3168–3172 *Nucleic Acids Research, 1999, Vol. 27, No. 15*

Faithful expression of a heterologous gene carried on an artificial macronuclear chromosome in *Euplotes crassus*

Jörg Bender, Matthias Kämpfer and Albrecht Klein*

Genetics, Department of Biology, University of Marburg, D-35032 Marburg, Germany

Received March 18, 1999; Revised and Accepted June 8, 1999

DDBJ/EMBL/GenBank accession no. AJ133692

ABSTRACT

Macronuclear chromosomes of hypotrichous ciliates are gene-sized molecules carrying the coding sequence flanked by short non-translated regions and bounded by telomeres. We have constructed artificial chromosomes for investigation of transcription in the macronucleus of *Euplotes crassus*. The *neo* gene was put under the control of the 5'-non-translated region of the *TBP* gene of *E.crassus*. These molecules were introduced into the cell with the help of liposomes. The cells were transformed and survived high concentrations of geneticin. The artificial chromosomes were kept in the macronucleus for at least 50 days at a copy number of about 200 per macronucleus. Expression of the gene was shown by reverse transcription of the *neo* messenger. The transcription start was mapped and found to coincide with that found on the natural macronuclear chromosome encoding TBP in *E.crassus*.

INTRODUCTION

Ciliated protozoa contain two types of nuclei in each cell, one or more generative micronuclei and vegetative macronuclei. These are formed from diploid precursors (anlagen). They are derived from a synkaryon resulting from the fusion of haploid meiotic products in the course of conjugation, a sexual interaction of cells exhibiting different mating types (1,2). In the case of hypotrichous ciliates the formation of the macronucleus from the anlage involves the removal of intragenic DNA sequences and fractionation of the chromosomes into gene-sized molecules, the macronuclear chromosomes (3). The macronuclear chromosomes are differentially amplified resulting in gene-specific copy numbers of 10^2–10^5 per macronucleus in the case of *Euplotes crassus* (4,5). The average number is about 2000 per macronucleus. The macronuclear chromosomes have short non-translated regions flanking the genes (5,6). They are bounded by telomeres (7). As centromeres are missing, the distribution of the chromosomes during amitotic division of the macronuclei during the vegetative cell cycle is stochastic. It is unknown to what extent the cell is able to adjust the copy numbers after nuclear division.

The differential amplification of the gene-sized macronuclear chromosomes has been viewed as a mechanism of dosage adjustment for genes the transcription of which is not regulated, e.g. housekeeping genes (8,9). Their products are needed in different amounts, which can be achieved by differential amplification of the genes even if the promoter strengths do not differ. Indeed, we have previously reported that transcription rates for different genes are similar among rRNA genes transcribed by different RNA polymerases, but that different transcription rates can be found among genes transcribed by RNA polymerase II in *E.crassus* (10). The gene encoding the TATA-binding protein TBP has a remarkably high transcription rate, which indicates that it has a strong promoter. Regulation of transcription also exists, as was found for Hsp70-encoding genes (11; U.Reidt and A.Klein, unpublished results). It follows that the ciliate cell has a wide spectrum of possibilities for control of gene expression. In addition, RNA stability varies (10), resulting in additional flexibility with regard to RNA steady-state concentrations.

We have been interested in the function of promoters in hypotrichous ciliates, especially on chromosomes with very short subtelomeric regions in front of the coding sequences that are likely to contain promoter elements. In a previous study (12) we found that the transcription start site in front of the *TP* gene (13) encoding the telomere-binding protein (14) is apparently directed from a downstream element either close to the 5'-end of the coding sequence or a short intron close to it. Given the different known gene structures, it is unlikely that this is the general case.

It is therefore essential to be able to do mutational studies on different promoters in order to probe their functional components. This requires the availability of a transformation system with which modified chromosomes can be introduced into the macronucleus, which are then maintained and transcribed faithfully. Transfection of ciliated protozoa has been performed before, using direct transformation (15), microinjection of DNA (16–19) or electroporation (20), and maintenance of the introduced DNA for different time periods has been demonstrated in several cases. These systems were used for genetic complementation (21), replication studies (22), knockout mutagenesis (23) and investigations concerning the processing of DNA in cells after conjugation (19). In this communication we describe a system using artificial chromosomes to show faithful expression of a heterologous gene under the control of a promoter taken from *E.crassus*.

