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Bias Caused by Template Annealing in the Amplification of Mixtures of 16S rRNA Genes by PCR†

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The PCR is used widely for the study of rRNA genes amplified from mixed microbial populations. These studies resemble quantitative applications of PCR in that the templates are mixtures of homologs and the relative abundance of amplicons is thought to provide some measure of the gene ratios in the starting mixture. Although such studies have established the presence of novel rRNA genes in many natural ecosystems, inferences about gene abundance have been limited by uncertainties about the relative efficiency of gene amplification in the PCR. To address this question, three rRNA gene standards were prepared by PCR, mixed in known proportions, and amplified a second time by using primer pairs in which one primer was labeled with a fluorescent nucleotide derivative. The PCR products were digested with restriction endonucleases, and the frequencies of genes in the products were determined by electrophoresis on an Applied Biosystems 373A automated DNA sequencer in Genescan mode. Mixtures of two templates amplified with the 519F-1406R primer pair yielded products in the predicted proportions. A second primer pair (27F-338R) resulted in strong bias towards 1:1 mixtures of genes in final products, regardless of the initial proportions of the templates. This bias was strongly dependent on the number of cycles of replication. The results fit a kinetic model in which the reannealing of genes progressively inhibits the formation of template-primer hybrids.

In many applications of the PCR the template is a mixture of homologous genes. Three examples are (i) the amplification of multigene families from the DNA of a single species, (ii) the amplification of genes coding for rRNA (rDNA) from genomic DNA extracted from natural communities of microbes, and (iii) many quantitative PCR assays employing internal standards (2, 13, 26). In each of these cases the product amplicons are derived from template DNA by a process involving complex chemical kinetics, and the relative abundance of homologs among the final reaction products is often a parameter of interest. Variability in the efficiency of PCRs is a common observation, but it would not be expected to influence product ratios in mixed-template reactions unless amplification efficiencies differed among homologs. Thus, information about the relative efficiency of gene amplification in mixed-template PCRs is crucial to the interpretation of such experiments, and, in particular, the lack of such information has impeded progress in microbial ecology (11, 25).

Although the mechanisms of gene amplification from complex mixtures are not well understood, PCR is nonetheless widely used for quantitative studies in molecular biology, including quantitative reverse transcriptase PCR (2, 5, 8, 24, 26). In such studies, the internal standard is added at a known concentration to a reaction mixture containing the gene of interest at an unknown concentration. Generally, the internal standard is presumed to have priming sites identical to those of the gene under study, but it differs from the target molecules either in size or in restriction endonuclease sites to allow easy detection. The concentration of the gene of interest is estimated from the ratio of the concentrations of the product amplicons from the gene of interest and the internal standard. The inclusion of internal gene standards is vital to such studies because it minimizes errors introduced by tube-to-tube variation in amplification efficiency. However, in such cases it is assumed that the amplification efficiencies of the gene of interest and the internal standards are the same. This assumption requires several corollary assumptions: (i) that the gene of interest and the gene standard are equally accessible to primer hybridization following denaturation, (ii) that primer-template hybrids form with equal efficiencies for both templates, (iii) that both templates are extended by the polymerase with the same efficiency, and (iv) that limitations caused by substrate exhaustion equivalently affect the extensions of both templates. Two recent papers (14, 18) discuss the problems associated with quantitative PCR. Raeymaekers (18) mentions that in several published studies, violations of assumption iv may have occurred, whereas Morrison and Gannon (14) examine a system in which the assumptions apparently hold.

Several factors might bias the relative frequencies of genes in PCR products from mixed-template reactions. One such factor in particular, the moles percent guanine-plus-cystosine (G+C) content of template DNA, has been reported to influence gene amplification by PCR (6, 20). In addition, different binding energies resulting from primer degeneracy (that is, a mixture of primers with nucleotide sequences corresponding to observed variation among homologs) and the influence of template folding are other plausible, but undocumented, sources of bias in PCR. In no case yet has the quantitative extent of template bias been well documented.

