Published in final edited form as:

J Mol Biol. 2007 October 19; 373(2): 296–307.

Episomally-driven antisense mRNA abrogates the hyperinducible expression and function of a unique cell surface Class I nuclease in the primitive trypanosomatid parasite, *Crithidia luciliae*

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Summary

Herein, we show that *Crithidia luciliae*, a primitive trypanosomatid, purine auxotroph, up-expressed its unique, bi-functional, surface membrane 3'-nucleotidase/nuclease (*Cl* 3'NT/NU) activity by ~1000-fold in response to purine-starvation. A second surface membrane phospho-monoesterase, *i.e.* a tartrate-resistant acid phosphatase (*Cl* MacP) was also found to be up-expressed in such purine-starved cells. In this report, we used homologous episomal-expression of an antisense construct of the *Cl* 3'NT/NU to dissect the functional expression of these two surface membrane enzymes. In antisense-transfected cells, a large excess of the antisense transcript was produced and no trace of any endogenous *Cl* 3'NT/NU sense message was detected. Further, the purine-starvation hyper-induced levels of 3'NT/NU enzyme activity were completely abrogated in these transfected cells versus controls. Moreover, such antisense transcription completely abolished the ability of these transfectants to grow in poly(A)-containing medium demonstrating the essential nature of the 3'NT/NU for the growth/survival of this parasite. In contrast, antisense-transcription had no apparent deleterious effects on either endogenous or purine-starvation-induced levels of MacP enzyme activity, its steady state mRNA levels, or the constitutive expression of house-keeping genes (*e.g.* *Cl* α -tubulin) in these transfectants.

Cumulatively, results of our antisense experiments demonstrated that the functional nuclease activity of the surface membrane *Cl* 3'NT/NU was, in fact, critical/essential for the growth and development of these primitive parasites.

Keywords

Auxotrophic parasite; Class I nuclease; phospho-monoesterase; protozoan; purine salvage; tartrate-resistant acid phosphatase

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Introduction

Trypanosomatid protozoa are a group of primitive parasitic organisms which are of medical and veterinary/agricultural importance as, many of them cause serious or fatal diseases in humans and domestic animals worldwide ¹. While all of these organisms are purine auxotrophs (*i.e.* require preformed purines for their growth, multiplication and survival), none of them, however, are capable of *de novo* purine biosynthesis ². Thus, these parasites require extra-cellular salvage mechanisms to acquire these preformed essential nutrients from their host environments. It has been postulated that the trypanosomatid purine salvage pathway involves several surface membrane bound phosphomono-esterase activities acting/working in concert: *e.g.* a bi-functional 3'-nucleotidase/nuclease (3'NT/NU) and a tartrate-resistant acid phosphatase (MACP) ^{3; 4; 5; 6; 7; 8; 9}. Interestingly, it was reported previously that in the insect trypanosomatid parasite, *Crithidia luciliae* (*Cl*) both of these enzyme activities were significantly up-expressed in response to purine-starvation conditions ^{3; 4; 10; 11; 12; 13; 14}. Of these *Crithidia* enzymes, however, only the *Cl*3'NT/NU has been purified and characterized biochemically ⁸. In that regard, previously we isolated and characterized, a gene encoding the 3'NT/NU from *C. luciliae* ¹⁵. This parasite enzyme is unique as, it is the only surface membrane-anchored member of the Class I family of nucleases ¹⁵. Further, in that report, we showed that at least three copies of the *Cl*3'NT/NU gene exist in the *C. luciliae* genome and that its specific mRNA was dramatically up-expressed (*i.e.* by ~100 fold) in response to purine-starvation conditions. To investigate this unusual expression pattern, we undertook to develop 3'NT/NU-deficient *C. luciliae* parasites. While it is feasible to disrupt/"knock-out" the genes of trypanosomatid parasites by homologous recombination ^{16; 17; 18; 19; 20; 21; 22}, our attempts to delete this multi-copy *Cl*3'NT/NU gene were unsuccessful presumably due to the apparent critical/essential nature of this salvage enzyme to these purine auxotrophs. Even though RNAi-based approaches have worked exceedingly well for gene-silencing in African trypanosomes *per se* (*e.g.* *Trypanosoma brucei*) ²³ they have been largely unsuccessful in other closely-related trypanosomatid genera and species infecting humans (*e.g.* *Trypanosoma cruzi* and *Leishmania* *sps.*) ^{23; 24; 25}. The failure of RNAi to silence genes in these related organisms is presumably due to the lack of some essential cellular component (s) required for such processing. The use of anti-sense RNA however, has shown some promise in down-regulating the expression of a small number of genes in *Leishmania* *sp.* parasites (*e.g.* A2; GP-63 and a cysteine protease) ^{26; 27; 28}. To date, however, an antisense approach has not been reported for any member of the primitive, monoxenous insect trypanosomatid group of *Crithidia* *sp.* parasites. In light of the foregoing, in the current report we used an "antisense-RNA"-based approach to specifically block the expression of the unique surface membrane 3'NT/NU of *Crithidia luciliae* and to demonstrate that this enzyme is critical/essential for the growth/survival of this parasite.

Results

Generation of Antisense Transfectants

The complete ORF of the *C. luciliae* 3'-nucleotidase/nuclease (*Cl*3'NT/NU) consists of 1134 nt ¹⁵. A construct of the *Cl*3'NT/NU was made in which the last 129 nt of the ORF were deleted (*Cl*3'nt/nu^Δ). This construct encodes a truncated *C. luciliae* 3'-nucleotidase/nuclease (nt +1 to nt 1005), which lacks both the trans-membrane anchor and C-terminal domains of the native enzyme. A diagrammatic representation of the *pKSNEO* episomal expression vector and both the *Cl*3'nt/nu^Δ and its antisense construct (*Cl*3'nt/nu^ΔR) are shown in Fig. 1. Results of both restriction endonuclease mapping and nt sequence analyses verified that the *Cl*3'nt/nu^ΔR was inserted in an antisense orientation in the *pKSNEO* vector. Plasmids containing this antisense construct (*pKSCl*3'nt/nu^ΔR) were electroporated into *C. luciliae* and the resulting transfectants

were selected by their growth under increasing concentrations of Geneticin (G-418, Invitrogen) up to 200 µg/ml.

