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DNA Probes Specific for *Entamoeba histolytica* Possessing Pathogenic and Nonpathogenic Zymodemes

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A number of DNA probes which hybridize to highly abundant DNA sequences of *Entamoeba histolytica* were developed. Variations in the hybridization patterns of different *E. histolytica* strains were detected with selected probes. Four types of restriction fragment length patterns were obtained. Of these, the first class belonged to *E. invadens* and *E. histolytica*-like var. Laredo. The next two classes consisted of various strains of *E. histolytica* which were originally isolated from symptomatic patients and possessed pathogenic patterns of isoenzymes (zymodemes), whereas the fourth group contained *E. histolytica* strains with nonpathogenic zymodemes obtained from asymptomatic carriers. DNA probes, based on DNA sequences specific to *E. histolytica* isolates with pathogenic and nonpathogenic zymodemes were isolated, and their nucleotide sequences were determined. These probes (P145 and B133) hybridized selectively to DNA of isolates possessing either pathogenic or nonpathogenic isoenzyme patterns. The newly developed probes could be useful for diagnostic purposes and could serve as tools to investigate the molecular basis of pathogenicity and the genetic mechanisms which regulate the variable aggressive behavior of the parasite.

Entamoeba histolytica, the protozoan parasite which is the causative agent of amebiasis, inhabits the intestinal tracts of nearly 500 million people worldwide (19) and leads to about 75,000 deaths per year (7). The majority of infected persons are described as asymptomatic carriers, and only about 10% develop symptoms of invasive amebiasis. *E. histolytica* isolated from symptomatic individuals have been shown to differ in isoenzyme electrophoretic patterns, or zymodemes, from those of amoebae obtained from most asymptomatic carriers (17). This distinction has been the basis for the designation of pathogenic and nonpathogenic strains. Trophozoites possessing pathogenic zymodemes could also be distinguished by their interaction with certain monoclonal antibodies (18).

The molecular basis for pathogenicity or nonpathogenicity is still unknown. In fact, there are two opposing hypotheses to explain the very nature of pathogenicity. One hypothesis proposes that pathogenic and nonpathogenic isolates represent distinct subspecies of *E. histolytica* (17). The opposing view is based on the recent observation that isoenzyme conversion from nonpathogenic to pathogenic can occur within a cloned culture of a strain of *E. histolytica* during the process of axenization under the appropriate growth conditions (13–15). To gain more insight into these questions, we have developed a number of DNA probes that distinguish between isolates of *E. histolytica* on the basis of restriction fragment length polymorphisms. This set of probes, which contains sequences specific for isolates possessing pathogenic or nonpathogenic zymodemes, shows potential for use in a rapid test for the identification of *E. histolytica* and may prove useful tools for the understanding of the molecular mechanisms which determine the pathogenic nature of a given isolate.

MATERIALS AND METHODS

***Entamoeba* isolates and culture conditions.** *E. histolytica* strains with pathogenic zymodemes used in this study were the following. Axenic strains HM-1:IMSS cl6, 200:NIH, and Rahman were obtained from L. S. Diamond, National Institutes of Health, Bethesda, Md. Another axenic culture of HM-1:IMSS was provided by J. I. Ravdin, University of Virginia, Charlottesville. Strains SAW 1734R clAR and CDC:0784:4 were axenized by L. S. Diamond from xenic cultures which originally possessed nonpathogenic zymodemes and had converted to pathogenic ones during the process of axenization (14, 15). The above-mentioned strains were all grown in axenic medium TYI-S-33 (4) in plastic flasks (40 ml). Two additional strains with pathogenic zymodemes, SAW 408 (zymodeme group II) and SAW 755 (zymodeme group XIV), originating from P. G. Sargeaunt, London School of Hygiene and Tropical Medicine, London, England), were grown together with their original bacterial associates in TYSGM-9 medium (3).

Xenic strains of *E. histolytica* with nonpathogenic zymodemes used in this study were the following: SAW 1734R clAR (group III), SAW 760 (group IX), CDC:0784:4 (group I), and WI:1285:1 (group I). The first two strains were originally isolated by and obtained from P. G. Sargeaunt. These strains were cultured in both TYSGM-9 medium (4) and TYI-S-33 medium (3), together with a reduced bacterial flora in the presence of an antibiotic mixture (cefotaxime, erythromycin, and amikacin at final concentrations of 50 μg/ml each) (14). The last two strains were grown together with their bacterial associates only in TYSGM-9 medium (3).

