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Misalignment-Mediated DNA Synthesis Errors

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Genetic information can be altered not only by substituting one base for another but also by adding or deleting one or more nucleotides. Just as for base-substitution mutations, frame-shift[1] mutations can be either beneficial or detrimental to an organism. Thus, understanding how these are generated or avoided is important. Included among possible mechanisms for producing frame-shift mutations is aberrant DNA polymerization. For over 2 decades, studies of the fidelity of DNA synthesis in vitro have focused heavily on base-substitution errors, primarily because several sensitive methodologies were developed for their detection [for review, see Loeb and Kunkel (1982) and Goodman (1988)]. Consequently, much of our understanding of how genetic material is replicated and maintained with high fidelity is flavored with the reasoning associated with base miscoding. Only within the past 5 years have assays been developed to study frame-shift fidelity during DNA synthesis in vitro. This review focuses on how information obtained from these frame-shift fidelity assays has influenced our understanding of mutagenesis resulting from template-primer misalignments during DNA synthesis.

Four Pathways for DNA Synthesis Errors. Starting with the simplest frame-shift mutation, a minus-one-nucleotide error, consider what can go wrong during a DNA polymerization reaction on the template-primer shown in Figure 1. The pathway that has received the most experimental attention over the past 2 decades begins with misincorporation of a nucleotide (pathway B, for *B*ase substitution). When this is followed by extension from the mispair (pathway BB) to fix the error, a base-substitution mutation can result. The subject of this review is the evidence for the other three pathways, all of which use a misaligned template-primer. For example, in the appropriate sequence context, i.e., when the misincorporated nucleotide is complementary to the next template nucleotide, pathway BF leads to a minus-one-nucleotide frame-shift error. Alternatively, if the initiating event is template-primer slippage (pathway F, for *F*rame shift), two possible outcomes can be envisioned. Continued polymerization from the misaligned template-primer (pathway FF) fixes the extra nucleotide and yields a minus-one-nucleotide error. Incorporation of a single correct nucleotide followed by realignment (pathway FB) generates a mispair which, if extended by further polymerization, yields a base-substitution error. Note that two of the four pathways are decidedly nonclassical: in pathway BF a frame-shift error is initiated by nucleotide misincorporation, and in pathway FB a base-substitution error is initiated by template-primer slippage. Thus, thinking about misalignment-mediated errors should not be limited to frame-shift errors.

Frame-Shift Fidelity Assays. The branch points in the pathways presented in Figure 1 suggest a balance for utilization of misaligned and mispaired substrates. This logic was extracted from studies of the fidelity of DNA synthesis in vitro by use of an assay that detects both base-substitution and frame-shift errors (Kunkel, 1985a). Circular DNA from bacteriophage M13mp2 is used to construct a double-stranded DNA substrate with a 390-nucleotide single-stranded gap. The gap contains the reporter gene for DNA synthesis errors, the wild-type *lacZ* gene sequence encoding the N-terminal amino acids (the α-peptide) of the enzyme β-galactosidase. Correct polymerization to fill the gap produces DNA that, when used to transfect an appropriate *Escherichia coli* host strain, will produce dark blue M13 plaques on an indicator plate containing the chromogenic indicator X-gal[2] via α-complemen-

[1] Although the term frame-shift mutation usually refers to changes in the number of base pairs in a protein-coding sequence that are not multiples of three, for convenience it refers here to mutations resulting from any difference in the number of base pairs and regardless of location.

[2] Abbreviations: X-gal, 5-bromo-4-chloro-3-indolyl β-D-thiogalactoside; Pol α, DNA polymerase α; Pol β, DNA polymerase β; Pol γ, DNA polymerase γ; RT, reverse transcriptase; AMV, avian myeloblastosis virus; MMLV, Moloney murine leukemia virus; HIV-1, type 1 human immunodeficiency virus; SSB, single-stranded DNA binding protein.

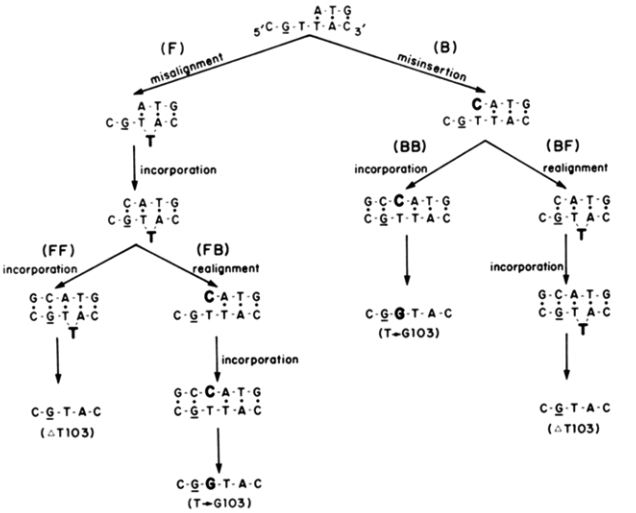

FIGURE 1: Pathways for single-base errors during DNA synthesis. F is for frame shift, B is for base substitution, and Δ indicates a one-base deletion. The underlined base is position 102, where position 1 is the first transcribed base of the *lacZ* gene. Adapted from Figure 1 in Kunkel and Soni (1988a).

tation of β-galactosidase activity within the infected host. Errors produced during DNA synthesis using the *lacZα* sequence as a template are scored as lighter blue or colorless plaques. Since this assay measures loss of a gene function that is not essential for phage production, a wide variety of sequence mutations at many different sites can be scored, recovered, and sequenced. This includes 221 different single-base substitutions at 114 different template positions (Kunkel & Alexander, 1986; Bebenek et al., 1990), single-base frame shifts at 150 positions (Kunkel, 1986), and a variety of larger and/or more complex frame shifts.

More recently, several assays have been designed to focus on particular subsets of frame-shift errors (de Boer & Ripley, 1988; Kunkel et al., 1989; Papanicolaou & Ripley, 1989; Bebenek & Kunkel, 1990). These assays use M13 DNA substrates that normally yield colorless plaques because they contain frame-shift mutations. DNA synthesis errors that restore the reading frame are detected upon transfection as blue revertants; hence, these are reversion assays.

Evidence of DNA Synthesis Errors Mediated by Misalignment. All DNA polymerases examined to data produce frame-shift errors during DNA synthesis in vitro. Frame-shift error rates are highly variable and depend on the DNA polymerase performing the synthesis as well as the position, composition, and symmetry of the error. The details of frame-shift error specificity are discussed here within the context of the misalignment pathways in Figure 1.

Pathway F, Then FF. Frame-shift mutations resulting from slippage of the two strands of DNA were first proposed by Streisinger to explain the observation that such mutations occurred in vivo more frequently at iterated[3] than at noniterated nucleotides (Streisinger et al., 1966). For errors arising during DNA polymerization, the logic is that as the length of the run increases the number of potential misaligned intermediates and the potential number of correct base pairs that could stabilize the misaligned intermediates both increase (Figure 2). Furthermore, the longer the run, the greater will

[3] A noniterated nucleotide is one having 5′ and 3′ neighbors that are not identical with the nucleotide considered. An iterated nucleotide has at least one identical neighbor. Iterated means repeated; reiterated means re-repeated.

