

## TROPHOZOITES OF *ENTAMOEBIA HISTOLYTICA* EPIGENETICALLY SILENCED IN SEVERAL GENES ARE VIRULENCE-ATTENUATED

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### Summary:

The human intestinal parasite *Entamoeba histolytica* causes amoebic colitis and amoebic liver abscesses. Three classes of amoebic molecules have been identified as the major virulence factors, the Gal/GalNAc inhibitable lectin that mediates adherence to mammalian cells, the amoebapores which cause the formation of membrane ion channels in the target cells and the cysteine proteinases which degrade the matrix proteins, the intestinal mucus and secretory IgA. Transcriptional silencing of the amoebapore (*Ehapa*) gene occurred after transfection of trophozoites with a plasmid containing a segment of the 5' upstream region of the gene. Transcriptional silencing of the *Ehapa* gene continued even after the removal of the plasmid and the cloned amoebae were termed G3. Transfection of G3 trophozoites with a plasmid construct containing the cysteine proteinase (*EhCP-5*) gene and the light subunit of the Gal-lectin (*Ehlg11*) gene, each under the 5' upstream sequences of the amoebapore gene, caused the simultaneous epigenetic silencing of expression of these two genes. The resulting trophozoites, termed RB-9, were cured from the plasmid and they do not express the three types of virulent genes. The RB-9 amoeba are virulence attenuated and are incapable of killing mammalian cells, they can not induce the formation of liver abscesses and they do not cause ulcerations in the cecum of experimental animals. The gene-silenced amoebae express the same surface antigens which are present in virulent strains and following intra peritoneal inoculation of live trophozoites into hamsters they evoked a protective immune response. Further studies are needed to find out if RB-9 trophozoites could be used for vaccination against amoebiasis.

**KEY WORDS :** *Entamoeba histolytica*, RB-9 trophozoite, virulence.

*Entamoeba histolytica* is an intestinal protozoan parasite of humans that causes amoebic colitis and amoebic liver abscesses which are diseases associated with significant levels of morbidity and mortality worldwide. The organism has a simple life cycle existing as the microaerophilic motile trophozoite or the detergent resistant cyst form. Infection begins by ingestion of cysts present in polluted water or vegetables. Cysts containing a chitin based rigid cell wall are formed in the descending colon and this protects the metabolically dormant cells from the adverse

conditions of the environment. Following ingestion, the cysts undergo an excystation process in the small intestine and the emerging motile trophozoites migrate and reside within the anaerobic confines of the human colon. The trophozoites reproduce by binary fission, they phagocytize large numbers of bacteria and food remnants. They lack mitochondria and derive energy from fermentation (Ravdin, 1995).

Epidemiological studies indicate that approximately 50 million people become infected with *E. histolytica* every year. Of these it is estimated that only 10 % of them show symptoms of invasive disease (dysentery and extra-intestinal lesions) (Stanley, 2003). This epidemiological data poses a number of important questions, for example why does only a fraction of the infected individuals develop symptomatic disease. One explanation for such a disparity in numbers is that there are considerable differences in the conditions found in the host and that these may be responsible in preventing the full expression of the trophozoite virulence factors needed to cause symptoms. Another reason could be differences in the pathogenicity of different strains of *E. histolytica*, some of which resemble the non pathogenic species *E. dispar* in their low levels of virulence (Burchard & Mirelman, 1988).

During the past two decades, the tools of molecular biology have greatly contributed to the understanding of *E. histolytica* pathogenesis. The publication of the *E. histolytica* genome (Loftus *et al.*, 2005) provides remarkable new insights into the biology of *E. histolytica* and has become a powerful source for the understanding of the requirements for intestinal parasitism as well as for the identification and characterization of the molecular weapons and mechanisms which the parasite uses to damage the host tissues and kill cells.

