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Repetitive DNA Elements Characteristic of Pathogenic *Entamoeba histolytica* Strains Can Also Be Detected after Polymerase Chain Reaction in a Cloned Nonpathogenic Strain

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Strains of *Entamoeba histolytica* which were isolated from symptomatic patients and which possess a characteristic pathogenic isoenzyme pattern (zymodeme) have extrachromosomal circular DNA molecules containing RNA genes and clusters of tandemly reiterated *Pvu*I elements. The nucleotide sequence of comparable reiterated *Bam*HI elements present in amebae with nonpathogenic zymodemes differs from that found in pathogenic ones. By using the polymerase chain reaction, it was demonstrated that the cloned, nonpathogenic *E. histolytica* strain SAW 1734R clAR also contains one or few of the tandemly repeated DNA *Pvu*I elements characteristic of the pathogenic amebae. Sequences were detected by hybridization with the P-145 probe after in vitro amplification. Because of technical difficulties, it was impossible to resolve whether single copies of the nonpathogenic *Bam*HI repetitive elements are present in pathogenic amebae. Our findings suggest that in the nonpathogenic amebae, the signal to start amplifying the *Pvu*I-type elements may be induced during the process of elimination of bacterial associates from their growth environment.

Numerous extrachromosomal circular DNA molecules containing rRNA genes as well as clusters of tandemly reiterated sequences have been found in *Entamoeba histolytica* (8). Recently, we have found that pathogenic (P) strains of *E. histolytica*, which possess a characteristic isoenzyme electrophoretic pattern (zymodeme) (16) have tandemly reiterated units different from those of nonpathogenic (NP) strains (6). The P repeats possess *Pvu*I elements, whereas the NP repeats have *Bam*HI fragments. By using the reiterated elements as DNA probes (P-145 for P strains and B-133 for NP strains), it was found that at high stringency, the probes hybridized specifically to the DNA of their own type of *E. histolytica* and not to the other. Examination of the hybridization specificity of the DNA isolated from the cloned NP strain SAW 1734R clAR revealed that it hybridized exclusively to probe B-133. On the other hand, the DNA obtained from the same strain hybridized with probe P-145 after its zymodeme had converted from NP to P during the process of elimination of the bacterial cells from the culture (10, 11). This finding raised the question of how the mutually exclusive reiterated elements can exist at different times in the same organism. The possibility that an unamplified copy of the alternative reiterated sequence also existed in the same cells was examined after in vitro amplification by the polymerase chain reaction (PCR) (15).

MATERIALS AND METHODS

Strains. The following *E. histolytica* strains were studied. The NP xenic strain used was SAW 1734R clAR. This strain was originally isolated and later cloned by P. G. Sargeaunt of the London School of Hygiene and Tropical Medicine. The NP strain was cultured in both TYSGM-9 medium (3) and TYI-S-33 medium (4) together with a reduced amount of bacterial flora (11). The P strains were HM-1:IMSS cl6 and the cloned SAW 1734R clAR, which originally possessed an NP zymodeme and had converted to P during the process of axenization (11). These strains were axenized by L. S. Diamond, National Institutes of Health, Bethesda, Md., and grown in TYI-S-33 axenic medium in plastic flasks (40 ml).

The P and NP isoenzyme patterns of each of the cultures were confirmed by the electrophoretic migration of the hexokinase and phosphoglucomutase as previously described (11). DNA from the *E. histolytica* strains were prepared as described previously (6).

DNA probes. Restriction enzyme digestion of clone H16 from a genomic library of pathogenic *E. histolytica* HM-1:IMSS (6) with *Pvu*I produced on agarose gel electrophoresis a ladder of fragments with a monomer of 145 base pairs (bp). The monomer fragment was removed from the agarose and used as a hybridization probe designated P-145. Restriction enzyme digestion of clone B3.0 from a genomic library of NP SAW 1734R clAR (6) with *Bam*HI produced on agarose gel electrophoresis a ladder of fragments with a monomer of 133 bp which was used as a probe designated B-133. DNA probes were labeled by random priming with a Boehringer GmbH (Mannheim, Federal Republic of Germany) labeling kit. Synthetic oligonucleotide primers, based on the sequence of the *Pvu*I fragment, for both sense and antisense directions were prepared with an automatic synthesizer. Primer P_I extended from nucleotide 9 to nucleotide 31; its structure is described in Fig. 1. Primer P_{II} was from the complementary strand (nucleotides 144 to 124) (Fig. 1). Primers were purified by gel electrophoresis before use. Synthetic oligonucleotide primers (B_I and B_{II} corresponding to sequences in nonhomologous areas of the shorter *Bam*HI fragment present in NP *E. histolytica* were also prepared (Fig. 1). Unfortunately, primers B_I and B_{II} were not useful because they dimerized in the PCR in the absence of DNA (not shown).