*To whom correspondence should be addressed. Tel: +49 6421 283013; Fax: +49 6421 286812; Email: aklein@mailer.uni-marburg.de

MATERIALS AND METHODS

Biological materials

Euplotes crassus, mating type Liv1, was a derivative of the original Liv1 isolate (24) kindly supplied by Dr P. Luporini (Camarino, Italy). The alga *Dunaliella tertiolecta* was used as feeding organism for the ciliate. It was also obtained from Dr P. Luporini. For cloning purposes *Escherichia coli* K12 DH5α (Life Technologies, Eggenstein, Germany) was used.

Isolation of macronuclear DNA

The previously described procedure (25) was modified as follows. *Euplotes crassus* cells (2000) were collected on a 10 μm gauze and washed twice with algae-free medium. They were transferred to 1 ml 250 mM NaCl, 100 mM EDTA, 10 mM Tris–HCl pH 7.5, 0.5% SDS, 400 μg RNase A and 200 μg proteinase K. After incubation for 45 min at 37°C the DNA was extracted with phenol and phenol/chloroform (26), precipitated and dissolved in 15 μl H_2O.

Isolation of RNA

RNA was isolated using a modification of previously described procedures (27). *Euplotes crassus* cells were collected by centrifugation and 2000 cells were resuspended in 100 μl RNA Clean solution (AGS, Heidelberg, Germany) at 4°C. The suspension was mixed vigorously for 15 s after addition of 0.1 vol chloroform. The mixture was kept on ice for 5 min and centrifuged for 15 min at 13 000 *g*. The RNA was precipitated from the aqueous phase with isopropanol after addition of 1 μg glycogen. The precipitate was dissolved in 100 μl 0.1 M sodium acetate and 5 mM $MgSO_4$, pH 5, and 20 U RNase-free DNase I were added. The solution was incubated for 60 min at 37°C and 100 μl RNA Clean solution were then added. The RNA was extracted as above, precipitated and sedimented. It was dissolved in 15 μl H_2O.

Primer extension

The determination of transcription start sites was performed by primer extension (26). Primer NeoPex (CAGTCATAGCCGAATAGC) was employed to map the 5'-end of the transcript of the artificial macronuclear chromosome (AMaC). This reaction was carried out using an unlabelled primer and [α-^{32}P]ATP (3000 Ci/mmol). Primer TBPPex (TTAGTCAAAGACGATAAGGAC) was used for determination of the 5'-end of the *TBP* transcript. This reaction was carried out with an IRD800-labelled primer and signals were detected with a LI-COR-Sequencer (MWG Biotech, Ebersberg, Germany).

Generation of the artificial macronucleus chromosome

The AMaC was generated with the help of two DNA clones. These clones were each built up of three PCR fragments comprising the 5'-end of the *TBP* gene, followed by the *neo* gene (28) and the 3'-end of the *TP* gene. They differed in that the first had a 28 bp double-stranded telomeric sequence at the 5'-end and a double-stranded 42 bp telomeric sequence at its 3'-end, while the second had the different telomeric sequences in the opposite positions. The 5'-end of the *TBP* gene was generated with primers TBP*Sac*I (CGAGCTCAAGGACTCCAAAGAAG) and TK (CGAATTCCCCAAAACCCCAAAACCCCAAAACCCC) or with primers TBP*Sac*I and TL (CGAATTCCAAAACCCCAAAACCCCAAAACCCCAAAACCCCC). These fragments were digested with *Eco*RI and *Sac*I. The 3'-end of the *TP* gene was generated with primer TP-*Bam*HI (GCGGATCCAGCTTAAGAATTGGATAATTC) and TK or with primers TP-*Bam*HI and TL. These fragments were digested with *Eco*RI and *Bam*HI. The *neo* gene was amplified with primers Neo*Sac*I (TTCGGAGCTCCATGATTGAACAAGATGGATTGC) and Neo-*Bam*HI (TTTGGATCCGCTTAGAAGAACTCGTCAAGAAGGCG). The PCR fragment of the *neo* gene was digested with *Sac*I and *Bam*HI.