### VIRULENCE OF *E. HISTOLYTICA*

*E. histolytica* invades tissue and causes clinical disease through a complex sequence of events (Leippe, 1997; Ackers & Mirelman, 2006). The amoebic trophozoite first adheres to the colonic mucus and epithelial cells through interaction of a (Gal/GalNAc)

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inhibitable lectin (Frederick & Petri, 2005). Other surface molecules appear to be involved in this process as well: a 220 kDa membrane protein, a serine-rich protein and a cysteine protease–adhesin dimer (Laughlin & Temesvari, 2005). Upon contact of the trophozoites with the intestinal epithelial tissue, cell death occurs within minutes (Ravdin *et al.*, 1980; Berninghausen & Leippe, 1997). Cytolysis is undertaken by the amoebapores that permeabilize the mammalian cells by forming pores (Leippe, 1997; Andra *et al.*, 2003). *E. histolytica* trophozoites can also kill mammalian cells by induction of programmed cell death (apoptosis). Host-cell apoptosis is detected in amoebic liver-abscess and intestinal disease in mice, suggesting that human intestinal epithelial or liver cells might undergo the same fate (Seydel & Stanley, 1998; Ragland *et al.*, 1994; Huston *et al.*, 2000; Boettner & Petri, 2005). Other factors that also play an important role in pathogenesis are the parasite secreted cysteine proteases (Que & Reed, 2000; Helberg, *et al.*, 2001). *E. histolytica* cysteine proteinases digest extracellular matrix proteins which facilitate trophozoite invasion into the submucosal tissues and enable their lateral spread (Tavares *et al.*, 2005). The final step in the pathogenesis of invasive amoebiasis involves the host response. Intestinal epithelial cells produce interleukin-1B, interleukin-8 and cyclooxygenase (COX)-2 in response to the *E. histolytica* infection. These mediators have several effects including attraction of neutrophils and macrophages to the site of amoebic invasion (Seydel *et al.*, 1997; Stenson *et al.*, 2001). As *E. histolytica* trophozoites enter into the bloodstream, the trophozoites interact with the complement system; a number of parasite surface molecules such as the complex lipophosphopeptidoglycans (Moody-Haupt *et al.*, 2000) and peroxiredoxin (Choi *et al.*, 2005), as well as proteases (Que & Reed, 2000; Hirata *et al.*, 2007) and the Gal/GalNAc-specific lectin (Braga *et al.*, 1992), are implicated in resisting this attack. Trophozoites reaching the liver create unique abscesses, and the lysis of neutrophils by *E. histolytica* trophozoites release mediators that lead to hepatocyte death (Burchard *et al.*, 1993).

## THE Gal/GalNAc INHIBITABLE LECTIN

The Gal/GalNAc lectin is a 260 kDa heterodimer consisting of disulfide-linked heavy (170 kDa) and light (31/35 kDa) subunits which are non-covalently associated with an intermediate subunit of 150 kDa (Petri *et al.*, 2002). Five distinct genes (termed *Ebhgl1* to *Ebhgl5*) encoding the lectin's heavy subunit have been identified and sequenced. The sequence of the *Ebhgl* genes is nearly completely conserved in isolates of *E. histolytica* from different continents (Beck *et al.*, 2002). The carbohydrate recognition domain

(CRD) is located within the cysteine-rich domain of the heavy subunit (Dodson *et al.*, 1999; Pillai *et al.*, 1999). Interestingly the intermediate subunit is part of a large family of trans-membrane kinases (Beck *et al.*, 2005). Disruption of the lectin by the inducible expression in the parasite of a dominant-negative mutant of the lectin, inhibited amoebic adherence, cytotoxicity and abscess formation in an animal model (Vines *et al.*, 1998; Coudrier *et al.*, 2005; Tavares *et al.*, 2005). Five genes (termed *Ebhgl 1-5*) were found to encode the 31/35 kDa light subunits of the lectin. Inhibition of expression of the light subunit genes by antisense mRNA did not significantly affect adhesion of the parasites to mammalian or bacterial cells but strongly inhibited cytopathic activity, cytotoxic activity and in the ability to induce the formation of liver lesions in hamsters (Ankri *et al.*, 1999a). Trophozoites transfected with a truncated *Ebhgl1* gene and expressing an lgl protein in which the 55 N-terminal amino acids were missing, showed a significant decrease in their ability to adhere to and kill mammalian cells as well as in their capacity to form rosettes or to phagocytose erythrocytes. In addition, the trophozoites showed an impaired ability to cluster or cap the lectin molecules to the uroid region of the amoeba (Katz *et al.*, 2002). These results indicate that the light subunit also has a role in virulence.

