The procedure for the microplate analysis of inhibitor effects on proteinases was modified from the substrate analysis procedure. Inhibitors (from Sigma Chemical and Calbiochem-Novabiochem, La Jolla, CA, USA) were dissolved in 100 µL of assay buffer and added individually to the top wells (row A) of a 96-well microplate at the concentrations indicated in the legend of Figure 2. Serial dilutions of 1:1 were made using a multichannel pipettor in 50 µL of assay buffer from rows A to G. Fifty microliters of trypsin were added at a concentration of 10 µg per well, and the plates were preincubated at 37°C for 15 min before addition of substrate. To initiate the reaction, 50 µL of a 1-mg/mL solution of a trypsin-specific substrate, N-α-benzoyl-L-arginine p-nitroanilide (BAPNA), were added to each well. The absorbance was monitored as in the substrate analysis procedure. Values obtained with the kinetic software program were converted using a transformation function in the program to percentage of control (no inhibitor). IC₅₀ values were estimated using linear regression analysis of the enzyme activity vs. the natural logarithm of the concentration of the inhibitor.

The data obtained from a single microplate analysis of different inhibitors of trypsin are shown in Figure 2. The most effective inhibitor was TLCK, with essentially 100% inhibition observed over the concentration range tested (10⁻³–10⁻¹ mM). Aprotinin and potato inhibitors I and II were also effective in preventing trypsin hydrolysis of BAPNA, with IC₅₀ values of 1.02, 1.46 and 2.33 µM, respectively. Antipain and leupeptin were slightly less effective in trypsin inhibition, and benzamidine was inhibitory only at high concentrations (mM). Chymotatin, APMSF, E-64 and TPCK did not inhibit trypsin hydrolysis of BAPNA at the concentrations tested. All inhibitors predicted to inhibit trypsin did so with the exception of APMSF. We attribute the lack of inhibition of trypsin by APMSF to the instability of the inhibitor at the pH of the buffer used in the assay. APMSF has a half-life of only 1 ms at pH 8.0 (1). Using a single microplate, eleven inhibitors were screened over the dose range, and the entire procedure required only about 2 h to complete.

This microplate technique was developed to analyze complex mixtures of proteinases. Substrates containing pNA-amino acid conjugates can be screened easily and quickly by this technique. In this assay, values obtained from a mixture of mammalian enzymes, each specific for a particular substrate, were similar to values obtained using individual enzymes. Inhibition curves were generated using serial dilutions of a variety of inhibitors in a single microplate. We have successfully used this method to partially characterize proteinases from Plodia interpunctella, an insect pest of stored products, and obtained data for the substrate specificity and inhibitor susceptibility of the major enzymes in gut extracts (3). The procedure can be used to study any enzyme that hydrolyzes a substrate that can be monitored spectrophotometrically.

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Isolation of Site-Specific Insert Probes from Chimeric YACs

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The yeast artificial chromosome (YAC) cloning system (3) and the development of “Mega YACs” with inserts greater than 1 Mb (1,4) have proven to be a powerful approach in the analysis of complex genomic regions (5). However, detailed analysis of YAC libraries showed that a significant proportion of clones contains two or more noncontiguous pieces of DNA (6). These so-called chimeric YACs, obtained either from somatic cell hybrid DNA or from total human DNA, can cause substantial problems with methods in genome research or with diagnostic applications in particular techniques such as cDNA selection or mapping of insert DNA by fluorescence in situ hybridization (FISH) analysis.

The method we describe offers a convenient and reliable approach to separate the YAC insert DNA from yeast DNA and to isolate DNA fragments from different genomic regions of chimeric YACs. It is based on the occurrence of two CpG dinucleotides in the restriction site of the rare cutting restriction enzyme MluI. HpaII tiny fraction (HTF) islands, which contain a high density of nonmethylated CpG islands, are clustered at the 5’ ends of human genes, where they are involved in the control of the transcription process (2). This is in contrast with the yeast genome, where CpGs are randomly distributed.
Four chimeric YACs (757g3, 911h10, 776a4 and 892d2; Research Genetics, Huntsville, AL, USA) were successfully used to prepare large site-specific probes for FISH analysis to human metaphase chromosome spreads. Following *Mlu* I digestion of YAC DNA and separation by pulsed field gel electrophoresis (PFGE), numerous small yeast DNA fragments (less than 100 kb) and up to five human DNA insert fragments (80–600 kb) can be obtained (Figure 1).