The amplification of rRNA genes from mixed genomic DNAs derived from natural microbial populations is analogous to quantitative reverse transcriptase PCR except that the gene mixtures are frequently more complex and the assumptions mentioned above must also hold for homologs with unknown sequences. In addition, universal primers employed for the amplification of rRNA genes often contain degeneracies which may influence the formation of primer-template hybrids. Also, the moles percent G+C composition of genomes in microbial populations may vary widely. Nonetheless, PCR-amplified

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TABLE 1. Sequences and specificities of the primers utilized

Primer	Sequence	Specificity
EubB (27F)	AGAGTTTGATCMTGGCTCAG	Bacteria
519F	CAGCMGCCGCGGTAATWC	Universal
338R	GCTGCCTCCCGTAGGAGT	Universal
1406R	ACGGGCGGTGTGTRC	Universal
1492R	GGTTACCTTGTTACGACTT	Bacteria
EubA (1522R)	AAGGAGGTGATCCANCCRCA	Bacteria

DNA has been used in numerous studies addressing the rDNA composition of mixed populations by applying analytical techniques to detect homologous genes, including gene cloning and sequencing (3, 4, 7), denaturing gradient gel electrophoresis (15), and chromatographic techniques such as capillary electrophoresis (1). Such techniques have potentially important applications to studies of spatiotemporal variations in microbial communities, wherein the ability to detect relative changes in gene concentrations is a foremost objective.

The study described here was undertaken to examine the potential introduction of biases by PCR in the amplification of rRNA genes from known mixtures. Our approach was to mix small-subunit ribosomal RNA genes (SSU rDNAs) from three different phylogenetic groups of marine bacteria. Pairwise mixtures in different proportions were used as templates for amplification by PCR. To measure bias produced by PCR, we compared the proportions of genes in the products with their proportions in the template mixture. Our goal was to understand mechanisms which might introduce PCR biases. Although the results described apply to a small subset of genes amplified under controlled conditions, they reveal information about mechanisms of PCR bias which can be used to identify conditions under which such biases are minimized.

MATERIALS AND METHODS

Templates. Since the accurate measurement of the template gene concentrations was critical to the assay, we chose to use either linearized plasmids containing cloned SSU rDNA inserts or the fragment of SSU rDNA generated with primers 27F and 1492R (27F-1492R fragment) by PCR. The sources of SSU rDNAs were clone libraries of bacterial SSU rDNAs (3, 12, 19). Briefly, 16S rRNA genes were amplified from DNA samples from the Bermuda Atlantic Time Series station in the Sargasso Sea (30°50′N, 64°10′W) via PCR using the bacterial primers 27F and 1522R (9) (Table 1). Libraries of SSU rDNAs were obtained by cloning the PCR products into the vector pCRII (Invitrogen, San Diego, Calif.), as previously described (3). Template DNA concentrations were measured spectrophotometrically with a UV160U spectrophotometer (Shimadzu Co., Kyoto, Japan).

Three clones for which the complete nucleotide sequences were known were chosen for this study: SAR432, SAR464, and SAR202. Clones SAR432 and SAR202 are affiliated with the gram-positive bacteria and the *Chloroflexus* and *Herpetosiphon* bacterial phyla, respectively (10, 12). SAR464 is a member of the SAR11 cluster of the α division of the class Proteobacteria (12). To avoid contamination of the cloned SSU rDNAs with genomic DNA from *Escherichia coli*, the plasmids were purified by alkaline lysis followed by CsCl-ethidium bromide density gradient centrifugation (22).

Two different sets of templates were used to evaluate the introduction of biases by PCR. The first consisted of a mixture of purified plasmids containing SSU rDNA from SAR202 and SAR464 and linearized by digestion with the restriction endonuclease *Not*I (Promega, Madison, Wis.). The second set of templates consisted of mixtures of the 27F-1492R fragments of SAR202, SAR432, and SAR464, each of which had been amplified separately by PCR from linearized plasmids. Except when noted, the proportions of each gene in paired mixtures were 0:1; 1:4, 2:3, 3:2, 4:1, and 1:0. All template mixtures were added to final concentrations of 0.1 ng/μl.