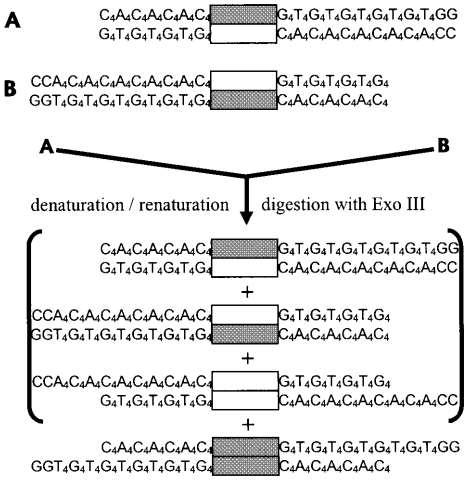

Figure 1. Generation of AMaCs with ends mimicking native telomeres of *E.crassus*. The two strands of products A and B represent complementary strands of the AMaC. Products A and B are *Eco*RI fragments after treatment with nuclease S1. The *Eco*RI recognition sequences were synthesised by PCR (see text for primer sequences). These PCR fragments had been cloned in *Eco*RI recognition sequences of the vector pBluescript II KS+. Denaturation and reannealing of the mixture of products A and B resulted in four different products. Three of them could be digested with exonuclease III (shown in brackets) while the fourth carries 3'-overhangs with the telomeric sequence. This type of product served for transformation.

PCR fragments were cloned in the direction TK/TBP*Sac*I–Neo*Sac*I/Neo*Bam*HI–TP-*Bam*HI/TL (clone A) and TL/TBP-*Sac*I–Neo*Sac*I/Neo*Bam*HI–TP-*Bam*HI/TK (clone B) in the *Eco*RI recognition sequence of the vector pBluescript II KS+ (Stratagene, Heidelberg, Germany). Generation of the artificial chromosome is outlined in Figure 1. Equal amounts of separated *Eco*RI-digested and nuclease SI-treated inserts were denatured for 10 min at 98°C and then slowly cooled to 25°C in 0.66 mM $MgCl_2$, 0.66 mM Tris–HCl, 1 mM 2-mercaptoethanol. After this procedure four different reannealed species of DNA molecules were obtained. They were treated with exonuclease III which left only the AMaC intact, since it had a 14 base 3'-overhanging telomeric sequence at each end. It was therefore protected against exonucleolytic digestion. (12).

Transformation of *E.crassus*

Euplotes crassus was transfected with the help of the non-liposomal lipid Effectene (Qiagen, Hilden, Germany). Well-fed *E.crassus* cells (2000) were collected on a 10 μm gauze and washed twice with algae-free medium and suspended in 1 ml 500 mM sorbitol, 0.5 mM Tris–HCl pH 7.0. The protocol

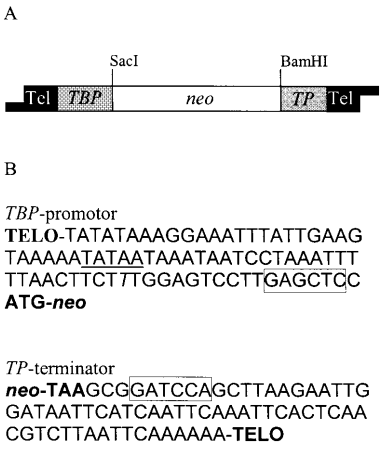

Figure 2. The AMaC. (**A**) Schematic representation of the structure. The telomeres (Tel) have 14 base 3'-overhanging ends. The neomycin resistance gene (*neo*) that confers resistance to geneticin (G418) is fused via a *Sac*I site to the 5'-non-translated subtelomeric region of the *TBP* gene. The 3'-non-translated region including the putative polyadenylation site of the *TP* gene was linked to the *neo* gene via a *Bam*HI site. (**B**) Sequences of the 5'- and 3'-non-translated regions. One A nucleotide of the *TBP* promoter was mutated to T (shown in italic) and a *Sac*I site was created downstream (framed sequence). The TGA stop codon of the *neo* gene was changed to a TAA codon, since TGA is decoded as cysteine in *E.crassus*. A *Bam*HI site (boxed) was created downstream of the stop codon and in the 3'-non-translated part of the *TP* chromosome (termed *TP* terminator) in order to link the two sequences. The *TP* terminator contains the polyadenylation site. The start and stop codons of the *neo* gene are shown in bold type. A putative TATA box within the *TBP* promoter is underlined. The *TP* gene sequence has been published (13). The complete *TBP* gene sequence is accessible as a GenBank file (accession no. AJ133692).