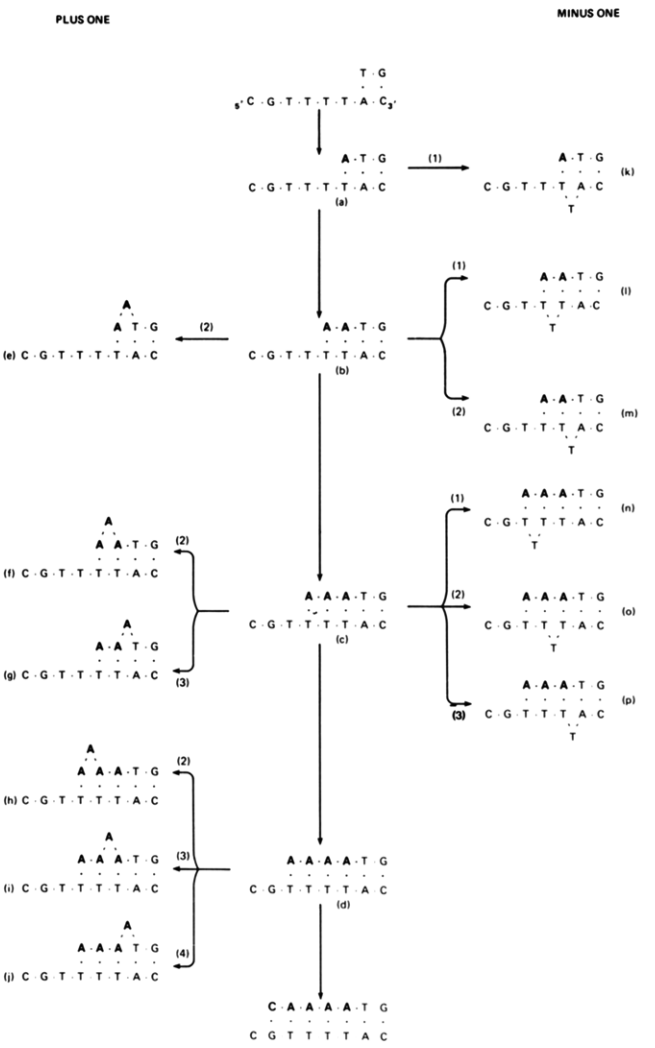

FIGURE 2: Possible intermediates for plus-one-base and minus-one base errors in a TTTT run. The numbers in parentheses are the number of base pairs that must be disrupted to produce the intermediate shown [from Kunkel (1986)].

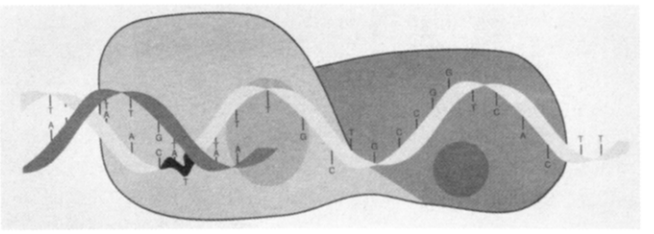

FIGURE 3: Schematic representation of the Klenow polymerase bound to a minus-one-base frame-shift intermediate. The 3′-OH primer terminus is within the active site (shaded circle) of the 46-kDa polymerase domain (on the left). The smaller, 22-kDa domain (on the right) contains the active site for exonucleolytic activity (shaded circle). This representation is based on data reviewed in Joyce and Steitz (1987) and on data presented in Cowart et al. (1989), Catalano et al. (1989), Allen et al. (1989), and Allen and Benkovic (1989).

be the distance between the extra nucleotide and the 3′-OH primer terminus, potentially reducing interference by the extra base in phosphodiester bond formation within the active site of the enzyme (Figure 3).

The strongest argument that some frame shifts at iterated nucleotides arise via misalignments during polymerization in vitro is that DNA polymerase frame-shift error rates, expressed per nucleotide polymerized (to correct for differences in the number of nucleotides in a run), increases as the length of the run increases. This has been observed with several forms of

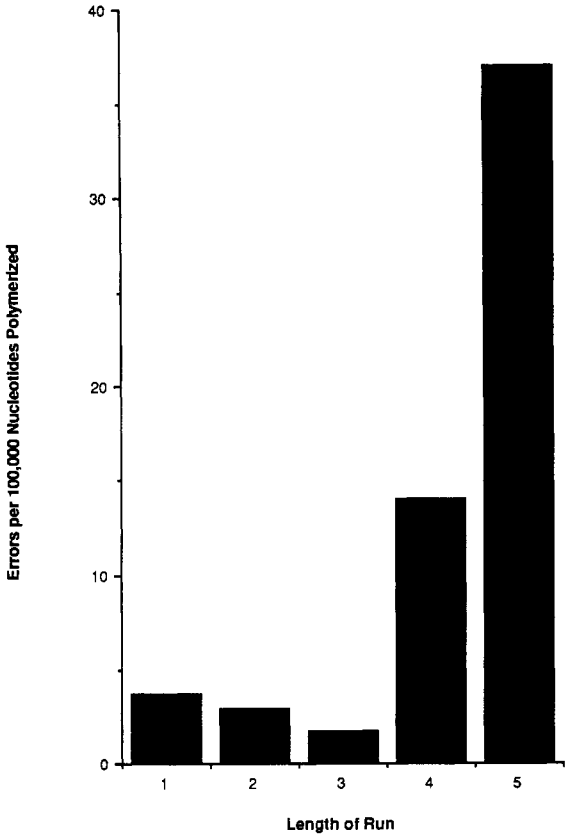

FIGURE 4: Minus-one-base frame-shift error rate versus run length for DNA polymerase α. The data are from Kunkel (1985b), with error rates calculated per detectable nucleotide polymerized, a minus-strand expression value of 60% being used (Kunkel & Soni, 1988b).

eukaryotic replicative Pol α (Figure 4; Kunkel, 1985b; Kunkel et al., 1989), HIV-1 RT (Bebenek et al., 1989), eukaryotic Pol β (Kunkel, 1985a), and three exonuclease-deficient derivatives of *E. coli* DNA polymerase I (Bebenek et al., 1990).

Additional in vitro evidence for slippage-mediated frame shifts comes from the analysis of a hot spot for minus-one-base errors by Pol β. This polymerase is particularly error prone for minus-T errors at a TTTT run at positions 70–73 in the *lacZ* coding sequence (Kunkel, 1985a). When the template sequence was altered by site-directed mutagenesis to TTCT or CTCT, the Pol β minus-one-base error rate at this four-nucleotide sequence decreased 4-fold and ≥30-fold, respectively (Kunkel, 1986). The data support the idea that most errors at the TTTT run result from misalignment.

