Table 1. Values (nmol·l^{-1}·s^{-1}) of the initial rate of reaction in Scheme 1 obtained by simulation of the progress curve of P and from eqn. (1) using K_m^s calculated from the equation indicated at the top of the column

In each case, we only vary the values of the rate constants k_{+1} (M^{-1}·s^{-1}), k_{-1} (s^{-1}) and k_{+2} (s^{-1}). In the last two columns, the values of $k_{+1}k'_{+X}k'_{-1}k_{-X}$ (in column A) and $k_{+X}k'_{+1}k'_{-X}k_{-1}$ (in column B) expressed in M^{-2}·s^{-4} are listed. Fixed values used: $[E]_T = 10^{-7}$ M, $[S] = 10^{-3}$ M, $[X] = 10^{-4}$ M, $k'_{+1} = 10^4$ M^{-1}·s^{-1}, $k_{+X} = 10^8$ M^{-1}·s^{-1}, $k_{-X} = 5 \times 10^3$ s^{-1}, $k'_{+X} = 2 \times 10^8$ M^{-1}·s^{-1}, $k'_{-X} = 10^4$ s^{-1}, $k'_{-1} = 0.10$ s^{-1}, and $k'_{+2} = 0.20$ s^{-1}.

Value			Initial rate					
k_{+1}	k_{-1}	k_{+2}	Simulation	(1)	Topham & Brocklehurst	Segel & Martin	A	B
200	1.00	10.0	220	220	21.1	6.19	2×10^{13}	1×10^{16}
300	0.20	1.00	42.9	42.9	15.4	9.33	3×10^{13}	2×10^{15}

is inserted into eqn. (7), the latter is transformed to the eqn. of Topham & Brocklehurst. However, the above condition is not necessary in Scheme 1, because it only is fulfilled when all of the four reversible steps in the general modifier mechanism are at quasi-equilibrium. Therefore, the initial rate of the reaction in Scheme 1 is described by eqn. (1) if K_m^s is given by eqn. (7), but not if the eqn. of Topham & Brocklehurst for K_m^s is used.

However, eqn. (1), with K_m^s given by the equation of Segel & Martin, has been obtained by Segel & Martin (1988) [their eqn. (38) which has been expressed in this form by Topham & Brocklehurst (1992)] by combining the steady state and equilibrium equations as follows: $d[E]/dt = 0$, which implies that:

$$(k_{-1}+k_{+2})[ES]+k_{-X}[EX] = k_{+1}[S][E]+k_{+X}[X][E] \quad (8)$$

From eqns. (8) and (5) they obtain:

$$(k_{-1}+k_{+2})[ES] = k_{+1}[S][E] \quad (9)$$

Eqns. (5) and (9) yield [ES] and [EX] in terms of [E]. Similarly, from $d[ES]/dt = 0$ and eqn. (6) they obtain [EXS] in terms of [E]. In this way, they have expressed the concentrations of all of the enzyme species in terms of [E]. From the above expressions, they obtain eqn. (1) with K_m^s given by the equation of Segel & Martin. The latter is, according to Topham & Brocklehurst (1992), incorrect. However, the cause of the error is not the one indicated by these authors but that Segel & Martin (1988) confuse the quasi-equilibrium assumption with that of a strict equilibrium, since eqns. (5) and (6) only are approximate and hence the symbol $\approx$, instead of $=$, is more correct. Since the terms $(k_{-1}+k_{+1})[ES]$ and $k_{+1}[S]$ are much smaller than both $k_{+X}[X]$ and $k_{-X}[EX]$, the insertion of eqn. (5) into eqn. (8) does not yield eqn. (9), even if in the latter the symbol $\approx$ is used instead of $=$. The following simple numerical example illustrates this fact: Let $A+a = B+b$ where $A = 99.9$, $B = 99.5$ (i.e. $A \approx B$ because $A/B \approx 1$), $a = 0.1$ and $b = 0.5$. Clearly, neither $a = b$ nor $a \approx b$ can be inferred from $A \approx B$.