PCR conditions. The primers and templates used and the number of amplification cycles varied; all other conditions were constant for all reactions. In a final volume of 100 μl, reaction mixtures contained 0.2 mM premixed deoxynucleoside triphosphates (dNTPs) (Stratagene, La Jolla, Calif.), 1.5 mM MgCl, 5% acetamide, and 2.5 U of *Taq* DNA polymerase (Promega). All reactions used the Ampliwax (Perkin Elmer-Cetus, Norwalk, Conn.) hotstart protocol and a PLT100 thermal cycler (MJ Research, Inc., Watertown, Mass.) programmed to 35 cycles (except for the one evaluating the effect of the number of cycles) of denaturation at 96°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 3 min.

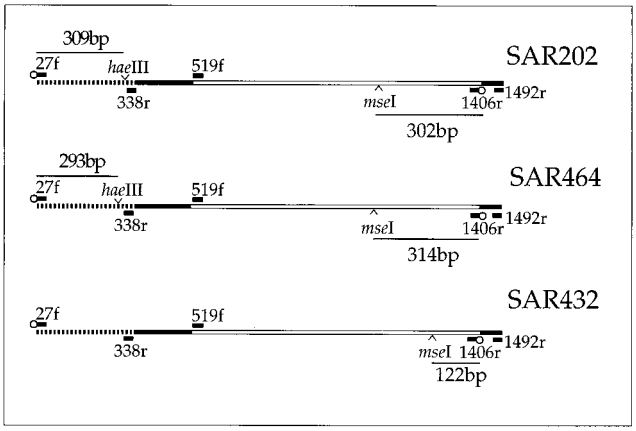

FIG. 1. Locations of PCR primers and cleavage sites of the restriction endonucleases used in this study.

PCR primers. Quantitative experiments used either the 27F-FAM–338R or the 519F–1406R-HEX primer pair. 27F-FAM, which was graciously supplied by Applied Biosystems Inc. (Foster City, Calif.), was 5′ end labeled with the phosphoramidite fluorochrome 5-carboxy-fluorescein (FAM). 1406R-HEX, purchased from Genset (San Diego, Calif.) was 5′ end labeled with the phosphoramidite fluorochrome 6-carboxy-2′4′7′,7-hexachlorofluorescein (HEX). All primers were added to final concentrations of 0.5 μM.

Detection of PCR products. Labeled PCR products were digested with restriction endonucleases producing fragments of different sizes (Fig. 1). FAM-labeled PCR products were digested with *Hae*III (Promega) (5 U of enzyme per μg of PCR product; 2 h; 37°C), and HEX-labeled PCR products were digested with *Mse*I (New England Biolabs, Beverly, Mass.) (5 U of enzyme per μg of PCR product; 2 h; 37°C). Labeled fragments (50 fmol) were chromatographically separated by polyacrylamide gel electrophoresis in an Applied Biosystems 373A automated sequencer in Genescan mode, which estimated both the sizes of fragments and the integrated fluorescence emissions of individual bands. Calibration curves with FAM- and HEX-labeled fragments were linear up to 50 fmol. Thus, the ratios of different PCR products were accurately represented by the ratios of peak areas.

Kinetic models. In order to better interpret the outcome of the quantitative PCR assays, a series of kinetic numerical models were created by using the modeling software Stella (High Performance Systems, Inc., Hanover, N.H.).

Effect of the number of cycles. The kinetic models developed to interpret PCR biases (see Results) predicted that the bias should increase with the number of cycles. To test this prediction, SAR202-SAR464 (4:1) template mixes were amplified as described above. The reactions were stopped by freezing after 10, 15, 25, or 35 cycles, and the ratio of the fragments was measured as described above.

RESULTS

We used two different primer pairs to amplify mixed templates that consisted of pairwise combinations of 16S rDNAs from three different bacteria. We observed biases which were strongly dependent on the choice of primers and dependent to a lesser extent on the templates. For one primer pair, we observed either little or no bias and a generally low yield of product. In contrast, for the second primer pair, we observed both a strong bias and a much higher molar yield of product. The following discussion will focus on the results obtained by using PCR products as templates. The results of assays using linearized plasmids as templates were similar.

