L-ATP is recognized by some cellular and viral enzymes: does chance drive enzymic enantioselectivity?

Annalisa VERRI*, Alessandra MONTECUCCO*, Gilles GOSSELIN†, Valérie BOUDOU†, Jean-Louis IMBACH†, Silvio SPADARI* and Federico FOCHER*¹

*Istituto di Genetica Biochimica ed Evoluzionistica, CNR, Via Abbiategrosso 207, I-27100 Pavia, Italy, and †Laboratoire de Chimie Bioorganique UMR CNRS-USTL 5625, Université Montpellier II, 34095 Montpellier cedex 5, France

We demonstrate that L-ATP is recognized by some enzymes that are involved in the synthesis of nucleotides and nucleic acids. L-ATP, as well as its natural D-enantiomer, acts as a phosphate donor in the reaction catalysed by human deoxycytidine kinase, whereas it is not recognized by either enantioselective human thymidine kinase or non-enantioselective herpes virus thymidine kinase. L-ATP strongly inhibits (K_i 80 μ M) the synthesis of RNA primers catalysed by DNA primase associated with human DNA polymerase α , whereas RNA synthesis catalysed by *Escherichia coli* RNA polymerase is completely unaffected. Moreover, L-ATP competitively inhibits ATP-dependent T4 DNA ligase (K_i 25 μ M), suggesting that it interacts with the ATP-binding site of

the enzyme. Kinetic studies demonstrated that L-ATP cannot be used as a cofactor in the ligase-catalysed joining reaction. On the other hand, L-AMP is used by T4 DNA ligase to catalyse the reverse reaction, even though a high level of intermediate circular nicked DNA molecules accumulates. Our results suggest that a lack of enantioselectivity of enzymes is more common than was believed a few years ago, and, given the absence of selective constraints against L-nucleosides in Nature, this may depend on chance more than on evolutionary strategy.

Key words: deoxycytidine kinase, DNA ligase, DNA primase, enantiomers.

INTRODUCTION

Enzymes are thought to be able to bind effectively to only one enantiomer of a chiral substrate. However, some exceptions to this rule have recently been identified among enzymes involved in the synthesis and polymerization of deoxyribonucleotides [1]. In fact, we have demonstrated that herpes virus [herpes simplex virus (HSV)-1, HSV-2 and pseudorabies virus (PRV)] thymidine kinases (TKs), human mitochondrial TK, cellular deoxycytidine kinase (dCK), human deoxynucleoside mono- and diphosphate kinases, and most cellular and viral DNA polymerases (such as DNA polymerase α , terminal deoxynucleotidyl transferase and, notably, HIV-1 reverse transcriptase) are able to recognize and metabolize L-nucleosides or L-nucleotides [2–9]. In particular, HSV TK and human dCK phosphorylate L-enantiomers of their natural substrates, thymidine and deoxycytidine respectively, with the same efficiency as the D-enantiomers [2,7].

The lack of enantioselectivity of HSV TK and human dCK represents the molecular basis for the development of L-thymidine and L-deoxycytidine analogues as anti-viral and/or anti-cancer drugs [1–3,7,8,10–22]. Indeed, although only one L-nucleoside analogue (3TC) has so far gained United States Food and Drug Administration approval for clinical use against HIV-1 [23,24], other L-enantiomers of nucleoside analogues that have shown anti-viral or anti-cancer activity in cell cultures are currently in clinical trials [1]. Their resistance to enantioselective enzymes such as thymidine phosphorylase [3], thymidylate synthase [3], and (deoxy)cytidine and dCMP deaminases [7], and their lower affinity for mitochondrial TK [6], might ensure higher selectivity and lower cytotoxicity compared with the corresponding natural D-enantiomers, and these properties could be exploited to solve some problems that arise during chemotherapy, such as metabolic inactivation, cytotoxicity and drug resistance.

From an evolutionary point of view, it is reasonable to find enantioselective enzymes especially when, in their environment, such enzymes encounter racemic substrates and must metabolize only one of them, i.e. the one with biological activity. However, when enzymes need not ‘choose’ between enantiomers, for example when metabolism makes available only the correct one, and therefore no selective pressure is present, we might expect that enantioselectivity as a necessary property may not occur in such cases.

