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Oligonucleotides with rapid turnover of the phosphate groups occur endogenously in eukaryotic cells

(hamster lung fibroblasts/*Tetrahymena*/^{32}P/HPLC/nucleases)

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ABSTRACT **Endogenous oligonucleotides were found in trichloroacetic acid extracts of hamster lung fibroblasts and *Tetrahymena* cells. Peaks of radioactivity that eluted with retention times similar to oligonucleotide markers (5- to 50-mer) were found by HPLC in cells labeled briefly with $^{32}P_i$. Only minute amounts of UV-absorbing material were detected, consistent with a rapid turnover of phosphate groups. The ^{32}P-labeled material also migrated as oligonucleotides on 20% polyacrylamide gels; it was not hydrolyzed by alkaline phosphatase but was digested by snake venom phosphodiesterase, S1 nuclease, and pancreatic RNase and was phosphorylated by T4 polynucleotide kinase. The ^{32}P-labeled material isolated by HPLC was alkali labile and the hydrolyzate ran as nucleotides on paper chromatography. It is concluded that the oligonucleotides are mainly oligoribonucleotides, but it is possible that oligodeoxynucleotides are also present.**

Uptake into living cells of synthetic oligonucleotides that are complementary to selected target sequences on viral or mRNA can modulate expression of the RNA markedly. In previous work, inhibition of the gene expression of Rous sarcoma virus (1, 2) and the acquired immune deficiency syndrome (AIDS) virus (HIV) (3) was described. This work describes acid-soluble cellular oligonucleotides that exist in cells in very small amounts. They are demonstrated by radioactive labeling with ^{32}P. Using ^{32}P-labeled cellular oligonucleotides, we show that they are substrates for specific and nonspecific nucleases.

The presence of endogenous cellular oligoribonucleotides of the same size class as the synthetic oligodeoxynucleotides used previously (5–30 nucleotides long) (3) is an unusual finding.

MATERIALS AND METHODS

Materials. Phosphomonoesterase (Worthington or Boehringer, bacterial alkaline phosphatase) purified free of nuclease activity was a gift from Earl Baril (Worcester Foundation). Pancreatic RNase, pancreatic DNase, and SP1 nuclease were from Pharmacia; oligodeoxynucleotide markers for electrophoresis, T4 polynucleotide kinase, and snake venom phosphodiesterase were from Boehringer. Radioactive nucleotides were from ICN or New England Nuclear. Oligodeoxynucleotides were synthesized as described (3) and used as markers for HPLC.

Cellular Material. Chinese hamster lung fibroblasts strain 023 (4) were grown in Dulbecco's modified Eagle's medium as described (5). All strains were found to be free of mycoplasma. Twenty hours before labeling, nearly confluent cultures were maintained in growth medium containing 20 mM glucose and 10% dialyzed fetal calf serum in 25 cm^2 Falcon flasks (6). *Tetrahymena* cells were grown in 2% proteose peptone broth at 28°C (7).

Preparation of the Acid-Soluble Cell Extract. To label the cell material, 0.75 mCi (1 Ci = 37 GBq) of carrier-free ^{32}P orthophosphate (HCl-free, ICN 64014) was added to each flask. After 10 min incubation at 37°C, the medium was poured off and the cells were washed gently five times with 10 ml of medium preheated to 37°C. The cells were then extracted with 2.4 ml of ice-cold 10% trichloroacetic acid, which was removed by extraction twice with 18% (wt/vol) trioctylamine in Freon (8). Then, 1.4 ml of the final upper phase was withdrawn from each sample and lyophilized. The lyophilized extract was dissolved in 500 μl of water, and 125-μl aliquots were removed for digestion with alkaline phosphatase, snake venom phosphodiesterase, and S1 nuclease. The digested and control aliquots were subjected to HPLC chromatography (8, 9).

Tetrahymena cells were grown to a density of 200,000 cells per ml and then diluted with 1 vol of proteose peptone broth, left for 1 hr, and then incubated with 1 mCi of carrier-free $^{32}P_i$ (New England Nuclear) for 90 min. The cells were pelleted by brief centrifugation and extracted with trichloroacetic acid as described above.