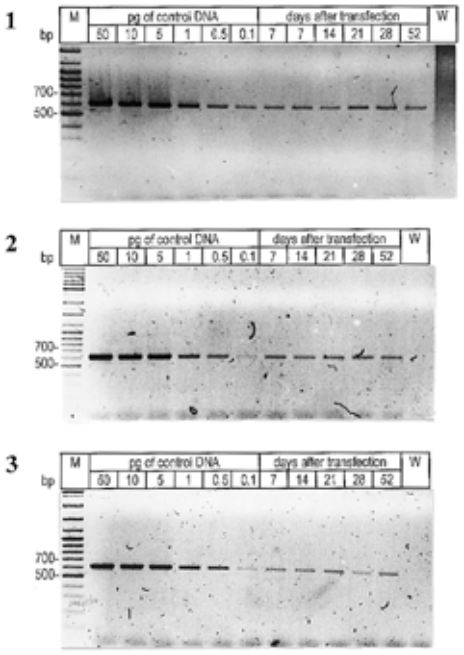

Figure 3. Stable maintenance of the artificial chromosome in transformed cells as shown by PCR amplification. Quantitative PCR with different amounts of plasmid template containing the complete artificial chromosome sequence were run for calibration as shown on the left side of each panel. The amounts of control plasmid DNA used as template are shown. The plasmid contained the complete artificial chromosome. 1 pg plasmid DNA equals 225 000 molecules. The 25 ng macronuclear DNA used are the equivalent of 500 macronuclei (1). A size marker (M) is given in bp. Lane W shows the products of a PCR reaction run with DNA from non-transformed cells. The reactions were carried out with three different clones (1–3). Lane W of the first panel shows the products of a PCR reaction run with 1.5 µg DNA from non-transformed cells. The reactions W of the second and third panel were carried out with 25 ng DNA.

of the supplier for formation of the DNA–Effectene complex was followed except for doubling the recommended amount of DNA from 0.4 to 0.8 µg AMaC DNA. One hour after addition of the DNA–Effectene complex 1 ml of algae-free medium was added. After another hour 1 ml algal suspension was added. An hour later the cells were harvested on a 10 µm gauze and washed twice with medium before releasing them into 2 ml medium with algae. The start of the selection for transformants with the antibiotic G418 (geneticin) followed 24 h later.

For selection 10 mg G418 were added to 1 ml *E.crassus*-containing medium with algae. Three days after addition of the antibiotic the non-transfected control cells died. The transformants were collected on 10 µm gauze and transferred to fresh medium. Typically, >90% of the cells survived treatment with the DNA–lipid mixture. Of the treated cells 5–10% withstood selection with the antibiotic.

Quantitative PCR

Quantitative PCR (29) was carried out with 25 ng macronuclear DNA which had been prepared from different transformants at various time points after transfection. As a standard DNA for the titration, plasmid clone 1 containing the AMaC sequence (see above) was used. The reactions contained 0.3 mM each nucleotide, 2 mM $MgCl_2$, 100 pmol of each primer NeoRT (GTCGATGAATCCAGAAAA) and Neo51 (GAACAAGATGGATTGCACGC) and 0.5 U *Taq* polymerase (Life Technologies, Eggenstein, Germany). Seventeen cycles of amplification were performed.

RT–PCR

RT–PCR (30) was carried out with 0.1 µg RNA using the Titan One Tube RT–PCR System (Boehringer Mannheim, Mannheim, Germany). The modified procedure suggested by the supplier was employed using NeoRT as downstream primer and Neo51 as upstream primer.

DNA hybridization

DNA was transferred to Nylon membranes using the alkaline downward transfer technique (31) and hybridised against suitable probes as described earlier (32).