In the present work we have studied the enantioselectivity for ATP of some enzymes involved in DNA synthesis, such as human dCK, human DNA primase, *Escherichia coli* RNA polymerase and T4 DNA ligase, and we demonstrate that the lack of enzymic enantioselectivity is more common than was believed a few years ago. Thus we suggest that the observed enantioselectivity of some enzymes, since it has not been subjected to natural selection, may be only a side property of the enzymes, depending more on chance than on evolutionary strategies.

MATERIALS AND METHODS

Chemicals

Commercially available reagents and solvents (analytical grade) were used unless otherwise stated. L-ATP and L-AMP were synthesized from enantiomerically pure L-adenosine (G. Gosselin, unpublished work; details available on application to the author) using standard phosphorylation methods. L-ATP and L-AMP were fully characterized by NMR (¹H, ³¹P), fast-atom-bombardment MS and UV spectroscopy; their purities were ascertained by HPLC. [³H]2'-Deoxycytidine (25 Ci/mmol), [³H]dUTP (25 Ci/mmol), [³²P]ATP (3000 Ci/mmol) and [³H]

Abbreviations used: HSV, herpes simplex virus; PRV, pseudorabies virus; TK, thymidine kinase; dCK, deoxycytidine kinase.

¹ To whom correspondence should be addressed (e-mail focher@igbe.pv.cnr.it).

dATP (50 Ci/mmol) were from Amersham. *E. coli* RNA polymerase, T4 DNA ligase and *E. coli* DNA polymerase (Klenow fragment) were from Boehringer.

Herpes virus TK assays

HSV-1, HSV-2 and PRV TKs were purified and assayed as described previously [5,25,26].

dCK assay

dCK was purified and assayed as described previously [7], using either D-ATP or L-ATP (1 mM) as phosphate donor.

E. coli RNA polymerase assay

E. coli RNA polymerase was assayed using poly[d(AT)] following the manufacturer's instructions (Boehringer).

DNA primase assay

Human DNA primase, associated with DNA polymerase α , was purified from HeLa cells as described previously for calf thymus DNA polymerase α [27] and assayed in 25 μ l of a mixture containing 50 mM Tris/HCl (pH 7.5), 1 mM dithiothreitol, 250 μ g/ml BSA, 10 mM MgCl₂, 0.5 μ g of poly(dT), 10 mM KCl and 50 μ M D-ATP. After 15 min of incubation at 37 °C, 40 μ M [³H]dATP (400 c.p.m./pmol) and 0.5 unit of *E. coli* DNA polymerase I (Klenow fragment) were added as the signal amplifier. The reaction was continued for 30 min at the same temperature. At the end, 20 μ l was spotted on to GF/C filters (Whatman), which were then washed three times in 5% (w/v) cold trichloroacetic acid and twice in ethanol. Filters were dried and the acid-precipitable radioactivity was measured in a β -radiation counter.

T4 DNA ligase assays

Adenylation reaction

T4 DNA ligase was incubated at 37 °C for 30 min in the presence of [³²P]ATP (400 Ci/mmol) in a reaction mixture containing 50 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 100 μ g/ml BSA, 1 mM EDTA and 5 mM dithiothreitol. Under these conditions, [³²P]ATP binds covalently to DNA ligase and becomes acid-precipitable.

Joining activity

This was measured using a method employing poly[d(AT)], as described in [28].

DNA relaxation

DNA relaxation of supercoiled pUC19 plasmid was performed as described previously [29].

Electromobility shift assay

The substrate for DNA binding experiments was a double-stranded oligonucleotide containing a single nick. The sequence and the reaction conditions were as described by Rossi et al. [30]. The extent of band shift was visualized by autoradiography of dried gels.

RESULTS

L-ATP acts as phosphate donor for human dCK, but not for HSV TKs

We have recently demonstrated that human dCK and herpes virus TKs are non-enantioselective enzymes, since they phosphorylate β -L-2'-deoxycytidine and β -L-2'-deoxythymidine, the enantiomers of their corresponding natural substrates [2,5,7] (for a review, see [1]). From an evolutionary point of view, this lack of enantioselectivity further supports the assumed common evolutionary origin of human dCK and herpes virus TKs [31].

