One-Lane Sequence Analysis of Oligodeoxyribonucleotides

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Treatment of 5'-end 32P-labeled oligodeoxyribonucleotides with 0.4 M aqueous piperidinium formate, pH 2, at 37°C for 6 h, followed by treatment with 1 M aqueous piperidine at 90°C for 6 h, produces, after electrophoresis through 27% polyacrylamide sequencing gels, one-dimensional distributions of radioactivity from which the base sequences can be deduced. The order of intensities for the bands signaling the various bases is G > A > C > T. The spacing from a given band to the next higher band in the ladder was base characteristic, the order of band spacings being G > T > A > C. In contrast to the one-cleavage one-lane DNA sequencing method reported earlier (B. J. B. Ambrose and R. C. Pless, 1985, Biochemistry 24, 6194–6200), which was based on treatment of end-labeled DNA with hot aqueous piperidine in the presence of sodium chloride, the present method produces a salt-free hydrolysate, thus minimizing electrophoretic irregularities in the fastest moving bands. © 1986 Academic Press, Inc.

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We have recently reported a new approach to sequencing of end-labeled DNA fragments involving a single chemical cleavage procedure and electrophoretic analysis in a single lane (1). One important area of application for this method should be sequencing of short synthetic oligonucleotides which are now routinely synthesized by automated or semiautomated methods (2). In principle, in such cases the sequence is determined by the succession of synthetic steps; nonetheless, a simple, rapid method for confirmatory sequencing of such synthetic oligonucleotides would be useful.

In the one-lane procedure for DNA sequencing based on the treatment of 5'-end labeled DNA with hot aqueous piperidine, it was found that an appreciable concentration of salt (0.3 M NaCl) was required to effect sufficient base selectivity to give rise to a one-lane radioactivity pattern readable in terms of DNA sequence (1). The presence of salt in the DNA hydrolysates, however, causes band distortions and irregularities in mobility for the shortest oligonucleotides, which comigrate with salt in the polyacrylamide sequencing gels. While this drawback is tolerable in the case of analysis of long sequences, where one may forego determination of the first several bases proximal to the labeled end, this problem becomes grave in the analysis of oligonucleotides, as the determination of a large proportion of the total positions in the short sequence will be adversely affected by salt. We have, therefore, developed a variant to the one-tube, one-lane method reported earlier (1), which produces a partial DNA hydrolysate free of salt. In this variant, the end-labeled DNA is first subjected to a mild acid treatment, to bring about partial depurination, followed by an extended treatment with hot aqueous piperidine, to cleave the DNA at the apurinic sites and to cause further cleavage at all positions. The method is, thus, similar to the (A + G) cleavage procedure of the method of Maxam and Gilbert (3), except that the treatment with hot aqueous

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piperidine is much extended, because in the one-lane procedure cleavage must not be limited to original purine sites, but must occur at all positions in the DNA.

EXPERIMENTAL

Materials. Chemicals were obtained from the following sources: tris(hydroxymethyl)aminomethane, ammonium persulfate, acrylamide, $N,N'$-methylenebisacrylamide, $N,N,N',N'$-tetramethylethylenediamine, formamide, and urea were from Bethesda Research Laboratories; piperidine was from Fisher Scientific Company; boric acid was from Allied Chemical; formic acid was from J. T. Baker Company; T4 polynucleotide kinase was from Pharmacia; and $[^{32}\text{P}]$ATP (3000 Ci/mmol) was from ICN. The oligonucleotides were synthesized on the 1 pmol scale on a controlled pore glass support using phosphite triester chemistry (4,5).

