Effects of a Trinucleotide Ethyl Phosphotriester, $G^mp(Et)G^mp(Et)U$, on Mammalian Cells in Culture†

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ABSTRACT: The nonionic 2′-*O*-methyribooligonucleotide ethyl phosphotriester, $G^mp(Et)G^mp(Et)U$, is complementary to the . . . ApCpC . . . sequence found in the amino acid accepting stem of most tRNAs and the anticodon region of $tRNA^{gly}$ and to the threonine codon of mRNA. $G^mp(Et)G^mp(Et)U$ forms hydrogen-bonded complexes with the amino acid accepting stem of $tRNA^{phe}_{yeast}$ and unfractionated tRNA *Escherichia coli* under physiological salt conditions at 37 °C as determined by equilibrium dialysis. The extent of phenylalanine aminoacylation of $tRNA^{phe}_{E.coli}$ is inhibited 39% by $G^mp(Et)G^mp(Et)U$ at 37 °C in solution. The triester is resistant to hydrolysis by serum nucleases and cell lysates. The triester is readily taken up by transformed Syrian hamster fibroblasts growing in monolayer. Within the cell, the triester is deethylated to give the trinucleotide species $G^mp(Et)G^mpU$, $G^mpG^mp(Et)U$, and G^mpG^mpU and is also hydrolyzed to dimeric and monomeric units. Treatment of transformed fibroblasts in monolayer with 25 μM $G^mp(Et)G^mp(Et)U$ results in a 40% inhibition of cellular protein synthesis with a concurrent slight increase in cellular RNA synthesis during the first 4 h. After 4 h, the rate of cellular protein synthesis begins to recover while RNA synthesis returns to that of the control. Our biochemical studies suggest that inhibition of cellular protein synthesis might be expected if $G^mp(Et)G^mp(Et)U$, $G^mp(Et)G^mpU$, $G^mpG^mp(Et)U$, and G^mpG^mpU, which have been taken up by or formed within the cell, physically bind to tRNA and mRNA and inhibit the function of these nucleic acids. The reversible inhibition of protein synthesis may be a consequence of further degradation of the trinucleotide species within the cell as well as to an increase in supply of RNA molecules involved in protein synthesis. The growth of the transformed fibroblasts is inhibited during the first 24 h of incubation with 25 μM $G^mp(Et)G^mp(Et)U$ after which growth proceeds at a normal rate. In cloning experiments, the number and size of colonies formed by the transformed fibroblasts after 5 days exposure to 25 μM triester is decreased by 50% relative to untreated controls. The temporary inhibition of cell growth may reflect the transitory inhibition of cellular protein synthesis caused by the triester.

Recent studies from our laboratory have demonstrated the utility of ethyl phosphotriester derivatives of oligodeoxyribonucleotides as probes of the structure and function of nucleic acids in biochemical experiments (Miller et al., 1974; Barrett et al., 1974). These neutral oligonucleotide analogues were shown to be capable of forming specific, hydrogen-bonded complexes with complementary anticodon or 3′-amino acid accepting stem regions of transfer RNA molecules at low temperature and ionic strength. As a consequence of these interactions, synthetase-catalyzed aminoacylation of tRNA was inhibited in a specific manner. Since oligonucleotide ethyl phosphotriesters are resistant to hydrolysis by nucleases found in mammalian sera and appeared to be capable of penetrating cellular membranes, we sought to determine if these oligonucleotide analogues could influence the biochemical or biological functions of living cells.

To this purpose, we have synthesized the 2′-*O*-methylribooligonucleotide ethyl phosphotriester, $G^mp(Et)G^mp(Et)U$,[1] whose sequence is complementary to the 3′-amino acid accepting stem of most tRNAs, the anticodon region of $tRNA^{gly}$, and the threonine codon of mRNA. Under physiological conditions in solution, this triester forms hydrogen-bonded complexes with tRNA and inhibits tRNA aminoacylation. Similar interactions between the triester and its target nucleic acids within the cell should have an inhibitory effect on the function of these nucleic acids which would ultimately be manifested as a decrease in cellular protein synthesis and perturbation of cell growth. In this report, we describe the synthesis of $G^mp(Et)G^mp(Et)U$, its interaction with tRNA in solution, and its effects on protein synthesis, RNA synthesis, and growth by mammalian cells in culture.

Materials and Methods

Materials. Guanosine and uridine 5′-phosphate were obtained from PL Biochemicals and were checked for purity by paper chromatography before use. 2′,3′-Di-*O*-acetyluridine 5′-phosphate was prepared by the procedure of Ukita et al. (1964). [^{3}H]Uridine 5′-diphosphate (specific activity 11 Ci/mmol), [^{3}H]leucine (specific activity 60 Ci/mmol), [^{3}H]uridine (specific activity 37.6 Ci/mmol), L-[^{3}H]amino acid mixture and L-[^{14}C]amino acid mixture were purchased from New England Nuclear. [^{14}C]Adenosine (specific activity 28.4 mCi/mmol) was a product of Amersham Searle. tRNA *Escherichia coli* was obtained from Schwarz Mann, Inc. Pu-

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[1] The system of abbreviations used to describe oligonucleotide ethyl phosphotriesters and protected oligonucleotide derivatives is same as that used by Miller et al. (1974). 2′-*O*-Methylribonucleotide ethyl phosphotriesters will be referred to as triesters in the text; additionally, the following abbreviations are used: DEAE, diethylaminoethyl; EDTA, (ethylenedinitrito)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; THF, tetrahydrofuran; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; UV, ultraviolet.

rified $tRNA^{phe}_{yeast}$ which accepted 1600 pmol of phenylalanine/A_{260} unit was a gift from Dr. Lou-Sing Kan. All solvents and reagents used in the chemical syntheses were purified as previously described (Miller et al., 1974).

Paper chromatography was performed on Whatman 3MM paper using the following solvent systems: solvent A, 2-propanol–concentrated ammonium hydroxide–water (7:1:2, v/v); solvent C, 1 M ammonium acetate–95% ethanol (3:7, v/v); solvent F, 1-propanol–concentrated ammonium hydroxide–water (50:10:35, v/v); or solvent I, 2-propanol–water (7:3, v/v). The high-pressure liquid chromatographic (HPLC) system has been described elsewhere (Leutzinger et al., 1977). Analyses were carried out on a column (0.21 × 100 cm) containing Pellionex AL WAX. A 30-mL linear gradient of ammonium acetate in 40% ethanol (pH 6) at a flow rate of 1 mL/min was used. Retention times are measured from the time of injection of the sample onto the column, and the gradient used is given.

General Synthetic Procedures. The syntheses of the required protected nucleosides and mononucleotides followed the basic procedures used in the syntheses of protected deoxyribonucleosides (Letsinger and Miller, 1969). The general procedures for chemical and enzymatic syntheses of oligonucleotides and their ethyl phosphotriester derivatives have been previously described (Miller et al., 1974). All operations were carried out at room temperature unless otherwise indicated. Digestion of oligonucleotides by snake venom phosphodiesterase and spleen phosphodiesterase was carried out at 37 °C using the conditions described by Miller et al. (1971).

Preparation of $MTrG^{m,iB}$. Guanosine (24 g; 84.8 mmol) was converted to G^m by reaction with diazomethane according to the procedure of Robins et al. (1974). Subsequent purifications by column chromatography on silica gel (5 × 60 cm) and Dowex 1-X2 (5 × 154 cm) gave 2.39 g (9.4% yield) of G^m.

