Guanosine 5′-Diphosphate 3′-Diphosphate Levels, Carbon Source, and Ribonucleic Acid Synthesis in a Mutant Strain of *Escherichia coli*†

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ABSTRACT: We have previously described a mutant strain of *Escherichia coli* (2S142) which shows a specific inhibition of stable RNA synthesis at 42 °C. The temperature-sensitive lesion mimics a carbon source downshift (diauxie lag). We therefore measured RNA synthesis and levels of ppGpp (guanosine 5′-diphosphate 3′-diphosphate) on a number of different carbon sources. There is a 6-fold variation in ppGpp levels at 42 °C, depending on the carbon source present. Much of the variation in ppGpp levels at 42 °C can be explained by variations in the decay rate of ppGpp at 42 °C. The rates of ribosomal RNA and total RNA synthesis also vary with the carbon source at 42 °C. Linear regression analysis shows only a moderately good correlation (correlation coefficient = 0.62, $P = 0.0001$) between the ppGpp level at 42 °C and the rate of rRNA synthesis at 42 °C. In fact, ppGpp levels are a slightly better predictor of the rate of total RNA synthesis (correlation coefficient = 0.69, $P = 0.0001$) at 42 °C. Other variables such as rate of carbon source uptake appear to have very little, if any, relationship to the rate of rRNA synthesis on the different carbon sources. Segmented linear regression analysis indicates that ppGpp levels and rates of RNA synthesis correlate best when the carbon sources are divided into two groups: 6- and 12-carbon sugars and other carbon sources. The rate of rRNA synthesis in 2S142 at 42 °C appears to be relatively insensitive to ppGpp levels with 6- and 12-carbon sugars as the carbon source. These data raise the possibility that carbon source may affect rRNA synthesis in a manner that is at least partially unrelated to ppGpp levels.

Stable RNA synthesis is regulated both during carbon source shift up and shift down (Nierlich, 1972) and during amino acid starvation (Ryan & Borek, 1971). The regulation of stable RNA synthesis in response to amino acid supply is fairly well characterized. The product of the relA gene of *Escherichia coli* mediates a drastic reduction of stable RNA synthesis in response to the accumulation of uncharged tRNA in the cell [reviewed by Gallant & Lazzarini (1976)]. This response is termed stringent control. Stringent strains (rel+) stop the synthesis of stable RNA during amino acid starvation while relaxed strains (rel–) continue to synthesize stable RNA. The relA gene product has been identified as a ribosome-bound nucleotide pyrophosphotransferase which catalyzes the synthesis of guanosine 5′-triphosphate 3′-diphosphate (pppGpp)[1] (Haseltine et al., 1972; Haseltine & Block, 1973; Lipmann & Sy, 1976) which is subsequently degraded to form guanosine 5′-diphosphate 3′-diphosphate (ppGpp) (Kari et al., 1977). A large body of evidence implicates ppGpp as the key effector molecule for regulation of stable RNA synthesis in the stringent response (Cashel & Gallant, 1974; Ryals & Bremer, 1982), in response to differences in growth temperature (Ryals et al., 1982a), and in response to differences in growth rate at the same temperature (Ryals et al., 1982b).

The mechanism governing the regulation of stable RNA synthesis during carbon source downshift is not as well understood. Both rel(+) and rel(–) cells accumulate ppGpp during a shift down (Lazzarini et al., 1971; Winslow, 1971). However, the accumulation of ppGpp is variable in rel(–) strains, i.e., the particular strain and the exact shift in carbon source affect the levels of ppGpp which accumulate (Lazzarini et al., 1971). A further uncertainty exists in that during carbon source downshift the cessation of RNA accumulation is usually immediate but the accumulation of ppGpp is sometimes slow (Gallant et al., 1972), suggesting that there might be another factor in addition to ppGpp which affects stable RNA synthesis during carbon source downshift.

We have previously described a mutant strain of *E. coli*, 2S142, which exhibits the following properties: (1) Stable RNA synthesis is markedly reduced at the restrictive temperature, 42 °C. 2) The levels of ppGpp rise at 42 °C but no pppGpp is detectable at any time (Chaney & Schlessinger, 1975). (3) The rate of ppGpp decay in glucose-grown cells is decreased at 42 °C (Harris et al., 1978). The changes in ppGpp metabolism which occur in 2S142 at 42 °C are very similar to those observed during carbon source downshift (Neidhardt, 1964; Gallant et al., 1972). Since 2S142 does appear to mimic a carbon source downshift at 42 °C, we have investigated the effect of different carbon sources on ppGpp levels and rRNA synthesis.

