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Genomic DNA differences between pathogenic and nonpathogenic *Entamoeba histolytica*

(coding DNA sequences/cDNA homology/amoebiasis/human anti-amoebae antibodies)

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ABSTRACT **cDNA libraries were constructed from pathogenic (HM-1:IMSS) and nonpathogenic (SAW 1734) isolates of *Entamoeba histolytica*. A cDNA clone (cEH-P1) specific for pathogenic amoebae was identified by screening with a pool of sera from patients with invasive amoebiasis that had been absorbed with nonpathogenic amoebae. This clone was used for the identification of a homologous clone (cEH-NP1) in the cDNA from nonpathogenic amoebae. Sequence analysis and comparison of the predicted amino acid sequences for both clones disclosed 12% evolutionary divergence in structure. Hybridization of both cDNA probes to genomic DNA from four pathogenic and five nonpathogenic *E. histolytica* isolates revealed two distinct Southern blot patterns, one characteristic for pathogenic amoebae and the other for nonpathogenic amoebae. Further, the complex pattern of restriction fragments hybridizing to an actin cDNA probe was also different between pathogenic and nonpathogenic isolates but was conserved within each group of amoebae. The results indicate that pathogenic isolates of *E. histolytica* are genetically distinct from nonpathogenic isolates.**

The protozoon *Entamoeba histolytica* is an intestinal parasite infecting 500 million people worldwide (1). In 10% of the infected individuals, *E. histolytica* invades the tissues and causes potentially life-threatening disease such as hemorrhagic colitis and extraintestinal abscesses. At present, the reason for the relatively low frequency of the development of disease is not clear. Either pathogenic and nonpathogenic *E. histolytica* exist as distinct forms, as has been proposed (2–5), or the expression of pathogenicity is inducible and depends on environmental conditions (6, 7). The question arises, therefore, as to whether *E. histolytica* can acquire pathogenicity or whether pathogenic and nonpathogenic forms are genetically distinct. The answer to this question will determine whether or not the large number of asymptomatic carriers of *E. histolytica* have to be treated (4, 8).

Here we report on the identification of two cDNA sequences, one specific for pathogenic and the other for nonpathogenic *E. histolytica*. Genomic DNA analysis of various isolates using these cDNA probes indicates that pathogenic and nonpathogenic *E. histolytica* represent genetically distinct forms.

MATERIALS AND METHODS

***E. histolytica* Isolates and Culture Conditions.** The pathogenic *E. histolytica* strain HM-1:IMSS was obtained from H. Scholze (Osnabrück, F.R.G.) and propagated in axenic medium TYI-S-33. *E. histolytica* strains with pathogenic zymodemes (i.e., isoenzyme patterns) (SAW 755, SAW 891, and SAW 1728) and with nonpathogenic zymodemes (SAW 142, SAW 760, SAW 1734, SAW 1798, and SAW 1799) were isolated and provided by P. Sargeaunt (London). Trophozoites of these *E. histolytica* isolates were xenically grown in media TYI-S-33 or TYSGM-9 as described by Diamond and co-workers (9, 10).

Construction of cDNA Libraries. Total cellular RNA from *E. histolytica* HM-1:IMSS and SAW 1734 was isolated from exponentially growing trophozoites by extraction with 4 M guanidinium isothiocyanate, followed by centrifugation through a 5.7 M CsCl cushion (11). Subsequently, poly(A)-RNA was purified by chromatography on oligo(dT)-cellulose (12). Double-stranded cDNA was prepared by using the method of Gubler and Hoffmann (13). The cDNAs were ligated with *Eco*RI linkers into the bacteriophage vector λgt11 and packaged *in vitro* (14). Recombinant phages were plated on *Escherichia coli* Y1090 and further maintained as liquid stocks.

