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Utility of Internal Markers to Improve the Accuracy of Cystic Fibrosis Genotype Analysis

ABSTRACT

DNA diagnostic tests often utilize restriction endonuclease digestion of PCR-amplified portions of genes under analysis. When partial digestion occurs, the resulting patterns may lead to error in diagnosis. To overcome such potential errors in cystic fibrosis testing, we have developed internal markers that can increase the precision and reliability of genotype assignments.

INTRODUCTION

Several methods have been devised to directly identify polymorphisms and mutations at the cystic fibrosis transmembrane regulator (CFTR) locus exploiting PCR amplification followed by restriction endonuclease digestion. Although these methods require only small amounts of template DNA and are easy, rapid and inexpensive, they have three general limitations. First, sequence information is required to design suitable primers for PCRs. Second, some mutations do not affect a specific restriction site. While these first two limitations can prevent diagnosis applications, the third limitation, incomplete digestion of PCR products by restriction enzymes, often leads to erroneous diagnoses. Sequencing data that identify new CFTR alleles and techniques that convert single-base changes to the gain or loss of a restriction site by modifying the primer's sequence provide potential solutions to the first two limitations (7). However, lack of restriction endonuclease cleavage of amplified products resulting from the concentration or composition of or impurities in PCRs or, alternatively, from changes in the digestion buffers or the incubation temperature and time used remain problematic.

When partial digestion occurs, some resulting DNA fragments may not accurately reflect the genotype of the individual being studied and lead to errors in diagnosis. Since convenient,

inexpensive methods to accurately monitor the effectiveness of restriction digestions are unavailable, we developed markers that can be used as internal controls to check the performance of restriction enzymes used in CFTR genotype analyses.

MATERIALS AND METHODS

A small piece of filter paper (1 mm²) was cut from a single Guthrie card blood spot and added to a 100- μ l standard PCR that contained 100 pM of each oligonucleotide primer (designed to flank the exon 11 sequences relevant for amplification of G551D and R553X and the sequences upstream to CFTR that flanked the KM19 and XV2C polymorphic loci), 10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM MgCl₂, 0.0001% gelatin and 200 μ M of each deoxyribonucleoside triphosphate (6). Three initial cycles at 96°C then 55°C for 3 min were done to free DNA and minimize inhibition by other released materials (4) in a Perkin-Elmer DNA Thermal Cycler (Norwalk, CT). Next, 1 unit of *Taq* DNA polymerase (Promega, Madison, WI) was added, and a 2-min extension was done at 72°C followed by 33 cycles of PCR amplification using denaturing, annealing and extension temperatures and time optimal for each primer set (1,2,5). Upon completion, a 10-min extension period was done at 72°C and the products were cooled on ice.

After amplification, we added 1 unit of each specific restriction endonuclease (*Pst*I, *Taq*I, *Hinc*II or *Mbo*I) enzyme and 9 μ l of corresponding internal marker to 40 μ l aliquots from each PCR. Samples were incubated for 30 min at 37°C. Next, samples were submitted to electrophoresis in 7% non-denaturing polyacrylamide gels in 1 \times TBE (90 mM Tris-borate, pH 8.0, and 1 mM EDTA) for 1 h at 100 mV and 2 h at 300 mV. The PCR products were then visualized by ethidium bromide staining and exposure to UV light (Figure 1).

DISCUSSION

Each marker is a synthetic sequence that contains only one recognition site for the restriction endonuclease used.