E. histolytica var. Laredo and *E. invadens* IP-1 were also obtained from L. S. Diamond and were grown axenically in TYI-S-33 medium at 27°C.

The pathogenic or nonpathogenic isoenzyme pattern of each of the cultures was confirmed by the electrophoretic

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migration of the hexokinase and phosphoglucomutase isoenzymes, as described previously (14, 15).

Construction of genomic libraries. DNA from *E. histolytica* HM-1:IMSS cl6 was prepared as described before (9), digested with *Eco*RI or *Hin*dIII, inserted into *Eco*RI- or *Hin*dIII-digested pUC18 DNA, and transformed into *Escherichia coli* DH1 (8). Clones containing highly abundant genomic DNA sequences were identified by colony filter hybridizations with nick-translated total genomic DNA as probe. Hybridizations were carried out as described previously (9). This screening yielded probes H6.6, R2.3, R715, and H4.4 with insert sizes of 6.6, 2.3, 0.9, and 4.4 kilobases (kb), respectively. An additional clone, H16, with a 14.3-kb insert contained a repetitive fragment of 145 base pairs (bp) which was released by digestion with *Sau*3AI and subcloned in the *Bam*HI site of pUC18. This subclone was designated P145.

DNA from the nonpathogenic strain *E. histolytica* SAW 1734R clAR was prepared as described previously (9) from trophozoites grown in the presence of reduced amounts of associated bacterial cells and starch particles. Xenic cultures of this organism were grown in TYI-S-33 medium (4) in 40-ml plastic flasks containing a mixture of antibiotics consisting of cefotaxime, erythromycin, and amikacin, at a final concentration of 50 μg/ml each. *Bgl*II-digested DNA was cloned in the *Bam*HI site of pUC8 and screened by hybridization with clone R715 (see Results), giving clone B2342. This clone had a 11.5-kb insert which contained a tandemly repeated *Bam*HI fragment of 133 bp. A 3.0-kb *Eco*RI fragment containing these reiterated units was subcloned in *Eco*RI-digested pUC8 as clone B3.0, and the 133-bp *Bam*HI repeat element was also subcloned in pUC8 and designated B133.

DNA sequence analysis. DNA sequencing was performed by the method of Sanger et al. (16) after subcloning of restriction fragments into phage vectors M13mp18 and M13mp19 (12). Sequence data were analyzed by using the MicroGenie program (Beckman Instruments) and the Fast P program of David J. Lipman and William R. Pearson (10).

Southern blot hybridizations. Genomic DNA (approximately 0.5 μg) isolated from the various *Entamoeba* strains was digested with *Eco*RI, size fractionated on 0.8% agarose gels in TAE buffer, pH 7.8 (100 mM Tris hydrochloride, 50 mM sodium acetate, 5 mM EDTA), transferred to nitrocellulose, and hybridized as described previously (9). DNA probes were labeled by nick translation. Exposures for 2 h, using two intensifying screens at −70°C, were generally sufficient to give strong signals. For reuse, blots were treated three times with buffer containing 0.01× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.01% sodium dodecyl sulfate for 5 min at 100°C to remove previously hybridized probe. Removal of probe was verified by autoradiography. DNA dot blot hybridizations were done under the same conditions.

Hybridizations of intact trophozoites on Zetapore filters. Trophozoites (2×10^5 cells) grown in either of the culture media used were harvested after chilling (5 min) and suspended in TYI-S-33 medium (5 ml without serum). The suspension containing the trophozoites was passed through a Zetapore membrane filter on a Büchner funnel, using a weak suction. The cells tended to concentrate into dots at locations on the membrane filter corresponding to the underlying holes in the Büchner funnel such that each dot contained about 4,000 cells. The cells were washed with phosphate-buffered saline, and the Zetapore filter was placed for 15 min on a Whatman 3MM filter saturated with a solution of detergent Nonidet P-40 (1%) in TAE buffer containing 25 μg of protease K per ml. The Zetapore filter was blotted several times over a dry Whatman 3MM filter and then placed on another Whatman 3MM filter saturated with a DNA denaturing solution consisting of 0.5 M NaOH and 1.5 M NaCl. Intermittent blotting was performed for 15 min, after which the Zetapore filters were neutralized by placing them on another Whatman 3MM filter saturated with Tris buffer, pH 7.4. After this step, the filters were washed, cut into halves, and hybridized with probe P145 and B133, as described above for Southern blots.

