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A new method for sequencing DNA

(DNA chemistry/dimethyl sulfate cleavage/hydrazine/piperidine)

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ABSTRACT **DNA can be sequenced by a chemical procedure that breaks a terminally labeled DNA molecule partially at each repetition of a base. The lengths of the labeled fragments then identify the positions of that base. We describe reactions that cleave DNA preferentially at guanines, at adenines, at cytosines and thymines equally, and at cytosines alone. When the products of these four reactions are resolved by size, by electrophoresis on a polyacrylamide gel, the DNA sequence can be read from the pattern of radioactive bands. The technique will permit sequencing of at least 100 bases from the point of labeling.**

We have developed a new technique for sequencing DNA molecules. The procedure determines the nucleotide sequence of a terminally labeled DNA molecule by breaking it at adenine, guanine, cytosine, or thymine with chemical agents. Partial cleavage at each base produces a nested set of radioactive fragments extending from the labeled end to each of the positions of that base. Polyacrylamide gel electrophoresis resolves these single-stranded fragments; their sizes reveal *in order* the points of breakage. The autoradiograph of a gel produced from four different chemical cleavages, each specific for a base in a sense we will describe, then shows a pattern of bands from which the sequence can be read directly. The method is limited only by the resolving power of the polyacrylamide gel; in the current state of development we can sequence inward about 100 bases from the end of any terminally labeled DNA fragment.

We attack DNA with reagents that first damage and then remove a base from its sugar. The exposed sugar is then a weak point in the backbone and easily breaks; an alkali- or amine-catalyzed series of β-elimination reactions will cleave the sugar completely from its 3′ and 5′ phosphates. The reaction with the bases is a limited one, damaging only 1 residue for every 50 to 100 bases along the DNA. The second reaction to cleave the DNA strand must go to completion, so that the molecules finally analyzed do not have hidden damages. The purine-specific reagent is dimethyl sulfate; the pyrimidine-specific reagent is hydrazine.

The sequencing requires DNA molecules, either double-stranded or single-stranded, that are labeled at one end of one strand with ^{32}P. This can be a 5′ or a 3′ label. A restriction fragment of any length is labeled at both ends—for example, by being first treated with alkaline phosphatase to remove terminal phosphates and then labeled with ^{32}P by transfer from γ-labeled ATP with polynucleotide kinase. There are then two strategies: either (*i*) the double-stranded molecule is cut by a second restriction enzyme and the two ends are resolved on a polyacrylamide gel and isolated for sequencing or (*ii*) the doubly labeled molecule is denatured and the strands are separated on a gel (1), extracted, and sequenced.

THE SPECIFIC CHEMISTRY

A Guanine/Adenine Cleavage (2). Dimethyl sulfate methylates the guanines in DNA at the N7 position and the adenines at the N3 (3). The glycosidic bond of a methylated purine is unstable (3, 4) and breaks easily on heating at neutral pH, leaving the sugar free. Treatment with 0.1 M alkali at 90° then will cleave the sugar from the neighboring phosphate groups. When the resulting end-labeled fragments are resolved on a polyacrylamide gel, the autoradiograph contains a pattern of dark and light bands. The dark bands arise from breakage at guanines, which methylate 5-fold faster than adenines (3).

This strong guanine/weak adenine pattern contains almost half the information necessary for sequencing; however, ambiguities can arise in the interpretation of this pattern because the intensity of isolated bands is not easy to assess. To determine the bases we compare the information contained in this column of the gel with that in a parallel column in which the breakage at the guanines is suppressed, leaving the adenines apparently enhanced.

An Adenine-Enhanced Cleavage. The glycosidic bond of methylated adenosine is less stable than that of methylated guanosine (4); thus, gentle treatment with dilute acid releases adenines preferentially. Subsequent cleavage with alkali then produces a pattern of dark bands corresponding to adenines with light bands at guanines.

Cleavage at Cytosines and Thymines. Hydrazine reacts with thymine and cytosine, cleaving the base and leaving ribosylurea (5–7). Hydrazine then may react further to produce a hydrazone (5). After a partial hydrazinolysis in 15–18 M aqueous hydrazine at 20°, the DNA is cleaved with 0.5 M piperidine. This cyclic secondary amine, as the free base, displaces all the products of the hydrazine reaction from the sugars and catalyzes the β-elimination of the phosphates. The final pattern contains bands of similar intensity from the cleavages at cytosines and thymines.

