© 1993 Oxford University Press

Nucleic Acids Research, 1993, Vol. 21, No. 2 191–195

The *ble* resistance gene as a new selectable marker for *Trypanosoma brucei*: fly transmission of stable procyclic transformants to produce antibiotic resistant bloodstream forms

D.Jefferies*, P.Tebabi, D.Le Ray[1] and E.Pays

Department of Molecular Biology, University of Brussels, 67 rue des chevaux, B1640 Rhode-St-Genese and [1]Prince Leopold Institute of Tropical Medicine, 155 Nationalestraat, B2000 Antwerp, Belgium

Received November 10, 1992; Accepted December 15, 1992

ABSTRACT

We describe here the stable transformation of *Trypanosoma brucei* using a new selectable marker for kinetoplastid protozoa, the Sh *ble*, or phleomycin, resistance gene. A plasmid containing this gene targeted to the tubulin gene locus by homologous sequences was introduced into procyclic trypanosomes by electroporation and cells selected for antibiotic resistance. Southern analysis of stable transformants showed that the plasmid had been integrated into the tubulin locus by homologous recombination. Analysis of bloodstream stage transformants, produced by transmission through the vector *Glossina*, showed that the resistance gene was conserved and expressed in these forms in the absence of selective drug pressure. In both procyclic and bloodstream forms, transcription of the *ble* gene appears to originate from the upstream tubulin promoter, despite the presence of a VSG promoter in the integrated construct. The generation of stable bloodstream transformants for the first time will facilitate the study of gene function and expression during the trypanosome life cycle, and aid in the investigation of genetic exchange in these organisms.

INTRODUCTION

Trypanosomes are kinetoplastid protozoa which frequently cause disease in Man and domestic animals. They exhibit a number of interesting and often novel mechanisms of gene expression and control, such as RNA editing, transsplicing and polycistronic transcription (for reviews see 1,2,3). *Trypanosoma brucei* is one of the most intensively studied of this group. It has a digenetic life cycle, alternating between a bloodstream stage in the mammalian host and a procyclic stage in the tsetse fly vector (4). Recently the techniques of stable transformation have been successfully applied to the kinetoplastid protozoa, predominantly members of the genera *Leishmania* (5–10) and *Trypanosoma* (11–16). When linearized DNA constructs are used, transformation occurs almost exclusively by homologous recombination, which allows highly specific targeting of a chosen gene locus (8–15). In the African trypanosome *T.brucei* two selectable marker genes have been used to achieve stable transformation of the procyclic life cycle stage; the neomycin (11–13, 15) and hygromycin B (14) antibiotic resistance genes. We descibe here the stable transformation of *T.brucei* using a further selectable marker, the Sh *ble*, or phleomycin, resistance gene (17), and the transmission of these transformants through the insect vector, *Glossina*, to produce antibiotic resistant bloodstream form trypanosomes for the first time.

MATERIALS AND METHODS

Trypanosomes

Maintenance of procyclic trypanosomes: *T.brucei* strain EATRO 1125 were grown at 25–27°C in SDM-79 (18), or Cunningham's, medium (19), supplemented with 15% inactivated foetal calf serum and 10 μg ml^{-1} gentamycin.

Fly transmission of stable transformants: Tsetse flies (*Glossina morsitans morsitans*) were infected by allowing them to feed on blood mixed with procyclic trypanosomes through artificial membranes (20). Twenty one days post-infection, flies were dissected and infected salivary glands were injected into mice. Blood from mice which developed infections was passaged repeatedly through mice and then rats at 3 day intervals to increase virulence, before cloning in mice.

