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Stable expression of biologically active recombinant bovine interleukin-4 in *Trypanosoma brucei*

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Abstract We have explored the potential of *Trypanosoma brucei* as a eukaryotic expression system. Procyclic forms, which correspond to an insect-adapted stage, can easily be cultured in vitro. The cells grow to densities ~10-fold greater than higher eukaryotic cells and are not infectious for mammals. An expression vector which can stably integrate into the genome was used to express high levels of recombinant bovine interleukin-4 (IL-4). Trypanosome-derived IL-4 is released into the medium and is biologically active. The recombinant protein down-regulates CD14 expression in human macrophages and inhibits NO production by stimulated bovine macrophages.

Key words: Expression system; Bioassay; Interleukin; Cytokine; Growth factor; Recombinant protein

1. Introduction

There is a general need for the production of recombinant proteins in sufficient quantities to study their functions under defined conditions. This is particularly true of molecules such as cytokines and growth factors, which are produced in trace amounts in a whole organism and must otherwise be purified from large amounts of tissue. Another reason why recombinant molecules might be preferable is that factors derived from cadavers, for example growth hormones from the pituitary glands, carry the risk of being contaminated with pathogenic agents such as viruses or prions.

A variety of bacterial and eukaryotic expression systems are available for the expression of recombinant proteins. Prokaryotic systems have the advantage of high yields and ease of handling, but there are several major drawbacks. Proteins are frequently insoluble and/or inactive. It has recently been demonstrated that the way in which proteins are folded in bacteria (post-translationally) is fundamentally different from the way in which they are folded in eukaryotic cells (co-translationally) and this may explain why many recombinant proteins assume the wrong conformation when they are expressed in bacteria [1]. In addition, bacteria are unable to glycosylate proteins, which may affect their biological activity, antigenicity and stability in vivo. Expression systems using mammalian cells are much more likely to produce correctly processed, functional proteins, but the yields are much lower. Intermediate between the bacterial and mammalian systems are the baculovirus/insect cell expression systems which give rise to partially glycosylated proteins.

An attenuated strain of *Trypanosoma brucei* offers several advantages as a eukaryotic expression system. The procyclic form can easily be cultured in defined media, grows rapidly and can attain densities ($>3\times10^7$ ml^{-1}) that are about 10-fold greater than higher eukaryotic cells. Procyclic culture forms are not infective for mammals and the subspecies *T. brucei brucei* is not pathogenic for humans at any stage of its life cycle. In contrast to bacteria, trypanosomes can glycosylate [2], tyrosine phosphorylate [3] and add glycolipid anchors to proteins [4,5]. Procyclic culture forms are covered by a glycoprotein coat of several million procyclin molecules [5,6]. The cells divide by binary fission, with a population doubling time of ~9–10 h in culture, and constitutively express high levels of procyclin in order to maintain the density of the coat. We have used a locus containing two procyclin genes as the basis for construction of the vector pGAPRONE (Fig. 1). This is a bicistronic vector in which the reporter gene and a selectable marker are flanked by the procyclin promoter and the necessary sequences for efficient processing of the precursor RNA into monocistronic mRNAs [7]. When linearised plasmids are introduced by electroporation, the vector integrates by homologous recombination of the flanking regions into a procyclin locus [8]. Six antibiotics that do not show cross-resistance [9,10] are available for sequential rounds of integration into defined sites in the genome. This potentially allows expression of multi-subunit complexes.

We have now used this system to produce stably transformed trypanosomes that express recombinant bovine interleukin-4 (rboIL-4) with a yield of approximately 3 million molecules per cell. The protein is released into the culture supernatant and is biologically active in assays performed with human and bovine macrophages.

