THE CLONING AND CHARACTERIZATION OF THE 3'-NUCLEOTIDASE/NUCLEASE IN *LEISHMANIA MEXICANA*

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by

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Earth's crammed with heaven
And every common bush afire with God;
But only he who sees takes off his shoes;
The rest sit around it and pluck blackberries.

Anon

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LIST OF ABBREVIATIONS

3'-nt	3'-nucleotidase/nuclease
5'-nt	5'-nucleotidase
CBP	calmodulin binding protein
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid (disodium salt)
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
Exo III	exonuclease III
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IPTG	β-D-isopropyl-thiogalactopyranoside
LB-amp	LB-ampicillin agar
ORF	open reading frame
pBs	pBluescript™ plasmid
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PKDL	post kala azar dermal leishmaniasis
PolyA	polyadenosine
RFLP	restriction fragment length polymorphism
RNase	ribonuclease
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SSC	standard saline citrate
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer

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ABSTRACT

The cloning and characterization of the 3'-nucleotidase/nuclease in *Leishmania mexicana.*

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Trypanosomatids are unable to synthesize purine nucleotides *de novo* and are therefore dependent on purine salvage pathways for their acquisition. The various means by which purines are salvaged from the protozoa's external environment represent possible targets for the development of chemotherapeutic agents against the medically important parasites of this family. The surface membrane 3'-nucleotidase/nuclease (3'-nt) may be a key enzyme in purine salvage in *Leishmania*. The 1134bp gene encoding this enzyme has been cloned in *L. mexicana* and shows 90% and 88% identity (at the nucleotide and amino acid levels respectively) to the previously described *L. donovani* gene. This is the second trypanosomatid 3'-nt gene to be cloned. In addition, the 3'-nt sequence showed similarities to the fungal nucleases S1 and P1, which also possess a dual nuclease/nucleotidase activity. The gene appears to be single copy in the *L. mexicana* genome. The cloned gene encodes a 40kDa protein that possesses 3'-nucleotidase and nuclease activities. Gene expression is stage-regulated, present in procyclic and metacyclic promastigote forms but not in amastigotes. Attempts to generate null mutants for 3'-nt were unsuccessful but recombinant protein was produced and anti-recombinant antibodies derived. Promastigote cultures *in vitro* were adapted to chemically defined medium, enabling the study of 3'-nt's function under different conditions of purine availability. In the procyclic promastigote, 3'-nt appears to have an important function in parasite growth and development, being upregulated under conditions of purine depletion. This upregulation was reversed in the presence of 3'-AMP, suggesting 3'-nt is regulated in response to external purine availability. In addition, other similar nucleotidase/nuclease activities were identified in these depleted cultures, possibly indicating a common upregulation response to starvation conditions. In contrast, axenic amastigote forms showed no response to artificially induced purine starvation conditions. It appears that 3'-nt may be specifically expressed in the promastigote as part of a linked purine salvage apparatus giving the parasite distinct competitive advantage over the insect host in terms of purine acquisition. The different environmental conditions of the amastigote form may negate the need for such a specific apparatus and so 3'-nt is not expressed. The insect stage specificity of this enzyme means it is not a suitable candidate for the development of chemotherapeutic agents against leishmaniasis.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Worldwide, the number of people thought to be at risk from infection by the protozoan parasite *Leishmania*, the causative agent of a number of clinical syndromes known as the leishmaniases, has been estimated by the World Health Organisation to be in excess of 350 million. The past decade has witnessed an expansion of traditionally endemic regions and sharp increases in the number of recorded cases of disease to an estimated 2 million new cases annually (WHO, 1990).

The rising significance of the leishmaniases is likely to be a product of many diverse factors. Thirteen of the eighty-eight countries affected are amongst the world's least developed and the costs of treatment and case detection inevitably hinder successful control efforts. The leishmaniases are primarily zoonotic in origin and as human communities colonise new land, particularly in the case of cleared South American jungle, they will often come into contact with animal reservoirs of the disease. In addition, leishmaniasis has become an increasingly common secondary infection associated with the immunosuppression of patients suffering from AIDS.

In any discussion of leishmaniasis control, the two prime aims appear to be to effectively control the disease in current endemic regions and to gain a better understanding of some of the unique biological characteristics of the parasite with a view to exploiting them. In terms of control, a number of ageing but nevertheless effective drugs do exist for the

treatment of both cutaneous and visceral leishmaniasis and the quest for an effective vaccine continues. In some endemic areas, control and elimination of both the sandfly vector and local reservoir hosts has been successful in decreasing the incidence of disease but it is generally acknowledged that in most cases vector control is almost impossible. In the sylvatic cycles of the disease particularly, not enough is known about the reservoir hosts or the breeding sites of the sandflies involved to practically address the problem.

So, chemotherapy remains the principal method of disease control and many targets for drug development exist in the specialised biology, biochemistry and host associations of the parasite. This work explores one such potential target in the New World parasite *Leishmania mexicana*: the 3'-nucleotidase/nuclease (3'-nt).

1.2 Clinical manifestations of leishmaniasis

Leishmaniasis disease may take a number of clinical forms, according to the species of infecting parasite. Visceral leishmaniasis (or kala-azar) involves parasite invasion of the liver, spleen and bone marrow with accompanying erythrocyte haemolysis leading to anaemia and irregular fever. Visceral leishmaniasis is characteristic of *L. donovani* infection. Post kala-azar dermal leishmaniasis (PKDL) is a frequent sequel of *L. donovani* infection following cure of visceral disease and can resemble diffuse cutaneous disease. Cutaneous leishmaniasis is characterised by the appearance of necrotic skin lesions around the site of an infected sandfly bite. Lesions are generally self-healing though infection may spread through the dermis in the less common condition of diffuse cutaneous leishmaniasis. Parasite species causing cutaneous leishmaniasis include *L. major* and *L. mexicana*. Mucocutaneous leishmaniasis (or espundia) also originates in

the skin dermis but may spread to the mucous membranes of the nasal septum, palate and pharynx. Gross disfigurement often results and secondary bacterial infection can be fatal. *L. braziliensis* is one species causing this condition.

1.3 Epidemiology of *Leishmania* infection

In 1992, approximately 100,000 cases of visceral disease and 300,000 cases of cutaneous disease were reported but it is considered that these numbers represent only a small proportion of actual cases (Ashford *et al.*, 1992). More up to date estimates posted on the WHO Division of Control of Tropical Diseases (CTD) world wide web site (WHO, 1998) put the annual incidence of new cases at 2 million, including 500,000 cases of visceral disease. Advances in taxonomy of *Leishmania* have shown that each species has distinctive epidemiological features, which may vary geographically with different vectors or reservoir hosts (Ashford, 1998). The leishmaniases may be split, according to species of infecting parasite, into the Old World leishmaniases and the New World leishmaniases.

The Old World diseases are visceral leishmaniasis (caused by *L. donovani* and *L. infantum*) and cutaneous leishmaniasis (caused by *L. major*, *L. tropica* and *L. aethiopica*). Visceral leishmaniasis is particularly prevalent in Northern India, Bangladesh, East Africa, North Africa, the Middle East and Southern Europe. Recent epidemics of kala azar have been reported in Southern Sudan and Eastern India. Many disease cases in Europe are reported to be the result of *Leishmania*/HIV co-infection (considered to be an emerging disease) in severely immunosuppressed AIDS patients (Desjeux *et al.*, 1996). PKDL is found in varying degrees in India and East Africa and may provide a reservoir of infection between epidemic periods (Addy & Nandy, 1992).

Cutaneous leishmaniasis in the Old World is found over a wide geographical area from Central Asia to North Africa, also in Sub-Saharan Africa, and is largely epidemic in nature. Some of the most striking increases in incidence of cutaneous disease over the past decade have been reported in Syria, Turkey and Tunisia (WHO, 1998) though the situation in countries where official data is not available (e.g. Afghanistan) may be far worse.

The New World diseases also include visceral leishmaniasis (*L. infantum* largely confined to Brazil) and cutaneous leishmaniasis (*L. mexicana*, *L. panamensis* and *L. amazonensis*) with the additional occurrence of mucocutaneous disease (caused by *L. braziliensis*). New World cutaneous disease is generally sporadic in many regions between Southern Mexico and Southern Brazil and mucocutaneous disease is restricted to regions of primary forest (Ashford, 1998).

The pathology associated with each of the forms of human leishmaniasis described above is dependent to varying degrees (according to the species of infecting parasite) on the immune response of the host (see section 1.5). This in turn may be influenced by genetic variability within the host population. The progress of disease is also influenced by the restriction of particular parasite species to specific parts of the body (Ashford & Bates, 1998). This is largely unexplained although the confinement of some species to the skin in cutaneous disease may simply be a matter of temperature sensitivity. Taking these factors into account, there is a considerable amount of generalisation involved in any description of the global epidemiology of leishmaniasis disease.

1.4 The *Leishmania* life cycle and the physiological environment of each stage

The *Leishmania* life cycle involves two major forms; the flagellated promastigote found in the gut of the sandfly vector and the aflagellate amastigote in the macrophages of the mammalian host. A number of morphologically similar but distinct promastigote stages have been identified in the sandfly (Bates, 1994b) but for the purposes of this research, these forms are limited to the generalised categories of procyclic promastigote (the multiplicative form) and metacyclic promastigote (the non-dividing infective form).

Amastigotes taken up in a blood meal by a feeding sandfly rapidly transform into procyclic promastigote forms (within 24 hours) which subsequently divide and develop. A proportion of these promastigotes ultimately gives rise to the non-replicative metacyclic promastigotes, which are the infective forms. This cyclopropagative mode of development occurs entirely within the gut lumen of the sandfly with all the associated environmental conditions. A peritrophic membrane is secreted around the blood meal between 12 and 36 hours after the feed. This porous chitin lattice encloses the meal during digestion and begins to disintegrate after 60 hours, when the products of digestion are excreted. This leaves a time period of approximately 36 hours in which developing promastigote forms must multiply sufficiently to ensure some parasites will escape excretion and establish an infection in the sandfly gut. It has been suggested that this process may involve secretion of chitinase by the parasites to accelerate their escape from the peritrophic membrane (Schlein *et al*, 1991). By and large, however, the initial multiplication of procyclic promastigotes occurs within the confines of the enclosed blood meal.

The temperature drop from mammalian blood to sandfly gut is likely to be a key factor in the transformation of amastigotes to promastigotes. Subsequently, the sandfly's digestion and absorption of nucleobases and sugars from the blood meal lead to starvation conditions in the gut, which contribute to the subsequent transformation into infective metacyclics (metacyclogenesis). In addition, promastigotes *in vitro* are known to acidify their culture medium during growth and a drop in pH of the digested blood meal may be another possible factor in the induction of metacyclogenesis (Bates & Tetley, 1993). Through their development, the promastigotes migrate forward within the gut and the metacyclic promastigotes are ultimately found in the sandfly mouthparts. These forms are apparently pre-adapted for survival in the mammalian host (Mallinson & Coombs, 1989) and injected into the blood when the sandfly feeds again.

Once they enter the mammalian host at the site of sandfly feeding, the metacyclic promastigotes are rapidly taken up by host macrophages in the skin. Their selective opsonisation by particular complement factors combined with the effect of sandfly saliva is thought to bypass the macrophage oxidative burst that would normally eliminate invading micro-organisms (Titus & Ribeiro, 1990; Wright & Silverstein, 1983). The metacyclic promastigotes are, therefore, able to transform to amastigotes within the phagosome of the macrophage (the transformation taking between 12 and 24 hours), a transformation probably mediated by the accompanied changes in temperature and pH with the transition from insect to mammal.

As the metacyclic promastigotes are transforming to amastigotes, digestive lysosomes fuse with the phagosome to form the acidic and hydrolytic environment of a phagolysosome. The amastigote form of the parasite is able to survive in the

phagolysosomal environment, thriving in the acidic conditions and possibly even actively maintaining them (Antoine *et al*, 1990). The expression of a surface membrane proton ATPase may be a key factor in preventing the acidification of the amastigote cell (Zilberstein & Shapira, 1994) and the parasite may also be capable of degrading some of the lysosomal enzymes secreted by the macrophage (Chaudhuri *et al*, 1988) thus avoiding destruction.

In *L. mexicana,* the resulting phagolysosome expands in the course of infection to form a large parasitophorous vacuole in which the amastigotes gradually multiply (Bray & Alexander, 1987). Vacuole formation is thought to be mediated in part by the secretion of proteophosphoglycan by the amastigotes and appears to be a parasite-induced phenomenon (Peters *et al*, 1997). In *L. amazonensis*, the delivery of several endocytosed ligands to the parasitophorous vacuole has been demonstrated (Rabinovitch *et al*, 1985) suggesting that the amastigotes gain a plentiful supply of nutrients via the endocytic network of their host cell. Indeed, it has been suggested that the efficiency of endosome delivery to the vacuole increases with age of infection (Russell *et al*, 1992), ensuring an adequate nutrient supply for the dividing population of amastigotes. In most other species, each amastigote is found in its own phagolysosome within the macrophage, the vacuole dividing with the amastigote. This may affect nutrient supply via the endocytic network in a different way.

1.5 Immunology of *Leishmania* infection

There is growing evidence that genetic variability in human hosts contributes to their immune response to infection with *Leishmania* and hence their susceptibility to disease (Shaw *et al*., 1995). The wide spectrum of clinical syndromes caused by apparently

identical strains of parasite certainly suggest a role for genetic variation and in mouse models, a number of genes have been identified as potentially involved in susceptibility to disease (Blackwell, 1996). Few human homologues have been described, though a natural resistance-associated macrophage protein (Nramp) has been characterised and is thought to provide resistance to a variety of intracellular infections in human macrophages (Cellier *et al.*, 1994). In addition to the genetic variability of host and parasite, factors such as number of infective sandfly bites received and size of parasite innoculum may also be important (Ashford & Bates, 1998). A full discussion of the nature of host immunological responses to the various species of *Leishmania* parasites is beyond the scope of this review, but there are a number of key aspects with general application.

In general, human cutaneous leishmaniasis is associated with a marked cell-mediated immune reaction, involving a delayed type hypersensitivity (DTH) response. Specific antibody may also be detected but the humoral response is generally weak. In contrast, visceral leishmaniasis patients show a strong, and largely non-specific, humoral response mediated by polyclonal B-cell activation and impaired cell-mediated immunity. Infection with the parasite will not always result in disease, and individuals with positive immunological reactions but no sign of clinical leishmaniasis can usually be found wherever there is parasite transmission (Ashford & Bates, 1998). Such cryptic or sub-clinical infection will often become patent when associated with immunosuppressive disease (for example, AIDS) and this is particularly observed in foci of *L. infantum* (Ashford & Bates, 1998).

In experimental mouse models, there has been much investigation into the dichotomy of Th1- and Th2-type responses. Intracellular amastigotes may be cleared by the activation of Th1-type T-cell responses in the host but the parasite appears to be able to suppress such activation. This may be achieved by the induction of inappropriate communication between infected macrophages and T-cells (possibly through the expression of aberrant antigen presentation molecules), the switching of T-cell responses to Th2-type (which suppress the effects of Th1-type responses) or by causing the infected macrophage to produce de-activating cytokines (Kaye, 1994). The route of T-cell differentiation to a Th1- or Th2-type pathway appears to be determined by the particular cytokines present at the initial stages of a cell-mediated response (Sad *et al.*, 1995).

In order to model better the immunology of murine leishmaniasis, genetically defined inbred strains of mice have been used in these investigations and it is difficult to assess the extent to which current results can be applied to the situation in humans. However, a similar dichotomy of cytokine profile and T-cell type can be demonstrated in human leishmaniasis (Kemp *et al.*, 1993) and research in mouse models has led to the development of some successful immunotherapy in human disease (see section 1.7).

1.6 Molecular aspects of *Leishmania*

There are a number of features of the molecular organisation of kinetoplastids that are unique to this group and offer possible targets for development of control strategies. The genome of *Leishmania* consists of nuclear and cytosolic compartments: the chromosomes and the independently replicating kinetoplast. The lack of chromosome condensation during mitosis makes assessment of ploidy difficult but most studies seem to point to *Leishmania* being largely diploid with some chromosomes being aneuploid

(Bastien, 1992; references cited in Lighthall & Giannini, 1992). Recent progress in mapping the *Leishmania* genome has established the existence of 36/37 linkage groups (Wincker, *et al.*, 1996) and corresponding chromosomal bands by pulsed field gel electrophoresis (PFGE). Individual chromosomes can show considerable size variations between species but the genetic organisation of homologues appears to be well conserved.

Rapid genome evolution is a factor of the trypanosomatid family, including *Leishmania* (observed most clearly in the processes of antigenic variation) and probably mediated by large numbers of repeated and transposable elements as well as the phenomenon of homologous recombination (Vickerman, 1994). Independent evolution of homologous chromosomes is also thought to be encouraged by the likely low importance of sexual reproduction in the trypanosomatid life cycle, so releasing the chromosomes from the restraints of regular pairing (Pays, 1993). There is circumstantial evidence for genetic exchange and a sexual cycle in *Leishmania* (Bastien *et al.*, 1992) but this has not been experimentally demonstrated and is certainly not an obligatory feature of the life cycle.

Another factor in the trypanosomatid genome is the existence of minichromosomes. These contain large numbers of tandem repeats which do not appear to possess any function in protein coding but have an important association with the variant surface glycoprotein apparatus, in the case of African trypanosomes. In *Leishmania*, these minichromosomes are circular and may be produced by the amplification of specific regions of the genome (Beverley, 1991). DNA amplification appears to be a general mechanism for increasing the copy number of key metabolic genes in response to stress and up to a 20-fold increase per cell has been observed (Hightower, 1988).

The cytosolic component of the genome, the kinetoplast, consists of a tightly interlocked network of circular DNA in the form of both maxi- and mini-circles (reviewed by Stuart & Feagin, 1992). The maxicircles, similar in structure to the mitochondrial DNA of other eukaryotes, contain genes associated with mitochondrial biogenesis and function. The minicircles, more heterogeneous in sequence and evolving rapidly within a particular population, transcribe the guide RNAs involved in RNA editing of maxicircle genes. RNA editing may be considered a primitive form of control for gene expression (Stuart & Feagin, 1992).

On the whole, gene regulation in trypanosomatids is controlled post-transcriptionally (Nilsen, 1994) and the family employ a number of unusual processing mechanisms. Trypanosomatid genes are often located in closely spaced tandem arrays apparently lacking individual promoter elements, such as exist in most other eukaryotes. Instead, there may be a single promoter for RNA polymerase binding with the genes being transcribed in multicistronic transcripts. Subsequent excision of mature mRNAs is mediated by the processes of *trans*-splicing and polyadenylation (Clayton, 1992). *Trans*-splicing enables the specific and precise joining of transcripts and is the process by which a 5' spliced leader RNA is added to each mRNA (Walder *et al*, 1986). The completion of transcript processing from the multicistronic unit is by polyadenylation of the 3' end.

There are several examples in *Leishmania* of developmentally regulated mRNAs as opposed to developmentally regulated transcription (observed in many eukaryotes). In these cases, the mRNA may be constitutively transcribed but subsequently activated (Bhaumik *et al*, 1991) or degraded (Argaman *et al*, 1994) in response to different

environmental cues. These different outcomes may be mediated by the mechanisms of *trans*-splicing or the transcription of specific nucleases to degrade the mRNA. In general, the three levels at which mRNA transcripts appear to be regulated are transport from the nucleus, stability of the transcript and efficiency of translation.

A differentially regulated gene family in *L. major* has been recently described as a potential model for polycistronic transcription and subsequent mRNA processing (Flinn & Smith, 1992), where untranslated 3' sequence elements are implicated in post-transcriptional control. Such elements may act as stem-loops, marking a particular transcript for processing or delaying its degradation. Untranslated 3' elements have also been shown to affect translation efficiency and steady-state levels of mRNAs in *Trypanosoma cruzi* (Nozaki & Cross, 1995) and *Trypanosoma brucei* (Blattner & Clayton, 1995). In *Leishmania* and trypanosomes, *trans*-splicing and polyadenylation appear to be coupled, mediated by splice sites of varying sequence but employing a fairly consistent intergenic space containing pyrimidine-rich elements (LeBowitz *et al*, 1993; Matthews *et al*, 1993). There may be a number of potential splice sites in the 3' untranslated region of a particular gene (as reported in *L. amazonensis;* Kawazu *et al*, 1997) and it is suggested that although one site seems preferably used, use of the other minor sites may produce transcripts of varying stability under differing environmental conditions.

In summary, the trypanosomatid genome appears to have quite a measure of flexibility with the ability to rapidly evolve using homologous recombination and gene amplification. Most gene regulation appears to occur post-transcriptionally, mediated by

the untranslated regions of polycistronic transcripts and the specific splicing of the mRNAs.

1.7 Chemotherapeutic targets in *Leishmania*

There have been few major developments in the chemotherapy of leishmaniasis in the past decade and many of the current drugs recommended for use are new formulations of older ones. Now almost eighty years old, antimonial drugs are still those of choice for treatment (Croft *et al*, 1997) but there is an increasing incidence of resistance reported (Olliaro & Bryceson, 1993). The need for new chemotherapeutic agents is great and a number of drug targets in *Leishmania* remain to be exploited.

Pentostam® and Glucantime®, both pentavalent antimonials, have been first line drugs for fifty years and their targets include glycolytic enzymes and fatty acid oxidation (Berman, 1991). Their mode of action is not fully understood, though the accumulation and retention of antimony by macrophages appears significant (Roberts *et al.*, 1995). In addition, the antimonials are preferentially concentrated in the liver and spleen (Bryceson, 1987) so are far less effective against cutaneous disease. The polyene antibiotic amphotericin B is a long-standing second choice drug, preferentially binding to the *Leishmania* membrane sterol, ergosterol, forming pores that cause subsequent ionic imbalance. This has been developed as a liposomal formulation, AmBisome®, and shown to be highly effective in the treatment of visceral leishmaniasis (Davidson *et al.*, 1994). Also available is Paromomycin, an aminoglycoside with broad antiprotozoal activity. This has been formulated into an ointment (together with methyl benzethonium chloride) and shown to be effective against cutaneous leishmaniasis (El-On *et al.*, 1992).

Future areas for drug development include sterol biosynthesis pathways, immunotherapy to activate macrophages and purine salvage pathways. The pathways in *Leishmania* for sterol synthesis have many features unique to the parasite and therefore represent potential targets. As mentioned, ergosterol, the predominant membrane sterol in *Leishmania*, is the target of amphotericin B, a drug already available. The biosynthesis of sterols is also sensitive to several antifungal agents, the effect either being accumulation of toxic intermediates or the blocking of essential end products (Chance, 1995; Olliaro & Bryceson, 1993). One such agent effective in the interruption of biosynthesis is ketoconazole (Goad *et al.*, 1985). However, it is possible that, *in vivo*, the parasite is able to compensate for loss of sterol synthesis by the uptake of host cholesterol (Haughan *et al.*, 1995). In contrast, clotrimazole has been shown to be effective clinically against *L. major* lesions (Larbi *et al.*, 1995).

The observation that macrophages activated by interferon-gamma (IFN-γ) are able to kill intracellular *Leishmania* amastigotes has led to its use as a chemotherapeutic agent. Studies have shown that the lymphokine is particularly effective when used in combination with antimonials for the treatment of visceral leishmaniasis previously resistant to antimonials alone (Badaro *et al.*, 1990; Sundar *et al.*, 1994). The pathways of purine salvage and metabolism also present possibilities for the development of new drugs. Allopurinol, an analogue of hypoxanthine, is metabolised by hypoxanthine-guanine phosphoribosyltransferase (HGPRT), a constituent enzyme of the purine salvage pathway. The resultant aberrant nucleotides are incorporated into RNA and interfere with protein synthesis (Chance, 1995). Clinical results are contradictory but these essential pathways warrant further investigation.

1.8 Gene targeting and its application in trypanosomatid research

Gene targeting is a powerful and increasingly routine tool used to assess the function and significance of a variety of trypanosomatid proteins for which the gene sequence has been elucidated. The procedure of gene targeting by which specific genes may be 'knocked out' of the genome is described more fully in Chapter 5.8 and is based on the phenomenon of homologous recombination. This process is thought to involve the association of distinct sections of homologous DNA sequence at the point of DNA replication with subsequent genetic exchange between sites. The natural tendency of trypanosomatid chromosomes to undergo such recombination has been utilised as one of several means by which manipulated sequence may be experimentally introduced into parasites in a stable transformation (Smith, 1990). In the unicellular protozoa, it is therefore possible to engineer an organism with mutant genotype by the transfection of a single cell.

Several different applications for gene targeting have been explored in the study of trypanosomatid infectivity and novel gene function. The successful creation of homozygous gene replacements by targeting both alleles with modified non-functional sequence in *Leishmania* has the effect of knocking out the native gene, resulting in a null mutant (Cruz *et al.*, 1991). The creation of null mutants in this way potentially enables the study of specific gene function in living parasite cells through assessing the phenotypic changes resulting from gene knockout. The advantages of employing this approach to study gene function include the specificity of targeting (which can be confirmed using straightforward molecular techniques) and the generation of a cloned cell line on which to experiment. Gene targeting also allows the undertaking of functional studies of proteins for which there are no specific inhibitors available and has

been used to develop an avirulent live vaccine for leishmaniasis in mice (Titus *et al.*, 1995).

Several different *Leishmania* proteins have been targeted in this way. These include alpha-tubulin (Delafaille & Wirth, 1992), dihydrofolate reductase-thymidylate synthase (dhfr-ts) (Cruz *et al.*, 1993), the DNA binding protein HEXBP (Webb & McMaster, 1994), cysteine proteinase (Souza *et al.*, 1994), trypanothione reductase (Dumas *et al.*, 1997), pteridine reductase 1 (Bello *et al.*, 1994), HGPRT and APRT (adenine phosphoribosyl-transferase) (Hwang *et al.*, 1996) and finally gp63 (Joshi *et al.*, 1998). Likewise, genes in other trypanosomatids have been targeted, for example the ubiquitin-fusion gene family of *Trypanosoma cruzi* (Gillespie *et al.*, 1993) and a mitochondrial DEAD-box protein and ornithine decarboxylase (ODC) in *Trypanosoma brucei* (Missel *et al.*, 1997; Li *et al.*, 1996).

A number of gene targeting experiments have resulted in some unpredicted outcomes and these highlight the limitations of the approach and have also illuminated some features of the molecular biology of *Leishmania*. It was found that in targeting the dhfr-ts gene locus in virulent *L. major*, double knockouts were obtained but null mutants could not be generated (Cruz *et al.*, 1993). The parasites appeared to compensate for the loss of one gene locus by increasing the chromosome number to a state of aneuploidy, retaining either one or two wild type dhfr-ts alleles. A similar compensation was observed in attempts to create LmmCRK1 null mutants in *L. mexicana* (Mottram *et al.*, 1996a) and plasticity in chromosome number is proposed as a means by which *Leishmania* avoids the deletion of essential genes. The process by which parasites increase gene copy number in this way may be by duplication of whole chromosomes as

above or by translocation of the particular gene locus to a different chromosome (Dumas *et al.*, 1997).

The inability to produce null mutants for a gene may be indicative of its importance to the parasite. However, if the gene being targeted is essential to the parasite, its knockout will result in a lethal phenotype that may not be recoverable. This highlights the importance of performing knockout experiments in the presence of a 'rescue phenotype'. A rescue phenotype may be provided either by supplementing the growth medium with the particular metabolite that a double knockout would deny the parasite (Bello *et al.*, 1994) or by co-transfecting the cell with an episomal copy of the targeted gene (Tovar *et al.*, 1998). The presence of metabolite or an episomal copy of the gene (which may then be induced in the null mutant) will prevent the double knockout being lethal, enabling the recovery of transfected parasites and possibly removing the pressure on the parasite to compensate for gene loss. Episomal expression of the targeted gene may also be used subsequent to a non-lethal knockout, to rescue the wild-type phenotype. This is regularly used to demonstrate that observed phenotypic changes are a result of gene targeting and not a factor of cell manipulation (Mottram *et al.*, 1996b; Papadopoulou *et al.*, 1996).

Another factor of the *Leishmania* genome highlighted by attempts to create null mutants is the multiplicity of copies of many genes. One example is the LmPCb cysteine proteinase of *L. mexicana,* which is present in the genome as an array of 19 tandemly repeated genes (Mottram *et al.*, 1996b). Likewise, the major surface protein of *Leishmania major*, gp63, is encoded by a family of seven genes (Joshi *et al.*, 1998). To produce null mutants, each copy of the gene must be knocked out and single copy genes, therefore, often make the best targets. Many knockouts do not produce a defined

phenotypic transformation and it is often unclear what the function of the target gene is in the parasite (Souza *et al*, 1994). Over-expression of such genes in episomes may demonstrate their activity more clearly. However, it is possible that the removal of a single protein (by targeting a single gene) from a complex cellular pathway may not always be sufficient to noticeably transform the phenotype, and the parasite often appears to be able to compensate for the loss, perhaps by modifying the targeted pathway or by employing a different one. One biochemical pathway in *Leishmania* with this apparent compensatory ability is the purine salvage pathway (Hwang & Ullman, 1997).

1.9 Purine salvage in trypanosomatids

As major constituents of nucleosides, purine bases have a variety of essential roles in the maintenance of cellular function. Not only are nucleosides the building blocks of nucleic acids but also the repositories of metabolic energy in their phosphorylated form and constituents of several key coenzymes (eg NADP). *Leishmania* promastigotes grown in continuous *in vitro* culture require an exogenous source of purines for growth (Steiger & Steiger, 1977). This observation suggested their inability to synthesise purine nucleobases *de novo* and hence their dependence on purine salvage pathways. The lack of purine synthesis was subsequently demonstrated in *L. donovani* and *L. braziliensis* (Marr *et al.*, 1978) and various purine salvage enzymes, indicating functional pathways, were described in a variety of species (references cited in Dwyer & Gottlieb, 1984).

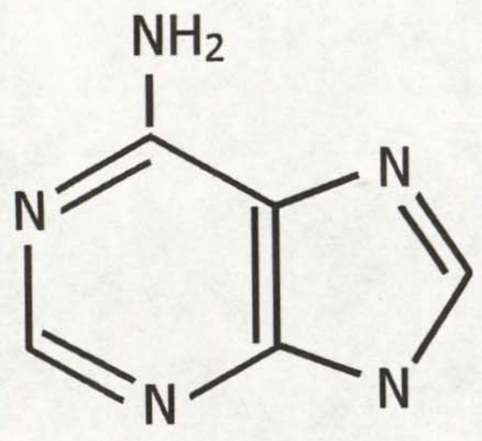
The two main components of purine salvage pathways are the transport of purines into the parasite cell and their subsequent metabolism into various usable forms. The trypanosomatids contain a variety of different kinase, transferase and phosphorylase activities for the manipulation of imported purine bases, suggesting the presence of

multiple potential pathways of salvage (references cited in Hammond & Gutteridge, 1984). Presented here is a brief summary of some of the biochemical pathways that may be used by trypanosomatids in purine salvage and a review of purine transport into the parasite cell. The basic chemical structures of the different purine forms mentioned are shown in Figure 1a and a summary of the various pathways described is outlined in Figure 1b.

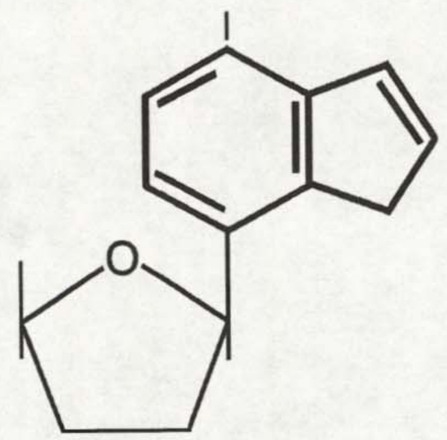
(a) Salvage pathways

One of the central molecules in purine salvage reactions is 5-phosphoribosyl-1-pyrophosphate (PRPP) which donates ribosephosphate to a purine base, resulting in the formation of a nucleotide (Stryer, 1988). This reaction may be catalysed by one of a number of phosphoribosyltransferases (PRTases), depending on the nature of the salvaged purine. Adenine, hypoxanthine/guanine and xanthine PRTase (APRT, HGPRT and XPRT respectively) activities have all been described in a number of species of *Leishmania* (Hassan & Coombs, 1986) and explored as potential drug targets. *Leishmania donovani* null mutants for APRT and HGPRT, however, have been shown to propagate normally in defined medium containing any single purine and also retain the ability to metabolise hypoxanthine to nucleotide (Hwang & Ullman, 1997). The non-essential nature of these individual proteins, even under conditions of limited source of purine, and the ability of the mutants to retain the salvage of hypoxanthine, seem to support the notion that a multiplicity of salvage pathways are available to these parasites.

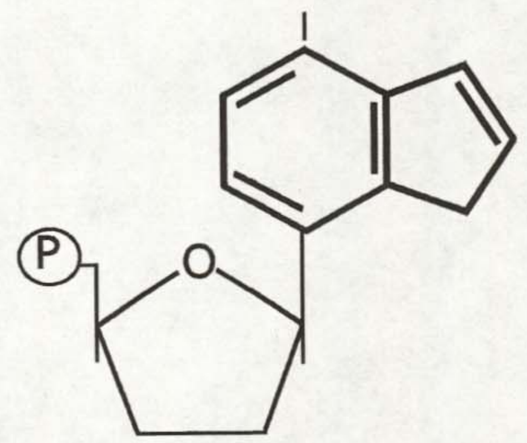
One such alternative route by which nucleotides may be formed from purine bases is via the nucleoside form, catalysed by the action of specific phosphorylases (Figure 1b). Adenosine phosphorylase and guanosine phosphorylase are both present in *Leishmania*



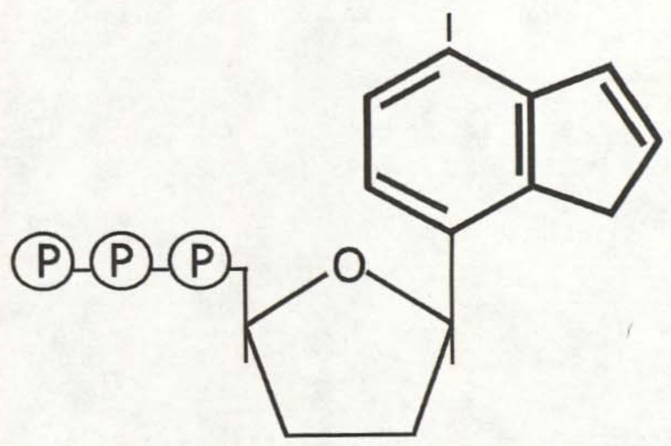
Purine base
(Adenine)



Nucleoside
(Adenosine)



Monophosphate nucleotide
(AMP)



Triphosphate nucleotide
(ATP)

Figure 1a Basic chemical structures of the purine forms involved in salvage pathways to illustrate the formation of triphosphate nucleotides. Details of chemical structure are not shown. The purine base is linked to ribose to form the nucleoside, which is then phosphorylated to the monophosphate and eventually the triphosphate form. P = phosphate molecule (PO_3^{2-})

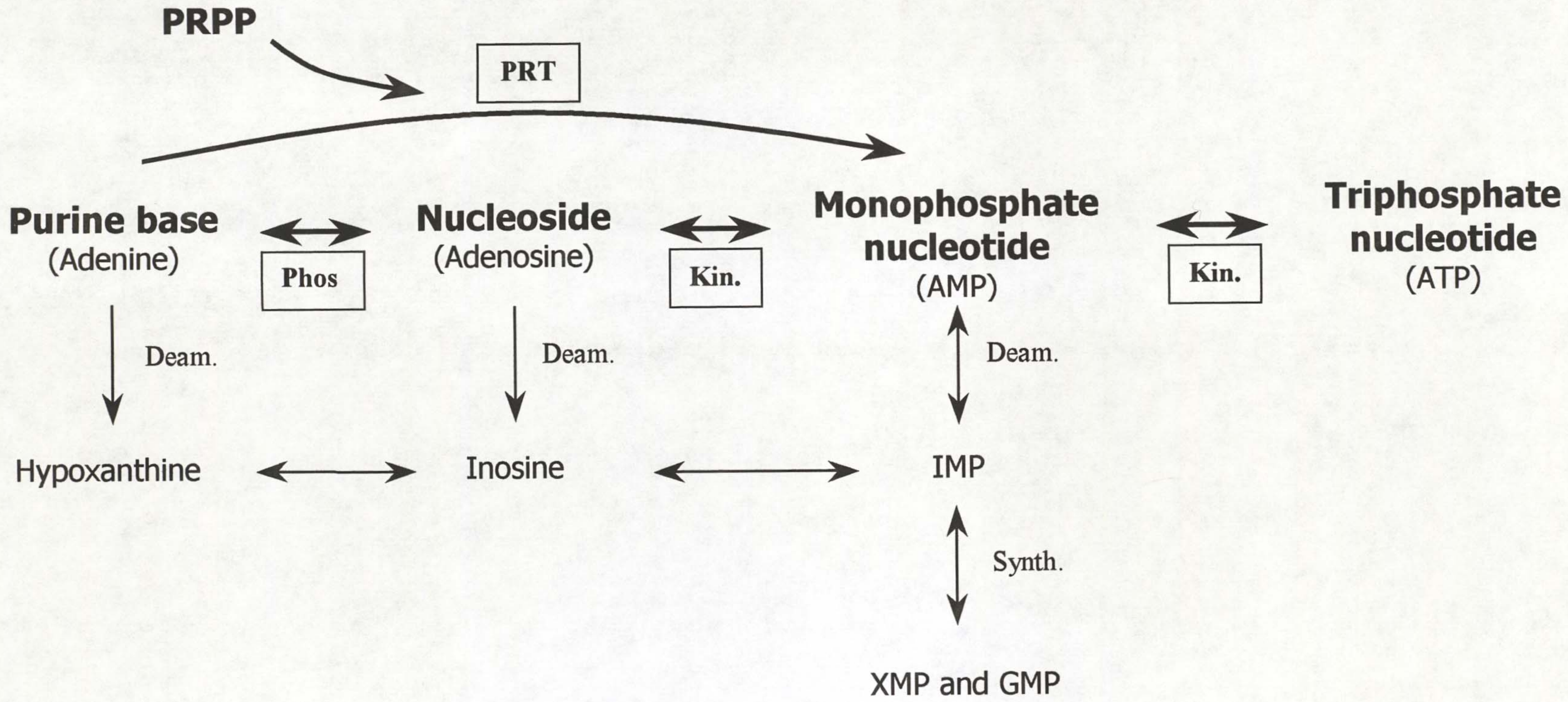


Figure 1b Summary of the central reactions involved in trypanosomatid purine salvage. The generalised pathway is shown in bold with the transformations of adenine shown in brackets to illustrate the different stages. Monophosphate nucleotide may be formed from nucleoside by the action of kinase (**Kin.**) and from purine base by the action of phosphorylase (**Phos.**) and kinase or directly by phosphoribosyltransferase (**PRT**) on phosphoribosylpyrophosphate (**PRPP**). Triphosphate nucleotide is also formed by the action of kinase. These two central pathways are present in trypanosomatids for adenine, guanosine, hypoxanthine and xanthine. In addition, the various forms in the adenine pathway may be converted to the corresponding hypoxanthine forms by the action of deaminases (Deam.) (shown by the vertical arrows) and inosine monophosphate (IMP) may be converted to AMP, XMP and finally GMP by the action of synthetases (Synth.). This summary illustrates general mechanism and not the specific enzymes involved. (Adapted from Hammond & Gutteridge, 1984).

and some *Trypanosoma* species (Davies *et al.*, 1983) and these may compensate for the loss of PRTase activity in the mutants above. Subsequent to nucleoside formation, *nucleotides* are formed by the action of a specific kinase. Adenosine, inosine, guanosine and xanthosine kinase activities have all been described in a number of species of *Leishmania* (Hassan & Coombs, 1986). Kinase activity was also targeted in the APRT/HGPRT null mutants described above (Hwang & Ullman, 1997) in an attempt to cut off this potential alternative route from base to nucleotide. As before, null mutants for APRT, HGPRT and adenosine kinase (AK) were able to grow on defined medium and retain the ability to salvage hypoxanthine. This result illuminates another pathway available to trypanosomatids; the capability for purine interconversion (reviewed in Hammond & Gutteridge, 1984).

Guanosine and adenosine monophosphate nucleotides (GMP and AMP, respectively) can both be synthesised from inosine monophosphate (IMP) and vice versa and the enzymes necessary for these conversions appear, generally, to be present in trypanosomatids (Ceron *et al.*, 1979). As above, IMP is formed by the salvage of purine base (hypoxanthine) and the donation of ribosephosphate from PRPP or by the sequential phosphorylation of hypoxanthine to inosine and then to IMP. Hypoxanthine is incorporated into IMP by HGPRT, whose presence in trypanosomatids has already been discussed. IMP may be the predominant nucleotide formed in trypanosomatid purine salvage as hypoxanthine and xanthine are continually produced in mammals as a result of nucleic acid metabolism and appear to be present in serum at higher concentrations than adenosine and guanosine (Hartwick *et al.*, 1979).

In addition to the interconversion of purine nucleotides, trypanosomatids also have the capability to interconvert nucleosides and purine bases within the parasite (Hammond &

Gutteridge, 1984). For example, adenine may be converted to hypoxanthine (catalysed by adenine deaminase), adenosine to inosine (by adenosine deaminase) and finally AMP to IMP (by AMP deaminase). There appear to be similar deaminases present for the interconversion of guanidine and xanthine. The presence of these various activities for interconversion mean that not only can trypanosomatids utilise various exogenous sources of purine to meet all their purine needs, but also that any of the pathways to nucleotide synthesis mentioned may be 'circumnavigated' should one of them break down. The observation above that a null mutant for AK could still synthesise AMP from exogenous adenine (and therefore grow), suggests that, with the conversion of adenosine to AMP blocked, adenosine was instead converted to inosine, to IMP and hence to AMP in a parallel pathway.

Once salvaged to form monophosphate nucleotides through the action of PRTase and/or phosphorylase, purine bases may be anabolised further to triphosphates for use in many cellular processes by the action of specific kinases, which add phosphate to the substrate molecules (Stryer, 1988). For example, adenosine monophosphate (AMP) is converted to adenosine di- and tri-phosphate (ADP and ATP) by the action of adenylate kinase. This is a similar reaction to that which has already been described to convert pre-formed nucleosides into monophosphate nucleotides (eg adenosine converted to AMP by AK).

So, trypanosomatids are able to produce nucleotides by salvage of both pre-formed nucleosides and free purine bases. Several enzymes thought to be involved in purine salvage pathways are either unique to the parasite (Shin & Wataya, 1995) or show significantly different kinetics from their mammalian counterparts (Martinez & Marr, 1992; Hendrickson *et al.*, 1993), making them potential chemotherapeutic targets.

Equally, it is evident that differences in metabolism do exist between different trypanosomatid species (Hassan & Coombs, 1986) so the development of anti-trypanosomatid drugs with wide application within the family may be difficult. In addition, the range of enzymes involved in purine salvage suggests that parasites have a broad adaptive capability if faced with purine stress.

(b) Purine transport

Mechanisms for importing nucleotides into the parasite cell may be less variable and therefore better targets for drug development. Studies of *L. panamensis* promastigotes provided evidence of the specific transport of purine bases and nucleosides and a distinct locus for adenosine import (Hansen *et al.*, 1982). Nucleosides also appeared to be transported more efficiently than purine bases. Using various purine analogues, purine transporters in *Trypanosoma* sp. have been closely studied both as drug targets (Cohn & Gottlieb, 1997) and as the means of drug uptake and delivery into the parasite cell (Byers *et al.*, 1992; Carter *et al.*, 1995; Sufrin *et al.*, 1996). There appears to be an established link between drug-resistance and the loss of purine transporter activity in *Trypanosoma* strains (for example, Ross & Barns, 1996) and in *Leishmania* (Kerby & Detke, 1993) and this may be mediated by gene amplification, perhaps of transport proteins with differing substrate specificities. Specific transporters for adenosine and inosine have also been described in *Trypanosoma cruzi* (Finlay *et al.*, 1988), *L. donovani* (Aronow *et al.*, 1987), *L. major* (Baer *et al.*, 1992) and the insect trypanosomatid *Crithidia luciliae* (Hall *et al.*, 1993). The purine transport system in *C. luciliae* has been best characterised to date.

C. luciliae is a close relation of *Leishmania* parasitising the alimentary canal of the blowfly, a physiological environment similar to that of *Leishmania* promastigotes in the

sandfly. It seems likely that the modes of purine salvage employed by the two parasites may also be similar. There are two distinct nucleoside transporters in *C. luciliae*, one for adenosine and one for guanosine and inosine (Hall *et al.*, 1993). The importance of these enzymes is indicated by the lack of transport of purine bases across the parasite cell membrane under normal circumstances (Kidder *et al.*, 1978). The presence of two independent transporters is in contrast to the situation in the host cells, which possess a single transporter with a broad range of substrate specificity (Jarvis, 1987). This difference raises the intriguing possibility that the high affinity of the two transporters for a specific substrate may enable *C. luciliae* to effectively compete with its host for available purines (Hall *et al.*, 1993). Similarly, the K_m values for the *L. donovani* transporters above (Aronow *et al.*, 1987) are two orders of magnitude lower than their mammalian counterparts, allowing the parasite to scavenge purines from the host environment even at low nucleoside concentrations.

Another factor of nucleoside transport in *C. luciliae* that may confer advantage over the host is the ability to specifically upregulate the rate of purine transport in response to purine stress or starvation (Alleman & Gottlieb, 1996; Gero *et al.*, 1997). It appears that adenosine transport is carrier mediated and increases on starvation, whereas adenine and hypoxanthine transport moves from simple diffusion in normal cells to carrier mediated in starved cells (Alleman & Gottlieb, 1996). The presence of a protein synthesis inhibitor (cycloheximide) prevented this increase, suggesting that increased levels of the transporters were responsible for the upregulation in transport (Gero *et al.*, 1997). The regulation was demonstrated to be in response to extracellular purine levels by supplementing the starved cells with adenosine. The levels of transport rapidly decreased, reversing the upregulation seen in starved cultures (Hall *et al.*, 1996),

suggesting the rate of purine transport could be tightly regulated according to purine availability. Another effect of cycloheximide was to sustain transport upregulation even in the presence of the adenosine supplement (Gero *et al.*, 1997), suggesting the downregulation of transporters in response to extracellular purine is dependent on the transcription of a further protein.

There is evidence that the observed upregulation of purine transport into the cell is linked with an increased purine metabolism in *C. luciliae* (Gero *et al.*, 1997), indicating that the specific transporters are part of the purine salvage pathway of this parasite. Also involved is the activity of a 3'-nucleotidase (3'-nt) on the parasite cell surface (Hall *et al.*, 1996). The co-ordinated upregulation of 3'-nt, purine transporters and purine metabolism suggests these are all linked in the role of purine salvage.

1.10 3'-nucleotidase in trypanosomatids

3'-nucleotidase activity (EC 3.1.3.6) was first described in *L. donovani* promastigotes (Gottlieb & Dwyer, 1982). The activity was associated with the cell surface membrane and cytochemical studies indicated the active site of the enzyme was externally oriented, suggesting a function in the dephosphorylation of external nucleotides (Dwyer & Gottlieb, 1984). The presence of similarly located 3'-nt activity has been demonstrated in several other trypanosomatids including *Trypanosoma rhodesiense*, *L. major* and *L. mexicana* (Hassan & Coombs, 1987).

