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Dimethyl Sulfoxide-Mediated Primer T_m Reduction: A Method for Analyzing the Role of Renaturation Temperature in the Polymerase Chain Reaction

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We report a method for optimizing the specificity of product formation in the polymerase chain reaction (PCR). This technique is based on the use of dimethyl sulfoxide (DMSO) and takes into account primer T_m. The reduction in T_m by DMSO is directly correlated with renaturation temperature such that a DMSO gradient reflects a temperature gradient. We use this relationship to show that optimum product formation usually occurs at or within several degrees of the midpoint T_m of a given primer pair. We illustrate these correlations using three examples deriving PCR products from a human cDNA library, representing the casein kinase II α and β subunits as well as the 5′ untranslated region for the β subunit. By following product formation as a function of renaturation temperature, we postulate rules for cycle design based on primer T_m. Implications for the use of degenerate primers are discussed. © 1993 Academic Press, Inc.

The polymerase chain reaction is one of the most important tools currently in use in molecular biology. However, amplification of heterologous substrates often requires optimization of the reaction by empirical methods. Satisfactory reaction products have been obtained by varying parameters such as the concentrations of magnesium ions, deoxyribonucleotides, enzyme, and primers (1–4). Variations in cycle parameters, in particular annealing temperature, have the potential to improve product specificity (1,3). Recently, a method has been reported for optimizing product yield and specificity by calculating the optimal renaturation temperature for a given reaction. This optimal value is derived from both primer and product T_m (5). The T_m of duplex nucleic acid is classically defined as the midpoint of melting or 50% denaturation as measured by a hyperchromic absorbance shift (6).

There have been reports (7–9) that the addition of cosolvents such as dimethyl sulfoxide (DMSO),[2] formamide, and tetramethylammonium chloride (TMACl) improves reaction yields and specificity. However, use of these reagents remains empirical, and it is not known which parameters of the reaction are affected. For example, these reagents may affect primer T_m, the catalytic activity of the enzyme, or template and product structure (1,9). In this report, we show that DMSO has its primary affect on primer T_m, and we demonstrate a linear relationship between DMSO concentration and the reduction of primer T_m. We exploit this correlation such that DMSO gradients become temperature windows where it is possible to follow product formation as a function of renaturation temperature. Examination of these windows shows a relationship between product formation and theoretical primer T_m. A method utilizing primer pair midpoint T_m to develop appropriate windows is presented.

We apply this procedure to the ubiquitous protein serine/threonine kinase, casein kinase II (CKII). Casein kinase II is present in all eukaryotic organisms (10); the holoenzyme is a tetramer with the subunit structure $\alpha_2\beta_2$ (10). Two forms of the catalytic subunit are known (11,12) and are designated α and α'. The noncatalytic subunit is designated β and is thought to be regulatory in nature (13,14). The method presented has allowed us

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[2] Abbreviations used: DMSO, dimethyl sulfoxide; TMACl, tetramethylammonium chloride; CKII, casein kinase II; PCR, polymerase chain reaction.

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to generate cDNAs for use as probes as well as for generating bacterial and mammalian expression constructs suitable for structure-function studies.

METHODS

Oligonucleotides

Oligonucleotides were synthesized by the solid state method on an Applied Biosystems Inc. 380A DNA synthesizer. After ammonium hydroxide cleavage and deprotection, oligonucleotides were dissolved in H_2O and purified using C_{18} Sep Paks (Waters/Millipore) according to a published method (15).

PCR Reactions

Reactions were carried out in 50 mM KCl, 10 mM Tris-HCl (pH 8.30 at room temperature), and 1.5 mM $MgCl_2$. Oligonucleotides were added to 50 pmol each, deoxyribonucleotides were added to 100 μM each, and template was 20 ng per reaction. Reaction volumes were 50 μl and contained 1 unit of *Taq*I polymerase (Perkin-Elmer/Cetus). For the puc5′ + CK2B2 primer pair experiment, 25-μl reaction volumes contained $\frac{1}{2}$ the amount of enzyme, oligonucleotides, and template. The template was a human glioblastoma λ ZAP (Stratagene) cDNA library constructed by Dr. Carmen Birchmeier (Max-Delbrueck-Laboratorium in der Max-Planck-Gesellschaft, Cologne, Germany).

