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Analysis of Long Pyrimidine Polynucleotides in HeLa Cell Nuclear DNA: Absence of Polydeoxythymidylate

(formic acid–diphenylamine hydrolysis/hydroxyapatite chromatography/thermal elution)

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ABSTRACT **HeLa cell nuclear DNA contains an unexpectedly large amount of long pyrimidine polynucleotides. These sequences were detected in DNA labeled with [^{3}H] thymidine by treatment with formic acid–diphenylamine and subsequent analysis by polyacrylamide gel electrophoresis. About 0.5% of the total thymine residues were found in polynucleotides that migrated more slowly than 4S RNA. No polynucleotides of comparable size were detected in *Escherichia coli* DNA. The pyrimidine polynucleotides contained no pure poly(dT) sequences (less than 0.0015% of the total residues), as judged by thermal chromatography of a complex formed with poly(A).**

When high molecular weight DNA is treated with acid under relatively mild conditions (1, 2), purine bases are selectively removed, the phosphodiester backbone is broken, and pyrimidine clusters having the general formula, $p(Pyp)_n$, are released. The largest pyrimidine oligonucleotides previously isolated from animal cell DNA contain 15–20 nucleotides (3–5). Small amounts of longer polynucleotides have been detected but not defined. Because relatively long polyadenylate segments (6–11) and uridylate-rich regions (12) have been found in cellular RNA, we have examined pyrimidine polynucleotides in HeLa cell nuclear DNA to see if corresponding sequences could be detected. Pyrimidine polynucleotides were separated according to chain length by polyacrylamide gel electrophoresis and, unexpectedly, a large amount of long polynucleotides was found. Since their size and amount differ very greatly from that predicted assuming a random distribution of pyrimidines, they may represent sequences of special significance in the genome. When annealed with commercial poly(A), almost no pure poly(dT) sequences could be detected.

MATERIALS AND METHODS

Preparation of [^{3}H]DNA. HeLa cells used in these experiments were grown in spinner culture in Eagle's minimal essential medium with 5% calf serum without antibiotics. 400 ml of cell suspension (3×10^5/ml) were incubated with [*methyl*-^{3}H]thymidine at a concentration of 1 μCi/ml, 0.5 μM, for 24 hr. Nuclei were isolated as described by Penman (13); the nuclear pellet was suspended in 0.5 ml of hypotonic buffer (10 mM NaCl–10 mM Tris·HCl–3 mM $MgCl_2$) and rapidly mixed with 8 ml of lysing solution [1 M LiCl, 0.1 M EDTA, 0.1 M Tris·HCl (pH 8.0), and 1% Na dodecyl sulfate]. The suspension was heated at 60° for 5 min, then cooled to room temperature and passed twice through a no. 26 needle to reduce its viscosity. The preparation was deproteinized by shaking at room temperature for 30 min with an equal volume of chloroform containing 0.1 ml *n*-octanol. The aqueous phase was recovered by centrifugation and the procedure was repeated three times more. DNA was precipitated with ethanol and dissolved in 2 ml of a solution containing 10 mM NaCl, 1 mM EDTA, 1 mM Tris·HCl (pH 7.4), and 0.2% Na dodecyl sulfate. NaOH was added to 0.15 N, and the DNA was heated at 60° for 10 min to hydrolyze RNA. The sample was neutralized with 3 N acetic acid, and the DNA was precipitated with ethanol. Recovery of DNA was 1.4 mg, and its specific activity was 10^5 cpm/μg. In control experiments with [^{3}H]poly(dT) (50 Ci/mol; General Biochemicals, Chagrin Falls, Ohio), the polymer was added to a similar preparation of unlabeled DNA after the deproteinization procedure.