Here we demonstrate that human dCK lacks enantioselectivity not only for the nucleoside substrate, but also for the phosphate donor. In fact, we found that both D-ATP and L-ATP, whose structures are shown in Figure 1, are used with the same efficiency

Figure 1 Stereo-pairs of D-ATP (A) and L-ATP (B)

'O' indicates the oxygen of the pentofuranosyl ring of 2'-deoxyribose.

Figure 2 Effect of L-ATP on human dCK activity

(A) Kinetics of human dCK activity in the presence of 1 mM D-ATP (■) and 1 mM L-ATP (○), and in the absence of ATP (●). (B) Kinetics of HSV-1 TK (□, ■), HSV-2 TK (○, ●) and PRV TK (△, ▲) activities in the presence of 1 mM D-ATP (open symbols) and 1 mM L-ATP (closed symbols).

by human dCK to phosphorylate 2'-deoxycytidine (Figure 2A). This is consistent with the recent observation of Tomikawa et al. [20], who found that L-ATP acts as phosphate donor for mouse dCK with an efficiency of 15–30% compared with the natural substrate D-ATP. Surprisingly, the herpes virus TKs (HSV-1, HSV-2 and PRV TKs), the gene sequences of which suggest a common origin with human dCK [31], are unable to utilize L-ATP as a phosphate donor (Figure 2B), despite their lack of enantioselectivity for the nucleoside substrate. On the other hand, human cytoplasmic TK is strictly enantioselective for both thymidine [2] and ATP (results not shown).

L-ATP inhibits human DNA primase

DNA primase is responsible for the synthesis of RNA primers on both leading- and lagging-strand DNA during semi-conservative DNA replication [32]. On the lagging strand, RNA primers are elongated by DNA polymerases to synthesize the Okazaki fragments, and are removed by RNase H before the ligation steps. When human DNA primase activity was assayed in the presence of 50 μ M D-ATP and different concentrations of L-ATP, we found that L-ATP strongly inhibited RNA primer synthesis, with an IC_{50} value of 25 ± 2 μ M (mean $\pm$ S.D.) (Figure 3A). Since the apparent K_m value of DNA primase for D-ATP is approx. 150 μ M (Figure 3B), the reported IC_{50} suggests that the

Figure 3 Effect of L-ATP on human DNA primase activity

(A) Effect of increasing concentrations of L-ATP on human DNA primase activity. (B) Lineweaver-Burk plot of the effect of L-ATP on the activity of human DNA primase in the presence of increasing concentrations of the natural substrate D-ATP. The enzyme was assayed as described in the Materials and methods section, in the presence of the following concentrations of L-ATP: □, 0 μ M; ◇, 100 μ M; ○, 200 μ M; △, 400 μ M. Each point is the mean of triplicate determinations.

enzyme efficiently recognizes the L-enantiomer of ATP. To determine the mechanism of inhibition by L-ATP of DNA primase, increasing inhibitor concentrations were tested at different substrate (D-ATP) concentrations. The Lineweaver-Burk plot reporting the results of this experiment (Figure 3B) demonstrates that L-ATP inhibits mammalian DNA primase by a competitive mechanism (K_i 80 ± 5 μ M). This enzymic behaviour indicates that the active site of the enzyme is non-enantioselective, being able to recognize both enantiomers of ATP with comparable efficiency. In contrast, *E. coli* RNA polymerase, which, like DNA primase, synthesizes RNA chains utilizing ATP as a substrate, is strictly enantioselective and does not recognize L-ATP at all (results not shown).

