Identification of a novel site specific endonuclease produced by *Mycoplasma fermentans*: discovery while characterizing DNA binding proteins in T lymphocyte cell lines

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ABSTRACT

We have discovered a new restriction endonuclease, MfeI, in nuclear extracts from T cells contaminated with *Mycoplasma fermentans*. This endonuclease was identified while studying proteins binding to the interleukin-2 receptor α chain gene promoter. MfeI cuts at the recognition sequence C'ATTG generating EcoRI compatible cohesive ends. Potential applications are discussed.

INTRODUCTION

Numerous laboratories are currently analyzing the 5' regulatory regions of genes for sequences that are specifically bound by nuclear proteins. Such assays often involve the use of the endonuclease, DNaseI (1,2), or exonucleases (3,4). Because of the importance of titrating these exogenously added nucleases, endogenous nuclease activity within the extracts presents a significant problem. During our analysis of the human interleukin-2 receptor α chain (IL-2Rα; also known as p55, Tac antigen) promoter region (5,6), we have discovered a novel restriction endonuclease, MfeI, whose activity was present in nuclear extracts derived from two T cell lines. The enzyme is produced by a strain of *Mycoplasma fermentans* contaminating these cell lines.

RESULTS

Two overlapping IL-2Rα promoter fragments (see legend, Figure 1) were labeled at both ends with Klenow according to standard protocol (7). The labeled fragments were then incubated with or without nuclear extracts (8) derived from HUT-102B2 or Jurkat human T cells. As shown in Figure 1, the extracts
Figure 1: Detection of site specific nuclease activity in Jurkat extracts. The EcoRI/PstI (-481/+109) (lanes 1-3; see reference 6 for numbering scheme) and PstI/PstI (-1242/+109) (lanes 4-6) IL-2RA promoter fragments were cloned in pUC13, and the plasmids were digested with EcoRI plus PvuII or XmaI plus PvuII, respectively, to liberate the promoter and the adjacent lac op region as previously described. The fragments (778 and 1562 bp, respectively) were end-labeled with Klenow and 32P dNTP's, the lac repressor/beta galactosidase fusion protein bound, and the fragments immunoprecipitated with anti-beta galactosidase antibodies (4). These labeled DNA fragments were then incubated with no extracts (lanes 1,4), or nuclear extracts from HUT-102B2, a T cell line transformed with HTLV-I (lanes 2,5), or Jurkat, an acute lymphocytic leukemia T cell line (lanes 3,6). Samples were then extracted with phenol/chloroform, ethanol precipitated, and analyzed on 8M urea, 6% polyacrylamide gels by autoradiography. Preparations from Jurkat cells contained a nuclease activity not present in those from HUT-102B2 cells. Each of the DNA fragments was cleaved once at a specific location suggesting site specific nuclease activity. The band of 470 bases was produced after cleavage of both DNA probes, indicating a shared 3' terminus. Extracts from several other cell types were evaluated to determine whether the activity was specific to Jurkat cells. Similar activity was detected in MT-2 T cells (shown in Figure 2, lane 16, with a different DNA construct) and in Jurkat cells induced with phorbol myristate acetate, but not in CEM T cells or HeLa cells (data not shown).
Figure 2: Optimal conditions for cleavage by MfeI. Oligo 2 (see Figure 4) was cloned into pUC13, excised with EcoRI and HindIII and filled in with $^{32}P$ dNTP's and Klenow. The probe was treated as follows: Lane 1, nuclear extracts from Jurkat treated with BM cycline; lanes 2 through 15, from Jurkat not treated with BM cycline; lane 16, from MT-2 an HTLV-I transformed T cell line; lane 17, no protein extracts. Nuclear extracts were subjected to the following conditions: Lane 2, proteinase K, 670 μg/ml at 37°C for 15 minutes; lane 3, 65°C for 15 minutes; lane 4, 5% glycerol; lane 5, 10% glycerol; lane 6, 50% glycerol; lane 7, 10 mM KCl; lane 8, 50 mM KCl; lane 9, 100 mM KCl; lane 10, 200 mM KCl; lane 11, 10 mM NaCl; lane 12, 50 mM NaCl; lane 13, 100 mM NaCl; lane 14, 200 mM NaCl; lane 15, 20 mM EDTA.