DMSO Additions

Aliquots of 83% DMSO (v:v) were diluted to different concentrations with water, and the other components of the reaction were combined and added subsequent to the DMSO dilutions. All manipulations were carried out in a dedicated area to avoid contamination, and pipettors were not exposed to products of reactions or to cloned DNA. Reactions minus template were utilized as controls to monitor for contamination. All experiments were carried out on a Perkin-Elmer/Cetus N801-0150 DNA thermocycler.

Calculations

The T_m of primers was calculated according to formulas: (i) that derived for large DNA molecules (6): $T_m = 69.3 + 0.41\ (GC)\% - 650/L$, where L is the number of nucleotides; and (ii) that derived by nearest neighbor base pair analysis of oligonucleotides (5): T_m primer = $(\Delta H/\Delta S - R \ln(c/4)) - 273.15 + 16.6 \log[K^+]$, where ΔH and ΔS are nearest neighbor enthalpy and entropy values respectively (16), R is the molar gas constant (1.987 cal/°C mol), $c/4$ = 250 fM, and $[K^+]$ is the potassium ion concentration. Primer T_m reduction was taken as 0.6°C/1% for DMSO (17). As an example, 1 μl of 83% DMSO in a 50-μl reaction corresponds to ($1/50 \times 0.83 \times 100 \times 0.60$) a 1.0°C reduction in T_m. Midpoint primer T_m was calculated as (primer 1 T_m + primer 2 T_m)/2. Optimal annealing temperatures were calculated according to (5): $T_a^{OPT} = 0.3T_m^{primer} + 0.7T_m^{product} - 14.9$, where $T_m^{product} = 81.5 + 0.41\ (GC)\% + 16.6 \log[K^+] - 675/l$, where l is the length of the DNA fragment. We note that the formulas of Rychlik *et al.* (5) contained three apparent errors: (i) $\Delta S + R \ln(c/4)$ should be $\Delta S - R \ln(c/4)$ in the denominator of their primer T_m formula; (ii) the value for $c/4$ should be 250 fM, not 250 pM; and (iii) the formula for product T_m requires the term 81.5 added to it, as described by Baldino *et al.* (18). These corrections were tested by actually using the values for ΔH and ΔS as listed by Breslauer *et al.* (16) to obtain the resulting T_m and T_a^{OPT} values listed in Rychlik *et al.* (5).

TABLE 1

α Subunit DMSO Addition Scheme

Reaction:	1	2	3	4	5	6	7	8	9	10
%DMSO (v:v)	0	1.7	3.3	5.0	6.6	8.3	10.0	11.6	13.3	15.0
T_m red. (°C)	0	1	2	3	4	5	6	7	8	9
Effective T (°C)	72	73	74	75	76	77	78	79	80	81

Cloning and Sequencing

Cloning and sequence analysis was done by established methods (19). The CKII α subunit fragment was phosphorylated and cloned directly into dephosphorylated *Sma*I-digested pUC118 vector (20). The CKII β subunit DNA was treated identically. The β 5′ untranslated region fragment was gel purified, digested with *Eco*RI (there exists a recognition site at position 205 (21)), and cloned into *Sma*I/*Eco*RI-digested pUC118. Plasmid DNA was denatured with 0.2 N NaOH and sequenced with appropriate combinations of primers using Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH). All DNA modification enzymes are from New England Biolabs.

RESULTS

Correlation between DMSO-Mediated T_m Reduction and Renaturation Temperature

Oligonucleotides CK2A1 and CK2A2 were designed to prime the coding region of the α subunit excluding carboxy terminal amino acid residues 302 to 391. These primers are 41mer and extend from nucleotide positions 154 to 194 and 1021 to 1061, respectively, of the human cDNA sequence (22). DMSO was added in increasing concentrations to 50-μl reaction volumes as indicated in Table 1. An initial denaturation step of 5 min at 95°C was followed by a two-step cycle routine denaturing at 95°C for 1 min 30 s and renaturing/elongating at 72°C