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Abbreviations: FBS, foetal bovine serum; GARP, glutamic acid/alanine-rich protein; PBMC, peripheral blood bovine mononuclear cells; (r)boIL-4, (recombinant) bovine interleukin-4; (r)huIL-4, (recombinant) human interleukin-4; UTR, untranslated region

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2. Materials and methods

2.1. Trypanosomes

Procyclic culture forms of *T. brucei* strain 427 and all derivatives were cultured at 27°C in SDM-79 medium [11] supplemented with 5% foetal bovine serum (FBS; Gibco BRL, Basel, Switzerland). Stable transformants were cultured in medium containing 50 μg ml^{-1} G-418. Before harvesting culture supernatants for rboIL-4 bioassays, the trypanosomes were washed twice in SDM-79 without G-418 and cultured for several generations in SDM-79 supplemented with 5% FBS. Supernatants were harvested from exponentially growing cultures and centrifuged for 10 min at $1200\times g$ followed by sterile filtration using a 0.25 μm sterile filter. The supernatants were aliquoted and stored at −70°C.

2.2. Construction of pG-boIL-4Δ164 and pG-mcsΔ164

The construction of the vector pGAPRONE-Δ164 (Fig. 1) is described in detail elsewhere [7]. This plasmid contains a gene for the trypanosome surface glycoprotein GARP (glutamic acid/alanine-rich protein) and a neomycin resistance gene as a selectable marker. The coding region of boIL-4 was amplified from a plasmid containing the complete cDNA sequence [12] using the following primers: boIL-4A (GGAAGCTTAATGGGTCTCAC) and boIL-4B (ACGGATCCTTCAACACTTGGA). Underlined sequences are synthetic *Hin*dIII and *Bam*HI restriction sites that were introduced to facilitate cloning. The GARP gene was excised from pGAPRONE-Δ164 and replaced by the amplified boIL-4 *Hin*dIII-*Bam*HI fragment, resulting in the expression vector pG-boIL-4Δ164 (Fig. 1). The prefix G indicates that the plasmid is derived from pGAPRONE and Δ164 refers to a truncated procyclin 3′ untranslated region (UTR) from which the first 164 nucleotides have been deleted [7].

In the second generation construct pG-mcsΔ164 (Fig. 1), a small multiple cloning site replaces the GARP gene. This was achieved by digesting pGAPRONE-Δ164 with *Hin*dIII and *Bam*HI and ligating it with overlapping complementary oligonucleotides (AGCTTGAATTCTCGAGCTCCCGG and GATCCCGGGAGCTCGAGAATTCA) that reconstituted the *Hin*dIII and *Bam*HI sites and introduced a further three unique sites (see Fig. 1).

2.3. Electroporation, G-418 selection and cloning

Procyclic culture forms of *T. brucei* 427 (5×10^7 cells) were electroporated with 5 μg of pG-boIL-4Δ164 that was linearised by digestion with *Kpn*I and *Not*I. Electroporation was carried out with a Genepulser (Bio-Rad) at 1.5 kV and 25 μF. Immediately after electroporation, the cells were transferred into SDM-79 medium containing 5% FBS and 50 μg ml^{-1} G-418 (Gibco BRL) and cultured at 27°C. G-418-resistant cells were cloned by limiting dilution in SDM-79 supplemented with 20% FBS.

2.4. Northern and Western blot analysis

RNA was isolated from exponentially growing cells and prepared for Northern blot analysis as described [13]. 10 μg of total RNA was denatured with glyoxal and dimethyl sulphoxide for 5 min at 50°C, separated on a 1.4% agarose gel and subsequently transferred to a nylon membrane. The following hybridisation probes were used: a PCR product corresponding to the 421-bp coding region of boIL-4 [12] and a genomic clone containing one copy each of α- and β-tubulin [14]. Post-hybridisation washes were performed in 1×SSC, 0.05% SDS at 65°C.

Western blot analysis was performed as follows: 15 μl culture supernatant from exponentially growing trypanosomes was mixed with protein sample buffer and boiled for 1 min. Alternatively, samples were denatured for 10 min at 100°C in the presence of 0.5% SDS, 1% β-mercaptoethanol then incubated for 2 h at 37°C in the presence or absence of 500 U PNGase F (New England Biolabs). Samples were separated by SDS–PAGE and transferred to nitrocellulose. The blot was incubated with 5 μg ml^{-1} of monoclonal anti-boIL-4 (9D1; [15]) at room temperature for 1 h and subsequently with horseradish peroxidase-conjugated rat anti-mouse antibody (Dako Immunoglobulins, Denmark) at a dilution of 1:1000. Antigen binding was visualised with an enhanced chemoluminescence (ECL) detection kit (Amersham, UK) according to the manufacturer's instructions.