## AMOEBAPORES AND THE SAPOSIN-LIKE FAMILY

Discovered in 1982 (Lynch *et al.*, 1982; Young *et al.*, 1982), the amoebapores are membrane-interacting proteins which display pore-forming activity toward liposomes (Leippe *et al.*, 1991). They have antibacterial activity (Leippe *et al.*, 1994a) and are cytotoxic to human cell lines *in vitro* (Leippe *et al.*, 1994b). The primary function of the pore-forming proteins *in vivo* is the killing of phagocytosed bacteria, but their cytolytic potency makes them a prominent virulence factor of the amoeba. The role of amoebapore in amoebic virulence was determined for the first time by inhibiting its expression with antisense mRNA. Trophozoites which expressed 60 % less amoebapore lost their ability to kill mammalian cells *in vitro* or to induce liver abscesses in the animal models (Bracha *et al.*, 1999; Zhang *et al.*, 2004a). Three amoebapore isoforms termed amoebapores A, B and C have been characterized. All of them are localized in cytoplasmic granules and show only some quantitative differences in their specific activities (Leippe *et al.*, 1994a). Structurally, the amoebapores belong to the family of saposin-like proteins (SAPLIPs) characterized by a conserved sequence motif of six cysteine residues

involved in three disulfide bridges and which comprises approximately 80 amino acid residues (Munford *et al.*, 1995). Although the members of the SAPLIP family have different biological functions, they are all able to interact with lipids. Amoebapores have a substantial sequence similarity with membrane-permeabilizing molecules of mammalian lymphocytes such as porcine NK-lysin and human granulysin (Leippe *et al.*, 1994a; Andersson *et al.*, 1995; Pena *et al.*, 1997; Bruhn *et al.*, 2003).

## CYSTEINE PROTEINASES (CPs)

As the name *E. histolytica* implies, amoebae are well known for their great capacity to destroy host tissue and to degrade extracellular matrix proteins. Compelling evidence is now available which clearly shows that CPs are one of the main culprits for the pathology caused by the pathogen. Some of the CPs have been shown to be involved in pathogenicity such as destruction of host tissue or triggering an inflammatory response in the infected individual (Que & Reed, 2000; Stanley, 2003). Other CPs were shown to be essential for intracellular protein turnover and degradation as well as for life cycle processes such as encystation and excystation (Makioka *et al.*, 2005). The mucous layer lining the colonic epithelium is the first line of host defense against invasive pathogens such as *E. histolytica*. Inhibition of cysteine proteinases by specific inhibitors such as E-64 or laminin, prevents the disruption of the intestinal mucus and the subsequent damage to the mucosal cells (Moncada *et al.*, 2003). Cleavage of the C3 fragment of complement as well as digestion of IgA and IgG were also attributed to the CPs (Reed *et al.*, 1989, 1995; Kelsall & Ravdin, 1993; Tran *et al.*, 1998). Treatment of *E. histolytica* trophozoites with sublethal doses of the CP inhibitor E-64 greatly reduced their ability to induce liver lesions in laboratory animals (Li *et al.*, 1995; Stanley, 1995). Furthermore, trophozoites in which the expression of CPs was inhibited (about 90 %) by the transcription of anti-sense mRNA, showed that they were incapable of forming liver abscesses in animal models and were also defective in their ability to invade through the intestinal mucosa (Ankri *et al.*, 1998, 1999b). An interesting and potentially useful inhibitor of CPs is the molecule Allicin which is the main biologically active component produced upon crushing of Garlic cloves (Ankri *et al.*, 1997). CPs appear to be a major contributor to gut inflammation and tissue damage in amoebiasis. Studies in a human intestinal xenograft model of disease (Zhang *et al.*, 2000) revealed that the trophozoites which were inhibited in the expression of CPs caused significantly less damage to the intestinal permeability barrier. The CP-deficient trophozoites also failed to induce the