The 3'-nt enzyme has been purified from *L. donovani* (Gbenle & Dwyer, 1992) and shown to have a broad pH optimum of 5.5-7.5, with a pI of 5.8. Nucleotidase activity is partially inhibited by 5mM EDTA, with the enzyme activity being restored by 5mM

$CoCl_2$ (Gbenle & Dwyer, 1992). The substrate specificity of the *L. mexicana* enzyme has been described (Bates, 1993b) and shows activity towards 3'-nucleotides, poly-nucleotides, RNA and single stranded DNA. Mobilities on SDS-PAGE show a co-migration of 3'-nt with nuclease activity and that the 3'-nt enzyme described actually has a dual catalytic function of nucleotidase and nuclease. The nuclease and nucleotidase activities in purified enzyme from *L. donovani* promastigotes show slightly different pH optima (Campbell *et al.*, 1991) and the nucleotidase catalytic function appears to show over three times the specific activity of the nuclease. It has been suggested that, although highly active as a nucleotidase, the *L. donovani* enzyme's physiological function is as a nuclease (Zlotnick *et al.*, 1985).

The substrate specificity studies above also distinguished two different 3'-nt activities in the promastigote and amastigote forms of *L. mexicana* (Bates, 1993b). Both forms have a doublet of nuclease activity at 29/31kDa though this is significantly greater (about 60-fold) in the amastigotes. In addition, the promastigotes have a significant band of nuclease activity at 40kDa and it is this band that shows high 3'-nt activity. Although lacking this 40kDa activity, the doublet in amastigotes shows some 3'-nt activity (but at a lower level than in promastigotes). The amastigote and promastigote forms of the *L. mexicana* enzyme had previously been shown to have differing pH optima of 7.5 and 8.0 respectively (Hassan & Coombs, 1987), suggesting these related activities may not be the same enzyme. The properties of the 40kDa 3'-nt/nuclease activity most nearly matched those of the *L. donovani* enzyme previously described (Gottlieb & Dwyer, 1982) and it was supposed that this band represented the *L. mexicana* surface-membrane 3'-nt.

The regulation of 3'-nt activity in response to external levels of purine has been demonstrated in both *L. donovani* and *C. luciliae* (Sacci *et al*., 1990; Alleman & Gottlieb, 1990). As with the components of *Crithidia*'s purine salvage pathway described above, the enzyme is upregulated in conditions of purine starvation and returns to baseline levels with the introduction of a source of purines in the culture medium. Such observation suggests the 3'-nt is an essential enzyme to the parasite. Gottlieb has proposed a model of purine acquisition in *Leishmania* involving the co-ordinated activity of the surface membrane 3'-nt, a related 5'-nucleotidase (5'-nt) and purine nucleoside transporters (Gottlieb, 1989). In this model, 3'-nt may either dephosphorylate 3' nucleotides, resulting in nucleosides that may be imported into the cell, or digest short nucleic acids to 5' nucleotides, which are similarly dephosphorylated by 5'-nt. The *L. donovani* 3'-nt gene has recently been cloned and characterised (Debrabant *et al*, 1995).

1.11 Aims of the Research

The aim of this research is to characterise the 3'-nt previously reported in *L. mexicana*, primarily by isolating and cloning the *L. mexicana* gene. The structure and function of the enzyme will be compared with those already described in other species by means of molecular and biochemical techniques and its significance to the various stages of the parasite assessed. It is hoped that study of this specialised feature of trypanosomatid purine salvage may further enhance our understanding of parasitic adaptation and illuminate potential areas of control for leishmaniasis.

CHAPTER 2
MATERIALS AND METHODS

2.1 Isolation of genomic DNA from *L. mexicana*

Leishmania mexicana (WHO designation MNYC/BZ/62/M379) promastigotes were cultured axenically at 26°C in M199 medium supplemented with 10% foetal calf serum. Cells were harvested in late log phase by centrifugation (3000×g for 10 minutes at 4°C) and the pellet washed in cold phosphate buffered saline (pH=7.2). The cell pellet was gently resuspended in a suitable volume of lysis buffer (50mM NaCl, 50mM EDTA, 50mM Tris-HCl, 1% SDS: pH=8.0) and incubated at 37°C overnight with proteinase K (fc 100µg/ml).

The released DNA was extracted from the cell lysate with an equal volume of phenol followed by an equal volume of a phenol/chloroform mix and a final extraction with chloroform alone. Each extraction was performed carefully to retain the integrity of the genomic DNA and the aqueous and organic layers separated by centrifugation (8000×g for 5 minutes at 4°C). Strands of genomic DNA precipitated out of solution on addition of $1/10^{th}$ volume of 3M sodium acetate (pH=5.2) and two volumes of ethanol. The visible aggregate of DNA was hooked out of solution and carefully washed with 70% (v/v) ethanol, air dried and resuspended in TE buffer (10mM Tris-HCl, 1mM EDTA: pH=7.2). Contaminating RNA was removed by the addition of heat-treated RNase A (fc 20ng/ml). Genomic DNA was stored at 4°C.

2.2 Restriction digest of DNA

The restriction endonucleases used were supplied by Boehringer Mannheim with optimised incubation buffers for their use. Where double digests were performed, the One-Phor-All™ buffering system (Pharmacia) was used. Unless stated otherwise, all digestions of genomic DNA and bacteriophage DNA were performed overnight at 37°C. All other digestions were performed for 1 hour at 37°C.

2.3 Separation of DNA by electrophoresis on agarose gels

Agarose gels were prepared using TAE buffer (Tris/acetate/EDTA) and run at 80 volts in TAE with ethidium bromide included (0.5μg/ml). Unless stated otherwise, 0.8% agarose gels were used to separate DNA fragments and the results photographed under ultraviolet illumination.

2.4 Southern transfer (blot)

Immediately following electrophoresis, DNA samples on agarose were transferred to charged nylon membranes (Boehringer Mannheim) by capillary blot under denaturing conditions (Sambrook *et al*, 1989). The gels were initially depurinated (in 250mM HCl for 5 minutes) to improve transfer of large DNA molecules and then denatured (0.5M NaOH, 1.5M NaCl) and neutralised (0.5M Tris-HCl, 3M NaCl: pH=7.5). The capillary blot was performed overnight using 20× SSC buffer (3M NaCl, 0.3M sodium citrate). The transferred DNA was fixed to the nylon membrane by UV cross-linking and used immediately or stored dry at 4°C.

2.5 Hybridisation of southern blots using the DIG system

The Digoxigenin (DIG) system supplied by Boehringer Mannheim is a non-radioactive labelling system for the preparation of probes used in nucleotide hybridisations. The DIG molecule is bound to dUTP, which may then be incorporated into a nucleic acid probe by random-primed Klenow extension (using the commercially available labelling mix, DIG High Prime) or polymerase chain reaction (PCR). These probes are used in standard hybridisations to bind to homologous nucleic acid sequence on fixed nylon membranes. The presence of hybridised probe on the blot is detected by an anti-DIG antibody-alkaline phosphatase conjugate that is allowed to bind to DIG residues incorporated into the probe. A chemiluminescent substrate for alkaline phosphatase allows probe detection on X-ray film.

Crosslinked nylon membranes were prehybridised in the optimised hybridisation solution available from Boehringer Mannheim, DIG Easy Hyb. Unless stated otherwise, prehybridisation was at 45°C for 30 minutes. Labelled probes were denatured by boiling and mixed with Easy Hyb at a concentration of between 10-20ng/ml of hybridisation solution. The concentration of each labelled probe was estimated by comparison to labelled standards provided with the kit (see DIG System User's Guide, Boehringer Mannheim) and the concentration used for each hybridisation experiment was optimised. Labelled probes were stored at -20°C ready mixed in Easy Hyb and used up to six times after subsequent denaturation at 60°C.

Following hybridisation, the probed membrane underwent four stringency washes; two at room temperature (2× SSC, 0.1% SDS for 5 minutes) and two at 50°C (0.1× SSC, 0.1% SDS for 15 minutes). Using the components of the commercial kit, the membrane was

blocked, incubated with antibody conjugate, washed and then incubated with CSPD® chemiluminescent substrate in a sealed plastic bag (see User's Guide for details of buffers used and times of incubation). Depending on the strength of the particular probe, a result could be achieved after 20 minutes exposure of the sealed blot to X-ray film. The chemiluminescent signal persisted for at least 24 hours and multiple exposures could be taken. The sealed blots were subsequently stored at 4°C. If required, blots were stripped for re-probing by incubating in just-boiled 0.1% SDS for 10 minutes prior to prehybridisation.

2.6 Purification of DNA from agarose gels using Prep-A-Gene®

The Prep-A-Gene® system (Bio-Rad Laboratories) uses a silica-based matrix for the purification of DNA from solution and from agarose. The agarose gel slices containing the desired DNA fragments were dissolved in the supplied binding buffer (6M sodium perchlorate, 50mM Tris (pH=8.0), 10mM EDTA) at 45°C and Prep-A-Gene matrix added to bind the solubilised DNA. The quantity of matrix used was according to the concentration of DNA estimated from the ethidium bromide stained agarose gel (see Instruction Manual). The matrix-bound DNA was pelleted by pulse centrifugation (30 seconds) and washed twice with the provided buffer (400mM NaCl, 20mM Tris (pH=7.5), 2mM EDTA, 50% ethanol (v/v)). DNA was eluted from the matrix with the provided elution buffer and pure enough for subsequent restriction digest or ligation.

2.7 Ligation and transformation

DNA restriction fragments purified from agarose gels were ligated into similarly digested plasmid vectors according to standard protocols (Sambrook *et al*, 1989). Ligations proceeded overnight at 12-14°C and recombinant plasmids used directly to

transform DH5α library competent cells (GibcoBRL) according to the supplier's instructions. Positive recombinants were selected on LB-ampicillin agar plates with X-gal using blue/white selection according to standard protocols (Sambrook *et al*, 1989).

2.8 Colony blots of positive transformants

This colony blotting method was used for both bacterial colonies and bacteriophage plaques. A charged nylon membrane (Boehringer Mannheim) was carefully laid onto the surface of the agar and left for a minute to make a print of the colonies/plaques beneath. The position of the membrane on the plate was carefully marked asymmetrically before removing. The membrane was sequentially denatured (with 0.5M NaOH, 1.5M NaCl, 1% SDS for 15 minutes), neutralised (1M Tris-HCl, 1.5M NaCl: pH=7.5 for 5 minutes) and incubated with 2× SSC (for 15 minutes) by laying it, colony-side uppermost, on a series of filter papers saturated with each of the solutions. The DNA released from the colonies by this process was fixed to the membrane by UV crosslinking and residual cellular debris washed from it by shaking at 68°C in a solution of dilute SSC (3× SSC, 0.1% SDS for 1 hour). The membrane was hybridised in the same way as a Southern blot (see section 2.5). The resultant autoradiogram was lined up with the original agar plate according to the marks made during blotting and any colonies showing positive hybridisation picked directly from the plate.

2.9 Isolation of plasmid DNA from recombinant colonies

Plasmid DNA was purified from bacterial genomic DNA and proteins using a silica-based miniprep system (QIAprep®, QIAGEN). Cell lysates from which genomic DNA and protein had been precipitated were spun through the silica-gel matrix immobilised in

a column. The bound plasmid DNA was washed and eluted in TE buffer (see QIAprep Handbook).

2.10 DNA sequencing and analysis

All sequencing was by an automated fluorescent-labelled system (ABI Prism 377), a service kindly provided by Dr Margaret Hughes (LSTM, Liverpool). Nucleotide sequence was analysed using Gene Jockey™ and DNAstar™ software and homology checked using the internet-based BLAST database (NIH, Bethesda).

2.11 Primer design and manufacture

All primers were designed by eye from previously derived nucleotide sequence and manufactured by GibcoBRL. The standard M13 vector primers were also purchased from GibcoBRL.

Designed primers:

pnucI:	TGTGCTCGTCCAACCTCA
pnucII:	GTCTTTCAGCTGTGACGAGT
pnuc2a:	CAGCGACTCGACGATAACTT
pnuc2b:	CCAAGATGCTACTGCACG
lm5':	TATTGCAAGCCTCAGC
pcr3'II:	GTCAAGCAGATCTCATGGCGTACACTGCTG
licnt5':	GACGACGACAAGATGGCTCGAGCTCGTTTCCTTCAGCTT
licnt3':	GGAACAAGACCCGTGTTTTACAGGGATACCGGCTC

2.12 Preparation of nested deletions using Erase-A-Base®

The Erase-a-Base® System (Promega) uses exonuclease III (Exo III) digestion of plasmid insert from a protruding 5' restriction site to prepare a series of unidirectional deletions that may be nested, so facilitating sequencing of large cloned inserts. 5' overhangs generated by a number of restriction enzymes are suitable for Exo III digestion (see Technical Manual) and likewise, a number of enzymes generate 3' overhangs resistant to Exo III digestion. Exo III also digests insert from nicks in the double strand so supercoiled plasmid was exclusively used in the process. Supercoiled plasmid was selectively isolated from solutions of recombinant plasmid by acid-phenol extraction (see Technical Manual). Supercoiled nuc1 clone was digested with *Kpn*I and *Bam*HI in preparation for exonuclease digestion.

Controlled digestion with Exo III proceeded at 30°C and aliquots of the reaction stopped periodically (approximately every 40 seconds) by removal into a solution of S1 nuclease, which removes the remaining single strands. S1 was inactivated at 70°C and the linear deletions re-ligated and used for the transformation of JM109 competent cells. Plasmid DNA from a selection of positive recombinants was prepared and analysed on an agarose gel to assess the degree of digestion of insert in each and hence allow the choosing of a number of suitably nested deletions for sequencing.

2.13 Polymerase chain reaction (PCR)

A standard polymerase chain reaction (PCR) method was used involving a cycle of 1 minute at 94°C, 1 minute at 55°C and 3 minutes at 72°C. This cycle was repeated 25 times. A denaturation step (5 minutes at 94°C) was included at the beginning of the reaction and a prolonged elongation step (7 minutes at 72°C) included at the end. The

method was modified for the amplification of phage DNA (72°C for 6 minutes instead of 3 minutes each cycle) and for the amplification of the 5' end (1 minute at 40°C instead of 55°C). A Perkin Elmer GeneAmp 2400 thermocycler was used for the reactions and Taq DNA polymerase, polymerase reaction buffer (with 15mM $MgCl_2$) and pre-mixed dNTP's (containing 10mM of each deoxynucleotide) were supplied by Boehringer Mannheim. Preparation of DIG-labelled probes by PCR followed the same method but replacing the dNTP's with DIG DNA Labelling Mix, containing a similar mixture of dNTP's but including DIG-dUTP (also supplied by Boehringer Mannheim).

2.14 TA cloning

Products of PCR may be directly cloned using the overhanging single adenosine residues added to the 3' end of each strand by *Taq* polymerase. Linearised plasmid bearing corresponding thymidine overhangs (pCR™2.1 vector) were supplied by Invitrogen (TA Cloning® System) and a standard ligation and transformation procedure followed using INVαF' One Shot™ competent cells provided with the kit (see TA Cloning Manual).

2.15 Preparation of bacteriophage DNA

The genomic library in phage DNA (Lambda ZAP, Promega) was expanded in LE392 strain overnight according to standard methods (Sambrook *et al*, 1989) and plated out in L-broth top agar. The plaques were allowed to grow overnight at 37°C and the plates chilled at 4°C before a colony lift was performed. Recombinant phage DNA was isolated from plaques according to standard methods.

2.16 Pulsed field gel electrophoresis (PFGE)

L. mexicana promastigotes were washed in TSE buffer (20mM Tris-HCl, 100mM NaCl, 50mM EDTA: pH=8.0) and set in plugs of low gelling temperature agarose (1.6% SeaPlaque agarose in TSE) at a density of 1×10^8/200µl. The immobilised cells were lysed *in situ* by incubating the plugs in 1% N-Lauroyl Sarcosine supplemented with 0.5M EDTA and washed in TE buffer. Samples of these plugs containing approximately 2×10^7 cells each were loaded onto a 1% agarose gel (pulsed field grade agarose, BioRad) in TBE and run in TBE for 60 hours at 135V with a switching time of 250. After running, the gel was stained with ethidium bromide and the separated chromosomes transferred to nylon and probed as for a standard Southern blot.

2.17 Culture of different parasite forms

L. mexicana amastigotes were isolated from lesions maintained in female CBA mice according to published methods (Bates *et al.*, 1992). Isolated amastigotes were maintained in axenic culture in Schneider's *Drosophila* medium (Gibco) supplemented with 20% foetal calf serum (FCS) and gentamicin sulphate (25µg/ml) at 32°C (Bates *et al.*, 1992). Optimal pH for the culture of amastigotes was 5.4. Promastigotes were obtained by the transformation of lesion amastigotes in M199 supplemented with 10% FCS and gentamicin sulphate (25µg/ml) at 26°C and pH=7.5. A population of metacyclic promastigotes was obtained by leaving promastigote culture sealed at 26°C to allow acidification of the medium.

2.18 Isolation of total RNA using TRIZOL® reagent

Total RNA was extracted from *Leishmania* cells in TRIZOL® (GibcoBRL), a phenolic solution of guanidine isothiocyanate. Cell pellets were lysed in TRIZOL (inactivating

RNases and dissolving cell components) and total RNA partitioned into an aqueous phase with chloroform (see supplier's instructions). Nucleic acid was precipitated from the aqueous phase with isopropyl alcohol, air-dried and re-dissolved in diethyl-pyrocarbonate (DEPC)-treated water (0.01% v/v). Yield of total RNA was estimated by spectrophotometry at 260nm.

2.19 Northern transfer

Samples of RNA were separated on agarose gels with glyoxal according to standard methods (Sambrook *et al*, 1989). Glyoxal was de-ionised using ion exchange resin (AG® 501-X8 Resin, BioRad). Samples of RNA were incubated with glyoxal (15% v/v) and phosphate buffer (10mM NaH_2PO_4/Na_2HPO_4: pH=7.0) at 50°C for 50 minutes before loading on agarose. The RNA samples underwent electrophoresis on 1.2% gels which were made and run in the same 10mM phosphate buffer. The glyoxal samples were loaded with bromophenol loading buffer and ethidium bromide (0.6% (v/v) of a 10mg/ml solution). Agarose gels were run at 110 volts with constant running buffer re-circulation using magnetic fleas. All solutions and apparatus used were treated with DEPC.

Following electrophoresis, the gels were photographed under UV light and the RNA transferred directly to a nylon membrane in 20× SSC (without prior denaturation) as for Southern transfer. Hybridisation with DIG-labelled DNA probes proceeded as for Southern blots.

2.20 Transfection of *Leishmania* promastigotes by electroporation

Plasmids used for transfection were sterilised by ethanol precipitation and linearised before introduction into *Leishmania* cells by electroporation. Late log phase promastigotes were washed and resuspended in electroporation buffer (21mM HEPES, 137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 6mM glucose, pH=7.5) at a concentration of 1×10^8/ml and chilled on ice (LeBowitz, 1994). Aliquots of 0.4ml of cells were transfected in sterile electroporation cuvettes (BioRad) using 50μg prepared DNA. Electroporation conditions were a single pulse (using a BioRad gene pulser) of 2.25kV/cm at a capacitance of 500μF. The cuvette was then incubated on ice for 10 minutes before transfer into fresh M199 medium. The transfected cells were grown in culture overnight to allow expression of the introduced genes and then plated on selective media plates. The plates for selection of positive transformants were prepared according to the method summarised by LeBowitz (1994) and consisted of a final concentration of 1% noble agar in M199 cell medium with hygromycin selective drug added (fc=50μg/ml). The plates were dried and equilibrated with CO_2 just prior to use (in an incubator containing 5% CO_2).

2.21 Western blots

Protein samples separated by SDS-PAGE were transferred to nitrocellulose by a 'wet' Western blot according to standard methods (Sambrook *et al.*, 1989). Following SDS-PAGE, the acrylamide gel was transferred to a sandwich stack soaked in transfer buffer (6g Tris base, 28.8g glycine, 400ml methanol in 2 litres dH_2O). The stack consisted of the gel laid on a nitrocellulose membrane slightly larger than it and sandwiched by two sheets of Whatman paper. This was sandwiched in the transfer plate of the wet transfer apparatus and run for 1 hour at 250mA. Successful transfer was confirmed by the use of

prestained protein markers (BioRad). Also used in SDS-PAGE were biotinylated markers, which were co-developed with the blot and enabled easy estimation of the sizes of identified proteins.

The blot was developed using the anti-rabbit Vectastain® ABC system (Vector Laboratories, USA) which uses an avidin/biotin complex conjugated with peroxidase. This complex binds to a biotinylated secondary antibody previously bound to the primary antibody recognising proteins on the Western blot. The blot was initially blocked with normal goat serum overnight. All antibody incubations and washes were performed in Tris buffered saline with Tween-20 (TTBS: 100mM Tris, 0.9% NaCl (w/v), 0.1% Tween-20 (v/v)). The presence of the peroxidase conjugate was demonstrated by a 4-chloro-1-napthol colourimetric substrate reaction.

2.22 Immunoprecipitation of 3'-nt activity

Polyclonal rabbit antiserum raised against recombinant *L. donovani* 3'-nt was used to immunoprecipitate 3'-nt activity from promastigote cell lysates according to a method adapted from Bates & Dwyer (1987). Washed promastigotes (wash buffer: 10mM HEPES, 145mM NaCl, pH=7) were lysed in a buffer containing non-denaturing detergent to preserve enzyme activity (38mM Tris, 100mM glycine, 1% Triton-X 100 (v/v), pH=8.5. Leupeptin added (fc 25µg/ml) just before use). Samples of lysate equivalent to 1×10^7 cells were incubated with 5µl of antiserum overnight at 4°C in a total volume of 100µl solubilisation buffer (38mM Tris, 100mM glycine, 0.1% Triton-X 100 (v/v), 25µg/ml leupeptin, pH=8.5). All non-solubilised material was removed from the lysate mix by centrifugation and the immune complex supernatant incubated with 100µl sepharose 4B beads (equilibrated in solubilisation buffer) for 1 hour at 4°C. The beads

were pelleted and the supernatant incubated for a further hour at 4°C with protein A-sepharose beads. The beads, with immune complexes bound, were pelleted (the residual lysate supernatant was kept), washed several times and finally resuspended in solubilisation buffer. Samples of the sepharose beads and the residual supernatant were tested for 3'-nt activity by tube assay.

2.23 Nucleotidase assay

3'-nucleotidase and 5'-nucleotidase activities were assayed using the staining of free inorganic phosphate by malachite green (Gottlieb & Dwyer, 1983). Cell samples were lysed in Tris/maleate/saline (TMS: 50mM Trizma-maleate, 145mM NaCl, pH=7.0) with Triton X-100 (0.2% v/v). Assays (of cell lysates as well as immunoprecipitated samples) were performed in 250µl TMS supplemented with $CoCl_2$ (1mM) and 3'-AMP (fc 2.5mM) or 5'-AMP (fc 5mM). Nucleotidase reaction conditions for 3'-nt in *L. mexicana* were optimised at 42°C for 40 minutes. The reaction was stopped by adding 250µl 2M HCl. Levels of released phosphate were assessed by the addition of 500µl malachite green reagent (3 parts 0.045% malachite green oxalate in dH_2O to 1 part 4.2% ammonium molybdate in 4M HCl. Allow to stand for 15 minutes and then add 0.05% Tween-20 (v/v)). The chromogenic product was measured at 630nm. A simultaneous calibration curve was plotted for each batch of malachite green reagent made using a dilution series of potassium phosphate.

2.24 Expression of recombinant 3'-nt

The system used for expression of recombinant 3'-nt was the Affinity™ LIC Cloning and Protein Purification kit (Stratagene). This used 'ligation independent cloning' to clone the desired gene into pCAL-n-EK expression plasmid in frame with a calmodulin

binding protein (CBP) gene. The eventual expression product would be a fusion protein with CBP that could subsequently be purified on a calmodulin affinity resin.

Initially, primers were designed to amplify the entire gene (licnt5' & licnt3'). Incorporated into these primers were 12 nucleotide lengths of sequence which, when digested with *Pfu* polymerase gave 5' overhangs complementary to the single strand overhangs of the expression vector pCAL-n-EK (see Instruction Manual). Annealing of the *Pfu*-treated PCR product to pCAL ensured the gene was in frame with CBP and immediately 3' to an enterokinase site in the plasmid which could be used to split CBP from the final expressed protein. To check successful cloning of the gene, the recombinant plasmids were first used to transform XL1-Blue competent cells, a standard strain of *E. coli*. Methods for PCR, purification of product from agarose and transformation were as described previously. The PCR products were annealed to the plasmid without ligase.

Positive recombinants were identified by ampicillin resistance and recombinant plasmid DNA prepared from these colonies according to methods already described. 0.1ng of purified plasmid was used to transform the expression strain of competent *E. coli* cells, BL21(DE3)pLysS as described in the Instruction Manual. Positive recombinants were selected for by ampicillin resistance as before.

A single positive colony was used to innoculate LB broth (supplemented with 50μg/ml ampicillin and 34μg/ml chloramphenicol) and grown in culture to an optical density of 0.6-1.0. At this point, a sample of cell culture was removed and frozen as a pre-induction control before IPTG (fc 1mM) was added to the remaining culture. The cells

were grown for a further 4 hours and then harvested by centrifugation (7,000×g for 15 minutes at 4°C). Soluble protein was prepared from the cells by lysozyme treatment and sonication before removing cell debris by centrifugation (14,000×g for 10 minutes at 4°C. The supernatant was stored at -80°C.

CBP fusion protein was purified from other bacterial proteins using calmodulin affinity resin. This was equilibrated in $CaCl_2$ binding buffer (50mM Tris-HCl, 150mM NaCl, 10mM β-mercaptoethanol, 1mM magnesium acetate, 1mM imidazole, 2mM $CaCl_2$: pH=8.0). In order to establish optimal binding and eluting conditions for the fusion protein, 50µl aliquots of resin were initially incubated with bacterial lysate in a total volume of 300µl binding buffer (4°C for 2 hours with constant rotation). The resin was pelleted by centrifugation and washed 4 times with binding buffer.

Protein was eluted from the resin by eight washes with 200µl elution buffer (50mM Tris-HCl, 10mM β-mercaptoethanol, 2mM EGTA: pH=8.0). The majority was eluted in the first two washes. Various conditions of binding and eluting involving increased salt concentrations and the addition of triton X-100 (see Instruction Manual) were tried in an attempt to obtain pure fusion protein but had no apparent impact. The method described above was finally used for a scaled-up preparation of the expressed 3'-nt/CBP protein from a calmodulin affinity resin column. All binding, washing and eluting steps were by gravity flow through the column.

The CBP tag could be removed from the purified expression protein by enterokinase digestion. The protein was diluted into cleavage buffer (50mM Tris-HCl, 50mM NaCl, 2mM$CaCl_2$, 0.1% Tween-20: pH=8.0) and 1 unit of enterokinase added per 100µg

protein to be digested. Digestion proceeded for 12 hours and the results analysed by SDS-PAGE.

2.25 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

10% polyacrylamide gels (using Acrylogel 2.6 acrylamide/bisacrylamide solution, BDH) were prepared according to manufacturer's instructions. Protein samples were boiled in reducing loading buffer (containing DTT) before loading, unless otherwise stated. Samples were run at 100V alongside standard low molecular weight markers (BioRad). Following electrophoresis, separated proteins were stained with coomassie blue according to standard procedures (Sambrook *et al.*, 1989).

2.26 Nuclease and nucleotidase substrate gels

Samples of cell lysate were run on SDS-PAGE and the separated protein bands renatured and assayed for nuclease and nucleotidase activities *in situ* (method as in Bates, 1993b). For nuclease activity, the polyA substrate was included in the acrylamide gel (at a concentration of 0.3mg/ml). For nucleotidase activity, the 3'-AMP substrate was included in the renaturation buffer. Unless otherwise stated, samples were run in loading buffer without reducing agents and without boiling. After electrophoresis, acrylamide gels were washed (4×15 minutes) in renaturation buffer (100mM HEPES, 0.1% Triton X-100, pH=8.5) and then incubated in the same buffer for 2 hours with regular agitation at 37°C. 3'-AMP (2.5mM) was included in the renaturation buffer used for 3'-nt activity substrate gels. After incubation, the nuclease gels were fixed in 7.5% acetic acid, washed in dH_2O (3× 10 minutes) and residual polyA in the gel stained with toluidene blue. The gels were destained in dH_2O overnight. The presence of nuclease activity was indicated by clear areas (where polyA had been digested) in an otherwise blue gel. After

incubation of the nucleotidase gels, these were briefly washed in dH_2O and stained in malachite green reagent (as above) without prior fixation. The malachite green reaction was stopped by transferring the gels to dH_2O. The presence of nucleotidase activity was indicated by areas of dark green precipitate (where phosphate had been released by nucleotidase digestion of 3'-AMP) in an otherwise yellowish gel.

2.27 Protein assay

Protein concentrations in cell lysates were measured spectrophotometrically using the Pierce Micro BCA Protein Assay Reagent (Pierce, USA). The bicinchoninic acid (BCA) reagent complexes with Cu^{1+} ions (generated from Cu^{2+} in the presence of protein) to form a colourimetric product that is measured at 530nm (see Instruction booklet). Calibration curves were prepared using bovine serum albumin.

2.28 Preparation of crude membrane extracts

Frozen pellets of *L. mexicana* promastigotes were lysed in TMS (see 2.23) by repeated freeze/thaw in an ethanol ice bath. Soluble components of the lysate were separated from membranes and other cellular debris by centrifugation (14,000×g for 30 minutes). Both supernatant and the subsequently washed pellet (three washes by resuspension in TMS) were analysed for nuclease and 3'-nt activity.

2.29 Growth of *L. mexicana* on defined medium

Normal promastigote cultures were adapted to chemically defined medium by a gradual process of diluting out FCS. M199 cultures (10% FCS) were mixed with an equal volume of supplemented RPMI 1640 (RPMI defined). The base medium (RPMI 1640 with HEPES and glutamine (Gibco catalogue number 52400-025)) was supplemented

with gentamicin sulphate (25μg/ml), folate (10μg/ml in 1M NaOH), BME vitamins, hemin (5μg/ml in 50% quadrol) and adenosine (25mM). In this way, the serum content of the medium was halved at each passage until the parasites were adapted to RPMI defined alone, without serum. Medium could then be prepared with or without adenosine. In addition, the adenosine could be replaced by another purine in the medium. In this way, cultures were adapted long term to polyA, 3'-AMP and 5'-AMP (all at 25mM).

2.30 Materials

All reagents not specifically mentioned were purchased from BDH Laboratory supplies (Poole, UK) and Sigma Chemicals (St Louis, USA).

CHAPTER 3
GENE CLONING

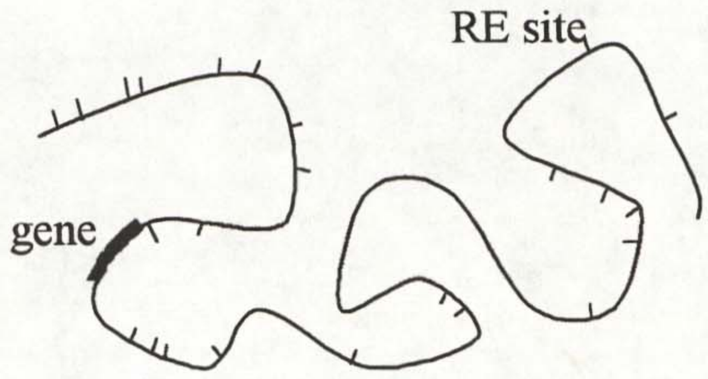
3.1 Introduction

The first step in describing and characterising the 3'-nucleotidase (3'-nt) in *L. mexicana* was to isolate and clone the gene responsible for encoding the enzyme. It was fortunate in that the first trypanosomatid 3'-nt gene had recently been isolated from *L. donovani* (Debrabant, 1995) and a homologous DNA probe to the gene sequence was available. The *L. mexicana* gene would be the second trypanosomatid 3'-nt to be isolated and so there was no information on comparative DNA sequence homology within the genus. For this reason, it was decided to attempt the isolation of the gene directly from genomic DNA rather than using a PCR-based strategy. The *L. donovani* probe would be used to pick out fragments of a suitable size for subsequent cloning from a range of different restriction digests of genomic DNA. Because of the large number of fragments generated by the average restriction digest of genomic DNA, single bands would not be resolved on an agarose gel and so a range of fragments around the size of the of the positively probed band would be purified and cloned. This mini-library of genomic fragments would then be screened with the same *L. donovani* probe until a single clone could be isolated (Fig. 3a).

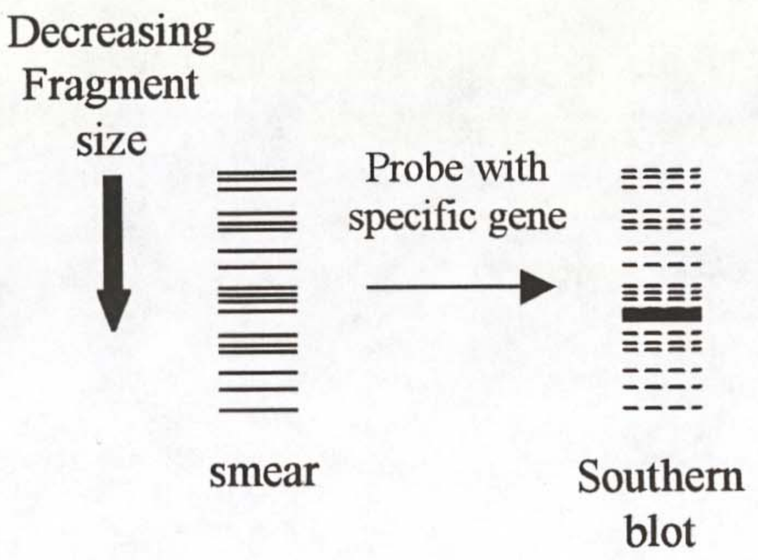
3.2 Initial Southern Blots

The form of labelling used for the DNA hybridisations in this work was the non-radioactive Digoxigenin (DIG) system. There were a number of advantages in using this system. These included speed of development, the ability to freeze and re-use labelled probes several times and the diminished experimental hazard as a result of eliminating

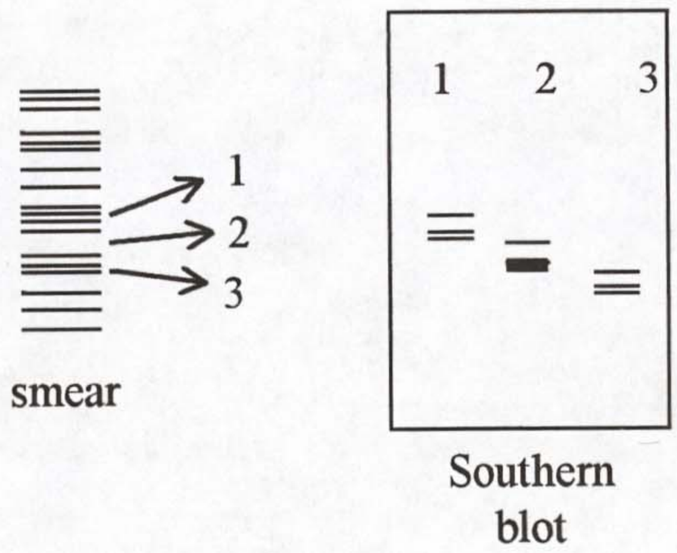
Each restriction enzyme will cut the genomic DNA at a large number of specific recognition sites (| RE site) giving a range of fragment sizes. Within one of these fragments will be the sought after gene (shaded block).



Running the restriction products on an agarose gel gives a 'smear' of different sized fragments which may be probed for the presence of the desired gene on a Southern blot



The digested DNA is then cut from several sections of gel around the site of positive probing and re-run on another Southern blot to check the presence of gene sequence



The mixture of fragments from the positive sample (in this case no. 2) is then cloned and the recombinant colonies again probed to identify recombinants bearing the gene

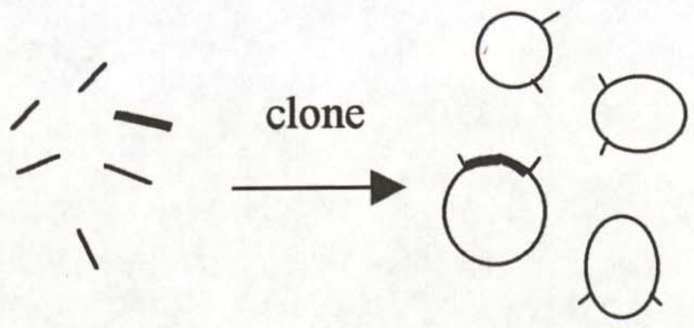


Figure 3a Schematic illustrating the chosen cloning strategy for *L. mexicana* 3'-nt.

radioisotopes from the procedures. A plasmid containing a 950bp fragment of the *L. donovani* 3'-nt gene sequence was kindly provided by Dr Alain Debrabant (NIH, Bethesda) and this was used as a template for Klenow enzyme to produce a probe, labelled by random primed incorporation of DIG-labelled dUTP's. The extent of incorporation of the labelled nucleotides into the new probe was checked according to the standards provided with the DIG system and the resultant probe named Ld950.

Genomic DNA was prepared from *L. mexicana* cultured promastigotes as described and digested with a variety of restriction enzymes overnight. The resultant restriction fragments were separated on an agarose gel and transferred to a nylon membrane for subsequent hybridisation with the prepared Ld950 probe (Southern hybridisation). A DIG-labelled DNA marker was run alongside the restriction fragments on agarose and, following hybridisation and development of the probe, this ladder also showed up on the blot, enabling easy estimation of the size of positively hybridising bands in the genomic digests. Taking the published *L. donovani* gene as a guide, the ideal restriction digest would yield a single positively probing fragment large enough to incorporate at least 1.5kb of gene sequence.

Amidst the first round of restriction enzymes used, only *Nco*I yielded a single positively probing fragment of suitable size. Many enzymes either yielded two fragments, showing the presence of a recognition site within the target gene sequence, or positively probing fragments that were too large to efficiently clone into a plasmid. The cloning vector used (pBluescript™ (pBs)) had no *Nco*I site in its multiple cloning site and attempts to blunt end the genomic fragments and clone them into a blunt-cut plasmid were unsuccessful. A second round of restriction enzymes compatible with the pBs multiple cloning site were

tried. Out of these, only *Sac*I yielded a suitably sized fragment of about 3kb (Fig. 3b) and this enzyme was subsequently chosen for the cloning procedure.

3.3 Extraction of genomic DNA from agarose gel

A scaled up digest of *L. mexicana* genomic DNA with *Sac*I was performed and the fragments run on an agarose gel, loaded into a single sample well spanning the gel's width. The same DIG-labelled marker was run alongside the digest to enable the excision of DNA fragments from the agarose around the site of positive hybridisation shown in Figure 3b without a further Southern blot. Size estimations from the ethidium bromide stained gel enabled the excision of three adjacent agarose slices across the width of the loading lane, containing the genomic fragments in the vicinity of the 3kb marker band. Each slice was about 1mm thick and cut into smaller sections with a scalpel blade to aid DNA extraction. The Prep-A-Gene® system was used to purify the separated DNA fragments from each gel slice.

A small aliquot of each purified sample was run on a further agarose gel (Fig. 3c) and another Southern hybridisation performed using the same Ld950 probe to check which gel slice contained the positively probing fragment. This further Southern blot was performed in an attempt to limit the number of irrelevant, but similarly sized fragments that would show as recombinants in the cloning experiment. The blot revealed that some of the probing sequence was found in each of the gel slices, demonstrating the limits of 0.8% agarose as an accurate size-delineating substrate (Fig. 3d). The majority of the hybridisation signal, however, appeared to be in the second slice taken and this sample was used for subsequent cloning.

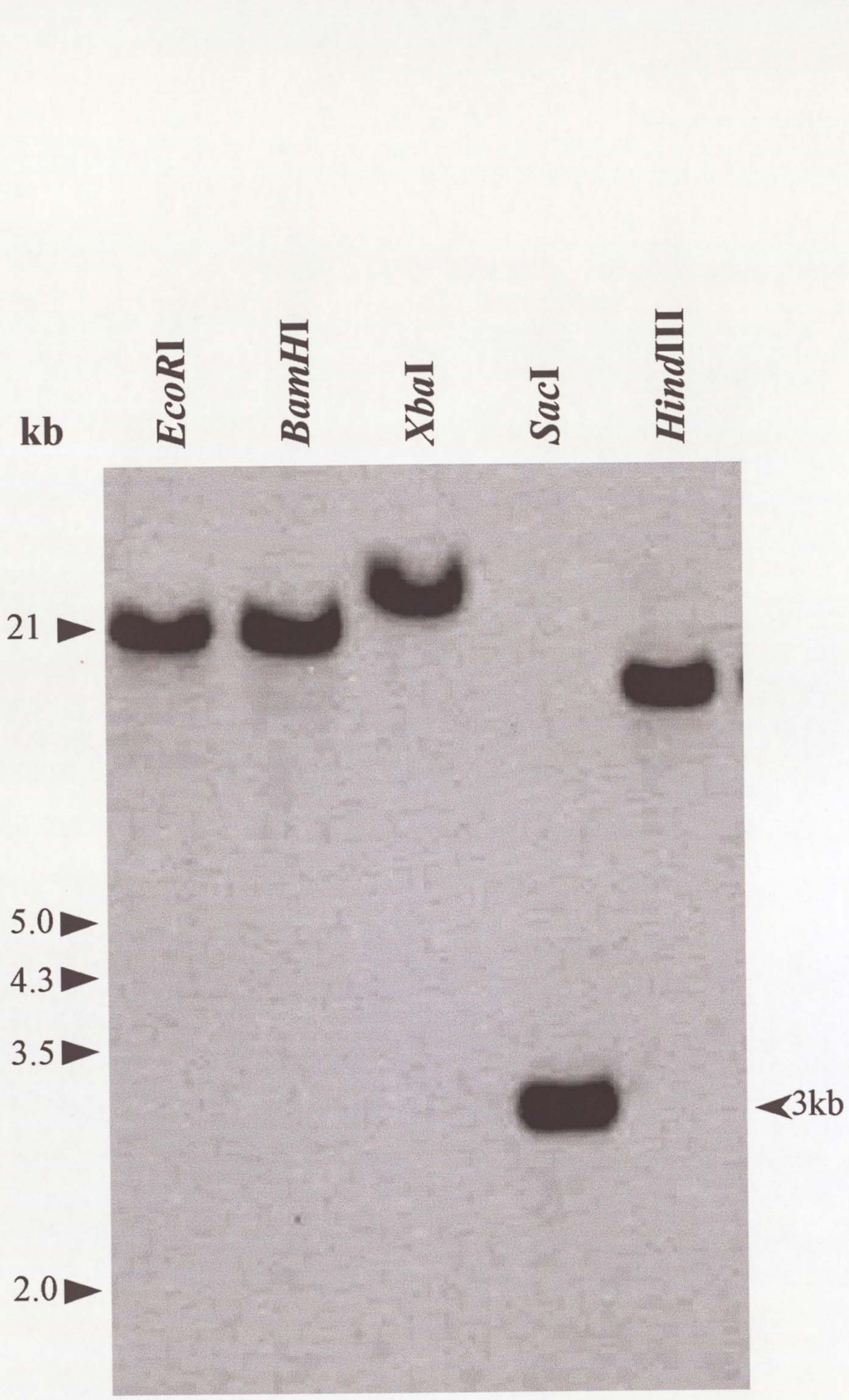


Figure 3b Southern hybridisation of *L. mexicana* genomic DNA. Total genomic DNA was digested with a variety of restriction enzymes and probed with the DIG-labelled Ld950 probe. DNA molecular mass markers are shown on the left and the approximate mass of the *Sac*I fragment shown on the right (all in kb). The labelled filter was developed using the chemiluminescent substrate CSPD (as described) and exposed to X-ray film for 20 minutes.

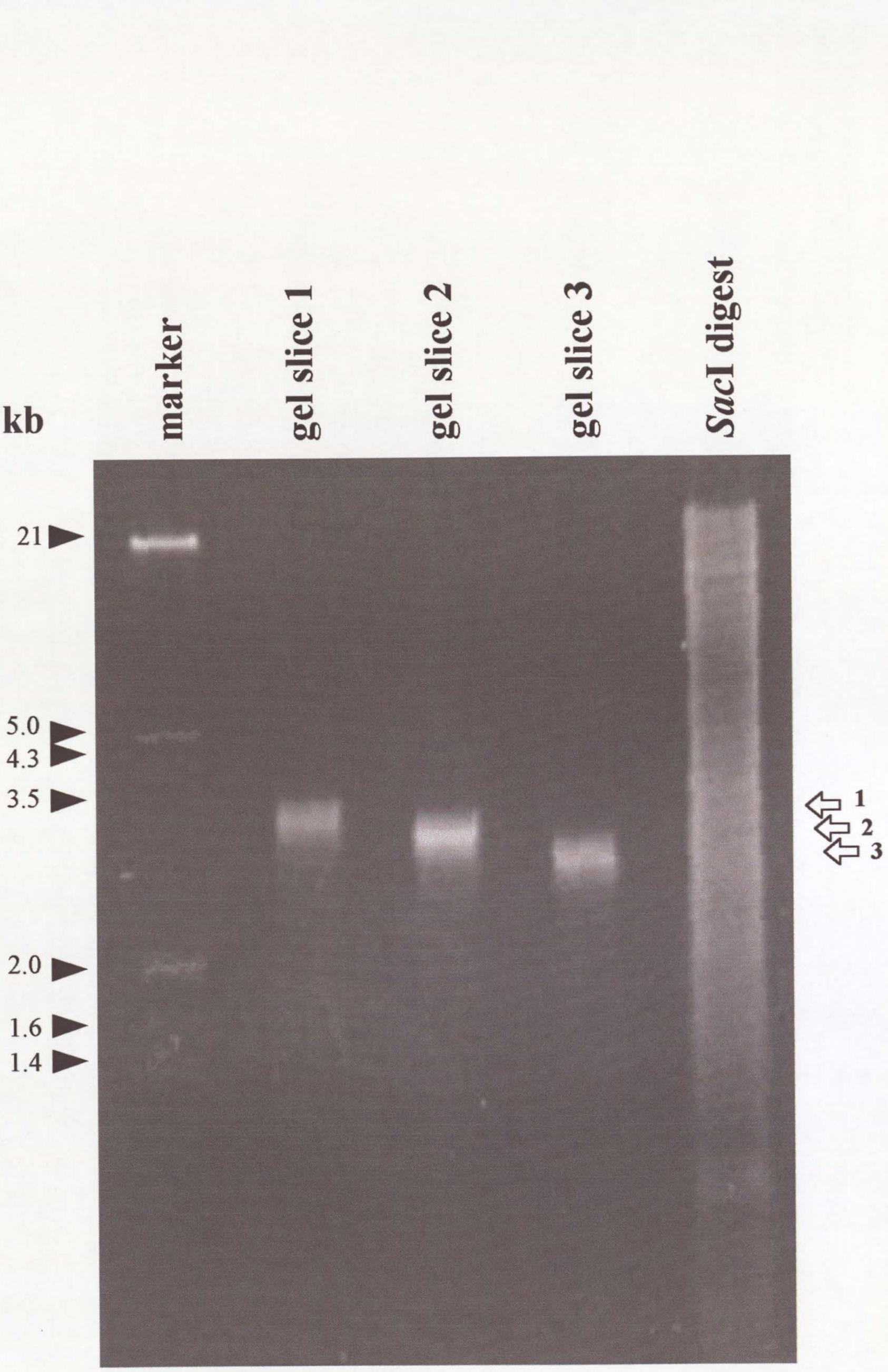


Figure 3c DNA samples extracted from three different slices (1-3) of a *Sac*I digest of genomic DNA. Adjacent agarose slices between the approximate sizes of 2.8 and 3.5kb (shown by ⇐) were taken from an electrophoresed sample of a *Sac*I digest similar to that shown (lane 5). The DNA was extracted from each slice and run on the agarose gel shown (lanes 2-4). The marker used was DIG-labelled and the mass of each fragment shown on the left in kb. Each lane consisted, therefore, of similarly sized DNA fragments bearing *Sac*I digested ends.