Effects of L-ATP and L-AMP on T4 DNA ligase reactions

The DNA ligase encoded by bacteriophage T4 is the most representative of the ATP-dependent DNA ligases [32]. The ligation reaction involves three successive nucleotide transfer reactions: (i) activation of the enzyme through the formation of a covalent protein-AMP intermediate, accompanied by the release of pyrophosphate, (ii) transfer of the nucleotide to a

Figure 4 Effect of L-ATP on the adenylation reaction of T4 DNA ligase

(A) Kinetics of T4 DNA ligase activity in the presence of increasing amounts of L-ATP. (B) T4 DNA ligase joining activity in the presence of different concentrations of D-ATP (●) and L-ATP (○). (C) Lineweaver-Burk plot of the effect of L-ATP on the adenylation activity of T4 DNA ligase in the presence of increasing concentrations of the natural substrate D-ATP. The enzyme was assayed as described in the Materials and methods section in the presence of the following concentrations of L-ATP: □, 0 μM; ◇, 12 μM; ○, 25 μM. Each point is the mean of triplicate determinations.

phosphorylated 5'-end of the nick to produce an inverted (5')-(5') pyrophosphate bridge structure; and (iii) catalysis of the transesterification reaction, resulting in joining of the nick and release of free AMP.

The first intermediate of the ligation reaction, namely the enzyme-AMP adduct, can be easily isolated by omitting the DNA substrate from the reaction mixture. When we analysed the ability of L-ATP to affect enzyme adenylation in the absence of DNA, we found that L-ATP inhibited the adenylation reaction of T4 DNA ligase with an estimated IC_{50} value of 4.2 ± 0.1 μM, indicating that L-ATP can interact with the enzyme (Figure 4A). The exponential curve suggests that L-ATP is not only an inhibitor of the adenylation step, but also a substrate; in fact it decreases both the utilization of labelled D-ATP and the

Figure 5 Effect of L-ATP on the DNA-binding properties of T4 DNA ligase

A 34 bp oligonucleotide was used in an electromobility-shift assay. Samples of 1 μg of T4 DNA ligase were tested in the presence of different cofactors (as indicated above the lanes) at a concentration of 1 mM. The position of the retarded band is indicated by an arrow, while the free oligomer is indicated by an arrowhead. Lig, DNA ligase.

Figure 6 Effect of L-AMP on the DNA relaxation activity of T4 DNA ligase

Plasmid pUC19 (0.2 μg) was used in DNA relaxation assays. (A) Shown is a 1% agarose gel, run in 1 × TAE electrophoresis buffer (40 mM Tris-acetate, pH 8, 1 mM EDTA). Under these experimental conditions, relaxed DNA substrate was resolved as several topoisomers. Lane 1, supercoiled pUC19; lane 2: supercoiled pUC19 in the presence of 1 mM D-AMP; lane 3: supercoiled pUC19 in the presence of 1 mM L-AMP. (B) The same reaction products were run in 1 × TAE electrophoresis buffer containing ethidium bromide. This intercalating agent allows better discrimination between nicked and relaxed DNA molecules; the relaxed DNA appears as the fastest-moving band. Abbreviations: n, nicked; sc, supercoiled; r, relaxed.

number of free enzyme molecules that can react with the natural substrate.

Although it is able to adenylate the enzyme, L-ATP is not effective as a cofactor in the joining reaction of poly[d(AT)] (Figure 4B). However, it competitively inhibits the D-ATP-dependent joining reaction, with an estimated K_i value of 24 ± 1 μM (Figure 4C).

We have demonstrated previously that T4 DNA ligase can form two different kinds of complex with DNA, depending on its adenylation state: a stable complex (S-complex) in the absence of ATP and a transient complex (T-complex) in the presence of ATP [30]. This behaviour seems to be due to a conformational change in the enzyme that occurs as a consequence of ATP binding [33]. Other cofactors, such as dATP or S-ATP, although

ineffective in the joining reaction [34], can affect the stability of the DNA binding of T4 DNA ligase [30]. The DNA–enzyme complex is sufficiently stable to allow DNA retardation in a electromobility-shift assay only in the absence of ATP. Therefore we tested L-ATP in comparison with D-ATP in this assay. As shown in Figure 5, no band retardation occurred in the presence of L-ATP, indicating that L-ATP was able to modulate the binding of DNA ligase to DNA in the same manner as the natural cofactor, D-ATP. This result further supports the suggestion that L-ATP can perform the adenylation step of the enzyme reaction.