The markers developed are useful in the direct detection of two mutations: G551D, which creates a *Mbo*I site, and R553X, which removes a normal *Hinc*II site. These two mutations are responsible for 1.4% and 3.2% of the North American CF alleles. We also developed markers which are useful in the detection of two polymorphisms that are in strong disequilibrium with CFTR alleles (KM19/*Pst*I and XV2C/*Taq*I) (Figure 1) (2-5). Addition of these internal markers to the PCR products prior to their digestion provides a convenient assessment of the performance of the restriction enzymes used as well as identification of partial digestions (Figure 1). For example, the R553X mutation is detected by the loss of the normal *Hinc*II restriction site when the amplified sequence is exposed to *Hinc*II. In the absence of an internal control, one is unsure if the un-

cleaved PCR products derived from DNA from a patient heterozygous for this mutation (lane 18) results from the absence of the restriction site for *Hinc*II or if a technical mistake, such as omission of the corresponding restriction enzyme or some other change resulting in less than optimal conditions for the enzyme, has occurred (lane 5). The addition of corresponding internal markers to PCR products prior to their digestion provides internal size markers, as well as verification of the completeness of digestion as seen in lanes 6, 11, 16 and 21. These internal markers are also useful to check the quality control of mutation analysis performed by PCR-mediated site-directed mutagenesis in which primers are designed to transform any alteration in DNA sequence into an allele-specific restriction enzyme recognition site (3). In summary, our results demonstrate

the utility of internal markers as controls in studies using PCR amplification coupled with restriction endonuclease digestion. The use of such control reagents can provide convenient and rapid assessment of the effectiveness of digestions, thereby increasing the precision and reliability of CFTR genotype analysis.

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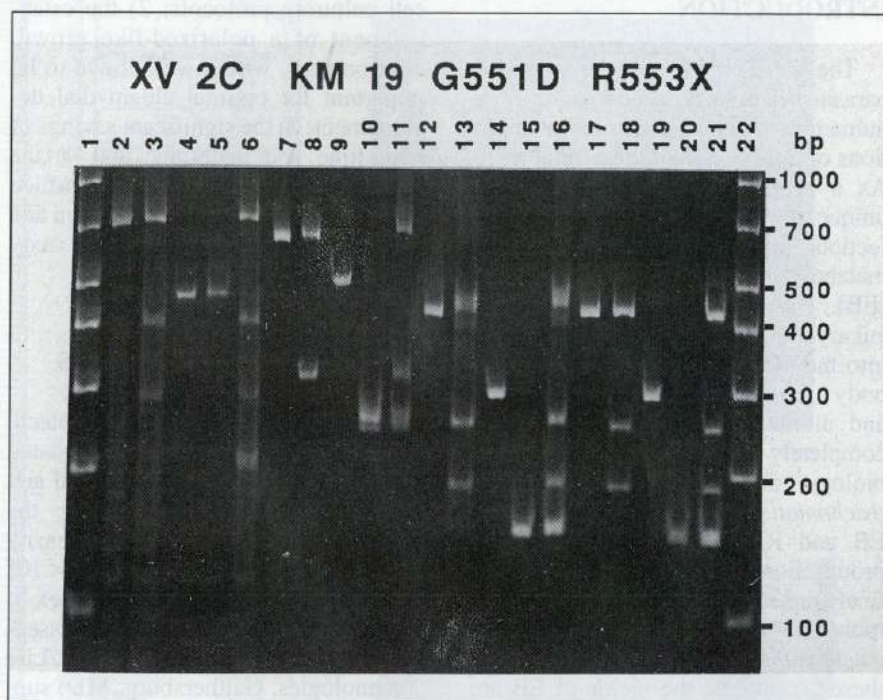


Figure 1. PCR-amplified fragments electrophoresed on a 6% acrylamide gel followed by ethidium bromide staining. The patterns shown are from DNA of CF subjects who are heterozygous for the XV-2C and KM-19 polymorphisms or heterozygous for the G551D or the R553X mutation, respectively. Lanes 1 and 22 are molecular weight standards (Biomarker™, BioVentures, Murfreesboro, TN). Lanes 2, 7, 12 and 17 are PCR products containing XV-2C, KM19, G551D and R553X sequences after 33 amplification cycles. Lanes 3, 8, 13 and 18 contain PCR products corresponding to XV-2C, KM19, G551D and R553X following digestion with *Taq*I, *Pst*I, *Mbo*I and *Hinc*II, respectively. Lanes 4 and 5, 9 and 10, 14 and 15 and 19 and 20 contain the allelic-specific internal markers prior to and following restriction endonuclease digestion with *Taq*I, *Pst*I, *Mbo*I or *Hinc*II (note no digestion in lane 5 due to accidental omission of enzyme). Lanes 6, 11, 16 and 21 contain PCR products of XV-2C, KM-19, G551D and R553X plus their corresponding internal standards after digestion with either *Taq*I, *Pst*I, *Mbo*I or *Hinc*II (note the absence of uncleaved internal markers and the doublets, representing complete cleavage of internal markers, as seen as the smallest fragment in each lane).