Plasmids

Plasmids were prepared by alkaline lysis and caesium chloride density gradient centrifugation (21). The construct pPT was derived from the plasmid pD5 (22) as follows: a 0.45 kb KpnI-

* To whom correspondence should be addressed at: Department of Genetics, University of Glasgow, Church Street, Glasgow G11 5JS, UK

NotI(blunt) fragment containing the Sh *ble* resistance (*ble*) gene from the plasmid pUT58 (17) was ligated into KpnI-HpaI digested pD5. A 5.3 kb BamHI fragment from the 3′ end of the *T.brucei* tubulin gene locus (23) was then inserted into a unique BglII site, just downstream of the *ble* gene. The resulting construct, pPT, contains the VSG gene promoter, the ESAG 7 3′ splice acceptor site (22) and the *ble* gene, followed by the tubulin sequences (see Figure 1B).

DNA and RNA analysis

The techniques of DNA and RNA isolation, Southern and Northern blotting, and cDNA cloning were as previously described (24). Sequencing of DNA fragments subcloned into M13 vectors was carried out by the method of Sanger *et al* (25), using a modified T7 DNA polymerase (Sequenase, United States Biochemical Corporation). Run-on transcription assays were performed using freshly isolated nuclei as described (26).

Transfection of trypanosomes

Procyclic trypanosomes were harvested in mid log phase, pelleted, washed once with Zimmerman's Post Fusion Medium (27), and resuspended in the same buffer at a concentration of 2.10^7 ml^{-1}. Plasmid DNA (5–10 μg in 10–20 μl of sterile TE buffer or distilled water), linearised at a unique PstI site at the 5′ end of the interrupted beta-tubulin gene (Fig. 1B), was added to 0.5 ml of this suspension in an electroporation cuvette (0.4 cm electrode gap) and mixed thoroughly. After several minutes incubation one or two pulses of 1.5 kv at 25 μF were delivered to the cuvette, using a BioRad gene pulser, resulting in 50–70% cell mortality. Following a further five minute incubation, the contents of the cuvette were transferred directly to a culture flask containing 4 mls of SDM-79 or Cunningham's medium. All electroporation steps were carried out at 4°C. Cells were left for 18–36 hours post-transfection to allow growth to resume, then diluted and the antibiotic phleomycin (Cayla, France) added, usually at a concentration of 1.0 μg ml^{-1} (the *ble* gene confers resistance to the bleomycin family of antibiotics, with phleomycin the most effective against eukaryotic cells (17)). Transformed cells could be positively identified after 7–10 days.