Cleavage at Cytosine. The presence of 2 M NaCl preferentially supresses the reaction of thymines with hydrazine. Then, the piperidine breakage produces bands only from cytosine.

AN EXAMPLE

Consider a 64-base-pair DNA fragment, cut from *lac* operon DNA by the *Alu* I enzyme from *Arthrobacter luteus*, which cleaves flush at an AGCT sequence between the G and the C (8). After dephosphorylation, the two 5′ ends of this fragment were labeled with ^{32}P. The autoradiograph in Fig. 1 shows that the two strands separate during electrophoresis, after denaturation, on a neutral polyacrylamide gel (1); they can be easily excised and extracted. For each strand, aliquots of the four

cleavage reactions (strong G/weak A, strong A, strong C, and C + T) were electrophoresed at 600–1000 V on a 40-cm 20% polyacrylamide/7 M urea gel. Twelve hours later, a second portion of each sample was loaded on the gel and electrophoresis was continued. Fig. 2 displays autoradiographs showing two regions of the sequence of each strand derived from this single gel: one close to the labeled end of the molecule in those samples that had been electrophoresed in a short time, and a region further into the molecule expanded by electrophoresis for a longer time. The sequence can easily be read from the pattern of bands. The spacing between fragments decreases (roughly as an inverse square) from the bottom toward the top of the gel. The slight variations in the spacing are sequence-specific and reflect the last nucleotide added, a T or G decreasing the mobility more than an A or C. The fragments on the gel end with the base just before the one destroyed by the chemical attack; the labels on the bands in the figure represent the attacked bases. In Fig. 2, 62 bases can be read for both strands, the last 2 bases at the two 5′ ends not being determined by this gel. The sequence of each strand is consistent with and confirms that of the other:

pXXGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAG
GACCGTGCTGTCCAAAGGGCTGACCTTTCGCCCGTCACTCGCGTTGCGTTAATTACACTCAAXX$_p$

Fig. 3 is an expansion of one region of the sequencing gel to show the base specificity of the cleavage reactions.

DISCUSSION

The chemical sequencing method has certain specific advantages. First, the chemical treatment is easy to control; the ideal chemical attack, one base hit per strand, produces a rather even distribution of labeled material across the sequence. Second, each base is attacked, so that in a run of any single base all those are displayed. The chemical distinction between the different bases is clear, and, as in our example, the sequence of both strands provides a more-than-adequate check.

We have chosen this specific set of chemical reactions to provide more than enough information for the sequencing. The *Techniques* section describes another reaction that displays the Gs alone as well as an alternative reaction for breaking at As and Cs. However, it is more useful to have a strong G/weak A display, in which there is generally enough information to distinguish both the Gs and the As, than just a pure G pattern alone, because redundant information serves as a check on the identifications. In principle, one could sequence DNA with three chemical reactions, each of single-base specificity, using the absence of a band to identify the fourth position. This would be a nonredundant method in which every bit of information was required. Such an approach is subject to considerable error, and any hesitation in the chemistry would be misinterpreted as a different base. For that reason we have chosen redundant displays, which increase one's confidence in the sequencing.

5-Methylcytosine and N^6-methyladenine are occasionally found in DNA. 5-Methylcytosine can be recognized by our method because the methyl group interferes with the action of hydrazine [thymine reacts far more slowly than does uracil (5)]; thus, a 5-methylcytosine cleavage does not appear in the pattern, producing a gap in the sequence opposite a guanine (observed in this laboratory by J. Tomizawa and H. Ohmori). However, we do not expect to recognize an N^6-methyladenine; the glycosidic bond should not be unstable, and an earlier methylation of adenine at the N^6 position should not prevent the later methylation at N3.

These methods work equally well on double- or single-stranded DNA. There are sequence-specific effects on the methylation reaction with double-stranded DNA that do not appear with single-stranded DNA: in the sequence GGA the reactivity of the middle G is suppressed; in the sequence AAA the reactivity of the central A is enhanced. Since these effects are absent with single-stranded DNA, they must arise through steric hindrance or stacking interactions; however, they do not interfere with sequencing because they appear equivalently in both displays of the base. Although in single-stranded DNA the N1 of adenine is exposed to methylation and should methylate as readily as the N7 of guanine, methylation at this position does not destabilize adenine on the sugar. Under our conditions the methyl group will migrate to the N^6 position and the extra charge will disappear (3).

