Geographic Diversity among Genotypes of *Entamoeba histolytica* Field Isolates

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It has been known that only 5 to 10% of those infected with *Entamoeba histolytica* develop symptomatic disease. However, the parasite and the host factors that determine the onset of disease remain undetermined. Molecular typing by using polymorphic genetic loci has been proven to aid in the close examination of the population structure of *E. histolytica* field isolates in nature. In the present study, we analyzed the genetic polymorphisms of two noncoding loci (locus 1-2 and locus 5-6) and two protein-coding loci (chitinase and serine-rich *E. histolytica* protein [SREHP]) among 79 isolates obtained from different geographic regions, mainly Japan, Thailand, and Bangladesh. When the genotypes of the four loci were combined for all isolates that we have analyzed so far (overlapping isolates from mass infection events were excluded), a total of 53 different genotypes were observed among 63 isolates. The most remarkable and extensive variations among the four loci was found in the SREHP locus; i.e., 34 different genotypes were observed among 52 isolates. These results demonstrate that *E. histolytica* has an extremely complex genetic structure independent of geographic location. Our results also show that, despite the proposed transmission of other sexually transmitted diseases, including human immunodeficiency virus infection, from Thailand to Japan, the spectra of the genotypes of the *E. histolytica* isolates from these two countries are distinct, suggesting that the major *E. histolytica* strains prevalent in Japan at present were likely introduced from countries other than Thailand. Although the genetic polymorphism of the SREHP locus was previously suggested to be closely associated with the clinical presentation, e.g., colitis or dysentery and liver abscess, no association between the clinical presentation and the SREHP genotype at either the nucleotide or the predicted amino acid level was demonstrated.

Entamoeba histolytica is the causative agent of an estimated 40 million to 50 million cases of amebic colitis and liver abscess and is responsible for up to 100,000 deaths worldwide each year (6, 28, 33, 41). It has generally been granted that a majority of individuals infected with *E. histolytica* do not develop symptomatic disease (2, 11, 12, 15, 17, 18, 27). In recent cohort studies in Bangladesh, only about 3% of the *E. histolytica*-infected children developed symptoms attributable to amebic dysentery (16, 32). However, the parasite and the host factors that determine the onset of disease, i.e., whether or not amebae initiate tissue invasion and thus cause symptoms, remain undetermined (3, 41). A high degree of heterogeneity in virulence has been demonstrated previously. Interstrain variations in the adhesion of *E. histolytica* trophozoites to human epithelium have been demonstrated for two *E. histolytica* strains (1, 10), in which underrepresentation of the 35-kDa light subunit of the Gal-GalNAc lectin in the avirulent Rahman strain was shown to be correlated with a lack of cytopathic activity (1). Variations in cysteine proteinase expression between highly virulent and avirulent strains were also reported (23). In addition, marked differences in the levels of lipophosphoglycan-like

and lipophosphopeptidoglycan molecules were demonstrated between virulent and avirulent strains of *E. histolytica* (24). Interstrain variations in the ability to produce liver abscesses in both gerbils and hamsters are also known. These interstrain variations in in vitro and in vivo virulence have prompted the World Health Organization's expert committee to recommend reinforced efforts through molecular epidemiological studies to determine whether some subgroups of *E. histolytica* are more likely than others to cause invasive disease (41). Another very puzzling question is why certain groups of infected individuals develop extraintestinal amebiasis without showing apparent intestinal symptoms. This observation also appears to be partially explained by interstrain variations in parasite virulence, i.e., tissue and organ tropisms, and host immune backgrounds, as suggested elsewhere (32). DNA typing of polymorphic genetic loci, recently developed by others (2, 7, 13, 43), helped us to closely examine the polymorphic structures of *E. histolytica* field isolates. While a majority of polymorphic genetic loci lack a correlation with virulent (or avirulent) phenotypes, Ayeh-Kumi et al. (2) recently showed that the serine-rich *E. histolytica* protein (SREHP) genotypes of clinical isolates from patients with liver abscesses were distinct from those of clinical isolates from patients with colitis and dysentery in Bangladesh, suggesting that an association between the SREHP genotypes (35) and clinical presentation may exist (2). Extensive genetic polymorphisms in both noncoding and cod-

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TABLE 1. Background and genotypes of the *E. histolytica* isolates used in this study