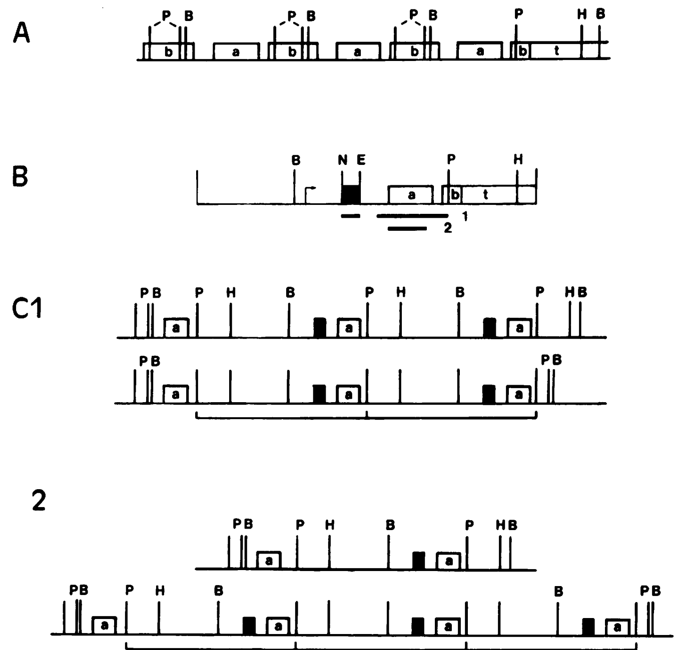

Figure 1. Restriction maps of integration construct pPT and tubulin locus. Abbreviations: a = alpha-tubulin gene, b = beta-tubulin gene, t = retrotransposon-like element, B = BamHI, E = EcoRI, H = HpaI, N = NcoI, P = PstI. Scale of A and B is twice that of C. **A)** Wild type tubulin gene array showing alpha- and beta-tubulin gene repeats, with the interrupted beta-tubulin gene at the 3′ extremity of the tubulin locus. **B)** Plasmid construct pPT used to target the tubulin array. The small horizontal arrow indicates the position of the VSG promoter. The solid black box represents the *ble* resistance gene. The black bars underneath the construct indicate the probes used in the Southern analyses (1—tubulin probe used in Fig. 2. 2—alpha-tubulin specific probe used in Fig.3. The same *ble* probe was used in both Figs.). The restriction sites EcoRI and NcoI were used to generate the *ble* gene specific fragment for the run-on transciption assays. **C)** Two possible interpretations of the Southern data. Both involve two separate integration events. The line under each of the two patterns indicates the extent of the integrated plasmids. C1) Pattern 1 shows two tandem copies of the construct integrated into each allele of the tubulin locus (2:2 arrangement). C2) Pattern 2 shows a single copy integrated at the 3′ end of the first allelic locus and 3 adjacent copies at an internal position on the second allele (3:1 arrangement).

RESULTS

In order to avoid disrupting essential genes, and to maximise the chances of successful integration (12), the construct containing the *ble* gene was targeted to the tubulin locus, which consists of a multiple array of alternating alpha- and beta-tubulin gene repeats (23, 28–30). Procyclic trypanosomes were electroporated with linearised plasmid DNA and selected for resistance to phleomycin (17).

In a preliminary analysis, hybridisation of a *ble* gene spcific probe to genomic DNA isolated from several transfected cell lines, showed that these trypanosomes contained the *ble* gene. One of these lines, ST3, was analysed in more detail (see Fig. 2). Digestion with PstI alone gave a single band of 10.4 kb with the *ble* probe, which also hybridised with the tubulin specific probe (probe 1, Fig. 1B). A BamHI + PstI digest gave a single band of 4.8 kb with the *ble* specific probe, consistent with the restriction map of the construct (Fig. 1B), and generated 2 novel tubulin specific bands; the 4.8 kb band and a second of 5.6 kb, which represents the remaining integrated sequences. The intensity of hybridisation to these bands suggested that there were several copies of pPT in the genome. This was confirmed by the BamHI digest, which revealed 3 *ble* specific bands of 10.4, 7.4 and 5.8 kb, and a fourth tubulin specific band of 8.4 kb. The hybridisation intensity of the *ble* specific bands (2:1:1) reveals that there are 4 copies of the integrated construct. In addition, the size of the bands indicates that at least one copy of the construct has been integrated into the interrupted beta-tubulin gene at the 3′ end of the tubulin locus (7.4kb fragment), and that a second integration event has occurred at a PstI site within an intact beta-tubulin gene (5.8 kb band). This suggests that either two separate integration events have occurred in the same trypanosome, or that the ST3 line is polyclonal. Two of the 3 possible arrangements of the integrated constructs are shown in Fig. 1C. The first (C1) shows two tandem copies of pPT in both alleles of the tubulin locus, two at the 3′ end and two internal (2:2 arrangement). The second (C2) has a single copy at the 3′ end and 3 internal copies (1:3 arrangement). The third (not shown), is a reversal of C2 (3 at the 3′ end and one internal; 3:1 arrangement).

To confirm that integration had occurred by homologous recombination, the unique BamHI genomic fragments of 5.8, 7.4 and 8.4kb, which span the junctions of inserted plasmid and

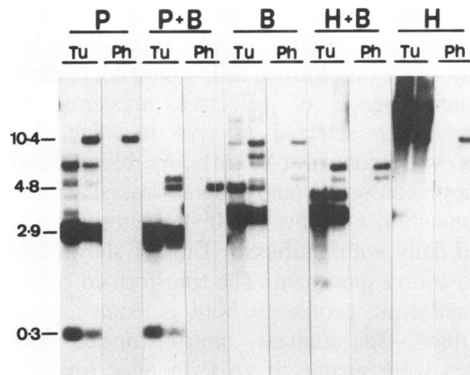

Figure 2. Southern analysis of transformed procyclic trypanosome line ST3. Genomic DNA from wild type (left hand lane) and transformed (right hand lane) procyclics, was digested with various restriction enzymes (indicated by the letters above the panel) and hybridised to tubulin (Tu) and *ble* gene (Ph) specific probes (probe fragments are shown in Fig. 1).