519F–1406R primer pair. When the template was mixed SAR202 and SAR464, the ratios of 519F-1406R PCR products did not differ from the ratios of the templates (Fig. 2). Similar results were obtained with template mixtures of SAR432 and SAR464 (results not shown). When mixtures of SAR202 and SAR432 were used as templates, a slight bias occurred; the proportion of SAR202 in the products was higher than its

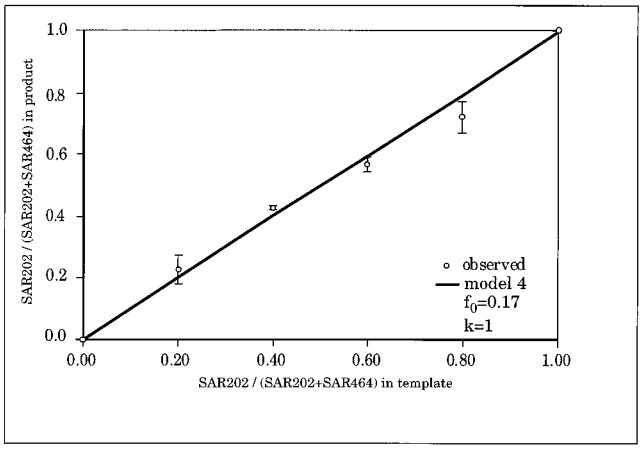

FIG. 2. Amplification of genes with 519F-1406R from mixtures of clones SAR202 (affiliated with the *Chloroflexus-Herpetosiphon* phylum) and SAR464 (belonging to the SAR11 cluster of the α division of the proteobacteria) and prediction of model 4, assuming that k equals 1 and f_0 equals 0.17 cycle^{-1}. Error bars indicate the standard deviations of triplicate PCRs.

proportion in the templates for all the template ratios (Fig. 3). Deviations from linearity seen with this template pair are consistent with predictions for templates differing in amplification efficiency as a consequence of primer preference, secondary structure, or G+C content. All reactions using the primer pair 519F-1406R had lower yields than reactions using the 27F-338R primer pair, producing 16 to 40 nM 888-bp HEX-labeled fragment after 35 cycles.

27F-338R primer pair. With the 27F-338R primer pair and mixtures of SAR202 and SAR464 as templates, we obtained the surprising result that the PCR products were biased towards a final ratio of 1:1 (Fig. 4) regardless of the initial ratio between the templates. This contrasted with the results obtained with the primer pair 519F-1406R and led directly to the formulation of the kinetic model presented below. In addition, the reactions using the primer pair 27F-338R had much higher yields than the reactions using the 519F-1406R primer pair, producing about 140 nM 312-bp FAM-labeled amplicons after 37 cycles.

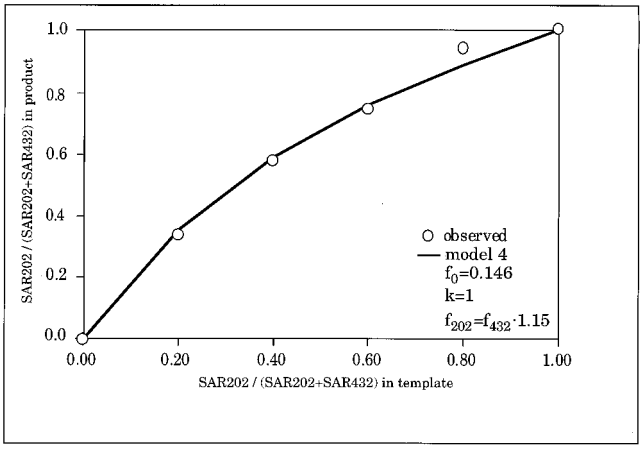

FIG. 3. Amplification of genes with 519F–1406R from mixtures of clones SAR202 (affiliated with the *Chloroflexus-Herpetosiphon* phylum) and SAR432 (a marine gram-positive bacterium) and prediction of model 4, assuming the indicated values for k, f_0, and f_{202}.