The sequencing method is limited only by the resolution attainable in the gel electrophoresis. On 40-cm gels we can, without ambiguities, sequence out to 100 bases from the point of labeling. If there is other information available to support the sequencing, such as an amino acid sequence, one can often read further. The availability of restriction endonucleases is now such that any DNA molecule, obtainable from a phage, virus, or plasmid, can be sequenced.

TECHNIQUES

[γ-^{32}P]ATP Exchange Synthesis (9). The specific activity routinely attains 1200 Ci/mmol. Dialyze glyceraldehyde-3-phosphate dehydrogenase against 3.2 M ammonium sulfate, pH 8/50 mM Tris·HCl, pH 8/10 mM mercaptoethanol/1 mM EDTA/0.1 mM NAD^+; and dialyze 3-phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) against the same solution minus NAD^+ (enzymes from Calbiochem). Combine 50 μl of the dialyzed dehydrogenase and 25 μl of the dialyzed kinase, sediment at 12,000 × g, and redissolve the pellet in 75 μl of twice-distilled water to remove ammonium sulfate. Dissolve 25 mCi (2.7 nmol) of HCl-free, carrier-free $^{32}P_i$ in 50 μl of 50 mM Tris·HCl, pH 8.0/7 mM $MgCl_2$/ 0.1 mM EDTA/2 mM reduced glutathione/1 mM sodium 3-phosphoglycerate/0.2 mM ATP (10 nmol); add 2 μl of the dialyzed, desalted enzyme mixture, and allow to react at 25°. Follow the reaction by thin-layer chromatography on PEI cellulose in 0.75 M sodium phosphate, pH 3.5, by autoradiography of the plate. At the plateau, usually 30 min, add 250 μl of twice-distilled water and 5 μl of 0.1 M EDTA, mix, and heat at 90° for 5 min to inactivate the enzymes. Then chill, add 700 μl of 95% ethanol, mix well, and store at −20°. The theoretical limit of conversion is 79%, and if this is achieved the [γ-^{32}P]ATP would have a specific activity near 2000 Ci/mmol.

Labeling 5′ Ends. 5′-Phosphorylation (10, 11) includes a heat-denaturation in spermidine which increases the yield 15-fold with flush-ended restriction fragments. Dissolve dephosphorylated DNA in 75 μl of 10 mM glycine·NaOH, pH 9.5/1 mM spermidine/0.1 mM EDTA; heat at 100° for 3 min and chill in ice water. Then add 10 μl of 500 mM glycine·NaOH, pH 9.5/100 mM $MgCl_2$/50 mM dithiothreitol, 10 μl of [γ-^{32}P]ATP (100 pmol or molar equivalent of DNA 5′ ends, 1000 Ci/mmol), and several units of polynucleotide kinase to a final volume of 100 μl. Heat at 37° for 30 min; add 100 μl of 4 M ammonium acetate, 20 μg of tRNA, and 600 μl of ethanol, mix well, chill at −70°, centrifuge at 12,000 × g, remove the supernatant phase, rinse the pellet with ethanol, and dry under vacuum.

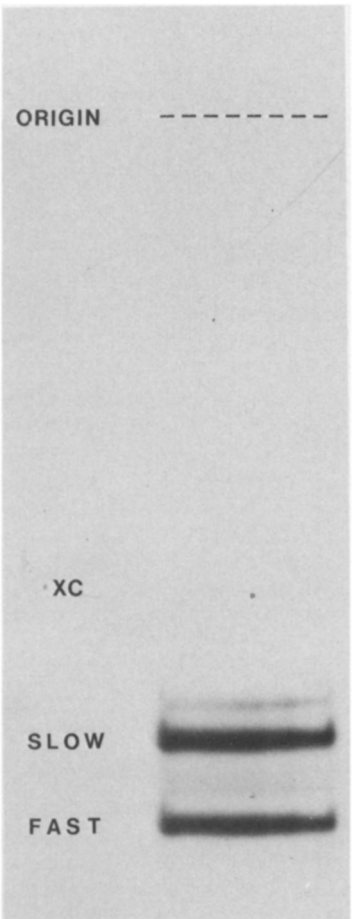

FIG. 1. Strand separation of a restriction fragment: 1.5 μg of a 64-base-pair DNA fragment (75 pmol of 5′ ends) was phosphorylated with [γ-^{32}P]ATP (800 Ci/mmol) and polynucleotide kinase, denatured in alkali, layered onto a 0.3 cm × 3 cm surface of an 8% polyacrylamide slab gel (see under *Techniques*), and electrophoresed at 200 V (regulated) and 20 mA (average), until the xylene cyanol (XC) dye moved 9 cm. The gel on one glass plate was then tightly covered with Saran Wrap and exposed to Kodak XR-5 x-ray film for 10 min.