$G^{m,iB}$ was prepared in 80% yield by reaction of G^m (1.5 g; 5 mmol) with isobutryl chloride (28 mmol) in 3 mL of anhydrous pyridine for 4 h, followed by hydrolysis of the *O*-isobutryl groups with 1 N NaOH in 50% aqueous pyridine for 7 min at −5 °C. After neutralization with Dowex-50X–pyridinium resin and evaporation of solvents, the $G^{m,iB}$ was precipitated from anhydrous pyridine solution by addition of ether, or was stored in anhydrous pyridine solution. The UV spectrum was identical to that of *N,O*$^{3'}$-diisobutryldeoxyguanosine 5′-phosphate (Weber and Khorana, 1972).

$G^{m,iB}$ (0.8 mmol) was reacted with monomethoxytrityl chloride (309 mg; 1 mmol) in 2 mL of anhydrous pyridine for 18 h. Water was added to the reaction mixture and the aqueous solution was extracted with chloroform. $MTrG^{m,iB}$ (269 mg, 53% yield) was obtained after purification by silica gel column chromatography. The UV spectrum of this material was identical to that of previously prepared d-$MTrG^{iB}$ (Miller et al., 1974), and the ^1H NMR spectrum was consistent with the assigned structure.

Preparation of $MTrG^{m,iB}p$. $MTrG^{m,iB}$ (200 mg; 0.32 mmol) was converted to $MTrG^{m,iB}pCE$ by reaction with β-cyanoethyl phosphate (2 mmol) and dicyclohexylcarbodiimide (8 mmol) in 2 mL of anhydrous pyridine for 3 days. After filtration and ether extraction, the aqueous solution was extracted with chloroform. The material in the chloroform extract was treated with 4 mL of 2 N NaOH in a solution containing 2 mL of pyridine and 2 mL of 95% ethanol for 20 min. The solution was neutralized with Dowex 50X pyridinium resin. $MTrG^{m,iB}p$ was obtained in 68% yield after precipitation from ether. The UV spectrum was identical to that of $MTrG^{m,iB}$. Complete removal of the protecting groups by sequential treatment with base and acid gave G^mp (R_f^A = 0.05, R_f^F = 0.44, cellulose TLC), the ^1H NMR spectrum of which was consistent with the assigned structure. G^mp was converted to G^m (R_f^A = 0.44, R_f^F = 0.68, cellulose TLC) by bacterial alkaline phosphatase.

Preparation of $G^{m,iB}pU(OAc)_2$. $MTrG^{m,iB}$ (760 mg; 1.2 mmol) and $pU(OAc)_2$ (1.13 g; 2 mmol) were reacted with triisopropylbenzenesulfonyl chloride (1.21 g; 4 mmol) in 10 mL of anhydrous pyridine for 5.5 h. The reaction mixture was treated overnight with 10 mL of water, and then extracted twice with 10 mL of ether and twice with 15 mL of chloroform. The chloroform extract contained 695 mg of pure $MTrG^{m,iB}pU(OAc)_2$ which was isolated in 53% yield by precipitation from ether. The dimer had an R_f = 0.28 on silica gel TLC (EtOAc–THF, 1:1) and an HPLC retention time of 12.8 min (0.001–0.5 M ammonium acetate).

$MTrG^{m,iB}pU(OAc)_2$ (695 mg; 0.633 mmol) was treated with 5 mL of 80% acetic acid for 2 h and then for 1 min on a steam bath. After removal of solvent, $G^{m,iB}pU(OAc)_2$ was isolated by precipitation from ether in 85% yield (451 mg). The pure dimer had an R_f = 0.41 (MeOH–$CHCl_3$, 1:1) on silica gel TLC and a retention time of 15.2 min (0.001–0.25 M ammonium acetate) on HPLC. Complete removal of protecting groups by treatment with base gave G^mpU with an HPLC retention time of 16.4 min (0.001–0.50 M ammonium acetate) and R_f values of 0.10 (solvent A), 0.42 (solvent C), and 0.42 (solvent F) on paper chromatography. The UV spectrum (water, pH 6.6) showed λ_{max} 257 nm, λ_{min} 228 nm, sh 267 nm. The dimer was hydrolyzed by snake venom phosphodiesterase to G^m and pU and by spleen phosphodiesterase to G^mp and U.

Preparation of G^mpG^mpU. $MTrG^{m,iB}p$ (184 mg; 0.21 mmol) and $G^{m,iB}pU(OAc)_2$ (187 mg; 0.22 mmol) were reacted with triisopropylbenzenesulfonyl chloride (212 mg; 0.70 mmol) in 0.5 mL of pyridine for 7 h. After overnight treatment with 0.5 mL of water, the reaction mixture was extracted with chloroform. The material in the chloroform extract was treated with 80% acetic acid for 2 h and for 2 min on a steam bath. The material was then applied to a DEAE-cellulose column (2.5 × 28 cm) in the acetate form. The column was eluted with 400 mL of 0.001 M ammonium acetate in 50% ethanol followed by a 4-L linear gradient of 0.001 to 1.0 M ammonium acetate in 50% ethanol. Pure $G^{m,iB}pG^{m,iB}pU(OAc)_2$ was eluted with 0.2 m ammonium acetate and had a retention time of 27.2 min (0.001–0.5 M ammonium acetate) on HPLC. The solution containing the trimer was diluted to 1000 mL (final [acetate] = 0.02 M) and was applied to a DEAE-cellulose column (2.5 × 8 cm) in the bicarbonate form. The column was eluted with 150 mL of 0.01 M triethylammonium bicarbonate in 50% ethanol followed by a 1 L linear gradient of 0.01 to 0.50 M triethylammonium bicarbonate in 50% ethanol. The eluted $G^{m,iB}pG^{m,iB}pU(OAc)_2$ (655 A_{257} units) was obtained in 7% yield after removal of buffer by evaporation and was stored in anhydrous pyridine at 0 °C. A UV spectrum gave λ_{max} 257 nm, λ_{min} 232 nm, sh 280 nm in 50% ethanol–water.

Removal of the protecting groups gave pure G^mpG^mpU which has R_f values of 0.02 (solvent A) and 0.28 (solvent F) on paper chromatography and a retention time of 37.5 min (0.001–1.0 M ammonium acetate, 50 mL gradient) on HPLC. The UV spectrum in water showed λ_{max} 255 nm, λ_{min} 227 nm, sh 270 nm. The trimer was digested to G^m, pG^m, and pU by snake venom phosphodiesterase and to G^mp and U by spleen phosphodiesterase.

