Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 7095–7098, July 1994
Medical Sciences

Transfection and transient expression of chloramphenicol acetyltransferase gene in the protozoan parasite *Entamoeba histolytica*

(amebiasis/electroporation/gene transfer/heterologous gene expression)

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Communicated by Hans J. Müller-Eberhard, April 4, 1994 (received for review January 31, 1994)

ABSTRACT **Hybrid plasmids were constructed and used for successful transfection and transient expression of the chloramphenicol acetyltransferase (CAT) gene in the protozoan parasite *Entamoeba histolytica*. Transfection was performed by electroporation of the amebae in a potassium phosphate-based buffer under conditions of 3000 V/cm and 25 μF, resulting in a time constant of 0.4 ms. Expression of CAT activity was achieved with constructs in which the CAT coding region was flanked by untranslated upstream and downstream sequences of *E. histolytica* genes. Highest activity was detected after culturing transfected cells for 48 hr. Activity was found to be proportional to the amount of DNA transfected.**

The enteric protozoan *Entamoeba histolytica* is the causative agent of human amebiasis. It causes about 50 million cases of colitis or extraintestinal abscesses annually, resulting in at least 50,000 fatalities (1).

During recent years the introduction of recombinant DNA technology into *Entamoeba* research has substantially increased the knowledge of *E. histolytica* strain variations, gene organization, and structure and function of amebic proteins potentially involved in human tissue destruction (for review, see ref. 2). Unfortunately, because of major difficulties in using classical genetic approaches, a number of questions concerning regulation of gene expression and further characterization of putative pathogenicity factors have not been addressed. To overcome such problems, a sufficient transformation system is required for *E. histolytica* as has been developed for several other protozoan parasites during the past few years (3–6).

Here we report on successful transfection via electroporation and transient expression of the prokaryotic chloramphenicol acetyltransferase (CAT) gene in *E. histolytica*.

MATERIALS AND METHODS

Plasmid Construction. Plasmids used for transfection were constructed by PCR-amplification of the respective DNA fragments and directional insertion into the multiple cloning site of pBluescribe (pBS; Stratagene). Fragments comprising the 5′ and 3′ flanking sequences of an actin coding region termed "5′-actin" and "3′-actin," respectively, were derived from a 10-kb clone of a genomic *E. histolytica* library (7). The fragments were amplified by using the two sets of synthetic oligonucleotide primers Eh-Ac5′-S25 (5′-TAGAATTCAAATGATGCTATATTTT)/Eh-Ac5′-AS25 (5′-CATGGTACCTGAATGTTCAATTCAG) and Eh-Ac3′-S26 (5′-GAGGATCCTAATTACTTTCTCATTTG)/Eh-Ac3′-AS27 (5′-CCTCTACTTCTCTCTCCTGTGTACACC), respectively. Fragment 5′-actin contains about 480 bp of the 5′ flanking sequence ending at position −6 relative to the actin translation initiation ATG. Fragment 3′-actin comprises about 600 bp of the actin 3′ flanking sequence starting 6 bp downstream of the actin stop codon. The oligonucleotide primers were designed such that fragment 5′-actin was flanked by *Eco*RI and *Kpn* I restriction sites and fragment 3′-actin was flanked by *Bam*HI and *Xba* I sites, allowing insertion of both fragments in a predicted orientation. A fragment containing the complete CAT coding sequence from ATG to the stop codon was amplified from the plasmid pSV2CAT (8) by using as primers CAT-S25 (5′-CAGGTACCATGGAGAAAAAAATCAC) and CAT-AS25 (5′-CTGGATCCTTACGCCCCGCCCTGCC), which contained the restriction sites *Kpn* I and *Bam*HI, respectively. This fragment was inserted later by using the *Kpn* I and *Bam*HI sites of 5′-actin and 3′-actin, respectively, to construct transfection vector pA5′A3′CAT. A second transfection vector termed "pL5′A3′CAT" was constructed by replacing 5′-actin of pA5′A3′CAT by a fragment of 1430 bp encompassing the 5′-upstream sequence of the gene encoding the 170-kDa lectin of *E. histolytica* (9). This fragment was released by *Alu* I/*Kpn* I-digestion of a 1710-bp fragment that had been obtained by PCR from DNA of the genomic clone gEh-170 (10) by primers Eh-Lec5′-S22 (5′-GACCTTGGATTATTTAAAAATC) and Eh-Lec5′-AS25 (5′-TTCGGTACCTGAATTTTTAAAGTTC). The 1430-bp fragment was linked to the CAT fragment by ligation into the *Ecl*136II/*Kpn*-site of pBS. All other plasmids used for transfection experiments were derived from pA5′A3′CAT and pL5′A3′CAT by combining the four amplified fragments in various combinations (Fig. 1*A*).