Screening of the HM-1:IMSS λgt11 cDNA Library with Antiserum. Serum from 33 patients with invasive amoebiasis was pooled and used to screen approximately 1×10^4 recombinant phages of the HM-1:IMSS cDNA library (14). Duplicate filter lifts were analyzed with a 1:300 dilution of the pooled serum and with the same pool absorbed with crude protein extract of the nonpathogenic *E. histolytica* SAW 1734. A horseradish peroxidase-conjugated anti-human IgG antibody was used to visualize positive plaques with chloronaphthol. Positive clones were purified to homogeneity by serial dilutions, and the DNA insert was released from the recombinant phages as an *Eco*RI fragment.

Isolation of Clone cEH-NP1 by Cross-Hybridization to cEH-P1. Approximately 5×10^4 recombinant phages of the SAW 1734 cDNA library were hybridized to radioactively labeled probe cEH-P1 (specific activity of 1×10^8 cpm/μg) under low-stringency conditions [2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) at 50°C]. Hybridizing phages were purified as described above.

Determination of Nucleotide Sequences. *E. histolytica* cDNA inserts were subcloned in the plasmid vector pTZ19R or the phage vectors M13mp18 or M13mp19 (15). Sequence analysis was performed by the dideoxy chain-termination method (16). The obtained sequence data were analyzed using the MicroGenie program (Beckman).

Isolation of Genomic DNA and Southern Blot Analysis. Cells were harvested in late logarithmic phase by chilling on ice for 10 min and low-speed centrifugation at 4°C for 5 min. Nuclei were obtained from washed cell pellets by lysis in 1% Nonidet P-40 and centrifugation at 500 × *g* at 4°C for 5 min. The nuclear pellet was resuspended, and DNA was released by treatment with proteinase K (1 mg/ml) in a buffer containing 100 mM NaCl, 10 mM Tris, 10 mM EDTA, and 0.5% *N*-lauroylsarcosine at 60°C for 2 hr. The DNA was extracted twice with phenol/chloroform, 1:1 (vol/vol), and once with chloroform and was precipitated with ethanol. High molecular weight genomic DNA was digested with different restriction endonucleases under conditions recommended by

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the supplier. The DNA was separated on agarose gels, transferred to a nylon membrane (Pall Biodyne A, 0.2 μm), and hybridized to the radioactively labeled probes (17). Probes were prepared by isolating the cDNA insert and labeling it by random priming with [^{32}P]dCTP using Multiprime labeling kit (Amersham). Filters were hybridized at 42°C for 4 hr in 50% (vol/vol) formamide/5× Denhardt's solution (18)/5× SSPE (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/0.2% SDS/denatured herring sperm DNA (200 μg/ml). Hybridizations were carried out in the same buffer at 42°C overnight. The probes were usually applied at a concentration of 1–3 × 10^6 cpm/ml at a specific radioactivity of 1 × 10^8 cpm/μg. Filters were washed for low-stringency conditions in 2× SSC at 50°C and for high-stringency conditions in 0.1× SSC at 65°C.

Immunoblot Analysis of Protein Extracts from *E. histolytica*. Proteins of *E. histolytica* isolates HM-1:IMSS and SAW 1734 were solubilized in 1% SDS, separated by SDS/PAGE (5–20%), and transferred to nitrocellulose by electroblotting. Proteins were stained on parallel strips with either pooled patient sera or the absorbed serum pool as described above.

RESULTS

Human Antiserum Specific for Pathogenic *E. histolytica*. Sera from patients with invasive amoebiasis were pooled and used as a source of antibodies to *E. histolytica*. By using this pool, immunoblots were performed on solubilized trophozoites of the pathogenic isolate HM-1:IMSS and of the nonpathogenic isolate SAW 1734. Although the immunoblot patterns of both isolates appeared similar, differences were detectable. Absorption of the serum pool with lysate of the nonpathogenic isolate SAW 1734, which eliminated virtually all reactivity with the homologous material, allowed detection of several antigens of the pathogenic isolate HM-1:IMSS (Fig. 1).

