One-Step, One-Lane Chemical DNA Sequencing by *N*-Methylformamide in the Presence of Metal Ions

BioTechniques 21:910-917 (November 1996)

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ABSTRACT

We report on a chemical method that allows DNA sequencing by a single reaction. It is based on treatment of 5¢-end-labeled DNA with N*-methylformamide in the presence of manganese. This method allows the manipulation of samples to be kept to a minimum and consists of a single chemical step that requires about 30 minutes to complete base degradation, phosphodiester bond cleavage and denaturation. Examples of one-treatment, one-lane DNA sequencing of both radioactively and fluorescently 5¢-end-labeled DNAs are reported.*

INTRODUCTION

Several one-lane chemical sequencing strategies have been developed; for their critical evaluation and comparison with enzymatic DNA sequencing procedures, see Reference 12. Previously we reported on the reaction of amides with DNA and exploited, for DNA sequencing purposes, the degradation of phosphodiester bonds by formamide (2,5,9,10). We determined that formamide reacts with DNA according to the following two-step mechanism: (*i*) degradation of the purine and pyrimidine bases and (*ii*) β-elimination of the sugar protons with consequent β-elimination of the phosphate residue. The 3′ β-elimination occurs first, causing an increase in acidity of the reactive proton Hγ in the α,β unsaturated sugar residue, thus allowing 5′ bond β-elimination.

The proposed general mechanism of DNA fragmentation by formamide is shown in Figure 1. During the first step, the base is degraded and removed (Figure 1, **1** and **2**); the order of decreasing sensitivity of the nucleic bases to degradation is I>G≥A>C>T (2). For purines, degradation occurs by nucleophilic attack in C(8), leading to degradative C(8)-ring opening of the imidazole ring; for pyrimidines, degradation occurs by nucleophilic attack in C(6), leading to degradative C(6)-ring opening of the pyrimidine ring. Alterations of the chemical structure of the natural nucleic bases [i.e., C(7) *deaza* modifications for purine residues and C(5) substitution with an electron-donating methyl group or with an electron-withdrawing bromine for the 2′-deoxy cytidine residues, respectively] markedly change the sensitivity of the nucleic bases to formamide attack (13).

In the second step (Figure 1, from **3** to **5**), degradation and removal of the heterocyclic purine or pyrimidine bases by hydrolysis of the β-nucleosidic bond induce, in the 2′-deoxy ribose moiety, an equilibrium between the hemiacetalic and the aldehydic form (Figure 1, **2** and **3**). The two reactive protons (Hα and Hγ, indicated in **3**), present in the aldehydic form, are available for reaction and are eliminated. This leads to cleavage of 3′ and 5′ phosphodiester bonds, respectively.

In a DNA sequencing procedure, the use of a nitrogenous base that efficiently carries out the first step is not necessarily as efficient in carrying out the second step. Actually, the opposite is often true (see below).

In the case of formamide (a weakly basic compound), cleavage of the 5′ bond occurs less efficiently than cleavage of the 3′ bond because of the lower acidity of proton Hγ. Therefore, this problem applies only to 5′-end-labeled DNA molecules, not to those labeled at the 3′ end. For a 5′-end-labeled DNA fragment, two bands are visible because incomplete cleavage of the DNA molecule at the 5′ phosphate yields two visible products derived from the 5′ end of the DNA molecule—that cleaved at both the 5′ and 3′ phosphate bonds and that cleaved only at the 3′ phosphate bond. Both are visible because the label remains at the 5′ end of the molecule and the two classes of labeled

molecules are produced.

For 3′-end-labeled DNA molecules, it is the 3′ DNA fragment that retains the label and is visible (Figure 2a), even though both products are still produced in the 5′ end of the DNA molecule. Elimination of double bands is mandatory to obtain clear profiles in 5′-end-labeled DNA sequencing. This can be achieved (5) by coupling the formamide reaction to the piperidine one. Piperidine is a strong base that β-eliminates very efficiently at both the 3′ and the 5′ phosphodiester bonds, and it can be used to remove—in the formamide one-lane sequencing of 5′-end-labeled DNA—the double bands produced by the formamide weak β-elimination (10). Piperidine solves the sequencing ambiguity created by these double bands; however, its use should be avoided because it is time-consuming, requires optimization, is toxic, must be removed before gel analysis and makes automated procedures difficult.

We report that *N*-methylformamide (NmF) maintains the base-selective cleavage and increases the efficiency of β-elimination at the 5′ bond. We describe its use in the selective degradation of nucleic bases in polynucleotide chains and its efficiency in carrying out β-elimination at the 5′ phosphodiester bond when coupled with certain divalent cations.