Preparation of $G^mp(Et)G^mp(Et)U$. $G^{m,iB}pG^{m,iB}pU(OAc)_2$

(132 A_{257} units) was acetylated in 0.5 mL of pyridine containing 0.5 mL of acetic anhydride to give $AcOG^{m,iB}pG^{m,iB}pU(OAc)_2$ which was isolated by precipitation from ether. The fully protected trimer was dissolved in a solution containing 0.2 mL of *N,N*-dimethylformamide, 0.1 mL of 2,6-lutidine, and 0.1 mL of anhydrous ethanol. *p*-Toluenesulfonyl chloride (15 mg) was added and after 1 h three subsequent additions of *p*-toluenesulfonyl chloride (15 mg) and ethanol (0.1 mL) were made at 1-h intervals. After addition of 1 mL of 50% aqueous pyridine, the reaction mixture was incubated with 1 mL of concentrated ammonium hydroxide for 2.5 days. The reaction mixture was chromatographed on paper using solvent I. $G^mp(Et)G^mp(Et)U$ (R_f = 0.38) was eluted from the paper chromatogram with 10% ammonium hydroxide and was then passed through a 2-mL DEAE-Sephadex column to remove a small amount of contaminating $G^mp(Et)G^mpU$, $G^mpG^mp(Et)U$ (R_f = 0.26). The pure $G^mp(Et)G^mp(Et)U$ (31 A_{255} units) was obtained in 27% yield. The triester was not retained on the HPLC Pellionex column and gave a UV spectrum in water at pH 6.6 with λ_{max} 255 nm, λ_{min} 227 nm, sh 270 nm.

Preparation of G^mpG^m. $MTrG^{m,iB}p$ (35 mg; 0.04 mmol) and $G^{m,iB}$ (22 mg; 0.06 mmol) were reacted with triisopropylbenzenesulfonyl chloride (30 mg; 0.1 mmol) in 0.4 mL of pyridine. After 6 h, an additional 18 mg (0.05 mmol) of $G^{m,iB}$ and 15 mg (0.05 mmol) of triisopropylbenzenesulfonyl chloride in 0.1 mL of pyridine was added. The reaction was terminated after 6 h and the protecting groups were removed. The dimer was purified by chromatography on a DEAE-Sephadex A-25 column (5 × 12 cm) using a 1 L linear gradient of 0.01–0.50 M ammonium bicarbonate. The dimer which was contaminated by small amounts of triisopropylbenzenesulfonic acid and G^mp was rechromatographed on the DEAE-Sephadex column. The pure dimer (285 A_{254} units, 0.12 mmol, 30% yield) had an R_f value of 0.37 (solvent C) on paper chromatography and a retention time of 8.8 min (0.001–0.50 M ammonium acetate) on HPLC. The UV spectrum in water (pH 6.6) showed λ_{max} 253 nm, λ_{min} 226, sh 275 nm. Digestion of the dimer with snake venom phosphodiesterase gave G^m and pG^m and with spleen phosphodiesterase gave G^mp and G^m.

Preparation of $G^mpG^mp[^3H]U$. $G^mpG^mp[^3H]U$ was prepared by primer-dependent polynucleotide phosphorylase catalyzed reaction of G^mpG^m with [^{3}H]UDP for 4 days at 37 °C using conditions described by Uhlenbeck et al. (1970). The enzyme was prepared by partial trypsin digestion of commercial polynucleotide phosphorylase using the method of Klee and Singer (1967) and was shown to be completely primer dependent. The incubation mixture contained 1.5 mM G^mpG^m, 1.0 mM [^{3}H]UDP (specific activity 1.3 Ci/mmol, 500 mCi/mmol, or 100 mCi/mmol), 10 mM $MnSO_4$, 0.2 M NaCl, 0.2 M Tris-HCl (pH 9.2), 0.31 mg/mL polynucleotide phosphorylase II, 0.2 mg/mL RNase A, 0.5 unit/mL RNase T_1. After further treatment with bacterial alkaline phosphatase, the reaction mixture was chromatographed on paper with solvent A. The trimer, R_f = 0.02 (solvent A), was rechromatographed on paper with solvent F (R_f = 0.28). The trimer migrated coincidentally with chemically synthesized G^mpG^mpU on paper chromatograms and was obtained in a 7–12% yield.

Preparation of $G^mp(Et)G^mp(Et)[^3H]U$. The pyridinium salt of $G^mpG^mp[^3H]U$ (1×10^7 cpm; specific activity 1.3 Ci/mmol) was acetylated in 0.2 mL of 50% pyridine–acetic anhydride overnight. The acetylated trimer was then treated with 5 mg of *p*-toluenesulfonyl chloride in a solution containing 10 μL of anhydrous *N,N*-dimethylformamide, 10 μL of anhydrous 2,6-lutidine, and 10 μL of anhydrous ethanol. Two subsequent additions of *p*-toluenesulfonyl chloride (5 mg), ethanol (25 μ L), and *N,N*-dimethylformamide (25 μL) were made at 2-h intervals. After treatment with 40 μL of concentrated ammonium hydroxide for 1 h at 55 °C, the reaction mixture was chromatographed on paper using solvent I. $G^mp(Et)G^mp(Et)[^3H]U$ was isolated in 41% yield (4.1×10^6 cpm) after elution from the chromatogram (R_f = 0.38) with 10% ammonium hydroxide. The radioactive triester migrated coincidentally with authentic $G^mp(Et)G^mp(Et)U$ on paper chromatography.

Equilibrium Dialysis Experiments. The equilibrium dialysis experiments were carried out as previously described (Miller et al., 1974). The buffer contained 0.1 M sodium chloride, 10 mM magnesium chloride, 10 mM Tris-HCl (pH 7.5), 0.01% sodium azide. tRNA at a concentration of 40–58 μM and oligomer at a concentration of 0.31–14 μM were placed in opposite chambers. Chambers were analyzed after 4 days of equilibration. The 3′-CA terminus of unfractionated tRNA *E. coli* was removed from tRNA *E. coli* with snake venom phosphodiesterase using methods described by Miller et al. (1974).

Aminoacylation Experiments. Phenylalanine aminoacylation of tRNA *E. coli* and inhibition of aminoacylation at 37 °C were carried out using the conditions of Barrett et al. (1974).

Cell Culture. A fibroblastic cell line established in this laboratory from benzo[*a*]pyrene transformed Syrian hamster embryo cells was used. Stock cultures were grown in 75-cm^2 Falcon flasks in monolayer using Eagles reinforced medium containing 10% Rehatuin filtered serum and 0.22% sodium bicarbonate in a 5% CO_2 atmosphere. For use in the various experiments described below, cells were removed from the 75-cm^2 flask 3 or 4 days after passage by incubation with 0.01% trypsin for 5 min at 37 °C. The cells were suspended in 10 mL of medium. Aliquots were diluted to the appropriate concentration and then seeded into culture vessels in which the experiment was to take place. The cells were then allowed to grow 3 to 4 days. The cell cultures used in these experiments were shown to be free of mycoplasma, by Microbiological Associates, Walkersville, Md.

Transport of $G^mp(Et)G^mp(Et)[^3H]U$ into Transformed Hamster Fibroblasts. Two separate experiments were performed. Cells were seeded in 16-mm test wells (4000 cells/well) or 6-mm test wells (2000 cells/well) and allowed to grow for 3 days. The medium was removed and fresh medium (0.15 mL in 16-mm wells or 20 μL in 6-mm wells) or medium containing 25 μM $G^mp(Et)G^mp(Et)[^3H]U$ was added. The cells were then incubated at 37 °C. The medium in the 16-mm wells was removed at 0, 40, 70, 130 min, and 24 h. The medium in the 6-mm wells was removed at 0, 2, 4, 7, 15, and 24 h. The cells were washed three times with phosphate-buffered saline. The medium and washings were counted in 10 mL of Hydromix. The cells were lysed in the well by addition of 0.5% sodium dodecyl sulfate solution, and then the well was rinsed twice with phosphate-buffered saline and the rinsings and lysate were combined. Aliquots of the lysate (50 or 100 μL) were counted in 10 mL of Hydromix, while the remaining lysate was stored at −20 °C for further analysis. Cell counts were determined as described below.

