Cellular Content of Chloroplast DNA and Chloroplast Ribosomal RNA Genes in *Euglena gracilis* during Chloroplast Development†

Barry K. Chelm, Patricia J. Hoben, and Richard B. Hallick*

ABSTRACT: The cellular content of chloroplast DNA in *Euglena gracilis* has been quantitatively determined. DNA was extracted from *Euglena* cells at various stages of chloroplast development and renatured in the presence of trace amounts of ^{3}H-labeled chloroplast DNA. From the kinetics of renaturation of the ^{3}H-labeled chloroplast DNA, compared with the kinetics of renaturation of excess nonradioactive chloroplast DNA, the fraction of cellular DNA represented by chloroplast DNA was calculated. The content of chloroplast DNA was found to increase from 4.9 to 14.6% of cellular DNA during light-induced chloroplast development. Correcting for the change in DNA mass per cell, the number of copies of chloroplast DNA is found to vary from 1400 to 2900 per cell. During this developmental transition, the cellular content of the chloroplast ribosomal RNA genes varies from 1900 to 5200 copies per cell. The ratio of the number of copies of rRNA genes to chloroplast genomes per cell remains in the range of 1–2 throughout chloroplast development, ruling out selective amplification of chloroplast rRNA genes as a means of regulation of rRNA gene expression. Direct measurement of the number of rRNA cistrons per 9.2×10^7 dalton genome yields a value of 1 or 2.

Euglena gracilis chloroplast DNA has been characterized as a covalently closed, superhelical duplex molecule with a molecular weight of 9.2×10^7 (Manning and Richards, 1972). Although it has been recognized that multiple copies of chloroplast DNA are present in *Euglena* cells (Brawerman and Eisenstadt, 1964; Edelman et al., 1964; Ray and Hanawalt, 1964), and that the chloroplast DNA content varies with the cell growth conditions (Gibson and Hershberger, 1975), quantitative data on the cellular content of chloroplast DNA are limited. Originally the DNA content per *Euglena* chloroplast was estimated at from 1.2×10^{-15} (Edelman et al., 1964) to 9×10^{-15} g (Brawerman and Eisenstadt, 1964), or between 8 and 60 copies per chloroplast, respectively. Recently the cellular chloroplast DNA content of stationary phase *Euglena* cells has been measured by the kinetics of renaturation of cellular DNA to ^{125}I-labeled chloroplast DNA (Rawson and Boerma, 1976). Chloroplast DNA content was reported to vary from 217 molecules/cell for dark adapted cells to 1014 molecules/cell for light-grown cells on an autotrophic medium.

In a recent study from this laboratory, the cellular content of the chloroplast ribosomal RNA genes was quantitatively measured by the kinetics of hybridization of cell DNA to purified ^{3}H-labeled chloroplast ribosomal DNA (Chelm et al., 1977). The content of this gene varied from 1900 to 5200 copies per cell, depending on the stage of chloroplast development. The number of copies of rRNA gene per 9.2×10^7 dalton genome has been estimated as one (Rawson and Haselkorn, 1973), three (Stutz and Vandrey, 1971), and three or six (Scott, 1973). It is apparent that the cellular content of rRNA genes (Chelm et al., 1977) is higher than one would have estimated from the reported values for chloroplast DNA content and rRNA cistron content per genome.

The high content of rRNA cistrons could be due either to a high content of the entire genome or a selective amplification of the rRNA genes. We have tested these two possibilities by measuring the cellular content of chloroplast DNA and chloroplast rDNA cistrons in *Euglena* at various times during light-induced chloroplast development. We find that the cellular content of chloroplast DNA in exponentially growing *Euglena* cells is higher than any of the previously reported values. There is no indication of selective amplification of the rRNA cistrons.

Methods

Growth of Cells. The preparation of dark-adapted cultures, the protocol for greening cultures, and the procedure for harvesting cells at various stages of chloroplast development have previously been described (Chelm and Hallick, 1976). The permanently bleached *Euglena* var. *bacillaris*, strain, W_8BHL (Schiff et al., 1971), obtained from Oliver C. Richards, was grown in a heterotrophic medium (Difco Euglena Broth).

