TEMPLATE FUNCTIONS IN THE ENZYMIC FORMATION OF POLYRIBONUCLEOTIDES, II. METAPHASE CHROMOSOMES AS TEMPLATES IN THE ENZYMIC SYNTHESIS OF RIBONUCLEIC ACID*

By Hsiang Ju Lin,† John D. Karkas, and Erwin Chargaff

CELL CHEMISTRY LABORATORY, DEPARTMENT OF BIOCHEMISTRY, COLUMBIA UNIVERSITY

A recent study from this laboratory[1] gave a preliminary account of experiments aiming at the isolation of chromosome preparations from synchronized HeLa cells in metaphase arrest. It appeared of interest to examine the template activity of these preparations in directing the incorporation of ribonucleotides by RNA polymerase, with respect to the amount and, more importantly, to the composition of the polymeric product formed; and to do this in comparison with the template behavior of the total free DNA isolated from the same HeLa cell cultures. The RNA normally present in the preparations of metaphase chromosomes is, similarly to cytoplasmic RNA, of a high GC-type, quite unlike the DNA of HeLa cells. It was one of the purposes of this study to ascertain whether the enzymic product formed with chromosomal preparations as templates resembled in any way the RNA component of the chromosomes. This proved not to be the case.

Materials and Procedures.—HeLa cells in suspension culture were grown in phosphate medium containing 0.3% (w/v) Methocel HG (Standard Grade, 4000 cP, Dow Chemical Co.) and 5% (v/v) whole horse serum.[2] The cells were synchronized and arrested at metaphase as described previously for stationary cultures.[1] Three cultures, each containing $1.2-1.5 \times 10^9$ cells of which about 40% were in metaphase, were fractionated so as to yield the preparations rich in chromosomes and free of nuclei and soluble DNA described previously as fraction C.[1] Two cycles of resuspension in 0.16 M NaCl solution and centrifugation ($1100 \times g$, 15 min, 4°) resulted in the recovery of 95% of the chromosomes, as judged from DNA assay. In the incorporation experiments the washed chromosomes were tested as a suspension in 0.16 M NaCl solution.

Four preparations of DNA were used. Preparations 1–3 were isolated from HeLa cells with the aid of Duponol[3] and purified in the usual manner (compare p. 327 of ref. 4). The fibers obtained by precipitation with ethanol were dissolved in, and dialyzed against, physiological saline, and the solution was stored in the frozen state at −35°. For the isolation of DNA preparation 4, another procedure described recently[5] was adapted. The preparation from HeLa cells comprising intact nuclei and aggregated chromosomes, designated as fraction P in a recent paper,[1] was washed twice by centrifugation ($1100 \times g$, 15 min, 4°) with 0.15 M NaCl–0.015 M sodium citrate (pH 7) and then treated with trypsin (0.3 mg/ml) in the same saline-citrate solution for 4 hr at 37°. After centrifugation the entire DNA was found in the supernatant layer which was then subjected to incubation with pronase and ribonuclease and to extraction with phenol, as described in the papers mentioned before.[5] The final preparation was essentially free of protein and RNA.

Two preparations of RNA were isolated with the use of a procedure applied previously to the preparation of RNA from HeLa cells.[6] The starting material for the specimen designated as cytoplasmic RNA was the cell lysate freed by centrifugation of nuclei and chromosomal particles.[1] The chromosomal RNA preparation was made from fraction C.[1]

The base composition of DNA was determined in the usual manner.[7] RNA was hydrolyzed to the mononucleotides and analyzed by the two-dimensional chromatography of the latter (isopropanol-NH₃, buffered isobutyrate) in the arrangement described before.[8]

RNA polymerase was prepared from frozen cells of *Escherichia coli*, strain W (Grain Processing Co., Muscatine, Iowa) by a procedure patterned after one described previously,[9] with the modifications mentioned in a recent paper.[10] Other enzymes used were commercial preparations.

The radioactive triphosphates of adenosine (8-C[14]), guanosine (8-C[14]), cytidine (2-C[14]), and uridine (2-C[14]) were supplied by Schwarz BioResearch, Inc. For use as precursors, they were diluted with the corresponding unlabeled nucleoside triphosphates. The specific activities of the precursors employed, which will be mentioned later in the tables, were determined with a precision of about 3% after purification through paper chromatography.[10]

The incorporation experiments were performed in 0.04 M Tris buffer of pH 7.9 at 37°. The total volume in each assay was 0.5 ml, with the following additions (as μmoles per 0.5 ml): 2 $MgCl_2$, 0.5 $MnCl_2$, six 2-mercaptoethanol. The concentrations of enzyme, template, and precursors will be stated in the tables. The enzymic reaction was stopped by the addition of 10 vol of 10% (w/v) trichloroacetic acid (4°). The products insoluble in acid, collected on membrane filters (Schleicher & Schüll, type B-6), and washed five times with 5% trichloroacetic acid were dissolved in 3% NH_3 (w/v), scintillation fluid[11] was added, and this was followed by assay in a liquid scintillation counter.

