 3% polyacrylamide gels. A 65-ml YEP vegetative culture was labeled with 65 μCi of [^{14}C]adenine (52.5 mCi/mmol) and then grown in excess unlabeled adenine before stationary phase. 50-ml portions of the sporulating culture were labeled with 5 μCi of [^{32}P]phosphate for 10 min. The cells were poured over ice and harvested. The RNA was isolated as described above and analyzed on 3% polyacrylamide gels.* (○) *[^{14}C]adenine presporulation label in 26S and 18S rRNAs;* (●) *^{32}P 10-min labeled sporulation RNA.*

synthesized during sporulation is not restricted to a single or limited number of isoacceptors. However, the resolution of this procedure is not sufficient to determine if the differences observed in the profiles are due to loss of reduced synthesis of some species.

DISCUSSION

Although it has been known for a number of years that there is a large (50%) increase in bulk RNA during the first 10 h of sporulation in *S. cerevisiae*, lack of knowledge of uptake of RNA precursors, pool dilution, and RNA turnover have limited investigation of the problem. Firstly, since the chemical amount of ^{32}P and [^{32}P]phosphate added is very small in the experiments reported here, the labeling of RNA precursors can be accomplished without changing the cell's internal nucleotide pools.

We have been able to show in these double-labeling experiments that the maximal rate of phosphate uptake occurs in advance of the maximal rate of RNA synthesis. Further, RNA synthesis does not occur in the early period of sporulation by mechanisms of pool utilization or turnover of previously synthesized RNA molecules. These experiments do not rule out the possibility that short-lived RNA molecules are made and degraded in such a manner that the $^{32}P/^{3}H$ ratio remains unchanged. If the RNA synthesized in vegetative growth was degraded and de novo synthesized RNA, using vegetative RNA as a source of precursors, one would expect that the $^{32}P/^{3}H$ ratio would increase as discussed above for the three-label experiment (Fig. 2).

Phosphate uptake can serve as a useful biochemical marker during sporulation. The uptake of ^{32}P is very efficient, considering that the chemical amount of phosphate added is very small. The cells do not immediately take up phosphate when placed under the starvation conditions of KAc sporulation, but begin at approximately $T_{2.5}$. Uptake then continues at a

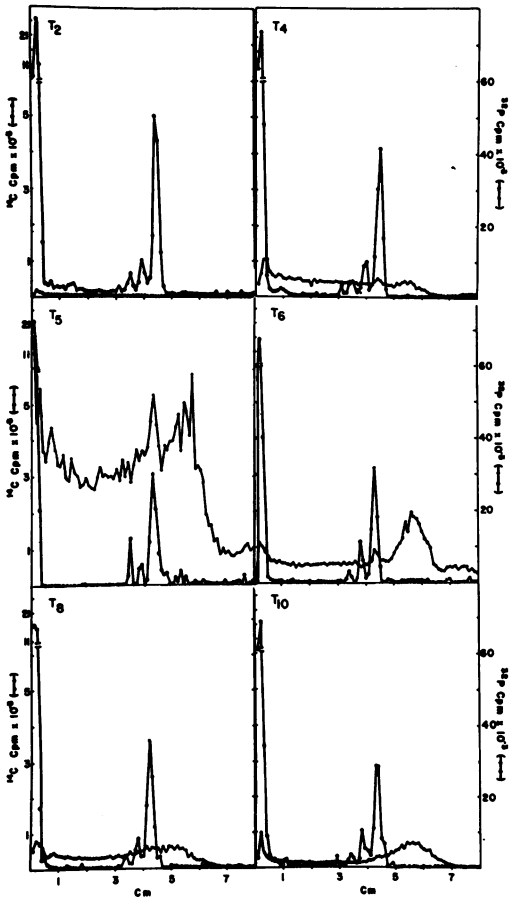

FIG. 5. *Analysis of sporulation RNA products on 4.5% polyacrylamide gels. See Fig. 6 for details. The ^{14}C label is primarily in 5.8S and 5S rRNAs and 4S tRNAs.*

TABLE 1. *Comparisons of base compositions of short-term labeled RNA*[a]

Sporulation medium (h)	Cytosine (%)	Adenine (%)	Guanine (%)	Uridine (%)
0				
2	12	22	27	39
4	21	23	25	30
6	22	24	26	28
8	23	24	26	29
10	21	23	29	27

[a] See Fig. 4 and 5 for details.