A single treatment of 5′-end-labeled DNA with NmF in the presence of manganese (Mn^{+2}) allows determination of its sequence.

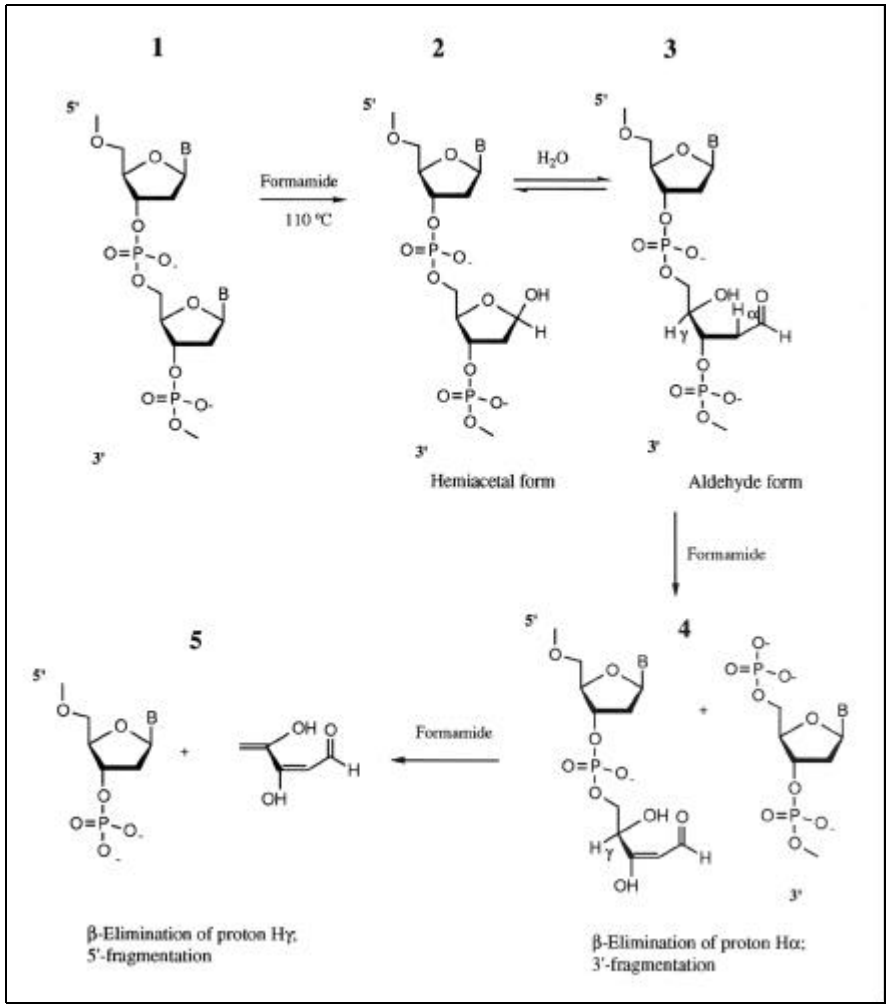

Figure 1. DNA fragmentation using formamide. The removal of a nucleic base by a weak amide (in the example: formamide) is described (**1** and **2**), leading to a hydrolytic equilibrium (**2** and **3**). In the presence of a weak base, the aldehydic form undergoes β-elimination of the Hα proton, causing cleavage of the 3′-phosphodiester bond **(4)**. β-Elimination of the Hγ proton, leading to cleavage of the 5′ bond, occurs with slower kinetics (**5**). This step is more efficient in the presence of a stronger base (i.e., NmF, *N*-dimethylformamide or piperidine).

MATERIALS AND METHODS

Materials and methods are as described (2,5,9,10), with the following specifications: NmF was obtained from several commercial sources (Sigma Chemical, St. Louis, MO, USA; Fluka Chemika-Biochemika, Buchs, Switzerland; and Carlo Erba Chimici, Milano, Italy). No appreciable differences were found. *N*-Dimethylformamide was from Fluka and Chelex® 100 resin was from Bio-Rad (Hercules, CA, USA). The synthetic deoxypolynucleotide used for the sequencing analyses in Figures 2 and 3 is a 34-mer whose sequence is 5′-GAAAAATAAAAATAAAAAAGCT-ACCGATTGCGCG-3′. Labeling was at the 5′-end position and was obtained by polynucleotide kinase and [γ-^{32}P] ATP.

