hectare basis, the biodynamic farms were just as often financially viable as their neighboring conventional farms and representative conventional farms.

REFERENCES AND NOTES

1. H. H. Koepf, *The Biodynamic Farm* (Anthroposophic Press, Hudson, NY, 1989), pp. 94–112.
2. Ministry of Agriculture and Fisheries, *Farm Monitoring Report: North Central Region* (MAF, Palmerston North, New Zealand, 1987–1991); *Farm Monitoring Report: North Region* (MAF, Hamilton, New Zealand, 1987–1991).
3. H. Jenny, *Factors of Soil Formation* (McGraw-Hill, New York, 1941), pp. 12–20. Fields not adjacent to the boundary between farms may differ not only in soil characteristics but in economic performance, limiting the economic component of studies with whole farms.
4. Ten pairs of paddocks were directly adjacent to each other; 5 to 6 soil samples were taken from each paddock. Two paddocks were in hill country and had to be sampled about 300 m apart to get the same slope and aspect; here 12 soil samples were taken from each paddock. Soil samples were collected in the spring of 1990 and the summer of 1990 to 1991 from the upper 10 cm.
5. Soil samples were analyzed for the following properties: total carbon, with the use of a Leco (Saint Joseph, MI) high-frequency induction furnace; extractable potassium, calcium, and magnesium, with the use of a semimicro leaching procedure; pH in a water suspension; extractable phosphorus and cation exchange capacity as described in L. C. Blakemore, P. L. Searle, and B. K. Daly, *New Zealand Soil Bureau Scientific Report 80* (Department of Scientific and Industrial Research, Lower Hutt, New Zealand, 1987)]; soil respiration, by manometric measurements of the respiratory uptake of gaseous oxygen by soil [W. W. Umbreit, R. H. Burris, J. F. Stauffer, *Manometric and Biochemical Techniques* (Burgess, Minneapolis, 1972)] and modified by A. N. Macgregor and L. M. Naylor [*Plant Soil* **65**, 149 (1982)]; mineralizable soil nitrogen, by incubation [D. R. Keeney and J. M. Bremner, *Soil Sci. Soc. Am. Proc.* **31**, 34 (1967)]; total nitrogen and phosphorus, with the use of a micro-Kjeldahl digestion of soil followed by nitrogen analysis [Technicon, *Industrial Method No. 329-74 W/A* (Technicon, Tarrytown, NY, 1976)] and phosphorus analysis [J. R. Twine and C. H. Williams, *Commun. Soil Sci. Plant Anal.* **2**, 485 (1971)]; and sulfate, by the automated Johnson and Nishita technique [B. Heffernan, *A Handbook of Methods of Inorganic Chemical Analysis for Forest Soils, Foliage, and Water* (CSIRO Division of Forest Research, Canberra, Australia, 1985)]. Soil profiles were analyzed in the field for the following properties: soil texture, structure, and consistence as described by standard New Zealand Soil Bureau procedures [N. H. Taylor and I. J. Pohlen, *Soil Bureau Bulletin 25* (Soil Bureau, Lower Hutt, New Zealand, 1962)]; bulk density with the use of thin-walled aluminum cores; and penetration resistance with the use of a Rimik (Toowoomba, Queensland, Australia) CP10 cone penetrometer.
6. R. G. McLaren and K. C. Cameron, *Soil Science: An Introduction to the Properties and Management of New Zealand Soils* (Oxford Univ. Press, Auckland, New Zealand, 1990), p. 132.
7. E. W. Russell, *Russell's Soil Conditions and Plant Growth* (Longman, Essex, England, 1988), pp. 472–499.
8. J. K. Syers and J. A. Springett, *Plant Soil* **76**, 93 (1984).
9. I. S. Cornforth and A. G. Sinclair, *Fertiliser Recommendations for Pastures and Crops in New Zealand* (MAF, Wellington, New Zealand, 1984); C. J. Clarke, G. S. Smith, M. Prasad, I. S. Cornforth, *Fertilizer Recommendations for Horticultural Crops* (MAF, Wellington, New Zealand, 1986).
10. A. Wright, in *Integrated Systems Analysis and Climate Impacts*, R. W. M. Johnson, Ed. (MAF Tech, Wellington, New Zealand, 1989), pp. 55–63.
11. M. D. Boehlje and V. R. Eidman, *Farm Management* (Wiley, New York, 1984), pp. 86–91.
12. *SAS Institute Inc., JMP User's Guide* (SAS Institute, Cary, NC, 1989).
13. *SAS/STAT User's Guide, Release 6.03 Edition* (SAS Institute, Cary, NC, 1988).

