

# DNA rehybridization during PCR: the 'C<sub>0</sub>t effect' and its consequences

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## ABSTRACT

The rate of amplification of abundant PCR products generally declines faster than that of less abundant products in the same tube in the later cycles of PCR. As a consequence, differences in product abundance diminish as the number of PCR cycles increases. Rehybridization of PCR products which may interfere with primer binding or extension can explain this significant feature in late cycles. Rehybridization occurs with a half-time dependent on the reciprocal of the DNA concentration. Thus, if multiple PCR products are amplified in the same tube, reannealing occurs faster for the more abundant PCR products. In RT-PCR using an internal control, this results in a systematic bias against the more abundant of the two PCR products. In RNA fingerprinting by arbitrarily primed PCR (or differentially display of cDNAs), very large or absolute differences in the expression of a transcript between samples are preserved but smaller real differences may be gradually erased as the PCR reaction proceeds. Thus, this 'C<sub>0</sub>t effect' may systematically cause an underestimate of the true difference in starting template concentrations. However, differences in starting template concentrations will be better preserved in the less abundant PCR products. Furthermore, the slow down in amplification of abundant products will allow these rarer products to become more visible in the fingerprint which may, in turn, allow rarer cDNAs to be sampled more efficiently. In some applications, where the object is to stoichiometrically amplify a mixture of nucleic acids, the bias against abundant PCR products can be partly overcome by limiting the number of PCR cycles and, thus, the concentration of the products. In other cases, abundance normalization at later cycles may be useful, such as in the production of normalized libraries.

## INTRODUCTION

The efficiency of each cycle of PCR amplification is a function of the efficiency of primer binding and the proportion of molecules that extend to completion. These factors are controlled

by many parameters, such as buffer type, polymerase type and concentration, primer concentration and the extent and stability of the primer match with the template. In practice, these variables are generally subsumed into a single adjustment in the base 'x' of the equation for amplification,  $x^n$ , where n is the number of PCR cycles. The value of the base, x, would ideally be 2, but even in the best reactions the base generally does not exceed 1.9. For earlier cycles of PCR the base hardly changes and this constancy is the foundation of many methods for calculating the relative or absolute amounts of nucleic acids (e.g., 1–4). At later cycles, the efficiency of PCR eventually declines. This decline can occur for a number of reasons, including a decline in primer and dNTP concentration as they are incorporated into PCR products and the accumulation of inhibitors, such as pyrophosphate, and competitors, such as primer dimer. These factors, and progression from polymerase excess to template excess, compounded by the gradual decline in functional enzyme, eventually result in a declining rate of amplification. These factors, however, do not sufficiently explain the non-exponential or 'plateau' effect.

During the late cycles of PCR a phenomenon occurs that has been largely neglected and which cannot be explained by the effects mentioned above. As the reaction proceeds, the PCR product strands become sufficiently concentrated that they reanneal to some extent during the time spent below the DNA melting temperature at each cycle, which is often a few minutes. Reannealing may interfere with primer binding. The phenomenon that we refer to as the 'C<sub>0</sub>t effect' was first suggested by Innis and Gelfand (24). Our data and that of Suzuki and Giovanonni (5) demonstrate this effect. This 'C<sub>0</sub>t effect' may be of particular importance when more than one PCR product is being amplified as, for example, in arbitrarily primed PCR fingerprinting of DNA (6,7) and RNA (8,9), where many products are being compared, side-by-side, between PCR reactions. These applications of PCR rely on the detection of differences of PCR product abundance between samples well beyond the exponential phase of the reaction. As a consequence, if reannealing takes place to a significant extent during late cycles, the more abundant products in the fingerprint systematically amplify less efficiently and a large difference in the abundances of template samples is reduced in the final fingerprint. However, differences between starting templates in the same sample are also diminished in the final product, allowing other products that are rarer to catch up. This may be advantageous, as will be explained.

