Inhibitors of Ribonucleic Acid Synthesis in *Saccharomyces cerevisiae*: Decay Rate of Messenger Ribonucleic Acid

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Daunomycin and ethidium bromide, two deoxyribonucleic acid-intercalating drugs, inhibit ribonucleic acid (RNA) and protein synthesis in *Saccharomyces cerevisiae*. Both agents rapidly curtail uptake of radioactive adenine, whereas the kinetics of radioactive leucine uptake after drug addition are consistent with translation of a pool of exponentially decaying messenger RNA. Messenger RNA half-life determinations from these experiments gave identical results over a range of drug concentrations; this value is 21 ± 4 min at 30 C. In a temperature-sensitive mutant in which RNA synthesis is curtailed at the nonpermissive temperature, a similar half-life for messenger RNA decay is found both in the absence and in the presence of either drug. This indicates that at the concentrations used in this study, neither daunomycin nor ethidium bromide has an appreciable direct effect on translation and do not increase the lability of messenger RNA.

Daunomycin (DMC) is known to inhibit DNA and RNA synthesis in a variety of organisms such as HeLa cells (25, 30), Ehrlich ascites tumor cells (7), and *Escherichia coli* (18, T. Tønnesen and J. D. Friesen, Mol. Gen. Genet., in press). In HeLa cells, DMC also reduces mitotic activity (25), causes disintegration of nuclei, and produces morphological changes of nucleoli (6). Ethidium bromide (EBR) has been reported to affect mitochondrial deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein synthesis in eukaryotes (2, 28), RNA synthesis in whole cells (27), and DNA, RNA, and protein synthesis in bacteria (34, Tønnesen and Friesen, in press). Both drugs seem to exert their effect by intercalating with DNA (4, 19, 36, 37). They have both been shown to inhibit *E. coli* RNA polymerase in vitro (36), and they also inhibit RNA tumor virus growth by inhibiting RNA-dependent DNA polymerase (1, 5, 14). EBR has been shown to be more effective against mitochondrial DNA polymerase than against the nuclear DNA polymerase from rat liver (26).

In *Saccharomyces cerevisiae*, EBR has been used for obtaining petite mutants (10, 31), and it has been shown that mitochondrial DNA, RNA, and protein synthesis in yeast are affected by the drug (8, 10, 33). Some evidence suggests that high concentrations of EBR are capable of

a severe inhibition of yeast nuclear RNA synthesis (17). DMC has also been shown to inhibit mitochondrial RNA and protein synthesis in yeast (29, 33). In view of these indications of the possible effectiveness of DMC and EBR and of the difficulties that we encountered in finding other agents capable of inhibiting transcription in yeast, we were encouraged to undertake an investigation of effects of the two drugs on transcription and translation in *S. cerevisiae*.

MATERIALS AND METHODS

Strains and media. *S. cerevisiae* strain 1493-10C (*α, his₄*) (20), a haploid, was kindly given to us by N. A. Khan. All experiments were carried out in 0.67% yeast nitrogen base (YNB) medium (Difco Laboratories, Detroit, Mich.) containing histidine (100 μg/ml) and 0.1% glucose. The doubling time was approximately 135 min. Cultures were grown at 30 C in baffled Erlenmeyer flasks in a New Brunswick gyratory shaker bath (New Brunswick Scientific Co., New Brunswick, N.J.). Growth was monitored by measuring the absorbance at 450 nm with a Zeiss PMQII spectrophotometer. Strain A364A and mutant *ts⁻* 136 (15) were kindly given to us by L. H. Hartwell. Strain *ts⁻* 136 was grown at 26 C in YM-5 medium (11, 15) with 1% glucose. The doubling time was approximately 190 min.

Chemicals. DMC was kindly given to us by F. Poulin (Poulenc, Montreal, P.Q.) either as a commercial drug, Cerubidine, conaining 5 parts of mannitol

to 1 part DMC, or as the pure compound. Either form of the drug is very soluble in water or in YNB medium (20 mg of DMC per ml). EBR was obtained from B.D.H. Chemicals, Toronto. All radioisotopes were obtained from New England Nuclear Corp., Boston, Mass.