Degradation of Guanine Residues in Deoxypolynucleotides by Photoreaction in the Presence of Methylene Blue

Three nanomoles of 5′-end-labeled deoxypolynucleotide (2000 cpm/pmol) were dissolved in 120 µL of water. Methylene blue was added to a final concentration of 0.025% (wt/vol) and the sample was layered on a small petri dish, placed on an ice-water bath to minimize evaporation and exposed to a 200-W white-light bulb at a distance of 10 cm for 1 h. The recovered sample was ethanol-precipitated twice.

Labeling, Purification and Sequencing Procedure

The 5S DNA used for DNA sequencing analysis (Figure 4) was obtained (2) as follows: 0.01 pmol of a plasmid carrying the *Saccharomyces cerevisiae* 5S rRNA repeat gene (1) was amplified by polymerase chain reaction (PCR) (15 cycles) using 30 pmol of two internal primers complementary to positions 100–120 (5′-end-labeled with [γ-^{32}P] ATP at a specific activity of 100 000 cpm/pmol, noncoding strand) and -11/+10 (unlabeled, coding) and a mixture of deoxyribonucleoside triphosphates containing 250 µM dATP, dCTP, dTTP

and 500 μM dITP. After amplification, the full-length product was gel-purified, ethanol-precipitated, resuspended in 80% NmF + 3 mM $MnCl_2$, incubated for 30 min at 110°C and diluted 1:1 with formamide. An aliquot was then loaded on a denaturing gel. The human wild-type and mutant TOP1 gene (8) DNAs were provided by P. Benedetti (CNR, Rome, Italy). The sequencing reaction on fluorescently labeled, PCR-amplified TOP1 fragments is reported in Figures 5 and 6. Amplification was from oligonucleotides complementary to positions 1386–1403 (labeled, noncoding strand) and 1051–1070 (unlabeled, coding). The oligonucleotides, labeled with FAM or HEX according to Reference 7, were custom-made by F. Riccobono (M-Medical, Florence, Italy). In particular, 6-FAM amidite and HEX amidite (Perkin-Elmer/Applied Biosystems Division, Foster City, CA, USA) were attached to the oligonucleotides in solid phase. An amount of plasmid (0.03 pmol) carrying the TOP1 gene was PCR-amplified (20 cycles) using 15 pmol of the pair of oligonucleotides, one of which was 5′-end-labeled with FAM or HEX fluorochromes. After amplification or terminal transferase (TdT) treatment (2), the samples were reacted with NmF + Mn^{+2}, typically as follows: 0.05 pmol of fluorescent FAM-labeled DNA was reacted 30 min at 110°C with 80% NmF + 3 mM $MnCl_2$, diluted 1:1 with formamide and loaded directly on a standard gel in an Applied Biosystems automated sequencer (Model 370A; provided by Ist. Biologia Cellulare, CNR, Rome, Italy). Before the NmF + Mn^{+2} reaction, the amplified fluorescent DNA was either gel-purified or treated with TdT (2) to prevent background due to premature amplification terminations.