2.5. Cytokine bioassays: NO generation by bovine macrophages

Peripheral blood bovine mononuclear cells (PBMC) were isolated from freshly drawn venous cattle blood by a modified Ficoll-hypaque (Ficoll-triosyl) method [16]. 4×10^6 PBMC ml^{-1} were sealed in custom-made bags from Teflon foil (type 100 A; Du-Pont de Nemours, purchased through Angst and Pfister, Zürich, Switzerland). The cells were cultured in Iscove's modified Dulbecco's medium (Seromed, Munich, Germany) with the following additives: 10 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamine, 1% v/v non-essential amino acids (Seromed), 1% v/v minimum essential medium (MEM) vitamin solution (Seromed), 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 10 μg ml^{-1} neomycin, and 20% heat-inactivated FBS low in endotoxin (Life Technologies, Basel, Switzerland). Bags were incubated in a humidified incubator at 37°C, 5% CO_2. After 1 week, the majority of lymphocytes disappeared and monocytes had differentiated into macrophages. Cells were harvested and subcultured in cell culture medium containing 5% FCS in flat-bottom 96-well plates in the presence of 200 μg ml^{-1} heat-inactivated (2 h at 60°C) *Listeria monocytogenes* (strain NCTC 10,527) or *Salmonella dublin* and serial dilutions of supernatants from trypanosome cultures. Twenty-four hours after stimulation, supernatants were collected and nitrite, a stable metabolite of NO was determined using the Griess reaction [17,18].

2.6. Down-regulation of CD14 in human macrophages

PBMC from human blood were isolated by isopycnic centrifugation on Ficoll-hypaque as described [19]. Monocytes were enriched by an adherence step using medium (see below) with 2% heat-inactivated human AB serum, followed by three rinses with RPMI 1640. After overnight culture in medium containing 10% heat-inactivated AB serum, adherent cells were dislodged by vortexing. Cells were washed, placed in Teflon bags and cultured at a density of 10^6 monocytes ml^{-1}. The cells were cultured in RPMI 1640 medium containing 10 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamine, 1% v/v non-essential amino acids, 1% v/v MEM vitamin solution, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 40 mg l^{-1} folic acid and heat-