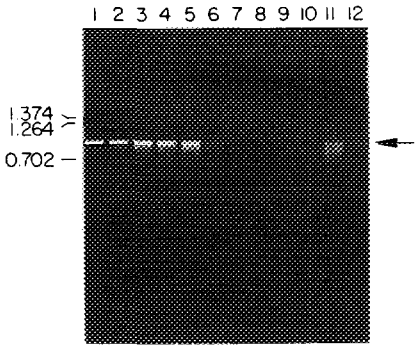

FIG. 1. Synthesis of the amino terminal coding region of the α subunit. Aliquots (10 μl) of reactions were run on a 1.5% agarose gel stained with ethidium bromide. Reactions were run as described in the text. Lanes 1–10 correspond to reactions 1–10, lanes 11 and 12 to the 77 and 78°C equivalent reactions. Selected λ *Bst*EII digest markers are indicated, and product is indicated by an arrow.

for 10 min. A total of 35 cycles were performed followed by a return to 4°C. Aliquots from reactions were subjected to agarose gel electrophoresis as described in the legend to Fig. 1. The expected product size was 907 bp and a band of this size is seen in lanes 1–6 of Fig. 1. Sequence data from one clone derived from a similar experiment showed identity except for a one-base substitution to the previously published sequence (22). This mutation was not reproducible and therefore was considered to be a *Taq*I misincorporation error.

We were interested in relating DMSO-induced T_m reduction to renaturation temperature. Therefore, we calculated percentages of DMSO (v:v) that would correspond to integral ΔT_m values. As shown in Table 1, incremental increases in DMSO are associated with cumulative theoretical T_m reductions of 1.0°C. A two-step cycle procedure was chosen because of its reduced complexity compared to other cycle routines. This was desirable for interpretation of reagent affects that can, in principle, occur at any step in a PCR cycle.

Figure 1 shows a decline in product synthesis as a function of added DMSO. A synthesis transition occurs between reactions 6 and 7 (lanes 6 and 7, Fig. 1), such that reaction 6 has low levels of product and reaction 7 exhibits no visible synthesis. This product formation transition could be due to either DMSO inhibition of the Taq polymerase (23) or to reagent affects on T_m. As shown in Table 1, reactions 6 and 7 correspond to T_m reductions of 5 and 6°C, respectively. We sought to reproduce these reactions by eliminating DMSO and raising the renaturation temperature by the same ΔT_m. The same cycle routine was performed as above; in separate experiments, renaturation/elongation temperatures were raised by 5 and 6°C to 77 and 78°C, respectively. These DMSO deficient reactions shown in lanes 11 and 12 of Fig. 1 show a high degree of similarity to their DMSO containing counterparts (lanes 6 and 7, Fig. 1).

TABLE 2

β Subunit DMSO Addition Scheme

Reaction:	1	2	3	4	5	6	7	8	9	10
%DMSO (v:v)	0	1.7	3.3	5.0	6.6	8.3	10.0	11.6	13.3	15.0
T_m red. (°C)	0	1	2	3	4	5	6	7	8	9
Effective T (°C), CK2B1 + CK2B2	68	69	70	71	72	73	74	75	76	77
Effective T (°C), puc5′ + CK2B2	63	64	65	66	67	68	69	70	71	72
Effective T (°C), puc5′ + CK2B3	63	64	65	66	67	68	69	70	71	72

Therefore, the product synthesis transition between reactions 6 and 7 is reproduced by temperature modification in the absence of DMSO. The bottom row of Table 1 indicates the renaturation temperatures produced by DMSO induced primer T_m reduction. These are referred to as effective renaturation temperatures and are theoretical values obtained by adding the calculated DMSO induced T_m reduction value (Methods, Table 1) in °C to the set renaturation temperature in the cycle routine.