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## MATERIALS AND METHODS

### RNA preparation and RAP-PCR fingerprints

Total RNA was prepared from different stages of the life cycle of *Trypanosoma brucei brucei* GUTat3.1. The procyclic stage (Pc), was cultured *in vitro*, while the slender (Sl) and stumpy (St) bloodstream forms were grown in mice. Cultures and recovery of parasite pellets were performed under standard conditions (10,11), and RNA was purified using the guanidinium thiocyanate–cesium chloride method (12). RNA arbitrarily primed PCR fingerprints were obtained as previously described (9,13), using either two different 10mer oligonucleotide primers of arbitrary sequences, or a combination of a 10mer arbitrary primer and a 11mer derived from the 5' mini-exon sequence of the trypanosomes mRNA (14,15). Differentially amplified RAP-PCR products were isolated using single-stranded conformation polymorphism (SSCP) gels and sequenced as previously described (16).

### Low stringency RT-PCR

Reverse transcription was performed on RNA at three concentrations per sample (500, 250 and 125 ng per reaction), using a mixture of three different anchored-dT primers [(T)<sub>12</sub>-G,A or C]. RNA (5 µl) was mixed with the same volume of RT mixture for a 10 µl final reaction containing 50 mM Tris pH 8.3, 50 mM KCl, 4 mM MgCl<sub>2</sub>, 10 mM DTT, 0.2 mM of each dNTP, 2 µM of primer and 13 U of MuLV–reverse transcriptase. Reverse transcription was performed at 37°C for 1 h (after a 5 min ramp from 25 to 37°C), the enzyme was inactivated by heating the samples at 94°C for 2 min, and the mixture containing newly synthesized cDNA obtained was diluted 4-fold in water. PCR was performed using two primers (19 or 20mers) chosen from the sequence characterized previously 5'-AATGAAAGTTACGAT-AGCGG and 5'-AAAGACAACGGAGATGGCA for the ESAG transcript (GenBank accession no. U53929), and 5'-TGAAGCA-GAAGACAATCAGG and 5'-AAAAATGCCAGTAGCAGGAC for the other transcript, called 'BET-2' (GenBank accession no. U49238). Diluted cDNA (5 µl) was mixed with 5 µl PCR mixture for a 10 µl final reaction containing 10 mM Tris pH 8.3, 10 mM KCl, 4 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 1.5 µM of each primer, 0.5 µCi [ $\alpha$ -<sup>32</sup>P]dCTP and 2 U of AmpliTaq polymerase Stoffel fragment (Perkin-Elmer-Cetus, Norwalk, CT). Thermocycling was performed with a GeneAmp PCR System 9600 thermocycler (Perkin-Elmer-Cetus), using a low stringency thermal profile. The same results were obtained using either of the two following cycling conditions: varying number of cycles of 94°C for 30 s, 35°C for 30 s and 72°C for 50 s or five cycles of 94°C for 30 s, 40°C for 30 s and 72°C for 50 s, followed by various numbers of cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 50 s, as described in the figure legends. Amplification products (5 µl) were mixed with 15 µl formamide dye solution, denatured at 68°C for 15 min, and 2.2 µl was loaded onto a 6% acrylamide–43% urea gel, prepared in 1× TBE buffer. Electrophoresis was performed using a sequencing apparatus at 50 W for ~3 h. The gel was dried under vacuum and placed on Kodak BioMax X-Ray film for ≤ 5 days.

### Densitometry

Autoradiograms were scanned and peaks displayed using the software NIH Image v1.57. Peak heights were determined and the

background from the negative control RNA lanes was subtracted. The peaks were measured for the lowest of the three RNA concentrations used at each data point. Deviations from a non-linear response in the autoradiogram were controlled for by using a low film exposure and a standard consisting of a serially diluted radioactive marker. Observed densities were adjusted using this calibration curve. Graphs of the data were plotted in Microsoft Excel 5.0 on a Power Mac 7100. The proportion of the RNA sample that generated the PCR product of interest was plotted on the X-axis. The Y axis plotted the observed to expected ratio of DNA synthesis. In Figure 3, the observed synthesis at each ratio was normalized against the synthesis seen in the slender RNA sample for cycle number. This normalized number (between 0 and 1) was then divided by the fractional ratio of the RNA sample that generated the PCR product of interest (i.e., 0, 0.11, 0.17, 0.33, 0.50, 0.67, 0.83 or 1) to obtain the deviation from expected.