(A) Variables Affecting Frame-Shift Error Rates at Iterated Sites. It is important to realize that, although frequency versus length correlations support the Streisinger model, such observations are not unequivocal. This is because comparisons must necessarily be made between different DNA sequences, and just as observed for base-substitution errors [e.g., see Mendelman et al. (1989); for review, see Bebenek and Kunkel (1988)], frame-shift error rates are sequence dependent. For example, Pol β and HIV-1 RT exhibit 5-fold and 43-fold differences, respectively, in the rate of minus-C errors at two different template CCC runs within the *lacZ* target sequence (Table I). Since position-dependent differences for runs of identical length can be as large as the difference shown in Figure 4, frequency versus length correlations must be interpreted with caution, especially when a small number of sites is being considered. Nevertheless, because the data in Figure 4 represent average values for a 150-nucleotide target and because the analysis of the Pol β frame-shift hot spot was performed with the same local DNA sequence context, both approaches provide excellent support for the Streisinger model.

Table I: One-Base Frame-Shift Error Specificities of Exonuclease-Deficient DNA Polymerases[a]

	DNA polymerase			
	Pol β	Pol α	HIV-1 RT	Klenow (exo$^-$)
minus:plus ratio	81:1	13:1	9:1	15:1
minus-one errors				
ratio for loss of pyr vs pu (in runs)	19:1	3:1	4:1	1:1
ratio of run to nonrun	31:1	2:1	104:1	1:1
error rate at				
TTTT at 70–73	1/57	1/2200	1/1400	≤1/75 000
CCC at 106–108	1/260	≤1/13 000	1/440	≤1/56 000
CCC at 166–168	1/1400	≤1/13 000	≤1/20 000	1/56 000

[a] The data are for Pol β (Kunkel, 1985a, 1986), human fibroblast (KB cell), Pol α (Kunkel, 1985b, 1986), HIV-1 RT (Bebenek et al., 1989), and the exonuclease-deficient form of the large Klenow fragment of *E. coli* DNA polymerase I (Bebenek et al., 1990). Error rates are expressed per detectable nucleotide incorporated, in order to correct for differences in lengths of the runs.

In addition to the surrounding sequence, the nucleotide composition of the run itself affects the frame-shift error rate. Thus, most exonuclease-deficient DNA polymerases make more errors within template pyrimidine runs within template purine runs (Table I). This may reflect weaker stacking interactions between adjacent pyrimidines than between adjacent purines, leading to more frequent formation of misaligned intermediates. As with any generalization, there are exceptions to this rule. For example, the HIV-1 RT is considerably less accurate at a GGG run than at several three-base pyrimidine runs in the *lacZ* target (Bebenek et al., 1989).

One rule that emerges from studies of frame shifts within runs is that minus-one-base errors occur much more frequently than do plus-one-base errors. As noted by Streisinger and Owen (1985), this could be due to equilibrium considerations for the aligned versus misaligned state. The intermediate for a plus-one-base error, containing an extra nucleotide in the primer, has lost one base pair relative to the intermediate for a minus-one-base error, which contains an extra nucleotide in the template strand. Also, for misaligned intermediates stabilized by an equal number of correct base pairs, the formation of a plus-one-base intermediate requires the disruption of one additional base pair relative to formation of a minus-one-base intermediate. For example, starting from intermediate c in Figure 2 and proceeding to a misaligned intermediate stabilized by two base pairs, it can be seen that three T·A base pairs must be disrupted to form the plus-one intermediate g, while only two need to be disrupted to form the minus-one intermediate o. This may partly explain the bias for minus-one-base errors.

Another explanation for this bias is that a minus-one-base intermediate may not be constrained by the DNA polymerase to the same extent as a plus-one-base intermediate (Figure 3). Differential structural constraints imposed by polymerases may also explain why frame-shift error rates and error specificities are so polymerase dependent. Polymerase error rates for the same mistaken can vary over 1000-fold (Table I). The extent of the bias for loss of a template pyrimidine rather than a template purine, and for minus-one rather than plus-one errors, is also polymerase dependent.

Fortunately, details of polymerase-mediated frame-shift specificity are emerging just as a wealth of new information is available on the structure and mechanisms of discrimination

during catalysis by DNA polymerases. For eample, Figure 3 depicts a hypothetical view of the large fragment of *E. coli* DNA polymerase I containing a minus-one-base frame-shift intermediate at a run of template T residues. This is based on X-ray crystallography data (Ollis et al., 1985) demonstrating that the Klenow polymerase consists of two domains, one of 46 kDa that contains the active site for polymerization (Freemont et al., 1986) and one of 22 kDa that contains the active site for the 3′ → 5′ exonuclease activity (Derbyshire et al., 1988). The large domain contains a cleft of the appropriate dimensions for binding about five to nine base pairs of a double-stranded B DNA helix. The polymerase and exonuclease active sites of this two-domain enzyme (shaded circles in Figure 3) are physically separated by 25–30 Å, a distance sufficient to account for contacts with about eight additional bases. This structural information [reviewed in Joyce and Steitz (1987)] and data from several other approaches that describe how the polymerase interacts with the template-primer (Ferrin & Mildvan, 1986; Cowart et al., 1989; Catalano et al., 1989; Allen et al., 1989; Allen & Benkovic, 1989) offer promise for eventual understanding of polymerase-dependent and neighboring-nucleotide-mediated effects on fidelity.

A current model for coordination of Klenow polymerase and exonucleolytic proofreading activities suggests that the template-primer DNA can slide between the two domains, with the terminal four base pairs melting out, providing single-stranded DNA for binding to the exonuclease active site (Joyce & Steitz, 1987; Freemont et al., 1988). Even in the absence of proofreading exonuclease activity (which is described below), physical separation of two DNA binding sites could have implications for frame-shift fidelity. Sliding, melting, and then re-forming correct base pairs may provide an opportunity to realign frame-shift intermediates. The observation that, for minus-one-base frame-shift errors with runs, the fidelity of the 46-kDa polymerase domain is lower than that for the two-domain, exonuclease-deficient Klenow polymerase (Bebenek et al., 1990) is consistent with this idea.

High-resolution crystallography data are not yet available for other DNA polymerases. However, the genes encoding other DNA polymerases have been cloned and sequenced, including all those shown in Table I. Comparison of the deduced amino acid sequences of DNA polymerases [e.g., Bernad et al. (1987), Wong et al. (1987), Hall (1988), and Bernad et al. (1989)] shows a number of conserved residues that may be important for catalysis and binding of template, primer, and dNTP substrates. Several DNA polymerases have now been overproduced from clones, so structural information may eventually be available.

In the meantime, descriptions of the DNA polymerases shown in Table I that may be relevant to their frame-shift fidelity have been obtained with other approaches [for review, see Fry and Loeb (1986)]. For example, Pol β is the smallest known polymerase, consisting of a single polypeptide of 40 kDa. Controlled proteolysis studies have shown that the recombinant rat β polymerase has an NH_2-terminal 8-kDa domain that binds single-stranded DNA (covering about eight bases) and a 31-kDa domain that could contain the polymerase active site (Kumar et al., 1990). Pol β binds tightly to nicked double-stranded DNA, polymerizes nucleotides in a distributive manner, and efficiently adds bases onto mismatched primer termini. These observations imply that Pol β can incorporate nucleotides without extensive or perfect contacts with the single-stranded template or the primer, and they help to explain its low frame-shift fidelity.