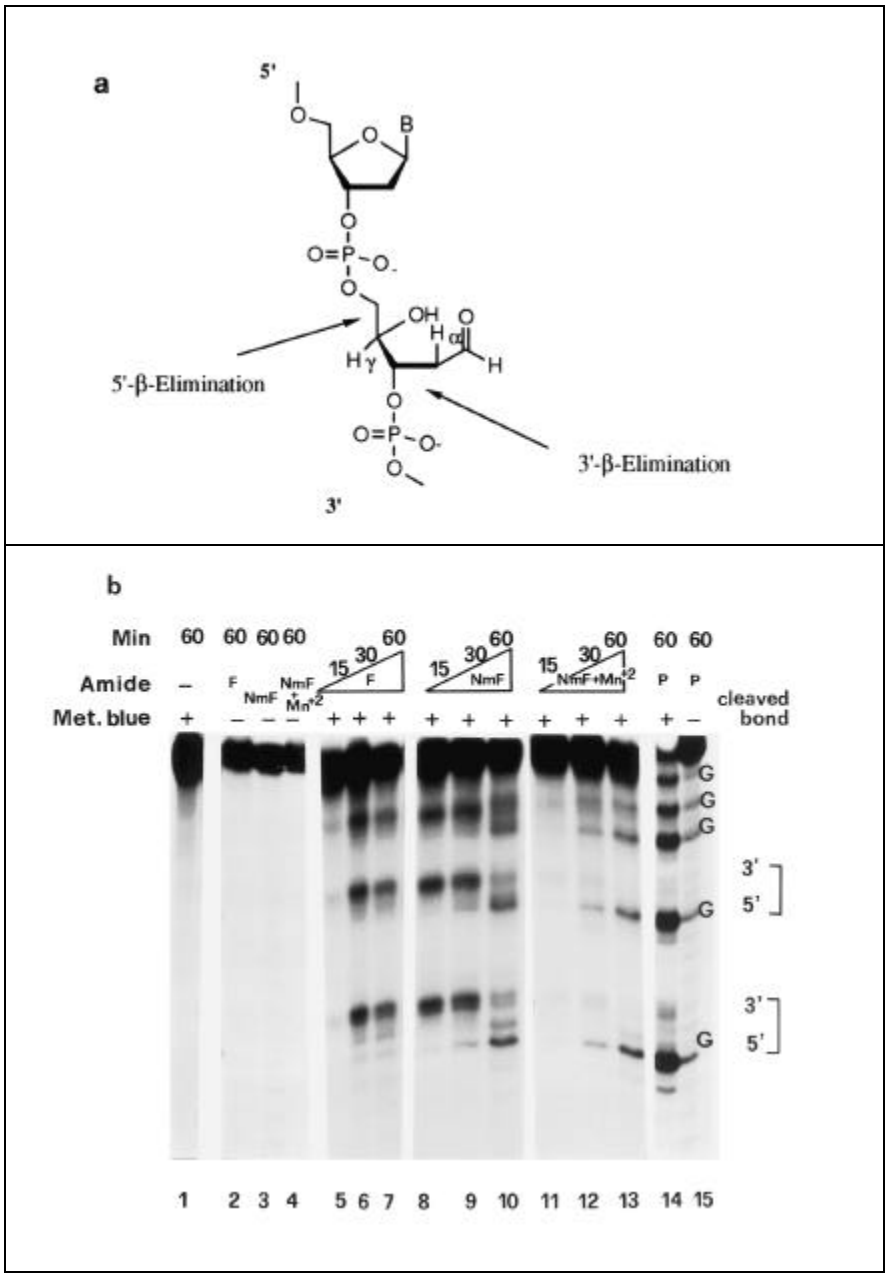

Figure 2. Differential cleavage of the 3′ and 5′ phosphodiester bonds using different amides. Panel a: Schematic representation of the degradation of the sugar moiety indicating the two different β-eliminations (see text). Panel b: Differential cleavage. Each sample, containing 0.015 μg (30 000 cpm) of 5′-end-labeled deoxypolynucleotide, was incubated at 90°C for the indicated times with the specified reagents. Sample in lane 1 was heated in water, dried, resuspended in 80% formamide, heated 1 min at 90°C and loaded on the gel. Samples in lanes 14 and 15, after incubation, were vacuum-dried, washed with 10 volumes of water and vacuum-dried again, resuspended in 80% formamide and loaded. All the other samples were added with one volume of 80% formamide, 0.1% xylene cyanol and 0.1% bromophenol blue and were loaded (this addition is necessary in the samples containing NmF because it increases density and favors layering). Lane 1: Control. Polynucleotide treated with methylene blue (see Materials and Methods) but not photoreacted; no treatment with amides. Lanes 2–4: Controls. Reaction of "normal" (not photoreacted in the presence of methylene blue) DNA with formamide (F, 80%, 60 min, lane 2), or *N*-methylformamide (NmF, 80%, same conditions, in the absence [lane 3] or in the presence [lane 4] of 3 mM Mn^{+2}). Lanes 5–13: Reaction, for the indicated times, of DNA samples that were previously depurinated at the G positions by the "methylene blue–light" treatment with formamide (F, lanes 5–7), *N*-methylformamide (NmF) (lanes 8–10) or NmF + 3 mM Mn^{+2} (lanes 11–13). Lanes 14 and 15: Reaction with piperidine (P, 10%) of "methylene blue–light" treated (lane 14) or untreated (lane 15) DNAs. The bands resulting from cleavage at the 3′ or 5′ bonds (as schematically illustrated in Figure 2a) are indicated on the right.

RESULTS AND DISCUSSION

Determination of Relative Efficiency of 3′ and 5′ Phosphodiester Bond Cleavages—Optimization of Cleavage at 5′ Bond

