Differential priming of RNA templates during cDNA synthesis markedly affects both accuracy and reproducibility of quantitative competitive reverse-transcriptase PCR

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Quantitative competitive reverse-transcriptase PCR is the most sensitive method for studying gene expression. To investigate whether the accuracy of the calculated target mRNA copy number is affected by the cDNA priming process, we utilized primers of different lengths, concentrations and primer sequences to prime cDNA synthesis reactions. Our results show a ≈ 19-fold increase in the calculated mRNA copy number from cDNA synthesis reactions primed with random hexamers ($P < 0.001$, $n = 4$), and a ≈ 4-fold increase in copy number with a specific hexamer ($P < 0.001$, $n = 4$) compared with that obtained with a 22-mer-sequence-specific primer. The increase in calculated mRNA copy number obtained by priming cDNA synthesis with the shorter specific and non-specific primers could be explained largely by the synthesis of truncated standard cDNA molecules lacking a requisite binding site for amplification with PCR primers. Since these truncated standard cDNA molecules could not be amplified and standard RNA is used to quantify target mRNA copy number, this phenomenon resulted in over-estimation of target mRNA copy number. In conclusion, accurate determination of target mRNA copy number is most likely if a long specific antisense primer is used to prime cDNA synthesis.

INTRODUCTION

Quantitative reverse-transcriptase (RT) PCR is the most sensitive technique for measuring mRNA copy numbers [1–3], and this technique is becoming increasingly popular in the study of gene expression. A search of Bids-Embase (an international biomedical information service) confirmed that there has been a rapid increase in the numbers of published papers utilizing quantitative RT PCR. A total of 17 papers were published using RT PCR between 1988 and 1990, 496 between 1991 and 1993, 1906 between 1994 and 1995, and 3811 between 1996 and 1997. However, there are many potential problems causing poor reproducibility and precision associated with the technique. If meaningful results are to be obtained that can be compared between laboratories, it is important to understand the factors that affect this methodology.

RT PCR can be undertaken semi-quantitatively to make relative comparisons of mRNA levels between different samples. To serve this purpose, non-competitive quantitative RT PCR has been used, where a 'housekeeping' gene, such as that coding for β_2-microglobulin, actin etc., is used as a reference [4,5]. The advantage of using a housekeeping gene is its simplicity, since no competitor molecules (either DNA or RNA) are required as an internal standard. The disadvantages are that this technique is based on assumptions that the expression level of the housekeeping gene is identical between samples, and that cDNA synthesis and PCR amplification efficiencies are identical between different reactions. However, expression of putative 'stable' housekeeping genes [e.g. those coding for dihydrofolate reductase (DHFR) or β-actin] may vary as much as that of the target gene [6,7].

Alternatively, a synthesized DNA sharing identical PCR primer binding sites with target mRNA molecules can be used as a competitor in quantitative competitive PCR [8,9]. The competitor DNA can be added either together with total RNA, and before or after the RT step. The advantage of using DNA as a competitor is that DNA molecules are more stable and easier to handle than RNA molecules. The disadvantage is that this methodology assumes that cDNA-synthetic efficiency is 100 % for precise quantification of target mRNA copy number. However, cDNA synthesis efficiency from total RNA is often less than 100 % [10]. A DNA competitor has been shown to underestimate the number of target mRNA molecules by 10-fold when compared with an RNA competitor [11]. However, the discrepancy between results obtained from using either a DNA competitor or an RNA competitor may be corrected if cDNA synthesis is undertaken at high temperature (70 °C) using a thermostable DNA polymerase [12]. Thus it is important that cDNA synthesis reaction conditions are carefully controlled to ensure good reproducibility.

Where synthesized RNA is used as a competitor [13–15], a known amount of synthesised RNA [standard RNA or recombinant RNA (*M*s)] is added to a known amount of total RNA [containing target RNA molecules (*M*t)], and cDNA synthesis reactions are undertaken. Synthesized cDNAs from both target RNA (M_0^t) and standard RNA (M_0^s) are amplified by PCR with a pair of target gene-specific primers. Since standard templates and target templates share the same binding sites, both templates are amplified by the same pair of PCR primers under identical conditions. After amplification, the original number of target RNA copies (*M*t) can be calculated from the relative intensities of DNA bands [absorbances or fluorescence

Abbreviations used: *M*s, standard RNA molecules; *M*t, target mRNA molecules; M_0^t, target cDNA molecules prior to PCR amplification; M_0^s, standard cDNA molecules prior to PCR amplification; *D*t, target DNA band intensity after PCR amplification; *D*s, standard DNA band intensity after PCR amplification; $\bar{R}$, the mean value of *D*t/*D*s; *K*s, ratio of standard RNA length to target RNA length; *T*, the amount of total RNA added to the reaction (in ng); RT, reverse transcriptase; M-MLV, Moloney murine leukaemia virus.

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intensities of the target DNA bands (*D*t) to the standard DNA bands (*D*s)].

A fundamental assumption of quantitative RT PCR is that cDNA synthesis efficiencies are identical for both target and standard RNA molecules. Thus, after reverse transcription, the ratio of target to standard cDNA molecules (M_0^t/M_0^s) should be equal to the initial ratio of target to standard RNA molecules (*M*t/*M*s). This ratio should remain unchanged throughout the PCR amplification process [8,15,16]. This assumption is crucial if quantitative RT PCR is to be used as a reproducible technique for precise measurement of mRNA copy number. We have shown that the initial ratio of target to standard RNA (*M*t/*M*s) may alter during cDNA synthesis (an altered initial ratio), despite equal amplification of both cDNA templates during the PCR process [17]. This result suggested to us that a change in the ratio of target to standard templates had occurred during reverse transcription, causing poor reproducibility and decreased accuracy in the calculation of target mRNA copy number [17]. Despite showing that an altered initial ratio caused poor reproducibility, the mechanism underlying this phenomenon was uncertain. To investigate whether variation in the cDNA priming process caused a change in the ratio of target to standard templates, and affected reproducibility and accuracy of the results, we have studied the effects of priming cDNA synthesis with primers of different sequences, lengths and concentrations. Our data show that variation in priming cDNA synthesis can markedly affect both the accuracy and reproducibility of results. We also show that the mechanism underlying the altered initial ratio can be predominantly explained by poor primer binding to the 3′ end of the standard RNA.

MATERIALS AND METHODS

Preparation of oligonucleotides

Primers for γ-fibrinogen [18] were used in the study. The design of the primers for primers 1, 3 and 5 has been described [19]. Primers 2 and 4 were designed from the sequence of standard RNA (Figure 1). The sequences for cDNA synthesis and PCR are listed in Table 1. All primers were synthesized by Genosys Europe, Cambridge, U.K. The primers were dissolved in TE solution (10 mM Tris/HCl/1 mM EDTA, pH 8.0) to a concentration of 80 μM. The sizes of PCR products amplified with each primer pair from both standard cDNA and target cDNA are shown in Figure 1.

Conditions for cDNA synthesis

cDNA synthesis reactions with Moloney-murine-Leukaemia-virus (M-MLV) RT

All cDNA synthesis reactions were undertaken with M-MLV unless otherwise specified. RNAs were denatured at 70 °C for 5 min and chilled in ice before added to a reverse-transcription solution containing 0.5 mM dNTPs, 5 μM random hexamer or primer as otherwise specified, 10 units/μl M-MLV RT (Promega, Southampton, U.K.), 1 unit/μl RNase inhibitor (Promega), 50 mM Tris/HCl, pH 8.3, 40 mM KCl, 10 mM dithiothreitol (DTT), 7 mM $MgCl_2$, 0.1 mg/ml BSA in a final volume of 20 μl. The cDNA-synthesis reaction was performed at 42 °C for 60 min. The reaction was stopped by denaturing at 95 °C for 5 min.

cDNA synthesis reactions with thermostable DNA polymerase

Standard RNA (0.2 nM) was added to a buffer with 10 mM Tris/HCl, pH 8.3 at 25 °C, 90 mM KCl, 1 mM $MnCl_2$, 0.2 mM dNTPs, 0.25 unit/μl *Tth* DNA polymerase (Promega), 7.5 μM antisense primer in a 10 μl of reaction volume overlaid with mineral oil. The cDNA synthesis reaction was undertaken with 3 min at 60 °C and 15 min at 70 °C, then 95 °C for 5 min to stop the reactions [12].

