A two-dimensional thin layer chromatographic procedure for the sequential analysis of oligonucleotides employing tritium post-labeling

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ABSTRACT

Two dimensional PEI-cellulose thin layer chromatography can resolve sequentially degraded oligonucleotide fragments of tRNA. This technique entails the sequential degradation of the oligonucleotide with snake venom phosphodiesterase in the presence of bacterial alkaline phosphatase, and periodate oxidation followed by tritiated sodium borohydride reduction of the 3' terminal nucleoside. Subsequently the tritiated oligonucleotide fragments were resolved by two dimensional PEI-cellulose TLC. The results of these experiments indicate that, in some cases, the complete nucleotide sequence of a large oligonucleotide fragment may be determined by interpretation of the observed mobility shifts, thereby eliminating the need for additional analysis of the oligonucleotide. In addition, the use of two-dimensional rather than one-dimensional resolution of the tritium labeled fragments allows for a complete separation of any interfering background spots from the sequentially degraded oligonucleotides.

This procedure was applied to the complete nucleotide sequence analysis of several ribonuclease T1 and ribonuclease A digestion products from human placenta tRNAp1.

INTRODUCTION

The tritium post-labeling technique (1,2) has been recently applied to the complete nucleotide sequence analysis of several tRNAs (3,4). An important part of this procedure entails the sequential degradation of purified oligonucleotide fragments produced by either complete ribonuclease T1 or ribonuclease A digestion of the intact tRNA. Due to batch variations in the commercial tritium labeled borohydride, we have recently had difficulty interpreting the results of the sequential degradation procedure when PEI-cellulose TLC was performed as reported (1,2). Therefore, during the course of our determination of the complete nucleotide sequence of human pla-
Valcenta tRNA$^{Val}_{1a}$ (14), we devised a two-dimensional PEI-cellulose TLC procedure for improved resolution of the sequentially degraded nucleotides. This procedure involves the development of the PEI-cellulose TLC plate in two dimensions using a stepwise gradient of unbuffered Li-chloride as the first dimension and a stepwise gradient of ammonium formate, pH 3.5, as the second dimension. The sequential degradation products were visualized by fluorography (5), digested in situ with ribonuclease T$_2$ (1), and subsequently analyzed for 3' terminal triitol on silica gel or cellulose TLC (1,6,7).

One major advantage of this two dimensional PEI-cellulose TLC technique is that since the sequential degradation fragments do not overlap with the non-nucleic acid background spots, there is a greater ease and reliability in interpreting the chromatogram. Furthermore, since the sequential degradation tract is highly reproducible, the mobility shifts can be correlated with the nucleotide sequence directly, and for some oligonucleotide fragments, it is possible to determine the complete nucleotide sequence without further analysis.

**MATERIALS**

Human placenta tRNA$^{Val}_{1a}$ was isolated to a purity of 1.2 nmoles per A$_{260}$ unit as previously described (8,9) and further purified by two additional RPC-5 column chromatographic steps at pH 7.6 (9). Complete digestion of the human placenta tRNA$^{Val}_{1a}$ with ribonuclease T$_1$ (Sanko) or ribonuclease A (Sigma), two dimensional resolution of the digestion products on PEI-cellulose, and subsequent elution of the ultraviolet visualized fragments were performed as described earlier for human placenta tRNA$^{Phe}$ (4).

Ribonuclease T$_2$ and bicine (N,N bis-[2-hydroxyethyl]glycine) were obtained from Calbiochem. Tritium labeled sodium borohydride (9.0 Ci/mmmole) from Amersham-Searle was dissolved in 0.1 M KOH, lyophilized and stored at -70° (10). Sodium metaperiodate (J. T. Baker) was dissolved in water to a concentration of 180 mM immediately prior to use. Bacterial alkaline phosphatase (Worthington) was dialyzed against water as described (6), and snake venom phosphodiesterase (Worthington) was pretreated with acid as reported (11). All other reagents were analytical grade and
all solutions were made from glass distilled-deionized water.

PEI-cellulose thin layer plates (20 x 20 cm without fluorescent indicator) from Brinkman Instruments were prewashed as described (12). Silica gel thin layer plates (10 x 20 cm) with fluorescent indicator from Eastman and cellulose thin layer plates (20 x 20 cm) without fluorescent indicator (E. Merck No. 5502) from Brinkman, were both used without pretreatment.