***In vitro* Growth of *C. luciliae* Transfectants**

Growth kinetics were carried out under replete (+Ado) culture conditions with *pKSCl3'nt/nu^ΔR* (antisense)-transfected and control *pKSNEO*-transfected *C. luciliae* to ascertain whether expression of the antisense message effected either the growth or multiplication of these transfectants. Results of these studies demonstrated that both the control *pKSNEO*-transfected cells (Fig. 2a) and the *Cl3'nt/nu^ΔR* antisense-transfectants (Fig. 2b) had virtually identical growth kinetics under replete (+Ado) culture conditions. In contrast, the growth of both transfected-cell lines was dramatically arrested under adenosine starvation (−Ado) conditions (cf. Fig. 2a and 2b).

Northern Blot Analyses

Experiments were carried out to determine whether the anti-sense construct was actually transcribed by *pKSCl3'nt/nu^ΔR*-transfected *C. luciliae* cells. In that regard, total RNA isolated from such transfectants grown under adenosine-replete (+Ado) conditions was separated by gel electrophoresis and blotted onto nylon membranes. The resulting blots were subsequently hybridized with either a single-strand “Sense”- or an “Antisense”- *Cl3'nt/nu^Δ* RNA probe. Results of such preliminary assays demonstrated that the *pKSCl3'nt/nu^ΔR*-transfected *C. luciliae* effectively transcribed the *Cl3'nt/nu^ΔR* antisense message (data not shown).

Previously, it was shown that *C. luciliae* inducibly up-expressed its endogenous 3'-nucleotidase activity by >1000-fold in response to purine starvation conditions ^{3; 4; 12; 13; 14}. Subsequently, we identified, cloned and characterized a gene (*Cl3'NT/NU*) encoding this *C. luciliae* enzyme ¹⁵. Further, in that study, we showed that the endogenous steady state levels of *Cl3'NT/NU* mRNA were elevated by ~100 fold when such *C. luciliae* cells were placed under adenosine-starvation conditions. In light of these observations, in the current study it was of interest to determine whether an antisense message (e.g. *Cl3'nt/nu^ΔR*) could obviate the up-expression of the endogenous *Cl3'NT/NU* sense message in adenosine-starved *C. luciliae*. To address this question, both *pKSNEO*- and *pKSCl3'nt/nu^ΔR* (antisense)-transfected *C. luciliae* cells were grown to log phase (~2×10⁷ cells ml^{−1}) in chemically-defined, adenosine replete medium (i.e. +Ado), harvested by centrifugation, washed once in such medium lacking adenosine (i.e. −Ado) and resuspended to the same cell density in such (−Ado) medium. These cells were incubated at 26°C in (−Ado) medium for various periods from T₀ up to 72 hrs (T₇₂). Control cell populations were handled identically except, that they were maintained in adenosine replete (+Ado) medium throughout the course of these experiments. Total RNA was isolated from cells maintained under either (+Ado) or (−Ado) conditions for 36 hrs as above and subsequently used for Northern blot analyses. Equivalent amounts of total RNA (15 µg) from each cell type was separated by agarose gel electrophoresis and stained with ethidium bromide to visually assess the quality and quantity of RNA loaded (Fig. 3a–d, lower panels). Such gels were blotted onto nylon membranes and the membranes were hybridized with either the DIG-labeled “Sense”- or “Antisense”- *Cl3'nt/nu^Δ* RNA probes, as above. In these Northern blot analyses, *pKSNEO*-transfected *C. luciliae* maintained under adenosine replete (+Ado) and adenosine starvation (−Ado) conditions were used as positive controls for hybridizations with the “Antisense”-RNA probe. Results of such analyses with the “Antisense”-probe showed that *pKSNEO*-transfected cells maintained in (+Ado) medium possessed a low level signal (i.e. typical of un-induced/non-starved cells) for the ~2.3 kb steady-state endogenous (“sense”) *Cl3'NT/NU* mRNA (Fig. 3a, lane 1). This endogenous sense message was dramatically up-expressed (~100 fold) in *pKSNEO*-transfected cells maintained for 36 hr under adenosine starvation (−Ado) conditions (Fig. 3a, lane 2). These observations are in good agreement with those reported previously concerning the up-expression of *C. luciliae* 3'NT/NU mRNA levels

in response to purine starvation¹⁵. In contrast, *pKSCl3'nt/nu^ΔR* (antisense)-transfectants showed no discernible signal with the “*Antisense*”-probe regardless of whether they were maintained under adenosine replete (+Ado) or adenosine starvation (–Ado) conditions (Fig. 3a, lanes 3 and 4, respectively). The latter results indicated that the amount of antisense message produced in the *pKSCl3'nt/nu^ΔR*-transfectants was more than sufficient to abrogate detection of the endogenous *Cl3'NT/NU* sense mRNA in these cells.

Duplicate blots of the same RNA samples as above were also hybridized with a single-strand “*Sense*” *Cl3'nt/nu^Δ* RNA probe. As anticipated, no detectable signal was observed with this “*Sense*”-probe in control *pKSNEO*-transfectants maintained under either adenosine replete (+Ado) or adenosine starvation (–Ado) conditions (Fig. 3b, lanes 1 and 2, respectively). In contrast, two major signals (*i.e.* ~2.0 and ~2.3 kb, respectively) of antisense mRNA message were readily detected with the “*Sense*”-probe in Northern blots of *pKSCl3'nt/nu^ΔR* (antisense)-transfectants maintained under (+Ado) conditions (Fig. 3b, lane 3). Based on the design of our antisense construct (*i.e.* lacking a stop codon), these signals presumably represent the predominant read-through products of the antisense mRNA message. The several smaller sized bands observed in these blots might represent degradation products of the above. Surprisingly, in such blots, the ~2.0 and ~2.3 kb antisense mRNA signals appeared to be even more pronounced (*i.e.* up-expressed) in *pKSCl3'nt/nu^ΔR* (antisense)-transfectants maintained under adenosine starvation (–Ado) conditions for 36 hrs (Fig. 3b, lane 4). The mechanism(s) responsible for such enhanced mRNA up-expression from this episomal construct under adenosine starvation conditions remains to be investigated further. Moreover, *pKSCl3'nt/nu^ΔR* (antisense)-transfectants also possessed mRNAs larger than 2.3 kb which apparently hybridized with this “*Sense*”-probe. Presumably, these signals could reflect the increased quantities of read-through products and/or multiple sized concatamers of the antisense message produced by these transfectants under adenosine starvation (–Ado) conditions. Taken together, these results demonstrated that the *C. luciliae pKSCl3'nt/nu^ΔR* (antisense)-transfectants produced, even under adenosine replete (+Ado) conditions, an excessive amount of the antisense mRNA compared to that of the endogenous *3'NT/NU* sense message. Such antisense mRNA expression was even more exaggerated when these transfectants were maintained under adenosine starvation (–Ado) conditions.