Preparation of DNA. DNA was extracted from *Euglena* cells, purified by preparative CsCl density gradient centrifugation, and sheared to a DNA fragment size of 500–600 bases as previously described (Chelm et al., 1977). Covalently closed, superhelical *Euglena* chloroplast DNA was isolated from purified chloroplasts by the method of Richards and Manning (1974). Chloroplasts were isolated from phototrophically grown cells by a previously described method (Manning et al., 1971). Chloroplasts were lysed at 0 to 4 °C with predigested Pronase (1 mg/ml) and 1% *N*-laurylsarcosine. The lysate was subsequently mixed with an equal volume of 7.14 M CsCl, 1.25 mg/ml ethidium bromide, 0.04 M Tris-HCl, pH 8.0. The resulting mixture was transferred to a cellulose nitrate centrifuge tube, overlayered with mineral oil, and centrifuged for 44 h at 44 000 rpm in a Spinco Type 65 rotor. Following centrifugation, DNA was visualized in two fluorescent bands when the centrifuge tube was illuminated by a Blak-Ray Model ML-49 ultraviolet lamp. The supercoil band, found approximately 8 mm below the larger main band, was collected by suction with a syringe fitted with a 16-gauge needle. The resulting DNA

† From the Department of Chemistry, University of Colorado, Boulder, Colorado 80309. *Received July 15, 1976.* This work was supported by Grant GM 21351 from the National Institutes of Health and Grant BMS 75-14541 from the National Science Foundation.

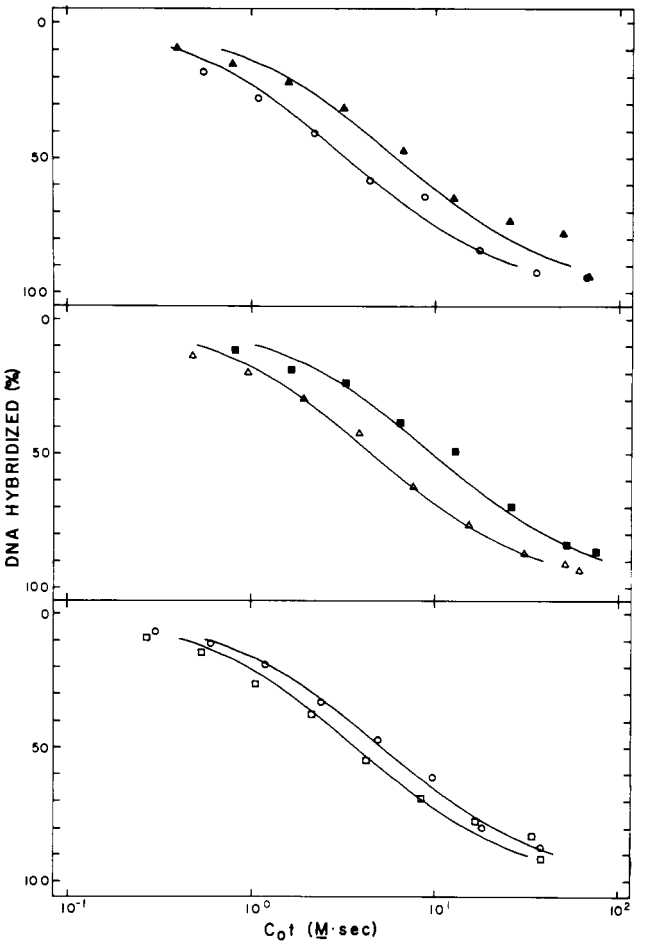

FIGURE 1: Hybridization of [^{3}H]ct DNA with DNA extracted from *Euglena* cells at different stages of chloroplast development. Top panel: (▲) 8 h of development; (○) 48 h of development. Middle panel: (■) 4 h of development; (△) 24 h of development. Bottom panel: (○) 0 h of development; (□) 72 h of development.

solution was extracted twice with 1-butanol and dialyzed extensively against 0.1 × SSC.[1]

Preparation of ^{3}H-Labeled Chloroplast DNA. Covalently closed, superhelical chloroplast DNA was labeled in vitro by previously described procedures (Chelm and Hallick, 1976). Rapidly renaturable ^{3}H-labeled chloroplast DNA, accounting for 16% of the in vitro labeled DNA, was removed by chromatography on hydroxylapatite (Chelm et al., 1977). The specific activity of the ^{3}H-labeled chloroplast DNA product was 5.8 μCi/μg of DNA.