14. We thank the 16 New Zealand farm families for donating the use of their farms to this study. Supported by the Fertiliser and Lime Research Centre at Massey University, a Prince and Princess of Wales Science Award by the Royal Society of New Zealand, the Massey University Research Fund, and International Program Development at Washington State University.

14 September 1992; accepted 4 March 1993

Transient Transfection and Expression in the Obligate Intracellular Parasite *Toxoplasma gondii*

Dominique Soldati and John C. Boothroyd*

Toxoplasma gondii **is a protozoan pathogen that produces severe disease in humans and animals. This obligate intracellular parasite provides an excellent model for the study of how such pathogens are able to invade, survive, and replicate intracellularly. DNA encoding chloramphenicol acetyltransferase was introduced into *T. gondii* and transiently expressed with the use of three vectors based on different *Toxoplasma* genes. The ability to introduce genes and have them efficiently and faithfully expressed is an essential tool for understanding the structure-function relation of genes and their products.**

Toxoplasma gondii is a ubiquitous parasite that can infect almost any warm-blooded vertebrate. In humans, it has long been recognized as a major cause of severe congenital disease. More recently, it has emerged as one of the most important opportunistic pathogens in patients with acquired immunodeficiency syndrome (AIDS) (*1*). In the laboratory, *T. gondii* is relatively easy to handle and maintain and consequently has become an important model for the study of how obligate intracellular parasites function. To date, however, such studies have been hampered by the absence of a method for introducing DNA into the parasites. In part, this lack has been due to the difficulty of transfecting one cell inside another: the many membranes that the transfecting DNA must cross represent a significant barrier, and the dependence on the host cell for survival can further preclude manipulations of the extracellular parasite. As a result, although transfection and stable transformation have been achieved for a range of trypanosomatids (*2–8*), such methodologies have not been reported for any of the obligate intracellular parasites, most notably members of the phylum Apicomplexa, which includes *Toxoplasma*, *Eimeria*, and *Plasmodium*, the causative agent of human malaria.

Electroporation has successfully been used to introduce DNA into many cell types. It is believed that pores are generated by reversible electrical breakdown of the cell membrane. Recent studies have shown that immediately after electroporation, cells are sensitive to the osmolarity and ionic composition of the medium and that the use of a potassium phosphate–based electroporation buffer (cytomix) that resembles the cytosol's ionic composition considerably increases cell survival (*9*). We chose, therefore, to use such a buffer in our initial transfection studies rather than culture medium or phosphate-buffered saline, which contain sodium ions at concentrations that are detrimental to the cells. We found that electroporation of *T. gondii* in cytomix buffer gives an extremely good rate of cell survival: an average of ~80% of the parasites are capable of invading host cells after electroporation as compared with the same population of parasites not subjected to an electric pulse.

For use as a reporter construct, a plasmid (SAG1/2 CAT) was made containing the chloramphenicol acetyltransferase (CAT) gene (*11*) and the upstream and downstream sequences of the *T. gondii* major surface antigen gene, p30 or *SAG1* (*12*) (Fig. 1). This was done by a two-step method. First, reverse polymerase chain reaction (PCR) (*13*) was performed with an SK+ Bluescript vector (Strategene) containing the complete *SAG1* gene with the use of primers that generate an Nsi I site at the second in-frame ATG and a Pac I site at the stop codon. Then, a CAT cassette with a Nsi I site embracing its ATG and a Pac I site encompassing its stop codon was generated by PCR and cloned into the corresponding Nsi I–Pac I sites of the *SAG1* expression vector.

Electroporation of this construct into

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305.

