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Novel Ribonucleic Acid Species Accumulated in the Dark in the Blue-Green Alga *Anacystis nidulans*

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In the dark, the obligately photoautotrophic blue-green alga *Anacystis nidulans* accumulates large relative amounts of two novel stable ribonucleic acid species (RNAs). These species are also made in illuminated cells but are unstable in them. When darkened cells are reilluminated, these RNAs are rapidly degraded; degradation is inhibited by chloramphenicol. Upon denaturation with heat or urea, one novel species (0.33×10^6 daltons) dissociates into two fragments that comigrate with the second novel species (0.16×10^6 daltons) on polyacrylamide gels. Both RNAs are associated with particles sedimenting between 30*S* and 50*S* through sucrose gradients and are removed from these particles at low magnesium concentration. The function(s) of these RNAs remains unknown.

Many blue-green algae (cyanobacteria) are obligate photoautotrophs, unable to increase in mass or number without light, even when supplied with glucose or other substrates. In their natural environment, such organisms are subjected to daily transitions between vigorous biosynthetic activity (in the light) and relative inactivity (at night). It is of interest to know whether these transitions, which are accompanied by changes in rates of net synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein (3, 4), also affect relative rates of accumulation of individual species of RNA and protein. In the present report we compare high-molecular-weight stable RNAs accumulated by the obligate photoautotroph *Anacystis nidulans* during rapid growth in the light and during incubation in the dark, when the growth rate is effectively zero. We find striking qualitative differences in the patterns of stable RNA in light and darkness. These can be attributed to differences in the stability, under these two conditions, of certain novel RNA species of unknown function.

MATERIALS AND METHODS

Organism and growth conditions. *A. nidulans* was originally obtained from J. Myers at the University of Texas. Conditions for maintenance of axenic cultures and growth in liquid medium were described previously (1, 2). At 37 to 39 C with air as the sole source of CO_2, generation times between 5 and 8 h were obtained. All experiments were performed with cultures in the early logarithmic phase (2×10^7 to 6×10^7 cells/ml).

Labeling. In all experiments, cells were "prelabeled" by exposure to [^{32}P]orthophosphate (New England Nuclear Corp.) at 1 μCi/ml for several hours in the light. This label was "chased" in the light for one or more generations after centrifugation and resuspension of the cells in fresh nonradioactive medium. Thus, all RNA preparations contained ^{32}P-labeled material corresponding to mature transfer RNA (tRNA) and ribosomal RNA (rRNA) and the two in vivo cleavage products of 23*S* rRNA previously identified (2). These served as molecular weight markers and internal controls for degradation of RNA during purification. Total ^{32}P radioactivity in high-molecular-weight RNA was also used in some experiments for normalization of ^{3}H radioactivity to unit recovery after extraction and gel electrophoresis.

[5,6-^{3}H]uracil (approximately 50 Ci/mmol; New England Nuclear Corp.) was used at 1 to 5 μCi/ml without added nonradioactive carrier. *A. nidulans* does not take up nucleic acid precursors readily (1, 12), and only a small fraction of the added label was assimilated in any experiment. Rates of uracil uptake appear to be independent of uracil concentration (unpublished data), and thus nucleic acids are most effectively labeled by the present procedure.

Lysis. Lysis was effected by one of two procedures.

(i) Lysozyme-EDTA treatment (1, 2). Cultures were harvested by centrifugation and resuspended in 0.1 volume of 0.03 M potassium phosphate buffer (pH 6.8) containing 0.5 M mannitol, 0.001 M sodium ethylenediaminetetraacetate (EDTA), and 0.5 mg of lysozyme (Worthington Biochemical Corp.) per ml. After 1 h at 37 C (in darkness), treated cells were centrifuged and either immediately lysed or resuspended in growth medium supplemented with 0.5 M mannitol and 0.1% sodium bicarbonate for further incubation and experimental manipulation before lysis. Lysis in either case was effected by resuspension

of a cell pellet in ice-cold distilled water containing 25 μg of deoxyribonuclease (ribonuclease free; Worthington Biochemical Corp.) per ml and the addition of sodium dodecyl sulfate (SDS) to 1.0%.