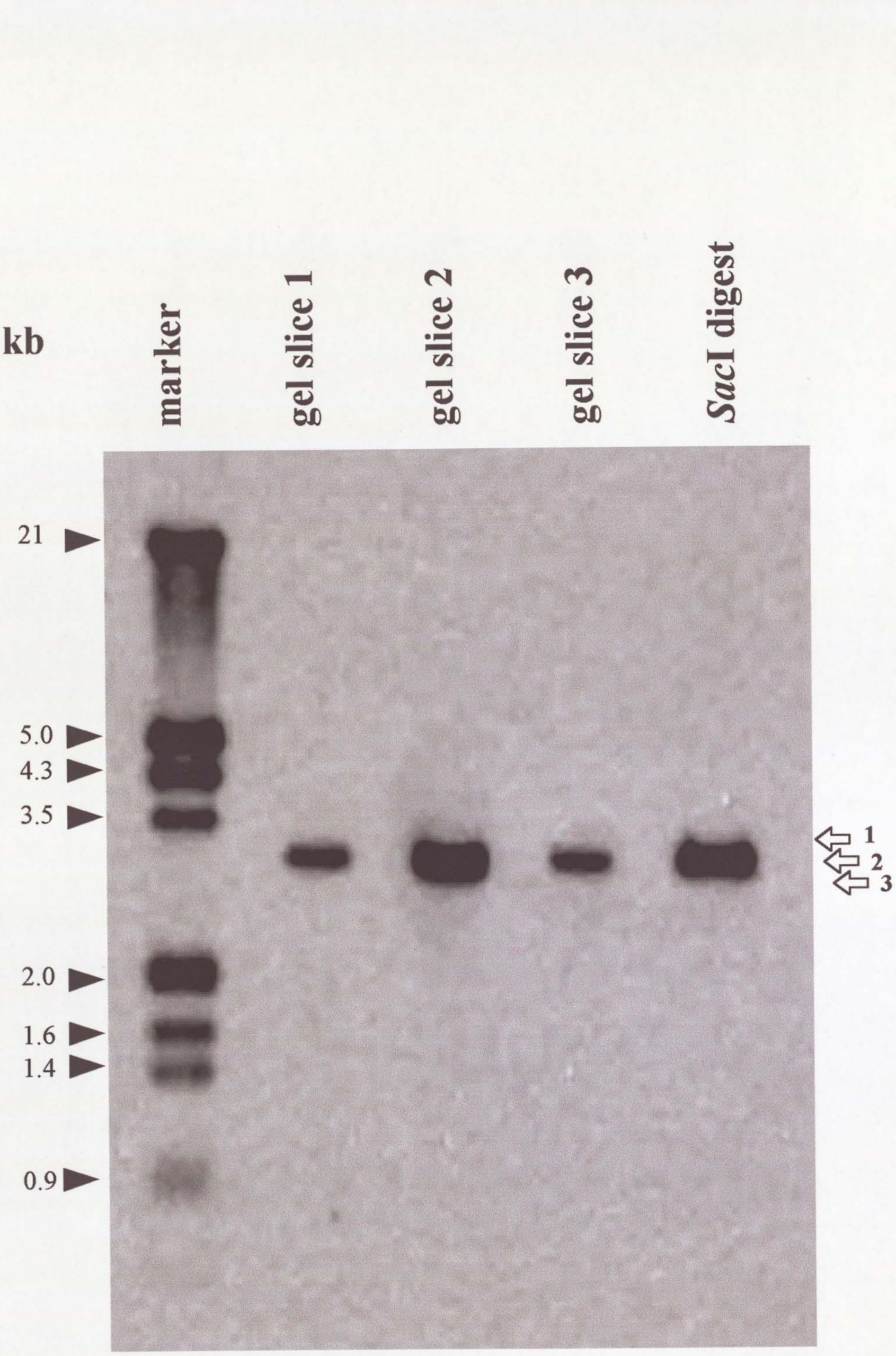


Figure 3d Southern hybridisation of Figure 3c using the DIG-labelled Ld950 probe. Following CSPD development, the blot was exposed to X-ray film for 30 minutes. The majority of positively hybridising sequence appears to be in the second gel slice taken. This is seen to be the same size (about 3kb) as the original fragment detected in the *Sac*I genomic digest (lane 5). DNA from this sample was used for subsequent cloning.

3.4 Screening Putative Positives

The purified DNA fragments from sample 2 were ligated into *Sac*I-digested pBluescript (pBs) plasmid vector and the resulting recombinants used to transform DH5α library-competent cells. The positive transformants were selected on ampicillin plates and using standard blue-white selection. The positively selected bacterial colonies effectively represented a mini genomic library of all the fragments (of about 3kb) generated when *L. mexicana* genomic DNA is digested by *Sac*I. Within this library should be a genomic fragment bearing the sequence picked up by the Ld950 probe. The frequency of this sequence was not known and so the proportion of positively probing colonies expected following plating out of the mini library was uncertain.

Initially, sixty of the resulting recombinant (white) colonies were picked from the plates using a sterile toothpick and transferred to a single LB-amp master plate. After 12 hours of further growth, a colony blot was performed on this master plate and probed, as before, with Ld950. Several putative positives were picked and plasmid DNA prepared by miniprep. This plasmid DNA was run on a further agarose gel but subsequent Southern blots failed to show a positive hybridisation with Ld950. Several different master plates were prepared and the process was repeated but failed to deliver a single positive clone.

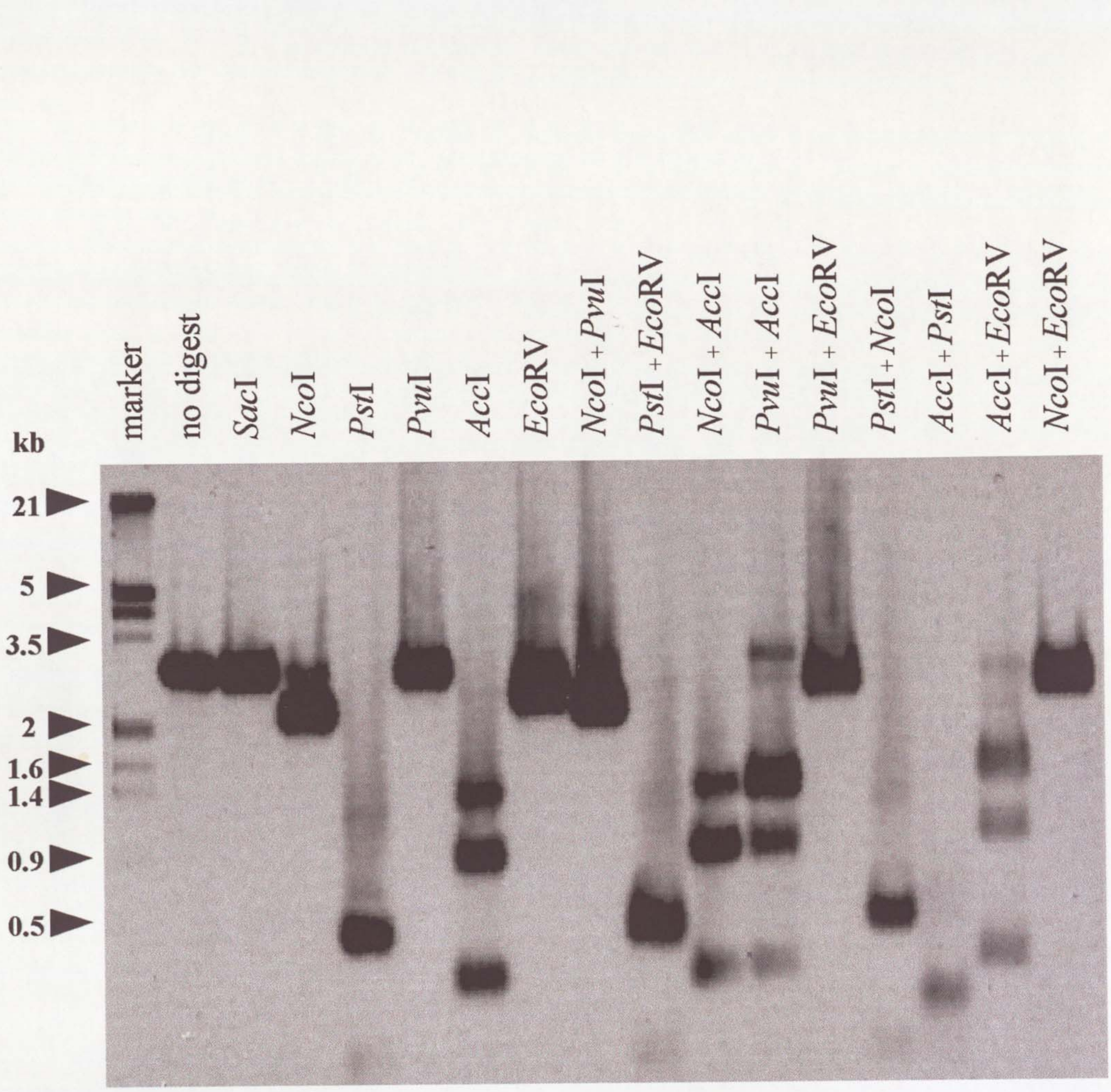
Because of the apparent problems of false positives resulting from re-plating on a master plate and the unknown frequency of the Ld950 probing sequence in the mini-library, it was decided to repeat the initial ligation and transformation, plate out the resulting library fresh and perform colony blots directly on the entire colony profile, both blue and white. This approach yielded a small number of strong positives spread across several plates. Lining up the developed autoradiogram with the original plate, smears of bacterial colonies

around each area of positive hybridisation on the film were taken with a sterile loop and the cells resuspended in small aliquots of fresh medium. These cell suspensions were then plated out separately and colony blots performed as before in a secondary screening. The secondary screen revealed an increased proportion of positively hybridising colonies and a tertiary screen in the same way enabled a single putatively positive colony to be picked.

3.5 Isolation of the Nuc1 Clone

Plasmid DNA was prepared from the single colony picked and a Southern blot (probed with Ld950) confirmed it was a recombinant bearing the genomic fragment initially picked out from the *Sac*I restriction digest of *L. mexicana* genomic DNA. This cloned plasmid was called nuc1. Restriction digest with *Sac*I, to excise the insert from the plasmid vector, yielded what appeared to be a single 3kb fragment (by electrophoresis on agarose). This was assumed, in fact, to be a doublet, the pBs cloning vector by chance being the same size as the genomic fragment inserted into it. A *Sac*I/*Pvu*I double digest was devised to distinguish the insert from the vector, using the presence of a *Pvu*I recognition site in the plasmid vector backbone to split this fragment into two, so leaving a single 3kb fragment on agarose assumed to be pure insert. In this way, it was possible to purify insert DNA (using Prep-A-Gene) and attempt to construct a restriction map of the fragment. This was done by digesting purified DNA with a range of restriction enzymes, performing a Southern blot of the resultant fragments and probing with Ld950 to assess which of them contained gene sequence (Fig. 3e).

Taking the size of each fragment showing positive hybridisation with the Ld950 probe, a rough map of the insert was constructed (Fig. 3e). Because the template was of known, finite size, it was possible to assess where each of the enzyme recognition sites fell in



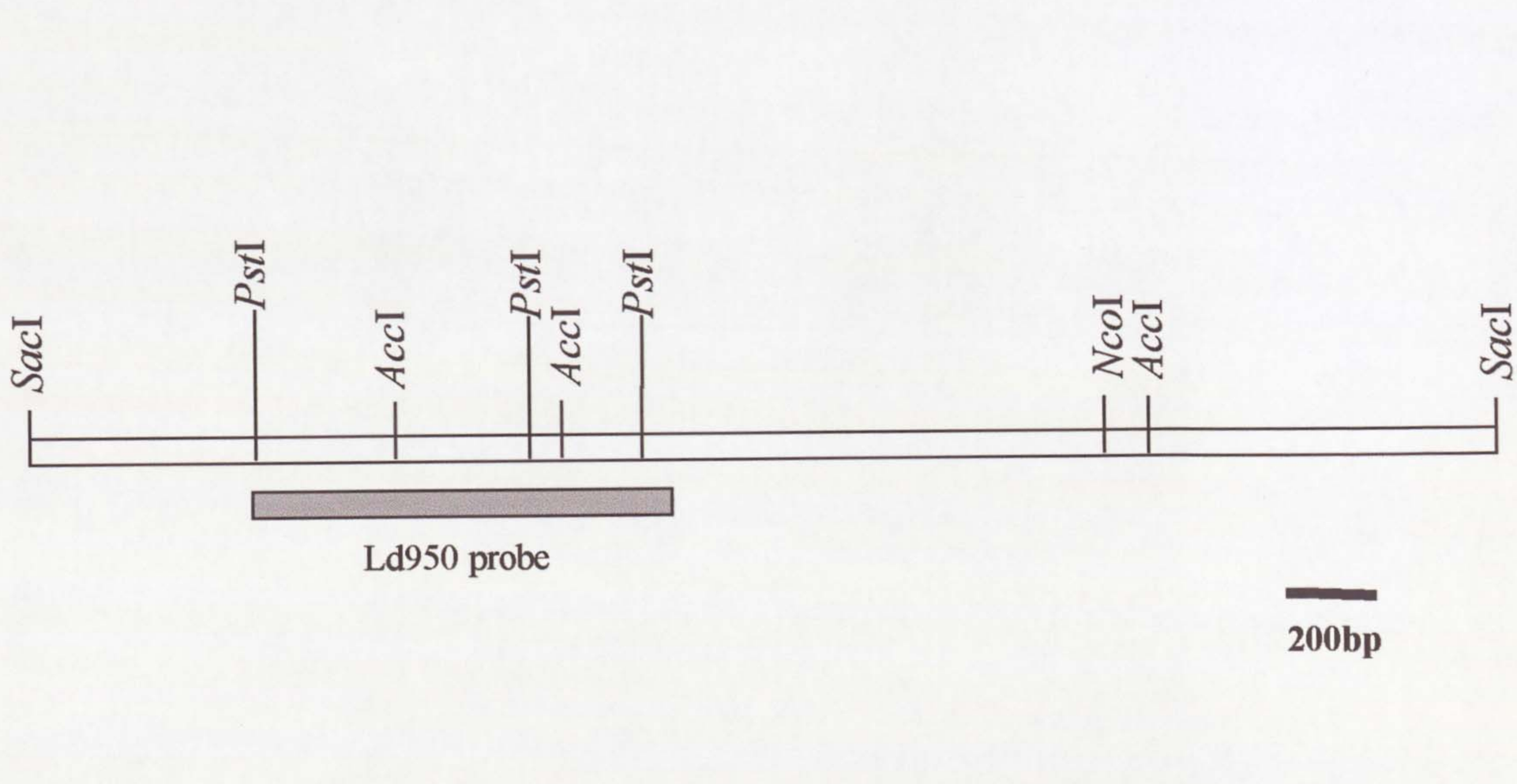


Figure 3e Southern hybridisation of the 3kb nuc1 fragment digested with a range of restriction enzymes and the resultant restriction map of the clone (schematic). The blot was developed on X-ray film for 30 minutes and the DNA molecular weight markers are shown on the left-hand side. Single and double restriction fragment sizes were estimated from the gel and used to build an approximate restriction map. The estimated position of the Ld950 probe sequence is shown as a grey block.

relation to the Ld950 probe. This map was used mainly to confirm the identity of nuc1 as containing the original genomic fragment (by comparing the positively hybridising fragment sizes of the original Southern blots of digested genomic DNA with those of the nuc1 insert), but was also useful in subsequent sub-cloning strategies.

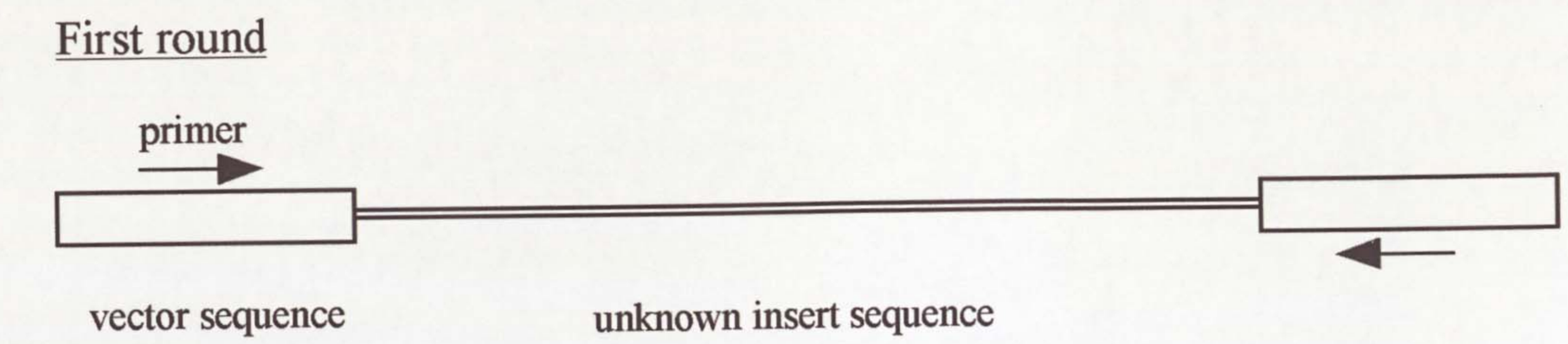
3.6 Preliminary Sequencing of Nuc1

The insert of the nuc1 plasmid was sequenced from either end, using primers complementary to the vector sequence adjacent to the cloning site. The first round of sequencing generated about 400bp from either end of the insert. This sequence showed no homology with the *L. donovani* 3'-nt gene or any clear open reading frame (ORF) that might suggest the presence of a coding sequence. From the *L. mexicana* sequence derived, further primers were designed (pnucI & pnucII) to sequence into the insert step by step (Fig. 3f). At the 5' end of the insert, the second round of sequencing still failed to show any homology or ORF. At the 3' end, the sequencing reaction repeatedly failed (under various reaction conditions) to extend beyond a particular motif of about 15 nucleotides, which was especially rich in guanosine residues. This appeared to constitute some form of secondary structure preventing the onward progression of the DNA polymerase in the sequencing reaction. It was decided to try and by-pass this region and continue upstream using other sequencing strategies.

3.7 Selective Exonuclease Treatment of Nuc1

One method of avoiding sequencing the insert by 'stepping' in from the ends is to prepare a series of clones each containing a smaller section of the full insert to be sequenced. This would by-pass any regions proving difficult to sequence through and may also identify target sequence more quickly. One way to do this is to prepare a set of nested deletions

(a)



(b)

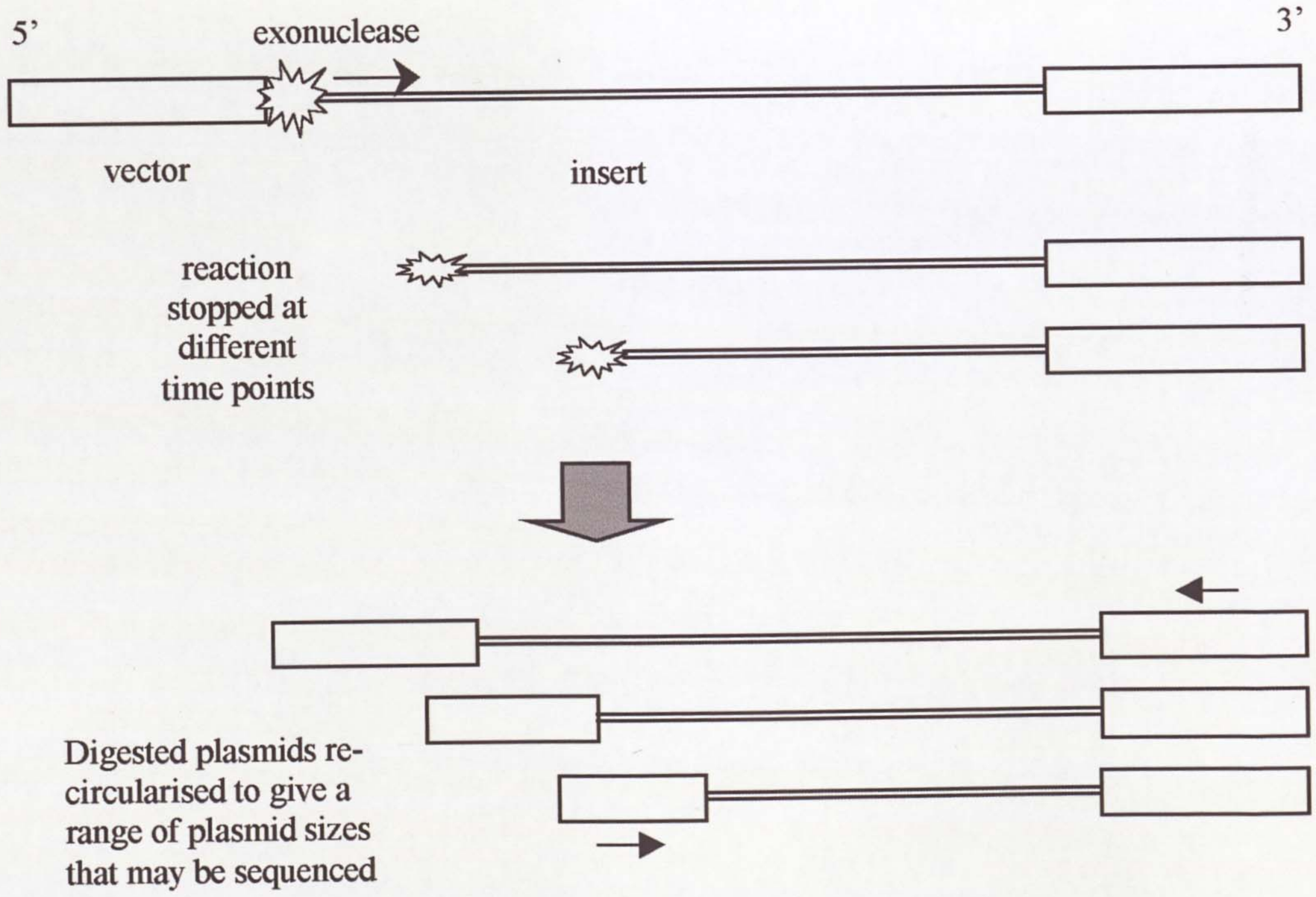


Figure 3f Schematic of initial sequencing strategies. (a) 'stepwise' sequencing from either end, designing new primers as new sequence is derived (shaded block). (b) selective exonuclease treatment over a time-course allowing internal sequence to be generated directly without 'stepping'.

using selective exonuclease treatment. The commercially available Erase-A-Base® system (Promega) was chosen. This system employs a unidirectional exonuclease digestion of a single DNA strand over a temperature-controlled time course.

Supercoiled recombinant plasmid was linearised at the 5' end of the insert sequence using *Bam*HI (giving a 5' overhang susceptible to exonuclease digestion) and the exposed 3' vector sequence digested with *Kpn*I (giving an exonuclease-resistant overhang). Exonuclease digestion of one strand of the insert sequence then proceeded from the 5' to the 3' end, the rate of digestion being controlled by the reaction temperature. The extent of digestion was controlled by removing aliquots of the reaction at regular time points, stopping their exonuclease digestion and digesting the trailing single strand with S1 nuclease. The linear fragments resulting from digestion were re-circularised into intact plasmids, representing a set of nested deletions. In each of these plasmids, the 3' insert sequence would be identical but the 5' sequence would represent a series of steps downstream from the original insert's 5' end (Fig. 3f).

Repeatedly poor results led to a dissection of the various steps in the methodology and it was discovered that the S1 nuclease solution, provided to remove the trailing single strands left by exonuclease digestion, was faulty, resulting in over-digestion of the insert sequence. The company supplying the system had received similar complaints from a number of researchers concerning that particular batch of the kit and, because of time, this approach was abandoned.

3.8 Sub-cloning restriction fragments of nuc1

With attempts to sequence the recombinant plasmid being so far unsuccessful, it was still not certain whether nuc1 actually contained the 3'-nt gene or just genomic sequence that cross-hybridised with the Ld950 probe. A priority was to show some homology with the *L. donovani* gene before sequencing the entire nuc1 insert. It was decided the most efficient way to do this would be to sub-clone one of the restriction fragments that showed hybridisation to Ld950 in previous Southern blots. The restriction map results already presented (Fig. 3e) show a number of *Acc*I recognition sites within the proposed gene sequence yielding restriction products of a suitable size for cloning. This enzyme is commonly used in cloning strategies and therefore included in the multiple cloning sites of most commercially available plasmid vectors. For these reasons, *Acc*I was chosen.

A gel-purified sample of the 3kb nuc1 insert was digested with *Acc*I and the products run on an agarose gel. The three fragments previously shown to hybridise to Ld950 (1.2kb, 0.9kb and 0.4kb) were purified from the gel (using Prep-A-Gene) and ligated into separate pBs plasmids digested with *Acc*I. Competent cells were transformed by the recombinant plasmids as before and plated on LB-amp agar. Subsequent colony blots of the positive transformants were probed with Ld950 and a number of positively hybridising colonies picked from those plates transformed by the smallest (0.4kb) *Acc*I fragment. Plasmid DNA was prepared by miniprep and subsequent *Acc*I digestion showed the presence of a 400bp insert to which Ld950 strongly hybridised in a Southern blot (Fig. 3g). One of these recombinant plasmids was chosen to be sequenced and called nuc2.

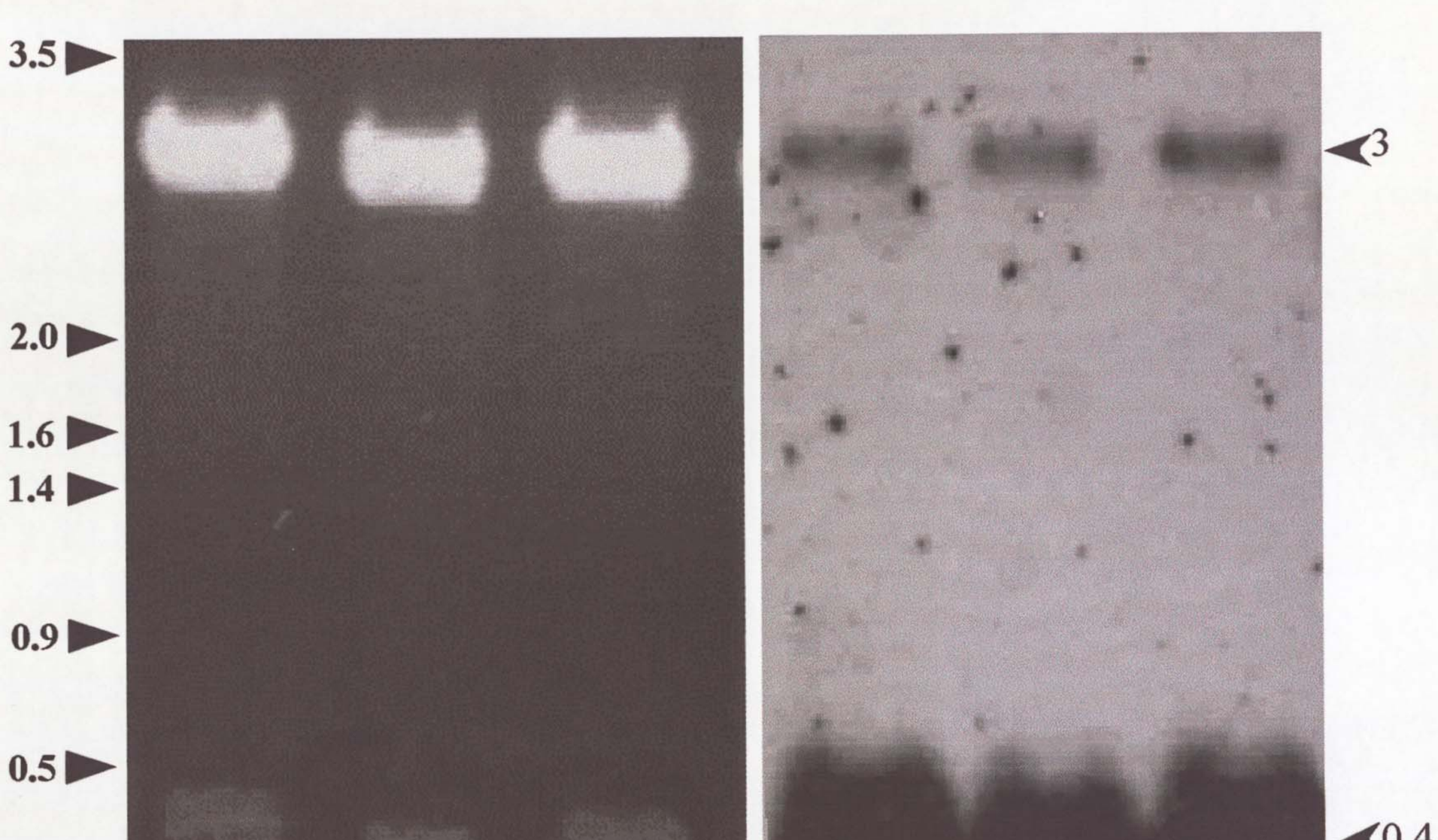


Figure 3g *Acc*I restriction digest of putative positive plasmids and the corresponding Southern hybridisation (probed with Ld950). The restriction digests show the excision of a 400bp fragment from each recombinant (left-hand panel) and these are strongly recognised by the Ld950 probe, compared with the 3kb plasmid vector DNA (right-hand panel). X-ray film exposure was for 60 minutes. DNA molecular weight markers are shown on the left and approximate fragment sizes on the right (in kb).

3.9 Nuc2 Sequence and Location in the Nuc1 Clone

The full insert was sequenced in both directions to reveal greater than 90% homology with the published *L. donovani* 3'-nt gene and corresponded to *L. donovani* sequence located about halfway through the gene. To estimate the location of nuc2 within the *L. mexicana* nuc1 clone, primers complementary to the reverse strand of the 5' end and the sense strand of the 3' end of nuc2 (pnuc2a and pnuc2b respectively) were designed. These were used in combination with flanking vector primers to PCR the insert sequence between nuc2 and the ends of nuc1 (see Fig. 3i for schematic).

A range of combinations of primers gave rise to some inexplicable fragment sizes and no clear, single products as would be expected from a single plasmid. It was suspected that nuc1 may actually be a combination of different fragments, by chance giving identical restriction patterns when digested with *Sac*I. Through more careful restriction mapping of the full plasmid (rather than the insert, as before), it was discovered that nuc1 was in fact a 12kb recombinant (as opposed to the expected 6kb). This giant plasmid contained at least one copy of the positively hybridising 3kb fragment, at least one copy of the pBs vector (also 3kb) and 6kb of unknown sequence giving fragments of 3kb on digestion with *Sac*I.

The multiple ligation event producing this plasmid may have incorporated three unrelated 3kb fragments from the original mixture of genomic fragments purified from agarose into one plasmid or been a combination of several copies of pBs vector with one genomic fragment. *Sac*I digestion resulted in four identically sized fragments that could not be distinguished on agarose, giving the impression of a 3kb doublet. Using the *Sac*I/*Pvu*I double digest as before to distinguish between plasmid and insert bands, the 3kb insert fragments generated were purified from an agarose gel and re-cloned into a new vector,

pGem-3zf (Promega). A colony blot was performed (using the Ld950 probe) on the resulting positive transformants and a number of putative positives chosen. Digestion with *Eco*RI (previously shown to have no recognition site within nuc1 (Fig. 3b) but a single site in the pGem vector) showed a number of these positives contained multiple inserts, the linear plasmid sizes being greater than the predicted 6kb of nuc1 plus vector. One recombinant of suitable size was chosen and a range of digestions performed to confirm its identity (Fig. 3h). The fragment sizes generated by digestion with *Acc*I and *Pst*I were as predicted from previous restriction mapping, confirming the new clone as a single copy of nuc1.

3.10 PCR Localisation of Nuc2

The previous PCR experiment was repeated to locate nuc2 within the newly cloned nuc1 insert. The primer combination of pnuc2a and M13 gave a very strong, single band of about 500bp (Fig. 3i). Taking into account the distance between the M13 primer site and the beginning of the insert, this indicated that the 5' sequence between the *Sac*I cloning site and the start of the nuc2 sequence was about 250bp. Presuming with such high homology that the *L. mexicana* gene would be similar in length to the *L. donovani* gene, this distance was compared to that of the published sequence. The comparison seemed to suggest that the nuc1 clone was shorter than expected and the *Sac*I cloning site actually fell within the gene sequence, downstream from the predicted start codon of the *L. mexicana* ORF.

To confirm this observation, the 500bp PCR product was cloned using a TA cloning strategy and sequenced in both directions. The sequence derived demonstrated a continuing level of at least 90% homology with the *L. donovani* gene and also revealed the predicted absence of a start codon at the 5' end. Comparison with the *L. donovani* gene at

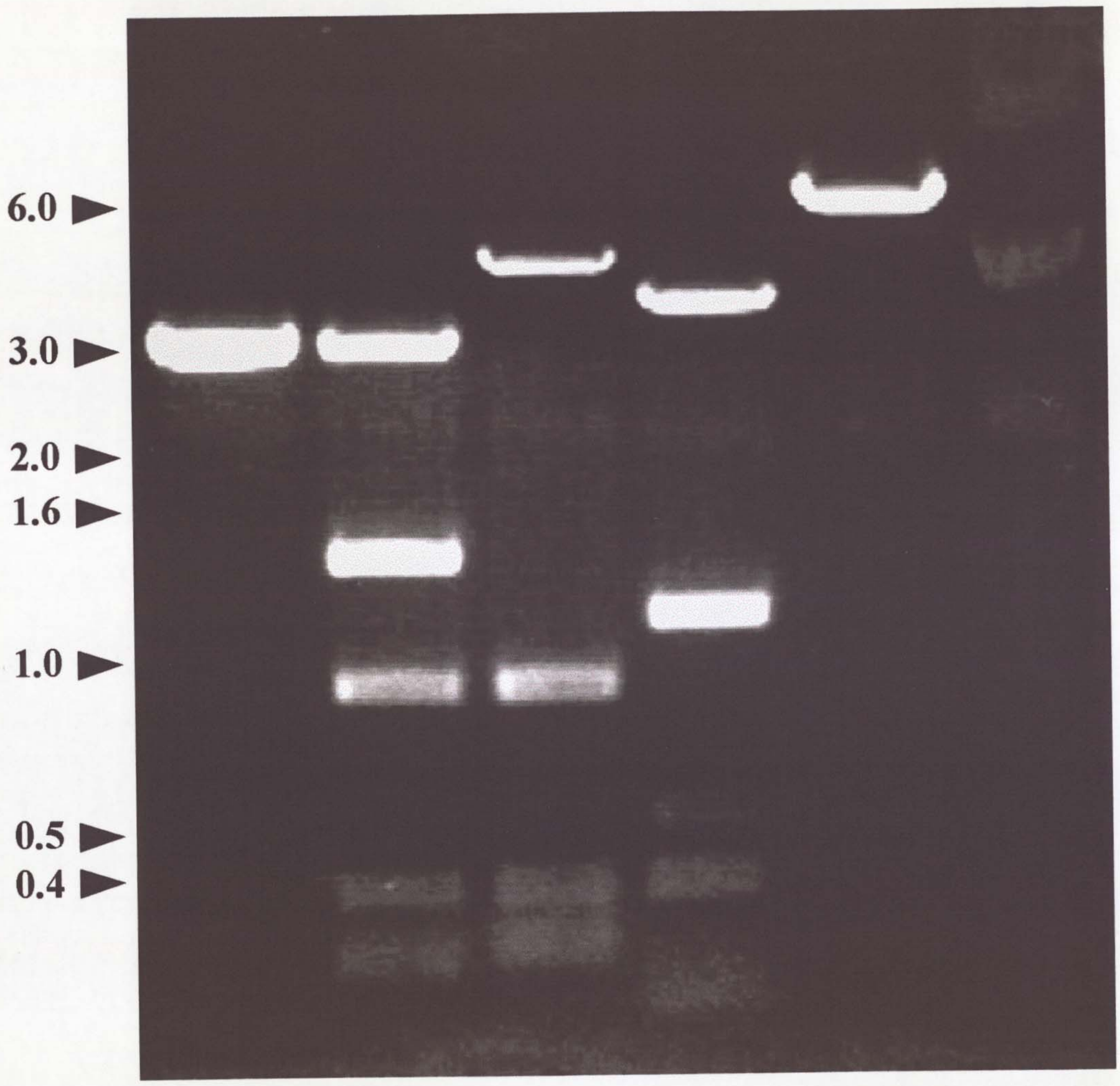
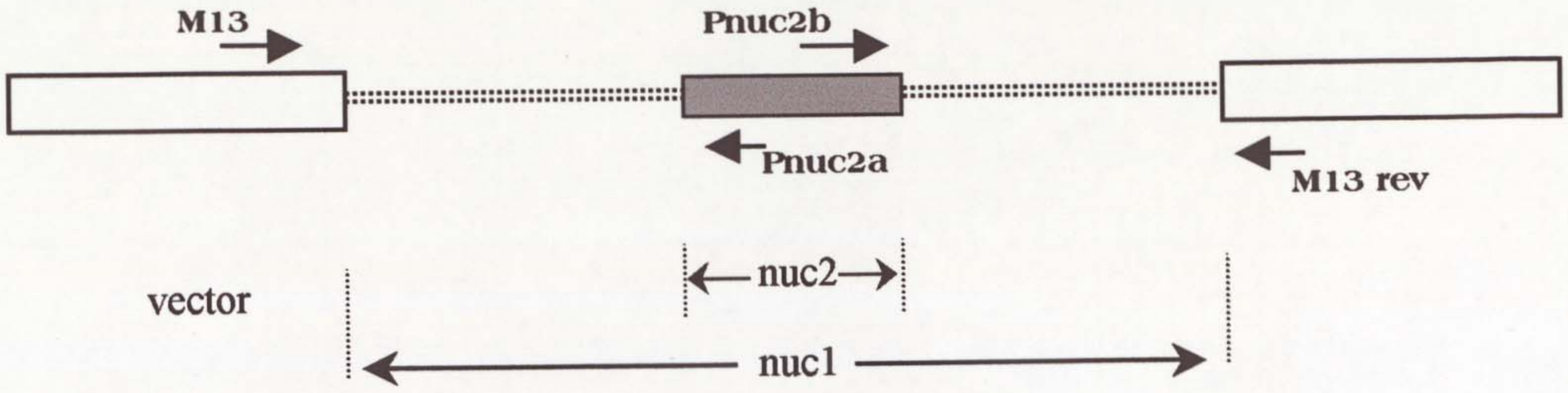
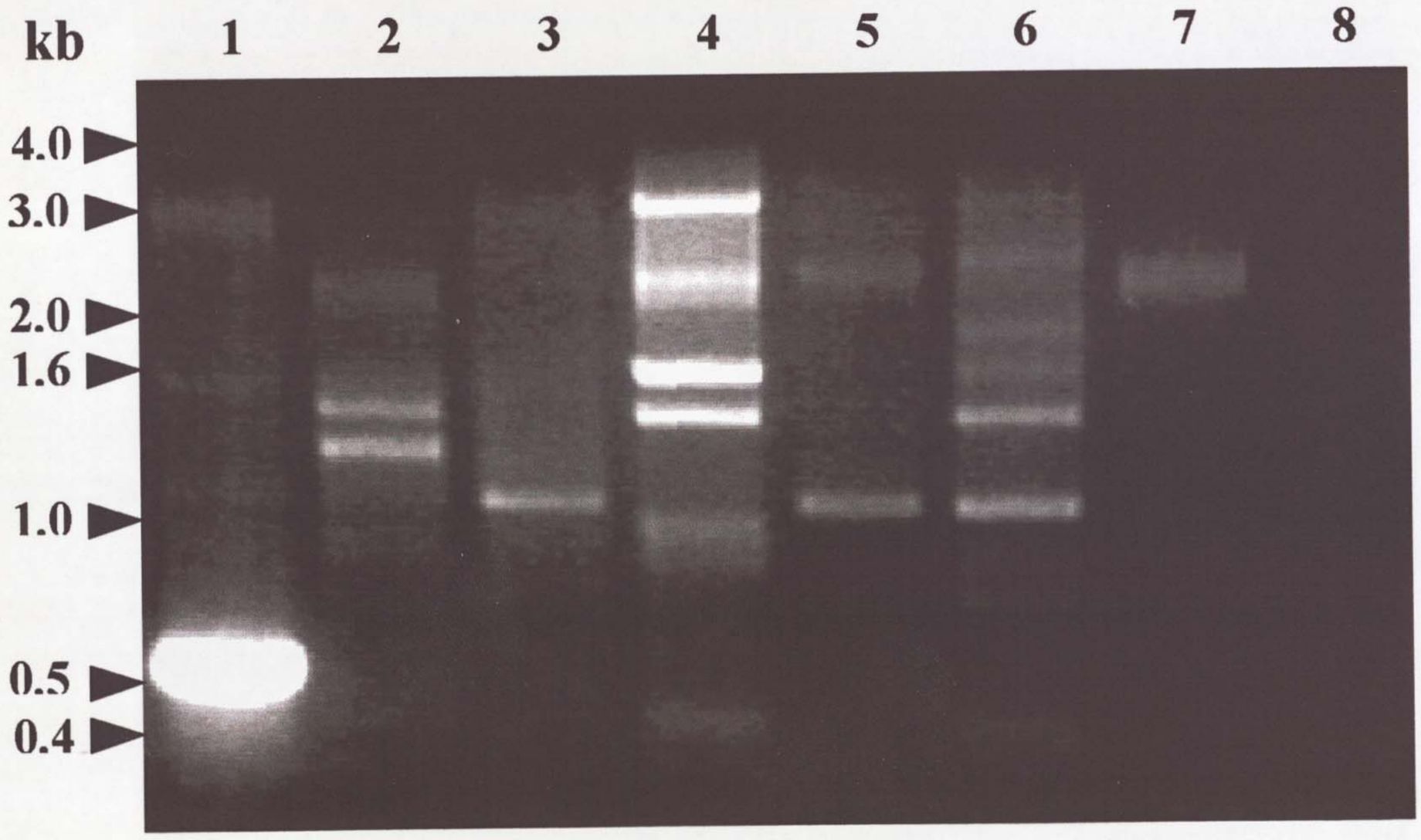


Figure 3h Fragments generated by the digestion of the re-cloned nuc1 plasmid with various enzymes. *Sac*I digestion resulted in two 3kb fragments: one of insert sequence and one of plasmid vector. The single *Eco*RI recognition site gave a 6kb fragment showing the presence of only one copy of the insert. DNA molecular mass markers are shown on the left.

(a)



(b)



1. pnuc2a + M13
2. pnuc2a + M13rev
3. pnuc2a + pnuc2b
4. M13rev + M13
5. pnuc2b + M13rev
6. pnuc2b + M13
7. no primer
8. no template DNA

Figure 3i PCR location of nuc2 within the nuc1 insert: (a) schematic of the primers used in an attempt to amplify the intervening nuc1 sequence between nuc2 and the 5' end cloning site (b) PCR products resulting from the listed primer combinations (1-8 loaded in lanes 1-8), showing the 500bp 5' fragment generated by pnuc2a + M13. The PCR reaction conditions were as described (Chapter 2.13) using 100ng of nuc1 recombinant plasmid as the template.

the nucleotide level seemed to suggest that only six nucleotides of gene sequence (upstream from the *Sac*I site) remained before the start codon.

3.11 Completion of available ORF sequencing

The remainder of the ORF *downstream* was sequenced by preparing two overlapping sub-clones (Fig. 3j). From the previous restriction mapping, *Bgl*II and *Pst*I digests were chosen to give overlapping fragments small enough to clone and sequence using standard vector primers. As before, the restriction fragments generated were separated on an agarose gel, excised and purified using Prep-A-Gene and ligated into plasmids cut with the same enzymes. In this way, continuous sequence was derived until the gene's stop codon was encountered. The position of this codon was identical to that of the *L. donovani* gene.

3.12 Completion of the 5' sequence

A number of strategies were used to try and obtain the remaining 5' end sequence. A genomic library prepared in lambda bacteriophage (kindly provided by Dr Jeremy Mottram, University of Glasgow) was plated out and probed, as with previous colony blots, using Ld950. Three positively hybridising bacteriophage plaques were picked after secondary screening of initial putative positives. Phage DNA was purified from these plaques and their identity as positive recombinants confirmed by Southern blot. Using this DNA, attempts were made to PCR amplify the intervening sequence (between the 3'-nt gene and the flanking lambda arms of the phage vector) and to sequence the lambda DNA directly using pnuc2a primer. Neither was successful. Finally, it was decided to clone restriction fragments of lambda DNA showing positive hybridisation after digestion of the recombinant phage.

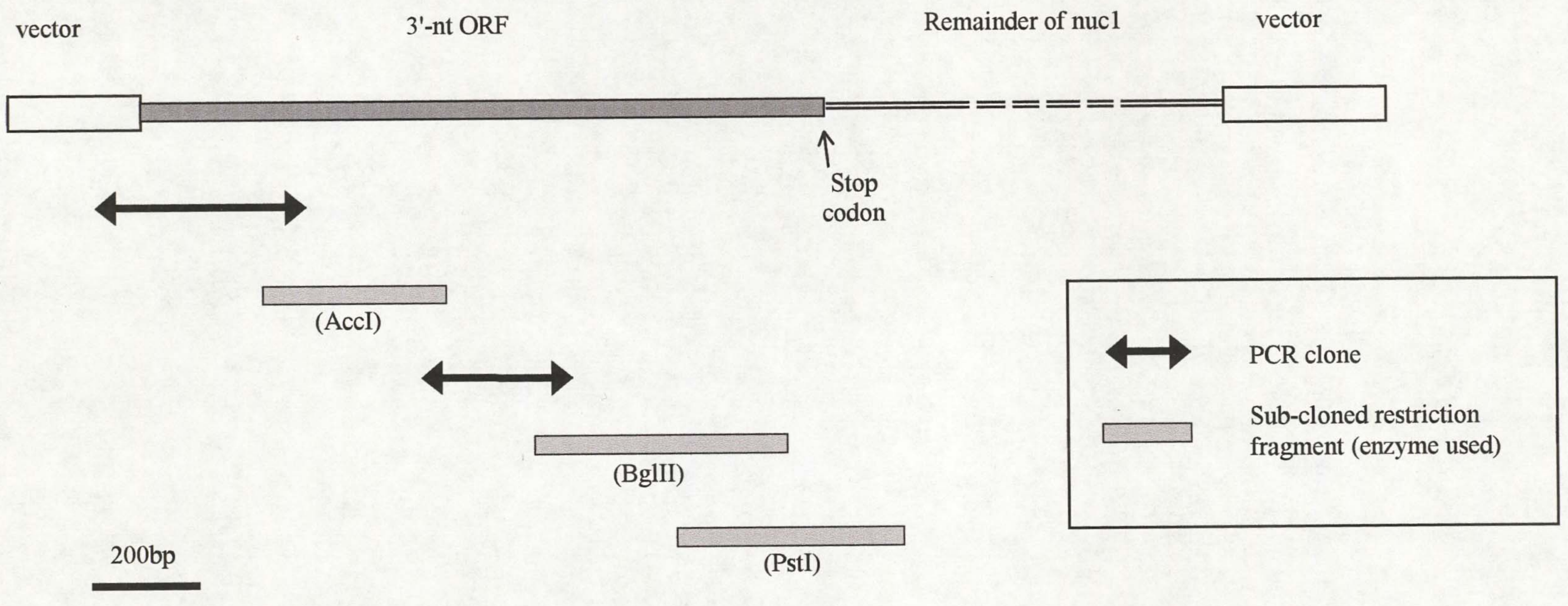


Figure 3j Summary schematic illustrating the sequencing of the *L. mexicana* 3'-nt open reading frame through the generation of several subclones. The double arrows represent sequence derived from the preparation of PCR subclones by the TA cloning of PCR products. The pale grey boxes represent subclones of specific nuc1 restriction fragments. These subclones could be sequenced more rapidly than the original nuc1 recombinant plasmid, and represent an overlapping series that incorporates the whole of the 3'-nt ORF.

From the known gene restriction map and using a new probe (DIG-labelled sequence from the 5' end of nuc1), it was possible to predict which positively hybridising restriction fragments on a Southern blot should contain the missing sequence. An *Acc*I digest was chosen and a positively probing 1.2kb fragment picked for cloning into an *Acc*I cut plasmid vector. This fragment proved to be of very low yield even in scaled up digests and several attempts to ligate it failed.