DNA-DNA Hybridization. The cellular content of chloroplast DNA at various stages of chloroplast development was determined from the kinetics of hybridization of excess total cellular DNA to trace amounts of ^{3}H-labeled chloroplast DNA. The procedures for hybridization reactions, detection of hybrids using an S1-nuclease criteria, and the determination of second-order rate constants have previously been described (Chelm et al., 1977).

Results

Cellular Chloroplast DNA Content during Chloroplast Development. To determine the fraction of cellular DNA represented by chloroplast DNA, cell DNA driven hybridizations to ^{3}H-labeled chloroplast DNA were performed. The kinetics of hybridization of total cellular DNA, extracted from *Euglena* cells 0, 4, 8, 12, 48, and 72 h after the onset of light-induced chloroplast development, were determined. The C_0t curves for the six DNA preparations, and also for purified chloroplast DNA, are shown in Figure 1. Data were analyzed by least-squares standard deviation fit to the second-order rate equation $C/C_0 = 1/(1 + kC_0t)$ (Britten and Kohne, 1968), where C_0 is the initial concentration of ^{3}H-labeled chloroplast DNA, C/C_0 is the fraction of the concentration of ^{3}H-labeled chloroplast DNA remaining single stranded, t is the time in s, and k is the rate constant in l. mol^{-1} s^{-1}. In each case the hybridization reactions followed the expected second-order kinetics. The rate constants for each reaction are listed in Table I.

TABLE I: Cellular Chloroplast DNA Content during Chloroplast Development.

Hours of Chloroplast Development	Second-Order Rate Constant (l. mol^{-1} s^{-1})	% of Total Cell DNA[a]
0	0.194 ± 0.009	9.3
4	0.103 ± 0.010	4.9
8	0.166 ± 0.020	7.9
12	0.228 ± 0.014	10.9
48	0.306 ± 0.021	14.6
72	0.269 ± 0.024	12.9
Chloroplast DNA	2.09 ± 0.19	

[a] $(k_{\text{cell DNA}}/k_{\text{ct DNA}}) \times 100$.

The rate constant for purified chloroplast DNA is 2.09 l. mol^{-1} s^{-1}, which corresponds to a $C_0t_{1/2}$ of 0.48 mol s/l. This value is consistent with the expected value for renaturation of DNA with a complexity of 139 kbp in 0.3 M NaCl (Britten and Kohne, 1968). The $C_0t_{1/2}$ also agrees with our previously reported value, when nonsuperhelical chloroplast DNA was used for ^{3}H-labeled chloroplast DNA preparation and driver (Chelm and Hallick, 1976).

From a comparison of the rate constant for total cell DNA driven reactions with that for purified chloroplast DNA, the fraction (f) of total cell DNA represented by chloroplast DNA can be calculated from the equation

$$f = k_{\text{cell DNA}}/k_{\text{ct DNA}}$$

The results on cellular chloroplast DNA content are summarized in Table I. In exponentially growing, dark-adapted cells, 9.3% of the cell DNA is chloroplast DNA. The chloroplast DNA content decreases to 4.9%, following the shift to an acid phototrophic medium and the onset of light-induced development, and subsequently increases to greater than 12% after 48 h of development. In a control experiment, DNA from the permanently bleached *Euglena* strain, W$_8$BHL, shows no renaturation with ^{3}H-labeled chloroplast DNA at C_0t of 100 mol s/l. The reactions for DNA from the dark-adapted cells or greening cells are all complete at a C_0t of 100, indicating that no nuclear DNA or ^{3}H-labeled chloroplast DNA self-renaturation has occurred. At a C_0t of 4200 mol s/l., 0.28 of the ^{3}H-labeled chloroplast DNA has renatured in the W$_8$BHL-driven reaction. The calculated C_0t for the ^{3}H-labeled chloroplast DNA in this reaction was 0.03 mol s/l., indicating that self-renaturation of ^{3}H-labeled chloroplast DNA may have contributed to the observed reaction.