Acid Hydrolysis of DNA. DNA was degraded to release pyrimidine oligonucleotides according to the procedure of Burton (2). The DNA (500–750 μg) was dissolved in 0.5 ml of water. 1.5 ml of 90% formic acid–2.7% diphenylamine was added. The mixture was incubated at 30° for 18 hr in a darkened, glass-stoppered tube. Formic acid and diphenylamine were removed by a modified ether extraction procedure since the standard method (2) led to preferential losses of high molecular weight DNA fragments. 1 ml of a solution containing 10 M urea, 1 M sodium acetate, and 100 μg of tRNA was added to the hydrolysis mixture; it was extracted three times with 5 ml of ether. The extraction tube was rinsed with 1 ml of 1 M sodium acetate at 60°, and the aqueous solutions were combined. Two volumes of ethanol were added and the precipitate was collected by centrifugation at 30,000 $\times$ *g* for 15 min at 0°; it was dissolved in 1 ml of NETS buffer [0.1 M NaCl–0.01 M Tris·HCl (pH 7.4)–0.2% Na dodecylsulfate] containing 100 μg of Pronase. After 30 min of incubation at 37°, the solution was clarified by centrifugation at 30,000 $\times$ *g* for 15 min at 20°. The pellet was discarded, and nucleotide material was precipitated with ethanol as before. Samples to be analyzed by gel electrophoresis were dissolved in 0.2 ml of NETS buffer and precipitated with ethanol once again.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was used for size separation of pyrimidine oligo- and polynucleotides released from DNA by acid hydrolysis. Gels were prepared according to Loening (14), with 8% acrylamide, 0.4% bisacrylamide in his "E buffer". Precipitated nucleotide material was dissolved in 50 μl of sample buffer (5% sucrose,

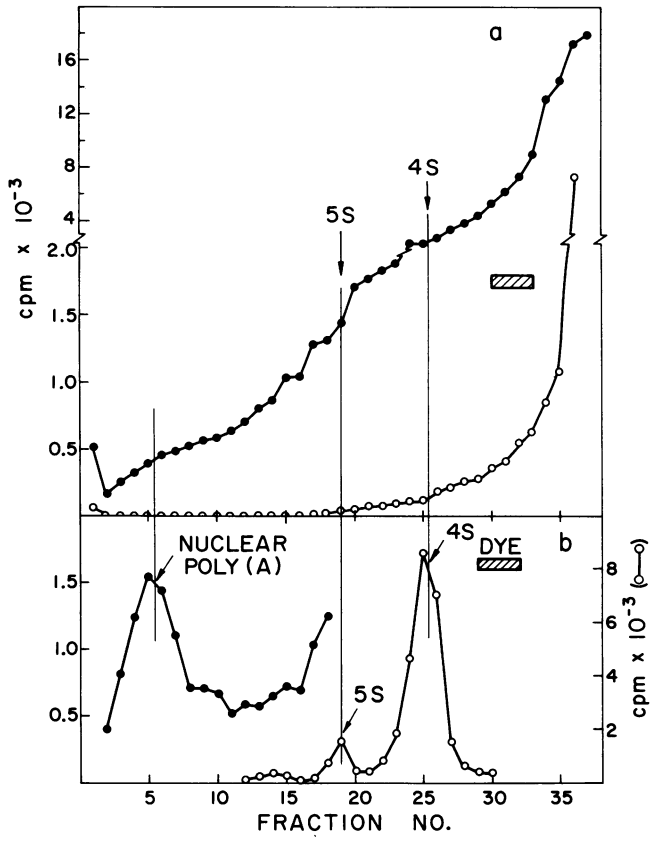

FIG. 1. Separation of polynucleotides by polyacrylamide electrophoresis. (*a*) [^{3}H]thymidine–polypyrimidine nucleotides released from HeLa cell nuclear DNA (●——●) or *E. coli* DNA (○——○) by formic acid–diphenylamine hydrolysis were precipitated with ethanol and subjected to electrophoresis on an 8% polyacrylamide gel. (*b*) Marker polynucleotides to indicate mobility of defined species. 4S and 5S RNA were prepared from HeLa cells labeled with [^{14}C]adenine. Nuclear poly(A) was prepared from [^{3}H]adenine–labeled, nucleoplasmic RNA by digestion with pancreatic and T1 RNases. The dye marker, bromphenol blue, was run 6.0 cm in all experiments.