Also attempted was a full scale re-cloning of another genomic DNA fragment (using a *Pvu*I restriction digest), which gave a 2kb positively probing fragment predicted to span the start of the ORF. Simultaneously, primers complementary to *L. donovani* sequence upstream of the start codon were used in an attempt to PCR and sequence across the missing 5' end of the *L. mexicana* gene (using genomic DNA as a template). The primers evidently did not show sufficient complementarity to the *L. mexicana* sequence and the 5' end remained elusive.

Finally, Dr Mat Yamage (NIH, Bethesda) completed the 5' sequence from complementary DNA (cDNA) using RT-PCR with a spliced leader primer. Using this sequence (kindly provided), a primer was designed upstream from the start codon of the ORF and used to sequence genomic DNA to confirm the cDNA result. The genomic sequence was identical and so the ORF sequence was complete.

3.13 The *L. mexicana* 3'-nt gene

The final gene sequence was 1.134kb nucleotides long and theoretically encoded a protein of 378 amino acids (Fig. 3k). The derived amino acid sequence was used to predict the potential secondary structure of the translated protein (Fig. 3l). This analysis showed the

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ATGGCTCGAGCTCGTTTCCTTCAGCTTCTGCTGGTGGCGCTGACGCTCCTCAGTATCGCTGCACTCCCTGTCAGC 78
 M  A  R  A  R  F  L  Q  L  L  L  V  A  L  T  L  L  S  I  A  A  L  P  V  S

GCGTGGTGGAGCAAGGGCCACATGTCCGTGGCGCTCATTGCAAAGCGGCATATGGGCGCGTCTCTCGTTGAAAAG 153
 A  W  W  S  K  G  H  M  S  V  A  L  I  A  K  R  H  M  G  A  S  L  V  E  K

GCCGAACTGGCTGCTAAGGTGCTGTCCTTGTCTGGACCCTATCCGAAAAGCCCAGACATGGTGCAGACCGCATCA 228
 A  E  L  A  A  K  V  L  S  L  S  G  P  Y  P  K  S  P  D  M  V  Q  T  A  S

TGGGCGGATGACATCAAGGCGATCGGCCTCACAACATTGTCGACGTGGCACTACATCACGACGCCGTACTACCCA 303
 W  A  D  D  I  K  A  I  G  L  T  T  L  S  T  W  H  Y  I  T  T  P  Y  Y  P

GACGAAAACTTCACCTTGGAGATCAGCCCGGTACAGACGGTGAACGTTGCCTCTGTCATTCCGATGCTGCAGACG 378
 D  E  N  F  T  L  E  I  S  P  V  Q  T  V  N  V  A  S  V  I  P  M  L  Q  T

GCGATAGAAAAGCCAACGGCTAACTCGGAAGTTATCGTCGAGTCGCTGGCCCTCTTGCTACACTTTGTGGGCGAC 453
 A  I  E  K  P  T  A  N  S  E  V  I  V  E  S  L  A  L  L  L  H  F  V  G  D

ATCCACCAGCCACTGCACAACGTCAACCTCTTCTCTAACCAGTACCCGGAAAGTGACCTCGGTGGCAACAAGCAG 528
 I  H  Q  P  L  H  N  V  N  L  F  S  N  Q  Y  P  E  S  D  L  G  G  N  K  Q

CGCGTCGTTATCGACTCCAAGGGAACCAAGATGCTACTGCACGCGTACTGGGACTCGATGGCGGAGGGCAAAGCT 603
 R  V  V  I  D  S  K  G  T  K  M  L  L  H  A  Y  W  D  S  M  A  E  G  K  A

GGGGAGGATGTGCCGCGTCCGTTGAGCAAGGCCGACTACGACGATCTGAACAAATTTGTCGACTACCTGGAGGCA 678
 G  E  D  V  P  R  P  L  S  K  A  D  Y  D  D  L  N  K  F  V  D  Y  L  E  A

ACGTACGCAGGCACGCTGACGGATAAAGAAAAGAATCTCGTGGACCCTATGAAGATCTCGGAGGAGACCTTCGAC 753
 T  Y  A  G  T  L  T  D  K  E  K  N  L  V  D  P  M  K  I  S  E  E  T  F  D

CTGGCGCTGAAGCACGCCTACCCCGGCGCCAAGAATGGCGCCACGCTCTCGGACGAATACAAGAAGAACGCGAAG 828
 L  A  L  K  H  A  Y  P  G  A  K  N  G  A  T  L  S  D  E  Y  K  K  N  A  K

AAGATCTCGGAGCGTCAGGTGCTGCTTGCCGGCTACCGCCTGGCCAAGATGCTGAATGCGACGCTGAAGCCGATC 903
 K  I  S  E  R  Q  V  L  L  A  G  Y  R  L  A  K  M  L  N  A  T  L  K  P  I

AGCACGGCAGCGATTCTGCAGGGTCTCAAGAACATCCAAAGCGAAGTGGACACGGAAAACAAGGCTGAGGTGTAC 978
 S  T  A  A  I  L  Q  G  L  K  N  I  Q  S  E  V  D  T  E  N  K  A  E  V  Y

AACCACTACGATCAGAAAGGTCTCAGTACTGCTGTGACGGCGATTGTCGCCGTGGCAGTCTTCATCGTCGGCATT 1053
 N  H  Y  D  Q  K  G  L  S  T  A  V  T  A  I  V  A  V  A  V  F  I  V  G  I

ATCATCGCCACCCTGGTGGTTCTGGTCCTCAAGTGTTACCTGCAGAAGCGCGACCGCTTTGTTTACTACGAGCCG 1128
 I  I  A  T  L  V  V  L  V  L  K  C  Y  L  Q  K  R  D  R  F  V  Y  Y  E  P