Radioactive labeling. Cultures of strain 1493-10C growing exponentially in YNB medium ($A_{450} = 0.30$) were labeled with a mixture of [^{3}H] adenine (11 μCi/ml; 2 μg/ml) and [^{14}C] leucine (0.6 μCi/ml; 2 μg/ml). At intervals 50-μliter samples were withdrawn and precipitated in 2 ml of ice-cold 5% trichloroacetic acid. After 5 to 10 min the precipitates were collected on nitrocellulose membrane filters (Sartorius, Göttingen, W. Germany) and washed three times with 3 ml of 5% trichloroacetic acid and once with 2 ml of distilled water to reduce quenching. Filters were dried and counted in a Beckman LS-233 liquid scintillation spectrometer.

Cultures of strain ts^- 136 growing exponentially in YM-5 medium ($A_{450} = 0.35$) were labeled by adding [^{3}H] adenine (10 μCi/ml) and [^{14}C] leucine (2 μCi/ml). Samples (50 μliters) were withdrawn at intervals and treated as described above.

Pulse labeling was carried out on 5-ml cultures of strain 1493-10C. At intervals 100-μliter samples of the culture were pipetted into a tube containing 10 μCi of [^{3}H] adenine for 4 min. The pulses were terminated by adding 2 ml of ice cold 5% trichloroacetic acid containing 100 μg of adenine per ml. The membrane filters used for collecting the precipitates were soaked

in a solution containing adenine (2 μg/ml), washed three times with 3 ml of 5% trichloroacetic acid containing 100 μg of adenine per ml, and afterwards treated as described above.

RESULTS

Effect of DMC and EBR on RNA and protein synthesis. Cultures of strain 1493-10C were incubated with varying concentrations of DMC; the rates of RNA and protein synthesis were measured by incorporation of [^{3}H] adenine and [^{14}C] leucine into trichloroacetic acid-precipitable material (Fig. 1A and B). As reported earlier (38), total [^{3}H] adenine incorporation is a measure of RNA synthesis, since less than 1% of the labeled adenine is incorporated into DNA. When DMC (313 μg/ml) was added to a culture of strain 1493-10C, the rate of adenine uptake was unaffected for the first 15 min and then continued at a drastically reduced although finite rate (Fig. 1A). All other concentrations of DMC used completely halted adenine uptake within 15 min of drug addition (Fig. 1A). The addition of 313 μg of DMC per ml decreased the rate of leucine uptake by approximately 50% (Fig. 1B), indicating that the residual RNA synthesis maintained in the presence of this concentration of DMC might primarily
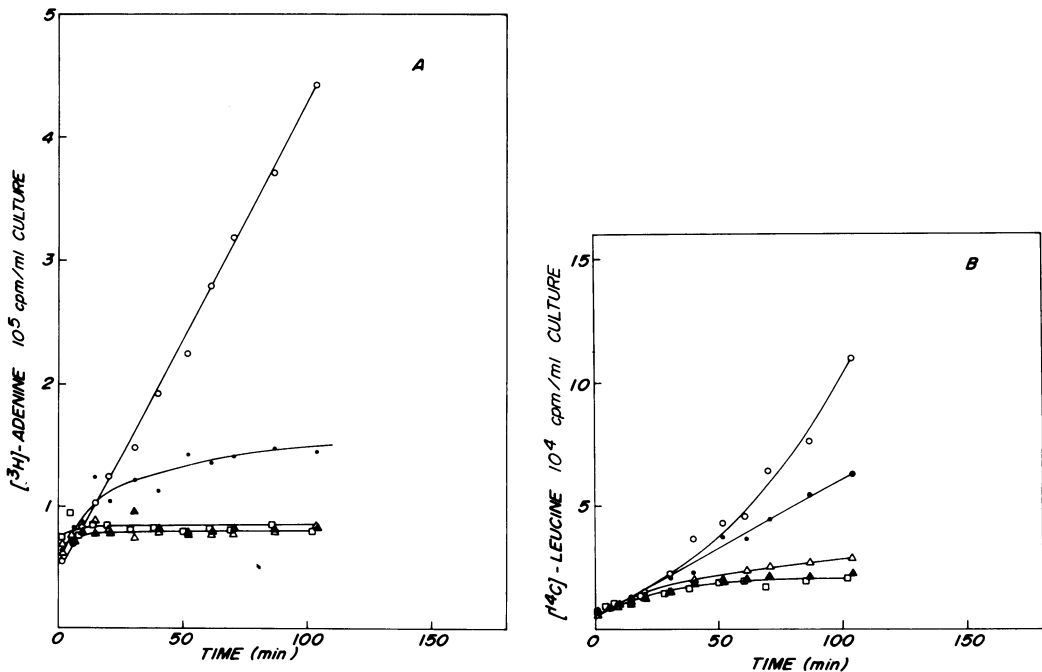