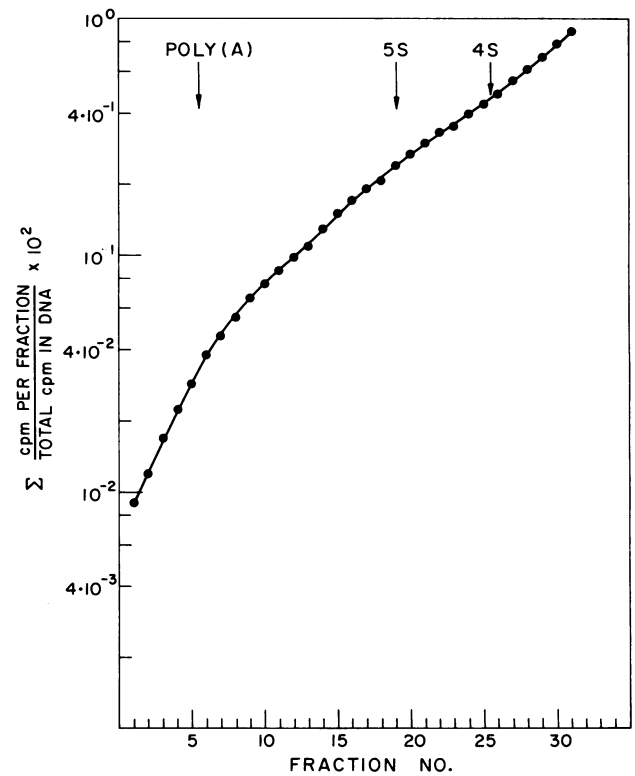

FIG. 2. Cumulative, semi-logarithmic plot of the distribution in a polyacrylamide gel of pyrimidine polynucleotides from HeLa cell nuclear DNA. The data are those of Fig. 1*a*, which have been corrected for efficiency of recovery (67%) of [^{3}H]poly(dT) in a control experiment (Fig. 3*a*). Cpm in each fraction are expressed as a percentage of total cpm in the DNA from which the polypyrimidine nucleotides were prepared. The cumulative plot is intended to show the percentage of total thymidine residues that are to be found in polypyrimidine sequences of a certain size and greater. For example, about 0.5% of the thymidine residues are present in polynucleotides that migrate more slowly than 4S RNA.

0.5 × E buffer, 0.02% bromphenol blue) and applied to the surface of a 0.625 × 7-cm gel. Electrophoresis was continued until the blue dye marker had migrated 6.0 cm. The gel was sectioned into 2-mm slices (15) which were collected in glass scintillation vials containing 1 ml of 0.7 M NH_4OH–9% H_2O_2. To solubilize the gel, the vials were secured with polyethylene-lined, aluminum caps (Amersham-Searle Corp.) and heated in an oven at 80° for 2–3 hr. The samples were cooled, and 10 ml of dioxane-based scintillation fluid (1 liter of dioxane, 155 g of naphthalene, 6 g of Omnifluor, 30 ml of water, and 10 ml of Nuclear-Chicago NCS reagent) was added. Recovery of ^{3}H cpm, as [^{3}H]poly(dT) added to a gel, was 84%. In addition to high efficiency, complete solubilization of sample, and low cost, this procedure does not give high backgrounds associated with other methods using H_2O_2 (16).

In some experiments, [^{3}H]polynucleotides were eluted from gel slices by shaking with 1 ml of 0.01 M Na_2CO_3 for 2 days. The nucleotide material was precipitated with cetyltrimethylammonium bromide (17). This step was useful in removing contaminants that were extracted from the polyacrylamide gel. These samples were either run again on another gel or hydrolyzed enzymatically for nucleoside analysis.