(ii) Lysis in a French pressure cell. Harvested cells were resuspended in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride-0.001 M $MgCl_2$ (pH 7.3), and lysed in a French pressure cell (American Instrument Co.) at 1,100 kg/cm².

When routine gel electrophoresis is performed in the absence of magnesium ions, as in the experiments reported here, the mobilities and relative abundances of all of the RNA species described are the same whether lysis is achieved by osmotic shock after lysozyme-EDTA treatment or by use of the French pressure cell with untreated cells suspended in buffer containing either magnesium ions or EDTA. We do observe, as have others (11, 13, 14), that the two fragments of 23*S* rRNA produced by post-maturational cleavage (2) remain associated only when lysis, RNA extraction, and electrophoresis are all performed in the presence of magnesium ions.

RNA extraction and polyacrylamide gel electrophoresis. RNA was extracted from 3-ml lysates obtained by either of the above procedures as described previously (1, 2) and subjected to electrophoresis on 2.8% polyacrylamide gels in E buffer (0.04 M Tris-hydrochloride, 0.02 M sodium acetate, and 0.001 M EDTA, made pH 7.2 with acetic acid) containing 0.1% SDS (1, 2, 10). Gels were sliced, digested with 30% hydrogen peroxide, and counted in Aquasol (New England Nuclear Corp.).

Sucrose density gradient centrifugation. Cultures labeled in the dark as described above were harvested, resuspended in 0.005 M Tris-hydrochloride, 0.01 M $MgSO_4$, and 0.06 M KCl (pH 7.3), and lysed in the French pressure cell. After centrifugation at 15,000 × *g* for 10 min to sediment unbroken cells and large debris, 2.0 ml of supernatant fluid was layered over a linear 15 to 30% sucrose gradient made in lysis buffer and centrifuged 7.5 h at 24,000 rpm in a Spinco SW25.1 rotor. Five-drop fractions were collected through a pinhole in the bottom of the gradient tube. The pellet was resuspended in 0.4 ml of lysis buffer. Samples (50 μliters) from each fraction and from the resuspended pellet were precipitated with ice-cold 5% trichloroacetic acid, collected on glass-fiber filters, and counted in toluene-base scintillation fluid. Selected fractions were pooled and made up to 2.4 ml with 0.01 M Tris-hydrochloride-0.001 M EDTA (pH 7.3), and RNA was extracted with phenol in the presence of 0.1% SDS and 0.02 M EDTA (1, 2).

RESULTS

RNA synthesis in the light and the dark. Kinetics of RNA synthesis in an illuminated culture are illustrated in Fig. 1. In addition to peaks representing known mature RNA species (23*S*, 1.05 × 10⁶ daltons; 16*S*, 0.55 × 10⁶ daltons [1, 6]) and a 16*S* rRNA precursor ("p16," 0.68 × 10⁶ daltons [1, 13, 14]), these gel profiles show peaks corresponding to RNAs of 0.45, 0.33 (peak A), and 0.16 (peak B) × 10⁶ daltons. These latter species are prominent only early in the labeling period and are absent from the "chased" material, indicating that they are less