GTATCCCTGTAA 1140
 V  S  L  •
```

Figure 3k Full open reading frame of the *L. mexicana* 3'-nt gene with predicted translated protein sequence. The ORF is 1134 nucleotides long and gives a translated peptide of 378 amino acids with a calculated molecular weight of 41665Da.

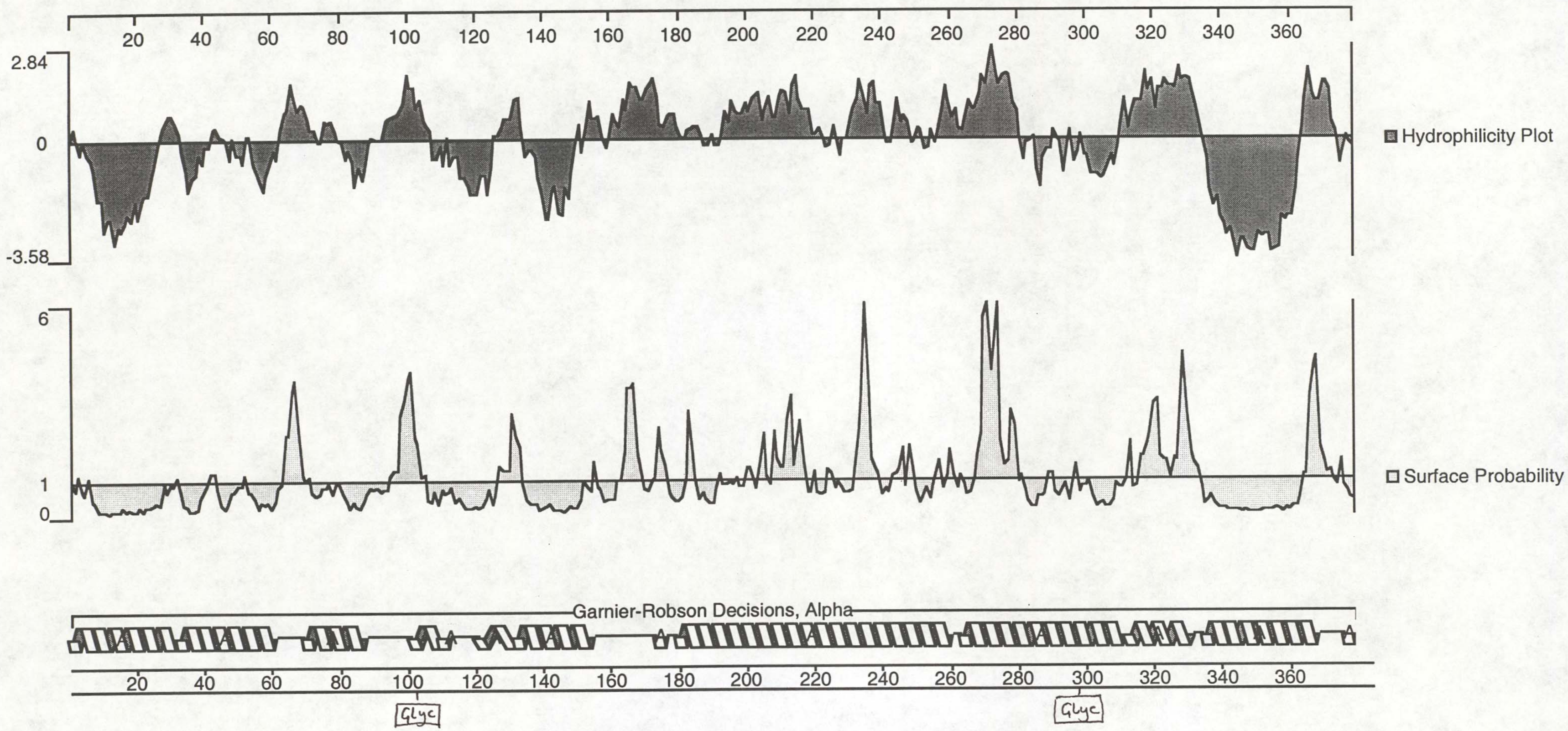


Figure 3l Features of the predicted secondary structure of the *L. mexicana* 3'-nt protein. Analysis of the predicted peptide sequence (Fig. 3k) reveals a predominance of alpha helices (Garnier-Robson prediction) in the 3'-nt protein. Several of these constitute hydrophobic regions (Kyte-Doolittle prediction, top), which correspond to stretches of sequence with a very low probability of being on the protein surface (Emini, below). Of particular note is the hydrophobic region between residues 335 and 360, which may constitute an intra-membranous anchor for the mature protein. The peptide sequence also contains two potential sites for *N*-linked glycosylation (marked 'Glyc'), suggesting the attachment of oligosaccharide chains to the mature protein. This would be consistent with its identity as a surface membrane protein.

3'-nt to be mainly comprised of alpha helical motifs with two very hydrophobic regions, at the C- and the N-termini. The hydrophobic C-terminus between residues 335 and 360 (Fig. 3l) may represent an intra-membranous domain, anchoring the mature protein to the cell surface membrane. There are two potential sites for *N*-linked glycosylation at residues 103 and 294, suggesting the attachment of oligosaccharide chains to the mature protein. Studies of the *L. donovani* enzyme have demonstrated the glycoprotein nature of 3'-nt (Campbell *et al.*, 1991). Glycosylation at these sites would be consistent with 3'-nt being a surface membrane protein.

The *L. mexicana* 3'-nt gene sequence showed 90% identity at the nucleotide level and 88% identity at the amino acid level to the *L. donovani* gene (Fig. 3m). This very high homology suggests that further members of the 3'-nt family could be isolated from other *Leishmania* species by PCR, using primers complementary to the most conserved regions of sequence. As with the *L. donovani* gene, the *L. mexicana* 3'-nt peptide sequence also shows some homology to a group of class 1 nucleases described in *Penicillium citrinium* and *Aspergillus oryzae* (Fig. 3n). The areas of conservation in the peptide sequence are close by the amino acids identified in these nucleases as key for substrate binding (Romier, *et al.*, 1998) and these residues are likely to be crucial in the formation of the enzyme active site and possibly the binding of Zn^{2+} co-factors.

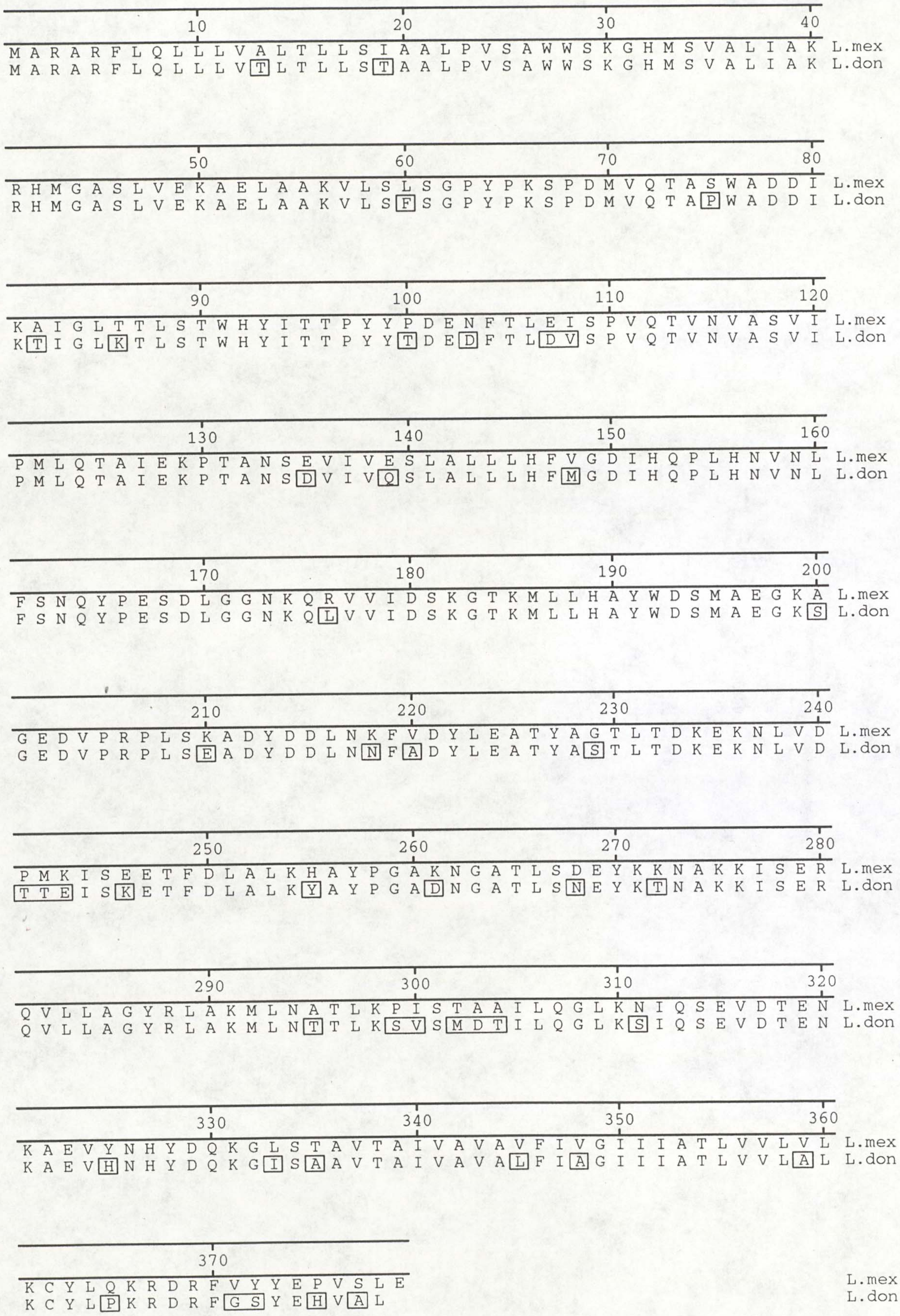


Figure 3m Comparison of the predicted amino acid sequence of *L. mexicana* 3'-nt with the *L. donovani* peptide sequence. The two sequences show 88% identity at the amino acid level. Those residues in the *L. donovani* sequence that do not match *L. mexicana* are boxed.

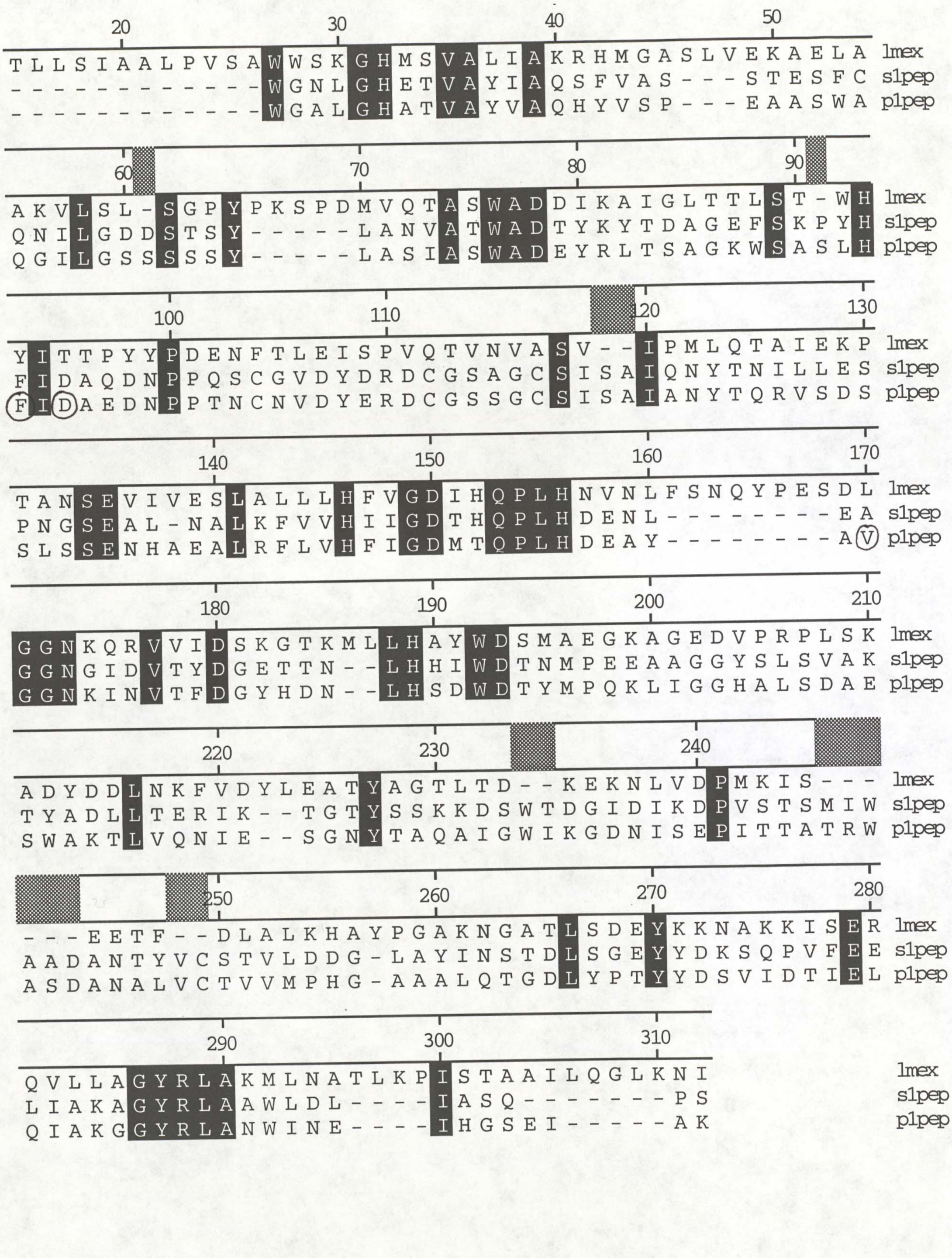


Figure 3n Comparison of the predicted amino acid sequence of *L. mexicana* 3'-nt with S1 and P1, nucleases of *Aspergillus oryzae* and *Penicillium citrinum*, respectively. There appear to be a number of regions of homology (blocked in black) that may represent significant structural motifs of the enzymes' catalytic site. The three residues shown to be crucial in the active site of P1 (Romier *et al.*, 1998) are circled in the P1 sequence.

CHAPTER 4
GENE EXPRESSION

4.1 Introduction

With the full sequence of the *L. mexicana* 3'-nt gene known, the first stages in functional analysis were to assess the copy number in the genome and to demonstrate expression in living parasites. This involved Southern and Northern blotting using a range of probes developed from the nuc1 clone.

4.2 Production of probes

Two different methods were used to prepare DIG-labelled DNA probes: random primed labelling and labelling by PCR. In the first instance, specific restriction digests released fragments of unknown sequence. The fragments were used as a template for a random primed Klenow reaction incorporating DIG-labelled dUTP residues (DIG-dUTP). The PCR method followed a standard PCR protocol where the sequence of the template DNA (and hence primer binding sites) were known, and incorporated DIG-dUTP through its inclusion in the standard PCR dNTP mix. These labelled probes had a high sensitivity and could be stored pre-diluted in hybridisation solution at -20°C. The advantage of this was the ability to re-use the same solution of probe several times in repeated hybridisation experiments.

Probes spanning different regions of the 3'-nt ORF and nuc1 clone were prepared according to the elucidated gene sequence and restriction enzyme recognition sites established by previous restriction mapping of nuc1 (see Fig. 4a).

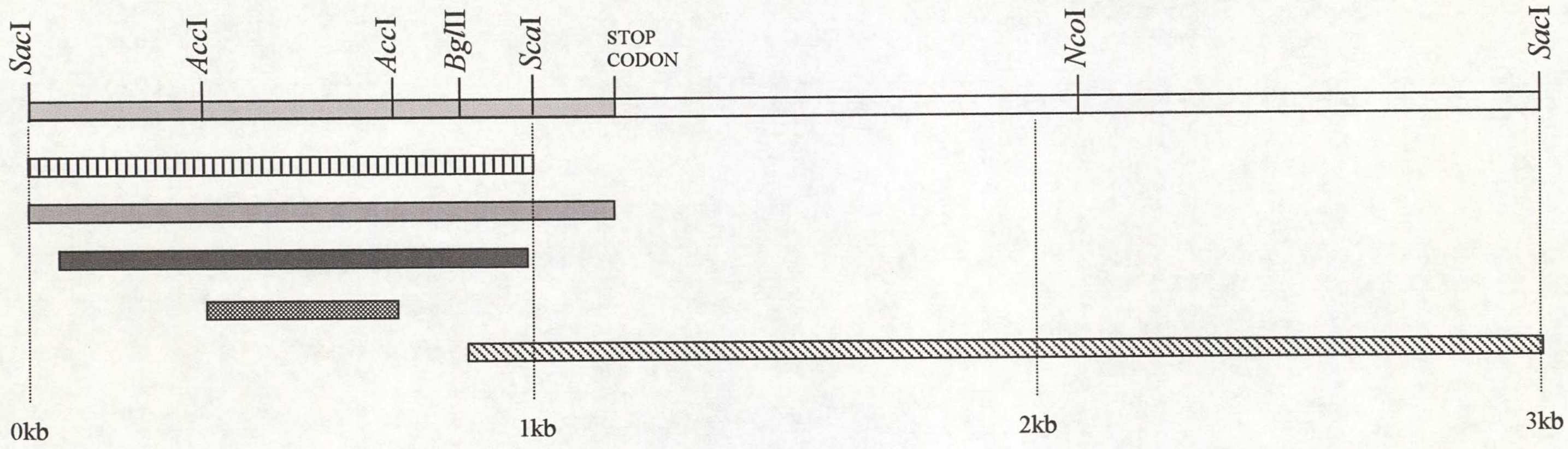


Figure 4a Summary schematic illustrating the position of probes manufactured for studies of gene copy number and Northern hybridisations. The top bar is the complete nuc1 insert (3kb long) with the ORF shaded grey. The position of each probe (approximately) is denoted by a separate bar, identified according to the probes key. Probe nomenclature is their approximate length in base pairs, prefixed by the initials of the *Leishmania* species to which they are homologous. The probe incorporating the whole open reading frame is denoted ORF.

4.3 Gene copy number

With such an array of probes, it was possible to predict the banding patterns of genomic DNA restriction digests and build up a restriction map incorporating about 27kb of sequence around the ORF site in the genome. Both single and double digests were used and the genomic restriction fragments run on agarose and Southern blotted (see Fig. 4b for one example). By probing and re-probing the same blots with a variety of the probes described above, a map was built up (Fig. 4c). With each subsequent digest tried, the array of fragments expected using a particular probe could be predicted according to the map constructed.

As this process continued, both frequent cutting and rare cutting enzymes were used and the fragment sizes were always as predicted. This suggested that there is only a single copy of the gene within the genome, as a distant second copy would also show positive hybridisation and give different (and unpredicted) extra banding patterns. The results using the frequent cutting enzymes showed there were no extra tandem copies of the gene adjacent in the genome, and the results from the rare cutters implied there were no distant copies (which may, for example, be on separate chromosomes).

The only unexpected result was using the restriction enzyme *Pvu*I. As seen from the restriction map in Figure 4c, there are two bands expected as a result of digesting genomic DNA with this enzyme and probing with the ORF probe. However, in the accompanying Southern blot (Fig. 4b), an extra band is seen at about 6kb. Double digests involving *Pvu*I showed this result would be consistent with the presence of a restriction fragment length polymorphism (RFLP) with the loss in one allele of the nearest *Pvu*I site downstream from the gene (shown boxed on the restriction map). No

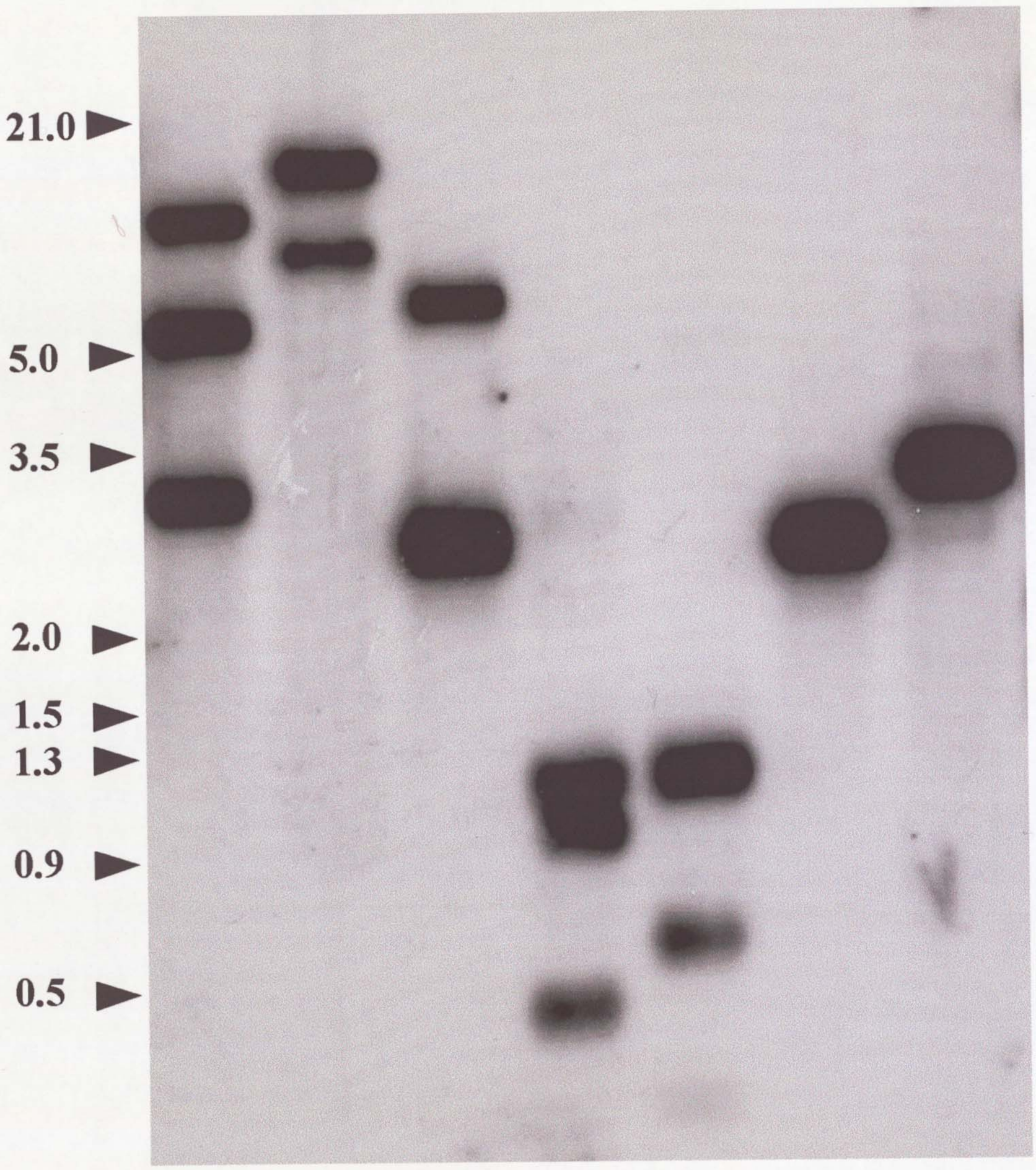


Figure 4b One of a number of Southern blots used to construct a restriction map for the 3'-nt locus. Genomic DNA (1μg each lane) was digested with the enzymes shown and the restriction products transferred to nylon. Hybridisation of the blot with the ORF probe detected these band sizes. Many similar experiments allowed the construction of a restriction map. DNA molecular mass markers are shown on the left. The blot was developed for 60 minutes.

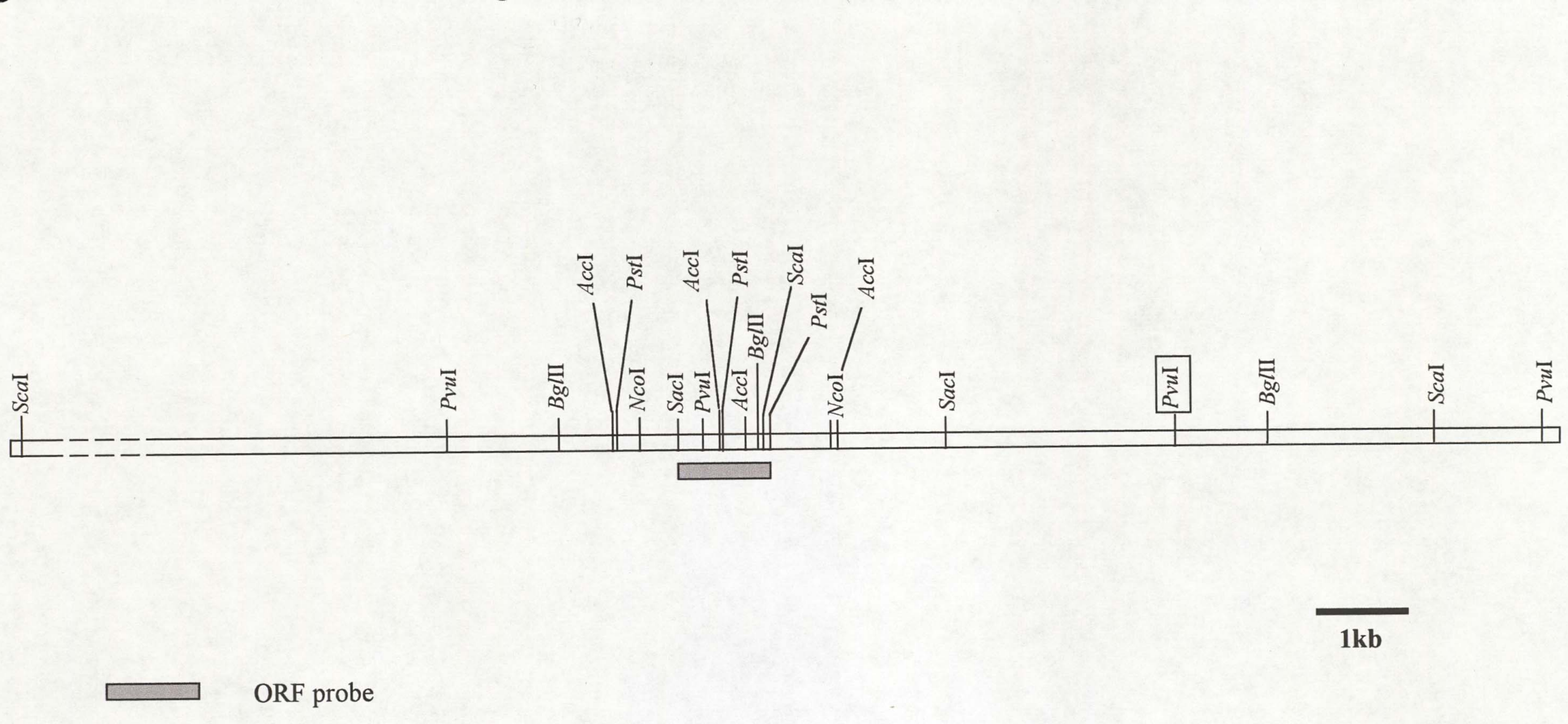


Figure 4c Summary schematic illustrating the approximate restriction map of the 3'-nt locus built up from the Southern blot in Fig. 4b. The shaded box indicates the position of the ORF sequence, used in this case as the DIG-labelled probe. The boxed *Pvu*I site indicates the position of a possible absent site, giving rise in one allele to a restriction fragment length polymorphism (RFLP). Several Southern blots (as in Fig. 4b) were performed using a variety of restriction enzyme single and double digests of genomic DNA and a variety of different probes. Data from each blot allowed the construction of a possible restriction map against which subsequent hybridisation results could be compared. In this way, it became clear that only one copy of the 3'-nt gene was present in about 27kb of flanking sequence at the gene locus.

other combination of enzymes and probes used suggested there was more than a single copy of the 3'-nt gene in the *L. mexicana* genome.

4.4 Southern blots of phage DNA

The restriction mapping of the genomic DNA was repeated in the positive phage recombinants picked from the library. The results showed identical banding patterns to those obtained using the full genome. This confirmed that the sequence around the phage recombinant copy of the gene (i.e. the pattern of restriction sites) was identical to that in the genomic DNA. This would not be expected if the phage recombinant bore a second copy of the gene from elsewhere in the genome. Thus, the gene-bearing fragment picked out of a genomic library appears to be the same as the fragment cloned from genomic DNA digests, suggesting this gene is the only copy.

4.5 Pulsed field gel electrophoresis (PFGE)

Cultured *L. mexicana* promastigotes were gently lysed under conditions that preserved the integrity of the chromosomes and run on an agarose pulsed field gel. The separated chromosomes were transferred to a nylon membrane by Southern blot and probed with a *L. donovani*-specific 3'-nt DNA probe (this work was done in Dr Debrabant's lab, NIH and the final result was kindly provided by Dr Debrabant). The results show a single positive hybridising band in the *L. mexicana* chromosomes (Fig. 4d) suggesting the 3'-nt gene is only present on one pair of chromosomes in this species. *L. donovani* chromosomes were also probed as a comparison and these too show a single band of hybridisation. Together with the available Southern blot data, the presence of 3'-nt sequence on a single pair of homologous chromosomes is consistent with there being a single gene locus bearing a single copy of the gene in *L. mexicana*.

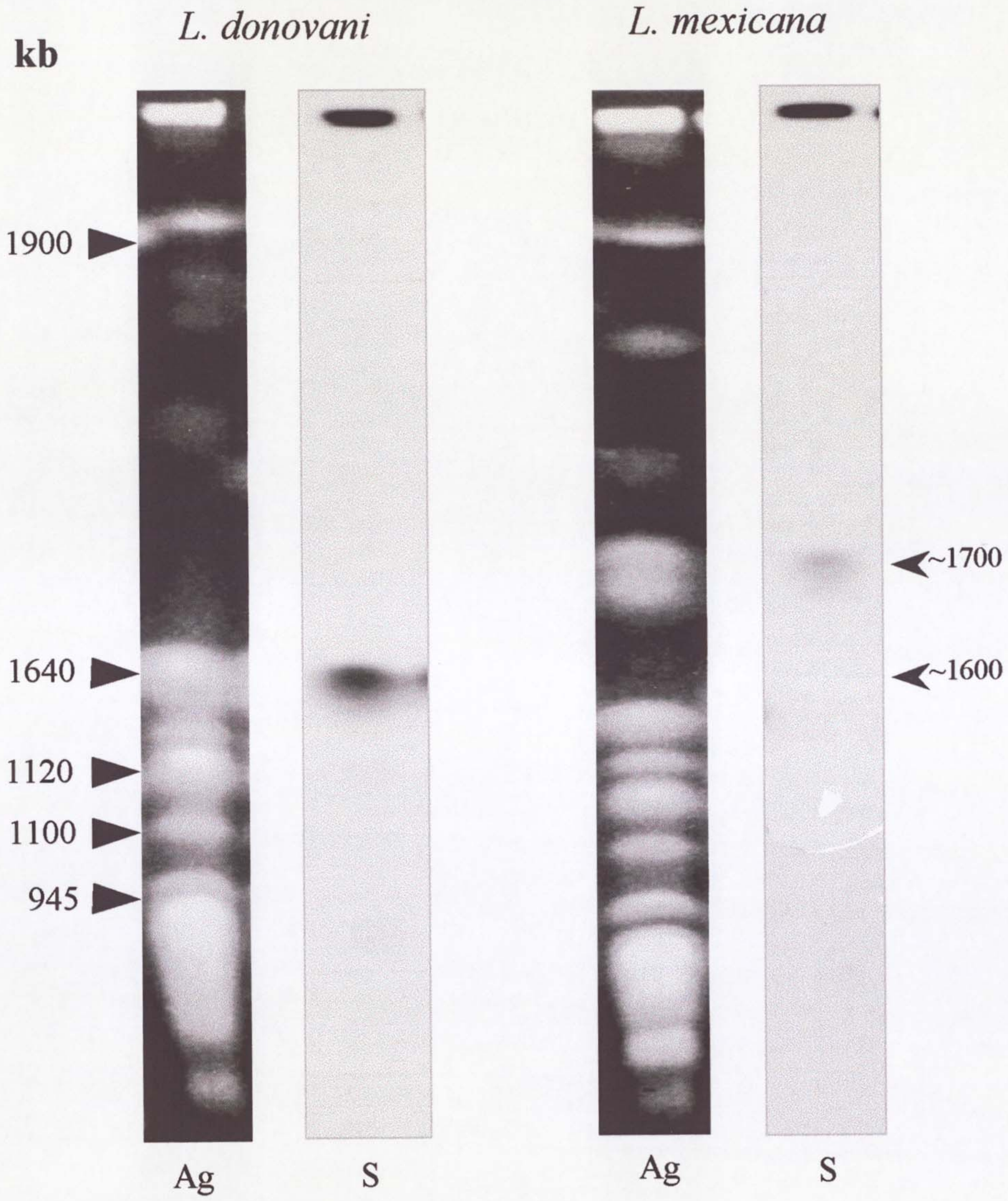


Figure 4d Pulsed field gel electrophoresis of *L. donovani* and *L. mexicana* chromosomes. Chromosomes of each species were separated on the same agarose gel, the relevant portions of which are shown (Ag). DNA transferred by Southern blot was then probed with the DIG-labelled 3'-nt ORF from *L. donovani*. The corresponding panels of the Southern blot are shown (S), aligned according to the approximate molecular mass markers shown on the left (in kb). At this level of measurement, the probe detected 3'-nt sequence at one site in each of the probed panels, strongly in *L. donovani* and more weakly in *L. mexicana*. These sites were taken to be single chromosomes, seen by ethidium bromide staining in the agarose panels, and their approximate sizes are shown on the right (in kb). The blot was developed overnight.

4.6 Gene expression through the life cycle

The three main developmental stages of the *L. mexicana* life cycle (the amastigote, the procyclic promastigote and the metacyclic promastigote) may be derived *in vitro* using various conditions of culture. It was of interest to establish which, if any, of these forms naturally expressed the elucidated 3'-nt gene. This was done by investigating the messenger RNA of each developmental stage.

Populations of axenic amastigotes, procyclic promastigotes and metacyclic promastigotes were prepared and total RNA isolated as described. The RNA was analysed by Northern blot and probed using the DIG-labelled ORF probe (Fig. 4e(a)). Estimating the intensity of the signal on the resultant autoradiogram by eye, the positive hybridisation was strongest in the procyclic sample, about three times weaker in the metacyclic sample and not apparent at all in the amastigote sample. These relative intensities are not precisely accurate, as the DIG detection process is more qualitative than quantitative, but are an indication of differential expression. The size of the single hybridising band was about 2.7kb. The equivalence of the levels of total RNA loaded on the agarose gel was measured spectrophotometrically and confirmed by ethidium bromide staining of the ribosomal bands prior to transfer (upper panel of Fig. 4e(a)).

To confirm that the axenic cultures truly reflected the situation *in vivo*, especially the absence of expression by amastigotes, it was necessary to repeat the Northern analysis using cells from the natural life cycle. Amastigotes were isolated directly from an experimentally induced lesion on a mouse. A sample of these cells was also placed in culture conditions that induced transformation to procyclic promastigotes. Without further passage, these procyclic forms were collected. This population represented the

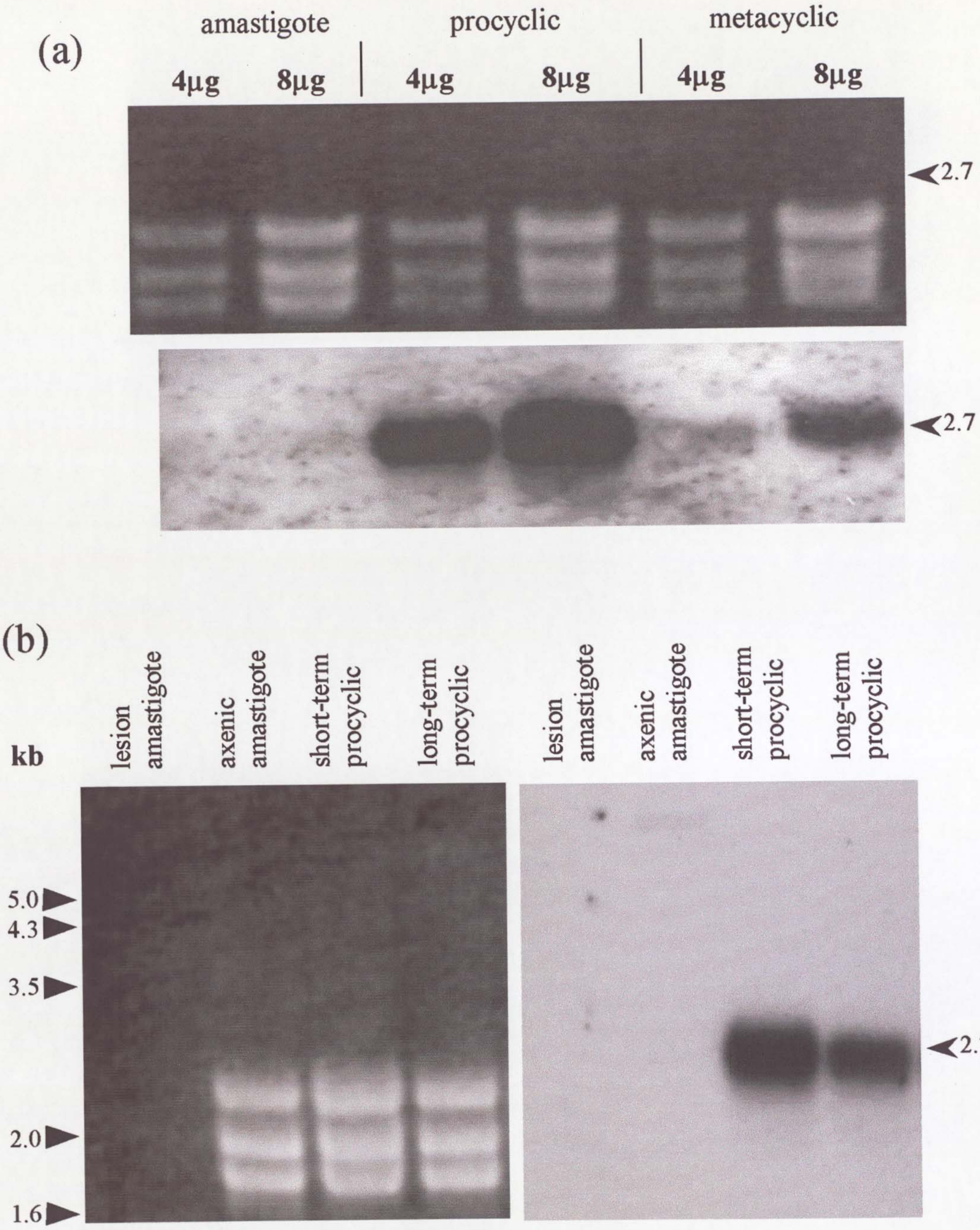


Figure 4e Northern hybridisations of different *L. mexicana* developmental forms. (a) total RNA (4µg and 8µg aliquots) from equal numbers of amastigotes, procylic and metacyclic promastigotes, derived *in vitro*, was separated on agarose (top panel). RNA was transferred to nylon and hybridised with the LmORF DNA probe (panel beneath). The approximate band size of the single hybridising message is shown on the right in kb. (b) total RNA (5µg each lane) from both *in vivo* (lesion) and *in vitro* (axenic) amastigotes was compared to RNA from short-term promastigotes (derived from lesion amastigotes) and long-term promastigotes (from standard axenic culture) in the same way. The agarose gel is shown on the left and the corresponding LmORF-probed blot on the right (aligned with DNA molecular weight markers). Blot (a) was developed for 3 hours and blot (b) for 60 minutes. Relative abundance of message in each lane was estimated by eye. Blots (a) and (b) were performed on different occasions and comparisons between them in terms of message abundance were not made.

procyclic promastigotes that would arise from the uptake of lesion amastigotes by a sandfly. It was assumed that the physiological status of these short-term procyclic forms would reflect the natural population more nearly than the procyclics arising from long-term continuous culture.

Total RNA was prepared from the lesion amastigote and procyclic populations described above and the Northern analysis repeated (Fig. 4e(b)) comparing 'axenic' and 'lesion' amastigote populations and the corresponding long-term and short-term promastigote populations. The previous results were confirmed with no signal in the amastigote samples (either lesion or axenic) although the ribosomal bands in the RNA sample from the lesion amastigote population were not as obvious on ethidium bromide staining as those in the axenic sample (left hand panel Fig. 4e(b)). The isolation was repeated using a fresh lesion. Although the amastigote and promastigote cell numbers were equivalent and the nucleic acid content equal spectrophotometrically, the image on agarose was still not clear. These cells were shown later to have various enzyme activities (Fig. 6b) and the isolation procedure appeared to be working effectively so it was assumed that the lack of satisfactory ribosomal bands was a factor of *Leishmania* lesion amastigotes. This may reflect the lower growth rate of amastigotes in a natural lesion compared with those in optimal conditions of axenic culture. Alternatively, the isolation procedure for amastigotes from lesion material may cause RNA degradation. The short-term procyclic promastigotes showed a signal about twice the intensity of that in the long-term sample (judged by eye).

4.7 Conclusions

Extensive Southern blotting, with a range of probes and using different combinations of restriction enzymes, allowed the construction of a partial restriction map of the 3'-nt gene locus. The number and size of bands observed on hybridisation with these probes was consistent with the gene being single copy within the genomic locus studied. The detection of a single hybridising band of 3'-nt sequence in the PFGE separation of *L. mexicana* chromosomes suggested the presence of a single locus for the gene in the genome. These combined observations suggest that 3'-nt may be a single copy gene in *L. mexicana*. Northern blots revealed 3'-nt gene expression in both procyclic and metacyclic promastigotes (although highest expression was in the procyclic population) but no expression in the amastigote form. This suggests that the gene sequenced encodes an enzyme which is stage-specific, developmentally regulated and of little importance to the amastigote stage of the life cycle.

CHAPTER 5

LINKING THE 3'-NT GENE WITH OBSERVED ACTIVITY

5.1 Introduction

The high homology between the *L. mexicana* gene sequence described and the previously published *L. donovani* 3'-nt gene is very clear evidence that this gene is in fact the functional 3'-nt. However, conclusive proof would come from linking the DNA sequence to the biochemically described activity of the 3'-nucleotidase enzyme in *Leishmania* cells.

5.2 Immunoprecipitation experiments

One way to link the described gene to enzyme activity was to use specific antibodies derived from recombinant 3'-nt protein in immunoprecipitation experiments. Recombinant protein could be prepared by artificially expressing the gene sequence derived *in vitro*. If antibodies raised against this protein could be shown to precipitate 3'-nt activity from native *L. mexicana* cells, this would be very strong evidence that, *in vivo*, the gene encoded the natural 3'-nt observed.

Antiserum raised against recombinant *L. donovani* 3'-nt was already available (kindly provided by Dr Alain Debrabant) and was shown to recognise three *L. mexicana* promastigote proteins in Western blots (Fig. 5a). 3'-nt has been reported to be upregulated under conditions of purine starvation in *L. donovani* (see 1.10) so the anti-recombinant antibody was used against both normal and starved cells. All procyclics contain a 52kDa protein recognised by the immune serum and the starved cells show an

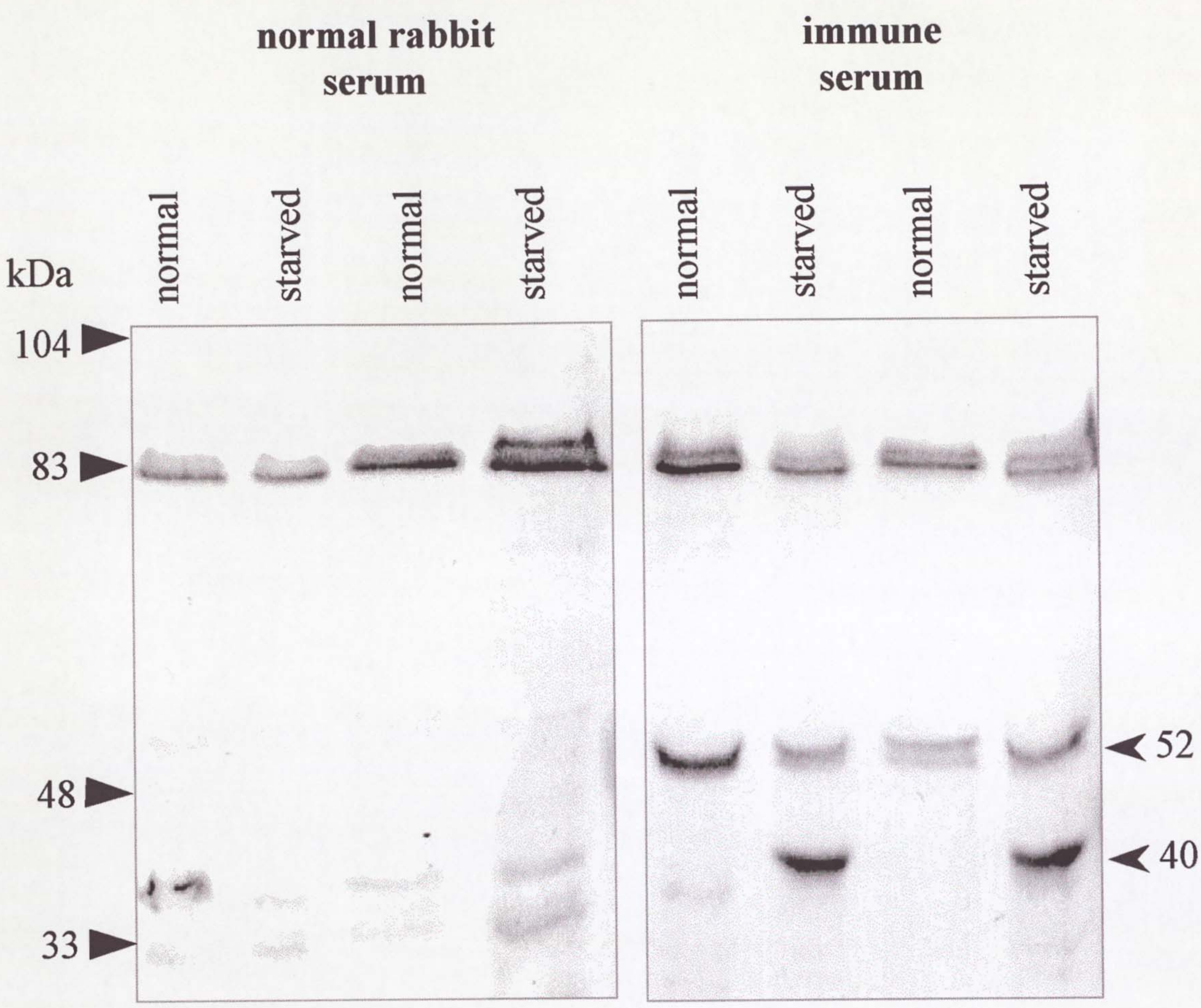


Figure 5a Western blots showing antigen recognition in *L. mexicana* procyclic promastigotes. Cell lysates of promastigotes grown under normal and purine starved conditions were run on SDS-PAGE and Western blotted using an antibody raised against recombinant *L. donovani* 3'-nt. Antigen was detected in the control (left panel) using normal rabbit serum. Protein markers are shown on the left and approximate antigen band sizes on the right (both in kDa). A band of about 83kDa was visible in all samples using both normal and immune serum and this was ignored. Two further bands were detected with immune serum, one of about 52kDa found in both normal and starved cells and one of about 40kDa only in purine-starved cells.

additional band of recognition at about 40kDa. In *L. donovani*, the serum recognised a single protein of 43kDa (personal communication, Dr Debrabant). This serum was used in an attempt to precipitate 3'-nt activity from axenically grown procyclic promastigotes.

The recombinant serum was incubated with *L. mexicana* cell lysate overnight and the antibodies then bound to protein A-sepharose beads in a microfuge tube. The beads were pelleted by centrifugation and washed several times. Both they and the residual supernatant were then tested for 3'-nt activity in a standard malachite green tube assay. A simultaneous control followed the same procedure but using normal, pre-immune serum in place of the derived antiserum. The recombinant antibody proved to be relatively poor at immunoprecipitation but under optimised conditions of binding, 11% of the total 3'-nt activity was precipitated from the lysate onto the beads with the anti-recombinant antiserum compared with only 0.17% with the normal serum.

In order to assess whether this observed 3'-nt activity corresponded to that previously described in *Leishmania*, an aliquot of each of the washed, antibody-bound beads were loaded on an SDS-PAGE substrate gel containing polyA. A single 40kDa band of nuclease activity was present on the immune serum beads and nothing on the normal control (Fig. 5b). This band corresponded to the uppermost band of nuclease activity seen in both *L. mexicana* and *L. donovani* promastigotes and also to the band of co-migrating 3'-nucleotidase activity observed on malachite green gels (see Fig. 6a). This result gave a very strong indication that the gene cloned does in fact encode the native 3'-nucleotidase.

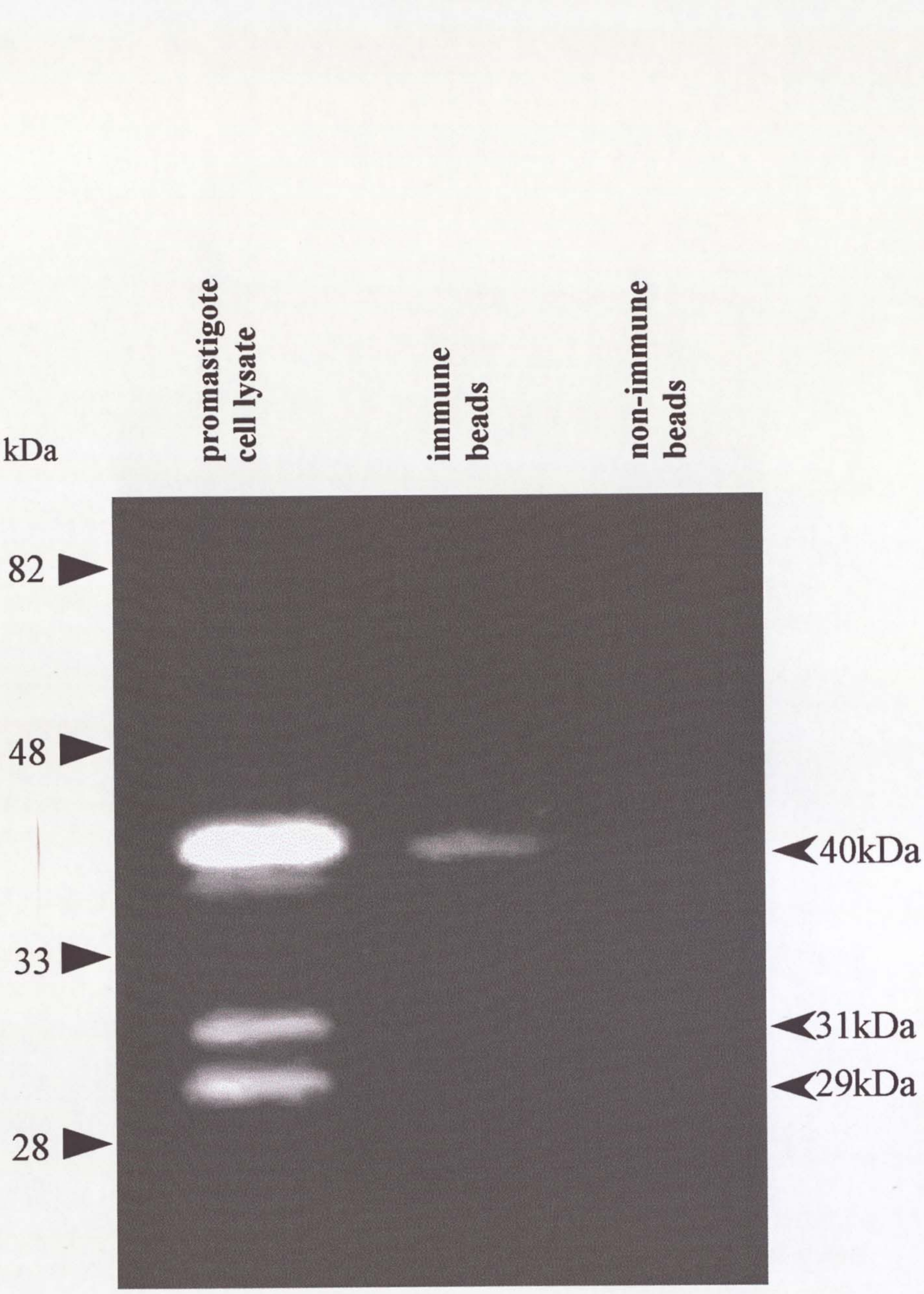


Figure 5b PolyA substrate SDS-PAGE gel comparing the nuclease activities of *L. mexicana* promastigotes with immunoprecipitated activity bound to immune and non-immune coated protein-A beads. Equal aliquots of coated beads (immune and non-immune with protein complexes attached) were boiled in reducing buffer and loaded directly into the gel lanes. Lysate of a roughly equivalent number of procyclic promastigotes to that in each aliquot of the immunoprecipitation was also run (left hand lane). Protein size markers are shown on the left and approximate sizes of the bands of nuclease activity seen are shown on the right (all in kDa).

5.3 Expression of the *L. mexicana* 3'-nt

Having established the gene's identity, it was decided to try and obtain recombinant protein and prepare antiserum specific for the *L. mexicana* enzyme. The complete open reading frame was ligated into a suitable expression plasmid vector (pCAL-n-EK, Stratagene). The vector chosen contained sequence that encoded calmodulin-binding protein (CBP) such that any recombinant protein expression would produce a CBP fusion protein. This protein could then be simply purified from bacterial proteins using calmodulin-coated resin.

The particular system used for 3'-nt expression also employed 'ligation independent cloning' whereby the gene to be expressed was PCR amplified using primers containing standard 5' sequence (licnt5' and licnt3') which, when digested, gave ends compatible with the supplied expression plasmid. The first transformation step used a standard bacterial strain as the host cell to check the successful formation of expression plasmids. Once these had been confirmed, the plasmids were then sub-cloned into the expression strain of bacterial cells, BL21(DE3)pLysS. As with standard transformation procedures, positive transformants were selected on the basis of ampicillin resistance, which had been engineered into the expression plasmids used. In addition, these plasmids bore a chloramphenicol resistant marker and an IPTG inducible sequence. Plasmid expression was induced with IPTG at an approximate cell density of OD=0.8 and the cells grown for a further four hours. At this point, the recombinant cells were harvested by centrifugation and a cell lysate prepared.

Initially, small aliquots of the induced cell lysate were incubated with calmodulin-coated resin beads to assess the optimal binding and washing conditions needed to obtain pure

recombinant protein. As a negative control, a number of aliquots of pre-induced cell culture had been taken before the addition of IPTG and these aliquots were taken through the same binding and washing procedures. Initial elutions from the washed calmodulin resin indicated that, in comparison with the uninduced control, IPTG had induced the expression of a 55kDa protein from the recombinant culture (Fig. 5c). Taking into account the size of the CBP fusion portion of the expressed protein, this corresponded to the predicted size of the protein encoded by the described 3'-nt sequence. Efforts were made to further purify the expressed protein by increasing the stringency of washing but the background bands of bacterial proteins seen in Figure 5c persisted.

The remainder of the induced cell lysate was loaded onto a column of calmodulin-coated resin and the bound protein washed and eluted according to the optimised conditions established for the small aliquots above.

5.4 Analysis of the expressed 3'-nt

The expression protein could be further purified by removing the CBP fusion peptide. A number of peptidase recognition sites had been engineered into the expression plasmid between the CBP and the cloning site. Enterokinase and thrombin were used in an attempt to cleave the CBP tag from the recombinant 3'-nt. Both digestions resulted in very low final yields of protein and it was surmised that internal sites for these peptidases might be present in the 3'-nt peptide sequence.

To confirm the recombinant's identity, it was decided to try and generate some peptide sequence of the expression product. The major 55kDa band was purified from acrylamide and micro-sequenced (service kindly provided by Dr Mark Wilkinson,

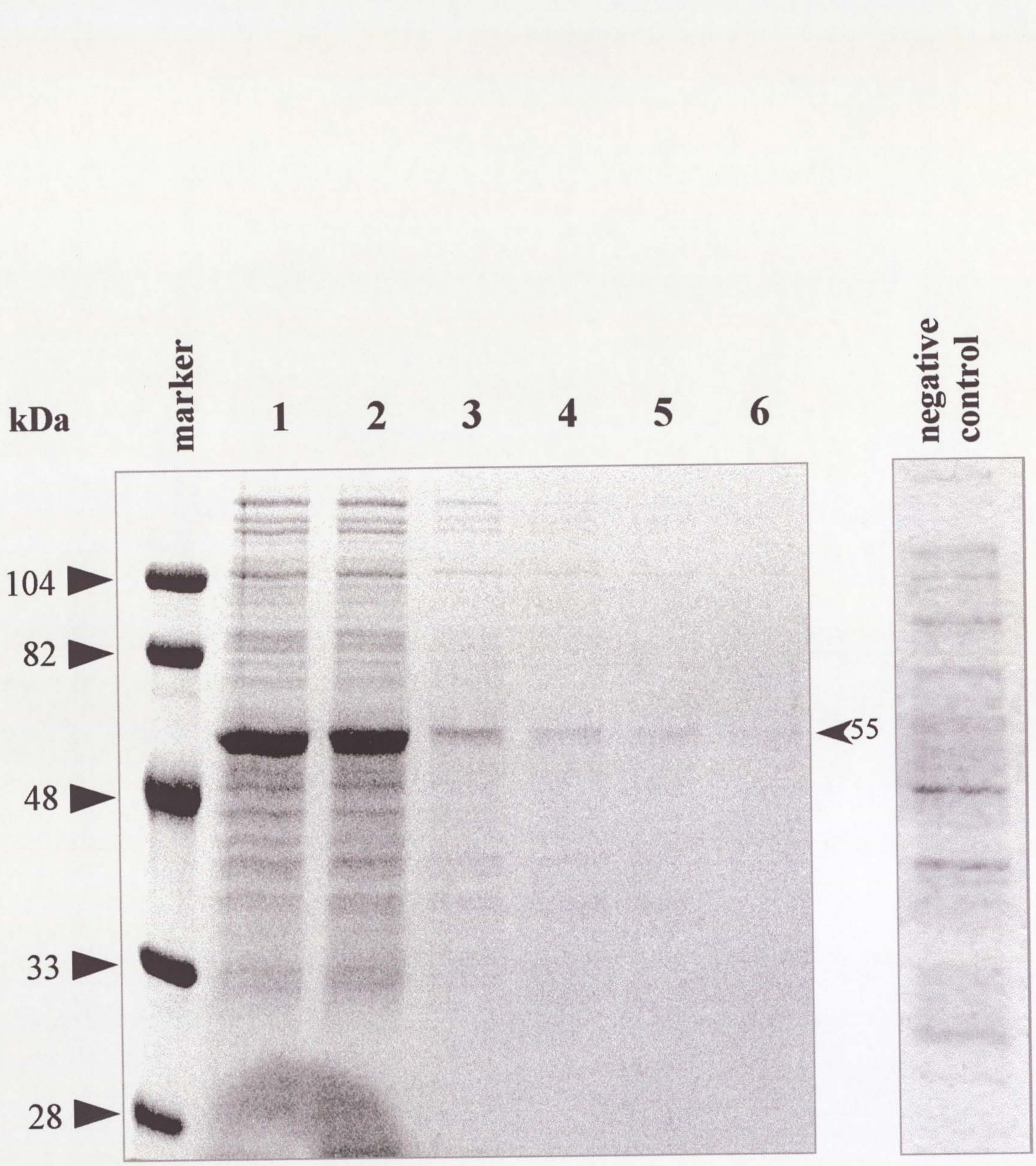


Figure 5c SDS-PAGE gel showing sequential elutions of recombinant CBP fusion protein from calmodulin-coated resin. Aliquots of the protein coated resin beads were washed with six pellet volumes of elution buffer and the eluates run on SDS-PAGE (lanes 1-6). The protein marker bands are also shown (in kDa). The major protein (55kDa) in the first two elutions approximately corresponds in size to recombinant CBP-fused 3'-nt. A negative control of protein from the same bacterial culture prior to induction with IPTG is shown in the right hand panel.

Liverpool). After several different approaches failed, the only sequence obtained (from a non-specific tryptic digest) was of a contaminating bacterial protein and it was considered too expensive and time consuming to continue.

Some of the recombinant protein was also analysed for activity on SDS-PAGE. An aliquot of expressed protein equivalent (in terms of protein content) to the samples of *Leishmania* cell lysates routinely run on SDS-PAGE, was loaded onto a polyA substrate gel to identify any nuclease activity. One of the minor contaminating bacterial protein bands showed some activity but the major 55kDa expression product showed none.

5.5 Generation of specific antibodies

In order to generate antibodies specific for the 3'-nt of *L. mexicana*, the remaining calmodulin-purified recombinant protein was used to immunise a rabbit. An initial pre-immune bleed was taken to provide non-immune normal rabbit serum (NRS). Eight weeks later, a second pre-immune bleed was taken. The first antigen challenge was made using 250μg recombinant 3'-nt emulsified in Freunds complete adjuvant. The first immune bleed was taken four weeks later. Two weeks subsequently, an antigen boost was made using 250μg 3'-nt in Freunds incomplete adjuvant. The second immune bleed was taken three weeks later.

5.6 Western blots using anti-recombinant antibodies

Initial Western blots of starved *L. mexicana* promastigotes showed no antigen recognition with normal or immune serum. There was good self-recognition of eluate from the CBP purification process, which demonstrated the effective functioning of the method, although the smaller bands of contaminating bacterial proteins from the column

were recognised with equal strength to the major 55kDa expression product. Time did not allow an attempted immunoprecipitation of 3'-nt activity using this serum.

5.7 Gene knockout

A powerful and increasingly routine tool used in the study of gene function in *Leishmania* is the production of 'null mutant' parasites by 'knocking out' specific genes. The knockout procedure employs the tendency of trypanosomatid chromosomes to undergo homologous recombination during cell division. Obtaining a *L. mexicana* null mutant for the 3'-nt gene sequenced would be another way of linking gene sequence and function and may also demonstrate the role of 3'-nt in the development and growth of the parasite.

5.8 Background to gene knockouts

The procedure of gene knockout involves the introduction of 'foreign' DNA (whether a replacement gene or a disrupted gene) into the genomic DNA of the 'host' cell, in this case *L. mexicana.* The foreign DNA must first be introduced intact into the host cell (the transfection step) and subsequently into the host genome (the incorporation step).

A number of different plasmid constructs have been developed as carrier molecules for the import of engineered DNA fragments into protozoa (the transfection step). These plasmids contain both the apparatus necessary for replication within the host cell and the foreign DNA, bearing an antibiotic resistance marker for selection of positive recombinants on antibiotic supplemented media. Transfection is most commonly by electroporation.

The incorporation step is a rare event whereby the replicated foreign DNA may become incorporated into the genome by homologous recombination. This process is thought to involve the association of homologous DNA sequence in some way at the point of DNA replication and its subsequent exchange. In the natural process of cell division, this may result in the swapping of homologous alleles or the exchange of identical sequence between two sites normally distant in the genome. If the foreign sequence introduced into a living cell by transfection is homologous with a native gene, it raises the possibility of gene replacement by this same process. Thus a plasmid bearing enough of a specific gene and its flanking sequence to target the native gene locus but disrupted so that the gene will not be transcribed, could be used to knockout the native gene. In a diploid organism such as *Leishmania*, both alleles of the chosen gene would need to be knocked out in separate events to give a null mutant.

5.9 Initial strategy

Samples of knockout cassettes used by Dr. Jeremy Mottram's group (University of Glasgow; Souza *et al.*, 1994) were kindly provided. The plasmids used were modified from the pX plasmid backbone (Cruz *et al.*, 1991) and each cassette contained a different antibiotic resistance gene. Each separate knockout event should lead to the incorporation of a different antibiotic resistance marker so that double knockouts could be distinguished from single knockouts using media containing both antibiotics. The marker chosen for the first knockout was hygromycin resistance. Each cassette had a standardised array of cloning sites included in the sequence so that flanking *Leishmania* sequence and the antibiotic resistance markers could be freely exchanged between the different plasmid vectors (Fig. 5d). The cassettes needed adapting for use as 3'-nt

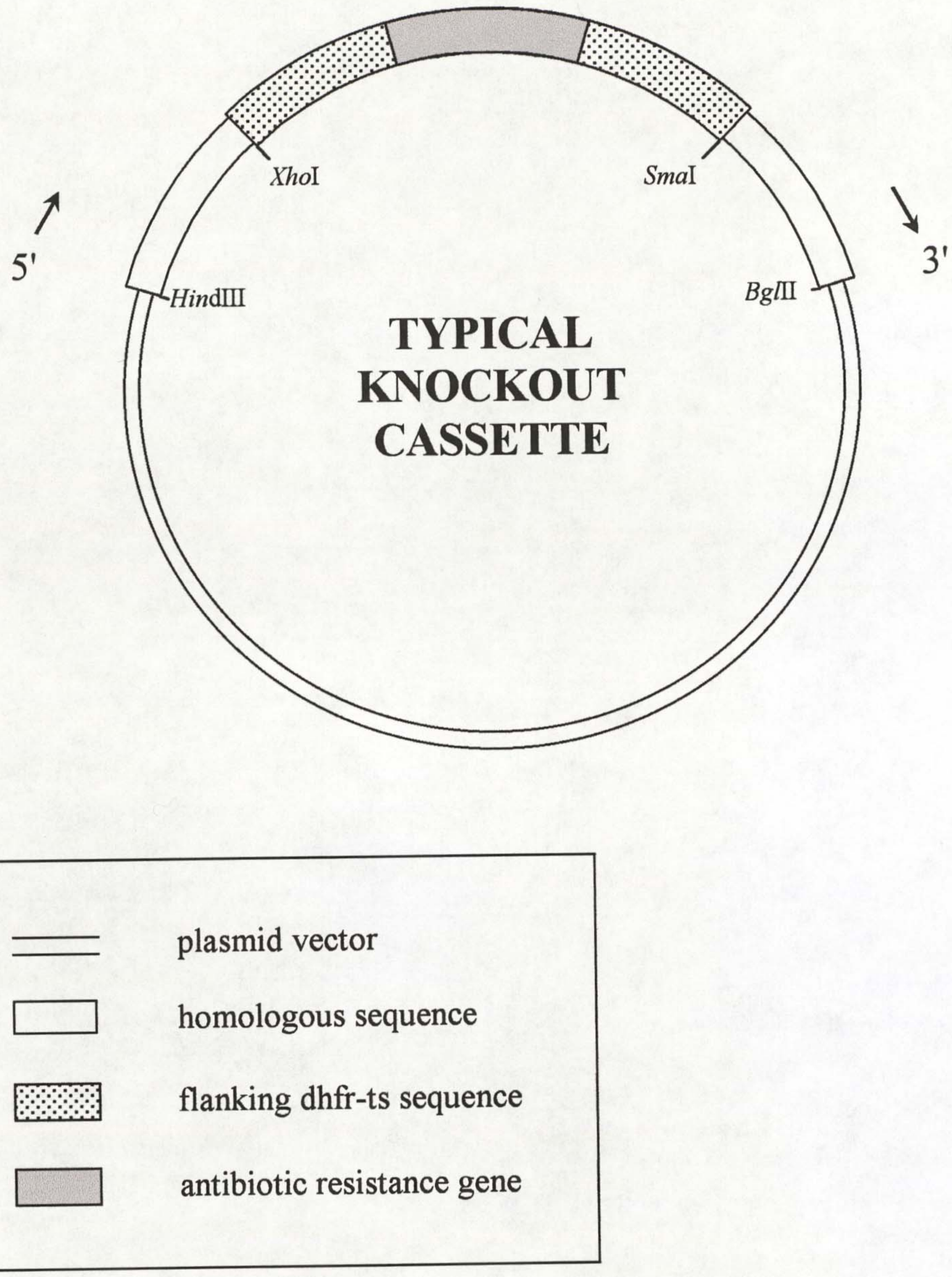


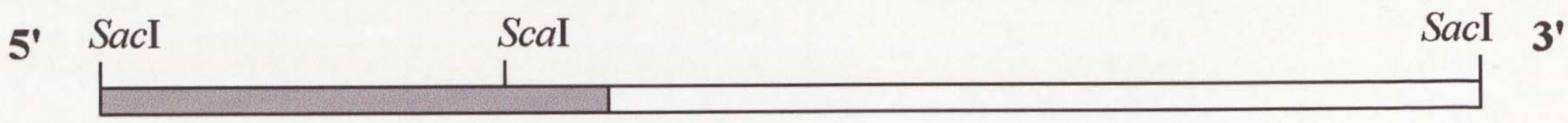
Figure 5d Simplified schematic of a typical knockout cassette plasmid showing the restriction sites used for cloning in flanking homologous sequence. The homologous sequence allows the specific targeting of the construct to host sequence. The antibiotic resistance marker allows the selection of positive recombinants. The backbone is based on the pSP6-T3 plasmid (adapted from Souza *et al.*, 1994) and contains an ampicillin resistance gene. In this figure, *Leishmania* dihydrofolate reductase-thymidylate synthase (dhfr-ts) sequences flank the antibiotic resistance gene to ensure its expression. For transfection, the section of homologous sequence bearing the antibiotic resistance marker is excised from the plasmid and introduced into the recipient cell as a linear fragment. On average, the length of flanking homologous sequence used in such constructs is about 1-2kb.

knockout vectors by the cloning in of *L. mexicana* 3'-nt sequence (at the sites of 'homologous sequence' in Fig. 5d). This could be achieved by PCR.

To clone *L. mexicana* 3' sequence into the cassette, flanking *Bgl*II and *Sma*I recognition sites were needed (as seen in Fig 5d). The section of nuc1 sequence (downstream from the 3'-nt gene) chosen for cloning already contained a *Sca*I recognition site (Fig 5e), which, when cut, gave a blunt end like *Sma*I. Therefore, a PCR product spanning this site would negate the need to engineer a *Sma*I site. The required 3' *Bgl*II site, however, was incorporated into the primer sequence of pcr3'II as a non-complementary 'tail' (Fig 5e). This primer was used in combination with pnuc2b to amplify a nuc1 fragment of approximately 2.3kb (Fig 5e). By performing a double digest using *Bgl*II and *Sca*I, a fragment with ends complementary to the similarly digested knockout cassette was obtained, and these could then be ligated.

Initial ligations were unsuccessful and it was thought that the efficiency of restriction digest of the PCR product was low. Successful digestion of the *Sca*I site was evident from the excision of a 0.4kb fragment that was visible on an agarose gel (Fig. 5e). *Bgl*II digestion, however, would only involve the cleaving of a few nucleotides from the 3' end of the PCR product and so much more difficult to evaluate. Longer digests were attempted and the fragment ethanol precipitated before using the second enzyme, to ensure each digest was performed in the optimal buffer conditions. There was still no ligation. In an attempt to improve the efficiency of digest, the fresh PCR product was first cloned using a TA cloning strategy, and the fragment subsequently excised from an intact plasmid. Although the desired fragment was generated in this way, positive transformants showing its ligation into the knockout cassette could still not be obtained.

In order to clone the sequence downstream from the ORF into the knockout cassette, it must be flanked by one blunt-ended site at the 5' end and a site compatible with *Bgl*II at the 3' end



nuc1 full sequence (3kb)

The blunt end could be provided by *Sca*I within the ORF by preparing a fragment spanning this site. The *Bgl*II site could be incorporated into a 3' primer (pcr3'II) as a non-complementary primer 'tail'.

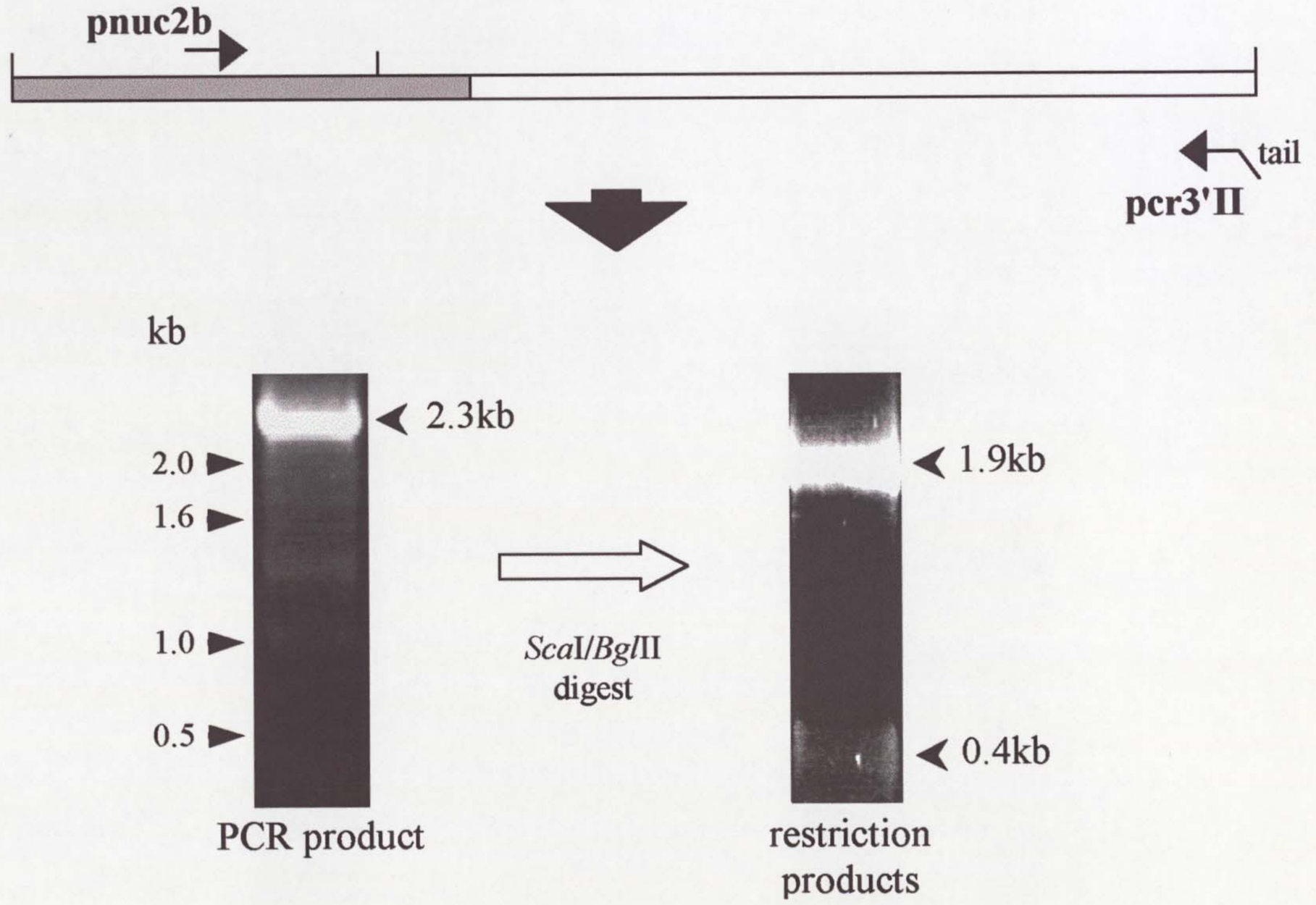


Figure 5e Schematic showing preparation of flanking sequence (by PCR) from the 3' end of nuc1. The resulting 2.3kb PCR product (shown on the bottom left panel) was digested with *Sca*I and *Bgl*II, as described, to give complementary ends for cloning. This digestion yielded a 1.9kb fragment (right hand panel) that was subsequently ligated into the prepared knockout cassette. In this figure, the two panels are aligned and the DNA molecular mass markers shown on the left.

5.10 Second strategy

At this point in time, knockout cassettes for the *L. donovani* 3'-nt gene became available (Fig. 5f), kindly provided by Dr Alain Debrabant (NIH, Bethesda). It was decided, on the basis of the high sequence identity between the two species and the obvious time commitment of preparing *L. mexicana* specific cassettes, to try these cassettes directly in *L. mexicana*. The section of the hygromycin cassette bearing the *Leishmania* sequence and the antibiotic resistance marker was excised using *Bam*HI and *Kpn*I and purified from agarose.