Hydroxyapatite Chromatography. Pyrimidine polynucleotides released from DNA labeled with [^{3}H]thymidine were analyzed for the presence of poly(dT) by annealing with commercial poly(A) (P-L Biochemicals, Milwaukee, Wis.) and determination of the specificity of the duplex structure by thermal chromatography on hydroxyapatite in 0.08 M sodium phosphate buffer (pH 6.8) (18). After incubation with 100 μg of poly(A) at 35° for 30 min, samples were applied to a hydroxyapatite bed prepared as follows. 300 mg of Bio-Gel HTP (Bio-Rad, Richmond, Calif.) was added to a 14-mm diameter jacketed-glass column containing a fine nylon screen bed support. The adsorbent was washed with boiling 0.08 M phosphate buffer and the top of the bed was protected with a close-fitting disk of Whatman no. 40 filter paper. After the sample was applied, the column bed was washed successively with 4-ml volumes of 0.08 M phosphate buffer at increasing temperature (18). For counting, each 4-ml fraction was mixed with 0.2 ml of 1.4 M phosphate buffer and 15 ml of PCS scintillation fluid (Amersham-Searle Corp.); efficiency of counting for ^{3}H was 24% in this system.

RESULTS AND DISCUSSION

Other workers have previously analyzed pyrimidine oligonucleotides in animal cell DNA by ion-exchange chromatography (3–5), a procedure suitable for separation of oligonucleotides of chain length up to about 15. In the present experiments, polyacrylamide gel electrophoresis has been used. With this technique, small amounts of much longer polynucleotides have been detected in HeLa cell nuclear DNA (Fig. 1*a* and 2). The presence of long pyrimidine polynucleotides (chain length greater than 30–40) was unexpected on the basis of a random distribution of pyrimidine nucleotides. For example, a random distribution would predict (5) that a pyrimidine polynucleotide of chain length 30 would account for 1.4×10^{-6}% of total pyrimidines; for chain length 40, the value is 1.8×10^{-9}%; and for chain length 50, the value is 1.8×10^{-21}%. By contrast, the data of Fig. 2 show that about *0.5*% of the thymine label was in pyrimidine polynucleotides of a size greater than transfer RNA. Despite uncertainties about the exact size-mobility relationship of these polynucleotides relative to the markers used, the amount of these polypyrimidines is clearly unexpectedly large.

Several lines of evidence suggest that the ^3H radioactivity was in fact in thymine-containing pyrimidine polydeoxynucleotides. (*i*) No such material was seen if the sample was