METHODS

The sequential degradation of individual ribonuclease T₁ or ribonuclease A fragments was performed using the partial snake venom phosphodiesterase (SVD) procedure in the presence of bacterial alkaline phosphatase (BAP) as described earlier by Sivaraman, et al. (2). Briefly, the incubation mixture (80 ul) contained the following: 3 ul of 0.25 M magnesium acetate, 2.5 ul of 0.6 M bicine-NaOH (pH 8.0), 2 ul of SVD (0.5 mg/ml), 6 ul of BAP (1.5 mg/ml) and 0.02 A₂₆₀ units of oligonucleotide. Reactions, which were begun by addition of oligonucleotide, were subsequently incubated for 0.5, 1.0, 2.0, 3.0, 5.0, 8.0, 12.0, and 18.0 minutes at 23°. At these times, aliquots of 10 ul were pipetted directly into 5 ul of 0.75 mM sodium periodate to terminate the reaction and incubated in the dark for at least 1 hour. After the time points had been collected and oxidized by periodate, the tritium label was introduced into the 3' terminus by the addition of 2 ul of 0.03 M tritiated sodium borohydride and incubated at 23° for 1 hour as described (2). After the excess borohydride was decomposed by the addition of acetic acid, and dried at room temperature in vacuo over solid KOH and phosphorous pentoxide, the residue was dissolved in 8 ul of water.

The partial SVD-BAP digestion fragments were then separated using a modification of the two dimensional PEI-cellulose TLC procedure reported earlier by Randerath, et al. (13). Aliquots (3 ul) of each time point were combined and applied to a prewashed PEI-cellulose plate in the lower left hand corner 1.5 cm from each edge. After soaking each plate in 300 ml of methanol: ammonium hydroxide (1000:1 v/v), a 5 x 20 cm wick (Whatman No. 1 filter paper) was stapled to the top edge. The plate was then washed by chromatographing with water to 23 cm (4 cm on the wick),
dried in a stream of cool air and developed in the first dimension using a stepwise gradient of unbuffered lithium chloride as follows: (a) water to the origin, (b) 0.05 M lithium chloride to 3 cm above the origin, (c) 0.1 M lithium chloride to 7 cm above the origin, (d) 0.2 M lithium chloride to 11 cm above the origin, (e) 0.35 M lithium chloride to 15 cm above the origin, and finally (f) 0.6 M lithium chloride to 1 cm on the paper wick. After drying in a stream of cool air, the wick was cut off, and the chromatogram was washed twice by soaking in 300 ml of methanol. Following air drying, the second dimension was developed using a stepwise gradient of ammonium formate (pH 3.5) as follows: (a) 0.02 M ammonium formate to 1 cm above the origin, (b) 0.7 M ammonium formate to 5 cm above the origin, (c) 1.0 M ammonium formate to 8 cm above the origin, (d) 1.3 M ammonium formate to 11 cm above the origin, (e) 1.6 M ammonium formate to 14 cm above the origin, and (f) 2.5 M ammonium formate to 18 cm above the origin. Following completion of the second dimension, the chromatogram was washed by soaking in 300 ml of methanol, air dried, coated with 7% PPO (5) and exposed to X-ray film at -70° for 3 days (6,7).

Parallel experiments were performed in the absence of oligonucleotide to determine the chromatographic mobility position of any background spots due either to the incorporation of tritium into non-nucleotide reaction mixture components or the presence of non-volatile tritiated borohydride breakdown products. The oligonucleotide fragments were located by fluorography, subjected to in situ ribonuclease T2 digestion (1), contact transferred to either silica gel or cellulose TLC plates after the addition of the nonradioactive triol standards, and subsequently developed as reported (1). The 3' terminal triol was then identified by liquid scintillation counting of the ultraviolet visualized standard markers or by fluorography after coating with 7% PPO (5). The 5' terminal nucleoside was determined in a separate experiment using the procedure described by Randerath (1).

RESULTS AND DISCUSSION

A fluorogram, which was obtained after a two dimensional PEI-cellulose TLC separation for the sequential degradation of the oligonucleotide fragment AAACACCA\textsubscript{OH}, is shown in Figure 1a. Another fluorogram, obtained from a parallel incubation lacking
Two dimensional PEI-cellulose TLC (20 x 20 cm) of (3H)-oligonucleotide-3' dialcohol obtained by partial snake venom phosphodiesterase/phosphatase digestion of (a) nucleotide fragment AAACACCA$_{0+}$ and (b) blank, followed by periodat oxidation and (3H)-NaBH$_4$ (9 Ci/mmole) reduction. The first dimension was developed from bottom to top with a 0-0.6M LiCl stepwise gradient while the second dimension was developed from left to right with a 0.02M-2.5M ammonium formate (pH 3.5) stepwise gradient (see Methods). Film detection by fluorography.
The added oligonucleotide is shown in Figure 1b. It can be seen that the background spots travel as a series of hyperbolic arcs which differ significantly from the mobility of the sequentially degraded oligonucleotides, up to at least a chain length of 10. Since the largest oligonucleotides usually obtained from a ribonuclease digestion of tRNA is 10 to 12 nucleotides (3,4), such resolution should be adequate for the complete sequential analysis required for tRNA structural studies. It should be noted that we have observed variations in the background spots depending on both the batch of tritiated sodium borohydride and the source of the enzymes used. Therefore, background chromatograms should be developed for each series of sequential degradation experiments performed.