Results obtained in the above Northern blots with the individual single-stranded “*Sense*”- and “*Antisense*”-probes were verified in duplicate control RNA blots hybridized with a double-stranded (“*ds*”) DNA probe (*i.e.* spanning nt+1 to nt+1005 of the *Cl3'NT/NU* ORF). In that regard, as anticipated, this “*ds*”-probe hybridized with both the endogenous sense mRNA message produced by *pKSNEO* control transfectants (Fig. 3c, lanes 1 and 2, cf. Figs. 3a, lanes 1 and 2) as well as, with the antisense mRNA expressed by *pKSCl3'nt/nu^ΔR* (antisense)-transfectants (Fig. 3c, lanes 3 and 4, cf. Figs. 3b, lanes 3 and 4).

In addition to the foregoing, it was of interest to ascertain whether expression of *pKSCl3'nt/nu^ΔR* (antisense) message had any “generalized” effects on altering mRNA levels of endogenous “house-keeping” genes. To that end, a *C. luciliae* alpha-tubulin gene was chosen as an example of a constitutively expressed parasite “house-keeping” gene. For these experiments, parallel RNA blots (identical to those above) were hybridized with a labeled *C. luciliae* α-tubulin probe (*Cl αTubulin*). In such blots, both the control *pKSNEO*- and *pKSCl3'nt/nu^ΔR* (antisense)-transfectants maintained under either adenosine replete (+Ado) or adenosine starvation (–Ado) conditions expressed virtually identical levels of a ~2.0 kb α-tubulin mRNA transcript (Fig. 3d, lanes 1–4). These results indicated that expression of the antisense-*Cl3'NT/NU* mRNA message *per se*, had no apparent adverse effects on the overall gene transcription in these cells. Further, they underscored the specificity of the *Cl3'nt/nu^ΔR* antisense message for down-regulating the levels of the endogenous *Cl3'NT/NU*-sense mRNA transcripts.

Effects of *Cl3'nt/nu^ΔR* Antisense Transcription on 3'-Nucleotidase/Nuclease Enzyme Activities

A. Spectrophotometric Assays—Both *pKSNEO*- control and *pKSCl3'nt/nu^ΔR* (antisense)-transfected *C. luciliae* cells were maintained under adenosine replete (+Ado) or adenosine starvation (−Ado) conditions for various periods up to 72 hrs. At given times, these cells were harvested, washed, lysed and the lysates analyzed for their 3'-nucleotidase activity in a colorimetric assay using 3'-AMP as substrate ^{8; 15}. Results of such assays showed that *pKSNEO* control-transfected cells maintained under replete (+Ado) conditions, typically expressed $\sim 3 \pm 1.5$ nmol of 3'NT activity $\text{min}^{-1} \text{mg}^{-1}$ of cell protein (Fig. 4a) (*i.e.* a value virtually identical to that obtained with wild-type, non-transfected *C. luciliae*, data not shown). However, when such cells were maintained for 24 or more hrs under adenosine starvation (−Ado) conditions they dramatically up-expressed their 3'-nucleotidase specific activity by >1000-fold (Fig. 4a). In stark contrast, *C. luciliae* transfected with the antisense *pKSCl3'nt/nu^ΔR*-plasmid failed to show any up-expression in 3'NT specific activity under such adenosine-starvation (−Ado) conditions (Fig. 4b). In fact, these transfectants showed only basal levels (*i.e.* $\sim 4.4 \pm 1.8$) of 3'-nucleotidase specific activity regardless of whether they were maintained under adenosine replete (+Ado) or adenosine-starvation (−Ado) conditions (Fig. 4b). These results demonstrated that the *pKSCl3'nt/nu^ΔR* (antisense)-transfectants were incapable of up-expressing their endogenous 3'-nucleotidase activity under adenosine-starvation (−Ado) conditions. In conjunction with our Northern blot results, these observations strongly suggested that the antisense message produced in *pKSCl3'nt/nu^ΔR* (antisense)-transfected cells was fully capable of abrogating the up-expression of both their endogenous 3'-NT mRNA levels and 3'-nucleotidase activity.

Similarly, lysates obtained from control *pKSNEO*- and *pKSCl3'nt/nu^ΔR* (antisense)-transfected *C. luciliae* maintained under (+Ado) and (−Ado) conditions were also quantitated spectrophotometrically for their total nuclease activities using poly(A) as substrate ¹¹. As anticipated from our 3'-nucleotidase results above, under adenosine-starvation (−Ado) conditions control *pKSNEO*-transfected cells significantly up-expressed their total nuclease activity compared to those maintained under adenosine replete (+Ado) conditions (data not shown). In contrast, the *pKSCl3'nt/nu^ΔR* (antisense)-transfectants maintained under adenosine-starvation (Ado) conditions showed no up-expression in their total nuclease activity. Thus, results of these nuclease activity assays were in full agreement with those obtained in the 3'-nucleotidase activity assays above.