Radioactive compounds in the cell lysates were characterized as follows. Column chromatography on Bio-Gel-P30 or on DEAE-cellulose was used to resolve high-molecular-weight RNA, oligonucleotides, and mononucleotides. Materials eluted from these columns were further characterized by enzymatic degradation, cellulose thin-layer chromatography, and com-

parison of chromatographic mobilities to those of known compounds.

Effect of $G^mp(Et)G^mp(Et)U$ on Cellular Protein Synthesis and RNA Synthesis. Cells were seeded in 6-mm test wells (2000 cells/well) and allowed to grow for 3 to 4 days. The medium was replaced with 20 μL of fresh medium as control or with 20 μL of medium containing the triester. The cells were then incubated for the appropriate time period. A 5-μL aliquot of amino acid ([^{3}H]leucine, 250 μCi/mL of medium; L-[^{14}C]amino acid mixture, 100 μCi/mL of medium; or L-[^{3}H]amino acid mixture, 200 μCi/mL of medium) or a 5-μL aliquot of [^{14}C]adenosine (150 μCi/mL of medium) was added to each well and the cells were further incubated. The medium was removed at 0, 10, 20, or 40 min, the cells were lysed with 100 μL of 0.5% sodium dodecyl sulfate solution, and the lysate was added to 1 mL of ice-cold 5% trichloroacetic acid. The well was washed with two (100 μL) portions of phosphate-buffered saline and the washings were added to the Cl_3CCOOH solution. When measuring protein synthesis the Cl_3CCOOH precipitate was heated at 75 °C for 20 min before filtering. When measuring RNA synthesis, the precipitate from the cold Cl_3CCOOH solution was filtered directly. Precipitates were collected on glass-filters, washed with four (1 mL) portions of 2 N hydrochloric acid and four (1 mL) portions of 95% ethanol, dried, and counted in toluene-based scintillation fluid. Cell counts were determined by incubating cells from control wells with 0.2 mL of 0.25% trypsin–0.01% EDTA for 20 min. The digest was counted in 9.8 mL of Isoton using a Coulter counter. For experiments involving prolonged incubation with triester, cell counts were obtained from control wells containing 25 μM $G^mp(Et)G^mp(Et)U$.

Effect of $G^mp(Et)G^mp(Et)U$ on Cell Growth. Cells were seeded into 16-mm wells (4000 cells/well). Three wells were seeded for each compound at each time point. After 24 h, the medium was replaced with either: (1) medium containing 0.25% dimethyl sulfoxide, (2) medium containing 25 μM G^mpG^mpU and 0.25% dimethyl sulfoxide, or (3) medium containing 25 μM $G^mp(Et)G^mp(Et)U$ and 0.25% dimethyl sulfoxide. The cells were incubated at 37 °C and cell numbers were determined at the times indicated in Figure 5. The error in determining the number of cells was 10%.

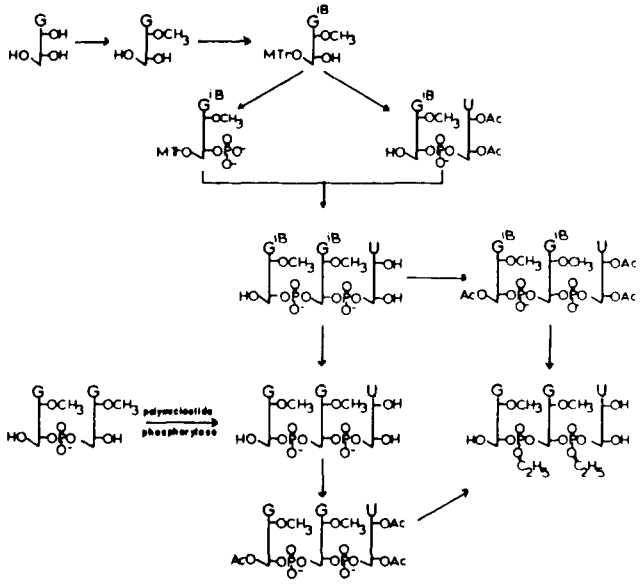
Effect of $G^mp(Et)G^mp(Et)U$ on Colony Formation. Aliquots (0.5 mL) of transformed hamster fibroblasts (200 cells/mL) suspended in medium containing either: (1) 0.3% dimethyl sulfoxide; (2) 0.3% dimethyl sulfoxide and 5, 10, or 25 μM G^mpG^mpU; (3) 0.3% dimethyl sulfoxide and 5, 10, or 25 μM $G^mp(Et)G^mp(Et)U$ were placed in 16-mm test wells. Six wells were seeded for each compound at each concentration. The cells were allowed to grow for 5 days. The cells were then fixed and stained, and the number and size of colonies formed were determined.

Results

Synthesis of $G^mp(Et)G^mp(Et)U$. The basic procedures involved in the synthesis of oligonucleotide ethyl phosphotriesters have been described in our previous paper (Miller et al., 1974). For the synthesis of $G^mp(Et)G^mp(Et)U$, the approach involved synthesis of a protected trinucleotide with the base sequence G^mpG^mpU followed by ethylation of the internucleotide phosphoryl groups. The sequence of reactions is outlined in Scheme I.

Stepwise condensation of suitably protected derivatives of 2′-*O*-methylguanosine and uridine 5′-phosphate and selective removal of protecting groups yielded two oligomers, $G^{m,iB}pG^{m,iB}pU(OAc)_2$ and G^mpG^m. The 5′-hydroxyl group

Scheme I



of the trinucleotide was then acetylated. After ethylation, the protecting groups were removed to give the triester $G^mp(Et)$-$G^mp(Et)U$.

Reaction of G^mpG^m with [^{3}H]uridine 5′-diphosphate catalyzed by primer-dependent polynucleotide phosphorylase yielded the labeled trimer G^mpG^mp[^{3}H]U. After temporary protection of the hydroxyl groups by acetylation, the trinucleotide was converted to its phosphotriester derivative, $G^mp(Et)G^mp(Et)$[^{3}H]U, which was identical to the triester produced by the other synthetic route.

Interaction of $G^mp(Et)G^mp(Et)U$ with tRNA in Vitro. The interaction of tritium-labeled G^mpG^mpU and its ethyl phosphotriester derivative with $tRNA^{phe}_{yeast}$, unfractionated tRNA *E. coli*, and unfractionated tRNA *E. coli* lacking the 3′-CpA terminus was measured by equilibrium dialysis (Miller et al., 1974). The binding constants for these oligomers at various temperatures in 0.1 M sodium chloride, 10 mM magnesium chloride are given in Table I.

The binding constant of G^mpG^mpU is largest at 0 °C and diminishes with increasing temperature. The triester appears to have similar binding constants at all temperatures studied. This effect may be due to self-aggregation of $G^mp(Et)$-$G^mp(Et)U$ at low temperatures, which would result in a decreased apparent binding to the tRNA.

Removal of the 3′-CpA nucleotides from unfractionated tRNA *E. coli* by treatment with snake venom phosphodiesterase results in reduction of the binding constants for both oligomers. This indicates that the major binding site for these oligomers is, indeed, the 3′-amino acid accepting end of the tRNA. The residual binding observed may be due to binding to other complementary single-stranded regions of the tRNA.