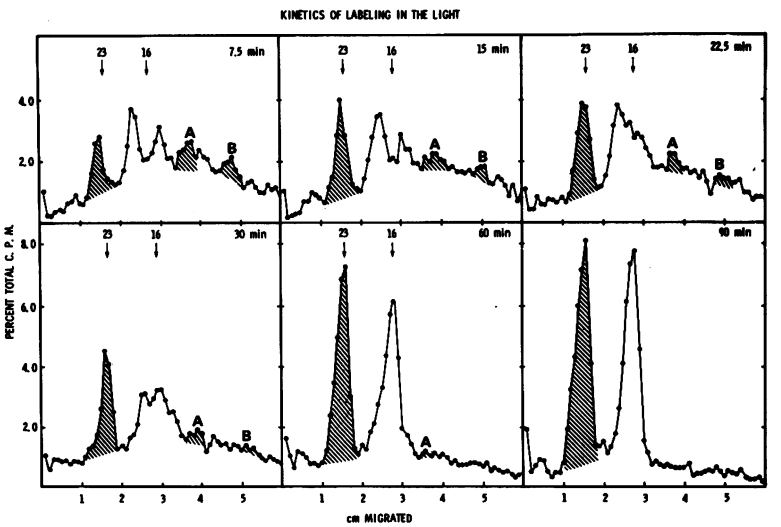

FIG. 1. *Kinetics of RNA labeling in the light. A logarithmically growing culture was exposed at zero time to 2 μCi of [³H]uracil/ml. Samples were removed at 7.5, 15, 22.5, and 30 min after the addition of label. At 30 min, nonradioactive uracil was added to 100 μg/ml, and further samples were taken at 60 and 90 min, this "chase" resulting in a 10-fold reduction in the rate of label incorporation. RNA extracted after lysis in a French pressure cell was resolved on 2.8% polyacrylamide gels. Positions of ³²P-"prelabeled" 23S and 16S rRNA present in each sample are indicated by arrows.*

stable than 23*S* and 16*S* rRNA in the illuminated culture.

Figure 2 presents the results of an experiment identical to that shown in Fig. 1, except that light was excluded from the culture 3 h before the addition of [^{3}H]uracil, and all subsequent steps were performed in darkness. (Exclusion of light results in an approximately 10-fold reduction in the rate of uracil incorporation in this obligately photoautotrophic organism.) The three novel RNA species were again detected, and two (those of 0.33×10^6 and 0.16×10^6 dalton) constituted an increasing fraction of total labeled material present at later times in the dark and thus can be considered stable RNAs. (All species are sensitive to ribonuclease in vitro.) The results of Fig. 1 and 2 are compared quantitatively in Fig. 3, in which relative radioactivity in 23*S* rRNA, 0.33×10^6 dalton, and 0.16×10^6 dalton material is plotted as a function of labeling time. (Total radioactivity after background subtraction corresponds to hatched areas in Fig. 1 and 2.)

In all experiments of this type, cultures had been "prelabeled" with [^{32}P]orthophosphate in the light and "chased" for several generations in nonradioactive medium before [^{3}H]uracil addition, to provide internal molecular weight markers. In no case was ^{32}P-labeled material observed migrating with the RNAs of 0.33 and 0.16×10^6 daltons. Thus, if these novel species are degradation products of rRNA, only newly synthesized rRNA is subject to such degradation.

Stability, upon subsequent illumination, of RNA labeled in darkness. We anticipated that RNA of 0.33×10^6 and 0.16×10^6 daltons accumulated in darkness might become unstable

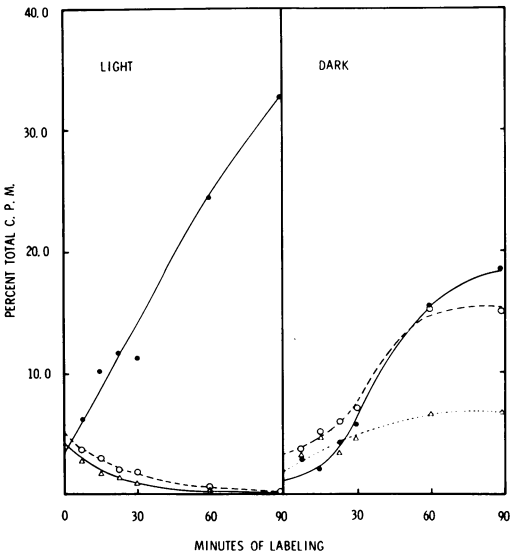

FIG. 3. *Comparison of RNA accumulation in light and darkness. Total radioactivities in regions of the gels of Fig. 1 and 2 that corresponded to 23S rRNA (●), 0.33 × 10^6 dalton RNA (○), and 0.16 × 10^6 dalton RNA (△) were determined by summation after subtraction of heterogeneous background radioactivity, as indicated by the hatched areas in Fig. 1 and 2. These values are plotted as a function of time after [^{3}H]uracil addition.*