We have indicated in the Introduction the chemical factors that cause the preferential cleavage of the 3′ phosphodiester bond by a weak nitrogenous

base relative to the one at 5′, based on known chemistry and on our previous observations (2,5,9,10). The experimental results depicted in Figure 2b show that: *(i)* formamide (F) actually causes only partial cleavage at the 5′ phosphodiester bond (lanes 5–7); *(ii) N*-methylformamide (NmF, lanes 8–10) cleaves better but still inefficiently at the 5′ bond; and *(iii)* addition of a divalent cation (lanes 11–13) causes complete cleavage of the 5′ bond (lane 13). This experiment consists of selective degradation (not shown) of guanosine residues by photoreaction of a 5′-end-labeled polynucleotide in the presence of methylene blue [based on the observation that methylene blue does not cleave phosphodiester bonds unless a successive treatment with a nitrogenous base at high temperature is performed (6,9, 15)]. The substrate so obtained makes it possible to separate the analysis of the two degradative steps (i.e., of the nucleic base and of the phosphodiester bonds). The analysis consists of observing the kinetics of the cleavage of the 3′ and 5′ phosphodiester bonds in the polynucleotide in which the guanosine residues were degraded under various conditions. Of the three results mentioned above, the third (*iii*) is relevant to sequencing procedures: NmF + Mn^{+2} causes complete 5′-fragmentation (lane 13). Reaction of this substrate with piperidine (known to cleave very efficiently both at the 3′ and the 5′ bonds, because of its strong-base character) served as markers (lanes 14 and 15).

N-Dimethylformamide was also tested and its behavior was found to be very similar to that of NmF, but it was more difficult to use because of its lower boiling temperature and density.

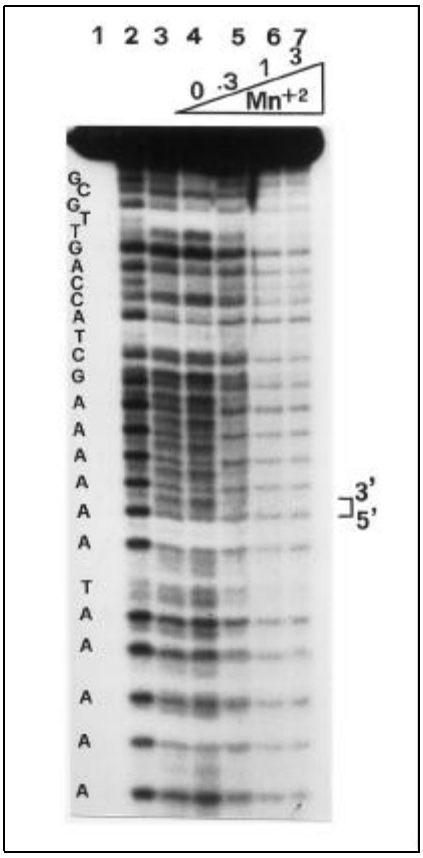

Figure 3. Effect of Mn^{+2} on DNA cleavage using NmF. 5′-end-labeled polynucleotide DNA (0.015 µg, 30 000 cpm/lane) was reacted for 20 min with 80% NmF at 110°C. The sample was diluted 1:1 with 90% formamide, 0.1% (wt/vol) xylene cyanol, 0.1% (wt/vol) bromophenol blue and loaded on a denaturating sequencing gel (16%). Lane 1, control, untreated; lane 2, commercial NmF (nonpurified, from Fluka); lane 3, NmF, same as in lane 2, but with addition of 5 mM EDTA; lanes 4–7, Chelex-purified NmF in the presence of the indicated concentrations (mM) of Mn^{+2}. An example of cleavage at the 3′ and 5′ bonds is indicated.

Different Cations Affect the Degradation by NmF Differently

To better characterize the effect of divalent cations on the cleavage of the phosphodiester bonds, we carried out several experiments. Our observations are outlined below.

Commercial NmF (from several sources) in the absence of divalent cations produces a blurred background (Figure 3, lane 2), due to incomplete cleavage at the 5′ bond (as also seen in Figure 2, lanes 5–13).

Nuclear magnetic resonance (NMR) analysis (data not shown) of NmF from various sources did not reveal the presence of organic contamination in appreciable amounts. The use of chelating agents (Figure 3, lane 3) and the purifi-

cation of NmF on Chelex resin (lane 4) strongly decrease the relative efficiency of the 5′ bond cleavage (lanes 3 and 4 vs. lane 2), pointing to an effect of one or more ions in the selective degradation. Addition of Mn^{+2} (lanes 5–7) actually allows complete cleavage at the 5′ bond (lane 7). The purpose of this experiment was to establish the role of Mn^{+2} in the phosphodiester bond cleavage by NmF.

The effect of zinc (Zn^{+2}) (data not shown) is quite similar: i.e., efficient cleavage of the 5′ phosphodiester bond and removal of the blurred background. However, the action of Zn^{+2} has the undesirable effect of decreasing base selectivity. By increasing the formamide attack of pyrimidines, Zn^{+2} increases cleavage at T residues and causes less of a difference between purines and C residues, substantially increasing errors if used in a sequencing protocol.