FIG. 1. *Effect of daunomycin (DMC) on adenine and leucine uptake in strain of 1493-10C. Cultures of strain 1493-10C were labeled as described in Materials and Methods. After 15 min of labeling various concentrations of DMC were added, and adenine and leucine uptake were measured as described in Materials and Methods. A, [^{3}H] adenine uptake. B, [^{14}C] leucine uptake. Symbols: O, control; ●, 313μg of DMC per ml; △, 521 μg/ml; ▲, 625 μg/ml; □, 833 μg/ml.*

be messenger RNA (mRNA). Addition of 521 µg of DMC per ml further reduced the rate of leucine uptake, although not ultimately to zero. The two higher concentrations of DMC resulted in a gradual decrease of the rate of leucine uptake, approaching zero 60 min after drug addition. This is the shape of the leucine uptake curve expected if DMC primarily inhibits transcription and allows translation of an exponentially decaying pool of mRNA (24).

The effects of EBR on adenine and leucine uptake are shown in Fig. 2. The effective concentration of this drug was much lower than for DMC. Even at a concentration as low as 19 µg/ml the rate of adenine uptake declined to zero within 70 min (Fig. 2A). Higher concentrations of the drug further reduced the time taken for complete inhibition of adenine uptake (within 15 min for EBR concentrations of 100 and 250 µg/ml). The shapes of the leucine uptake curves (Fig. 2B) were correlated with the degree of residual adenine uptake after EBR addition (Fig. 2A). Thus in the presence of low concentrations of the drug (which allowed a considerable period of adenine uptake before the slope of that curve approached zero), there was also an appreciable rate of protein synthesis; even after long incubation periods the rate of leucine uptake did not approach zero. The two highest EBR concentrations completely inhibited adenine uptake within 15 min and yielded a typical "run out" of leucine uptake

consistent with translation of an exponentially decaying pool of mRNA (24).

The inhibition of adenine uptake in the presence of DMC and EBR was studied in greater detail in a pulse-labeling experiment. Cells were incubated with DMC (625 to 833 µg/ml) or EBR (100 to 250 µg/ml), and at various times during the incubation samples were withdrawn and given a 4-min pulse of [³H] adenine as described in Materials and Methods. The rate of adenine uptake during the 4-min pulses was immediately reduced (Fig. 3). After about 5 min of incubation in the presence of either drug there was at least a 75% decrease in rate with either DMC concentration used or with 100 µg of EBR per ml. A decrease of 90% in the rate of adenine uptake was observed within 2 min in the presence of 250 µg of EBR per ml.

The data shown in Fig. 3 (pulse label) are in good agreement with those shown in Fig. 1A and 2 (continuous label). The pulse-labeling experiment is much more sensitive, but suffers from the possible disadvantage that drug addition might alter the rate of equilibration of exogenous adenine with the intracellular nucleotide pool. Nevertheless, both kinds of experiments indicate virtually complete inhibition of adenine uptake within 10 to 20 min of drug addition.

