

Transfection and continuous expression of heterologous genes in the protozoan parasite *Entamoeba histolytica*

(amoebiasis/electroporation/gene transfer/neomycin phosphotransferase/chloramphenicol acetyltransferase)

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ABSTRACT To provide tools for functional molecular genetics of the protozoan parasite *Entamoeba histolytica*, we investigated the use of the prokaryotic neomycin phosphotransferase (NEO) gene as a selectable marker for the transfection of the parasite. An *Escherichia coli*-derived plasmid vector was constructed (pA5'A3'NEO) containing the NEO coding region flanked by untranslated 5' and 3' sequences of an *Ent. histolytica* actin gene. Preceding experiments had revealed that amoebae are highly sensitive to the neomycin analogue G418 and do not survive in the presence of as little as 2 µg/ml. Transfection of circular pA5'A3'NEO via electroporation resulted in *Ent. histolytica* trophozoites resistant to G418 up to 100 µg/ml. DNA and RNA analyses of resistant cells indicated that (i) the transfected DNA was not integrated into the amoeba genome but was segregated episomally, (ii) in the amoebae, the plasmid replicated autonomously, (iii) the copy number of the plasmid and the expression of NEO-specific RNA were proportional to the amount of G418 used for selection, and (iv) under continuous selection, the plasmid was propagated over an observation period of 6 months. Moreover, the plasmid could be recloned into *E. coli* and was found to be unrearranged. To investigate the use of pA5'A3'NEO to coexpress other genes in *Ent. histolytica*, a second marker, the prokaryotic chloramphenicol acetyltransferase (CAT) gene under control of an *Ent. histolytica* lectin gene promoter was introduced into the plasmid. Transfection of the amoebae with this construct also conferred G418 resistance and, in addition, allowed continuous expression of CAT activity in quantities corresponding to the amount of G418 used for selection. When selection was discontinued, transfected plasmids were lost as indicated by an exponential decline of CAT activity in trophozoite extracts.

Entamoeba histolytica, the causative agent of human amoebiasis, is an enteric protozoan parasite capable of invading the mucosa of the colon and entering the blood circulation to reach various extraintestinal organs (1). Fifty million people suffer from amoeba-induced colitis or extraintestinal abscesses resulting in ≈50,000 deaths annually (2).

During recent years our knowledge of the structure and function of amoeba proteins potentially involved in human tissue destruction has increased substantially. At least three functions of the amoebae are considered essential for pathogenic tissue invasion: (i) adherence of the amoebae to host cells, predominantly mediated by a galactose- and N-acetylgalactosamine-inhibitable lectin, (ii) killing of host cells by a pore-forming peptide known as amoebapore, and (iii) proteolysis of the host's extracellular matrix mediated by cysteine proteinases (for review, see ref. 3). However, because of major difficulties in using classical genetic approaches, the various molecules could only be studied by *in vitro* analyses and their functional importance could not be addressed *in vivo*. To

overcome such problems, a sufficient stable transformation system is required for *Ent. histolytica*.

Recently, transfection and transient expression of two prokaryotic genes in *Ent. histolytica* were described (4, 5). Here we report on an *Ent. histolytica* transformation system allowing continuous and regulated expression of heterologous genes.

MATERIALS AND METHODS

Cultivation of Parasites. For all experiments, the axenically cultured *Ent. histolytica* isolate HM-1:IMSS was grown in TYI-S-33 medium (6).

