REFERENCES

1. **Abbott, G.W., F. Sesti, I. Splawski, M.E. Buck, M.H. Lehmann, K.W. Timothy, M.T. Keating, and S.A.N. Goldstein.** 1999. MiRP1 forms I_{Kr} potassium channels with HERG and is associated with cardiac arrhythmia. Cell *97*:175-187.
2. **Curran, M.E., I. Splawski, K.W. Timothy, G.M. Vincent, E.D. Green, and M.T. Keating.** 1995. A molecular basis for cardiac arrhythmia: HERG mutations cause Long QT Syndrome. Cell *80*:795-803.
3. **Goldin, A.L. and K. Sumikawa.** 1992. Preparation of RNA for injection into Xenopus oocytes. Methods Enzymol. *207*:279-297.
4. **Grunnet, M., B.S. Jensen, S.P. Olesen, and D.A. Klaerke.** 2001. Apamin interacts with all subtypes of cloned small-conductance Ca^{2+}-activated K^{+} channels. Pflugers Arch. *441*:544-550.
5. **Gurdon, J.B.** 1974. The Control of Gene Expression in Animal Development. Oxford University Press, Oxford.
6. **Kozak, M.** 1987. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. *15*:8125-8148.
7. **Krieg, P.A. and D.A. Melton.** 1984. Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. Nucleic Acids Res. *12*:7057-7070.
8. **Lane, C.D.** 1981. The fate of foreign proteins introduced into *Xenopus* oocytes. Cell *24*:281-282.
9. **Shih, T.M., R.D. Smith, L. Toro, and A.L. Goldin.** 1998. High-level expression and detection of ion channels in Xenopus oocytes. Methods Enzymol. *293*:529-556.
10. **Soreq, H.** 1985. The biosynthesis of biologically active proteins in mRNA microinjected *Xenopus* oocytes. Crit. Rev. Biochem. *18*:199-238.
11. **Stefani, E., L. Toro, E. Perozo, and F. Bezanilla.** 1994. Gating of Shaker K+ channels: I. Ionic and gating currents. Biophys. J. *66*:996-1010.
12. **Tinel, N., S. Diochot, M. Borsotto, M. Lazdunski, and J. Barhanin.** 2000. KCNE2 confers background current characteristics to the cardiac KCNQ1 potassium channel. EMBO J. *19*:6326-6330.
13. **Wallner, M., P. Meera, M. Ottolia, G.J. Kaczorowski, R. Latorre, M.L. Garcia, E. Stefani, and L. Toro.** 1995. Characterization of and modulation by a β-subunit of a human maxi K_{Ca} channel cloned from myometrium. Receptors Channels *3*:185-199.

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Simultaneous Cycle Sequencing Assessment of $(TG)_m$ and T_n Tract Length in CFTR Gene

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ABSTRACT

The lengths of the dinucleotide $(TG)_m$ and mononucleotide T_n repeats, both located at the intron 8/exon 9 splice acceptor site of the cystic fibrosis transmembrane conductance regulator (CFTR) gene whose mutations cause cystic fibrosis (CF), have been shown to influence the skipping of exon 9 in CFTR mRNA. This exon 9-skipped mRNA encodes a nonfunctional protein and is associated with various clinical manifestations in CF. As a result of growing interest in these repeats, several assessment methods have been developed, most of which are, however, cumbersome, multi-step, and time consuming. Here, we describe a rapid method for the simultaneous assessment of the lengths of both $(TG)_m$ and T_n repeats, based on a nonradioactive cycle sequencing procedure that can be performed even without DNA extraction. This method determines the lengths of the $(TG)_m$ and T_n tracts of both alleles, which in our samples ranged from TG_8 to TG_{12} in the presence of T_5, T_7, and T_9 alleles, and also fully assesses the aplotypes. In addition, the repeats in the majority of these samples can be assessed by single-strand sequencing, with no need to sequence the other strand, thereby saving a considerable amount of time and effort.

INTRODUCTION

Cystic fibrosis (CF), the most common severe autosomal recessive disease in the Caucasian population, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (1,21). Since the genomic DNA sequence of the CFTR gene, with its exon-intron boundaries, was completed by Zielenski et al. (26), the existence of some polymorphic sequences has been detected. Two of these are the dinucleotide $(TG)_m$ repeats and the adjacent polythymidine IVS8-T tract (T_n), both located at the end of intron 8, at the exon 9 splice acceptor site, with the $(TG)_m$ that precedes the T_n. Chu and co-workers (5,6) demonstrated that the shorter the T_n tract, the higher the proportion of CFTR mRNA without exon 9 in the respiratory epithelium, and that the shortest T_5 allele is the main factor causing exon 9 skipping. Subsequent studies demonstrated that, in addition to the effect of the T_n tract, exon 9 skipping is also influenced by the $(TG)_m$ repeat, which thus can explain the partial penetrance of the T_5 allele as a disease mutation (8,19). In particular, the longer the $(TG)_m$ is, the higher the proportion of exon 9 defective CFTR mRNA. This mRNA encodes a nonfunctional protein (5,23) and is associated with variable clinical manifestations in CF (2,4,7,8,12). Growing interest in the involvement of these repeats in the production of variable CF phenotypes has led to the development of several assessment methods, most of which are, however, cumbersome, multi-step, and time consuming.