Rate of mRNA decay. The effects of DMC and EBR on *S. cerevisiae* observed in the experiments reported in Fig. 1 and 2 are qualita-
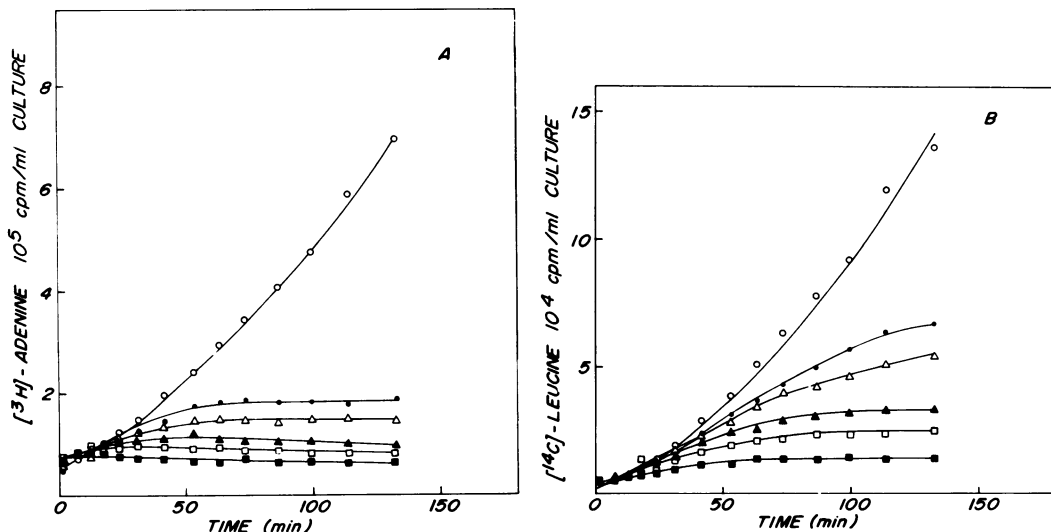

FIG. 2. *Effect of ethidium bromide (EBR) on adenine and leucine uptake in strain 1493-10C. Cultures of strain 1493-10C were grown and labeled as described in Materials and Methods. After 15 min of labeling various concentrations of EBR were added and adenine and leucine uptake were measured as described in Materials and Methods. A, [³H] adenine uptake. B, [¹⁴C] leucine uptake. Symbols: O, control; ●, 18.8 µg of EBR per ml; △, 37.5 µg/ml; ▲, 75 µg/ml; □, 100 µg/ml; ■, 250 µg/ml.*
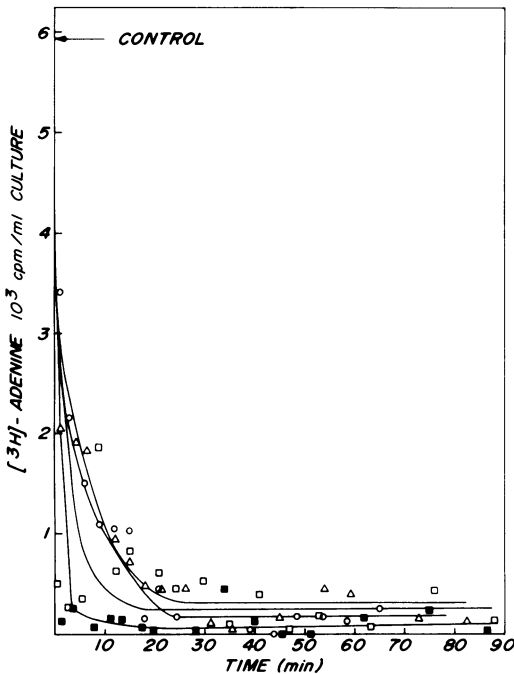
FIG. 3. *Inhibition of adenine uptake by daunomycin (DMC) or ethidium bromide (EBR). Cultures of strain 1493-10C were grown as described in Materials and Methods and incubated with various concentrations of DMC or EBR, and samples were withdrawn and pulse-labeled as described in Materials and Methods. The control value is determined from pulse-labeling of an uninhibited culture. Symbols:* △, *625 µg of DMC per ml;* ●, *833 µg of DMC per ml;* □, *100 µg of EBR per ml;* ■, *250 µg of EBR per ml.*

tively very similar to the effects of these two drugs on transcription and translation in *E. coli* (TØnnesen and Friesen, in press). In particular, the kinetics of leucine uptake after antibiotic addition to *E. coli* are consistent with the notion that an exponentially decaying pool of mRNA continues to be translated after cessation of transcription (a so-called "run out" of protein synthesis). By analogy we shall assume a similar effect of DMC and EBR on transcription and translation of *S. cerevisiae.* An analysis of the kinetics of leucine uptake (see reference 24) then yields a value for the half-life of decay of overall or "average" yeast mRNA based upon the assumption that at each point along the run out curve, the rate of protein synthesis is proportional to the existing amount of functional mRNA (Fig. 4). It should be noted that no significant protein turnover in the absence of protein synthesis has been observed (35).