In contrast to Pol β, Pol α is a multisubunit enzyme whose catalytic subunit is much larger [180 000 kDa; for review, see Kaguni and Lehman (1988)]. This polymerase does not bind at nicks or fill gaps to completion, is more processive, requires single-stranded DNA for binding, and needs at least three to five correctly paired bases for efficient catalysis [for review, see Fry and Loeb (1986)]. The more extensive DNA–protein interactions implied by these data may result in greater and/or different constraints on either the frequency of formation or the subsequent stability of misaligned template-primers. Such interactions may explain the fact that most of the Pol α frame shifts within runs are in runs of three or more bases, while Pol β produces a high frequency of minus-one events even in two-base runs.

(*B*) *Processivity.* One property of polymerization that may be relevant to frame-shift fidelity, at least for one-base errors at runs, is processivity, the number of nucleotides the polymerase incorporates per association/dissociation cycle with the template-primer. This idea was first suggested by the observation that Pol α is both more accurate and more processive than Pol β (Kunkel, 1985b). The observation that polymerization by Pol β is about 5-fold more processive in reactions activated by Mn^{2+} than in reactions activated by Mg^{2+} (Wang & Korn, 1982) prompted a further examination of frame-shift fidelity in Pol β reactions activated with each divalent metal cation. The minus-T error rate at the TTTT hot spot for Pol β was lower in Mn^{2+}-activated reactions, consistent with the possibility that higher processivity was responsible for higher fidelity. This result is particularly striking because Mn^{2+} is highly mutagenic rather than antimutagenic for both base-substitution errors [for review, see Loeb and Kunkel (1982)] and frame-shift errors by Pol γ (Kunkel, 1985b).

More recently, the correlation between one-base frame-shift fidelity in runs and the processivity of the reaction has been examined quantitatively, by use of the HIV-1 reverse transcriptase (Bebenek et al., 1989). This enzyme is exceptionally inaccurate for one-base frame shifts within some but not all template runs in the *lacZ* target sequence. Comparison of the error rates at each run to the probability that the polymerase would cease synthesis within that run demonstrated that one-base frame-shift error rates were indeed higher at template runs where there is a higher probability of termination.

The correlation is not quantitative; the highest error rates are not necessarily at the sites exhibiting the highest termination probability. Also, the termination probability was always much higher than the error rate. For example, the enzyme terminates synthesis at position 70 approximately half the time, yet the error rate for plus-one errors at the TTTT run is 0.06%. Given the number of parameters known to infuence the error rate, such quantitative differences are not inconsistent with the concept that processivity is important for fidelity. Other explanations are also possible. For example, mutations may result from some other low-frequency event which cannot be detected by existing methods, or termination probability and error rate may be independently influenced by a third phenomenon.

Pathway F, Then FB. Misalignment followed by incorporation of an additional correct nucleotide provides an intermediate that can ultimately be processed into a base-substitution error via pathway FB in Figure 1. If, prior to another incorporation to further stabilize the misalignment, the extra template nucleotide resumes its position in the helix, a terminal mispair is created, yielding a base-substitution mutation. This is referred to as dislocation mutagenesis, by analogy to a dislocated shoulder joint that pops out of alignment but ul-

timately resumes a normal position.

The dislocation hypothesis is not new. It was presented 16 years ago as a hypothetical explanation for base-substitution mutations in an *E. coli* mutator strain (Fowler et al., 1974). However, experimental support for the model awaited DNA sequence analysis of mutants produced during DNA synthesis in vitro using the M13mp2 forward mutation assay that could score both base-substitution and frame-shift errors at sites where the interplay between these errors could be inferred. The model was suggested to explain base-substitution errors at two hot spots for errors by Pol β (Kunkel, 1985a). The two sites were related in their template DNA sequences and error specificities. At the sequence 5′-C-G-T-T-T-T-A-C-3′, a high frequency of minus-T frame shifts was observed. At a lower (but still high) frequency, T → G transversions were observed at the 5′-most T in the run (position 70, underlined), having a 5′-nearest-neighbor template G residue. The second hot spot, 33 bases away, had a similar template sequence, 5′-C-G-T-T-A-C-3′ (the sequence shown in Figure 1). Again, both minus-T frame shifts and T → G transversion at the 5′-most T in the run (position 103, underlined) were observed, but the relative frequencies of the two errors were reversed; the frame-shift frequency was much lower, and T → G errors predominated. The specificity of the base substitutions, exclusively T → G transversion implying T·dCMP mispairing, was unexpected. Considering the chemistry and structure of mispairs and measurements of misincorporation of pyrimidines opposite pyrimidines by prokaryotic DNA polymerases, direct misinsertion (pathway BB in Figure 1) might be expected to occur only rarely, while the other two base substitutions that are detectable at these positions might be expected to occur at equal or higher frequencies. However, neither T → C transitions (via a potentially more favorable T·dGMP intermediate) nor T → A transversions (via a T·dTMP mispair) were observed. The unusual base-substitution specificity, the fact that the base-substitution errors occurred at the 5′-most T in a run of T residues followed by a template G residue, and the tendency of Pol β to produce minus-one-base frame shifts within runs of a common base led to the dislocation model.

Since this transient misalignment pathway suggests that a nucleotide at one position codes for a mutation at another position, the model was tested (Kunkel & Soni, 1988a) by introducing G to A base changes into the target sequences as new 5′ neighbors to the sites for the T → G errors. The modified template was then used to reexamine the error specificity of Pol β at the hot spots. The base-substitution specificity of DNA polymerase β at the adjacent nucleotide switched from T → G transversion to T → A transversions. The cumulative change in frequency, represented by the disappearance of the T → G events and the appearance of the T → A events, was >300-fold. This result, predicted by the dislocation model, suggested that misalignment initiated the base-subsitution error.

The model was also tested by determing nucleotide misinsertion rates on the original and modified templates by use of a polycarylamide gel electrophoresis assay (Boosalis et al., 1989). Here the distinction between dislocation and miscoding mutagenesis is based on significant differences in apparent K_m and V_{max} values governing nucleotide insertion. This approach provided direct biochemical evidence that the transversion hot spots observed with Pol β resulted from dislocation.

Neither test of the dislocation model is unequivocal. It is formally possible that the results reflect neightboring nucleotide effects on direct miscoding (pathway BB). However, the dislocation model is attractive because it precisely explains striking and unexpected error specificities.

Recent data suggest that dislocation mutagenesis may also operate during DNA-dependent DNA synthesis in vitro by the HIV-1 RT (Bebenek et al., 1989). This polymerase is error prone not only for one-base-errors with runs but also for single-base substitutions at the ends of runs. In fact, within the spectrum of errors generated by this enzyme there are seven base-substitution hot spots whose specificity is exactly as predicted by the dislocation model.

As for frame shifts within runs, the error rate for base substitutions that may result from dislocation depends on the DNA sequence and the polymerase. Sequence context effects are clearly illustrated with the HIV-1 RT, where (putative dislocation) error rates vary more than 50-fold depending on the location. Furthermore, the error rate for putative dislocation base substitutions by Pol β at position 103 of the *lacZ* sequence is reduced at least 12-fold by changing a single neighboring template nucleotide (Boosalis et al., 1989). The effect of the polymerase is illustrated by the fact that not a single T → G error was generated by HIV-1 RT at position 103, which is the above-mentioned hot spot for this transversion in reactions catalyzed by Pol β (Kunkel, 1985a). Paradoxically, rat Pol β committed no T → C errors at position −36, while this was an extreme hot spot for such errors by the HIV-1 RT. The explanations for these sequence- and polymerase-mediated differences await a more complete understanding of the interactions between the enzyme and template-primer.

