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Disruption of the *Crithidia fasciculata RNH1* gene results in the loss of two active forms of ribonuclease H

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ABSTRACT

Both prokaryotic and eukaryotic cells contain multiple forms of ribonuclease H, a ribonuclease that specifically degrades the RNA strand of RNA–DNA hybrids and which has been implicated in the processing of initiator RNAs and in the removal of RNA primers from Okazaki fragments. The *Crithidia fasciculata RNH1* gene encodes an RNase H and was shown to be a single-copy gene in this diploid trypanosomatid. The *RNH1* gene has been disrupted by targeted gene disruption using hygromycin or G418 drug-resistance cassettes. Major active forms of RNase H (38 and 45 kDa) were observed on activity gels of extracts of wild-type cells or cells in which one allele of *RNH1* was disrupted. Both the 38 and 45 kDa activities were absent in extracts of cells in which both alleles of *RNH1* were disrupted indicating that both forms of the *C.fasciculata* RNase H are encoded by the *RNH1* gene.

INTRODUCTION

RNase H specifically degrades the RNA strand of RNA–DNA hybrids and was first described by Hausen and Stein (1) in calf thymus extracts. Two or more forms of RNase H of various sizes have been found in most eukaryotic cells (2). RNases H from mammalian cells appear to be of two classes: RNase HI with a molecular mass of 60–100 kDa and RNase HII with a molecular mass of 30–40 kDa. Multiple species have also been observed in yeast including an enzyme with a molecular mass of ~70 kDa and two additional forms with molecular masses of ~55 and 42 kDa (3). An RNase H from *Drosophila* embryos has been purified to near homogeneity and was found to have a molecular mass of 180 kDa and to be a tetrameric protein with two 49 kDa and two 39 kDa subunits (4). In general the significance and the relationships of the various forms of eukaryotic RNase H are unknown. Bacterial enzymes have been found to be only ~20 kDa and in *Escherichia coli* two forms (RNases HI and HII) have been identified and are encoded by distinct genes (*rnhA* and *rnhB*) that have no significant sequence similarity (5,6).

A role for RNase H in the removal of RNA primers from Okazaki fragments has been suggested from studies of discontinuous DNA synthesis by purified murine proteins although the mouse RNase H-1 was unable to remove the residual one or two ribonucleotides at the primer/DNA junction (7). Using RNase HI purified from calf thymus, initiator RNA in a model Okazaki fragment was shown to be cleaved specifically 1 nt 5′ of the RNA–DNA junction (8). In both cases a 5′-exonuclease was required to remove the remaining ribonucleotide(s). The *Drosophila* RNase H represents the most highly purified eukaryotic enzyme and appears to be associated with a DNA polymerase α–primase complex and to stimulate DNA synthesis (4). Unlike the murine and calf thymus enzymes, the *Drosophila* enzyme is able to remove the last ribonucleotide from the 5′ end of a DNA strand. A novel feature of the stimulation of polymerization by the *Drosophila* RNase H is that it is specific for a coupled reaction in which both priming and chain elongation are catalyzed by the polymerase–primase. It is suggested that the RNase H stimulates synthesis by the polymerase–primase by enhancing its recycling capacity and thereby increasing the rate of primer synthesis.

The structure–function relationships and physiological roles of *E.coli* RNase HI are understood in considerably greater detail than those of the many eukaryotic enzymes. RNase HI consists of a single polypeptide chain of 155 residues and is encoded by the *rnhA* gene (5). Three acidic residues (Asp10, Glu48 and Asp70) have been found to be essential for catalytic activity by site directed mutagenesis (9). The three-dimensional structure of this enzyme determined by X-ray analysis shows that these three residues form a catalytic triad in the active site of the enzyme (10,11). The *E.coli* RNase HI has been shown to play a role in initiation of replication of the ColE1 plasmid (12). Replication from a single unique origin on the ColE1 plasmid involves the formation of an RNA–DNA hybrid with a displaced DNA strand and cleavage of the RNA strand to generate a short RNA primer that is used for initiation of replication by DNA polymerase I. (13) The role of the *E.coli* RNase HII is unknown.