Concentrations of DMC ranging from 625 to 1,042 µg/ml yielded almost identical half-lives

(19.0 to 19.8 min) in the exponential portion of the curve (Fig. 4A). With increasing DMC concentrations the shoulder preceding the exponential portion of the decay curve is slightly smaller, reflecting the fact that the higher DMC concentration inhibited adenine uptake somewhat more rapidly, leading to a shorter lag before onset of the reduction of the rate of protein synthesis as seen from the leucine uptake curve. The data shown in Fig. 4B, from an experiment in which EBR was used to inhibit RNA synthesis, are similar to those obtained with DMC. In many cases the data points near the foot of the decay curves deviate from linearity since this is the region where the determination of the slope of the leucine uptake curve is most subject to error. In the data shown in Fig. 4, the initial rate of protein synthesis in the drug-inhibited cultures relative to the control cultures should ideally be 100%. This variation is probably due to uncertainty in the estimation of the initial slope of the control culture but does not interfere with the half-life estimations. A summary of mRNA half-lives determined from a large number of experiments similar to those reported above is shown in Table 1.

Within experimental error, inhibition of RNA synthesis with either DMC or EBR yielded identical mRNA half-life estimations. Moreover, within experimental error the half-life was independent of drug concentration, and no effect of an increasing period of labeling before drug addition was observed. This last point is important because incomplete equilibration of the leucine pool (i.e., within 15 min) could result in distorted half-life estimations.

Effect of DMC and EBR on RNA synthesis and protein synthesis in a temperature-sensitive mutant. The analysis of the data leading to estimations of mRNA half-life in yeast is based on the assumption that DMC and EBR inhibit transcription and are without direct effect on protein synthesis. Hutchison et al. (15) have isolated a temperature-sensitive mutant (ts⁻ 136) of *S. cerevisiae* in which RNA synthesis is severely restricted at the nonpermissive temperature and a consequent "run out" of protein synthesis is observed. Although the effect of this mutation is not clearly understood (15), it can be used to provide some further indication of the action of DMC and EBR in yeast.

Figures 5A and B show adenine and leucine uptake in strain ts⁻ 136 grown at 26 C and shifted to the restrictive temperature (37 C), both in the absence and the presence of DMC or EBR. When a culture was shifted to 37 C, the rate of adenine uptake was reduced to a very low
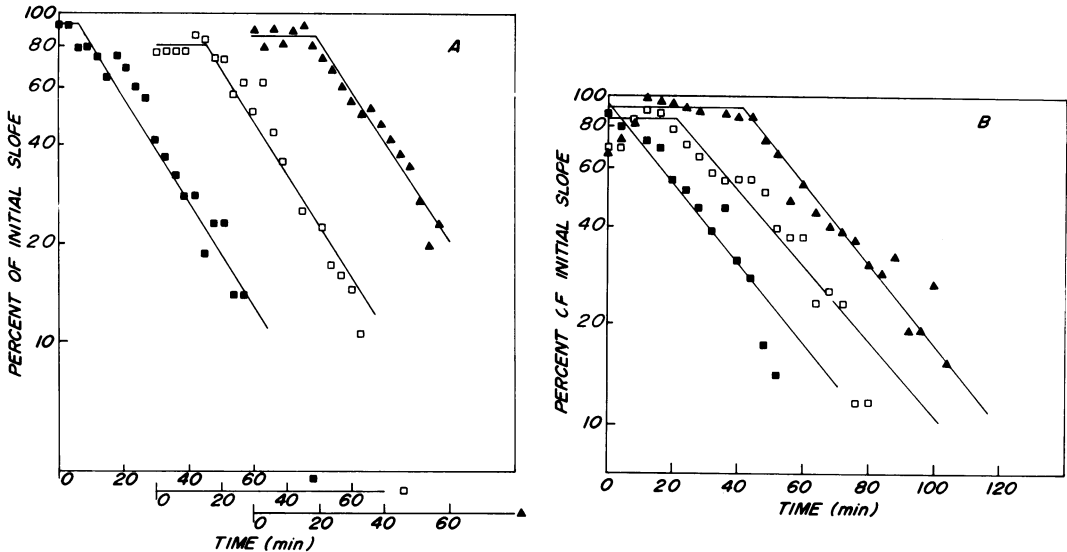
FIG. 4. *Decay of messenger RNA in cultures of S. cerevisiae inhibited by daunomycin (DMC) or ethidium bromide (EBR). A, Decay of mRNA in the presence of DMC. At intervals along leucine uptake curves the slopes were measured relative to the initial slope (0–5 min) of the same curve (as explained in the text). Symbols: ▲, 625 μg of DMC per ml (Fig. 1); ○, 833 μg/ml, (Fig. 1); ■, 1.042 μg/ml (from a similar experiment). B, Decay of mRNA in presence of EBR. Calculations were as described above, and all experimental values are from Fig. 2. Symbols: ▲, 75 μg of EBR per ml; □, 100 μg/ml; ■, 250 μg/ml.*