Transfection of *E. histolytica* Trophozoites. The *E. histolytica* isolate HM-1:IMSS was cultured in axenic medium TYI-S-33 in sealed plastic tissue culture flasks (11). Trophozoites in late logarithmic growth phase were detached by chilling on ice for 10 min, decanted, pelleted by centrifugation at 500 × *g* at 4°C for 10 min, and washed twice in ice-cold phosphate-buffered saline (PBS) and once in cytomix buffer consisting of 10 mM K_2HPO_4/KH_2PO_4 (pH 7.6), 120 mM KCl, 0.15 mM $CaCl_2$, 25 mM Hepes, 2 mM EGTA, and 5 mM $MgCl_2$. Subsequently, 1×10^7 trophozoites were resuspended in 0.8 ml of cytomix buffer supplemented immediately before use with 4 mM adenosine triphosphate and 10 mM glutathione, transferred to an electroporation cuvette (4-mm gap) (Eurogentec, Seraing, Belgium), and incubated in the presence or absence of cesium chloride-purified circular plasmid DNA with or without electroporation. All electroporations were performed with the Bio-Rad Gene Pulser under conditions of 3000 V/cm and 25 μF, with a time constant of 0.4 ms. Electroporated cells were transferred into

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Abbreviation: CAT, chloramphenicol acetyltransferase.

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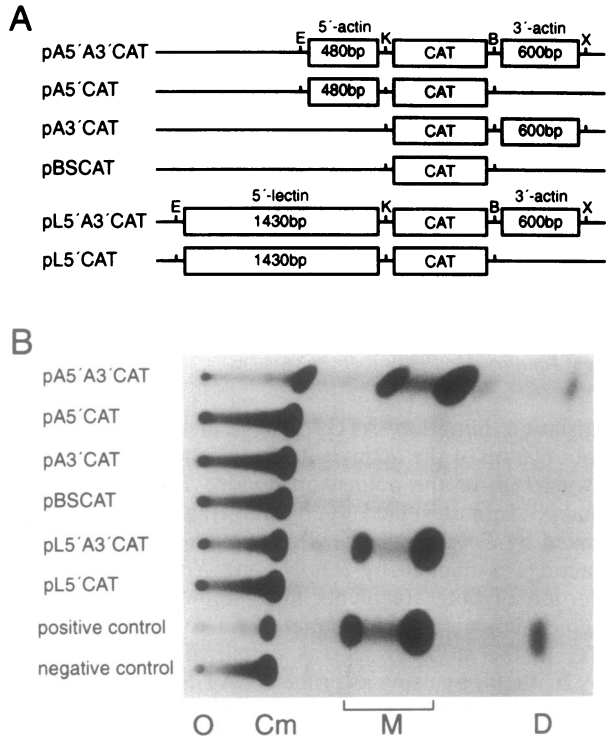