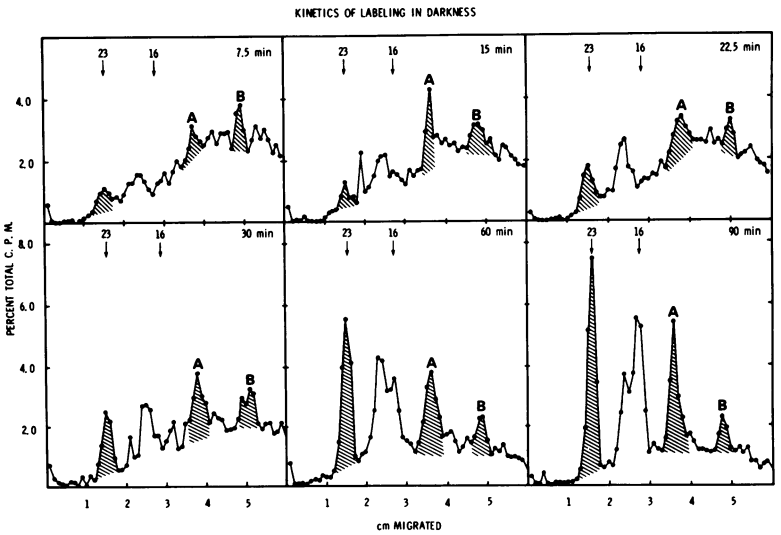

FIG. 2. *Kinetics of RNA labeling in the dark. The protocol of Fig. 1 was followed, except that the culture was darkened 3 h before [^{3}H]uracil addition, and all procedures through cell lysis were performed in the dark.*

upon subsequent illumination of the culture. This is in fact so, as indicated by experimental results summarized in Fig. 4. ^{32}P-"prelabeled" and -"chased" cells were darkened and, 1 h later [^{3}H]uracil was added to 10 μCi/ml). After 12 h in the dark (during which the rate of label incorporation was constant), the cells were rendered susceptible to osmotic shock by lysozyme-EDTA treatment in the presence of 100 μg of nonradioactive uracil/ml. Cells were centrifuged and resuspended in mannitol-supplemented medium containing either rifampin (50 μg/ml; Pittman-Moore) or rifampin plus chloramphenicol (100 μg/ml; Calbiochem) and incubated at 37 C for 30 min, all procedures to this point being performed in darkness. Fractions of each subculture were set aside as "dark controls," and the remainder of each was illuminated. Samples were withdrawn at intervals, and the cells were immediately centrifuged and lysed for RNA extraction. After resolution on 2.8% polyacrylamide gels, ^{3}H radioactivity in each slice was normalized to unit recovery of the ^{32}P-labeled rRNA present as "chased prelabel." Areas under peaks corresponding to high-molecular-weight rRNA (23*S*, 16*S*, and the previously identified 0.88 $\times$ 10^6 dalton 23*S* rRNA fragment [2]) and under the peak corresponding to 0.33 $\times$ 10^6 dalton material were individually determined, as described for Fig. 3. Total normalized radioactivity in 0.33 $\times$ 10^6 dalton RNA and in the combined high-molecular-weight rRNA species is plotted as a function of time after illumination in Fig. 4. Although the amount of rRNA remained constant after illumination, 0.33 $\times$ 10^6 dalton material was lost exponentially, with a half-time of 6 min in the absence of chloramphenicol and 14 min in its presence. (All RNA species were stable in the unilluminated "dark controls.") Radioactivity from 0.33 $\times$ 10^6 dalton material did not appear elsewhere on the gels, and we believe it was degraded to mononucleotides or small RNA fragments migrating more rapidly than tRNA. Dark-accumulated RNA of 0.16 $\times$ 10^6 daltons also disappeared after illumination, but was present in amounts too small to permit quantitative estimation. This low-molecular-weight species did not appear to be an intermediate in the light-stimulated degradation of the larger novel RNA. The two molecules may be related, however, since mild denaturing treatment (4.8 M urea, 20 min at 25 C) resulted in quantitative conversion of 0.33 $\times$ 10^6 dalton RNA into material comigrating with 0.16 $\times$ 10^6 dalton RNA on polyacrylamide gels.

