

Stimulation of Oligodeoxyribonucleotide Synthesis by Cytoplasmic Factors in the Isolated Nuclei of Leukemic Cells¹

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SUMMARY

Nuclei were isolated by hypotonic shock and homogenization from freshly drawn, nonstimulated leukemic leukocytes and were pulse labeled with deoxythymidine triphosphate-³H. Labeled oligodeoxyribonucleotides were separated from the bulk of DNA and proteins by membrane cone filtration and were further purified by alkaline hydrolysis and diethylaminoethyl cellulose chromatography. In parallel experiments, cytoplasmic factors, obtained after removal of insoluble material from the cytoplasm by high-speed centrifugation, were included. More than a twofold increase of label incorporation into oligodeoxyribonucleotides was observed.

INTRODUCTION

In the past, radioactive oligodeoxyribonucleotides were isolated from pulse-labeled Chinese hamster cells (9), HeLa cells (7), *Escherichia coli* (8), and *Bacillus subtilis* (11). These molecules, composed of about 10 nucleotides (8, 9), satisfy the requirements for priming DNA synthesis by the following criteria. They possess 3'-hydroxy ends and covalently incorporate into Okazaki segments and larger DNA upon a chase with nonradioactive medium (7-9, 11); *in vitro* they hybridize to single-stranded DNA templates and elongate to the size of the Okazaki segments, 1000 to 2000 nucleotides long (7). However, further evidences are necessary for the final confirmation of a priming role of oligodeoxyribonucleotides.

Isolated nuclei, obtained for instance from HeLa cells (3) or from rat liver cells (1), continue to synthesize DNA. In the former system, it was found that DNA synthesis largely, but not entirely, is comprised of an elongation of the sites already initiated *in vivo*. However, the addition of CF,² proteins obtained from late-S-phase cells, promoted initiation *in vitro* on DNA molecules that did not yet begin to replicate *in vivo* in the early-S-phase nuclei (3, 6). In the latter system a calcium-dependent initiation is described. According to the authors Ca²⁺ activates a nuclease or nu-

cleases which nick the nuclear DNA so that it can serve as a primer for the incorporation of precursors. When calcium is removed, however, the nuclear system can synthesize DNA only by elongating the chains that were growing *in vivo* (4). Also, in the isolated nuclear system of rat liver, it was recently found that serum factors stimulate DNA synthesis (5).

If the oligodeoxyribonucleotides in question play a role in priming DNA synthesis and CF are required for initiation on new sites, then these latter proteins may exert a stimulatory effect upon oligomer synthesis because at the sites of initiation molecules with the properties of the oligomers may be required. This communication describes experiments conducted to test whether or not CF affect the synthesis of oligodeoxyribonucleotides in pulse-labeled nuclei of nonstimulated leukemic cells.

MATERIALS AND METHODS

Cells. Leukemic white blood cells, originally drawn from a 506-day-old germ-free Fischer 344 rat on January 8, 1971, bearing a spontaneous mononuclear leukemia, were serially passaged by this laboratory in conventional Fischer 344 rats. Blood was drawn into a heparinized syringe by cardiocentesis. The erythrocytes were sedimented and the leukocytes were pipetted off after mixing each 1 ml of blood with 0.4 ml of 6% dextran in phosphate-buffered saline (10).

Buffers and Chemicals. Buffer A, used for induction of hypotonic swelling of the cells, contained 3 mM MgCl₂, 2 mM 2-mercaptoethanol, and 10 mM of KH₂PO₄, pH 7.7. Buffer B, used for stabilization of nuclei after homogenization, contained 22 mM glucose, 2 mM 2-mercaptoethanol, 3 mM CaCl₂ when indicated, 0.12 M NaCl, and 0.12 M Tris-HCl, pH 8.0. Buffer C, for washing nuclei, contained Buffer A, bovine serum albumin (15 mg/ml), and 3 mM CaCl₂ when indicated. Buffer D, incubation mixture, contained 9 mM MgCl₂; 1 mM EDTA; 22 mM 2-mercaptoethanol; 5 mM KH₂PO₄; 22 mM glucose; 5 mM ATP; 0.5 mM each of dATP, dGTP, dCTP, and dTTP; and 0.12 M Tris-HCl, pH 8.0. ³H-Labeled dTTP (12.5 Ci/mole) was obtained from International Chemical and Nuclear Corporation, Irvine, Calif.