Experimental Procedures

Strains and Media. 2S142 (met, ilv, rns, rel, ts) has been described previously (Chaney & Schlessinger, 1975; Harris & Chaney, 1978; Harris et al., 1978). The parental strain, D10, has been described by Gesteland (1966). Cells were grown on M9 medium (Adams, 1966) or low-phosphate medium (Cashel, 1969) supplemented with 0.2% carbon source and 0.05 mg/mL of each of the 20 amino acids. The low-phosphate medium was also supplemented with 1×10^{-5} M $FeCl_3$, 1.5×10^{-5} M $MgCl_2$, and 2.5×10^{-5} M $CaCl_2$.

Methods. The levels of ppGpp were determined by a modification (Chaney et al., 1977) of the procedure described by Cashel (1969). Rates of RNA synthesis and percentage rRNA synthesis were determined as described previously (Chaney et al., 1977). The measured rates of RNA synthesis

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[1] Abbreviations: ppGpp, guanosine 5′-diphosphate 3′-diphosphate; pppGpp, guanosine 5′-triphosphate 3′-diphosphate; f_r, rate of ribosomal RNA synthesis/rate of total RNA synthesis; μ, growth rate in doublings per hour.

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were corrected for slight permeability effects by EDTA treatment, essentially as described previously (Chaney et al., 1977). Control studies have shown that no more than 10–15% of the ppGpp is lost and that f_r values are changed very little following EDTA treatment. Sugar uptake was measured essentially as described by Saier et al. (1976). Statistical analysis of the data was performed by the University of North Carolina Biometric Consulting Laboratory.

Results

Responses of 2S142 and D10 to Change in Growth Temperature. Previous studies in our laboratory have established that the mutant strain, 2S142, undergoes some very predictable metabolic changes when shifted to 42 °C. There is an initial burst in RNA synthesis for the first 10–15 min at the elevated temperature, followed by a much slower, linear accumulation of RNA for an extended period of time (Chaney et al., 1977). Cell density follows a similar pattern. Following the burst in RNA synthesis, there is not only a decrease in the rate of total RNA synthesis but also a preferential decrease in the synthesis of stable RNAs (Chaney & Schlessinger, 1975; Chaney et al., 1977). ppGpp levels increase gradually over the first 2 h at the restrictive temperature (Chaney et al., 1977; Harris et al., 1978).

These metabolic responses to 42 °C appear to be solely the property of the mutant strain. The isogenic parental strain, D10, grows normally, synthesizes ribosomal RNA normally (Chaney & Schlessinger, 1975; Chaney et al., 1977), and accumulates little or no ppGpp (Chaney & Shlessinger, 1975; Harris et al., 1978) at 42 °C. Since all of these previous studies have been carried out with glucose grown cells only, it seemed desirable to extend these observations to other carbon sources. Figure 1 shows the response of 2S142 to the restrictive temperature on three different carbon sources. Two observations are immediately apparent from this figure. First, while the length of the initial burst may vary somewhat depending on carbon source, both RNA accumulation and growth completed their adjustment to the restrictive temperature by 30 min. Second, while ppGpp levels never reach a definitive steady-state value at 42 °C, the kinetics of the increase in ppGpp levels is very similar on all carbon sources for the first 60 min. While the corresponding data for the other carbon sources are not shown, they are very comparable. On the basis of these data, we decided to make our detailed comparisons between carbon sources at 45 min following the shift to 42 °C. This is a point in time at which RNA accumulation has reached a steady-state value and the kinetics of ppGpp accumulation is very similar on all carbon sources.

Figure 1 also shows the response of glycerol-grown D10 to the restrictive temperature. As previously shown with glucose-grown D10, growth and RNA accumulation are logarithmic and little ppGpp accumulates. Subsequent studies have confirmed this behavior on all of the carbon sources tested (data not shown).

Bremer et al. (1973) have shown that for wild-type *E. coli*, the parameter f_r (rate of rRNA synthesis/rate of total RNA synthesis) should vary in a linear manner with μ (growth rate expressed as doublings/h). In fact, we see exactly such a correlation for 2S142 grown at 30 °C and D10 grown at both 30 and 42 °C on a variety of carbon sources (Figure 2A). The major exceptions are glucose-grown 2S142 at 30 °C (μ = 0.58, f_r = 0.53), fructose-grown 2S142 at 30 °C (μ = 0.27, f_r = 0.28), fructose-grown D10 at 30 °C (μ = 0.92, f_r = 0.46), and fructose-grown D10 at 42 °C (μ = 1.75, f_r = 0.57). It is somewhat more difficult to make a similar comparison for 2S142 at 42 °C. Since the growth rate is linear at 42 °C