Results.—We give first, in Table 1, the results of the quantitative analyses. The data on the cellular concentrations of DNA and RNA are in accord with our previous findings.[1] The base composition of DNA may best be evaluated in the light of what is known about the DNA of human cells. The most extensive direct analyses have yielded a dissymmetry ratio (A + T/G + C) of 1.53 for human DNA.[12] Except for a slight shift in the purine values, the present results are in good agreement with the previous observations. The agreement with other analytical data on HeLa-DNA,[13, 14] which also do not agree with each other, however, is less satisfactory. The two RNA preparations investigated here—one of nuclear, the other of cytoplasmic origin—differ little in composition: they are both of an extreme GC-type, guanylic and cytidylic acids comprising about two thirds of all nucleotide constituents.

The template activities, in the polymerase reaction, of unbound HeLa-DNA and of DNA forming part of metaphase chromosomes from HeLa cells are compared in Table 2 at four levels of DNA concentration. It can be seen that, with equivalent amounts of DNA-phosphorus, the template activity of free DNA exceeded that of the chromosomal preparations, as has recently also been observed by others.[15] In our hands, the chromosomal preparations incorporated between one eighth and one half of the precursor quantity taken up in the presence of free DNA. The pretreatment of the chromosome specimens with crystalline pancreatic deoxyribonuclease (free of ribonuclease) abolished the incorporation of ribonucleotides. Two additional experiments suggested themselves. As the chromosomal preparations contain also RNA, in a RNA/DNA ratio of about 0.6,[1] the effect of a corresponding

TABLE 1

CONCENTRATION AND COMPOSITION OF NUCLEIC ACIDS OF HELA CELLS*

Preparation	Picograms/ metaphase cell	Moles/100 gm-Atoms of Nucleic Acid Phosphorus					Molar Ratios		
		A	G	C	T	U	(A + T)/ (G + C)	(A + U)/ (G + C)	6-Am/ 6-K
DNA	17 (2.5)	29.6	20.4	19.9	30.1	...	1.48	...	0.99
RNA (chromosomal)	11 (2.4)	17.6 (0.3)	32.0 (0.4)	34.4 (0.5)	...	15.9 (0.2)	...	0.50	1.09
RNA (cytoplasmic)	43 (6.0)	17.8 (0.5)	33.2 (0.6)	32.2 (0.3)	...	16.8 (0.5)	...	0.53	1.00

* The figures for the concentration of DNA are based on nine determinations, those for chromosomal and cytoplasmic RNA on five and seven, respectively. The nucleotide composition of the RNA preparations is derived from the analysis of four specimens of each preparation. The standard deviation is given in parentheses. Abbreviations: A, G, C, T, U, designate adenine, guanine, cytosine, thymine, uracil, respectively; 6-Am stands for the sum of 6-amino nucleotides (adenylic and cytidylic acids); 6-K for the 6-keto nucleotides (guanylic and thymidylic or uridylic acids).

TABLE 2

RELATIVE EFFICIENCIES OF DNA AND CHROMOSOMAL PREPARATIONS AS TEMPLATES
FOR ENZYMIC RNA SYNTHESIS*

Expt. no.	DNA-P as mμatoms in assay	Nucleotides as mμmoles Incorporated in Assay		Relative efficiency of chromosomal preparations, as % of template activity of pure DNA
		DNA	Chromosomal preparation	
	2.75	2.45	1.30	53
1	8.25	2.30	1.20	52
	15.0	2.45	1.05	43
	27.5	2.50	0.85	34
	2.75	2.10	0.30	14
2	8.25	2.30	0.35	15
	15.0	2.50	0.30	12
	27.5	2.75	0.85	31

* Each assay was carried out in 0.5 ml of incubation mixture containing 45 μg of enzyme protein and 170–200 mμmoles each of the four C^{14}-labeled nucleoside triphosphates (sp. act. of each, 200 cpm/mμmole). Incubation for 25 min at 37°. The same DNA preparation (prep. 2), but different chromosomal preparations were employed in the two experiments. The results are corrected for an uptake of about 0.1 mμmole of nucleotide in the absence of template.

