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Reverse Transcription and DNA Amplification by a *Thermus thermophilus* DNA Polymerase

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ABSTRACT: A recombinant DNA polymerase derived from the thermophilic eubacterium *Thermus thermophilus* (*Tth* pol) was found to possess very efficient reverse transcriptase (RT) activity in the presence of $MnCl_2$. Many of the problems typically associated with the high degree of secondary structure present in RNA are minimized by using a thermostable DNA polymerase for reverse transcription, and predominantly full-length products can be obtained. The cDNA can also be amplified in the polymerase chain reaction (PCR) with the same enzyme. The *Tth* pol was observed to be greater than 100-fold more efficient in a coupled RT/PCR than the analogous DNA polymerase from *Thermus aquaticus* (*Taq* pol). The sensitivity of the reactions performed by *Tth* pol allowed for the detection of ethidium bromide stained products starting with as little as 100 copies of synthetic cRNA. Similar results were also obtained with RNA from a Philadelphia-chromosome positive cell line. Detection of IL-1α mRNA was possible starting with 80 pg of total cellular RNA. The ability of *Tth* pol to perform both reverse transcription and DNA amplification will undoubtedly prove useful in the detection, quantitation, and cloning of cellular and viral RNA.

The standard methods for the detection and analysis of RNA molecules include in situ hybridization, Northern, dot, or slot blot analysis, S1 nuclease analysis, and RNase protection assays. These protocols are limited in their usefulness because of either the large quantity of RNA required or the inherent difficulty of some of the procedures. The technique of DNA amplification by the polymerase chain reaction (PCR) (Saiki et al., 1985, 1988; Mullis & Faloona, 1987) has been extended to include RNA as the starting template by first converting RNA to cDNA by either avian myeloblastosis virus reverse transcriptase (RT) or Moloney murine leukemia virus RT (Powell et al., 1987; Kawasaki et al., 1988; Frohman et al., 1988). The process of RT/PCR has proved invaluable for detecting gene expression (Kawasaki et al., 1988; Rappolee et al., 1988), for amplifying RNA sequences for subcloning and analysis (Veres et al., 1987; Todd et al., 1987), and for the diagnosis of infectious agents (Byrne et al., 1988; Gama et al., 1988; Hart et al., 1988) or genetic diseases (Gibbs et al., 1989). However, a significant problem in using RNA as a template is the inability of mesophilic viral RTs to synthesize cDNA through stable secondary RNA structures (Buell et al., 1978; Kotewicz et al., 1988). Several methods have been described to destabilize regions of complementarity with methylmercury (Bailey & Davidson, 1976), dimethyl sulfoxide (Bassel-Duby et al., 1986), and increased reaction temperatures (Huibregste & Engelke, 1986; Shimomaye & Salvato, 1989). Another approach suggested was simply to avoid areas of potential secondary structure predicted by computer analysis (Pallansch et al., 1990).

Escherichia coli DNA polymerase I (*E. coli* pol I) has been shown to exhibit limited RT activity (Karkas et al., 1972; Karkas, 1973; Loeb et al., 1973). Early studies with *E. coli* pol I suggested that higher levels of RT activity were achieved by using Mn^{2+} as the divalent metal ion when nuclease-treated poly(C)·oligo(I) was used as a template (Karkas, 1973). This phenomenon appears to be highly template dependent, however, since approximately equal levels of incorporation were observed by using either Mg^{2+} or Mn^{2+} and poly(A)·poly(dT) as template (Karkas et al., 1972). Additionally, the ability of *E. coli* pol I to copy natural RNAs was demonstrated to be completely dependent on the presence of Mg^{2+} (Loeb et al., 1973). Overall, the general applicability of this enzyme for cDNA synthesis has been minimal due to the large quantity of enzyme required and the small size of the products syn-

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thesized (Gulati et al., 1974).