*To whom correspondence should be addressed.

freshly purified, extracellular *T. gondii*, followed by incubation for 16 hours in medium, resulted in substantial levels of CAT activity (Fig. 1). Neither the host cells [human foreskin fibroblasts (HFF)] nor *T. gondii* had any detectable intrinsic CAT activity, and there was no significant level of expression of SAG1/2 CAT in electroporated HFF cells alone (this was a necessary control because the parasites were grown in HFF cells, which could have been contaminating the final preparation of parasites). Invading parasites could not passively have delivered the plasmid to host cells because no CAT activity was seen when plasmid, parasites, and host cells were incubated together in the absence of electroporation.

The possibility that expression is due to transfection of a contaminant (such as bacteria) in the medium or buffer can be excluded on several grounds. First, electroporation of host cell cultures treated as though infected gave no activity (Fig. 1). Second, all reagents, including electroporated parasites, were plated on rich (L broth) agar, and no growth of any contaminating organisms was seen after incubation for 2 days. Third, no CAT activity was detectable when transfected parasites were incubated in medium containing 100 μg of cycloheximide per milliliter (*10*), indicating that expression was exclusively dependent on eukaryotic translational machinery (*14, 15*).

Because the parasites subjected to the electric pulse were still capable of invading host cells, we compared the CAT activity generated by electroporated parasites incubated in Dulbecco's minimum essential medium (DMEM) containing 20% Nu serum (Gibco) for 16 hours to that of parasites introduced onto a fibroblast monolayer 2 hours after transfection and then incubated for 14 hours. Both conditions gave the same CAT activity, which implies that extracellular parasites are biosynthetically active for an extended period.

No CAT was detectable immediately after electroporation; the earliest detectable activity was at about 4 hours after electroporation and rose steadily through the first 24 hours (*10*). Hence, CAT enzyme was not contaminating our plasmid DNA preparation which was, in any case, phenol-extracted and purified by cesium chloride banding.

On the basis of these controls, we conclude that measuring CAT enzymatic activity reflects both DNA uptake and expression by *T. gondii*. The enzymatic activity was proportional to the number of parasites present, over a range of 10^6 and 5.10^7 parasites. It was also proportional to the amount of plasmid in the 5- through 150-μg range. In practice, a readily detectable signal can be obtained 16 hours after electroporation, with 10^6 parasites and as little as 5 μg of plasmid. Optimal electroporation parameters were found to be 2.0 kV and 48 ohm, with an extremely low time constant of 0.40 to 0.45 ms owing to the salt composition of the cytomix buffer (*10*).

The transience of the transfection has been addressed by measurement of the longevity of CAT expression (Fig. 2, A and B) and by slot-blot quantitation of the presence of the transfecting plasmid (Fig. 2, C and D) for a 9-day period after electroporation. During this period, the culture was passed with a 1:5 to 1:10 dilution on days 1, 3, 5, and 7. By 7 days after electroporation, there was no longer any detectable plasmid DNA. Traces of CAT activity remained, however, as would be expected given the unusual stability of the CAT protein. These results showed that the introduced DNA was gradually diluted out of the parasite population, with the slope indicating no significant replication of the introduced plasmid.

The efficiency of the transfection, in terms of the proportion of the parasites expressing the transfected gene, could not be assessed with the CAT construct (it is not possible to measure the CAT activity of individual parasites, and commercial antibodies to CAT cross-react with a range of *Toxoplasma* antigens). A clear indication of this efficiency, however, could be judged from experiments in which 100 μg of a construct bearing the intact *SAG1* gene is transfected into a $SAG1^-$ mutant. Using a cell sorter and a monoclonal antibody specific for the *SAG1* gene product, we found that ~15% of the electroporated parasites were expressing the transfected gene 1 day after electroporation (*16*).

