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MS primers and were absent from the yeast control. Sequence analysis of these clones indicated that 15 contained MS. Inverse PCRs corresponding to 8 of these clones provided the sequence of the entire MS and flanking region.

Among the MS repeats we identified, five were simple (GA)_n or (CA)_n tandem repeats, while three had a more complex repeat structure (Table 1). Using specific primers for each MS (Figure 1, p1 and p3), we mapped back all 8 MS to the original YAC (Table 1). This result confirms that all the isolated MS are derived from the YAC and not from yeast genomic DNA. Finally, we tested the MS repeats for polymorphism between the BALB/cByJ and CAST/EiJ mouse strains, and determined that all were polymorphic. Data relating to these polymorphisms have been submitted to the Mouse Genome Database (www.informatics.jax.org; D12Lip1-7, MGI Accession IDs 1342092–1342098). The identification of these new polymorphic MS repeats in a BALBxCAST cross segregating the *fld* mutation was instrumental in reducing the critical genomic region and in the subsequent positional cloning of the underlying gene (5).

In summary, we developed a simple PCR-based procedure for the identification of novel MS from YACs and other large-insert clones [e.g., bacterial artificial chromosome (BAC) and P1 artificial chromosome (PAC)]. Although we have demonstrated the use of the technique by isolating several polymorphic markers from a mouse YAC clone, the procedure could be easily adapted to human DNA by substituting the B1/B2-specific primers with primers derived from the human *Alu* repeat. A major advantage of the new method over existing ones is its species specificity, which eliminates the need to gel-purify YACs and enables analysis from total yeast DNA preparations.

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Denaturing Gradient Gel Electrophoresis Resolves Virus Sequences Amplified with Degenerate Primers

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Denaturing gradient gel electrophoresis (DGGE) is a versatile technique for studying genetic variation in populations. The technique is based on the separation (melting) of double-stranded (ds) DNA as a function of temperature and concentration of denaturant (i.e., urea and/or formamide) (9). As a result of the formation of molecules with complex structure, partial separation of strands inhibits the electrophoretic mobility of dsDNA. Resolution of dsDNA fragments of the same length that differ in sequence occurs by running the DNA in a polyacrylamide gel that has a continuously increasing concentration of denaturant. Because dsDNA molecules that differ in sequence partially melt at different concentrations of denaturant, rates of migration are inhibited at different positions in a denaturing gradient gel. Furthermore, the attachment of a G + C-rich sequence (i.e., GC clamp) to one end of the dsDNA fragments that are to be separated increases the sensitivity of DGGE. The G + C-rich region creates a high melting temperature domain that allows detection of single base pair substitutions in lower melting domains (11). Attachment of this GC clamp to DNA fragments is easily achieved by the incorporation of a GC-rich region into one of the primers used for PCR (10,11). This modification has become standard in most applications of DGGE.

Commonly, PCR with non-degenerate primers with a GC clamp is used to amplify short DNA fragments ranging from 150–400 bp in length (5,7,8,16). While use of a GC clamp can greatly enhance the sensitivity of DGGE and allow the detection of single base pair changes, it may not always be advantageous or necessary. In this report, we demonstrate that DGGE can be used to resolve longer PCR products without the use of a GC clamp primer. In addi-

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tion, PCR with degenerate primers and DGGE with a broad gradient resolved similar sized products that were amplified from related but relatively dissimilar virus templates.

The discovery that viruses are abundant, dynamic components of marine ecosystems and important players in the mortality of microorganisms (6,13) and in geochemical cycles (15) has led to a need to characterize natural virus communities. Recent methodological developments have allowed us to use PCR to examine some groups of viruses that infect unicellular algae. Specifically, the design of algal virus-specific primers (AVS-1 and AVS-2) that amplify a highly conserved DNA polymerase gene fragment has facilitated examination of natural virus community diversity without the need for culture (1–3).