Isolation of Nuclei. Essentially the same method was used as described by Kidwell and Mueller (3) and modified by Friedman (2). All operations were done at 0-2°

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² Abbreviation used is: CF, cytoplasmic factors.

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and all centrifugations were at $350 \times g$ for 5 min. Chilled leukocytes were suspended in Buffer A (10^8 cells /50 ml) and then resuspended in same (7×10^7 cells/ml) and allowed to swell for 10 min. Homogenization was done in a 0.0045-inch-clearance Teflon-glass homogenizer by 5 up and down strokes. An equal volume of Buffer B, containing Ca^{2+} , was rapidly added to the homogenate. The nuclei were washed with Buffer C containing Ca^{2+} , then with Buffer B again without Ca^{2+} , and subsequently with Buffer C without Ca^{2+} . At this point, the material was equally distributed into portions required by the experiment. Finally, a 60% yield was harvested that was free of cytoplasmic tags as determined by phase-contrast microscopy.

Preparation of CF. The method of Kidwell and Mueller was used (3). The CF were suspended in Buffer B without Ca^{2+} and were concentrated to one-fourth of the original volume with the aid of Centriflo filter cones (exclusion limit, 50,000; Amicon Corp., Lexington, Mass.).

Preparation of Labeled Oligodeoxyribonucleotides. Approximately 7×10^7 nuclei in each individual assay were suspended in 1.50 ml of the appropriate incubation mixture and were warmed to 37° for 10 min. dTTP- ^3H ($50 \mu\text{Ci/ml}$) pulses of 4-min duration were delivered at 37° . The incorporation of label was terminated by pouring the incubation mixtures into equal volumes of ice-cold 0.04 M KCN. The nuclei were sedimented in a clinical centrifuge and lysed with 3 ml of 0.3 M NaOH containing 1 mM EDTA. The pH of the lysates were 12.5. The preparations were incubated at 37° for 16 hr and then dialyzed against 200 times their volumes of 10 mM Tris-HCl and 1 mM EDTA, pH 7.8, for 6 hr at 4° . A_{260} was measured for each cell lysate, and all the radioactive results were adjusted to $A_{260} = 1$, in order to account for an equal amount of nucleic acids in each experiment. The variation between samples was not greater than 10%. Aliquots of the dialysates were precipitated with 10 times their volumes of 10% ice-cold trichloroacetic acid. The total radioactivity that incorporated into DNA was measured by counting the precipitated material on 0.22- μm Millipore filters (Millipore Corp., Bedford, Mass.). The major portion of each dialyzed sample was filtered through Centriflo cones and DEAE-cellulose chromatography was carried out as described (9) for the isolation and purification of the oligomers. The same column (1×6 cm) was utilized for each series of experiments interrupted by a wash with 2 M NaCl between each run. Elutions were with a linear NaCl gradient of 0.02 M to 0.20 M concentrations. Chromatography of the labeling material alone showed that dTTP- ^3H eluted in the same position as ATP and contained some dTMP and smaller soluble material, probably deoxythymidine and thymine. When incubated in 0.3 M NaOH:1 mM EDTA at 37° for 16 hr, about 9% of dTTP remained intact, 39% was degraded to dTDP, and 27% was degraded to dTMP; the rest of the label eluted earlier.

Assay for Radioactivity. Filters were counted in 5 ml scintillation fluid containing 4 g of Omnifluor (New England Nuclear, Boston, Mass.) per liter of toluene. Aqueous DNA samples, 0.5 ml, obtained from the fractions of DEAE-cellulose chromatography, were counted in 2.5 ml Insta-Gel (Packard Instrument Co., Downers Grove, Ill.).

RESULTS AND DISCUSSION

Leukemic leukocytes continue to divide *in vitro* when incubated in an equal volume of Eagle's minimal essential medium at 37° with adequate aeration, *i.e.*, vigorous shaking. Such cultures increase in number during the 1st 2 hr of incubation. Viability studies, staining with trypan blue, indicated that all of the 1:1 mixtures of cells with Eagle's minimal essential medium remained alive and infectious