Labeling 3′ Ends. To adenylate with [α-^{32}P]ATP and terminal transferase (12), dissolve DNA in 70 μl of 10 mM Tris·HCl, pH 7.5/0.1 mM EDTA, heat at 100° for 3 min, and chill at 0°. Then add, in order, 10 μl of 1.0 M sodium cacodylate (pH 6.9), 2 μl of 50 mM $CoCl_2$, mix, 2 μl of 5 mM dithiothreitol, 10 μl of [α-^{32}P]ATP (500 pmol, 100 Ci/mmol), and several units of terminal transferase to a final volume of 100 μl. Heat at 37° for several hours, add 100 μl of 4 M ammonium acetate, 20 μg of tRNA, and 600 μl of ethanol, and precipitate, centrifuge, rinse, and dry the DNA as described above. Dissolve the pellet in 40 μl of 0.3 M NaOH/1 mM EDTA, heat at 37° for 16 hr, and either add glycerol and dyes for strand separation or neutralize, ethanol precipitate, and renature the DNA for secondary restriction cleavage.

Strand Separation. Dissolve the DNA in 50 μl of 0.3 M NaOH/10% glycerol/1 mM EDTA/0.05% xylene cyanol/0.05% bromphenol blue. Load on a 5–10% acrylamide/0.16–0.33% bisacrylamide/50 mM Tris·borate, pH 8.3/1 mM EDTA gel and electrophorese. The concentration of DNA entering the gel is critical and must be minimized to prevent renaturation. Use thick gels with wide slots (0.3- to 1-cm-thick slabs with 3-cm to full width slots), and run cool (at 25°).

Gel Elution. Insert an excised segment of the gel into a 1000 μl (blue) Eppendorf pipette tip, plugged tightly with siliconized glass wool and heat-sealed at the point. Grind the gel to a paste with a siliconized 5-mm glass rod, add 0.6 ml of 0.5 M ammonium acetate/0.01 M magnesium acetate/0.1% sodium dodecyl sulfate/0.1 mM EDTA (and 50 μg of tRNA carrier if the DNA has already been labeled); seal with Parafilm and hold at 37° for 10 hr. Cut off the sealed point, put the tip in a siliconized 10 × 75 mm tube, centrifuge for a few minutes, rinse with 0.2 ml of fresh gel elution solution, and alcohol precipitate twice.

Partial Methylation of Purines. Combine 1 μl of sonicated carrier DNA, 10 mg/ml, with 5 μl of ^{32}P-end-labeled DNA in 200 μl of 50 mM sodium cacodylate, pH 8.0/10 mM $MgCl_2$/0.1 mM EDTA. Mix and chill in ice. Add 1 μl of 99% (10.7 M) dimethyl sulfate, mix, cap, and heat at 20° for 15 min. To stop the reaction, add 50 μl of a stop solution (1.0 M mercaptoethanol/1.0 M Tris·acetate, pH 7.5/1.5 M sodium acetate/0.05 M magnesium acetate/0.001 M EDTA), 1 mg/ml of tRNA, and mix. Add 750 μl (3 volumes) of ethanol, chill, and spin. Reprecipitate from 250 μl of 0.3 M sodium acetate, rinse with alcohol, and dry.

Strong Guanine/Weak Adenine Cleavage. Dissolve methylated DNA in 20 μl of 10 mM sodium phosphate, pH 7.0/1 mM EDTA, and collect the liquid on the bottom of the tube with a quick low-speed spin. Close the tube and heat in a water bath at 90° for 15 min. Chill in ice and collect the condensate with a quick low-speed spin. Add 2 μl of 1.0 M NaOH, mix, and draw the liquid up into the middle of a pointed glass capillary tube, seal with a flame, and hold at 90° for 30 min. Open the capillary and empty into 20 μl of urea-dye mixture, heat, and layer on the gel.