level, although not to zero. The kinetics of leucine uptake showed a normal run out consistent with earlier observations (15). Addition of DMC or EBR to strain *ts⁻* 136 at the nonpermissive temperature suppressed adenine incorporation slightly below the level due to the effect of the mutation alone. Consistent with this is the observation that the plateau of the leucine uptake curve was somewhat lower in the presence of either drug than in its absence, most likely due to suppression of a residual amount of mRNA synthesis in the mutant at 37 C.

mRNA decay rates in strain *ts⁻* 136 with or without EBR or DMC are shown in Fig. 5C. Half-life estimations from four similar experiments are shown in Table 2. Within experimental error, DMC and EBR do not alter the mRNA decay rate already determined as a result of transcription inhibition due to the temperature-labile effect of mutant *ts⁻* 136. This we take as an indication that neither drug increases the lability of mRNA in yeast.

The observed mRNA half life in strain *ts⁻* 136 at 37 C (Table 2) is only approximately 10% less than that in strain 1493-10C at 30 C (Table 1). This somewhat surprising result might be a reflection of the fact that the growth rate of wild-type *S. cerevisiae* does not vary significantly over the temperature range 30 to 37 C (unpublished data), and one might expect that

other temperature-dependent processes (such as mRNA decay) would be relatively invariant over this temperature range.

DISCUSSION

In the present experiments yeast cultures were grown in glucose medium, in which mitochondrial-associated metabolism is suppressed (31). Therefore the measurements reported here primarily refer to nuclear transcription and cytoplasmic translation of nuclear transcripts, and this is implicit in the ensuing discussion. Our results show that DMC and EBR act as potent inhibitors of RNA synthesis in yeast, in agreement with results obtained with other organisms (7, 17, 18, 25, 27, 30, Tønnesen and Friesen, in press).

An appreciable lag before complete inhibition of RNA synthesis in yeast by DMC and EBR was frequently observed; this could be due to the time taken to build up the appropriate intracellular concentration of the drug. It is therefore impossible on the basis of the present data to tell whether the drugs inhibit chain initiation or chain elongation in the transcription process. However, the intercalative mode of action of DMC and EBR (4, 19, 36, 37) strongly suggests inhibition of RNA polymerase translocation, which in turn suggests inhibition of chain elongation. Alternatively, the shoulder on

TABLE 1. *Messenger RNA half-life in Saccharomyces cerevisiae strain 1493-10C*

Inhibitor	Time of labeling before drug addition (min)	Concentration of inhibitor (μg/ml)	Messenger RNA half-life (min)
Ethidium bromide	15	75	24.5
	60	75	26.0
	15	87.5	21.8
	15	100	17.8; 26.2
	60	100	26.2
	15	150	16.2; 17.0; 17.8; 19.9; 26.2
	60	150	13.2
	15	250	15.8; 17.8; 20.3; 21.0; 21.5; 21.8; 24.5; 24.5
	60	250	24.0
	15	500	20.5
Mean values			21.1 ± 3.9
Daunomycin	15	600	19.8
	15	625	20.8; 23.0; 24.5; 28.5
	60	625	20.8; 22.0 27.2
	15	729	19.8
	15	833	18.5; 19.5
	60	833	20.5
	15	900	19.6
	60	900	18.8
	15	938	18.8
	15	1042	18.3
Mean values			21.3 ± 3.2