The need for a thermostable RT to possibly alleviate some of the problems associated with cDNA synthesis has been recognized (Chien et al., 1976). The elevated reaction temperature required for an optimally active thermostable enzyme would increase the specificity of primer extension and presumably destabilize some of the secondary structure present in the RNA template. An early study using a polymerase activity from *Thermus aquaticus* (Kaledin et al., 1980) reported synthesis with a poly(rA)·oligo(dT)$_{10}$ template, but did not detect cDNA synthesis with mRNA·oligo(dT)$_{10}$ or poly(rC)·oligo(dG)$_{12-18}$ templates. Similarly, one of three DNA polymerase activities isolated from *Thermus thermophilus* had a limited capacity to incorporate deoxyribonucleotides at 37 °C into acid-precipitable material with poly(rA)·(dT)$_{10}$ as template (Rüttimann et al., 1985). However, characterization of the reaction products was not performed. The three isoenzymes were reported to have approximate molecular masses of 110 kDa, as determined by glycerol gradient centrifugation. In contrast, polypeptides of 77, 40, and 17 kDa were observed when fractions of the glycerol gradient coincident with polymerase activity were analyzed by electrophoresis on a denaturing polyacrylamide gel. The polymerases were found to lack exonuclease activity and apparently were optimally active at low salt concentrations. The *Tth* pol used in the present study is a single 94-kDa polypeptide[1] that has 5′→3′ exonuclease activity[2] and is optimally active at high salt concentrations. Given the dissimilarities in the physical and enzymological characteristics reported by Rüttimann et al. (1985) and the data presented below, it is doubtful that the *Tth* pol used in the present study is derived from the proteins previously reported.

The large amplification obtained by the PCR can partially compensate for a less efficient reverse transcription reaction. The use of *Taq* pol for both cDNA synthesis and subsequent amplification in a PCR has been reported (Jones & Foulkes, 1989; Shaffer et al., 1990). However, neither the reverse transcription reaction nor the PCR amplification was well characterized and large amounts (1–5 μg) of RNA were used. A more comprehensive study reported that *Taq* pol was capable of reverse transcription and amplification from as little as 40 ng of starting RNA, although Southern analysis was required for detection of low target levels (Tse & Forget, 1990). We have investigated the use of DNA polymerases from both *T. aquaticus* and *T. thermophilus* for their applicability in a coupled reverse transcription reaction and PCR amplification. The *Tth* pol was found to be considerably more efficient than *Taq* pol in this study, and the sensitivity of the method was determined to be at least 200-fold greater than the limit of detection previously reported for *Taq* pol (Tse & Forget, 1990). The high degree of specificity and sensitivity, in addition to the convenience of *Tth* pol performing both cDNA synthesis and PCR amplification, should find wide applicability in diagnostics and molecular biology.

Experimental Procedures

Enzymes. AmpliTaq DNA polymerase (330 units/μL) and recombinant *Tth* pol (1697 units/μL, 13.9 units/pmol) were provided by Perkin-Elmer Cetus Instruments. Unit definitions provided by Perkin-Elmer Cetus are based upon DNA polymerase activity utilizing activated salmon sperm DNA as template. These enzymes were diluted in storage buffer [20 mM Tris-HCl (pH 7.5), 100 mM KCl, 50% glycerol (v/v), 0.1 mm EDTA, 1 mM dithiothreitol, and 0.2% Tween 20] to obtain concentrations that provided one-tenth reaction volume as enzyme.

DNA, Oligodeoxynucleotides, and Nucleotides. A 1-kb DNA ladder was obtained from BRL and radiolabeled as suggested by the manufacturer with [γ-^{32}P]ATP and T4 polynucleotide kinase. Oligodeoxynucleotides d(T)$_{16}$, DM152 (5′-CATGTCAAATTTCACTGCTTCATCC-3′), TM01 (5′-GCTTGCAAGCTTTATTTAGTTATGACTGATAACACTC-3′), and DM151 (5′-GTCTCTGAATCAGAAATCCTTCTATC-3′) were synthesized on a Biosearch 8700 DNA synthesizer, purified by polyacrylamide gel electrophoresis, and stored in distilled H_2O. 2′-Deoxynucleoside 5′-triphosphates were obtained from Perkin-Elmer Cetus Instruments.