PCR. The PCR (15) was carried out as follows. Reaction

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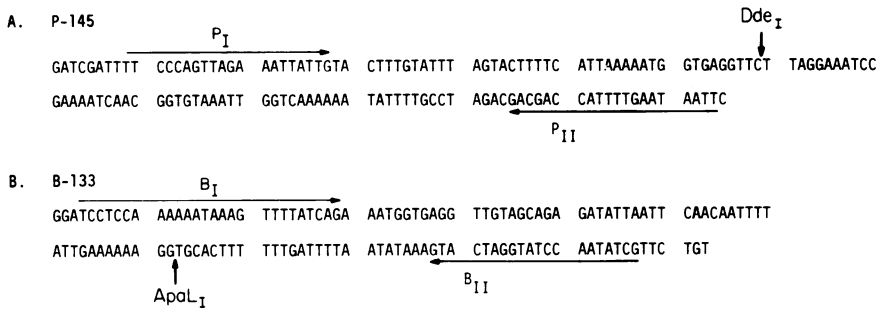

FIG. 1. Nucleotide sequence of the reiterated element P-145 (A) found in *E. histolytica* P strain HM-1:IMSS and element B-133 (B) from cloned NP strain SAW 1734R clAR (2). A cluster of 15 tandemly reiterated elements of this type has been located on palindromic circular DNA episomes of approximately 26 kilobases (6, 8). Synthetic oligonucleotide primers in sense and antisense directions (P_I, P_{II}, B_I, and B_{II}) were prepared with an automatic synthesizer. Restriction enzymes (*Dde*I for P-145 and *Apa*LI for B-133) which have only one cleavage site along each of the monomeric elements were selected for analysis of DNA fragments generated by PCR.

mixtures (100 μl) containing ameba DNA template (1 μg), *Thermus aquaticus* DNA polymerase (2.5 U), appropriate primers (50 pmol), deoxynucleotide triphosphates (20 nmol each), and the necessary buffers and additives were prepared by the procedure devised by Cetus Corp., Emeryville, Calif. (14). As controls, samples without DNA or containing foreign DNA template (a 500-nucleotide segment of bacteriophage lambda DNA; Cetus) were included. The amount of *E. histolytica* DNA added was 1 μg. A thermal cycler (MJ Research, Inc., Cambridge, Mass.) was programmed as follows: initial melting for 90 s at 94°C followed by cycles of 1 min at 37°C, 1 min at 72°C, and 1 min at 94°C. The gradual temperature increase used was 15°C/min. This cycle was repeated 25 times, after which the last step was 8 min at 72°C, and the reaction mixture was cooled to 4°C.

After the reaction, samples were size fractionated on agarose gels (1% or 1.5%) and transferred to Zetapore (CUNO Inc., Meriden, Conn.) nylon membrane blots. After the blots were baked, hybridization with radiolabeled probe P-145 or B-133 was carried out under high-stringency conditions (50% formamide, 42°C, 16 h) followed by stringent washings. The washing procedure began with several washings with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate. The washings were then continued with 0.1× SSC and 0.1% sodium dodecyl sulfate at 65°C for 20 min and were continued further with the same buffer at room temperature. The blots were exposed to X-ray film for 16 h. Other samples from the PCR products were digested with restriction enzyme *Dde*I or *Apa*LI before fractionation on agarose gels. The size markers used were commercially available *Hin*dIII digests from lambda or *Hae*III digests from φX174 phage.

