RNA: a method to specifically inhibit PCR amplification of known members of a multigene family by degenerate primers

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ABSTRACT

The polymerase chain reaction (PCR) is a versatile method to amplify specific DNA with oligonucleotide primers. By designing degenerate PCR primers based on amino acid sequences that are highly conserved among all known gene family members, new members of a multigene family can be identified. The inherent weakness of this approach is that the degenerate primers will amplify previously identified, in addition to new, family members. To specifically address this problem, we synthesized a specific RNA for each known family member so that it hybridized to one strand of the template, adjacent to the 3′-end of the primer, allowing the degenerate primer to bind yet preventing extension by DNA polymerase. To test our strategy, we used known members of the soluble, nitric oxide-sensitive guanylyl cyclase family as our templates and degenerate primers that discriminate this family from other guanylyl cyclases. We demonstrate that amplification of known members of this family is effectively and specifically inhibited by the corresponding RNAs, alone or in combination. This robust method can be adapted to any application where multiple PCR products are amplified, as long as the sequence of the desired and the undesired PCR product(s) is sufficiently distinct between the primers.

INTRODUCTION

Gene families are best defined by related functions of individual gene products. In the absence of functional data, gene family members can be identified by amino acid sequence homology. The two main methods to identify new family members within an organism, short of a complete genome sequence, are amplification by polymerase chain reaction (PCR) with degenerate primers (1,2) and low stringency hybridization to screen libraries (3,4). If continuous amino acid sequences (>5) are highly conserved within a gene family, the former method is feasible. Low stringency hybridization does not require such concentrated stretches of conserved sequence, but it does not have the intrinsic advantage of PCR: selection coupled with amplification.

Each of these approaches has an inherent shortcoming: because the search for new gene family members is based on the sequence of previously identified members, they are inevitably re-identified. This fundamental flaw can make it difficult, if not impractical, to sift through a large number clones of known family members, in order to find new members. This problem is exacerbated if any known family member is abundant and/or the family is diverse. We sought a general method to select against the known family members, without interfering with the identification of possible new members.

Our strategy takes advantage of the linkage between recognition (annealing) and amplification (extension) during PCR. We devised a method that allowed degenerate primers to anneal to all gene family members, but prevented extension only in those members that were already known. Our strategy is distinct from restricted PCR (5,6), where annealing of a non-extendable, specific oligonucleotide prevents annealing of the extendable, degenerate oligonucleotide to the template. Restricted PCR has a narrow range of success, where the specific inhibitory primer is ineffective at low concentrations and interferes with annealing of the degenerate primer to other templates at higher concentrations.

We could have overcome the problems of restricted PCR by designing similar non-extendable oligonucleotides to hybridize adjacent to the 3′-end of the degenerate primer (7,8). This approach requires that the non-extendable oligonucleotide hybridizes to a sequence that is divergent enough within the gene family to ensure that PCR amplification was specifically inhibiting the corresponding gene family member. Instead we chose a more robust strategy that can be used for any gene family, regardless of the properties of the degenerate primers and intervening sequence, illustrated in Figure 1. We demonstrate that a specific RNA corresponding to a known gene family member, which does not interfere with the annealing of degenerate primer, effectively inhibits the amplification of this known gene family member. The specificity of this inhibition allows RNA inhibitors to be used in combination, with the aim of inhibiting all known gene family members.

To test our strategy, we used degenerate primers to amplify a subfamily of guanylyl cyclases. The soluble, heterodimeric guanylyl cyclases require an α- and a β-subunit for activity, and the predominant form is α1β1, which is found in most

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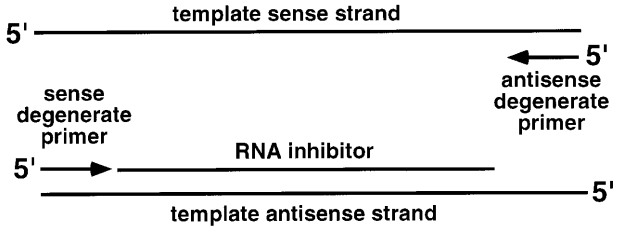

Figure 1. Rationale for RNA as an inhibitor of PCR amplification by degenerate primers. RNA is synthesized by *in vitro* transcription so that it binds specifically to one of the template strands (in this case, the antisense strand). The 5′-end of the RNA is adjacent to the 3′-end of the degenerate primer (in this case, a sense degenerate primer) so that it can still bind to the template strand, but extension from this degenerate primer is prevented by the bound RNA.

mammalian cell types. In mammals only two other subfamily members have been identified: β2 from rat kidney and α2 from human fetal brain. Because different sets of degenerate primers had been used to identify β2 and α2, we started our search for any novel α- or β-subunits in rat kidney with another set of degenerate primers based on all four subfamily members. To prevent reamplification of subfamily members known to exist in rat kidney, we synthesized specific RNA inhibitors to prevent the amplification of α1, β1 and β2 (α2 is not found in kidney), in order to amplify any other α- or β-subunits that may exist in rat kidney.