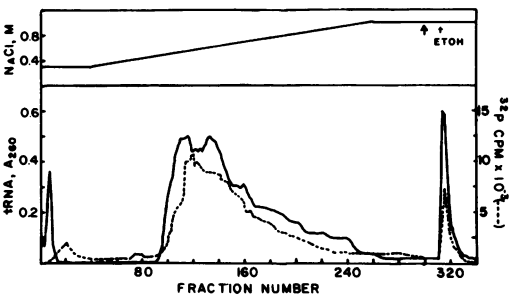

FIG. 6. *Chromatography of tRNA on BD-cellulose. A 300-ml sporulating culture was labeled with [^{32}P]phosphate at 10 μCi/ml for 12 h. The tRNA was prepared as described. Fifty A_{260} units of unlabeled vegetative tRNA was added to fractionated [^{32}P]tRNA and applied to a 1-ml (0.2 by 21 cm) BD-cellulose column and eluted with a 20-ml linear gradient of 0.3 M to 1.0 M NaCl, followed by an ethanol purge. (——) A_{260} (- - -) counts/min ^{32}P.*

maximal rate until approximately T_6, after which the rate declines and by T_8 to T_9 ceases. This is in agreement with previous observations on RNA synthesis (3, 9, 14). This impermeability to [^{32}P]phosphate was also evident in unsuccessful attempts to label DNA made after T_{10}. In contrast, haploid strains which cannot undergo meiosis take up phosphate immediately when placed under the same conditions, and continue until the medium is exhausted of phosphate. The uptake of phosphate of the diploid of this strain did not show a pH dependence. In fact, when the pH is optimal for adenine uptake (9), the cells have not yet started to take up phosphate. Once phosphate uptake begins it continues at maximal rate although the pH has risen to its final level before uptake diminishes.

The evidence presented here is a direct indication that significant RNA synthesis probably does not occur for the first 3 to 4 h after the cells are placed in sporulation medium. The synthesis of RNA which begins at T_4 reaches peak activity at T_5 to T_6. The RNA synthesis then apparently decreases and by T_{10} has dropped back to the T_4 level. Thus, RNA synthesis during sporulation begins at a definite point and may be completed nearly 5 h before the first appearance of asci and 30 h before the complete maturation of ascospores. The majority of protein synthesis begins before T_5 shortly after transfer to sporulation medium (T_0 to T_5) and continues until approximately T_{16} (3, 9, 15). The short-term labeled RNA which is synthesized during T_4 to T_8 is unstable and turns over as previously shown (4). The short-term labeled RNA, as analyzed on polyacrylamide gels, indicates that the majority of the RNA synthesized had *S* values between 16*S* and 8*S*. There is also significant but lesser amount of RNA made of higher molecular weight. The base ratios of material made at 4 and 6 h reveal that this RNA has base compositions unlike that of ribosomal RNA species (13). The bulk of the radioactivity was found to be in those regions of the gel profile not involved with rRNA, or rRNA precursor. Therefore, if the RNA made early in the sporulation process was mRNA, then a mechanism for discontinuity between transcription and translation is suggested. This system has already been found to hold during development in *D. discoidium* (S. J. Sogin et al., Exp. Cell Res., in press).

The pattern of elution from the BD-cellulose column has demonstrated that although most tRNAs are apparently present there may be some degree of difference in relative amounts. All isoacceptor groups appear to be represented although no attempt was made at quantitation. In *Neurospora crassa* for example, it was found that one of the leucine tRNA isoaccepting species was absent during the stationary phase of growth (9). If tRNA is likely to play a role in regulation, then it might be likely that a subtle difference in isoaccepting species levels is the determining factor.

ACKNOWLEDGMENTS

This investigation was supported by a Public Health Service grant AI-010610 from the National Institute of Allergy and Infectious Diseases (Harlyn O. Halvorson), a Research Scholar Award from the Massachusetts Cancer Society (Steven J. Sogin) and support for a postdoctoral traineeship on a Graduate Training in Developmental Biology grant 3T01-HD00022 from the National Institute of Child Health and Human Development (W. LaJean Chaffin).

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