Preparation of standard RNA

Total RNA was prepared as described in [20]. A 1 μg portion of total RNA was reverse-transcribed into cDNA and amplified by PCR with primer 1 and primer 3 for synthesis of standard RNA in a total volume of 50 μl. The DNA product (40 μl) was checked on a 2 %-(w/v)-agarose gel. The desired DNA band was excised and purified by QIA Gel Extraction Kit (Qiagen, Crawley, West Sussex, U.K.). The purified DNA was dissolved in aqueous diethyl pyrocarbonate (DEPC), and added to DTT (10 mM), rNTPs (0.5 mM), T7 RNA polymerase buffer [Promega; 40 mM Tris/HCl (pH 7.9)/6 mM $MgCl_2$/2 mM spermidine/10 mM NaCl]/RNase (1 unit/μl) inhibitor/T7 RNA polymerase (1 unit/μl; Promega) and T7 Primer (1.25 μM) to a total volume of 20 μl. The reaction was incubated at 37 °C for 1 h, and the DNA digested by incubation with RNase-free DNase (1 unit/μl) at 37 °C for 30 min. The synthesized standard RNA was extracted with chloroform/3-methylbutan-1-ol (49:1, v/v), precipitated with 100 % (v/v) ethanol and washed with 70 % (v/v) ethanol. The standard RNA pellet was vacuum-dried, dissolved in DEPC/water and quantified by spectrophotometry.

PCR amplification and quantification of PCR products

PCR amplification conditions

PCR amplification was performed in a total volume of 25 μl per reaction using Ready-To-Go PCR beads (Pharmacia Biotech). A 1 μl portion of freshly synthesized cDNA was added to each Ready-To-Go PCR bead, which contain ≈ 1.5 units of *Taq* DNA polymerase, 10 mM Tris/HCl, pH 9.0 at room temperature, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM sense and antisense primers, and was overlaid with ≈ 40 μl of mineral oil. Amplification was undertaken by denaturation at 95 °C for 50 s, annealing at 57 °C for 50 s and extension at 72 °C for 1 min with a final 7 min extension time at 72 °C.

TC-type RT PCR

Each RNA sample was analysed using the TC RT PCR technique (Table 2) [17] unless otherwise specified. Briefly, four separate cDNA-synthesis reactions were undertaken with increasing amounts of RNA from one liver RNA preparation and a fixed amount of standard RNA in successive cDNA reactions. Each of the four completed cDNA reactions was divided into three aliquots after adding the PCR master mix, and individual aliquots amplified either 21, 22 or 23 cycles [19].

Consequently, results from 12 RT PCR reactions were obtained from each total RNA sample that was examined. To analyse the results: (1) the mean values of ratios ($\bar{R}\pm$S.D., $n=3$) of target DNA band intensity (*D*t) to standard DNA band intensity (*D*s) were calculated from each of the original cDNAs amplified from the three consecutive cycles; (2) each of these mean values (±S.D.) of *D*t/*D*s was plotted against amount of total RNA contained in the original cDNA-synthesis reaction, and (3) log values of the mean values of *D*t/*D*s were plotted against log values of the amounts of total RNA contained in the original cDNA synthesis reaction [21]. With this methodology we have shown that the inter-assay and intra-assay coefficients of variation are ≈ 10 and ≈ 8 % respectively, and that we can reproducibly detect ≈ 20 % differences in mRNA copy number between samples [17].

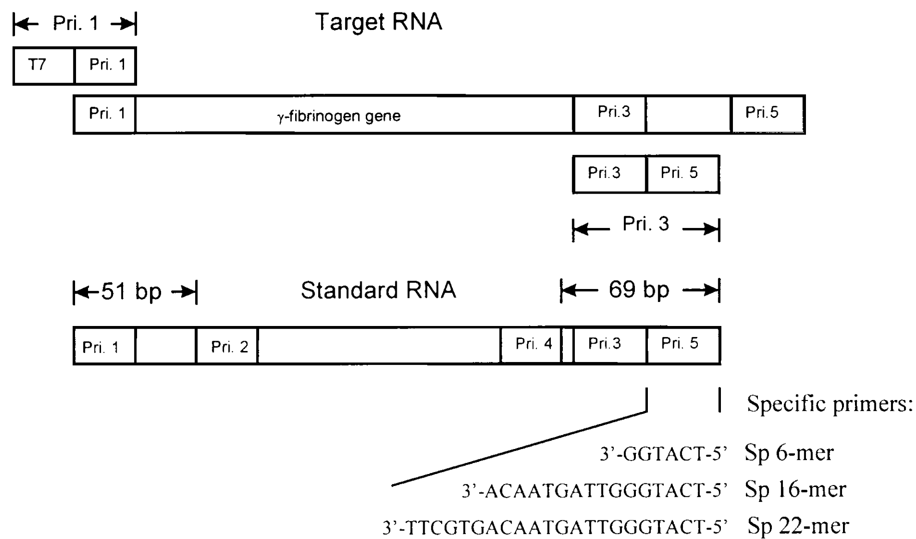


Figure 1 Design of primers

Specific primers used in cDNA synthesis are taken from the primer-5-binding sequence for PCR amplification. Sequences of three specific primers (specific hexamer, 16-mer and 22-mer) and binding positions are illustrated in the Figure. The binding sites for PCR amplification with primers 1, 2, 3, 4 and 5 are also shown in the Figure. The sequences of these primers are listed in Table 1. Primer pairs used for PCR amplification include primers 1 + 5; primers 2 + 5 and primers 1 + 4. The sizes of DNA products amplified with different pairs of primers are listed below.

Primer pair	Product size (bp)	
	Standard	Target
1 + 5	303	394
1 + 4	234	234
2 + 5	232	323

Table 1 Sequences of primers used in cDNA synthesis and PCR amplification

Primer	Sequence
cDNA primers	
Specific hexamer	5′-TCA TGG-3′
Specific 16-mer	5′-TCA TGG GTT AGT AAC A-3′
Specific 22-mer	5′-TCA TGG GTT AGT AAC AGT GCT T-3′
PCR primers	
Primer 1	5′-TAA TAC GAC TCA CTA TAG GGT TTC ATT CTA TGC TGG GCG CT-3′
Primer 2	5′-ACA CTG CTA CTA GAG ATA ACT G-3′
Primer 3	5′-TCA TGG GTT AGT AAC AGT GCT TAC CTG GAT GGC TTT AAT CAG TT-3′
Primer 4	5′-AGC TCG TTG TAA GAT GTT TTC C-3′
Primer 5	The same as specific 22-mer

Quantification of RT PCR products

Quantification of RT PCR products was undertaken as previously described [19]. Briefly, RT PCR products were analysed in a 2%-agarose gel containing ethidium bromide. DNA bands were photographed using an Eagle Eye II video system (Stratagene, Cambridge, U.K.). The image was exported in a TIFF file and DNA bands were quantified using NIH Image 1.55 software for Macintosh personal computers. The quantification value of the bands in the gel was designated the 'optical density' (pixels). In each case, 'optical densities' from target template (*D*t) and standard templates (*D*s) were obtained, and only values within the linear range of the software were acceptable and used to calculate mRNA copy number.