TABLE 3

HeLa-DNA AS TEMPLATE FOR ENZYMIC RNA SYNTHESIS:
EFFECT OF ADDED RNA*

mμAtoms P in Assay		Nucleotides as mμmoles incorporated in assay
DNA	RNA	
6.05	0	3.95
6.05	3.63	3.70
12.10	0	4.80
12.10	7.26	4.40

* The DNA specimen used was HeLa-DNA, prep. 4; a preparation of cytoplasmic RNA from HeLa cells was employed (compare Table 1). The conditions of the assay were as in Table 2, with the exception that of the four nucleoside triphosphates only guanosine triphosphate was labeled with C^{14} (sp. act. about 1200 cpm/mμmole). For the calculation of the uptake the products were assumed to contain 20% guanylic acid.

quantity of RNA on the template activity of HeLa-DNA was studied as shown in Table 3: there was only a slight diminution. Moreover, in the concentration range of free DNA studied in Table 2, no increase in incorporation was observed with increasing amounts of template; and this was reminiscent of observations in the literature[16] on degraded templates. A DNA specimen (prep. 4), prepared from HeLa cells by a different procedure,[5] was, therefore, tested in comparison with a DNA preparation from calf thymus (Fig. 1). The activity of this preparation. though it exhibited in small amounts some dependence upon concentration, also soon leveled off.

The composition of the products formed by RNA polymerase in the presence of the DNA or of a chromosomal preparation from HeLa cells is summarized in Table 4. Four experiments with HeLa-DNA as the template and two experiments with a chromosomal preparation are presented. When the ribonucleotide composition of any of the products formed in a single experiment is compared with the composition of the DNA serving as the template (see Table 1), the agreement will be seen to be far from excellent. With free DNA as the template (expts. 1–4), only one product (no. 1) shows a dissymmetry ratio approximating that of the template; in three cases it is much higher. Complete base pairing between adenine and uracil is achieved only in experiment 4; a fair degree of pairing of guanine and cytosine is encountered in experiments 1 and 2. None of the products resembles the DNA template entirely. It is, however, peculiar that the mean of the four determinations shows acceptable base pairing, though still a divergent dissymmetry ratio.
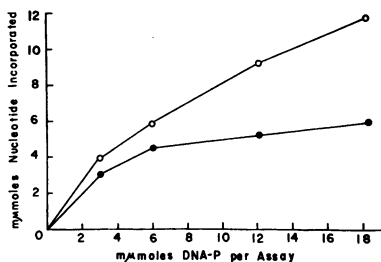
FIG. 1.—Enzymic polyribonucleotide synthesis as a function of template concentration. Open circles, calf thymus DNA; full circles, HeLa-DNA, prep. 4. For experimental conditions, see Table 2.

A similar trend is observed with the chromosomal specimen as the template (nos. 5 and 6 in Table 4). The agreement between the composition of HeLa-DNA and that of one of the enzymic products—experiment 5—is in fact quite good. Two conclusions are obvious. (*a*) The RNA product formed enzymically on metaphase chromosomes as the template in no way resembles the RNA component of chromosomal preparations. (*b*) It is similar in composition to the HeLa-DNA.

Discussion.—If it is assumed, as most workers are inclined to do, that enzymes of the type studied here are instrumental in the synthesis of RNA in the cell, then it must be acknowledged that it is difficult to reconcile the composition of most cellular species of RNA with that of the polyribonucleotide products formed by the enzyme *in vitro*. In animal cells, the nuclear RNA is of a pronounced GC-type,[17–19] and the same is true of the bulk of cytoplasmic RNA;[8, 17, 18] and this despite the fact that, so far as we are aware, all DNA species from animal cells belong to the AT-type.[4] On the other hand, as concerns the action of RNA polymerase *in vitro*, it has frequently been stated that the base composition of the enzymic product is dictated by the base composition of the DNA used as the template; and—with some good will—this can be said to be more or less borne out by the analytical data compiled for several polyribonucleotide products formed by the enzyme in the presence of different DNA templates.[20]