Before the increased interest in the $(TG)_m$, several methods that were specific for the analysis of the T_n tract alone had been developed. They were usually based on a preliminary PCR amplification of exon 9 and its intronic boundaries and subsequent evaluation of the PCR product (see Discussion section). In addition to these widely used methods, a number of less common methods for the characterization

of the T_n tract, based on radioactive single-strand conformation polymorphism (SSCP) analysis (16), capillary zone electrophoresis (13), or primer oligonucleotide base extension and mass spectrometry (3), have also been described. Some multi-step, sequencing-based methods that can potentially analyze both the $(TG)_m$ and T_n sequences have also been proposed, though it is only more recently that some authors have focused on the simultaneous characterization of both repeats (see Discussion section).

The present work describes a rapid method for the simultaneous assessment of the length of both $(TG)_m$ and T_n repeats, based on a nonradioactive cycle sequencing procedure. This method can be performed without DNA extraction, starting directly from blood or semen samples; the latter possibility may be of particular interest given the growing body of evidence suggesting a correlation between CFTR and reproductive disorders. This method allows the resolution of the sequences of interest with the determination of the length of the $(TG)_m$ and T_n tracts of each allele, as well as the aplotype characterization. In most samples, these repeats can be assessed by single-strand sequencing, with no need to sequence the other strand, thereby saving a considerable amount of time and effort.

MATERIALS AND METHODS

We performed the analysis on blood or semen samples, both of which were subjected to DNA extraction or direct boiling. PCR amplification was achieved using three different *Taq* DNA polymerases, namely AmpliTaq Gold® (Applied Biosystems, Foster City, CA, USA), Super Taq (HT Biotechnology Ltd., Cambridge, UK), and *Taq* DNA polymerase (Stratagene GmbH, Heidelburg, Germany), which showed comparable performances.

Sample Collection

Blood samples were collected as 2-mL aliquots in the presence of 35 µL EDTA (from a stock solution at a 100 mg/mL concentration). The spermatozoa concentration in the semen samples was assessed by the superimposed imaging analysis system (SIAS) (17,18). The semen was washed twice in Tyrode's solution by centrifugation at 600× *g* for 10 min; spermatozoa were pelletted and resuspended in Tyrode's solution at a concentration of 20 million/mL. Both types of samples were freshly processed or frozen at -20°C until used.

Sample Preparation by DNA Extraction

The DNA extraction was performed on 2 mL of either a blood sample or resuspended spermatozoa (as described above) using the QIAamp® DNA Blood Midi Kit (Qiagen S.p.A., Milan, Italy) according to the manufacturer's instructions. For semen processing, the quantity of Qiagen protease (stock solution 20 mg/mL) was raised from 200 µL (the quantity used for blood) to 400 µL. The subsequent amplification was performed on 50 ng DNA obtained from blood or semen.

Sample Preparation by Boiling

Peripheral blood (3 µL) or 10 µL resuspended spermatozoa (as described above) were added respectively to 34.5 or 38 µL of the buffer for EDTA Blood manufactured by Applied Biosystems (Cystic Fibrosis Sample Preparation Module). After treatment at 97°C for 40 min, each sample was centrifuged at 16 000× *g* for 10 min. The subsequent amplification was performed on 15 µL supernatant obtained from blood or semen.

Amplification Protocols

The following primers were used for the amplification of CFTR exon 9 and its intronic boundaries (GenBank® accession no. M57509): CF9P1 5′-TGAAAATATCTGACAAACTC-3′ (nucleotides 350–369; the CF9 primer of Reference 11) as the forward primer, and CF9M1 5′-CCTTCCAGCACTACAAACTA-3′ (nucleotides 665–684; the GCCF9 primer of Reference 11, without the GC tail) as the reverse primer. PCR was performed in a total volume of 50 µL containing 20 pmol each primer, 175 µM dNTPs, and the

sample volume described above. After an initial 5-min denaturation at 95°C, the appropriate number of cycles (indicated below for each protocol) of 1 min at 95°C, 1 min at 55°C, and 2 min at 68°C, followed by a final extension of 10 min at 68°C, were performed using a GeneAmp® PCR System 9600 (Perkin Elmer, Gaithersburg, MD, USA).