RESULTS

The artificial macronuclear chromosome

The AMaC shown in Figure 2 consists of three moieties. The 5'-region is taken from the TBP-encoding gene of *E.crassus*. It includes the telomere and the subtelomeric region up the start codon. It is followed by the *neo* gene encoding neomycin phosphotransferase. The very short 3'-region was taken from the *TP* gene. It contains a well-defined polyadenylation site (13). The *TBP* promoter is relatively strong and shows

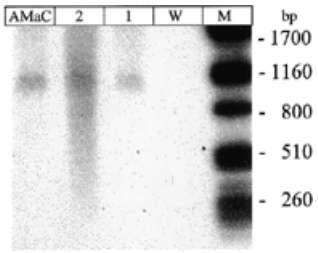

Figure 4. Stable maintenance of AMaCs as shown by Southern hybridisation. The blot was carried out with 5 µg of macronuclear DNA of transformed clones 1 (140 days after transfection) and 2 (60 days after transfection), non-transformed cells (W) and AMaC DNA as a positive control. *Pst*I digested λ DNA was used as marker (M). The blot was probed with labelled DNA of the *neo* gene and phage λ.

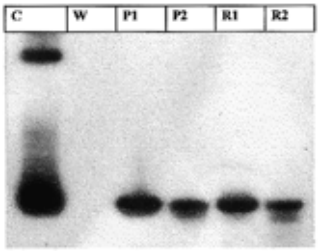

Figure 5. Transcription of the artificial chromosome. PCR (lanes P1 and P2) or RT–PCR (lanes R1 and R2) products were blotted and probed with a radioactive probe for the *neo* gene. Two different transformant clones (clones 1 and 2) were used. In the control lanes plasmid DNA containing the artificial chromosome (C) or the PCR products obtained with DNA from non-transformed cells (W) was blotted and probed with the same probe.

sequences reminiscent of a TATA box, a key element of many promoters transcribed by RNA polymerase II in eukaryotes.

Stable transformation of *E.crassus*

Clones raised from three independently transformed cells were tested for the presence of the AMaC. First, DNA from only a few cells was amplified by PCR before the analysis. After gel electrophoresis a blot was analysed with a probe derived from the *neo* gene. As shown in Figure 3, signals were obtained only from DNA of transformed cells. In order to find out whether the artificial chromosome remained stable in the cells, the analysis was done up to 52 days after transformation. The signal appeared to remain almost constant, indicating maintenance of the AMaC without selective pressure. To confirm the results obtained after PCR, Southern blot analyses of the transformed clones were carried out using a *neo* gene probe. The results are shown in Figure 4.

Copy number determination

Both the TBP- and TP-encoding genes of *E.crassus* have copy numbers of about 2000 per macronucleus in *E.crassus* cells. It was of interest to determine the copy number the artificial chromosome reached in the transformed cells. A quantitative PCR was therefore performed which included standard DNA in different amounts. Care was taken that the PCR had not reached saturation. As seen in Figure 3, the copy number can be estimated to be approximately 200 copies of the AMaC per cell.

Expression of the heterologous gene on the artificial chromosome

In order to be able to study promoter function with the help of transformed cells, genes carried on artificial chromosomes must be transcribed. To visualise transcription products, RT–PCR was performed on RNA extracted from transformed cells and the product obtained was probed with the same probe used to detect the artificial chromosome. Figure 5 shows the results of experiments with two independent transformed clones. Clear signals were obtained with the products of RT–PCR with RNA from transformant clones, while no signals were obtained with RNA from non-transformed cells. This shows that the *TBP* promoter was able to drive transcription of the heterologous *neo* gene.

Identical transcription start sites are found on the macronuclear *TBP* chromosome and on the AMaC

So far, it is largely unknown how the transcription start site is determined on macronuclear chromosomes. It was therefore interesting to map the 5'-ends of the transcripts of the natural TBP-encoding chromosome and the AMaC, on which the *neo* gene is under the control of the same promoter. Figure 6 shows the results. In spite of the different sequences following the promoter region the main transcription start sites were detectable at the same distance with respect to the telomeres in both cases. The occurrence of two adjacent start points of transcription on the natural chromosome is not unusual and has been seen before in *E.crassus* (12). Note that on the artificial chromosome the second base on which the minor start signal occurred on the original chromosome was changed.