Double-Gradient Anion Exchange HPLC. This was performed on Waters Associates Partisil (Whatman) strong anion exchange (SAX) cartridges in a Waters Z-compression module using a Waters or a Perkin–Elmer two-pump chromatograph with gradient former. The effluent was monitored for absorbance at 254 nm and for radioactivity with a Berthold (Nashua, NH) LB 505DC HPLC monitor (see Fig. 1) or with a Cerenkov-radiation monitor (a Teflon spiral placed in front of a Geiger–Muller monitor wired to a recorder) (see Fig. 5). The strong anion exchange column was first eluted with a potassium phosphate/KCl linear gradient from 0% to 100% in 45 min as described (8). When GTP had appeared, the high-salt solvent was washed out with the low-salt solvent for at least 10 min, and then a second gradient was run from 0.0032 M to 0.64 M potassium phosphate (pH 6.8) in 20% acetonitrile over 1 hr at 1.5 ml/min. In later experiments (see Fig. 5), a linear gradient of ammonium formate [0.1–4 M (pH 6.8)] in 20% methanol was used.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed on 20% vertical slabs (21 × 23 cm; 1.5 mm thick) containing 8 M urea at pH 8.0 (10). The gels were run at 30 W constant power for 1 hr and exposed to x-ray films at room temperature for 1–24 hr.

RESULTS

Endogenous Oligonucleotides Are Found in Acid-Soluble Cellular Extracts. The initiation of a search for naturally occurring oligonucleotides of 10–30 monomers in cells was suggested by our previous experience in inhibiting the gene expression of RNA viruses (1–3). HPLC analysis of the oligonucleotide domain (starting at 18 min in the second

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gradient) using cellular extracts containing 1–2 mg of protein showed no significant difference in UV absorption from that obtained with the medium alone. A brief labeling *in vivo* with $^{32}P_i$, however, revealed the existence of peaks of radioactivity that traveled with retention times corresponding to oligonucleotides. Fig. 1 shows a 10% trichloroacetic acid extract of hamster cells that was analyzed by strong anion exchange HPLC using double-gradient elution as described. We had labeled the cells *in vivo* by an exposure to $^{32}P_i$ followed by extensive washing with medium preheated to 37°C. The retention time of inorganic phosphate is 14–17 min in the first gradient of this system. By 80 min, the last of the nucleoside triphosphates (GTP) had appeared, and the strong salt solvent was then washed out with the initial solvent. The second gradient was started as indicated by the arrow, and after 20 min two conspicuous peaks of radioactivity, S1 and S2, were recorded as well as some minor peaks (Fig. 1). From our previous unpublished experience with the double-gradient system, it was known that HPLC of standard oligonucleotides (5- to 20-mer) elute with retention times >18 min in this system. There seems to be no temporal relationship between the peaks of radioactivity and the UV peaks; conspicuous peaks of UV-absorbing material were found by analysis of larger amounts of cellular extracts (corresponding to 10 mg of protein), as shown in Fig. 2; in Fig. 2 the labeling of the cellular material was performed *in vitro* with T4 polynucleotide kinase as described.

The Oligonucleotides Migrate as Expected in Polyacrylamide Gel Electrophoresis. Electrophoresis of acid-soluble extracts of fibroblasts labeled *in vivo* with ^{32}P is shown in Fig. 3. The extract was first desalted by adsorption on a Waters C_{18} Sep-Pak cartridge and elution with 60% methanol in water. Electropherograms of cell extracts show a clear intense band running close to a 12-mer standard and some additional weaker bands.