The following DNA polymerase-dependent protocols were used: (*i*) for Super Taq, 35 cycles for the DNA samples and 40 cycles for the boiled samples, in the presence of 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton® X-100, 0.01% (w/v) gelatin, and 1 U Super Taq DNA polymerase; (*ii*) for AmpliTaq Gold, 40 cycles for the DNA samples and 45 cycles for the boiled samples, in the presence of 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, and 1 U AmpliTaq Gold DNA polymerase; and (*iii*) for Stratagene *Taq* DNA polymerase, 35 cycles for the DNA samples and 40 cycles for the boiled samples, in the presence of 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, and 1 U *Taq* DNA polymerase.

Aliquots (5 µL) of the PCR products were examined by electrophoresis in 1.5% agarose gels. Each of these protocols yielded a fragment of about 335 bp.

Sequencing Protocols

PCR products were purified by Centricon-100™ columns (Applied Biosystems). For the subsequent sequencing step, 25 ng PCR-amplified DNA, as measured by gel electrophoresis and comparison with a known amount of molecular weight marker, were used in a final total volume of 20 µL. Two sequencing procedures were used, with comparable results, both based on a dichlororhodamine cycle sequencing method (either dRhodamine Terminator Cycle Sequencing Kit or BigDye® Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosytems), with dye-labeled ddNTPs as terminators and with AmpliTaq DNA polymerase FS, according to the manufacturer's instructions. The sequencing primers used were the CF9P1 for forward sequencing, and either the CF9M1 (also used for PCR amplification) or the internal IVS8TM1 primer 5′-GTTTTGTTTTGCTTTCTC-3′ (nucleotides 439–456; the IVS8TR primer of Reference 25) for reverse sequencing. Twenty-five cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C were performed. After the sequencing step was completed, the excess dye terminators were removed by Centri-Sep™ spin columns (Princeton Separations, Adelphia, NJ, USA) according to the manufacturer's instructions.