DISCUSSION

The procedure for transformation of the hypotrichous ciliate *E.crassus* described here exploits the fact that DNA complexed with lipophilic material is taken up through biological membranes. This transfection method avoids the damage to the cells which can be caused by both electroporation and microinjection, two widely used methods for the transformation of ciliates. The complexed DNA is apparently ingested by *E.crassus* (our unpublished observation) and thus concentrated inside the cell. This might explain the observed high efficiency of transformation. Antibiotic resistance markers have been used for selection of transformed ciliates before. In our case we also used the *neo* gene as a reporter for the activity of a promoter transcribed by RNA polymerase, known to have a comparatively high activity. However, we found that the introduced AMaC remained stable in the cell even without selection pressure. Therefore, in view of the high transformation efficiency obtained using our approach, it might be possible in the future to use screening instead of selection of transformants, which might make a resistance marker dispensable.

Transcription of protein-encoding genes in eukaryotes by RNA polymerase II involves the formation of highly organised initiation complexes from at least five proteins, mostly consisting of several subunits (reviewed in 33). Central to the process is TATA-binding protein, which is part of the transcription factor TFIID and is required in most cases even if the promoter

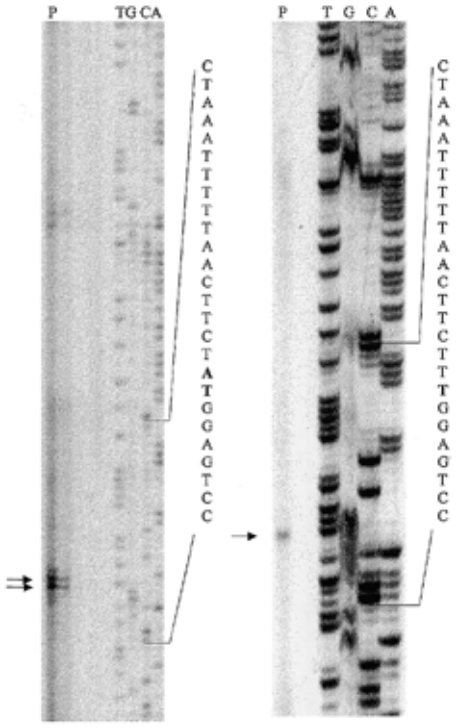

Figure 6. Determination of the transcription start sites on the artificial chromosome in comparison with the macronuclear chromosome carrying the *TBP* gene. Primer extension reactions were performed on RNA isolated from non-transformed cells (**left**) using a primer located in the *TBP* gene or from transformed cells (**right**). In the latter case the primer was located in the *neo* gene sequence. Sequencing reactions with the same primers are shown next to the primer extension reactions (P). Note that the start point is located close to the first potential start codon of a continuous open reading frame. However, sequence comparisons with other *TBP* genes indicate that the effective start codon is located 117 bp downstream.

lacks a TATA box. It anchors the transcription complex on the DNA and/or leads to assembly of the initiation complex. The other basic transcription factors interact with TFIID and/or RNA polymerase to effect transcription initiation. Many additional proteins usually bind upstream of the promoter and enhance or down-regulate initiation frequency (34–36), which generally determines the transcription rate. The macronuclear chromosomes of hypotrichous ciliates are short and frequently possess very short non-translated regions in front of the genes. Therefore, regulation of transcription initiation cannot mainly depend on protein factors interacting with the basic transcription apparatus in the way of enhancer- or silencer-binding proteins. Rather, the promoter structure itself is likely to set the level of transcription, along with the fixed copy number of the respective gene. These promoters would then be model systems for basic transcription.

We have previously shown that the promoter of the TP-encoding gene most likely encompasses an element downstream of the transcription start (12). In the case of the *TBP* promoter incorporated into the artificial chromosome described here this appears highly unlikely, since it works faithfully in front of a heterologous gene sequence and a 3'-region taken from the *TP* chromosome. It will be interesting to see whether in this case the putative TATA box is the key element for positioning of the transcription initiation complex.