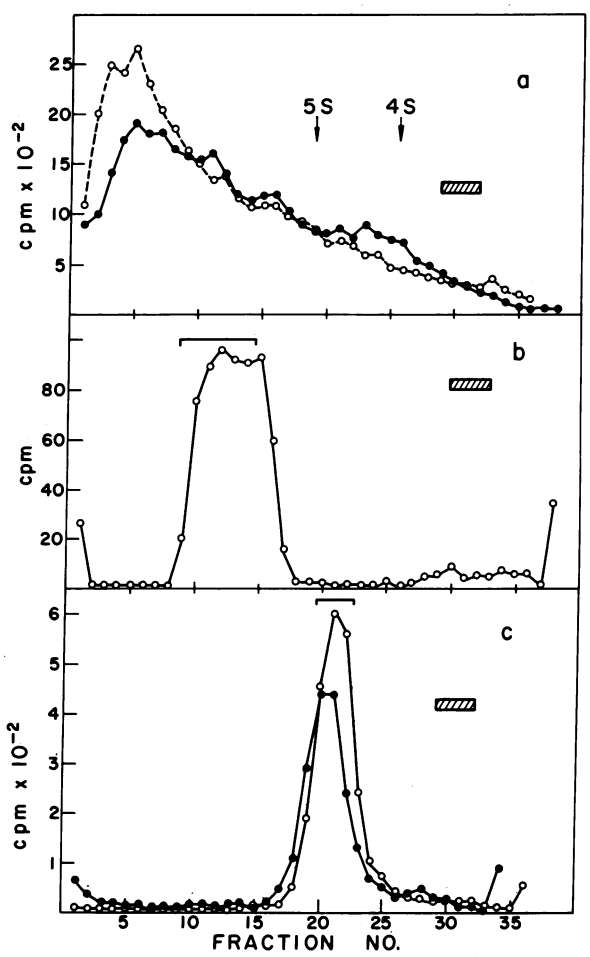

FIG. 3. (*a*) Electrophoresis of [^3H]poly(dT) before (O—·—O) and after (●—·—●) treatment with formic acid–diphenylamine. Marker compounds as in Fig. 1. (*b*) Pyrimidine polynucleotides from HeLa DNA were prepared and subjected to electrophoresis as in Fig. 1*a*, then eluted from gel slices indicated by the *horizontal bracket*. After being precipitated as described in the *text*, the polynucleotides were again subjected to electrophoresis as before. (*c*) Repeat electrophoresis of polynucleotides eluted from gel slices as in (*b*). Eluted material was subjected to electrophoresis either directly (O—·—O) or after a second 18-hr treatment with acid–diphenylamine (●— —●).

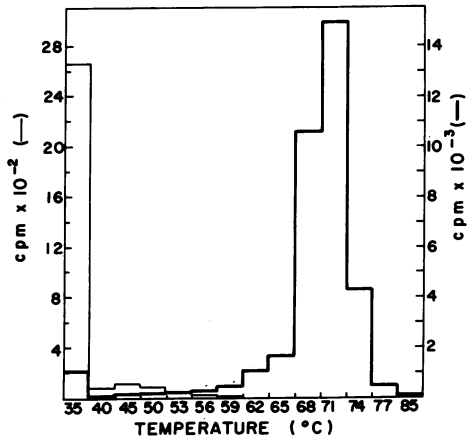

FIG. 4. Thermal chromatogram of [^3H]pyrimidine polynucleotide·poly(A) complex. (——), Pyrimidine polynucleotides derived from [^3H]thymidine labeled HeLa cell DNA (fractions 2–19, Fig. 1*a*) were annealed with poly(A) and eluted from a hydroxyapatite column with 0.08 M phosphate buffer at increasing temperature. (——), Synthetic [^3H]poly(dT) annealed with poly(A) and analyzed in the same way.

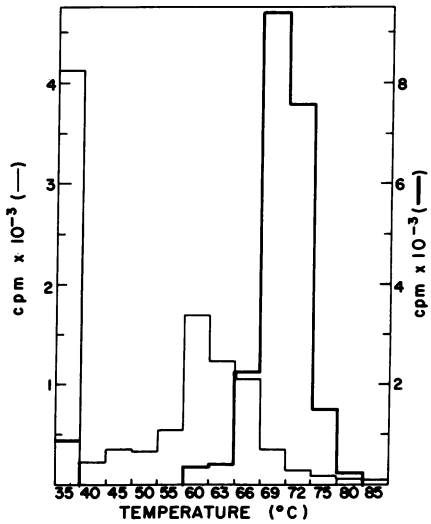

FIG. 5. Thermal chromatogram of [^3H]pyrimidine polynucleotide·poly(A) complex. (——), [^3H]thymidine-labeled HeLa cell DNA (2.9×10^7 cpm) was treated with acid and precipitated with alcohol. The sample was annealed with 100 μg of unlabeled poly(A) and applied to a hydroxyaptite column. The column was washed with 32 ml of 0.08M phosphate buffer at temperatures up to 58°. Material that eluted with 4 ml of 0.08 M phosphate buffer at 80° was again annealed with unlabeled poly(A) and applied to a second hydroxyapatite column. Radioactivity eluted with 4 ml of 0.08 M phosphate buffer at the specified temperature is shown. [——], [^3H]poly(dT) (4×10^4 cpm) was mixed with about 500 μg of unlabeled HeLa cell DNA and treated as above.