B. *In situ* Activity Gels Analyses—In order to further assess the effects of adenosine starvation on control *pKSNEO*- and *pKSCl3'nt/nu^ΔR* (antisense)-transfected *C. luciliae*, lysates of such cells were separated by SDS-PAGE gels, renatured and subsequently stained *in situ* for their 3'-nucleotidase activity using 3'-AMP as substrate ^{8; 15; 29}. Parallel aliquots of these cell lysates were also analyzed for their nuclease activities in SDS-PAGE gels containing poly (A) as substrate ¹⁵. In these gel assays, only a single ~ 43 kDa band of 3'-nucleotidase (3'NT) enzyme activity was detected in *pKSNEO*-transfected cells maintained under adenosine-replete (+Ado) conditions (Fig. 5a, lane 1). The latter result visually reflects the basal levels of endogenous 3'-nucleotidase activity expressed by these cells under such conditions. By comparison, *pKSNEO*-transfected cells maintained under adenosine-starvation (−Ado) conditions for 24 or more hrs showed a much more pronounced 43 kDa band of 3'NT enzymatic activity (Fig. 5a, lane 2). These results visually reflect the dramatic up-expression of 3'-nucleotidase specific activity in these *pKSNEO*-transfected cells in response to adenosine-starvation. The latter results are virtually identical to those obtained in parallel experiments with wild-type, non-transfected *C. luciliae* under similar culture conditions (data not shown). In contrast, however, no band of 3'-nucleotidase activity was detectable in lysates of *pKSCl3'nt/nu^ΔR* (antisense)-transfected *C. luciliae* maintained under either adenosine-replete (+Ado)

or adenosine-starvation (–Ado) conditions (Fig. 5a, lanes 3 and 4, respectively). The latter results demonstrated the diminution/virtual abrogation of endogenous 3'-nucleotidase activity in these antisense-transfected cells. In parallel experiments, virtually identical results were obtained with these cell samples in gels stained *in situ* to visualize their endogenous nuclease activities (Fig. 5b, lanes 1–4). Taken together, these results demonstrated that both of the bi-functional enzyme activities of the *Cl3'NT/NU* (*i.e.* 3'-nucleotidase and nuclease) were induced/up-regulated under adenosine-starvation conditions and that the expression of an antisense mRNA (*i.e.* *pKSCl3'nt/nu^ΔR*) negated/abrogated this process. Further, results obtained in these *in situ* activity-gel assays are in good agreement with those obtained in the spectrophotometric enzyme assays above.

Effect of *Cl3'nt/nu^ΔR* Antisense Transcription on the Growth of *C. luciliae* in polynucleotide-containing medium

Previously, we found that *C. luciliae* parasites could readily grow to high cell densities (*i.e.* $\geq 5 \times 10^7 \text{ ml}^{-1}$) in chemically-defined, RPMI-1640 medium supplemented with 50 μM poly(A) as a sole source of purines (Dwyer, unpublished). To determine whether antisense expression had any effect on the ability of these parasites to grow and multiply in such polyA-containing medium, growth kinetic experiments were set-up with *pKSCl3'nt/nu^ΔR* (antisense)- and *pKSNEO* control -transfected *C. luciliae*. Results of those experiments demonstrated that the control *pKSNEO*-transfected parasites, similar to wild-type cells, routinely grew to very high cell densities (*i.e.* $\geq 6 \times 10^7 \text{ ml}^{-1}$) in the poly(A)-containing medium (Fig. 6). In contrast, however, the *Cl3'nt/nu^ΔR* antisense-transfectants failed to show any growth in this polynucleotide-containing medium (Fig. 6).

These results in conjunction with those of the enzyme activity assays above, showed that antisense expression clearly abolished the functional enzymatic activities of the parasite 3'NT/NU and that such activity was essential for the growth of this organism in medium containing a complex polynucleotide as a sole source of purines.

Effects of *Cl3'nt/nu^ΔR* Antisense Transcription on Surface Membrane Acid Phosphatase

A. MACP Enzyme Activity—In addition to the 3'nucleotidase/nuclease, *C. luciliae* also possesses a second phosphomono-esterase activity on its cell surface, *i.e.* a membrane-bound acid phosphatase³. Previously, total cellular acid phosphatase (AcP) activity (presumably reflecting both membrane-bound and secretory AcP isoforms) was shown to be up-expressed in adenosine-starved *C. luciliae*³. Recently, we found that the *C. luciliae* surface membrane-bound (M)AcP was highly resistant to inhibition by sodium tartrate (2.5 mM) while its secretory (S)AcP was completely inhibited by this compound (Yamage, unpublished). In light of these observations, it was of interest to determine whether MACP expression might be linked with that of the surface membrane 3'nucleotidase/nuclease. In that regard, lysates of both control *pKSNEO*- and *pKSCl3'nt/nu^ΔR* (antisense)-transfected *C. luciliae* maintained under (+Ado) and (–Ado) conditions were analyzed for their tartrate-resistant MACP activities. Results of those assays showed that *pKSNEO*-transfected cells maintained for 24 or more hrs under adenosine-starvation (–Ado) conditions, up-expressed their membrane acid phosphatase specific activity by more than 2–3 fold compared to those maintained under adenosine replete (+Ado) conditions (Fig. 7a).

Similarly, *pKSCl3'nt/nu^ΔR* (antisense)-transfectants maintained for 24 or more hrs under adenosine-starvation (–Ado) conditions also up-expressed their MACP specific activity (*i.e.* by ~3 fold) compared to those kept under adenosine replete (+Ado) conditions (Fig. 7b). Thus, these results indicated that the induction/up-expression of membrane acid phosphatase enzyme activity under (–Ado) conditions was not impeded by the antisense message produced in the *pKSCl3'nt/nu^ΔR* (antisense)-transfectants. Further, these results also suggested that MACP

expression *per se* was not directly linked to that of the 3'NT/NU. Moreover, these observations demonstrated the specificity of the 3'NT/NU-antisense message in abrogating the expression of 3'-nucleotidase/nuclease activity but not altering MACP expression in these cells.

B. MACP mRNA Expression—In order to substantiate our observations on MACP activity above, the steady-state levels of membrane acid phosphatase mRNA were also examined in both *pKSNEO*- and *pKSCI3'nt/nu^ΔR* (antisense)-transfected *C. luciliae*. To that end, total RNA was isolated from such transfectants maintained under adenosine-replete (+Ado) and adenosine-starvation (−Ado) conditions for 36 hrs and used for Northern blot analyses, as above. Equivalent amounts of total RNA (15 μg) from each cell type was separated by agarose gel electrophoresis and stained with ethidium bromide for visualization (Fig. 8a and b, lower panels, lanes 1–4). Blots of these gels were hybridized with either: a probe specific for the *C. luciliae* membrane-acid phosphatase gene (*ClMACP*) or the *C. luciliae* alpha-tubulin-specific probe (*Cl α-tubulin*) as a control. Results of such Northern blot analysis showed that both *pKSNEO*- and *pKSCI3'nt/nu^ΔR* (antisense)-transfected cells maintained under (+Ado) conditions expressed a single distinct band of *MACP* message (Fig. 8a, lanes 1 and 3, respectively). This *MACP* message was apparently up-expressed by ~2–3 fold in both control and antisense-transfected cells when they were maintained under adenosine-starvation (−Ado) conditions for 24 or more hr (Fig. 8a, lanes 2 and 4, respectively). In contrast to the membrane acid phosphatase message, the alpha-tubulin mRNA appeared to be constitutively expressed in both of these transfectants regardless of whether they were maintained under adenosine-replete (+Ado) or adenosine-starvation (−Ado) conditions (Fig. 8b, lanes 1–4).