The CKII β Subunit Coding Region is Produced Utilizing a Three-Step Cycle in the Presence of DMSO

The β subunit of casein kinase II was used to test if specific product formation would occur with a three-step cycle using a similar DMSO procedure as described for the α subunit. CK2B1 and CK2B2 were constructed as 36mer extending from nucleotide positions 134 to 169 and 752 to 787 of the human cDNA sequence (21). The oligonucleotides were designed to include both the initiation and the termination codons and therefore, the full length coding region product. DMSO was added in increasing concentrations as indicated in Table 2. Denaturation for 5 min at 95°C was followed by a three-step

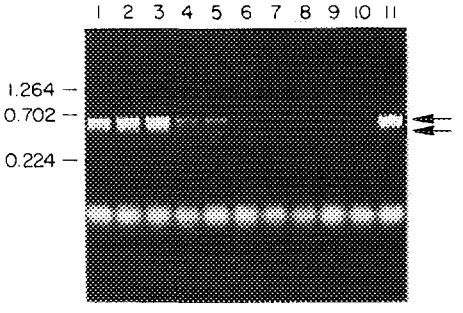

FIG. 2. Synthesis of the coding region for the β subunit. Aliquots (10 μl) of reactions were run on a 1.5% agarose gel stained with ethidium bromide. Reactions were run as described in the text. Lanes 1–10 correspond to reactions 1–10, lane 11 corresponds to the 72°C equivalent reaction. Selected λ *Bst*EII digest markers are indicated. The upper arrow corresponds to β subunit product, the lower arrow to an unknown product.

TABLE 3

Oligonucleotide Sequences and T_m Values for Primers and Primer Pairs

Primer	Primer sequences (5′ to 3′)	T_m[a] (°C)	T_m[b] (°C)
CK2A1	ATGTCGGGACCCGTGCCAAGCAGGGCCAGAGTTTACACAGA	77.5	81.8
CK2A2	TTGTCCAGGAAATCCAAGGCCTCAGGGCTGACAAGGTGCTG	76.5	87.5
CK2B1	ATGAGCAGCTCAGAGGAGGTGTCCTGGATTTCCTGG	74.0	74.5
CK2B2	AGGGAATCAGCGAATCGTCTTGACTGGGCTCTTGAA	71.7	74.4
puc5′	CACACAGGAAACAGCTATGACCATG	63.0	57.2
CK2B3	ATCCACTTCACAGAAGAATTCATTGCCACGGAGCCC	71.7	74.5

	Midpoint T_m (°C)[c]	T_a^{OPT} (°C)[d]
CK2A1 and CK2A2	77.0	63.0
CK2B1 and CK2B2	72.9	63.6
CK2B2 and puc5′	67.4	59.5
CK2B3 and puc5′	67.4	59.7

[a] Primer T_ms calculated according to the method of Marmur and Doty (6).
[b] Primer T_ms calculated according to the method of Rychlik *et al.* (5).
[c] Midpoint T_ms calculated as shown under Methods.
[d] T_a^{OPT} values calculated according to the method of Rychlik *et al.* (5).

cycle routine that consisted of denaturation at 95°C for 1 min 30 s, renaturation at 68°C for 2 min, and elongation at 75°C for 8 min. A total of 35 cycles were carried out, followed by a return to 4°C. Product size was predicted at 653 bp, and a band of this size is seen in lanes 1–6 of Fig. 2 (corresponding to reactions 1–6, Table 2). Following the strategy utilized for α subunit formation, we took the T_m reduction corresponding to reaction 5 (lane 5, Fig. 2 and Table 2) and reset the renaturation temperature from 68 to 72°C. DMSO was eliminated and the same three-step cycle routine was carried out. The desired 653-bp product appears in lane 11 of Fig. 2. In contrast to the previous experiment, this experiment exhibits intense primer dimer formation. The fragment was cloned and the complete sequence analysis of one clone established identity to the published sequence (21). The theoretical midpoint T_m of this primer pair is 72.9°C (Methods, Table 3).