Calculation of mRNA copy numbers

mRNA copy numbers were calculated from the regression line of the plotted results using equations:

$Mt = \bar{R} \times Ks \times Ms/T$ [19]

Table 2 TC-type RT PCR assay

Reaction no. . . .	(1)	(2)	(3)	(4)
cDNA reactions				
Known concentrations of total RNA (μl) . . .	0.5	1.0	1.5	2.0
Known concentrations of standard RNA (μl) . . .	2.0	2.0	2.0	2.0
PCR reactions				
21 Cycles	Ratio (1)	Ratio (1)	Ratio (1)	Ratio (1)
22 Cycles	Ratio (2)	Ratio (2)	Ratio (2)	Ratio (2)
23 Cycles	Ratio (3)	Ratio (3)	Ratio (3)	Ratio (3)
Mean ratios . . .	$\bar{R}$ (1) ± S.D.	$\bar{R}$ (2) ± S.D.	$\bar{R}$ (3) ± S.D.	$\bar{R}$ (4) ± S.D.

$\bar{R}$ is the mean values of ratios Dt/Ds; Ks is the ratio of standard RNA length to target RNA length; T is the amount of total RNA added to the reaction (in ng); Ms is the number of molecules of standard RNA added to the reactions. Ms is calculated from mol of standard RNA added to reactions multiplied by Avogadro's constant (6×10^{23}). mRNA levels are expressed as the number of mRNA copies/ng of total RNA.

Statistical analysis

All statistical calculations were performed using Stat-works statistical software. Differences in mean mRNA copy numbers between reactions primed with different cDNA synthesis primers were examined by unpaired student's *t* test. Results are presented as means ± S.D.

RESULTS

Effect of primer sequence on cDNA synthesis and on the accuracy of target mRNA copy number calculated from quantitative competitive RT PCR

We investigated whether priming cDNA synthesis with primers of different characteristics affected the calculated target mRNA copy number from results of quantitative RT PCR.

Effects of random hexamers and a specific 6-mer on priming of cDNA synthesis

Calculated target mRNA copy numbers were compared from results obtained with quantitative competitive RT PCR in which the cDNA-synthesis reactions were primed with either random hexamers or a gene-specific hexamer. The sequence of the gene-specific hexamer was taken from the 5′ end of the PCR primer 5 sequence (Table 1; see the Materials and methods section), and this primer was designed to match perfectly sequence in the primer-5-binding site from both standard and target RNA. For each of the two primers studied, four cDNA-synthesis reactions were undertaken, with increasing amounts of total RNA added to fixed amounts of standard RNA. Each of the cDNA reactions was subdivided into three aliquots and each aliquot amplified a given number of PCR cycles (Table 2; see the Materials and methods section). Thus 12 RT PCR reactions were analysed for each of the two types of primers used in both cDNA-synthesis reactions. The measured ratios of target DNA band intensity (Dt) to standard DNA band intensity (Ds) between different numbers of cycles were similar, indicating parallel amplification of both standard and target templates (Figure 2a, cDNA synthesis primed with random hexamers; Figure 2b, cDNA synthesis primed with a specific 6-hexamer). The intercept values of the regression line from the non-log plot were close to 0 [17] and the slope values of the regression line from the log–log plot were close to 1, ensuring reproducible results [17,21]. Thus the results satisfied the criteria required for calculation of the target mRNA copy number (Mt) for reactions primed either with random hexamers or specific hexamer [17]. From the same total and standard RNA samples, the Mt value obtained with random hexamers reactions was ≈ 5-fold greater than that obtained with the specific hexamer ($P < 0.001$, $n = 4$). The mRNA copy numbers were calculated from $Mt = \bar{R} \times Ks \times Ms/T$ (see the Materials and methods section). Since values of Ks, Ms and T were identical between the random-hexamer and specific-hexamer reactions, the discrepancy in Mt values was due to the difference in the $\bar{R}$ values, i.e., the ratio of Dt/Ds obtained with random hexamer reactions was greater than the ratio Dt/Ds obtained with specific-hexamer reactions. This discrepancy in Dt/Ds values was not caused by PCR amplification, since we observed parallel PCR amplification of both standard and target templates with both cDNA synthesis primers across each of the three consecutive PCR cycles. Thus it was likely that the discrepancy in Dt/Ds occurred prior to PCR amplification. The results suggested that a change in the ratio of target molecules to standard molecules had occurred during cDNA synthesis, and thus the ratio of target cDNA molecules to standard cDNA molecules did not equal the initial ratios of target RNA molecules to standard RNA molecules.

Differences in the ratios of Dt/Ds may occur between reactions either due to relative increases or relative decreases in Dt/Ds. For example, in reactions in which cDNA synthesis was primed by random hexamers, Dt/Ds was greater than in reactions primed by the specific hexamer. Because we were able to monitor PCR amplification, we were able to infer that relative changes in Dt/Ds were produced because a change in the ratio of target to standard cDNA molecules (M_0^t/M_0^s) had occurred during cDNA synthesis. A relative increase in the ratio M_0^t/M_0^s may occur as a result of decreased synthesis of standard cDNA molecules (M_0^s) (e.g. in reactions primed with random hexamers) resulting in an increased value of M_0^t/M_0^s and therefore an increased value of Dt/Ds [even though the synthesis of target cDNA molecules (M_0^t) remained unchanged]. Alternatively a relative decrease in the Dt/Ds ratios caused by a corresponding decrease in the value of M_0^t/M_0^s during cDNA synthesis (e.g. in reactions primed with the specific hexamer) may occur as a result of decreased target cDNA synthesis (M_0^t) in the specific-hexamer primed reactions.

With the data that were available to us from the results of these experiments, it was not possible to determine whether the relative change in M_0^t/M_0^s ratios with the two cDNA synthesis primers was due to one or other of the above phenomena. [However, our later results (see Figure 5 below and the subsection 'The mechanism underlying discrepancies in mRNA copy numbers when different primers are used to prime cDNA synthesis', sub-

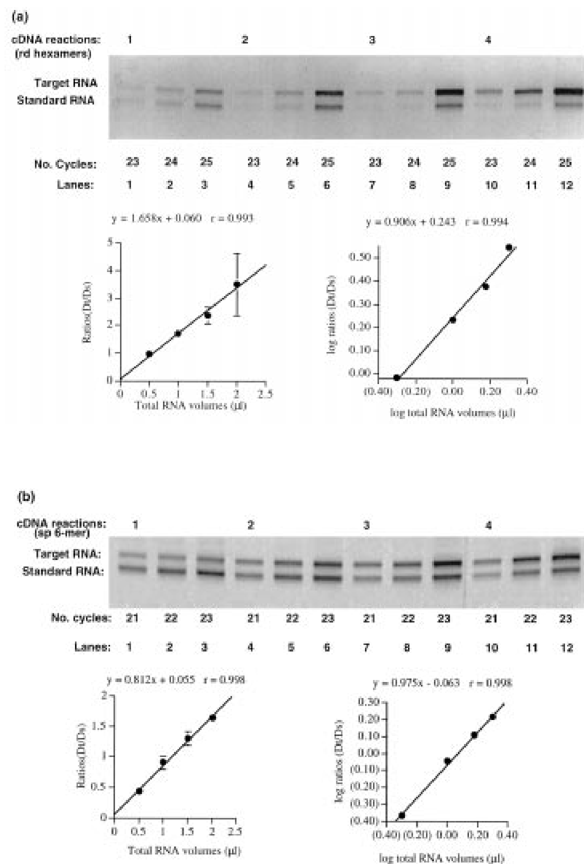


Figure 2 Analysis of quantitative RT PCR results with either random-hexamer- or specific-hexamer-primed cDNA-synthesis reactions

