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MicroReview

The role of RNA polymerase in transcriptional fidelity

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Summary

The overall transcription of DNA has previously been demonstrated to proceed at extremely high levels of accuracy. We review the evidence that the process of transcription is subject to proof-reading in the Hopfield sense. In addition, we speculate that the proof-reading activity associated with transcription is subject to cyclical phase transitions. That is, during periods of low processivity associated with initiation, RNA synthesis is relatively imprecise. The transition to the elongation phase of RNA synthesis, characterized by a shift to high processivity, is accompanied by enhanced proof-reading. A model for the damping of transcriptional errors, based on a PPi-mediated processive pyrophosphorolysis reaction, is discussed in terms of pausing during transcription.

Introduction

In all biological systems the faithful expression of any gene is critically dependent upon the fidelity of the enzymes associated with the replication and transcription of DNA, as well as accurate translation of the mRNA. Although errors in DNA replication are permanent, and thus potentially the most damaging type of error, errors in the primary transcription product may have significant physiological consequences for the cell. Most errors introduced into the primary structure of a mRNA alter the primary, and consequently, secondary structure of its encoded polypeptide, and thus its sequence-dependent functions. In addition, since individual protein encoding mRNAs are translated about 40 times, a significant burst of erroneous proteins could result. Such an event could in itself become catastrophic for a cell if the erroneously produced polypeptide were to affect an essential cellular function.

It has been demonstrated that *in vitro*, the *Escherichia coli* DNA-dependent RNA polymerase exhibits error rates of approximately 1 to 2 $\times$ 10^{-5} for poly (d(A-T) poly d(A-T))-direct misincorporation of non-complementary GMP (Blank *et al.*, 1986; Springgate and Loeb, 1975). Although other reported transcriptional error rates vary substantially, only the latter two studies are consistent with *in vivo* estimates of the frequency of amino acid replacements in proteins. The frequency of transcriptional error must be less than the frequency with which incorrect amino acids appear in proteins because the latter is due either to errors in transcription or to ribosomal errors in translating correct messages. The average frequency of amino acid substitutions in proteins (averaged over many positions) has been estimated to be about 10 to 40 $\times$ 10^{-5} (Loftfield and Vanderjagt, 1972; Edelmann and Gallant, 1977; Ellis and Gallant, 1982), and most of this is likely to be of translational origin. Only the values of Blank *et al.* (1986) and Springgate and Loeb (1975) for transcriptional error are low enough to be realistic.

In vivo, estimates of transcriptional error levels have been made using extremely polar nonsense mutations early in the *lacZ* gene of *E. coli*. Such mutants produce an extremely low background level of β-galactosidase which probably results mainly from transcriptional, rather than translational, error (Rosenberger and Foskett, 1981; Rosenberger and Hilton, 1983). This inference follows from the nature of polarity: when the lead ribosome encounters a nonsense codon and dissociates from the mRNA, the transcription apparatus detaches from the gene some distance downstream, so that only a fragment of the gene is transcribed. As a result, mistranslation of a nonsense codon by all ribosomes behind the lead ribosome cannot contribute to active enzyme synthesis because they have only a fragment of message to translate in any case. Thus, extreme polarity reduces the translational component of background leakiness by a factor equal to the average number of ribosomes per mRNA, revealing the residual error frequency due to transcriptional error. The background leakiness of extremely polar nonsense alleles early in the *lacZ* gene is in the range of 1 to 10 $\times$ 10^{-5} (Blank *et al.*, 1986; Rosenberger and Foskett, 1981; Rosenberger and Hilton, 1983; J. A. Gallant, unpublished), in rough agreement with the *in vitro* estimates cited above.

The low value of these estimates suggests that fidelity of transcription is maintained by one or more proof-reading step(s). It is the purpose of this review to discuss the

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rather limited evidence on the existence of transcriptional proof-reading and to speculate about additional error-avoidance mechanisms.