Application to the One-Sided Polymerase Chain Reaction

Using the methods described above, we followed product formation generated by the one-sided polymerase chain reaction (24). We chose to generate the 5′ untranslated region of the CKII β subunit in two rounds of amplification using two sequence specific primers and one nonspecific primer. For the 1st round of amplification, carboxy terminal primer CK2B2 (above) and vector arm primer puc5′ were used. Oligonucleotide puc5′ is a 25mer, homologous to m13 reverse sequencing primers, and will hybridize to any vector containing the appropriate *Lac*I region, including λ ZAP (Stratagene). DMSO was added as standard (Table 2); in this case, reaction volumes were 25 μl and 41.5% DMSO was substituted. The same three-step cycle routine that was used for the β subunit was executed except that renaturation was set at 63°C. As shown in Fig. 3a, there is no visible synthesis at the predicted 882 position in any reaction. A prominent lower molecular weight band of approximately 250 bp appears in lanes 1–5 (reactions 1–5). This product is unaccounted for and bears no obvious relationship to the design of the experiment. Synthesis ceased in reaction 6; this corresponds to an effective temperature of 68°C (lane 6, Fig. 2, and Table 2). The midpoint T_m for this primer pair is 67.4°C. Reaction 5, which corresponded to an effective renaturation temperature of 67°C (Table 2) and to the midpoint T_m, was diluted by 1000-fold and subjected to reamplification. An aliquot of 1 μl was added per reaction and reactions were primed using puc5′ and CK2B3. Oligonucleotide CK2B3 is an antisense amino terminal 36mer corresponding to nucleotide positions 177 to 212 (21). DMSO was added as indicated in Table 2. The cycle routine was identical to the 1st round of amplification and 30 cycles were carried out. Full length product formation is predicted at 308 bp. As shown in Fig. 3b, a band of approximately this size appears in lanes 1–8 (reactions 1–8). This fragment was cloned and sequenced; it is homologous to the known sequence (21) and is truncated by 18 nucleotides 3′ to the *Not*I cloning site.

DISCUSSION

The objective of this study was to relate the effects of DMSO to renaturation temperature and to establish a correlation between the amount of DMSO added and T_m reduction in degrees. To determine this relationship, formation of the CKII α subunit fragment was exam-

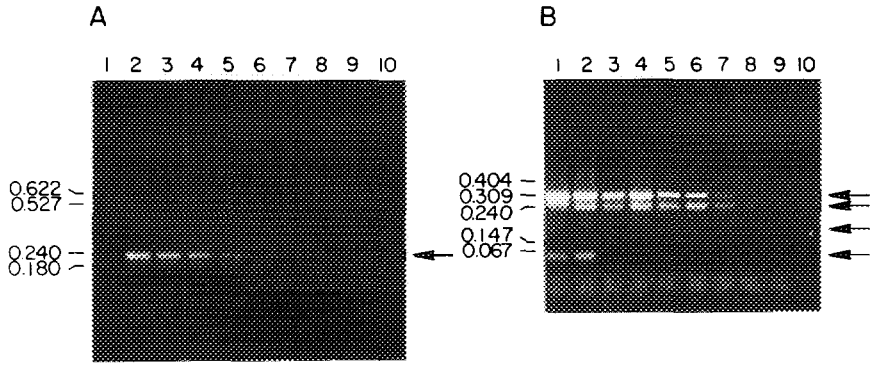

FIG. 3. Synthesis of the 5′ untranslated region of the β subunit. (a) Aliquots (10 μl) of reactions were run on 1.5% agarose gel stained with ethidium bromide. Reactions were run as described in the text. Lanes 1–10 correspond to reactions 1–10. Selected pBR322 *Msp*I digest markers are indicated. (b) Aliquots (10 μl) of reactions were run on a 2.0% agarose gel. Reactions were run as described in the text. Lanes 1–10 correspond to reactions 1–10. Selected pBR322 *Msp*I digest markers are indicated. Arrow in (a) indicates 250-bp product. Arrows in (b) indicate specific 308-bp β 5′ untranslated product, β 5′ untranslated anomalous product, region of nonspecific product formation, and unknown 70-bp product, respectively.

ined at 72°C in various percentages of DMSO (Fig. 1). Within this concentration range of DMSO a steep transition in the levels of product was observed. We questioned whether the transition from low product synthesis to no detectable synthesis was due to DMSO inhibition of the *Taq*I polymerase or to reagent effects on primer T_m. To distinguish between these possibilities, we calculated a putative value for the DMSO-induced ΔT_m using a temperature reduction formula (17). The renaturation temperature was reset accordingly for reactions in the absence of DMSO. A two-step cycle was used to simplify the interpretation because of possible effects of the solvent on a third and separate elongation step in the cycle routine. For these two reactions, the final reaction temperatures were closely predicted by the calculation based on the DMSO data. The results shown here indicate that there is a critical DMSO concentration at which synthesis ceases, and that this product formation transition can be replicated by raising the annealing temperature by a calculated DMSO-induced ΔT_m and eliminating DMSO from the reactions. This experiment indicates that DMSO is not a requirement for the shift in synthesis and that DMSO has its primary effect on primer T_m reduction.