No.	Isolate	Isolation		Clinical diagnosis	Serology result ^a	Zymo-deme	DNA origin	Type		Chitinase	SREHP
		Location	Date					Locus 1-2	Locus 5-6		
1	TM19	Thailand	1987	Dysentery and colitis	+	II	Xenic	D	A6/Cv	C	12
2	TM20	Thailand	1987	Dysentery and colitis	+	II	Xenic	D	A6/Cv	C	12
3	TM21	Thailand	1987	Dysentery and colitis	+	II	Xenic	B	A6/Cv	C	6
4	TM23	Thailand	1987	Colitis	+	II	Xenic	D	A11v	A	5
5	TM24	Thailand	1987	Dysentery and colitis	+	II	Xenic	I	A7	C	8/16
6	TM25	Thailand	1987	Colitis	+	II	Xenic	D	A11v/A9	A/C	7
7	TM27	Thailand	1987	Colitis	+	II	Xenic	K	A5	C	14
8	TM28	Thailand	1987	Dysentery and colitis	+	II	Xenic	D	A7/Cv	C	9
9	TM29	Thailand	1987	Dysentery and colitis	+	II	Xenic	D	A7/Cv	C	ND
10	TM35	Thailand	NA ^b	ALA	+	ND ^c	Pus	B	A7	E	Irr ^d
11	TM36	Thailand	NA	ALA	+	ND	Pus	B	A6/Cv	C	Irr
12	TM37	Thailand	NA	ALA	+	ND	Pus	L	A7	B	Irr
13	TM51	Thailand	1992	ALA	+	II	Axenic	B	A6/Cv	C	3/F
14	TM53	Thailand	1988	Colitis	+	II	Xenic	D	A7/Cv	C	10/L
15	TM54	Thailand	1988	Colitis	+	II	Xenic	D	A7/Cv	C	10/L
16	TM55	Thailand	1988	Colitis	+	II	Xenic	D	A7/Cv	C	10/L
17	TM58	Thailand	1989	Colitis	+	II	Xenic	D	A7/Cv	C	10/L
18	TM59	Thailand	1989	Dysentery and colitis	+	II	Xenic	D	A7	C	G
18	TM60	Thailand	1989	Colitis	+	II	Xenic	B	A7	E	G
20	TM61	Thailand	1989	Colitis	+	II	Xenic	B	A7	C	G
21	TM62	Thailand	1989	Dysentery and colitis	+	II	Xenic	B	A7	E	G
22	TM63	Thailand	1989	Dysentery and colitis	+	II	Xenic	L	A6/Cv	C	G
23	TM64	Thailand	1989	Colitis	+	II	Xenic	L	A6/C7	C	10/L
24	TM65	Thailand	1989	Dysentery and colitis	+	II	Xenic	D	A9v/A7	E	10/L
25	TM67	Thailand	1989	Colitis	+	II	Xenic	D	A7/Cv	C/F	10/L
26	TM83	Thailand	2001	Dysentery and colitis	+	II	Xenic	D	A7/Cv	C	11
27	TM84	Thailand	2001	Colitis	+	II	Pus	D	A7	C/F	L
28	TM40	Bangladesh	2000	NA	+	ND	Xenic	D	A6	E	15
29	TM41	Bangladesh	2000	NA	+	ND	Xenic	D	A5	C	Mix ^e
30	TM42	Bangladesh	2001	NA	+	ND	Xenic	D	A6	E	13
31	TM43	Bangladesh	2001	NA	—	ND	Xenic	D	A9	C	13
32	TM44	Bangladesh	2001	NA	+	ND	Xenic	D	A8/A5	C	1/4
33	KU6	Ghana	1994	Asymptomatic	+	II	Xenic	L	Cv	C	F
34	KU12	Cambodia	1995	Asymptomatic	+	II	Xenic	D	A5	C	2/17/18
35	PK1	Indonesia	2002	Colitis	ND	ND	Stool	M	A9v/A7	G	H
36	KU33	Institution E ^f	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
37	KU34	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
38	KU35	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
39	KU36	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
40	KU37	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
41	KU38	Institution E	June, 2002	Asymptomatic	—	II	Xenic	F	A5v/Cv	C	K
42	KU39	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
43	KU40	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K
44	KU41	Institution E	June, 2002	Asymptomatic	+	VII	Xenic	F	A5v/Cv	C	K
45	KU42	Institution E	June, 2002	Asymptomatic	+	II	Xenic	F	A5v/Cv	C	K