We next determined the optimal conditions for digestion with the site specific endonuclease. The enzymatic activity was destroyed by proteinase K treatment (Figure 2, lane 2), and by heating to 65°C for 15 minutes (lane 3). The enzyme cleaved more efficiently at 5% glycerol than at 10% or 50% glycerol (lanes 4-6). More complete digestion was obtained at 10 mM NaCl or KCl than at higher salt concentrations (lanes 7-14), and the enzyme could be inactivated by 20 mM EDTA (lane 15), demonstrating a requirement for Mg$^{2+}$ or perhaps other divalent cations.
Figure 3: Specificity of cleavage site for MfeI isolated from purified M. fermentans. The pUC 13/oligo 2 construct containing the CAATTG recognition site was digested with EcoRI and PvuII, labeled, and run as a positive control (lanes 1 and 2). Oligonucleotides 3, 4, and 5 (Figure 4) were cloned into the BamHI site of pGem 4Z (Promega Biotec) and then excised with EcoRI and HindIII and filled-in with $^{32}$P dNTP's and Klenow. Lanes 2, 4, 6, and 8 were treated with extracts of purified M. fermentans made by a modification of a published procedure (8). Lanes 1, 3, 5, and 7 were untreated controls.

It was formally possible that this endonuclease activity was produced by certain eucaryotic cells. However, since all known restriction enzymes are derived from procaryotes and since our cultures were not obviously contaminated with bacteria, we hypothesized that this activity was derived from contaminating mycoplasma. When the cell lines being carried in the laboratory were tested for mycoplasma, only the Jurkat and MT-2 cell lines were contaminated, correlating precisely with the presence of the nuclease activity. Typing of the mycoplasma in both cell lines (by Microbiological Associates, Inc., Rockville, MD) revealed it to be M. fermentans, a human and primate pathogen encountered occasionally as a contaminant in cultured cells.

In order to determine whether the M. fermentans was the source of the nuclease activity, we eliminated it from our Jurkat cell line by using BM-cycline (Boehringer Mannheim) according to the manufacturer's directions. Following this treatment, the Jurkat cells were retested and determined to be negative for
Figure 4: Sequences of the IL-2Ra promoter region from -195 to -150 containing the cleavage site, and synthetic oligonucleotides sharing (oligo 1) or not sharing (oligo 2) homology with the promoter beyond the palindrome CAATTG. The radiolabeled bases after filling in the 5' overhanging sequences are indicated by asterisks. The remaining sequences (oligos 3, 4, and 5) each contain three different derivative recognition/cleavage sites based on purine/pyrimidine substitutions.

mycoplasma. Evaluation of nuclear extracts from the uninfected cell line indicated that the nuclease activity was no longer present (Figure 2, lane 1 vs. lane 4). In addition the M. fermentans from the MT-2 cells was purified by cloning. This isolate, which we designate M. fermentans MT-2/NJW, was grown in 500 ml of broth medium in the absence of T cells for 4 days and sedimented by centrifugation at 33,000 x g for 20 minutes.

Protein extracts made from the mycoplasma pellet contained the restriction enzyme activity (Figure 3, lane 2) confirming that the activity was derived from this strain of M. fermentans. We have named this enzyme MfeI in keeping with traditional nomenclature.