Mutations that inactivate the *E.coli rnhA* gene eliminate the requirement for replication from *oriC* (14). It is speculated that RNase H may normally inhibit initiation of replication from sites other than *oriC* by degradation of any RNA strand outside of the *oriC* region that happens to remain hybridized to DNA. Interestingly, *rnhA* mutants also have been found to require a functional RecBCD protein for viability (15,16). Mutant strains carrying a disruption of the *rnhA* gene together with either the

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recC271 or *recB270* mutations have a ts growth phenotype which is suppressed by supplying RNase H in *trans* (17). These strains have provided a powerful tool for cloning genes encoding RNase H from other bacterial species including *Salmonella typhimurium* and *Thermus thermophilus* and from the yeast *Saccharomyces cerevisiae*.

During the course of a search for enzymes that might be involved in the removal of RNA primers from kinetoplast minicircle DNA replication intermediates we recently cloned an RNase H gene from the trypanosomatid *Crithidia fasciculata* by complementation of such an *E.coli* mutant (18). The *C.fasciculata RNH1* gene contains an open reading frame encoding 494 amino acid residues. The carboxyl half of the protein has a high degree of sequence identity to bacterial and retroviral RNases H and to the *S.cerevisiae* RNase H. Much of the uncertainty concerning the structure and function of RNase H in eukaryotes stems from the unknown contribution of proteolysis to the formation of various forms of the enzyme and the lack of molecular genetic studies. With the availability of the cloned *C.fasciculata RNH1* gene and through the use of *in vitro* mutagenesis and gene replacement methods for trypanosomatids (19–21), it is now possible to answer important questions regarding the biogenesis and function of different forms of this novel enzyme. In this work we show that two active forms of RNase H are encoded by a single gene in the trypanosomatid *C.fasciculata*.

MATERIALS AND METHODS

Southern blots

For Southern blots, restriction digests of total DNA (2 μg per lane) were fractionated on 0.6% agarose gels containing 0.5× TBE and transferred to nylon membranes (MagnaGraph, Micron Separations, Inc.) by vacuum blotting as directed by the manufacturer. DNA was cross-linked to the membranes by UV irradiation using a Stratalinker (Stratagene) per the manufacturer's instructions. A 1.45 kb *Nco*I–*Nhe*I fragment derived from the *RNH1* ORF (18) was labeled with fluorescein-12-dUTP by random priming using a Fluorescein dUTP Labeling Kit (DuPont NEN). Membranes were hybridized with the labeled probe and washed at 65°C in 0.1× SSC and 0.1% SDS. Hybridizing fragments were detected using the anti-fluorescein HRP conjugate and Nucleic Acid Chemiluminescence Detection Reagent (DuPont NEN).

Plasmids

Plasmids expressing resistance to G418 and hygromycin were derived from the *Leishmania major* pX vectors (19,20) in which sequence from the 5′ and 3′ flanking regions of the *L.major* dihydrofolate reductase-thymidylate synthase (DHFR-TS) are used for mediating expression of neomycin phosphotransferase (NEO) or hygromycin phosphotransferase (HYG). Plasmid pRNH-NEO was constructed by cloning the NEO gene with *L.major* 5′ and 3′ flanking sequences as a 3.25 kb *Bsp*120I–*Not*I fragment from plasmid pX.2-KO (22) into a unique *Not*I site in the middle of the *RNH1* gene in plasmid p6His-1 (18). Plasmid pRNH-HYG was constructed by blunt-end cloning of the HYG gene with *L.major* 5′ and 3′ flanking sequences as a 3.44 kb *Eco*RV fragment from plasmid pHYG-KO into the *Not*I site in p6His-1 after filling in the ends with the Klenow fragment of DNA polymerase I. The pHYG-KO plasmid was constructed by cloning an *Xba*I–*Eco*RV–*Cla*I–*Kpn*I polylinker into unique *Hin*dIII and *Bgl*II sites flanking the hygromycin phosphotransferase drug-resistance cassette of pX63HYG (20). *C.fasciculata* cells were grown and transformed by electroporation as described (22).