RESULTS

Upon digestion of HM-1:IMSS DNA with *Eco*RI and size fractionation on agarose gels, a distinct series of bands was visible after staining with ethidium bromide. *Hin*dIII digestion similarly resulted in a number of distinct bands. These bands indicate the presence of highly abundant DNA sequences. A number of these elements were cloned as a first step toward understanding the DNA sequence organization of *E. histolytica*. Using the procedures detailed in Materials and Methods, we obtained several clones, of which H16, H6.6, R2.3, R715, and H4.4 are described in this paper. Clone B3.0 was subcloned from a highly abundant *Bgl*II fragment from the DNA of the nonpathogenic *E. histolytica* isolate SAW 1734R clAR.

DNA of several strains of *E. histolytica* with pathogenic and nonpathogenic zymodemes was digested with *Eco*RI, size fractionated on agarose gels, and transferred to nitrocellulose filters. Figure 1 shows the hybridization pattern obtained after probing one such filter with H6.6, a probe derived from a genomic library of *Hin*dIII-digested *E. histolytica* HM-1:IMSS cl6 DNA in plasmid pUC18. The autoradiogram shown in Fig. 1 contains DNA from several isolates of *E. histolytica* as well as DNA from *E. histolytica* var. Laredo, a variant of *E. histolytica* with altered growth characteristics and generally regarded as a species different from *E. histolytica* (2, 5), and *E. invadens*, a low-temperature-growing species which infects reptiles. Two main trends are immediately apparent in Fig. 1. First, all of the *E. histolytica* isolates had two bands (0.8 and 0.9 kb) in common. These bands were absent in *E. invadens* and *E. histolytica* var. Laredo strains. Second, there was variability in the hybridization patterns obtained from the various *E. histolytica* strains. One class of isolates consisted of the axenically grown strains HM-1:IMSS, HM-1:IMSS cl6, and CDC:0784:4 (lanes 2, 4, and 7), all of which possessed pathogenic zymodeme group II. Strains Rahman and NIH:200 (lanes 1 and 3), which also had pathogenic zymodeme group II, comprised a second class. Strains SAW 1734R clAR and WI:1285:1, which possessed nonpathogenic zymodemes (groups III and I, respectively; lanes 5 and 6), comprised a third class.

Some of our probes highlight specific subsets of the bands visible in Fig 1. Thus, probe R2.3, also derived from HM-1:IMSS cl6, gave the pattern seen in Fig. 2A. This pattern, although simpler than that of Fig. 1, still allows the classification of the strains into the same three groups. Another probe derived from HM-1:IMSS cl6, R715, hybridized to only one band common to all of the *E. histolytica* strains (Fig. 2B). This probe did not hybridize to *E. histolytica* var. Laredo or *E. invadens* (latter not shown).

Probe H4.4, which was also derived from strain HM-1:IMSS cl6, did not hybridize to DNA from *E. histolytica* SAW 1734R clAR and WI:1285:1, which have nonpathogenic zymodemes (Fig. 3A). The striking absence of hybrid-