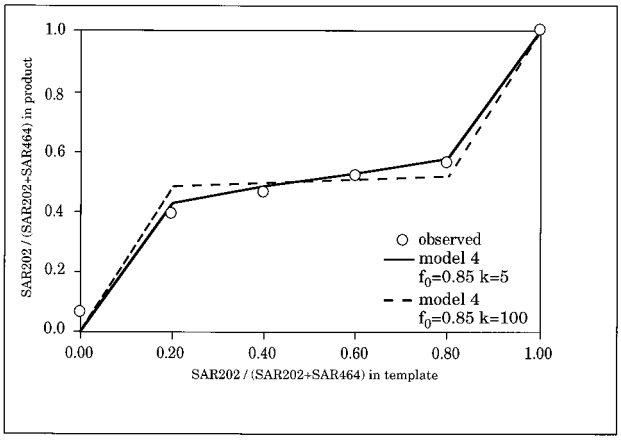

FIG. 4. Amplification of genes with 27F-338R from mixtures of clones SAR202 and SAR464 and predicted results for model 4, in which reannealing of templates inhibits the formation of primer template hybrids (for the dashed line, $k = 100$ and $f_0 = 0.85$ cycle^{-1}; for the solid line, $k = 5$ and $f_0 = 0.85$ cycle^{-1}).

Kinetic models. For the 27F-338R primer pair, the shapes of the curves displaying the observed biases as a function of initial ratios of template molecules were quite unlike the curves predicted by simple models based on G+C bias or primer preference; hence, we developed a kinetic model which took into account the possibility that templates might reanneal and thereby exclude primers (21, 23). This model matched the observed bias and also provided the testable hypothesis that the bias should be a function of the number of cycles, which was verified by further experiments.

The simplest model for a PCR assumes that in each cycle of replication all copies of the genes are replicated. In this case, after n replication cycles, the final molarity of the gene, M, will be equal to

$$M = M_0 \cdot 2^n \tag{1}$$

where M_0 is the initial concentration of template genes.

However, this model predicts an unrealistic final product concentration. A more realistic model assumes that only a fraction of the template is amplified in each replication cycle. This fraction is defined by the parameter f, the efficiency of replication per cycle:

$$M = M_0 \cdot e^{f \cdot n} \tag{2}$$

A modification of equation 2 can also be used to predict the outcomes of PCRs which favor one of the templates, as for G+C bias or primer preference. In such cases, a unique efficiency can be assigned to each template, leading to bias increasing the proportion of the product with the higher amplification efficiency. This bias is described as

$$\frac{M_1}{M_2} = \frac{M_{1_0} \cdot e^{f_1 \cdot n}}{M_{2_0} \cdot e^{f_2 \cdot n}} = \frac{M_{1_0}}{M_{2_0}} \cdot e^{(f_2 - f_1) \cdot n} \tag{3}$$

where M_1 is the molarity of the first template, M_2 is the molarity of the second template, f_1 is the amplification efficiency of the first template, and f_2 is the amplification efficiency of the second template.

The models described by equations 2 and 3 assume that the amplification efficiency, f, remains constant as the cycle number changes. Replication efficiency may actually decrease over successive cycles because of decreasing concentrations of primers and dNTPs or decreasing enzyme activity (21, 23). De-

TABLE 2. Concentrations of combined PCR products measured after 35 amplification cycles or calculated by models 2 and 4 using the listed parameters as described in the text

Templates	Primers	Method of determination	Fixed parameter(s)	Calculated parameter	Molarity of combined products
SAR202-SAR464	519F-1406R	Observed			$3.79 \cdot 10^{-8}$
		Model 2		$f = 0.17$	$3.79 \cdot 10^{-8}$
		Model 4	$k = 1$	$f_0 = 0.17$	$3.74 \cdot 10^{-8}$
SAR202-SAR432	519F-1406R	Observed			$1.69 \cdot 10^{-8}$
		Model 2			$1.69 \cdot 10^{-8}$
		Model 4	$k = 1, f_0 = 0.146$	$f_{202} = 1.15 \cdot f_{464}$	$2.58 \cdot 10^{-8}$
SAR202-SAR464	27F-338R	Observed			$1.42 \cdot 10^{-7}$
		Model 2		$f = 0.20$	$1.42 \cdot 10^{-7}$
		Model 4	$f_0 = 0.85$	$k = 5$	$4.98 \cdot 10^{-7}$
		Model 4	$f_0 = 0.85$	$k = 100$	$1.53 \cdot 10^{-7}$

creases in the concentrations of dNTPs or enzyme activity should affect the amplification efficiencies of different templates equally. Thus, equations 2 and 3 probably provide good estimations of product ratios despite these effects.