FIG. 1. CAT gene expression in *E. histolytica* trophozoites transfected by electroporation with 100 μg of different hybrid plasmids. (*A*) Schematic description of the six constructs used for transfection. They were generated by cloning four different DNA fragments or combinations thereof into the multiple cloning site of plasmid vector pBluescribe (pBS). Fragments 5'-actin and 3'-actin represent untranslated upstream and downstream sequences of an *E. histolytica* actin gene. Fragment 5'-lectin contains the upstream region adjacent to the *E. histolytica* lectin coding region, and the CAT fragment encompasses the complete coding sequence from ATG to the TAA stop codon of the bacterial CAT gene. The lengths of the different fragments are given in base pairs (bp). Restriction sites used for subcloning the different fragments are indicated. E, *Eco*RI; K, *Kpn* I; B, *Bam*HI; X, *Xba* I. (*B*) Thin-layer chromatography of acetylated forms of radioactively labeled chloramphenicol after incubation in lysates of cells transfected with the different constructs as indicated. As controls, chloramphenicol-containing lysates of untransfected cells were supplemented with (positive) or without (negative) purified CAT from *Escherichia coli*. Migration of unacetylated chloramphenicol (Cm), the two monoacetylated forms (M), and the diacetylated form (D) relative to the origin (O) are indicated.

TYI-S-33 medium and incubated for 48 hr unless otherwise indicated.

Assay for CAT Activity. Transfected trophozoites were harvested and washed twice in PBS as described above. The resulting pellet of about 200 μl was resuspended in 200 μl of lysis buffer consisting of 0.5% Triton X-100 and 0.5 M Tris·HCl (pH 7.6). After 5 min of incubation at room temperature, the lysate was diluted with 400 μl of 0.25 M Tris·HCl (pH 7.6), and cleared by centrifugation in an Eppendorf Microfuge (14,000 × *g*) for 5 min. The cleared lysate was incubated at 67°C for 10 min to inactivate possible transacetylases and was cleared again by centrifugation in a Microfuge for 5 min. The lysate was subsequently dialyzed overnight against an excess volume of 0.25 M Tris·HCl (pH 7.6). One hundred microliters of the dialyzed material was assayed for CAT activity in 300 μl (final volume) containing 0.25 M Tris·HCl (pH 7.6), 0.5 mM acetyl coenzyme A, 1 μCi (37 kBq) of [^{14}C]chloramphenicol (50–60 mCi/mmol; Amersham). The reaction mixture was incubated overnight (about 16 hr) at 37°C, extracted with 1 ml of ethyl acetate, and dried. The pellet was resuspended in 20 μl of ethyl acetate and spotted on a thin-layer chromatography aluminum sheet (Merck). After development for 2 hr with chloroform/methanol, 95:5 (vol/vol), the sheet was dried and exposed overnight to an x-ray film for analysis.

RESULTS

Electroporation Conditions. Previous studies have indicated that electroporation is a suitable method to introduce DNA into protozoan parasites. It requires, however, relatively harsh conditions such as an exponential discharge of 2000–4000 V/cm and a time constant as low as 0.4 ms. Therefore, an appropriate buffer system has to be used, which allows the survival of at least 50% of the cells after electroporation. Good results had been obtained with a potassium phosphate-based buffer (cytomix buffer) (12), which had been applied successfully for the transfection of *Toxoplasma gondii* and *Plasmodium falciparum* (5, 6). We investigated cytomix buffer for electroporation of *E. histolytica* trophozoites and found survival of >50% under an exponential discharge of up to 3000 V/cm at a capacitance of 25 μF, resulting in a pulse length of 0.4 ms. Further augmentation of the discharge values drastically reduced the numbers of viable amebae (Table 1).

Construction of Transient Expression Vectors. Several hybrid plasmids were constructed containing the sequence encoding CAT and were used as reporter for transient transfection of *E. histolytica*. This was accomplished by PCR amplification of various DNA fragments, which were ligated into the multiple cloning site of the plasmid vector pBS. The following fragments were used: (*i*) the coding sequence of the bacterial CAT gene; (*ii*) approximately 480 bp and 600 bp of the sequences upstream and downstream, respectively, of an *E. histolytica* actin coding region; and (*iii*) a 1430-bp fragment of the 5' sequence adjacent to the region coding for the 170-kDa lectin of *E. histolytica*. Restriction sites for certain endonucleases were introduced into the primer sequences used for PCR, allowing rapid subcloning of the fragments in a predicted orientation and connection of them in close proximity to each other in various combinations (Fig. 1*A*).