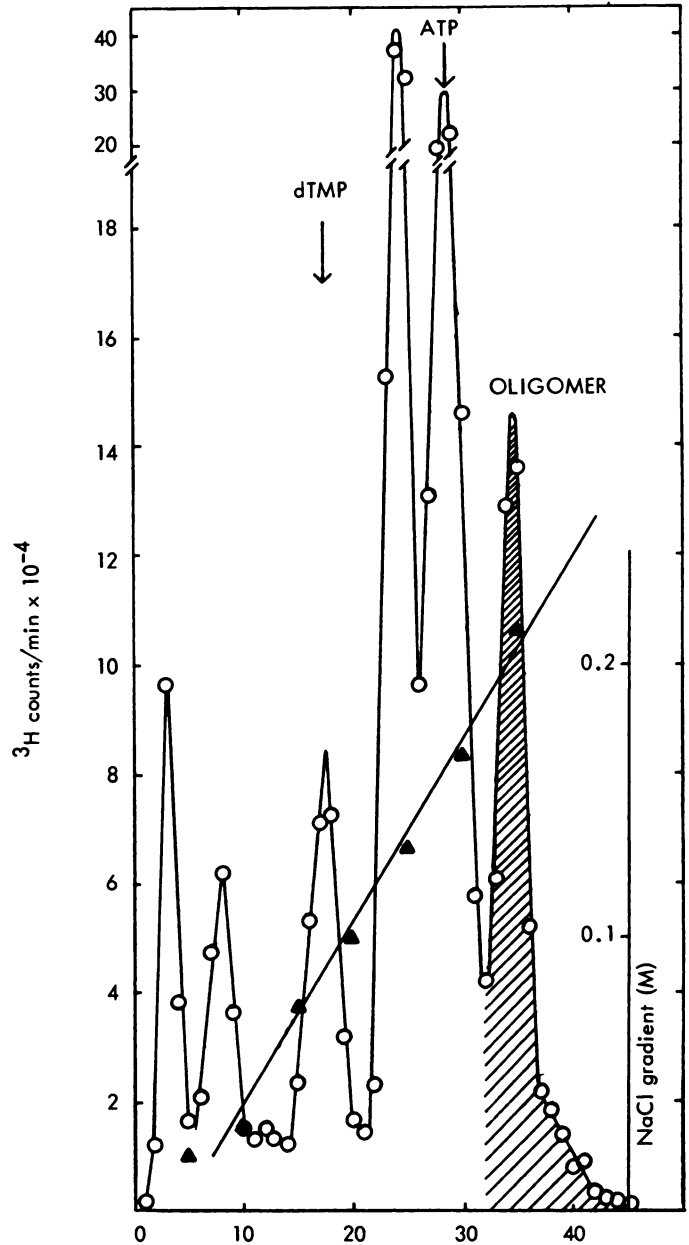


Chart 1. DEAE-cellulose chromatography at 80° of oligodeoxyribonucleotides isolated from the nuclei of leukemic leukocytes, pulse-labeled for 4 min with dTTP- ^3H . dTMP was added to the solution containing the oligomers prior to chromatography. The DNA-synthesizing system was composed of 1 ml of Buffer D plus 0.5 ml of Buffer B. For the details of isolation, labeling, and lysing nuclei and the oligomer-containing solution, see "Materials and Methods." Fractions of 2.6 ml were collected at the rate of 1 ml/min.

for 24 hr when stored at 4°. In all experiments, however, freshly drawn cells were used which remained viable for at least the length of time required for the performance of the experiments.

Oligodeoxyribonucleotides of one kind, with respect to length, were isolated from the whole cells after labeling for 2 min with ³H-labeled thymidine, in a way similar to that of already reported cases (7-9), but of major interest was the isolation of these molecules from the intracellular site of DNA synthesis, the nuclei. The DEAE-cellulose elution pattern of oligodeoxyribonucleotides, obtained from the separated nuclei of the cells, is depicted in Chart 1. Radioactive dTTP was used for the delivery of a 4-min pulse. Six peaks can be seen. The 1st 2 peaks are due to small soluble materials; the 3rd is dTMP and the 5th is dTTP, whereas the 6th represents the oligomers. The oligodeoxyribonucleotide nature of this peak is witnessed by its resistance to alkaline hydrolysis, the specificity of the labeled precursor used, and its elution profile from DEAE-cellulose. Judging from the elution profile, the size of the oligomers was similar to the size of those oligomers that were measured (8, 9), i.e., in the neighborhood of 10 nucleotides long. Larger DNA's, including the Okazaki segments, are retained by Centriflo filter cones and they elute from DEAE-cellulose at much higher NaCl concentrations than do the oligomers. The lysing of nuclei with 0.25% (w/v) sodium dodecyl sulfate:0.015 M EDTA:0.05 M Tris, pH 8.0, and the omission of alkaline hydrolysis did not alter the elution profile of the oligomers from DEAE-cellulose columns. Peak 4 probably contains dTDP. Rechromatography of the material present in Peak 6 resulted in an identical elution pattern, i.e., between 0.20 and 0.21 M NaCl concentrations. Apparently, to some extent, the labeling material, dTTP, and its phosphorylated breakdown products survived the alkaline treatment to which the nuclear lysates