RNA Preparation. The plasmid pAW109 contains a synthetic linear array insert of primer sequences for multiple target genes and a polyadenylated sequence. The insert in pAW109 is identical to the insert described for plasmid pAW108 (Wang et al., 1989). Run-off transcription of *Bam*HI-linearized pAW109 by T7 RNA polymerase and purification of pAW109 cRNA were performed as previously described (Wang et al., 1989). pAW109 cRNA dilutions were made with 30 μg/mL *E. coli* rRNA included as carrier. Total cellular RNA from the K562 cell line (Lozzio & Lozzio, 1975) was isolated as described (Sambrook et al., 1989).

RT Assay. A mixture (12 μL) containing 16.7 mM Tris-HCl (pH 8.3), 150 mM KCl, 0.5 pmol of pAW109 cRNA, and 2.5 pmol of oligodeoxynucleotide TM01 was preincubated for 5 min at 70 °C. Reactions were initiated by the addition of a mixture (8 μL) containing 2.5 mM $MnCl_2$, 500 μM each of dATP, dGTP, dTTP, and [α-^{32}P]dCTP (2.5 Ci/mmol), and the indicated amounts of *Tth* pol. Final reactions (20 μL) were incubated for 10 min at 70 °C and terminated by chilling on ice and adding 30 μL of 60 mM EDTA. The radiolabeled cDNA was then separated from unincorporated [α-^{32}P]dCTP by centrifugation through Sephadex G-50 (Penefsky, 1977). Incorporation of [α-^{32}P]dCMP was detected by Cerenkov radiation. The product size was then determined by electrophoretic separation of aliquots containing approximately equal amounts of radioactivity on a denaturing 6% polyacrylamide/7 M urea gel and analysis by autoradiography.

RT/PCR Coupled Reactions. RT reactions (20 μL) containing 10 mM Tris-HCl (pH 8.3), 90 mM KCl (40 mM for reactions containing AmpliTaq DNA polymerase), 1.0 mM $MnCl_2$, 200 μM each of dATP, dCTP, dGTP, and dTTP, 15 pmol of either primer TM01 or DM152 or 50 pmol of d(T)$_{16}$, 5 units of either *Tth* pol or AmpliTaq DNA polymerase, and the indicated amounts of target template were overlaid with 75 μL of mineral oil and incubated for 15 min at 70 °C (samples containing d(T)$_{16}$ were incubated for 5 min at 42 °C followed by 10 min at 70 °C). Following the RT reaction, 80 μL of a solution containing 10 mM Tris-HCl (pH 8.3), 100 mM KCl (50 mM for reactions containing AmpliTaq DNA polymerase), 1.88 mM $MgCl_2$, 0.75 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA), 5% glycerol (v/v), and 15 pmol of primer DM151 were added [15 pmol of primer DM152 was also added to reactions that utilized d(T)$_{16}$ for the RT reaction]. The samples (100 μL) were then amplified in a Perkin-Elmer Cetus Instruments DNA Thermal Cycler by using four linked files as follows: File 1, STEP-CYCLE 2 min at 95 °C for 1 cycle; file 2, STEP-CYCLE 1 min at 95 °C and 1 min at 60 °C for 35 cycles; file 3, STEP-CYCLE 7 min at 60 °C for 1 cycle; file 4, SOAK 5 min at 4 °C.

[1] D. H. Gelfand, unpublished data.
[2] R. D. Abramson, unpublished data.