intestinal epithelial cell production of the inflammatory cytokines IL-1 $\beta$  and IL-8 and caused significantly less intestinal inflammation and tissue damage (Zhang *et al.*, 2000).

More than twenty full-length CP genes have been identified to date in the *E. histolytica* genome but only about half of them have been found to be expressed in trophozoites cultured under axenic conditions (Bruchhaus *et al.*, 2003; Tillack *et al.*, 2006, 2007). Four of the CPs (*EbCP-1*, *EbCP-2*, *EbCP-3* and *EbCP-5*) have been purified and found to account for approximately 90 % of the total CP activity present in lysates of axenically cultured trophozoites (Scholze & Tannich, 1994; Bruchhaus *et al.*, 1996, 2003; Jacobs *et al.*, 1998). *EbCP-5* which has significant homology to cathepsin L appears to be of special importance for pathogenicity because it is the only CP that was found to be present on the surface of the trophozoite (Jacobs *et al.*, 1998). Interestingly in contrast to all the other cysteine proteinases, it was found that functional genes corresponding to *EbCP-1* and *EbCP-5* are absent in *E. dispar*. The elucidation of the precise role of each of the various CP enzymes is a major challenge and in view of their important contribution to pathogenesis, their characterization will help us understand some of their unique properties which may be useful for the design of new therapies.

## REGULATION OF GENE EXPRESSION

The lack of a suitable reverse genetic system in *E. histolytica* and the inability to use targeted gene deletions and gene knockout by homologous recombination have prompted investigators to use other methods to down or up-regulate gene expression. As mentioned above valuable information was obtained with transfectants that transcribed either dominant-negative or antisense RNA to certain virulence genes (Bracha *et al.*, 1999; Moncada *et al.*, 2006). The RNAi pathway appears to be in place based on the presence of AGO-like proteins (Abed & Ankri, 2007) and RdRP-like proteins (De *et al.*, 2006). Proteins involved in transcriptional gene silencing such as a DNA methyltransferase (Fisher *et al.*, 2004; Bernes *et al.*, 2005), histone acetyltransferase and histone deacetylase (Ramakrishnan *et al.*, 2004) have also been identified and were demonstrated to be operational. Initial reports have demonstrated the feasibility of using double-stranded RNA (dsRNA) to down regulate gene expression (Kaur & Lohia, 2004; Vayssie *et al.*, 2004). *Entamoeba* appears to encode a protein with only a single RNaseIII domain (Abed & Ankri, 2007). This enzyme may perhaps act as a dimer to assume a catalytic core similar to that of Dicer (Zhang *et al.*, 2004b; Macrae *et al.*, 2006). Alter-

natively, the Dicer-like sequence might be so divergent that it is no longer recognizable by primary sequence searches. Putative candidates of microRNAs were also identified using a bioinformatic approach (De *et al.*, 2006), but still await experimental validation.