An additional interesting activity of T4 DNA ligase concerns the so-called 'reverse reaction', namely the AMP-dependent relaxation of supercoiled DNA [35]. DNA relaxation occurs through a nicking/closing mechanism and results in a progressive decrease in superhelical turns, according to a rather processive mode of action [35]. We analysed the ability of T4 DNA ligase to use L-AMP in the relaxation reaction. As shown in Figure 6, in the presence of L-AMP a significant amount of nicked circular DNA accumulated as a reaction product, indicating that the nicking/closing reaction is largely impaired. In particular, the ability to reseal the nick seems most affected.

From these experiments, it appears that T4 DNA ligase can recognize and bind L-ATP, but that the catalytic activity of the enzyme is inhibited.

DISCUSSION

The enantioselectivity of enzymes, namely their property of recognizing and metabolizing only one of the two enantiomers of chiral molecules, is related to the chiral structure of the enzymes, reflecting the three-dimensional folding of the polypeptide backbone and the orientation of the amino-acid side chains in the folded molecule. Because of the chirality of amino acids (L), the chemistry of life should be highly sensitive to different enantiomers of chiral substrates. However, since the emergence of enzymic behaviour depends on natural selection that favours binding of the enantiomer with the better fit, we propose that enantioselectivity could have been very important at the origin of life, when different choices were possible. But now, in a world consisting only of D-nucleosides and L-amino acids, enantioselectivity may be purely a side property and not a vital property. For instance, in the case of HSV-1 TK [2] and human dCK [7,20], enzymes that phosphorylate D-nucleosides but require no enantioselectivity (since the environment does not provide L-nucleosides), the enzymes need not be enantioselective at all. It is noteworthy that, although they are genetically related enzymes, HSV TKs and human dCK [31] show different behaviour: HSV TKs are non-enantioselective only with respect to the substrate, whereas human dCK is non-enantioselective for both the substrate and the phosphate donor. In the present work we have also demonstrated that other enzymes, such as human DNA primase, *E. coli* RNA polymerase and T4 DNA ligase, which use the same substrates (DNA and ribonucleoside triphosphate), show completely different stereoselective behaviours with respect to ATP recognition.

From a structural point of view, these findings could be interpreted by an extension of Koshland's 'induced fit' theory of enzyme activity (which introduced the concept of enzyme flexibility, describing the mutual fitting of the interacting partners using the 'hand-in-glove' metaphor [36]). In fact, in our case the interactions of D- and L-enantiomers with a particular non-enantioselective enzyme, rather than fitting the 'hand-in-glove' metaphor, could be better represented by a 'hand-in-mitten' metaphor [37], in which the thumb would be the

amino-acid pocket interacting with the adenine moiety of ATP, while the rest of the mitten would fit the sugar triphosphate moiety equally well as a right or a left hand.

Our findings suggest that enantioselectivity, probably required at the origin of life when both enantiomers of the substrate could have been present in the primordial 'soup', is now more dependent on chance than on pressing evolutionary strategies. Certain enzymes either may have evolved after enantiomeric substrates predominated, in which case enantioselectivity was unnecessary, or may have lost enantioselectivity through otherwise conservative mutations that permit less strict substrate binding. Therefore enantioselectivity of enzymes metabolizing chiral substrates could be considered as an incidental property or a vestigial property which is maintained as far as its linkage with other essential enzymic properties is maintained. Of course, enantioselectivity is strictly required for enzymes that specifically create chiral centres, and synthesize only one of the two possible chiral products.

In conclusion, our present study shows that the enantioselectivity of enzymes is less common than was believed a few years ago. In the field of nucleotide and nucleic acid biosyntheses, for instance, several non-enantioselective enzymes and enzymes with relaxed enantioselectivity have been identified; not only nucleoside kinases, DNA polymerases, terminal transferase and viral reverse transcriptases [1], but also DNA ligases and DNA primases are able to recognize in their active site the L-enantiomer of the natural substrate (D-ATP). On the basis of these findings, non-enantioselective enzymes could in some cases be potential targets of L-nucleoside analogues for anti-cancer and anti-viral chemotherapy [1], since these drugs could find the target enzymes highly indifferent to chiral choices.

