ANALYTICAL BIOCHEMISTRY 208, 171–175 (1993)

Solid Phase DNA Minisequencing by an Enzymatic Luminometric Inorganic Pyrophosphate Detection Assay

Pål Nyrén, Bertil Pettersson, and Mathias Uhlén

Department of Biochemistry and Biotechnology, The Royal Institute of Technology, S-100 44 Stockholm, Sweden

Received June 8, 1992

A solid phase DNA sequencing method for nonradioactive detection of single base changes without the need for electrophoresis is presented. The concept relies on the detection of DNA polymerase activity by an enzymatic luminometric inorganic pyrophosphate detection assay (P. Nyrén, 1987, *Anal. Biochem.* 167, 235–238). Immobilized DNA fragments amplified with the polymerase chain reaction are used as template. A detection primer is annealed in front of the mutation and four aliquots of this mixture are incubated with DNA polymerase and one of the four different dideoxynucleotides. The presence or absence of an incorporated dideoxynucleotide is thereafter monitored by the release of inorganic pyrophosphate during the following primer extension step. We show that the concept can be used for sequencing of single bases as well as stepwise analysis of several subsequent bases.

The two most commonly used methods for DNA sequencing are the enzymatic chain-termination method of Sanger *et al.* (1) and the chemical cleavage technique by Maxam and Gilbert (2). Both methods require gel electrophoresis to resolve, according to their size, DNA fragments produced from a larger DNA segment.

Techniques enabling the rapid detection of single DNA base changes are also important tools in genetic analysis. In many cases, detection of a single base or a few bases is of great help since several genetic diseases and certain cancers can be related to minor mutations (3,4). Recently, a minisequencing (analysis of the first base in a DNA fragment) protocol based on a solid phase principle (5) was described (6). The incorporation of a radiolabeled nucleotide was measured and used for analysis of the three-allelic polymorphism of the human apolipoprotein E gene.

The aim of the present work was to develop a simple nonradioactive method for rapid DNA sequence analysis. The sensitivity of the enzymatic method developed for continuous monitoring of the inorganic pyrophosphate (PP_i)[1] released as a result of the specific activity of the DNA polymerase (7) was utilized as a detection system for solid phase DNA minisequencing. The PP_i formed in the DNA polymerase reaction is rapidly converted to ATP by ATP sulfurylase, which is subsequently determined by the luciferase–luciferin assay (8). The reactions occurring in the assay are

$$(DNA)_n + dNTP \xrightarrow{\text{DNA polymerase}} (DNA)_{n+1} + PP_i \ (n = \text{number of residues}) \quad [1]$$

$$PP_i + \text{adenosine 5'-phosphosulfate} \xrightarrow{\text{ATP sulfurylase}} ATP + SO_4^{2-} \quad [2]$$

$$ATP + \text{luciferin} + O_2 \xrightarrow{\text{Luciferase}} AMP + PP_i + \text{oxyluciferin} + CO_2 + h\nu. \quad [3]$$

The light emission is essentially time independent (decay rate <1%/min) and proportional to the ATP concentration over the range 10 pM to 1 μM.

A sequencing method based on this pyrophosphate detection has been theoretically outlined by Hyman (9). Here, we show that the enzymatic luminometric inorganic pyrophosphate detection assay (ELIDA) can be used for minisequencing using DNA bound to paramagnetic beads as a template. To increase the sensitivity of the assay, a dideoxynucleotide approach was used. This means that instead of measuring a single pyrophos-

[1] Abbreviations used: PP_i, inorganic pyrophosphate; ELIDA, enzymatic luminometric inorganic pyrophosphate detection assay; HIV, human immunodeficiency virus; FPLC, fast protein liquid chromatography; PCR, polymerase chain reaction; DTT, dithiothreitol; BSA, bovine serum albumin;

0003-2697/93 $5.00

phate release per template the release of many pyrophosphate molecules per template is detected.