This DNA fragment was sterilised through an ethanol precipitation and used to transfect *L. mexicana* promastigotes by electroporation according to the standard protocol. The transfected cells were stabilised in culture, plated out on medium-supplemented agar (containing hygromycin) and grown at 26°C. After one week, it was obvious that the plates had become fungally infected. The process was repeated using fresh DNA, cells and reagents but after three weeks of observation, there were no hygromycin resistant colonies evident.

5.11 Conclusions

Using antibodies generated from recombinant 3'-nt gene sequence, this gene has been linked to native 3'-nucleotidase activity in *L. mexicana*. The gene has also been specifically linked by immunoprecipitation to the 40kDa band of nuclease/nucleotidase activity seen in *L. mexicana* and *L. donovani*. This supports studies of the *L. donovani* 3'-nt gene, which show it to encode the previously described surface membrane enzyme thought to be involved in the acquisition of extracellular purines. The failure to produce single knockouts of 3'-nt in *L. mexicana* using the *L. donovani* cassettes may be due to

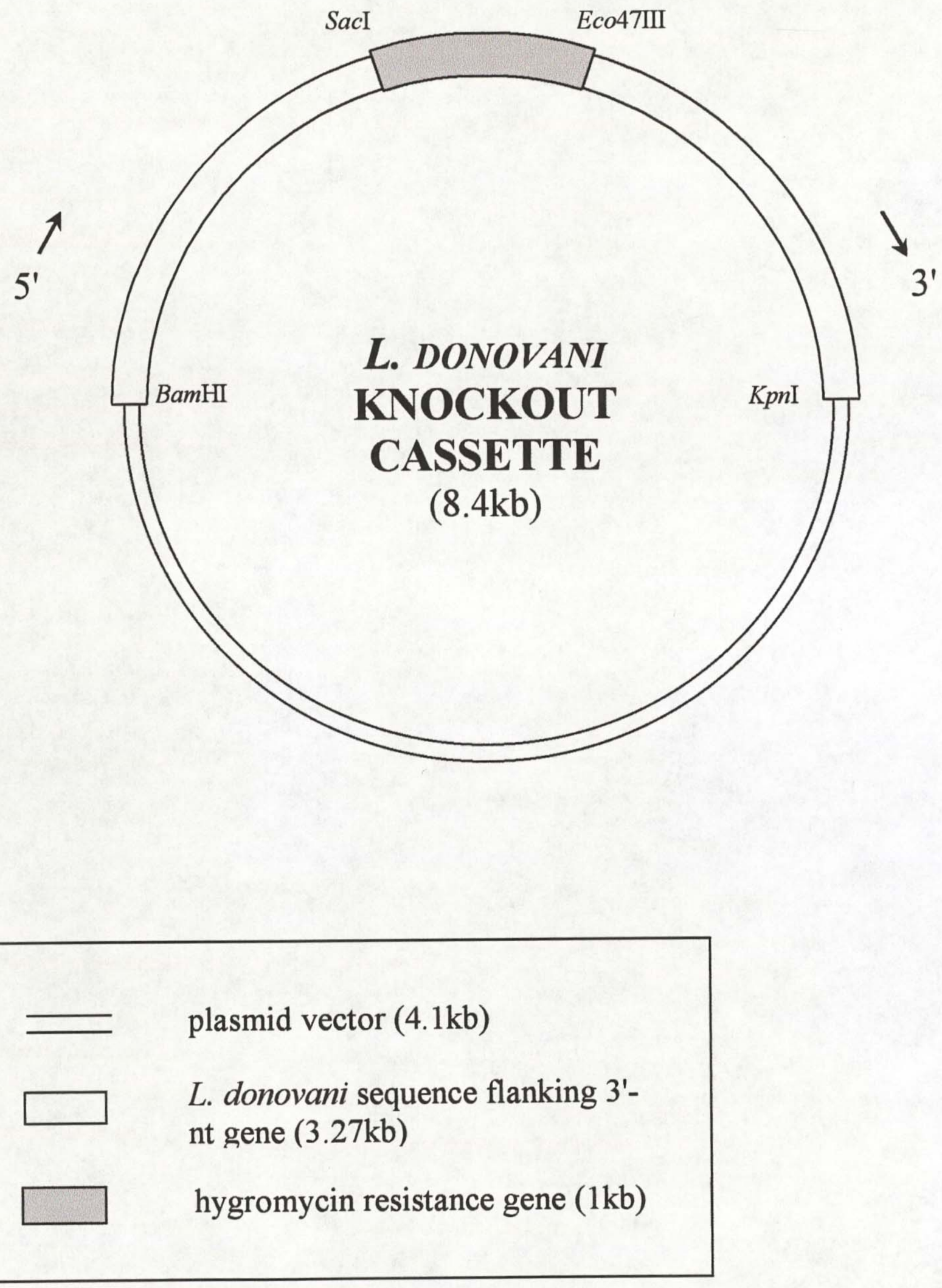


Figure 5f Simplified schematic of the *L. donovani* knockout cassette plasmid. The plasmid backbone is the same as that in Fig. 5d. A gene for hygromycin resistance is incorporated directly into a 3.27kb fragment of genomic sequence, cloned from the *L. donovani* 3'-nt locus. This construct contained no additional dhfr-ts sequence. As with the constructs previously described, the homologous sequence bearing the antibiotic resistance gene was introduced into recipient *Leishmania* cells as a linear fragment.

insufficient quantity of DNA for transfection or a low rate of transfection. It may be that the gene locus in each species is sufficiently different to prevent homologous recombination between the two. Due to restrictions of time, it was decided to abandon the attempts to produce null mutants of the gene and use other methods to assess the function of the 3'-nt protein encoded for by the reported gene sequence.

CHAPTER 6

FUNCTION AND REGULATION OF THE 3'-NUCLEOTIDASE

6.1 Introduction

The function and regulation of 3'-nt in the *L. mexicana* life cycle could be assessed by means of enzyme assay. Assays for nuclease and nucleotidase activities in *Leishmania* are well established and these methods were used to investigate the function of the 3'-nt in terms of its activity in different forms of the parasite and under various conditions of culture.

6.2 The basis of the enzyme assays used

General nuclease activity was assessed in terms of polyA digestion. PolyA stains a deep blue colour in the presence of toluidene blue. In order to identify particular nuclease activities within cell lysate mixtures, proteins were separated by SDS-PAGE on polyacrylamide gels containing polyA. Following electrophoresis, the gels were incubated under conditions of optimal nuclease activity. Subsequent staining with toluidene blue reveals a background of stained polyA with cleared stain at points of nuclease activity where the polyA has been digested. In this way, particular bands of protein with nuclease activity may be identified within cell lysates.

To identify specific 3'-nt activity in similar SDS-PAGE gels, the unstained polyacrylamide was incubated in the presence of 3'-AMP (again under optimal digestion conditions). Free phosphate was released at those sites of 3'-AMP digestion and detected by malachite green staining. A dark green precipitate indicated the presence of free

phosphate (in this case equivalent to 3'-nt activity as 3'-AMP was the only substrate). 3'-nt activity could also be measured spectrophotometrically using the same malachite green reaction in a microfuge tube assay. This reaction only measured the total 3'-nt activity of a sample whereas the gel-based assay could distinguish different bands of protein activity.

6.3 Nuclease and 3'-nt activities in different life cycle stages

Cell pellets of the three main stages of the *L. mexicana* life cycle (amastigote, procyclic promastigote and metacyclic promastigote) were prepared as previously. Nuclease and 3'-nt activities in whole cell lysates of each stage were assessed by substrate SDS-PAGE, using both polyA gels and malachite green staining. Equivalent concentrations of protein from each lysate were run on the gels.

The results (Fig. 6a) show a nuclease activity doublet of 29/31kDa common to all stages of the life cycle, though apparently greatest in the amastigote form, and a second major activity of about 40kDa only in the two promastigote forms, but greatest in the procyclic. These results support previous observations of a gradual appearance of the 40kDa nuclease activity as amastigotes transform to promastigotes and then a decrease again through the metacyclic stage back to amastigotes (Bates, 1994a). The 40kDa promastigote activity appears to be a doublet but corresponds to a single band of 3'-nt detected in the malachite green gel (no 3'-nt activity was detected in metacyclic promastigotes). If the mature 3'-nt protein is glycosylated as suggested by the translated gene sequence, this doublet may correspond to the glycosylated and non-glycosylated forms of the enzyme. The nature of substrate gels makes it difficult to clearly define

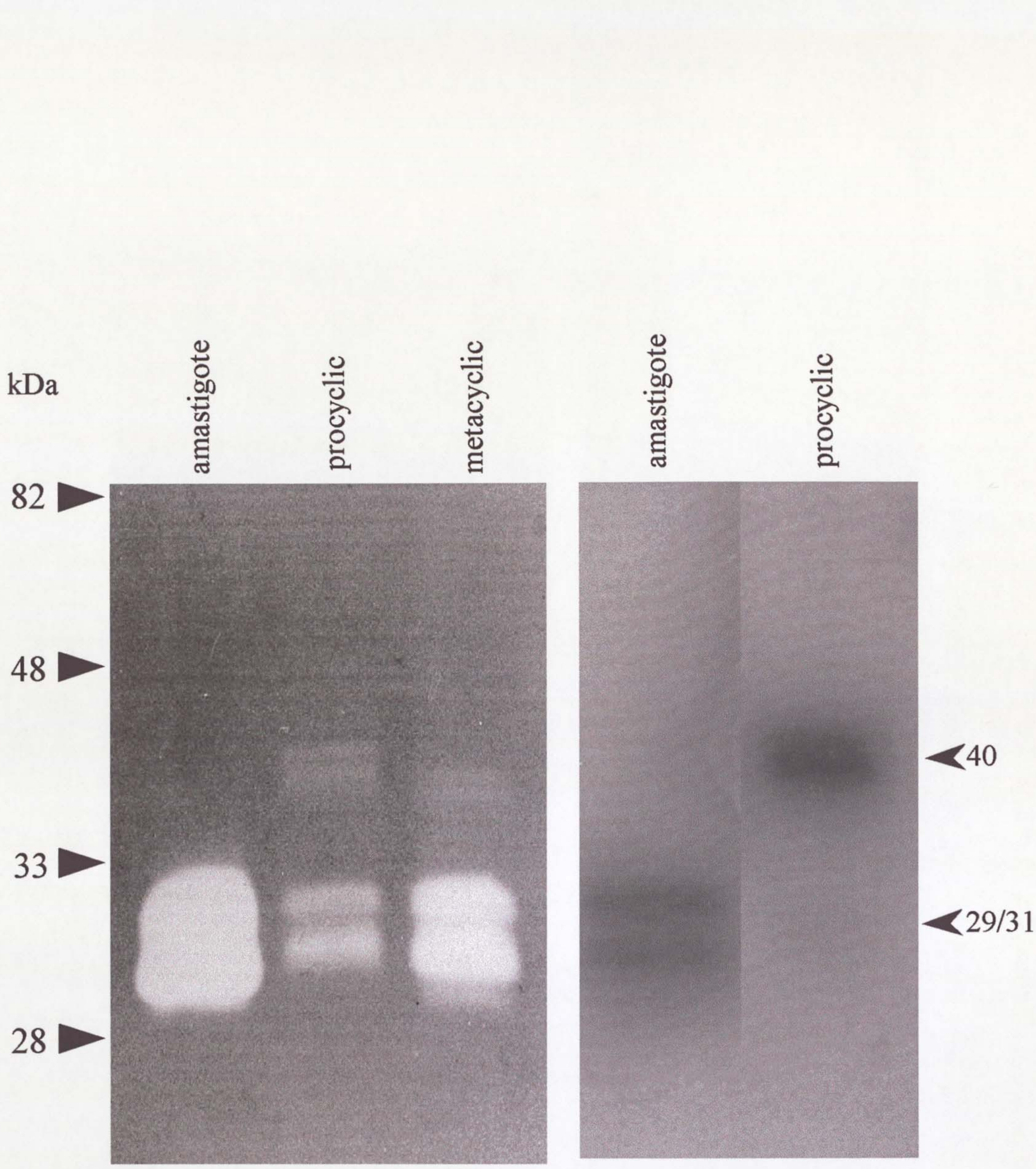


Figure 6a SDS-PAGE substrate gels showing nuclease and 3'-nt activities in different life-cycle stages of *L. mexicana.* Identical cell lysates of amastigote and pro- and metacylic promastigote samples (from *in vitro* culture) were run on both nuclease and 3'-nucleotidase gels. The gels were run simultaneously in the same electrophoresis chamber. Protein markers are shown on the left and the approximate sizes of bands of activity detected shown on the right (all in kDa). The gels are aligned in this figure and the 3'-nucleotidase activity observed (none of which was detected in metacyclic promastigotes) appears to be associated in size with bands of nuclease activity.

bands of precise activity on the malachite green gel, and the apparent single band of 3'-nt activity at 40kDa may conceivably be a close doublet.

The doublet of 3'-nucleotidase activity in the amastigote cell lysate is much clearer at about 29/31kDa. Both regions of nucleotidase activity (in the right panel of Fig. 6a), in promastigotes and amastigotes, correspond in size to regions of nuclease activity (left panel). At this level of measurement, the 29/31kDa doublet of 3'-nucleotidase activity seen in amastigotes is not seen in promastigotes. The strength of signal at 29/31kDa in the nuclease gel, compared to the corresponding 3'-nucleotidase activity, suggests that this is the predominate activity of this band in amastigotes. However, in the promastigote sample, it is the 3'-nucleotidase activity of the 40kDa band (compared to a relatively weak corresponding nuclease activity) that appears to predominate. Size predictions of the translated sequence of the 3'-nt gene support this upper band's identity as the *L. mexicana* 3'-nt, suggested by the previous immunoprecipitation experiments. The lower band of 3'-nucleotidase activity seen in the amastigote may be associated with a different gene, suggested by the absence of 3'-nt message in the amastigote form (shown previously by northern blot).

6.4 Comparison of enzyme activities with other cultures

These results were compared with those obtained in *L. donovani* and with fresh *L. mexicana* material. Previously published results from *L. donovani* suggest that the 3'-nt is constitutively expressed in this species through all forms of the life cycle, in contrast to the stage-specific results obtained for *L. mexicana*. The *L. donovani* cells used in these experiments had been derived from axenically cultured promastigotes and transformed into the various forms artificially. Measuring nuclease and 3'-nt activity in

amastigotes isolated directly from an infected mouse, the situation in *L. donovani* actually appeared to mirror that in *L. mexicana* more nearly than previously thought (Fig. 6b).

The nuclease activity of *L. donovani* amastigotes appears very similar to that in *L. mexicana*. There is a large doublet of activity at about 29-31kDa, comparable in magnitude to that of axenically generated *L. mexicana* amastigotes (Fig. 6a). These lower bands of nuclease activity in the fresh *L. mexicana* lesion amastigote material (Fig. 6b) are stronger and more diffuse than in the axenically derived cells and this was also seen to be the case in promastigotes derived from fresh lesion material.

The fresh *L. donovani* amastigotes also appear to have a faint band of nuclease activity at 40kDa, equivalent in size to that seen in the promastigote form of both species. In axenically cultured forms, this doublet is much stronger and led to the belief that 3'-nt was constitutively expressed in *L. donovani*. In order to assess whether this nuclease band did in fact have 3'-nt activity, the same samples of fresh amastigote material were run on 3'-AMP substrate gels and stained with malachite green (Fig. 6b). Again, the situation in *L. mexicana* and *L. donovani* appears to be more similar than originally thought. There is no 3'-nt activity evident at 40kDa but a single lower band of similar size to the doublet already described in *L. mexicana*.

The similar pattern of nuclease and nucleotidase activities in *L. mexicana* lesion amastigotes and axenic amastigotes suggest these cells are similar and that exposure to long-term artificial culture does not significantly alter the properties and expression of these enzymes. However, the apparently elevated activities of the enzymes in cells

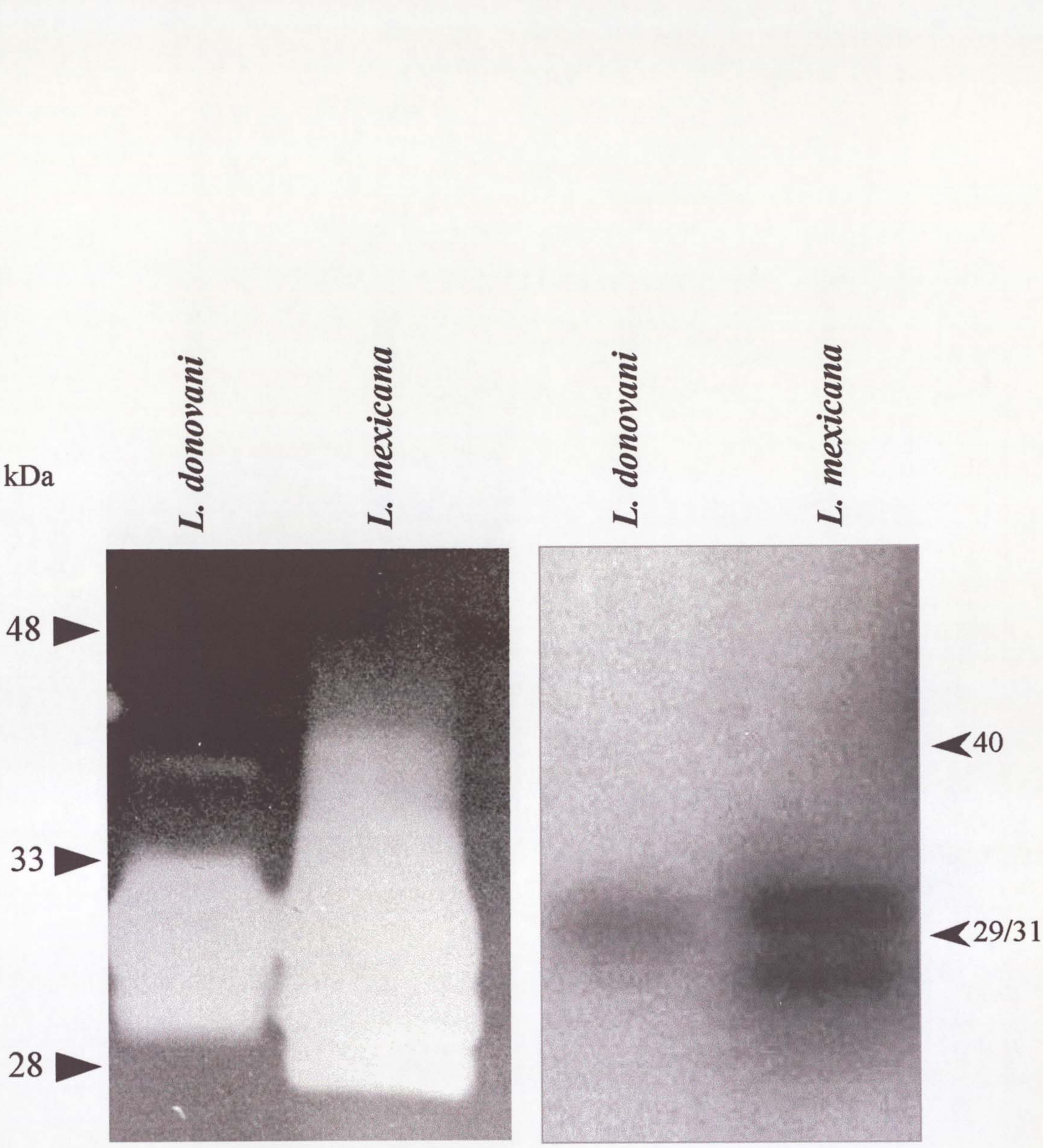


Figure 6b SDS-PAGE substrate gels showing nuclease and 3'-nt activities in *L. mexicana* and *L. donovani* amastigotes. Amastigotes of the two species were isolated directly from lesions in a mouse and hamster respectively. As in Fig. 6a, the gels are aligned and the markers and band sizes are shown (in kDa). Equivalent numbers of cells from each species were lysed and run on the gels. Although there is a distinct 40kDa band of nuclease activity apparent in *L. donovani* cells (left panel), it does not appear to possess 3'-nt activity (right panel) as previously thought.

prepared from fresh lesions, though not studied in detail, may indicate a downregulation of activity in response to optimal culture conditions. Contradictory to previous assessments, the observed stage-specificity of 3'-nt in *L. mexicana* appears to be maintained in the *L. donovani* enzyme.

6.5 Localisation of 3'-nt activity in cell membranes

It has been suggested that the 3'-nt is situated in the cell membrane and this position may be indicative of its function as a supplier of free purine nucleosides to the *Leishmania* cell. This was tested using a crude membrane preparation of *L. mexicana* procyclic promastigotes. The cells were lysed by repeated freeze/thaw in dry ice and the fragmented cell membranes precipitated by centrifugation. This crude method separated the cytosolic components of the procyclic forms from the membrane-associated components and samples of each were run on a nuclease substrate SDS-PAGE gel (Fig. 6c). The results show a large proportion of the 40kDa 3'-nt band partitioning into the membrane fraction. Comparison of 3'-nt activity (by tube assays) in intact and lysed promastigotes suggested approximately 70% of total cellular activity was found on the cell surface (results not shown). Aliquots of the same protein samples were also run on a 3'-nucleotidase substrate gel (Fig. 6c) and this confirmed the partitioning of 3'-nt activity to the membrane fraction.

6.6 The effect of purine starvation on 3'-nt activity

It is reported in the trypanosomatid *Crithidia luciliae* that 3'-nt activity is upregulated 1000-fold under conditions of purine starvation. This observation is one of the main sources of evidence to suggest the importance of 3'-nt to the parasite. Activity upregulation is also described in *L. donovani* and *L. mexicana*. To investigate this

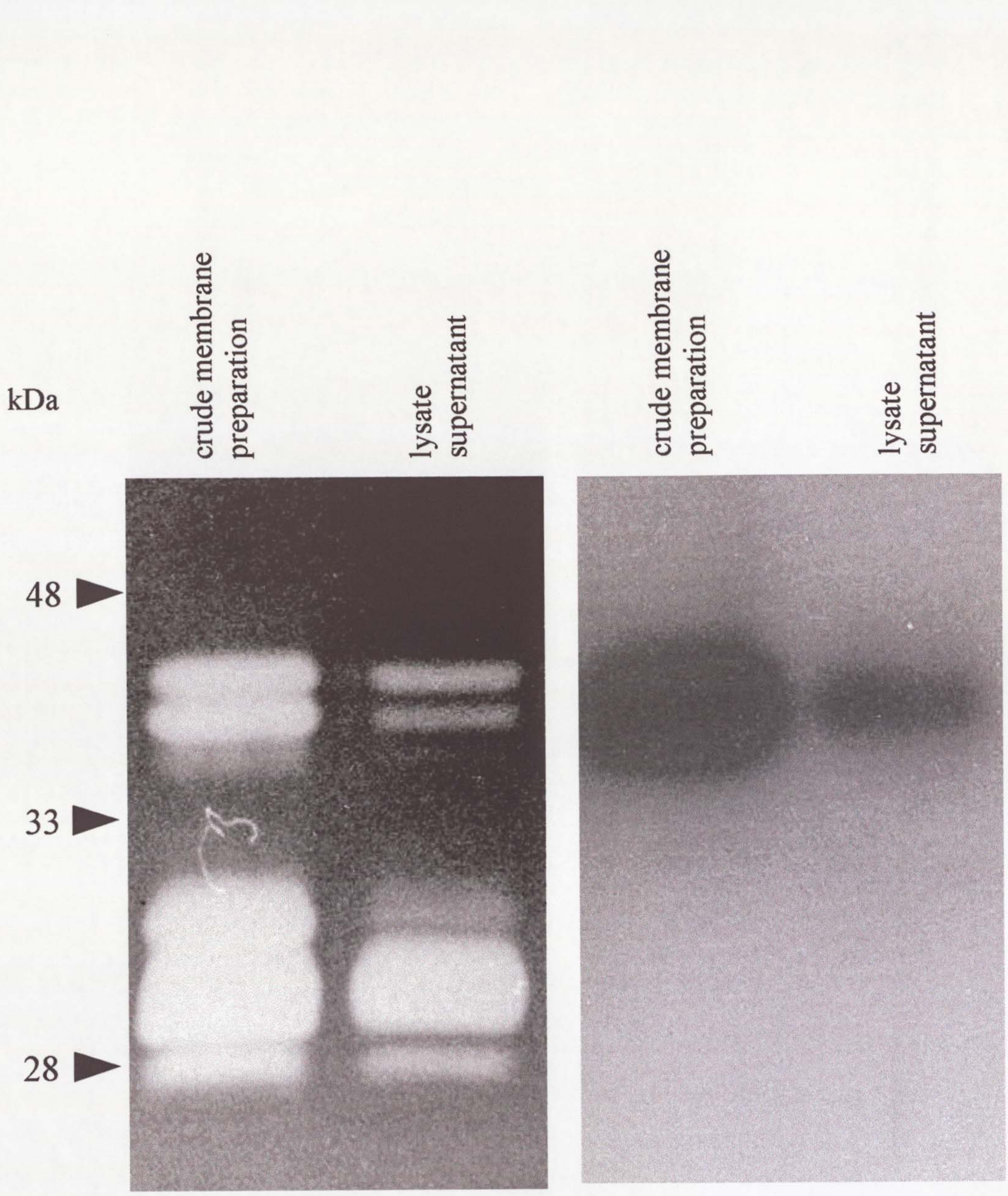


Figure 6c SDS-PAGE substrate gels comparing nuclease and 3'-nt activities in crude membrane extracts of *L. mexicana* promastigotes with activities in the residual lysate supernatants. Equivalent concentrations of protein were loaded in each lane. As previously, the samples were run simultaneously on the nuclease and nucleotidase gels and the relevant panels aligned in this figure with the protein markers. A large proportion of the 40kDa nuclease doublet (left hand panel) appears to have partitioned to the crude membrane extract. This can be seen to correspond to the partitioning of 3'-nucleotidase activity also (right hand panel), which is greatly enriched in the membrane fraction.

observation further, cultured *L. mexicana* procyclic promastigotes were adapted to serum-free, chemically defined medium. The source and nature of purines supplied to the cultures could thus be controlled. Routinely, the defined medium was supplemented with adenosine for normal growth.

In order to assess the effect of starvation, aliquots of late log phase promastigote culture were passaged into defined medium (without adenosine) and cell growth and 3'-nt activity compared with routinely passaged adenosine-supplemented cultures. The cells passaged were not washed prior to their transfer into purine free medium so there was initially residual adenosine available to the cells. It was hoped that this initial source of purine would enable sufficient cell growth for enzyme activity measurements but with subsequent starvation as the source was not renewed.

Cell pellets of normal and purine-starved procyclics were collected over a number of days and their respective 3'-nt and nuclease activities compared using substrate gels (Fig. 6d) and tube assays (Fig. 6e). There is a marked and persistent upregulation of 3'-nt activity in the purine starved cultures over seven days of growth and a corresponding decrease in growth rate. The purine-replete cultures show initial expression of 3'-nt activity which, on average (from a number of similar experiments), begins to decrease within 48 hours of passaging, finally becoming negligible after about four days. It can be seen from Figure 6e that this constitutive expression begins to decrease at the onset of log phase growth.

To study this initial expression of 3'-nt activity, a closer time course was measured for starved and replete cultures between zero and 168 hours (Fig. 6f). The results were

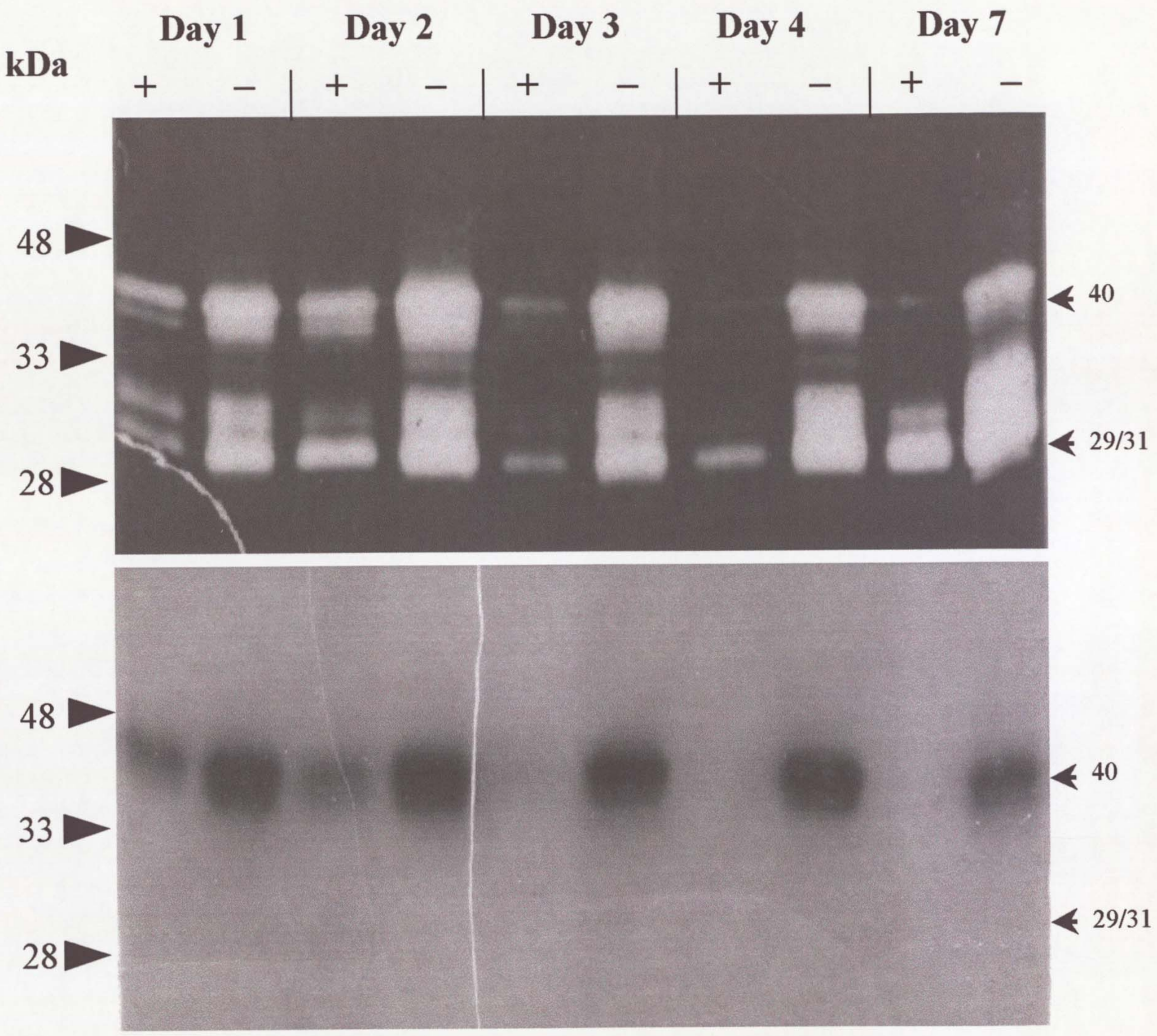


Figure 6d SDS-PAGE substrate gels comparing nuclease and 3'-nt activities in *L. mexicana* promastigotes grown in normal (+) and purine-depleted (-) medium. Equal aliquots of cells were removed from culture each day for 7 days after passage into the respective media. These aliquots were frozen until use for SDS-PAGE. Equivalent concentrations of protein were run in each lane. Nuclease (top panel) and 3'-nucleotidase (lower panel) activities were assessed for each sample. Protein markers are shown on the left and approximate band sizes on the right. Upregulation of the 40kDa nuclease and corresponding 3'-nucleotidase activity appears to be sustained in the purine-depleted cultures throughout the 7 day period.

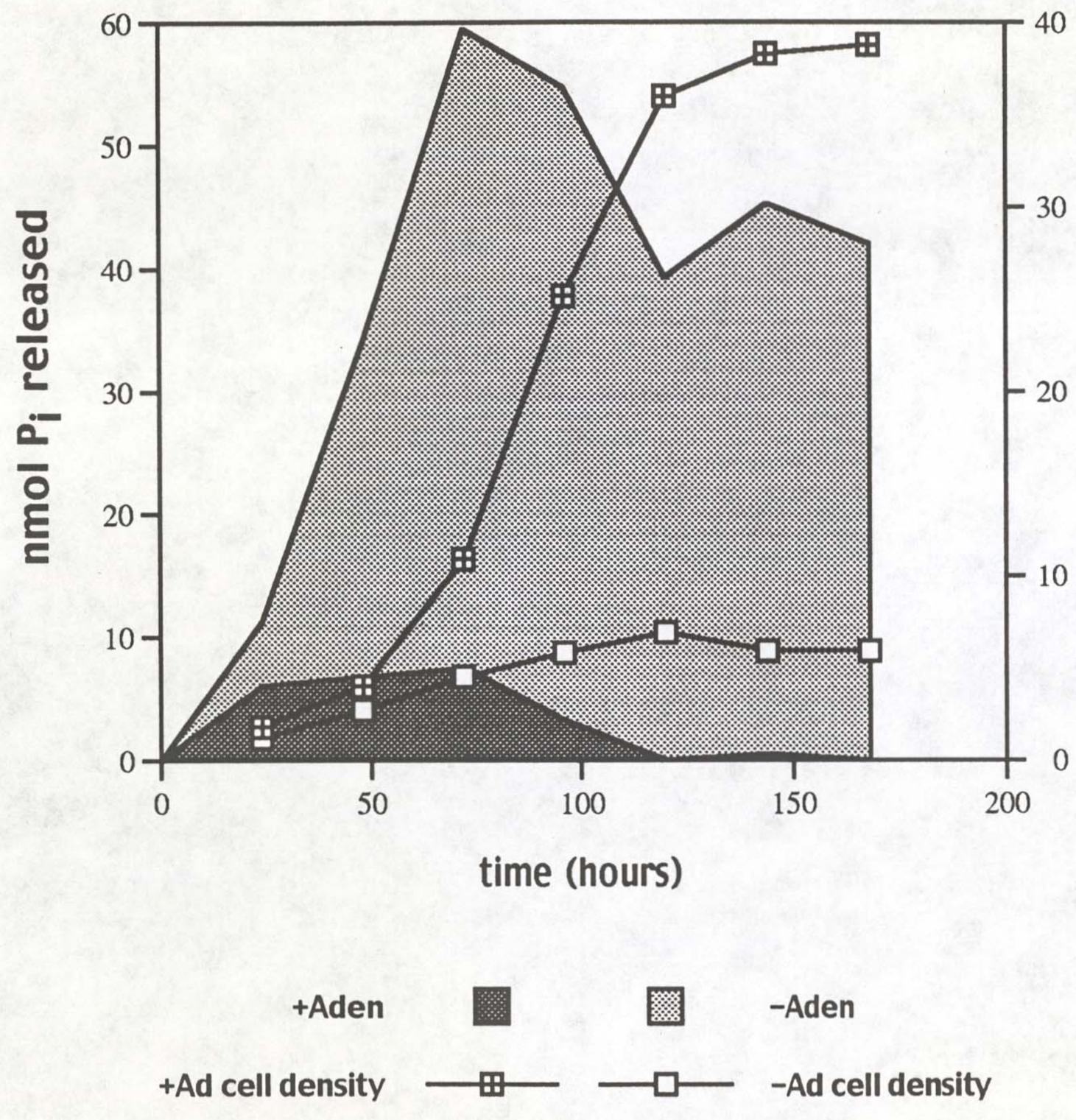


Figure 6e Tube assay results comparing 3'-nt activities in *L. mexicana* promastigotes grown in normal (+Aden) and purine-depleted (-Aden) medium over 168 hours. 3'-nt activity is denoted by the levels of inorganic phosphate (P_I) released during the assay and measured at 630nm. Also plotted are the cell densities of each culture.

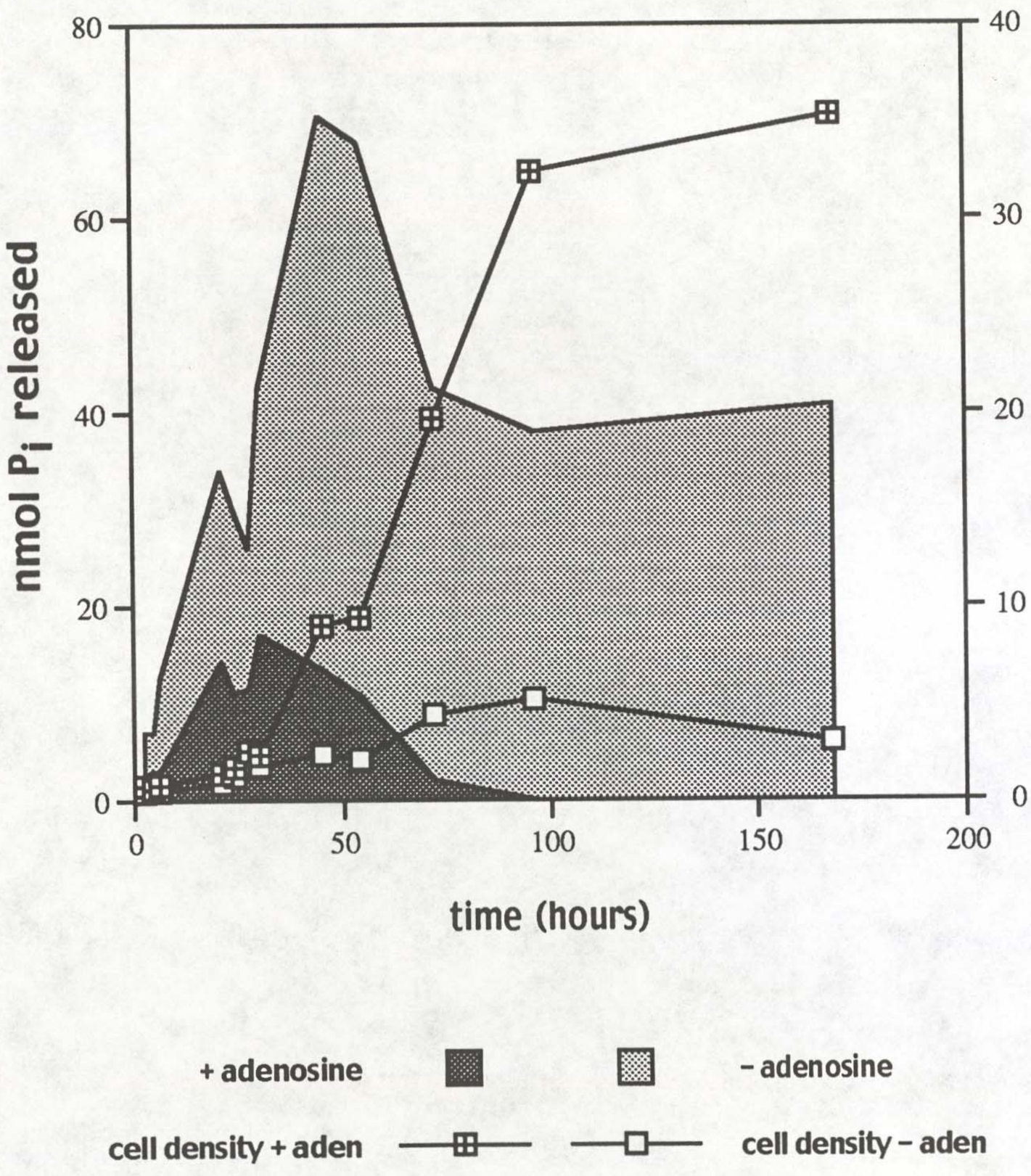


Figure 6f Tube assay results comparing 3'-nt activities in *L. mexicana* promastigotes grown in normal (+adenosine) and purine-depleted (-adenosine) medium over 168 hours. Aliquots were taken at frequent intervals between 0 and 48 hours. Also plotted are the cell densities of each culture.

confirmed by this time course which also showed that initial growth rates and nucleotidase activities were very similar in normal and starved cells for the first 24 hours of measurement. Although sustained at elevated levels throughout the 7-day period, 3'-nt levels in the two starved cultures (Figs. 6e & 6f) also show, in common with the purine-replete cultures, an initial peak of activity.

The initial expression of 3'-nt even in conditions of plentiful and available purines suggests it is a constitutive enzyme, possibly downregulated in the presence of purine nucleosides. This was tested by adding 3'-AMP (at the concentration normally used in supplemented medium) back to starved cultures and measuring the effect on 3'-nt activity by tube assay (Fig. 6g). The results show that the observed upregulation of activity in starved cultures was reversed by the addition of purine. This reverse was not observed in cultures where free phosphate was added back, indicating the importance of the purine component of digested 3'-AMP and not the phosphate. This suggests *L. mexicana* is able to regulate 3'-nt expression in response to its environment by some sort of feedback mechanism.

Northern blots of the starved promastigotes show an increased level of 3'-nt message after 48 hours, compared with purine replete promastigotes (Fig. 6h). At 48 hours, the abundance of 3'-nt message is almost equal in the two cultures but at 72 and 94 hours, there appears to be an upregulation of 3'-nt expression. This increase of 3'-nt transcribed message approximately correlates with the upregulation of 3'-nt activity described in starved promastigotes and may be indicative of gene expression being regulated by the modulation of mature mRNA levels.

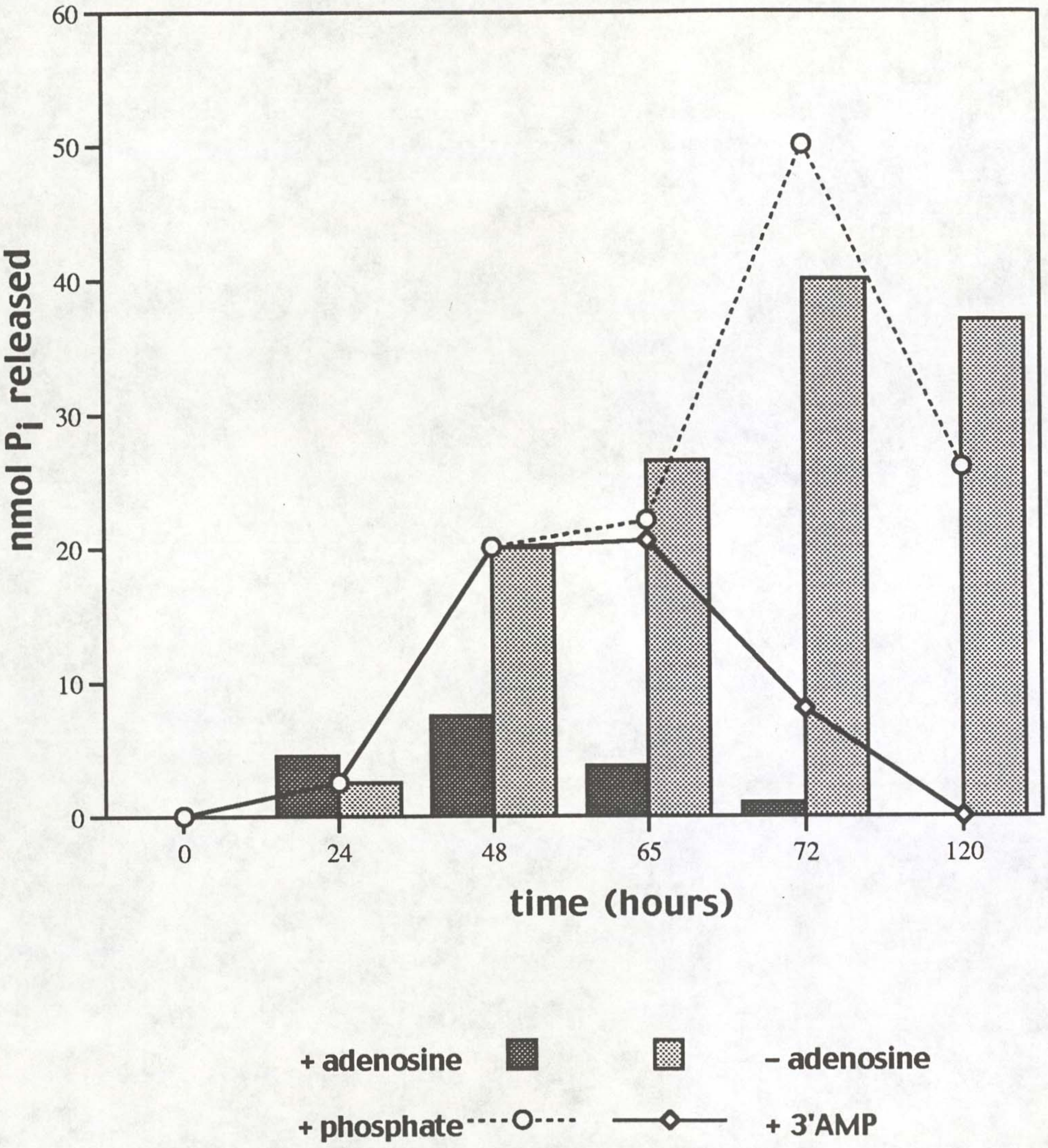


Figure 6g Tube assay results comparing 3'-nt activities in *L. mexicana* promastigotes grown in normal (+adenosine) and purine-depleted (-adenosine) medium over 120 hours. After 48 hours, the depleted culture was split into three. One part was unchanged (continuing bars), one part was supplemented with P_I (dashed line) and one part supplemented with 3'-AMP (solid line).

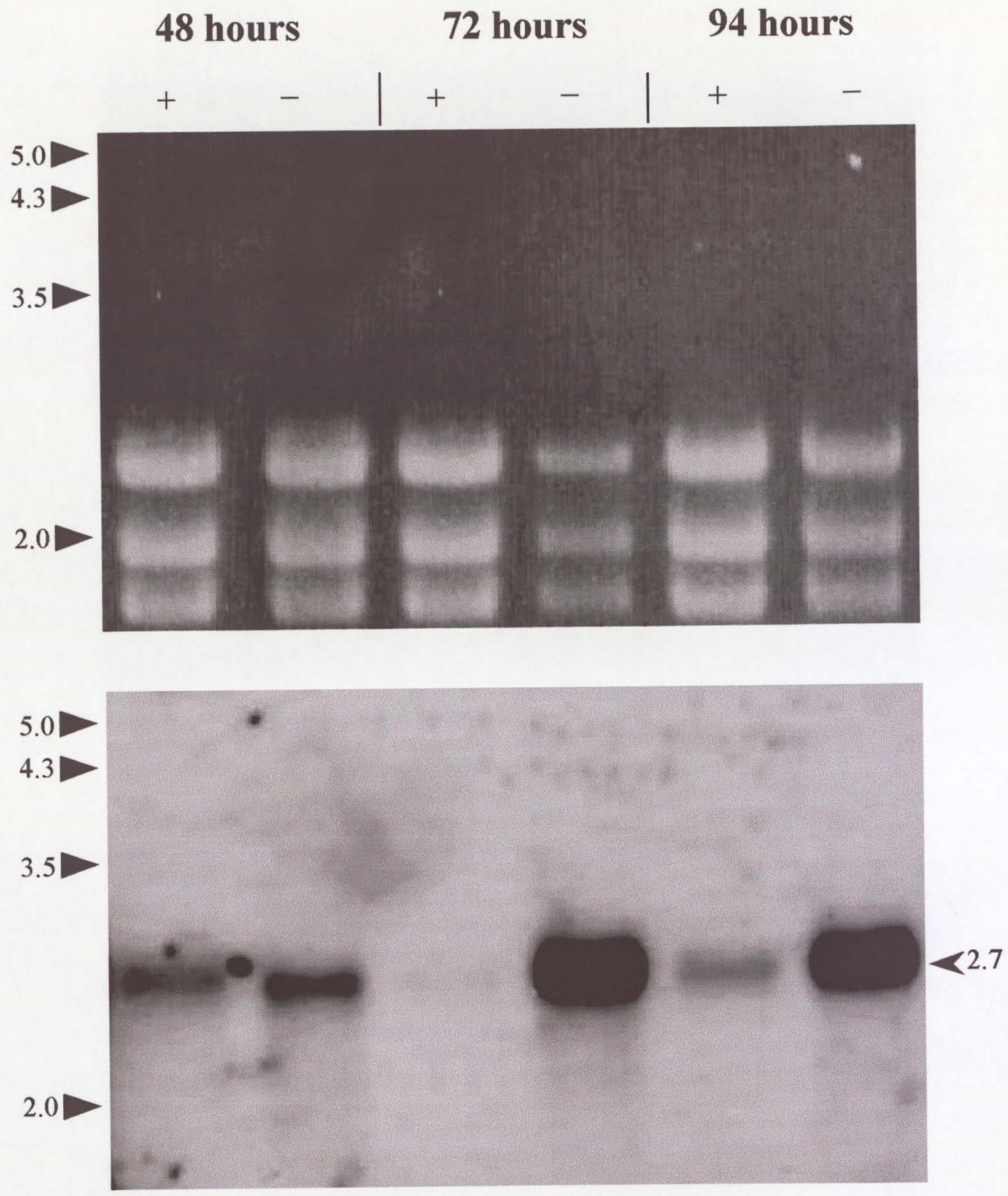


Figure 6h Northern blot showing 3'-nt expression in purine depleted *L. mexicana* promastigote cultures. Total RNA was isolated from equal numbers of adenosine replete (+) and adenosine depleted (-) cells over a period of 94 hours. Equal concentrations of RNA (5μg) were loaded in each lane of the agarose gel and the ribosomal bands stained with ethidium bromide (top panel). After transfer to a nylon membrane, the blot was hybridised with DIG-labelled LmORF (lower panel). The blot was developed for 90 minutes and the comparative abundance of each message estimated by eye. DNA molecular weight markers are shown on the left and the approximate hybridised band size on the right.

6.7 Behaviour of *L. mexicana* on different sources of purine

In an attempt to discover how tightly the 3'-nt was regulated in response to environment, defined cultures of procyclics were adapted to a variety of purine sources in supplemented media. In addition to adenosine, cultures were adapted to, and regularly passaged in, defined medium containing polyA, 5'-AMP and 3'-AMP. PolyA requires nuclease digestion in order for purine nucleosides to be available for import into the cell and 5'-AMP and 3'-AMP require 5'-nt and 3'-nt digestion respectively. After several months of continuous culture, samples of each of these cell populations were measured for 3'-nt activity. The cell samples were taken from cultures 48 hours after passaging and the nuclease profiles of each adapted cell line appeared identical on substrate gel (Fig. 6i). Total RNA was also prepared from these cells and Northern blots showed equal transcription of 3'-nt message (judged by eye) in all the adapted cultures (Fig. 6j).

To monitor activity more closely, samples of each adapted culture were taken over a period of four days and 3'-nt activity measured for each. The growth rates of each culture were similar although the 5'-AMP-adapted cells grew most rapidly. Levels of 3'-nt activity were measured by tube assay (Fig. 6i). All the adapted cultures, in common with the cells grown in defined medium with adenosine, showed initial 3'-nt activity coinciding with the onset of log phase growth. This activity peaked about one day into the log phase and then decreased again to a baseline level. The rate of activity decrease in each culture, however, varied according to the purine source. The culture adapted to 5'-AMP appeared to lose 3'-nt activity most rapidly, then the 3'-AMP culture and finally the polyA culture, which appeared to maintain 3'-nt activity at peak levels for at least a further 24 hours.

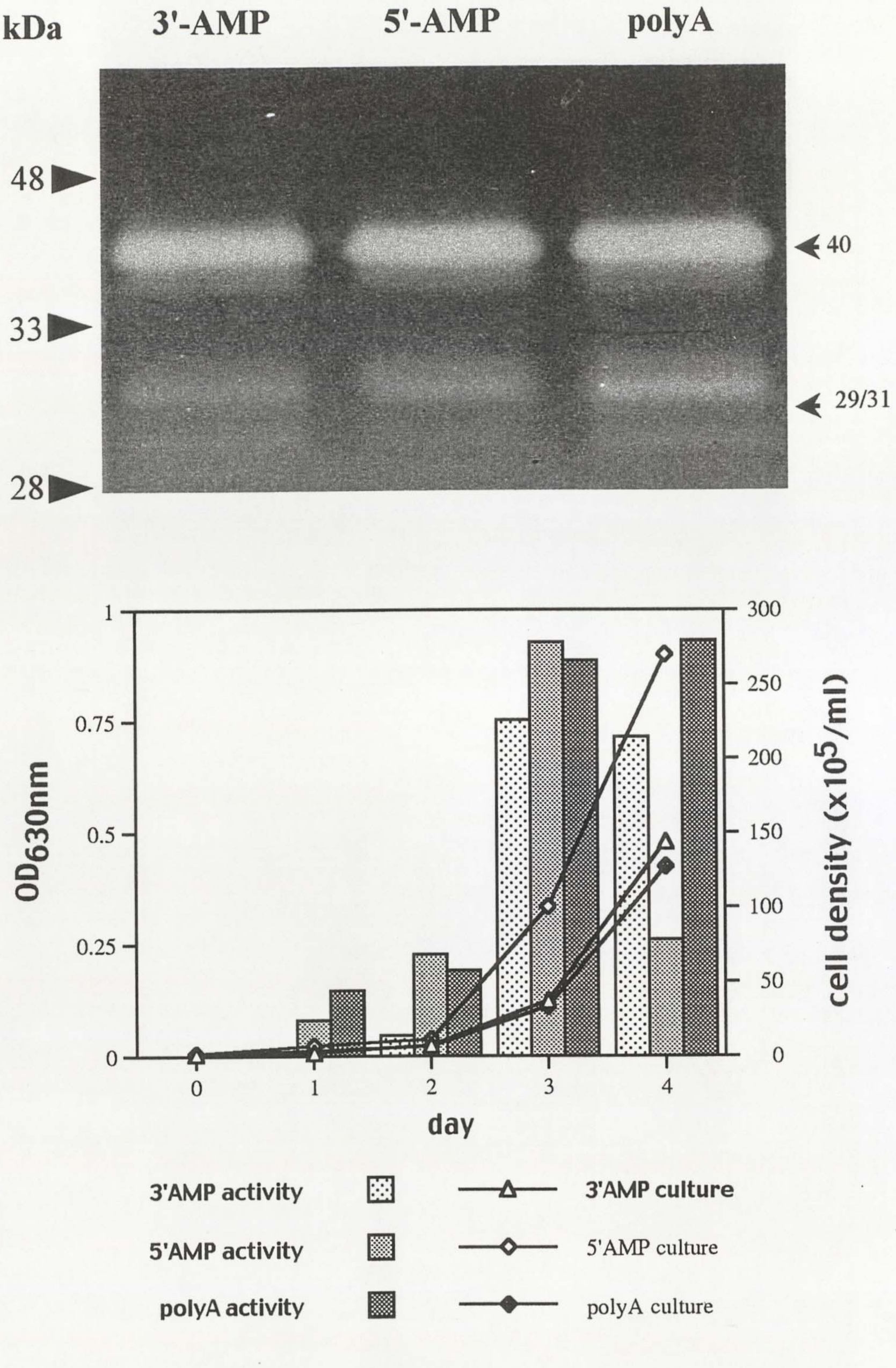


Figure 6i SDS-PAGE substrate gel comparing nuclease activities in *L. mexicana* promastigotes adapted to culture medium with various sole sources of purine. The nuclease profiles appear very similar to each other and to adenosine-adapted cultures. The corresponding graph of 3'-nt activity over four days of growth in each culture show some differences. Also plotted are the cell densities of each culture over this time period. (NB. $OD_{630} \equiv P_I$ released)

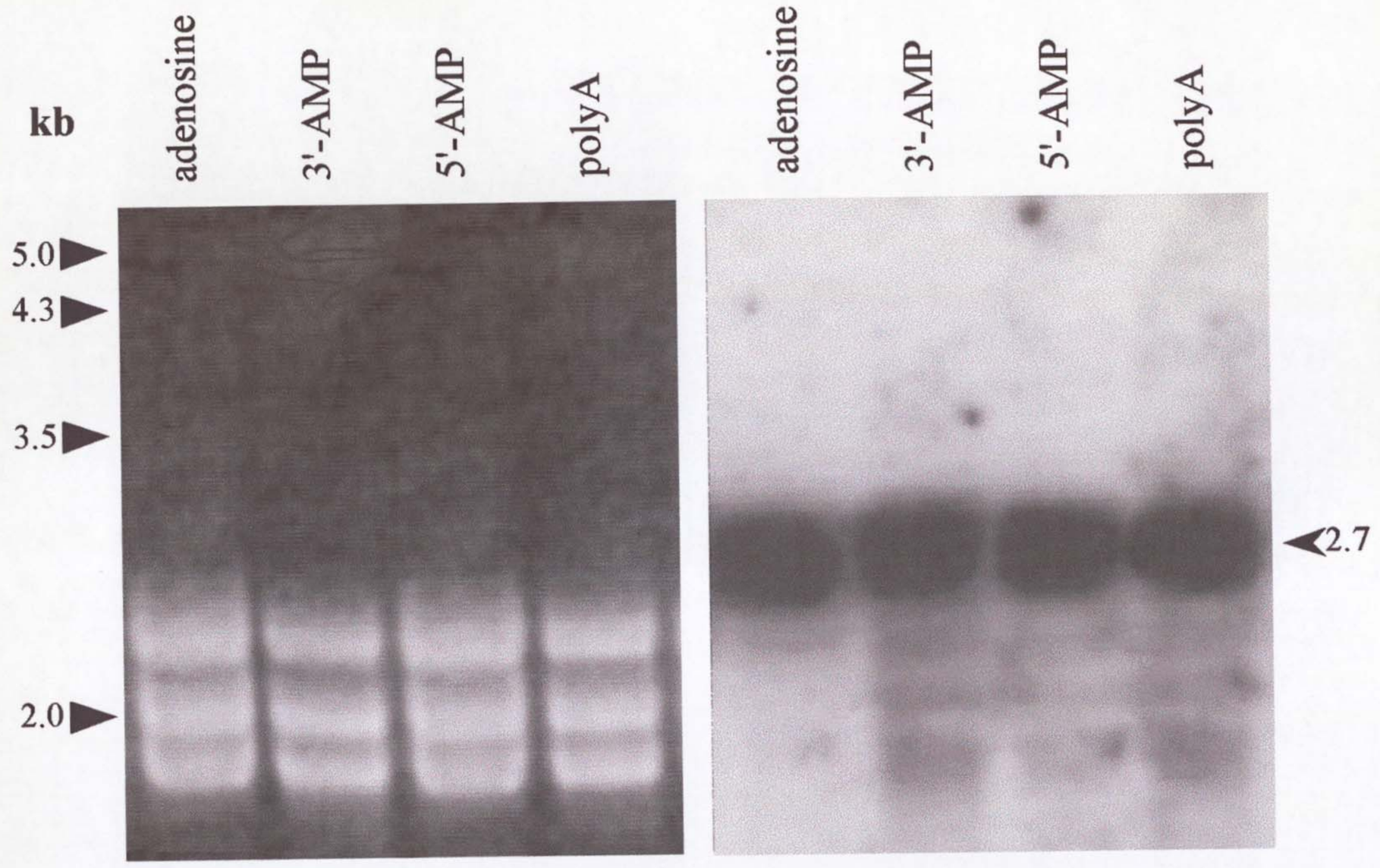


Figure 6j Northern blot showing 3'-nt expression in *L. mexicana* promastigotes adapted to different sources of purine. Total RNA was isolated from equal numbers of log phase promastigotes adapted to adenosine (lane 1), 3'-AMP (lane 2), 5'-AMP (lane 3) and polyA (lane 4) as the sole source of cell medium purine (using the same cultures as in Fig. 7i). Equal concentrations of RNA (5μg) were loaded in each lane of the agarose gel and the ribosomal bands stained with ethidium bromide (left panel). After transfer to a nylon membrane, the blot was hybridised with DIG-labelled LmORF (right panel). The blot was developed for 90 minutes. DNA molecular weight markers are shown on the left and the approximate hybridised band size on the right (all in kb).

These results suggest the regulation of 3'-nt is in some way dependent on the type of purine available to the parasite. The enzyme is initially expressed regardless of purines available, possibly as part of a constitutive response to the changed environment through passage. Subsequent to this, the maintenance of 3'-nt activity appears to be linked to the purines available. The presence of adenosine (which is freely imported with no digestion or other modification required) leads to the rapid downregulation of nucleotidase activity within 48 hours of passage. A similar situation is seen in the presence of 5'-AMP. It is possible that cells adapted to 5'-AMP would have no need for a membrane bound 3'-nt, using instead the 5'-nt enzyme described in trypanosomatids to prepare nucleotides for cell import. This may be the reason for its rapid downregulation. However, with 3'-AMP as the only source of purine (as in the 3'-AMP adapted culture), it may be expected that the role of 3'-nt in the acquisition of purines would become more significant and this is reflected in the less rapid downregulation of activity in these cultures.

The most persistent 3'-nt activity following the initial constitutive expression is seen in the culture adapted to polyA. For purines to be utilised from this source, the substrate must first be digested by a nuclease and then a nucleotidase thus releasing nucleosides that can be imported. As described previously, the 3'-nt enzyme characterised here appears to possess both nuclease and nucleotidase activities. The persistence of enzyme activity in the polyA-adapted culture may be a reflection of the increased significance of the nuclease activity to these cells. The fact that sustained nuclease activity is accompanied by sustained *nucleotidase* activity (measured here) supports the evidence that both these activities are functions of the same enzyme.

6.8 Attempts to regulate 3'-nt activity by long-term culture

It was of interest to see if long-term adaptation to culture conditions not requiring 3'-nt activity would actually change the expression and regulation of the enzyme. Initial measurements of 3'-nt activity in 5'-AMP-adapted cultures had shown very little present. Aliquots of the 5'-AMP-adapted culture in mid-log growth phase were transferred to fresh defined medium supplemented with 3'-AMP, 5'-AMP or polyA and allowed to grow for three days. Cell densities and 3'-nt activities were measured for each culture over these three days (Fig. 6k).

The rate of cell growth and levels of 3'-nt activity in each of the cultures were very similar. The long-term adaptation to using 5'-AMP as sole purine source did not seem to effect the parasites and their expression of 3'-nt. It might have been expected that such cells would adapt to utilising other pathways of purine acquisition in the absence of adenosine and 3'-AMP and hence grow less efficiently when transferred to conditions with 3'-AMP as the only purine source. Both cultures transferred to polyA and 3'-AMP, however, showed growth and 3'-nt activity almost identical to the positive control of cells continued in 5'-AMP. In addition, when transferred to purine free medium, all the adapted cultures showed a similar 3'-nt upregulation response to that described in the adenosine culture.

From the results of these experiments artificially controlling the extracellular environment of *Leishmania* promastigotes, it appears that 3'-nt is important to the parasite. Despite the various options for purine acquisition presented to the promastigote cells and even in the absence of any substrate requiring 3'-nucleotidase or nuclease digestion, 3'-nt activity is retained. In addition, in situations of nutritional stress, the

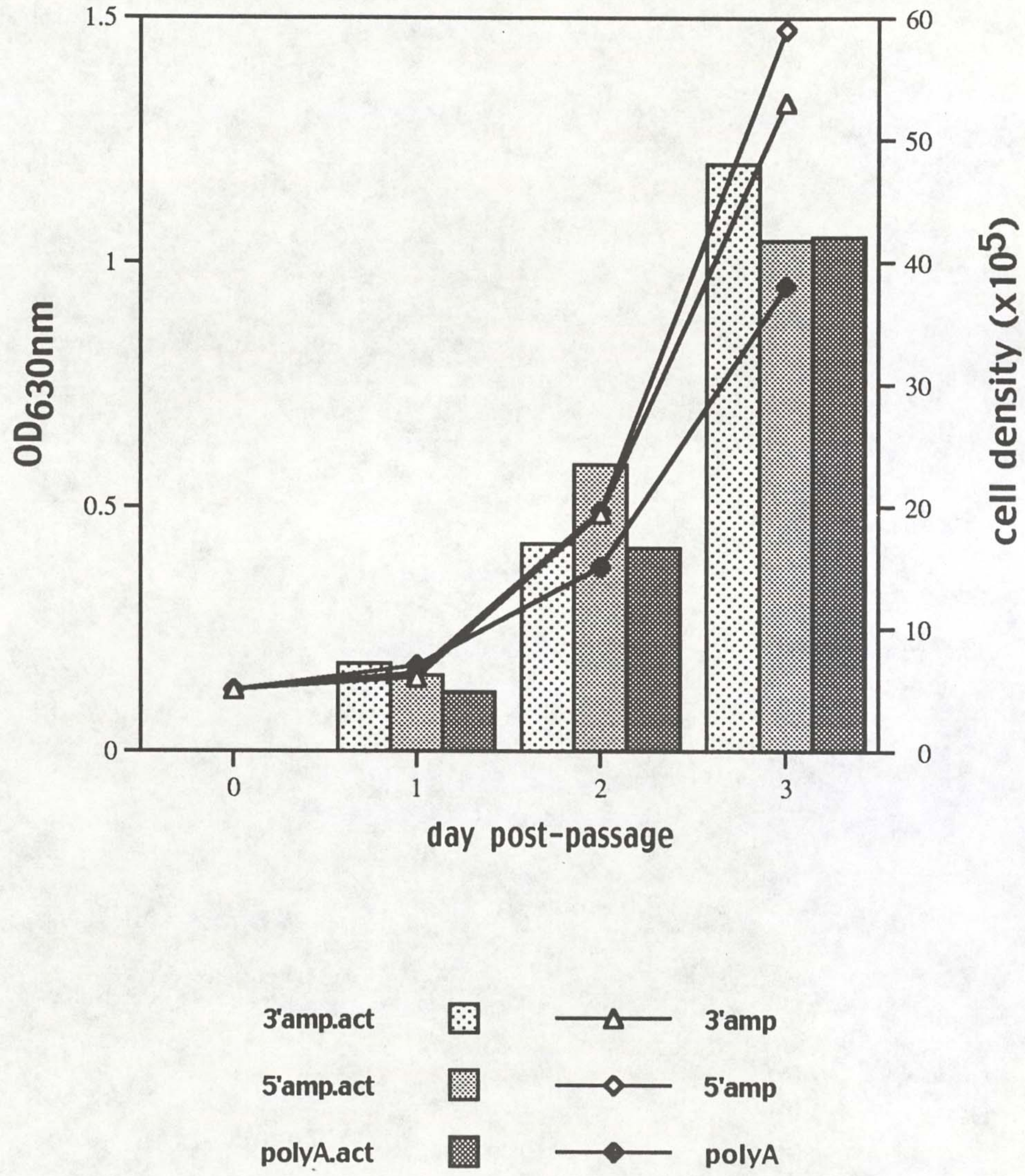


Figure 6k Tube assays comparing 3'-nt activities (bars) in 5'-AMP-adapted *L. mexicana* promastigotes (log phase) which have been transferred to other sources of purine (3'-AMP, 5'-AMP and polyA). Over three days post-passage, the activities appear to be identical. Also plotted are the cell densities (lines) of each culture over this time period. (NB. $OD_{630} \equiv P_i$ released).

enzyme is upregulated, even in cells adapted to survive without a requirement for 3'-nucleotidase activity.

6.9 The co-ordinated activity of 5'-nt and 3'-nt

In order to investigate the respective contributions to purine salvage of the 5'-nt and the 3'-nt activities described in *L. mexicana*, malachite green tube assays to measure 5'-nt activity were used. In equivalent numbers of promastigotes, 5'-nt activity appeared to be at a much lower level than 3'-nt. In addition, there did not appear to be any significant difference between levels of 5'-nt activity in each of the adapted cell cultures, the cells dependent on 5'-AMP as sole purine source showing the same levels of activity as those adapted to 3'-AMP and polyA. However, under conditions of purine starvation, 5'-nt activity showed a similar upregulation to that of 3'-nt with the increase in activity most pronounced in the 5'-AMP cultures. The maximum upregulation of 5'-nt activity observed was about 2-fold, compared with the 6- or 7-fold increase in 3'-nt activity under the same starvation conditions. The assay for 5'-nt activity was adapted from the 3'-nt assay and was not optimised in these experiments so may be less sensitive. Therefore, the precise levels of 5'-nt and its regulation in the promastigote would need confirming.

It was of interest to see if inhibition of 5'-nt activity would result in the upregulation of 3'-nt to compensate. Ammonium molybdate is a specific inhibitor of 5'-nt that has no effect on 3'-nt activity. A concentration of 100μM of molybdate was chosen (the maximum concentration tested that did not appear to adversely affect promastigote growth). On inclusion of this inhibitor in the culture medium, both 3'-nt and 5'-nt activities in purine starved cells appeared to decrease and it was therefore difficult to draw any clear conclusions.

It is highly likely that molybdate had multiple effects on the cells unrelated to nucleotidase activities and only its use in a more defined system would enable investigation of the possible co-ordination of 3'-nt and 5'-nt activities at the promastigote cell surface. The similar co-upregulation of 5'-nt and 3'-nt activity under conditions of purine starvation, however, suggests these enzymes may be part of functional unit that is upregulated or simply two of a number of key enzymes upregulated in a general response to nutritional stress.

6.10 Properties of other observed nucleotidase activities

During the course of purine starvation experiments, it became obvious that a third major nuclease activity was present in starved promastigotes. In addition to the 29/31kDa doublet and the 40kDa 3'-nt activity, a band of about 80kDa appeared on polyA substrate gels within 48 hours of transfer to purine depleted medium. This band was also shown to have 3'-nt activity on a 3'-AMP substrate gel (Fig. 6l). This activity was unaffected by reducing agents (DTT) and by boiling and appeared to partition, along with the surface membrane 3'-nt, to the membrane fraction of crude membrane extracts (Fig. 6m).

No extra bands of 3'-nt message were observed on Northern blots of starved promastigotes and it was thought that this larger band may represent either a dimer of 3'-nt or an unconnected surface membrane enzyme with nucleotidase activity. If nucleotidase activity *is* a cell function that is upregulated as part of a general promastigote response to nutritional stress (as suggested above), the expression of another nuclease with nucleotidase activity under such conditions would be a likely explanation of this band. It is not clear whether the upregulation is specifically in response to purine stress or a more general nutritional stress.

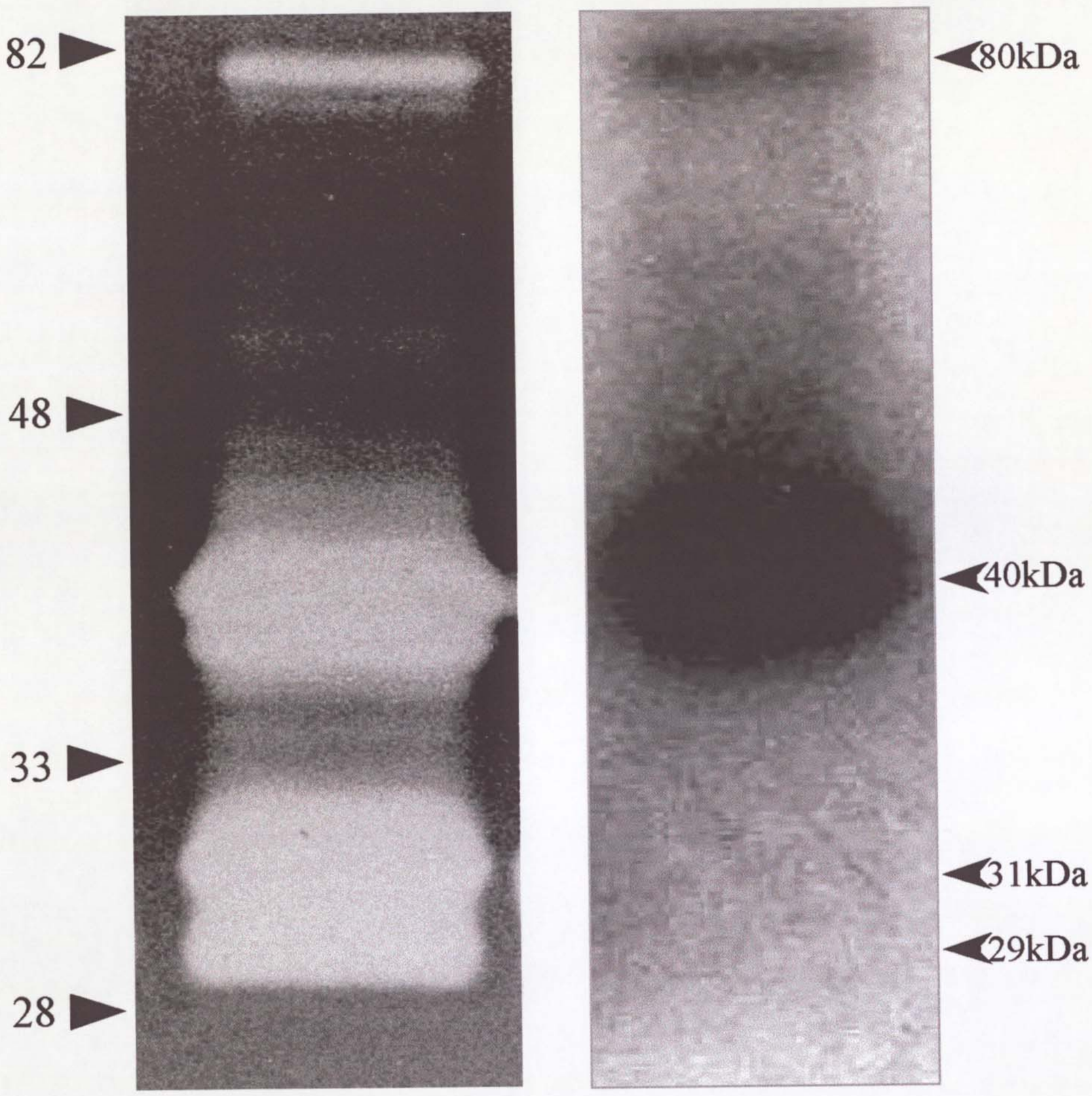


Figure 6l SDS-PAGE substrate gels showing nuclease and 3'-nucleotidase activities in purine-starved *L. mexicana* promastigotes. Aliquots of cell lysates containing equal concentrations of protein were simultaneously run on nuclease and 3'-nucleotidase substrate gels. The two panels are aligned in this figure with protein molecular weight markers shown on the left and approximate activity band sizes on the right. After approximately 48 hours in purine depleted culture (samples shown here), an 80kDa band of nuclease activity, additional to the usual bands, appeared in cell samples (left panel) and this is seen to correspond to a similar band of 3'-nucleotidase activity (right panel).

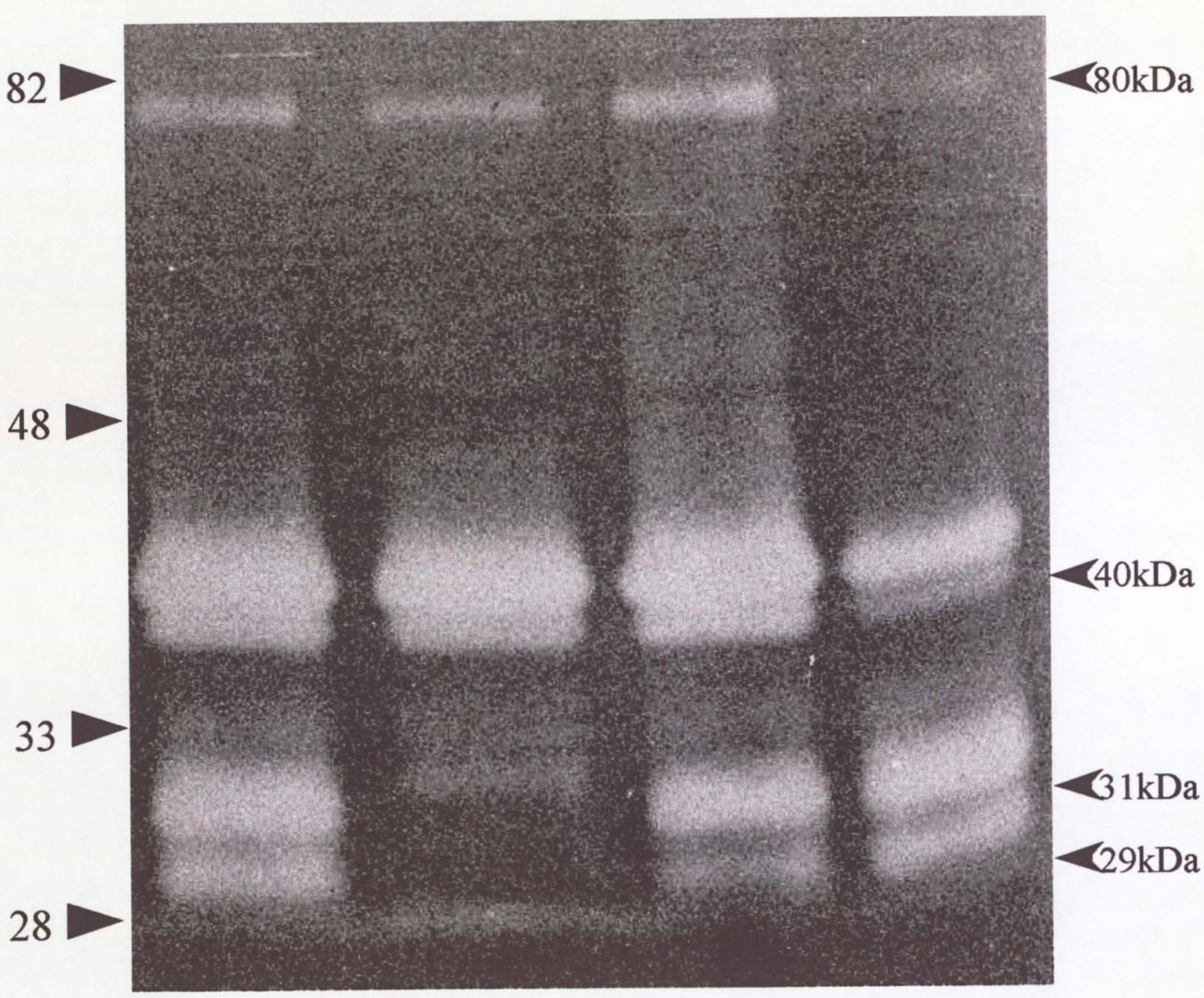


Figure 6m SDS-PAGE substrate gel showing nuclease activities in purine-starved promastigote cell pellets treated in a variety of ways. Identical aliquots of purine-starved promastigote cell lysates were; boiled for 10 minutes prior to loading (lane 1), loaded in reducing buffer containing DTT (lane 2) and taken through a crude membrane extraction by freeze-thawing as previously (lanes 3 and 4, showing membrane fraction and residual supernatant activities respectively). Equal concentrations of protein were loaded in each lane. The 80kDa nuclease/nucleotidase activity previously described (Fig. 6l) is unaffected by boiling and reduction with DTT and appears to partition into the membrane fraction of a crude membrane preparation. DTT appears to inhibit the 29/31kDa nuclease activity. Protein molecular weight markers are shown on the left (kDa) and approximate activity band sizes on the right.

In one sample of starved cells, taken during the course of a standard purine starvation experiment, this 80kDa band was absent and apparently replaced by a fourth band of nuclease activity about 35kDa in size (Fig. 6n). This band was also shown to have 3'-nt activity and assumed to be related in some way to the 80kDa nuclease/nucleotidase described above. Boiling the cell sample before loading onto the SDS-PAGE substrate gel restored the nuclease activity at 80kDa and slightly decreased the activity at 35kDa (Fig. 6o). This was also confirmed on a 3'-nucleotidase gel (Fig. 6o). The significance of this observation is uncertain.

In this particular culture sample there may have been an inhibitor present (possibly another protein) that inhibited the activity of the 80kDa band. To compensate, the purine-starved cells may have expressed or upregulated a further nuclease/nucleotidase (the 35kDa band, which was not seen in any other substrate gels run). Boiling the sample removed the inhibition (either denaturing it or dislodging it from the 80kDa nuclease active site) so restoring the 80kDa activity. In subsequent cell samples (taken on subsequent days from the same flask of starved culture), the 35kDa activity is seen to decrease as the 80kDa enzyme is restored. It is not known what may have caused this transient expression of another nuclease/nucleotidase activity or whether it is connected to the three others previously mentioned. Its appearance indicates again the versatility of *Leishmania* in its acquisition of essential purines.

6.11 Function of 3'-nt in the amastigote

As demonstrated previously, *L. mexicana* amastigotes do not express the 3'-nt gene described but do possess nuclease and 3'-nucleotidase activities. These activities correspond in size to the cytoplasmic nuclease activities found in promastigotes and it is

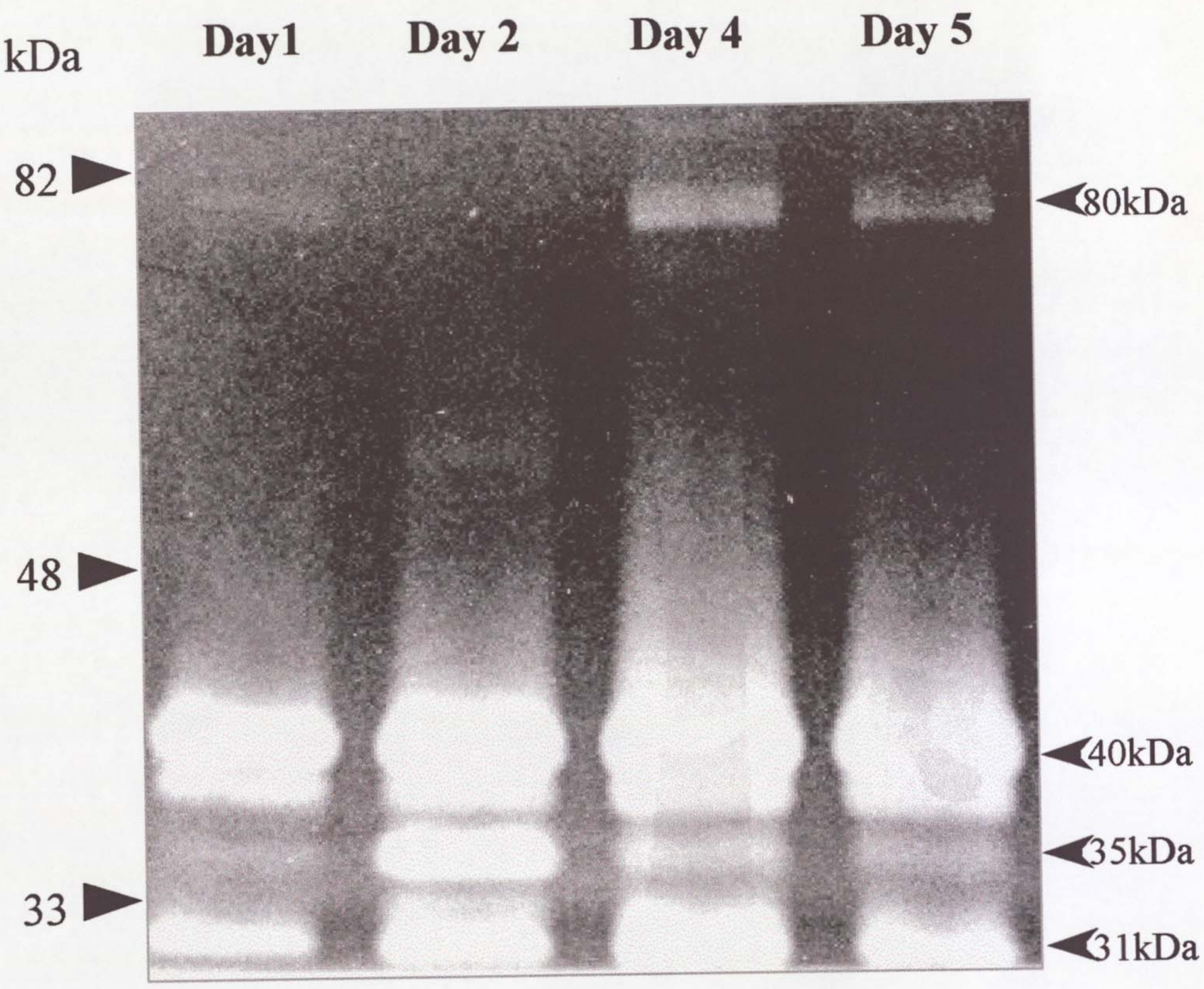


Figure 6n SDS-PAGE substrate gel showing nuclease activities in purine-starved promastigote cell pellets over five days of culture. Cell aliquots were taken from the culture flask each day and the cells pelleted and frozen. After collection of all the samples, cell lysates were prepared and run on SDS-PAGE. Protein molecular weight markers are shown on the left and approximate activity band sizes on the right. Equal concentrations of protein were run in each lane. In this particular sequence of samples, the 80kDa nuclease/nucleotidase band of activity is absent in the day 2 sample, in which a previously unseen 35kDa nuclease band appears. The 80kDa activity is restored in cells taken on subsequent days.

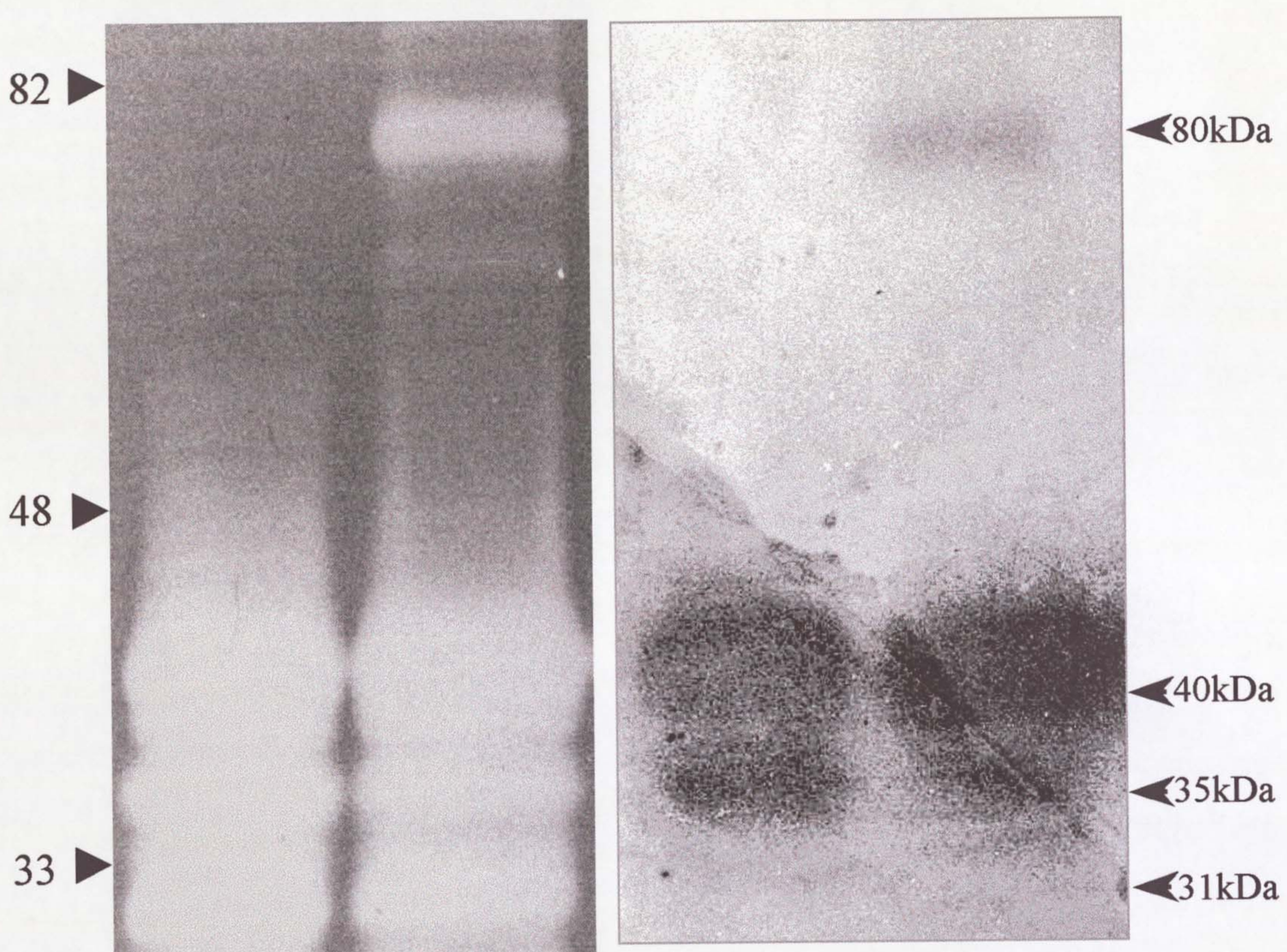


Figure 6o Substrate gels comparing nuclease and 3'-nucleotidase activities in the 'day2' starved culture (Fig. 6n) before and after boiling. Aliquots of the day2 lysate containing equal concentrations of protein were loaded on a further SDS-PAGE nuclease substrate gel (left-hand panel). One of the samples was boiled for 10 minutes prior to loading (lane 2). Duplicate samples were simultaneously run on a 3'-nucleotidase substrate gel (right-hand panel). The two panels are aligned in this figure with protein molecular weight markers on the left and the approximate band sizes of activity are shown on the right. The previously described 35kDa nuclease activity, corresponds with a similarly sized band of 3'-nucleotidase activity. Boiling appears to restore the 80kDa band of nuclease/ nucleotidase activity.