### Quantitation of PCR products

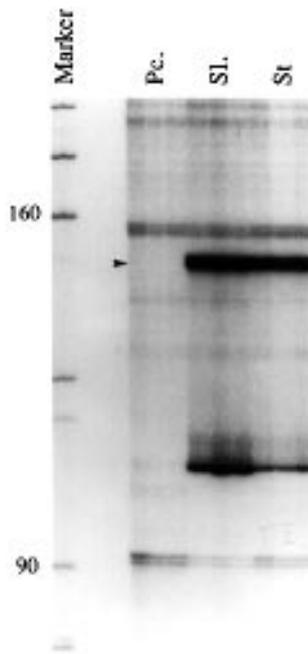
PCR products were cut from the acrylamide gel and disintegrations of <sup>32</sup>P were counted in EcoScint scintillation fluid for 5 min in a Packard Scintillation counter. The molarity of the product was then determined directly, based on the radioactivity, the number of cytosines in the product, and the specific activity of the dCTP in the reaction.

## RESULTS

### Low stringency RT-PCR for semi-quantitative analysis of RNA

These experiments were initially prompted by a desire to develop a simpler method to determine the relative abundance of specific RNA transcripts between samples. Methods for determining the relative abundance of RNAs between samples include Northern blots, S1 protection, primer extension and RT-PCR using internal controls and/or sampling a PCR reaction in the exponential phase. These methods are very effective but quite labor intensive. We speculated that if specific primers for RT-PCR were used for PCR at low stringency, in addition to the desired product many reliable arbitrary products would be generated, as occurs in arbitrarily primed PCR of DNA (6,7) and of RNA (8,9). These extra products might lead to stoichiometric sampling of the specific product in the context of largely invariant PCR products derived from transcripts that are not differentially regulated. Unlike a typical PCR reaction in which there is only one desired product, any individual product in a fingerprint contributes only a small fraction to the total mass. Thus, as the reaction becomes saturated for the limiting component (usually the enzyme), all the products should be affected to the same degree so that the reaction slows down for all the products equally. This would, in principle, preserve the differences for any individual product between samples.

There was considerable precedent for this possibility. In the case of DNA fingerprints, differences in the abundance of PCR products between samples correlate with differences in the amount of the template DNA, as evidenced by the lower intensity of polymorphic bands in the DNA fingerprints of F1 mice versus two different inbred parentals (17). This observation has been extended to detect chromosomal amplifications and deletions in cancers (18,19). Similarly, RNA fingerprinting using arbitrarily primed PCR and differential display on an acrylamide gel has been

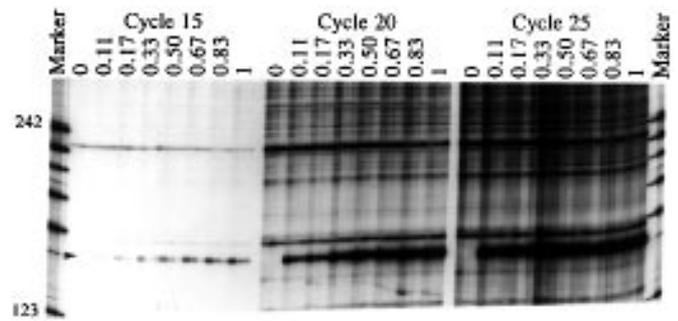


**Figure 1.** Detection of ESAG transcript in different life stages using low stringency RT-PCR. The RNA samples were from the insect procyclic stage 'Pc' and mammalian bloodstream slender 'Sl' and stumpy 'St' stages of the *T.brucei* life cycle. Each RNA template (500, 250 and 125 ng) was reverse transcribed using a mixture of anchored-dT primers, and the cDNA was PCR amplified at low stringency (35°C) for 35 cycles with two ESAG-specific primers (see Materials and Methods). The product at 147 bp is the size expected. The product at ~100 bp is probably an internally primed variant.

used extensively to detect differentially expressed RNAs. It has generally been assumed that differences in the intensity of a cDNA product between two RNA fingerprints from an isogenic source is due to corresponding differences in the sampled RNA, even beyond the exponential phase of the PCR reaction. These observations were consistent with the generally held opinion that all PCR products are similarly affected by the limitation of reagents as PCR exits from the exponential phase, while preserving any intrinsic sequence-dependent differences in amplification efficiency and the bias against longer PCR products (e.g. 20,21).