[1] Abbreviations used: SSC, 0.15 M NaCl-0.015 M sodium citrate; kbp, kilo basepair; C_0t, DNA nucleotide concentration in M × t in s; rRNA, ribosomal RNA; X_{pSC101}, kinetic complexity of pSC101 DNA; $X_{\text{ct rDNA}}$, kinetic complexity of chloroplast rRNA cistrons; $X_{\text{ct DNA}}$, kinetic complexity of chloroplast DNA; Tris, tris(hydroxymethyl)aminomethane.

TABLE II: Number of Copies of Chloroplast DNA and Chloroplast rRNA Genes per *Euglena gracilis* Cell during Light Induced Chloroplast Development.

Hours of Development	Copies of Chloroplast DNA	Copies of rRNA Genes	Ratio of rRNA Genes/ Chloroplast DNA
0	2800	3800	1.4
4	1400	3000	2.1
8	1500	1900	1.3
12	2100	3700	1.8
48	2900	4000	1.4
72	2500	5200	2.1

Number of Copies of Chloroplast DNA and Chloroplast Ribosomal RNA Genes. It is possible to calculate the number of copies of chloroplast genome per *Euglena* cell from these cellular DNA content data. The hybridization reactions described above were done simultaneously, and with the same DNA preparations, as were used for our study on chloroplast ribosomal RNA gene content (Chelm et al., 1977). Therefore we can use the experimentally determined data on total DNA mass per cell at various stages of chloroplast development (Chelm et al., 1977) to calculate the average number of copies of 9.2×10^7 dalton genome present in the cell. These values are listed in Table II. For comparison, the previously determined values for ribosomal RNA gene content and the ratio of copies of ribosomal RNA cistron to chloroplast genome are also shown.

From the data in Table II, the number of copies of chloroplast DNA per *Euglena* cell is seen to vary from 1400 to 2900 for the growth conditions employed in this study. The ratio of the number of copies of chloroplast rRNA cistrons to chloroplast genomes remains in the range of 1–2 throughout chloroplast development. It is apparent that the previously observed high content of rRNA genes (Chelm et al., 1977) is due to a high cellular content of the chloroplast genome, and not a selective amplification of chloroplast rRNA genes.

Number of Ribosomal RNA Cistrons per Euglena Chloroplast Genome. The availability of ^{3}H-labeled chloroplast DNA, ^{3}H-labeled chloroplast ribosomal DNA, and covalently closed, superhelical DNA affords the possibility of estimating the rRNA cistron content from the kinetics of DNA–DNA hybridizations in solution. Isolation of superhelical chloroplast DNA for this experiment assures that all cistrons are present in the DNA in the same stoichiometry that obtains in the intact genome. This is of particular importance in studying *Euglena* chloroplast ribosomal cistrons because the rRNA cistrons can be separated as a satellite DNA ($\rho = 1.692$ g/cm^3) in buoyant density centrifugation of chloroplast DNA (Vandrey and Stutz, 1973; Rawson and Haselkorn, 1973).

The number of copies of the rRNA cistrons can be estimated from a comparison of the kinetics of hybridization of an excess superhelical chloroplast DNA to trace amounts of either ^{3}H-labeled chloroplast DNA or ^{3}H-labeled chloroplast ribosomal DNA. The C_0t curves for this experiment are shown in Figure 2. The best fit second-order rate constant for the reaction with ^{3}H-labeled chloroplast ribosomal DNA ($k_{\text{ct rDNA obsd}}$) is 5.67 ± 0.67 l. mol^{-1} s^{-1}. The rate constant for the reaction with ^{3}H-labeled chloroplast DNA ($k_{\text{ct DNA}}$) is 2.09 ± 0.20. At any C_0t value, the effective C_0t for the reaction to the ^{3}H-labeled chloroplast ribosomal DNA is in principle an integral number times the C_0t for the reaction to the ^{3}H-labeled chloroplast DNA. The integral number or