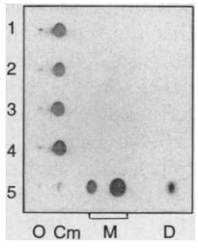

Fig. 1. Thin-layer chromatography of acetylated forms of radioactively labeled chloramphenicol after incubation in lysates of cells transfected with the CAT expression construct SAG1/2 CAT (*21, 22*). Migration of unacetylated chloramphenicol (Cm), the two monacetylated forms (M), and the diacetylated form (D) relative to the origin (O) are indicated. Lane 1, HFF not transfected; lane 2, HFF transfected with the plasmid; lane 3, HFF mixed with *T. gondii* parasites and the plasmid but without electroporation; lane 4, *T. gondii* parasites mixed with plasmid but without electroporation; lane 5, *T. gondii* parasites transfected with the plasmid. The SAG1/2 CAT plasmid contains the CAT gene flanked by about 800 bp of sequence upstream of the first ATG and 313 bp downstream of the stop codon from the *T. gondii SAG1* gene (*17*) and was constructed as described in the text.

We compared the ability of 5′ regions from two other *T. gondii* genes to mediate expression of CAT: those from *TUB1* (*17*), encoding α-tubulin, and from *ROP1* (*18*), encoding a protein found in the specialized secretory organelles of the apical complex called rhoptries. The constructs are schematically represented in Fig. 3A. SAG1/1 CAT was generated in essentially the same way as SAG1/2 CAT except that the upstream reverse PCR primer extended from the first in-frame ATG of the *SAG1* gene, and hence this is the ATG driving CAT. SAG1′/2 CAT is identical to SAG1/2 CAT except that it is missing a region of about 400 bp of the region upstream of *SAG1* that was found in other experiments to have no effect on CAT expression. Plasmids ROP1/1 CAT and ROP1/2 CAT were generated by replacement of the 5′ flanking region of the *SAG1* gene in the SAG1′/2 CAT construct with PCR-generated fragments of the upstream sequences of *ROP1*. These fragments terminated at either the first or the second ATG of *ROP1* for the ROP1/1 and ROP1/2 constructs, respectively. In plasmid TUB1 CAT, the upstream sequence of SAG1′/2 CAT has

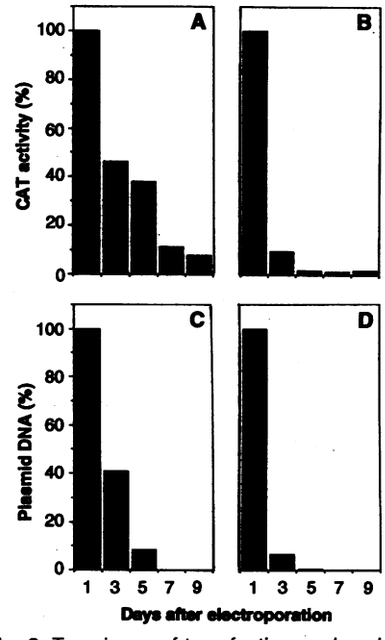

Fig. 2. Transience of transfection analyzed on two populations of recombinant parasites electroporated with 25 pmol of either TUB1 CAT (**A** and **C**) or ROP1/2 CAT (**B** and **D**) plasmids (*23*). (A and B) Equal numbers of successive generations of parasites transfected with TUB1 CAT and ROP1/2 CAT at days 1, 3, 5, 7, and 9 after electroporation were assayed for CAT activity (in the linear range) with the solvent phase-partition method as described (*24*). (C and D) As for (A) and (B), except that the parasites were analyzed by slot-blot hybridization for the presence of the transfecting plasmid (*25, 26*).

been replaced by about 3 kb of 5′ flanking region of the *TUB1* gene. In all cases the sequences immediately upstream of the start codon and downstream of the stop codon are derived from the indicated *T. gondii* gene. In plasmid TUB1/inv. CAT, a segment of about 500 bp immediately upstream of the ATG, including the transcription start site, is inverted.

These constructs were transfected into *T. gondii*, and the resulting CAT activities were assayed (Fig. 3B). Although the expression vectors derived from all three genes were highly active, quantitation of these results from assays in the linear range reproducibly showed that the TUB1 CAT and ROP1/2 CAT constructs yield about eightfold and fourfold more CAT than SAG1′/2 CAT, respectively (Fig. 3B). This difference is likely due to some combination of differences in promoter strength, mRNA stability, and translation efficiency. The current data do not allow us to determine the relative contribution of these parameters. The CAT cassette present in a plasmid vector that had no *T. gondii* sequences (SK CAT) showed virtually no activity. Addition of the 3′ sequences of *SAG1* downstream of the CAT cassette in SK CAT gives the same minimal activity (*10*).