Addition of comparable concentrations of magnesium (Mg^{+2}) slightly inhibits the entire β-elimination process and is, for the rest, essentially inactive (data not shown). *cis*-Platinum and copper ions were also tested. In both instances, NmF base degradation was no longer selective at 110°C and its action was not analyzed further. In conclusion, of all the divalent cations tested, only Mn^{+2} ions have the appropriate properties for complementing the cleavage reaction of phosphodiester bonds carried out by the selected amide.

A hypothetical, qualitative model for the interpretation of the effect of different transition-metal ions (Mn^{+2}, Mg^{+2} and Zn^{+2}) on DNA degradation by NmF can be proposed. The ability of metal ions to react with a variety of electron-donor sites on polynucleotides has been established (4,11,14). Evidence was obtained that Mn^{+2} ions form inner-sphere complexes with single-stranded poly(A). In these complexes a single Mn^{+2} ion coordinates directly with two phosphate oxygens and with the nitrogen atom of one of the heterocyclic bases (16). With Mg^{+2} ions, only outer-sphere complexes, by means of a water bridge, are formed with the phosphate (3). On the basis of these data, it is conceivable that the inner-sphere complexes formed by Mn^{+2} ions might favor the anti-periplanar conformation necessary for the β-anti 5′-elimination. Mg^{+2} ions, which do not form inner-sphere complexes, induce no detectable effect. Finally, Zn^{+2} ions, which partially form inner-sphere complexes (3), show a behavior similar to that of Mn^{+2}.

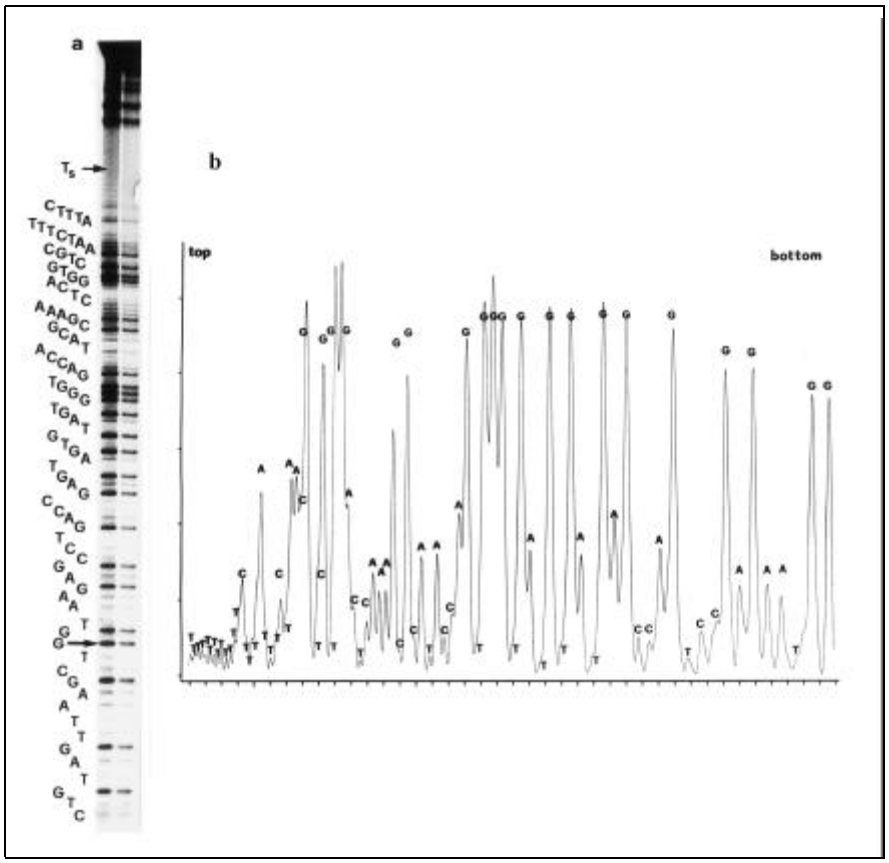

Figure 4. One-lane sequence determination of radioactively 5′-end-labeled 5S gene DNA using NmF + Mn^{+2}. (a) Sequencing procedure: 5′-end-labeled, PCR-amplified DNA sequenced with Chelex-purified NmF in the presence of 3 mM Mn^{+2} (see Materials and Methods). Left lane, 120 000 cpm; right lane, 40 000 cpm. (b) Scanning analysis: The profile (obtained on a Bio-Rad GS-670 Imaging Densitometer) refers to the tract encompassed in Panel a between the two arrows.