With these assumptions, we designed a set of experiments to test RNA fingerprinting with specific primers used at low stringency as a strategy to measure the relative abundance of an RNA between samples. In essence, one or more arbitrary products would act as the internal control in RT-PCR, obviating the need to construct such a control and add it in known amounts to each tube. Furthermore, no sampling would be needed at multiple points during the exponential phase. Thus, while only relative abundances could be measured between samples, the method would be simple to execute.

To test this strategy we used one cDNA from a collection of cDNAs that we believed to be differentially expressed during the life cycle of *T.brucei* (the agent of sleeping sickness) based on differential amplification in RNA arbitrarily primed PCR (RAP-PCR; F. Mathieu-Daudé, manuscript in preparation). The life cycle of this kinetoplastid protozoa involves a 'procyclic' stage in the insect vector and bloodstream 'slender' and 'stumpy' stages in the mammalian host. The gene we selected for these experiments was a new member of a gene family (Expression Site Associated Gene, ESAG) associated with the variable surface



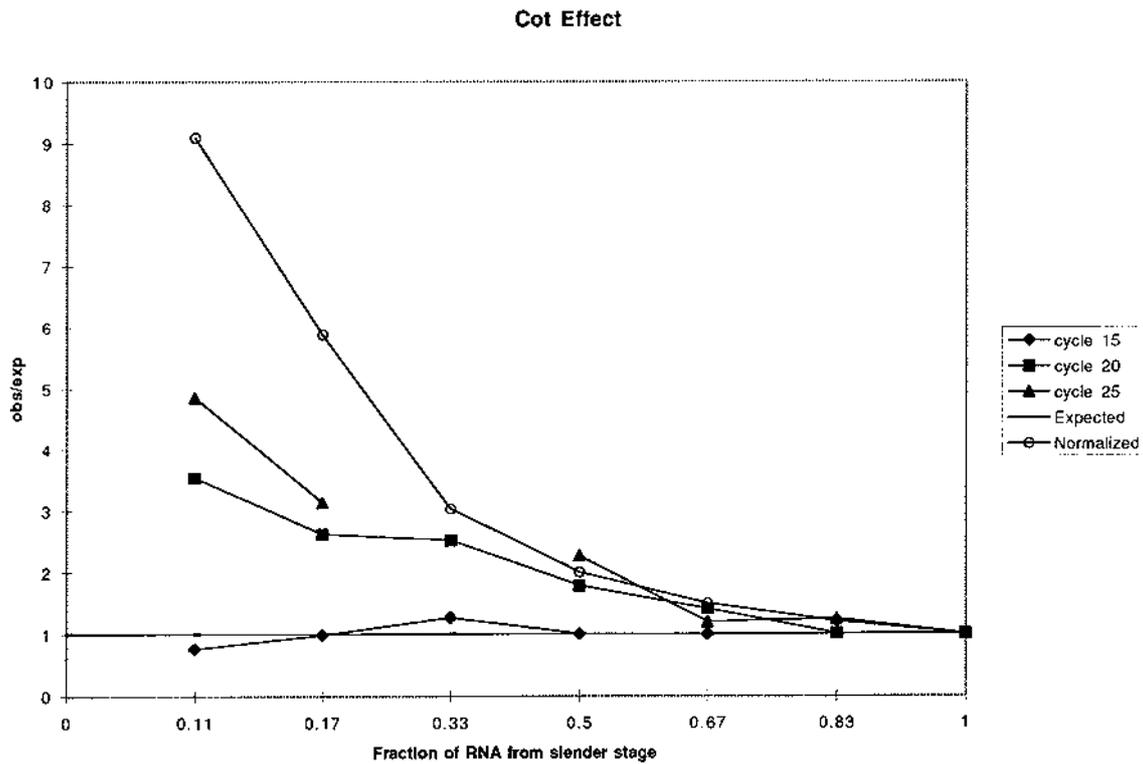
**Figure 2.** Test of how stoichiometry changes with cycle number. RNA from the bloodstream stage 'Sl' was mixed with the RNA from the insect stage 'Pc' in the proportions shown. Amplification conditions of ESAG transcript were as in Figure 1 except that five cycles of low stringency PCR (40°C) were followed by 10, 15 and 20 cycles of higher stringency (60°C).