Plasmid Construction. Transfection vector pA5'A3'NEO was constructed by replacing the chloramphenicol acetyltransferase (CAT) coding sequence of transient transfection vector pA5'A3'CAT (4) by the neomycin phosphotransferase (NEO) coding sequence. The NEO sequence with addition of *Kpn* I and *Bam*HI restriction sites was generated by PCR using pSV2neo (7) as template and synthetic oligonucleotides NEO-S25 (5'-GGG-GTA-CCA-TGA-TTG-AAC-AAG-ATG-G-3') and NEO-AS23 (5'-GGG-GAT-CCT-TAG-AAG-AAC-TCG-TC-3') as primers. The CAT sequence of pA5'A3'CAT was removed by the restriction endonucleases *Kpn* I and *Bam*HI and subsequently the PCR-generated NEO sequence was introduced. The final transfection vector is a pBS (Stratagene)-derived plasmid containing the NEO coding sequence flanked by 480 bp of untranslated 5' sequence and 600 bp of untranslated 3' sequence of an *Ent. histolytica* actin gene (Fig. 1A). A second transfection vector, pEhNEO/CAT, was constructed by introducing a 1750-bp fragment into *Hind*II/*Hind*III-digested pA5'A3'NEO (see Fig. 5). This 1750-bp fragment was excised by digestion with the restriction endonucleases *Eco*RV and *Hind*III from transient expression plasmid pL5'A3'CAT (4, 8). It comprises the CAT coding region flanked by 485 bp of untranslated 5' sequence of an *Ent. histolytica* lectin gene and by 600 bp of untranslated 3' sequence of an *Ent. histolytica* actin gene.

Transfection of *Ent. histolytica* Trophozoites. Transfections were performed by electroporation as described (4), with the minor modification that instead of one, two subsequent pulses were applied, each with an exponential discharge of 3000 V/cm at a capacitance of 25 µF, with the Bio-Rad Gene Pulser. We generally transfected 1×10^7 trophozoites with 200 µg of circular plasmid DNA per cuvette. Drug selection was started 48 hr after transfection, using G418 at 10 µg/ml unless otherwise indicated.

DNA and RNA Analysis. Genomic DNA and total cellular RNA from *Ent. histolytica* were prepared as described (9). Plasmid DNA from *Escherichia coli* was extracted by the alkaline lysis method (10). Southern and Northern blots,

Abbreviations: CAT, chloramphenicol acetyltransferase; NEO, neomycin phosphotransferase.

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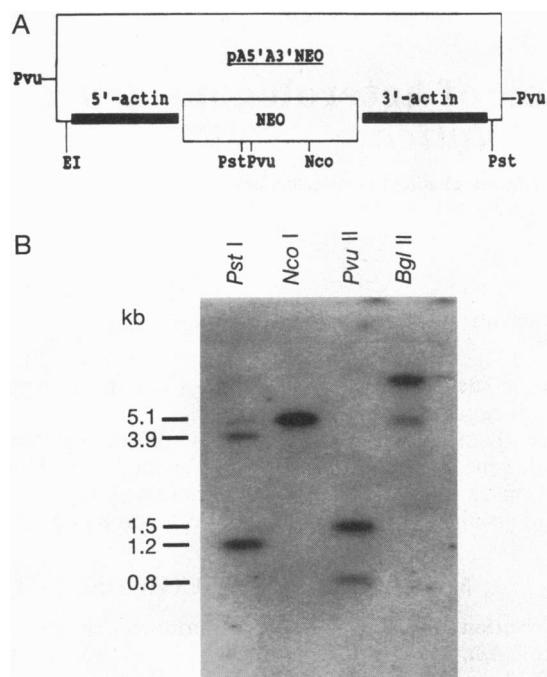


FIG. 1. Detection of pA5'A3'NEO sequences in transfected, G418-resistant *Ent. histolytica*. (A) Schematic depiction and partial restriction map of the 5.1-kb transfection plasmid pA5'A3'NEO. This vector is a pBS-derived plasmid containing the NEO coding region from ATG to the stop codon, flanked by untranslated 5' and 3' sequences of an *Ent. histolytica* actin gene (5'-actin, 3'-actin). EI, *EcoRI*; Nco, *Nco I*; Pst, *Pst I*; Pvu, *Pvu II*. (B) Autoradiograph of a Southern blot of total genomic DNA isolated from pA5'A3'NEO-transfected, G418-resistant trophozoites. Ten micrograms of DNA was digested with restriction endonucleases as indicated, separated in an agarose gel, blotted, and hybridized with the purified radiolabeled NEO coding sequence. Size markers are indicated at left. Note: The sizes of the hybridizing fragments are identical to those predicted from the pA5'A3'NEO restriction map. *Bgl II* does not cleave within pA5'A3'NEO. The hybridizing fragments obtained with *Bgl II*-digested DNA correspond to relaxed and concatameric circular plasmids.

hybridization, and washings were done by standard protocols (10).