DNA transformations

For disruptions of cellular *RNH1* genes cells were transformed with plasmid DNAs in which the *RNH1* genes were disrupted by insertion of either the NEO or HYG cassettes. Prior to transformation the disrupted *RNH1* gene was released from the vector by digestion with *Hin*dIII and *Dra*I (for pRNH-NEO) or with *Msc*I and *Nhe*I (for pRNH-HYG). Removal of the vector fragments was found to be unnecessary in these experiments. Single colonies were picked from BHI plates containing 50 μg G418/ml or 80 μg hygromycin/ml and grown subsequently in liquid BHI medium containing 25 μg/ml G418 or 40 μg/ml hygromycin.

Activity gels

Cells were grown at 27°C in BHI medium containing 20 μg/ml hemin and 100 μg/ml streptomycin and harvested by centrifugation in a microcentrifuge for 1 min at 3000 r.p.m. upon reaching a cell density of 3–6 × 10^7 cells/ml. A total of 6 × 10^7 cells were harvested and washed once in buffer containing 137 mM NaCl, 2.7 mM KCl and 25 mM Tris pH 8.0. Cell pellets were resuspended in 50 μl 50 mM Tris pH 6.8 and immediately lysed by the addition of an equal volume of 2× SDS gel loading buffer (50 mM Tris pH 6.8, 4% SDS, 17% glycerol, 5% β-mercaptoethanol and 0.025% bromophenol blue) and boiling for 5 min. RNase H activity gel analysis was performed as described (23) with renaturation in the presence of 10 mM $MgCl_2$. Approximately 2 × 10^7 cell equivalents of cell lysate were run in each lane. Following electrophoretic separation the denaturing agent was washed out allowing the proteins to renature and leading to the degradation of the embedded substrate at positions where proteins with RNase H activity had renatured. Undegraded substrate was precipitated and degradation products were removed by washing the gel in TCA. The gel was subsequently dried and exposed to autoradiographic film. A lane containing molecular weight markers was cut off the gel prior to the renaturation treatment, stained with Coomassie Blue and was subsequently dried along side the activity gel.

RESULTS

RNH1 is a single-copy gene

Although highly expressed genes are often tandemly repeated in trypanosomes, genes involved in DNA replication in *C.fasciculata* have been found to be single copy thus far (22,24–28). Southern blots of *C.fasciculata* genomic DNA digested with various restriction enzymes and probed with an *RNH1* gene probe indicate that *RNH1* is also a single copy gene (Fig. 1). The enzymes used for these digestions were known not to cut within coding sequence of *RNH1*. In each digest only a single band was found to hybridize to the labeled probe consistent with the presence of only a single copy of *RNH1* on each chromosome of the chromosome pair encoding *RNH1* in this diploid organism.

Disruption of *RNH1*

We have used targeted gene disruption to determine the phenotypes of strains in which one or both alleles of *RNH1* have

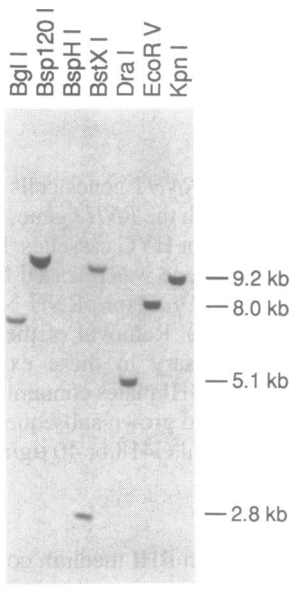

Figure 1. Southern blot of *C.fasciculata* genomic DNA. Total DNA (2 µg per lane) was digested with the indicated restriction enzymes, fractionated on a 0.6% agarose gel and transferred to a nylon membrane. The blot was hybridized to a fluorescein-12-dUTP-labeled probe derived from the *RNH1* coding sequence and detected using an HRP-labeled antibody conjugate and a chemiluminescence detection reagent.