On the basis of the DNA sequence analysis (*17, 18*), there are two plausible ATG start codons for *SAG1* and *ROP1*. Placement of the CAT cassette adjacent to the first ATG of either gene gave rise to no significant activity as compared with the SK CAT control, whereas placement immediately downstream of the second ATG gave rise to high activity for both (Fig. 3B). These data are consistent with predictions (*17, 18*) of which ATG functions in vivo based on ATG context and distance from the predicted (*ROP1*) or known (*SAG1*) signal peptide cleavage site (both proteins pass through the secretory pathway). That the effect is posttranscriptional in at least the SAG1-based constructs is apparent from primer extension analysis on RNAs isolated from parasites transfected with the two constructs: use of CAT gene–specific antisense oligonucleotide primers shows comparable amounts of transcript of the expected size in parasites receiving SAG1/1 CAT and SAG1′/2 CAT (*10*).

Recently, the potential of *T. gondii* as a genetic system has been realized through the analysis of phenotypic mutants (*19*) and the creation of a low-resolution genetic map that has been used to localize mutant genes of interest (*20*). The development of transfection complements this more classical genetic approach and permits "reverse" genetics to be exploited in this system. Although stable transfection clearly remains an important objective, the efficiency of the transient expression described here already makes possible detailed studies on the expression of genes important to the processes of invasion, drug resistance, and other aspects of the host-parasite interaction. It may also facilitate progress along similar lines in related genera such as *Plasmodium*.

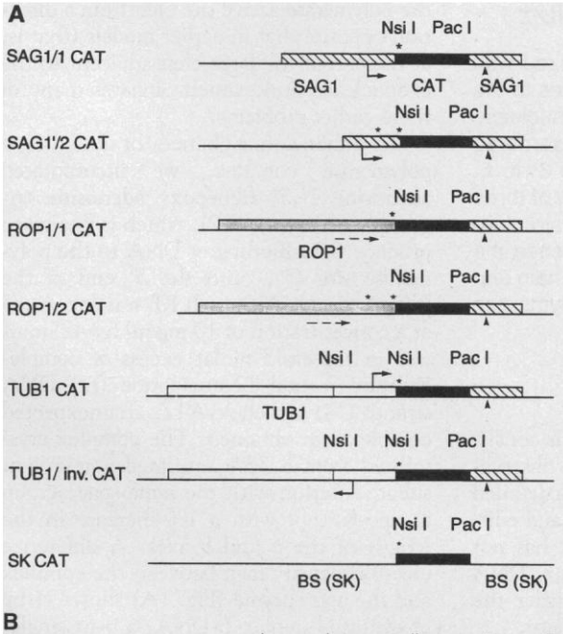

Fig. 3. CAT gene expression in 10^7 extracellular *T. gondii* parasites transfected by electroporation with 50 pmol of three different *T. gondii* expression vectors. Conditions for electroporation and CAT assay were otherwise as described (*21, 22*). (**A**) Schematic description of six expression plasmids (not to scale). The black box indicates the CAT coding region. A thin line represents Bluescript vector sequence. Hatching represents *SAG1* sequences comprising either the region upstream of an ATG start codon (marked with an asterisk) or downstream of the TAG stop codon and including the transcription start site (bent arrow) and polyadenylate-addition site (triangle). Grey boxing indicates sequences from *ROP1* (*18*), and the open box represents sequences from the *TUB1* region (*17*), beginning with an ATG start codon and proceeding upstream for ~1.35 kb and ~3 kb, respectively. The precise transcription start point for *ROP1* is not known. The number after the slash indicates whether the first or second in-frame ATG of the *SAG1* or *ROP1* gene is at the start of the CAT cassette. TUB1/inv. CAT has the Nsi I fragment that includes the transcription start site inverted. (**B**) CAT assay (*22*) results in which lysates of parasites transfected with SAG1/1 CAT (lane 1), SAG1/2 CAT (lane 2), ROP1/1 CAT (lane 3), ROP1/2 CAT (lane 4), TUB1 CAT (lane 5), TUB1/inv. CAT (lane 6), and SK CAT (lane 7) were analyzed by thin-layer chromatography.