CAT Assay. CAT activity was analyzed by the two-phase diffusion assay (11) using 10 μg of trophozoite extract and [^{14}C]butyryl-CoA (DuPont/NEN). CAT activity was determined in the linear range of the assay.

RESULTS

Transfection of *Ent. histolytica* Trophozoites Conferring G418 Resistance. Several sequence motifs within the upstream and downstream regions have been found to be highly conserved in various *Ent. histolytica* genes (12). In addition, transient transfections of *Ent. histolytica* have demonstrated that expression of heterologous genes is dependent on the presence of specific amoebic 5' as well as 3' flanking sequences (4, 5, 8). Therefore, we used pA5'A3'CAT, a hybrid plasmid able to drive transient CAT expression in *Ent. histolytica*, for construction of a transfection vector, pA5'A3'NEO, in which the CAT coding sequence was replaced by the coding sequence of the prokaryotic NEO gene. Therefore, pA5'A3'NEO is an *E. coli*-derived plasmid comprising the NEO coding region flanked by 480 bp of upstream sequence and 600 bp of downstream sequence of an *Ent. histolytica* actin gene (Fig. 1A). Earlier studies had indicated that cultured *Ent. histolytica* trophozoites were highly sensitive to G418 and did not survive in the presence of G418 at $>2 \mu\text{g}/\text{ml}$. Transfection by elec-

troportionation of $\approx 10^7$ trophozoites with 200 μg of circular plasmid pA5'A3'NEO and subsequent selection with G418 at 10 $\mu\text{g}/\text{ml}$ resulted in a substantial number of resistant clones which became visible after 8–10 days of selection. In contrast, no resistant amoebae were obtained in mock-transfected cultures or when pA5'A3'NEO was introduced in a linearized form. After several weeks of G418 selection, total DNA of resistant trophozoites was analyzed for the presence of NEO sequences. Southern blot analysis showed that the transfected DNA was not integrated into the amoeba genome but remained episomally as unrearranged circular plasmids (Fig. 1B). The integrity of pA5'A3'NEO within the amoebae was confirmed by recloning the plasmid from total genomic DNA of resistant trophozoites into *E. coli*. Restriction analysis revealed no rearrangement of the recloned plasmid. Taken together, the results indicate that pA5'A3'NEO is suitable for stable transfection of *Ent. histolytica* and that it provides a useful shuttle vector between *Ent. histolytica* and *E. coli*.

Replication of Transfected Plasmid DNA. Within an observation period of >6 months, G418-resistant amoebae grew well under continuous selection pressure, with a generation time equivalent to that of control cultures. The transfected plasmid within resistant amoebae remained episomal. These results strongly suggest that pA5'A3'NEO was being replicated. To address this question more directly, DNA methylation of the plasmid was studied before and after transfection (Fig. 2). Plasmid DNA of pA5'A3'NEO-transformed *E. coli* DH5 α (*dam*⁺) and total genomic DNA of transfected, G418-resistant *Ent. histolytica* were isolated and digested with the isoschizomers *Dpn I* and *Nde II*, which distinguish the DNA methylation states of the sequence GATC. The restriction enzyme *Dpn I* will cleave the sequence only if the A residue is methylated, whereas *Nde II* will not because it is sensitive to the *E. coli* *Dam* methylation system. As expected, the plasmid isolated from *E. coli* was not digested with *Nde II* but was digested with *Dpn I*. In contrast, DNA analysis of G418-resistant amoebae which had been transfected with *E. coli*-derived, *Dam*-methylated pA5'A3'NEO revealed the converse cutting order for the plasmid. The long-term maintenance of