Table 1

Effect of CF upon the incorporation of label into acid-precipitable DNA of isolated leukemic nuclei

Nuclei, 7 × 10⁷ assay, were pulse-labeled with dTTP-³H for 4 min at 37°. The incubation was terminated and the nuclei were lysed as described in "Materials and Methods." Aliquots of 0.05 ml in duplicates were taken from the lysates and were assayed for label incorporation by precipitation with trichloroacetic acid as described in "Materials and Methods."

Experiment	CF (A ₂₆₀ unit)	Ca ²⁺ (mM)	dTTP- ³ H incorporated (cpm/0.05 ml acid-precipitated sample) ^a	Relative label incorporation
1			1120	1.0
		1	1860	1.7
	0.70		3450	3.1
2			470	1.0
	0.35		540	1.2
	0.70		640	1.4

^a The average of the duplicate values is represented where the individual values agreed within 10% of the mean value.

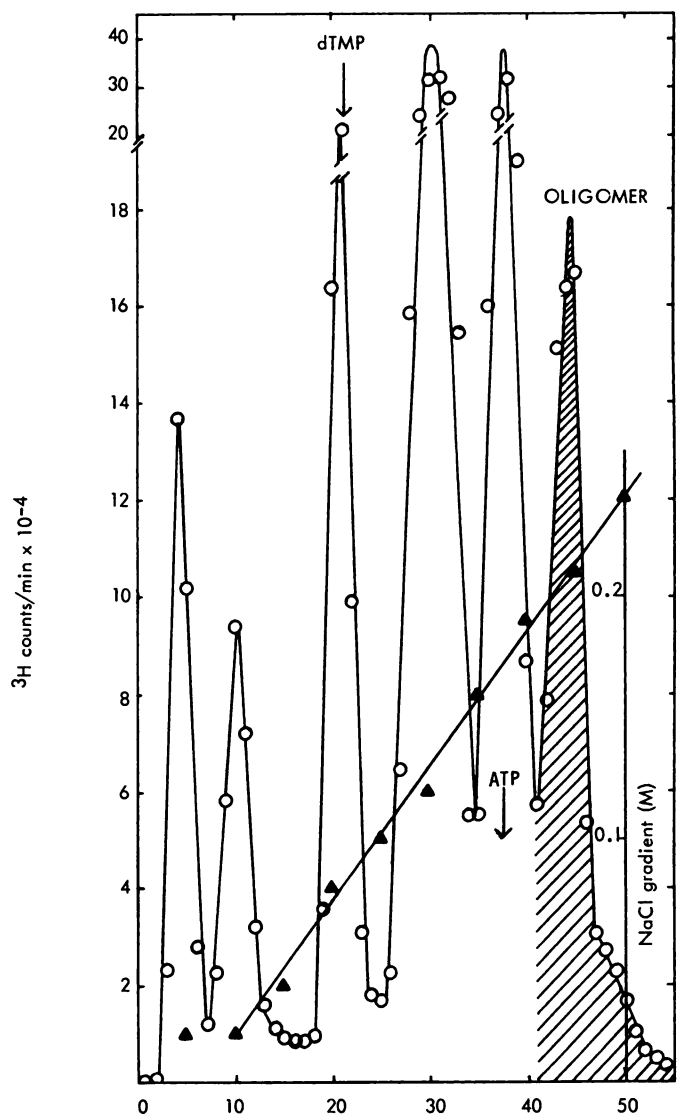


Chart 2. DEAE-cellulose chromatography of oligodeoxyribonucleotides synthesized in the isolated nuclei of leukemic cells at the presence of cytoplasmic factors. All details are the same as those of Chart 1 except that the DNA-synthesizing system was composed of 1 ml of Buffer D, 0.5 ml of A₂₆₀ = 0.70 unit of CF suspended in Buffer B. ATP marker was used. Fractions of 2.6 ml were collected at the rate of 1 ml/min.

were exposed for the removal of RNA prior to chromatography.