Isolation and Characterization of a cDNA Clone from the Pathogenic *E. histolytica* HM-1:IMSS Isolate. λgt11 cDNA libraries of the pathogenic isolate HM-1:IMSS and the nonpathogenic isolate SAW 1734 were constructed; each comprised about 500,000 independent recombinants. Double filter lifts of 10,000 recombinant phages of the HM-1:IMSS library were screened in parallel with the unabsorbed and the absorbed serum pool. Forty clones reacted positively with the unabsorbed serum and only 3 of them reacted with the absorbed serum. These 3 clones were considered specific for the pathogenic form of *E. histolytica*. One of the clones, designated cEH-P1, containing a 1.9-kilobase (kb) *Eco*RI insert was selected for further studies because the fusion protein expressed by this clone was recognized by each of the 33 patient sera contained in the serum pool. In addition to the terminal *Eco*RI restriction sites, internal sites were found for the restriction enzymes *Xmn* I at residues 450 and 1489 and for *Taq* I at residues 88 and 1363 (Fig. 2).

Mapping of Genomic Sequences in Pathogenic and Nonpathogenic Amoebae Hybridizing with cDNA Clone cEH-P1. Genomic HM-1:IMSS DNA was digested with the restriction enzymes *Eco*RI, *Xmn* I, and *Taq* I and hybridized to the clone cEH-P1. The size of the hybridizing genomic *Eco*RI fragment equals that of the probe, indicating the absence of intervening sequences within this part of the gene. This notion was supported by the finding that one of the genomic *Xmn* I and one of the *Taq* I fragments corresponded in size to the internal cEH-P1 fragments predicted by the cDNA restriction map (Fig. 2). For comparison, genomic DNA of the nonpathogenic isolate SAW 1734 was analyzed with cEH-P1. By Southern blot analysis, cEH-P1 revealed hybridization bands that differed in size from those obtained with HM-1:IMSS DNA. Moreover, they were faint and detectable only under reduced stringency (2× SSC, 50°C). As a control, the blot was reprobed with A-1, a 760-base-pair fragment of actin cDNA of *E. histolytica*, and the intensity of the observed hybridization signals demonstrated that comparable DNA quantities of both the pathogenic and the nonpathogenic isolates were present (Fig. 2). Thus, the restriction enzyme patterns of the genomic DNA demonstrated the absence of intervening sequences, and they suggested that cEH-P1 is part of a single copy gene. In addition, the comparison between the patterns obtained with DNA from pathogenic and nonpathogenic *E. histolytica* suggested striking genomic differences between the two forms.

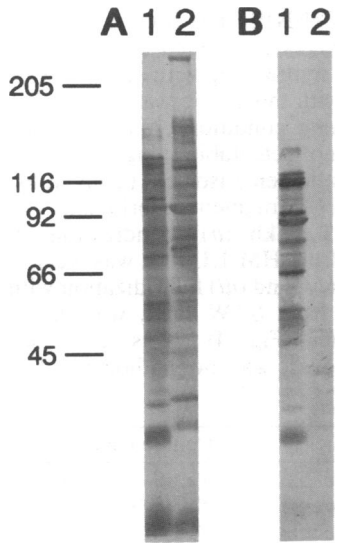

FIG. 1. Immunoblot analysis of *E. histolytica* proteins by using as antibody source a serum pool from patients with invasive amoebiasis. Proteins from *E. histolytica* HM-1:IMSS (lanes 1 in *A* and *B*) and from *E. histolytica* SAW 1734 (lanes 2 in *A* and *B*) were immunostained with pooled patients serum (*A*) or with serum pool previously absorbed with *E. histolytica* SAW 1734 (*B*). Size markers (in kDa) are indicated.