Pathway B, Then BF. Single-base-substitution errors can obviously result from direct insertion of an incorrect nucleotide (pathway B) followed by extension from the mispaired terminus (pathway BB). An alternative pathway is that, following misinsertion but before addition of the next correct nucleotide, a misaligned template-primer forms due to complementarity between the misinserted nucleotide and the next template nucleotide (pathway BF). Extension from the misaligned substrate will further stabilize the misalignment, ultimately yielding a minus-one-base frame shift. In this model, the frame-shift error is initiated not by a misalignment but by a misinsertion.

This model was suggested by four in vitro specificity observations. First, during synthesis by Pol β (Kunkel, 1985a), Pol α (Kunkel, 1985b), yeast DNA polymerase I (Kunkel et al., 1989), AMV RT (Roberts et al., 1989), and the exonuclease-deficient Klenow polymerase (Bebenek et al., 1990), the error rate for minus-one-base errors at noniterated template positions was surprisingly high, similar to rates for single-base-substitution errors. Second, frame-shift errors at nonrun sites and at two-base runs were mostly loss of a template purine that has a template pyrimidine as a 5′ nearest neighbor. Third, DNA polymerases frequently misinsert dAMP and dGMP opposite template purines (Kunkel & Alexander, 1986; Kunkel et al., 1989; Roberts, et al., 1989; Mendelman et al., 1989). Fourth, template-primers containing terminal purine–purine mispairs are sometimes poor substrates for further incorporation by DNA polymerases (Perrino & Loeb, 1989; Joyce, 1989; Mendelman et al., 1990; Bebenek et al., 1990). Thus, some of the frame-shift errors at nonrun or two-base runs may have been initiated by misinsertion of either dAMP or dGMP opposite template purines that had a pyrimidine as a 5′-template neighbor. These "difficult-to-extend" terminal mispairs may have realigned (e.g., pathway BF), such that synthesis would proceed from a substrating containing an extra nucleotide in the template strand but a correct base pair(s) at the terminus.

The model has been tested in two ways. First, a gapped circular M13mp2 DNA substrate was constructed, containing a terminal T·dCMP mispair as shown in Figure 1 (Roberts et al., 1989). Polymerization from the mispair yields base-substitution errors (pathway BB) that are scored as light blue M13mp plaques, while polymerization from the misaligned substrate (pathway BF) yields frame-shift errors that are scored as colorless plaques. During gap-filling DNA synthesis with this substrate, exonuclease-deficient Pol β (Roberts et al., 1989), yeast DNA polymerase I (Kunkel et al., 1989), AMV, MMLV, and HIV-1 RTs (Roberts et al., 1989; Bebenek, et al., 1989), and the exonuclease-deficient Klenow polymerase (Bebenek et al., 1990) all produced the minus-one-base frame shift at a significant frequency. Furthermore, transfection of this substrate without prior in vitro synthesis also yielded frame-shift mutations (Bebenek & Kunkel, 1990), suggesting that misincorporation can lead to frame shifts in vivo. Changing the neighboring template nucleotide from a "complementary" G (underlined in Figure 1) to a "noncomplementary" A reduces the frame-shift errors both in vitro (Kunkel et al., 1989) and in vivo (Bebenek et al., 1990), consistent with the requirement for a correctly base-paired terminus in the misaligned substrate.

The second approach examined the model during a reaction requiring that both misincorporation and misalignment occur during ongoing polymerization rather than starting with a performed mismatched substrate. By use of a frame-shift reversion assay that detects minus-one-base errors at 38 template positions, nucleotide substrate pool imbalances that are known to increase the rate of misincorporation were shown to also increase minus-one-base frame-shift error rates by the exonuclease-deficient derivative of the large fragment of *E. coli* DNA polymerase I (Bebenek & Kunkel, 1990). Examining the errors produced in reactions with different dNTP pool imbalances revealed that the template nucleotide that was preferentially lost had as a 5′ neighbor a nucleotide complementary to the dNTP provided in excess. Thus, when excess dATP was included in the reaction, minus-one-base frame shifts occurred for those template positions where the next correct template base was T, while when excess dGTP was included, the template base lost had as a neighbor a template C. Conversely, an antimutagenic response was observed for the loss of template G residues when excess dCTP was included. Both the mutagenic and antimutagenic responses support the model that frame-shift errors can result from misincorporation.

This mechanism is in principle possible at any template position and is not limited to the production of minus-one-base errors. Thus, frame-shift errors at template runs as well as plus and minus errors of varying numbers of nucleotides may initiate by misincorporation. This model may also apply to incorporation opposite damaged nucleotides. Figure 5 shows an example for a single-base frame shift, one of the most frequent mutations observed in a recent study of the mutagenic consequences of the carcinogen *N*-acetoxy-2-(acetylamino)-fluorene in *E. coli* (Schaaper et al., 1990). In this instance, incorporation of dCMP opposite a template G containing an AAF-generated adduct creates a substrate that is difficult to extend (Moore et al., 1981; Rabkin & Strauss, 1984). In the appropriate sequence context, misalignment may create a correctly paired primer terminus, yielding a frame shift. In fact, in vitro DNA synthesis catalyzed by a modified form of T7 DNA polymerase using a template containing aminofluorene adducts yielded a high proportion of minus-one-base and minus-two-base frame-shift mutations (Strauss, 1989).

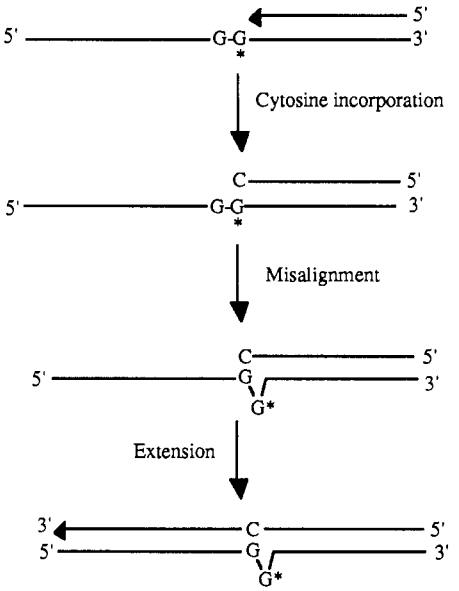

FIGURE 5: Model for minus-one-base errors resulting from misalignment due to synthesis on DNA containing an acetylaminofluorene adduct. Adapted from Figure 2 in Schaaper et al. (1990).

In principle, the model is not limited to correct incorporation opposite a damaged base but could involve misinserted nucleotides or insertion opposite noncoding lesions. Since a number of DNA damaging agents generate lesions that terminate incorporation either one nucleotide before or directly opposite the lesion [for review, see Strauss (1985)], the choice of whether to extend from a mispaired or a misaligned intermediate, depicted at both branch points in Figure 1, may provide a framework for considering how a variety of DNA adducts could induce both base substitutions and frame shifts.