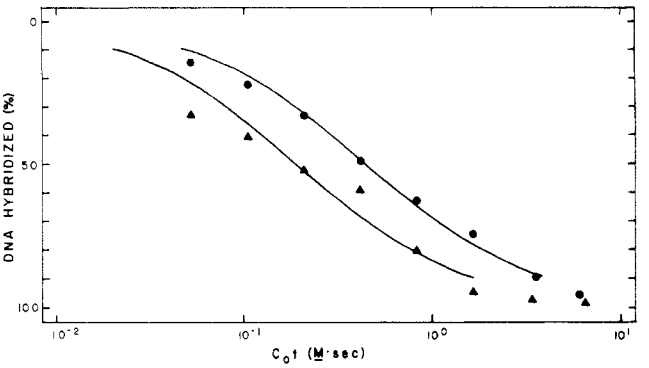

FIGURE 2: Hybridization of chloroplast DNA isolated as supercoils to [^{3}H]ct DNA and [^{3}H]ct rDNA. (●) [^{3}H]ct DNA; (▲) [^{3}H]ct rDNA.

repetition frequency can be calculated from the above k values. Since $k = 1/(C_0t_{1/2})$ (Britten and Kohne, 1968), the number of copies of rRNA cistron per genome (N) is then estimated from the equation

$$N = k_{\text{ct rDNA obsd}}/k_{\text{ct DNA}}$$

yielding a value of 2.7. This value may be higher than the true value because of the difference in base composition between the rRNA cistrons, which are 51 mol % G + C base pairs (Rawson and Stutz, 1969) and the chloroplast genome, which is 25 mol % G + C base pairs. The second-order rate constant for DNA renaturation is known to increase with increasing G + C base content (Wetmur and Davidson, 1968).

Alternatively the rRNA cistron content may be determined by comparing $k_{\text{ct rDNA obsd}}$ to the k of an appropriate hybridization standard with a base composition similar to the rRNA cistrons. The *E. coli* plasmid pSC101 DNA, whose base composition and kinetic complexity (51 mol % G + C, 8.8 kbp) are similar to those of chloroplast ribosomal RNA cistrons (51 mol % G + C, 4.8 kbp), renatures with a $k_{\text{pSC101}} = 71.6 \pm 5.1$ l. mol^{-1} s^{-1} (Chelm et al., 1977). The fraction of chloroplast DNA driving the hybridization reaction is given by the equation

$$f = k_{\text{ct rDNA obsd}}/k_{\text{ct rDNA pure}}$$

The value of $k_{\text{ct rDNA pure}}$ is calculated from a comparison with k_{pSC101}. Since the rate constant for renaturation is inversely proportional to complexity (Wetmur and Davidson, 1968)

$$k_{\text{ct rDNA pure}} = \frac{(k_{\text{pSC101}})(X_{\text{pSC101}})}{(X_{\text{ct rDNA}})}$$

where X_{pSC101} and $X_{\text{ct rDNA}}$ are the kinetic complexities of pSC101 DNA and chloroplast rRNA cistrons, respectively. Therefore, the fraction of chloroplast DNA representing rRNA cistrons is given by the equation

$$f = (k_{\text{ct rDNA obsd}})(X_{\text{ct rDNA}})/(k_{\text{pSC101}})(X_{\text{pSC101}})$$

Since N can be related to f by the equation

$$N = (f)(X_{\text{ct DNA}})/(X_{\text{ct rDNA}})$$

where $X_{\text{ct DNA}}$ is the complexity of the chloroplast genome, the number of copies of rRNA cistrons per genome is given by

$$N = (k_{\text{ct rDNA obsd}})(X_{\text{ct DNA}})/(k_{\text{pSC101}})(X_{\text{pSC101}})$$

This calculation yields a value of 1.2 rRNA cistrons per genome. The fact that this calculation yields a lower value than the value of 2.7 copies described above is consistent with the expected effects of base composition on $k_{\text{ct DNA}}$. The two

methods of estimating the number of rRNA cistrons per genome give values of either 1 or 2, the same range as was observed for the cellular ratio of rRNA genes to chloroplast genomes at various stages of chloroplast development (Table II). Therefore the entire cellular content of rRNA cistrons can be accommodated in the 139 kb chloroplast genomes.