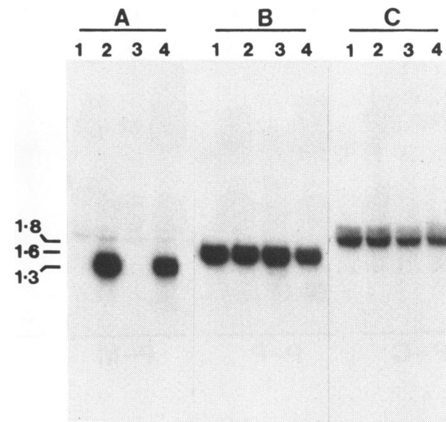

Figure 4. Transcripts from the *ble* gene. Northern blots of poly $[A]^+$ RNA from transformed (lanes 2 & 4) and untransformed (lanes 1 & 3) procyclic (lanes 1 & 2) and bloodstream (lanes 3 & 4) form trypanosomes were hybridised with probes specific for the *ble* gene (panel A), the actin gene (panel B) and the alpha-tubulin gene (panel C).

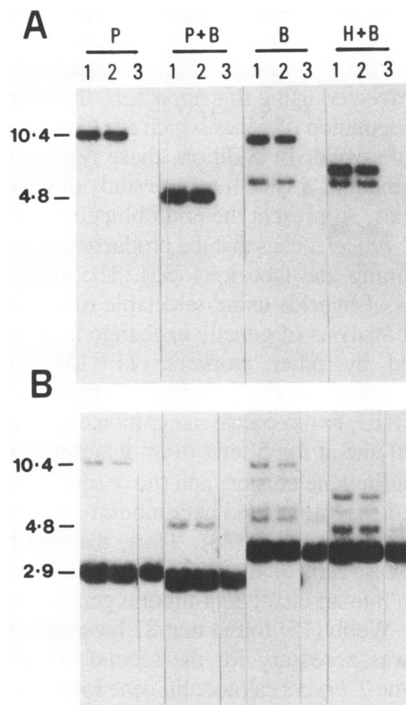

Figure 3. Southern analysis of the fly transmitted trypanosome line ST3. Genomic DNA from transformed mixed (1), transformed cloned (2) and wild type (3) populations, was digested with various restriction enzymes (above panel) and hybridised to *ble* gene (**panel A**) and alpha-tubulin gene (**panel B**) specific probes.

genomic sequences, were cloned in lambda EMBL12, and the regions around the PstI and terminal BamHI sites sequenced over a distance of 2–300 bps. This confirmed that these junctions were unaltered at the nucleotide level (data not shown), indicating that integration had indeed occurred by homologous recombination.

This transformed trypanosome line, ST3, was then transmitted through tsetse flies and the resulting bloodstream forms cloned in mice. Southern analysis of both the original population emerging from the tsetse fly and the cloned line showed an identical pattern of restriction fragments, consistent with the integration of 3 copies of the PT plasmid into a beta-tubulin gene at an internal position in the tubulin locus (Fig 3A); the diagnostic evidence being the 2:1 ratio of the 10.4 and 5.8 kb BamHI *ble* specific bands, and the absence of the 7.8 kb band indicative of a 3′ end insertion. The alpha-tubulin specific probe (probe 2; Fig. 1B) also hybridises to these two bands, in addition to a third 8.4 kb band generated from the 5′ end of the integration (Fig. 3B). The fourth hybridising band, closest to the strongly hybridising 3.8 kb tubulin repeat unit, is generated from the 3′ end of the wild type tubulin locus, and is also present in the controls. This confirms that the original procyclic line ST3 was not clonal, and that the line originating from the fly transmission, as well as the clone derived from this line, contained 3 copies of the plasmid integrated into an intact beta-tubulin gene, as represented by the lower half of diagram C2 in Fig. 1.