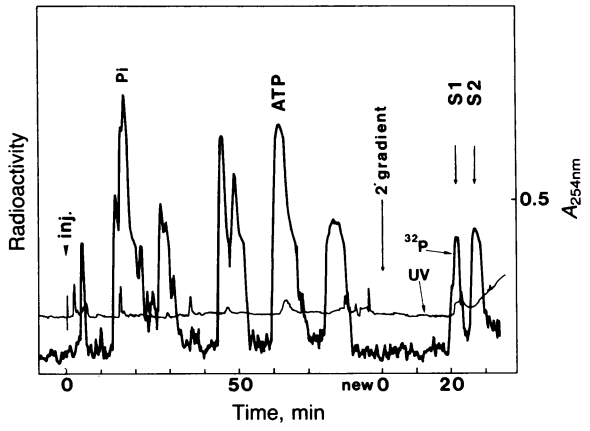

FIG. 1. HPLCs of acid-soluble extracts of strain 023 hamster lung fibroblasts previously incubated with $^{32}P_i$ *in vivo* were analyzed on a Waters SAX 10 cartridge. Elution with the double-gradient system. First gradient: low salt solvent, 0.007 M potassium phosphate (pH 4.1); high salt solvent, 0.25 M potassium phosphate/0.5 M KCl, pH 4.5, linear gradient 0–100% in 45 min. Second gradient: low salt solvent, 0.0032 M potassium phosphate in 20% acetonitrile (pH 6.8); high salt solvent, 0.64 M potassium phosphate in 20% acetonitrile, pH 6.8, linear gradient 0–60% in 60 min. Light tracing, absorbance at 254 nm; heavy tracing, ^{32}P radioactivity monitored with a Berthold 505 monitor fitted with a ^{32}P scintillation cell. No scale is given for the radioactivity deflection because the rate meter of the Berthold monitor switched the range automatically by a factor of 10 when the reading exceeded full deflection. Elution with the high salt solvent was changed to isocratic low salt at retention time 75 min until the start of the second gradient (marked with an arrow). The start of the second gradient was defined as 0 time for the second gradient (the new 0). The flow rate was 1.5 ml/min in all HPLC analyses.

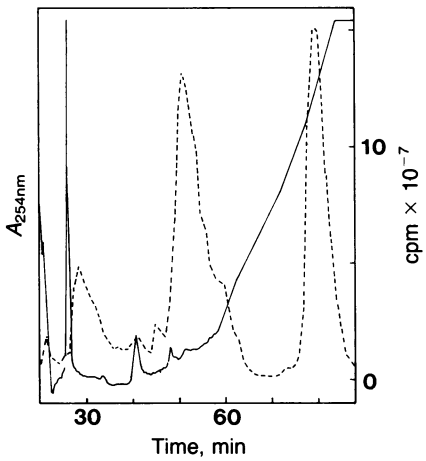

FIG. 2. Second part of double-gradient HPLC analysis of the acid-soluble extract of cells from eight 9-cm dishes of nearly confluent hamster lung 023 fibroblasts that was 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (10, 11). Solvent system as in Fig. 1. Solid line, UV absorption at 254 nm; broken line, ^{32}P radioactivity of 10-μl aliquots measured by liquid scintillation counting. Only the second gradient is shown. Time 0 equals new 0 in Fig. 1.

The ^{32}P Oligonucleotides Are Resistant to Alkaline Phosphatase. The double-gradient HPLC procedure was used to separate mono- from oligonucleotides. In Fig. 4, an aliquot of the extract used in Fig. 1 was treated with phosphodiesterase-free alkaline phosphatase and then chromatographed. All compounds with retention times >34 min in the first gradient, which, amongst others, include the mononucleoside di- and triphosphates, were completely degraded by alkaline phos-

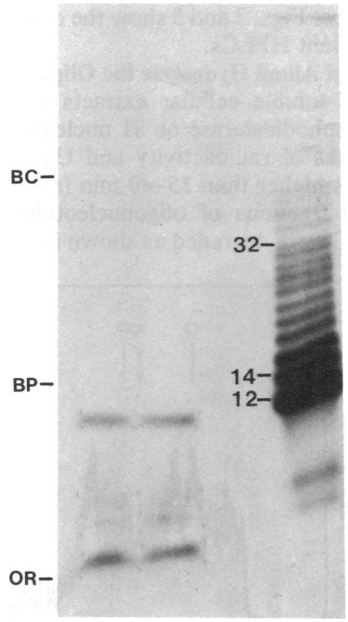

FIG. 3. Electrophoresis of acid-soluble extracts of 023 hamster lung fibroblasts. Material from cells in one 9-cm Petri dish was used for each lane. Each cell culture had been incubated for 10 min with 1 mCi of $^{32}P_i$. The extracts, which had been desalted on C_{18} Sep-Pak cartridges, were run on 1.5-mm-thick 20% polyacrylamide gels containing 8 M urea (pH 8). The positions of xylene cyanol (BC), bromophenol blue (BP), and orange G (OR) that were used as markers are given. An oligonucleotide ladder, prepared by 5′-end-labeling of oligonucleotide markers with [γ-^{32}P]ATP and T4 polynucleotide kinase, was run in the right lane. The intense slowest band of the 023 extract migrates as the 12-mer of the marker mixture.