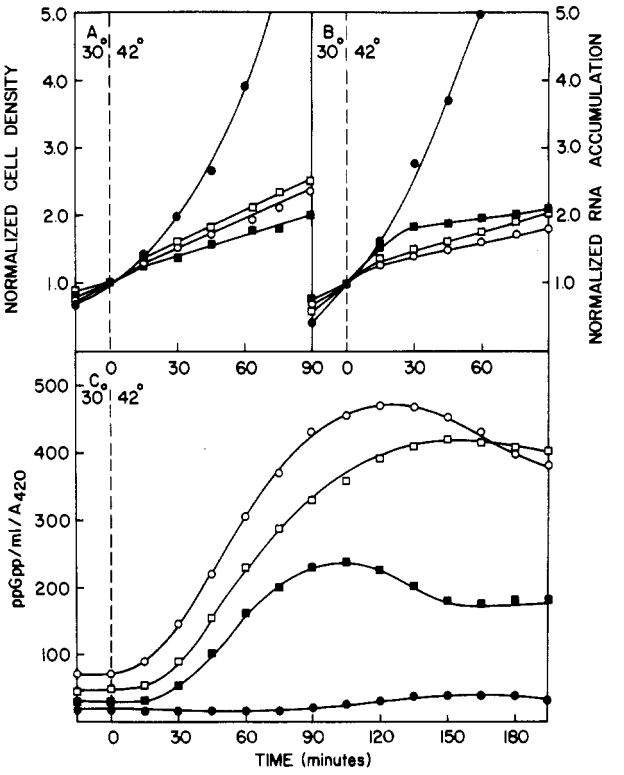

FIGURE 1: Response of 2S142 and D10 to growth at 42 °C. 2S142 was grown at 30 °C as described under Strains and Media with either glucose (○), mannitol (□), or glycerol (■) as the carbon source. D10 was grown under similar conditions with glycerol (●) as a carbon source. At 1 × 10^8 cells/mL, each culture was shifted to 42 °C. Cell density was monitored with a Klett-Summerson Model 800-3 colorimeter by using a green filter. RNA accumulation was measured by orcinol determination as described previously (Chaney et al., 1977). ppGpp levels were determined as described under Methods. Both cell density and RNA amounts were normalized to give a value of 1.0 at the time of the shift to 42 °C. (A) Cell density vs. time at 42 °C; (B) RNA accumulation vs. time at 42 °C; (C) ppGpp levels vs. time at 42 °C.

rather than exponential, the parameter μ cannot be used. However, if one compares the linear growth rate at 42 °C with f_r (Figure 2B), no correlation can be seen. f_r for 2S142 at 42 °C also shows no correlation with μ at 30 °C (correlation coefficient = 0.11, P = 0.01).

ppGpp Levels and RNA Synthesis. The effects of 13 different carbon sources on ppGpp levels in 2S142 are shown in Table I. ppGpp levels are low but detectable on all carbon sources at 30 °C. However, there is a 6-fold variation in the levels of ppGpp which accumulate in 2S142 at 42 °C depending on the carbon source in the growth medium.

Previous data suggested that the elevation of ppGpp in 2S142 at 42 °C was caused by a decrease in the rate of ppGpp decay (Harris et al., 1978). Figure 3 shows that the rate of ppGpp decay at 42 °C in 2S142 varies with the carbon source used. Since the rate of decay varies inversely with ppGpp levels at 42 °C, the observed variations in ppGpp decay rate are probably sufficient to explain the variation in ppGpp levels.

We next wished to determine whether the difference in ppGpp levels resulted in a corresponding change in the rate of RNA synthesis in 2S142 at 42 °C. Table II shows the rate of total RNA and ribosomal RNA synthesis in 2S142 at 30 and 42 °C on the same carbon sources used to determine ppGpp levels. Visual inspection of the data shows no apparent correlation between the rate of rRNA synthesis and ppGpp levels at either 30 or 42 °C. If the effects of ppGpp levels on rRNA synthesis in this system could be explained on the basis