The effects of cytoplasmic factors were studied upon label incorporation into acid-insoluble counts, i.e., total DNA, and into oligodeoxyribonucleotides in the isolated nuclei of leukemic leukocytes. It was found that incorporation was stimulated by CF and the magnitude of increase depended upon whether or not freshly prepared CF were used. Either storage at -68° or freezing and thawing reduced the stimulative effect. Table 1 shows the relative incorporation of label into acid-precipitable DNA. Experiment 1 depicts the results obtained with fresh CF and Experiment 2 shows those after freezing, storing at -68° for 1 week, and thawing at room temperature. Fresh nuclei were prepared prior to each experiment. Total

Table 2
Effect of CF upon oligodeoxyribonucleotide synthesis in the isolated nuclei of leukemic leukocytes

CF (A ₂₈₀ unit)	Ca ²⁺ (mM)	ΣdTTP- ³ H incorporated (cpm × 10 ³)	Relative label incorporation (arbitrary units)
	1	4.01 ^a	1.0
		3.21 ^b	0.8
0.35		5.37 ^c	1.3
0.70		6.77 ^d	1.7
0.70 ^e			2.2 ^f

^a Data were taken from the results of Chart 1.

^b Methods and all conditions used were similar to those of Chart 1 except that 0.5 ml of Buffer D contained 1 mM Ca²⁺.

^c Data were obtained as described in the text.

^d Data were taken from the results of Chart 2.

^e Same, but fresh CF were used as in the other 2 experiments of this table; see text for details.

^f The number expresses label incorporation in comparison with a control experiment that did not contain CF and was designated as 1.0.

DNA synthesis tripled in the experiment where freshly prepared CF (A₂₈₀ = 0.70), were used. Ca²⁺ also was stimulatory in this respect, as reported (1), by 1.7-fold. However, it is difficult to evaluate the effect of calcium in these preparations because it was necessary to include it in some of the buffers used for the preparation of nuclei. Omission resulted in clumping. A lesser stimulation, 1.2- and 1.4-fold, was observed in the experiments in which frozen then thawed CF, A₂₈₀ = 0.35 and 0.70, respectively, were used.

The inclusion of CF in the nuclear DNA-synthesizing system resulted in an increased amount of label incorporation into oligodeoxyribonucleotides as well. Chart 2 shows the results of such an experiment in which A₂₈₀ = 0.70 unit of CF were added to the incubation mixture. The elution pattern of the oligomers is the same as that represented in Chart 1. However, the area under the curve designated *OLIGOMER* is larger due to the synthesis of more molecules.

Table 2 summarizes the effect of CF upon oligomer synthesis in the isolated nuclei. The same CF preparation was used in all experiments described in this paper except that, as indicated, it was used either immediately after preparation or after freezing at -68°, storage for 1 week, and thawing at room temperature. The results represented in Chart 1 were taken as control, *i.e.*, no CF. The data represented in Chart 2 were used to express the effect of A₂₈₀ = 0.70 unit of CF. The chart of the experiment in which A₂₈₀ = 0.35 unit of CF is not shown because of its similarity to Charts 1 and 2. However, the *OLIGOMER* peak occupied an intermediate area between that of Charts 1 and 2. Table 2 shows the total number of cpm detected in the shaded area of Charts 1 and 2 and of the experiment in

which A₂₈₀ = 0.35 unit of CF. A linear relationship can be seen between the amount of CF used and the label incorporated into oligodeoxyribonucleotides, *i.e.*, 1.3- to 1.7-fold increase of incorporated label, was observed. The stimulatory effect of CF was the greatest when fresh preparations were used. A 2.2-fold increase of incorporated radioactivity was observed in comparison with the parallel experiment without CF. Apparently, as observed earlier, CF are unstable. Contrary to the results obtained for total DNA synthesis, Ca²⁺ did not stimulate oligomer synthesis in the nuclear system.

The relatively low stimulatory effect of CF can be attributed to asynchrony of the nuclei obtained from an asynchronous leukocyte population. The kinetics of cell division of leukemic leukocytes in 50% Eagle's minimal essential medium suggests that less than 1% of the cells (nuclei) were in the exact same stage of the cell cycle at any given time; thus only a limited number of nuclei were in early S phase with sites available for the initiation of synthesis.

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