A 0.5, 1.0, 1.5 or 2.0 μl portion of total RNA was added to 2.0 μl (10 fmol/μl) of standard RNA in four cDNA-synthesis reactions. The concentration of total RNA (32.2 μg/ml) used in random-hexamer-primed reactions was diluted by 5-fold from the concentration (161 μg/ml) used in specific-hexamer-primed reactions; otherwise the amplified standard DNA bands were too weak to detect. Each of the four synthesized cDNAs were divided into three aliquots, and each aliquot amplified with primer $1+5$, either for 23, 24 or 25 cycles for random-hexamer reactions (e.g. lanes 1, 2 and 3, **a**) or for 21, 22 or 23 cycles for specific-hexamer reactions (e.g. lanes 1, 2 and 3, **b**). Thus 12 RT PCR reactions were analysed with either random hexamers or the specific hexamer. The mean ratios of target DNA band intensities (*Dt*) to standard DNA band intensities (*Ds*) amplified from each of the four cDNA reactions with three consecutive cycles were calculated. The data were analysed with both non-log and log–log plots. The mean ratios ($Dt/Ds \pm$ S.D.) were plotted against the volumes of target RNA (μl) in a non-log plot and the log values of the ratios (Dt/Ds) was plotted against the log values of the total RNA volumes (μl) in a log–log plot.

subsection 'Detection of truncated standard cDNA molecules') showed that synthesis of full-length standard cDNA molecules was differentially decreased in reactions primed with random hexamers, resulting in a relative increase in the M_0^t/M_0^s ratio after the RT step, a parallel increase in the ratio Dt/Ds and, consequently, an overestimation of the calculated target mRNA copy number.]

Effects of random hexamers and non-specific 6-mers on priming of cDNA synthesis

To investigate changes in the ratio of target to standard templates when cDNA synthesis was primed with the above two types of hexamers, we undertook additional experiments to examine the priming process. There are two possible scenarios for random hexamers binding to templates. The first is that a small amount of the random hexamer mixture containing a perfect match to a complementary sequence in the standard and target sequence binds to this sequence in both templates. Since theoretically random hexamers contain every possible hexamer combination of sequences, the other and most likely scenario is that binding occurs only between a few bases (e.g. three or four bases) of the hexamer and each template. In order to test which of these binding processes occur, we used three non-specific hexamers of random sequence to prime standard cDNA synthesis. The sequences of the three primers were: non-specific primer 1, 5′-CCT TAC-3′; non-specific primer 2, 5′-GCA CAC-3′ and non-specific primer 3, 5′-CGT GGA-3′. We examined whether each of these three non-specific hexamers had any sequence matching PCR primer 5 sequence by computer analysis, since only cDNA synthesized from standard RNA containing the PCR-primer-5-binding site could be amplified by PCR with primers $1+5$. When no, or only one, mismatch was specified, there were no matches between any of the three non-specific primers and the primer-5 sequence. When two-base mismatches were specified, 1 match (three base-pair matches in a row, with one base pair gap and another match) was present in one of the three primers. However, when three mismatches were allowed, three base-pair matches in a row were present in all three primers. Therefore, theoretically, if cDNA synthesis were to be primed with any of these three primers, it was highly likely that binding would occur between only three bases from each primer and the primer-5-binding site in standard RNA.

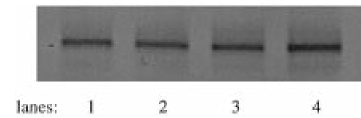


Figure 3 Comparison of different hexamer primers in priming standard cDNA synthesis

Either random hexamers or three non-specific hexamers (all at 5 μM concentrations) were added to 2 nM standard RNA in cDNA-synthesis reactions. The sequences of the cDNA primers are random hexamers (lane 1), 5′-CCT TAC-3′ (lane 2), 5′-GCA CAC-3′ (lane 3) and 5′-CGT GGA-3′ (lane 4). Synthesized cDNA from each of the primers was amplified with primers $1+5$ for 33 cycles.

Figure 3 shows DNA bands that were amplified from each of the cDNAs primed by the three non-specific primers. There were no significant differences in the intensities of amplified DNA bands between those obtained with each of the non-specific primers and those obtained with random hexamers (Figure 3). These results suggested that it was not essential for all six bases of the primer to match the template in order for cDNA synthesis to occur with either random hexamers or non-specific hexamers. Subsequently we refer to the phenomenon of a few bases of a primer, rather than the whole primer, binding to complementary sequence in the template and priming cDNA synthesis as 'non-specific binding'. The results were consistent with the prediction that not all of the six bases within the hexamer were required to prime cDNA synthesis. As the melting temperature for three bases is approx. 9 °C and cDNA synthesis was undertaken at 42 °C, these results suggested that cDNA priming can be a dynamic process with constant annealing to, and melting off, of the primer from the template, throughout the incubation process.

Effects of length of primer on non-specific binding to RNA template

To investigate further the phenomenon of non-specific binding during cDNA synthesis, we undertook RT PCR experiments

using five long non-specific primers. These primers included a coding strand sequence primer from the γ-fibrinogen gene (40-mer), and four primers of different sequences taken from other genes [those coding for peroxisome proliferator-activated receptor-α (40-mer), glucocorticoid receptor (40-mer), α- and β-fibrinogen genes (39- and 40-mer)]. After cDNA synthesis, all cDNAs were amplified with the same primers used above (primers 1 and 5; see the Materials and methods section). The results supported, and were consistent with, the non-specific binding phenomenon described above. Each of the longer non-specific primers used for cDNA synthesis was also capable of priming cDNA synthesis and, after PCR amplification, a DNA band of similar intensity with each primer was produced (results not shown).

Control experiments were also undertaken by PCR amplification of total RNA or standard RNA without prior cDNA synthesis. No bands were visible from these reactions, confirming that the DNA bands observed above were amplified from cDNA synthesized as a result of non-specific binding.

Effects of primer concentration on quantitative competitive RT PCR results

Different concentrations of hexamer

The discrepancy in mRNA copy number obtained with random hexamers and the specific hexamer could have been due to differences in the concentration of sequence-specific primer contained within 5 μM random hexamers, compared with the concentration of the specific hexamer. Theoretically, random hexamers contain 4^6, i.e. 4096, different combinations of hexamer primer sequences including one hexamer that perfectly matches the specific hexamer sequence. Thus the concentration of the specific hexamer within 5.0 μM of random hexamers would be $5.0\ \mu M \div 4096 = 1.22$ nM. If the concentration of the specific hexamer is therefore diluted to a similar concentration (≈ 1 nM) to prime cDNA-synthesis reactions, the mRNA copy numbers generated with the specific hexamer at this concentration should be similar to the mRNA copy number obtained with 5 μM random hexamers in cDNA-synthesis reactions.

The mRNA copy numbers obtained with both 5 nM and 1 nM specific hexamer primer were $(83 \pm 6) \times 10^6$ ($n = 4$) copies/ng of total RNA and $(82 \pm 5) \times 10^6$ ($n = 4$) copies/ng of total RNA respectively compared with the mRNA copy numbers of $(490 \pm 45) \times 10^6$ copies/ng of total RNA obtained with 5 μM random hexamers (for 5 nM specific hexamer versus 5 μM random hexamers, $P < 0.001$, $n = 4$; for 1 nM specific hexamer versus 5 μM random hexamers, $P < 0.001$, $n = 4$). Thus the discrepancy in mRNA copy numbers obtained with random hexamers and the specific hexamer was still present. These results suggested that the concentration of sequence-specific hexamer contained within the mixture of random hexamers was not important in modulating primer binding to standard RNA sequence, whereas the number of bases in the specific hexamer primer matching the templates affected primer binding and cDNA synthesis.