Taken together, these results indicated that expression of the *MACP* gene was independently regulated and not directly-coupled to that of the 3'-nucleotidase/nuclease (*i.e.* 3'NT/NU antisense expression did not abrogate the up-regulation of either the endogenous *MACP* message or its functional enzyme activity)

Discussion

The primitive, monoxenous insect trypanosomatid protozoan, *Crithidia luciliae*, like other trypanosomatid parasites are purine auxotrophs (*i.e.* they are incapable of synthesizing the purine ring *de novo*) and therefore require extra-cellular salvage mechanisms to satisfy their need for these essential nutrients². It has been suggested that its unique bi-functional, surface membrane-anchored, 3'-nucleotidase/nuclease (*Cl3'NT/NU*), presumably plays an important role(s) in the purine salvage pathway of these parasites^{3; 4; 12; 13}. In that regard, previously, we isolated and characterized a gene (*Cl3'NT/NU*), which encodes this parasite enzyme¹⁵. In that report, we showed that at least three copies of the *Cl3'NT/NU* gene were present in the diploid genome of this organism. Further, we showed that in response to purine-starvation conditions, these parasites dramatically up-expressed the *Cl3'NT/NU* message by ~100 fold which resulted in an ~1000-fold increase in their 3'NT/NU specific enzyme activity. The latter results corroborated at the molecular level, earlier observations showing that these parasites up-expressed their 3'-nucleotidase enzyme activity in response to purine starvation conditions^{3; 8}. In the current report, we demonstrated that, in addition to this dramatic up-expression in 3'-nucleotidase/nuclease activity, a tartrate-resistant, surface membrane acid phosphatase (*ClMACP*) was also up-expressed (by ~2–3 fold) in purine-starved *C. luciliae*. These results were verified by our Northern blot analyses which demonstrated that the *ClMACP*-specific message was also up-expressed by approximately two fold in these purine-starved cells. Taken together, the apparent synchronous up-expression of these two surface membrane phosphomono-esterase activities (*i.e.* in response to purine starvation) suggested that their genes might be part of a “common” regulatory mechanism. In an attempt to disrupt the expression of one of these enzymes (*i.e.* the *Cl3'NT/NU*), we used an antisense-based approach. This approach was chosen rather than one based on RNAi because, the latter has not been successfully used for

silencing genes in several groups of trypanosomatid parasites (e.g. *Leishmania sp.* and *Trypanosoma cruzi*)^{23; 24; 25}. Therefore, for the current study, we generated a plasmid construct for episomal expression of a specific antisense *Cl3'NT/NU* mRNA. Transfection of parasites with this *Cl3'NT/NU* antisense construct had no apparent adverse effects on the overall growth of these cells compared to controls. Northern blot analyses demonstrated that a large excess of the antisense transcript was in fact produced in *C. luciliae* transfected with this antisense plasmid. However, no *Cl3'NT/NU* sense message could be detected in these transfectants under adenosine replete conditions or even when they were maintained under purine starvation (i.e. hyper-inducible) conditions. Moreover, in agreement with the latter, the dramatic up-expression in 3'-nucleotidase/nuclease activities, which are typically induced under purine starvation conditions, were completely abrogated in these antisense-transfected parasites. These observations demonstrated the efficacy of this antisense approach. Of greater significance, however, was our observation that such antisense transcription completely abolished the ability of these transfected parasites to grow in medium containing a complex polynucleotide as the sole source of purines. The latter results demonstrated the critical/essential nature of this enzyme to parasite growth and development.

In contrast to the abrogation in 3'NT/NU enzyme activity, the parasite tartrate-resistant surface membrane acid phosphatase (MACP) activity was in fact up-elevated (i.e. up-expressed by ~2–3 fold) in antisense-transfected *C. luciliae* maintained under purine starvation conditions. This elevation in MACP activity was virtually identical to that observed with control cells maintained under similar conditions. The latter results are in good agreement with our Northern blot analyses. These results indicated that *Cl3'NT/NU* antisense expression had no apparent effects on either the endogenous- or the “purine starvation-induced” up-expression of the parasite surface membrane MACP. In parallel to investigating the cell surface membrane phospho-monoesterases, we also evaluated the expression of α -tubulin (i.e. a typical house-keeping gene) in these parasites. Results of those experiments demonstrated that the constitutive expression of α -tubulin mRNA was unaltered in control- as well as in antisense-transfected *C. luciliae* regardless of whether they were maintained under purine replete or purine starved conditions. Taken together, these observations indicated that *Cl3'NT/NU* antisense expression did not appear to cause any generalized suppression of gene expression *per se* in these transfected parasites.

In summary, in this report we showed that an episomally-driven *Cl3'NT/NU* antisense message could specifically and completely abrogate the expression and functional activity of the *C. luciliae* surface membrane 3'-nucleotidase/nuclease. Further, our results demonstrated that this surface membrane enzyme, was in fact, critical/essential for the growth and development of this parasite. In a more general context, the 3'NT/NU might prove to be a logical target for therapeutic intervention against the pathogenic relatives of these primitive trypanosomatid organisms.

Materials and Methods

Reagents

All chemicals and reagents used in this study, unless otherwise specified, were of analytical grade and were obtained from Sigma-Aldrich Chemical Co. Enzymes used for molecular studies were purchased as indicated; DNA and RNA molecular mass standards were from Invitrogen, Inc. and protein molecular mass standards were purchased from GE Healthcare Bio-Sciences Corp. (Amersham).