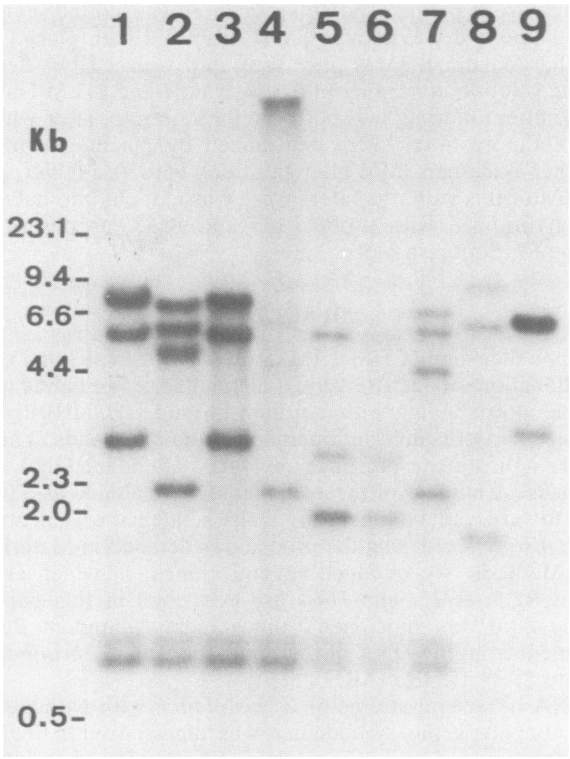

FIG. 1. Classification of *E. histolytica* isolates based on hybridization patterns, using probe H6.6. The Southern blot shown contained *Eco*RI-digested DNAs from the following sources. Lanes 1 to 7, *E. histolytica* isolates: lane 1, Rahman; lane 2, HM-1:IMSS (from J. I. Ravdin); lane 3, 200:NIH; lane 4, HM-1:IMSS cl6 (from L. S. Diamond); lane 5, SAW 1734R clAR (from P. G. Sargeaunt); lane 6, WI:1285:1; lane 7, CDC:0784:4 (axenic culture from L. S. Diamond). Lane 8, *E. histolytica* var. Laredo. Lane 9, *E. invadens*.

ization by H4.4 to DNA of SAW 1734R clAR and WI:1285:1 indicates that these isolates lack some of the DNA sequences common to all the other pathogenic *E. histolytica* strains tested. Moreover, the probe did not hybridize to *E. histolytica* var. Laredo DNA (lane 8), *E. invadens* DNA (lane 9), or rat DNA (not shown). That this probe did not hybridize to certain *E. histolytica* strains was not due to lack of amebic DNA on the blots or to dilution by bacterial DNA originating from the xenic cultures. Sufficient amebic DNA was present, as seen by reprobing the same blots with probe R715, which hybridized to the common bands (0.8 and 0.9 kb, Fig. 2B), or probe B3.0 (Fig. 4).

Restriction enzyme analysis of clone H16 revealed a *Pvu*I fragment of 145 bp which was repeated about 15 times. When this fragment, released by digestion with *Sau*3AI, was subcloned into the *Bam*HI site of pUC18 and used as a hybridization probe, the pattern shown in Fig. 3B was obtained. Clone P145 hybridized to a subset of the bands visualized by clone H4.4 (Fig. 3A). Interestingly, clone H4.4 had no *Pvu*I sites and hybridized very weakly with clone P145 (not shown). In addition, hybridization with P145 was observed with dot blots of DNA of xenically grown strain SAW 755 which has pathogenic zymodeme group XIV, but not with that from the nonpathogenic strain SAW 760 or CDC:0784:4 (Fig. 5 and 6).

This exciting finding of sequences specific to isolates of *E. histolytica* with pathogenic zymodemes prompted us to search and test DNA probes derived from isolates with nonpathogenic zymodemes for their specificity.

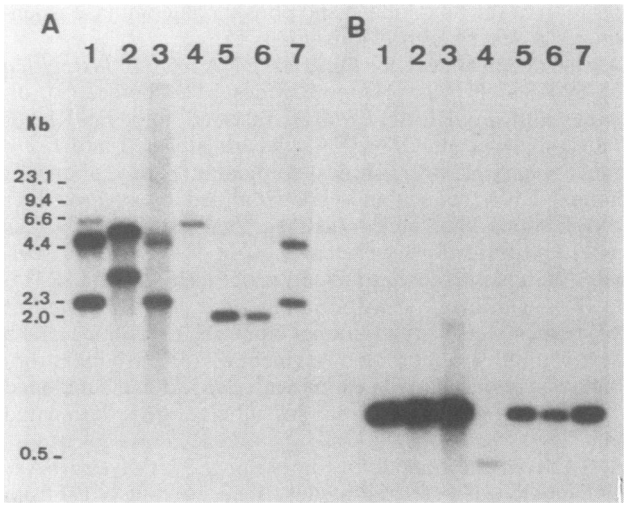

FIG. 2. Probes which demonstrate differences (A) and similarities (B) between isolates. Southern blot hybridizations were both prepared from the same blot after removal of probes as described in Materials and Methods. (A) Probe R2.3; (B) probe R715. *E. histolytica* isolates used (except where noted) were: lane 1, HM-1:IMSS; lane 2, NIH:200; lane 3, HM-1:IMSS cl6; lane 4, *E. histolytica* var. Laredo; lane 5, SAW 1734R clAR; lane 6, WI:1285:1; lane 7, CDC:0784:4 (axenic).