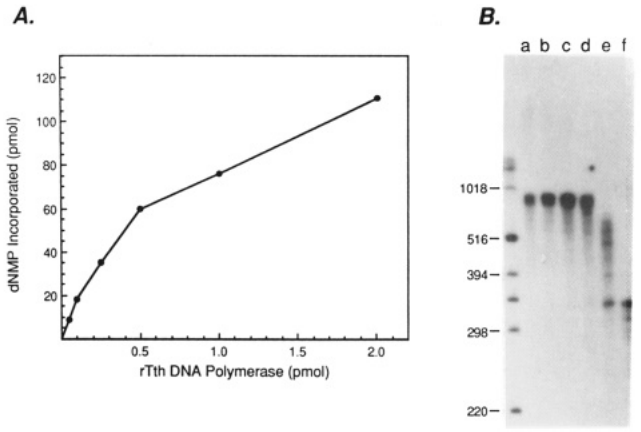

FIGURE 1: Reverse transcription by *Tth* pol. cDNA synthesis with increasing levels of *Tth* pol was carried out on TM01-primed pAW109 cRNA (0.5 pmol) as described under Experimental Procedures. Reverse transcription levels were measured by the incorporation of [α-^{32}P]dCMP into cDNA (panel A). Aliquots of the RT reactions were then analyzed by electrophoresis on a denaturing 6% polyacrylamide gel (panel B). Lanes a–f correspond to 2, 1, 0.5, 0.25, 0.1, and 0.05 pmol, respectively, of *Tth* pol present during the RT reaction. A 1-kb ladder was used as a size marker.

Aliquots (5 μL) of the PCR amplifications were analyzed by electrophoresis on a 2% (w/v) NuSieve/1% (w/v) SeaKem agarose (FMC) gel stained with ethidium bromide.

Results and Discussion

Initial characterization of RT activity in *Tth* pol was performed by determining the relative amounts of incorporation of dNMP into cDNA and the length of product formed with the synthetic pAW109 cRNA as a template. This RNA (963 nucleotides in length) provides a template of 889 nucleotides when the primer TM01 is utilized for cDNA synthesis. The use of recombinant *Tth* pol eliminated concerns about contaminating thermoactive RTs or RNases. We found that $MnCl_2$ was far superior for reverse transcription than either $MgCl_2$ or $CoCl_2$ (data not shown). However, a significant amount of the cDNA being synthesized was much shorter than full-length. The accumulation of these smaller framents increased proportionately with time, temperature, and $MnCl_2$ concentration. This phenomenon was determined to be due to divalent metal ion catalyzed hydrolysis of the RNA, which created smaller templates that became available for further cDNA synthesis (data not shown). Although the ability of metal ions to cause degradation of RNA has been described (Brown, 1974), the process becomes increasingly more problematic at the elevated temperatures required for the thermoactive enzymes.

In order to minimize the extent of RNA hydrolysis, relatively high concentrations of enzyme, moderate $MnCl_2$ concentrations, and short reaction times were used for the reverse transcription reaction. A near linear response in dNMP incorporation during cDNA synthesis is observed when the molar ratio of *Tth* pol to template is less than 1 (Figure 1A). The data suggests that *Tth* pol utilizes an RNA template more efficiently than *E. coli* pol I, since it has been reported that *E. coli* pol I requires a 4-fold molar excess of enzyme over template for reverse transcription (Gulati et al., 1974). Analysis of the cDNA products by denaturing polyacrylamide gel electrophoresis revealed that in order to achieve predominantly full-length cDNA, the enzyme to template ratio needed to be near 1 (Figure 1B). The presence of a specific banding pattern at the lower concentrations of *Tth* pol suggests that the enzyme is only moderately processive on RNA templates.

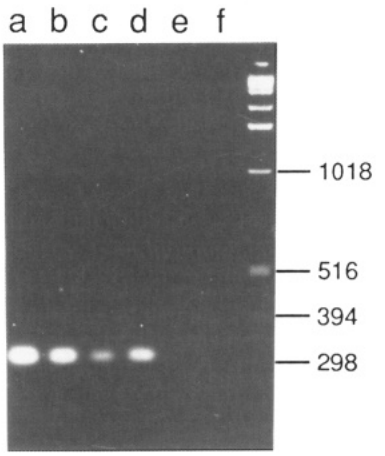

FIGURE 2: Reverse transcription and PCR amplification by either *Tth* pol or AmpliTaq. cDNA synthesis using DM152-primed pAW109 cRNA and subsequent amplification with primer pair DM152/DM151 were performed as described under Experimental Procedures. *Tth* pol (lanes a–c) or AmpliTaq DNA polymerase (lanes d–f) were used for both cDNA synthesis and PCR amplification from 10^6, 10^5, or 10^4 copies of pAW109 cRNA (lanes a and d, b and e, and c and f, respectively). PCR amplifications were analyzed by electrophoresis on 2% (w/v) NuSieve/1% (w/v) SeaKem agarose stained with ethidium bromide.