We support the validity of our conclusions by showing some numerical examples in Table 1. The values for the initial rate equation of Scheme 1 were obtained from simulated progress curves of P for two sets of rate constants and initial concentrations which satisfy fitting condition (4). These values are compared with those obtained by eqn. (1) in which K_m^s is given by eqn. (7) and those of Topham & Brocklehurst and of Segel & Martin. The agreement between the results obtained by numerical integration and those obtained using eqn. (1) with K_m^s given by eqn. (7) is complete, whereas no agreement exists if K_m^s is evaluated either from the eqn. of Topham & Brocklehurst or from that of Segel & Martin. The simulations were carried out with the predictor-corrector algorithms of Adams–Moulton, starting with the fourth order Runge–Kutta method (Gerald, 1989). The algorithm for the numerical integration and our own computer program were implemented in Turbo PASCAL 6.0. The above program was run on a Tandon 386 SX/20 computer with an Intel 80387 arithmetic coprocessor.

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Inhibition of primer extension by single mismatches during asymmetric re-amplification

Hybridization of short oligonucleotides to DNA is a specific method for the detection of small mutations (for example point mutations). This technique is based on the decrease in melting temperature (T_m) caused by mismatches within a hybrid [1]. Manipulating the conditions of hybridization allows these changes in T_m to be detectable by determining the extent of oligonucleotide annealing [2].

Conventional symmetric PCR does not allow easy discrimination between the DNA amplified from mismatched and normal primers. Although the efficiency of mismatched primer hybridization during the early cycles of PCR is low, there is inevitably some annealing followed by logarithmic amplification to produce DNA which includes the mismatched primer(s) and complementary sequences. The size and amount of this amplified DNA may thus be indistinguishable from the DNA which is produced by perfectly matched primers.

A different PCR approach, using 3′ mismatched primers, has been used for the detection of small mutations [3–5]. This method relies on a conformational change occurring at the site of extension by *Taq* polymerase and this enzyme is inhibited as a result. It has however been found that because of the efficiency of logarithmic DNA amplification during PCR, a single 3′

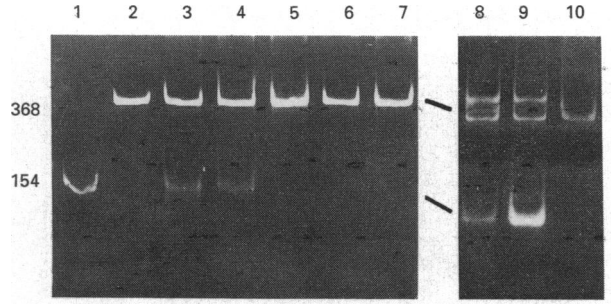

Fig. 1. Electrophoretic separation (ethidium-bromide-stained 6% polyacrylamide non-denaturing gel) of amplified and re-amplified hepatitis B virus (HBV) X-gene DNA

DNA depicted in lanes 1 and 2 represents the control size markers with their base lengths indicated on the left of the Figure. The band in lane 2 was prepared by standard symmetric amplification of an SP64 X-gene plasmid using primers 1 and 2 (HBV nucleotide sequences 1420–1443 and 1788–1765 respectively). Lane 1 represents DNA which was amplified using primer 2 and primer 3 (sense sequence 1634–1648, 5′ AGATCCTGCCCAAGG 3′). An aliquot (2%) from a PCR amplification reaction of DNA prepared as for lane 2 was re-amplified asymmetrically for 15 cycles using only primer 3 (normal sequence) and annealing temperatures of 50 °C (lane 3) and 60 °C (lane 4). Similar re-amplification with primer 3 sequences mismatched at the eighth base (C, A or T instead of G) is resolved in lanes 5–7. DNA which was produced after symmetric re-amplification using a mismatched primer (similar to that used in lane 5) and normal primer 2 at annealing temperatures of 50 °C and 60 °C is depicted in lanes 8 and 9 respectively. The DNA in lane 10 depicts separation of DNA re-amplified with the primer 3 sequence mismatched at the 3′ end (C instead of G). Two similar gels run independently (lanes 1–7 and 8–10) are depicted in the Figure.

mismatch is not always sufficient to decrease DNA synthesis significantly. More than one mismatch at the 3′ end of a primer is often required to detect a mutation [6].