However, for decreases in primer concentration the approximations provided by equations 2 and 3 may not hold, since as the concentration of products increases, the single strands formed at each denaturation step may reanneal to their homologous complements during the annealing step and so inhibit the formation of primer-template complexes (21, 23). From the second-order kinetics of such competing reactions, we derived the following equation for the decrease in the amplification efficiency during each replication cycle caused by the reannealing inhibition effect:

$$f(n) = f_0 \cdot \left(\frac{P(n)}{k \cdot M(n) + P(n)} \right) \quad (4)$$

where $P(n)$ and $M(n)$ are the molarities of the primer and templates, respectively, at the start of each replication cycle, f_0 is the theoretical maximum amplification efficiency, and k is the ratio between the rate constants of the reannealing and priming reactions. In this model we also assume that in a PCR mixture containing mixed templates, each template reanneals only to its homologous complement and so does not inhibit the priming reaction of the other template.

Calculating the values of *f* and *k*. We performed sensitivity analyses to estimate the values of f_0 and k in equation 4 which resulted in the best predictions of observed final product concentrations and ratios among different PCR products. In these analyses, the value of one of the parameters was fixed and a search was made for a best fit value of the second parameter. For experiments with the 27F-338R primer set, data from experiments in which the number of cycles was varied were also considered.

The calculated values for f, obtained by using equation 2, were 0.17 cycle^{-1} for the SAR202-SAR464 template mixture and 0.15 cycle^{-1} for the SAR202-SAR432 template mixture amplified with 519F-1406R.

The sensitivity analyses, which assumed that k equaled 1 for the reactions using SAR202-SAR464 and SAR202-SAR432 template mixtures amplified with 519F-1406R, resulted in the same values for f_0 (Table 2) as were calculated with equation 2. A value of 1 for k assumes that the hybridization of a primer to a single-stranded homolog will occur at the same rate as the reannealing of the homologs when primers and single-stranded homologs have the same molarities. Thus, choosing a value of 1 for k compensates for the decrease in efficiency as well as the bias caused by template reannealing described by equation 4. The low value for f_0 estimated by the sensitivity analysis suggests that the reactions which use the primer pair 519F-1406R have an inherently low initial efficiency.

Since the reaction using the SAR202-SAR432 template mixture amplified with 519F-1406R produced a slight bias with SAR202 as the favored template, to evaluate the extent of such bias a sensitivity analysis assuming that k equaled 1 and f_0 equaled 0.15 was performed. Under such conditions, the best fit (Fig. 3) was attained when the initial efficiency of amplification of SAR202 (f_{202}) was 15% higher than the initial efficiency of amplification of SAR432 (f_{432}).

The integrated value of f calculated with equation 2 for the SAR202-SAR464 reaction using 27F-338R was 0.20 cycle^{-1}. Equation 4 predicted the unusual shape of the bias curve and also predicted that a decrease in the number of cycles would reduce bias which resulted from template reannealing by preventing amplicon concentrations from rising to a critical level. The influence of cycle number on bias is shown in Table 3 and Fig. 5. The integrated amplification efficiency was calculated by equation 2 from the molarity of products after n cycles. The integrated amplification efficiency during the initial 10 cycles was higher (0.62 cycle^{-1}) than the integrated efficiency for 35 cycles (0.21 cycle^{-1}) (Table 3), indicating that the efficiency decreased with time. In fact, the integrated efficiency for the 30th cycle to the 35th cycle was only 0.02 cycle^{-1}. Also, as predicted by model 4, the bias was not constant but increased towards a 1:1 product ratio as the number of cycles increased (Fig. 5).

To find the best fit of model 4 to the observed data, we took into consideration the high initial efficiencies; therefore, we

TABLE 3. Effect of the number of amplification cycles on the concentration of products, amplification efficiency, and ratios among templates

No. of cycles	Molarity of products	Integrated f (cycle^{-1})[a]	Ratio of SAR202 templates to SAR202 + SAR464 templates
0	$1.03 \cdot 10^{-10}$		0.80
10	$5.75 \cdot 10^{-8}$	0.632	0.68
15	$1.08 \cdot 10^{-7}$	0.125	0.50
25	$1.49 \cdot 10^{-7}$	0.032	0.50
35	$1.90 \cdot 10^{-7}$	0.024	0.50

[a] The integrated amplification efficiency was calculated by equation 2.