^a The gel diffusion precipitin test and enzyme-linked immunosorbent assay were used for serology.^b NA, not available.^c ND, not determined.^d Irr, irrelevant PCR fragments.^e Mixed, likely a mixture judged by sequencing.^f Institution E is located in Yamagata Prefecture, Japan.

ing loci, including the SREHP locus, were previously demonstrated among *E. histolytica* isolates obtained from two social populations (mentally handicapped individuals and homosexual men) in a limited geographic area (domestic cases only in Japan) (14). In the present study, we extend our previous study to answer three specific questions: (i) how polymorphic are the Southeast Asian *E. histolytica* isolates? (ii) how similar or dissimilar are the genotypes of the Southeast Asian strains in comparison to those of the Japanese strains? and (iii) does a correlation exist between the genotypes of the isolates and the clinical presentations that they cause? The results of the present study not only support the previous finding of extensive genetic diversity among *E. histolytica* isolates (2, 7, 13, 14, 43,

44) but also fail to demonstrate a notable association between SREHP genotypes and clinical presentation or geographic origin.

MATERIALS AND METHODS

Clinical specimens. A total of 79 *E. histolytica* isolates, including 45 that were newly isolated, were analyzed in this study (Table 1). Thirty-four strains reported previously (14) were also used in the present study for comparison. Among the 45 new isolates, 10 isolates were obtained from stool samples from asymptomatic but seropositive individuals, with one exception, in an institution for mentally handicapped individuals in Yamagata Prefecture, Japan. Twenty-seven isolates were collected from either stool or liver aspirates from patients who visited outpatient clinics of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Five specimens were kindly provided by Rashidul Haque, Dhaka, Bangladesh, through Mahidol University. Three additional strains were

also isolated from stool samples from two Japanese workers who previously worked in Ghana and Cambodia and a domestic patient from Manado, Indonesia. Four patients had amebic liver abscesses (ALAs), and 24 patients had amebic dysentery and/or colitis. Twelve patients did not show any notable symptoms and thus were considered asymptomatic cyst passers. Identification of individual isolates as *E. histolytica* and not *Entamoeba dispar* was verified as described previously (14) by PCR with *E. histolytica*- and *E. dispar*-specific oligonucleotide primers (see below). A past or present history of invasive amebiasis was verified for 40 patients by serology by the gel diffusion precipitation test (26) and enzyme-linked immunosorbent assay (36). The clinical status of patients infected with five isolates (isolates TM40 to TM44) were not determined. All clinical specimens were collected after informed consent was obtained from the patients.

Cultivation. Xenic and axenic in vitro cultures were established by using Robinson's medium and BI-S-33 medium, respectively, as described previously (9, 29). Most xenic and axenic strains were cryopreserved by the method of Diamond (8) after xenic and axenic cultures were established and were revived 1 to 3 months prior to the present study to minimize possible changes, if any, in the genotypes.

DNA preparation, PCR, and sequence analysis. Total genomic DNA from trophozoites and/or cysts was purified from either cultured trophozoites or clinical specimens as described previously (14). Identification of *E. histolytica* and exclusion of *E. dispar* were verified by PCR with two sets of primers (primers Hsp1 and Hsp2 for *E. histolytica* and primers Dsp1 and Dsp2 for *E. dispar*) under the conditions described previously (44). Individual *E. histolytica* isolates were classified by PCR amplification of four previously described loci, i.e., locus 1-2 and locus 5-6 (43) and the chitinase and SREHP loci (13), by using four sets of oligonucleotides under the PCR conditions described previously (14), except that an annealing temperature of 50°C was used for all four loci. Loci 1-2 and 5-6 are present as tandemly linked multicopies within a >20-kb region (43) and contain tRNA genes (C. G. Clark, personal communication). No polymorphism in the nucleotide sequences was found among individual repeat units in the genome database (data not shown), which is consistent with the finding that PCR fragments containing these loci are homogeneous. Chitinase and SREHP are each apparently present as a single copy per haploid genome; only one copy of chitinase and SREHP each was found in the HM1 genome database. Therefore, although the ploidy of *E. histolytica* has not been determined, each of these genetic markers can be considered to be present as a single copy (per haploid genome). PCR products containing these loci were directly sequenced with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction II kit (PE Applied Biosystems, Foster City, Calif.) on an ABI PRISM 310 Genetic Analyzer. In cases in which multiple (more than one) bands were recognized after separation by 2% agarose gel electrophoresis, each DNA fragment was excised and sequenced separately, as described previously (14). The expected frequency of mutations with HotStar TaqDNA polymerase (Qiagen, Tokyo, Japan) was 2×10^{-5} (data not shown). We also tried to minimize the cycle numbers to avoid the accumulation of PCR products, which is known to increase the chance of introduction of mutations. Thus, when the lengths of the PCR fragments amplified in this study are considered (120 to 490 bp), the chance that mutations were introduced by PCR was negligible. The sequences obtained were manually edited and aligned by using DNASIS (version 3.7; Hitachi, Yokohama, Japan).