Initial evidence of the diversity of algal viruses was revealed when five different sequences were obtained from the PCR products of a single environmental sample. The heterogeneity of al-

gal virus sequences amplified in a single PCR was resolved by cloning, restriction fragment length polymorphism (RFLP) analysis and sequencing (3). While this technique is effective, it is relatively time-consuming and cumbersome. As a result, we sought alternative methods that would allow direct examination of PCR products amplified from seawater samples to genetically "fingerprint" subsets of natural virus communities. Recently, we modified existing DGGE methods to facilitate the rapid analysis of PCR products amplified from seawater samples to follow changes in the genetic diversity of virus communities (12). Here, we demonstrate that a mixture of PCR products of

the same length that have been amplified using degenerate primers can be clearly resolved by DGGE without using a GC clamp.

DNA polymerase gene fragments were amplified from three cultured viruses that infect microalgae and a naturally occurring virus community. Template DNA from the viruses MpV-SP1 and MpV-PB8, which infect the marine phytoplankton *Micromonas pusilla*, were prepared as previously outlined (4). Template DNA from the virus CVA-1, which infects the freshwater alga *Chlorella* sp. (14), was provided by Y.P. Zhang and J.L. Van Etten. The target sequences from MpV-SP1 and MpV-PB8 share 81% identity with each

Figure 1. Gel electrophoresis of PCR products amplified from algal viruses. (A) Agarose gel electrophoresis of algal virus samples. Lanes correspond to PCR amplification products from (1) combination of MpV-SP1, MpV-PB8 and CVA-1 templates, (2) MpV-SP1, (3) MpV-PB8, (4) CVA-1, (5) negative control and (6) Jericho Pier community (negative control). Sizes, in base pairs, are shown to the left of the appropriate bands of the molecular weight marker. (B) DGGE of the same PCR products. Lanes are labeled as above and the duration of electrophoresis is shown above the appropriate lanes.

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other and 55% identity with the sequence from CVA-1 (1). The natural virus sample was obtained from Jericho Pier, Vancouver, BC, Canada, and template DNA was prepared as previously outlined (3). Target sequences from all templates were amplified using the degenerate (8192-fold and 4096-fold, respectively) 23-mer oligonucleotide primers AVS-1 and AVS-2 that are specific for the Phycodnaviridae (2).

For reactions with cultured and naturally occurring virus templates, approximately 100 ng of viral DNA were added to 50 μ L of a PCR mixture containing *Taq* DNA polymerase assay buffer (50 mmol/L KCl, 20 mmol/L Tris-HCl, pH 8.4), 1.5 mmol/L $MgCl_2$, 0.16 mmol/L of each dNTP, 60 pmol of each primer and 0.625 U of *Taq* DNA polymerase (Life Technologies, Gaithersburg, MD, USA). Two drops of mineral oil were added to each tube.

PCR was also performed on a combined template sample containing all the same reagents by adding approxi-

mately 50 ng of each cultured virus DNA to the reaction mixture. Negative controls contained all reagents except template. PCR was carried out using a Model PTC-150 MiniCycler™ (MJ Research, Watertown, MA, USA) with the following cycle parameters: denaturation at 95°C for 2 min, 30 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min; 29 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. Following 30 rounds of amplification, 8 μ L of the PCR products were electrophoresed in 1.5% LE agarose (FMC BioProducts, Rockland, ME, USA) in 0.5× TBE buffer (0.045 mol/L Tris-borate, 1 mmol/L EDTA, pH 8.0) at 90 V for 1 h, 20 min. For molecular weight standards, 0.25 μ L of 100 bp ladder (Life Technologies) were loaded into a separate lane. The gels were stained with ethidium bromide and visualized on a UV transilluminator.