REFERENCES AND NOTES

1. L. H. Kasper and J. C. Boothroyd, in *Immunology of Parasitic Diseases*, K. Warren, Ed. (Blackwell Scientific, Oxford, United Kingdom, 1992), pp. 264–299.
2. V. Bellofatto and G. A. M. Cross, *Science* **244**, 1167 (1989).
3. A. Laban and D. F. Wirth, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9119 (1989).
4. M. G.-S. Lee and L. H. T. Van der Ploeg, *Science* **250**, 1583 (1990).
5. C. M. Coburn *et al.*, *Mol. Biochem. Parasitol.* **46**, 169 (1991).
6. J. Eid and B. Sollner-Webb, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2118 (1991).
7. J. H. LeBowitz, C. M. Cobrun, D. Mc Mahon-Pratt, S. M. Beverley, *ibid.* **87**, 9736 (1990).
8. J. F. Tobin, A. Laban, D. F. Wirth, *ibid.* **88**, 864 (1991).
9. M. J. B. Van den Hoff, A. F. M. Moorman, W. H. Lamers, *Nucleic Acids Res.* **20**, 2902 (1992).
10. D. Soldati and J. C. Boothroyd, unpublished results.
11. C. M. Gorman, L. F. Moffat, B. H. Howard, *Mol. Cell. Biol.* **2**, 1044 (1982).
12. J. L. Burg, D. Perelman, L. H. Kasper, W. P. L. Ware, J. C. Boothroyd, *J. Immunol.* **141**, 3584 (1988).
13. T. Triglia, M. G. Peterson, D. J. Kemp, *Nucleic Acids Res.* **16**, 8186 (1988).
14. T. G. Obrig, W. J. Culp, W. L. McKeehan, B. Hardesly, *J. Biol. Chem.* **246**, 174 (1971).
15. H. G. Elmendorf, J. D. Bangs, K. Haldar, *Mol. Biochem. Parasitol.* **52**, 215 (1992).
16. K. Kim and J. C. Boothroyd, unpublished results.
17. S. D. Nagel and J. C. Boothroyd, *Mol. Biochem. Parasitol.* **29**, 261 (1988).
18. P. N. Ossorio, J. D. Schwartzman, J. C. Boothroyd, *ibid.* **50**, 1 (1992).
19. L. H. Kasper, *Parasite Immunol.* **9**, 433 (1987).
20. L. D. Sibley, A. J. Leblanc, E. R. Pfefferkorn, J. C. Boothroyd, *Genetics* **132**, 1003 (1992).
21. *Toxoplasma gondii* tachyzoites (RH strain) were grown in HFF cells until the infected cultures had spontaneously lysed. They were then purified by passage through Whatman CF-11 cellulose [K. Tanabe, I. Kimata, M. Iseki, S. Takada, *Jpn. J. Parasitol.* **26**, 113 (1977)] and harvested by centrifugation at 1000*g* for 15 min. The resulting material was washed twice by resuspension in electroporation buffer [120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4–KH_2PO_4 (pH 7.6), 25 mM Hepes (pH 7.6), 2 mM EDTA (pH 7.6), and 5 mM $MgCl_2$] (*9*) and spinning as before. Either 5×10^6 HFF or 1×10^7 *T. gondii* tachyzoites were resuspended in 0.8 ml of cytomix buffer supplemented, just before use, with 2 mM adenosine triphosphate (pH 7.6) and 5 mM glutathione and mixed with 0.1 ml of cytomix buffer alone or containing 25 pmol of SAG1/2 CAT circular plasmid isolated from a cesium chloride gradient (see below). The entire mixture was then transferred to an electroporation cuvette (4-mm gap) (BTX, San Diego, CA) and exposed to an electric pulse with an electroporator (BTX Electro Cell Manipulator 600) in the high-voltage mode ("2.5 kV/RESISTANCE"), the charging voltage set to 2.0 kV, the resistance set at 48 ohm ("R3"). The resulting discharge