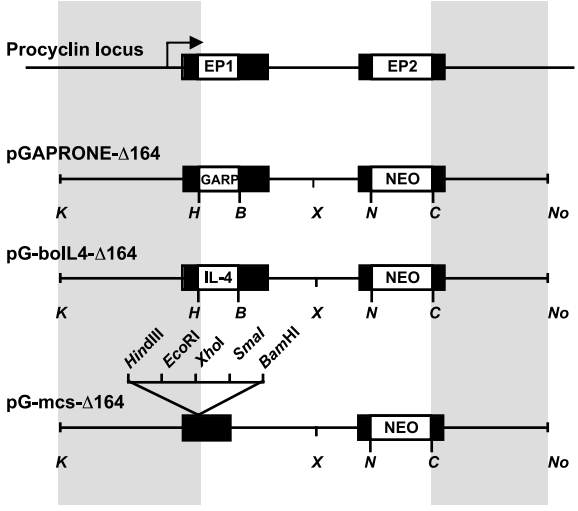

Fig. 1. Vectors for stable expression in *T. brucei*. The plasmids pG-boIL4-Δ164 and pGmcs-Δ164 are derivatives of pGAPRONE-Δ164 in which the GARP gene has been replaced by the boIL4 coding region and a multiple cloning site, respectively. The vectors contain a procyclin promoter (arrow), 5′ UTR and flanking sequences necessary for *trans*-splicing, a truncated procyclin 3′ UTR and intergenic regions responsible for the polyadenylation of the IL-4 mRNA. The neomycin resistance gene (NEO) is preceded by procyclin 5′ UTR sequences and followed by a truncated 3′ UTR and intergenic region. All modules can easily be exchanged due to unique restriction sites: K = *Kpn*I, H = *Hin*dIII, B = *Bam*HI, X = *Xba*I, C = *Cla*I, N = *Nhe*I and No = *Not*I. The neomcycin resistance gene can be replaced by cassettes conferring resistance to phleomycin, hygromycin, nourseothricin and blasticidin [9,10]. The *Eco*RI, *Xho*I and *Sma*I sites in pGmcs-Δ164 are unique. Integration occurs by homologous recombination (in the regions shaded in grey) replacing the two procyclin genes *EP1* and *EP2*. Coding regions are denoted by open boxes and UTRs by black boxes.

inactivated human AB serum. After cultivation for 7 (±1) days the cell population was highly enriched in macrophages (purity >95%, viability >90%). Two days before harvesting, trypanosome supernatants were added to the Teflon bags. Cells were harvested and labelled on ice with antibodies specific for surface markers such as anti-CD11b (Becton-Dickinson, San Jose, CA, USA), anti-CD14 (Becton-Dickinson and Dako, Glostrup, Denmark) and isotype control antibodies (Coulter, Hialeah, FL, USA) at saturating concentrations. Some antibodies were directly fluorescence-labelled; others were stained by an indirect procedure, using phycoerythrin-labelled anti-mouse IgG $F(ab')_2$ fragments (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Stained cells were subjected to flow cytometry, using a FACScan analyser (Becton-Dickinson), and the PC-LYSYS software. The level of surface marker expression was expressed as mean fluorescence of cells within the macrophage gate, as determined by light scatter properties of the cells.

3. Results and discussion

3.1. Production of stable transformants expressing boIL-4

The GARP open reading frame in the expression vector pGAPRONE-Δ164 was replaced by boIL-4 to create the plasmid pG-boIL4-Δ164 (Fig. 1). Procyclic forms of *T. brucei* were transfected by electroporation with linearised pG-boIL-4Δ164, subjected to selection with G-418 and cloned by limiting dilution. To verify that boIL-4 mRNA was expressed, a Northern blot was hybridised simultaneously with probes for boIL-4 and tubulin (Fig. 2A). RNA from trypanosomes stably transformed with the original pGAPRONE-Δ164 and pGAPRONE-wt vectors was used as a negative control. IL-4 transcripts were clearly detected in cells transformed with pG-boIL-4Δ164 (lane G-boIL-4), but not in the negative controls (lanes G-wt and G-Δ164).

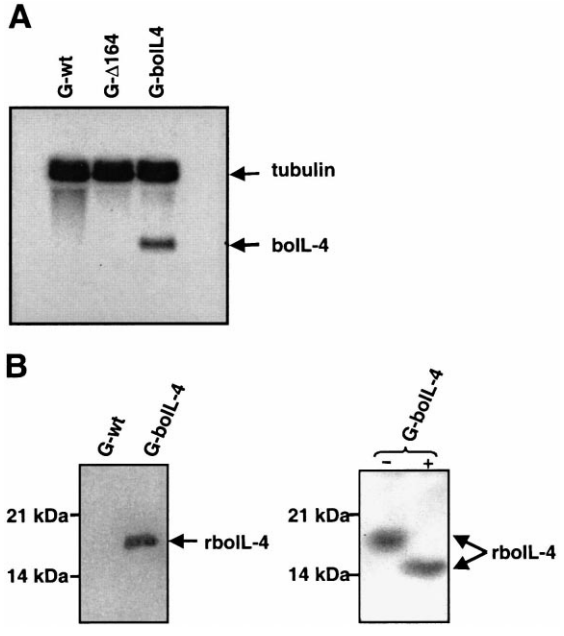

Fig. 2. A: IL-4 expression by *T. brucei*. 10 μg of total RNA per lane was subjected to Northern blot analysis using radiolabelled IL-4 and tubulin probes. RNA was isolated from cells transformed with pG-boIL-4Δ164 (G-boIL4). Two cell lines transformed with pGAPRONE derivatives (G-wt and G-Δ164) serve as negative controls. B: Trypanosome-derived boIL4 is secreted and glycosylated. Left panel: Western blot with untreated supernatants. Proteins were separated on a 15% SDS–polyacrylamide gel. G-boIL4: cells expressing boIL4. As a negative control, supernatants were harvested from cells that had been stably transformed with the construct pGAPRONE (G-wt). Right panel: mock-treated (−); incubated with PNGase F (+).