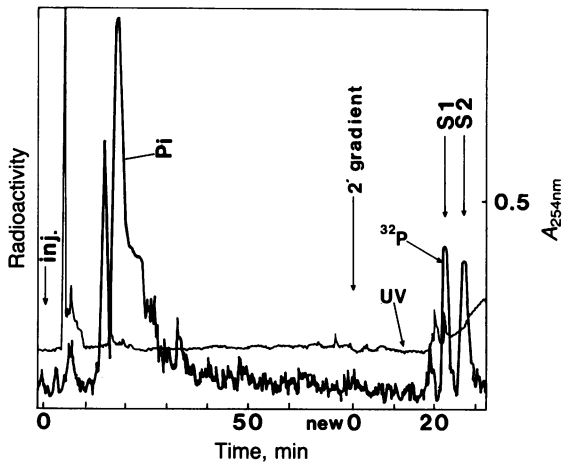

FIG. 4. HPLC of alkaline phosphatase digest of an acid-soluble extract of 023 hamster lung fibroblast. To an aliquot (150 μl) of the same extract used in Fig. 1 was added 15 μl of 1 M Tris·HCl (pH 8.5) and 1 μl (0.15 unit) of bacterial alkaline phosphatase free of phosphodiesterase. After 15 min at 37°C, the incubation mixture was injected on a Whatman Partisil SAX cartridge and chromatographed as described in Fig. 1, except that the change from the high salt solvent to isocratic low salt was initiated at 60 min when it was evident that no ATP was left undegraded.

phatase. The peaks in the oligonucleotide region of the second gradient survived the enzyme treatment.

The Oligonucleotides Can Be Labeled *in Vitro* Using T4 Polynucleotide Kinase and [γ-^{32}P]ATP. Incubation of an acid-soluble extract with [γ-^{32}P]ATP and T4 polynucleotide kinase and subsequent double-gradient HPLC shows a pattern of radioactive labeling that is similar to the results obtained by *in vivo* labeling with P_i shown in Fig. 1 insofar as the peaks labeled have retention times as 5- to 50-mer oligonucleotides. Figs. 2 and 5 show the oligonucleotide part of double-gradient HPLCs.

Nucleases and Alkali Hydrolyze the Oligonucleotides. Incubation of acid-soluble cellular extracts with either snake venom 5′-phosphodiesterase or S1 nuclease resulted in the loss of all peaks of radioactivity and UV absorption with retention times higher than 35–40 min (results not shown). Also, isolated fractions of oligonucleotides from double-gradient HPLC were degraded as shown in Figs. 5 and 6. An

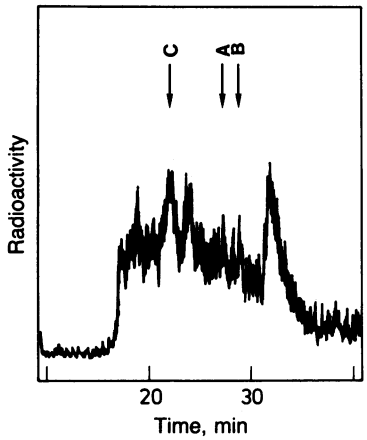

FIG. 5. HPLC of an acid-soluble extract of *Tetrahymena* cells after 5′-end-labeling *in vitro* with [γ-^{32}P]ATP and T4 polynucleotide kinase. Material from 15 ml of *Tetrahymena* cell culture (100,000 cells per ml) was used. The Cerenkov radiation of the eluate was recorded as described. Only the last 40 min of the HPLC is included. Fractions were collected every minute (1.5 ml each); A, B, and C are fractions that were processed further (Fig. 6).