Asymmetric PCR provides an alternative approach to the detection of small mutations. Here we have shown that linear re-amplification using only one primer improves the distinction between amplification from mismatched and normal primers. Using this procedure, incorporation of mismatched oligonucleotides and their complementary sequences into the template DNA does not occur. The data in Fig. 1 on the amplification of an X-gene fragment of the hepatitis B virus demonstrate this. In this set of experiments, a 368 bp X-gene fragment, amplified using a standard PCR protocol, was used as template source for reamplification from a set of five internal primers (see legend to Fig. 1 for details). After linear asymmetric amplification from a perfectly matched internal primer at a concentration of 0.25 μM (lanes 3 and 4), there is production of easily detectable extended product after 15 cycles—the 154 base fragment. This band was detectable when using an annealing temperature as high as 60 °C (lane 4). All three mismatches at the eighth base of the primer resulted in a failure to detect extension (lanes 5–7), as did a primer which had a 3′ mismatch (lane 10). In each of the experiments using mismatched primers the annealing temperature was 50 °C. Symmetric re-amplification with two primers (one mismatched) at equal concentrations does result in significant amplification of the smaller fragment (lanes 8 and 9 of Fig. 1). The extent of symmetric re-amplification was similar when using the fully complementary or any of the other mismatched primers. The additional larger DNA band seen in lanes 8 and 9 is an artefact which was often produced during symmetric logarithmic PCR using the conditions described here.

Decreased melting temperature or conformational changes at the site of extension by *Taq* polymerase are probably responsible for the observed differences between symmetric and asymmetric re-amplification. The inhibitory effect of single base mismatches on primer extension during asymmetric re-amplification is a useful property which can be exploited to assess oligonucleotide and template DNA sequence complementarity.

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Mitochondrial cyclophilins

The cyclophilins, a family of proteins with peptidylprolyl *cis–trans* isomerase activity, were first identified as abundant cytoplasmic proteins, with about 165 amino acid residues and very strong sequence conservation. Bovine and porcine cyclophilin-A are identical, while the human enzyme differs at only three residues [1–3]. There are seven conservative differences between human and rat cyclophilin-A, plus one additional amino acid at the *C*-terminal end of the human protein [4]. Proteins with strong homology are present in the cytoplasm of all eukaryotic organisms studied and in *Escherichia coli*.

More recently a second family of cyclophilins with an *N*-terminal signal sequence has been reported in species ranging from *E. coli* to man [5–7]. The *E. coli* cyclophilin-B is retained in the periplasm, while the mammalian proteins in this group have a *C*-terminal decapeptide which may direct them to an endoplasmic reticulum-associated compartment [8]. These cyclophilins-B have a lower level of similarity to the cytoplasmic cyclophilins-A. In man there are 59 amino acid differences, many not conservative, in addition to the *N*- and *C*-terminal extensions of cyclophilin-B.

A mitochondrial form of cyclophilin has been reported in *Neurospora crassa*, encoded by the same genes as the cytoplasmic enzyme and arising by alternative splicing [9]. There is much evidence for cyclosporin-sensitive peptidylprolyl isomerases in mammalian mitochondria (see [10]), but no evidence for additional exons available for alternative splicing in the human cyclophilin-A gene [11]. Now Connern & Halestrap [10] have reported the *N*-terminal amino acid sequence of a rat mitochondrial cyclosporin-sensitive peptidylprolyl isomerase which shows clear homology with the known rat and human cyclophilins but has seven amino acid differences from rat cyclophilin-A and 12 differences from rat cyclophilin-B in the first 16 amino acids. However, this partial rat mitochondrial sequence shows