Accuracy and the initiation of RNA synthesis

Although high fidelity of transcription appears to be essential during the elongation phase of RNA synthesis, the same is not necessarily true for the initiation phase. The hypothesis that initiation of RNA synthesis is sloppy and may not precisely start at a specified sequence, is supported by the following experimental observations: when the *lacZ* gene is transcribed from cAMP–CRP-independent mutant promoters *in vitro*, distinct heterogeneity is observed in the 5′ terminus of the mRNA (Carpousis *et al.*, 1982). The observed heterogeneous transcripts were found to contain two major, as well as two minor, 5′ termini following partial sequence analysis of the mRNAs. These results have been interpreted by Carpousis *et al.* (1982) to mean that all the transcripts were derived from the same, rather than overlapping, sets of promoters. This interpretation has been further strengthened by experiments which clearly demonstrated that variations in transcriptional start sites were influenced by alterations in the ratios of rNTPs. Moreover, Maizels (1973) and Majors (1975) in a separate set of experiments have similarly demonstrated the ambiguous nature of initiation using templates containing single promoters: while two major transcripts were observed to initiate at the G1 and A1 positions of the *lac* promoter (Carpousis *et al.*, 1982), the frequency of initiation from the two sites varied, and could be altered by varying the rNTP levels.

Thus, at the *lac* promoter initiation region, transcription initiates at four distinct purines within a six base-pair sequence. Such levels of ambiguity are never tolerated when the enzyme enters the elongation phase of RNA synthesis. These rather limited studies of transcriptional initiation fidelity suggest that the initiation complex has relatively loose primary sequence requirements for the position at which to initiate RNA synthesis. Rather, the transcription apparatus may seek to initiate at sites with similar secondary structure. Although this strategy may appear to be quite error-prone, it may be harmless because the translational intiation signal is generally located some distance downstream of the 5′ termini of the transcript, and therefore errors due to initiation site heterogeneity or base substitutions occurring upstream of the AUG codon would be expected to have limited adverse affects. Needless to say, if a functional role does exist for the 5′ end of the mRNA, errors in this region may have significant detrimental effects on the cell.

The fact that initiation proceeds with far less fidelity than elongation initially led to the notion that DNA polymerase I may undergo a rapid proof-reading 'warm-up' phase (Lecomte and Ninio, 1987). RNA polymerase may be envisaged as undergoing a similar type of warm-up transition; that is, as the enzyme enters the elongation mode, and becomes more processive, its proof-reading capability may become more efficient.

Accuracy during RNA polymerization

During the elongation stages of RNA synthesis both cognate and non-cognate rNTPs continuously interact with sites on the polymerization complex. Selection of the correct rNTP for incorporation into the nascent RNA chain is, in part, a consequence of the free energy difference between the binding of correct versus incorrect rNTPs at the elongation site; that is, incorrect rNTPs form weaker, more unstable contacts with the elongation complex than do the correct rNTPs. Thus the instability associated with such non-cognate rNTP binding may serve as a signal for the initiation of RNA 'editing' in *E. coli*. Molecular processes associated with the improvement of transcriptional accuracy are likely to encompass mechanisms which function to actively avoid, as well as remove, erroneous rNTPs from the nascent transcript. Thus, the overall fidelity of RNA synthesis is probably achieved by means of a concerted effort involving error avoidance as well as error correction.

RNA polymerase has been demonstrated to convert all four rNTPs into their corresponding rNDP, while incorporation into the nascent transcript has been shown to be strictly dependent upon the presence of the template-specific complementary base (Ninio *et al.*, 1975). The incorporation or hydrolysis of rNTPs has been suggested by Ninio *et al.* (1975) to represent two alternative pathways resulting from the interaction of an rNTP with a polymerase–template complex. Moreover, the generation of rNDPs is observed only when transcription is allowed to proceed in the presence of non-cognate rNTPs. Furthermore, rNDP formation has been found not to be due to a polynucleotide-phosphorylase-like attack of the *de novo* transcript (Ninio *et al.*, 1975). Thus, the conversion of rNTPs to rNDPs during transcription involving non-cognate precursors may represent a Hopfield-like kinetic proof-reading scheme for reducing errors (Hopfield, 1974). A mechanism for the reduction of transcriptional errors has been further supported by experiments that have demonstrated the existence of a nucleotide triphosphate phosphohydrolase (NTPase) activity associated with RNA polymerase. The NTPase activity demonstrated by Volloch *et al.* (1979) was found to be capable of hydrolysing all four rNTPs to nucleoside diphosphates. However, during poly(dC)- or poly(dT)-directed *in vitro* transcription, only the non-complementary rNTP (of the same heterocyclic class) was hydrolysed. Moreover, a 30 to 50-fold increase was observed in the incorporation of