Methods. The oligonucleotides were labeled at their 5'-ends by established procedures (3) using T4 polynucleotide kinase and $[^{32}\text{P}]$ATP. Samples of labeled oligomer (approx. 20,000 cpm) were treated at 37°C with 0.4 M aqueous piperidinium formate, pH 2.0, containing 0.2 mg/ml of sonicated salmon sperm DNA, in reaction volumes of 5 μl contained in 1.5-ml polypropylene snapcap tubes (Sarstedt). After the indicated reaction times, samples were taken to dryness in a Savant Speed Vac, redissolved in 20 μl of water, and again taken to dryness. The residues were taken up in 10 μl of 1 M aqueous piperidine and heated in glass capillaries at 90°C for 6 h. The piperidine solvent was evaporated, and the residues were electrophoresed through thin 27% polyacrylamide gels made up in 100 mM Tris-borate, pH 8.3, 2 mM EDTA, 7 M urea.

Lanes 1, 2, 3, 5, and 6 in Fig. 1 constitute two sets of autoradiograms, one for an experiment in which the acid treatment was performed for 2, 3, and 4 h (lanes 1, 2, and 3, respectively), and a second one for a separate experiment in which acid treatments of 6 and 8 h were used (lanes 5 and 6). In all cases, the G-bands are predominant, while the T-bands are weakest. Bands for C-sites and A-sites are intermediate, their relative intensities depending on the duration of the acid treatment compared to the solvolysis time in hot aqueous piperidine. It is seen that the shorter acid treatment times (2, 3 h) are not sufficient to ensure a reliable preponderance of A-band intensities over C-band intensities. With acid treatment times of 4 h or longer, the radioactivity distributions ultimately obtained show A-bands clearly stronger than C-bands. The order of decreasing band intensities as seen on the film thus becomes G > A > C > T.
FIG. 1. Autoradiograms of the gel electrophoretograms of hydrolysates obtained from the 5'-end-labeled hexadecamer d-CACCTTCGTGATCATG by acid treatment at pH 2.0, 37°C, for 2 h (lane 1), 3 h (lane 2), 4 h (lanes 3 and 4), 6 h (lane 5), and 8 h (lane 6), followed by treatment with 1 M aqueous piperidine, at 90°C, for 6 h. For lane 7, acid treatment for 4 h was followed by treatment with 0.5 M aqueous piperidine at 90°C for 17 h. For lane 4, treatment with hot aqueous piperidine was performed in a polypropylene snapcap tube; in all other cases, glass capillaries were used in this step. The dashed lines indicate the position of the starting line.

Figure 1, lane 7, shows the result of a separate experiment with this hexadecamer in which acid treatment for 4 h was followed by a 17-h solvolysis at 90°C in an aqueous solution of only 0.5 M piperidine. The result is qualitatively similar to that seen in lanes 3, 5, and 6.

We have generally carried out the solvolysis in hot aqueous piperidine in borosilicate glass capillaries. Figure 1 shows, in lane 4, the radioactivity distribution obtained in an experiment identical to lane 3, except that for lane 4 the piperidine treatment was carried out in a 400-μl polypropylene snapcap tube. The patterns in lanes 3 and 4 are very similar, except for stronger T-bands in lane 4.

For a quantitative assessment of the relative reactivities of the various bases, the densitometric scan for lane 7, Fig. 1, is shown in Fig. 2.