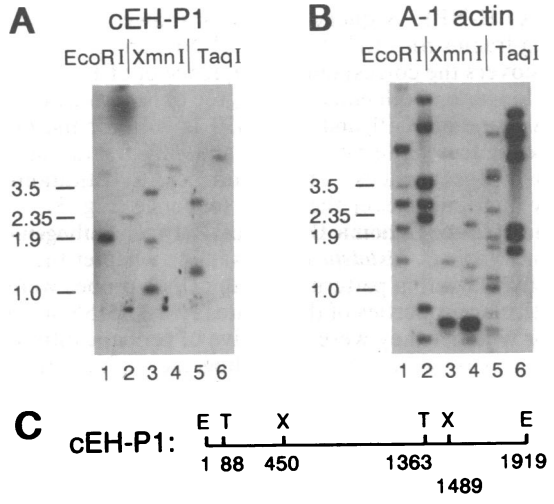

FIG. 2. Comparison of genomic DNA fragments of *E. histolytica* HM-1:IMSS and SAW 1734 probed with cEH-P1 and actin cDNA. Approximately 3 μg of HM-1:IMSS DNA (lanes 1, 3, and 5) and SAW 1734 DNA (lanes 2, 4, and 6) were digested with the indicated restriction enzymes and hybridized to cEH-P1 (*A*) or A-1, a partial actin cDNA of *E. histolytica* (*B*). Sizes (in kb) are indicated at left. (*C*) Partial restriction analysis of clone cEH-P1 is shown for three relevant enzymes. Numbers refer to nucleotides in the cDNA. E, *Eco*RI; T, *Taq* I; X, *Xmn* I.

```
 P1  IQVGIGKCNHNPSDQWL NVNNWSNDRMPIDSIGFDLGLNTTQPYIINDTFKIGSPFGGMI
NP1  V--S----------D-ISR----------I----Q-----S----------I----

     YLRSDTTFTNSFYVTFSNVGRAPIINYNITTNEEWNSVLRNAPGNVAEIRTPGNRLVLTSR
     ---F----------Y-----S-------M--------K----------------F---

     NIRSLEDAQYISDFWLKAISISNYAVTLENIPITLNFDQRVDAGAAVAYVDRWFTQNPSDW
     H--N---------Y----------------------------------F-------H----

     AAACVGKDGLINYGNWGPLHEMNHHMQGPYLKGGNWGISNPGEETNNVMTSINYILYTNIA
     -SG--N-E----S----------------R------KE--------------------

     GHRNQGLSGWNYVSDGYSTIYKILKGENDQPHLRSYVNMAHAFGTDTLIALVKSYYGLWYE
     ----------------------N----------I----------------------

     NNFESKYSIKRDSTSAFCLLAALVTKRDTRYLCSLFKYDIQSNVSEAIKNMNYPTYYPFFN
     --Y-GE----------------IA----------------Q------------------

     LYAMSYNGNYYGRPYKIPYGRTRLNFTATCSIDPKATSVSYTIKSGLTKGKLERVEDNVYD
     V----------T------T--------TA---S----------------Q--E----

     YTPFFGIEENDTFVLNIDCVVNGEKVHIEQEGTFELDPHQVEYEVYKDVQTRDMAQALNII
     ---N--AD----------I----------D----------------K-K--E----T-

     QNKTANDTGRASFFDIGTYNDGSMQSMLVEKGKLIVPKSGYYTLFMKADDLGRLLLNITGE
     ----S-Y--TST-----N-D--T--------------T------------------VN--

     YEQLLDVKTYLGGYSKTLNGSYATVKLEKDVGYPFILYNLNTGGQGFIRIGYCYHGTEESS
     -----N----------I--T---------TE--------------------Q--DQ--

     VDVSKCSVSDIGSSMVLNEKVKTGAKEPEF
     -N------L------I------A-------
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FIG. 3. Comparison of amino acid sequences deduced from cDNA clones cEH-P1 (P1) and cEH-NP1 (NP1). The sequences were derived from the only open reading frame in each clone. The corresponding parts of the clones are presented. Identical residues are shown by dashes below the translated sequence of cEH-P1. For optimal alignment a deletion has been introduced into the cEH-P1 sequence as indicated by a space.

Isolation and Characterization of a cDNA Clone from the Nonpathogenic *E. histolytica* SAW 1734. To isolate the DNA of the nonpathogenic *E. histolytica* SAW 1734 that was responsible for the cross-hybridization with cDNA clone cEH-P1, ≈50,000 recombinant phages of the SAW 1734 cDNA library were plated out and screened with this probe. Seven clones were detected; one of them containing an internal *Eco*RI fragment of 2.35 kb was purified and designated cEH-NP1. Sequence analysis showed unique open reading frames for cEH-P1 and cEH-NP1 and that the latter clone covers the corresponding sequence of cEH-P1 entirely and is longer on both ends. The degree of homology between cDNA clone cEH-P1 and cEH-NP1 is 90% for the nucleic acid sequences (data not shown) and 88% for the deduced amino acid sequences. The amino acid substitutions are evenly distributed over the entire sequence (Fig. 3).