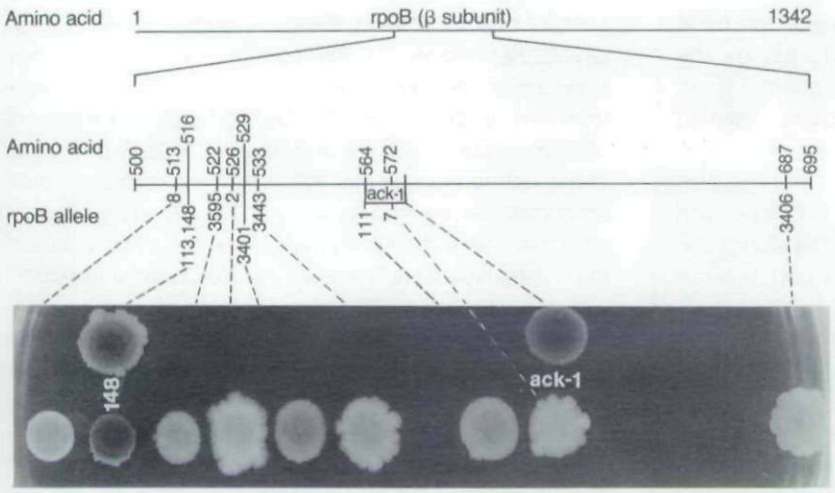

Fig. 1. Map co-ordinates of mutants displaying reduced transcriptional fidelities. Strains of *E. coli* DS410.1 (F^-, *ara, azi, tonA*^{R}, *minA, minB, str, xyl, mtl, thi*) bearing the polar U118 ochre muation in *lacZ* was used as the recipient strain during P1-mediated transduction of various *rif*^{R} lesions (Jin and Gross, 1988; Libby *et al.*, 1989), and mapped by deletion plasmid analysis according to the procedure of Jin and Gross (1988). Map positions of the individual mutants within the *rpoB* gene are as indicated. The photograph below demonstrates the level of suppression of the U118 ochre mutation by mutants *113, 148* and *ack-1* (and absence of suppression in the others) following growth on minimal M63 plates containing 5mM cAMP, 2 mM IPTG, and 1.2 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) for five to seven days at 37°C. (See colour picture on front cover.)

non-complementary rNTPs during *in vitro* poly(dC)-directed transcription with RNA polymerase preparations devoid of NTPase activities.

Volloch *et al.* (1979) proposed that selection of rNTPs is based on two tests of fitness. The first test would evaluate random, weak binding of all possible precursors at a site on the enzyme common to both cognate and non-cognate rNTPs. Correct precursors would be selected by their ability to induce a conformational shift in the enzyme, resulting in high-affinity binding of the cognate rNTP at the elongation site. Once in the elongation site, the appropriateness of fit would be tested a second time by the NTPase system. Incorrect rNTPs, which occasionally bind to the elongation site, would be hydrolysed to their corresponding rNDP, and thus 'tagged' for ejection from the elongation complex. The occasional incorrect rNTP which escapes the primary error-avoidance system would be either incorporated into the nascent transcript, or removed post-transcriptionally (see below). Intriguing as the results were, however, neither Volloch *et al.* (1979) nor Ninio *et al.* (1975) demonstrated a direct link between the observed NTPase activity and the enzyme itself.