antigen expression site. Transcripts of this family are expressed almost exclusively in the mammalian host, as part of a strategy to avoid the immune system. Specific primers were devised for this new RNA transcript, as well as for five other transcripts. These primers were 19–24 nt long and the calculated  $T_m$ s were  $\geq 58^\circ\text{C}$ . The primers were first used in conventional RT-PCR with a 58–65°C annealing temperature and shown to amplify the expected cDNA fragment (data not shown). The primers were then used with the same protocol except with a lower stringency annealing temperature of 35°C. As expected, in addition to the specific product, a number of other products were generated, the vast majority of which did not show any evidence of variation among life stages of the trypanosome (Fig. 1). Consistent with our original RAP-PCR data, the transcript was not detected in the procyclic stage and was apparently equally expressed in the slender and stumpy bloodstream stages.

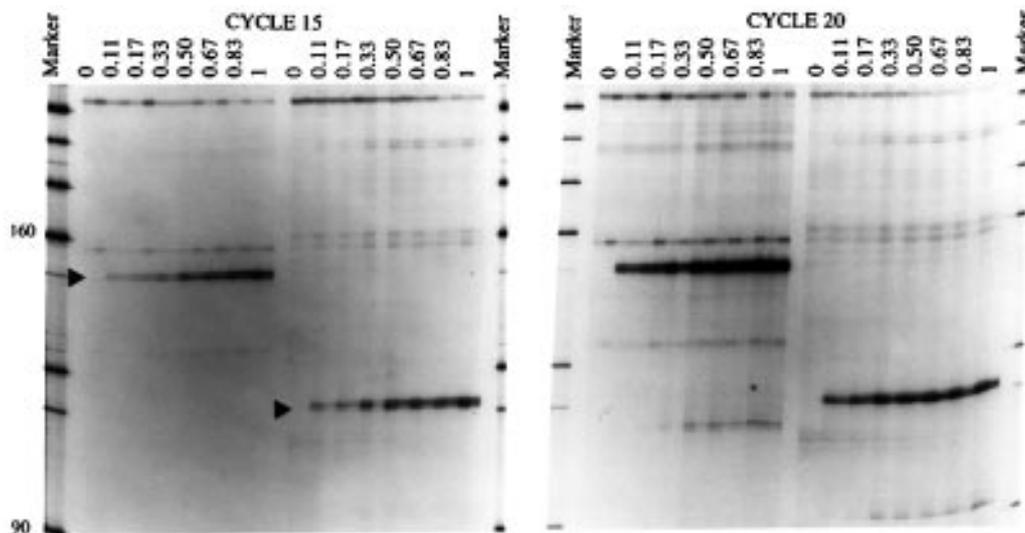
To determine whether the relative abundances observed in these fingerprints could be interpreted linearly, a reconstruction experiment was performed in which procyclic RNA was mixed in various ratios with RNA from the slender mammalian stage. The specific primers for ESAG were again used at low stringency and amplification was performed for 15, 20 and 25 cycles. While this product was not amplified from the procyclic form, unexpectedly, even 1/8th a mass of the mammalian stage RNA was enough to give a signal almost equal to that of pure mammalian stage RNA after 25 cycles of PCR. Even at cycles prior to saturation of the amplification, the PCR signal was not linear with respect to the amount of ESAG RNA (Fig. 2 and data plotted in Fig. 3).

#### Normalization at late cycles is promoted by rehybridization

There were few plausible explanations for the lack of stoichiometric amplification of the titrated ESAG product. Despite a lack of claims to have demonstrated this effect in the literature perhaps, the amount of PCR product was sufficient to allow it to reanneal, thus inhibiting amplification and allowing products of lower concentration to catch up. At lower DNA concentrations in earlier cycles the difference between samples would still be present and would gradually disappear as the reaction progressed (Fig. 2). This phenomenon was also seen using different specific primers directed against ESAG, and specific primers directed against another novel differentially expressed gene, Bloodstream expressed transcript-2 (BET-2) (Fig. 4).



**Figure 3.**  $C_{0t}$  effect. The experimental data was obtained using the ESAG-specific primers at low stringency, Figure 2. The proportion of slender stage RNA in a mixture with procyclic RNA is plotted against the observed/expected ratio, as explained in the Materials and Methods.