Application to DNA Sequencing Procedures

A common application of sequencing procedures is its use with DNA fragments obtained by PCR amplification. Therefore, efficient one-lane chemical sequencing of 5′-end-labeled DNA is an important technique whose use has so far been hindered by the low cleavage efficiency of the 5′ phosphodiester bond. In order to solve this problem, we defined a chemical rationale (see Introduction) and used it to program the testing of nitrogenous bases besides formamide (too weak) and piperidine (requiring a second reaction). The advantageous properties of NmF and the complementary action of Mn^{+2} were identified in this way, thus obtaining efficient cleavage of the phosphodiester bond in correspondence with a selectively degraded nucleic base (G≥A>C>T). In order to increase the selectivity of the cleavage reaction, we have extended these results by complementing them with the incorporation of base analogues (2) in the PCR-produced DNA to be sequenced.

Selectivity of degradation is high between purines and pyrimidines and low within each class. For purines, a way to increase the sensitivity of guanines is to substitute them with inosines (2), and to decrease the sensitivity of adenines is to substitute them with their *deaza* derivatives (13). For pyrimidines, the cytosine signal may be modulated in various ways (data not shown), the most convenient being its increase by 5-bromination. Therefore, quantitative

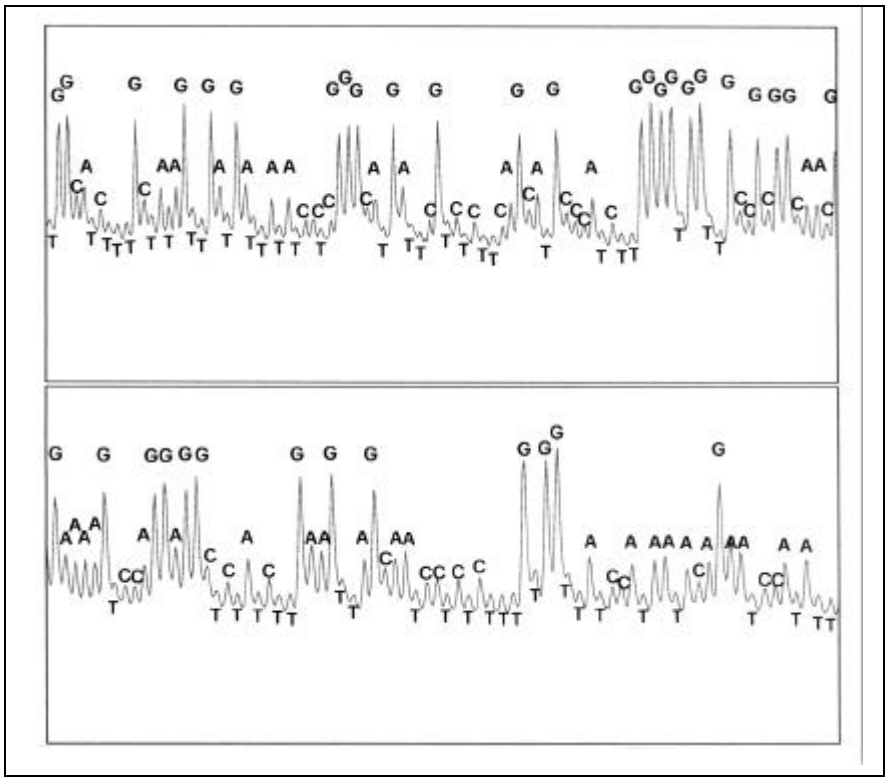

Figure 5. One-lane sequence determination of fluorescently (FAM) 5′-end-labeled mutant human TOP1 gene DNA using NmF + Mn^{+2}. Shown is the raw sequencing profile produced by the analysis program of the Applied Biosystems apparatus (Model 370A). The sequence goes from position 1383 to 1228.