RESULTS AND DISCUSSION

Recent results obtained with DNA probe P-145, which is specific for *E. histolytica* strains having P zymodemes, and probe B-133, which is specific for *E. histolytica* strains having NP zymodemes, have shown that these probes were able to discriminate among more than 80 different clinical isolates and correlated perfectly with the P and NP zymodemes of the strains (2, 6). As previously mentioned (6), faint hybridization signals were observed with probe P-145 in DNA blots of NP *E. histolytica* SAW 1734R clAR after prolonged exposure (30 days) of the blots to X-ray films. The observed signals were several orders of magnitude weaker than those obtained with probe B-133, but they suggested that the NP strain may contain, in addition to the abundant *Bam*HI repetitive sequences, a copy or a few copies of the *Pvu*I element, which is characteristic of P strains (2, 6). One possibility that was considered was that perhaps *E. histolytica* strains possess similar genomic repertoires containing copies of both types of extrachromosomal circular DNA molecules, but under metabolic or physiological conditions, such as growth in the presence or absence of bacteria, the amebae may selectively amplify either one of the extrachromosomal elements. Such a hypothesis could be tested by using PCR to search the DNA of an NP organism for the unamplified copies of the characteristic *Pvu*I reiterated sequences of P amebae.

Incubations of DNA isolated from the cloned xenically grown NP strain SAW 1734R clAR together with the sense and antisense primers, P_I and P_{II} (Fig. 1), yielded products that positively hybridized on Southern blots under stringent conditions with the radiolabeled probe, P-145, which is specific for P organisms (Fig. 2). Because of the concatemeric nature of the sequences of the reiterated elements, priming most likely occurred at various locations, resulting in a ladderlike formation of reaction products (Fig. 2). As expected, PCRs containing primers P_I and P_{II} greatly enhanced the presence of the *Pvu*I concatemeric elements in the DNA of both of the pathogenic strains tested (HM-1:IMSS and axenic SAW 1734R clAR). The P-145 monomeric subunit was used as an amplification control (Fig. 2). In the absence of DNA template, the primers P_I and P_{II} did not dimerize after PCR, and no hybridization signal was observed. Also, no signal was obtained when the B-133 monomeric subunit was used as a DNA template together with the P_I and P_{II} primers. Additional controls containing as a template a 500-nucleotide fragment of lambda DNA, together with primers P_I and P_{II}, also failed to give any hybridization signals with P-145 (Fig. 2). Moreover, no hybridization with P-145 was observed when primers P_I and P_{II} were added to the DNA of the NP strain that was not subjected to the PCR (not shown). As previously shown (2, 6), hybridization of the DNA from the NP SAW 1734R clAR with radiolabeled probe B-133 gave a distinct signal, but this probe did not hybridize with the DNA of the P strain HM-1:IMSS or SAW 1734R clAR (2, 6).

Verification that the sequences in the various amplified DNA elements produced in the PCR experiment were identical to the sequences originally identified was obtained by restriction enzyme analysis of the DNA fragments generated by the PCR. Digestion of the P_I and P_{II}-primed PCR products with *Dde*I, which has a cleavage site in the center of the