Parasites and Culture Conditions

Crithidia luciliae cells (strain: ATCC No. 30285)³⁰ were grown at 26°C in chemically defined-medium M199 (Invitrogen), pH 6.8 as described previously³¹. For transfection experiments parasites were grown in the above medium but supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, Woodland, CA). A clone (MY₁₀₁) was isolated by limiting-dilution from such cultures and used for all subsequent experiments¹⁵. For adenosine-starvation experiments, replete cells were grown and maintained in chemically-defined medium, RPMI-1640³¹ supplemented with 100 µM adenosine (*i.e.* +Ado) as the sole source of purines and adenosine-starved cells were incubated in such medium lacking added adenosine (–Ado) for various periods up to 72 hr. For some growth kinetics experiments, parasites were also cultured in RPMI-1640 medium as above but containing 50 µM polyadenylic acid (poly (A), Sigma) as the sole source of purines. In all experiments, log-phase cultures ($2-4 \times 10^7$ cells ml⁻¹) were harvested by centrifugation as previously described³². The resulting cell pellets were washed three times in ice-cold phosphate buffered saline (PBS, 10mM sodium phosphate, 145 mM NaCl, pH 7.4) by centrifugation as above and finally resuspended in buffers appropriate for the extraction of RNA or proteins (see below).

Construction of Antisense Expression Plasmid and Transfection

To make the antisense construct, a truncated fragment (nt+1 to nt+1005) of the *Crithidia luciliae* 3'-nucleotidase/nuclease gene was generated by PCR using 0.1 µg of a plasmid (pCl-3) containing the full length *Cl3'NT/NU* gene [*i.e.* nt +1 to nt +1134, *GenBank*TM accession no. AF140355,¹⁵] as template. Such reactions contained 10 µM each of dATP, dCTP, dGTP and dTTP, 2 units of *Pfu* DNA polymerase (Stratagene), 3 units of Taq DNA polymerase (Sigma), 1X TaqPlus Long Low salt buffer (Stratagene) and 20 pmol each of a mixture of four oligonucleotide primers each containing an artificial restriction site for *SpeI* (underlined); Fwd-1: 5'-agt gtc act agt ATG ATG CGT GCC GTT CTC C-3'; Fwd-1.1: 5'-agt gtc act agt ATG ATG CGT-3'; Rev-1: 5'-act aga cta gtC TAG TTG TG AGG CCC TTC TG-3' and Rev-1.1: 5'-act aga cta gtC TAG TGG TG-3'. PCR conditions consisted of a single cycle at 99°C for 1 min and 53°C for 10 min followed by 37 cycles at: 94°C for 15 sec, 53°C for 1 min and 72°C for 2 min with final terminal extension at 72°C for 7 min. The resulting fragment (*Cl3'nt/nu*^Δ), which contained a portion of the *Cl3' NT/NU* ORF (*i.e.* nt +1 to nt +1005), was ligated into the pCRII vector (Invitrogen). Subsequently, the latter was digested with *SpeI* and the resulting fragment ligated into the *SpeI* site of the *pKSNEO* leishmanial episomal expression vector^{26; 33}. The resulting plasmid construct, *pKSCl3'nt/nu*^{ΔR}, contained a truncated insert of the *Cl3'NT/NU* gene in reverse orientation with respect to the normal direction of transcription of this expression vector. Both restriction endonuclease mapping and nt sequence analyses were used to verify that the *Cl3'nt/nu*^{ΔR} was inserted in an antisense orientation in the (*pKSNEO*) vector. This antisense plasmid (*pKSCl3'nt/nu*^{ΔR}) as well as the *pKSNEO* control plasmid were electroporated into *C. luciliae* cells as previously described¹⁵. Following an overnight recovery in medium M199+³¹ with 10% FBS at 26°C, both the *pKSCl3'nt/nu*^{ΔR} (antisense-) and *pKSNEO* (control-) transfectants were selected for growth under increasing concentrations of Geneticin (G-418, Invitrogen) up to 200 µg ml⁻¹.

Growth Kinetics of Transfected Parasites

Both the *pKSNEO* (control) and *pKSCl3'nt/nu*^{ΔR} (antisense) transfected parasites were monitored at regular intervals during the course of their growth *in vitro* under various culture conditions (*i.e.* in +Ado, –Ado or in Poly(A)-containing media). For experiments, triplicate cultures of both transfectants were initiated at $1-2 \times 10^6$ cells ml⁻¹ (*i.e.* from stock cultures in their exponential phase of growth). Aliquots from such cultures were taken at regular intervals, diluted appropriately and counted using a Coulter[®] counter (Model Z1, Beckman-Coulter) as previously described³⁴.

Generation of Probes and Northern Blot Analyses

The *Cl3'nt/nu^Δ* fragment above was flanked by artificial promoter sequences for T7 and *Sp6* RNA polymerases at the 5'- and 3'-ends, respectively. This was done by PCR amplification as above but using 20 pmol of each of the following primers: (T7 primer 1) 5'-atc gta taa tac gac tca cta tag ggc gaA TGA TGC GTG CCG TTC CCT GCC GCT GCT GT-3'; (T7 primer 2) 5'-atc gta taa tac gac tca cta-3'; (*Sp6* primer 1) 5'-agt cag att tag gtg aca cta tag aat acG TTG CTG AGG CCC TTC TGC ACG TAG TGG TTC ACC AC-3' and (*Sp6* primer 2) 5'-agt cag gat tta ggt gac act at-3' (promoter sequences are underlined). The resulting 1 kb fragment was purified using GFX PCR Gel Band Extraction Kit (GE Healthcare Bio-Sciences) and blunt-ended using the *Klenow* fragment of *E. coli* DNA polymerase (Roche Molecular Biochemicals). This template was used to synthesize our single-strand specific RNA probes. In such reactions, either the T7 (Roche) or *Sp6* RNA polymerase (Promega Corp., Madison, WI) was used in the presence of 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.65 mM UTP, and 0.35 mM DIG-11 UTP (Roche). The resulting DIG-labeled sense-strand probe ("*Sense*") was generated with T7 and the DIG-labeled antisense-strand probe ("*Antisense*") was generated with *Sp6*.

A DIG-labeled double-stranded DNA probe ("*ds*") for the *Cl3'nt/nu^Δ* was also prepared using PCR reactions as above but in the presence of 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP, 0.065 mM dTTP, and 0.035 mM DIG-11 dUTP (Roche) and the following primers: (Primer 3) 5'-ATG ATG CGT GCC GTT CTC C-3' and (Primer 4) 5'-GTT GCT GAG GCC CTT CTG-3'.

