

Isolation of a Strain-Specific *Entamoeba histolytica* cDNA Clone

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Entamoeba histolytica is an intestinal parasite causing significant morbidity and mortality worldwide. More tools are needed to understand the epidemiology and molecular pathogenesis of amebiasis. A cDNA library was constructed by using poly(A)⁺ RNA isolated from an axenic strain of *E. histolytica*, HM1:IMSS, which expresses a pathogenic isoenzyme pattern (zymodeme). Differential screening of the library yielded a strain-specific 3' polyadenylated cDNA clone, C2, possessing nine 26-nucleotide tandem repeats. RNA and DNA transfer blot analysis of four axenic strains of *E. histolytica* possessing the same pathogenic zymodeme revealed that the gene is present and expressed in pathogenic *E. histolytica* HM1:IMSS and 200:NIH but is not present in pathogenic strains HK-9 and Rahman. In addition, Southern blot analysis using the C2 clone showed heterogeneity of genomic organization between HM1:IMSS and 200:NIH. DNA dot blot hybridization analysis demonstrated that cDNA clone C2 was also able to distinguish xenically cultured *E. histolytica* strains possessing pathogenic zymodemes from those possessing nonpathogenic zymodemes and could detect as few as 100 amebic trophozoites. We conclude that C2 is a strain-specific *E. histolytica* cDNA clone that, in conjunction with other *E. histolytica*-specific probes, could serve as a useful epidemiologic tool.

The intestinal protozoan *Entamoeba histolytica* infects about 500,000,000 people worldwide; however, only about one-tenth develop clinically overt disease (8). More tools are necessary to understand the epidemiology of the disease and to understand why nine-tenths of those infected are asymptomatic. One of the critical questions in amebiasis has been whether the large number of asymptomatic carriers reflects differences in the pathogenic potential of the infecting strain or variation in the host response to infection.

Evidence for inherent differences in virulence among *E. histolytica* isolates has been provided by Sargeant and colleagues. They have demonstrated the existence of differences in isoenzyme patterns (zymodemes) between xenically cultured *E. histolytica* isolates from patients with invasive disease versus those obtained from asymptomatic carriers (17). This system of isoenzyme analysis, based on the study of isoenzyme patterns from thousands of clinical isolates obtained around the world, enables one to predict whether a given isolate can cause disease and can provide information on the geographic origin of the strain (16). In general, only strains possessing a pathogenic zymodeme have been cultivated in axenic culture. While attempting to culture a nonpathogenic strain of *E. histolytica* axenically, Mirelman and colleagues observed that its zymodeme pattern changed from nonpathogenic to pathogenic (13).

Recently, *E. histolytica*-specific DNA probes which distinguish pathogenic from nonpathogenic clinical isolates (2, 7, 20) and may serve as valuable diagnostic reagents (15) have been developed. A strain-specific cDNA probe could prove useful for rapid typing of *E. histolytica* strains and might provide further insight into the epidemiology and molecular pathogenesis of amebic disease. We report here the isolation of a strain-specific *E. histolytica* cDNA clone that distinguishes between axenically cultured strains pos-

sessing the same pathogenic zymodeme. Furthermore, this clone hybridizes specifically to DNAs extracted from xenically cultured *E. histolytica* strains possessing pathogenic zymodemes while not hybridizing to DNAs extracted from organisms possessing nonpathogenic zymodemes.

MATERIALS AND METHODS

Entamoeba strains and culture conditions. Axenic *E. histolytica* HM1:IMSS, HK-9, 200:NIH, and Rahman (all belonging to pathogenic zymodeme II); the *E. histolytica*-like Laredo strain; *E. invadens*; and *E. moshkovskii* were obtained from the American Type Culture Collection (Gaithersburg, Md.) and cultivated in TYI-S-33 medium (4). The *E. histolytica* strains were grown at 35°C, and the other amebic species were grown at 24°C. Clinical isolates of *E. histolytica* (Table 1) were all obtained from stool or liver abscess fluid. They were isolated and maintained in Robinson's medium (14) (except strain FAT957, which was originally isolated in Locke egg medium [22] before transfer to Robinson's medium). Zymodeme analysis was performed as described by Sargeant et al. (17).