voltage was ~1.4 kV with a pulse length of 0.40 to 0.45 ms. Electroporated cells were incubated at room temperature for 15 min and transferred into an enriched culture medium (DMEM) (*12*) with 20% Nu serum (Collaborative Research, Inc., Bedford, MA) containing 0.04% gentamicin and incubated for 16 hours at 37°C. This material was then harvested and washed at 4°C in 0.25 M tris-HCl (pH 7.8). After resuspension in 100 μl of 0.25 M tris-HCl (pH 7.8), the cells were frozen and thawed three times. The lysate was cleared by centrifugation in an Eppendorf Microfuge (10,000*g*) for 10 min.

22. CAT activity was assayed in a mixture of 0.25 M tris-HCl (pH 7.8), 1 mM acetyl coenzyme A, 0.3 μCi of [^{14}C]chloramphenicol (50 to 60 mCi/mmol; Amersham) in a final volume of 100 μl. The reaction mix was incubated at 37°C for 16 hours, extracted with ethylacetate, and dried. The pellet was resuspended in 27 μl of ethylacetate and spotted on a thin-layer chromatography plate (PE SIL G, Whatman). After development for 2 hours with chloroform-methanol (95:5), the plates were dried and analyzed with a Phosphor Imager (Molecular Dynamics).
23. Parasites (5×10^7) were electroporated with 25 pmol of either TUB1 CAT or ROP1/2 CAT plasmids and inoculated into monolayers of HFF. One day later, lysis of the host cell was complete, and the recombinant parasites were harvested and counted. One-fifth to one-tenth (usually ~1×10^7 parasites) of the population was inoculated and expanded in HFF until they lysed again 2 days later. In parallel, cell lysates were prepared from 5×10^7 parasites and assayed for CAT activity, and total DNA was extracted from 5×10^7 parasites and subjected to Southern (DNA) blot analysis. The same procedure was repeated until day 9 after electroporation.
24. J. R. Neumann, C. A. Morency, K. O. Russian, *BioTechniques* **5**, 444 (1987).
25. Total DNA was extracted from 10^7 parasites and transferred by slot blotter, in duplicate, to nylon membranes. The presence of CAT DNA sequences was detected by Southern blot analysis with a 600-bp DNA fragment encoding the CAT sequence. Signals for each point were normalized to the same amount of total DNA with signals obtained by hybridization of the duplicate with a single-copy *Toxoplasma* gene probe. The probes were labeled by the random primer method with ^{32}P-labeled deoxycytidine 5′-triphosphate, and the spots were quantified by Phosphor Imager.
26. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, ed. 2, 1989).
27. We thank D. Wirth, T. Soldati, and our colleagues for helpful suggestions; S. Pfeffer for use of the Phosphor Imager; and K. Kim and S. Tomavo for critical reading of the manuscript. Supported by the National Institutes of Health, the MacArthur Foundation, the Burroughs Wellcome Fund molecular parasitology award, a European Molecular Biology Organization fellowship, and a Swiss National Foundation fellowship.

20 October 1992; accepted 14 January 1993

Structure of DNA Polymerase I Klenow Fragment Bound to Duplex DNA

Lorena S. Beese,* Victoria Derbyshire,† Thomas A. Steitz‡

Klenow fragment of *Escherichia coli* DNA polymerase I, which was cocrystallized with duplex DNA, positioned 11 base pairs of DNA in a groove that lies at right angles to the cleft that contains the polymerase active site and is adjacent to the 3′ to 5′ exonuclease domain. When the fragment bound DNA, a region previously referred to as the "disordered domain" became more ordered and moved along with two helices toward the 3′ to 5′ exonuclease domain to form the binding groove. A single-stranded, 3′ extension of three nucleotides bound to the 3′ to 5′ exonuclease active site. Although this cocrystal structure appears to be an editing complex, it suggests that the primer strand approaches the catalytic site of the polymerase from the direction of the 3′ to 5′ exonuclease domain and that the duplex DNA product may bend to enter the cleft that contains the polymerase catalytic site.