Since most applications involving the use of a reverse transcriptase typically have relatively low levels of specific target template present, we felt that modest concentrations of *Tth* pol would provide sufficient RT activity and that the RT reaction could be coupled to a PCR for amplification of the resulting cDNA.

The pAW109 cRNA contains primer binding sites constructed such that "upstream" primers are followed by complementary sequences to their "downstream" primers in the same order (Wang et al., 1989). Amplification of the segment specific for IL-1α primers DM151 and DM152 generates a 308 base pair (bp) PCR product. We found that the concentration of *Tth* pol required for optimal RT activity with samples containing relatively high concentrations of nontarget nucleic acids (up to 250 ng) was excessive for PCR amplification, and a large amount of nonspecific product was generated (data not shown). Only specific products were observed if the RT reaction was diluted 5-fold prior to PCR amplification (Figure 2). The buffer, KCl, and glycerol concentrations were kept constant for both the RT reaction and the PCR amplification, and no additional dNTPs were added. The addition of a 3-fold molar excess of EGTA was included to preferentially chelate Mn^{2+}, since EGTA has a much lower affinity for Mg^{2+} than Mn^{2+} (Blanchard, 1984). $MgCl_2$ was added to a concentration optimal for this primer/template (1.5 mM).

Since *Taq* pol had previously been reported to synthesize cDNA inefficiently with Mg^{2+} as the divalent metal ion (Jones & Foulkes, 1989; Shaffer et al., 1990; Tse & Forget, 1990), we compared the amount of product formation when either *Tth* pol (Figure 2, lanes a–c) or *Taq* pol (Figure 2, lanes d–f) was used in coupled RT/PCR amplifications of pAW109 cRNA with Mn^{2+} as the divalent metal ion. The only difference between the *Tth* pol and *Taq* pol reactions was the KCl concentration, which was 100 and 50 mM for the two enzymes, respectively, in each reaction. These salt concentrations were found to give optimal reverse transcription and PCR amplification under these conditions for the two enzymes (data not shown). The *Tth* pol gave a detectable signal starting with 10^4 copies of target pAW109 cRNA (Figure 2, lane c). Product was not observed for the *Taq* pol at even 10^5 copies,

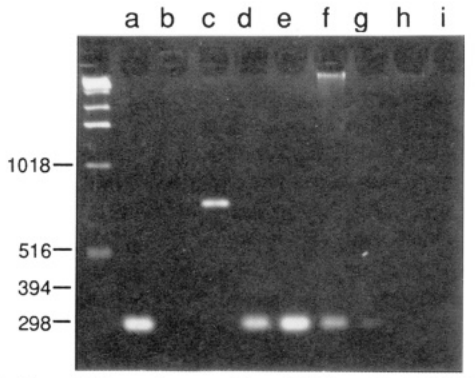

FIGURE 3: Sensitivity and versatility of *Tth* pol in RT/PCR with synthetic cRNA. cDNA synthesis and amplification were performed as described under Experimental Procedures. Reverse transcription reactions were performed by using either primer DM152 (lanes b and e–i), TM01 (lane c), or $d(T)_{16}$ (lane d). Amplification was performed by using either primer pair DM152/DM151 (lanes a, b, and d–i) or TM01/DM151 (lane c). Reverse transcription reactions were performed on 10^4 copies (lanes b–e), or 1000, 500, 100, or 0 copies of pAW109 cRNA (lanes f–i, respectively). A control reaction for contaminating DNA was performed by incubating the RT reaction at 4 °C followed by standard PCR amplification (lane b). PCR amplification was also performed on 10^4 copies of pAW109 plasmid DNA (lane a). Analysis of PCR products was performed as described for Figure 2.