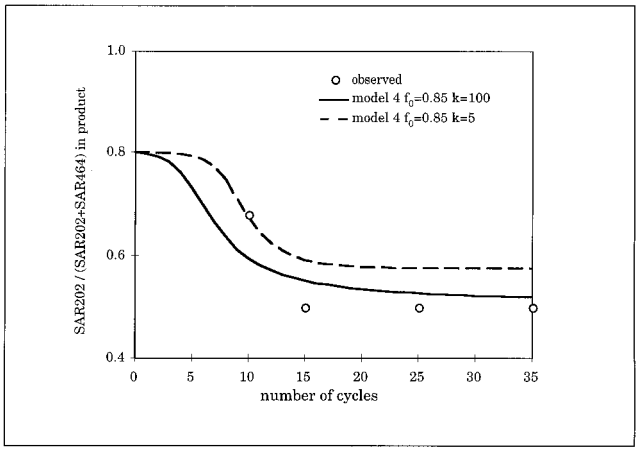

FIG. 5. Ratio of SAR202 genes to the total PCR products (SAR202 plus SAR464) as a function of the number of cycles for the 27F-338R primer pair and prediction by model 4. Deviations from the original ratio of genes (0.80) increased with the number of cycles and converged to a value of 0.5 for each gene.

sought the optimum value of k for a fixed value of f_0, 0.85 cycle^{-1} (23). This is a tractable procedure which avoids the complexity of fitting both the total product concentration (which decreases as k increases) and the bias (which increases as k decreases). For an f_0 of 0.85 cycle^{-1}, the value of k for which model 4 best predicted the total PCR product concentration was 100, which created somewhat more bias than was observed (Fig. 4). When, however, k was set equal to 5, the model 4 bias prediction was more accurate but the predicted final product concentration was about two times the observed values.

The value of k can also be approximated directly from the relative lengths of DNA molecules by the method of Wetzmur and Davidson (27). By this approach, we independently calculated a value of 17 for k, which agrees well with experimental observations and falls between the values of 5, the estimated value for k which most accurately predicted the bias, and 100, the estimated value for k which most accurately predicted the yield of PCR product.

DISCUSSION

Several hypothetical mechanisms which might contribute to the selective amplification of some templates from mixtures of more than one template and the resulting misrepresentation of gene abundance in final PCR products have been described. One example is the selective amplification of templates with low G+C content (low-G+C templates). Because high-G+C genes dissociate into single-stranded molecules with lower efficiency than low-G+C templates, low-G+C templates may be overrepresented in the population of single-stranded molecules available for hybridization with primers, resulting in a bias in their favor. The introduction of acetamide into PCRs has been suggested as one method of reducing the melting point of template hybrids, thereby making it possible for high-G+C genes to compete more effectively (20).

Figure 4 provides an example of a type of bias not predicted by the selection models described above. In these experiments we observed that the final concentrations of genes tended towards a 1:1 mixture independent of the concentrations of genes in the original reaction mixture. It was not possible to explain these results by invoking models in which the G+C content of genes or primer selection created biases in the representation of genes. A kinetic model which explained the results was developed. The central feature of this model is that as the concentrations of product molecules increase, the rate of the bimolecular reaction in which homologous single-stranded template molecules hybridize with each other will increase as a function of the product concentrations. Since single-stranded molecules must react with free primer to initiate extension reactions, the rate of formation of primer-template hybrids will be influenced by the proportion of template molecules in a single-stranded state. This mechanism has been previously mentioned (21, 23), but not in the context of mixed templates. In reactions with mixed templates and high amplification efficiency, the template with the higher initial concentration in the starting mixture reaches inhibitory concentrations sooner while the second template continues to undergo amplification efficiently, and thereby the original difference in concentrations decreases until a 1:1 ratio is achieved. The results of quantitative PCR assays using the primer pair 27F-338R fit the predictions of this model. The reactions had high initial amplification efficiencies, which resulted in high concentrations of products in early cycles of replication. The final product concentrations were biased toward a 1:1 ratio. As predicted by the model and demonstrated by subsequent experiments, this bias was strongly dependent on the number of cycles. As predicted by model 4 for reactions with low amplification efficiencies, the 519F-1406R reaction did not show the bias; the molarity of the products of the 519F-1406R amplification after 35 cycles was five times lower than that of the 27F-338R products.