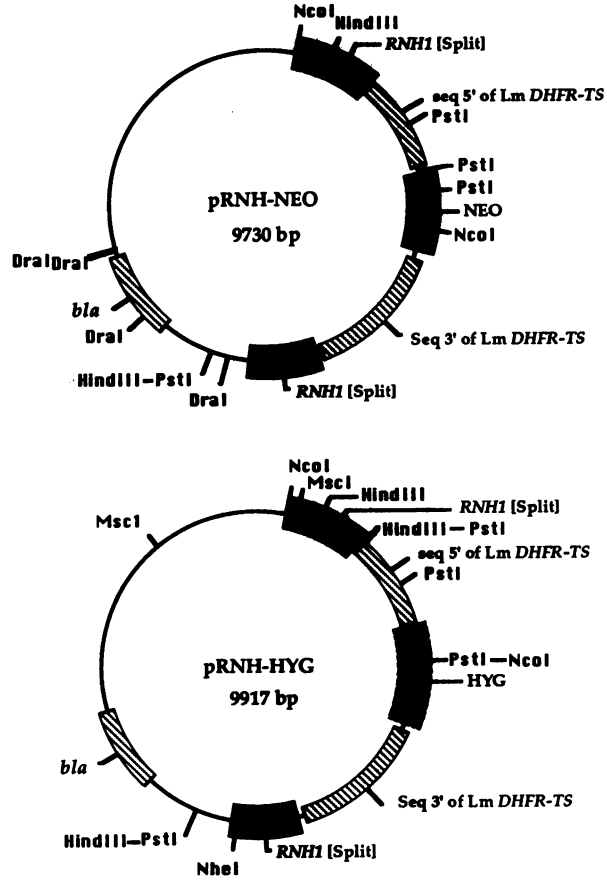

Figure 2. Plasmid constructs used for disruption of the *RNH1* gene. Drug-resistance cassettes expressing either the neomycin phosphotransferase gene (NEO) or the hygromycin phosphotransferase gene (HYG) were cloned into a unique *Not*I site in the middle of the *RNH1* coding sequence. Disruption of the *RNH1* gene was achieved by transforming *C.fasciculata* with pRNH-NEO DNA (digested with *Hin*dIII and *Dra*I) or with pRNH-HYG (digested with *Msc*I and *Nhe*I).

been disrupted. Drug-resistance cassettes conferring resistance to either neomycin (NEO) or hygromycin (HYG) were cloned into a unique *Not*I site in the middle of the *RNH1* ORF to create disrupted genes for transformation into *C.fasciculata* (Fig. 2). To create disruptions of a single allele of *RNH1*, *C.fasciculata* was transformed with either pRNH-HYG digested with *Msc*I and *Nhe*I or pRNH-NEO digested with *Hin*dIII and *Dra*I. Resulting clones resistant to either hygromycin B or G418 were analyzed by Southern blotting of *Bsp*HI fragments of genomic DNA from representative isolates. One of the G418-resistant clones was transformed subsequently with the pRNH-HYG-digested DNA to obtain clones resistant to both G418 and hygromycin. Clones resistant to both drugs resulted from the disruption of both alleles of *RNH1*. A Southern blot showing digests from each of the single disruption strains and from a double disruption strain are shown in Figure 3. In *C.fasciculata* wild-type cells the *RNH1* probe hybridized to a 2.8 kb *Bsp*HI fragment. Disruption of one allele of *RNH1* by either the NEO or HYG cassettes resulted in the appearance of a second hybridizing fragment of 6.1 or 6.3 kb, consistent with insertion of the 3.25 kb NEO cassette or the 3.44 kb HYG cassette into the *RNH1* gene. In the strain expressing resistance to both G418 and hygromycin the 2.8 kb fragment was absent and both the 6.1 and 6.3 kb fragments were observed on the genomic Southern blot indicating that both alleles of the *RNH1* gene had been disrupted. Neither the single nor the double disruption mutants show any significant alteration in growth rate or cell morphology.

RNase H activity gel analysis

Extracts of wild-type cells and cells carrying disruptions of one or both alleles of the *RNH1* gene were analyzed by electrophoresis on an SDS activity gel containing the substrate poly([^{32}P]rA)·poly(dT). The results of such an activity gel analysis are shown in Figure 4. Extracts from wild-type cells or cells having a single wild-type allele and a single disrupted allele (either HYG or NEO) showed two major RNase H activities with mobilities corresponding to polypeptides of ~38 and 45 kDa. However, in the double disruption strain both activities were absent indicating that both forms of the enzyme are expressed by the *RNH1* gene. A minor, faster migrating species was observed in all of the strains and may represent an activity encoded by a different gene. This or other activities not detected by such renaturation gel analysis may be responsible for the survival of the double disruption strain.