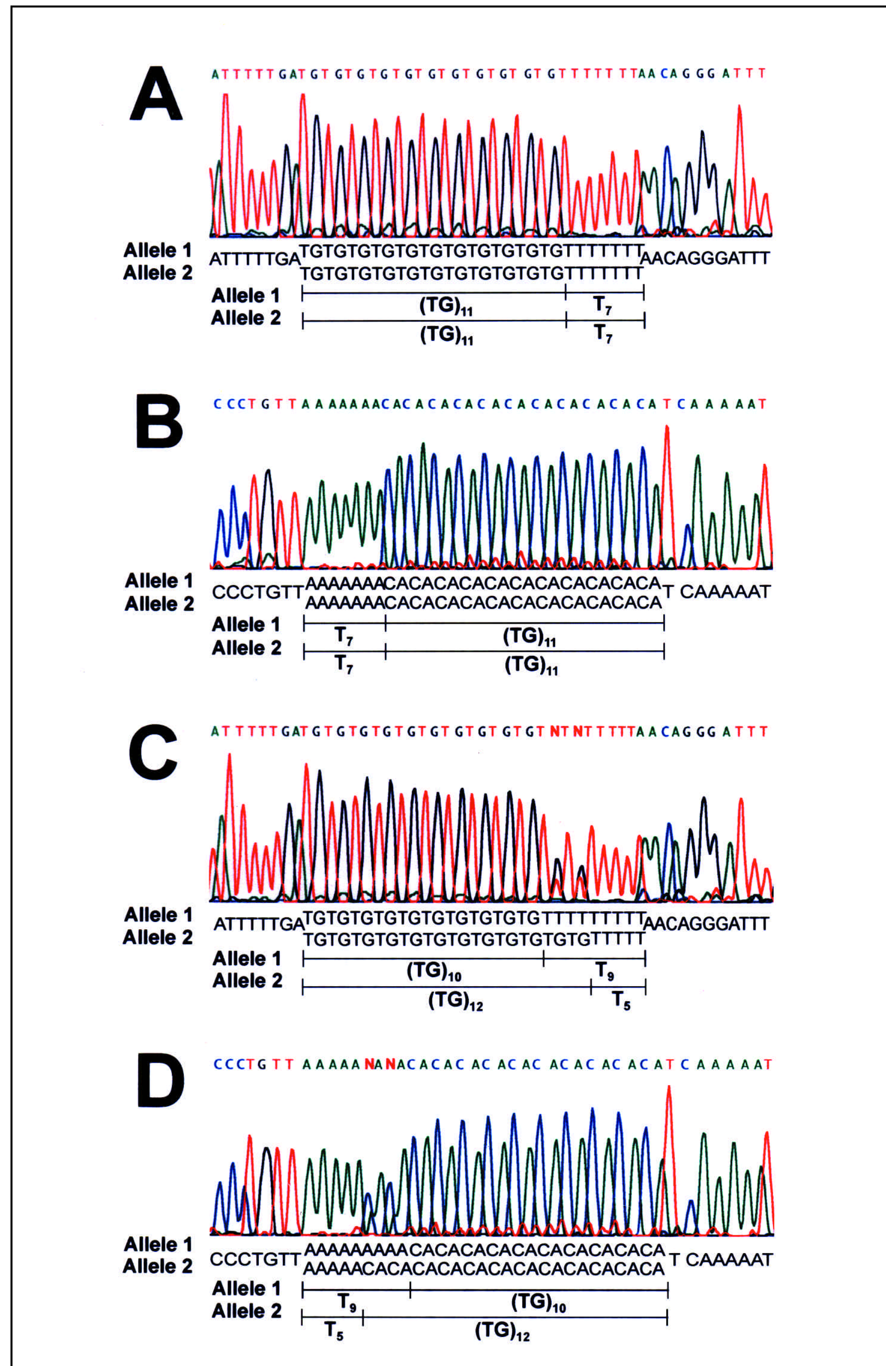

Figure 1. Examples of electropherograms of genotypes leading to in-frame sequences. (A and B) In-frame sequences both inside and outside to the $(TG)_m$ - T_n zone: $(TG)_{11}T_7/(TG)_{11}T_7$ genotype. (C and D) Sequences that return to being in-frame only downstream of the $(TG)_m$ - T_n zone: $(TG)_{10}T_9/(TG)_{12}/T_5$ genotype. In parts A and C, a forward primer (upper strand sequence) was used, while in parts B and D, a reverse primer (lower strand sequence) was used.

Capillary Electrophoresis

After using the BigDye method, 5 µL of the 20 µL of the sequencing reaction mixture were added to 20 µL template suppression reagent (TSR; Applied Biosystems). After using the dRhodamine method, the entire volume of the sequencing reaction mixture was dried in a vacuum centrifuge, and the pellet was resuspended in 12 µL TSR. A denaturation step at 95°C for 2 min was performed in both cases. Denatured samples were subjected to capillary electrophoresis and spectrofluorimetric analysis by the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Method Validation

The T_n genotypes were confirmed by a commercial method (InnoLipa™; Innogenetics, Ghent, Belgium) that uses a reverse hybridization principle (24). The $(TG)_mT_n$ aplotypes were confirmed by cloning (TA Cloning®; Invitrogen, Leek, The Netherlands) and sequencing (methods described above) of several individual plasmids, with each plasmid, obtained from the same PCR products subjected to direct sequencing and representative of only one allele.

RESULTS

Four representative sequences were chosen as examples. The results shown in Figure 1, A and B, were obtained for a $(TG)_m$ and T_n homozygous subject [genotype: $(TG)_{11}T_7/(TG)_{11}T_7$]. The sequences of the two alleles in this sample are identical: the simplest electrophoretic, so-called in-frame, patterns are shown by both forward (Figure 1A) and reverse (Figure 1B) primers. The results shown in Figure 1, C and D, were obtained for a subject heterozygous for both the $(TG)_m$ and the T_n [genotype: $(TG)_{10}T_9/(TG)_{12}T_5$]. In this case, the sequences of the two alleles are different, and some double peaks in the electrophoretic patterns, which are limited to the $(TG)_m$ - T_n zone and reveal a limited out-of-frame zone in the sequence, are shown. However, owing to the identical overall length of the $(TG)_m + T_n$ tract in both alleles [first allele: $(TG)_{10}$ = 20 bases + T_9 = 9 bases, amounting to a total of 29 bases; second allele: $(TG)_{12}$ = 24 bases + T_5 = 5 bases, amounting to a total of 29 bases], after the out-of-frame zone, which is internal to the $(TG)_m$ - T_n segment, the sequence returns to being in-frame, as shown by both the forward (Figure 1C) and reverse (Figure 1D) primers. The results shown in Figure 2 refer to more complex cases, in which