Previously, we cloned a gene (*Clat*) encoding an α -tubulin from *C. luciliae* (Yamaguchi, unpublished). This clone was used as template in PCR reactions as above to generate a DIG-labeled double-stranded DNA probe for α -tubulin using the following primers: (*Clat*1) 5'-TGC GTG AGG CTA TCT GCA T-3'; (*Clat*2) 5'-CTT GCC GGA CAC CAG CTG-3'. Similarly, we also cloned a gene encoding a *C. luciliae* surface membrane acid phosphatase (*ClMacP*, Yamaguchi, unpublished). It was used as template in PCR reactions as above to generate a DIG-labeled double-stranded DNA probe for the *C. luciliae* surface membrane acid phosphatase using the following primers: (*ClMacP*1) 5'-TCG ACG CGC GCC TTG TC-3'; (*ClMacP*2) 5'-CGT TGA AGT CCG TGC ACA G-3'. Both the *C. luciliae* α -tubulin (*Cl α -Tubulin*) probe and surface membrane acid phosphatase (*ClMacP*) probe were used in Northern blot hybridizations.

For Northern blot analyses, both *pKSNEO* (control-) and *pKSCl3'nt/nu^{ΔR}* (antisense-) transfected *C. luciliae* cells were grown at 26°C under adenosine-replete (+Ado) or maintained under adenosine-starvation (-Ado) conditions for up to 36 hr. Total RNAs were isolated from such cells using STAT60 (TEL-TEST, Inc. Friendswood, TX) according to manufacturer's recommendations. Equivalent amounts (15 μ g) of these RNAs were separated by agarose gel electrophoresis³⁵, transferred onto nylon membranes (HyBond-N, GE Healthcare) and cross-linked by UV irradiation as previously described³⁶. Blots were hybridized at high stringency with each of the above probes using Genius Detection System (Roche) as per manufacturer's recommendations. Subsequent to washing at high stringency (0.1 $\times$ SSC, 0.1% SDS at 65°C), the hybridized fragments were visualized using an anti-DIG antibody in conjunction with a chemiluminescent substrate (Roche). Images were captured from such blots using XOMAT-ARTM X-ray film (Kodak) and analyzed using the NIH Image densitometry software package (available from NIH at <http://rsb.info.nih.gov/>). Such images were processed using Adobe Photoshop 5.5 (Adobe Systems Inc).

Enzyme Assays

3'-nucleotidase enzyme activity was determined in cell lysates in test tube assays using adenosine 3'-monophosphate (3'-AMP) as substrate as described previously^{8; 15}. One unit of enzyme activity is defined as 1 nmol of Pi released from 3'-AMP min⁻¹ mg⁻¹ of cell lysate protein. Protein concentrations were determined using the bicinchoninic acid method (BCA, Pierce Chemical Co). Total nuclease activity was determined using poly(A) as substrate as previously described¹¹. Tartrate-resistant acid phosphatase activity was determined in the presence of 2.5 mM sodium tartrate using 10 mM *p*-nitrophenyl phosphate as substrate in 50 mM sodium acetate buffer (pH 4.8) as described previously³⁷.

In situ Detection of Enzyme Activity in SDS-PAGE Zymogram Gels

Cell lysates of *C. luciliae* (20 µg/lane) were separated in SDS-PAGE (8 × 8 cm.) mini-gels. Subsequent to renaturation, these gels were processed for *in situ* staining of their 3'-nucleotidase activity using 3'-AMP as substrate and malachite green/ammonium molybdate as a phosphate capture reagent as previously described^{8; 15; 29}. In these gels, 3'-nucleotidase activity was apparent as a dark-green band of precipitated product on a faint green background. Duplicate aliquots of such cell lysates were also separated in SDS-PAGE gels containing Poly (A) as substrate. Following renaturation and staining with Toluidine Blue O, nuclease activity was visualized in these gels as a clear/colorless zone of substrate hydrolysis in an otherwise uniformly-stained, dark blue background of un-hydrolyzed poly(A) substrate^{15; 38 39}.

Acknowledgements

This research was supported by the intramural research program of the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, NIH. Dr. Mat Yamage was an NIH Visiting Fellow supported by a postdoctoral fellowship from the Fogarty International Center and the NIAID, NIH and Dr. Manju Joshi was supported by an appointment to the Oak Ridge Institute for Science and Education (ORISE), Research specialist Program at NIH. The program is administered by ORISE through an inter-agency agreement between the US Dept. of Energy and the NIH. We thank Dr. Greg Matlashewski (Dept. of Microbiology and Immunology, McGill University, Montreal, Canada) for kindly providing the *pKSNEO-Leishmania* expression vector and Duane Bartley of the Johns Hopkins DNA Analysis Facility, (Baltimore, MD) for assistance with nucleotide sequencing. We also thank Drs. Michael Gottlieb (Foundation for the National Institutes of Health, NIH), Alain Debrabant (Division of Emerging and Transfusion Transmitted Diseases, CBER, FDA, Bethesda, MD), Alison Shakarian (Salve Regina University, Newport, RI) and Elodie Ghedin (The Institute for Genomic Research, Rockville, MD and Dept. of Medicine/ Infectious Diseases, University of Pittsburgh School of Medicine, Pittsburgh, PA) for their advice and helpful suggestions during the course of these studies.

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Abbreviations

aa	amino acid
Ado	adenosine
3'-AMP	adenosine 3'-monophosphate
bp	base pair
DIG	digoxigenin
FBS	fetal bovine serum
MAcP	membrane-bound acid phosphatase
3'NT/NU	

3'-nucleotidase/nuclease

ORF

open reading frame

PCR

polymerase chain reaction

poly(A)

poly adenylic acid

SDS-PAGE

sodium dodecyl sulfate polyacrylamide gel electrophoresis

Figure 1. Diagrammatic representation of the [pKSNEO] vector containing the truncated antisense *CI3'*nt/*nu*^AR fragment

A fragment (*CI3'nt/nu*^A), which spans nt+1 to nt+1005 of the *CI 3'NT/NU* gene, was ligated into the *pKSNEO* vector in the reverse orientation (*CI3'nt/nu*^A**R**). The direction of transcription in the *pKSNEO* vector is shown (arrow). Relevant restriction sites are shown and the entire *CI3'NT/NU* ORF is depicted below in its sense orientation.