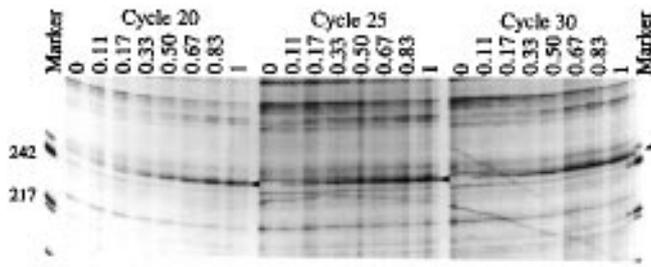


**Figure 4.** Test of how stoichiometry changes with cycle number. The two highest concentrations of the RNA mixture from Figure 2 were used in this experiment. Amplification conditions were as in Figure 1, with 15 and 20 cycles of low stringency PCR (35°C). Two different transcripts were amplified with specific primers: the ESAG transcript (147 bp product) on the left, and a novel transcript (113 bp product), called 'BET-2', on the right (see Materials and Methods for primer sequences).

The rehybridization prediction is different from the trivial circumstance in which a PCR reaction contains a PCR product that, because of limiting reaction components, will amplify to the same plateau mass, regardless of starting template concentration. In contrast, rehybridization lowers the relative mass of the more abundant PCR product in a mixture of PCR products so that the

ratios of products change, which cannot be explained by limiting reaction components.

Densitometry was performed to quantitate the deviation of amplification ratios from expected ratios. An example is presented in Figure 3. The deviation from a stoichiometric proportion is not detected at 15 cycles in this experiment but increases as the



**Figure 5.** Effect of the number of cycles on RAP-PCR. The same RNA samples as in Figure 2 were used in a reaction involving the combination of arbitrary primers originally used to find the ESAG and 'BET-2' transcripts. The RAP-PCR reaction was performed using an arbitrarily chosen 10mer primer (5'-GGGGCACCAG) for the reverse transcription, 4  $\mu$ M of an 11mer primer derived from the 5' mini-exon sequence of the trypanosomes mRNA (5'-AACAGTTTCTG) for the PCR step, and 20, 25 and 30 cycles of low stringency PCR (35°C). The relative mass of the PCR product derived from the 'BET-2' gene (240 bp) is lower and the effect of increased cycles on stoichiometry is less than in Figure 4, right hand panel.

reaction progresses. Similar data were obtained with other primers directed toward different genes. In addition, inspection of Figure 2 shows that as the number of cycles of PCR increases, the ratio of ESAG PCR product to other products on the gel loses its dependence on the amount of mammalian stage RNA added. This cannot be explained by limiting reaction components.

If there were a larger number of different PCR products in the fingerprint, the concentration of the product of interest may be limited by competition to the point where rehybridization has no observable effect. Under such circumstances, the normalization phenomenon would be moderated. To test this hypothesis we used the same combination of arbitrary primers initially used for RAP-PCR to detect fragments of the two genes (ESAG and BET-2) in a reconstruction experiment. In the resulting RAP-PCR fingerprint (Fig. 5) the differentially expressed products represent a very much smaller fraction of the total DNA than when specific primers were used (e.g., Figs 2 and 4). A product from the BET-2 gene (Fig. 5) represents ~5% of the molecules in the RAP-PCR versus 30% of the molecules in the low stringency PCR with specific primers. This is expected because the specific primers have a perfect match with the template, whereas the arbitrary primer initially has an imperfect match such that other arbitrary products can compete effectively. At each sampled cycle (20, 25 and 30 cycles) the difference in the ESAG and BET-2 PCR products between the lane containing a 0.11 fraction of mammalian stage RNA and a lane containing only mammalian stage RNA, is better conserved than at the same number of cycles for the same transcript using specific primers. Densitometry indicated that the BET-2 product at the 0.11 dilution was 2.6-fold above expected after 20 cycles and 3.5-fold over expected after 30 cycles in the RAP-PCR (Fig. 5). For comparison the excess was ~1.8- and 4.9-fold after the 15th and 20th cycles when specific primers were used (Fig. 4, right half of each panel).