The data that support the misincorporation model do not exclude other explanations for frame-shift errors. For example, a nucleotide may simply assume a position during polymerization in which it neither instructs incorporation nor interferes with its neighbor's ability to do so. This possibility is supported by structural studies with oligonucleotides demonstrating that an extra base can exist in conformations that do not disrupt hydrogen bonding of adjacent base pairs [Patel et al., 1982; Hare et al., 1986; Roy et al., 1987; Woodson & Crothers, 1988; Joshua-Tor et al., 1988; Miller et al., 1988; for review, see Patel et al. (1987)]. Extra bases may be stabilized by interactions with amino acids in the DNA polymerase (Kunkel, 1986; de Boer & Ripley, 1988; Miller et al., 1988), perhaps by formation of hydrogen bonds with specific nucleotides (Hendry et al., 1981, 1984; Lacey & Mullins, 1983), which might exclude them from pairing with the other strand.

Loss or Gain of More Than a Single Nucleotide. Included in the collections of frame-shift errors produced by a number of DNA polymerases during in vitro DNA synthesis are those involving the loss or gain of more than a single nucleotide. A substantial proportion of these can be explained by invoking the principles of Streisinger slippage between directly repeated DNA sequences separated by a variable number of intervening nucleotides. The model is shown in Figure 6, with as an example a 317-base deletion between two 5′-C-C-C-G-C-3′ sequences that was recovered repeatedly from reactions catalyzed by Pol β (Kunkel, 1985a). It involves the following steps: (1) The first step is synthesis of nine nucleosides starting from the provided 3′-OH primer and proceeding through the first C-C-C-G-C template repeat and then disruption of (at least) the five G·C primer-terminal base pairs. (2) This is followed by rearrangement of the DNA with re-formation of

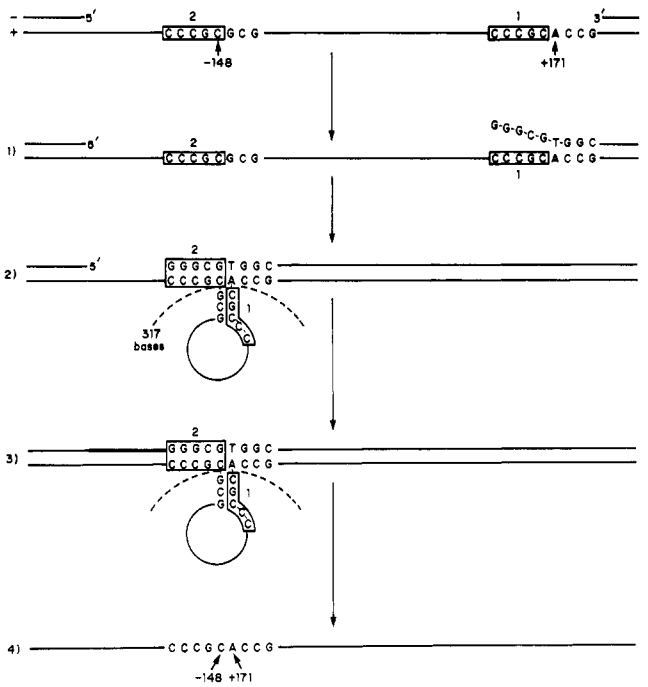

FIGURE 6: Model for deletions between direct repeats. Adapted from Figure 3 in Kunkel and Soni (1988a).

five hydrogen-bonded G·C base pairs involving the newly made DNA and the second copy of the direct repeat, 317 bases downstream. This intermediate may be further stabilized by three G·C base pairs that can potentially form a stem within the resulting loop of the heteroduplex intermediate. Through this step, this mechanism is formally equivalent to formation of a frame-shift intermediate (pathway F, Figure 1) but involves more nucleotides, greater distances, and a larger misaligned heteroduplex. (3) Continued synthesis from the intermediate (equivalent to pathway FF in Figure 1) produces the heteroduplex, which upon transfection and expression of the minus strand (step 4) yields the deletion.

The collection of mutants generated by rat Pol β also contained a complex mutant in which 123 bases were deleted and three base changes were present at one end of the deletion. This complex deletion can be precisely explained by a transient misalignment model involving four blocks of DNA sequences spread over the entire 390-base single-stranded gap [see Figure 3C in Kunkel and Soni (1988a)]. The explanation is formally equivalent to that for base substitutions by dislocation in that a misalignment occurs, followed by a limited amount of correct incorporation from this misaligned intermediate and then realignment followed by continued synthesis from the second heteroduplex intermediate. The result is that a block of 11 nucleotides has been moved to a position 253 nucleotides distant.

Models to explain the sequences of simple and complex frame-shift mutations recovered from reactions catalyzed by *E. coli* DNA polymerase I and its large fragment (de Boer & Ripley, 1988; Papanicolaou & Ripley, 1989) and yeast DNA polymerase I (Kunkel et al., 1989) also have invoked transient misalignment, but to different positions. In those studies, aberrant synthesis was suggested to involve strand switching [e.g., see Figure 4 in Papanicolaou and Ripley (1989)] or primer loop-back [e.g., see Figure 3 in Papanicolaou and Ripley (1989), Figure 4 in Kunkel et al. (1989), and Figure 3 in Schaaper et al. (1990)]. Consistent with previous models for generating complex frame shifts recovered in vivo (Ripley, 1982; Ripley & Glickman, 1983; Glickman & Ripley, 1984), the ends of some complex deletions produced in vitro suggest the involvement of palindromic DNA sequence [again, see Figure 4 in Papanicolaou and Ripley (1989) or Figure 4 in Kunkel et al. (1989)].

Just as for one-base frame shifts, the frequency of polymerization-dependent errors involving more than one nucleotide depends on several variables. Duplication errors are much less frequent than are deletion errors, perhaps partly reflecting the larger number of base pairs which must be disrupted to generate a duplication intermediate. While extensive deletion error specificity data are not available for most polymerases, in a collection of 51 deletion mutants generated by yeast DNA polymerase I that could be explained by a direct repeat model, an increase in error rate was observed as the length of the direct repeat increased from three to five (Kunkel et al., 1989). Beyond this length the error rate did not increase. This relationship may reflect the frequency of initial formation of the misalignment and the requirement for a stable intermediate that is acceptable to the polymerase for continued synthesis.

The error rate should thus depend not only on the length of the direct repeat but also on its position and nucleotide composition and on the polymerase. Unusual specificities are readily apparent from the limited data available. For example, the 317-base deletion shown in Figure 6 was generated at 100-fold higher frequency by DNA polymerase β (Kunkel, 1985a) than by the exonuclease-deficient form of the Klenow polymerase (Bebenek et al., 1990). Among the deletion mutants generated by yeast DNA polymerase I, this same 317-nucleotide deletion was recovered 20 times, while deletions between a variety of other direct repeats of identical length and similar nucleotide composition were not seen once.

Differences in the spectra of complex frame shifts that can be explained by strand-switching versus looping-back synthesis were observed when the fidelity of intact *E. coli* DNA polymerase I was compared to that of its large fragment (Papanicolaou & Ripley, 1989). It was suggested that these specificity differences reflect the ability to generate and/or use intermediates during nick-translation versus strand-displacement synthesis.