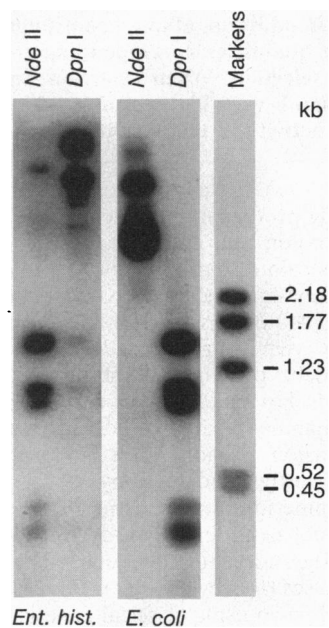


FIG. 2. Changes in the methylation state of transfected plasmid DNA in *Ent. histolytica*. Total genomic DNA of parasites transfected with pA5'A3'NEO (Left) and plasmid DNA of pA5'A3'NEO-transformed *E. coli* DH5 α (*dam*⁺) (Right) were isolated, digested with the restriction endonucleases *Nde II* and *Dpn I*, electrophoresed in an agarose gel, blotted, and hybridized with radiolabeled pA5'A3'NEO.

the plasmid, as well as the changes in the methylation pattern, indicated that pA5'A3'NEO was replicating in *Ent. histolytica*. In addition, the comparison between pA5'A3'NEO from *E. coli* and *Ent. histolytica* demonstrated that in amoebae the plasmid did not form a supercoil. Undigested plasmid isolated from *E. coli* revealed three bands indicative of supercoiled, relaxed, and concatameric DNA, whereas undigested plasmid of resistant *Ent. histolytica* revealed only two bands, corresponding to relaxed and concatameric plasmid (Fig. 2).

Copy Number of pA5'A3'NEO and Expression of NEO-Specific Transcripts in Response to Increased G418 Pressure. Autonomous replication of extrachromosomal circular DNA elements has been reported for other protozoan parasites (13, 14). In drug-resistant *Leishmania* it was found that such elements were amplified relative to the drug concentration used for selection, resulting in an increase in the respective transcript. Therefore, we selected pA5'A3'NEO-transfected trophozoites with various amounts of G418, from 5 $\mu\text{g/ml}$ up to 100 $\mu\text{g/ml}$. Subsequent Southern and Northern blot analyses of the various drug-resistant amoebae revealed that, indeed, the copy number of the plasmid as well as the amount of NEO-specific transcripts increased depending on the amount of G418 added to the cultures (Figs. 3 and 4).

Transfection of *Ent. histolytica* Trophozoites with pEhNEO/CAT, a Plasmid Containing Both the NEO Gene and the CAT Gene. Since pA5'A3'NEO replicated autonomously in *Ent. histolytica* trophozoites and increased its copy number in response to G418 pressure, we asked whether this vector would be suitable to overexpress a second gene incorporated in the plasmid. For this purpose, we ligated L5'A3'CAT into pA5'A3'NEO to obtain the expression vector pEhNEO/CAT (Fig. 5). L5'A3'CAT, which has been used successfully for transient expression of CAT in *Ent. histolytica* (4, 8), comprises