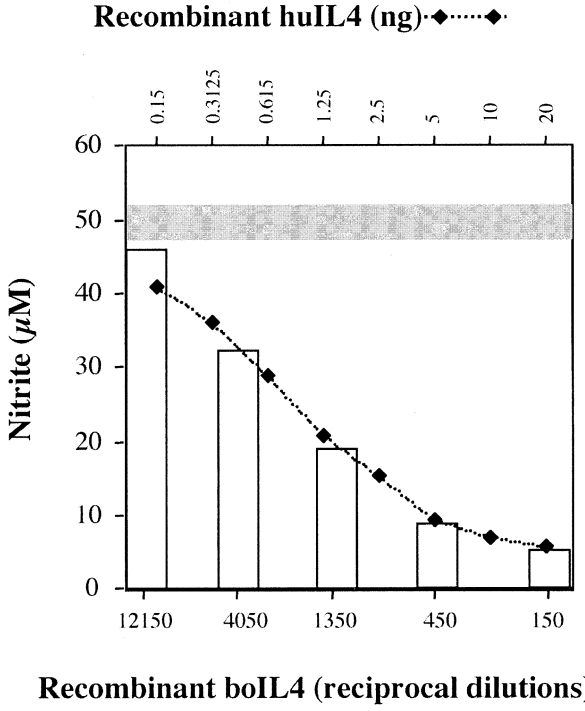

Fig. 3. IL-4-mediated down-regulation of NO generation by bovine monocyte-derived macrophages. Cells were exposed to *S. dublin* (200 μg ml^{-1} and to the indicated concentrations of supernatants from G-boIL-4 transformants (open bars) or with recombinant human IL-4 (dotted line). Nitrite, the stable metabolite of NO, was determined by the Griess assay. The shaded area indicates the level of nitrite formation in the presence of a control supernatant from G-wt trypanosomes.

Since the amplified IL-4 sequence encoded the entire protein including the bovine signal peptide, we analysed whether rboIL-4 was released into the supernatant. Culture supernatants from G-boIL-4 trypanosomes and control trypanosomes (G-wt) were subjected to Western blot analysis using a monoclonal anti-boIL-4 antibody [15]. As shown in Fig. 2B recombinant bovine IL-4 (M_r ~17 kDa) was detected in supernatants from G-boIL-4 transformants. The antibody reaction was specific as no proteins of this size were detectable in the supernatant of control cells even after long exposure. Trypanosome-derived boIL4 is larger than the predicted size of the polypeptide backbone minus the signal peptide (12.6 kDa [12]), suggesting that carbohydrates are linked to the protein. Treatment with PNGase F caused a shift to M_r 14 kDa confirming that this is indeed the case (Fig. 2B). From these results we conclude that *T. brucei* is able to express a glycosylated form of rboIL-4 and that the bovine IL-4 signal peptide is sufficient for direct release of the cytokine into the culture supernatant.

3.2. Biological activity of recombinant bovine IL-4

To assess the biological activity of trypanosome-derived rboIL-4, two different types of bioassay were performed. It has been demonstrated that mouse and bovine macrophages produce NO when exposed to Gram-negative bacteria, and