We thank G. E. Wright for a critical reading of the manuscript. This work was supported by grants from CNR PF Biotecnologie (EC TMR-ERBMRXCT 970125), Associazione Italiana per la Ricerca sul Cancro (AIRC) and Agence Nationale de Recherche sur le SIDA (ANRS; France). A.V. was supported by a fellowship provided by Fondazione A. Buzzati-Traverso.

REFERENCES

- Spadari, S., Maga, G., Verri, A. and Focher, F. (1998) *Expert Opin. Invest. Drugs* **7**, 1285–1300.
- Spadari, S., Maga, G., Focher, F., Ciarrocchi, G., Manservigi, R., Arcamone, F., Capobianco, M., Carcuro, A., Colonna, F., Iotti, S. and Garbesi, A. (1992) *J. Med. Chem.* **35**, 4214–4220.
- Spadari, S., Ciarrocchi, G., Focher, F., Verri, A., Maga, G., Arcamone, F., Iafate, E., Manzini, S., Garbesi, A. and Tondelli, L. (1995) *Mol. Pharmacol.* **47**, 1231–1238.
- Spadari, S., Maga, G., Verri, A., Bendiscioli, A., Tondelli, L., Capobianco, M., Colonna, F., Garbesi, A. and Focher, F. (1995) *Biochimie* **77**, 861–867.
- Maga, G., Verri, A., Ponti, W., Bonizzi, L., Poli, G., Garbesi, A., Niccolai, D., Spadari, S. and Focher, F. (1993) *Biochem. J.* **294**, 381–385.
- Verri, A., Priori, G., Spadari, S., Tondelli, L. and Focher, F. (1997) *Biochem. J.* **328**, 317–320.
- Verri, A., Focher, F., Priori, G., Gosselin, G., Imbach, J.-L., Capobianco, M., Garbesi, A. and Spadari, S. (1997) *Mol. Pharmacol.* **51**, 132–138.
- Focher, F., Maga, G., Bendiscioli, A., Capobianco, M., Colonna, F., Garbesi, A. and Spadari, S. (1995) *Nucleic Acids Res.* **23**, 2840–2847.
- Maga, G., Amacker, M., Hübscher, U., Gosselin, G., Mathé, C., Faraj, A., Sommadossi, J.-P. and Spadari, S. (1998) *Nucleosides Nucleotides*, in the press.
- Iotti, S., Colonna, F. P., Garbesi, A., Spadari, S., Focher, F., Ciarrocchi, G. and Arcamone, F. (1990) *I Brevetti CNR, Italy 18-A11, patent 22032A/90*.
- Balzarini, J., De Clercq, E., Baumgartner, H., Bodenteich, M. and Griengl, H. (1990) *Mol. Pharmacol.* **37**, 395–401.
- Chang, C.-N., Skalski, V., Zhou, J. H. and Cheng, Y. (1992) *J. Biol. Chem.* **267**, 22414–22420.
- Coates, J. A. V., Cammack, N., Jenkinson, H., Mutton, I., Pearson, B., Storer, R., Cameron, J. and Penn, C. (1992) *Antimicrob. Agents Chemother.* **36**, 202–205.
- Furman, P. A., Davis, M., Liotta, D. C., Paff, M., Frick, L. W., Nelson, D. J., Dornsife, R. E., Wurster, J. A., Wilson, L. J., Fyfe, J. A. et al. (1992) *Antimicrob. Agents Chemother.* **36**, 2686–2692.