Recently, however, Libby *et al.* (1989) have demonstrated a genetic connection between NTPase activity and the beta subunit of RNA polymerase, on the one hand, and the accuracy of transcription on the other. The NTPase activity described by Libby *et al.* (1989) was found specifically to convert non-cognate rNTPs into their corresponding rNDP *in vitro* in a template-dependent manner. When RNA polymerase was purified from an *rpoB* rifampicin-resistant (Rif^R) mutant which displayed reduced transcriptional accuracy, designated *ack-1* (see Fig. 1), it was found to be completely devoid of non-cognate NTPase activity (Libby *et al.*, 1989). The lesion(s) resulting in the Rif^R, $NTPase^-$ phenotype was found to map to the 'E' deletion interval, representing amino acid positions 565–576 of the beta subunit. It remains to be determined, however, whether this region of *rpoB* represents a unique domain within the beta subunit which participates in the maintenance of accuracy.

Thus the irreversible nature of the conversion of non-cognate rNTPs to rNDPs allows the proof-reading step to be independent of the preceding discrimination step, as required for a formal proof-reading mechanism (Ninio, 1975; Hopfield, 1974). The identification of a Rif^R mutant which is devoid of NTPase activity, as well as displaying reduced transcriptional accuracy strengthens the link between the hydrolytic activity associated with the enzyme and proof-reading. In accordance with Hopfield's predictions of kinetic proof-reading, the mutant enzyme was found to transcribe the *lacZ* gene at a slightly faster rate than the wild-type enzyme.

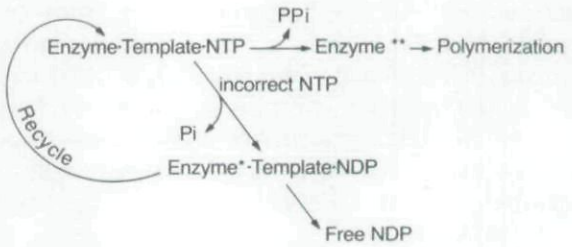

Fig. 2. Polymerization and error-avoidance schemes during RNA synthesis. As depicted in the scheme above, RNA polymerase forms an initiation complex with the template and NTP. Following this association event, PPi is released when a cognate NTP occupies the elongation site, resulting in a conformational shift in the enzyme to a tight binding configuration followed by polymerization of the NTP into nascent RNA. When a non-cognate NTP enters the elongation site and is recognized by the NTPase proof-reading system it is hydrolysed to Pi + NDP. The 'tagged' NDP is subsequently ejected from the elongation complex, allowing the enzyme–template complex to reform the ternary complex.

Thus, in *E. coli* one of the major pathways responsible for transcriptional error damping is mediated by the NTPase activity. The damping of transcriptional errors may be envisaged as proceeding in a manner consistent with the nucleotide incorporation scheme of Ninio *et al.* (1975), as outlined in Fig. 2. As such, a ternary complex would form initially between the enzyme, template, and nascent transcript. If the incoming rNTP at the elongation site is judged to be cognate, the enzyme would undergo an isomerization step to a tight binding configuration, followed by release of PPi, and incorporation of the rNMP into the growing chain. However, when the elongation site encounters a non-cognate rNTP it is hydrolysed to its rNDP form while releasing Pi. During subsequent steps, free rNDP is released which may be rephosphorylated, thus reforming the rNTP.

Post-transcriptional error correction

Although the NTPase system described above probably represents one of the major pathways for damping transcriptional errors, other mechanisms also may be operative to ensure the accurate transcription of genetic information. In the case of DNA polymerases I (Pol I) and III (Pol III), for example, the accurate replication of the *E. coli* genome is maintained, in part, through a 3′→5′ exonucleolytic reversal of polymerization, i.e. the preferential excision of incorrectly incorporated nucleotides from the growing 3′ end of the chain (Brutlag and Kornberg, 1972; Fersht and Knill-Jones, 1983). Furthermore, the 3′→5′ exonucleolytic activity has been shown to enhance the fidelity of DNA polymerization by 10- to 200-fold for Pol I and Pol III, respectively. To date, little information exists concerning whether or not RNA polymerase possesses the same type of error-correction activity.