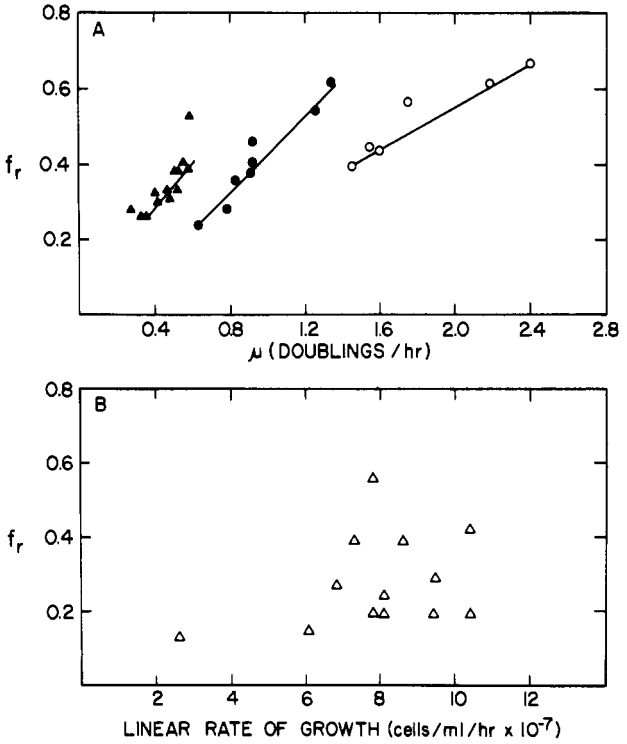

FIGURE 2: f_r as a function of growth rate. When growth was exponential, the growth rate was expressed as μ (doublings/h) and was obtained graphically from semilog plots of cell density vs. time for each strain and each carbon source. When growth was linear (2S142 at 42 °C), the growth rate was expressed as the linear increase in the number of cells per milliliter per hour and was obtained from linear plots of cell density vs. time. Cell density was monitored with a Klett-Summerson Model 800-3 colorimeter by using a green filter. f_r (rate of rRNA synthesis/rate of total RNA synthesis) was obtained from data such as that shown for 2S142 in Table II. (A) f_r vs. μ for 2S142 at 30 °C (▲), D10 at 30 °C (●), and D10 at 42 °C (○); (B) f_r vs. linear growth rate for 2S142 at 42 °C (△).

Table I: Effect of Carbon Source on ppGpp Levels in 2S142 at 30 and 42 °C[a]

carbon source	ppGpp levels (pmol mL^{-1} OD_{420}^{-1}) 30 °C	42 °C
glucose	79 ± 8	209 ± 18
fructose	52 ± 12	233 ± 18
mannitol	67	158 ± 56
mannose	70 ± 12	234 ± 5
maltose	56 ± 6	174 ± 28
succinate	15	129 ± 20
galactose	18 ± 2	100 ± 26
glycerol	33 ± 12	101 ± 7
lactose	34	200 ± 19
gluconate	17 ± 11	89 ± 9
lactate	17 ± 10	68 ± 11
arabinose	17 ± 3	38 ± 7
acetate	24 ± 5	122 ± 11

[a] 2S142 was cultured at 30 °C in low-phosphate medium as described under Strains and Media and labeled with 100 μCi/mL $[^{32}P]P_i$ for the entire growth period. At 3 × 10^8 cells/mL, aliquots were removed and the culture was shifted to 42 °C for 45 min. Aliquots were then removed again and ppGpp levels were determined as described under Methods. ppGpp values are reported as ± standard error of the mean.

of simple reversible inhibition kinetics, one would expect a single-reciprocal Dixon plot to give a linear transformation of the data. For example, Fiil et al. (1972) have measured the rate of RNA accumulation and ppGpp levels during amino acid starvation in *E. coli* using a single carbon source. A

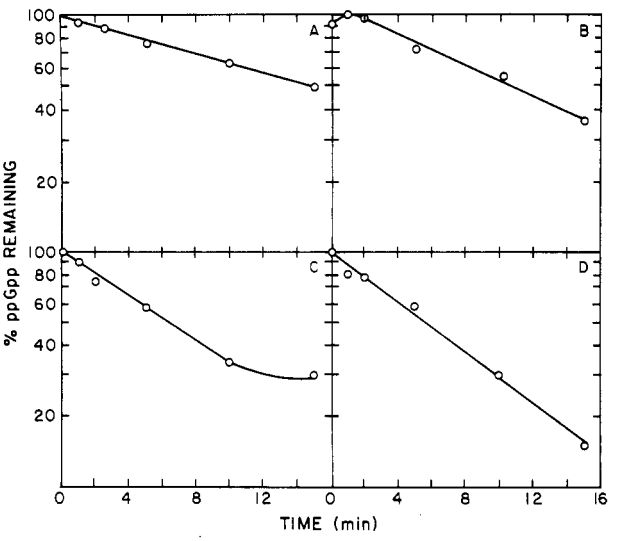

FIGURE 3: Effect of carbon source on ppGpp decay at 42 °C. 2S142 was grown at 30 °C on low-phosphate medium as described under Strains and Media with either glucose, succinate, galactose, or lactate as a carbon source. At 0.5 × 10^8 cells/mL, 100 μCi/mL $[^{32}P]P_i$ was added. At 1 × 10^8 cells/mL the culture was shifted to 42 °C. After 45 min 100 μg/mL chloramphenicol was added. 100-μL aliquots were removed prior to addition of chloramphenicol and at the indicated times following the addition. ppGpp levels were then determined as described under Methods. All the values are expressed as a percentage of the value obtained before the addition of chloramphenicol. (A) Glucose; (B) succinate; (C) galactose; (D) lactate. $T_{1/2}$ for ppGpp decay at 42 °C: glucose = 15 min; succinate = 9.2 min; galactose = 5.8 min; lactate = 4.5 min.