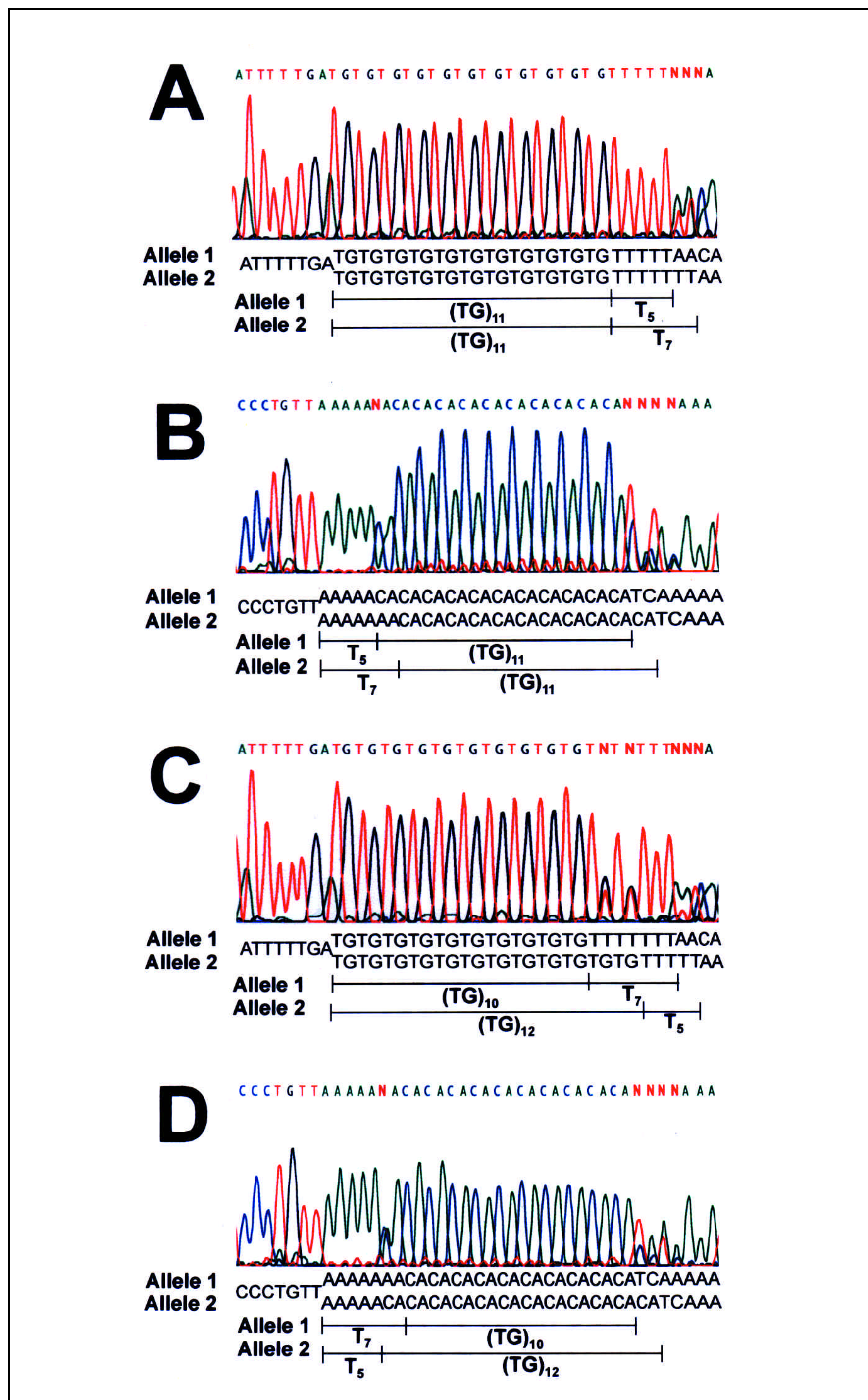

Figure 2. Examples of electropherograms of genotypes leading to out-of-frame sequences. (A and B) Sequences partially out-of-frame: $(TG)_{11}T_5/(TG)_{11}T_7$ genotype. (C and D) Sequences completely out-of-frame: $(TG)_{10}T_7/(TG)_{12}T_5$. In parts A and C, a forward primer (upper strand sequence) was used, while in parts B and D, a reverse primer (lower strand sequence) was used.

the sequences on the two alleles show differences that generate out-of-frame electrophoretic patterns even outside the $(TG)_m$ - T_n zone. The first case (Figure 2, A and B) is that of a subject with a homozygous $(TG)_m$ tract but a heterozygous T_n tract [genotype: $(TG)_{11}T_5$/ $(TG)_{11}T_7$], which generate, both by forward and reverse sequencing, a first in-frame segment followed by a second out-of-frame segment extending beyond from the $(TG)_m$ - T_n zone. The results shown in Figure 2, C and D, were obtained for a $(TG)_m$ heterozygous and T_n heterozygous subject [genotype: $(TG)_{10}T_7/(TG)_{12}T_5$]. In this sample, the repeats of the two alleles are different for both the $(TG)_m$ and the T_n tracts; the sequencing generates complex electrophoretic out-of-frame patterns, both by the forward (Figure 2C) and reverse (Figure 2D) primers, as well as inside and outside the $(TG)_m$ - T_n zone.

A total of 123 subjects (all from Central Italy) were analyzed. The method proposed in this study allows the complete characterization of all the $(TG)_m$ and T_n length combinations found: $(TG)_8T_7$, $(TG)_9T_9$, $(TG)_{10}T_7$, $(TG)_{10}T_9$, $(TG)_{11}T_5$, $(TG)_{11}T_7$, $(TG)_{11}T_9$, $(TG)_{12}T_5$, and $(TG)_{12}T_7$. The method proved to possess excellent specificity, as can be inferred from the comparison with results obtained by the aforementioned validation procedure. This comparison was performed at least in triplicate for each T_n genotype, with 23 total samples subjected to comparison by reverse hybridization. Aplotype confirmation was performed at least in triplicate, with clones for each aplotype taken from three different subjects with the same aplotype [except for $(TG)_8T_7$ and $(TG)_9T_9$ aplotypes, which were found only once], amounting to 54 sequenced clones (from 18 subjects $\times$ 2 alleles). These procedures revealed, in all the cases examined, a perfect match between the genotypes determined by the method proposed in this study and those determined by the validation methods, corresponding to a 100% specificity (in our study) of the method proposed.