ACKNOWLEDGEMENTS

We thank our colleagues for the gift of strains and Dr S. Curtenaz for critically reading the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

1. Orias,E. (1986) In Gall,J.G. (ed.), *The Molecular Biology of Ciliated Protozoa*. Academic Press, New York, NY, pp. 45–84.
2. Heckmann,K. (1964) *Z. Vererbungsl.*, **95**, 114–124.
3. Klobutcher,L.A. and Prescott,D.M. (1986) In Gall,J.G. (ed.), *The Molecular Biology of Ciliated Protozoa.* Academic Press, New York, NY, pp. 111–154.
4. Baird,S.E. and Klobutcher,L.A. (1991) *J. Protozool.*, **38**, 136–140.
5. Prescott,D.M. (1994) *Microbiol. Rev.*, **58**, 233–267.
6. Ghosh,S., Jaraczewski,J.W., Klobutcher,L.A. and Jahn,C.L. (1994) *Nucleic Acids Res.*, **22**, 214–221.
7. Klobutcher,L.A., Swanton,M.T., Donini,P. and Prescott,D.M. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 3015–3019.
8. Kaufmann,J. and Klein,A. (1992) *Nucleic Acids Res.*, **20**, 4445–4450.
9. La Terza,A., Miceli,C. and Luporini,P. (1995) *Dev. Genet.*, **17**, 272–279.
10. Brandt,A. and Klein,A. (1995) *J. Eukaryot. Microbiol.*, **42**, 691–696.
11. Anderson,R.C., Lindauer,K.R. and Prescott,D.M. (1996) *Gene*, **168**, 103–107.
12. Bender,J. and Klein,A. (1997) *Nucleic Acids Res.*, **14**, 2877–2882.
13. Wang,W., Skopp,R., Scofield,M. and Price,C. (1992) *Nucleic Acids Res.*, **20**, 6621–6629.
14. Price,C.M., Skopp,R., Krueger,J. and Williams,D. (1992) *Biochemistry*, **31**, 10835–10843.
15. Wünning,I.U. and Lipps,H.J. (1983) *EMBO J.*, **10**, 1753–1757.
16. Tondravi,M.M. and Yao,M.C (1986) *Proc. Natl Acad. Sci. USA*, **83**, 4369–4373.
17. Godiska,R., Aufderheide,K.J., Gilley,D., Hendrie,P., Fitzwater,T., Preer,L.B., Polisky,B. and Preer,J.R.,Jr (1987) *Proc. Natl Acad. Sci. USA*, **84**, 7590–7594.
18. Kahn,R.W., Andersen,B.H. and Brunk,C.F. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 9295–9299.
19. Wen,J.P., Eder,C. and Lipps,H.J. (1995) *Nucleic Acids Res.*, **23**, 1704–1709.
20. Gaertig,J. and Gorovsky,M.A. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 9196–9200.
21. Harumoto,T. and Hiwatashi,K. (1992) *Dev. Genet.*, **13**, 118–125.
22. Blomberg,P., Randolph,C., Yao,C.H. and Yao,M.C. (1997) *Mol. Cell. Biol.*, **17**, 7237–7247.
23. Gaertig,J., Gu,L., Hai,B. and Gorovsky,M.A. (1994) *Nucleic Acids Res.*, **22**, 5391–5398.
24. Valbonesi,A., Ortenzi,C. and Luporini,P. (1992) *J. Protozool.*, **39**, 45–54.
25. Ammermann,D., Steinbrück,G., von Berger,L. and Hennig,W. (1974) *Chromosoma*, **45**, 401–429.
26. Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds) (1996) *Current Protocols in Molecular Biology*. John Wiley & Sons, New York, NY.
27. Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156–159.
28. Beck,E., Ludwig,G., Auerswald,E.A., Reiss,B. and Schaller,H. (1982) *Gene*, **19**, 327–336.
29. Dickover,R.E., Donovan,R.M., Goldstein,E., Dandekar,S., Bush,C.E. and Carlson,J.R. (1990) *J. Clin. Microbiol.*, **28**, 2130–2133.
30. Berghöfer,Y., Agha-Amiri,K. and Klein,A. (1994) *Mol. Gen. Genet.*, **242**, 369–373.
31. Chomczynski,P. (1992) *Anal. Biochem.*, **201**, 134–139.
32. Church,G.M. and Gilbert,W. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 1991–1995.
33. Roeder,R.G. (1996) *Trends Biochem. Sci.*, **21**, 327–335.
34. Ptashne,M. (1988) *Nature*, **235**, 683–689.
35. Dynan,W.S. (1989) *Cell*, **14**, 1–4.
36. Ogbourne,S. and Antalis,T.M. (1998) *Biochem. J.*, **331**, 1–14.