Having observed that the mRNA copy number was not affected by decreasing the concentrations of specific hexamer, we investigated whether changing concentrations of random hexamers had any effect on calculated mRNA copy number. Despite the observation that mRNA copy numbers obtained between concentrations of 0.05 μM and 20 μM with specific hexamer-primed cDNA synthesis were similar (Table 3); mRNA copy numbers obtained with random hexamers were different depending on their concentration. mRNA copy number varied from $(147 \pm 26) \times 10^6$ ($n = 4$) copies/ng of total RNA at 0.05 μM random hexamers to $(633 \pm 99) \times 10^6$ ($n = 4$) copies/ng of total RNA at 20.0 μM random hexamer (Table 3). The calculated mRNA copy number obtained with random hexamers suggested that synthesis of standard cDNA did not increase with increasing concentration of cDNA primer, whereas, in contrast, there was an increase in synthesis of target cDNA, resulting in a greater Dt/Ds ratio and therefore an increase in calculated mRNA copy number. At 0.05 μM random-hexamer concentration, target cDNA synthesis was limited by the amount of primer available, resulting in a decreased calculated mRNA copy number $[147 \pm 26) \times 10^6$ copies/ng of total RNA], which was close to that obtained with specific hexamer at the same primer concentration $[(106 \pm 11) \times 10^6$ copies/ng of total RNA].

Table 3 mRNA copy numbers obtained with either random-hexamer- or specific-hexamer-primed cDNA-synthesis reactions

TC RT PCR reactions were undertaken as described in the Materials and methods section. Data were analysed as shown in Figure 2. mRNA copy numbers (mean $\pm$ S.D. $\times 10^6$ copies/ng of total RNA) were calculated from the mean ratio of target DNA band intensities to standard DNA band intensities ($n = 12$) for each of the primer concentrations (from 0.05 to 20.0 μM).

[Primer] (μM)	mRNA copies (random hexamer)	mRNA copies (specific hexamer)
0.05	147 ± 26	106 ± 11
0.5	193 ± 20	123 ± 20
5.0	490 ± 45	94 ± 7
10.0	604 ± 12	117 ± 34
20.0	633 ± 99	101 ± 18

Effects of varying sequence-specific primer length on cDNA synthesis

Since our results suggested that the number of primer bases that were complementary to RNA templates affected the calculated mRNA copy number, we used a specific 16-mer and a specific 22-mer to prime cDNA synthesis. To test the hypothesis further we compared the calculated mRNA copy number obtained with the specific hexamer to that obtained with either the 16-mer or the 22-mer. The specific 22-mer was the antisense primer (primer 5) used in PCR amplification (Table 1; the Materials and method section), and the specific 16-mer shared identical sequence with the 5′ end of the specific 22-mer. Both primers were perfect complementary matches to the relevant sequence in both RNA templates. The melting temperature of the specific 16-mer was $\approx$ 44 °C, and slightly above the cDNA synthesis reaction temperature. There was a trend towards an increased mRNA copy number with the specific 16-mer compared with the specific 22-mer ($P = 0.16$, $n = 4$); however, the mRNA copy number obtained with the specific 6-mer was $\approx$ 4-fold greater than that obtained with the specific 22-mer ($P < 0.001$, $n = 4$). For comparison mRNA copy number obtained with 5 μM random hexamers was $\approx$ 19 fold greater than that obtained with specific 22-mer ($P < 0.001$, $n = 4$; Figure 4). These data suggested that the number of bases that were perfect complementary matches to the RNA templates markedly affected the results obtained with quantitative RT PCR. As the length of specific primers increased, the calculated mRNA copy number decreased. These results also further supported the difference in mRNA copy number observed between reactions primed with either random hexamers or the specific hexamer (Table 3).

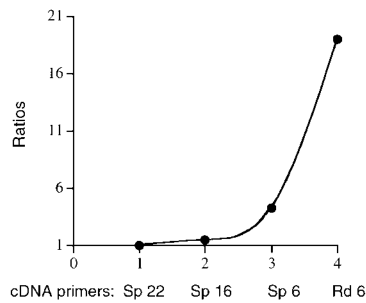


Figure 4 Relationship between primer length and mRNA copy numbers

mRNA copy numbers were calculated from RT PCR reactions in which each of the four types of primers was used in cDNA-synthesis reactions. Ratios of mRNA copy numbers obtained with each of the four types of primer to that obtained with the specific 22-mer were plotted against each type of primer (Sp 22, specific 22-mer; Rd 6, random hexamers).

The mechanism underlying discrepancies in mRNA copy numbers when different primers are used to prime cDNA synthesis

The mechanisms underlying the difference in calculated mRNA copy number obtained with random hexamers compared with specific primers was further investigated. To clarify whether the difference in calculated mRNA copy number was due to a decrease in the number of standard cDNA molecules produced during reverse transcription (with random hexamers) or was due to a decrease in the number of target cDNA molecules produced during reverse transcription (with the specific 22-mer), we designed experiments to test two fundamental assumptions of quantitative competitive RT PCR that could potentially cause marked variation in the results. These assumptions are: (1) all synthesized cDNAs from either the standard or target RNAs can be amplified, i.e., all cDNAs possess the requisite primer-binding sites for PCR amplification; (2) all RNA molecules added to cDNA-synthesis reactions can be reverse-transcribed into cDNA or, alternatively, the same percentage of both target and standard RNA molecules are reverse-transcribed into cDNA.

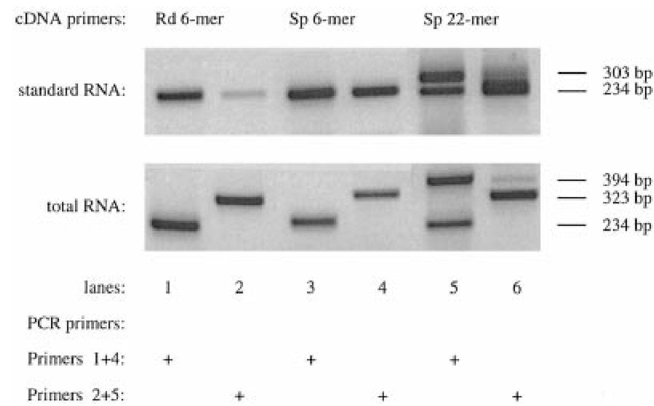


Figure 5 Comparison of DNA products amplified from cDNAs with two pairs of PCR primers