Effect of Proofreading and Polymerase Accessory Proteins. In vivo studies of frame-shift fidelity demonstrated that mutations in the gene encoding the bacteriophage T4 DNA polymerase produce altered frame-shift mutation rates (Ripley & Shoemaker, 1983). When alleles that had classical antimutator phenotypes for A:T → G:C transitions were examined, those that encoded polymerases having increased exonuclease activity relative to DNA polymerase activity were found to have antimutator effects on frame-shift mutagenesis within runs of A:T base pairs (Ripley et al., 1983). One explanation is that exonucleolytic proofreading enhances frame-shift fidelity.

This idea is supported by frame-shift fidelity measurements with the wild-type Klenow polymerase and its exonuclease-deficient derivatives. Proofreading enhances fidelity 3-fold for frame shifts within runs (Bebenek et al., 1990) and 6–15-fold for frame shifts at non-reiterated template positions (Bebenek et al., 1990; Bebenek & Kunkel, 1990). The lesser proofreading effect for errors in runs compared to nonruns may result from protection of the misaligned heteroduplex from digestion by the exonuclease by the correct base pairs possible in a run. This logic comes from studies of proofreading of base-substitution errors, where correctly paired bases protect mispairs from exonucleolytic digestion (Sinha, 1987). The greater contribution of proofreading to frame-shift fidelity at nonrun positions is consistent with lesser protection and the possibility (discussed above) that some frame shifts may result

from misinsertions that can be proofread.

While the contribution of proofreading to frame-shift fidelity has only been described for the Klenow polymerase, it is likely that proofreading enhances frame-shift fidelity for other polymerases as well. For example, Pol γ has high frame-shift fidelity (Kunkel, 1986) and also contains an associated proofreading exonuclease (Kunkel & Soni, 1988b).

Little information is available about the contribution of polymerase accessory proteins to frame-shift fidelity. A nonessential, 38-kDa single-stranded DNA binding protein from *Saccharomyces cerevisiae* enhanced the fidelity of DNA polymerase I from the same organism (Roberts et al., 1989b). The effect was specific for minus-one-base frame shifts at noniterated template sites (13-fold) and for deletions between direct repeats ($\geq$14-fold). Studies of this type have not yet been reported for prokaryotic or higher eukaryotic SSBs that are known to be essential for DNA recombination, repair, or replication. That accessory proteins may influence frame-shift fidelity during replication is suggested by the observation that the frame-shift fidelity of SV40-origin-dependent semiconservative replication in extracts of human HeLa cells is more accurate than that of purified replicative DNA polymerase α (Roberts & Kunkel, 1988).

Concluding Comments. Thirty years ago Fresco and Alberts (1960) discussed the possibility that deletion and addition mutations might arise from a "helix-with-loops". Twenty-four years ago George Streisinger and his colleagues proposed that frame-shift mutations occur preferentially within runs and at the ends of molecules (Streisinger et al., 1966). Studies performed over the last 5 years demonstrating that frame-shift errors are common during DNA synthesis in vitro provide strong support for these ideas. The models reviewed here and the in vitro data supporting them extend the original ideas and are consistent with the possibility that misalignment-mediated errors during DNA synthesis may contribute significantly to spontaneous and damage-induced frame-shift mutations, and possibly to base-substitution mutations as well. Fortunately, methodologies are now available for testing these models and for addressing the many unanswered questions posed by the recent work.

References

Allen, D. J., & Benkovic, S. J. (1989) *Biochemistry 28*, 9586–9593.

Allen, D. J., Darke, P. L., & Benkovic, S. J. (1989) *Biochemistry 28*, 4601–4607.

Bebenek, K., & Kunkel, T. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4946–4950.

Bebenek, K., Abbotts, J., Roberts, J. D., Wilson, S. H., & Kunkel, T. A. (1989) *J. Biol. Chem. 264*, 16948–16956.

Bebenek, K., Joyce, C. M., Fitzgerald, M. P., & Kunkel, T. A. (1990) *J. Biol. Chem.* (in press).

Bernad, A., Zaballos, A., Salas, M., & Blanco, L. (1987) *EMBO J. 6*, 4219–4225.

Bernad, A., Blanco, L., Lazaro, J. M., Martin, G., & Salas, M. (1989) *Cell 59*, 219–228.

Boosalis, M. S., Mosbaugh, D. W., Hamatake, R., Sugino, A., Kunkel, T. A., & Goodman, M. F. (1989) *J. Biol. Chem. 264*, 11360–11366.

Catalano, E., & Benkovic, S. J. (1989) *Biochemistry 28*, 4374–4382.

Cowart, M., Gibson, K. J., Allen, D. J., & Benkovic, S. J. (1989) *Biochemistry 28*, 1975–1983.

de Boer, J. G., & Ripley, L. S. (1988) *Genetics 118*, 181–191.

Derbyshire, V., Freemont, P. S., Sanderson, M. R., Beese, L., Friedman, J. M., Joyce, C. M., & Steitz, T. A. (1988) *Science 240*, 199–201.

Ferrin, L. J., & Mildvan, A. S. (1986) *Biochemistry 25*, 5131–5145.

Fowler, R. G., Degnen, G. E., & Cox, E. C. (1974) *Mol. Gen. Genet. 133*, 179–191.

Freemont, P. S., Ollis, D. L., Steitz, T. A., & Joyce, C. M. (1986) *Proteins 1*, 66–73.

Freemont, P. S., Friedman, J. M., Beese, L. S., Sanderson, M. R., & Steitz, T. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8924–8928.

Fresco, J. R., & Alberts, B. M. (1960) *Proc. Natl. Acad. Sci. U.S.A. 46*, 311–321.

Fry, M., & Loeb, L. A. (1986) in *Animal Cell DNA Polymerases*, CRC Press, Boca Raton, FL.

Glickman, B. W., & Ripley, L. S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 512–516.

Goodman, M. F. (1988) *Mutat. Res. 200*, 11–20.

Hall, J. D. (1988) *Trends Genet. 4*, 42–46.

Hare, D., Shapiro, L., & Patel, D. L. (1986) *Biochemistry 25*, 7456–7464.

Hendry, L. B., Bransome, E. D., Hutson, M. S., & Campbell, L. K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7440–7444.

Hendry, L. B., Bransome, E. D., Hutson, M. S., & Campbell, L. K. (1984) *Perspect. Biol. Med. 27*, 623–651.

Joshua-Tor, L., Rabinovich, D., Hope, H., Frolow, F., Appella, E., & Sussman, J. L. (1988) *Nature 334*, 82–84.

Joyce, C. M. (1989) *J. Biol. Chem. 264*, 10858–10866.

Joyce, C. M., & Steitz, T. A. (1987) *Trends Biochem. Sci. 12*, 288–292.

Kaguni, L. S., & Lehman, I. R. (1988) *Biochim. Biophys. Acta 950*, 87–101.

Kumar, A., Widen, S. G., Williams, K. R., Kedar, P., Karpel, R. L., & Wilson, S. H. (1990) *J. Biol. Chem. 265*, 2124–2131.