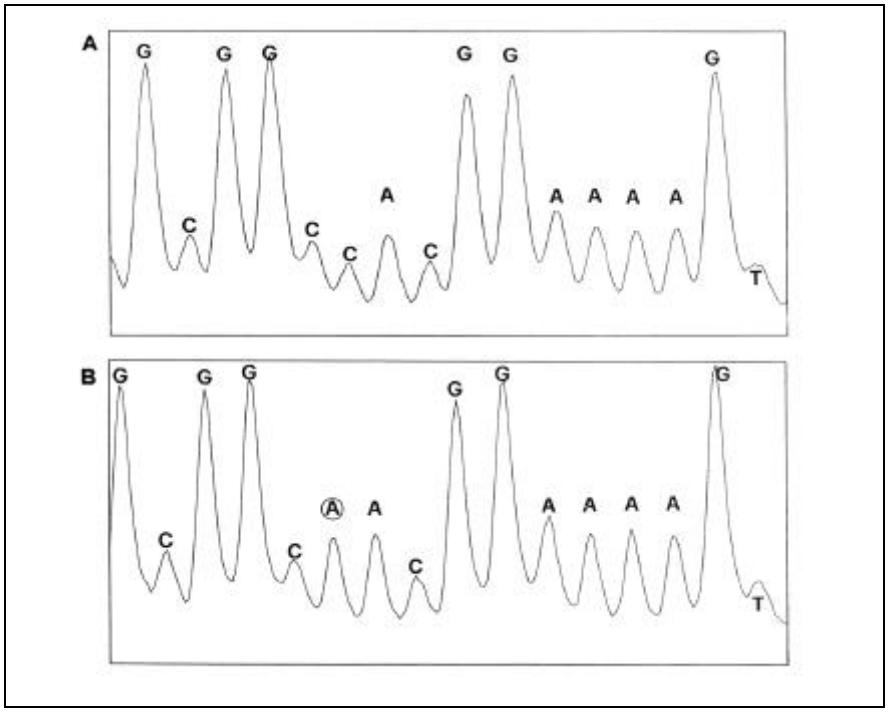

Figure 6. Comparison of human wild-type and mutant TOP1 sequences, as obtained using NmF + Mn^{+2}. Panel A: The 1304 to 1289 portion of the wild-type human TOP1 DNA, PCR-amplified and labeled with HEX. Panel B: The mutant (C→A at position 1299) TOP1 DNA labeled with FAM. The mutated position (verified by Sanger sequencing—data not shown) is circled.

modulation of signals is complete and is sufficient for exploitation in the analysis of heterozygotic sequences (to be detailed elsewhere). These base analogues are not used in the sequencing examples reported below, with the exception of the use of inosine instead of guanosine (see Figure 4).

We provide here two examples of the application of the NmF sequencing procedures on long DNA tracts.

5S-Gene Sequencing by the NmF + Mn^{+2} Protocol

Figure 4 shows the DNA sequence of a PCR-produced, radioactively 5′-end-labeled DNA reacted with NmF + Mn^{+2}. The two gel lanes (Panel a) differ only in the intensity of the radioactive signal: the more intense (left) shows the difference between weak signals (Cs and Ts); the less intense (right) shows the difference between the strong signals (As and Is).

Single-Lane Sequence Analysis of DNA Fragments Labeled with FAM or HEX Fluorochromes

Figure 5 shows a portion of the electrophoretogram (raw data) of the NmF + Mn^{+2} reaction on the PCR-amplified, fluorescently labeled human TOP1 gene, from position 1383 to 1221, carrying a C→A mutation in position 1299. The sequence, as determined by this method, matches that previously determined (8) using the Sanger method. Figure 6 shows that the NmF + Mn^{+2} protocol promptly identifies the mutation.

In our method the sequence is determined by evaluation of the intensities of the bands, which are, in decreasing order, G>A>C>T, as detailed previously (2,5,9,11). When fluorescently labeled DNA is analyzed, such evaluation is provided by the peak heights of the *raw data* densitogram produced by the automated sequencer. In the absence of an appropriate software program to determine the baseline and to call the bases as a function of the peak heights, these operations were performed manually. We found the peak heights to be locally reproducible.

We have so far determined 3870 sequence positions in 18 different sequencing experiments of miniprep plasmid DNA with a rate of uncertainties/errors of 1.55%. These uncertainties occurred mostly at positions where a pyrimidine was next to an inosine residue. In this case, the intense signal of inosine may cause "shoulder" effects consisting of an overestimation of the height of the neighboring pyrimidine peak. This problem is more relevant in the analysis of ^{32}P-labeled DNA densitograms (Figure 4) because of their intrinsic lower resolution and could be solved by decreasing the intensity of the inosine signal (obtained by mixing I with G in the PCR). In our experience the average reading length has been 215 bp per lane.

The fact that complete sequence information is obtained in a single lane potentially allows the determination of *four* different sequences in the same lane. This could be achieved, in principle, by using four different fluorochromes to label four different oligonucleotides and amplifying four different DNA tracts. The practice of using four fluorochromes in the same lane, one for each Sanger reaction, aiming for the determination of *one* sequence per lane, is already in use in commercial apparatuses from various manufacturers.

ACKNOWLEDGMENTS

This work was supported by P.F. Ingegneria Genetica (C.N.R., Italy), by G.P. d'Ateneo (Università di Rome, Italy) and by MURST. We gratefully acknowledge the creative collaboration with Dr. R. Matteoni in adapting the 370A Sequencer to our needs and thank F. Riccobono for providing the fluorescent oligonucleotides.

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Received 27 November 1995; accepted 17 May 1996.

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