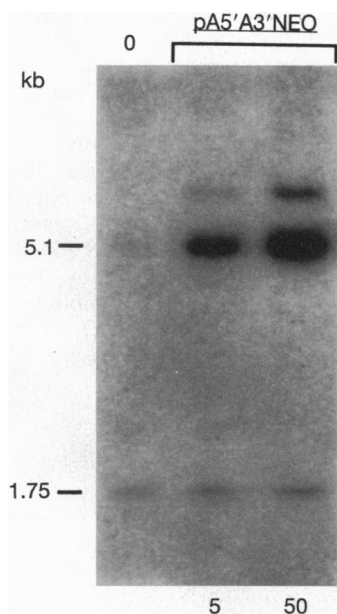


FIG. 3. Southern blot analysis of untransfected (0) or pA5'A3'NEO-transfected *Ent. histolytica* trophozoites. The latter were selected in the presence of G418 at 5 $\mu\text{g/ml}$ or 50 $\mu\text{g/ml}$. Total DNA of respective amoebae was extracted and digested simultaneously with the restriction endonucleases *EcoRI* and *EcoRV*. Subsequently, digested DNA was electrophoresed in an agarose gel, blotted, and hybridized with a purified radiolabeled 3' actin sequence (see Fig. 1). Sizes of the hybridizing fragments are indicated. The 1.75-kb band in each track corresponds to the endogenous *Ent. histolytica* actin genes, whereas the 5.1-kb band corresponds to the linearized pA5'A3'NEO (*EcoRV* does not cleave and *EcoRI* cleaves once within pA5'A3'NEO). The high molecular weight DNA represents undigested concatameric pA5'A3'NEO.

the coding sequence of the prokaryotic CAT gene flanked by 485 bp of untranslated 5' sequence of an *Ent. histolytica* lectin gene and 600 bp of untranslated 3' sequence of an *Ent. histolytica* actin gene. Transfection of *Ent. histolytica* trophozoites with pEhNEO/CAT and subsequent selection with G418 at 25 $\mu\text{g/ml}$ resulted in drug-resistant amoebae expressing extraordinarily high levels of CAT activity. Further increases of the G418 concentration to 50 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$ resulted in 2-fold and 3-fold increases of the CAT activity, respectively (Table 1).

In addition, transfection with pEhNEO/CAT was used to monitor the stability of CAT expression by subsequent culturing of drug-resistant amoebae in the absence of G418. Trophozoites were harvested at various time points up to 24 days after discontinuation of drug pressure, and extracts were examined for CAT activity. The results indicated an exponential decline of CAT activity over time. After 5–6 days, $\approx 50\%$ of CAT activity was lost, suggesting that the stability or at least the maintenance of high copy numbers of the plasmid was incomplete without further selection pressure (Fig. 6).

DISCUSSION

In a previous study (4) we reported on conditions, including the construction of specific vectors, for transfection and transient expression of a heterologous gene in the protozoan parasite *Ent. histolytica*. We constructed a specific vector and used the CAT gene as a reporter (4). In an attempt to develop a stable transformation system for *Ent. histolytica*, we used similar conditions and a modified vector, pA5'A3'NEO, in which the CAT coding sequence was replaced by that of the NEO gene, resulting in amoebae resistant to the drug G418. The NEO gene was chosen because preceding experiments had revealed that compared with other eukaryotes, amoebae are extraordinarily sensitive to G418. Trophozoites made resistant by transfection with pA5'A3'NEO were found to contain the circular plasmid in a high copy number as autonomously replicating, extrachromosomal DNA as long as G418 selection was maintained. Various circular DNA molecules are known to be present in *Ent. histolytica*, but no origin for replication of these circles has been defined (15–18). Whether a specific origin for replication active in amoebae is accidentally present on the transfection plasmid or whether the inclusion of such an element would increase stability of the plasmid after cessation of G418 selection still needs to be determined. However, it may