Construction and screening of an HM1:IMSS cDNA library. A pUC13 cDNA library derived from HM1:IMSS poly(A)⁺ RNA was constructed in *Escherichia coli* DH5 α [genotype: F⁻ endA1 hsdR17 (r_K⁻ m_K⁺) supE44 thi-1 λ ⁻ recA1 gyrA⁹⁶ relA1 Δ (argF-lacZYA)U169 ϕ 80dlacZ Δ M15] as described previously (18). Duplicate replica filters representing approximately 10,000 of the recombinants were probed with ³²P-labeled single-stranded cDNAs from HM1:IMSS and the *E. histolytica*-like Laredo strain, respectively.

DNA probes. An actin cDNA probe was isolated by using oligonucleotides based on the sequence of HM1:IMSS actin cDNA previously described (5). Clone C1, which encodes a unique *E. histolytica* membrane antigen, has been previously described (18). A DNA probe encoding *E. histolytica* rRNA (15) was kindly provided by John Samuelson and Dyann

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TABLE 1. DNA dot blot hybridization analysis of xenically cultured *E. histolytica* using the C2 cDNA clone

<i>E. histolytica</i> strain	Zymodeme ^a	Clinical background ^b	Hybridization ^c
SAW1453	XIV (P)	ALA	+
SD53	II (P)	Colitis	+
FAT957	II (P)	ALA	+
SAW990	XIV (P)	Colitis	+
SD92	II (P)	Colitis	+
FAT1014	II (P)	ALA	+
FAT967	II (P)	ASX ^d	+
SD11	I (NP)	ASX, GM	-
SD126	III (NP)	ASX, GM	-
SD130	I (NP)	ASX, AIDS	-
SD116	I (NP)	ASX, AIDS	-
SD107	I (NP)	ASX, AIDS	-
FAT1010	I (NP)	ASX	-

^a Roman numerals designate zymodeme groups as defined by Sargeant et al. (17). P, Pathogenic zymodeme; NP, nonpathogenic zymodeme.

^b Abbreviations: ALA, amebic liver abscess; ASX, asymptomatic; GM, homosexual male.

^c +, Hybridization; -, no hybridization.

^d South African child with positive amebic serology.

Wirth, Harvard School of Public Health, Boston, Mass. Oligonucleotide probes were synthesized by using phosphoramidite chemistry and an Applied Biosystems 380B DNA synthesizer (1). cDNA was labeled with ³²P by the random primer method (6) to a specific activity of 1,000 cpm/pg. Oligonucleotide probes were labeled with T4 polynucleotide kinase and [γ -³²P]dATP (10).

RNA transfer blot analysis. RNA was isolated from trophozoites by the guanidinium thiocyanate method as previously described (18). Northern (RNA) blots were prepared by electrophoresing 20 μ g of total cellular amebic RNA from each axenic strain through a 1.5% agarose gel containing formaldehyde (21) and transferring it to a nylon membrane (Nytran; Schleicher & Schuell, Inc., Keene, N.H.). The blots were hybridized with cDNA probes in 50% formamide-6 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-1.5 \times Denhardt's solution (10)-0.05 M sodium phosphate-0.001 M EDTA-100 μ g of salmon sperm DNA per ml-0.1 g of dextran sulfate per ml at 42°C overnight with 2 \times 10⁶ cpm of labeled cDNA probe. Filters were washed twice with 1 \times SSC-0.1% sodium dodecyl sulfate (SDS) at ambient temperature and then twice with 0.1 \times SSC-0.1% SDS at 55°C. The blots were hybridized with 5 \times 10⁶ cpm of each oligonucleotide probe under the same conditions at 37°C. These were washed twice with 1 \times SSC-0.1% SDS at room temperature and then twice again at 37°C. All autoradiographs were exposed for 24 h.

Southern blot analysis. Nuclear DNA was prepared by the agarose block method as described by Edman et al. (5). Genomic DNAs from all of the axenic amebic strains were digested with *Eco*RI, size fractionated, and transferred to a nylon membrane as described by Maniatis et al. (10). Hybridization methods were the same as those used for Northern blots, except that the hybridization solution was composed of 6 \times SSC-4 \times Denhardt's solution-0.1% SDS-100 μ g of salmon sperm DNA per ml. Washing and autoradiography conditions were identical to those used for Northern blots.

Nucleotide sequence analysis. The cDNA clones were sequenced by the technique of Maxam and Gilbert (12).