[a] With ethidium bromide, experiments similar to those in Fig. 2 were analyzed, and half-lives were determined as described in Fig. 4. With daunomycin, experiments similar to those in Fig. 1 were analyzed, and half-lives were determined as above.

the mRNA decay curves could indicate the existence of a "pretranslation" pool of mRNA which supplies transcripts to the polysomes (22). After cessation of transcription, this pool would have to be exhausted before exponential decay of mRNA could be revealed. This notion is not supported by the data of Fig. 5C, which show that exponential decay of protein synthetic capacity sets in immediately after temperature shift and cessation of transcription.

Both DMC and EBR have been reported to bind to RNA (4, 39), and both are capable of degrading or disassembling bacterial ribosomes subjected to thermal or ionic stress (39). However, in view of the data presented in Fig. 5 it seems unlikely that any such possible direct effects on translation occur in vivo in *S. cerevisiae*.

The estimations of mRNA half-life based on the inhibition of transcription by DMC and EBR in *S. cerevisiae* are in agreement with those derived from polysome degradation in spheroplasts (15) and from the kinetics of appearance of newly synthesized RNA on spheroplast polysomes (13). Decay rates of tenaciously bound DNA-like RNA isolated from methylated albumin kieselguhr columns have been measured after a uracil pulse chase and half-life estimations ranging from 15 to 20 min were observed (16), and were in good agreement with our estimations. Both the present analysis and that based on decay of polysomes in mutant *ts⁻* 136 as the nonpermissive temperature (15) measure essentially the functional aspect of mRNA. In the former case the actual in vivo capacity of mRNA to program translation is determined, and in the latter the intactness of the functional translation apparatus is measured. The chemical isolation of a DNA-like RNA (16), on the other hand, could quite likely include other species of RNA, those not destined to leave the nucleus (28). We have also attempted to measure decay of mRNA in a pulse-chase experiment (unpublished data) and were unsuccessful, presumably because of the inability of exogeneous uracil to enter rapidly the intracellular nucleotide pool. The fact that the ultimate slopes of the leucine uptake curves are nearly zero at the appropriate drug concentration (Fig. 1B, 2B, and 5B) is a good indication that there is less than 5 to 10% stable mRNA in *S. cerevisiae*. The observation that the present data for mRNA decay fit well a negative exponential function (Fig. 4 and 5C) indicates random degradation of message molecules, in agreement with observations in prokaryotes, and suggests a similar basic mechanism of degradation (9).