Restriction length polymorphism (RFLP) analysis of SREHP locus. Approximately 0.1 µg of the SREHP PCR products was digested with 3 U of *AluI* (Takara, Tokyo, Japan) in a volume of 20 µl at 37°C for 2 to 16 h. About 5 µl of the *AluI*-digested material was electrophoresed in 12% polyacrylamide gels (30). To visualize the DNA, the gels were stained by use of a silver staining kit (Pharmacia Biotech, Tokyo, Japan).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in the present work have been submitted to the GenBank/EMBL/DBJ database under accession numbers AB096653 to AB096676.

RESULTS

High-resolution genotyping of *E. histolytica* field isolates and identification of new genotypes. It was previously shown that the levels of genetic polymorphism of the four polymorphic genetic loci mentioned above among *E. histolytica* isolates in Japan are extremely high (14). However, it is unknown if genetic polymorphisms also exist among the amebic isolates within the areas of developing countries where *E. histolytica* is endemic, e.g., Southeast Asian countries, and, if so, to what

extent. Thus, we conducted high-resolution genotyping of these polymorphic loci for a large number of the *E. histolytica* isolates obtained from the area of endemicity. We attempted to answer the following questions: (i) how polymorphic are the Southeast Asian *E. histolytica* strains? (ii) how similar or dissimilar are the genotypes of the Southeast Asian ameba strains in comparison to those of the Japanese strains? and (iii) does any correlation exist between the genotypes of the isolates and the clinical presentations that they cause? We chose Thai isolates for analysis since it has been demonstrated that some of the human immunodeficiency virus (HIV) strains present in Japan were imported from Thailand (5, 20, 40). We amplified the four loci by PCR and sequenced individual fragments from 45 clinical isolates (27 isolates from Thailand; 5 isolates from Bangladesh; 10 isolates from Japan; and 1 isolate each from Cambodia, Indonesia, and Ghana). The profiles of the PCR fragments on agarose gels are shown in Fig. 1A for representative isolates (only data for new genotypes of SREHP are shown; see reference 14 for the previously identified genotypes). After sequencing, we identified among these 45 isolates 3 novel genotypes for locus 1-2 (genotypes K, L, and M), 2 novel genotypes for locus 5-6 (genotypes A11v and A9v), 1 novel genotype for chitinase (genotype G), and 18 novel genotypes for SREHP (genotypes 1 to 18) (a schematic diagram of all SREHP genotypes only is shown in Fig. 2; those of the other loci are not shown; all the sequence information was deposited in the GenBank/EMBL/DBJ database). When these data are combined with previous data (14), we have identified among our 79 isolates and 4 previously reported isolates (13, 39) 13 different genotypes in locus 1-2, 15 different genotypes in locus 5-6, 9 different genotypes in the chitinase locus (data not shown), and 37 different genotypes in the SREHP locus (Fig. 2). The deduced peptide sequences of the chitinase and SREHP loci were also analyzed. The total number of SREHP genotypes based on the predicted amino acid sequences (31) was only slightly smaller than the number of SREHP genotypes based on the nucleotide sequences of individual PCR fragments (37) (data not shown), whereas the total number of chitinase genotypes was identical between the nucleotide and the predicted amino acid sequences. Among 27 Thai isolates, we identified 5, 7, 6, and 13 distinct genotypes for locus 1-2, locus 5-6, the chitinase locus, and the SREHP locus, respectively (4 isolates were excluded from the analysis of the SREHP locus for the reason explained in footnotes c and d of Table 1), suggesting that the extent of polymorphism is comparable between the isolates from Thailand and those from the Japanese homosexual men.

