The Use of Oligodeoxyribonucleotides Isolated from Pulse-labeled HeLa in DNA Synthesis in Vitro

Emil K. Schandl

Department de Biologie, Université Laval, Québec 10, Province du Québec, Canada

SUMMARY

When isolated from monolayer cultures of pulse-labeled HeLa cells by membrane cone filtration and DEAE-cellulose chromatography, oligodeoxyribonucleotides increase in size to that of a gene in DNA polymerase assays in vitro, as measured by sedimentation in sucrose gradients.

INTRODUCTION

Relatively little is known of the priming role of short-chain oligomers and their assembly into DNA in the cell. The isolation and characterization of oligodeoxyribonucleotides from pulse-labeled Chinese hamster cells have been reported by Schandl and Taylor (8). More recently we have found similar oligomers in pulse-labeled Escherichia coli (9). The oligomers from the mammalian source have an estimated length of 12 nucleotides, whereas those of bacterial origin have 2 distinct sizes, one of 8 and the other of 12 nucleotides. The fact that these oligomers become labeled as a result of short pulses of radioactive DNA precursors, rapidly incorporate into large DNA upon a cold chase with nonradioactive medium, and are susceptible to a 3'-OH end-specific exonuclease (EC 3.1.4.1) suggests that they may play a priming function in DNA replication. Suggestive evidence for this is found in the following report which describes the isolation of oligodeoxyribonucleotides from pulse-labeled HeLa cells and their utilization in DNA synthesis in vitro.

MATERIALS AND METHODS

Monolayer cultures of rapidly dividing asynchronous HeLa S-3 cells were grown at 37°C in plastic bottles with Eagle's basal medium supplemented with Earle's balanced salt solution and 10% fetal bovine serum. The cells were pulsed at 37°C for 2 min with 3H-labeled thymidine, 50 μCi/ml (15.9 Ci/m mole; New England Nuclear, Boston, Mass.), and the label was washed off with ice-cold growth medium. The lysates were incubated at 37°C for 30 min and deproteinized by chloroform:isoamyl alcohol (25:10, v/v). The oligomers were separated from the bulk of the DNA either by centrifugation in alkaline sucrose gradients (5 to 25.6% sucrose), with the use of a SW 25.1 rotor, by hydroxyapatite chromatography, or by membrane cone filtration followed by DEAE-cellulose chromatography. These methods were described elsewhere (8), with the exception that the sedimentation velocities were calculated for the linear gradients according to Burgi and Hershey (2) and Studier (10). The centrifuged samples were fractionated and precipitated by an equal volume of ice-cold trichloroacetic acid. The acid-precipitable counts were assayed for radioactivity on 0.2-μm pore-size nitrocellulose filters provided by Sartorius-Membrane-Filter GmbH, Göttingen, W. Germany. Each sample was washed with 5 ml of 70% ethanol.

Earlier work (8) revealed that the acid-precipitable material in the 2 to 3 S region of alkaline sucrose gradients, in the eluents of 0.05 M phosphate buffer concentration of hydroxyapatite chromatography, and in the filtrates of DNA through membrane cone filters, and the counts obtained after drying Fraction I (Fraction II was not present) of DEAE-cellulose chromatography are identical, i.e., the oligodeoxyribonucleotides in question. Also, a discontinuity in size was shown between the oligomers and the Okazaki segments.

The conditions used in the DNA polymerase assays were essentially the same as described by Richardson et al. (6), except single-stranded DNA was used for template and 3H-labeled oligodeoxyribonucleotides for primer. The enzyme was obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

RESULTS

HeLa cells contain oligodeoxyribonucleotides, as indicated by the sedimentation profile of a pulse-labeled DNA sample in an alkaline sucrose gradient shown in Chart 1. It can be seen that most of the acid-precipitable radioactivity remained near the top of the gradient, whereas the labeled precursors passed through the nitrocellulose filters used in the assay. The peak at 6.5 S may represent the discontinuous Okazaki segments which were described in HeLa cells by Painter and Schaefer (5) and which have also been observed in other mammalian...
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Chart 1. Sedimentation profile of pulse-labeled HeLa DNA. An asynchronously growing monolayer of cells was pulse labeled with $^3$H-labeled thymidine for 2 min, lysed, deproteinized, and layered on top of an alkaline sucrose gradient. Centrifugation was carried out at 24,500 rpm for 20 hr. Fractions of 0.3 ml were collected, beginning at the top of the tube, precipitated by trichloroacetic acid, and captured on nitrocellulose filters for assay of radioactivity.