MATERIALS AND METHODS

Synthesis and purification of nucleotides. The oligonucleotides RIT331 (5'CCCGAATTCGATGGAGTTCATAACCCATCCAAAG), RIT332 (5'CATCTGTTGAAGTGGGGACTT), and RIT333 (5'CCCGGATCCATACAATACTCCAGTATTTGC) which are complementary to a region in the active site of the HIV reverse transcriptase gene [encoding bases 625 to 1165; see Ref. (10)] were synthesized by phosphoramidite chemistry in an automated DNA synthesis apparatus (Gene Assembler Plus, KABI Pharmacia AB, Sweden). Purification was performed with a fast-protein liquid chromatography (FPLC) pepRPC 5/5 column (KABI Pharmacia AB, Sweden). PCR primers for amplification of cloned material were obtained from the Primer Set A solution from the Template Preparation Kit for DNA Sequencing (Dynal AS, Norway).

Template preparation. A HIV reverse transcriptase gene fragment from a patient showing zidovudine resistance was PCR-cloned into the vector pRIT28 (11) by using the primers RIT331 and RIT333. The *Escherichia coli* strain RR1ΔM15 (12) was used and blue/white selection was thus possible. PCR amplification was carried out by lysing a bacterial colony in 10 μl 20 mM Tris–HCl (pH 8.7) at 99°C for 5 min. Then 1 μl of the lysate was added to 5 pmol Primer set A, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 20 mM Tris–HCl (pH 8.7), 2 mM $MgCl_2$, 0.1% Tween 20, and 0.5 units Ampli Taq DNA polymerase (Cetus, CA) making up a total volume of 50 μl. The temperature profile included a 0.5-min denaturation step at 95°C and a 1.5-min annealing/extension step at 70°C; these steps were repeated 30 times. A GeneAmp PCR System 9600 (Perkin–Elmer, Palo Alto, CA) was used for both lysing the bacterial colony and running the reactions. The PCR product was immobilized (5) on paramagnetic beads (13) with covalently coupled streptavidin, Dynabeads M280. The beads were used as described by the manufacturer (Dynal AS, Norway). Single-stranded DNA was obtained by removing the supernatant after incubation of the immobilized PCR product in 0.10 M NaOH for 5 min. The immobilized single-stranded DNA was washed once with 20 μl 0.10 M NaOH and once with 50 μl 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 2 M NaCl, followed by 50 μl 10 mM Tris–HCl (pH 7.5). After washing, 20 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, and 5 pmol sequencing primer was added to a final volume of 13 μl. The mixture was incubated at 65°C for 5 min and then cooled to room temperature.

Minisequencing. The dideoxynucleotide incorporation reactions were performed in a mixture of 1 μl ($\frac{1}{13}$ of a 50-μl PCR amplification reaction) of the template/primer fragment immobilized on paramagnetic beads, 0.13 units Sequenase version 2.0 (U.S. Biochemical, Cleveland, OH), 5 pmol of a single ddNTP, and a buffer containing 25 mM Tris–HCl (pH 7.5), 12.5 mM $MgCl_2$, and 2.5 mM DTT in a final volume of 10 μl. After incubation at room temperature for 5 min, the beads were washed with 50 μl 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 2 M NaCl, 1% Tween 20 followed by 50 μl 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 2 M NaCl, and finally with 50 μl 10 mM Tris–HCl (pH 7.5). The volume was adjusted to 5 μl with 10 mM Tris–HCl (pH 7.5). Control fragments were incubated with DNA polymerase in the absence of ddNTPs and zero control fragments in the presence of all ddNTPs. The different samples were subsequently analyzed with the ELIDA.