The effect of G^mpG^mpU and $G^mp(Et)G^mp(Et)U$ on aminoacylation of $tRNA^{phe}_{E.coli}$ at 37 ° is shown in Table I. The aminoacylation of unfractionated tRNA *E. coli* catalyzed by a mixture of synthetases isolated from *E. coli* was studied. The percent inhibition of the extent of the aminoacylation reaction (Barrett et al., 1974) is the same for both oligomers.

Transport of $G^mp(Et)G^mp(Et)U$ into Transformed Hamster Fibroblasts. Cells growing during log phase were incubated with $G^mp(Et)G^mp(Et)U$ at an initial concentration of 25 μM. The amount of radioactivity in the medium and in the cell lysate was determined at various times for a 24-h period

TABLE I: Interaction of $G^mp(Et)G^mp(Et)U$ with Transfer RNA.

	Temp (°C)	$K(M^{-1})^a$ $tRNA^{Phe}_{yeast}$	$K_{app}(M^{-1})^a$ $tRNA_{E.\ coli}$	$K_{app}(M^{-1})^a$ $tRNA_{E.\ coli\text{-}CA}$	% Inhibition of Phe[b] Aminoacylation
$G^mp(Et)G^mp(Et)U$	0	3 100	9 300	1600	
	25	3 100	1 900		
	37	1 700	2 000		39
G^mpG^mpU	0	63 500	103 000	4000	
	25	5 300	12 300		
	37	750	1 100		39

[a] 0.1 M NaCl, 10 mM $MgCl_2$, 10 mM Tris, pH 7.5. [b] 2 μM tRNA, 50 μM oligomer.

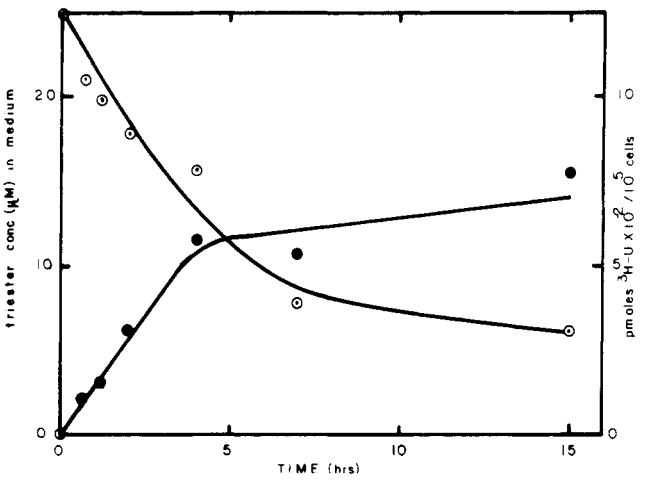
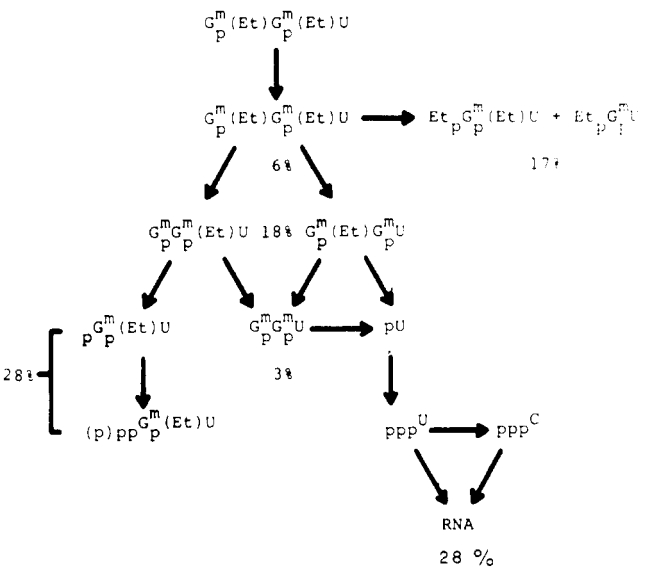


FIGURE 1: Transport of $G^mp(Et)G^mp(Et)[^3H]U$ into transformed hamster fibroblasts growing in monolayer. Extracellular triester concentration: (○) μM. Intracellular amount of [³H]U from triester and its metabolites (●). The initial triester concentration was 25 μM.

Scheme II



as shown in Figure 1. During the first 4 h, the triester concentration in the medium decreases in a linear manner and then falls more slowly to a final concentration of 7 μM at 24 h. The decrease in extracellular triester concentration is accompanied by an increase in radioactivity found in the cell lysate.

The radioactivity recovered from both the medium and the cell lysate was analyzed by gel filtration chromatography to determine if any degradation of the triester had occurred. Only intact $G^mp(Et)G^mp(Et)[^3H]U$ was found in the medium. However, a substantial amount of the radioactivity from the cell lysate was found to be incorporated into high-molecular-weight RNA as uridine and cytidine. After 40 min of incubation, 22% of the radioactivity was found in the RNA, while, after 24 h, 83% of the radioactivity was found in the RNA.

Radioactive material in the cell lysate was further characterized by a combination of DEAE-cellulose chromatography, cellulose thin-layer chromatography, digestion of isolated products with nuclease enzymes, and comparison of chromatographic mobilities with known compounds. The results of this analysis are outlined in Scheme II, which shows the radioactive products found in the cell lysate after 2 h of incubation with $G^mp(Et)G^mp(Et)[^3H]U$. Approximately 27% of the radioactivity occurs in trinucleotide species with the sequence G^mG^mU, 28% is incorporated as uridine or cytidine into high-molecular-weight RNA, and the remainder is found in various mono- and dimeric species.

In contrast to the behavior of $G^mp(Et)G^mp(Et)U$, the diester G^mpG^mpU was rapidly hydrolyzed in the culture medium. After 1 h of incubation, only uridine and uridine 5′-phosphate were recovered from the medium. Radioactivity recovered from the cell lysate was found in either high-molecular-weight RNA or as the 5′-triphosphates of uridine or cytidine. No intact $G^mpG^mp[^3H]U$ was found in the cell lysate.

Effect of $G^mp(Et)G^mp(Et)U$ on Cellular Protein Synthesis and RNA Synthesis. The effect of increasing concentrations of triester on the kinetics of protein synthesis by transformed hamster fibroblasts is shown in Figure 2. In these experiments, cells growing in monolayer were preincubated with triester for 1 h before addition of radioactive amino acid. The amount of radioactivity in hot trichloroacetic acid precipitable material from the cell lysate was then determined. Incorporation of amino acid into protein is linear for at least 40 min. The rate of incorporation decreases proportionally with increasing added concentration of triester over a triester concentration range of 100 μM. This inhibition is not due to a decreased ability of the cells to transport amino acid from the medium. Preincubation of cells with 100 μM triester had no effect on the transport of leucine into the cell lysate. During the preincubation period, the cells showed no morphological changes, indicating cell death as determined by microscopic examination. The parent diester, G^mpG^mpU, had no significant effect on incorporation of [³H]leucine into cellular protein, even at an initial concentration of 200 μM (Table II).