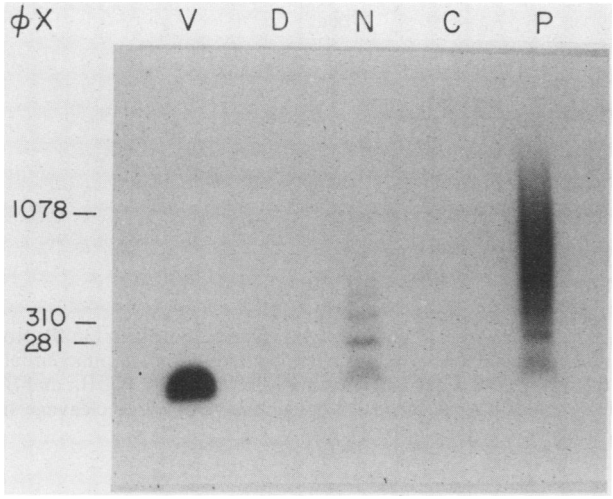

FIG. 2. Hybridization on Southern blots with ^{32}P-translated DNA probe P-145 (P) of the PCR products. Incubation mixtures (100 μl) contained DNA (1 μg), sense and antisense primers (50 pmol), and a mixture of the four nucleotides (deoxynucleoside triphosphates) (25 nmol of each). PCR was performed for 25 cycles with *T. aquaticus* DNA polymerase (2.5 U; Cetus) as described in Materials and Methods. PCR mixtures contained the following: V, monomeric *Pvu*I fragment isolated from DNA digests of strain HM-1:IMSS (6) together with P_I and P_{II} primers; D, DNA template, a 500-nucleotide fragment from phage lambda (Cetus) together with primers P_I and P_{II}; N, DNA from xenic cultures of *E. histolytica* NP strain SAW 1734R clAR (11) together with primers P_I and P_{II}; C, Primers P_I and P_{II} without any DNA template; P, DNA from axenic cultures of *E. histolytica* P strain SAW 1734R clAR (11) together with primers P_I and P_{II}. Size markers used were digests from lambda and φX174 phages. Reaction products were separated on an agarose gel (1.5%), blotted on Zeta filter paper (Zetapore; CUNO), and probed under stringent conditions as described elsewhere (6).

*Pvu*I fragment (Fig. 1), yielded one major product, which was smaller than the monomer (Fig. 3). No digestion was observed with *Apa*LI, which has a cleavage site only in the NP fragment B-133 (Fig. 1 and 3).

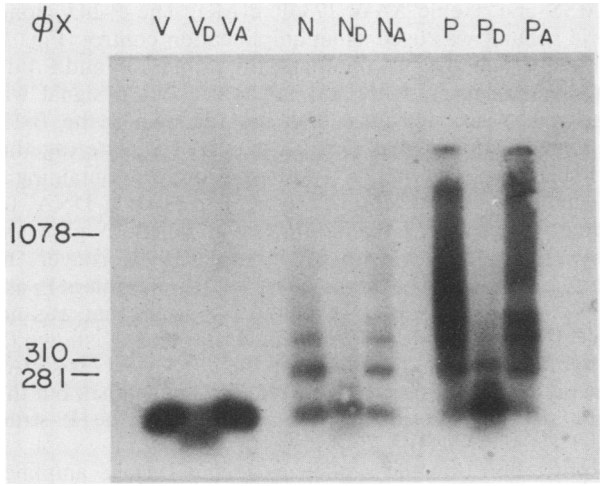

FIG. 3. Hybridization on Southern blots with ^{32}P-labeled P-145 of PCR products (Fig. 2) after digestion with either *Dde*I (D) or *Apa*LI (A). Samples were taken from reactions V, N, and P as described in the legend to Fig. 2. Size markers were digests of φX174.

The second question, whether sequences specific for NP *E. histolytica* also exist in P strains, was not resolved because of artifacts encountered in the PCR when the synthetic oligonucleotide primers B_I and B_{II}, specifically prepared for sequences of the reiterated element B-133, were used (Fig. 1). These primers were not useful because they dimerized in the PCR reaction even in the absence of DNA and also gave positive signals with a template of lambda DNA. Because of the considerable homology (43.7%) which exists between the DNA sequences of subunits B-133 and P-145 (6), it may be difficult to choose other primers that will not cross-hybridize.

A number of NP *E. histolytica* strains have been converted into strains with P zymodemes during the process of elimination of bacterial associates from trophozoite cultures (1, 9–11). The newly converted amebae had all the characteristics of p amebae, such as virulence (11), specific antigens (17; W. A. Petri, T. F. H. G. Jackson, and D. Mirelman, Proc. Meet. Am. Soc. Trop. Med. Hyg., abstr. no. 378, 1989), and hybridized with DNA probe P-145 (2, 6). Our finding that, in addition to the numerous copies of the NP reiterated sequences, an unamplified copy (or copies) of the P sequence exists in trophozoites of the cloned NP *E. histolytica* strain SAW 1734R clAR, suggests that this ameba can selectively amplify one type of extrachromosomal element while retaining a master copy of the other. Structurally distinct stage-specific ribosomes as well as selective gene amplifications from chromosomal templates have been described for other systems (5, 8, 12–14). The signal to start amplifying the P type of extrachromosomal molecules may be induced by the metabolic and physiological changes that occur during the process of axenization and the drastic alterations in the amebic growth conditions. Further characterization of this switch, including molecular analysis of the mechanisms involved, may further our understanding of the pathogenic process in amebiasis.

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