Strong Adenine/Weak Guanine Cleavage. Dissolve methylated DNA in 20 μl of distilled water. Chill to 0°, add 5 μl of 0.5 M HCl, mix, and keep the sample at 0° in ice, mixing occasionally. After 2 hr, add 200 μl of 0.3 M sodium acetate and 750 μl of ethanol, chill, spin, rinse, and dry. Then dissolve in 10 μl of 0.1 M NaOH/1 mM EDTA and heat at 90° for 30 min in a sealed capillary. Add contents to urea-dye mixture, heat, and layer.

An Alternative Guanine Cleavage. Dissolve methylated DNA in 20 μl of freshly diluted 1.0 M piperidine. Heat at 90° for 30 min in a sealed capillary. [This reaction opens 7-MeG adjacent to the glycosidic bond (13), displaces the ring-opened product from the sugar, and eliminates both phosphates to cleave the DNA wherever G was methylated.] Return the contents of the capillary to the reaction tube, lyophilize, wet the residue, and lyophilize again. Finally, dissolve the last residue in 10 μl of 0.1 M NaOH/1 mM EDTA and prepare for the gel.

An Alternative Strong Adenine/Weak Cytosine Cleavage. Combine 20 μl of 1.5 M NaOH/1 mM EDTA with 1 μl of sonicated carrier DNA (10 mg/ml) and 5 μl of ^{32}P end-labeled DNA, and heat at 90° for 30 min in a sealed capillary. [The strong alkali opens the adenine and cytosine rings (13); then, the ring-opened products can be displaced and phosphates eliminated with piperidine.] Rinse the capillary into 100 μl of 1.0 M sodium acetate, add 5 μl of tRNA (10 mg/ml), add 750 μl of ethanol, chill, spin, rinse, and dry. Dissolve the pellet in 20 μl of freshly diluted 1.0 M piperidine, and heat at 90° for 30 min in a sealed capillary. Lyophilize twice, dissolve the last residue in 10 μl of 0.1 M NaOH/1 mM EDTA, and add urea-dye mixture.

Cleavage at Thymine and Cytosine. Combine 20 μl of distilled water, 1 μl of sonicated carrier DNA (10 mg/ml), and 5 μl of ^{32}P end-labeled DNA. Mix and chill at 0°. Add 30 μl of