During the early days of RNA polymerase characterization, Maitra and Hurwitz (1967) described a small but detectable pyrophosphorolysis of RNA (or reversal of the incorporation reaction) associated with RNA–DNA hybrids. Similar processive pyrophosphorolysis of RNA has been demonstrated by Rozovskaya *et al.* (1980) which is catalysed by RNA polymerase itself. Moreover, it was demonstrated that the sequential DNA-dependent pyrophosphorolysis required an active ternary complex of enzyme, DNA, nascent RNA, Mg^{2+}, and PPi (Rozovskaya *et al.*, 1982).

Recently, the reversal of the incorporation reaction has been examined in an effort to determine whether it can influence the fidelity of transcription: using the chain-terminating CTP analogue, 3′-*O*-methyl-cytidine-5′-triphosphate during *in vitro* transcription of synthetic templates, Kahn and Hearst (1989) demonstrated that full-length transcripts are produced only following exonucleolytic removal of the CTP analogue from the 3′ end of the nascent chain. Removal of the analogue was found to be strictly dependent on the presence of PPi. In other experiments, removal of PPi (following addition of PPase) from the transcription reaction led to an increase in the rate of misincorporation (Kahn and Hearst, 1989). Experiments of this nature indicate that PPi, which is a normal product of nucleotide incorporation (see Fig. 2), may play an important role in the maintenance of accuracy by means of the reversal reaction. However, the direct exchange of PPi with non-cognate rNTPs during *in vitro* or *in vivo* transcription has yet to be established. Kahn and Hearst (1989) clearly demonstrated that PPi does exchange with all four NTPs during *in vitro* transcription of templates containing all four deoxyribonucleotides, but with such a system it is difficult to determine whether or not the reaction is specific for non-cognate rNTPs. Synthetic templates lacking one (or several) species of deoxyribonucleotides could be used to determine whether or not PPi specifically exchanges with non-cognate rNTPs.

Nonetheless, the reversible incorporation of rNTPs from the 3′ end of the nascent chain would provide an essential feature for error correction. It has been postulated that the maximum free energy difference (ΔG_{max}) of binding of correct versus incorrect rNTPs at the elongation site sets an upper limit on the accuracy of transcription in the absence of proof-reading (Kahn and Hearst, 1989). Thus, the reversible incorporation of rNTPs from the nascent transcript is necessary in order to take full advantage of the ΔG_{max}.

Although PPi appears to play a role in improving the accuracy of transcription, it may have an opposite effect on other polymerizing enzymes, such as DNA polymerase. For example, kinetic proof-reading during DNA replication has been proposed to involve a branched pathway for the rejection of errors (Hopfield, 1974; 1980; Ninio, 1975; Bernardi *et al.*, 1979). These models, however, predict that PPi should reduce the fidelity of DNA replication by inhibiting the error discrimination/rejection process. To date, the effect of PPi on the fidelity of DNA replication has generated conflicting results: Kunkel *et al.* (1986) demonstrated that PPi enhanced the level of Polymerase-I-directed misincorporation errors in copying ΦX-174 DNA. However, they also demonstrated that PPi was not mutagenic with avian myeloblastosis virus (AMV) DNA polymerase or DNA polymerase α and β from animal cells (enzymes that lack 3′→5′ exonuclease activity). Similarly, Abbotts and Loeb (1985) observed that high concentrations of PPi led to an increase in the fidelity of replication by DNA polymerase α. This apparently paradoxical situation suggests that the increased misincorporation observed with *E. coli* Pol I is not due to a PPi-stimulated increase in polymerase errors, but rather to inhibition of the 3′→5′ exonucleolytic proof-reading activity by PPi, as suggested by Kunkel *et al.* (1986) and Kuchta *et al.* (1987).

DNA and RNA polymerases appear to have quite different target sites for PPi, and thus the effect of PPi on DNA polymerase fidelity may not directly relate to RNA polymerase. The processivity of DNA and RNA polymerases are sufficiently different that it would not be surprising to find that PPi has opposite effects on the fidelity of transcription and DNA replication.