Preparation of dot blots. DNA was extracted from tropho-

zoites as previously described by Samuelson et al. (15). Axenic and xenic trophozoites were suspended in 0.5 M EDTA-0.01 M Tris chloride buffer (pH 8.0) (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 10⁶ trophozoites per ml. The suspension was thrice repetitively freeze-thawed on dry ice and in a 37°C water bath, with vigorous vortexing after each thaw. This suspension was then centrifuged at 4°C at 13,000 \times g for 15 min. The supernatant was serially diluted to obtain a range of 10⁵ to 10⁰ trophozoites per 100 μ l. With a Minifold apparatus (Schleicher & Schuell), 100- μ l samples were applied to nylon membranes. The DNA was denatured by sequentially placing the nylon filters on a Whatman 3MM filter saturated with 0.5 M NaOH, another Whatman 3MM filter saturated with 0.5 M NaOH, a Whatman 3MM filter saturated with Tris-chloride buffer (pH 8.0), and a Whatman 3MM paper saturated with 1.0 M Tris-chloride (pH 8.0)-1.5 M NaCl. The nylon filters were air dried and baked under a vacuum for 1 h at 80°C. The filters were probed, washed, and autoradiographed in the same manner as the Southern blots.

All blots were stripped before repeat hybridization by boiling in 0.01 M Tris-chloride (pH 8.0)-0.001 M EDTA for 20 min.

RESULTS

Isolation of *E. histolytica* cDNA clone C2. We screened 10,000 recombinants from the pUC13 cDNA library derived from pathogenic *E. histolytica* HM1:IMSS (an axenic strain of *E. histolytica*) mRNA with radiolabeled single-stranded cDNA transcribed from poly(A)⁺ RNA from HM1:IMSS and the nonpathogenic *E. histolytica*-like Laredo strain, respectively. Four unique clones that hybridized to HM1:IMSS cDNA but not to Laredo cDNA were isolated. One of these clones, designated C2, was chosen for further study.

Differential expression of the C2 gene among axenic strains of *E. histolytica* possessing the same zymodeme. We used RNA blot hybridization analysis to study the expression of the gene for C2 among four axenic *E. histolytica* strains (HM1:IMSS, HK-9, 200:NIH, and Rahman), the *E. histolytica*-like Laredo strain, and two non-*E. histolytica* species, *E. invadens* and *E. moshkovskii*. The C2 clone hybridized to RNAs isolated from *E. histolytica* HM1:IMSS and 200:NIH (Fig. 1, lanes 2 and 4) but not to RNAs isolated from *E. histolytica* HK-9 and Rahman (lanes 3 and 5). The predominant transcripts were at 0.7 kb in HM1:IMSS and 1.0 kb in 200:NIH. Larger transcripts were also observed at 1.35, 2.1, and 3.5 kb in RNA isolated from strain HM1:IMSS. C2 did not hybridize to RNA from the *E. histolytica*-like Laredo strain, *E. invadens*, or *E. moshkovskii* (lanes 6 to 8, respectively). Hybridization of the same Northern blot with an *E. histolytica* actin cDNA probe confirmed the presence of intact mRNA in all of the lanes (data not shown).

To determine whether the gene corresponding to the C2 clone was present in ameba not expressing C2 RNA, we used the C2 clone to probe Southern blots of *Eco*RI-digested genomic DNAs isolated from *E. histolytica* HM1:IMSS, HK-9, 200:NIH, and Rahman; the *E. histolytica*-like Laredo strain; *E. invadens*; and *E. moshkovskii* (Fig. 2). C2 hybridized with 23- and 6.6-kb fragments of HM1:IMSS DNA (lane 2) and to 23-, 10.0-, and 3.7-kb fragments of 200:NIH DNA (lane 4). C2 did not hybridize to DNAs isolated from *E. histolytica* HK-9 and Rahman (lanes 3 and 5) or to DNAs isolated from the other three other *Entamoeba* species, Laredo, *E. invadens*, and *E. moshkovskii* (lanes 6 to 8, respectively). Hybridization of the same Southern blot with