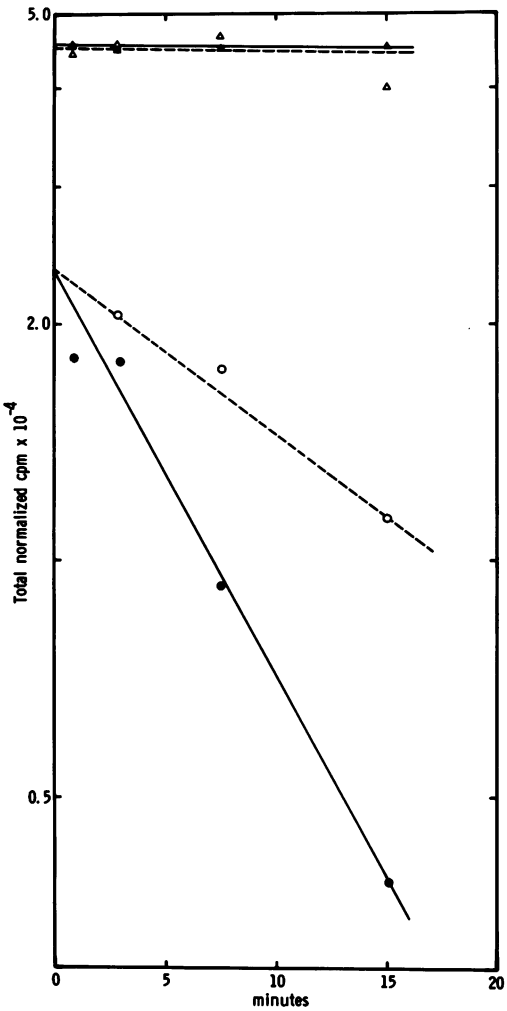

FIG. 4. *Degradation of dark-accumulated 0.33 $\times$ 10^6 dalton novel RNA in the light. Areas of normalized gel profiles corresponding to 0.33 $\times$ 10^6 dalton RNA, and combined areas under the peaks corresponding to high-molecular-weight rRNA were added together and plotted as a function of time after illumination. RNA (0.33 $\times$ 10^6 dalton) in chloramphenicol-treated* (O---O) *or untreated* (●——●) *cells; high-molecular-weight rRNA in the same chloramphenicol-treated* (Δ---Δ) *or untreated* (▲——▲) *cells.*

Subcellular localization of novel RNAs accumulated in darkness. Preliminary experiments suggested that the novel RNA species were not tightly associated with membranous debris, since the material pelleted from crude French pressure cell lysates in short, 15,000 $\times$ *g* centrifugations was not enriched for these RNAs. However, centrifugation that pelleted ribosomes (75 min at 230,000 $\times$ *g*) also sedimented the 0.33 $\times$ 10^6 and 0.16 $\times$ 10^6 dalton novel RNA species. These observations led us to

determine the sedimentation characteristics of these RNAs by sucrose density gradient centrifugation.

Figure 5 is the distribution of label in a lysate of cells "prelabeled" with ^{32}P in the light and then labeled with [^{3}H]uracil in the dark for 16 h before lysis. Newly synthesized, ^{3}H-labeled RNA sediments not only with 70*S* and 100*S* monoribosomes and ribosomal dimers, but also as a polydisperse band between 30*S* and 50*S*.