Northern blots of poly$[A]^+$ RNA isolated from transformants before and after fly transmission revealed the presence of a 1.3 kb transcript, which hybridised with a *ble* specific probe (Fig. 4A, lanes 2 and 4), which was absent from control cells(Fig. 4A, lanes 1 and 3). Transcript abundance appeared to be similar in both life cycle stages (RNA loading checked using the actin gene (31) as a control probe (Fig. 4B)). Hybridisation with a tubulin probe showed that transcript levels were largely unaffected by the presence of the endogenous sequences (Fig. 4C). The size of the transcript suggested that the polyadenylation site of the *ble* gene mRNA was located within the tubulin intergenic region. This was checked by cDNA cloning and sequencing of the 3′ end of the transcript, which showed that polyadenylation had occurred at the wild type beta-tubulin polyadenylation site (position 1710 of the sequence in Kimmel et al (30); data not shown).

Run-on transcription assays in isolated nuclei showed that both the plasmid and the *ble* gene were transcribed in an alpha-amanitin sensitive manner, in both procyclic and bloodstream stage transformants (Fig. 5; compare lanes c and a in P-P and B-P). As the VSG gene promoter directs alpha-amanitin resistant polymerase activity (32), this suggested that transcription of the

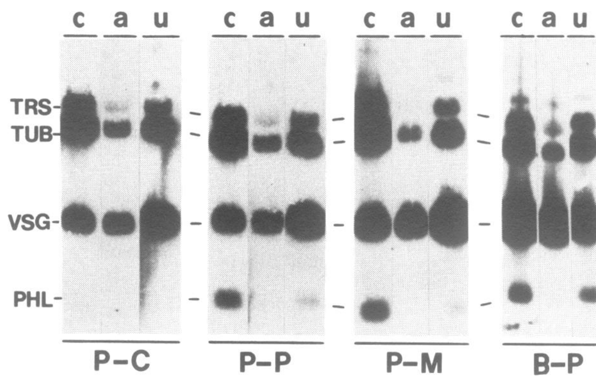

Figure 5. Run-on transcription assays of the *ble* gene. Run-on transcription assays were performed with nuclei from control procyclic forms (P-C), procyclic transformants (P-P: integration at PstI site; P-M: integration at MluI site), or bloodstream transformants (B-P), obtained after fly transmission of P-P (see text). Lane c: nuclei incubated in the absence of alpha-amanitin. Lane a: nuclei incubated in the presence of 1mg ml^{-1} alpha-amanitin. Lane u: cells irradiated with 60 Joules of UV light prior to isolation of the nuclei (incubated as for control cells). Run-on transcripts were hybridised to Southern blots of BamHI+EcoRI+NcoI digested pPT (see Fig. 1). The interpretation of the bands is as follows: TRS = 4.6kb NcoI-BamHI fragment containing the TRS and plasmid sequences. TUB = 3.6kb EcoRI-NcoI fragment containing the tubulin sequences. VSG = 1.6kb BamHI-NcoI fragment containing the VSG promoter and ESAG 7 splice site. PHL = 0.6kb NcoI-EcoRI fragment containing the *ble* resistance gene.