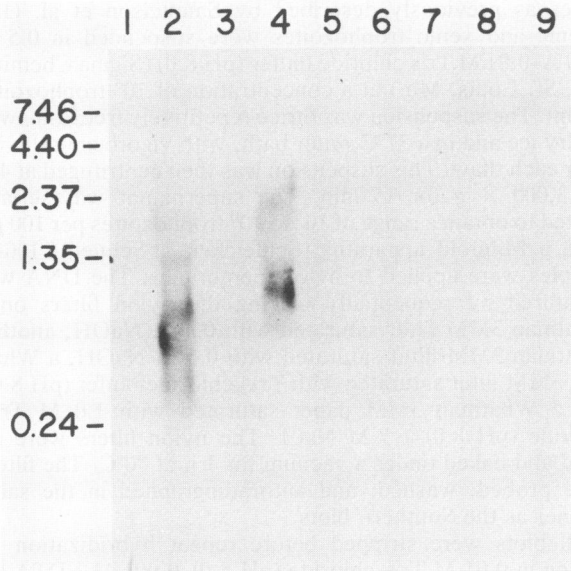


FIG. 1. Northern blot of total amebic RNA probed with C2. C2 hybridizes specifically to transcripts from strains HM1:IMSS and HK-9. Lanes: 1, molecular weight standards; 2, HM1:IMSS; 3, HK-9; 4, 200:NIH; 5, Rahman; 6, Laredo variant; 7, *E. invadens*; 8, *E. moskovskii*; 9, molecular weight standards. The numbers on the left indicate molecular sizes in kilobases.

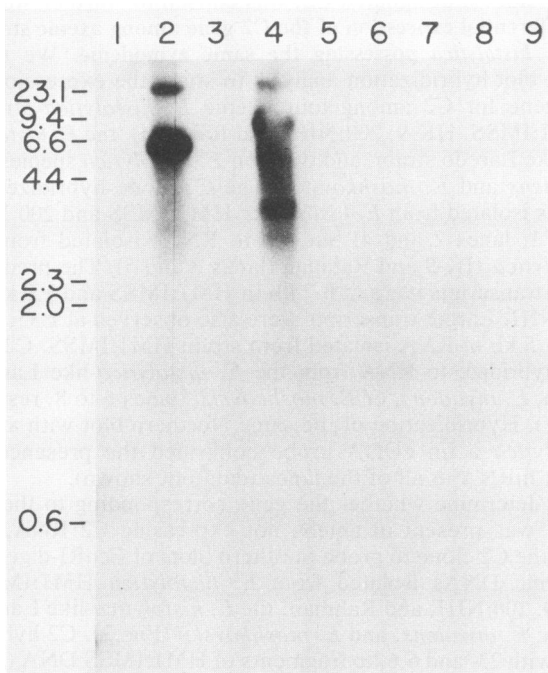


FIG. 2. Southern blot analysis of *EcoRI*-digested genomic amebic DNA. C2 hybridized specifically to restriction fragments from HM1:IMSS and HK-9. Lanes: 1, molecular weight standards; 2, HM1:IMSS; 3, HK-9; 4, 200:NIH; 5, Rahman; 6, Laredo variant; 7, *E. invadens*; 8, *E. moskovskii*; 9, molecular weight standards. The numbers on the left indicate molecular sizes in kilobases.

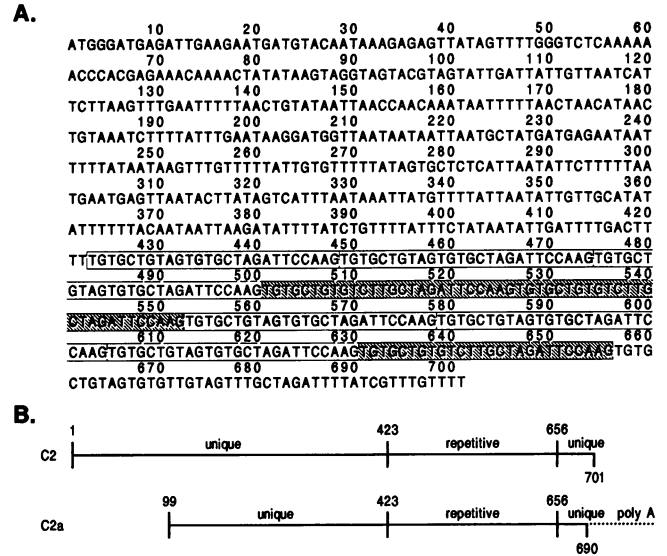


FIG. 3. (A) Complete nucleotide sequence of C2 showing the nine 26-nucleotide tandem repeats from nucleotides 423 through 656. Two species of repeats which differ by four nucleotides are represented by shaded and unshaded boxes. (B) Schematic of C2 showing unique and repetitive areas of DNA. C2a has a homologous sequence from residues 99 through 690 with a poly(A)⁺ tail on the 3' end.

an *E. histolytica* actin cDNA probe confirmed the presence of DNA in all lanes (data not shown).