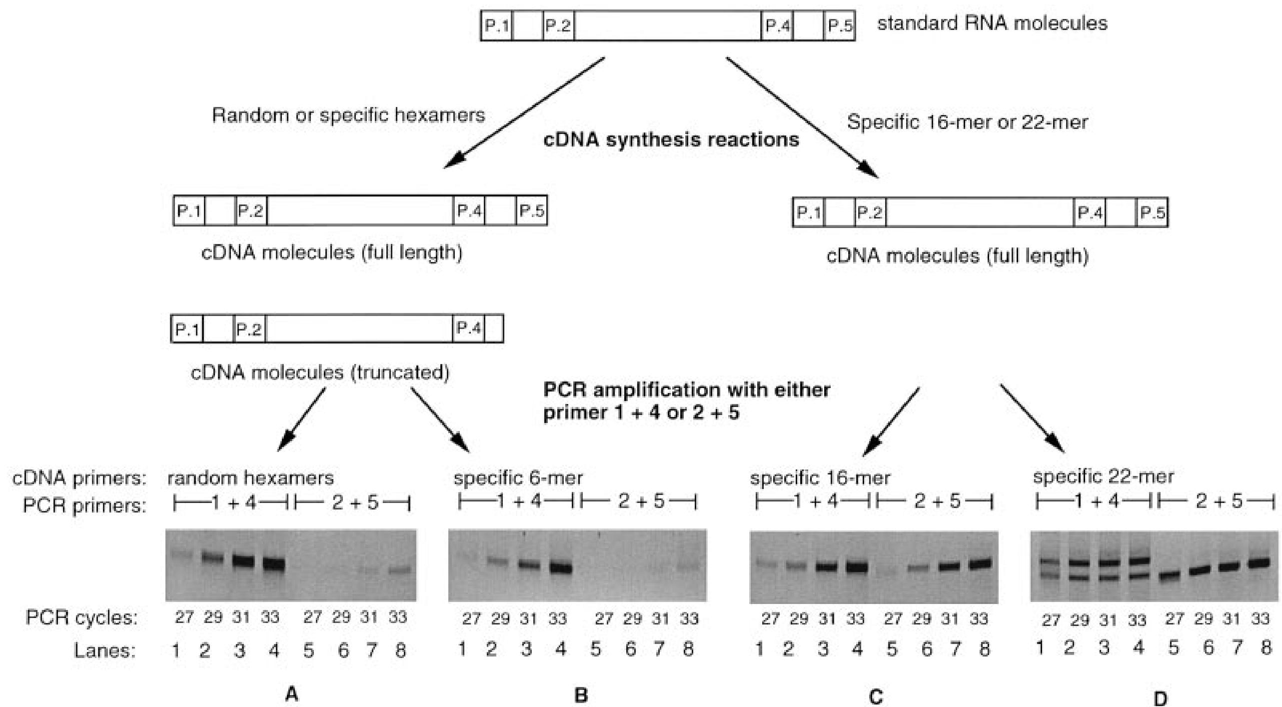
Standard RNA (2.0 nM) (upper panel) or 60 μg/ml total RNA (lower panel) was added to cDNA reactions primed with 5.0 μM primers [either random hexamer (rd 6-mer); or specific hexamer (sp 6-mer); or specific 22-mer (sp 22-mer)] in a 10 μl reaction volume. A 1 μl portion of the synthesized cDNA (e.g. produced after priming with random hexamers) was amplified with 0.4 μM of either PCR primer pair $1+4$ or PCR primer pair $2+5$ for 29 cycles in a 25 μl reaction volume. The extra band shown in lane 5 (303 bp in the upper panel and 394 bp in the lower panel) was due to the presence of primer 5 competing with primer 4 to amplify cDNA during the PCR process, since primer 5 was also used to prime cDNA synthesis. Thus both primer 5 and primer 4 amplified cDNA when combined with primer 1. Owing to the lower melting temperatures when the specific hexamer was used to prime cDNA synthesis, no extra band was amplified during PCR at 57 °C annealing temperature (although the specific hexamer could theoretically also compete with primer 4 during PCR amplification).

Detection of truncated standard cDNA molecules

Given that cDNA molecules are synthesised from the 3′- to the 5′-end of a RNA template and the phenomenon of non-specific binding occurs at multiple sites along the RNA templates, we anticipated that cDNAs (of varying lengths) were synthesized and truncated at their 5′ end. Truncated standard cDNA molecules would possibly lack the primer-5-binding-site sequence and therefore not be amplified by primer pair $1+5$. However, we reasoned that these truncated cDNA species would probably be amplified by primer pair $1+4$, since the primer-4-binding site was 69 bases internal to the primer-5-binding site (see Figure 1 and the Materials and methods section). To exclude the possibility that smaller PCR products would be preferentially amplified due to size differences between products amplified by primers 1 and 5 (303 bases) and PCR products amplified by primers $1+4$ (234 bases), we compared DNA products obtained with primer pair $2+5$ (232 bp) with those obtained with rimer pair $1+4$ (234 bp; see Figure 1).

When random hexamers were used to prime standard cDNA synthesis, the PCR band amplified with primers 1 and 4 was of much greater intensity than that amplified with primers 2 and 5 (Figure 5, upper panel, lanes 1 versus 2). The same results were obtained when the three non-specific hexamers (for their sequences, see under 'Effects of random hexamers and non-specific hexamers on priming of cDNA synthesis') were used in cDNA synthesis (results not shown). Since the two products were amplified from the same cDNA preparation, and the sizes of the amplified DNA products were almost identical, these results suggested that more standard cDNA molecules contained the primer-1-binding site than the primer-5-binding site, because the DNA band obtained after amplification with primer pair $1+4$ was of a much greater intensity than that obtained with primer pair $2+5$. Consequently these results suggested that a decreased number of standard cDNA molecules were synthesized possessing the primer-5-binding site when cDNA synthesis was primed with random hexamers. In contrast, when total RNA only was used in a cDNA-synthesis reaction and was primed with random hexamers, there was no difference between DNA products amplified with both pairs of PCR primers (Figure 5, lower panel, lanes 1 versus 2). These results suggested that synthesized cDNAs from total RNA primed by random hexamers contained both primer-1- and primer-5-binding sites.

If the specific hexamer was used to prime cDNA-synthesis reactions, the difference between standard DNA products amplified with either PCR primer pair $2+5$ or primer pair $1+4$ was smaller, in comparison with the same DNA bands produced when cDNA synthesis was primed with random hexamers (Figure 5, upper panel, lanes 3 and 4 versus 1 and 2). Therefore more standard cDNA molecules were synthesized containing the primer-5-binding site after priming with the specific hexamer, compared with standard cDNA molecules synthesized containing the same primer-binding site after priming with random hexamers. Whereas similar results were observed with total RNA primed with the specific hexamer during cDNA synthesis (Figure 5, lower panel, lanes 3 and 4 versus 1 and 2), suggesting that cDNA synthesis from total RNA was not affected. This was consistent with the above results obtained with random hexamers.



Scheme 1 Explanation of standard DNA results obtained from RT PCR with different primers during cDNA synthesis

Standard RNA (2.0 nM) was used in the cDNA-synthesis reaction, primed with 7.5 μM of either random hexamers, specific hexamer, specific 16-mer or specific 22-mer cDNA-synthesis reactions were undertaken at 70 °C, with thermostable DNA polymerase (see the Materials and methods for detailed conditions) in a 10 μl reaction volume. cDNA synthesized with each primer was amplified by PCR for either 27, 29, 31 or 33 cycles with either primer pair 1 + 4 or primer pair 2 + 5 (gels A, B, C and D). The reason two bands were amplified in gel D is explained in the legend to Figure 5. To assist in an interpretation of these results (lower panel, gels A–D) and the results shown in Figure 5 (upper panel), a schematic Figure is presented (upper panel and middle panel). In the upper panel, the binding sites for different PCR amplification primers (primer 1, 2, 4 and 5) are marked. The sequences for specific hexamer, 16-mer and 22-mer are given in Figure 1 and Table 1. The middle panel (left) shows that two types of cDNA molecules may be synthesized, either full-length cDNA molecules containing the PCR primer-5-binding site or truncated molecules that do not contain the PCR primer-5-binding site. The middle panel (right) represents full-length synthesized cDNA molecules containing the intact primer-5-binding site. If only full-length cDNA molecules are synthesized, no differences in DNA products should be observed between cDNAs amplified by primer 1 + 4 and those amplified by primer 2 + 5 (gels C and D). However, if synthesized cDNA molecules are truncated, these truncated molecules cannot be amplified by PCR primers 2 + 5, but can be amplified by PCR primers 1 + 4 (gels A and B). Truncated cDNA molecules may vary in size, but, for simplicity, only one type of truncated molecule lacking the primer-5-binding site is shown.