Table 1 shows the 3' terminal nucleoside analysis for the sequential degradation fragment given in Figure 1a. Since this oligonucleotide contained three cytosine and five adenosine residues with one adenosine as the 5' terminus, the complete nucleotide sequence of $\text{AAACACCA}_{\text{OH}}$ can readily be deduced from the sequential analysis data. In addition, a comparison of the observed mobility shifts for the fragments which lack one cytosine or adenosine indicates that the removal of an adenosine causes a greater mobility shift than does the removal of a cytosine.

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Table 1: Radioactivity (cpm) of 3'-termini released by in situ RNase T$_2$ treatment on PEI-cellulose of (3H)-oligonucleotide-3'-dialcohol spots from Fig. 1(a).

*Total nucleoside analysis indicated as base composition of C$_3$A$_5$ while 5' terminal analysis yielded A.
Figure 2 shows a composite drawing of the mobility data obtained for several large oligonucleotides whose sequence was determined during the course of our studies on human placenta tRNA$^{\text{Val}}_{1a}$ (14). In Figure 2, track 1 represents the sequential degradation analysis for the oligonucleotide fragment GGAAACp, while track 2 and 3 represent the sequential degradation analysis data obtained for the oligonucleotides GAAAGm$^7$GD$p$ and CCUCACACG$p$, respectively. As can be seen from this composite figure, the removal of a guanosine residue causes a much larger mobility shift than the removal of a cytosine. In addition, the removal of the modified nucleoside m$^7$G causes the normally smooth hyperbolic arc to be shifted to a new track. The conclusions reached from an analysis of the data obtained in Figures 1 and 2 as well as several other plates (data not shown) indicates that the removal of a purine residue causes a larger mobility shift than does the removal of a pyrimidine. We have also observed that the removal of a guanosine causes a larger mobility shift than does the removal of an adenosine. However, since sufficient
data has yet to be collected on the pyrimidines, we cannot at present differentiate between mobility shifts caused by the removal of either a cytosine or a uridine.

Several other observations can be made from the plates shown in Figures 1, 2, and unpublished results from other two dimensional PEI-cellulose sequential degradation maps. Figures 1 and 2 illustrate that the tritiated dinucleotides corresponding to CpG', GpA', ApA' and CpC' travel with increased mobility in both solvents as the purine (guanosine) content decreases and the pyrimidine content increases. We have also observed (unpublished data) that the dinucleotide DpD', which travels with the solvent front, cannot be resolved on these two dimensional maps. In addition, when an oligonucleotide fragment contains only purines such as the trinucleotides GGA' and GAA' (see Figure 2), the adenosine rich fragment has a greater mobility than the guanosine rich fragment in both solvents. Similar correlations between nucleotide content and mobility on PEI-cellulose have been reported earlier by others (15,19-21).

Figure 3 shows the resolution of several nucleoside tri-alcohols on two dimensional silica gel TLC employing acetonitrile:tertiary amyl alcohol:ammonia (2:1:1) as the solvent for the first dimension and tertiary amyl alcohol:methyl ethyl ketone:water (3:6:1.2) as the solvent for the second dimension, as described by Randerath, et al (1). The major difference between the mobilities for the triols resolved on either silica gel, or cellulose TLC (6) lies in the relative positions of A', U', rT' and m^1G'. Although in some cases separation for several triols (such as G' from I', C' from m^5C', and A' from D' or m^6A') on cellulose TLC (6) is superior to that obtained on the smaller size silica gel TLC plates, this resolution could be improved by using a larger silica gel TLC plate. However, for the nucleoside analysis of most oligonucleotide fragments, the smaller (10 x 20 cm) silica gel TLC plate has the advantage of rapid development with satisfactory resolution. Another advantage of TLC on silica gel is that the batch dependent variations in resolution obtained with cellulose TLC plates (6) have not been observed for silica gel TLC plates. However, cellulose is superior to silica gel TLC as it gives enhanced resolution for the complex mixtures of nucleoside triols obtained during the complete base
Two-dimensional TLC of nucleoside-trialcohols on silica gel plate (20 x 10 cm). The first dimension was developed with acetonitrile/tert. amyl alcohol/conc. ammonia (2:1:1) to 15 cm above the origin, and the second dimension with tertiary amyl alcohol/methyl ethyl ketone/water (3:6:1.2) to 8 cm above the origin.

In conclusion, we have described a procedure for the resolution of sequentially degraded large oligonucleotide fragments of tRNA employing two dimensional TLC on PEI-cellulose thin layers. This technique has been used in determining the complete nucleotide sequence of human placenta tRNA Val (to be reported in detail elsewhere). Documentation of several observations of mobility shifts indicate that such a procedure could be used to determine directly nucleotide sequences in a manner similar to that reported by Sanger (16-18). Finally, we are reporting the two dimensional nucleoside trialcohol map on silica gel thin layers, hopeful that it will be helpful to other investigators.
ACKNOWLEDGEMENTS

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