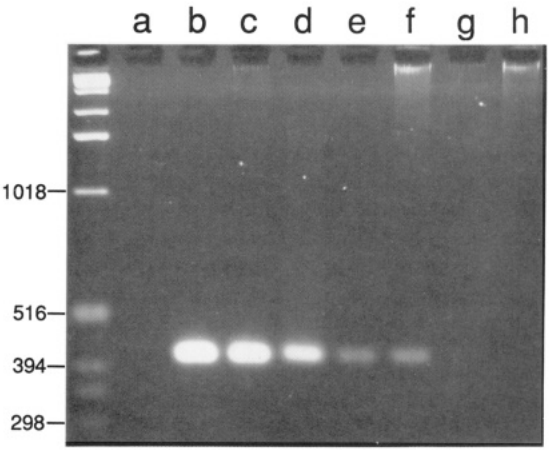

FIGURE 4: *Tth* pol RT/PCR of IL1-α mRNA from total cellular RNA. Reactions were performed as described under Experimental Procedures. RT reactions contained 250, 250, 50, 10, 2, 0.4, 0.08, and 0 ng of K562 total cellular RNA (lanes a–h, respectively), and primer DM152 was used. The sample in lane a was incubated at 4 °C (vs 70 °C) during the RT reaction. Amplification was performed by using primer pair DM152/DM151. Analysis of PCR products was performed as described for Figure 2.

although lower limits of detection would be expected if hybridization of labeled oligonucleotides was used for detection. The lanes shown in Figure 2 are representative of reactions that were performed in triplicate to control for any possible well-to-well variation in the thermocycler. No contaminating pAW109 DNA could be detected in the pAW109 cRNA when amplified by *Tth* pol (see below and Figure 3, lane b). These results clearly demonstrate that the product results from RNA rather than contaminating DNA and that, under similar reaction conditions, the *Tth* pol provides at least 100-fold greater sensitivity than the analogous *Taq* pol in a coupled reverse transcription/PCR amplification. Amplification of pAW109 DNA by *Taq* pol is comparable to or possibly better than that by *Tth* pol (data not shown), suggesting that the enhanced sensitivity of the coupled RT/PCR achieved by *Tth* pol results from increased RT activity and not merely an increase in PCR amplification efficiency.

The explanation for the differential RT activities observed for *Tth* pol and *Taq* pol remains unclear. Significant amino acid sequence similarity exists between *Tth* pol (834 amino acids) and *Taq* pol (832 amino acids). One possible sequence alignment yields 93% similarity and 88% identity between the two polymerases.[1] Both *Taq* pol (Gelfand, 1989; Longley et al., 1990) and *Tth* pol[2] possess a 5′→3′ exonuclease activity. *Taq* pol (Tindall & Kunkel, 1988; Lawyer et al., 1989) does not have 3′→5′ exonuclease activity, and on the basis of amino acid sequence comparisons, *Tth* pol is also presumed to lack a proofreading exonuclease. Although these two enzymes are very similar in amino acid sequence and enzymatic properties, several characteristics such as salt sensitivity and the ability to utilize RNA templates appear to differ. These differences may result from minor differences in amino acids in or around conserved regions or domains responsible for divalent metal ion binding or primer/template binding.

The strong signal produced by the *Tth* pol coupled RT/PCR in Figure 2 suggested that the detection limit was much lower than 10^4 copies. Figure 3, lanes e–h, demonstrates that products are clearly visible (by ethidium bromide staining) from 10^4 to 100 copies of pAW109 cRNA. The use of primer TM01 for reverse transcription and the primer pair TM01/DM151 for PCR amplification generates a specific product of 735 bp, demonstrating the ability to synthesize longer cDNA (Figure 3, lane c). A negative control lane (Figure 3, lane i) indicates that product was not formed when pAW109 cRNA was omitted and only *E. coli* rRNA was used. To ensure that pAW109 DNA was not present in the cRNA preparations, a sample containing 10^4 copies of cRNA was incubated at 4 °C rather than 70 °C and then thermocycled (Figure 3, lane b vs lane e). In order to generate a PCR product, the cDNA synthesis reaction must be performed at elevated temperatures, demonstrating that the products generated from the cRNA were in fact from RNA and not from contaminating DNA. Although we cannot rule out that some contaminating pAW109 DNA is present in the cRNA, we have been unable to detect product following PCR amplification of similar mock RT reactions with up to 10^8 copies of cRNA (data not shown). The ability of *Tth* pol to form detectable PCR product from 100 copies of RNA with $MnCl_2$ vs the absence of detectable product when $MgCl_2$ and standard PCR conditions are used implies a $>10^6$-fold increase in sensitivity imparted by $MnCl_2$. The product resulting from the amplification of 10^4 copies of pAW109 cRNA (Figure 3, lane e) is comparable to 10^4 copies of pAW109 DNA (Figure 3, lane a), although precise quantitation would require measurements during the exponential phase of amplification (Wang et al., 1989).