TABLE 4

COMPOSITION OF POLYRIBONUCLEOTIDES FORMED ENZYMICALLY WITH HeLa-DNA OR
CHROMOSOMAL PREPARATIONS AS TEMPLATES*

Expt. no.	Template	Nucleotides incorporated (mμmoles)	Nucleotide Composition of Products (mole %)				Molar Ratios			
			A	G	C	U	(A + U)/(G + C)	A/U	G/C	6-Am/6-K
1	DNA	1.1	27.6	19.1	20.2	33.1	1.54	0.83	0.95	0.92
2	"	1.4	28.8	19.2	18.4	33.5	1.70	0.86	1.04	0.90
3	"	1.3	35.3	16.3	17.6	30.7	1.95	1.15	0.93	1.13
4	"	1.3	31.8	20.4	16.1	31.7	1.74	1.00	1.27	0.92
Av. nos. 1–4	"	1.3	30.9	18.8	18.1	32.3	1.71	0.96	1.04	0.96
5	Chromosomal prep.	0.67	29.7	19.4	19.9	31.0	1.54	0.96	0.97	0.98
6	"	0.47	33.3	20.7	20.5	25.6	1.43	1.30	1.01	1.16
Av. nos. 5 and 6	"	0.57	31.5	20.1	20.2	28.3	1.48	1.11	1.00	1.07
Av. nos. 1–6	—	—	31.1	19.2	18.8	30.9	1.63	1.01	1.02	1.00

* Each experiment consisted of four sets, each set of four tubes containing 0.5 ml of incubation mixture with three of the four nucleotide precursors unlabeled and one—different in each set—labeled with C¹⁴. The specific activities of the nucleoside triphosphates were determined in each case; they varied from 1050 to 1460 cpm/mμmole. In expts. 1–4 purified HeLa-DNA (prep. 3) corresponding to 48 mμatoms DNA-P, 20 μg of enzyme, and 100 mμmoles of each nucleotide precursor were added to each tube; in expts. 5 and 6, the additions were the chromosomal preparation (21 mμatoms DNA-P), 40 μg enzyme, and 200 mμmoles of each nucleoside triphosphate. The incubation was carried out at 37° for 15 min. In each experiment the RNA composition, expressed as mole % of nucleotide, was calculated, based on determinations in quadruplicate for each C¹⁴-nucleotide (cf. ref. 10.).

The extent to which these observations are confirmed by the present experiments with purified HeLa-DNA as the template can be gauged from the results recorded in experiments 1–4 in Table 4. A certain similarity between product and template is unmistakable: all products are of a marked AU-type; but we should hesitate to consider them as replicas of the template. It should, however, be remembered that recent experiments in this laboratory have in fact yielded enzymic products that showed excellent agreement of composition with intact calf thymus DNA serving as the template.[10] It is not impossible that DNA specimens of different cellular origin differ in the degree of perfection with which they are copied in the polymerase system.

It appeared conceivable that chromosomal preparations, in which the DNA occurred as an undegraded nucleoprotein, would differ in their template action from isolated DNA. This is, however, not the case so far as the composition of the product is concerned (Table 4). Here, again, a polyribonucleotide of a pronounced AU-type was produced. As was shown in Table 2, chromosome preparations under comparable conditions are, however, less active than DNA as templates; but the polymers formed do not differ significantly. It will be noticed that the type of RNA formed by RNA polymerase in the presence of chromosomal preparations (Table 4) is entirely different from the RNA existing preformed in these preparations (Table 1).

One could, therefore, question whether enzymes of the type studied here are really instrumental in the synthesis of the RNA species comprising the bulk of nuclear and cytoplasmic RNA encountered in the cell. The literature is not devoid of examples of the biosynthesis of RNA varieties that do not show the base pairing found in the products of the action of the template-dependent RNA polymerase, e.g., in segments of the giant chromosomes of *Chironomus*,[21] in HeLa cells,[22] etc. The evidence for the direct involvement of DNA as the template in the biosynthesis of all forms of cellular RNA has been reviewed recently.[23] But we are still left with the curious discrepancy between the nucleotide proportions of the products of the enzymic reaction *in vitro* and those of the preponderant portion of cellular RNA; and we are also left with a baffling problem: how does a DNA with a G + C content of 40 per cent give rise to a RNA with a G + C content of 66 per cent? The usual explanation that only one DNA strand is being copied during the biosynthesis of RNA is of no help, for it stands to reason, as a consequence of the base-pairing regularities, that the G + C content of one strand must equal that of the complementary strand as well as that of the intact DNA. The role of DNA, if any, in directing the formation of cellular RNA with a high G + C content must therefore express itself either through the as yet unrevealed presence[24] of a DNA species with an equally high concentration of G + C, or through the existence of very long tracts on the DNA molecule having these base characteristics and being copied at a high rate. In bacteria, there exists some evidence[25, 26] that the latter is the case; but the situation prevailing in cells having a distinct nuclear apparatus remains to be investigated in sufficient detail.

Summary.—Metaphase chromsoomes from HeLa cells support the synthesis *in vitro* of polyribonucleotides by the RNA polymerase of *E. coli*, although the rate of synthesis with chromosomes as template is lower than that obtained with free HeLa-DNA. The composition of the enzymic products formed with both types

of template is of the AU-type and does not resemble that of the normal RNA component of the chromosomes.