systems (7, 11). The other 2 peaks are due to oligonucleotides. It should be noted that very small fractions must be collected in order to resolve these peaks. Chart 2 shows the results of DEAE-cellulose chromatography of the oligomers after their separation from larger DNA by filtration through membrane cones. Oligomers of 2 sizes were eluted. Possible contaminants of RNA and nucleoside di- and triphosphates were degraded to mononucleotides in the process of alkaline hydrolysis, which was carried out prior to the DEAE-cellulose chromatography in 0.3 M NaOH for 16 hr. Judging from the data obtained by the use of similar DEAE-cellulose columns which were calibrated with oligomers of known chain length (9), one of the oligonucleotides may be about 11 nucleotides long and the other may be approximately 13.

The incorporation of oligomers to larger DNA was studied by in vitro DNA polymerase assays. Tritium-labeled oligomers were isolated by filter cone separation, alkaline hydrolysis, and DEAE-cellulose chromatography. The 2 radioactive peaks obtained from DEAE-cellulose chromatography, similar to those shown in Chart 2, were mixed with single-stranded HeLa DNA template, nonradioactive precursors, cofactors, and DNA polymerase, Fraction VII from E. coli. In the control experiments, buffer was substituted for the enzyme. Incubations were carried out at 37° for 1 hr in a shaking water bath. After stopping the reaction by lowering the pH to 3.5 and cooling the incubation mixture to 0°, the products were analyzed by hydroxyapatite chromatography, as described elsewhere (7). These results are presented in Chart 3. The radioactivity at 0.05 M phosphate buffer represents oligomers that did not elongate, i.e., 65 to 70% of the total radioactivity used in an experiment. Single-stranded larger polynucleotides eluted around 0.14 M phosphate buffer concentration. Double-stranded DNA could be recovered between 0.22 and 0.50 M phosphate buffer concentrations. In the control experiments, 85 to 90% of the total radioactivity was recovered at 0.05 M phosphate concentration. The remainder of the counts was eluted between 0.18 and 0.24 M phosphate buffer concentrations, suggesting the double-stranded nature of the DNA. These data indicate that a large proportion of the oligomers hybridized to template DNA and increased in length. However, as reflected from the control runs, much of the oligonucleotides detached from the template during the experimental procedure. For determination of the size of the polymerized oligomers, samples of the fractions obtained by hydroxyapatite chromatography were layered on top of alkaline sucrose gradients and sedimented. Chart 4 shows the sedimentation profile of the sample that eluted from hydroxyapatite at 0.5 M phosphate buffer. A well-defined peak can be seen with the calculated sedimentation coefficient of 6.3 S, i.e., the same as the 3rd peak shown in Chart 1. The profiles obtained for the 0.14 and 0.22 M phosphate buffer samples indicated the presence of 2 to 10 S DNA's.

DISCUSSION

The stimulation of the template activity of DNA by oligomers with DNA polymerase has been well described. (For a literature survey, see Ref. 8.) Baltimore and Smoler (1) have recently found that DNA polymerases from 2 oncogenic RNA viruses, avian myeloblastosis and mouse leukemia virus, require...
Chart 2. DEAE-cellulose chromatography of pulse-labeled HeLa DNA after filtration through a membrane cone. Following a 2-min pulse, delivered with $^3$H-labeled thymidine, the cells were lysed in 0.3 M NaOH, and the DNA was deproteinized. The deproteinized solution was then filtered through a membrane cone similarly to that of Chart 1 (exclusion limit, 50,000 for globular molecules). The filtrate, pH 12.8, was incubated at 37°C for 16 hr and dialyzed in 3/8-inch diameter tubes obtained from Union Carbide Corp., Chicago, Ill. The dialysis was carried out for 8 hr against a large volume (1:1,000) of 10 mM Tris.HCl, pH 7.8, containing 1 mM EDTA. The dialyzed sample was subjected to DEAE-cellulose chromatography at 80°C in a column (1 x 6 cm) washed with a linear gradient of 0.02 to 0.20 M NaCl buffered with 10 mM of Tris:HCl at pH 7.8. Nonradioactive dTMP and dTTP were added to the sample (1 drop of each of 4 mg/ml solutions) as absorbance markers. Fractions of 3.6 ml were collected (1.2 ml/min), and dried aliquots of 1 ml were assayed for radioactivity.