not clear whether amastigotes possess a surface membrane 3'-nucleotidase. In order to determine whether amastigotes might at any time express the promastigote 3'-nt, it would be beneficial to be able to control the levels of purine in amastigote culture. This could be most easily achieved by adapting amastigotes to chemically defined medium with a removable source of purines.

Repeated attempts were made to adapt axenic amastigotes to serum-free growth conditions but without success. Commercially available dialysed serum was also used but could not be guaranteed free of purines. Finally it was decided to try simply incubating amastigotes grown in purine-replete medium in purine-free medium rather than trying to grow them under purine-free conditions. Late log phase amastigotes grown from a fresh lesion sample were harvested and washed in serum-free Schneiders medium (the same medium used for normal amastigote culture) and resuspended at a density of 2×10^6/ml in serum-free medium containing no purines. A positive purine control was also included where the same density of washed amastigotes was incubated in serum-free medium supplemented with adenosine (at normal concentration). Several aliquots of cells were harvested over a ninety-hour time period and checked for nuclease and nucleotidase activity on substrate gels (Fig. 6p).

The nuclease profile of the amastigotes over the period of 90 hours shows no difference between the cells exposed to purine starvation and those supplemented with adenosine. The strong 29/31kDa band of nuclease activity persists with no appearance of any extra bands as have been described in starved promastigotes. Over the time period assessed, the cell densities did not alter, the amastigotes appearing to neither grow nor die.

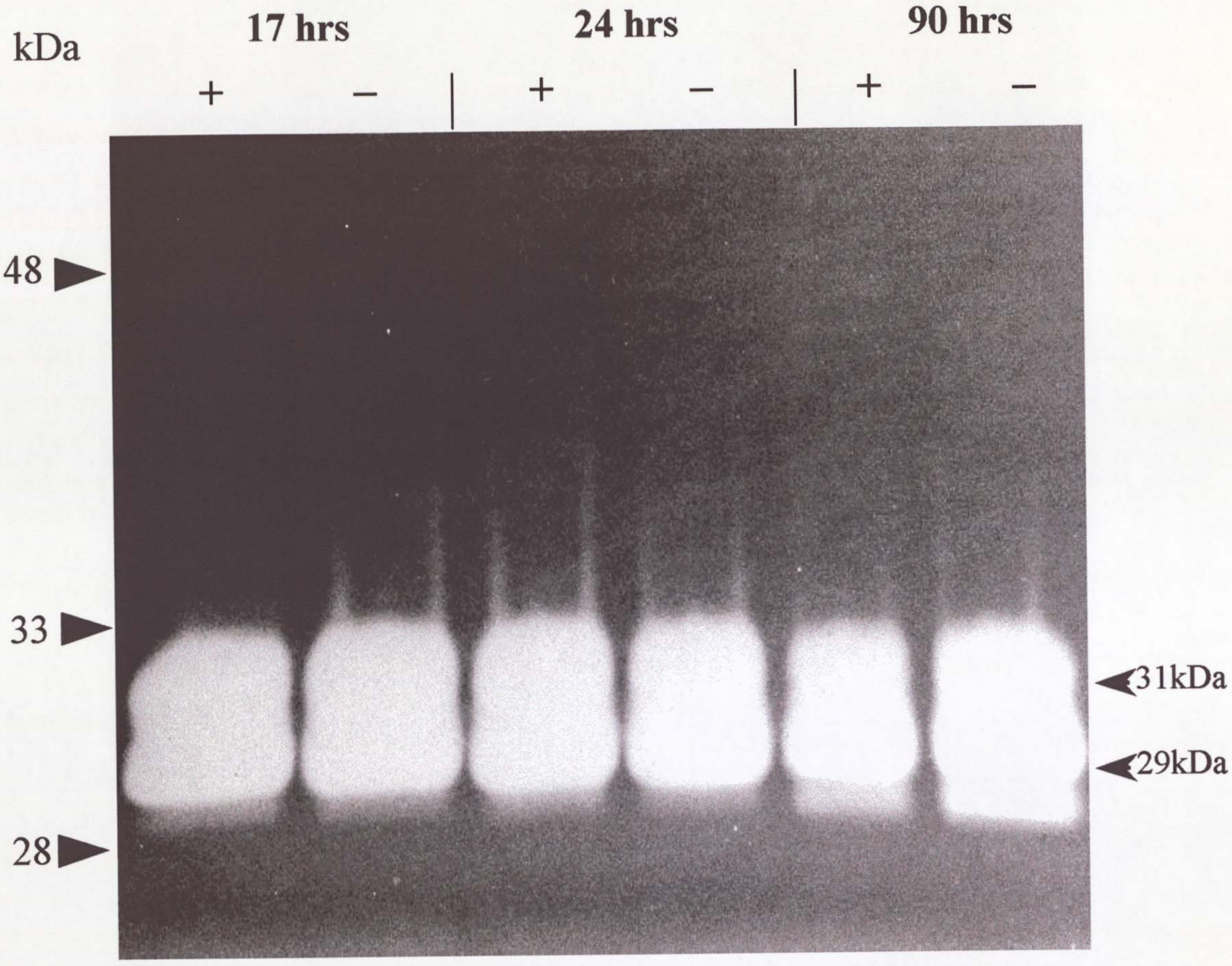


Figure 6p Substrate gel comparing nuclease activities in amastigotes incubated in medium with (+) and without (-) adenosine over a period of 90 hours. Short term culture amastigotes were harvested from standard amastigote culture medium at late log phase and washed in identical medium but without serum. The cells were incubated in fresh serum-free medium with and without adenosine for 90 hours and aliquots of cells were harvested after 17, 24 and 90 hours. Harvested amastigotes were lysed and run on a standard SDS-PAGE nuclease substrate gel. Equal numbers of cells were run in each lane. Protein molecular weight markers are shown on the left and activity band sizes on the right.

Because this incubated amastigote population appeared to be a static one and so perhaps not a good measure for the development of 3'-nt activity in amastigotes, it was decided to try and induce amastigote-like forms by placing purine-adapted promastigote cultures at 32°C and measuring nuclease and nucleotidase activities as before. Promastigotes grown in the presence of adenosine and promastigotes conditioned to using 3'-AMP as their sole source of purines were used. Promastigote cultures in late log phase were transferred from 26°C to 32°C (the temperature at which amastigotes develop). The cells remained at 32°C for 6 days with aliquots occasionally removed to measure nuclease activity. After 6 days, a proportion of the cell cultures was returned to 26°C and also to purine-free medium at 32°C. Nuclease substrate gels were used to investigate any changes in the adenosine and 3'-AMP-adapted cultures over this time (Fig. 6q).

Morphologically, the stimulus of increased temperature led to the transformation of promastigotes into rounded amastigote-like forms. The nuclease profiles observed support this transformation. Within 48 hours, the 40kDa 3'-nt activity has almost completely disappeared from both cultures and the upregulation of the 29/31kDa band characteristic of amastigotes is seen. Even in the 3'-AMP-adapted cells, conditioned to utilise 3'-AMP as their sole source of purine, there is no evidence of 3'-nt activity being retained during this transformation to amastigote-like forms, suggesting the amastigotes have an alternative means by which to acquire purines from the medium.

Transferring aliquots of induced amastigotes back to 26°C had no effect on the 3'-AMP culture but after 48 hours, the adenosine culture showed the re-appearance of the 40kDa 3'-nt. This suggests the 3'-AMP culture growth may have been retarded by the formation of amastigotes with less efficient means of purine acquisition, meaning the subsequent

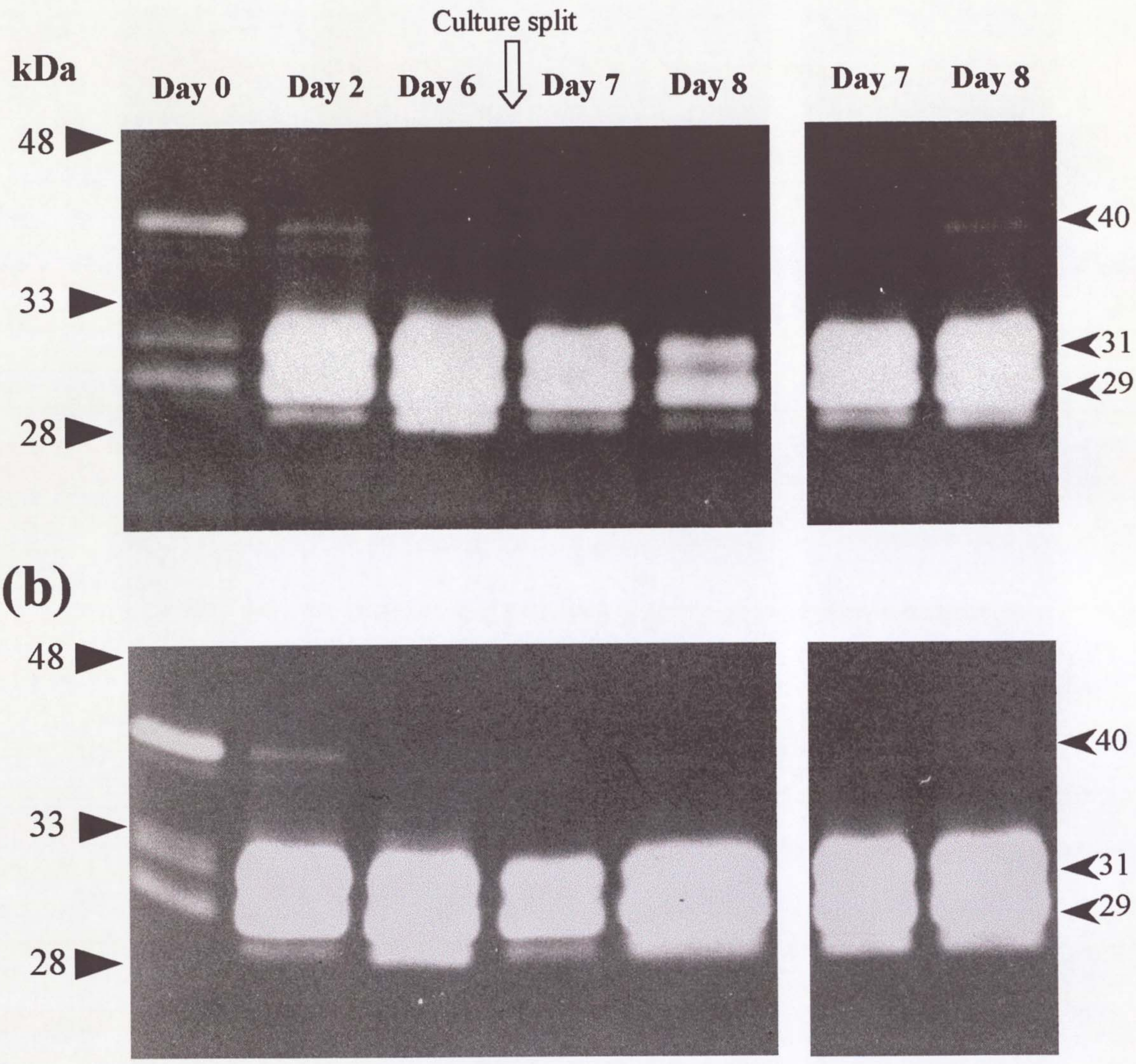


Figure 6q Substrate gels showing nuclease activities in procyclic promastigotes incubated at 32°C. Procyclic promastigotes previously adapted to (a) adenosine and to (b) 3'-AMP were transferred, at late log phase, from 26°C to 32°C and incubated for 6 days. Cells were harvested after 2 and 6 days and their nuclease profiles compared to the Day0 26°C promastigote cells (left-hand panels). After a six day incubation, each culture ((a) & (b)) was split. Half was transferred back to 26°C (right-hand panels) and half left at 32°C and incubated for a further two days (days 7 & 8). Equal concentrations of protein were loaded in each lane. Protein molecular weight markers are shown on the left and approximate band sizes on the right.

transformation back to promastigotes is slower than that in the adenosine cultures, which have a constant supply of available purines whatever their form. The induced amastigotes transferred to purine-free medium at 32°C (results not shown) appeared identical to those remaining in supplemented medium. There was no detectable 3'-nt activity in these induced amastigotes when measured by tube assay.

Having demonstrated the artificial transformation of promastigotes to amastigote-like forms using temperature, a final attempt to induce 3'-nt expression in amastigotes was made by transferring purine-starved promastigotes to 32°C and following their progress over a period of 7 days. As above, induced amastigotes (after 3 days at 32°C) were also transferred back to 26°C to check their viability. The process was repeated for adenosine-adapted cells to compare promastigote to amastigote transformation with and without purine. Cell growth was measured over this time period and cell samples run on nuclease substrate gels as before (Fig. 6r).

Over the first 48 hours, the physiological transformation of promastigotes with and without adenosine to amastigotes appears very similar. The starved promastigotes seem to retain a higher level of 3'-nt activity (40kDa) after 48 hours but the nuclease profile of both cultures on day 3 is identical. After this point, a proportion of both cultures (with and without adenosine) was transferred back to 26°C. At 32°C, the completed transformation to amastigote-like forms is seen with the disappearance of the 40kDa band of nuclease activity. It was seen from the cell densities that disappearance of this band coincides with an apparent stasis of growth, the amastigote forms no longer able to multiply without the presence of serum. Although this may be linked with purine

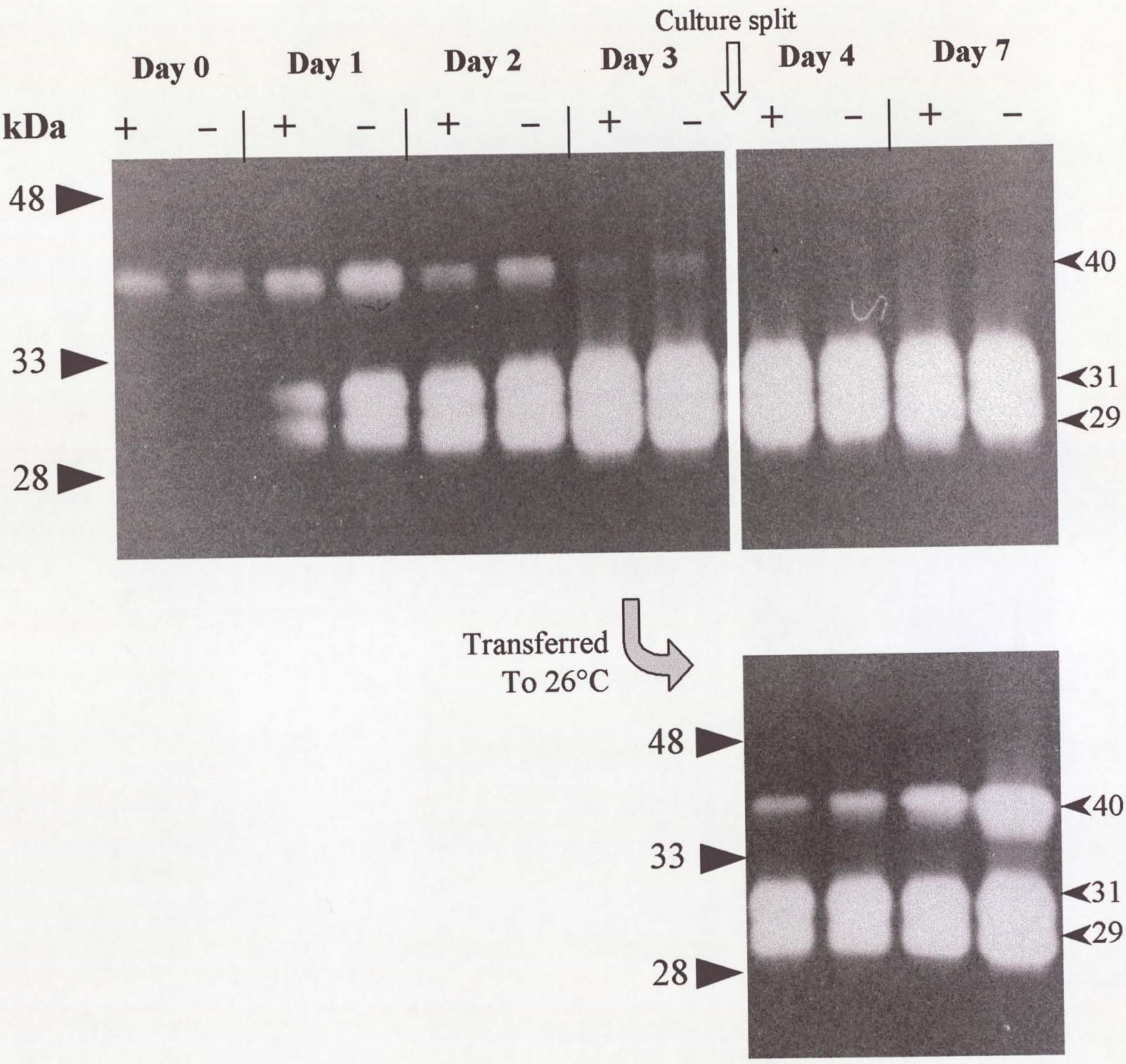


Figure 6r Substrate gels showing nuclease activities in normal (+) and purine-starved (-) procyclic promastigotes incubated at 32°C. Promastigote cultures were transferred, at late log phase, from 26°C to 32°C and incubated for 3 days. Nuclease activities of cells harvested each day were compared to the Day0 26°C promastigote cells (left-hand panel). After 3 days, each culture (with and without adenosine) was split, half the volume being transferred back to 26°C (lower right panel) and half left at 32°C (upper right panel). The cultures were incubated for a further four days and cells harvested on days 4 and 7. Equal concentrations of protein were loaded in each lane. Protein molecular weight markers are shown on the left and approximate activity band sizes on the right (all in kDa).

availability, the lack of growth is seen in both adenosine-replete and adenosine-starved cultures so is probably due to other factors.

With the transfer of cells back to 26°C, the 40kDa band began to reappear and the cells began to multiply again. After 4 days at 26°C (on day 7), the effects of starvation could be seen with the upregulation of the 40kDa band compared to the adenosine-replete culture. This demonstrated that the purine-starved culture had not been changed over the time period at 32°C, subsequently responding to a lack of purines as expected. Therefore, the lack of response (in terms of 3'-nt activity) to purine starvation while at 32°C suggests that these amastigote forms may have a reduced need for purines. Alternatively, amastigotes may have a means of generating purines in times of stress that does not involve 3'-nucleotidase activity or simply have no capability by which to increase purine uptake under such conditions.

6.12 Conclusion

Although both promastigote and amastigote forms show 3'-nucleotidase activity, the surface membrane 3'-nt (40kDa band of activity) appears to be promastigote-specific. In this form of the parasite, it is constitutively expressed and upregulated under conditions of purine starvation and may be an important component of the cellular purine salvage apparatus. Purine starvation also induces the expression of other nuclease/nucleotidase activities in *L. mexicana*, which may be linked to the 40kDa 3'-nt or constitute novel enzymes. Their expression in response to low purine is an indication of the importance of purine salvage to the promastigote form. In contrast, the amastigote forms show no change in nuclease activity under similar conditions of starvation. The 3'-nt gene described here appears to have no function in the amastigote form of the parasite and the

amastigotes may have different means of acquiring purines, possibly solely mediated by nuclease activity. Alternatively, the requirement for purines may differ between the amastigote and promastigote forms.

CHAPTER 7

GENERAL DISCUSSION

7.1 Characterisation of 3'-nt gene sequence and deduced protein

A 3'-nucleotidase/nuclease gene sequence (3'-nt) has been isolated and characterised in *L. mexicana*. The sequence encodes an open reading frame (ORF) of 1134 nucleotides that can be conceptually translated into a 378 amino acid protein of approximately 41.7kDa. This is the second 3'-nt enzyme to be fully cloned and sequenced within the family Trypanosomatidae and the gene sequence derived shows very high homology with the previously described *L. donovani* gene (Debrabant *et al.*, 1995). The deduced *L. donovani* ORF is the same length and gives a putative translated protein of 41.5kDa (Debrabant *et al.*, 1995). The identity between the two genes at the nucleotide level is 90% and at the amino acid level, 88%. The differences observed between the putative translated proteins are mainly a result of single amino acid substitutions and the longest continuous stretch of differing sequence is only three amino acids long (Fig. 3m). These differences are spread throughout the length of the ORF.

This similarity between the two species of *Leishmania* suggests the 3'-nt sequence is highly conserved within the genus. In fact, preliminary investigations of *L. major* (Kirk, 1997) revealed another 3'-nt-like gene sequence (400 nucleotides of an ORF showing 93% identity at the nucleotide level). Sequence with a similarly high level of identity has also been found in the 3'-nt gene of the related trypanosomatid *Crithidia luciliae*, a monogenetic parasite of insects (Yamage *et al.*, 1997). The similarity of these gene sequences within the family suggests they have been conserved through evolution and may indicate an important role for this gene in the parasitic lifestyle.

The predicted peptide sequence of the 3'-nt gene described contains a region of high hydrophobicity at the C-terminal between residues 336 and 360 (Fig. 3l). This is also a feature of the *L. donovani* 3'-nt peptide sequence previously described (Debrabant *et al.*, 1995) and may indicate the presence of an intra-membranous peptide motif in the mature enzyme. Previous studies of the localisation of the *L. donovani* 3'-nt enzyme *in vivo* show it to be anchored in the cell surface membrane with its active site externally oriented (Dwyer & Gottlieb, 1984). Further expression studies in *L. donovani* have shown that preparation of truncated recombinant 3'-nt proteins (excluding the hydrophobic C-terminus) results in a soluble form of the enzyme which is exported from the parasite cell surface (Dr Alain Debrabant, personal communication).

Many surface membrane proteins expressed in *Leishmania*, including the 5'-nucleotidase, are anchored by glycosylphosphatidylinositol (GPI) lipid anchors (Ferguson, 1997), which are added in the endoplasmic reticulum as a result of post-translational modification. This form of membrane attachment appears to predominate in the coat proteins of single-celled eukaryotes (Ferguson & Williams, 1988) and is mediated by particular recognition sequences in the newly synthesised protein. The recognition sequences of known GPI-anchored proteins have been compared (Gerber *et al.*, 1992) and although there is no consensus sequence as such, there appears to be a common pattern consisting of a hydrophobic C-terminal of at least 18 residues and five key amino acids either side of the peptide cleavage site at which the GPI is attached. Using site-directed mutagenesis, the potential GPI attachment amino acid and the two residues immediately downstream have been found to be limited to small amino acids (Gerber *et al.*, 1992).

Apart from these key amino acid residues, evidence that a hydrophobic C-terminus will be replaced with GPI rather than constitute an intra-membranous domain, rests with the length of the domain and the presence or absence of a putative cytoplasmic region of hydrophilic (polar) amino acids at its C-terminus. Many proteins destined for GPI anchoring have completely hydrophobic C-termini or hydrophobic domains considered too short to span the membrane (Ferguson & Williams, 1988).

It is not clear from the studies of truncated *L. donovani* 3'-nt clones whether the export of soluble enzyme is due to the removal of a protein motif necessary for GPI addition or the removal of an intra-membranous membrane-anchoring region in the mature protein. The predicted peptide sequence of the *L. donovani* gene does not appear to support the attachment of GPI (Debrabant *et al.*, 1995) and, given the high homology between the two genes, the *L. mexicana* peptide would not be expected to either. Even so, the *L. mexicana* amino acid sequence does differ from *L. donovani* by several residues in the region of putative GPI anchor attachment, immediately upstream from the hydrophobic C-terminus, between residues 320 and 340 (Fig. 3m). The differing amino acids in the *L. mexicana* peptide, however, are not sufficiently small to constitute a suitable GPI attachment site. In addition, a transmembrane peptide attachment for 3'-nt is supported by the presence of an unbroken, hydrophobic α-helix between residues 336 and 360 (Fig. 3l) and a C-terminus region of hydrophilicity that may constitute a cytoplasmic domain.

Studies of the *L. mexicana* surface membrane in this work support the localisation of the 3'-nt on the parasite surface. The majority of 3'-nt enzyme activity partitions to crude membrane extracts and 70% of measured activity was detected on the surface of intact cells compared to whole cell lysates. So, the enzyme is anchored in the surface

membrane, most likely by a transmembrane domain in the mature protein, although a GPI attachment cannot be ruled out. Experiments labelling the C-terminus of the protein prior to its export to the surface membrane could be performed to confirm this.

No work has been done to investigate whether 3'-nt exists in the membrane as a single molecule or has connections with any other membrane proteins. There is a single cysteine residue (amino acid 362 in the open reading frame; Fig. 3k) with the potential for forming a disulphide bridge with another cysteine, either stabilising the 3'-nt structure or covalently binding it to an adjacent protein. This residue is located, however, on the putative cytosolic face of the enzyme and therefore in a reducing environment in which disulphide bonds rarely form (Creighton, 1988). Another feature of transmembrane proteins that may suggest an association with other protein molecules is the presence of polar amino acid side chains in the intramembrane domain. These hydrophilic residues will tend to associate with other polar side chains in the hydrophobic environment of the lipid bilayer and this may lead to the formation of such structures as membrane pores, consisting of several similar subunits electrostatically bound together. In the 3'-nt peptide sequence described (Fig. 3k), there is only a single uncharged polar amino acid (threonine; residue 354) in the otherwise nonpolar putative membrane-spanning region described above, and this is unlikely to sustain such electrostatic associations.

Apart from the corresponding gene in *L. donovani*, the predicted protein sequence of *L. mexicana* 3'-nt shows greatest similarity to the S1 nuclease of *Aspergillus oryzae* (Iwamatsu *et al.*, 1991) and the P1 nuclease of *Penicillium citrinum* (Kazama *et al.*, 1990), both of which also show 3'-nucleotidase activity. There are several regions of amino acid sequence conservation between these enzymes (Figure 3n) and such motifs

may represent key structural and physiological components of the nuclease/nucleotidase catalytic site. S1 and P1 activities are both classified as EC 3.1.30.1, pertaining to their physiological function as endonucleases of single-stranded nucleic acids. They are related in function to the plant nuclease I enzymes (EC 3.1.30.2) described in barley and pea seedlings (Brown & Ho, 1986; Naseem & Hadi, 1987) and in *Lentinus edodes* (Shimada *et al.*, 1991; Kobayashi *et al.*, 1995). The plant nucleases also possess 3'-nucleotidase activity and although they show no sequence homology with 3'-nt, they, together with P1 and S1, represent possible models for the characterisation of its structure and function.

The barley nuclease is reported to be a single polypeptide of 36kDa with a pH optimum of 5.0–6.5 (Brown & Ho, 1986). The nuclease from pea seeds, however, appeared to consist of two subunits and show optimum activity at neutral pH (Naseem & Hadi, 1987). Also reported were differing rates of thermal inactivation for the component nuclease (optimum activity at 45°C) and nucleotidase (optimum activity at 60°C) activities, suggesting two different catalytic sites. This theory was further supported by the effect of EDTA, which inhibited the nucleotidase but had no effect on nuclease activity (Naseem & Hadi, 1987). In addition, the presence of DNA was stimulatory for 3'AMP hydrolysis, and this was shown to be independent of DNA digestion, suggesting that the stimulation was mediated by the allosteric binding of the nucleic acid. Naseem and Hadi postulate that the binding of DNA, perhaps in a site formed by the fusion of the two subunits, changes the conformation of the distinct nucleotidase site, thus increasing its efficiency.

Out of the nucleases described, the *Penicillium* nuclease (P1) appears to show the greatest similarity to *Leishmania* 3'-nt. P1 is described as a single polypeptide, of similar size to the barley enzyme, with a pH optimum range between 6.5 and 8.0 for the nucleotidase activity (Kazama *et al.*, 1990). There is no evidence that the *Leishmania* 3'-nt has more than one subunit, as postulated for the pea seed nuclease above, and it shows a similarly broad pH range of activity (between 5.5 and 7.5; Gbenle & Dwyer, 1992). Both P1 and 3'-nt activities are inhibited by EDTA and there is some sequence homology between the genes as shown in Figure 3n. The characteristics of P1, therefore, rather than those of the plant nucleases, may represent the best model for the characteristics of 3'-nt.

Crystals of the P1 enzyme complexed with substrate analogues have recently been derived and show two nucleotide binding pockets linked by a channel in the protein structure (Romier, *et al.*, 1998). Only one binding pocket appears to be critical in phosphate hydrolysis (being conserved in both S1 and P1 nucleases) and the key catalytic and binding residues identified are a phenylalanine (amino acid 61 in the P1 peptide sequence), an aspartic acid (amino acid 63) and a valine (amino acid 132). As seen in Figure 3n, although not conserved between the nucleases and the nucleotidases, these residues are adjacent to regions of particular homology between the four sequences. This conservation of sequence motifs in the region of key catalytic residues may indicate a similar form of active site in the 3'-nt as that of P1.

Indeed, the substitutions in *L. mexicana* of the three amino acids mentioned are equivalent. The aromatic ring of phenylalanine (phe61) in P1 is replaced in 3'-nt by tyrosine (tyr93), also with an aromatic ring and this is thought to be critical for the

correct stacking of the target purine ring (Romier *et al.*, 1998). Valine (val132) in P1, a small nonpolar amino acid, is replaced by leucine (leu170), another nonpolar residue. Finally, the carboxyl group of aspartic acid (asp63) thought to hydrogen bond to the target nucleotide, is replaced in 3'-nt by a threonine (thr95) with a similar capacity for hydrogen bonding.

The structural and physiological similarities between the *L. mexicana* 3'-nt and the *Penicillium* nuclease P1 may indicate a common catalytic function and mechanism. Defined as a nucleotidase on account of its high level of 3'-nucleotidase specific activity (Gottlieb & Dwyer, 1983; Campbell *et al.*, 1991), the 3'-nt described also catalyses the hydrolysis of single and double stranded polynucleotides (Bates, 1993b). It's catalytic function is as a phosphomonoesterase (Hassan & Coombs, 1987), cleaving a single phosphate ester bond, whereas the P1 nuclease is described as a phosphodiesterase with phosphomonoesterase activity (Fujimoto *et al.*, 1974). Hydrolysis of polynucleotides by these enzymes and the other plant nucleases mentioned results in 5'-nucleotide products.

The common biochemical function of these enzymes, the *Leishmania* 3'-nucleotidase with associated exonuclease activity and the plant endonucleases with associated 3'-nucleotidase activity, suggests that this uncommon dual activity may have an important biological role in these very distinct species (and is therefore conserved). Alternatively, this dual function may simply be a factor of similar protein structure, and particularly active site structure, an explanation that appears to be supported by the peptide sequence similarities discussed above. Although classed as distinct enzymes, it is possible that the observed 3'-nucleotidase activity of each enzyme is simply a co-incidental factor of the physiological nature of the nuclease catalytic site, as discussed below.

The crystal structure of the substrate-bound active site of P1 nuclease (Romier, *et al.*, 1998) shows a channel between two nucleotide binding pockets, which may be significant in the orienting of polynucleotides for 3'-phosphate bond cleavage. On account of their smaller size, this site could also incorporate single 3'-nucleotides with ease and hence function dually as a nuclease and nucleotidase. It is interesting that there are no 'professional' (ie sole function) 3'-nucleotidases reported (as with 5'-nucleotidase) but that all described 3' activity is associated with a nuclease. This seems to suggest that 3'-nucleotidase activity is a coincidental by-product of the nuclease 1-type active site.

The theory that *Leishmania* 3'-nt is in fact a nuclease with co-incidental nucleotidase activity, as appears to be the case with the plant nucleases, carries the assumption that it is a single polypeptide with a single active site, as suggested by the sequence similarities with P1. This could be established by crystallising 3'-nt or by studying the inhibition of 3'-nucleotidase activity in the presence of polynucleotide analogues that bind to the nuclease site but are not cleaved. If this were the case, it would raise questions as to the significance of the 3'-nt enzyme to *Leishmania* and also the reason for its dramatic and specific upregulation in response to purine stress in the promastigote form. These questions and the possible biological role of 3'-nt are discussed further in section 7.4.

Using a number of *L. mexicana*-specific probes derived from the gene sequence presented, restriction mapping of the 3'-nt gene locus suggested the gene was single copy in the *L. mexicana* genome. A 10kb region of nucleotide sequence surrounding the gene was examined and no other 3'-nt gene or related sequence was detected by Southern hybridisation in the adjacent genomic DNA. Subsequently, the presence of a single hybridising band on a pulsed-field gel electrophoresis blot (probed with the labelled open

reading frame of the enzyme) suggested that the gene was also only present on one pair of homologous chromosomes. From the available evidence, therefore, the enzyme appears to be a suitable candidate for gene knockout, where a null mutant for 3'-nt could be engineered through the sequential disruption of the two allelic copies of a single gene locus that is associated with one pair of homologous chromosomes. The methods used to establish the copy number of 3'-nt in *L. mexicana*, however, would not have detected the presence of aneuploidy and the flexibility of the *Leishmania* genome suggests that an important gene may be amplified under such conditions of stress, making knockout difficult.

Unfortunately, due to the technical difficulties encountered in preparing suitable constructs for gene replacement, attempts to create a null mutant for 3'-nt in *L. mexicana* could not be completed. The cassettes used successfully to replace the 3'-nt gene in *L. donovani* were not effective in *L. mexicana*, illustrating the sensitivity of the process of homologous recombination. This is supported by studies of gene targeting frequency in relation to a number of parameters, including isogenicity of DNA sequence (Papadopoulou, 1997). The heterogeneous nature of the cultured cell population used in the knockout experiments may also have decreased the efficiency of the electroporation procedure.

The identity of the cloned gene as the 3'-nt previously described in *L. donovani* and *L. mexicana* was confirmed using antiserum raised against the recombinant *L. donovani* protein. In Western blots with *L. donovani* promastigotes, this serum recognises a single 43kDa band (Debrabant, unpublished observation). When used with *L. mexicana* promastigotes grown under normal conditions, an equivalent band was not detected.

However, in starved promastigote cultures this serum recognised a band of about 40kDa, which was assumed to be the nuclease/nucleotidase upregulated by purine starvation (Fig. 5a). The immune serum used also detected a 52kDa band in both normal and starved promastigotes whose identity is unclear. There are two putative sites for *N*-linked glycosylation in the predicted 3'-nt protein sequence (Fig. 3l) and discrepancies in the rate of migration of the enzyme during SDS-PAGE may be expected to result from glycosylation, but perhaps not to this extent. It is possible that this band may constitute some form of inactive pro-enzyme that is subsequently cleaved to its active 40kDa form.

The initial description of the *L. donovani* 3'-nt gene was of a 1431 nucleotide ORF that was translated into a 477 amino acid protein with a calculated molecular mass of 52.16kDa (Debrabant *et al.*, 1995). Because this calculated mass did not agree with the 43kDa band of 3'-nt activity observed in *L. donovani*, it was deduced that the gene was directly translated into an active 43kDa protein, starting at an ATG codon further downstream (at nucleotide 300) in the published ORF. This ATG corresponds to the start codon described here for the *L. mexicana* gene. 5' flanking sequence of the *L. mexicana* gene locus has not been derived but it may be that *L. mexicana* also possesses another potential start codon further upstream that could lead to the expression of the 52kDa protein detected in Western blots.

The use of ATG 300 as the start site for 3'-nt translation in *L. donovani* was supported by the presence of a putative signal peptidase cleavage site and N-terminal signal sequence in the preceding translated sequence (Debrabant *et al.*, 1995). The potential for the expression of a 52kDa inactive precursor using the original ATG in *L. donovani* was considered, but dismissed on account of the lack of any reported evidence in previous

work and the absence of a detectable 52kDa band in Western blots (Debrabant *et al.*, 1995). It appears that in *L. mexicana* such a precursor may exist. It is possible that this pro-enzyme may be cleaved to the active 40kDa form in response to purine stress, so increasing 3'-nt activity in starvation conditions. Alternatively, the recombinant serum may simply be more cross-reactive with other unrelated proteins in *L. mexicana* than in *L. donovani*.

In order to assess whether native enzyme could be recognised by the immune serum, an immunoprecipitation was attempted using the same anti-recombinant serum. The 3'-nt activity precipitated onto protein A-sepharose beads was shown on a nuclease substrate gel to correspond to the 40kDa band of nuclease activity seen in promastigote cell lysates. No activity was seen at 52kDa. This evidence confirms that the 3'-nt gene cloned here is expressed as a 40kDa nuclease/nucleotidase in promastigotes.

7.2 Expression of 3'-nt in the different life cycle stages

Analysis of RNA in Northern hybridisations revealed that 3'-nt expression is stage-regulated in *L. mexicana*. Transcripts of the cloned gene appeared to be most abundant in the procyclic promastigote stage of the life cycle and absent in the amastigote form, with a lower level present in metacyclic than in procyclic promastigotes. The method of probe detection used in the Northern blots was not sufficiently quantitative to assess the precise comparative levels of message but a similar pattern of protein expression was revealed by substrate SDS-PAGE. Thus, a strong 40kDa band of activity was seen in procyclic promastigotes, a weaker band in metacyclic promastigotes and no detectable activity in amastigotes. The correlation between levels of mRNA and levels of 3'-nt activity in each of the life cycle stages indicates that regulation of expression is achieved

mainly by modulation of the level of mature mRNA and not by some form of pro-enzyme activation, as suggested above.

Given that gene transcription in all *Leishmania* genes investigated is constitutive with no examples known of transcriptional regulation, this stage-specificity of 3'-nt expression is almost certainly regulated post-transcriptionally (Nilsen, 1994). Such regulation may be mediated by the processing of transcripts through the adding of 5' spliced leader sequence and/or a polyA tail. Incorrect processing in the amastigote may interfere with these additions and lead to the degradation of the transcript by nucleases. Alternatively, the 3'-nt gene transcript may be correctly processed in all stages of the life cycle but degraded in the amastigote form by an amastigote-specific nuclease transcribed in response to a particular environmental cue (Argaman *et al.*, 1994). Another possibility is that the 3'-nt transcript may be unstable in amastigotes with greater stability being conferred in the promastigote form by the use of an alternative splice site (Kawazu *et al.*, 1997). In any of these cases, the amastigote transcript would be unstable and so not visible on a Northern blot whereas the promastigote transcript would be stabilised and result in expression of 3'-nt activity. Gene expression could be studied in more detail using RT-PCR to pick up transient and rare transcripts and further study of the 3' untranslated region of the gene in *L. mexicana* may reveal the means by which 3'-nt is regulated in each life cycle stage.

The difference in 3'-nt expression between the different life cycle stages of *L. mexicana* may reflect the function of the enzyme *in vivo*. If 3'-nt has a function in the acquisition of purine nucleosides, as it has been suggested (Gottlieb, 1989), then its activity would be expected to be most significant in those cell stages which are rapidly growing and

dividing, whose requirement for nucleic acids and nucleotides is greatest. This appears to be supported by the difference of expression in procyclic and metacyclic promastigotes. In terms of both enzyme activity and levels of mRNA transcript, the metacyclic forms show significantly lower levels of 3'-nt expression than the procyclics.

The metacyclic promastigotes are non-replicative forms that have been described as pre-adapted for survival in the mammalian host (Mallinson & Coombs, 1989). The absence in these forms of active cell division may be the reason for reduced purine acquisition, reflected in decreased expression of 3'-nt. In addition, the nature of metacyclics as pre-adapted for the mammalian host may mean, in a sense, they are enzymatically between promastigote and amastigote. The nuclease profile of the metacyclic forms certainly appears to be intermediate between promastigote and amastigote, with decreasing 40kDa activity and increasing 29/31kDa activity. This is more clearly shown over 14 days of continuous culture (Bates, 1994a). The possible reasons for the observed stage-specificity of 3'-nt expression are discussed further in section 7.4.

7.3 Regulation of 3'-nt expression in response to purine availability

The regulation of the native 3'-nt cloned was investigated using nuclease and 3'-nucleotidase assays. In promastigotes grown on normal medium (supplemented with adenosine), the 40kDa band of 3'-nt activity is initially expressed following passage to fresh medium but is subsequently downregulated after about 48 hours. This suggests the enzyme is expressed constitutively in this stage but is regulated in response to available purines. By removing purines from the promastigote medium (subjecting the cells to purine depleted conditions), it can be seen that the 3'-nt activity continues to be expressed and is upregulated. This has been reported previously in other

trypanosomatids (Sacci *et al.*, 1990; Alleman & Gottlieb, 1990). The upregulation coincides with the onset of log phase growth in promastigote cultures and is maintained in the cell population for as long as there is a lack of purines (measured up to 7 days here). The 29/31kDa band of nuclease activity is also upregulated under starvation (Fig. 6d) but shows no nucleotidase activity at this level of measurement.