Characteristic Genomic Differences Between Pathogenic and Nonpathogenic *E. histolytica*. To examine whether the differential hybridization patterns of both cDNA probes were due to particular properties of the isolates HM-1:IMSS and SAW 1734 or whether they were indicative of genomic differences between pathogenic and nonpathogenic *E. histolytica* in general, seven additional isolates from various parts of the world were tested. They were selected to represent several different isoenzyme patterns, old and very recent clinical samples, and samples grown under different culture conditions. Three of them were isolated from patients with amoebic disease, and four were isolated from asymptomatic carriers (Table 1).

Genomic DNA digested with *Eco*RI was probed with cEH-P1 and cEH-NP1. The Southern blots showed that all pathogenic isolates resembled HM-1:IMSS in that (*i*) the DNA fragment hybridizing with cEH-P1 was also 1.9 kb, (*ii*) hybridization with this probe was strong and resisted highly stringent washing conditions, and (*iii*) hybridization with cEH-NP1 was not detectable at high stringency. On the other hand, all nonpathogenic isolates behaved like SAW 1734 in that (*i*) the DNA fragment hybridizing with cEH-P1 and cEH-NP1 was 2.35 kb; (*ii*) hybridization with cEH-P1, the probe derived from HM-1:IMSS, was weak and disappeared at high stringency; and (*iii*) hybridization with cEH-NP1, the probe derived from SAW 1734, was intense and resisted stringent washes (Fig. 4). Thus, a consistent difference between pathogenic and nonpathogenic *E. histolytica* was

Table 1. Isolates of *E. histolytica* employed for genomic DNA analysis

Isolate	Origin	Zymodeme*	Pathogenicity	Culture condition
HM-1:IMSS	Latin America	II	Pathogenic	TYI-S-33/axenic
SAW 142	India	III	Nonpathogenic	TYS-9/xenic
SAW 755	India	XIV	Pathogenic	TYI-S-33/xenic
SAW 760	Europe	IX	Nonpathogenic	TYI-S-33/xenic
SAW 891	Latin America	XII	Pathogenic	TYSGM-9/xenic
SAW 1728	Africa	XIX	Pathogenic	TYSGM-9/xenic
SAW 1734	Africa	III	Nonpathogenic	TYI-S-33/xenic
SAW 1798	Europe	I	Nonpathogenic	TYSGM-9/xenic
SAW 1799	Europe	I	Nonpathogenic	TYSGM-9/xenic

*Roman numerals refer to the isoenzyme patterns as defined by Sargeaunt (4).

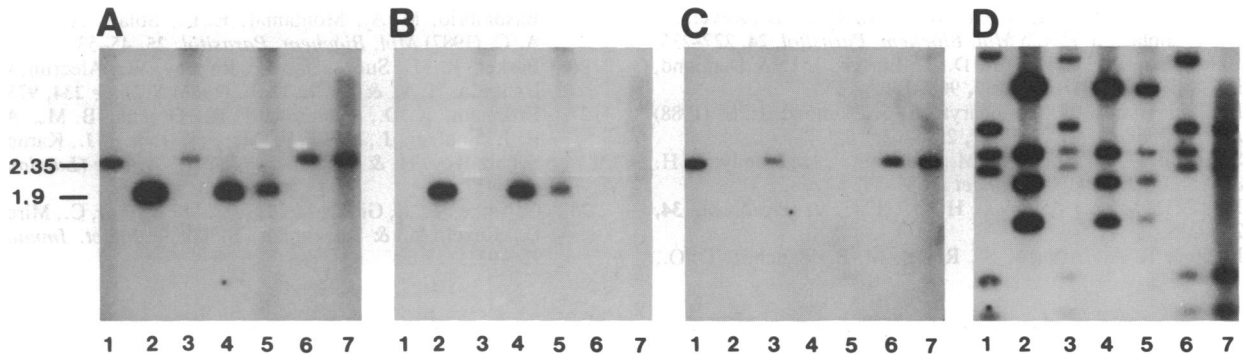

FIG. 4. Southern blot analysis of pathogenic and nonpathogenic isolates of *E. histolytica* hybridized with cEH-P1, cEH-NP1, and actin cDNA. DNAs were digested and applied to the gel in the following order: lane 1, SAW 142; lane 2, SAW 755; lane 3, SAW 760; lane 4, SAW 891; lane 5, SAW 1728; lane 6, SAW 1798; lane 7, SAW 1799. The blot was probed with cEH-P1 under low-stringency (*A*) and high-stringency (*B*) conditions. The probe was removed, and the blot was subsequently rehybridized with cEH-NP1 and stringently washed (*C*). Again the probe was removed, and the blot was rehybridized with the actin probes A-1, at high stringency (*D*).

demonstrated within a coding DNA sequence. Corresponding results were obtained when the restriction enzyme *Xmn* I was used for DNA digestion (data not shown).