Table 1. *In vivo* phleomycin resistance of bloodstream form trypanosomes containing the *ble* resistance gene

	No. days post-infection				
Treatment	2	4	6	8	10
T (+P)	+	+++	+	0	+
T (−P)	+	+++	+	0	+
C (+P)	0	0	0	0	0

Groups of 3 mice were inoculated with 2.10^6 trypanosomes (C—control, T—transformed; (+P)—phleomycin treated, (−P)—untreated) and injected daily (day 0 to day 10) with phleomycin in aqueous solution (dose rate 3mg kg^{-1}). Thin blood smears from each mouse were checked daily for trypanosomes and the parasitaemia scored using a simplification of the method of Lumsden *et al* (45). (0 = no trypanosomes in microscope field, + = 1−10, ++ = moderate numbers, +++ = teeming). Selection experiments were repeated several times with similar results. Control, non-transformed, trypanosomes were shown to be infective for mice.

integrated plasmid was not originating from the internal VSG promoter, but from the upstream tubulin promoter. In order to verify this, we measured the effect of UV irradiation on transcription of the *ble* gene (see 26, for a detailed explanation of this effect). Briefly, if this gene was transcribed from a promoter located just upstream, there would be an accumulation of *ble* gene transcripts due to a transient blocking of RNA decay under these conditions (26, 33, 34). However, as shown in Fig. 5, there is an apparent inhibition of transcription following UV irradiation (Fig. 5; P-P (u) and B-P (u)), as would be expected for a trypanosome gene transcribed by a distant promoter. Thus, the VSG promoter present in the construct was either inactive, or its activity was too low to be detected. The same result was obtained with a transformed procyclic line in which integration was achieved using a unique MluI restriction site in the tubulin intergenic region (Fig. 5; P-M).

Attempts to establish bloodstream stage transformants in culture, in order to determine phleomycin resistance levels, were not successful as the transformed cells would not multiply *in vitro*, even in the absence of selective pressure. Therefore, trypanosomes were selected *in vivo* in mice. Preliminary experiments with control (AnTat1.3A) bloodstream forms, revealed that phleomycin would suppress parasitaemia, provided the initial inoculum was low (2.10^6 trypanosomes), and mice were treated daily with antibiotic. Table 1 shows the result of a typical selection experiment. The transformed cells showed a similar parasitaemic profile in both phleomycin treated and untreated mice. In contrast, untransformed bloodstream trypanosomes were unable to grow in mice treated with this concentration of antibiotic.

DISCUSSION

We have shown here that the *ble* gene can be used as a selectable marker for procyclic *T.brucei*, and that these trypanosomes continue to express the gene after transmission through the tsetse fly vector. This is of particular importance for the study of differential gene expression during the trypanosome life cycle using transgenic techniques. The difficulty of transfecting bloodstream form trypanosomes (22; DJ unpublished data) may also be circumvented using this approach, in order to study the function and regulation of genes which are expressed only at this stage of the life cycle. In addition, these results open the way to the development of a system for the study of genetic exchange in trypanosomes. At present the non-obligatory nature of sexual exchange in *T. brucei* means that the production of genetic hybrids is time consuming and laborious (35). The ability to generate large numbers of hybrids using selectable markers will allow a more detailed analysis of genetic exchange in these organisms.

As reported by other workers (11−15), integration of exogenous sequences was by homologous recombination. In the procyclic line ST3, two separate integration events had occurred. One at the PstI site at the 5′ end of an intact beta-tubulin gene, within the tubulin gene cluster; and the second into the PstI site at the 5′ end of the interrupted beta-tubulin gene, present at the 3′ end of the tubulin locus (23). Thus, the 700 base pairs of homology downstream of the PstI site (23, 30) was sufficient for integration into an intact beta-tubulin gene to occur. Indeed, Eid & Sollner-Webb (15) found that 87 base pairs of homology was all that was necessary for the second crossover event of insertion into the *T.brucei* calmodulin gene locus, as long as there was an extensive region of homology (6−7 kb) for the first crossover.