The method by which 3'-nt activity is upregulated in response to purine availability appears, as in the enzyme's stage-specificity, to be mediated by the level of transcribed mRNA. Northern blots of starved and replete promastigotes showed an increase in starved culture 3'-nt mRNA levels (after 3 days of culture) compared to replete levels. Subsequently, the amount of 3'-nt transcribed dropped to the same levels of expression seen in purine-replete cells. This suggests the increase in 3'-nt activity under starvation conditions is mediated by increased gene transcription or by the increased stability of transcript. In either case, the upregulation of 3'-nt activity correlates with increased protein expression, rather than an activation of the expressed enzyme in response to purine stress. This is supported by evidence from *Crithidia luciliae*, where the increase in 3'-nt activity due to purine starvation is prevented by cycloheximide and actinomycin D, inhibitors of protein and RNA synthesis respectively (Gottlieb *et al.*, 1988). The presence, therefore, of an inactive 3'-nt in promastigotes that is cleaved to the active form in response to starvation is unlikely.

The observed upregulation is reversed by the addition of 3'-AMP to the cell medium of purine-depleted culture. The addition of inorganic phosphate (the other product of 3'-nucleotidase digestion) to identical culture had no effect on the levels of 3'-nt activity expressed. This evidence indicates that native 3'-nt expression is specifically regulated

according to the availability of free nucleoside bases to the promastigote cell. The method by which purine levels regulate 3'-nt expression was not investigated, but in the insect parasite *Crithidia*, inhibitors of protein synthesis prevented the reversal of upregulation seen above, suggesting 3'-nt regulation is controlled by the expression of other specific proteins (Hall *et al.*, 1996).

This is supported in *L. mexicana* by the tube assay 3'-nt activity results (Figs. 6e & 6f). In starved cells, 3'-nt activity is initially expressed as in normal cultures, but at an elevated level. After about 50 hours, both cultures exhibit a drop in activity: normal cultures to a negligible level and starved cultures to a sustained upregulated level. It is possible that in both cultures, the decrease in 3'-nt activity (in response to available purines in the culture medium) is mediated by the expression of a further protein. Expression of this regulatory protein may be sustained by positive feedback in the presence of available purines (ie in normal cultures) but downregulated in a situation of depleted purine (ie in starved cultures). This would result in a rapid decrease of 3'-nt activity in a situation of purine abundance but a continuing activity in purine scarcity, as demonstrated by the results above.

The constitutive nature of 3'-nt expression in *L. mexicana* was further tested using cultures adapted to a variety of purine sources. Promastigote cultures adapted to using 3'-AMP, 5'-AMP and polyA as their sole sources of purine, were used to assess whether 3'-nt expression was permanently regulated in response to external purine supply over long periods of time. It was considered that promastigotes conditioned to meet their purine needs solely from 5'-AMP may, after a sufficiently long period, stop expressing

3'-nt altogether and cells similarly conditioned to 3'-AMP may prolong 3'-nt expression beyond the 48 hour period observed in adenosine-adapted cultures. Neither was the case.

Enzyme activities and levels of expressed mRNA were equal in all the adapted cultures tested. The only significant difference observed was the delayed downregulation of constitutive 3'-nt expression in cultures adapted to polyA as their sole purine source. In these cultures, 3'-nt activity was downregulated after 72 hours in fresh culture as opposed to after the 48 hour period routinely seen. This delayed regulation of 3'-nt in response to purine supply may be a reflection of the increased complexity of polynucleotide digestion compared with that of single nucleotides. Polyadenosine must undergo both nuclease and nucleotidase digestion to release nucleosides that can be subsequently imported. The persistence of 3'-nt activity in the polyA-adapted culture may be due to an increased significance of the nuclease function of this enzyme to the cells. Since 3'-nt has a dual nuclease/nucleotidase activity, an upregulation of the nuclease will result in a corresponding upregulation of nucleotidase activity (measured in these cells).

Under starvation conditions, each of the adapted cultures also behaved in a similar manner to each other. All the promastigote cultures showed similar upregulation of 3'-nt activity in response to purine starvation, regardless of whether 3'-AMP was a substrate available to them or not. In addition, 5'-nt assays of the same cultures showed upregulation (though to a lesser extent) of this cell-surface enzyme. It has been hypothesised that the two nucleotidase activities function in tandem to supply the cell with importable nucleosides (Gottlieb, 1989) and this co-ordinated upregulation of both activities suggests they may both be part of a general response in the promastigote to conditions of nutritional stress. The upregulation of 3'-nt activity regardless of the purine

source available to the promastigote seems to indicate a significant role for this enzyme in the promastigote form of the parasite.

7.4 The role of 3'-nt in the biology of *Leishmania*

Trypanosomatid parasites appear to possess several diverse means by which to salvage purines from the host environment (Chapter 1.9). Both nucleobases and nucleosides may be used to synthesise all purine requirements by the process of interconversion along several different pathways (Fig. 1b). For this reason, it is unlikely that any single enzyme or transporter is vital for parasite survival although in combination, their function is obviously critical.

Nucleotides may be formed directly from imported nucleobases by the action of several phosphoribosyltransferases (PRTases). There is evidence that the transport of these bases is specifically upregulated, in *Crithidia*, from simple diffusion to carrier-mediated import under conditions of purine starvation, indicating the importance of this particular pathway of salvage (Alleman & Gottlieb, 1996). A similar situation of facilitated transport taking over from diffusion has been described in *L. braziliensis*'s uptake of adenine and hypoxanthine bases under conditions of purine starvation (Hansen *et al.*, 1982) and this may be a common mechanism in trypanosomatids.

The importance of nucleobase transport and salvage pathways to the parasites may be explained by the observation that the most abundant sources of purine in mammalian serum, as a result of host metabolism of nucleic acid, are the bases xanthine and hypoxanthine (Hartwick *et al.*, 1979). However, though more abundant, there is evidence that the import of nucleobases is less efficient than that of nucleosides (Hansen

et al., 1982), from which nucleotides are formed in the parasite by the action of kinase. In *Crithidia*, hypoxanthine and adenosine may compete for the same transporter (Day & Gero, 1997) although increased uptake of both is reported under conditions of low purine. In general, these two pathways of nucleobase and nucleoside salvage (Fig. 1b) appear to be capable of functioning independently of each other but are both maximised under artificial conditions of purine stress.

The 3'-nucleotidase described here functions as a phosphomonoesterase (cleaving the 3' phosphate bond of a 3'-nucleotide to give a nucleoside) and an exonuclease (cleaving the 3' phosphate bond of the terminal residue of a polynucleotide to give a 5'-nucleotide). These two catalytic functions ultimately provide substrate for the parasite's nucleoside transporters (the resulting 5' nucleotides being further digested by 5'-nucleotidase) and so potentially supply the kinase pathway of purine salvage. In a cell with the capability to import purine bases, therefore, the action of 3'-nt would not be expected to be essential. Even in a situation where the PRTase pathway of salvage is not functioning, it seems the parasite could still supply all its purine requirements from imported nucleobases, through the action of phosphorylase and kinase activities (Fig. 1b). In addition, the 3'-nucleotidase activity described here may only be a co-incidental factor of the nuclease active site in 3'-nt and not its prime function.

Despite the logical conclusion that 3'-nt activity would not be expected to hold a position of critical function in *Leishmania*, there are several points of evidence to suggest the contrary, at least in the promastigote form of the parasite. For instance, the enzyme appears to be constitutively expressed in promastigotes (section 7.3), even after long-term adaptation to sources of purine whose utilisation would not require its activity. In

addition, there is a dramatic and specific upregulation of 3'-nt activity in response to artificially induced purine depletion in the cell medium. This has been demonstrated in *L. donovani* (Sacci *et al.*, 1990), here in *L. mexicana* and also in *Crithidia luciliae* (Alleman & Gottlieb, 1990). The co-ordination of 3'-nt and purine transporter activities has been studied most extensively in *C. luciliae*.

This study in *C. luciliae* has demonstrated that the observed upregulation of 3'-nt activity in purine-starved culture is concomitant with an increase in specific adenosine and guanosine transport into the cell (Hall *et al.*, 1996). This appears to be a specific response to purine starvation and is not accompanied by a general increase in metabolite transport in the purine-starved cells (Gero *et al.*, 1997). This co-ordinated increase of available nucleosides (through the actions of 3'-nt and 5'-nt) and their increased transport in response to a specific environmental cue (purine stress) appears to be a unique function in parasite biochemistry (Gero *et al.*, 1997). Because purine transport is regulated concomitantly with the provision of purines in an importable form, the system of purine salvage in *Crithidia* has a distinct competitive advantage over the insect host. In addition, host purine transport is mediated through a single transporter with a broad substrate specificity (Jarvis, 1987), which further increases the parasite's competitive advantage.

Evidence suggests (Aronow *et al.*, 1987; Baer *et al.*, 1992) that a similar system of specific nucleoside transport exists in *Leishmania*. If so, it seems likely that a similar co-ordination of 3'-nt activity and nucleoside transport is also present. The advantage such a co-ordinated system would provide for *Leishmania* procyclic promastigotes developing in the sandfly gut may be significant in ensuring the establishment of an infection.

As has been discussed (Chapter 1.4), the population of procyclic promastigotes developing in the sandfly gut (from amastigotes taken up in a blood meal) faces environmental conditions of limited nutritional resources. The limited time available to the parasites before digestion and excretion of the blood meal would greatly favour the development of any competitive advantage over the host in terms of nutrient acquisition. The 3'-nt appears to be a central component of such a system, enabling the accelerated acquisition of purines from the surrounding blood meal. This acquisition subsequently enables the accelerated division of promastigote forms, even in conditions of low nutrient availability, to a population from which a significant proportion are able to develop further into the infective metacyclic forms.

It would be of interest to assess the rate at which the sandfly is able to fully digest a typical blood meal and so determine the precise environment in which the procyclic forms develop. It may be that, *in vivo*, the cells are perpetually in a state of purine stress and the 3'-nt naturally functions in its upregulated state. The downregulation of constitutive 3'-nt expression in 'normal' (purine-replete) cultured forms may only be a factor of prolonged cell culture in artificial conditions. Conversely, the population of developing procyclic promastigotes may initially become established in purine-replete conditions and the co-ordinated upregulation of 3'-nt and transporters only become significant in cells developing later, so giving a final 'boost' to the promastigote population as purines become depleted in the sandfly gut.

The increased transcription of 3'-nt in short-term promastigotes (derived from lesion amastigotes) and the increased nuclease/nucleotidase activity of lesion amastigotes (Chapters 4.6 and 6.4) suggest the *in vivo* forms of the parasite exist in conditions where

the significance of purine acquisition is greater than in artificial culture conditions. This is as expected, as culture conditions are designed to be optimal and it may indicate that 3'-nt activity *in vivo* is likely to be functioning in an upregulated state. The initial constitutive expression and subsequent downregulation of the enzyme and the notable contrast between expression in normal and starved cultures may be exaggerated, therefore, as a result of artificial culture conditions and not as significant for the biological function of the enzyme as is suggested experimentally.

The most abundant sources of purine in human serum are reported to be the bases xanthine and hypoxanthine, as already mentioned (Hartwick *et al.*, 1979). In addition, the majority of general cellular nucleases (of any organism) characterised cleave the 3' phosphate bond linkages of polynucleotides and nucleic acids, resulting in 5'-nucleotides, the most common biochemical form for nucleotides. These are then converted to importable nucleosides by 5'-nt, a surface membrane enzyme found in a very wide variety of species as diverse as humans and trypanosomatids. Taking this into account, the purine composition of mammalian blood taken up by a feeding sandfly will predominately consist of a mixture of 5'-nucleotides (resulting from cell lysis and mammalian degradation of nucleic acids in the serum), free purine bases (for example, hypoxanthine) and polynucleotide chains in various stages of digestion. Thus, the necessary supply of purines required for *Leishmania* growth in the sandfly gut could be met by the combined action of cell surface nucleases, 5'-nt and nucleoside/nucleobase transporters.

Given this, the significance of the 3'-nt enzyme described is most likely to be as a surface membrane nuclease in *Leishmania* with coincidental 3'-nucleotidase activity. There is a

report that, in an investigation of 3'-AMP levels in various rat tissues, 3'-nucleotides are particularly prevalent in the spleen (Bushfield *et al.*, 1990), and this may provide a role for 3'-nt in visceral species of *Leishmania*. In general, however, the expression of a specific 3'-nucleotidase activity would not be expected to significantly contribute to the parasite's purine acquisition. This appears to be supported by the absence of activity in the amastigote form. However, as part of a co-ordinated uptake system (as described in *Crithidia*), the 3'-nt may have a specific role in the promastigote, increasing purine import in competition with the host where there is a limited purine supply.

The significance of the described 3'-nt activity to the promastigote form of the parasite appears to be further supported by the expression of another 3'-nucleotidase/nuclease activity (under conditions of purine-starvation) with very similar properties (Figs. 6l & 6m). This 80kDa activity appeared to be unconnected to the 3'-nt gene sequenced. Although its size suggested a dimerisation of the 40kDa 3'-nt, the activity was unaffected by either boiling or the reducing agent DTT. In addition, a third band of 3'-nucleotidase/ nuclease activity (35kDa: Fig. 6n) appeared in one population of starved promastigotes which, for an unknown reason, failed to express the expected 80kDa activity. Boiling the cell sample prior to SDS-PAGE restored the 80kDa activity, which may have been inhibited in that particular sample of promastigotes by a heat-labile inhibitor. Subsequent aliquots of the same starved culture also showed the restoration of the 80kDa, possibly due to the diluting out of the inhibitor present at day 2. Interestingly, with the re-appearance of the 80kDa activity, there is an apparently corresponding decrease in the 35kDa form.

One explanation of these observations which, unfortunately, could not be reproduced, is that the starved promastigote cells possess a variety of different enzymes or different forms of the same enzyme which may be expressed in response to purine stress to increase the parasite's capacity for purine acquisition. What is interesting is that both forms described also showed the dual 3'-nucleotidase/nuclease activity of the cloned 3'-nt, suggesting either that they are simply forms of the same enzyme or that the co-function of nucleotidase and nuclease is particularly significant in the salvage pathway. Again, this may simply be a factor of the form of the active site involved in nuclease activity or it may signify an as yet undiscovered significance for such a dual activity. It would be tempting to suppose that the combination of nuclease and nucleotidase activities in one enzyme allows the efficient processing of polynucleotides to nucleosides in one or two steps. However, in this case, of course, the nucleotides formed from such nuclease activity have 5' phosphates and the nucleotidase portion of the enzyme has no activity towards 5'-nucleotides (Bates, 1993b). The particular significance of the 3'-nucleotidase/nuclease association remains uncertain.

With the role of 3'-nt appearing to be as a boost for nutritionally limited *L. mexicana* promastigotes to enable more effective competition for purines, it was of interest to establish whether enzyme expression could be induced by purine starvation in the amastigote form. Unfortunately, it was not possible to adapt amastigotes to culture conditions where the levels of purine could be so tightly controlled as for the experimental promastigote cultures and so a number of other strategies were tried in order to obtain artificially starved cells.

Incubation of axenic amastigotes in purine-free medium simply arrested cell growth and no changes in nuclease profile were observed. Promastigote cultures transferred from 26°C to 32°C led to the induction of amastigote-like forms that showed both morphological and nuclease characteristics of amastigotes. Promastigotes adapted to adenosine and 3'-AMP were both transformed in this way and identical nuclease profiles were seen over 7 days (Fig. 6q). The eighth day showed a decrease in nuclease activity in the adenosine-adapted culture but sustained activity in the 3'-AMP-adapted cells. This may indicate some upregulation of the 29/31kDa nuclease in response to purine depletion. The adenosine culture had a ready supply of importable purines in the culture medium whereas purine acquisition for the 3'-AMP cells required a nucleotidase digestion. It was previously demonstrated that the 29/31kDa nuclease of amastigotes also possesses some 3'-nt activity (Fig. 6a) and this may be sufficient for the provision of nucleoside for these cells. However, as purine availability decreases (after 8 days), those cells without a ready supply of purine may sustain the 29/31kDa nuclease activity to compensate. The experiment was not repeated and the result may only be a factor of the limited quantitative nature of substrate SDS-PAGE.

In addition to the cells monitored at 32°C, a proportion of each culture was transferred back to 26°C after 6 days. The adenosine-adapted culture is seen to begin the transformation back to the promastigote form by the re-appearance of the 40kDa nuclease activity within 48 hours of the transfer. In comparison, the 3'-AMP culture shows no transformation within this time. The time of transformation from amastigote to promastigote in this artificial system may again be a reflection on the availability of importable purine to each population of cells. Nucleotides would be necessary for this transformation as the amastigote cells divide and develop into promastigotes.

Amastigote forms with a ready supply of nucleoside (the adenosine-adapted culture) may be expected to transform more quickly than those with an unavailable supply of nucleoside (the 3'-AMP-adapted culture). The experiment was not extended to assess whether these forms would have the capability to transform at all but if the 3'-nt activity of the 29/31kDa nuclease band persists in these amastigotes, it would be reasonable to suppose that they would eventually, but at a slower rate.

This experiment was repeated to investigate the properties of amastigotes induced from purine-depleted promastigotes. Purine starvation did not appear to affect the promastigotes' ability to develop into amastigote-like forms at 32°C and the final replete and depleted forms have an identical nuclease profile. Those cells transferred back to 26°C show the re-appearance of the upregulated 40kDa band generally seen in starved promastigotes, indicating that they represented the same population of cells as those originally transformed at 32°C. This also indicated that the cells were still in starvation conditions (the cultures were not changed at all except the incubation temperature). These results confirmed that, at least *in vitro*, amastigote forms do not respond to external levels of purine in the same way that promastigotes do.

Experiments thus far seemed to suggest that there is no role in amastigotes for the 40kDa 3'-nt activity described here, even when exposed to conditions where purine is not readily available, conditions under which promastigotes show a significant upregulation of 3'-nt activity. The reason for this may be that the amastigotes have a reduced requirement for purines or an efficient alternative means of acquiring them, which does not involve the 40kDa 3'-nt. The presence of 3'-nucleotidase activity in the 29/31kDa nuclease activity bands suggests that there is some requirement for 3'-nt activity in the

amastigote form but perhaps not to the same extent. This 29/31kDa 3'-nt activity appears to be amastigote-specific. Even in 3'-nucleotidase activity gels that have been significantly overloaded with promastigote cell samples, there is no 3'-nt activity evident in the 29/31kDa nuclease bands of promastigotes. Similarly, promastigotes grown in purine-depleted medium show an upregulation of the nuclease bands at 29/31kDa but no evidence of a corresponding 3'-nt activity band of this size, as seen in amastigotes (Figs. 6d & 6l).

Studies of purine requirement in *L. mexicana* amastigotes suggest a generally slower rate of purine metabolism than is observed in other life cycle stages (Hansen *et al.*, 1984). This may be due to the fact that amastigotes are more slowly dividing forms (and this is certainly seen in *in vitro* culture) with a lower general rate of metabolism and not restrained by the same time limits (in terms of population development) as are the promastigotes. In addition, by virtue of their position in the host macrophage, they may obtain a far more ready supply of purines and other nutrients than is available to the promastigote forms in the insect gut. A chief role of the macrophage in the host blood circulation is to scavenge cellular debris, micro-organisms and damaged cells from the serum and these are degraded through the lysosomal pathways of the cell. The parasitophorous vacuole (PV) of *L. mexicana* is fusigenic in nature and pulse chase experiments have established the targeting of lysosome components to it (Russell *et al.*, 1992). The rapid uptake and exploitation of purines by the amastigotes may even serve to divert cellular trafficking of cytosolic purines down a concentration gradient to them, the PV acting as a kind of 'purine sink'. The abundance of nucleotides resulting from the macrophage's scavenging would therefore be available to the parasites.

Furthermore, the acidic conditions of the phagolysosome in which amastigotes reside may encourage greater purine transport. The acidification of the PV appears to be an active process (Antoine *et al.*, 1990), implying the importance of acid conditions for amastigote survival and growth. In the related bloodstream parasite *Trypanosoma brucei brucei*, hypoxanthine transport has been shown to be proportional to the proton motive force (the energy generated by the flow of H^+ ions down a concentration gradient) (DeKoning & Jarvis, 1997a). This suggested that nucleobase transport may be mediated by a nucleobase/proton symporter and a similar model has also been proposed for adenosine transport (DeKoning *et al.*, 1998). The hypoxanthine symporter appears to be in addition to another nucleobase transporter with differing uptake properties independent of proton motive force (DeKoning & Jarvis, 1997b). The characterisation of these two transporters suggests that the normal uptake of purines by bloodstream forms of *T. b. brucei* may be aided in conditions of low pH by the activity of the symporter.

If a similar mechanism of purine/proton symport was present in *L. mexicana* amastigotes, it could be argued that the maintenance of a steep pH gradient across the parasite cell membrane might serve to generate a sustained protonmotive force. If so, the acidic conditions of the PV would increase the efficiency of purine uptake by the amastigote. In *L. donovani* promastigotes, a similar proton motive force-driven symporter mechanism has been described for the uptake of D-glucose and L-proline (Zilberstein & Dwyer, 1985). Proton symport mechanisms for a number of nutrients and ions also operate in the amastigote form of *L. donovani* (Zilberstein, 1993) and the presence of a similar system in *L. mexicana* is likely.

A purine/proton symport mechanism may significantly contribute to the amastigote form's acquisition of purines but is still dependent on the supply of nucleobases and nucleosides to the parasite cell surface. As described here, there is an abundance of nuclease activity present in the amastigote compared to the promastigote form (Fig. 6a; Bates, 1993b) and these nucleases may be sufficient for such supply. Hypothetically, if the nucleic acids and nucleotides targeted to the PV have been trafficked through the phagolysosomal digestive system of the macrophage or directly from the macrophage cytosol, they will already be in a degraded form and more easily imported than those possibly confronting the promastigotes in whole serum in the sandfly gut.

Since the PV is essentially a physically limiting environment around the amastigotes, secretion of digestive enzymes by the parasites is also an effective strategy for preparing nutrients for import. This is illustrated by the secretion of a potent nucleotidase by *Toxoplasma gondii* tachyzoites, another PV-bound intracellular parasite (Sibley *et al.*, 1994). To date, there are no definitive reports of amastigote digestive enzymes that are released from the cell surface, though amastigotes have been shown to secrete proteophosphoglycan, which may contribute to the formation of the *L. mexicana* PV (Peters *et al.*, 1997).

Taking into account these specialised circumstances of its intracellular niche, the amastigote form's expression of a surface membrane enzyme with a specific role in purine acquisition may be negated, and so 3'-nt expression is shut down until it is required. If expression continued through the amastigote stage of the life cycle but was rapidly and constitutively downregulated, it may be expected that the forced starvation of

these forms may induce 3'-nt expression. This was not seen and it seems that amastigotes are unable or unwilling to express the 40kDa enzyme.

There is a suggestion that the stage-specificity of 3'-nt may be explained in terms of parasite evolution (Hall *et al.*, 1996). The simplest trypanosomatids, typified by *Crithidia* spp., may have been monogenetic, dwelling entirely in the insect gut environment with a perpetually fluctuating level of purines. In this situation, the development of a specifically inducible system for purine acquisition that allows the parasite to compete effectively with the host for nutrient supplies (especially at low concentrations) would be highly favourable. Such a system may be present in the *L. mexicana* promastigote, as discussed. With the evolution of a digenetic life cycle and the relatively stable environment of the PV within host macrophages, levels of purine are likely to be less variable. In this case, the need for such an inducible system is reduced and may lead to its downregulation and eventual disappearance. This may explain the absence of 3'-nt expression in amastigotes.

7.5 Conclusions and suggested further work

It appears, therefore, that the 3'-nt enzyme described is a promastigote-specific enzyme with a particular function in providing these parasite forms with a competitive advantage for nucleoside acquisition over their insect hosts, particularly in conditions of low purine availability. The amastigote form of the parasite appears to be sufficiently supplied with purines through its situation in the host cell and by the action of its expressed nuclease activities (29/31kDa) and shows no expression of 3'-nt even under starvation conditions.

The significance of the dual nuclease and 3'-nucleotidase activities of the described enzyme is unknown. It is a feature conserved in several distinct nuclease enzymes and is shown in this work to be upregulated in response to purine stress by the expression of further similar enzymes. However, the dual nature of 3'-nt's catalytic activity may only be a factor of the structure of the nuclease active site, as suggested by the characterisation and crystallisation of *Penicillium* nuclease P1.

It is apparent, therefore, that the 3'-nt of *L. mexicana* does not constitute a favourable target for chemotherapy, being absent from the human stage of the life cycle. Although of obvious importance to the promastigote stage, the range of different purine acquisition enzymes and pathways available to the parasite suggest that it may have the capability to compensate for the loss of 3'-nt, should a successful knockout be achieved. This may equally be the case for the amastigote salvage enzymes, as demonstrated by the knockout of PRTases (Hwang & Ullman, 1997), and the successful development of any chemotherapeutic agents targeting the purine salvage apparatus would probably require the simultaneous interruption of a number of different pathways.

The most effective therapeutic approach would perhaps be the simultaneous targeting of the specific purine transporters and the key cytosolic components of the purine salvage pathway. These enzymes have relatively narrow substrate specificities and have been shown to be accessible to purine analogues, both on the surface of the cell and in the cytosol. Central salvage enzymes such as HGPRT may either be targeted directly using drugs that inhibit catalysis or employed to metabolise purine analogues, giving products toxic to the parasite (Ullman & Carter, 1995). For instance, the metabolism of the hypoxanthine analogue allopurinol (a pyrazolopyrimidine drug already in use) by

HGPRT results in aberrant nucleotides being incorporated into nucleic acid (Marr and Berens, 1983). This strategy of parasite conversion of non-toxic analogues is also illustrated by *Leishmania*'s metabolism of 6-methylpurine 2'-deoxyriboside to the potent adenine antimetabolite 6-methylpurine (Carson & Chang, 1981) and the metabolism of formycin B (Nolan *et al.*, 1984).

An example of the use of a combined therapeutic approach is the simultaneous targeting of nucleoside transporter and nucleotide formation within the cell (Ogbunude & al Jaser, 1992). The combination of a transport inhibitor and formycin A was shown to be more effective against *L. major* in hamsters than each agent in isolation. Again, this involved the formation of aberrant nucleotides, a strategy that is possibly more effective in blocking salvage pathways than inhibition of enzyme activities. With the targeting of single enzyme activities and even multiple activities involved in salvage, a pressure is applied to the parasite to compensate by using other pathways, of which there appear to be several in *Leishmania.* If enzyme activities are not targeted, however, and the pathway blocked by means of substrate conversion to toxic products, whatever pathways of purine metabolism are open to the parasite, the outcome remains the same. If the raw materials of nucleotide metabolism are flawed, no amount of pathway development or circumnavigation will be able to compensate. This strategy would avoid the need for inhibition of all the possible pathways of purine interconversion and transport by multiple knockouts or combination therapy. A number of purine analogues have been identified as potential drug candidates (Marr, 1991).

Chemotherapy aside, further work on the characterisation of *L. mexicana* 3'-nt could include a more detailed investigation of its relationship to purine transport and

metabolism pathways in the promastigote. The possibility of a co-ordinated system for purine salvage regulated by external purine supply, as that suggested in *C. luciliae*, is a biologically fascinating one. In addition, investigating the relationship between the various forms of the dual nucleotidase/nuclease activity demonstrated in starved promastigotes, amastigotes and the 40kDa 3'-nt, may illuminate the role of this catalytic function and its possible significance in purine salvage. Of particular interest would be the amastigote-specific 29/31kDa activity and the cloning and characterising of this gene may contribute to an understanding of the amastigote form's specialised adaptation to its intracellular environment.

The means by which purine levels are able to regulate 3'-nt activity, whether by expression of a specific inhibitor or by affecting mRNA stability, would also be an interesting investigation with a broad application to the understanding of gene regulation in trypanosomatids. The expression of an inactive pro-enzyme (for example, the 52kDa protein in Western blots) may be significant in this process and an investigation of the potential 5' splice sites available to the 3'-nt gene may reveal its means of regulation. The stage specificity and ease of study of the *L. mexicana* 3'-nt (in terms of established enzyme assay, abundant product and ease of *in vitro* culture) make it a useful potential model for the study of trypanosomatid gene regulation in response to environmental cues.

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