Probe B3.0 derived from a genomic library of strain SAW 1734R clAR, which has a nonpathogenic zymodeme, hybridized only to DNA of the xenically grown nonpathogenic strains SAW 1734R clAR and WI:1285:1 and not to DNA of any of the pathogenic strains tested (Fig. 4).

Restriction enzyme analysis of clone B3.0 revealed a *Bam*HI fragment of 133 bp which was repeated about 14 times. When this fragment (B133) was released, subcloned into the *Bam*HI site of pUC8, and used as a probe, it hybridized only to DNA of strains SAW 1734R clAR, WI:1285:1, SAW 760, and CDC:0784:4, which had nonpathogenic zymodemes (Fig. 5 and 6). Probe B133 did not hybridize to any of the strains that possessed pathogenic zymodemes, including the xenically grown strain SAW 755. Patterns of hybridization could be observed on either nu-

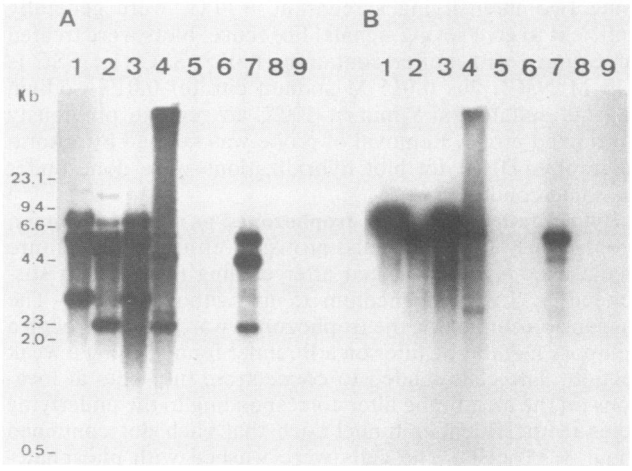

FIG. 3. Hybridization of (A) probe H4.4 and (B) probe P145 exclusively to DNA from pathogenic isolates of *E. histolytica*. Samples are the same as given in the legend to Fig. 1.

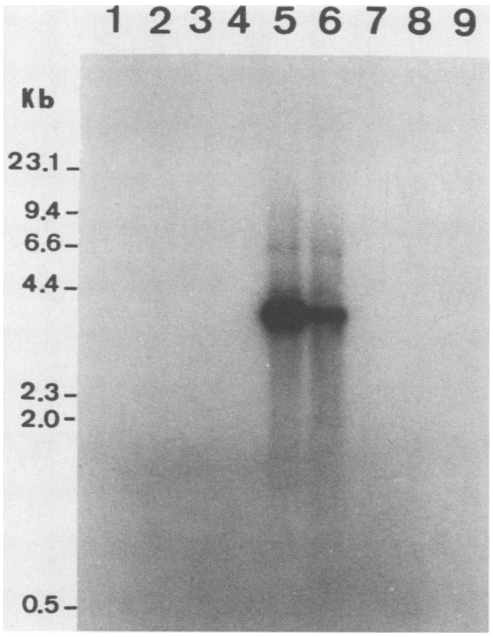

FIG. 4. Hybridization of probe B3.0 exclusively to DNA from nonpathogenic isolates of *E. histolytica*. Samples are the same as given in the legend to Fig. 1.

clear DNA dot blots (Fig. 5) or Southern blots of *Eco*RI-digested DNA (not shown) or with DNA from intact *E. histolytica* trophozoites which were present at a maximum concentration of 4,000 cells per dot after filtration through a Zetapore membrane filter (Fig. 6). Countertesting of the same filters with probe P145 revealed that this probe hybridized only with the strains that had pathogenic zymodemes (HM-1:IMSS, SAW 755, and SAW 408) and not with the nonpathogenic ones. The sensitivity of these hybridization tests indicates that the abundant sequences can be detected in less than 4,000 trophozoites after an 18-h exposure. It remains to be seen, however, whether the sensitivity of our present probes will be sufficient for the direct detection of *E. histolytica* trophozoites from human stools without culture enhancement.