Figure 3 shows the incorporation of radioactivity into cold trichloroacetic acid precipitable material when transformed

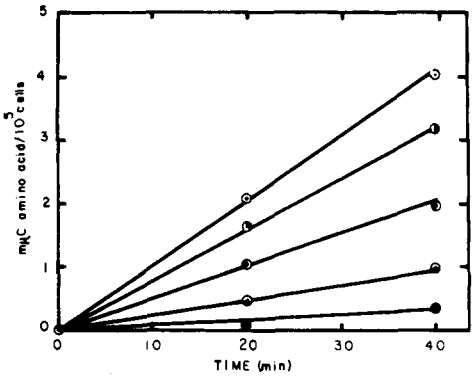


FIGURE 2: Effect of 0 (○), 10 (◑), 25 (◐), 50 (◒), and 100 μM (●) $G^mp(Et)G^mp(Et)U$ on protein synthesis in transformed hamster fibroblasts growing in monolayer. Cells were preincubated with triester for 1 h, a mixture of L-[^{14}C]amino acids was added, and the cells were lysed at the indicated times. Protein was precipitated in hot 5% Cl_3CCOOH.

TABLE II: Effect of Oligomers on Protein Synthesis in Syrian Hamster Transformed Fibroblast Cells.

	Oligomer Concn (μM)	% Inhibition[a]
$G^mp(Et)G^mp(Et)U$	200	73
	100	53
	50	39
G^mpG^mpU	200	8
	100	4
	50	12

[a] % Inhibition of incorporation of [^{3}H]Leu into protein at 37 °C. Hamster fibroblasts in monolayer were preincubated with oligomer for 1 h before addition of [^{3}H]Leucine.

hamster fibroblasts are incubated with [^{14}C]adenosine for various periods of time. The material in the precipitate consisted entirely of RNA, as shown by its complete susceptibility to hydrolysis by ribonucleases A and T_1. After an initial lag, the rate of RNA synthesis is linear over a 30-min period. When cells were preincubated with 25 μM $G^mp(Et)G^mp(Et)$ before addition of [^{14}C]adenosine, the rate of RNA synthesis during the linear portion of incorporation appeared to be slightly increased relative to the control. Preincubation of cells with 100 μM triester had no effect on transport of [^{3}H]uridine into the cells.

The effect of prolonged incubation of triester on cellular protein synthesis and RNA synthesis is shown in Figure 4. In these experiments, the cells were preincubated with triester at an initial concentration of 25 μM for various lengths of time. Labeled amino acid or adenosine was then added and the amount of radioactivity incorporated after 40 min into protein or RNA per 10^5 cells was determined. Values obtained were compared with similar values from untreated controls. As shown in Figure 4, the rate of protein synthesis is inhibited 40% for the first 4 h of triester incubation. During the next 11 h, the rate increases until at 15 h it is 30% greater than that of the control. The rate of RNA synthesis appears to increase slightly during the first 4 h of triester treatment and then returns to the level of the control.

Inhibition of Cell Growth and Colony Formation by $G^mp(Et)G^mp(Et)U$. Cells were seeded at an initial density of 8000 cells/mL and were allowed to attach for 24 h. The medium was then replaced with medium containing 25 μM $G^mp(Et)G^mp(Et)U$ or 25 μM G^mpG^mpU, and cell growth was

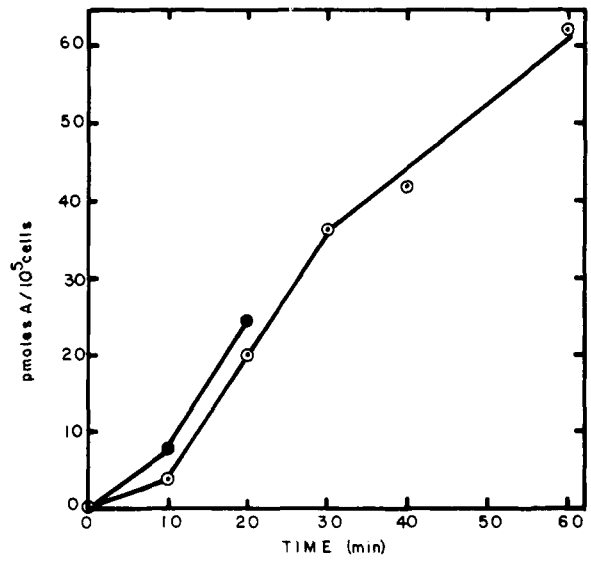


FIGURE 3: Effect of 0 (○) and 25 μM (●) $G^mp(Et)G^mp(Et)U$ on RNA synthesis in transformed hamster fibroblasts growing in monolayer. Cells were preincubated with triester for 1 h, [^{14}C]adenosine was added, the cells were lysed at the times indicated, and RNA was precipitated with cold 5% Cl_3CCOOH.

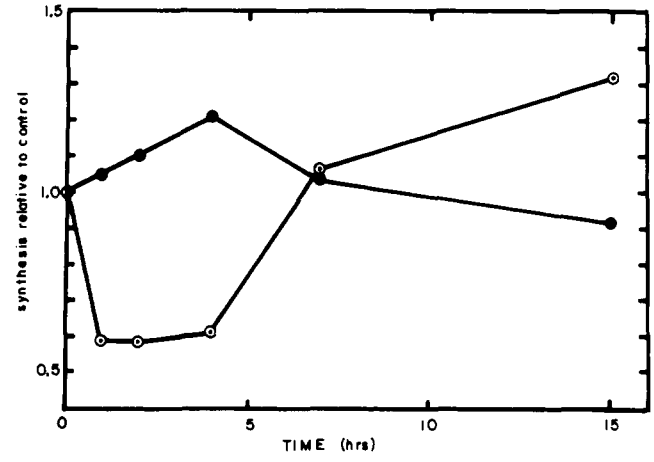


FIGURE 4: Effect of 25 μM $G^mp(Et)G^mp(Et)U$ on protein synthesis (○) and RNA synthesis (●) in transformed hamster fibroblasts growing in monolayer. Cells were preincubated with triester for the indicated time periods. L-[^{3}H]Amino acids or [^{14}C]adenosine was added and the amount of radioactivity incorporated into protein and RNA was determined after 40 min.

monitored as indicated in Figure 5. The diester, G^mpG^mpU, had no effect on the growth of the cells. The triester, on the other hand, inhibited cell growth for approximately 24 h. After this time, the cells began to grow at the same rate as the untreated controls.

A different effect was observed on formation of colonies by the cells. Cells were seeded at a low density in medium containing various concentrations of $G^mp(Et)G^mp(Et)U$ or G^mpG^mpU. After 5 days, the plates were fixed and stained and the number of colonies was counted. As shown in Table III, 25 μM $G^mp(Et)G^mp(Et)U$ caused a 50% reduction in the number of colonies formed relative to the control. Furthermore, the size of the colonies formed was approximately 50% smaller than that of the control. This effect was dependent upon the initial concentration of $G^mp(Et)G^mp(Et)U$ in the medium. The diester, G^mpG^mpU, had no significant effect on the number or the size of colonies formed.