One can calculate the number of times an average mRNA molecule is translated in yeast before it is degraded (24). The total amount of protein synthesized after inhibition of transcription by the highest concentrations of drugs used is equal to about 16.5 min of protein synthesis in the control culture (Fig. 1 and 2). Since the generation time of the culture (doubling time/ln2; 195 min), the amount of RNA and protein per cell in minimal medium (RNA, 0.782 × 10⁻⁶ μg/cell; protein, 3.30 × 10⁻⁶ μg/cell; K. W. Boehlke and J. D. Friesen, manuscript in preparation), and the amount of mRNA (12.0% determined by hybridization of uniformly labeled RNA; Boehlke and Friesen, manuscript in preparation) are known, the average number of times a yeast mRNA mole-
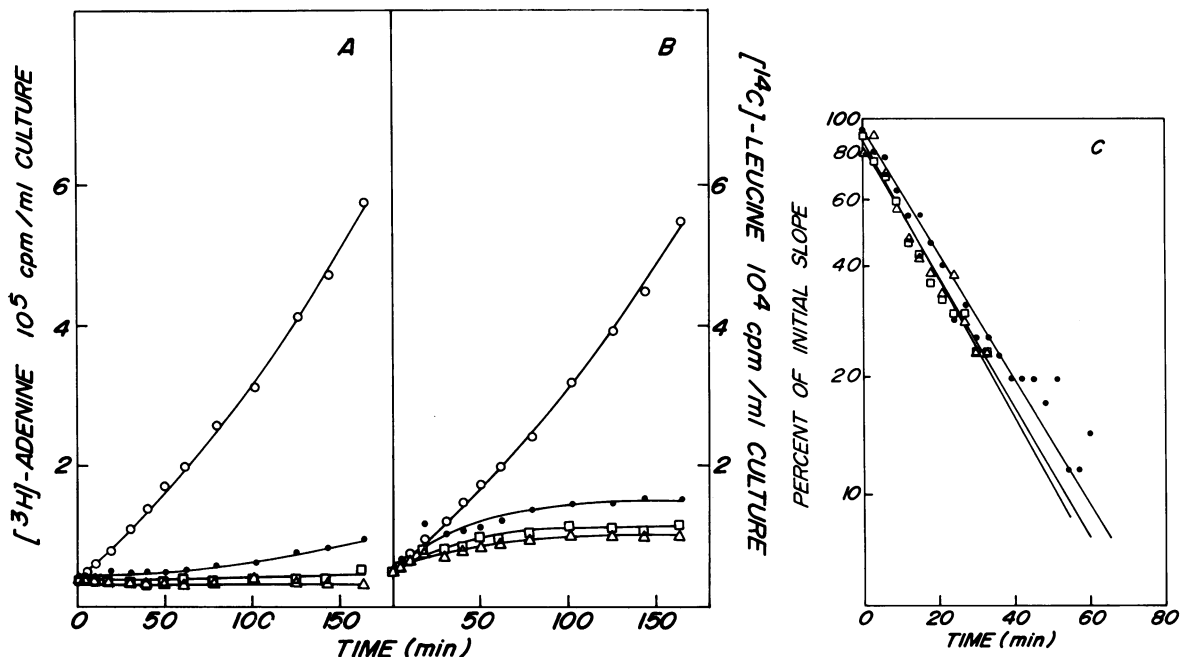
FIG. 5. *Effect of temperature shift and of daunomycin (DMC) and ethidium bromide (EBR) on adenine and leucine uptake and on mRNA decay rates in strain ts⁻ 136. Cultures of strain ts⁻ 136 grown and labeled as described in Materials and Methods. After 20 min labeling portions of the cultures were shifted to 37 C and DMC or EBR was added. Adenine and leucine uptake were measured as described in Materials and Methods. A, [³H] adenine uptake. B, [¹⁴C] leucine uptake. C, Decay of mRNA at the nonpermissive temperature and in cultures inhibited by DMC or EBR. Calculations were as described for Fig. 4A. Symbols: ●, 37 C; △, 37 C plus 250 μg of EBR per ml; □, 37 C plus 800 μg of DMC per ml.*

TABLE 2. *Messenger RNA half-life in strain ts⁻ 136 at 37 C in the absence and presence of daunomycin (DMC) or ethidium bromide (EBR)[a]*

Expt no.	Culture conditions		
	37 C	37 C plus DMC (800 μg/ml)	37 C plus EBR (250 μg/ml)
1	18.5	16.5	16.2
2	19.6	22.0	21.0
3	25.5	20.3	20.0
4	20.0	19.2	18.5
Mean values:	20.9 ± 2.1	19.5 ± 2.3	19.4 ± 2.6

[a] Experiments similar to Fig. 5 were analyzed. Numbers refer to half-life of mRNA decay in minutes determined as described in Fig. 4.

cule is translated can be calculated (24). This value is 27 translations per molecule. It is interesting to note that the half-life of mRNA is approximately 10 times greater in yeast than in *E. coli* (24), but the average number of times an mRNA functions in the two organisms is approximately the same (3).

From a knowledge of the functional half-life for mRNA and the number of protein copies made per mRNA molecule, it can be calculated that ribosomes in yeast initiate translation of each mRNA molecule on the average every 67 s. This indicates that the frequency of translation initiation in yeast is approximately 16 times slower than in bacteria (3). However, it should be noted that the validity of these calculations rests on the reliability of DNA-RNA hybridization as a true measure of the cellular content of mRNA in yeast (Boehlke and Friesen, manuscript in preparation). If an appreciable fraction of the RNA defined as mRNA in that assay actually belonged to a class of DNA-like RNA molecules that never engaged in translation (such as Hn-RNA; 28), estimation of the actual amount of functional mRNA in yeast would have to be reduced, consequently raising the ribosome-to-mRNA ratio in the above calculations.

ACKNOWLEDGMENTS

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