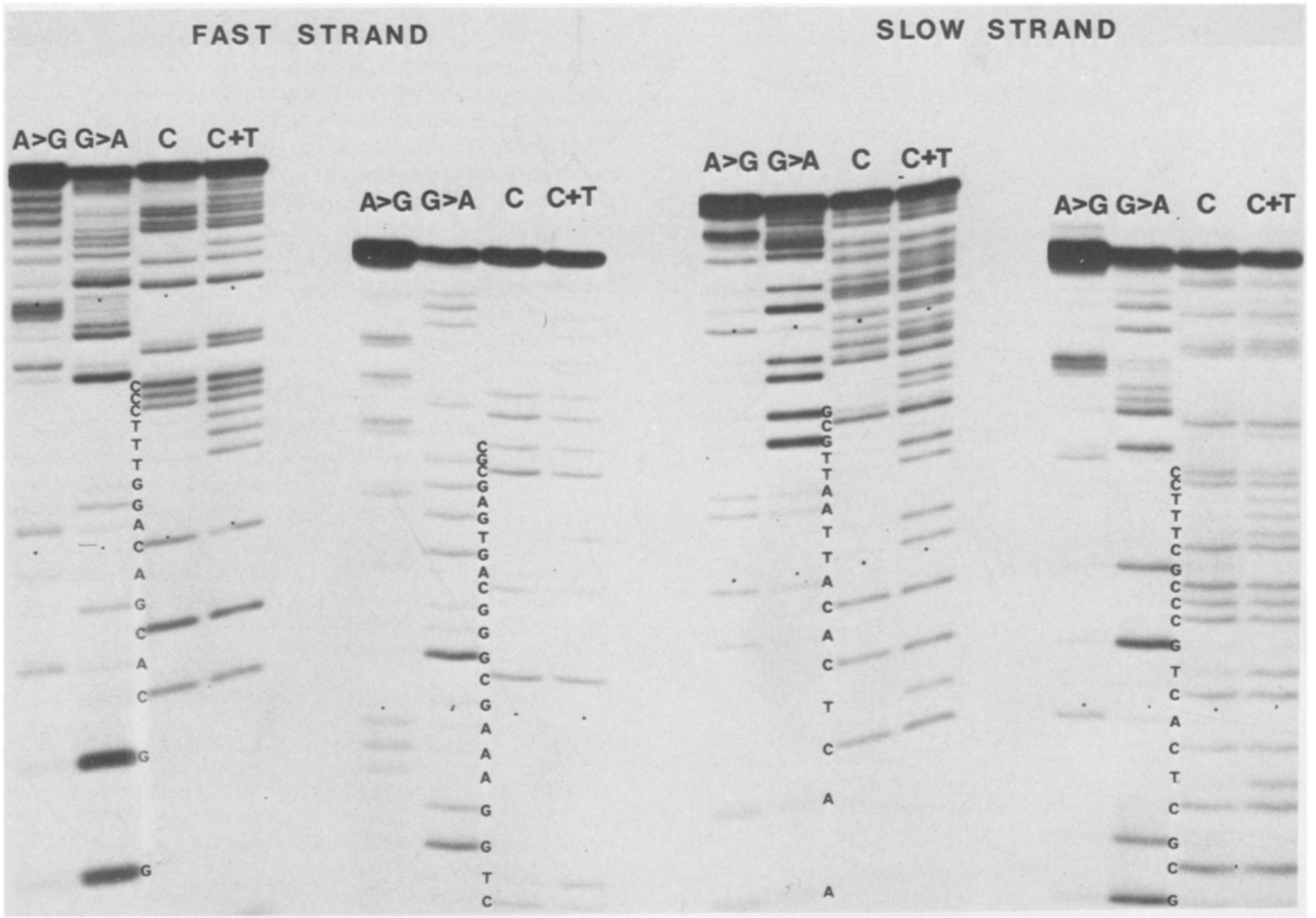

FIG. 2. Autoradiograph of a sequencing gel of the complementary strands of a 64-base-pair DNA fragment. Two panels, each with four reactions, are shown for each strand; cleavages proximal to the 5′ end are at the bottom on the left. A strong band in the first column with a weaker band in the second arises from an A; a strong band in the second column with a weaker band in the first is a G; a band appearing in both the third and fourth columns is a C; and a band only in the fourth column is a T. To derive the sequence of each strand, begin at the bottom of the left panel and read upward until the bands are not resolved; then, pick up the pattern at the bottom of the right panel and continue upward. One-tenth of each strand, isolated from the gel of Fig. 1, was used for each of the base-modification reactions. The dimethyl sulfate treatment was 50 mM for 30 min to react with A and G; hydrazine treatment was 18 M for 30 min to react with C and T and 18 M with 2 M NaCl for 40 min to cleave C. After strand breakage, half of the products from the four reactions were layered on a 1.5 × 330 × 400 mm denaturing 20% polyacrylamide slab gel, pre-electrophoresed at 1000 V for 2 hr. Electrophoresis at 20 W (constant power), 800 V (average), and 25 mA (average) proceeded until the xylene cyanol dye had migrated halfway down the gel. Then the rest of the samples were layered and electrophoresis was continued until the new bromphenol blue dye moved halfway down. Autoradiography of the gel for 8 hr produced the pattern shown.

95% (30 M) hydrazine*, mix well, and keep at 0° for several minutes. Close the tube and heat at 20° for 15 min. Add 200 μl of cold 0.3 M sodium acetate/0.01 M magnesium acetate/0.1 mM EDTA/0.25 mg/ml tRNA, vortex mix, add 750 μl of ethanol, chill, spin, dissolve the pellet in 250 μl of 0.3 M sodium acetate, add 750 μl of ethanol, chill, spin, rinse with ethanol, and dry. Dissolve the pellet and rinse the walls with 20 μl of freshly diluted 0.5 M piperidine. Heat for 30 min at 90° in a sealed capillary. Lyophilize twice, dissolve in 10 μl of 0.1 M NaOH/1 mM EDTA, add urea-dye mixture, heat, and layer on gel.

Cleavage at Cytosine. Replace the water in the hydrazinolysis reaction mixture with 20 μl of 5 M NaCl, and increase the reaction time to 20 min. The freshness and the concentration of the hydrazine are critical for base-specificity.

Reaction Times. The reaction conditions provide a uniformly labeled set of partial products of chain length 1 to 100. To distribute the label over a shorter region, increase the reaction time, and vice versa.

* CAUTION: Hydrazine is a volatile neurotoxin. Dispense with care in a fume hood, and inactivate it with concentrated ferric chloride.