Table II: Rates of RNA Synthesis in 2S142 at 30 and 42 °C[a]

carbon source	rate of total RNA synthesis (pmol mL^{-1} min^{-1} OD_{420}^{-1}) × 10^4 30 °C	42 °C	rate of rRNA synthesis (pmol mL^{-1} min^{-1} OD_{420}^{-1}) × 10^4 30 °C	42 °C
glucose	1005	14.4	533	0.939
fructose	911	19.89	255	1.4
mannitol	1658	17.66	630	1.15
mannose	1802	34.59	721	2.96
maltose	2003	14.74	641	0.72
succinate	1303	83.20	391	8.15
galactose	1239	27	409	1.74
glycerol	938	42.27	244	2.75
lactose	508	17.01	132	0.76
gluconate	566	79.31	215	11.11
lactate	1233	131	407	24.5
arabinose	741	92.26	289	12.78
acetate	1660	44.88	847	4.36

[a] Cells for measurement of rates of total RNA and rRNA synthesis were grown in M9 medium at 30 °C as described under Strains and Media. At a cell density of 3 × 10^8 cells/mL, the culture was split into two aliquots with one aliquot being incubated at 30 °C and the other at 42 °C. After 45 min, 5 mL of each culture was pulse labeled for 2 min with 25 μCi of [^{3}H]uridine at the proper temperature. RNA was extracted, purified, and percent rRNA synthesis was measured by gel electrophoresis as described previously (Chaney et al., 1977). Rates of total RNA synthesis and rRNA synthesis were calculated from total Cl_3AcOH-precipitable counts corrected for permeability effects (Chaney et al., 1977) and percent rRNA.

single-reciprocal plot of their data gave an excellent linear fit. However, a single-reciprocal Dixon plot (Figure 4) of the rate of rRNA synthesis at 42 °C vs. ppGpp levels at 42 °C shows only a partial correlation (linear correlation coefficient = 0.64, $P = 0.0001$), suggesting that the relationship between carbon source, ppGpp levels, and rRNA synthesis is complex in our strain.

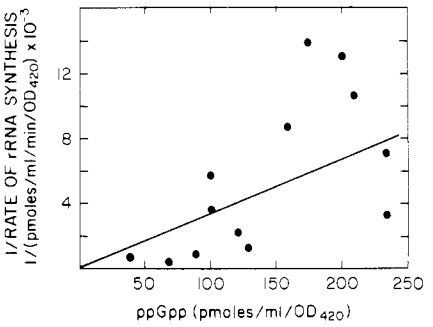

FIGURE 4: Rate of rRNA synthesis vs. ppGpp levels at 42 °C in 2S142. The rates of rRNA synthesis were measured as described in Table II. ppGpp levels were measured as described in Table I. The solid line indicates the best fit by linear regression analysis. The data points represent average values. Intercept and slope were calculated by linear regression analysis using total data points.

Table III: Rate of Uptake of Various Carbon Sources by 2S142[a]

carbon source	rate of uptake at 30 °C [μmol min^{-1} (g dry wt)$^{-1}$]	rate of uptake at 42 °C [μmol min^{-1} (g dry wt)$^{-1}$]	rel rate of uptake (42 °C/30°C)
glucose	14.55 ± 0.63	9.65 ± 2.45	0.65 ± 0.09
fructose	10.66 ± 0.20	7.83 ± 0.10	0.72 ± 0.03
mannitol	14.98 ± 0.50	10.81 ± 1.25	0.74 ± 0.03
mannose	123	92	0.76
maltose	8	7	0.88
galactose	11.13 ± 0.42	16.08 ± 0.81	1.39 ± 0.04
arabinose	2.48 ± 0.26	3.13 ± 0.35	1.27 ± 0.06

[a] Cells for measurement of carbon source uptake were grown at 30 °C in M9 medium plus 0.2% carbon source to a cell density of 3 X 10^8 cells/mL. At that point the culture was then split into two aliquots with one being incubated at 30 °C and the other at 42 °C. After 45 min, both cultures were chilled rapidly on ice and collected by centrifugation at 2400*g* for 15 min at 4 °C. The cells were then washed once in cold M9 medium (minus carbon source) and resuspended in cold M9 medium (minus carbon source) at approximately 5 X 10^8 cells/mL. The uptake of the ^{14}C-labeled carbon sources was measured essentially as described by Saier et al. (1976). The values for relative rate of carbon source uptake are expressed as ± standard error of the mean.