Figure 2. Growth kinetics of *C. luciliae* transfectants under adenosine replete (Ado+) and adenosine starvation (Ado-) conditions

C. luciliae transfected with either the *pKSNEO* control vector (A) or with the *CI3'nt/nuΔR* antisense plasmid (B) were cultured in the presence (v) or absence (θ) of 100μM adenosine and monitored for their growth *in vitro*. Cultures were initiated at $1-2 \times 10^6$ cells ml⁻¹ and aliquots were taken for cell counting at various time points as shown. Values represent the mean ($\pm$ S.E.) of triplicate samples from three separate experiments.

Figure 3. Northern blot analyses of *C. luciliae* 3'NT/NU sense and antisense mRNAs
C. luciliae transfected with either the *pKSNEO* control vector (*pKSNEO*) or with the *pKSCl3'nt/nu Δ R* antisense plasmid (*Cl3'nt/nu Δ R*) were maintained *in vitro* at 26 °C under either adenosine replete (Ado+, 100 μ M) or adenosine starvation (Ado-) conditions for 36 hrs. Subsequently, total RNA (15 μ g) from (Ado+) and (Ado-) control *pKSNEO*-transfected *C. luciliae* (lanes 1 and 2, respectively) and from (Ado+) and (Ado-) *pKSCl3'nt/nu Δ R* (antisense)-transfected *C. luciliae* (lanes 3 and 4, respectively) were separated by agarose gel electrophoresis and stained with ethidium bromide (lower panels in A–D). Blots of these gels were hybridized with: (A) a DIG-labeled single-strand 3'nt/nu single-strand Antisense-RNA probe; (B) a DIG-labeled 3'nt/nu single-strand Sense-RNA probe; (C) a DIG-labeled double-stranded (ds) 3'nt/nu -DNA probe and (D) a DIG-labeled control *C. luciliae* (α -) Tubulin-specific probe. Arrow at the left denotes the ~2.3 kb endogenous/native 3'NT/NU sense message and the arrow at the right denotes the ~2.0 kb endogenous α -tubulin message.

Figure 4. Analysis of 3'-nucleotidase activity in control and anti-sense transfected *C. luciliae*
 The 3'-nucleotidase activities of control *pKSNEO*-transfected (**A**) and *pKS-Cl3'nt/nuΔR* (antisense)-transfected (**B**) *C. luciliae* maintained for various periods under adenosine replete (Ado+, [v]) or adenosine starvation (Ado-, [θ]) conditions were measured in cell lysates (at the time points shown) using 3'-AMP as substrate. Specific activities are given as nmols min⁻¹ mg⁻¹ cell protein. Values shown at each time point represent the mean (± S.E.) of three separate experiments.

Figure 5. *In situ* zymogram analyses of 3'-nucleotidase and nuclease activities in control and antisense transfected *C. luciliae*

pKSNEO-transfected and *pKS-Cl3'nt/nuΔR* (antisense)-transfected *C. luciliae* were maintained under adenosine replete (Ado+, lanes 1 and 3, respectively) or adenosine starvation (Ado-, lanes 2 and 4, respectively) conditions for 36 hr. (A) Cell lysates (20 μg/lane) from each of these transfectants were separated in SDS-PAGE gels and stained *in situ* for their 3'-nucleotidase activity using 3'-AMP as substrate and malachite green/ammonium molybdate as a phosphate capture reagent. In these gels, 3'-nucleotidase activity was apparent as a single dark-green band of precipitated product on a faint green background. (B) Identical aliquots of such cell lysates were also separated in SDS-PAGE gels containing poly(A) as substrate and subsequently stained *in situ* for their nuclease activity. Following renaturation and staining with Toluidine Blue O, nuclease activity was visualized in these gels as a clear/colorless zone of substrate hydrolysis in an otherwise uniformly-stained, dark blue background of unhydrolyzed poly(A) substrate. The arrow on the left of each panel denotes the ~43 kDa band of endogenous 3'NT (A) and nuclease activity (B), respectively.

Figure 6. Growth kinetics of *C. luciliae* transfectants in medium containing poly(A) as a sole source of purines

C. luciliae transfected with either the *pKSNEO* control vector (v) or with the *Cl3'nt/nuΔR* antisense plasmid (θ) were cultured in chemically defined RPMI-1640 medium containing 50μM poly(A) as a sole source of purines and monitored for their growth *in vitro*. Cultures were initiated at $1-2 \times 10^6 \text{ cells ml}^{-1}$ and aliquots were taken for cell counting at various time points as shown. Values represent the mean ($\pm$ S.E.) of triplicate samples from three separate experiments.

Figure 7. Analysis of tartrate-resistant acid phosphatase activity in control and anti-sense transfected *C. luciliae*

The tartrate-resistant acid phosphatase activities of control *pKSNEO*-transfected (**A**) and *pKS-Cl3'nt/nuΔR* (antisense)-transfected (**B**) *C. luciliae* maintained for various periods under adenosine replete (+Ado, [v]) or adenosine starvation (−Ado, [θ]) conditions were measured in cell lysates using *p*-nitrophenyl phosphate as substrate. Specific activities are given as nmols min^{−1} mg^{−1} cell protein. Values shown at each time point represent the mean (±S.E.) of three separate experiments.

Figure 8. Northern blot analyses of *MACP* and α -tubulin mRNAs in control and anti-sense transfected *C. luciliae*

pKSNEO-transfected and *pKS-Cl3'nt/nuΔR* (antisense)-transfected *C. luciliae* were maintained under adenosine replete (+Ado, lanes 1 and 3, respectively) or adenosine starvation (−Ado, lanes 2 and 4, respectively) conditions for 36 hr. Total RNA (15 μ g) from such cells were separated by agarose gel electrophoresis and stained with ethidium bromide (lower panels in **A** and **B**). Blots of these gels were hybridized in (**A**) with a DIG-labeled probe specific for a *C. luciliae* membrane acid phosphatase gene (*CIMAcP*) and in (**B**) with a *C. luciliae* α -tubulin (*Cl* α -tubulin)-specific DIG-labeled probe.