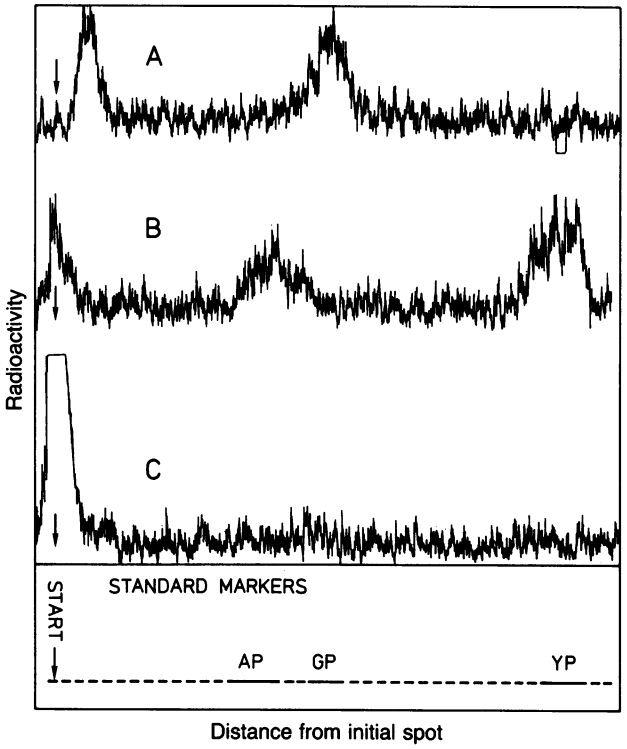

FIG. 6. Scans of the radioactivity on paper chromatograms of fractions A, B, and C from the HPLC in Fig. 5 after treatment with various enzymes. The HPLC fractions were dried in a Savant centrifuge under reduced pressure at 45°C. If salt remained, the residue was dissolved and taken to pH 8.2 with NH_4OH, precipitated by addition of 10 μl of 1 M barium acetate and 2 vol of ethanol in a 1.5-ml Eppendorf tube. The precipitate was collected by centrifugation and dissolved in 0.5 M HCl to give a clear solution; the pH would then be ≈4.5. Ten microliters of 1 M H_2SO_4 was added to precipitate the barium, and the pH of the supernatant was adjusted as required. About 50% of the radioactivity was lost by the barium precipitation procedure. Chromatography was done on Whatman no. 1 paper with the descending technique for 18 hr in 0.1 M potassium phosphate in saturated ammonium sulfate and 2% *n*-propanol (pH 6–5). Marker standards were located by UV light, and radioactivity was located by scanning with a Berthold LB 2722 TLC scanner. The arrows at left indicate the origins. (*A*) Fraction 27 incubated with RNase A for 30 min. (*B*) Fraction 29 treated with 0.05 M NaOH for 20 min at room temperature. (*C*) Fraction 24 incubated with DNase I for 30 min at room temperature. Standards: GP, GMP; AP, AMP; YP, CMP and UMP.

acid-soluble extract of *Tetrahymena* cells (in early logarithmic growth phase) was 5′-end-labeled with ^{32}P using polynucleotide kinase and [γ-^{32}P]ATP. The oligonucleotides were isolated by HPLC, treated with alkali or nuclease, and then chromatographed on paper in a solvent system that retained oligonucleotides near the origin, ran the orthophosphate with the front, and mononucleotides at intermediate R_f values. All 10 runs gave results similar to the 3 shown in Fig. 6. Material remaining at the origin may indicate either incomplete degradation of oligoribonucleotides or the additional presence of oligodeoxynucleotides.

DISCUSSION

The data described indicate that eukaryotic cells in culture contain oligonucleotides with a rapid turnover of ^{32}P. The existence of these compounds was revealed by development of a specialized technique to separate mono- and dinucleotides from oligonucleotides on anion exchange HPLC columns loaded with ^{32}P-labeled acid-soluble cellular extracts,

as herein described. Subsequent elution with solvents that are generally used for purification of synthetic oligonucleotides (8, 9) eluted peaks of ^{32}P radioactivity with retention times characteristic of oligonucleotides. That an initial removal of the mononucleotides is needed in this analysis is shown in Fig. 1. This figure demonstrates that mononucleotides are so abundant that the low level of oligonucleotides would not be detectable otherwise in cellular extracts. The mononucleotides run in the oligonucleotide domain in the phosphate/acetonitrile gradient described in Fig. 1; ATP, for example, runs with a 15- to 30-mer.

The ratio of oligonucleotide to mononucleotides is so disproportionally small that our evidence for their identity is based on behavior of the labeled oligonucleotides when subjected to our double HPLC, plus electrophoresis and treatment with specific enzymes. Fig. 6 shows evidence for the identity of the oligonucleotides. Paper chromatograms demonstrate that hydrolysis with RNase or alkali causes the radioactivity to chromatograph with R_f values that correspond to mononucleotides, in agreement with the expected degradation of the 5′ end to 3′, 5′-nucleoside diphosphates (12). All tests confirmed that S1 and S2 in Fig. 1 behave like oligonucleotides.

The results indicate that oligoribonucleotides exist free in eukaryotic cells. The amounts of cellular material at hand and the methods of separation used have not enabled us as yet to isolate pure oligonucleotides for sequence determination; that several of the oligonucleotides are oligoribotides seems clear from the results exemplified in Fig. 6, since the requirement for a free 2′-hydroxyl group is necessary for base hydrolysis of oligonucleotides, irrespective of their size.

It is perhaps premature to discuss the possible function of the cellular oligonucleotides herein described. It is, however, worth considering that they may play a regulatory role in intracellular metabolism and may conceivably travel from one cell to another in a similar role.

Note Added in Proof. A hamster fibroblast mutant DS-7, unable to use glucose for the maintenance of high ATP levels (6), showed only a diminutive S-1 peak, the S-2 peak being unaffected.

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