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SRASKIN



Eukaryotic Cells Grown on Microcarrier Beads Offer a Cost-Efficient Way to Propagate *Chlamydia trachomatis*

ABSTRACT

The use of microcarrier cell culture as a method for the *in vitro* propagation of the obligate intracellular bacterial parasite, *Chlamydia trachomatis*, is described. The microcarrier beads proved to be a more cost-effective means to propagate *C. trachomatis* than traditional tissue culture flasks or roller bottles without sacrificing yields or infectivity. In addition, microcarrier cell culture was found to be a much simpler technique to study the intracellular development of these bacteria.

INTRODUCTION

The obligate intracellular bacterial parasite *Chlamydia trachomatis* is a human pathogen responsible for infections of the eye and of the genital tract. As a genus, the *Chlamydia* have a unique developmental cycle. The infectious form of the *Chlamydia* is a metabolically inert elementary body (EB). Upon internalization by the eukaryotic host cell, the EB develops into the metabolically active reticulate body (RB). The RB, in turn, divides and ultimately converts into EB. To completely understand the complex biology and pathogenicity of *C. trachomatis*, large amounts of purified EB and RB are necessary. *In vitro* propagation of *C. trachomatis* is routinely done in tissue cell lines grown as monolayers on plastic. While *C. trachomatis* grows quite well under these conditions, the yields of EB are dictated by numbers of tissue culture flasks or roller bottles. In contrast, large yields of *Chlamydia psittaci* and the lymphogranuloma venereum biovar of *C. trachomatis* can be obtained from L-929 suspension cell cultures.

Because of the expense and man-hours of propagating *C. trachomatis* in flasks or roller bottles, a cost-effective alternative was sought. In addition, we wanted a method that would permit a

polarized-like growth of the eukaryotic host cells because our previous studies have shown EB progeny from these cells to be more infectious than EB progeny from the same inoculum but propagated in host cells cultured on plastic surfaces (6). Over the last decade, the use of microcarrier beads as a support for the growth of eukaryotic cell monolayers has become more common. Microcarrier bead cultures, which have many of the positive attributes of cells grown in suspension, as well as the capability for the cells to feed naturally from their basolateral membranes, have been used to produce large amounts of various cellular products and to support the growth of many viruses (4). We have found the microcarrier bead culture technology to also be a feasible system in which to grow *C. trachomatis*. The advantages include: 1) the ease with which the beads can be adapted to existing eukaryotic cell culturing protocols; 2) the establishment of a polarized-like growth environment, which we believe to be important for optimal chlamydial development; 3) the significant savings of both time and materials; and 4) the simplicity and rapidity with which multiple samples can be withdrawn and processed in experiments that study chlamydial development.

MATERIALS AND METHODS

Cytodex® 3 (Pharmacia Biotech, Uppsala, Sweden), a collagen-coated microcarrier bead, was rehydrated and sterilized as recommended by the manufacturer. The final bead density was adjusted to approximately 1×10^4 beads/ml (ca. 2.5 mg/ml of Cytodex 3) in 125 ml of Eagle's minimum essential medium (MEM; GIBCO BRL/Life Technologies, Gaithersburg, MD) supplemented with 5% bovine calf serum (BCS) and 2 mM glutamine in a 125-ml slow speed stirring vessel (Model No. 26501125; Corning Glass, Corning, NY). The beads were stirred overnight at 60 rpm at 37°C to allow the beads to equilibrate prior to inoculating with cells.

McCoy cells (CRL 1696; ATCC, Rockville, MD), grown on plastic tissue culture flasks in MEM