The method proved, in addition, to possess a very high degree of sensitivity, as can be deduced from the limited quantity of the sample needed for the execution. Good results, using this method, were also obtained with less than 0.5 ng starting DNA and less than 1/500 of the quantity normally used of boiled sample, the latter corresponding to less than 20 cells.

DISCUSSION

The methods specific for the analysis of the T_n tract alone were usually two-step methods based on a preliminary PCR amplification of exon 9 and

its intronic boundaries, with specific primers located in introns flanking exon 9 (26). The subsequent evaluation of the PCR product was performed by one of two methods: (*i*) allele-specific oligonucleotide (ASO) hybridization by oligonucleotidic probes that recognized the presence of 5, 7, or 9 thymidines and subsequent (in general radioactive) detection (14,15) or (*ii*) a nested PCR performed using the forward nested primer with a TG repeat of some extent to the 5′ end and a T stretch to the 3′ end, corresponding to the first 5T of the T_n tract, followed by *Xmn*I restriction and ethidium bromide visualization after nondenaturing polyacrylamide gel electrophoresis (4). Although the first PCR step also amplified the $(TG)_m$ repeat, the specificity of ASO in the former method and the peculiarity of the forward nested primer in the latter omitted the analysis of the adjacent $(TG)_m$ repeat. A variant method based on single-step non-nested PCR, with the forward primer showing a partial overlap with the $(TG)_m$ repeat and the first thymidine residues of the T_n tract, was also used (25). Another method (12) consisted of an allele-specific PCR assay performed with a common forward primer and three reverse primers, each specific for the T_5, T_7, or T_9 alleles. The amplification was followed by ethidium bromide visualization on 4% agarose gel, with the different alleles being distinguished by size. This method was also proposed in an enhanced version with three specific reverse primers, each labeled with a different fluorescent tag (22). This method also allows, to some degree, the detection of the allelic variability in the $(TG)_m$ repeat.

When the importance of $(TG)_m$ emerged, there arose a need for methods able to analyze both the $(TG)_m$ and T_n sequences. It is for this purpose that some sequencing-based methods have been proposed. These methods are, in general, multi-step procedures that require a preliminary amplification step, performed with a first primer pair surrounding the exon 9 and comprising part of the intronic regions; this preliminary step is followed by a nested PCR performed with a second primer pair, in which one of the primers is biotinylated for the recovery of ssDNA. This strand is subsequently sequenced either by radioactive-based sequencing using an additional internal primer (6) or by an internal FITC-labeled primer (9). For the analysis of both $(TG)_m$ and T_n, other authors (10) have performed heteroduplex analysis of symmetric PCR products, followed by selective strand amplification by asymmetric PCR (using only one primer) and subsequent radioactive sequencing. Some researchers (2,20) have used a two-step procedure that involves the method of Chillon and co-workers (4) for the preliminary identification of the T_n alleles and direct sequencing of a PCR product relative to the intron 8/exon 9 junction for the subsequent determination of the $(TG)_m$ length. In other methods (7,20), the DNA region containing exon 9 and adjacent intron sequences has been amplified by primers suitable for denaturing gradient gel electrophoresis (DGGE) analysis (11); in these studies, the $(TG)_mT_n$ polymorphic tract produced several distinctly recognizable DGGE patterns, subsequently characterized by direct DNA sequencing.

The method proposed in this study specifically addresses the need for a rapid, simultaneous assessment of $(TG)_m$ and T_n tract length. The results obtained show that the sequences of interest can be resolved, thereby offering the possibility of determining the $(TG)_m$ and T_n tract length of each allele, and consequently of defining the aplotypes and complete genotype for all samples analyzed, ranging from $(TG)_8$ to $(TG)_{12}$, along with the T_5, T_7, and T_9 alleles. The different combinations of $(TG)_m$ and T_n and the repetition number on each allele yield sequences with different levels of interpretational difficulty. In particular, we classified two main types of sequences: (*i*) with no double peaks in the electropherograms (or with double peaks limited to a restricted electropherogram zone)—the so-called in-frame sequences (Figure 1)—and (*ii*) with double peaks starting from an electropherogram point and extending downstream from that point—the so-called out-of-frame sequences (Figure 2). The in-frame sequences were easier to interpret. The sequencing of a single strand can, as shown in both examples in Figure 1, lead to an unequivocal result, with no need to sequence the other strand as well. The out-of-frame sequences yielded more complex electropherograms. In the example shown in Figure 2, A and B, the sequence of the $(TG)_m$ and T_n tracts of both alleles, with the corresponding aplotypes, can be determined by sequencing only one strand, though the sequence of the $(TG)_m$ is more easily assessed by forward sequencing (Figure 2A) and that of the T_n by reverse sequencing (Figure 2B). On the contrary, in the case of Figure 2, C and D, the sequencing of both strands is required to establish the aplotype of both alleles and the complete genotype.