Expression of CAT in *E. histolytica*. One hundred micrograms of the various hybrid plasmids were transfected into 10^7 *E. histolytica* trophozoites by electroporation in cytomix buffer at 3000 V/cm for 0.4 ms (25-μF capacitor), and CAT activity was assayed after culturing the transfected cells for 48 hr. Activity was found in trophozoites transfected with pA5'A3'CAT and pL5'A3'CAT, the plasmids containing either the 5'-actin or the 5'-lectin sequence in conjunction with the CAT gene and the 3'-actin fragment. No activity was detected with constructs lacking either the 5' or the 3' flanking sequences (Fig. 1*B*). In addition, no CAT activity was found when cells were incubated with pA5'A3'CAT or pL5'A3'CAT without electroporation (Fig. 2 *A* and *B*).

Addition of fresh, reduced glutathion into the electroporation buffer was found to be essential for survival of the amebae and for the expression of CAT. Therefore, we enhanced the amount of glutathion from 5 mM, as recommended in the original description of cytomix buffer, to 10

Table 1. Survival of *E. histolytica* trophozoites in cytomix buffer under various electroporation conditions

Electroporation conditions		Survived amebae, % of total
V/cm	μF	
2000	25	60
3000	25	55
3250	25	50
3500	25	35
4000	25	10

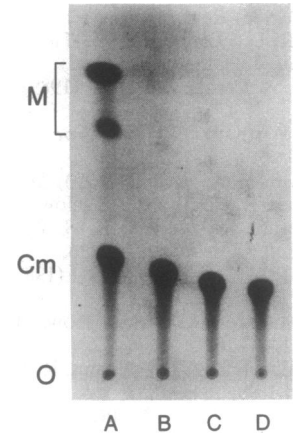

FIG. 2. CAT expression in *E. histolytica* or culture medium with or without electroporation. *E. histolytica* trophozoites (*A* and *B*) or the corresponding culture medium (*C* and *D*) were incubated in the presence of 100 μg of pL5'A3'CAT with (*A* and *C*) or without (*B* and *D*) electroporation. Subsequently, parasites or medium were cultured for 48 hr and assayed for CAT activity as described in the legend to Fig. 1.

mM to get more reproducible results. Without glutathion or in the presence of oxidized glutathion, CAT expression was reduced by >80% (data not shown). To rule out the possibility that CAT activity was expressed by other organisms that might have been transfected while contaminating the *E. histolytica* cultures, two control experiments were carried out as follows: (*i*) aliquots of 500 μl of the transfected cultures as well as the pellets of the entire supernatant volumes were plated on Luria broth, blood, or McConkey agar plates, and no growth of microorganisms was detected; and (*ii*) plasmid DNA electroporated in culture medium or supernatant in the absence of amebae did not result in the production of CAT activity (Fig. 2 *C* and *D*). When trophozoites were transfected with various amounts of pL5'A3'CAT DNA (1–400 μg), it was found that relatively high amounts (>25 μg of DNA) have to be added to obtain reasonable CAT activity and that CAT expression was proportional to the amount of DNA used (Fig. 3).

To determine the time course of CAT expression, parasites were transfected with 100 μg of pL5'A3'CAT, and lysates were assayed after culturing the amebae for 12, 24, 48, 72, or 96 hr. Activity was highest after 48 hr and later on decreased substantially (Fig. 4).

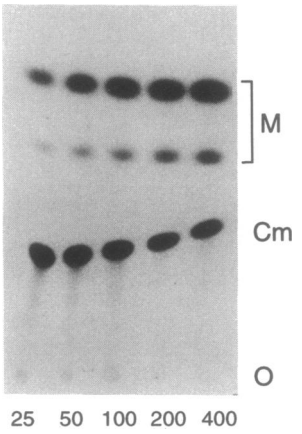

FIG. 3. Time course of CAT expression. *E. histolytica* trophozoites were transfected with 100 μg of pL5'A3'CAT. Subsequently, parasites were cultured for different periods of time as indicated in hours, and lysates were assayed for CAT activity as described in the legend of Fig. 1.