It is important to note that the midpoint T_m for primer pair CK2A1 and CK2A2 is 77.0°C (Methods, Table 3). The product formation transition observed at 77 and 78°C suggests that the midpoint T_m for any primer pair represents an important parameter in the polymerase chain reaction. We typically set up experiments so that the midpoint T_m of a given primer pair is in the middle of the DMSO gradient. This allows us to explore flanking temperatures as well as the midpoint T_m.

The CKII β subunit DNA was generated by a three-step cycle routine, and the renaturation temperature was set at 68°C. The midpoint T_m for this primer pair is 72.9°C (Table 3). In this case, product obtained at 68°C with a DMSO-induced reduction of 4°C did not replicate precisely at a renaturation temperature of 72°C (compare lanes 5 and 11, Fig. 2). Relatively high levels of product formation may reflect lack of general DMSO inhibition on the Taq polymerase (23). There are two anomalies in the experiment. The faint lower band present in the DMSO experiment (lower arrow, Fig. 2) is not reproduced in the equivalent reaction in the absence of DMSO. Second, we do not know why β subunit product migrates as a doublet; we have obtained only one type of cloned DNA from this reaction. Specific product formation is generated by renaturing at approximately the midpoint T_m of primers CK2B1 and CK2B2 using a three-step cycle routine in the absence of DMSO.

The 5′ untranslated region for the β subunit required two rounds of amplification according to an established method (24) since the first round of amplification did not yield the predicted 882-bp product. The midpoint T_m for this reaction is 67.4°C (Table 3), and synthesis ceased at the effective temperature of 68°C (reaction 6, lane 6, Fig. 3a). Because reaction 5 corresponding to an effective temperature of 67°C, exhibited product formation, this reaction was chosen as the highest effective temperature suitable for reamplification. Reamplification yielded good product formation, and in this case synthesis continued to an effective temperature of 70°C (reactions 1–8, lanes 1–8, Fig. 3b, and Table 2); this is above the midpoint T_m of 67.5°C (Table 3). In addition, the synthesis transition was less marked at the predicted midpoint T_m than in previous reactions. This experiment also contained an artifact band migrating at position 250 (arrow, Fig. 3b) which was gel purified and sequenced directly (27). The sequence appeared to be identical to the 308-bp product, and we cannot explain the anomalous mobility of this band on electrophoresis.

Nonspecific products were cloned at 140 bp (arrow, Fig. 3b), and a product migrating at 70 bp (arrow, Fig. 3b) is unknown. Artifact formation arises sporadically, and at the moment we cannot distinguish between library construction and PCR artifacts.

Three formulas can be applied to the problem of determining optimal renaturation temperatures in PCR: (i) Wu *et al.* (28) empirically derived a formula for determining optimal annealing temperatures based on the ability of short oligonucleotides to prime synthesis in PCR; (ii) Rychlik *et al.* (5) used nearest neighbor base pairing thermodynamics to determine oligonucleotide T_ms, and this value was subsequently used in an empirically derived formula for calculating optimal renaturation temperatures; and (iii) Marmur and Doty (6) determined the base composition of various genomic DNAs by measuring absorbance shifts on denatured DNAs and assigning T_m values for various GC contents.

According to the method of Wu *et al.* (28), the optimal renaturation temperature is given by the formula $T_p = 22 + 1.46\ (L_n)$ where L_n = 2 (No. Gs and Cs) + (No. *A*s and *T*s) in a given primer. This formula was empirically determined and the optimal renaturation temperature is set by using the T_p value for one primer of the primer pair. We encountered two problems when calculating optimal renaturation temperatures for our primers: (i) the formula is not applicable for primers much longer than 25 nucleotides (28) (T_p values for our 41mer are well in excess of 100°C); and (ii) T_p for puc5′, a 25mer, is 76°C, a value that is 13°C higher than our calculated T_m. We are not able to obtain synthesis above 70°C using this primer paired with another 25mer with a similar GC content (data not shown). T_p values may be most relevant using comparatively short primers of 20 nucleotides or less.