Discussion

We have made use of total cell DNA driven hybridization reactions to ^{3}H-labeled chloroplast DNA from *Euglena* to quantitate the cellular content of chloroplast DNA at various stages of chloroplast development in the unicellular algae *Euglena gracilis*. The experimental values of 5 to 13% of cellular DNA as chloroplast DNA are higher than previous estimates based on the recovery of the chloroplast DNA satellite band (ρ = 1.685 g/cm^3) on equilibrium CsCl density gradients (Edelman et al., 1964; Brawerman and Eisenstadt, 1964; Scott, 1973). The values from these previous studies would be expected to be minimal estimates since they are based on an indirect criteria, the quantitative recovery of DNA from partially purified chloroplasts. In contrast, the procedure used in this study is based on a direct measurement of chloroplast DNA content in whole cell DNA. Our measurements on chloroplast DNA content are also higher than those of Rawson and Boerma (1976), who found that cellular chloroplast DNA content varied from 1 to 5% in cultures of *Euglena* grown to stationary phase. Rawson and Boerma also employed hybridization kinetics in their work. The differences may be accounted for by the different culture conditions in the two studies. We are in agreement with Rawson and Boerma that significant quantitative differences do occur in cellular chloroplast DNA content under different growth conditions. In the present study cellular chloroplast DNA content doubles between the early and late stages of chloroplast development in an exponential culture growing on a phototrophic medium.

Manning and Richards (1972) have studied the replication and turnover of chloroplast DNA during exponential growth in a phototrophic medium. Chloroplast DNA was found to replicate by a semiconservative mechanism at a rate 1.5 times as fast as nuclear DNA. At the same time chloroplast DNA turns over with a half-life of 1.6 generations. Therefore the observed changes in chloroplast DNA content could be due to an increase in the replication rate with respect to nuclear DNA, or a decrease in the turnover rate.

The observation that the ratio of rRNA cistrons to chloroplast genomes remains constant throughout development deserves comment. Work in several laboratories has led to the speculation that segments of the chloroplast DNA may be amplified under certain conditions of cell growth. The minicircular DNA first observed by Nass and Ben-Shaul (1972) is one candidate for an amplified segment. It has been observed that the buoyant density of this DNA is similar to the chloroplast ribosomal DNA satellite (Nass and Ben-Shaul, 1972). It has also been reported that a chloroplast satellite band increases during logarithmic cultures compared with stationary cultures (Manning and Richards, 1972), and conversely increases when the cells entered stationary phase of growth (Gibson and Hershberger, 1975). These results have led to the speculation that the chloroplast rRNA cistrons are capable of being amplified. Our results, however, rule out the possibility of rRNA cistron amplification under the cell growth conditions employed in this study. In addition, if any other large (>10 kbp) segment of the chloroplast genome were amplified, we would likely have observed deviation from single component second-order kinetics in whole cell driven reactions to ^{3}H-labeled chloroplast DNA. We would therefore suggest that, if amplified segments of chloroplast DNA do exist, they must be non-rRNA cistrons and either small DNA segments or chloroplast DNA sequences not present in the intact 9.2×10^7 dalton chloroplast genome.