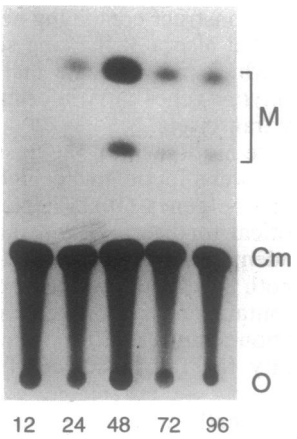

FIG. 4. CAT expression in relation to the amount of DNA being transfected. *E. histolytica* trophozoites were transfected with different amounts of expression vector pL5'A3'CAT as indicated in micrograms. After culturing the transfected cells for 48 hr, lysates were assayed for CAT activity as described in the legend of Fig. 1.

DISCUSSION

In previous years, DNA-mediated gene transfer has been used widely as a powerful tool for the genetic manipulation of cells or organisms. Transfection and expression of DNA in protozoan parasites was found to be more difficult than in higher eukaryotic cells, since the protozoan parasites require particular growth conditions and possess a number of peculiarities in gene organization and control of gene expression.

In an attempt to develop a transformation system for the human pathogen *E. histolytica*, we have adapted conditions that have been applied successfully to the transfection of other parasites. The bacterial CAT gene was used as a reporter and put under the control of noncoding upstream sequences of *E. histolytica* actin or lectin genes. These two upstream sequences were selected for the construction of expression vectors because actin mRNA has been found to be one of the most abundant transcripts in *E. histolytica* trophozoites (13), and the lectin upstream sequence is the only one so far positively identified as a promoter region in *E. histolytica* (10). Although the conditions we used to transfect *E. histolytica* trophozoites were rather similar to those applied to other parasites, some steps were found to be different but critical to achieve reasonable CAT expression. (*i*) Addition of fresh, reduced glutathion into the electroporation buffer was essential for survival of the amebae and for the expression of CAT. *E. histolytica* trophozoites have a very reduced oxygen tolerance and do not possess any catalase or peroxidase (14, 15). Therefore, glutathion may be important for the detoxification of reactive oxygen species or for reducing proteins and lipids that may be oxidized during electroporation. (*ii*) Relatively high amounts of DNA have to be transfected; <25 μg did not result in substantial CAT activity. The most likely explanation is that the transfected DNA is degraded by endogenous nucleases, which are known to be present in considerable quantities inside the amebae. (*iii*) Reasonable CAT expression was not found until 48 hr after transfection. This may be explained by the following observations. During the first 12–24 hr after electroporation, trophozoites appeared to remain in a temporary resting state because no cell division was observed, and a high proportion (about 30%) of the cells developed into cysts. After 24 hours, cysts disappeared, and cell growth and the rate of cell division became normal.

Both 5' flanking regions used as promotor sequences were able to drive CAT expression. This supports our previous finding that transcription in *E. histolytica* is monocistronic rather than polycistronic (10). In addition, expression of CAT

activity required a construct containing not only one of the two 5′ sequences but also a 3′ flanking sequence of an *E. histolytica* gene, which may indicate that the downstream regions of *E. histolytica* genes carry information important to stabilize primary transcripts.

Several sequence motifs within the upstream and downstream sequences were found to be highly conserved in various *E. histolytica* genes (10). These sequence motifs appear to be critical for transcription initiation, termination, and polyadenylylation, respectively. It has to be mentioned that both transfection vectors, pA5′A3′CAT and pL5′A3′CAT, contain these conserved sequence motifs. Further transfection analyses with respective mutant constructs will clarify the relevance and function of these motifs.

In summary, the methods described here allow transient expression of reporter genes in *E. histolytica* and provide the basis for further analyses of gene regulation in this protozoan. In addition, they may facilitate the development of a stable transformation system for *E. histolytica*.

We thank R. D. Horstmann for critical review of the manuscript and H. Buß, H. W. Hagel, and W. Raabe for skillful technical assistance. This work was supported by the Bundesministerium für Forschung und Technologie.

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