It is noteworthy that the height of the double peaks present in both the in-frame and out-of-frame sequences is about half that of the peaks that represent only one base. This is an additional element that often helps in the interpretation of electropherograms. It should also be noted that a careful electropherogram analysis can reveal the correct sequence also in electropherograms with a low resolution (such as the sixth and eighth A bases (green) of the T_n tract in Figure 1D and the sixth A base of the T_n tract in Figure 2B).

In the population analyzed in this study (Central Italy), it was possible to resolve over 70% of the genotypes by the sequencing of a single strand, with a consequent further savings of the effort normally required for aplotype and complete genotype determination. No ambiguities or discrepancies emerged in any of the cases sequenced, and the specific sequences were always clear (if necessary after the sequencing of both strands).

If compared with the existing methods used for the genotyping of these repeats, the main advantage of the method described in this study is the ease and rapidity of execution, which is due above all to the fact that it does not require DNA extraction and that it markedly reduces both the overall time and number of steps involved. Another notable advantage is that it is based on commercial sequencing methods, which are, as such, subjected to high standardization, optimization, and upgrading. In conclusion, this method is suited to the rapid management of a large number of samples and may help to clarify the role of $(TG)_m$ and T_n repeats in the CF genotype-phenotype relationship.

REFERENCES

1. **Akabas, M.H.** 2000. Cystic fibrosis transmembrane conductance regulator. Structure and function of an epithelial chloride channel. J. Biol. Chem. *275*:3729-3732.
2. **Arduino, C., M. Gallo, A. Brusco, S. Garnerone, M.R. Piana, S. Di Maggio, G. Gerbino Promis, M. Ferrone, A. Angeli, and E. Gaia.** 1999. Polyvariant mutant CFTR genes in patients with chronic pancreatitis. Clin. Gen. *56*:400-404.
3. **Braun, A., D.P. Little, and H. Koster.** 1997. Detecting CFTR gene mutations by using primer oligo base extension and mass spectrometry. Clin. Chem. *43*:1151-1158.
4. **Chillon, M., T. Casals, B. Mercier, L. Bassas, W. Lissens, S. Silber, M.-C. Romey, J. Ruiz-Romero et al.** 1995. Mutations in the cystic fibrosis gene in patients with congenital absence of the vas deferens. New Engl. J. Med. *322*:1475-1480.
5. **Chu, C.-S., B.C. Trapnell, S. Curristin, G.R. Cutting, and R.G. Crystal.** 1993. Genetic basis of variable exon 9 skipping in cystic fibrosis transmembrane conductance regulator mRNA. Nat. Genet. *3*:151-156.
6. **Chu, C.-S., B.C. Trapnell, J.J. Murtagh, Jr., J. Moss, W. Dalemans, S. Jallat, A. Mercenier, A. Pavirani et al.** 1991. Variable deletion of exon 9 coding sequences in cystic fibrosis transmembrane conductance regulator gene mRNA transcipts in normal bronchial epithelium. EMBO J. *10*:1355-1363.
7. **Costes, B., E. Girodon, N. Ghanem, E. Flori, A. Jardin, J.C. Soufir, and M. Goossens.** 1995. Frequent occurrence of the CFTR intron 8 $(TG)_n$ 5T allele in men with congenital bilateral absence of the vas deferens. Eur. J. Hum. Genet. *3*:285-293.
8. **Cuppens, H., W. Lin, M. Jaspers, B. Costes, H. Teng, A. Vankeerberghen, M. Jorissen, G. Droogmans et al.** 1998. Polyvariant mutant cystic fibrosis transmembrane conductance regulator genes. The polymorphic $(TG)_m$ locus explains the partial penetrance of the T5 polymorphism as a disease mutation. J. Clin. Invest. *101*:487-496.
9. **Cuppens, H., P. Marynen, C. De Boeck, and J.-J. Cassiman.** 1993. Detection of 98.5% of the mutations in 200 Belgian cystic fibrosis alleles by reverse dot-blot and sequencing of the complete coding region and exon/intron junctions of the CFTR gene. Genomics *18*:693-697.
10. **Dork, T., R. Fislage, T. Neumann, B. Wulf, and B. Tummler.** 1994. Exon 9 of the CFTR gene: splice site haplotypes and cystic fibrosis mutations. Hum. Genet. *93*:67-73.