Heterozygosity of chitinase and SREHP. Although the chitinase locus was previously found to be homozygous, with one exception (type A/C) (14), we have found three additional isolates with two distinct chitinase genes (previously identified as genotype A/C and a new genotype, C/F). The demonstration of double SREHP genotypes in several isolates (i.e., isolates TM24, TM44, TM51 to TM58, and TM64 to TM67) strongly argues for the heterozygosity of this gene, as suggested previously (2, 13, 14). However, one isolate (isolate KU12) showed triple SREHP fragments on agarose gel electrophoresis corresponding to genotypes 2, 17, and 18.

Intergeographic differences in distributions of genotypes of each polymorphic locus. To examine the similarities and dif-

FIG. 1. Profiles of SREHP fragments amplified by PCR from clinical specimens and electrophoresed on agarose gels. All genotypes reported previously (14) and reported by Ghosh et al. (13) are given letter designations (A to S [14; this study]), and new genotypes designated in the present study are given number designations (1 to 18) in this and the other figures. (A) Agarose gel electrophoresis of undigested SREHP PCR fragments from representative *E. histolytica* isolates. Only the results for representative isolates that belong to each genotype (presented in parentheses) are shown. (B) Polyacrylamide gel electrophoresis of PCR-amplified and *AluI*-digested SREHP fragments from selected isolates. (C) Schematic representation of *AluI* digests of all genotypes. Note that the individual genotype is designated for each DNA fragment. Double asterisks indicate that more information can be found in reference 13.

ferences among the isolates from Japan, Thailand, and other countries, the distributions of the genotypes of the four loci for all 79 isolates analyzed in our previous and present studies, together with those of four strains reported by others (13, 43), were examined (Fig. 3). We excluded 16 of 21 Japanese isolates from mentally handicapped individuals from these analyses because the mass infections were likely attributable to single strains (14), and therefore, inclusion of these genotypes would likely bias the outcomes of the analyses; e.g., locus 1-2 genotype F, locus 5-6 genotype A5v/Cv, chitinase locus genotype C, and SREHP locus genotype K were found in 16 isolates from mentally handicapped individuals. Marked differences in the histograms of locus 1-2, locus 5-6, and the SREHP locus were readily recognized, whereas the distributions of the chitinase locus genotypes were similar among the three groups of isolates. Some differences were very striking; e.g., genotype B of locus 1-2 represents a dominant type among the Japanese isolates (about 40%), while genotype D of locus 1-2 is dominant among the isolates from Thailand and other countries (about 50%) (Fig. 3A). Marked differences in histograms were found not only in a homozygous locus, i.e., locus 1-2, but also in a heterozygous noncoding locus, i.e., locus 5-6. A histogram showing the frequencies of locus 5-6 (Fig. 3B) showed that locus 5-6 genotype A5v or A7 was detected in about 20% of the Japanese isolates, whereas genotypes A6/Cv, A7, and A7/Cv were dominant (about 30%) among the Thai isolates. The results were almost similar when the allelic types, but not combinations of alleles, of locus 5-6 were compared (data not shown). Locus 5-6 allelic genotypes A5, A5v, and A7 were dominant and were found in about 60% of the Japanese isolates, while allelic type A7 was dominant (45%) among the Thai isolates. In contrast to the notable differences in the genotype distributions of loci 1-2 and 5-6, the chitinase locus genotypes showed similar distributions among the three groups (Fig. 3C). The extent of genetic variation of the SREHP locus is much higher than those of the other three loci (Fig. 3D); only one SREHP locus genotype (genotype 7) was shared by the Japanese and Thai isolates.