In Figure 4, the top sequence is that of the IL-2Ra
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promoter from -195 to -150. Based on the sizes of the fragments generated (Figures 1 and 3), we mapped the putative cleavage site to the palindrome CAATTG. To confirm that this six base sequence was sufficient for recognition and cleavage, we prepared two synthetic double stranded oligonucleotides using a DNA synthesizer (Applied Biosystems Model 381A). These oligonucleotides either share several base pairs of homology (Figure 4, oligo 1) or no homology (oligo 2) with the IL-2Rα sequence on either side of the putative cleavage sequence, CAATTG (boxed). Each double stranded oligonucleotide was ligated via its BamHI/XbaI compatible cohesive ends into the pUC13 polylinker. The second and third sequences in Figure 4 represent the synthetic oligonucleotides plus the associated pUC13 polylinker resulting from digestion with EcoRI and HindIII and filling in with 32p-dNTPs and Klenow. The synthetic oligonucleotides exclusive of the pUC13 polylinker sequences are underlined. These oligonucleotides were incubated with nuclear extracts from Jurkat T cells. Both oligo 1 (not shown) and oligo 2 were digested (Figure 3, lane 2), suggesting that the six base palindrome CAATTG was sufficient for both recognition and cleavage.

In order to exclude the possibility that four or five base pair sequences (i.e., CAAT, AATT, ATTG, CAATT, or AATTG) rather than six were sufficient for recognition and cleavage or that purine/pyrimidine substitutions (i.e., PyAATTPu, CPuATPyG, or CAPuPyTG) would suffice, we prepared three synthetic double stranded oligonucleotides, each of which contained three possible derivative recognition sequences (Figure 4, oligos 3, 4, and 5). Labeled DNA fragments were treated with extracts containing the restriction endonuclease activity. In contrast to the fragment containing the control sequence, CAATTG, none of these oligos were digested (Figure 3) demonstrating that the sequence CAATTG is both necessary and sufficient for cleavage. Although this sequence is the predominant recognition and cleavage site for MfeI, we cannot rule out the possibility that alternate recognition sites are being digested at a much lower efficiency. No enzyme has previously been identified with this recognition sequence (9).
To determine the exact bases where cleavage occurred, we next performed a primed synthesis assay. For this assay a radioactive second strand was synthesized from the M13 universal primer annealed to a template containing the restriction enzyme cleavage site (oligo 2, Figure 4). This double-stranded DNA was then treated either with extracts containing the MfeI enzyme activity (Figure 5, lane 1) or with a suitable control enzyme, HindIII (lane 3), which also cleaved the DNA. Following inactivation and phenol extraction, half of each reaction was treated with Klenow and unlabeled dNTP's to fill in any cohesive ends created by cleavage. HindIII cleavage occurred between the two A's in the sequence AAGCTT (Figure 5, lane 3), and filling in the cohesive ends created by digestion added an additional four bases (lane
4). A similar pattern of digestion and filling in was seen for Mfel at the recognition site CAATTG (lanes 1 and 2). Thus, Mfel cleavage occurred between the C and the A on each strand to generate four bases of 5' overhanging sequence:

C'A A T T G
G T T A A'C

DISCUSSION

The restriction endonuclease described, which we have named Mfel in keeping with traditional nomenclature, appears to be a typical type II enzyme in that it recognizes a short palindromic sequence and cleaves at a site within the recognition site (10). It represents the third reported restriction enzyme derived from the class Mollicutes. The others, SciNI and Uur960I, are produced by Spiroplasma citri (a helical wall-free plant pathogen) and Ureaplasma urealyticum (a mollicute of human origin), respectively (11,12). It is therefore reasonable to suspect that additional mycoplasma-derived restriction enzymes will continue to be discovered. Further, it is conceivable that Mfel can be used to develop a useful mycoplasma typing assay based on the detection of the activity of the enzyme or on the detection of the gene encoding it.

Mfel itself is potentially an extremely valuable enzyme for the molecular biologist since it cuts at a unique six base pair recognition site and is the only known restriction enzyme to generate cohesive ends which are compatible with EcoRI ends. As such, further evaluation of this enzyme appears warranted.

In summary, we have identified a restriction enzyme from M. fermentans MT-2/NJW which cleaves at the site C'AATTG. This activity was fortuitously noted while evaluating proteins capable of binding to the IL-2Rα promoter. Investigators should be aware of the possibility of such enzymatic activity contaminating extracts, especially if they are derived from mycoplasma contaminated cell lines.

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