We further investigated the phenomenon of truncated cDNA molecules using a thermostable DNA polymerase, which reverse-transcribes RNA into cDNA at high temperature (70 °C) [12], to determine if it were possible to improve cDNA synthesis from standard RNA molecules. When random hexamers and a specific hexamer were used to prime cDNA-synthesis reactions, PCR products amplified from primer pair 1 + 4 were much stronger than those amplified from primer pair 2 + 5, and this difference was consistent across different numbers of PCR amplification cycles, suggesting that the PCR amplification process had not caused these results (Scheme 1, lower panel, gels A and B, lanes 1–8). The results suggested that part of the cDNA synthesized by priming with these two hexamers lacked the primer-5-binding site, and could not be amplified by PCR when primer 5 was used, but could be amplified by PCR when primer 4 was used. Interestingly, these results were consistent with previous results obtained when cDNA-synthesis reactions were undertaken at 42 °C with M-MLV RT enzyme (Figure 5, lanes 1 and 2, upper panel). Since the melting temperatures of both hexamer primers were much lower than the cDNA-synthesis reaction temperature (18 versus 70 °C), both hexamer primers did not anneal to templates at 70 °C (Promega; product usage information). However, the results (Scheme 1, gel A, lanes 1–4; gel B, lanes 1–4) show that PCR amplification of cDNA occurred, supporting the notion that cDNA priming is a dynamic process with constant annealing to, and melting off, of the primer from the template, (see under 'Effects of random hexamers and non-specific hexamers on priming of cDNA synthesis').

When cDNA synthesis was primed with the specific hexamer at 70 °C, more synthesised standard cDNA molecules were truncated compared with standard cDNA molecules synthesized with the same primer using M-MLV RT at 42 °C (compare Figure 5, lanes 3 and 4, with Scheme 1, gel B, lanes 1–4 and 5–8).

When a specific 16-mer was used to prime cDNA synthesis, the amount of amplified DNA products was similar to PCR amplification primers 1 + 4 and 2 + 5 (Scheme 1, gel C, lanes 5–8 versus lanes 1–4), suggesting that fewer synthesized standard cDNA molecules were truncated. This was consistent with results obtained at a lower cDNA-synthesis reaction temperature with M-MLV RT (Figure 4). When the specific 22-mer (i.e. PCR primer 5) was used to prime cDNA-synthesis reactions with the thermostable enzyme at 70 °C, strong bands were amplified with both PCR primers 1 + 4 and primers 2 + 5 (Scheme 1, gel D, lanes 1–4 and 5–8), suggesting that full-length cDNA molecules were synthesized. A comparison of DNA products amplified from the two pairs of PCR primers was not possible with this cDNA-synthesis primer, because two bands were amplified by PCR primers 1 + 4. The extra upper band was produced because cDNA was also amplified by primer 1 and primer 5, since some primer 5 remained from cDNA-synthesis reactions.

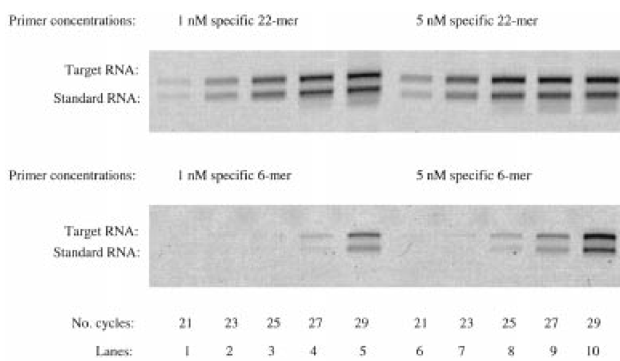


Figure 6 Semi-quantification of cDNA synthesis efficiencies

Standard RNA (2.0 nM) and a 60 μg/ml (upper panel) or 15 μg/ml (lower panel) portion of total RNA was added in cDNA-synthesis reactions (in 10 μl reaction volumes), primed either with both 1 nM and 5 nM specific 22-mer (upper panel), or with both 1 nM and 5 nM specific hexamer (lower panel). A 5 μl portion of cDNA from each of the synthesized cDNAs was added to 120 μl of water containing primer pair $1+5$ (see the Materials and methods section for reaction conditions), and divided into five aliquots of 25 μl. Each of the 25 μl aliquot was added to a Ready-To-Go PCR bead, and amplified either 21, 23, 25, 27 or 29 cycles (see the Materials and methods section for amplification conditions).

The results obtained from cDNA-synthesis reactions undertaken at either 42 °C with M-MLV or 70 °C with thermostable DNA polymerase suggested that the percentage of full-length standard cDNA molecules synthesized during the RT step with different primers contributes to the difference in calculated mRNA copy numbers. The percentage of synthesised full-length standard cDNA was related to the type and length of primer used to prime cDNA synthesis (specific 22-mer or 16-mer > specific hexamer > random or non-specific hexamer).

Semi-quantitative measurement of cDNA synthesis efficiencies

To test the second assumption of quantitative competitive RT PCR, that all RNA molecules added to cDNA synthesis reactions can be reverse-transcribed into cDNA, or, alternatively, the same percentage of both target and standard RNA molecules are reverse-transcribed into cDNA, we designed a semi-quantitative method to estimate cDNA synthesis efficiencies by restricting the amount of primer used in the cDNA-synthesis reactions. The concentration of specific primer was decreased to such an extent that any further decrease diminished the amount of cDNA synthesis, and resulted in a decrease in band intensity after PCR amplification with a fixed number of cycles. With the assumption that each RNA template requires at least one molecule of primer to initiate cDNA synthesis, the efficiency of cDNA synthesis was estimated by dividing the number of molecules of primer by the number of molecules of template RNAs in the reaction. Figure 6 shows the results of PCR amplification from cDNA primed by 1 nM specific 22-mer (upper panel, lanes 1–5) and 5 nM specific 22-mer (upper panel, lanes 6–10). Weak DNA bands were observed after amplification with 21 cycles (Figure 6, upper panel, lane 1). However, these bands were stronger when the concentration of cDNA primers were 5 nM or above (Figure 6, upper panel, lane 6) after amplification with the same number of PCR cycles. These data suggest that synthesized cDNA molecules were limiting PCR amplification at cDNA primer concentrations of approx. 1 nM. Similar results were observed when the specific hexamer was used in cDNA synthesis reactions (Figure 6, lower panel, lanes 3 versus lanes 8). Therefore, the results suggested that approx. 1 nM specific 22-mer was sufficient to prime all possible cDNA molecules, and greater concentrations of the primer did not increase cDNA-synthesis efficiency, because there was little increase in the intensity of DNA bands after PCR amplification with the same number of PCR cycles. Since the concentration of standard RNA in the cDNA synthesis reaction was 2.0 nM, and the concentration of target RNA was approx. $1.6\times 2.0 = 3.2$ nM (1.6 was the ratio of 'optical density' of the target DNA band to the standard DNA band after PCR), the total concentration of relevant RNA molecules that could be bound by primer was approx. $2.0+3.2 = 5.2$ nM. Therefore, we estimated that the cDNA synthesis efficiency was approx. $1\ \text{nM} \div 5.2\ \text{nM} \times 100 = 20\,\%$.

When a specific hexamer was used to prime cDNA synthesis, a greater number of PCR cycles (25–29) was required to amplify cDNA. At identical numbers of PCR amplification cycles and identical concentrations of cDNA primers, the intensities of DNA bands amplified from cDNA primed with the specific 22-mer (Figure 6, upper panel) were greater than the intensities of DNA bands amplified from cDNAs primed with the specific hexamer (figure 6, lower panel). Thus the data suggested that cDNA synthesis efficiencies were lower when priming with the specific hexamer than cDNA synthesis efficiencies after priming with identical concentrations of the specific 22-mer.