The fractions indicated by the shading in Fig. 5 were pooled, and RNA was extracted and subjected to electrophoresis as described. Figure 6 shows gel profiles of the interesting regions of the gradient. The 0.33×10^6 dalton RNA species is found mainly in the "40*S*" region of the gradient, with a smaller amount of the total in the pooled "50*S*," "30*S*," and "20*S*" material. From these data it appears that the 0.33×10^6 dalton species is bound to particles sedimenting between 20*S* and 50*S*. Other experiments have shown that the 0.16×10^6 dalton RNA species is also particulate, with a sedimentation coefficient between 30 and 50*S* (data not shown).

When ribosomes and particles containing these novel RNA species were sedimented by centrifugation, resuspended in lysis buffer with reduced (10^{-4} M) $MgSO_4$ concentration to dissociate ribosomes to subunits (15), and centrifuged through sucrose gradients made in this buffer, an altered sedimentation profile was obtained. Ribosomal material sedimented as dissociated 50*S* and 30*S* ribosomal subunits, whereas RNA with molecular weights of the novel RNA species sedimented at about 10*S*. Thus, in reduced magnesium ion concentrations the particles containing these novel RNA species are disrupted.

(Figure 6 also shows that ^{32}P-labeled RNA of 0.88×10^6 and 0.17×10^6 daltons are found in regions of the gradient containing 50*S* subunits, 70*S* ribosomes, and 100*S* dimers. This confirms our previous identification of these species as the products of very slow in vivo cleavage of mature 23*S* rRNA [2] and suggests that ribosomes containing fragmented 23*S* rRNA remain intact.)

DISCUSSION

None of the above experiments identifies a function for the novel RNAs accumulated in the dark in *A. nidulans*. Hypotheses we consider most likely are: (i) the novel RNAs are in vivo degradation products of an unstable fraction of newly synthesized rRNA (probably 23*S* rRNA) that are rapidly destroyed in the light but stable in the dark. Several workers have recently

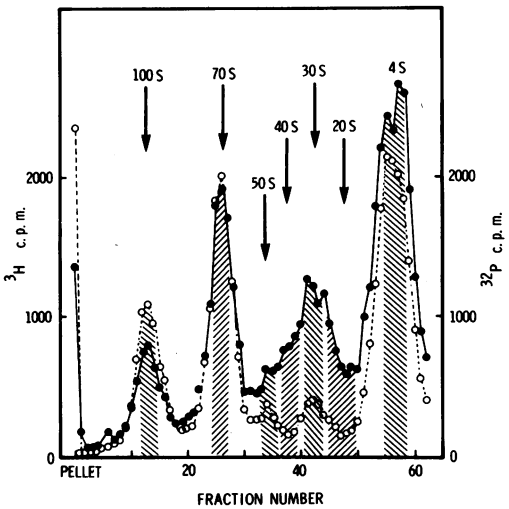

FIG. 5. *Sedimentation pattern of RNA labeled in the dark. Clarified lysates of cells "prelabeled" with ^{32}P in the light and then labeled with [^{3}H]uracil in the dark for 16 h before lysis were sedimented through a 15 to 30% sucrose gradient, and trichloroacetic acid precipitates of samples from each fraction were counted.* ^{3}H (●——●); ^{32}P (O- - -O).

emphasized the importance of degradation of newly synthesized RNA in controlling ribosome abundance in bacteria (8, 9). (ii) The novel RNAs are precursors of tRNA, the final processing of which is light dependent. (iii) The novel RNAs are "stored" messenger species stabilized by association with ribosomal particles in the dark and degraded concomitantly with translation in the light. Chloramphenicol thus retards degradation (Fig. 4) through its effect on translation, as observed in bacterial systems (5). Decision between these hypotheses must await preparation of ^{3}H- or ^{32}P-labeled novel RNAs of sufficiently high specific activity in sufficient quantity for DNA-RNA hybridization competition experiments of T1 ribonuclease oligonucleotide fingerprint analysis. This will be difficult because (i) *A. nidulans* incorporates ^{3}H-labeled RNA precursors at rates at least two orders of magnitude lower than those at which *Escherichia coli* incorporates such precursors (1, 12), and (ii) [^{32}P]orthophosphate incorporated in the dark appears largely in high-molecular-weight polyphosphate (7) that cannot readily be separated from the novel RNAs.