Complementary oligodeoxyribonucleotides for synthesis on polyribonucleotide templates.

The data represented here indicate that oligodeoxyribonucleotides can be isolated from pulse-labeled HeLa cells. In addition to the information obtained from the other systems (8, 9), it was found that the oligomers attach to template DNA and are utilized by DNA polymerase for chain growth by the addition of deoxyribonucleoside triphosphates. The largest size of DNA attained in the experiments is 1000 to 2000 nucleotides long, i.e., similar to the length of the Okazaki segments (3), and originally isolated from the cells (Chart 1). The polymerase used is specific for a 3'-OH ending on a priming chain of deoxyribonucleotides; therefore it can be assumed that the tritium-labeled oligomers possess available 3'-OH ends and are at the origin of the 6.3 S DNA segments of Chart 4. The possibility that elongation was due not to polymerization but to the action of polynucleotide ligase contaminant in the commercial enzyme preparation is excluded in view of the fact that this enzyme, when obtained from E. coli, has an absolute requirement for NAD (4, 13). Thus the oligodeoxyribonucleotides isolated from pulse-labeled cells can fulfill the role of an in vivo primer molecule, if existent. The results obtained are in agreement with discontinuous DNA replication that requires 2 enzymes for the
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Chart 3. Hydroxyapatite chromatography of oligodeoxyribonucleotides after incubation with DNA polymerase. The most radioactive 3 fractions of Peaks I and II, obtained by DEAE-cellulose chromatography as described in Chart 2, were mixed and dialyzed against an excess volume of H₂O containing 1 mg Chelex 100 per ml (a chelating resin by Bio-Rad, Richmond, Calif.) for 48 hr. Template DNA was obtained by lysing HeLa cells in 0.3 M NaOH and 2 mg Chelex 100 per ml and incubating the lysate at 37° for 16 hr. After alkaline hydrolysis, the solution was deproteinized and adjusted to pH 6.8 by the addition of 1 M HCl. Hydroxyapatite chromatography was carried out to separate small oligomers from the large, single-stranded cellular DNA. The peak fractions, which eluted at 0.16 to 0.18 M phosphate buffer concentrations, were dialyzed as described above for the oligomers, heated at 100° for 5 min, cooled quickly, mixed with the oligomers, and concentrated to dryness. Samples were incubated with DNA polymerase. The incubation mixture contained in 0.45 ml, 7 µg template DNA; 3,000 cpm mixture of Peaks I and II; 30 µmoles of potassium phosphate (pH 7.4); 3 µmoles of MgCl₂; 0.45 µmoles of 2-mercaptoethanol; dATP, dGTP, dTTP, and dCTP 20 µmoles each; and 1 µg of DNA polymerase. Incubation was for 1 hr. We stopped the reaction by chilling the mixture and lowering the pH to 3.5 by the addition of 0.3 ml of 0.1 M HCl. The resultant mixture was adjusted to 0.05 M phosphate buffer concentration, pH 6.8, and hydroxyapatite chromatography was carried out as described elsewhere (7). Fractions of 2 ml were collected and a 0.4 ml- aliquot of each fraction was dried and counted for radioactivity for 100 min in a Packard Tri-Carb liquid scintillation counter. The values obtained were adjusted for the total volume of the sample.

Chart 4. Sedimentation profile of the 0.5 M phosphate buffer eluant of Chart 3. The fraction that eluted at 0.5 M phosphate buffer concentration was dialyzed against an excess of H₂O, containing Chelex 100 (1 mg/ml), for 2 hr at 0° and layered on top of an alkaline sucrose gradient. Centrifugation was carried out at 23,600 rpm for 20 hr. Fractions of 0.6 ml were collected beginning at the top of the tube, precipitated by trichloroacetic acid, and were captured on nitrocellulose filters for assay of radioactivity. Each sample was counted for 100 min.

production of larger DNA chains. Yudelevich et al. (12) showed that in E. coli the repair and chain growth of pulse-labeled discontinuous DNA segments can occur in an in vitro assay only in the simultaneous presence of polymerase and ligase.

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REFERENCES


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