Oligo dT is often used as a primer for reverse transcription of poly(A+) RNA. Although the *Tth* pol is only marginally active at temperatures low enough for $d(T)_{16}$ to anneal, sufficient RT activity is present to extend $d(T)_{16}$ at 42 °C. Following a brief incubation at 42 °C, the reaction temperature can be raised (Figure 3, lane d). The use of oligo(dT) for reverse transcription does not appear to generate as much product as the specific primer DM152, but the difference appears to be less than a factor of 10.

The coupled RT/PCR was extremely sensitive with the highly purified synthetic RNA, and this template enabled the optimal reaction parameters for both the reverse transcription and PCR amplification to be determined for *Tth* pol. The ability to reverse transcribe more complex samples was then tested. The primer pair DM152/DM151 was used to detect IL-1α mRNA in total cellular RNA isolated from the K562 cell line (Figure 4). A 420-bp product was clearly visible when

starting with 400 pg of total RNA (lane f), and a weak signal was detectable at 80 pg (lane g), which is approximately 10 cell equiv. The detection level may be even lower if carrier nucleic acid is added to the RNA samples to prevent nonspecific binding of the RNA to tubes. Another consideration is that only 5% of the PCR amplification was analyzed on the gel. The sensitivity of the procedure could undoubtedly be increased beyond the 80-pg detection limit (when stained with ethidium bromide) by increasing the percentage of the PCR analyzed and/or employing hybridization detection techniques. The lack of a detectable signal after incubation of the RT reaction at 4 °C (lane a) demonstrates that, in addition to the stimulatory effect that Mn^{2+} has on reverse transcription, contaminating PCR product, cDNA, or genomic DNA is not being amplified with the primer pair DM152/DM151.

The experiments presented here demonstrate that *Tth* pol is useful for the reverse transcription and PCR amplification of very low levels of RNA. The *Tth* pol appears to be more efficient at cDNA synthesis than the homologous DNA polymerase from *T. aquaticus*. The coupled RT/PCR previously reported for *Taq* pol (Jones & Foulkes, 1989; Shaffer et al., 1990; Tse & Forget, 1990) is considerably less sensitive than the methodology described here with *Tth* pol. The increased efficiency in reverse transcription gained by using $MnCl_2$ as the divalent metal ion may need to be weighed against a possible decrease in the fidelity of cDNA synthesis. The negative effect of Mn^{2+} on the fidelity of DNA synthesis has been well documented for *E. coli* pol I (Beckman et al., 1985). However, the fidelity of viral reverse transcriptases has also been shown to be rather low (Roberts et al, 1989). Templates containing large amounts of secondary structure are often difficult, if not impossible, for viral RTs to reverse transcribe. The ability of *Tth* pol to synthesize cDNA on these RNA templates at elevated reaction temperatures may compensate for any loss in fidelity. The need for highly accurate cDNA in molecular cloning suggests that sequencing of several separate RT/PCR amplifications is advisable. The addition of EGTA to preferentially chelate Mn^{2+} would be expected to minimize any effects that the Mn^{2+} may have on the fidelity of the enzyme during the PCR amplificaton. Additionally, the low dNTP and Mg^{2+} concentrations described in these experiments may increase the fidelity of *Tth* pol during PCR amplification since similar conditions were demonstrated to increase the fidelity of *Taq* pol (Eckert & Kunkel, 1990). We are currently further characterizing the RT activity of *Tth* pol and other thermoactive DNA polymerases, in addition to investigating the issues pertaining to the fidelity of reverse transcription and PCR amplification.