Two factors might cause lower amplification efficiencies for the 519F-1406R primer pair. First, the 519F-1406R fragment is about three times longer than the 27F-338R fragment. *Taq* DNA polymerase may not amplify a long fragment as efficiently as it amplifies a short fragment. Second, the 1406R primer is a 15-mer, which should anneal at 55°C with an efficiency lower than that of the 20-mer 27F or that of the 18-mer 338R.

The estimated best fit values of k for model 4 were of the same order as the values predicted independently by an equation which considered the influence of DNA length on hybridization kinetics. The observation that the reaction of reannealing of PCR products is more efficient than the priming reaction is consistent with hybridization theory. The rate-limiting step in DNA annealing reactions is the initial formation of short regions of double-stranded DNA at correctly paired positions (27); longer DNA molecules recognize complementary strands at a higher rate than do shorter strands, explaining the fact that the efficiency of the reannealing reaction is higher than that of the priming reaction.

The model described by equation 4 is, to our knowledge, the first model to quantitatively estimate the effect of template reannealing. Previous studies have attempted to model quantitative PCR (16–18) for the measurement of gene concentrations on the basis of the measurement of internal standards. Raeymaekers's model (17, 18) is very similar to the model described by equation 4 except that it assigns two different constants (one for the gene of interest and one for the standard) to factor differences in the drop in efficiency for the different templates; also, the model does not take into consideration the template reannealing effect. The models described by Nedelman and colleagues (16) assume that the amplification efficiencies for the standard and the gene of interest are the same, and they also do not take into account the template reannealing effect.

In his discussion of quantitative PCR, Raeymaekers (18) proposes that differences in amplification efficiencies between

standards and templates, as well as variations in such differences among different dilutions of the standard, may explain the deviations from linearity observed in some studies applying quantitative PCR. He suggests that differences in the amplification efficiencies of sequences containing the same priming sites may be caused by differences in the kinetics of product accumulation in the nonexponential phase of PCR (18). Our model agrees with this prediction and explains how product accumulation in the nonexponential phase of PCR causes amplification efficiencies to differ between standards and genes of interest containing the same priming sites. The model also explains why these differences may not be the same for all dilutions of the standard.

Morrison and Gannon (14) examined the outcome of a competitive PCR assay in which they investigated the effect of different concentrations of target, holding the concentration of one template constant while performing a 10-fold serial dilution of the other template. They thus asked a question different from ours, as the largest initial template ratio in our experiments was 4:1. In their experiments, when one of the templates was several orders of magnitude less concentrated than the other, it did not undergo amplification at all. Their method of assaying the outcomes of the competitive PCRs, ethidium bromide staining on agarose gels, is well suited for the detection of the presence and absence of product but not sufficiently quantitative to detect smaller differences in yield. Their conclusion that the ratio of targets will be preserved in the ratio of products, since factors inhibiting PCR act equally on the amplification of both targets, is contradicted by our results with the 27F-338R primer pair as well as by the predictions of equation 4.

The only case in which we observed preferential amplification of one of the templates was the experiment using the pair SAR202-SAR432, amplified with 519F-1406R (Fig. 3), with the calculated initial amplification efficiency for the fragment from SAR202 being 15% higher than that for the fragment from SAR432. The moles percent G+C content of SAR202 was higher (58%) than that of SAR432 (52%), and the priming sites of both templates present the same bases at the degenerate positions of the primers 519F and 1406R. Thus, neither of these models seemed to explain the data. An alternative explanation for the preferential amplification of the SAR202 template is differences in secondary structure affecting either the availability of the priming sites or the polymerization reaction.

The results presented here have implications for studies using PCR to amplify complete 16S rDNAs from DNA samples from natural ecosystems. If the environmental DNA sample contains highly diverse templates, it seems likely that the PCR-produced bias described by model 4 will be small, since it is unlikely that the amplification of any particular 16S rDNA will produce products at a concentration which is high enough to produce the reannealing inhibition effect. In addition, since the amount of this bias is dependent on the number of cycles, it can be reduced by keeping the number of cycles low. However, the complex chemistry of the PCR and phenomena which might result in the differential amplification of homologs from complex DNA mixtures will require further study before final conclusions about the quantitative potential of this method can be reached.

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