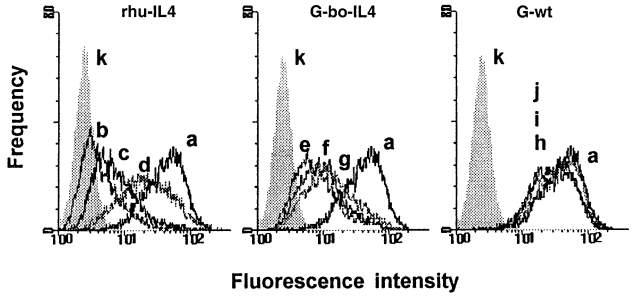

Fig. 4. Down-regulation of CD14 by rhuIL-4 and rboIL-4. Human macrophages were exposed to the indicated concentrations of rhuIL-4 and dilutions of G-boIL-4 and G-wt (control). After 2 days cells were harvested and tested for CD14 expression using a FITC-labelled anti CD14 antibody. a: CD14 expression by untreated cells (all panels). Left panel: Treatment with rhuIL-4; b: 10 ng ml^{-1}; c: 2 ng ml^{-1}; d: 0.4 ng ml^{-1}. Middle panel: Treatment with supernatant from G-boIL-4 transfected cells; e: 20-fold; f: 100-fold; g: 500-fold dilution. Right panel: Treatment with control supernatants; h: 20-fold; i: 100-fold; j: 500-fold dilution. Shaded areas (k) represent staining of untreated cells with an isotype control antibody. Incubation with rhuIL-4 and rboIL-4 did not alter expression of other surface markers such as CD18 (data not shown).

that this can be inhibited by IL-4 [19,20]. Monocyte-derived bovine macrophages were exposed to heat-inactivated *S. dublin* in the presence of culture supernatants from G-boIL-4 trypanosomes. Twenty-four hours after stimulation, the macrophages were harvested and the concentration of nitrite, a stable metabolite of NO, was determined. A representative dose–response curve is shown in Fig. 3. A 1:4050 dilution of the G-boIL-4 supernatant already caused a significant reduction in NO production, and a 1:150 dilution reduced nitrite levels more than 10-fold. To ensure that this effect was not caused by other factors released by the trypanosomes, a control supernatant from G-wt trypanosomes was also tested in the assay. This supernatant did not cause any inhibition of NO production by macrophages, confirming that the biological activity was due to rboIL-4. To estimate the amount of rboIL-4 in the culture supernatant, an assay was also performed with defined amounts of human recombinant IL-4 (rhuIL-4; Fig. 3, curve). This allowed us to estimate the amount of rboIL-4 in the trypanosome culture supernatant as being equivalent to 1–2 μg ml^{-1} huIL-4.

Another effect of IL-4 is to down-regulate the surface marker CD14 on human macrophages [21,22]. This was exploited in a second bioassay in which human macrophages were exposed to different concentrations of rhuIL-4 and trypanosome-derived rboIL-4. CD14 expression was monitored by flow cytometry using an anti-CD14 monoclonal antibody (Fig. 4). Compared to untreated cells (left panel) human macrophages exposed to 10 ng ml^{-1} or 2 ng ml^{-1} rhuIL-4 showed a significant reduction in CD14 expression. Exposure of the cells to various dilutions of G-boIL-4 supernatant reduced the CD14 expression in a similar manner (Fig. 4, middle panel) whereas the control supernatant from G-wt did not have any effect on CD14 expression (Fig. 4, right panel). The expression of other surface molecules, for example CD18, was not modulated by rhuIL-4 or rboIL-4 (data not shown). These results indicate that trypanosome-derived rboIL-4 must bind the huIL-4 receptor and activate the appropriate signal transduction pathway.

3.3. Yield of IL-4

Recombinant boIL-4 has been transiently expressed in three heterologous eukaryotic expression systems, COS cells [23], bovine herpesvirus [24] and vaccinia virus [25]. In terms of activity, the yields obtained from stably transformed trypanosomes are comparable to those obtained from the bovine herpesvirus system and are 10–100-fold higher than from COS cells and vaccinia virus. The amounts of cytokine produced (1–2 mg l^{-1}) are in the same range as for other cytokines, for example human leukaemia inhibitory factor, in Chinese hamster ovary cells and mouse erythroleukaemia cells [26,27]. In both cases, the yield could be scaled up to approximately 20 mg l^{-1} when the cells were cultured in roller bottles, but 3–9 months were required to obtain 10 mg crude protein. The advantage of the trypanosome system is that once stable transformants are obtained they can be cultured without antibiotic selection and their rapid growth allows the production of milligram amounts of protein within days. In addition, they may prove a useful alternative for the expression of proteins that are toxic for mammalian cells.