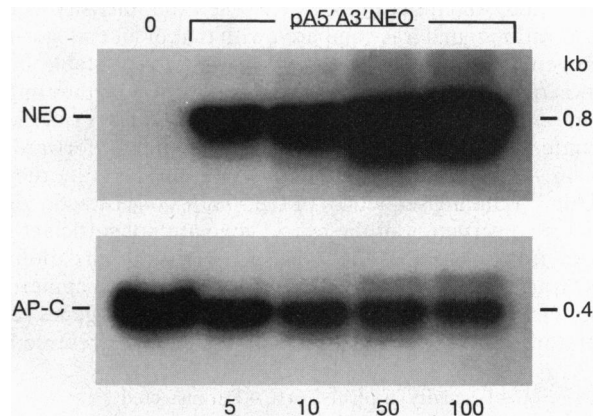


FIG. 4. Northern blot analysis of untransfected (0) or pA5'A3'NEO-transfected *Ent. histolytica* trophozoites. The latter were selected in the presence of G418 at 5, 10, 50, or 100 $\mu\text{g/ml}$ as indicated. Total cellular RNA of respective amoebae was isolated, electrophoresed in a formaldehyde/agarose gel, blotted, and sequentially hybridized with radiolabeled NEO coding sequence and AP-C, an *Ent. histolytica*-derived cDNA probe encoding the pore-forming peptide C. Size markers are indicated at right.

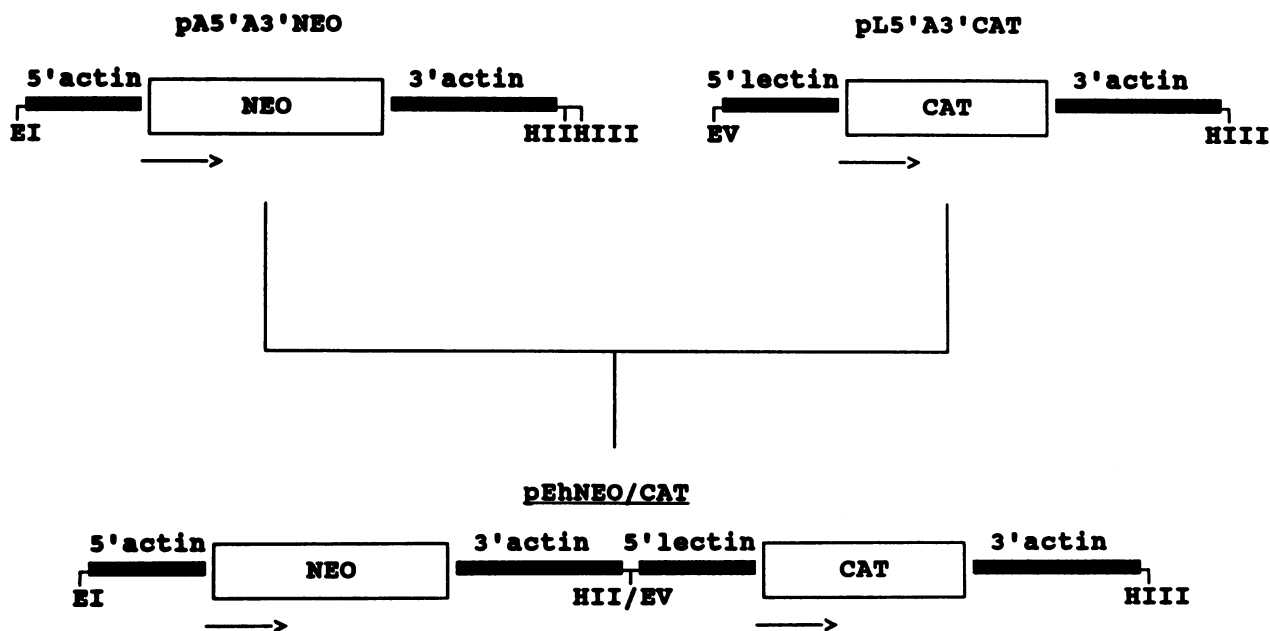


FIG. 5. Construction of transformation vector pEhNEO/CAT. The plasmid was prepared by inserting a 1750-bp *EcoRV*–*HindIII* fragment into pA5'A3'NEO digested with *HindII* and *HindIII*. The 1750-bp fragment comprises the CAT coding region flanked by 485 bp of untranslated 5' sequence of an *Ent. histolytica* lectin gene (5'lectin) and 600 bp of the untranslated 3' sequence of an *Ent. histolytica* actin gene (3'actin), EI, *EcoRI*; EV, *EcoRV*; HII, *HindII*; HIII, *HindIII*.

also be possible that a distinct origin for replication is not required, as replication of bacterial vectors in a eukaryotic organism would not be unique to *Ent. histolytica*. Plasmids containing only bacterial sequences have been shown to replicate well in *Xenopus* eggs or *Leishmania* (14, 19, 20).