Nucleotide sequence of the C2 cDNA clone. The C2 cDNA sequence consisted of 701 bp (Fig. 3A) and contained no large open reading frame. Beginning at nucleotide 423, there is a series of nine tandem repeats. The repeats include two nearly identical 26-nucleotide sequences represented by shaded and unshaded boxes. To determine which strand was transcribed, Northern blots were probed with an oligonucleotide corresponding to either strand (oligo1, CTCTTTAT TGTACATCAT; oligo2, AGAAACAAAACATATAAGT AGGTATGACGTAGTATTGAT). Only oligo1 hybridized with HM1:IMSS and 200:NIH RNA (Fig. 4, lanes 2 and 4). Note that the transcripts that hybridized with oligo1 (which hybridized to the 5' end of the sense strand of C2) were larger than the predominant transcript that hybridized to the C2 clone itself. Oligo1 hybridized predominantly to 3.5- and 5.2-kb transcripts, in addition to a 0.7-kb transcript, suggesting that C2 represents part of a much larger transcript that is processed.

To isolate cDNA clones that overlap C2 at the 5' and 3' ends, the HM1:IMSS cDNA library was rescreened by using oligo3 (GTAGTTTGCTAGATTTTATC), which hybridized with the 3' end of the sense strand, and oligo1. A second clone, C2a, which was obtained by screening the cDNA library with oligo3, corresponds exactly to nucleotides 99 to 690 of C2 but is polyadenylated (Fig. 3B). Neither of two additional clones that hybridized with oligo1 contained an additional 5' sequence.

A search of the GenBank and National Biomedical Research Foundation data bases revealed no previously reported DNA sequences with significant homology to cDNA clone C2.

Sensitivity of the C2 clone for detection of *E. histolytica* trophozoites. The sensitivity of C2 as a DNA probe was determined by serial dilutions of DNAs extracted from

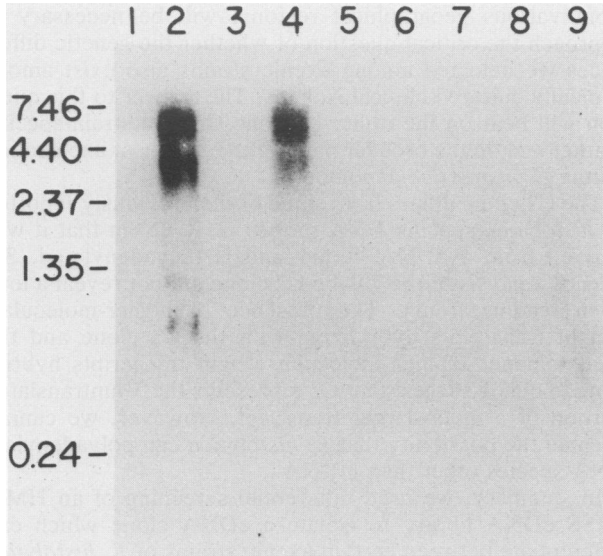


FIG. 4. Northern blot of total amebic RNAs probed with oligo1. The oligonucleotide hybridized only to transcripts from strains HM1:IMSS and 200:NIH. Lanes: 1, molecular weight standards; 2, HM1:IMSS; 3, HK-9; 4, 200:NIH; 5, Rahman; 6, Laredo variant; 7, *E. invadens*; 8, *E. moshkovskii*; 9, molecular weight standards. The numbers on the left indicate molecular sizes in kilobases.

axenic strains of *E. histolytica*. C2 detected DNA extracted from as few as 10^2 HM1:IMSS trophozoites after 24 h of exposure of the autoradiograph (Fig. 5). By increasing the exposure time to 72 h, the sensitivity was increased by another order of magnitude. No hybridization with HK-9 trophozoites was seen.