## TRANSCRIPTIONAL GENE SILENCING IN *E. HISTOLYTICA*

An unexpected transcriptional gene silencing (TGS) event was found to occur following transfection of trophozoites of a virulent strain with a plasmid containing the 5' and 3' regulating sequences as well as the ORF of the amoebapore gene. The 5' upstream segment of the *Ehap-a* gene which was used (473 bp) was found to contain in its distal end a truncated part (140 bp) of a transcribed neighbouring SINE (short interspersed nuclear element) retroposon element (Anbar *et al.*, 2005) (Fig. 1 – plasmid scheme showing also gene silencing on Northern blots). It was subsequently found that silencing of the *Ehap-a* gene could be induced by plasmids containing only the 473 bp 5' upstream segment. Omission of the 140 bp SINE sequences prevented gene silencing. Silencing of the *Ehap-a* gene was at the transcription level and remained in effect even after removal of the plasmid. Omission of the selective drug (G418) from the cultures of the transfectant for over 90 days drastically reduced the plasmid content of the trophozoites but did not cause any reversal of the *Ehap-a* silencing effect. Three independent clones (G1 to G3) were isolated from the cul-

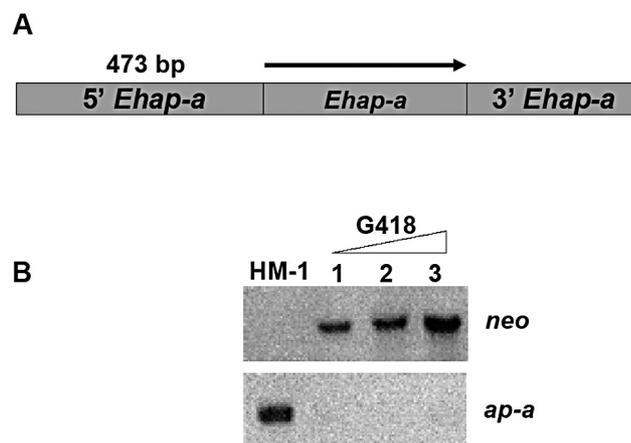


Fig. 1. – (A) Schematic diagram of plasmid psAP-1. The plasmid contains the *Ehap-a* gene with its 5' upstream region of 473 bp and its 3' regulatory region. The distal region of the 5' 473 bp segment contains the truncated SINE element that is transcribed in the opposite orientation (Anbar *et al.*, 2005). (B) Northern blot analysis of amoebic RNA extracts. Lanes 1-3 are HM-1:IMSS trophozoites transfected with plasmid psAP-1 and grown in the presence of increasing concentrations of the neomycin derivative, G418, used as the selective marker. The probes used are as indicated.

tures growing without G418, and even though all three of them were found to be devoid of plasmid, they still showed no AP-A protein expression, even after four years in culture indicating that the gene silencing mechanism is inherited in the progeny amoeba. Other researchers (Irmer & Tannich, 2006) have recently confirmed the silencing of the amoebapore gene by other constructs. They have found that the plasmids used for silencing of the amoebapore gene require the presence of a tRNA array (Clarck *et al.*, 2006) in addition to the regulating sequences of the amoebapore gene, and that removal of such arrays prevents silencing of the gene. We have now shown that silencing of additional genes, such as the light subunit of the Gal/GalNAc lectin and CP-5, can be induced in the plasmid-less G3 trophozoites that had already been silenced in the amoebapore gene using plasmid constructs that contain the second gene directly ligated to the above-mentioned 473 bp 5' upstream segment (Bracha *et al.*, 2006) (see below).

## CHARACTERIZATION OF THE AMOEBAPORE DEFICIENT (G3) TROPHOZOITES

Treatment of the G3 silenced trophozoite cultures with inhibitors of DNA methylation, such as 5-azacytidine (venkatasubbarao *et al.*, 2001), or of histone deacetylation, such as trichostatin A (Selker, 1998), did not restore the expression of the *Ehap-a* gene (Bracha *et al.*, 2003). ChIP analysis with an antibody against methylated lysin 4 of histone H3 (H3K4) showed a demethylation of K4 at the domain of the *Ehap-a* gene, indicating transcriptional inactivation (Anbar *et al.*, 2005). Transfection of the silenced, plasmidless G3 trophozoites with a plasmid containing the *Ehap-a* gene under the control of a different promoter failed to express the AP-A protein. This inability was only restricted to the *Ehap-a* gene, as transfection of G3 trophozoites with an analogous plasmid in which the *Ehap-a* gene was replaced by the chloroamphenicol acetyl transferase (CAT) gene, transcribed and produced the CAT protein. Other, unrelated genes, such as those encoding ribosomal protein L-21, actin, lectin, and cysteine proteinase, showed no changes in expression due to the silencing.