**Protocol for Insert Preparation from Chimeric YACs**

Single yeast colonies were grown in AHC medium (20% glucose, 6.7% yeast nitrogen base, 10% casein hydrolysate acid, 1% adenine hemi-sulfate) at 30°C, and DNA was prepared in agarose blocks as described previously (3). Agarose plugs were washed three times for 30 min in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) to remove the storage solution (0.5 M EDTA, pH 8.0). Each block (20 µL) containing about 5 × 10⁷ cells in 0.75% low-melting-point agarose (Sea-Plaque GTG®, FMC BioProducts, Rockland, ME, USA), was digested after equilibration in *Mlu* I restriction buffer (Boehringer Mannheim GmbH, Mannheim, Germany) with 40 U *Mlu* I for about 14 h in a total volume of 300 µL. The restriction enzyme (Boehringer Mannheim GmbH) was used according to the recommendations of the manufacturer. Preparative PFGE was carried out using the CHEF Mapper® PFGE System (Bio-Rad, Hercules, CA, USA). Size-fractionation of digested DNA was performed in 1.0% low-melting-point agarose gels in 0.5× TBE buffer (0.045 M Tris-borate, 0.001 M EDTA, pH 8.0) to 14°C at 6 V/cm for 23 h with pulse time varying from 6 to 40 s to obtain the best resolution of fragments from 50 to 500 kb (Figure 1). After excision of the 600-, 320- and 270-kb insert bands from YAC 911h10, 1-µL aliquots were amplified in 50-µL reaction mixtures by a slightly modified degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR) (7), compared to previous protocols (9).

The amplification products (10–20 ng) were labeled in a secondary PCR using digoxigenin-11-dUTP (Boehringer Mannheim GmbH). PCR labeling comprised: 4 cycles at 94°C for 1.6 min, 58°C for 1.6 min and 72°C for 1.6 min, followed by 8 cycles at 94°C for 1.6 min, 58°C for 15 s and 72°C for 15 s, and completed by 16 cycles at 94°C for 1.6 min, 58°C for 10 s and 72°C for 10 s. This type of reaction leads to a preferential amplification of smaller PCR products because of a decreasing time for primer extension in subsequent cycles, thereby creating labeled FISH probes in an optimized size range of 100–600 bp. For each hybridization, about 100 ng of the amplified probe and 3–5 µg Cot-1™ DNA (Life Technologies, Gaithersburg, MD, USA) were re-suspended in 8 µL hybridization mixture (Master Mix 1 [MM1]: 10% dextran sulfate, 2× standard saline citrate [SSC], 50% formamide) (8), denatured at 70°C for 8 min and pre-annealed at 37°C for 30 min. Metaphase spreads, prepared according to standard procedures, were denatured in 70% formamide, 2× SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) at 70°C for 2 min and dehydrated in a cold ethanol series. Probes were hybridized overnight in a moist chamber at 37°C. After three washes in 50% formamide, 2× SSC, 0.5% Tween® 20 (pH 7.0) for 2 min at 42°C and one in 4× SSC, 0.5% Tween 20 for 2 min at 42°C, slides were rinsed two times at room temperature. The digoxigenin-labeled probe was detected using rhodamine-conjugated anti-digoxigenin antibodies (Boehringer Mannheim GmbH). Counterstaining was performed using 4’,6-diamidino-2-phenylindole-dihydrochloride (DAPI; 0.5 µg/mL) in anti-fade solution (VECTASHIELD®, Vector Laboratories, Burlingame, CA, USA). Hybridization signals were observed using a microphot-SA microscope (Nikon, Chiyoda-ku, Tokyo, Japan) equipped with a triple band-pass filter, and photomicrographs were recorded on Ektachrome 400 daylight film (Eastman Kodak, Rochester, NY, USA).

The 600- and 320-kb fragments from the YAC 911h10 produced signals only on the short arm of chromosome seven. The 270-kb insert fragment mapped also to this genomic site, but this caused another, stronger signal on the long arms of chromosome nine to be visible. The same combination of signals was achieved when the whole YAC DNA was used (Figure 2). We did

**Figure 1.** PFGE analysis of the following YAC clones: 757g3, 911h10, 776a4 and 892d2 (lanes 1–4, respectively) undigested (a) and digested with the restriction endonuclease *Mlu* I (c). The corresponding Southern blot hybridization results obtained with human Cot-1 DNA as a probe are shown in panels b and d, respectively. For FISH analysis, the 600-, 320- and 270-kb insert bands of YAC 911h10 were used.
Figure 2. FISH results obtained with DOP-PCR probes from YAC 911h10 on normal human metaphase chromosomes. The whole YAC shows signals on chromosomal regions 7p (red arrowheads) and 9q (black arrowheads) (a). The 600-kb MluI fragment maps only to the short arm of chromosome 7 (b), and the 270-kb fragment gives similar results as in panel a, although relative signal intensity at 7p region is obviously weaker (c).
not attempt to further dissect this remaining 270-kb chimeric probe, although it can easily be done by a second digestion step of the isolated fragment. Since DOP-PCR allows generation of complex amplification products from miniscule amounts of DNA, high-quality FISH probes can be obtained very reliably. A major proportion of YAC inserts (approximately 80% according to PFGE bands compared with autoradiographic results obtained using human Cot-I DNA as a probe [Figure 1]) can be isolated and purified from yeast DNA by this approach.

In conclusion, the method described can efficiently utilize chimeric YACs as valuable resources for various applications including gene and genome analysis.

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