treated with pancreatic DNase before electrophoresis. (*ii*) Increasing the time of formic acid–diphenylamine hydrolysis to 40 hr did not change the amount of ^{3}H cpm recovered. (*iii*) No such material was found in acid digests of *E. coli* [^{3}H]DNA (Fig. 1*a*). (*iv*) Material eluted from a gel and treated again with formic acid–diphenylamine had a similar mobility on a second gel (Fig. 3*b*, and *c*). (*v*) Treatment of eluted material with pancreatic DNase, venom phosphodiesterase, and alkaline phosphatase converted all of the label to a form that coincided with thymidine on paper chromatography. (*vi*) In a similar experiment with [^{3}H]cytosine-labeled HeLa DNA, about the same proportion of ^{3}H cpm was detected in this region of a gel. (*vii*) When synthetic [^{3}H]poly(dT) was mixed with unlabeled HeLa DNA and treated with acid, its profile on a polyacrylamide gel showed only slight evidence of degradation (Fig. 3*a*). Taken together, these data indicate that small amounts of long pyrimidine polynucleotides do indeed exist in HeLa cell nuclear DNA. The exact origin of these sequences is unknown, but the improbability of their occurrence leads us to speculate that they may be of special significance. It would be of interest to establish if they form part of a discrete satellite DNA band or whether they are broadly distributed throughout the genome. In this connection, it should be noted that known initiating and terminating codons contain both purines and pyrimidines and, therefore, cannot be accommodated in these sequences.

As a preliminary step in the further characterization of HeLa cell pyrimidine polynucleotides, annealing with synthetic poly(A) and analysis by hydroxyapatite chromatography was used. This method was intended to determine if long poly(dT) sequences could be detected amongst the polypyrimidines. [^{3}H]Polynucleotide material, eluted from fractions 2–19 (Fig. 1*a*), was annealed with commercial poly(A) and applied to a hydroxyapatite column. The temperature elution profile of this sample is compared with that of synthetic [^{3}H]poly(dT) in Fig. 4. Most of the [^{3}H]polypyrimidine material passed through the column, indicating little complex formation with poly(A). About 10% was eluted between 40 and 59°. Less than 1% was eluted between 65 and 74°, the temperature at which authentic poly(dT)·poly(A) was released. Since polynucleotides from fractions 2–19 accounted for about 0.2% of the ^{3}H cpm (Fig. 2), then this result suggests that less than $1\% \times 0.2\% = 0.002\%$ of the thymidine residues in the DNA occur in poly(dT) sequences.

The near absence of long poly(dT) sequences in HeLa cell nuclear DNA has significance with regard to the mechanism by which polyadenylate segments become attached to cellular RNA. It supports the postulate (11) that a *post*-transcriptional mechanism for attachment of polyadenylate segments to RNA is operative. To confirm these findings, further experiments to assess the maximal amount of poly(dT) that could be present in [^{3}H]thymidine-labeled HeLa cell nuclear DNA were done. The entire alcohol-precipitable fraction (after acid treatment) was annealed to poly(A) and charged onto a hydroxyapatite column. Material that eluted between 58 and 80° was annealed again with 100 μg of poly(A) and applied to a second hydroxyapatite column. The thermal elution profile of this column is shown in Fig. 5. As a control, authentic [^{3}H]poly(dT) was mixed with unlabeled HeLa DNA, treated with acid, and analyzed in the same way. The overall recovery of ^{3}H cpm from [^{3}H]poly(dT) in the 69–75° fractions was 52.6%. Recovery of ^{3}H cpm from [^{3}H]DNA in the same fractions was only 0.002% (Fig. 5). Using the recovery of synthetic [^{3}H]poly(dT) to correct for losses, we estimate a maximum of 0.0044% of thymine residues or 0.0015% of total residues in DNA to be in poly(dT) sequences. The data are incompatible with a transcriptional origin of polyadenylate segments in cellular RNA. Poly(A) segments are considered to be about 200 nucleotides long (8, 11, 21) and are associated with the majority of mRNA molecules (19, 20). By contrast, the entire haploid genome, which has been estimated to contain 30,000 or more structural genes (22, 23), can contain no more than 200–400 sites of poly(dT) of a size corresponding to cellular poly(A).

Pyrimidine polynucleotides that form an unstable complex with poly(A) are detectable in both Figs. 4 and 5. In the experiment of Fig. 5, about 0.02% of the thymine in DNA was recovered in the 55–66° fractions. In the first hydroxyapatite column of the same experiment (data not shown), 0.5% of the ^{3}H cpm were recovered in the 35–58° fraction. These probably represent thymidylate-rich regions and may be complementary to deoxyadenylate-rich sequences that have been detected in mammalian DNA (24).

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