As expected, there was no apparent disruption of tubulin transcription due to the inserted sequences. Indeed, run-on analysis indicated that transcription of the tubulin locus continued through the plasmid, and that the VSG promoter within the plasmid was silent, even after fly transmission. This is in contrast to the results obtained by Lee & van der Ploeg (12) with a plasmid containing the procyclin/PARP gene promoter, inserted into the tubulin locus of *T.brucei* procyclic forms. Transcription of the exogenous sequences was shown to be alpha-amanitin resistant, which indicates that the procyclin/PARP promoter was active. The reason for this discrepancy is not clear, as the VSG promoter has been shown to be active in procyclic, as well as in bloodstream form trypanosomes (22, 33). Insertion of the plasmid PT into the tubulin locus at the MluI intergenic restriction site

used by Lee & van der Ploeg (12), gave the same result as the PstI integration (Fig. 5). However, in the transformed procyclic line studied by these authors, there were at least 16 copies of the plasmid inserted into the tubulin locus. Thus, it may be that, in our case, the level of transcription from the VSG promoter was too low to be detected. Alternatively, the procyclin/PARP promoter used in (12) may contain an upstream transcription termination region, not present in the VSG promoter fragment. This hypothesis is consistent with two observations: firstly, the VSG promoter region is smaller than that of the procyclin/PARP promoter (22, 36−38); secondly, the procyclin/PARP promoter appears to be positioned just downstream of a region transcribed by a different RNA polymerase, which terminates in close proximity to the procyclin/PARP promoter (33, 39, 40). In contrast, the VSG promoter does not appear to be associated with any upstream transcription units (41−44).

ACKNOWLEDGMENTS

This work was carried out while DJ was the holder of a Wellcome Trust Training Fellowship in Tropical Medicine. Support was also received from the Belgian 'Fonds de la Recherche Scientifique Medicale', the 'Fonds Special de la Recherche de l'Universite Libre de Bruxelles' and through a research contract with the 'Communaute Francaise de Belgique' (ARC 89/94-134). It was also funded by the Agreement for Collaborative Research between ILRAD (Nairobi) and Belgian Research Centres.