As an alternative, we used the method of Rychlik *et al.* (5) to determine primer T_ms and optimal annealing temperatures, and these are listed in Table 3. We note two trends when comparing our values with those obtained using this alternative method. First, T_m values obtained with either method are similar within several degrees; the largest difference is that of 11.0°C obtained with primer CK2A2. Second, T_a^{OPT} values are 7–14°C lower than our midpoint T_m values. It should be noted that the formula for determining T_a^{OPT} was empirically determined and utilizes the lower T_m primer of the pair. We acknowledge that higher levels of synthesis may be realized at temperatures lower than our midpoint T_ms. However, the greatest specificity is obtained at the highest temperatures where synthesis continues to occur. In support of this, Kainz *et al.* (29) have had difficulty reproducing amplification of 10-kb λ phage DNA fragments using T_a^{OPT} values, they found that an increase in renaturation temperature from 57 to 65°C was required for product formation.

Although the T_m formula of Marmur and Doty (6) was not designed for predicting oligonucleotide melting temperatures because it was determined using absorbance shift analysis on sheared genomic DNA, it has the interesting and coincidental property of predicting synthesis transition temperatures for a given PCR primer pair. Temperatures in this range are most likely to drive specificity in the polymerase chain reaction and are better suited to balance primers with unequal T_ms. We have noted that the primer T_m formula that we use (6) does not contain a term to account for ionic strength, although this relationship was originally derived in a solvent containing 0.165 M sodium ions (6). The components in our PCR buffer that contribute to its ionic strength are magnesium and potassium. Magnesium ions are 100-fold more efficient at maintaining duplex DNA at room temperature than are sodium ions (25,26). Therefore, 1.5 mM Mg^{2+} is equivalent to 150 mM Na^+. Adding 50 mM K^+ would bring the total equivalent Na^+ concentration to 200 mM, similar to that originally employed (6).

In order to generate cDNAs as reagents to study CKII function we chose to generate sequences *de novo* by PCR. Primer design based on published sequences facilitated our ability to generate desired cDNAs. Use of the DMSO method improved our ability to rapidly generate working polymerase chain reactions. Development of temperature windows has yielded insights into the relationship between primer T_m and renaturation temperature: with either two- or three-step cycles specific product formation occurs at or within several degrees Celcius of the midpoint T_m of a given primer pair. We have chosen not to examine temperature reduction with respect to either elongation or denaturation temperatures. In principle, a DMSO-mediated reduction of 5°C should give an effective elongation temperature of 80°C with set temperature of 75°C; a set denaturation temperature of 95°C corresponds to an effective temperature of 100°C. The direct correlation between effective renaturation temperatures calculated in the presence of DMSO and their corresponding temperatures realized in the absence of DMSO shows that the primary effect of DMSO is to reduce primer melting temperatures.

To date, reports on the use of DMSO as a PCR specificity enhancing reagent have been largely anecdotal. Here we report a method that predicts the effect of DMSO on the polymerase chain reaction. Further, the use of DMSO allows a critical evaluation of product formation as a function of renaturation temperature and therefore greatly eases empirical determination of this parameter. The method described here consists of three steps: (i) the theoretical midpoint T_m of the given primer pair is calculated; (ii) a DMSO gradient is set up as indicated under Methods and in the Tables; and (iii) a cycle routine is set up so that the set renaturation temperature falls 4–5°C below the calculated primer pair

midpoint T_m. As an alternative fourth step, product that appears at a given effective temperature can be reproduced by raising the renaturation temperature by the corresponding ΔT_m increment and eliminating DMSO. Since thermocyclers are designed to accept a large number of samples at the same temperature, this method should prove advantageous over direct temperature modulation. The method also may have application for the use of degenerate primers. For a degenerate primer pair, the midpoint T_m maximum is known, and therefore, windows can be developed that explore regions below and approaching the midpoint T_m. In theory, higher temperatures favor higher homology and the most desirable products may occur at the highest temperatures.

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