¹ Lin, H. J., and E. Chargaff, *Biochim. Biophys. Acta*, **91**, 691 (1964).

² McLimans, W. F., F. E. Giardinello, E. V. Davis, C. J. Kucera, and G. W. Rake, *J. Bacteriol.*, **74**, 768 (1957).

³ Kay, E. R. M., N. S. Simmons, and A. L. Dounce, *J. Am. Chem. Soc.*, **74**, 1724 (1952).

⁴ Chargaff, E., in *The Nucleic Acids*, ed. E. Chargaff and J. N. Davidson (New York: Academic Press, 1955), vol. 1, p. 307.

⁵ Massie, H. R., and B. H. Zimm, these PROCEEDINGS, **54**, 1636, 1641 (1965).

⁶ Scherrer, K., and J. E. Darnell, *Biochem. Biophys. Res. Commun.*, **7**, 486 (1962).

⁷ Chargaff, E., R. Lipshitz, C. Green, and M. E. Hodes, *J. Biol. Chem.*, **192**, 223 (1951).

⁸ Lipshitz-Wiesner, R., and E. Chargaff, *Biochim. Biophys. Acta*, **76**, 372 (1963).

⁹ Chamberlin, M., and P. Berg, these PROCEEDINGS, **48**, 81 (1962).

¹⁰ Karkas, J. D., and E. Chargaff, these PROCEEDINGS, **56**, 664 (1966).

¹¹ Bray, G. A., *Anal. Biochem.*, **1**, 279 (1960).

¹² Chargaff, E., and R. Lipshitz, *J. Am. Chem. Soc.*, **75**, 3658 (1953).

¹³ Harris, H., H. W. Fisher, A. Rodgers, T. Spencer, and J. W. Watts, *Proc. Roy. Soc. (London)*, **B157**, 177 (1963).

¹⁴ Scherrer, K., H. Latham, and J. E. Darnell, these PROCEEDINGS, **49**, 240 (1963).

¹⁵ Johnson, T. C., and J. Holland, *J. Cell Biol.*, **27**, 565 (1965).

¹⁶ Fox, C. F., and S. B. Weiss, *J. Biol. Chem.*, **239**, 175 (1964).

¹⁷ Elson, D., L. W. Trent, and E. Chargaff, *Biochim. Biophys. Acta*, **17**, 362 (1955).

¹⁸ Elson, D., and E. Chargaff, *Biochim. Biophys. Acta*, **17**, 367 (1955).

¹⁹ Harris, H., in *Progress in Nucleic Acid Research*, ed. J. N. Davidson and W. E. Cohn (New York and London: Academic Press, 1963), vol. 2, p. 19.

²⁰ Hurwitz, J., and J. T. August, in *Progress in Nucleic Acid Research*, ed. J. N. Davidson and W. E. Cohn (New York and London: Academic Press, 1963), vol. 1, p. 59.

²¹ Edström, J. E., and W. Beermann, *J. Cell Biol.*, **14**, 371 (1962).

²² Penman, S., K. Scherrer, Y. Becker, and J. E. Darnell, these PROCEEDINGS, **49**, 654 (1963).

²³ Prescott, D. M., in *Progress in Nucleic Acid Research and Molecular Biology*, ed. J. N. Davidson and W. E. Cohn (New York and London: Academic Press, 1964), vol. 3, p. 33.

²⁴ HeLa DNA preparation 4 was analyzed by means of centrifugation in a CsCl density gradient. Densitometer tracings of the DNA banding as a single peak at equilibrium were used to estimate the proportion present in regions of the gradient corresponding to a mean G + C-composition of 60% or more. Less than 1.5% of the sample was estimated to exhibit these extremely high densities in CsCl. This calculation required the following assumptions: (1) the position of the DNA molecules in the gradient at equilibrium was related only to its mean G + C-content; (2) the mol wt dispersion in this preparation did not significantly alter the DNA distribution at equilibrium; (3) the effect of diffusion of DNA did not alter the shape of the distribution curve; (4) the preparation was devoid of single-stranded DNA. In view of the tenuous nature of some of these assumptions, the value of 1.5% is probably an overestimate of the proportion of HeLa-DNA molecules having a mean G + C-content corresponding to that of cytoplasmic RNA. We wish to thank Dr. H. S. Rosenkranz for this analysis.

²⁵ Rudner, R., B. Prokop-Schneider, and E. Chargaff, *Nature*, **203**, 479 (1964).

²⁶ Rudner, R., E. Rejman, and E. Chargaff, these PROCEEDINGS, **54**, 904 (1965).