The large proteolytic fragment of DNA polymerase I (Pol I) from *E. coli* [Klenow fragment (KF)] contains a 5′ to 3′ polymerase activity and a 3′ to 5′ exonuclease activity that edits mismatched bases. The two active sites that catalyze these reactions are separated by about 35 Å in the crystal structure (*1*). A specific proposal for how these sites work together to enhance the fidelity of DNA replication (*2–4*) is consistent with biochemical and kinetic observations (*5–8*). The establishment of a detailed structural basis for polymerization and editing has been hindered because it has not been possible to visualize duplex DNA bound to DNA polymerase in either the polymerase or exonuclease active site.

A cocrystal structure of KF with an 8-bp DNA duplex showed the 3′ end of a single-stranded region three nucleotides in length bound in the exonuclease active site, but no duplex DNA was seen (*4*) even though biochemical analysis of these cocrystals demonstrated a 1:1 stoichiometry of DNA:KF. Comparison of the apo-enzyme and cocrystal structures indicated that a "thumb-like" protrusion consisting of two long helices (H and I) from one side of the polymerase cleft changed conformation upon DNA binding and that the DNA was too disordered to be seen.

The catalytic site for the polymerase reaction is located at the bottom of the large cleft. This region contains a tight cluster of the most highly conserved residues in the polymerase sequences that have been aligned (*1, 9*), a binding site for the deoxynucleoside triphosphates (*10*), and residues whose mutations eliminate polymerase, but not exonuclease, activity (*11*). On the basis of the results of footprinting studies that showed protection of 8 bp of duplex product (*2, 12*) and electrostatic calculations (*13*), a primer-template model was built into the cleft, with the primer strand entering it from the end farthest from the 3′ to 5′ exonuclease active site (*1*). However, this model failed to explain several more recent observations. The location of catalytically important side chains (*11*) and the location of deoxynucleotide triphosphate (dNTP) bound in the crystal structure (*10*) position the catalytic site deeper into the cleft than had been previously suspected; this makes the placement of the 3′ end of the model-built DNA near the catalytic site sterically impossible and inconsistent with the DNA footprinting results. The location of duplex DNA in the complex reported here suggests that the primer strand enters the polymerase active site cleft from a direction opposite that in earlier models (that is, at the end of the large cleft adjacent to the exonuclease site), which obviates many of these earlier problems.

To enhance our chances of obtaining a polymerase complex, we incorporated adenosine 2′,3′ riboepoxy adenosine triphosphate (epoxyATP), which is known to produce tight binding of DNA to the polymerase site (*5*), onto the 3′ end of the primer strand. Although KF was incubated at a concentration of 10 mg/ml for 48 hours with a threefold molar excess of complementary 7- and 12-nucleotide (nt) DNA strands (*14*) and epoxyATP, an unexpected complex was obtained. The complex crystallized from a 35% saturated ammonium sulfate solution with the same space group as apo-KF but with a 1% increase in the length of the *a* and *b* axes. A difference electron density map between the complex and the apo enzyme (Fig. 1A) shows 11 bp of distorted duplex B-DNA, a 3-nt single-stranded DNA overhang with its 3′ terminal nucleotide bound at the 3′ to 5′ exonuclease active site, and one nonstandard base pair at the junction between single-stranded and double-stranded regions. The 5′ terminal nucleotide of the 12-nt strand is not base-paired and binds at the entrance to the polymerase cleft. The conformations of the four nucleotides (1 to 4) of the primer strand bound to the exonuclease active site

Departments of Molecular Biophysics and Biochemistry and Chemistry and the Howard Hughes Medical Institute, Yale University, New Haven, CT 06511.

*Present address: Department of Biochemistry, Duke University Medical Center, Durham, NC 27710.
†Present address: David Axelrod Institute of Public Health, New York State Department of Health, Wadsworth Center for Laboratories and Research, Albany, NY 12201.
‡To whom correspondence should be addressed.