Enzymatic luminometric inorganic pyrophosphate detection assay. Samples from the minisequencing preincubation described above were assayed for full primer extension with the ELIDA. The assay was performed by using an LKB 1250 luminometer and a potentiometric recorder. The luminometer was calibrated to give a response of 10 mV for the internal light standard. The luminescence output was calibrated by the addition of a known amount of ATP or PP_i. The reaction was carried out at room temperature. The standard assay volume was 0.2 ml and contained the following components: 0.1 M Tris–acetate (pH 7.75), 2 mM EDTA, 10 mM magnesium acetate, 0.1% BSA, 1 mM DTT, 0.4 mg/ml polyvinylpyrrolidone (360 000), 2 μM of each dNTP, 100 μg/ml D-luciferin (BioOrbit, Finland), 4 μg/ml L-luciferin (BioOrbit, Finland), 0.3 units/ml ATP sulfurylase (Sigma, USA) and purified luciferase (Enzymatix, UK). The amount of luciferase used gave a response of 1 V for 100 pmol ATP in a volume of 1 ml. After 5 min of preincubation, adenosine 5'-phosphosulfate, NaF, and dNMP (all four deoxynucleoside monophosphates) were added to final concentrations of 2 μM, 5 mM, and 0.4 mM, respectively. The reaction was started after the addition of 5 μl of template/primer fragments (from the incorporation of dideoxynucleotides), by the addition of 0.13 units of Sequenase. The reaction was completed within 5 min.

Limited primer extension. It is possible to make limited primer extension of the initial template/primer fragment in the presence of the deduced complementary dNTP or mixtures of two or three different dNTPs. Eight microliters of the template/primer fragment ($\frac{8}{13}$ of a 50 μl PCR amplification reaction) was preincubated for 5 min with 10 pmol of the deduced complementary dNTP or a mixture of dNTPs, 0.26 units Sequenase, and 10 μl of a buffer containing 25 mM Tris–HCl (pH 7.5), 12.5 mM $MgCl_2$, and 2.5 mM DTT in a final volume of 20 μl. The beads were then washed as described above. The volume was finally adjusted to 15 μl with 10 mM Tris–HCl (pH 7.5). The new template/primer frag-

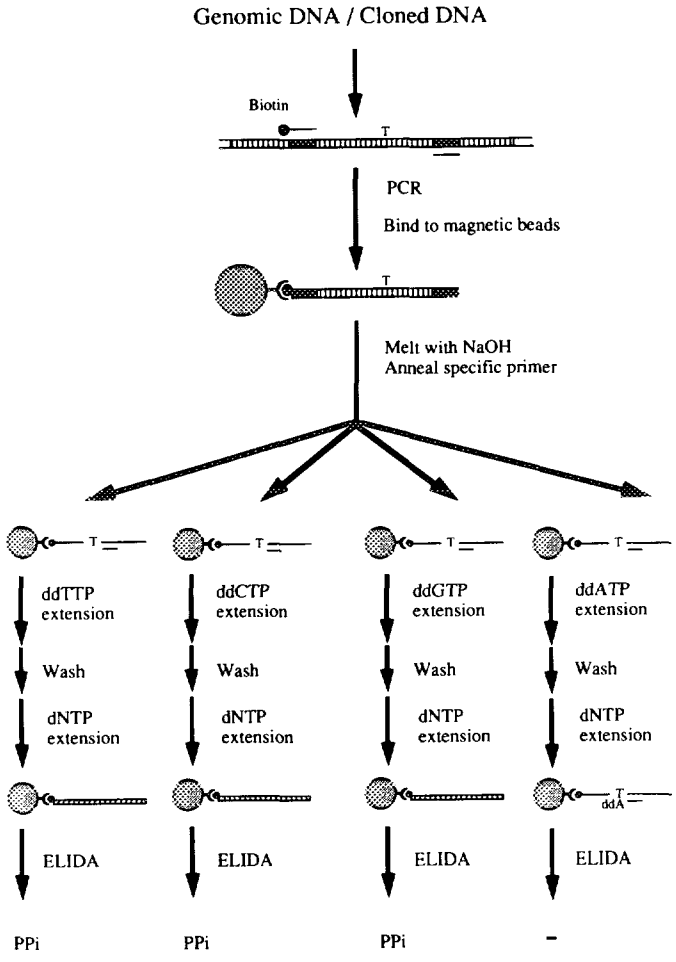

FIG. 1. A graphic representation of the basic concept of DNA minisequencing with the ELIDA. See the text for further details.

ments obtained after the limited primer extension reaction were then used for a second cycle of minisequencing.