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References

[1] Netzer, W.J. and Hartl, F.U. (1997) Nature 388, 343–349.
[2] Parodi, A.J. (1993) Glycobiology 3, 193–199.
[3] Das, A., Peterson, G.C., Kanner, S.B., Frevert, U. and Parsons, M. (1996) J. Biol. Chem. 271, 15675–15681.
[4] Ferguson, M.A., Low, M.G. and Cross, G.A. (1985) J. Biol. Chem. 260, 14547–14555.
[5] Ferguson, M.A., Murray, P., Rutherford, H. and McConville, M.J. (1993) Biochem. J. 291, 51–55.
[6] Clayton, C.E. and Mowatt, M.R. (1989) J. Biol. Chem. 264, 15088–15093.
[7] Furger, A., Schürch, N., Kurath, U. and Roditi, I. (1997) Mol. Cell. Biol. 17, 4372–4380.
[8] ten Asbroek, A.L., Mol, C.A., Kieft, R. and Borst, P. (1993) Mol. Biochem. Parasitol. 59, 133–142.
[9] Ruepp, S., Furger, A., Kurath, U., Renggli, C.K., Hemphill, A., Brun, R. and Roditi, I. (1997) J. Cell Biol. 137, 1369–1379.
[10] Brooks, D.R., McCulloch, R., Coombs, G.H. and Mottram, J.C. (2000) FEMS Microbiol. Lett. 186, 287–291.
[11] Brun, R. and Schonenberger, M. (1979) Acta Trop. 36, 289–292.
[12] Heussler, V.T., Eichhorn, M. and Dobbelaere, D.A. (1992) Gene 114, 273–278.
[13] Roditi, I. et al. (1989) J. Cell Biol. 108, 737–746.
[14] Seebeck, T., Whittaker, P.A., Imboden, M.A., Hardman, N. and Braun, R. (1983) Proc. Natl. Acad. Sci. USA 80, 4634–4638.
[15] Weynants, V. et al. (1998) Vet. Immunol. Immunopathol. 66, 99–112.
[16] Jungi, T.W., Brcic, M., Sager, H., Dobbelaere, D.A., Furger, A. and Roditi, I. (1997) Clin. Exp. Immunol. 109, 431–438.
[17] Adler, H., Peterhans, E., Nicolet, J. and Jungi, T.W. (1994) Biochem. Biophys. Res. Commun. 198, 510–515.
[18] Adler, H., Frech, B., Thony, M., Pfister, H., Peterhans, E. and Jungi, T.W. (1995) J. Immunol. 154, 4710–4718.
[19] Jungi, T.W. and Hafner, S. (1986) Immunology 58, 131–137.
[20] Perretti, M., Szabo, C. and Thiemermann, C. (1995) Br. J. Pharmacol. 116, 2251–2257.

[21] Landmann, R., Ludwig, C., Obrist, R. and Obrecht, J.P. (1991) J. Cell Biochem. 47, 317–329.
[22] Lauener, R.P., Goyert, S.M., Geha, R.S. and Vercelli, D. (1990) Eur. J. Immunol. 20, 2375–2381.
[23] Estes, D.M., Hirano, A., Heussler, V.T., Dobbelaere, D.A. and Brown, W.C. (1995) Cell. Immunol. 163, 268–279.
[24] Kühnle, G., Collins, R.A., Scott, J.E. and Keil, G.M. (1996) J. Gen. Virol. 77, 2231–2240.
[25] Von Beust, B.R., Brown, W.C., Estes, D.M., Zarlenga, D.S., McElwain, T.F. and Palmer, G.H. (1999) Vaccine 17, 384–395.
[26] Geisse, S., Gram, H., Kleuser, B. and Kocher, H.P. (1996) Protein Express. Purif. 8, 271–282.
[27] Geisse, S. and Kocher, H.P. (1999) Methods Enzymol. 306, 19–42.