REFERENCES

1. Borst, P. (1986) Ann. Rev. Biochem. 55, 701−732.
2. Pays, E. and Steinert, M. (1988) Ann. Rev. Genet. 22, 107−126.
3. Cross, G.A.M. (1990) Ann. Rev. Immunol. 8, 83−110.
4. Tetley, L. and Vickerman, K. (1985) J. Cell Science 74, 1−19.
5. Kapler, G.M., Coburn, C.M. and Beverly, S.M. (1990) Mol. Cell. Biol., 10, 1084−1094.
6. Laban, A., Tobin, J.T., deLafaille, M.A.C. and Wirth, D.F. (1990) Nature, 343, 572−574.
7. Lebowitz, J.H., Coburn, C.M., McMahon-Pratt, D. and Beverly, S.M. (1990) Proc. Natl. Acad. Sci. USA., 87, 9736−9740.
8. Cruz, A. and Beverly, S.M. (1990) Nature 348, 171−173.
9. Cruz, A., Coburn C.M. and Beverly, S.M. (1991) Proc. Natl. Acad. Sci. USA., 88, 7170−7174.
10. Tobin, J.F. and Wirth, D.F. (1992) J. Biol. Chem. 267, 4752−4758.
11. ten Asbroek, A.L.M.A., Ouellette, M. and Borst, P. (1990) Nature 348, 174−175.
12. Lee M.G.S.and van der Ploeg L.H.T. (1990) Science, 250, 1583−1587.
13. Eid, J. and Sollner-Webb, B. (1991) Proc. Natl. Acad. Sci. USA., 88, 2118−2121.
14. Lee, M.G.S. and van der Ploeg, L.H.T. (1991) Gene 105, 255−257.
15. Eid, J. and Sollner-Webb, B. (1992) Gen. Develop. 5, 2024−2023.
16. Kelly, J.M., Ward, H.M., Miles, M.A. and Kendall, G. (1992) Nucl. Acids Res. 20, 3963−3969.
17. Drocourt, D., Calmels, T., Reynes, J.P., Baron, M., Tiraby, G. (1990) Nucleic Acids Res. 18, 4009.
18. Brun, R. and Schoenenberger, M. (1979) Acta Trop. 36, 289−292.
19. Cunningham, I. (1977) J. Protozool. 24, 325−329.
20. Le Ray, D., Barry, J.D., Easton, C. and Vickerman, K. (1977) Ann. Soc. Belge Med. Trop. 57, 369−381.
21. Maniatis, T., Fritsch, E.F., Sambrook, J. (1982) Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.
22. Jefferies, D., Tebabi, P. and Pays, E. (1991) Mol. Cell. Biol. 11, 338−343.
23. Affolter, M., Rindisbacher, L. and Braun, D. (1989) Gene 80, 177−183.
24. Pays, E., Delronche, M., Lheureux, M., Vervoort, T., Bloch, J.,Gannon, F. and Steinert, M. (1980) Nucleic Acids Res. 8, 5965−5981.
25. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463−5467.
26. Coquelet, H., Steinert, M. and Pays, E. (1991) Mol. Biochem. Parasitol. 44, 33−42.
27. Bellofatto, V. and Cross, G.A.M. (1989) Science 244, 1167−1169.
28. Seebeck, T., Whittaker, P.A., Imboden, M.A., Hardman, N. and Braun, R. (1983) Proc. Natl. Acad. Sci. USA. 167, 4634−4638.
29. Thomashow, L.S., Milhausen, M., Rutter, W.J. and Agabian, N. (1983) Cell 32, 35−43.
30. Kimmel, B.E., Samson, S., Wu, J., Hirschberg, R. and Yarbrough, R. (1985) Gene 35, 237−248.
31. Ben Amar, M.F., Pays, A., Tebabi, P., Dero, B., Seebeck, T., Steinert, M. and Pays, E. (1988) Mol. Cell. Biol. 8, 2166−2176.
32. Kooter, J.M. and Borst, P. (1984) Nucleic Acids Res. 12, 9457−9472.
33. Pays, E., Coquelet, H., Tebabi, P., Pays, A., Jefferies, D., Steinert, M., Koenig, E., Williams, R.O. and Roditi, I. (1990) EMBO J. 9, 3145−3151.
34. Coquelet, H., Tebabi, P., Pays, A., Steinert, M. and Pays P. (1989) Mol. Cell. Biol., 9, 4022−4025.
35. Sternberg, J. and Tait, A. (1990) Trends Genet. 6, 317−322.
36. Zomerdijk, J.C.B.M., Kieft, R., Shiels, P.G. and Borst, P. (1991) Nucleic Acids Res., 19, 5153−5158.
37. Sherman, D.R., Janz, L., Hug, M. and Clayton, C.E. (1991) EMBO J. 10, 3379−3386.
38. Brown, S.D., Huang, J.D. and van der Ploeg, L.H.T. (1992) Mol. Cell. Biol. 12, 2644−2652.
39. Koenig, E., Delius, H., Carrington, M., Williams, R.O. and Roditi, I. (1989) Nucleic Acids Res. 17, 8727−8739.
40. Clayton, C.E., Fueri, J.P., Itzhaki, J.E., Bellofatto, V., Sherman, D.R., Wisdom, G.S., Vijayasarathy, S. and Mowatt, M.R. (1990) Mol. Cell. Biol. 10, 3036−3047.
41. Kooter, J., van der Spek, H.J., Wagter, R., d'Olivera, C.E., van der Hoeven, F., Johnson, P.J. and Borst, P. (1987) Cell 51, 261−272.
42. Pays, E., Tebabi, P., Pays, A., Coquelet, H., Revelard, P., Salmon, D. and Steinert, M. (1989) Cell 57, 835−845.
43. Gottesdiener, K.M., Chung, H.M., Brown, S.L., Lee, G.S. and van der Ploeg, L.H.T. (1991) Mol. Cell. Biol. 11, 2467−2477.
44. Zomerdijk, J.C.B.M., Kieft, R., Duyndam, M., Shiels, P.G., Borst, P. (1991) Nucleic Acids Res., 19, 1359−1368.
45. Lumsden, W.H.R., Herbert, H.R. and McNeillage, G.J.C. (1973) Techniques with trypanosomes. Churchill Livingstone, London.