It is also possible to ask whether the effect exerted by ppGpp levels in 2S142 at 42 °C is specific for rRNA synthesis or is a global effect on RNA synthesis. A linear regression analysis of the rate of total RNA synthesis at 42 °C (Table II) vs. ppGpp levels gives a correlation coefficient of 0.69 (P = 0.0001). This suggests that ppGpp levels are a slightly better predictor of the rate of total RNA synthesis than of rRNA synthesis under our experimental conditions.

Effect of Carbon Source on RNA Synthesis at 42 °C. We have reported elsewhere that cAMP levels, ATP levels, GTP levels, adenylate energy charge, rate of glucose uptake, and levels of glycolytic intermediates do not appear to be involved in the restriction of stable RNA synthesis in 2S142 at 42 °C (Reynolds & Chaney, 1983). However, restriction of glucose uptake at 42 °C in 2S142 does appear to affect ppGpp levels (Harris et al., 1978; Reynolds & Chaney, 1983), so uptake of several different carbon sources at both 30 and 42 °C was determined in 2S142 (Table III). The rate of carbon source uptake in 2S142 at 42 °C varies greatly depending on the carbon source and obviously does not correlate well with the restriction of stable RNA synthesis. Of course, since the various carbon sources are utilized with different efficiencies, it is possible that the restriction of carbon source uptake at 42 °C relative to the uptake at 30 °C might be the parameter which most closely correlated with rRNA synthesis. While

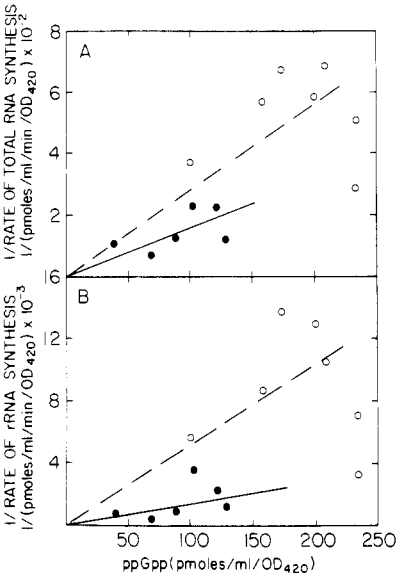

FIGURE 5: Segmented linear regression analysis of RNA synthesis vs. ppGpp levels in 2S142 on different carbon sources. The rates of rRNA synthesis were measured as described in Table II. ppGpp levels were measured as described in Table I. (○) 6- and 12-carbon sugars; (●) other carbon sources. The dashed line indicates the best fit for the 6- and 12-carbon sugars and the solid line indicates the best fit for the other carbon sources by segmented linear regression analysis. The data points represent average values. Intercept and slopes were calculated by segmented linear regression analysis using total data points. (A) Total RNA synthesis vs. ppGpp levels; (B) rRNA synthesis vs. ppGpp levels.

the relative rate of carbon source uptake (42 °C/30 °C) does have a slight predictive value for the rate of rRNA synthesis at 42 °C (linear correlation coefficient = 0.54, P = 0.21), it adds very little to the predictive value of ppGpp levels alone by multiple linear regression analysis. When just the seven carbon sources for which data are available for all three variables are used, ppGpp levels are predictive of the rate of rRNA synthesis with a correlation coefficient of 0.52. Addition of the relative rate of carbon source uptake as a second independent variable only increases the correlation coefficient to 0.55.

While there does not appear to be an extremely strong correlation in general between ppGpp levels at 42 °C and the rate of stable RNA synthesis, there is one very interesting observation concerning the different types of carbon sources. Visual inspection of the data in Figure 5 suggests that 6- and 12-carbon sugars behave differently than other carbon sources. This is confirmed by segmented linear regression analysis. Separation of the data into these two separate data sets improves the overall correlation coefficients from 0.64 to 0.79 for rRNA synthesis vs. ppGpp levels (P = 0.0001) and from 0.69 to 0.85 for total RNA synthesis vs. ppGpp levels (P = 0.0001). It is interesting to note that the correlation between rRNA or total RNA synthesis and ppGpp levels appears to be worse for the 6- and 12-carbon sugars than for the other carbon sources tested.