An argument for rehybridization as a plausible cause of this phenomenon can be made based on the work of Wetmur (22) and Wetmur and Sninski (23), who present a calculation that can be applied to the reannealing rates of PCR products. Based on the nucleation rate constant they measured for PCR buffer  $t_{1/2} = 20/(C \times L^{0.5})$  where C is the concentration of the PCR product (in  $\mu$ M) and L is the length of the PCR product. We observed >30 ng

(0.03  $\mu$ M) of the 147 bp ESAG PCR product after 25 cycles of the low stringency PCR in a reaction volume of 10  $\mu$ l (see Materials and Methods). At this concentration the expected  $t_{1/2}$  is ~55 s.

The rehybridization rate constant was confirmed by preparing a large quantity of the ESAG product by high stringency PCR and determining the time course of reannealing by measuring hypochromicity at 260 nm in PCR buffer. At a concentration of 0.035  $\mu$ M this product rehybridized with a half life of ~40 s (versus 47 s predicted from the equation above). Thus, the extent of rehybridization is likely to be considerable in the later cycles of PCR. Even though the rate of primer annealing may be as fast or faster, it is quite possible that the rehybridized molecules may displace a significant fraction of short nascent strands.

Given that a PCR reaction is typically below the  $T_m$  of the PCR product molecules for a  $\geq 1$  min per cycle, rehybridization may displace more than half of priming events at the late cycles. It should be noted that a small difference between an amplification efficiency of 1.50 and 1.55 results in a 40% difference in product yield after 10 cycles. So the effect of even a small amount of rehybridization in the last ten cycles can be profound.

## DISCUSSION

We set out to develop a PCR strategy for determining the relative abundance of specific transcripts among RNA samples using specific primers at low stringency. In addition to a product from the transcript of interest, at low stringency a series of other PCR products representing many other transcripts are generated, mostly from RNAs that do not differ between the samples. These other products represent internal controls. At a lower number of cycles or when the product of interest represents a small fraction of the total amount of DNA in the fingerprint, the estimates of relative abundance are more reliable. The low stringency PCR strategy with specific primers is much simpler than other methods for measuring relative levels of transcripts, as long as the number of cycles is limited. However, we also showed that when approaching saturating levels of amplification there is a normalization phenomenon that affects relative abundance.

Rehybridization appears to be responsible for the bias against PCR of abundant products in the late cycles of PCR. There seem to be few other plausible alternatives but even if rehybridization is not the cause, the phenomenon has some interesting consequences that are worthy of discussion. This phenomenon complicates use of PCR fingerprints to look for differences in DNA content, such as has been done for cancer, because the sensitivity of detecting subtle differences in ploidy would be diminished. Nevertheless, differences are actually seen (e.g., 18,19). If enough fingerprints are generated then there is a good chance that even a small change in the copy number of a chromosome or subchromosomal region will be represented in a relatively rare PCR product. Because rarer products are at lower concentration the normalization process would take place less efficiently. Thus, real differences in abundance of sequences between samples may remain reflected in these less prominent products.

In the case of RNA arbitrarily primed PCR fingerprinting and differential display the situation is more complex. For the more prominent PCR products in the fingerprint, modest differences in the abundance of the source transcript between RNA samples are reduced as the reaction progresses. It therefore appears that, for prominent cDNA products, the differences between RNA samples must be large to be observed.

We have previously argued that if two different RNA sequences in the same tube prime and amplify with equal efficiency but have a 100-fold difference in abundance this would result in PCR products that preserved the starting ratio of 100:1 (9,20). This would result in a very severe bias against sampling rarer transcripts in the fingerprint. The data presented here indicates that the slowing of amplification of the more abundant PCR products would allow the rarer PCR products in the same tube an opportunity to amplify. Thus, the ratio might be somewhat normalized, which works in favor of sampling rare RNAs because rare cDNA PCR products have a chance to 'catch up', but places restrictions on quantitative interpretation. The extent of the remaining bias against rare transcripts is unknown.

Overall, we would predict that in both DNA fingerprinting and RNA fingerprinting the trend would be for differences in the fingerprints to underestimate the difference in the original templates. This underestimate would be most severe for the more prominent products in the gel.