G3 trophozoites were found to be non-virulent in both *in vitro* and *in vivo* tests. The trophozoites were incapable of killing BHK cells in suspension or of destroying monolayers of BHK cells grown in tissue cultures. Phagocytosis was not impaired, but the disruption of the ingested cells was much slower and many bacteria remained undigested even after 24 hours (Bracha *et al.*, 2003). The absence of the amoebapore genes also caused a difficulty in the digestion of ingested red blood cells and round RBC's could be clearly seen in

trophozoites 24 h following ingestion. The RBC's were however slowly degraded upon further incubation suggesting that degradation also occurs by other mechanisms. G3 trophozoites did not induce the formation of liver lesions in hamsters, even at inoculations of one million trophozoites/liver (Bracha *et al.*, 2003). Nevertheless, G3 trophozoites could still cause inflammation and tissue damage in severe combined immunodeficient mouse-human intestinal xenograft (SCID-HU-INT), which is a well-established model of amebic colitis (Zhang *et al.*, 2004b). Preliminary studies using an i.p. vaccination of live G3 trophozoites in hamsters was effective in evoking the production of IgG antibodies that cross reacted with *E. histolytica* HM-1:IMSS membrane antigens and ELISA tests showed a clear rise in antibody levels in the vaccinated group as compared to the control group (Bujanover *et al.*, 2003). A significant variability was observed, however, in the anti-amebic IgG levels of the vaccinated group which might be due to small experimental variations during the injection of the trophozoites into the intraperitoneal cavity. Interestingly, the group of hamsters which had the higher titers of antibody were found to be protected following intra hepatic challenge with virulent trophozoites of strain HM-1:IMSS.

## MULTIPLE GENE SILENCED TROPHOZOITES

Simultaneous silencing of additional genes was achieved in G3 trophozoites transfected with a plasmid in which the 5' upstream *Ehap-a* fragment was directly ligated to the second gene of choice, one that encodes the light subunit of the Gal/GalNAc inhibitable lectin (Igl1) and the other, the cysteine proteinase 5 (CP-5) (Bracha *et al.*, 2006). Following this we have succeeded in the simultaneous silencing of both Igl1 and CP-5 by using a plasmid that contained the two genes, each ligated, as before, to the 5' upstream region of the *Ehap-a* gene (pRB9, Fig. 2). Transfection of this plasmid into the G3 trophozoites, which was already silenced in the *Ehap-a* gene, created a triple gene silenced trophozoite (Fig. 2) (Bracha *et al.*, 2007). Transcriptional silencing occurred in both, the transgenes and the chromosomal genes. Multiple gene silencing as observed before, only occurred if the plasmid construct contained the truncated sequences of the SINE1 element that is upstream to the *Ehap-a* gene. Another critical factor was the need of a direct connection between the 5' upstream sequences of the *Ehap-a* gene and the beginning of the open reading frame of the other gene. Furthermore, multiple gene silencing occurred only in substrain G3 in which the transcription of the amoebapore gene was already suppressed and did not occur in the parent strain, HM-1:IMSS, with any one of the plasmid constructs (Bracha *et al.*, 2007).

Trophozoites (termed RB-9), in which the transcription of three families of genes (amoebapores, cysteine proteinase 5 and light subunits of the Gal-lectin) became silenced, grow quite well in culture and were found to be incapable of killing mammalian cells and were non virulent in various animal models of disease such as the hamster liver and the mice cecum.