DISCUSSION

In the work described here two active forms of *C.fasciculata* RNase H have been identified by activity gel analysis. One of these corresponds in size to a 45 kDa RNase H found in *C.fasciculata* extracts earlier (29). A second major activity observed on activity gels is estimated to have a molecular mass of ~38 kDa and has not been described previously. Since the molecular masses of the two

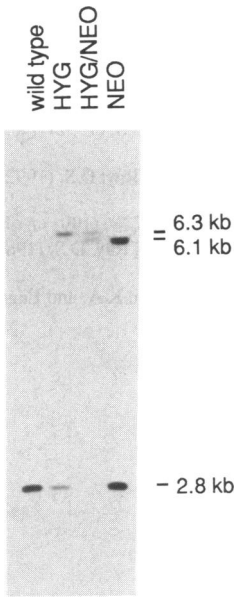

Figure 3. Southern blot of *Bsp*HI digests of *C.fasciculata* DNA from wild-type cells and from cells in which one allele of *RNH1* was disrupted by insertion of either the NEO or HYG cassettes or cells in which both alleles were disrupted (HYG/NEO).

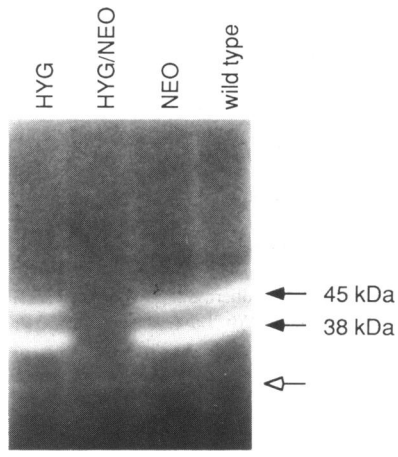

Figure 4. RNaseH activity gel analysis of extracts of wild-type and disruption mutant strains (HYG,NEO and HYG/NEO). Cell extracts were electrophoresed through SDS gels containing the substrate poly([^{32}P]rA)·poly(dT). Following renaturation of the proteins in the gel and repeated washes with TCA the gel was dried and exposed to autoradiographic film. A minor activity present in all lanes is indicated by the open arrowhead.

active forms of the *C.fasciculata* RNase H observed on activity gels are both less than the 54.7 kDa predicated for a protein encoded by the *RNH1* ORF (18), it was of interest to determine whether one or both of these enzymes is encoded by the *RNH1* gene.

With the recent development of gene disruption techniques for trypanosomatids it became possible to address this question by disruption of *RNH1*. Since some genes are tandemly repeated in trypanosomatids, it was necessary to determine whether or not the *RNH1* gene is reiterated. Southern blots of *C.fasciculata* DNA digested with several different restriction enzymes and probed with an *RNH1* gene probe indicated that *RNH1* is a single copy gene. Sequential disruption of the two alleles of *RNH1* in this diploid organism also supports this conclusion. In the double disruption mutant one allele was disrupted by insertion of a cassette expressing resistance to G418 and the other allele was disrupted by insertion of a cassette expressing resistance to hygromycin.

Surprisingly, both of the major active forms of RNase H detected on activity gels are absent in the double mutant indicating that both of these forms of RNase H are encoded by *RNH1*. The ability of these cells to survive and grow at a rate similar to wild-type cells and the possible existence of enzymes that escape detection by this method suggests that still other genes encoding RNase H may remain to be found in this trypanosomatid.

The relationship of the 38 and 45 kDa proteins to the *RNH1* ORF remains to be established. Purification of each protein and determination of their amino terminal sequences will be essential for distinguishing between various possibilities. Interestingly, initiation at the second ATG in the ORF would give rise to a protein of 343 residues and a molecular mass of 37.6 kDa, a size close to that observed for the 38 kDa species on activity gels. The 45 kDa active species might possibility result from proteolytic processing of the predicted 54.7 kDa protein or from a post-translational modification of the 38 kDa species. In light of the need for an RNase H activity to remove RNA primers present on replication intermediates of mitochondrial DNA minicircles in the single mitochondrion (kinetoplast) of these organisms (30,31), it will be of interest to determine the relationship between the two active polypeptides expressed by *RNH1* and their intracellular locations.