Specificity of C2 clone for *E. histolytica* isolated from patients with invasive amebiasis. Clone C2 was then used to probe dot blots of DNAs extracted from xenically cultured *E. histolytica* clinical isolates (Table 1). The isolates were obtained from patients with invasive amebiasis, as well as from asymptomatic cyst passers. C2 hybridized specifically with DNAs extracted from *E. histolytica* strains isolated

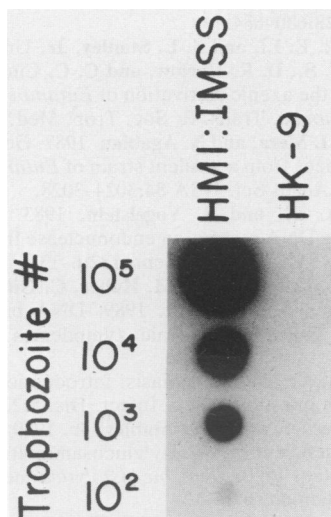


FIG. 5. DNA dot blot trophozoite dilution analysis. C2 detected as few as 100 trophozoites after 24 h of exposure.

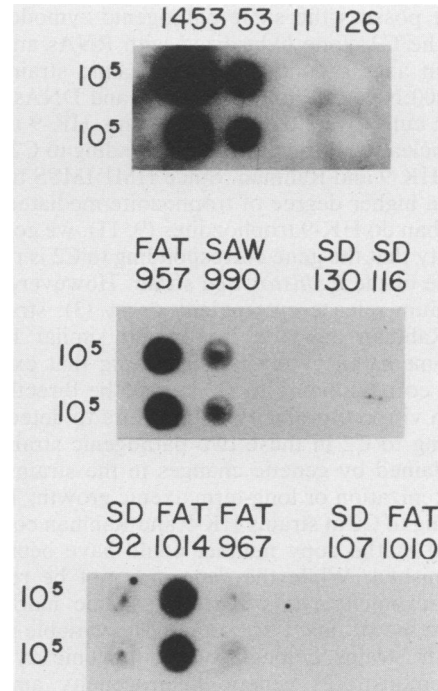


FIG. 6. Xenic strain DNA dot blot hybridization analysis. Trophozoites from each strain ($n = 10^5$) were probed in duplicate. Only strains possessing a pathogenic zymodeme produced signals.

from patients with invasive disease, including SAW1453, SD53, FAT957, SAW990, SD92, FAT1014, and FAT967 (Fig. 6). These isolates all expressed pathogenic zymodemes. Positive signals were observed after overnight exposure, although the signals varied in intensity. The samples yielding no hybridization signal after a minimum of 3 days of exposure included strains SD11, SD126, SD130, SD116, SD107, and FAT1010, all possessing nonpathogenic zymodemes and from patients without evidence of invasive disease. Three of the seven positive isolates were from patients with colitis, three were from patients with liver abscesses, and the seventh was from an asymptomatic child with positive serology. Of the six negative strains, five were from homosexual men, three of whom had AIDS. The sixth was from an asymptomatic South African child.

To control for the presence of DNA in the six isolates that did not hybridize to C2, a DNA probe encoding *E. histolytica* rRNA (15) was used. A positive signal was seen in each of the six blots, confirming the presence of DNA in each sample (data not shown).

DISCUSSION

A number of studies have suggested that biochemical and immunological differences exist between *E. histolytica* isolated from patients with invasive amebiasis and *E. histolytica* isolated from asymptomatic carriers (17, 19). Recently molecular probes have confirmed differences at the genetic level between pathogenic and nonpathogenic isolates (7, 20). Less well explored have been potential differences between pathogenic strains of *E. histolytica*, especially those bearing identical zymodemes. The ability to recognize certain strains might be particularly useful in epidemiologic studies. In this report, we describe the isolation of a cDNA clone, C2, that detects genetic differences among axenic strains of *E. his-*