Silencing of the light Gal-lectin genes did not simultaneously suppress the transcription of all them. Although the five light subunit genes of the Gal lectin share significant homology, two of them, *Ehlg 4,5* have a deletion and are shorter by about 40 bp. Plasmids which contained the *Ehlg1* gene under the 5' upstream segment of the *Ehap-a* gene caused the silencing of *Ehlg1* as well as that of *Ehlg2* and 3 but not *Ehlg4* and 5. As a matter of fact the expression of *Ehlg4,5* was significantly upregulated in such transfectants and thus compensated for the absence of Igl's 1-3 (Bracha *et al.*, 2007). Plasmids which contained the *Ehlg5* sequence silenced also the *Ehlg4* gene but failed to silence the *Ehlg's 1-3*. Trophozoites which were devoid of Igl's 1-3 had a defect in the clustering or capping of the Gal-lectin molecules to the uroid region of the amoeba. Interestingly trophozoites lacking *Ehlg4* and 5 were not affected in their capping indicating that only Igl's 1-3 are responsible for the capping phenomenon.

Our results so far indicate that the multiple gene silenced trophozoites are incapable of producing damage in animal models of disease. As mentioned above, the virulence attenuated trophozoites were capable of evoking an immune response. There is no doubt however that many more experiments are needed before any

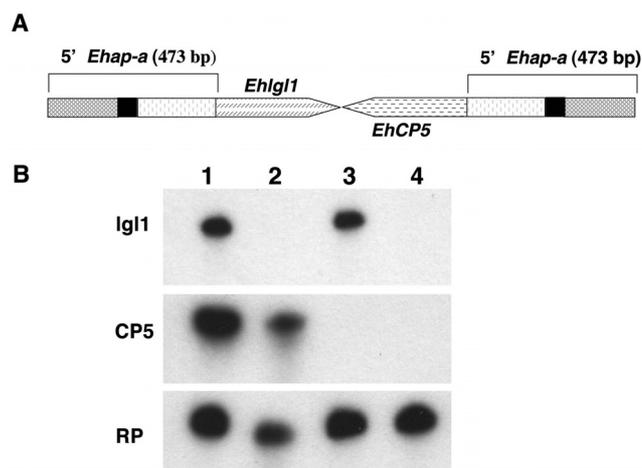


Fig. 2. – (A) Schematic diagram of plasmid pRB9 enabling the simultaneous silencing of two additional genes, *Ehlg1* and *Ehcp-5*, in G3 *Ehap-a*-silenced trophozoites. (B) Northern blot analysis of amoebic RNA extracts. Lanes: 1, plasmid-less G3 clone; 2, RBV clone silenced in *Ehlg1*; 3, RB8 clone silenced in *EhCP-5*; 4, triple-gene-silenced RB9 clone silenced in both *Ehlg1* and *EhCP-5*. The probes used are as indicated. Trophozoites that are multiple gene silenced (Bracha *et al.*, 2006) are virulence-attenuated and could be prime candidates for the development of a live vaccine.

conclusions can be made with respect to the potential value of these virulence attenuated trophozoites in eliciting a protective immune response. It certainly will be very interesting to test these new, multiple silenced trophozoites for their immunoprotective effect in monkeys.

## CONCLUDING REMARKS

Genetic approaches using anti-sense mRNA or plasmids that generate dsRNA to reduce expression of a targeted *E. histolytica* gene are not very effective. This is due to their inability to completely block gene expression and the need to maintain the levels of plasmid coded anti-sense mRNA or dsRNA by selection with drugs. Our discovery that expression of amoebapore genes as well as additional genes could be completely and stably silenced at the transcription level provides a new tool for analyzing the role of genes that code for virulence factors and for the possible development of a live vaccine against amoebiasis. The main disadvantage of our system is that the silencing of additional genes can be done only in the G3 trophozoites which are already silenced in the amoebapore gene and have an avirulent phenotype. This creates limitations for the investigation of the possible contributions of other genes to the virulence mechanisms of the parasite. On the other hand, the silenced trophozoites may provide an excellent biological model for studying the molecular mechanism by which the G3 trophozoites transfer and spread the epigenetic machinery to suppress the transcription of additional genes in other genomic locations.

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