Mnemonic aspects of proof-reading

It was pointed out earlier that initiation events proceed with far less fidelity than the elongation phase of RNA synthesis. This suggests, *a priori*, that as the transcription complex translocates down a template, its ability to discriminate and/or remove incorrect rNTPs from the transcript improves. Thus, we suggest that the phenomenon of accuracy improvement may be due to 'warm up' of the proof-reading function and thus results in a transition from a low processive to a highly processive state. Memory effects have previously been suggested for DNA replication (Papanicolaou *et al.*, 1984; 1986; Lecomte and Ninio, 1987), as well as transcription (Job *et al.*, 1988) and translation (Ninio, 1986).

In the case of DNA polymerase α isolated from calf thymus it has been observed that the misincorporation events at the initial position differed qualitatively and quantitatively from errors incorporated at subsequent positions (Grosse *et al.*, 1983). These observations led Pananicolaou *et al.* (1984) to speculate that yeast DNA Polymerase I is capable of switching between two exonuclease states: the low exonuclease state is associated with low processivity (i.e. low polymerization), while the high exonuclease state is associated with the highly processive (i.e. high polymerization) state. Following disassociation from the template, Papanicolaou *et al.* (1984) further speculated that the enzyme drifts towards the low exonuclease state, while recovery of the high exonuclease state requires a return to the highly processive mode. These speculations have been supported through experiments which have demonstrated that the 3′→5′ excision activity associated with Pol I waxes when the enzyme enters the highly processive state, and wanes following dissociation from the template (Papanicolaou *et al.*, 1986). Again, this is indicative of a mnemonic aspect associated with the error correction function of DNA polymerase.

RNA polymerase, on the other hand, has not been demonstrated to be capable of switching between high and low exonuclease states. However, it has been proposed that the transcription complex is capable of undergoing various mnemonical phase transitions which may be generated by the sequence and/or conformation of the DNA template (Job *et al.*, 1988).

In view of the fact that transcription undergoes an accuracy improvement upon entering the elongation phase, we speculate that accuracy improvement 'warms up' as the enzyme becomes more processive. Moreover, inasmuch as 3′-mRNA heterogeneity is observed at termination sites (Lewin, 1983; Platt, 1986) a similar, but opposite, accuracy transition may occur: that is, the decline in processivity observed as the enzyme encounters termination signals may result in yet another accuracy 'cool down' transition. Thus, the enzyme may be constantly undergoing cyclical accuracy transitions.

Concluding comments

Following the pioneering work of Ninio *et al.* (1975), who showed that non-cognate rNTPs may be converted to their corresponding rNDPs prior to their ejection from the elongation site, recent work has demonstrated a genetic connection between NTPase activity, accuracy, and the beta-subunit of RNA polymerase. It was shown that genetic alterations in the beta-subunit leading to increased transcriptional error (Blank *et al.*, 1986) result in a corresponding deficiency of non-cognate NTPase activity (Libby *et al.*, 1989).

The occasional non-cognate ribonucleotide which escapes this editing mechanism may be subjected subsequently to an additional error-damping pathway: misincorporated bases may be removed via the 3′→5′ exonucleolytic proof-reading system (Kahn and Hearst, 1989). Removal of the terminal nucleotide from the nascent transcript may proceed in a non-specific manner, yet still provide accuracy improvement during transcription. That is, the exonucleolytic removal step may be equally effective in removing both correct as well as incorrect nucleotides from the 3′ end of the nascent chain. Error reduction may be accomplished, however, if mismatched rNMP:dNMP base pairs inhibit addition of the next ribonucleotide, resulting in a 'paused' transcription complex. Pausing by the enzyme at these sites could allow for additional editing time by the exonuclease system. This hypothesis must be tested experimentally to determine whether non-cognate ribonucleotides are removed at defined pause sites.

The co-operative action of both error-avoidance and error-correction systems appears to be primarily responsible for ensuring high fidelity of transcription. Additional experimentation relating error rates to position within a transcript might elucidate further mechanisms utilized by RNA polymerase in the maintenance of optimal accuracy. The energetic considerations underlying this optimization have yet to be addressed in detail in the case of transcription.

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