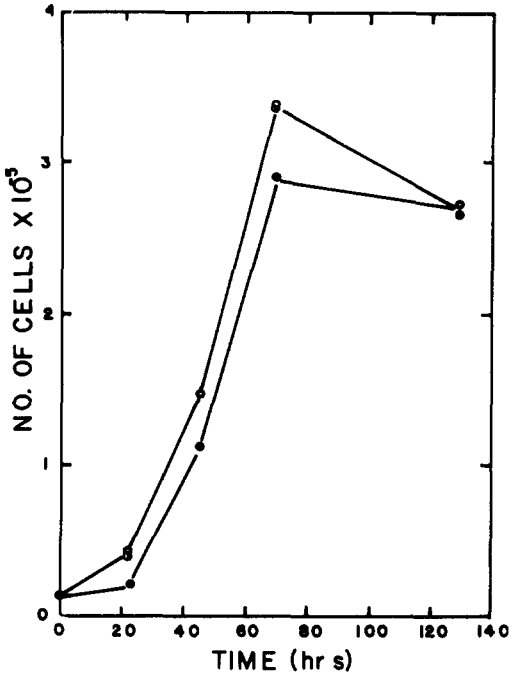


FIGURE 5: Growth of transformed hamster fibroblasts in monolayer in the absence (○) and in the presence of 25 μM $G^mp(Et)G^mp(Et)U$ (●) and 25 μM G^mpG^mpU (◑). The oligomers were applied at 0 h.

TABLE III: Effect of Oligomers on Colony Formation by Transformed Hamster Fibroblast Cells.

	Oligomer Concn (μM)	% Control[a]
$G^mp(Et)G^mp(Et)U$	25	50
	10	61
	5	89
G^mpG^mpU	25	89
	10	94
	5	94

[a] Average result of two experiments. The control contained 18 colonies after 5 days.

Discussion

The present study was undertaken with three objectives in mind: (1) to synthesize an oligonucleotide ethyl phosphotriester complementary to a cellular target nucleic acid and to study its physical binding with the target nucleic acid at physiological conditions; (2) to investigate triester transport into mammalian cells in culture and to study the fate of the triester within the cell; (3) to measure effect of the triester on the biochemical processes and the growth of living cells.

The target nucleic acids in this study are tRNA and mRNA. The oligonucleotide ethyl phosphotriester is $G^mp(Et)$-$G^mp(Et)U$ which is complementary to the 3'-ApCpCp ... amino acid accepting end of most tRNAs, the anticodon region of $tRNA^{gly}$, and the threonine codon of mRNA. Our previous study (Miller et al., 1974) showed that complementary ribooligonucleotides form complexes with tRNA with binding constants 8 to 20 times higher than corresponding oligodeoxyribonucleotides. Since phosphotriester derivatives of ribooligonucleotides are readily hydrolyzed in aqueous solution (Shooter et al., 1974; Singer and Fraenkel-Conrat, 1975), the 2'-*O*-methylribooligonucleotide ethyl phosphotriester, $G^mp(Et)G^mp(Et)U$, was prepared. In this analogue, the methylated 2'-hydroxyl groups are prevented from participating in cleavage of the phosphotriester moiety, while the basic conformational features of the ribonucleotide-type backbone are maintained (Alderfer et al., 1974).

The synthesis of $G^mp(Et)G^mp(Et)U$ followed the basic procedures used to synthesize oligodeoxyribonucleotide ethyl phosphotriesters (Miller et al., 1974), with the following exception. In the previous syntheses, the oligonucleotide chain was built in a stepwise manner with ethylation of each phosphodiester linkage as it was formed after mononucleotide addition. In the present approach, the oligonucleotide was first synthesized by either a completely chemical procedure or by a combination of chemical and enzymatic methods. The phosphate groups in the phosphodiester linkage of the completed oligonucleotide were then ethylated. This approach has the advantage of decreasing the number of synthetic steps required and can be applied to oligomers which have been prepared enzymatically.

Both $G^mp(Et)G^mp(Et)U$ and G^mpG^mpU form hydrogen-bonded complexes with $tRNA^{Phe}_{yeast}$ and unfractionated tRNA *E. coli* in 0.1 M sodium chloride, 10 mM magnesium chloride solution at 37 °C (Table I). At 37 °C, the binding constants of these oligomers are approximately fivefold greater than that of GpGpU (K = 200 M^{-1}) under the same conditions. The binding was reduced by 6- to 20-fold after the oligomer binding site was partially removed by treatment of tRNA *E. coli* with snake venom phosphodiesterase. The residual binding most likely results from complex formation with the anticodon region of $tRNA^{gly}$ and other complementary single-stranded sequences in the unfractionated tRNA. Although the binding constants of G^mpG^mpU show the expected temperature sensitivity, those of the triester appear to be much less affected by increasing temperature. This may be due to self-aggregation of the triester at low temperature. Oligomers with G-rich sequences are particularly prone to self-aggregation (Uhlenbeck, 1972) and this effect is probably enhanced in the case of the triester, since charge repulsion between oligomers has been eliminated by ethylation of the phosphate backbone (Miller et al., 1971, 1974).

The extent of synthetase-catalyzed aminoacylation of $tRNA^{Phe}_{E.coli}$ is inhibited by 39% by both 50 μM $G^mp(Et)G^mp(Et)U$ and 50 μM G^mpG^mpU, at 37 °C. On the basis of the binding results in Table I, it may be calculated that approximately 10% of the free tRNA in the aminoacylation reaction mixture is complexed with $G^mp(Et)G^mp(Et)U$. The magnitude of binding of the triester to the synthetase-tRNA complex is not known. If there is an appreciable increase in complex formation between the triester and synthetase bound tRNA, this could account for the higher extent of inhibition of aminoacylation than predicted on the basis of simple binding of triester to free tRNA. Our previous investigation did not indicate that inhibition is due to inactivation of the synthetase enzyme by the triester. The inhibition of aminoacylation by d-Tp(Et)Gp(Et)G was shown to be a consequence of binding of the triester to the tRNA and not due to inhibition of the synthetase enzyme (Barrett et al., 1974).

Oligodeoxyribonucleotide ethyl phosphotriesters are resistant to nuclease hydrolysis by serum nucleases (Miller et al., 1974) and are taken up by Syrian hamster embryo cells growing in monolayer (Barrett, 1974). Similarly, we found that $G^mp(Et)G^mp(Et)U$ is totally resistant to hydrolysis when incubated with cell culture medium supplemented with 10% bovine serum both in the absence or presence of cells, while G^mpG^mpU is rapidly hydrolyzed to monomeric units. The triester is taken up by transformed Syrian hamster fibroblasts

growing in monolayer in the manner shown in Figure 1. After 4 h, the concentration of triester within the cell based on uptake of radioactivity into the cell lysate is calculated to be approximately 4 mM assuming a cell volume of 1.5×10^{-9} cm^3 (Hempling, 1972).

Since the calculated triester concentration within the cell far exceeds the extracellular concentration, the uptake data suggested the triester is metabolized by the cells. Analysis of the radioactivity recovered from the cell lysate by chromatographic procedures showed that substantial degradation of the triester took place. After 24 h of incubation, 83% of the labeled uridine from the triester was found in high-molecular-weight RNA as uridine or cytidine, indicating that triester degradation products ultimately enter biosynthetic pathways (Sutton and Kemp, 1976).

The most reasonable mechanism to account for these degradation products is hydrolysis of the phosphotriester linkage, followed by nuclease hydrolysis of the resulting phosphodiesters. Phosphotriesterase activities are known to occur in mammalian sera (Dudman and Zerner, 1973; Aldridge, 1953). The substrate specificity of these enzymes has not been well characterized. The activity responsible for the initial phosphotriester hydrolysis may reside in a cellular structure, since incubation of the triester for 24 h with a crude lysate prepared from the transformed hamster fibroblasts resulted in no detectable degradation of the triester.