histolytica that possess the same pathogenic zymodeme. We found that the C2 clone hybridized with RNAs and DNAs derived from axenic cultures of pathogenic strains HM1:IMSS and 200:NIH but not with RNAs and DNAs isolated from axenic cultures of pathogenic strains HK-9 and Rahman. It is unclear why the gene corresponding to C2 was not detected in HK-9 and Rahman. Since HM1:IMSS trophozoites exhibit a higher degree of trophozoite-mediated cytolysis in vitro than do HK-9 trophozoites (9, 11), we considered the possibility that the gene corresponding to C2 is related to the virulence of the *E. histolytica* strain. However, in an in vitro chromium release cytotoxicity assay (3), strains 200:NIH and Rahman appeared to exhibit similar levels of trophozoite-mediated cytolysis, suggesting that expression of the gene corresponding to C2 cannot be directly correlated with in vitro cytotoxicity. Our failure to detect a gene corresponding to C2 in these two pathogenic strains could also be explained by genetic changes in the strains associated with axenization or long-term axenic growth. The gene corresponding to C2 in strains HK-9 and Rahman could have been deleted or the copy number could have been significantly diminished. While the gene may not be related to amebic pathogenicity, it is a strain-specific marker with respect to axenic strains. Furthermore, its variable presence in these four strains belonging to pathogenic zymodeme group II demonstrates genetic heterogeneity among axenized strains which possess the same pathogenic zymodeme.

Note that in addition to distinguishing HM1:IMSS and 200:NIH from HK-9 and Rahman, the C2 clone could also differentiate between HM1:IMSS and 200:NIH on Southern blot analysis, since the sizes of the *Eco*RI fragments differed between the two strains. This is consistent with a previous study, in which an analysis of restriction fragment length polymorphism using genomic DNA and a genomic DNA probe demonstrated that 200:NIH and HM1:IMSS possessed different patterns (7). In contrast to our findings, the genomic DNA probe used in that study revealed no differences between Rahman and 200:NIH. This provides additional evidence for genetic heterogeneity among axenic strains possessing the same zymodeme and suggests that multiple probes may be necessary to detect differences among pathogenic *E. histolytica* strains.

To examine the sensitivity and specificity of the C2 clone for detection of *E. histolytica* DNA from xenically cultured clinical isolates, we performed DNA dot blot analysis. The C2 clone was a relatively sensitive probe, with a capability of detecting DNA from as few as 10^2 trophozoites. In contrast, two other cDNA clones, one encoding actin (5) and the other encoding an *E. histolytica*-specific antigen (18), were unable to detect DNA extracted from 10^5 trophozoites on DNA dot blot analysis (data not shown). The sensitivity of C2 thus compares favorably with the reported sensitivity of probes derived from genomic DNA (15). The high sensitivity of the C2 cDNA clone may be due in part to the nine 26-nucleotide repeats present in its 3' end.

Dot blot analysis of DNAs extracted from xenically cultured *E. histolytica* trophozoites revealed that C2 distinguished strains with pathogenic zymodemes from those with nonpathogenic zymodemes. Hybridization signals could be detected in all seven of the xenic strains bearing pathogenic zymodemes that hybridized with the C2 clone. Thus, in this small sample we could not distinguish strain differences in the expression or presence of the gene corresponding to C2 among xenically cultured clinical isolates which possessed pathogenic zymodemes. A larger sample of clinical isolates,

from various geographical regions, will be necessary to approach the critical question of whether the genetic differences we detected among axenic strains also exist among xenically cultured clinical isolates. The answer to this question will bear on the utility of clone C2 as a strain-specific marker and on the broader point of the effects of axenization on the *E. histolytica* genome.

The C2 clone differs from some of the previously reported *E. histolytica*-specific DNA probes (2, 7, 15) in that it was derived from a cDNA library and is polyadenylated. Sequence analysis of the 701-bp C2 clone did not reveal a long open reading frame. The presence of higher-molecular-weight transcripts hybridizing with the C2 clone and the predominance of high-molecular-weight transcripts hybridizing to oligo1 suggest that C2 represents the 3'-untranslated portion of a much larger transcript. However, we cannot exclude the possibility that *E. histolytica* can polyadenylate RNA species other than mRNA.

In summary, we used differential screening of an HM1:IMSS cDNA library to isolate a cDNA clone which can differentiate between certain axenic strains of *E. histolytica* bearing the same zymodeme and appears to be a sensitive and specific probe for detection of pathogenic *E. histolytica* in xenic culture. If the strain specificity exhibited by C2 for axenic strains can be demonstrated for clinical isolates, the C2 clone, in conjunction with other molecular probes, could provide new insights into the epidemiology of invasive amebiasis.

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