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REFERENCES

1 Hausen, P. and Stein, H. (1970) *Eur. J. Biochem.*, **14**, 278–283.
2 Crouch, R.J. (1990) *New Biol.*, **2**, 771–777.
3 Karwan, R. and Wintersberger, U. (1988) *J. Biol. Chem.*, **263**, 14970–14977.
4 DiFrancesco, R.A. and Lehman, I.R. (1985) *J. Biol. Chem.*, **260**, 14764–14770.
5 Kanaya, S. and Crouch, R.J. (1983) *J. Biol. Chem.*, **258**, 1276–1281.
6 Itaya, M. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8587–8591.
7 Goulian, M., Richards, S.H., Heard, C.J. and Bigsby, B.M. (1990) *J. Biol. Chem.*, **265**, 18461–18471.
8 Turchi, J., Huang, L., Murante, R., Kim, Y. and Bambara, R. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 9803–9807.
9 Kanaya, S., Kohara, A., Miura, Y., Sekiguchi, A., Iwai, S., Inoue, H., Ohtsuka, E. and Ikehara, M. (1990) *J. Biol. Chem.*, **265**, 4615–4621.
10 Yang, W., Hendrickson, W.A., Crouch, R.J. and Satow, Y. (1990) *Science*, **249**, 1398–1405.
11 Katayanagi, K., Miyagawa, M., Matsushima, M., Ishikawa, M., Kanaya, S., Ikehara, M., Matsuzaki, T. and Morikawa, K. (1990) *Nature*, **347**, 306–309.
12 Itoh, T. and Tomizawa, J. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 2450–2454.
13 Dasgupta, S., Masukata, H. and Tomizawa, J. (1987) *Cell*, **51**, 1113–1122.
14 Ogawa, T., Pickett, G.G., Kogoma, T. and Kornberg, A. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1040–1044.
15 Itaya, M. and Crouch, R.J. (1991) *Mol. Gen. Genet.*, **227**, 433–437.
16 Kanaya, S. and Crouch, R.J. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3437–3451.

17 Itaya, M., McKelvin, D., Chatterjie, S.K. and Crouch, R.J. (1991) *Mol. Gen. Genet.*, **227**, 438–445.
18 Campbell, A.G. and Ray, D.S. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 9350–9354.
19 LeBowitz, J.H., Coburn, C.M., McMahon-Pratt, D. and Beverley, S.M. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9736–9740.
20 Cruz, A., Coburn, C.M. and Beverley, S.M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 7170–7174.
21 Coburn, C.M., Otteman, K.M., McNeely, T., Turco, S.J. and Beverley, S.M. (1991) *Mol. Biochem. Parasitol.*, **46**, 169–179.
22 Pasion, S.G., Brown, G.W., Brown, L.M. and Ray, D.S. (1994) *J. Cell Sci.*, **107**, 3515–3520.
23 Frank, P., Cazenave, C., Albert, S. and Toulme, J.J. (1993) *Biochem. Biophys. Res. Commun.*, **196**, 1552–1557.
24 Brown, G.W., Hines, J.C., Fisher, P. and Ray, D.S. (1994) *Mol. Biochem. Parasitol.*, **63**, 135–142.
25 Pasion, S.G., Hines, J.C., Aebersold, R. and Ray, D.S. (1992) *Mol. Biochem. Parasitol.*, **50**, 57–67.
26 Brown, G.W. and Ray, D.S. (1992) *Nucleic Acids Res.*, **20**, 3905–3910.
27 Brown, L., Hines, J.C. and Ray, D.S. (1992) *Nucleic Acids Res.*, **20**, 5451–5456.
28 Brown, G.W., Melendy, T.E. and Ray, D.S. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 10227–10231.
29 Vonwirth, H., Köck, J. and Büsen, W. (1991) *Experientia*, **47**, 92–95.
30 Birkenmeyer, L., Sugisaki, H. and Ray, D.S. (1987) *J. Biol. Chem.*, **262**, 2384–2392.
31 Ntambi, J.M., Shapiro, T.A., Ryan, K.A. and Englund, P.T. (1986) *J. Biol. Chem.*, **261**, 11890–11895.