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Articles

Biological Thiols Elicit Prolactin Proteolysis by Glandular Kallikrein and Permit Regulation by Biochemical Pathways Linked to Redox Control†

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ABSTRACT: Rat glandular kallikrein (GK), a trypsin-like serine protease, cleaves rat prolactin (PRL) in vitro to novel forms detectable in vivo and likely to be of physiological significance. PRL proteolysis by GK is thiol-dependent, with thiols acting upon PRL to refold the molecule into novel conformations that are GK substrates. This study compared several natural and synthetic thiols for their ability to elicit PRL proteolysis by GK. Rat PRL was incubated with rat GK in the presence of various thiols and 0.5% Triton X-100, which enhances thiol-elicited proteolysis. Cleavage was analyzed by gel electrophoresis under reducing and nonreducing conditions. In the presence of Triton X-100, all low molecular weight thiols elicited PRL cleavage by GK. The order of potency was dithiothreitol > mercaptoethanol > lipoic acid > cysteamine = glutathione (GSH) = coenzyme A > cysteine. In the absence of Triton, however, dithiothreitol, coenzyme A, and mercaptoethanol were most effective in eliciting GK proteolysis. Triton X-100 enhanced PRL cleavage by 4-19-fold, depending upon the thiol used. Folding isomers of processed PRL observed following cleavage included disulfide-liked homodimers, oxidized monomers, reduced monomers and mixed disulfides; the folding isomers generated varied depending upon the thiol used. GSH potency in eliciting PRL proteolysis increased 10-fold in the presence of biochemical pathways shuttling reducing equivalents to GSH disulfide (GSSG). PRL cleavage by GK could be controlled by substrates, enzymes, and cofactors making up the reducing shuttle when GSSG was used. Thioredoxin (a protein disulfide oxidoreductase) potently elicited PRL proteolysis by GK in the presence of a reducing shuttle and Triton X-100. Thioredoxin was about 400 times more potent than GSSG under such conditions. The results document that biological thiols can elicit PRL proteolysis by GK and permit control of the reaction by biochemical pathways linked to redox control.

Glandular kallikrein (GK;[1] EC 3.4.21.35) is the prototypical member of a distinct family of serine proteases that appears to function in the specific biosynthetic processing of bioactive peptides. Other members of this family include the γ-subunit of nerve growth factor and epidermal growth factor binding proteins (MacDonald et al., 1988). GK is a major estrogen-induced enzyme in the rat anterior pituitary (Powers & Nasjletti, 1984; Clements et al., 1986; Powers, 1986; Chao et al., 1987; Hatala & Powers, 1987). Anterior pituitary GK has been hypothesized to process prolactin (PRL) to novel forms with unique hormonal roles. This hypothesis has evolved from evidence documenting unique tissue-specific regulation paralleling PRL (Powers & Hatala, 1986; Powers, 1987; Hatala & Powers, 1988), a subcellular localization ideally suited for a role in precursor processing (Golgi apparatus and secretory granules) (Hatala & Powers, 1989), and a cellular localization in PRL-producing cells but not other anterior pituitary cell types (Vio et al., 1990; Kizuki et al., 1990; Kitagawa et al., 1990). However, pituitary GK predominantly exists as a latent zymogen that can be activated with trypsin (Powers, 1986) and does not undergo activation during estrogen or dopamine regulation (Powers, 1986; Powers & Hatala, 1986; Kitagawa et al., 1990), during secretion (Chao & Chao, 1988), or within specific organelles (Hatala & Powers, 1989). Such latency has obscured the role of GK in the pituitary.

In an effort to resolve the role of anterior pituitary GK, we recently examined the ability of active GK (purified from rat urine) to cleave PRL in vitro. GK did not cleave standard rat PRL, but proteolysis was elicited with the thiols dithiothreitol and mercaptoethanol, and the thiol effects were enhanced by nonionic detergents such as Triton X-100 (Powers & Hatala,

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[1] Abbreviations: GK, glandular kallikrein; GSH, glutathione; GSSG, glutathione disulfide; PRL, prolactin.

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