- 15 Shewach, D. S., Liotta, D. C. and Schinazi, R. F. (1993) *Biochem. Pharmacol.* **45**, 1540–1543
- 16 Lin, T. S., Luo, M. Z., Liu, M. C., Balakrishna Pai, S., Dutschman, G. E. and Cheng, Y.-C. (1994) *J. Med. Chem.* **37**, 798–803
- 17 Lin, T.-S., Luo, M.-Z., Liu, M.-C., Pai, S. B., Dutschman, G. E. and Cheng, Y.-C. (1994) *Biochem. Pharmacol.* **47**, 171–174
- 18 Grove, K. L., Guo, X., Liu, S. H., Gao, Z., Chu, C. K. and Cheng, Y.-C. (1995) *Cancer Res.* **55**, 3008–3011
- 19 Kukhanova, M., Liu, S.-H., Mozzerin, D., Lin, T.-S., Chu, C. K. and Cheng, Y.-C. (1995) *J. Biol. Chem.* **270**, 23055–23059
- 20 Tomikawa, A., Kohgo, S., Ikezawa, H., Iwanami, N., Shudo, K., Kawaguchi, T., Saneyoshi, M. and Yamaguchi, T. (1997) *Biochem. Biophys. Res. Commun.* **239**, 329–333
- 21 Pelicano, H., Pierra, C., Eriksson, S., Gosselin, G., Imbach, J.-L. and Maury, G. (1997) *J. Med. Chem.* **40**, 3969–3973
- 22 Kotra, L. P., Xiang, Y., Newton, M. G., Schinazi, R. F., Cheng, Y. C. and Chu, C. K. (1997) *J. Med. Chem.* **40**, 3635–3644
- 23 Eron, J. J., Benoit, S. L., Jemsek, J., MacArthur, R. D., Santana, J., Quinn, J. B., Kuritzkes, D. R., Fallon, M. A. and Rubin, M. (1995) *N. Engl. J. Med.* **333**, 1662–1669
- 24 Pluda, J. M., Cooley, T. P., Montaner, J. S., Shay, L. E., Reinhalter, N. E., Warthan, S. N., Ruedy, J., Hirst, H. M., Vicary, C. A. and Quinn, J. B. (1995) *J. Infect. Dis.* **171**, 1438–1447
- 25 Focher, F., Hildebrand, C., Freese, S., Ciarrocchi, G., Noonan, T., Sangalli, S., Brown, N. C., Spadari, S. and Wright, G. (1988) *J. Med. Chem.* **31**, 1496–1500
- 26 Focher, F., Sandoli, D., Hildebrand, C., Sangalli, S., Ciarrocchi, G., Rebuzzini, A., Pedrali-Noy, G., Manservigi, R., Wright, G., Brown, N. and Spadari, S. (1989) *Methods Find. Exp. Clin. Pharmacol.* **11**, 577–582
- 27 Focher, F., Spadari, S., Ginelli, B., Hottiger, M., Gassmann, M. and Hübscher, U. (1988) *Nucleic Acids Res.* **16**, 6279–6295
- 28 Modrich, P. and Lehman, I. (1970) *J. Biol. Chem.* **245**, 3626–3631
- 29 Ciarrocchi, G., Lestingi, M., Wright, G. and Montecucco, A. (1993) *Nucleic Acids Res.* **21**, 5934–5939
- 30 Rossi, R., Montecucco, A., Ciarrocchi, G. and Biamonti, G. (1997) *Nucleic Acids Res.* **25**, 2106–2113
- 31 Harrison, P. T., Thompson, R. and Davison, A. J. (1991) *J. Gen. Virol.* **72**, 2583–2586
- 32 Kornberg, A. and Baker, T. A. (1991) *DNA Replication*, Freeman and Co., New York
- 33 Hakansson, K., Doherty, A. J., Shuman, S. and Wigley, D. B. (1997) *Cell* **89**, 545–553
- 34 Montecucco, A., Lestingi, M., Pedrali-Noy, G., Spadari, S. and Ciarrocchi, G. (1990) *Biochem. J.* **271**, 265–268
- 35 Montecucco, A. and Ciarrocchi, G. (1988) *Nucleic Acids Res.* **16**, 7369–7381
- 36 Koshland, D. E. J. (1994) *Angew. Chem. Int. Ed. Engl.* **33**, 2375–2378
- 37 Garbesi, A., Hamy, F., Maffini, M., Albrecht, G. and Klimkait, T. (1998) *Nucleic Acids Res.* **26**, 2886–2890

Received 13 July 1998/1 October 1998; accepted 6 November 1998