RESULTS

Principle of the Minisequencing Method

The principle of the minisequencing method is outlined in Fig. 1. As an example, the presence or absence of a T residue in a specific position is investigated. The DNA fragment of interest is amplified with PCR with one of the primers biotinylated in the 5′ end. The PCR-amplified DNA is immobilized onto a solid support with covalently coupled streptavidin and subsequently converted into single-stranded form by treatment with NaOH. A primer is annealed to the single-stranded DNA in front of the base of interest. The template/primer fragments are then divided into four aliquots, which are separately treated with one of the four ddNTPs in the presence of the DNA polymerase. After the reaction, the resulting fragments are washed and used as substrate in a primer extension reaction (dNTP reaction) with all four dNTPs present (Fig. 1). The progress of the DNA-directed polymerization reactions are monitored with the ELIDA. Incorporation of a dideoxynucleotide in the first reaction will prevent the formation of pyrophosphate during the subsequent dNTP reaction. In contrast, no dideoxynucleotide incorporation in the first reaction gives extensive pyrophosphate release during the dNTP reaction and this will lead to generation of light through the ELIDA reactions. From the ELIDA results, the first base after the primer is easily deduced (Fig. 2). It is also possible to include both a negative control, which is preincubated with all ddNTPs before the dNTP reaction, and a positive control, which is preincubated with DNA polymerase in the absence of all four ddNTPs.

Minisequencing of a Specific DNA Fragment

As a model, a short stretch of the HIV-1 *pol* gene (the structure of the target gene after the RIT332 annealing site: 5′ATGTTTTTTGTCTGGTGTGGT), containing a specific mutation which correlates to increased resistance to zidovudine (14), was chosen. Figure 2 shows a schematic illustration of typical traces from ELIDA of the HIV-1 *pol* fragment with the annealed primer RIT332. Incorporation of a single ddNTP was observed only when the complementary dideoxynucleotide (ddATP) was present during the first reaction in the presence of DNA polymerase. PP_i formation detected by the ELIDA during the dNTP reaction was only ob-

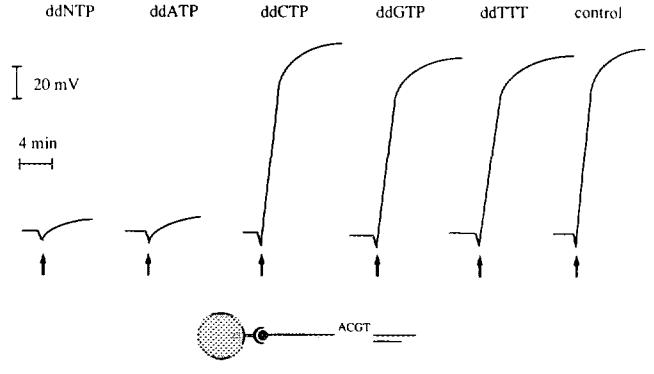

FIG. 2. A schematic representation of typical traces from ELIDA DNA minisequencing. A short stretch of the HIV-1 *pol* gene with the annealed primer RIT332 was chosen as the target DNA. The DNA primer/template fragments were divided into six different samples, which were preincubated for 5 min with the indicated dideoxynucleotides. After washing, primer extension reactions were performed in the presence of all four deoxynucleotides. The reactions were started by the addition of DNA polymerase (arrows). The PP_i formed was continuously monitored with the ELIDA (traces). Twenty millivolts corresponds to about 4 pmol PP_i. The experimental conditions were as described under Materials and Methods.

TABLE 1

Results from DNA Minisequencing of DNA Primer/Template Fragments Obtained before and after Limited Primer Extension Reactions

Nucleotides present during the limited primer extension	Amounts of PPi formed (pmol) during the primer extension of the specified fragments after treatment with the indicated ddNTP					
	None	All ddNTPs	ddATP	ddCTP	ddGTP	ddTTP
None	26.9	0.3	1.8	26.9	27.6	26.3
dATP	24.9	0.2	23.8	2.9	21.8	23.1
dATP + dCTP	18.0	0.8	15.5	19.0	2.9	19.0
dATP + dCTP + dGTP	20.4	0.1	19.0	19.1	19.3	1.3

Note. Experimental conditions were as described in the legend to Fig. 2 and under Materials and Methods. See the text for further details.

served when template/primer fragments were incubated with noncomplementary bases (ddCTP, ddGTP, and ddTTP, respectively) in the preceding reaction. It is important to note that a DNA polymerase with low or no exonuclease activity, such as T7 polymerase, must be used to obtain low background signals (not shown). It is also important to use low concentrations of nucleotides (0.05–5 μM) to avoid incorporation of noncomplementary bases (data not shown).