In contrast to results obtained by stable transformation of other protozoan parasites or higher eukaryotes, we were unable to achieve integration of transfected DNA into the amoeba genome, even when linearized plasmid or excised A5'A3'NEO fragment was introduced. We do not have an explanation for the exclusion of the transfected DNA from the amoeba genome, but Southern blot analyses suggested that a high copy number of the transfected hybrid gene was required to mediate G418 resistance. Trophozoites transfected with pA5'A3'NEO and selected with G418 at a concentration as low as 5 $\mu\text{g}/\text{ml}$ were found to contain about 10–20 copies of the plasmid, which became evident when the intensity of the hybridization signal was compared with that of an endogenous actin gene (see Fig. 3). Therefore, if there were stable integration of a single or a few copies of the NEO gene they might fail to result in expression of enzyme concentrations sufficient to confer drug resistance. Two reasons can be put forward to explain the requirements for high copy numbers: (i) the 5' and/or 3' flanking sequences of the *Ent. histolytica* actin gene used for construction of the hybrid gene are not sufficient for proper transcription of the NEO gene or (ii) translation of NEO transcripts is incomplete because they contain numerous codons found to be absent or rare in *Ent. histolytica* genes (21). Therefore, corresponding tRNAs may be underrepresented in

Ent. histolytica, resulting in a low translation efficiency of the heterologous gene.

Nevertheless, even without integration, transfected DNA remained stable within the amoebae as long as G418 selection was maintained. Therefore, the transformation system described provides a tool with promising applications for the genetic manipulation of *Ent. histolytica* trophozoites. pA5'A3'NEO has been proven to be a useful shuttle vector between *Ent. histolytica* and *E. coli*, and as shown for the example of the CAT gene, it can be used for the expression of additional genes. Therefore, pA5'A3'NEO may provide a vector for the construction of expression libraries to target genes conferring certain *Ent. histolytica* phenotypes. In addition, the finding that the expression of plasmid-coded genes can be titrated by the amount of G418 added to the cultures will allow regulated overexpression or downregulation of selected genes. The latter may be accomplished by introducing antisense constructs into pA5'A3'NEO.

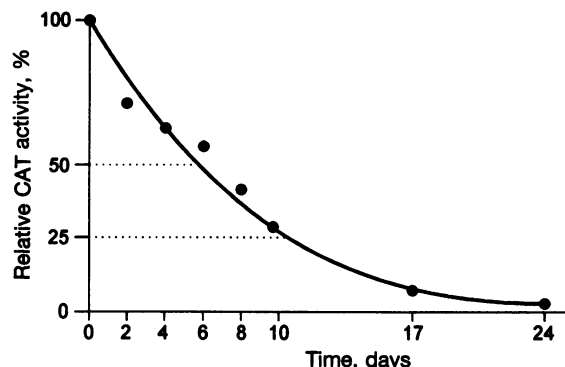


FIG. 6. Time course of CAT expression in pEhNEO/CAT-transfected *Ent. histolytica* after discontinuation of G418 selection pressure. Trophozoites were transfected with pEhNEO/CAT and cultured for several weeks in the presence of G418 at 10 $\mu\text{g}/\text{ml}$. Subsequently, amoebae were grown in the absence of G418. Cells were removed at various time points as indicated, and extracts were analyzed for CAT activity. Activity is expressed relative to the activity obtained with extracts of cells cultured in the presence of G418.

Table 1. CAT activity in pEhNEO/CAT-transfected *Ent. histolytica* in response to G418 selection

G418 selection, $\mu\text{g}/\text{ml}$	CAT activity,* counts per sec	Relative value
25	1582	1.0
50	3467	2.2
100	4523	2.9

*In 0.2 μg of amoeba lysate.

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