RFLP analysis of *AluI* digests of the SREHP locus. Ayeh-Kumi et al. (2) recently demonstrated, using RFLP analysis of the *AluI* digests of the SREHP PCR fragments (7), polymorphic patterns among clinical isolates from Bangladesh. They also reported that the majority (92%) of isolates from liver abscesses showed patterns distinct from those of the intestinal isolates. On the basis of these data, they proposed that particular SREHP locus genotypes and RFLP patterns may be closely associated with virulence. To further test this hypothesis, we first conducted a computational RFLP analysis based on the nucleotide sequences of the SREHP loci of all isolates that we obtained (Fig. 1C; only representative patterns are shown). Notable differences in the RFLP patterns were seen among these genotypes; the number of the patterns, however,

decreased significantly compared to the number obtained by genotyping based on nucleotide sequences ($n = 34$ to 24 patterns) (Fig. 2). We also found that the histograms of the RFLP patterns between Japanese and Thai isolates differed significantly (data not shown), which was similar to the observation for the comparisons at the nucleotide level. However, we were unable to find any RFLP patterns that correlated with clinical presentations (e.g., ALA, colitis, or cyst carrier), the backgrounds of the patients (e.g., homosexual men or mentally handicapped individuals), or geographic origin. These computational RFLP analyses of the SREHP locus were also verified by *AluI* digestion and polyacrylamide gel electrophoresis analyses of the PCR fragments from several representative isolates (Fig. 1B).

DISCUSSION

Using high-resolution genotyping based on the nucleotide sequences of four polymorphic loci of *E. histolytica*, we were able to demonstrate that this parasite from an area of endemicity in Southeast Asia has an extremely polymorphic genetic structure; e.g., 21 different combinations of genotypes were found among the 27 isolates obtained from Thailand. In combination with previous results (14), 53 combinations of genotypes were observed among 63 isolates. (Note that 16 isolates from institutions for mentally handicapped individuals [e.g., KU13, KU19 to KU22, KU28, and KU29 [14] and KU34 to KU42] were excluded for the reason described in Results.) This, together with previous work (14), in which an extensive polymorphism of the amebic strains from Japanese homosexual men was shown, reinforces the premise that *E. histolytica* has an extremely complex genetic structure independent of geographic location.

On the basis of the close social and economic relationship between Japan and Thailand and the fact that (i) sexual intercourse between homosexual men is closely associated with both HIV and amebic infections and (ii) comparison of genotypes between Thai and Japanese HIV strains indicates that a proportion of Japanese HIV strains were imported from Thailand (19, 21, 25, 37, 40), we hypothesized that the Japanese and Thai *E. histolytica* isolates might reveal a similar spectrum of genotypes that is indicative of similarities in the population structures of the *E. histolytica* strains between the two countries. However, our results appeared to argue against this hypothesis. Although notable similarities in the genotypes of locus 1-2, locus 5-6, and the chitinase locus were found between the Japanese and Thai isolates, polymorphisms in the SREHP locus have been found to be very extensive: only one of the Thai isolates showed an SREHP genotype identical to that of the Japanese isolates. When the genotypes of all four loci were combined, none of the Thai isolates had genotypes identical to those of the Japanese strains. Extensive polymor-

FIG. 2. Schematic representation of the polymorphisms in the repeat-containing region of the SREHP gene demonstrated among all isolates analyzed in the previous (14) and present studies and reference strains. *AluI* restriction sites are depicted by vertical lines. The numbers shown correspond to the nucleotides of strain HM1 (accession no. M80910). Gaps were manually introduced to optimize alignments. Conserved regions are highlighted with gray rectangles. The nucleotide and deduced amino acid sequences of tri-, tetra-, octa-, and nonapeptide repeats are shown below. Also note that a previously unidentified tripeptide repeat unit is also included here. Asterisks next to the genotypes indicate that more information can be found in reference 13.

FIG. 3. Histograms showing genotype distributions of locus 1-2 (A), locus 5-6 (B), the chitinase locus (C), and the SREHP locus (D) from representative isolates from Japan, Thailand, and other countries. Only results for representative isolates of each genotype are shown. Asterisks indicate that more information can be found in references 13 and 43. Note that 16 of 21 Japanese isolates obtained from mentally handicapped individuals, which showed identical genotypes for all four loci, were excluded from these analyses (as explained in Results). Also note that 11 isolates showing either mixed or irrelevant PCR bands as judged by sequencing were excluded from analysis of the SREHP locus.

phism of the SREHP locus at the nucleotide level was shown previously (14); e.g., six distinct genotypes were found among 11 isolates from Japanese male homosexual men. Although genetic polymorphisms in a restricted geographic location in the area of endemicity have been reported by Ayeh-Kumi et al. (2), it is conceivable that those investigators underestimated the degree of polymorphism due to a lack of resolution of their analytical methods (PCR amplification of only the SREHP locus, followed by RFLP analysis on agarose gels). On the basis of the nucleotide sequences of all SREHP fragments and computational analyses of virtual RFLPs, we found that several SREHP genotypes would have been indistinguishable by RFLP analysis (e.g., genotypes D, 15, and J and genotypes 10, B, P, L, 17, Q, and 18; Fig. 1C). We should also mention that a histogram of the SREHP genotypes of the Thai isolates was also significantly different from that of the isolates from neighboring countries, including Bangladesh and Indonesia (Fig. 3D), although the number of isolates from those countries was too small to draw a definitive conclusion.