Although hydrolysis of $G^mp(Et)G^mp(Et)U$ occurs within the cells, 27% of the products occur as trinucleotide species with the sequence G^mG^mU. Each of the four trinucleotide species can potentially bind the same complementary single-stranded regions of tRNA and mRNA. The binding of $G^mp(Et)G^mpU$ and $G^mpG^mp(Et)U$ would be expected to be similar to that of $G^mp(Et)G^mp(Et)U$, since both $G^mp(Et)G^mp(Et)U$ and G^mpG^mpU have similar binding constants with tRNA.

Incubation of transformed hamster fibroblasts with $G^mp(Et)G^mp(Et)U$ results in a decreased rate of cellular protein synthesis, while G^mpG^mpU has no effect. During prolonged incubation with the triester, protein synthesis is inhibited during the first 4 h and then resumes at approximately the same time when triester uptake begins to level off. In contrast, the rate of cellular RNA synthesis increases slightly during the first 4 h and then returns to the control level.

Three important points emerge from these experiments. First, incubation of the fibroblasts with triester results in inhibition of a cellular biochemical process involving tRNA and mRNA target nucleic acids, namely, protein synthesis. These results might be expected from our biochemical study if $G^mp(Et)G^mp(Et)U$, $G^mp(Et)G^mpU$, $G^mpG^mp(Et)U$, and G^mpG^mpU which have been taken up or formed within the cell physically bind to tRNA and mRNA and thereby inhibit the function of these target RNAs within the cell. Second, the inhibition of protein synthesis is reversible. This reversibility could arise from degradation of the trinucleotide inhibitors within the cell. As the uptake of triester begins to plateau at 4 h, the level of trinucleotide species within the cell must begin to level off and subsequently decrease. The reversibility may also arise from increased levels of RNA molecules, mRNA, tRNA, ribosomal RNA involved in protein synthesis. Third, during the period of inhibition of protein synthesis, the rate of RNA synthesis is slightly stimulated. The apparent increased rate of RNA synthesis during the first 4 h may reflect an attempt by the cell to compensate for reduced levels of protein synthesis. Both triester degradation and increased RNA synthesis would decrease the effective concentrations of trinucleotide species within the cell available for inhibition of protein synthesis.

Cell growth is inhibited during the first 24 h of treatment of the cells with 25 μM $G^mp(Et)G^mp(Et)U$, after which time the cells divide at the same rate as untreated controls. This effect is most likely related to the decreased rate of protein synthesis which occurs during the first 4 h of triester treatment and is consistent with the data on the transport and intracellular degradation of the triester. After 24 h, the extracellular triester concentration has decreased to approximately 7 μM, while most of the triester which has entered the cells has been degraded and the uridine moiety incorporated into RNA. The reversible nature of protein synthesis inhibition and cell growth inhibition complement one another and show that the triester degradation products do not exert a permanent cytotoxic effect on the cells. The diester, G^mpG^mpU, has no effect on cell growth, which further demonstrates that 2′-*O*-methylguanosine and uridine are not toxic to the cells.

$G^mp(Et)G^mp(Et)U$ exerts a more permanent effect on colony formation by fibroblasts. When a suspension of cells at very low density is treated with 25 μM triester for 5 days, the number of colonies formed and the size of these colonies are both reduced by 50%. This result suggests that the reduction in protein synthesis rates as a result of triester treatment perturbs the ability of single cells to attach and form colonies during a critical period. Those cells which do form colonies show a decreased growth rate in the continued presence of the triester. When cells are treated with 25 μM G^mpG^mpU under similar conditions, essentially no effect on colony formation or size is observed. This result is expected, since the diester is rapidly degraded to monomers.

The triester $G^mp(Et)G^mp(Et)U$ is complementary to target nucleic acids involved in cellular protein synthesis and has a transitory effect on the cells which is then relieved by its degradation. Such a transitory effect of inhibition may provide a convenient and useful way of imposing a temporary interruption of cellular functions. On the other hand, neutral oligonucleotide analogues with longer chain lengths and a greater resistance to hydrolysis would be expected to achieve a greater specificity and effectiveness in regulating the function of target nucleic acids inside the cell over a long time period. The observations in the present study lay the groundwork for continued investigation into the use of neutral oligonucleotide analogues as probes and regulators of nucleic acid function within living cells.

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Chemical Synthesis of an Octanucleotide Complementary to a Portion of the Cohesive End of P2 DNA and Studies on the Stability of Duplex Formation with P2 DNA†

R. Padmanabhan‡

ABSTRACT: A pyrimidine octanucleotide complementary to one of the cohesive ends of P2 DNA was chemically synthesized. Its sequence, d(C-T-T-T-C-C-C-C-OH), was verified by labeling it at the 5′ end, followed by partial enzyme digestion and separation by a two-dimensional fingerprinting system. A single ribo-G residue was added to its 3′ end using calf thymus deoxynucleotidyl terminal transferase. The resulting nonanucleotide primer was used in a detailed study on the stability of the duplexes formed in the partial as well as complete repair synthesis catalyzed by DNA polymerase I, at 5 °C in the presence of 70 mM potassium phosphate and 70 mM NaCl. The nonanucleotide primer was able to form a stable duplex with P2 DNA template only in the presence of DNA polymerase I. When the chain lengths of pyrimidine oligonucleotides were varied from 4 to 8 to test their abilities to serve as primers for the enzymatic repair synthesis, it was revealed that the minimum length required for the primer function is 8. Using the nonanucleotide as the primer and the right-hand cohesive end of the DNA as the template, repair synthesis was initiated simultaneously at the 3′ end of the primer as well as at the right-hand 3′ end of the DNA. This resulted in a decrease in the efficiency of repair synthesis at the 3′ end of the primer, possibly due to the displacement of the primer by the enzyme. The enzyme was unable to displace the primer, when the primer was extended to a 13-mer prior to the initiation of repair synthesis at the 3′-OH end of the DNA. These data suggest that the strand displacement by DNA polymerase I at 5 °C in the presence of 70 mM potassium phosphate and 70 mM NaCl is not significant when the duplex is at least 13 nucleotides long. The efficiency of the repair synthesis at the 3′-OH end of the DNA–primer duplex could be increased by blocking the repair synthesis at the 3′-OH end of the DNA by converting it to 3′- phosphate. This method could be useful in DNA sequence analysis, where such specfic repair synthesis is desired.

The complete sequences of the cohesive ends of *Escherichia coli* bacteriophage DNA molecules such as λ, 186, φ80, and P2 have been reported previously (Wu and Taylor, 1971; Padmanabhan and Wu, 1972; Bambara et al., 1973; Padmanabhan et al., 1974a; Murray and Murray, 1973). These DNA molecules have been very useful as models to test new methods for DNA sequencing or to study the properties of enzymes such as polymerases or nucleases (Ghangas and Wu, 1975; Uyemura et al., 1975).

Binding studies involving short synthetic oligonucleotide duplexes have been published previously (Gupta and Khorana, 1968; Kleppe et al., 1970, 1971). It was found that the minimum length of short oligonucleotides required to form stable duplexes with complementary oligo- or polynucleotides varied from 6 to 12 depending on the composition and concentration of the oligonucleotides used. Duplexes with uneven 3′-hydroxyl ends can be repaired by DNA polymerase (Goulian et al., 1973).

A method was proposed for DNA sequence analysis using a short oligonucleotide primer which could be bound to a specific location on the single-stranded region of a DNA molecule (Wu et al., 1972; Wu, 1972). The nucleotide sequence beyond the 3′ end of the primer could be determined

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