Although this method for estimation of cDNA-synthesis efficiencies was crude and only semi-quantitative, the data consistently showed that fewer cDNA molecules from both standard and RNA were produced when the specific hexamer was used to prime cDNA synthesis compared with cDNA molecules produced after priming with the specific 22-mer.

DISCUSSION

The novel results of the present study are that choice of primer used to prime cDNA synthesis can cause marked variation in calculated mRNA copy numbers. Compared with the specific 22-mer and the specific 16-mer, the specific hexamer may over-estimate mRNA copy number by $\approx$ 4-fold and random hexamers by up to $\approx$ 19-fold.

Our data suggest that the underlying mechanism causing variation in calculated mRNA copy number is due to variation in priming standard cDNA synthesis and that the cDNA priming process is most likely a balance between specific and non-specific binding processes. Our data also suggest that cDNA synthesis from target RNA was less affected by variation in the cDNA priming process compared with the synthesis of standard cDNA molecules.

We refer to perfect binding of the whole cDNA primer to the RNA template as 'specific' binding, and binding of only a few primer bases to a template as 'non-specific' binding. The co-existence of specific and non-specific binding processes was suggested by results obtained from amplifying the same synthesized cDNA with two different types of PCR primers (Scheme 1). Both truncated cDNA molecules synthesized as a result of non-specific binding, and full-length cDNA molecules synthesized as a result of specific binding, could be detected by PCR amplification using two sets of PCR primers.

Specific binding and non-specific binding

The equilibrium of non-specific binding to specific binding in a cDNA-synthesis reaction may be affected by reaction temperature, types of cDNA primers (non-specific or specific) and length of the specific primer. When non-specific cDNA primers were used (such as random hexamers and non-specific primers), non-

specific binding occurs, resulting the synthesis of truncated cDNA. When specific primers were used in cDNA synthesis, the priming process favours specific binding, but the equilibrium is also affected by the reaction temperature and by the length of the specific primer. For example, at 42 °C, most of the cDNA molecules synthesized by priming with the specific hexamer may be due to specific binding, since the difference between DNA bands amplified with different pairs of PCR primers was small (Figure 5). However, at 70 °C, most of the cDNA molecules synthesized with the same cDNA primer may be due to non-specific binding, as there was a marked difference between DNA products amplified with the two pairs of PCR primers (Scheme 1, gel B). As the length of the specific primer was increased from a specific hexamer to a specific 22-mer, even at 70 °C, the equilibrium of specific to non-specific binding is likely to shift in favour of specific binding, since there was little difference in DNA products amplified with both pairs of primers (Scheme 1, gels C and D). This result also suggested that most of the synthesized cDNA molecules were of full length.

Non-specific binding and quantitative RT PCR

As the calculation of target mRNA copy number is based on the ratio of target DNA to standard DNA products after RT PCR, it is crucial that all standard RNA molecules can be effectively synthesized into cDNA molecules and amplified by PCR. Therefore synthesis of truncated forms of standard cDNA molecules decreases the number of standard cDNA molecules that can be amplified by PCR, resulting in overestimation of target mRNA copy numbers. The phenomenon of differential decrease in standard cDNA synthesis can be partially corrected by the use of short (hexamer) specific primers (at 42 °C reaction temperature) or corrected to a greater extent by the use of longer (16-mer or 22-mer) specific primers. These results confirm that both the accuracy and the reproducibility of quantitative RT PCR can be markedly affected by the priming process during cDNA synthesis. Although a $\approx$ 19-fold variation in target mRNA copy number may occur with quantitative RT PCR when priming cDNA synthesis is undertaken with different types of cDNA primers, smaller variations in mRNA copy number may also occur between RT PCR reactions, even though the same primer is used to prime cDNA synthesis [17]. Thus we suggest that variation in primer binding to the standard RNA template may cause alteration of the initial ratio of target to standard RNA molecules during cDNA synthesis, resulting in variation in the calculated mRNA copy number and poor reproducibility of results.

cDNA synthesis efficiency

cDNA synthesis efficiency is usually estimated by incorporation of radioactive nucleotides such as [^{32}P]dCTP into synthesized cDNA. Consequently, the technique does not allow for different lengths of newly synthesized cDNAs to be detected. With quantitative competitive RT PCR, is it essential that synthesized cDNA molecules contain both sense and antisense-PCR-primer-binding sites in order that cDNA can be amplified. Our data suggest that not all of the synthesized standard cDNA is full-length, because some molecules do not contain the antisense-primer-binding site. Therefore we suggest that cDNA-synthesis efficiency may be defined as the number of synthesized standard or target cDNA molecules, containing both primer binding sites for PCR amplification, divided by the total number of the standard RNA or total RNA molecules, $\times 100$. For accurate quantification of target mRNA copy number, it is crucial that equal efficiencies of cDNA synthesis are maintained between target and standard RNA templates. Our data show that the percentage of full-length cDNA synthesized from standard RNA may differ according to the priming process, whereas target cDNA synthesis was less affected, suggesting that the relative efficiencies of target and standard cDNA synthesis can be different. Using longer specific primers to prime cDNA synthesis increased the amount of full-length cDNA molecules, and the difference in cDNA synthesis efficiencies between the target and standard cDNA synthesis was greatly decreased.

Decreased standard cDNA-synthesis efficiency with random hexamers probably occurs because of the standard RNA design. Standard RNA can be synthesized with or without extra sequence attached to either end of the PCR primer-binding sites [13,19,22,23]. Our results suggest that random hexamer primer binding to standard RNA sequences was decreased compared with random hexamer binding to target mRNA molecules. It is likely that standard cDNA synthesis would be improved by the presence of additional sequence 3′ to the primer-5-binding sequence in the standard RNA template. Alternatively, as we have shown, it is possible to circumvent the problem of differential cDNA synthesis between target and standard templates by choosing to prime cDNA-synthesis reactions with a longer antisense primer of specific sequence to the gene under study [24,25].

Theoretical rationale for undertaking TC RT PCR

Since the ratio of target to standard molecules may be altered during cDNA synthesis, we consider that it is important to undertake TC RT PCR to detect this problem (Table 2). When only C-type RT PCR is performed, a cDNA-synthesis reaction is undertaken and the cDNA amplified using a different number of cycles [9,26]. By measuring DNA bands produced after amplification with different numbers of PCR cycles, the process of PCR amplification can be monitored. As the two cDNA products can be amplified during PCR with different efficiencies [21,27,28], any variation in results occurring because of differential amplification of cDNA templates during PCR can be detected. However, if only one cDNA-synthesis reaction is undertaken, there are no other reference points to detect if an altered ratio of target to standard molecules has occurred in this single reaction. Alternatively, if only T-type RT PCR is undertaken, increasing amounts of RNA (total/liver RNA) are added to a fixed amount of standard RNA, in separate cDNA synthesis reactions. Each of the synthesized cDNAs is amplified a fixed number of PCR cycles [14,19,22]. Therefore, if variation in RT PCR results occur, it is impossible to detect whether variation originates from either differences occurring during PCR amplification or from differences occurring during cDNA synthesis. Thus by combining both T-type and C-type RT PCR, reference points in two directions are established. Different ratios of total RNA to standard RNA concentrations allows detection of variation in cDNA-synthesis reactions and, secondly, several PCR cycles allows detection of variation during PCR amplification [17].

In conclusion, we have shown that choice of primer used to prime cDNA synthesis can cause marked variation in calculated target mRNA copy numbers, and the data suggest that this most likely occurs because of the co-existence of non-specific and specific binding during cDNA-synthesis reactions, resulting in synthesis of truncated standard cDNA molecules that lack a requisite primer-binding site for PCR amplification. Accurate determination of target mRNA copy number is most likely if a long specific antisense primer is used to prime cDNA synthesis.

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