Kunkel, T. A. (1985a) *J. Biol. Chem. 260*, 5787–5796.

Kunkel, T. A. (1985b) *J. Biol. Chem. 260*, 12866–12874.

Kunkel, T. A. (1986) *J. Biol. Chem. 261*, 13581–13587.

Kunkel, T. A., & Alexander, P. S. (1986) *J. Biol. Chem. 261*, 160–166.

Kunkel, T. A., & Bebenek, K. (1988) *Biochim. Biophys. Acta 951*, 1–15.

Kunkel, T. A., & Soni, A. (1988a) *J. Biol. Chem. 263*, 14784–14789.

Kunkel, T. A., & Soni, A. (1988b) *J. Biol. Chem. 263*, 4450–4459.

Kunkel, T. A., Hamatake, R. K., Motto-Fox, J., Fitzgerald, M. P., & Sugino, A. (1989) *Mol. Cell. Biol. 9*, 4447–4458.

Lacey, J. C., & Mullins, D. W. (1983) *Origins Life 13*, 3–42.

Loeb, L. A., & Kunkel, T. A. (1982) *Annu. Rev. Biochem. 52*, 429–457.

Mendelman, L. V., Boosalis, M. S., Petruska, J., & Goodman, M. F. (1990) *J. Biol. Chem. 264*, 14415–14423.

Mendelman, L. V., Petruska, J., & Goodman, M. F. (1990) *J. Biol. Chem. 265*, 2338–2346.

Miller, M., Harrison, R. W., Wlodawer, A., Appella, E., & Sussman, J. L. (1988) *Nature 334*, 85–86.

Moore, P. D., Bose, K. K., Rabkin, S. D., & Strauss, B. S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 110–114.

Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G., & Steitz, T. A. (1985) *Nature 313*, 762–766.

Papanicolaou, C., & Ripley, L. S. (1989) *J. Mol. Biol. 207*, 335–353.

Patel, D. J., Kozlowski, S. A., Marky, L. A., Rice, J. A., Broka, C., Itakura, K., & Breslauer, K. J. (1982) *Biochemistry 21*, 445-451.
Patel, D. J., Shapiro, L., & Hare, D. Q. (1987) *Rev. Biophys. 20*, 35-112.
Perrino, F. W., & Loeb, L. A. (1989) *J. Biol. Chem. 264*, 2898-2905.
Rabkin, S. D., & Strauss, B. S. (1984) *J. Mol. Biol. 178*, 569-594.
Ripley, L. S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4128-4132.
Ripley, L. S., & Glickman, B. W. (1983) *Cold Spring Harbor Symp. Quant. Biol. 47*, 851-861.
Ripley, L. S., & Shoemaker, N. B. (1983) *Genetics 103*, 353-366.
Ripley, L. S., Glickman, B. W., & Shoemaker, N. B. (1983) *Mol. Gen. Genet. 189*, 113-117.
Roberts, J. D., & Kunkel, T. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7064-7068.
Roberts, J. D., Preston, B. D., Johnson, L. A., Soni, A., Loeb, L. A., & Kunkel, T. A. (1989) *Mol. Cell. Biol. 9*, 469-476.
Roberts, J. D., Hamatake, R. K., Fitzgerald, M. S., Sugino, A., & Kunkel, T. A. (1990) in *Mutation and the Environment*, Part A, Basic Mechanisms, pp 91-100, Wiley-Liss, Inc., New York.
Roy, S., Sklenar, V., Appella, E., & Cohen, J. S. (1987) *Biopolymers 26*, 2041-2052.
Schaaper, R. M., Koffel-Schwartz, N., & Fuchs, R. P. P. (1990) *Carcinogenesis* (in press).
Sinha, N. K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 915-919.
Strauss, B. S. (1985) *Cancer Surveys 4*, 493-516.
Strauss, B. S. (1989) *Ann. Ist. Super. Sanita 25*, 177-190.
Streisinger, G., & Owen, J. E. (1985) *Genetics 109*, 633-659.
Streisinger, G., Okada, Y., Emrich, J., Newton, J., Tsugita, A., Terzaghi, E., & Inouye, M. (1966) *Cold Spring Harbor Symp. Quant. Biol. 31*, 77-84.
Wang, T. S.-F., & Korn, D. (1982) *Biochemistry 21*, 1597-1608.
Wong, S. W., Wahl, A. F., Yaun, P.-M., Arai, N., Pearson, B. E., Arai, K., Korn, D., Hunkapiller, M. W., & Wang, T. S.-F. (1988) *EMBO J. 7*, 37-47.
Woodson, S. A., & Crothers, D. M. (1988) *Biochemistry 27*, 3130-3141.

Accelerated Publications

Self-Cleavage of Hepatitis Delta Virus Genomic Strand RNA Is Enhanced under Partially Denaturing Conditions†

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ABSTRACT: Self-cleavage of a polyribonucleotide containing an autocleaving sequence from the genomic strand of hepatitis delta virus was enhanced by conditions that destabilized RNA structure. Self-cleavage of the transcripts used in this study required Mg^{2+} (or another divalent cation), and in the absence of denaturants, maximum cleavage was observed at very low Mg^{2+} concentrations (0.05–0.1 mM). However, at 37 °C and in the presence of 2–10 mM Mg^{2+} the rate of cleavage was increased as much as 50-fold with the addition of urea to 5 M or formamide to 10 M. Cleavage was prevented by higher concentrations of the same reagents (9.5 M urea or 22.5 M formamide), presumably because a structure required for self-cleavage is disrupted by strongly denaturing conditions. In contrast to a previous report [Wu, H.-N., & Lai, M. M. C. (1989) *Science 243*, 652–654], we find that chelating Mg^{2+} with EDTA terminates the cleavage reaction without promoting measurable amounts of ligation of the cleavage products. The ability of denaturants to promote rapid self-cleavage in vitro raises the possibility that an unidentified factor could have a similar effect in vivo.

Specific sequences of RNA can fold to mediate both intramolecular catalysis, such as self-splicing (Kruger et al., 1982; Cech & Bass, 1986; Peebles et al., 1986; van der Veen et al., 1986) or self-cleavage (Prody et al., 1986; Hutchins et al., 1986; Buzayan et al., 1986; Forster & Symons, 1987; Epstein & Gall, 1987), and the catalytic cutting or joining of other RNA substrates (Guerrier-Takada et al., 1983; Zaug & Cech, 1986; Zaug et al., 1986). Self-cleaving domains of RNA may represent examples of the smallest and least embellished RNA structures that mediate cleavage reactions (Forster & Symons, 1987). Several self-cleaving RNAs have been harbingers of small catalytic RNAs which have been engineered to site specifically cleave substrate RNAs (Uhlenbeck, 1987; Haseloff & Gerlach, 1988; Hampel & Tritz, 1989). A common and well-characterized motif of secondary structure for self-cleaving RNAs is the hammerhead (Hutchins et al., 1986), a three-stemmed structure that can be drawn for sequences flanking the cleavage sites of certain single-stranded RNA plant pathogens (Forster & Symons, 1987) and the transcript of a newt satellite DNA (Epstein & Gall, 1987). However, many other RNA structures are also

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