Stepwise Monitoring of Several Bases

After one minisequencing cycle it is possible to make limited primer extension of the initial template/primer fragment in the presence of the deduced complementary deoxynucleotide or in the presence of mixtures of two or three different deoxynucleotides.

After the first base was deduced in the model system presented above and in Table 1 (first row), a limited incorporation of the complementary base (dATP) was performed on the initial template/primer fragment (as the DNA is bound to paramagnetic beads it is possible to reuse the fragments from the first mini-sequencing round if a new primer is annealed to the fragment after NaOH treatment). After washing, the template/primer fragment was used for another minisequencing step. As shown in Table 1 (second row), the next base was deduced to be a G. It is noteworthy that it is not possible to distinguish between the existence of one or several identical bases in each step.

A limited primer extension reaction could also be performed in the presence of mixtures of two or three different deoxynucleotides. The third row of Table 1 shows an additional minisequencing step of the template/primer fragment obtained after a limited primer extension of the initial template/primer fragment in the presence of both dATP and dCTP. The next base in the sequence, after the extension, was determined to be C. In the last row, a limited polymerization on the initial template/primer fragment in the presence of dATP, dCTP, and dGTP was performed. Now 20 bases (T:s, G:s, and C:s) have been passed before the enzyme stopped in front of an A, which shows the accuracy of the polymerase. Thus, three minisequencing steps were performed, after three different types of limited primer extension reactions, with low background signal in each step (see Table 1).

Sensitivity

In the experiments presented above $\frac{1}{13}$ of a normal (2–3 pmol DNA as determined by agarose gel electrophoresis) 50 μl PCR amplification reaction was used per ELIDA test. However, both lower and higher amounts can be used. In Fig. 3, the initial rate and the extent of PP_i formation during primer extension of a 161-base-long DNA fragment as a function of DNA concentration is shown. Both the initial rate and the extent of PP_i formed in the ELIDA are proportional to the DNA con-

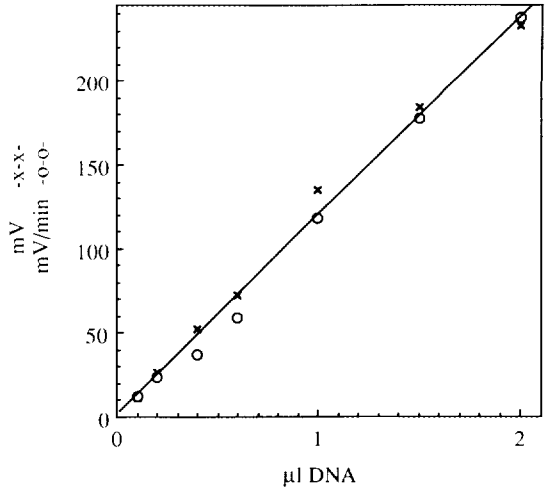

FIG. 3. The extent and the initial rate of PP_i synthesis during a primer extension of a 161-base-long single-stranded DNA fragment as a function of the DNA concentration. One microliter DNA corresponds to $\frac{1}{13}$ of a normal PCR amplification reaction. The experimental conditions were as described under Materials and Methods.

centration in the interval tested ($\frac{1}{130}$ to $\frac{2}{13}$ of a 50-μl PCR amplification reaction). The amount of DNA could be further increased as well as the binding capacity of the solid support to increase the signal of the assay. The upper limit for the assay (in a total volume of 200 μl) is 200 pmol PP_i formed. The lower limit is mainly determined by the length of the DNA fragment used (since the signal is proportional to the amount of nucleotides incorporated during the primer extension reaction), by the volume used, and by the contamination of PP_i in the different solutions.