DGGE of PCR products was conducted using 0%–60% (100% denaturant is defined as 7 mol/L urea and 40% deionized formamide) linear denaturing gradient 6% polyacrylamide gels. Six microliters of individual products were loaded into wells, while 18 μ L of the combined products were loaded. Electrophoresis of individual and combined products was carried out for 4, 6 and 8 h in a denaturing gradient gel. In another gel, 30 μ L of natural products were electrophoresed for 7 h. Both gels were run in 1× TAE (40 mmol/L Tris-base, 20 mmol/L sodium acetate, 1 mmol/L EDTA, pH 8.5) at 135 V at a constant temperature of 58°C using the DCode™ electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA). Gels were stained in a 1× SYBR® Green I (Molecular Probes, Eugene, OR, USA) solution overnight and visualized on a UV transilluminator.

Figure 1A shows that PCR amplification resulted in the production of bands approximately 700 bp in length. The expected size of the PCR products was 692, 683 and 683 bp for the templates CVA-1, MpV-PB8 and MpV-SP1, respectively. PCR products in the combined reaction were all resolved by DGGE. Bands corresponding to those in lanes loaded with PCR products from single template reactions can be ob-

served in the lane loaded with PCR products from the combined template reaction (Figure 1B). Several distinct bands can be observed in the gel loaded with PCR products from the natural sample (Figure 1B). From these results, it is apparent that a broad gradient (0%–60%) of denaturant was adequate for the resolution of these PCR products.

After 6 h, the PCR products from cultured viruses had focused in the gel, and longer electrophoretic duration did not result in further migration of any bands. All of the amplified fragments from cultured viruses focused at different locations in the gradient gel and several bands were resolved in the gel with the natural sample. Therefore, it can be concluded that incorporation of a GC clamp was unnecessary to resolve sequence differences in gene fragments amplified from cultured algal viruses or a natural virus community.

As previously shown, amplification of algal virus sequences with the primers AVS-1 and AVS-2 results in the production of approximately 690 bp PCR products. It appears that products of this length focus in a denaturing gradient gel with no need for a GC clamp. It could be postulated that as these DNA molecules migrate towards increasing concentrations of denaturant, partial melting of different domains results in DNA conformations that do not migrate in the gel. Once migration of the bands halts, no further denaturation can occur. Therefore, the dsDNA does not completely denature. Consequently, no single-stranded DNA is produced and the complex structure of the dsDNA allows the bands to focus at specific locations in the gel.

DGGE has become a widely used technique as a result of its flexibility and adaptability for many applications. For most applications, electrophoretic conditions must be determined empirically. While this makes the technique slightly less desirable for new applications, it does imply that this technique lends itself to a certain degree of fine-tuning. As demonstrated by our use of DGGE to resolve differences in algal virus polymerase sequences, the technique is applicable to virtually any study of sequence heterogeneity in related PCR products. PCR combined with DGGE can be used to assess spa-

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tial and temporal dynamics in the genetic diversity of algal virus populations amplified with the primers AVS-1 and AVS-2. These results demonstrate that DGGE can be used to rapidly assess the genetic diversity of algal virus communities in nature.

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Improved Determination Method for Plasmid Copy Number Using Pulsed Field Gel Electrophoresis

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The common methods of determining plasmid copy numbers calculate plasmid copies per cell (12). However, this is difficult because not all microorganisms grow as single cells (e.g., mycelial growth of actinomycetes or chain-forming streptococci). Therefore, the use of chromosomal DNA as a reference to calculate copies per chromosome is the preferred method. In most approaches, DNA is purified (1,5,7,9,13,17). As the isolation procedures involve a significant loss of DNA (6,11), all results are approximate values.

Although there are methods that use chromosomal DNA as an internal standard, precise results are obtainable only when the fragment pattern is reproducible. In the case of using strain-specific nucleases (8), a complete reproducible digestion cannot be proved. To optimize the assessability of the chromosomal fragments following the digestion by restriction endonuclease(s), the wide size range separation by pulsed field gel electrophoresis (PFGE) is most practical. One approach using Southern hybridization of the gel is described (10).

We describe a new method based on both the reproducible separation of chromosomal restriction fragments as an internal standard and the linearized plasmid DNA by means of PFGE. When