Many RNA processing and degradation events appear to be light stimulated in *A. nidulans*. Post-maturational cleavage of 23*S* rRNA is stimulated twofold by light (2), and cleavage of p16 to produce mature 16*S* rRNA

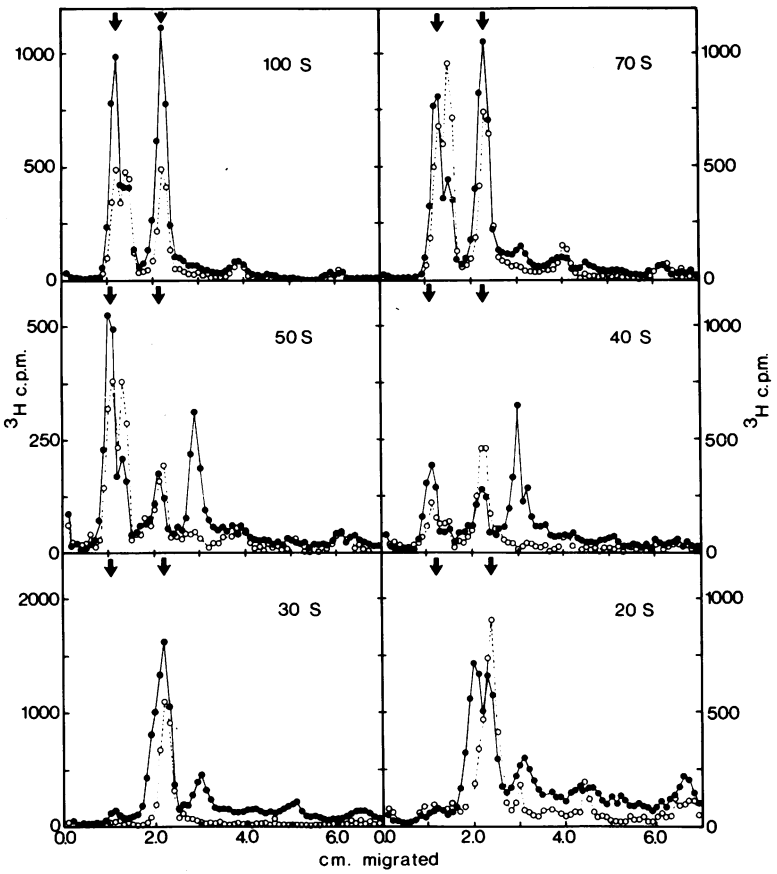

FIG. 6. *Dark-accumulated RNA from different regions of the gradient. Fractions of the sucrose gradient indicated by the hatched areas in Fig. 5 were pooled and extracted with phenol, and the RNA was resolved on 2.8% polyacrylamide gels. Positions of 23S and 16S rRNA are indicated by arrows. Ordinates for ^{32}P counts per minute are not shown, but are for "100S," 1; "70S," 0.5; "50S," 0.4; "40S," 0.2; "30S," 0.5; and "20S," 0.2 times those ordinates shown for ^3H counts per minute. ^3H (●——●); ^{32}P (O- - -O).*

also appears more rapid in the light than in the dark (cf. Fig. 1 and 2). Conversion of precursor 5*S* rRNA to mature 5*S* rRNA is blocked in darkness (Dobson and Doolittle, unpublished data), as is the degradation of the novel RNAs of unknown function described in this report. Light stimulation of RNA cleavage or degradation events appears to be mediated through photosynthesis, since inhibitors of photosynthetic electron flow or photophosphorylation mimic darkness in their effect on these events (unpublished data).

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