Moreover, pathogenic and nonpathogenic isolates could also be distinguished when the actin probe was used. Probe A-1, a part of the actin cDNA of *E. histolytica*, hybridized to multiple *Eco*RI fragments, which reflects the known multiplicity of actin genes within the amoebic genome (19, 20). The restriction fragment patterns again revealed differences between pathogenic and nonpathogenic isolates but were consistent within each group of amoebae (Fig. 4*D*), except for the minor variation of HM-1:IMSS, which showed a single additional band of ≈3.5 kb (Fig. 2*B*).

DISCUSSION

Attempts to classify and define differences between pathogenic and nonpathogenic isolates of *E. histolytica* have used biological assays, isoenzyme analysis, monoclonal antibodies, and repetitive DNA probes (21–23). Particularly, the distribution of different electrophoretic patterns of isoenzymes of the carbohydrate metabolism has been widely used to characterize the pathogenic and nonpathogenic forms of *E. histolytica* (4, 24). Also, monoclonal antibodies have been described that recognize antigens that are presumably specific for pathogenic strains (23). Since these analyses are limited to the definition of protein differences between the various isolates, it remained unclear whether these findings reflect genetic differences or whether they indicate epigenetic or phenotypic modulations of otherwise identical organisms. This latter notion is supported by recent reports that, during the process of axenization, conversion from nonpathogenic to pathogenic isoenzyme patterns has occurred in two isolates (6, 7). The high complexity of different zymodemes, their apparent instability, and the fact that no causal relationship of these metabolic enzymes with pathogenicity has ever been demonstrated led us to search for other parameters.

It has been recently shown that DNA probes can be successfully applied for the detection and typing of parasites, such as trypanosomes (25), leishmania (26), filaria (27), and *E. histolytica* (22, 28). In these studies highly repetitive and polymorphic DNA probes have been used, which do not allow the detection of subtle differences between various sequences. In the present study, the identification of two single-copy cDNA probes that exhibit similar but not identical structures is described. These probes represent homologous genes in two strains of *E. histolytica*. They allow for the subclassification of isolates according to their pathogenic or nonpathogenic properties as demonstrated on four pathogenic and five nonpathogenic isolates.

The detection of a pair of evolutionarily related but distinct genes in pathogenic and nonpathogenic *E. histolytica* and the consistency with which they are present in these isolates argues for genetically defined subspecies of *E. histolytica*. It therefore seems likely that pathogenicity of *E. histolytica* is dependent on genes that are specific for the pathogenic forms. We cannot, however, rule out the possibility that phenotypic conversion contributes to virulence.

The isolation of DNA probes should not only facilitate the investigation of the genomic organization of *E. histolytica* but also should help to answer questions concerning the molecular basis of pathogenicity expressed by this parasite.

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