Discussion

One might expect that differential responses to carbon source could be explained most readily on the basis of the efficiency of carbon source utilization (measured most conveniently by comparing the growth rate, μ, on the different carbon sources). In fact, assuming relatively constant efficiency of ribosome utilization, one can predict that f_r (rate of rRNA synthesis/rate of total RNA synthesis) should vary linearly with μ at any given temperature (Bremer et al., 1973;

Ryals et al., 1982b). This relationship, for the most part, appears to hold true for 2S142 at 30 °C, D10 at 30 °C, and D10 at 42 °C (Figure 2A). The one major exception to the linear correlation between f_r and μ was the carbon source fructose. Invariably, fructose grown cells had higher f_r's than would appear to be necessary to support their growth rate. The correlation between f_r and growth rate also clearly breaks down when 2S142 is shifted to 42 °C. There is no apparent correlation between f_r at 42 °C and either the linear growth rate at 42 °C (Figure 2B) or μ at 30 °C (data not shown).

Neither f_r nor the rate of rRNA synthesis appear to correlate with ppGpp levels during steady-state growth in 2S142 at 30 °C. This is in contrast to a recent report by Ryals et al. (1982b) showing an inverse correlation between f_r and ppGpp during the steady-state growth of isogenic relA$^+$/relA strains on various media. Possibly this difference may reflect a partial expression of the mutant phenotype in 2S142 at 30 °C. 2S142 does grow significantly slower than D10 on all carbon sources at 30 °C. However, Ryals et al. (1982b) also used only two different carbon sources (glycerol and glucose) compared to the 13 carbon sources used in this study. Most of the differences in media in their experiments were ones affecting the amino acid supply.

The elevation in ppGpp levels and restriction in rRNA synthesis observed in 2S142 at 42 °C are clearly the effects of the previously described mutation in this strain. No significant elevation of ppGpp levels are seen in 2S142 at 30 °C (Table I) or in the isogenic parental strain D10 at either 30 or 42 °C (Figure 1; Harris et al., 1978). Previous studies have suggested that the temperature shift from 30 to 42 °C in 2S142 mimics a carbon source downshift (Harris et al., 1978). While synthesis of rRNA is restricted and ppGpp levels increase on all carbon sources at 42 °C, there is a relatively poor correlation between the rate of rRNA synthesis and ppGpp levels at the restrictive temperature (Figure 4). Fiil et al. (1972) have observed an excellent correlation between rRNA synthesis and ppGpp levels during amino acid starvation on a single carbon source (glucose). Yet in these experiments, mimicking carbon source downshift on 13 different carbon sources, only a partial correlation is observed.

Detailed statistical analysis suggested that neither carbon source uptake at 42 °C nor the restriction of uptake at 42 °C relative to the rate at 30 °C had any significant effect on the rate of rRNA synthesis. In these experiments the rate of uptake of carbon source (transport plus utilization) was measured since nonmetabolizable analogues were not available for all carbon sources. However, previous experiments have shown that restriction of glucose uptake and methyl α-D-glucoside transport at 42 °C are comparable (Reynolds & Chaney, 1983).

The most interesting aspect of these studies were the observations that (1) when the carbon sources were divided into two categories (6- and 12-carbon sugars vs. all other carbon sources), a statistically significant improvement in the overall correlation between ppGpp levels and the rates of RNA synthesis at 42 °C was observed and (2) ppGpp levels appeared to be a less effective predictor of rRNA or total RNA synthesis for cells grown on 6- and 12-carbon sugars at 42 °C compared to other carbon sources (Figure 5). The reason for this apparent difference between 6- and 12-carbon sugars and other carbon sources is not known at present but does not appear to relate to the method of sugar transport or the point of entry into cellular metabolism. For example, the 6- and 12-carbon sugars which we have investigated are transported by a variety of different and independent transport systems and enter cellular metabolism at points ranging from glucose 6-phosphate to glyceraldehyde 3-phosphate.

While the effect of carbon source on rRNA synthesis could be peculiar observation with our mutant strain, it is interesting to note that almost all the previous studies on control of stable RNA synthesis during amino acid starvation and carbon source deprivation have used glucose as a sole or primary carbon source. Preliminary data from our laboratory indicate that ppGpp levels vary widely when carbon source downshifts are carried out by using succinate as the secondary carbon source and a wide variety of sugars other than glucose as the primary carbon source. We feel that our data warrant further investigation of these phenomena using a variety of carbon sources, as the control of RNA synthesis may be affected by the carbon source present.

Acknowledgments

Statistical analysis of the data was carried out by Dr. Dennis Gillings of the University of North Carolina Biometric Consulting Laboratory.

Registry No. ppGpp, 32452-17-8; glucose, 50-99-7; fructose, 57-48-7; mannitol, 69-65-8; mannose, 3458-28-4; maltose, 69-79-4; succinic acid, 110-15-6; galactose, 59-23-4; glycerol, 56-81-5; lactose, 63-42-3; gluconic acid, 526-95-4; lactic acid, 50-21-5; arabinose, 147-81-9; acetic acid, 64-19-7.