A high degree of genetic polymorphism in the repeat-containing region of SREHP raised a number of questions regarding the function of this protein and its association with the virulence and pathophysiology of *E. histolytica*. One of the most obvious questions is why the degree of polymorphism of SREHP is higher than those of the chitinase and noncoding loci. Although Ayeh-Kumi et al. (2) suggested that certain SREHP genotypes are more likely associated with ALA, this premise was not supported in the present study. We did not find any particular SREHP type, at either the nucleotide sequence level or the predicted amino acid sequence level, in association with clinical presentation. This is not likely due to either a lack of resolution of their analytical methods or differences in geographic backgrounds; the heterogeneity of RFLP patterns that Ayeh-Kumi et al. (2) reported (34 distinct patterns among 54 isolates) was comparable to the polymorphism of the SREHP locus at the nucleotide level that we report here. It has previously been demonstrated (31, 34) that SREHP is highly immunogenic because it possesses a number of conserved epitopes and that more than 80% of the individuals with ALA possess antibodies to SREHP, highlighting this protein as an important vaccine candidate. Together with the remarkable polymorphisms within the repeat-containing region of SREHP, as shown in this and other studies, these data strongly suggest that this polymorphism likely has a biological role, including immune evasion, as suggested elsewhere (34, 45, 46). However, the nucleotide and amino acid polymorphisms of the SREHP locus are too extensive to discuss the biological significance of these polymorphisms and their constraints on SREHP as a functional protein.

To verify the stability of the genotype observed for each isolate, we examined the nucleotide sequences of the four loci from four different isolates (isolates KU14, KU18, and KU26 [14] and isolate KU36) using xenic cultures, monoxenic cultures (cultivation with *Crithidia fasciculata*), and axenic cultures. We found no change in the genotypes of any of the four loci in these four strains (data not shown), indicating, together with previous findings (7, 44), that the nucleotide sequences of these loci are stable under a variety of conditions, e.g., long-term cultivation, axenization, cell cloning, and animal passage.

We should also note that the genotypes of the isolates ob-

tained from institution E (locus 1-2, genotype F; locus 5-6, genotype A5v/Cv; the chitinase locus, genotype C; and the SREHP locus, genotype K) are identical to those of isolates from two other institutions for mentally handicapped individuals (institutions B and C) (14). These isolates were obtained from independent mass infection events at remote geographic locations (Kanagawa, Shizuoka, and Yamagata Prefectures in Japan, approximately 540 km apart) at different times (1994, 2000, and 2002). This finding further supports the premise that the genotypes of the *E. histolytica* isolates are stable after human transmission.

We also present further evidence of the heterozygosity of the chitinase and SREHP loci. The presence of multiple isoenzymes showing distinct affinities for substrates and the inhibitor allosamidin was demonstrated in *Entamoeba invadens* (38), posing the question of why only a small proportion (5%) of *E. histolytica* isolates possess multiple chitinase isoenzymes. The presence of multiple chitinase isoenzymes may be beneficial for the amoeba since a broader substrate range may be covered by isoenzymes possessing distinct properties, as shown for two isoforms from *Serratia marcescens* (4), *E. invadens* (38), and *Plasmodium gallinaceum* (39). In contrast to chitinase genes, the SREHP locus was found to be heterozygous in approximately 29% of all isolates, suggesting the biological significance of heterozygosity in this gene. The presence of the triple SREHP genes in isolate KU12 cannot be due to a mixed culture or cross contamination since (i) none of these three bands were found in the other isolates and (ii) none of the other loci, i.e., locus 1-2, locus 5-6, and the chitinase locus, showed mixed patterns. This is inconsistent with the previous finding indicating that the SREHP gene appears to be present in a single copy (22). Thus, this isolate may represent a triploid or aneuploid, although the ploidy of reference strain HM1 was previously suggested to be at least four (42).

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