11. **Fanen, P., N. Ghanem, M. Vidaud, C. Besmond, J. Martin, B. Costes, F. Plassa, and M. Goossens.** 1992. Molecular characterization of cystic fibrosis: 16 novel mutations identified by analysis of the whole cystic fibrosis transmembrane conductance regulator (CFTR) coding regions and splice site junctions. Genomics *13*:770-776.
12. **Friedman, K.J., R.A. Heim, M.R. Knowles, and L.M. Silverman.** 1997. Rapid characterization of the variable length polythymidine tract in the cystic fibrosis (CFTR) gene: association of the 5T allele with a selected CFTR mutations and its incidence in atypical sinopulmonary disease. Hum. Mut. *10*:108-115.
13. **Gelfi, C., M. Perego, P.G. Righetti, S. Cainarca, S. Firpo, M. Ferrari, and L. Cremonesi.** 1998. Rapid capillary zone electrophoresis in isoelectric histidine buffer: high resolution of the poly-T tract allelic variants in intron 8 of the CFTR gene. Clin. Chem. *44*:906-913.
14. **Jarvi, K., S. McCallum, J. Zielenski, P. Durie, E. Tullis, M. Wilchanski, M. Margolis, M. Asch et al.** 1998. Heterogeneity of reproductive tract abnormalities in men with absence of the vas deferens: role of cystic fibrosis transmembrane conductance regulator gene mutations. Fert. Steril. *70*:724-728.
15. **Kiesewetter, S., M. Macek, Jr., C. Davis, S.M. Curristin, C.-S. Chu, C. Graham, A.E. Shrimpton, S.M. Cashman et al.** 1993. A mutation in CFTR produces different phenotypes depending on chromosomal background. Nat. Genet. *5*:274-278.
16. **Lissens, W., K.Z. Mahmoud, E. El-Gindi, A. Abdel-Sattar, S. Seneca, A. Van Steirteghem, and I. Liebaers.** 1999. Molecular analysis of the cystic fibrosis gene reveals a high frequency of the intron 8 splice variant 5T in Egyptian males with congenital bilateral absence of the vas deferens. Mol. Hum. Reprod. *5*:10-13.
17. **Mazzilli, F., T. Rossi, M. Delfino, and I. Nofroni.** 1999. Application of the upgraded image superimposition system (SIAS) to the assessment of sperm kinematics. Andrologia *31*:187-194.
18. **Mazzilli, F., T. Rossi, L. Sabatini, and F. Dondero.** 1995. Superimposed image analysis system (SIAS) software: a new approach to sperm motility assessment. Fertil. Steril. *64*:653-656.
19. **Niksic, M., M. Romano, E. Buratti, F. Pagani, and F.E. Baralle.** 1999. Functional analysis of *cis*-acting elements regulating the alternative splicing of human CFTR exon 9. Hum. Mol. Genet. *8*:2339-2349.
20. **Pallares-Ruiz, N., S. Carles, M. Des Georges, C. Guittard, F. Arnal, C. Humeau, and M. Claustres.** 1999. Complete mutational screening of the cystic fibrosis transmembrane conductance regulator gene: cystic fibrosis mutations are not involved in healthy men with reduced sperm quality. Hum. Reprod. *14*:3035-3040.
21. **Riordan, J.R.** 1993. The cystic fibrosis transmembrane conductance regulator. Annu. Rev. Physiol. *55*:609-630.
22. **Strasberg, P.M., K.J. Friedman, L. McGlynn-Steele, J. Zielenski, and P.N. Ray.** 1997. Rapid characterization of both the variable length 5, 7, or 9 polythymidine (T) tract in intron 8 and the adjacent CA repeat unit of the CFTR gene: use in DNA diagnostics. Am. J. Hum. Genet. Suppl. *61*:A348.
23. **Strong, T.V., D.J. Wilkinson, M.K. Mansoura, D.C. Devor, K. Henze, Y. Yang, J.M. Wilson, J.A. Cohn et al.** 1993. Expression of an abundantly alternatively spliced form of the cystic fibrosis transmembrane conductance regulator (CFTR) gene is not associated with a cAMP-activated chloride conductance. Hum. Mol. Genet. *2*:225-230.
24. **Stuyver, L., R. Rossau, A. Wyseur, M. Duhamel, B. Vanderborght, H. Van Heuverswyn, and G. Maertens.** 1993. Typing of hepatitis C virus isolates and characterization of new subtypes using a line probe assay. J. Gen. Virol. *74*:1093-1102.
25. **Tuerlings, J., B. Mol, J. Kremer, M. Looman, E. Meuleman, G. te Meerman, C. Buys, H. Merkus, and H. Scheffer.** 1998. Mutation frequency of cystic fibrosis transmembrane regulator is not increased in oligozoospermic male candidates for intracytoplasmic sperm injection. Fert. Steril. *69*:899-903.
26. **Zielenski, J., R. Rozmahel, D. Bozon, B.-S. Kerem, Z. Grzelczak, J.R. Riordan, J. Rommens, and L.-C. Tsui.** 1991. Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Genomics *10*:214-228.

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