The criterion of relative band spacing for base identification, used in the one-lane sequence analysis of polynucleotides with gels of 20% or lower polyacrylamide content (1), was also found to be of value in the present application, which involves very short fragments and very dense gels (27% polyacrylamide). If one takes into account the overall systematic decrease in band spacings as one moves upward in the gel, one sees that G-bands are characterized by particularly large spacings to the next higher band in the ladder, while C-bands have particularly short spacings. Figure 3, upper panel, shows a plot of Δ(1/m) (i.e., the change in inverse mobility in going from the band in question to the next higher band in the ladder) vs chain length L (equal to the number of phosphate moieties) of the oligonucleotide in question, for lane 7 in Fig. 1. Figure 3, lower panel, shows the corresponding plot for another oligonucleotide, the 5'-32P-labeled dodecamer d-CGAATTCGAGCT, also analyzed by treatment with mild acid followed by treatment with hot aqueous piperidine and electrophoresis through a 27% polyacrylamide gel. As already observed on 20% polyacrylamide gels (1), the simple mathematical expedient of calculating inverse mobilities largely compensates for the effect of the systematic decrease in band spacing with increasing chain length. An exception to this are the shortest bands (C at L = 1 in Fig. 3, upper panel, and C at L = 1 and G at L = 2 in Fig. 3, lower panel) for which uncommonly large values of Δ(1/m) are obtained (as already observed on 20% polyacrylamide gels, see Ref. (1)) and the very last bands (G at L = 16 in Fig. 3, upper panel, and T at L = 12 in Fig. 3, lower panel), which again show extraordinarily large Δ(1/m) values. These
latter exceptions are expected in view of the fact that the oligonucleotide signaling the last base differs from the next higher band (i.e., the parent peak) by one nucleotide unit, while all other members of the ladder differ from the next higher band by one nucleotide unit. For the other regions in the electrophoretograms, the local $\Delta(1/m)$ values are ordered as $G > T > A > C$. It is noteworthy that the ratio of $\Delta(1/m)$ for $G$-bands to $\Delta(1/m)$ for $C$-bands in these 27% polyacrylamide gels is about 1.3 or larger; it is, thus, larger on the average than observed in 20% polyacrylamide gels (1). This may be a reflection of a direct interaction of the guanine moieties with the polyacrylamide matrix.

**DISCUSSION**

Compared to the sequencing procedure described earlier (1), the following changes were introduced in this adaptation of the method to the sequencing of short oligonucleotides:

1. A change to a sequencing gel of higher polyacrylamide content (27% instead of 20%). This was to retard diffusion of the very short fragments and to minimize interference by any adventitious salt with the electrophoretic resolution of the shortest fragments.

2. A doubling of piperidine concentration from 0.5 to 1.0 M. The rationale for this change was to make the procedure more rapid. Even so, the reaction time used for the solvolysis in 1.0 M aqueous piperidine at 90°C (6 h) was slightly longer than the reaction time we used in our analysis of polynucleotides (5 h) by treatment with 0.5 M aqueous piperidine containing 0.3 M sodium chloride at 90°C (1). This was done because the solvolysis in hot aqueous piperidine is slower in the absence of salt (1), and because in sequencing by chemical cleavage it is customary to use longer degradation times for short fragments.

3. The substitution of a sequential treatment first with mild acid and then with hot, salt-free aqueous piperidine for the procedure involving only digestion with hot aqueous piperidine in the presence of sodium chloride, in order to obtain a hydrolysate essentially free of salt. The order of band intensities achieved with this method was $G > A > C > T$. This
result must be a composite effect of the two treatments to which the oligonucleotides are subjected: the acid hydrolysis, which is essentially the (A + G)-specific cleavage procedure of Maxam and Gilbert (3), should ultimately give rise to only G- and A-bands, while the prolonged treatment with hot aqueous piperidine in the absence of added salt should give approximately equal cleavage at G-, A-, and C-sites, and little cleavage at T-sites (see the band intensities for the shortest fragments in Fig. 3, panel A1, in Ref. (1)). It is because of this differential effect of the two treatments on the various bases that the relative duration of the two treatments is important: prolonged initial treatment with acid will ensure preponderance of A-bands over C-bands.

The characteristic large spacing for G-bands in 27% polyacrylamide gels should be of advantage in those cases where A-bands might be obtained of similar intensity as G-bands. This could occur due to adventitious salt in the oligonucleotide sample, which would favor A-cleavage over G-cleavage in the hot aqueous piperidine treatment (see the band intensities for the shortest fragments in Fig. 3, lanes A2 and A3, in Ref. (1)). In such a case, the purine positions would still be clearly signaled by the strongest bands, and A-bands and G-bands would be distinguishable by the criterion of spacing.

It is important to realize that this type of analysis, while useful to identify the main sequence in an oligonucleotide sample of reasonable purity, will not serve to detect relatively small levels of contaminating sequences. This is due to the fact that the single electrophoretic lane is crowded by the display of all bands of the major sequence. For a statement on the absence of impurities valid at the level of several percent, a standard sequencing analysis with multiple lanes (6), or a two-dimensional analysis (7) is required.

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