Very faint signals were observed in dot blots of DNA from xenically grown strain CDC:0784:4 upon hybridization with probe P145 (Fig. 5) and with strain SAW 755 probed with B133. The total amount of radioactivity found upon counting of the dot blots was <1% of that obtained with the other probes (H6.6, P145, or B133,), using approximately the same amounts of DNA.

Previous investigations had shown that, during the process of axenization of two isolates possessing nonpathogenic zymodemes (CDC:0784:4 and SAW 1734R clAR), a conversion to pathogenic zymodemes occurred (14, 15). Analysis of the hybridization patterns of the DNA from trophozoites of the cloned strain SAW 1734R clAR grown either under axenic conditions (where it has pathogenic zymodemes) or from xenic cultures (where it has nonpathogenic zymodemes) with probe H6.6 on Southern blots (Fig. 7) revealed that the DNA pattern from axenic cultures resembled that of pathogenic strain 200:NIH, whereas the xenic one was similar to that of nonpathogenic strain WI:1285:1 (Fig. 1). Moreover, the DNA from the axenically grown strain CDC:0784:4 (which had a pathogenic zymodeme) also displayed a hybridization pattern similar to that of a strain with pathogenic zymodemes (Fig. 1), whereas the DNA from xenically

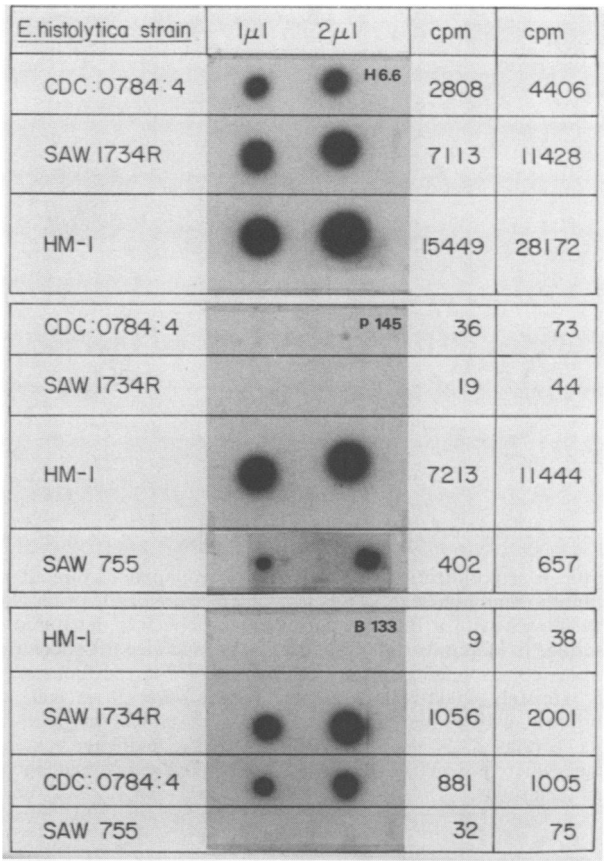

FIG. 5. DNA dot blot hybridization. DNAs from various strains of *E. histolytica* were hybridized on Zetapore membranes with probes H6.6, P145, and B133. Strains CDC:0784:4 and SAW 1734R clAR had nonpathogenic zymodemes, whereas strains HM-1:IMSS and SAW 755 had pathogenic ones. Strains CDC:0784:4 and SAW 755 were grown with their bacterial flora in Diamond's TYSGM-9 culture medium. After exposure (16 h) to X-ray film, the spots were cut and counted in a scintillation counter. Counting results given are those obtained after subtraction of background (18 cpm).

cultured trophozoites of the same strain, which had a nonpathogenic zymodeme, hybridized with probe B133 and not with P145 (Fig. 5).