There have been several previous determinations of the number of ribosomal RNA cistrons per intact chloroplast genome (Stutz and Vandrey, 1971; Scott, 1973; Rawson and Haselkorn, 1973). These estimates were based on the amount of [^{32}P]- or [^{3}H]rRNA that would hybridize to purified chloroplast DNA previously immobilized on cellulose nitrate filters. Scott reported that 6.2% of the chloroplast DNA is complementary to chloroplast rRNA, corresponding to 3.6 copies per 9.2×10^7 dalton genome. Our data are not compatible with this value nor with the observation (Scott, 1973) that rRNA cistrons are not detected in dark-adapted *Euglena* cells. Stutz and Vandrey (1971) reported that 6% of chloroplast DNA hybridized with chloroplast rRNA. Although this report is difficult to evaluate, it also appears incompatible with our results. Rawson and Haselkorn (1973) found that 1.9% of chloroplast DNA hybridized at saturation with ^{3}H-labeled chloroplast RNA, corresponding to 1.1 rRNA cistrons per genome. These authors state the reservation that their measurement is based on the assumption that immobilized DNA has a 100% efficiency of hybridization at rRNA saturation. Our data on rRNA cistron content per chloroplast genome, based on a solution hybridization procedure, are most consistent with a value of one or two, in agreement with Rawson and Haselkorn (1973). We do not consider this an unequivocal answer. It is doubtful whether hybridization experiments can provide the final answer to this question. A different procedure, perhaps based on physical mapping of rRNA cistrons on the genome, seems to be required to solve this problem.

Acknowledgment

We thank Patrick W. Gray for help in the preparation of covalently closed, superhelical DNA, and Dr. Marvin Caruthers for the generous gift of *E. coli* DNA polymerase I. We also thank Dr. Oliver C. Richards, Dr. James R. Y. Rawson, and Dr. Jerome A. Schiff for the communication of results prior to publication.

References

Brawerman, G., and Eisenstadt, J. M. (1964), *Biochim. Biophys. Acta 91,* 477.

Britten, R. J., and Kohne, D. E. (1968), *Science 161,* 529.

Chelm, B. K., and Hallick, R. B. (1976), *Biochemistry 15,* 593.

Chelm, B. K., Hoben, P. J., and Hallick, R. B. (1977), *Biochemistry 16,* preceding paper in this issue.

Edelman, M., Cowan, C. A., Epstein, H. T., and Schiff, J. A. (1964), *Proc. Natl. Acad. Sci. U.S.A. 52,* 1214.

Gibson, W. H., and Hershberger, C. L. (1975), *Arch. Biochem. Biophys. 168,* 8.

Manning, J. E., and Richards, O. C. (1972), *Biochemistry 11,* 2036.

Manning, J. E., Wolstenholme, D. R., Ryan, R. S., Hunter, J. A., and Richards, O. C. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1169.

Nass, M. M. K., and Ben-Shaul, Y. (1972), *Biochim. Biophys. Acta 272,* 130.

Rawson, J. R., and Stutz, E. (1969), *Biochim. Biophys. Acta 190,* 368.

Rawson, J. R. Y., and Boerma, C. (1976), *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Rawson, J. R. Y., and Haselkorn, R. (1973), *J. Mol. Biol. 77*, 125.
Ray, D. S., and Hanawalt, P. C. (1964), *J. Mol. Biol. 9*, 812.
Richards, O. C., and Manning, J. E. (1974), Colloques Internationaux du CPNRS No. 240-Les Cycles Cellulaires et Leur Blocage Chez Plusieurs Protistes, p 231.
Schiff, J. A., Lyman, H., and Russel, G. K. (1971), *Methods Enzymol. 23*, 143.
Scott, N. S. (1973), *J. Mol. Biol. 81*, 327.
Slavik, N. S., and Hershberger, C. L. (1975), *FEBS Lett. 52*, 171.
Stutz, E., and Vandrey, J. P. (1971), *FEBS Lett. 17*, 277.
Vandrey, J. P., and Stutz, E. (1973), *FEBS Lett. 37*, 174.
Wetmur, J. G., and Davidson, N. (1968), *J. Mol. Biol. 31*, 349.