DISCUSSION

A simple and rapid method for detection of single base changes has been developed. In the new approach we have combined two techniques: solid phase technology (DNA bound to paramagnetic beads) and the ELIDA. The method can be used for detection of point mutations responsible for both acquired and inherited diseases, identify DNA polymorphisms, and differentiate between drug-resistant and drug-sensitive strains of viruses or bacteria. The method avoids the need for centrifugations, filtrations, extractions, or electrophoresis. The assay is thus suitable for large-scale applications where many samples are screened for known nucleotide variations. No radioactivity is used and obviously any position of the amplified fragment can be sequenced provided that a suitable sequencing primer is synthesized. Alternatively, it might be possible to "walk" several nucleotides away from the primer position using stepwise additions of defined nucleotides to reach a new nucleotide position used for the ELIDA analysis (Table 1).

The DNA template bound to the solid support can be used for several subsequent minisequencing steps. After an analysis, the primer extended strand can be eluted simply by 0.15 M alkali and the amplified fragment is thus converted back to single-stranded form. A new primer can then be annealed to a new site for analysis.

The sensitivity of the assay makes it necessary to detect a dNTP extension reaction in which many pyrophosphate molecules are released per template. The question arises whether it might be possible to increase the sensitivity of the luminometric assay to allow detection of a single pyrophosphate release per template. In this case, a stepwise sequencing protocol can be envisioned. It remains to be seen if such an assay could be used for on-line sequencing of unknown DNA sequences. The fact that casting and loading of electrophoresis gels would be avoided might make such a procedure useful for large scale sequencing efforts, such as the human genome project.

In conclusion, a new minisequencing protocol suitable for large scale analytical applications has been developed. Automated on-line methods with multiple samples in parallel can be envisioned. The assay has been designed for minisequencing of a specific base, but stepwise monitoring of several bases is possible.

ACKNOWLEDGMENTS

The authors thank Thomas Hultman, Johan Wahlberg, Dr. Åke Strid, and Dr. Stefan Ståhl for helpful discussions and critical comments. This work was supported by grants from Magnus Bergvalls Stiftelse, Carl Tryggers Stiftelse för Vetenskaplig Forskning, Åke Wibergs Stiftelse, Stiftelsen Lars Hiertas Minne, Procordias forskningsstiftelse, Riksbankens Jubileumsfond, Roos' fond, and the Swedish Board for Technical Development.

REFERENCES

1. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
2. Maxam, A. M., and Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 560–564.
3. Antonarakis, S. E. (1989) *N. Engl. J. Med.* **320,** 153–163.
4. Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F. S., Weston, A., Modali, R., Harris, C. C., and Vogelstein, B. (1989) *Nature* **342,** 705–708.
5. Hultman, T., Ståhl, S., Moks, T., and Uhlén, M. (1988) *Nucleic Acids Res.* **17,** 4937–4946.
6. Syvänen, A-C., Aalto-Setälä, K., Harju, Leena, Kontula, K., and Söderlund, H. (1990) *Genomics* **8,** 684–692.
7. Nyrén, P. (1987) *Anal. Biochem.* **167,** 235–238.
8. Nyrén, P., and Lundin, A. (1985) *Anal. Biochem.* **151,** 504–509.
9. Hyman, E. D. (1988) *Anal. Biochem.* **174,** 423–436.
10. Myers, G., Korber, B., Berkofsky, J. A., Smith, R. F., and Pavlakis, G. N. (1991) Human Retroviruses and AIDS 1991, Los Alamos National Laboratory, Los Alamos, NM.
11. Hultman, T., Ståhl, S., Moks, T., and Uhlén, M. (1988) Nucleosides and Nucleotides **7,** 629–638.
12. Rüther, U. (1982) *Nucleic Acids Res.* **10,** 5765–5772.
13. Lea, T., Vartdal, F., Nustad, K., Funderud, S., Berge, A., Ellingsen, T., Schmid, R., Stenstad, P., and Ugelstad, J. (1988) *J. Mol. Recognit.* **1,** 9–18.
14. Larder, B. A., and Kemp, S. D. (1989) *Science* **246,** 1155–1158.