The DNA sequences of both reiterated fragments P145 and B133 were determined after subcloning into phage M13mp18 (12) (Fig. 8). Comparison of the sequences (Fig. 9) shows only about 44% best-fit alignment between the two fragments.

DISCUSSION

DNA probes have been shown recently to be useful in the diagnosis of trypanosome varieties (11), leishmania, malaria (1), and onchocerciasis (6). DNA probes that can specifically detect *E. histolytica* have also been reported recently by Bhattacharya et al. (2). The DNA probes described in the present study allow for not only the specific detection of *E. histolytica* but also their subclassification according to their pathogenic or nonpathogenic zymodemes. This finding was confirmed in nine different isolates of *E. histolytica* possessing pathogenic (five strains) and nonpathogenic zymodemes. Based on the hybridization patterns obtained on Southern blots, our set of probes could also distinguish between two subsets of isolates with pathogenic zymodemes. The origin

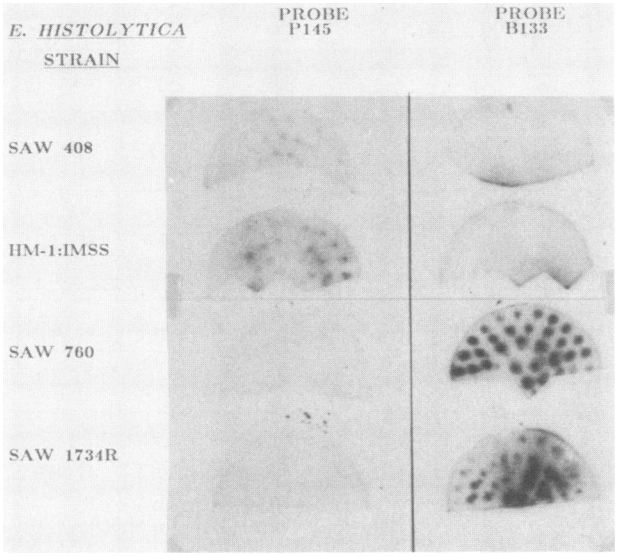

FIG. 6. Hybridization of *E. histolytica* trophozoites directly on Zetapore membrane filters. Suspensions of trophozoites (2×10^5) were filtered with a Büchner funnel, and DNA was denatured as described in Materials and Methods. The cells tended to concentrate in spots corresponding to the underlying holes in the funnel with approximately 4,000 cells per spot. Filters were cut in half and hybridized with probe P145 and B133 as described for Southern blots. Strains SAW 408 and HM-1:IMSS had pathogenic zymodemes, whereas SAW 1734R clAR and SAW 760 had nonpathogenic zymodemes.

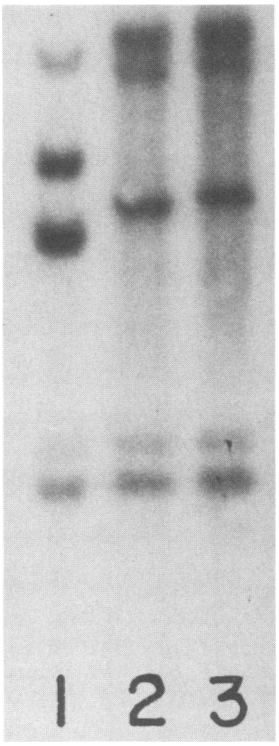

FIG. 7. Southern blot hybridization with probe H6.6. *Eco*RI-digested DNA from (1) xenic cultures of DNA from *E. histolytica* SAW 1734R clAR originally obtained from P. G. Sargeaunt; (2) axenic cultures of *E. histolytica* SAW 1734R clAR obtained from L. S. Diamond after they had converted to pathogenic zymodeme; (3) axenic cultures of *E. histolytica* HM-1:IMSS cl6.

of the subsets among the pathogenic isolates is unclear. There may be subtle differences in the pathogenicity of the two subsets, or the differences may be entirely unrelated to pathogenicity. Indeed, as with the whole question of zymodemes (13), we do not know yet whether the specificity of probes H4.4 and P145 for isolates with pathogenic zymodemes and probes B3.0 and B133 for isolates with nonpathogenic zymodemes is causally connected in any way with the degree of pathogenicity of these isolates. Yet the correlation exists, as observed with various isolates from each class (Fig. 3 to 7), and if further studies confirm our present results, a rapid assay that uses short (20-bp) synthetic oligonucleotide probes can be developed and used as a tool to help in the diagnosis of amebic infections and to control the high worldwide incidence of amebiasis (19).