It would be helpful to be able to model the effect of rehybridization. However, rehybridization generally becomes important at the same stage in the PCR amplification as many other factors that cause the reaction to become non-exponential. This phase of the reaction is difficult to model. The coincidence of non-exponential amplification and rehybridization at late stages of PCR is probably the reason why rehybridization has been largely ignored as a factor in the plateau of PCR amplification: there were already plenty of explanations for the decline in PCR at this stage in the process. Only the differential effect on products of different molarities in the same tube distinguishes rehybridization from these other factors that cause non-exponential amplification in late cycles.

#### Data from the literature that support the rehybridization hypothesis

If the observations we have made are generally true then presumably they would be seen in the data of others, particularly in the data from PCR-based quantitation methods that involve an internal control. For example, in a recent study by Borriello *et al.* (4), exogenous globin mRNA was titrated against endogenous TNF- $\alpha$  RNA and the product ratios were shown to be generally linear at the 24th, 26th and 28th cycle. A starting ratio of 2:1 resulted in a product ratio that increased from 0.2 to 0.25 to 0.3 as the cycles increased from 24 to 26 to 28 (fig. 2 in that reference). This would be predicted by the rehybridization hypothesis; the ratios would deviate towards 1.0 as the number of cycles increase.

In the study by Gilliland *et al.* (2), GM-CSF RNA was titrated versus increasing amounts of a plasmid carrying the GM-CSF gene. After 40 cycles (near enzyme and substrate saturation) the ratio of plasmid DNA product to cDNA product was not linear and deviated in the direction we would predict; with increasing amounts of plasmid DNA the ratio does not increase as fast as otherwise expected (fig. 4 in that reference). Similarly, in the paper by Bouaboula *et al.* (3), where they meticulously validate a multispecific internal control for RT-PCR, the ratio of the starting template and standard are linearly reflected in their product ratios except, as we would predict, when they differ markedly in starting ratios. In their data the deviation occurs at both ends of the titration; the ratio systematically favors the less abundant products, the template at one end of the curve and the

standard at the other. Their data also show that at large ratios of template and standard the differences in products are diminished between the 25th and 30th cycles, also as we would predict (fig. 5 in that reference).

In all the cases discussed, and in many others, the authors were aware that their quantitation was best in a certain range around a product ratio of 1:1. Thus their conclusions are not compromised by the phenomenon we observe in their data. Indeed, observation of the phenomenon in their data is confirmation of the quality of their experiments. However, experiments that involve more than one PCR product in a reaction and proceed into the non-exponential phase need not always display the rehybridization effect. This  $C_{ot}$  effect should be reduced if the number of cycles is limited or if the amount of primer or some other component has been limited so that the final product does not accumulate to a concentration above  $\sim 0.01 \mu\text{M}$  where rehybridization becomes a factor. Indeed, limiting the total molarity of PCR products is wise in quantitation experiments where product ratios are expected to be large and where PCR extends into the non-exponential phase.

Suzuki and Giovanonni (5) have independently demonstrated a particularly dramatic example of this phenomenon, which they observed when amplifying a mixture of two rRNA gene templates. They showed that the results fit a kinetic model in which the reannealing of genes progressively inhibits the formation of template-primer hybrids.

#### Consequences, mitigation and possible exploitation of the $C_{ot}$ effect

The normalization phenomenon is certainly a disadvantage when one wants to preserve the stoichiometry among initial template products, such as when surveying nucleic acids from mixtures of organisms. One way to minimize alterations in stoichiometry would be to ensure that the products remain as dilute as is practical. However, in a case where one actually wants to normalize a sample, such as when the extent of diversity in a sample is the question of interest, the rehybridization phenomenon can be exploited and even enhanced. For example, the reaction could be held at each cycle at above the  $T_m$  of the primers but below the melting point of the PCR products. This would allow reannealing to proceed for a chosen time, only limited by the half life of the enzyme and other components which could be replenished if necessary. Such limits will put a practical cap on the complexity of the DNA that can be reannealed. Nevertheless, assuming crossover PCR does not garble the results, it is possible that hundreds or perhaps thousands of separate PCR products could be partly normalized in a single tube in a few hours or days. Applications to normalized libraries from DNA and RNA fingerprints and from complex nucleic acid samples can be envisioned.

In summary, PCR seems to involve a rehybridization or ' $C_{ot}$  effect' at high DNA concentrations that has implications for the design and interpretation of PCR amplification experiments that contain multiple PCR products and proceed to high product concentrations.

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