Reaction Vessels. We use 1.5-ml Eppendorf conical polypropylene tubes with snap caps, treated with 5% (vol/vol) dimethyldichlorosilane in CCl_4 and rinsed with distilled water.

Alcohol Precipitation, Wash, and Rinse. Unless otherwise specified, the initial ethanol precipitation is from 0.3 M sodium acetate/0.01 M magnesium acetate/0.1 mM EDTA, with 50 μg of tRNA as carrier. Add 3 volumes of ethanol, cap and invert to mix, chill at −70° in a Dry Ice-ethanol bath for 5 min, and spin in the Eppendorf 3200/30 microcentrifuge at 15,000 rpm (12,000 × *g*) for 5 min. Reprecipitate with 0.3 M sodium acetate and 3 volumes of ethanol, chill, and spin. Rinse the final pellet with 1 ml of cold ethanol, spin, and dry in a vacuum for several minutes.

Gel Samples. All samples for sequencing gels are in 10 or 20 μl of 0.1 M NaOH/1 mM EDTA to which is added an equal volume of 10 M urea/0.05% xylene cyanol/0.05% bromphenol blue. Heat the sample at 90° for 15 sec, then layer on the gel.

Sequencing Gels. These are commonly slabs 1.5 mm × 330 mm × 400 mm with 18 sample wells 10 mm deep and 13 mm

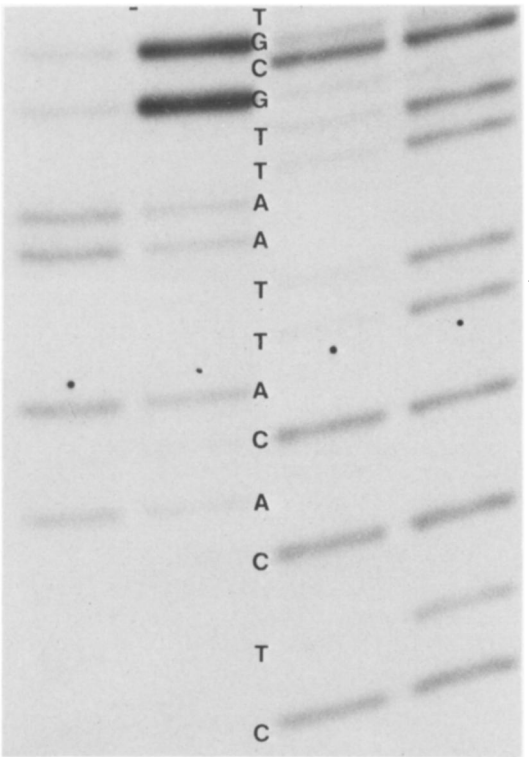

FIG. 3. Detail of the sequence gel. The four lanes are (from left to right) A > G, G > A, C, C + T; the dots show the position of the bromphenol blue dye marker, between fragments 9 and 10 long.

wide separated by 3 mm (fitting on a 35.5 × 43 cm x-ray film). They are 20% (wt/vol) acrylamide (Bio-Rad)/0.67% (wt/vol) methylene bisacrylamide/7 M urea/50 mM Tris·borate, pH 8.3/1 mM EDTA/3 mM ammonium persulfate; 300 ml of gel solution is polymerized with TEMED within 30 min (generally 50 μl of TEMED). Age the gel at least 10 hr before using it. Electrophorese with some heating (30–40°), to help keep the DNA denatured, between 800 and 1200 V. Load successively whenever the previous xylene cyanol has moved halfway down the gel. Bromphenol blue runs with 10-nucleotide-long fragments, xylene cyanol with 28. With three loadings at 0, 12, and 24 hr, a 1000-V run for 36 hr permits reading more than 100 bases. To sequence the first few bases from the labeled end, use a 25% acrylamide/0.83% bisacrylamide gel in the usual urea buffer and pre-electrophorese this gel for 2 hr at 1000 V.

Autoradiography. Freeze the gel for autoradiography. Remove one glass plate, wrap the gel and supporting plate with Saran Wrap, and mark the positions of the dyes with ^{14}C-containing ink. Place the gel in contact with film in a light-tight x-ray exposure holder (backed with lead and aluminum) at −20° under pressure from lead bricks.

Special Materials. Dimethyl sulfate (99%) was purchased from Aldrich Chemical Co., hydrazine (95%) from Eastman Organic Chemicals, and piperidine (99%) from Fisher Scientific; these were used without further purification.

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