Two of the *E. histolytica* strains investigated in this study, CDC:0784:4 and SAW 1734R clAR, possess nonpathogenic zymodemes when grown in xenic cultures together with bacteria. We have found that, during the process of axenization, an unexpected conversion to pathogenic zymodemes occurred with these two strains of ameba (14, 15). Analysis of the hybridization patterns obtained with the various DNA probes with each of the ameba in the two forms of growth medium (xenic or axenic) shows that it coincided with the respective zymodeme of the culture; i.e., the bacterium-ameba cultures from both strains hybridized identically to the other nonpathogenic strains tested, whereas the axenically grown amebae hybridized like the pathogenic ones. As previously stated (14), the molecular mechanisms involved in the conversions and changes in zymodemes are not yet known, and although they were also observed with a cloned culture (15), at present we still cannot totally exclude the possibility that they were due to the selection of a previously existing but undetectable subpopulation. It is tempting to speculate, however, that perhaps all *E. histolytica* strains contain copies of the same sequences in their genomes, but changes in certain conditions of growth may cause the ameba to amplify different elements and express modified amebic components and behaviors which would remain undetected under other culture conditions of growth. Although the present sensitivity of our probes is not sufficient to allow for the detection of one or two genomic master copies which may be present in all *E. histolytica* strains

A

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        10         20         30         40         50         60
GATCGATTTT CCCAGTTAGA AATTATTGTA CTTTGTATTT AGTACTTTTC ATTAAAAATG

        70         80         90        100        110        120
GTGAGGTTCT TAGGAAATCC GAAAATCAAC GGTGTAAATT GGTCAAAAAA TATTTTGCCT

       130        140
AGACGACGAC CATTTTGAAT AATTCGATC
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B

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        10         20         30         40         50         60
GGATCCTCCA AAAAATAAAG TTTTATCAGA AATGGTGAGG TTGTAGCAGA GATATTAATT

        70         80         90        100        110        120
CAACAATTTT ATTGAAAAAA GGTGCACTTT TTTGATTTTA ATATAAAGTA CTAGGTATCC

       130
AATATCGTTC TGTGGATCC
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FIG. 8. DNA sequences of (A) probe P145 isolated from *E. histolytica* HM-1:IMSS cl6 and (B) probe B133 obtained from *E. histolytica* SAW 1734R clAR, possessing nonpathogenic zymodemes. Sequences were determined by the dideoxy chain termination method of Sanger et al. (16) as described in Materials and Methods.

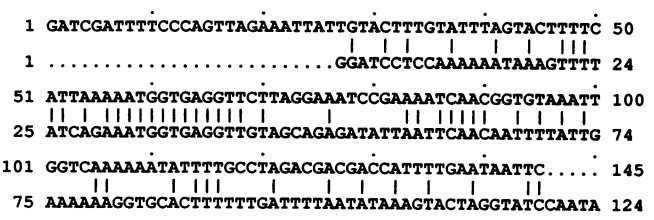

FIG. 9. Best-fit sequence alignment between P145 and B133. The calculated similarity between the two sequences was 43.6%.

under all circumstances, a faint hybridization signal was observed with probe P145 in DNA blots of the nonpathogenic strain CDC:0784:4 and with probe B133 in DNA blots of the pathogenic strain SAW 755 (Fig. 5). The observed signals were at least severalfold weaker than those obtained with the other probe, and this suggests that these particular strains may contain, in addition to the amplified sequences of their own, several copies of the other nonamplified sequence.

Very little is still known about the genome organization in *E. histolytica*. The origin and identity of the various DNA probes and the differences detected in the nucleotide sequences of the reiterated elements P145 and B133, described in this study, are currently under investigation (9a). Aside from the question of pathogenicity, the existence of restriction fragment length polymorphism in *E. histolytica* isolates poses important questions as to the mechanism of variation and fixation of these DNA patterns in populations of an organism in which sex is believed not to exist. The probes we describe may aid in answering those questions.

ACKNOWLEDGMENT

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