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Characterization of Deoxyribonucleic Acid Synthesis in Reconstituted Nuclear Systems†

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ABSTRACT: Reconstituted nuclear systems for the analysis of mammalian cell DNA replication were characterized in detail. These in vitro systems were made by adding back nuclear salt extracts to salt-treated nuclei which were deficient in some steps of DNA replication. In the reconstituted nuclear system consisting of 0.2 M KCl treated nuclei, which were deficient in the function to synthesize high molecular weight DNA, and their complementary nuclear extract, the function to convert Okazaki fragments to high molecular weight DNA was restored. The reconstitution between 0.3 M KCl treated nuclei, which had little capacity to synthesize DNA, and 0.3 M KCl nuclear extract from 0.2 M KCl treated nuclei resulted in the restoration of the ability to synthesize Okazaki fragments. DNA synthesis in these reconstituted nuclear systems required ATP, magnesium ion, and four deoxyribonucleoside triphosphates and was markedly inhibited by aphidicolin and by 1-β-D-arabinofuranosylcytosine 5′-triphosphate but not by 2′,3′-dideoxythymidine triphosphate. By use of 0.2 M KCl treated nuclei and a reconstituted nuclear system consisting of 0.2 M KCl treated nuclei and their complementary nuclear extract, the requirement of ATP in the processes of DNA replication was investigated. A high level of ATP was required for the synthesis of Okazaki fragments, and it was substituted by a high level of dATP and ADP completely and partially, respectively. The Okazaki fragments synthesized in 0.2 M KCl treated nuclei in the presence of a high level of ADP were converted to high molecular weight DNA with a low level of ATP in the presence of the complementary nuclear extract, whereas in the presence of a high level of dATP, the conversion was not observed even in the presence of a low level of ATP and the complementary nuclear extract.

Biochemical studies of DNA replication of mammalian cells have been difficult not only due to the complex nature of the replicative processes but also due to the absence of suitable systems for detecting enzymes and factors involved in the processes. The establishment of systems, which enable us to identify enzymes and factors and to evaluate their roles in connection with the processes of DNA replication, is an important step toward the elucidation of the molecular mechanism of mammalian DNA replication.

Although isolated nuclei from mammalian cells have been proved a useful experimental system for the study of the mechanism of DNA replication (Friedman & Mueller, 1968; Lynch et al., 1970; Hershey et al., 1973; Krokan et al., 1975a,b; Benbow & Ford, 1976; Jazwinski et al., 1976; Hooton & Hoffbrand, 1977; Seale, 1977; Wist, 1979; Oguro et al., 1980; Nagata et al., 1981), the nuclear system is too complex to study the factors involved in the processes of DNA replication. Seki & Mueller (1976) have demonstrated the dissociation and reconstitution of a nuclear system by mild salt treatment. By using a similar system, Brun & Weissbach (1978) provided the evidence suggesting that RNA polymerase I was involved in the priming event for DNA synthesis.

We have shown (Tanuma et al., 1980) that by mild salt treatment of isolated nuclei at appropriate concentrations, factors necessary for some steps of DNA replication can be selectively solubilized from isolated nuclei, leaving salt-treated nuclei capable of carrying out other steps of DNA replication. Therefore, the reconstituted nuclear system using the salt-treated nuclei as a "natural template" may become a valuable tool for the identification and subsequent characterization of factors involved in DNA replication.

We describe here the characterization of reconstituted nuclear systems made by adding back nuclear salt extracts to salt-treated nuclei. In addition, by using salt-treated nuclei and a reconstituted nuclear system, we have investigated the roles of ATP in the processes of the synthesis and subsequent joining of Okazaki fragments.

Materials and Methods

Materials. Aphidicolin was kindly supplied by Dr. S. Ikegami (University of Tokyo) and Dr. M. Ohashi (Tokyo Metropolitan Institute of Gerontology).

Cell Culture and Synchronization. HeLa S3 cells were maintained in a monolayer culture in Eagle's minimal essential medium supplemented with 10% calf serum. Synchronization was carried out by exposing cells to 1 mM hydroxyurea for 16 h.

Isolation of Nuclei. Nuclei were isolated from HeLa S3 cells 3 h after the release from the hydroxyurea block as described previously (Tanuma et al., 1979). Cells were suspended at a concentration of 2×10^7/mL in buffer A (10 mM Tris-HCl,[1] pH 7.8, 3 mM $MgCl_2$, 1 mM Na_2EDTA, 2 mM

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[1] Abbreviations: araCTP, 1-β-D-arabinofuranosylcytosine triphosphate; ddTTP, 2′,3′-dideoxythymidine triphosphate; EDTA, ethylendiaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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