Different Modes of Inhibition of Purified Ribonucleic Acid Directed Deoxyribonucleic Acid Polymerase of Avian Myeloblastosis Virus by Rifamycin SV Derivatives†

Corrado Gurgo* and Duane P. Grandgenett

ABSTRACT: The mechanism by which several rifamycin SV derivatives inhibit the purified $\alpha\beta$ DNA polymerase of avian myeloblastosis virus was investigated. The derivatives C-27 (rifamycin SV with dicyclohexylalkyl-substituted piperidyl ring at the 3 position), AF-013 (*O-n*-octyloxime of 3-formylrifamycin SV), and AF/DNFI (3-(2,4-dinitrophenylhydrazonemethyl)rifamycin SV), with major structural differences in their hydrophobic side chains, were selected for study from among the most potent inhibitors. The effect of drug was studied under two different assay conditions: when drug was added before template-primer, in order to examine the inhibition of an initial step(s) of DNA synthesis, or during the reaction of polymerization, to examine the inhibition of elongation and/or reinitiation. The ability of C-27, AF-013, and AF/DNFI, in order of decreasing activity, to inhibit the viral DNA polymerase at an initial step(s) was directly related to the lipophilicity of the compounds. When inhibition of later steps was examined, no correlation was observed. C-27 was the least inhibitory of the three derivatives when added during polymerization; analysis of the mode of inhibition demonstrated that reinitiation, but not chain elongation, was inhibited. Incorporation of triphosphates into chains initiated prior to drug addition continued in the presence of C-27 and was progressively blocked at later times, while immediate, complete inhibition of triphosphate addition to new primer molecules followed drug addition. Polyacrylamide gel profiles of poly(dT) synthesized in the presence and absence of the drugs were compared. The amount of product synthesized in the presence of C-27 was decreased, but there was no effect on the size distribution. Both the amount and the size of the product were decreased in the presence of AF-013, suggesting an effect on chain elongation as well as initiation. Kinetic evidence indicated that AF/DNFI had a mode of action similar to that of AF-013. All three derivatives appear to inhibit the viral enzyme with a strong cooperative interaction. However, when the initial rate of polymerization measured at different drug concentrations was analyzed according to Hill, different plots were observed. A straight line with a slope of 6.4 was obtained in the presence of C-27, and a biphasic plot with n values of 2.2 and 6.2 was observed with AF/DNFI, with the change in slope occurring at 65% inhibition. The results of our study are discussed in terms of different mechanisms of interaction of rifamycin SV derivatives with the viral DNA polymerase.

The ability of several rifamycin derivatives to inhibit oncornavirus reverse transcriptase, and to various extents other DNA and RNA polymerizing enzymes, has been reported (Gurgo et al., 1971, 1972, 1975; Yang et al., 1972; Green et al., 1971, 1972; Meilhac et al., 1972; Chamberlin and Ring, 1972; Gerard et al., 1973; Rose et al., 1975) and attributed to the presence of a hydrophobic side chain in the basic structure of the parental antibiotic rifamycin SV. On the basis of this observation, more selective derivatives, able to distinguish between viral reverse transcriptase and other polymerases, have been synthesized (Tischler et al., 1973; Thompson et al., 1974).

Inhibition of cell transformation and/or oncornavirus replication by rifamycin derivatives has been reported by several laboratories (Barlati and Vigier, 1972; Hackett et al., 1972; Ting et al., 1972; Hackett and Sylvester, 1972; Green et al., 1972, 1974; Bissel et al., 1974; Smith and Hackett, 1974; Shannon et al., 1974; Szabo et al., 1976). Thus far, it appears that when cytotoxic effects are avoided the antiviral activity observed may be the result of the inhibition of the reverse transcriptase and/or interference with other postpenetrational viral functions. MuLV[1] replication and cell transformation by

† From the Institute for Molecular Virology, St. Louis University Medical Center, St. Louis, Missouri 63110. *Received September 21, 1976.* This investigation was supported by Research Grant CA16266 from the National Institutes of Health.

[1] Abbreviations used are: MuLV, murine leukemia virus; MSV, murine sarcoma virus; AMV, avian myeloblastosis virus; NP-40, Nonidet P-40; AF-013, *O-n*-octyloxime of 3-formylrifamycin SV; AF/DNFI, 3-(2,4-dinitrophenylhydrazonemethyl)rifamycin SV; AF/ABDMP, 2,5-dimethyl-4-*N*-benzyldemethylrifampicin SV; C-27, rifamycin SV with dicyclohexylalkyl substituted piperidyl ring at the 3 position; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.