MATERIALS AND METHODS

Oligonucleotides

The antisense degenerate primer (5′-GAATTCTCGAG-GATCCRAAIARRCARTAICIIGGCAT-3′) was based on an amino acid sequence MPRYCLF that is highly conserved in all guanylyl cyclases, as previously described (9). The sense degenerate primer (5′-CCCGCGGAATTCAAGCTTMRIGG-ICARATGRTI-3′) was based on an amino acid sequence KGQMI that is conserved in soluble, heterodimeric guanylyl cyclase α- and β-subunits (9–14), but not membrane forms of guanylyl cyclase (15–20). Degenerate primers were synthesized and purified by HPLC (Operon).

For α1, β1 or β2 *in vitro* transcription templates, the following primer pairs were synthesized (Ransom Hill) with a T7 promoter at the 5′-end of one primer: α1, 5′-CAGTAATAC-GACTCACTATAGGGCTACATCGTTGAATCCA-3′ (T7 α1 sense) and 5′-CTTCACTCCGACAACT-3′ (α1 antisense); β1, 5′-CAGTAATACGACTCACTATAGGGCTATTTACCGGA-AGCAG-3′ (T7 β1 sense) and 5′-CCGCTGTCCAATCAC-3′ (β1 antisense); β2, 5′-CAGTAATACGACTCACTATAG-GGCTGGATGGAGTCTCTG-3′ (T7 β2 sense) and 5′-ATC-TTGTCTCCCACAAC-3′ (β2 antisense). These oligonucleotides are designed so that the 5′-end of the transcribed RNA is within 1 or 2 bases of the 3′-end of the sense degenerate primer (Fig. 1).

In vitro transcription

Templates for *in vitro* transcription were amplified from rat α1 (21), β1 (21) or β2 (9) cDNA by PCR using 0.5 μM of α1 T7 sense/α1 antisense, β1 T7 sense/β1 antisense or β2 T7 sense/β2 antisense, respectively. In the presence of 50 mM Tris pH 8.3, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 2% sucrose, 0.25 mg/ml BSA and 0.1 mM cresol red, PCR was performed with a mixture of KlenTaq (Ab Peptides) and *Pfu* (Stratagene) DNA polymerases (22) in an Air Thermal Cycler (Idaho Technology) for 30 cycles of 94°C (0 s), 55°C (0 s) and 72°C (15 s), followed by a 2 min incubation at 72°C. After agarose gel electrophoresis and purification by Qiaex resin (Qiagen), *in vitro* transcription was performed with a T7 Megascript kit (Ambion) and DNA was removed by DNase treatment. Synthesized RNA was confirmed to be a single 750 bp band and RNA concentrations were determined by spectrophotometry.

PCR with degenerate primers

cDNA was obtained from plasmids containing rat α1, β1 or β2 or synthesized from rat kidney poly(A)$^+$ RNA (Ambion) with displayTHERMO-RT (Display Systems Biotech). PCR was performed as described above, except that *Pfu* DNA polymerase was omitted, BSA was 0.75 mg/ml, annealing temperature was 50°C and degenerate sense and antisense primers were used. After PCR each sample was incubated for 15 min with 1 Kunitz U RNase A (Roche) and subjected to agarose gel electrophoresis. Southern blot analysis was also performed on rat kidney cDNA amplified by PCR, with downward transfer to Nytran membranes (S&S), hybridization at 65°C in ExpressHyb (Clontech) with random-prime labeled probe (Amersham Pharmacia) and washed at 65°C with 0.1× SSC, 0.2% SDS and 0.1% sodium pyrophosphate.

RESULTS

We first tested RNA as an inhibitor of PCR amplification by using pure α1, β1 or β2 templates. Relatively non-stringent PCR conditions were selected to enhance the amplification of these templates, so that RNA would have to be a robust inhibitor. Under these relaxed PCR conditions, this degenerate primer pair amplified α1 and β2 well, with the expected 750 bp bands clearly visible by ethidium bromide staining (Fig. 2A and C). However, β1 was amplified less efficiently; ~100-fold more β1 template was required to see an equivalent amount of PCR product (Fig. 2B). These low stringency PCR conditions also resulted in non-specific amplification, with bands at 400 bp (α1 template), 250 bp (β1 template) and 300/400 bp (β2 template) (Fig. 2A, B and C, respectively). Amplification of α2 was extremely inefficient; a 750 bp band was barely detectable, even if 100–1000-fold more α2 was used (data not shown).

Each template was amplified in the presence of increasing amounts of the corresponding RNA. Amplification of the α1 template was progressively inhibited by α1 RNA, with half-maximal inhibition at ~10 nM α1 RNA (Fig. 2A). A residual band was visible at 50 nM α1 RNA (Fig. 2A, lane 5), which is attributable to RNA. The weakly staining band was present without added template (Fig. 2A, lane 6) and the band was not visible after RNase treatment (Fig. 2A, lane 7). β1 RNA was a slightly less effective inhibitor, requiring between 10 and 25 nM RNA for half-maximal inhibition of amplification of the β1 template (Fig. 2B). Similarly, between 10 and 25 nM β2 RNA gave half-maximal inhibition of β2 amplification (Fig. 2C). α1 and β2 RNA inhibited amplification of their

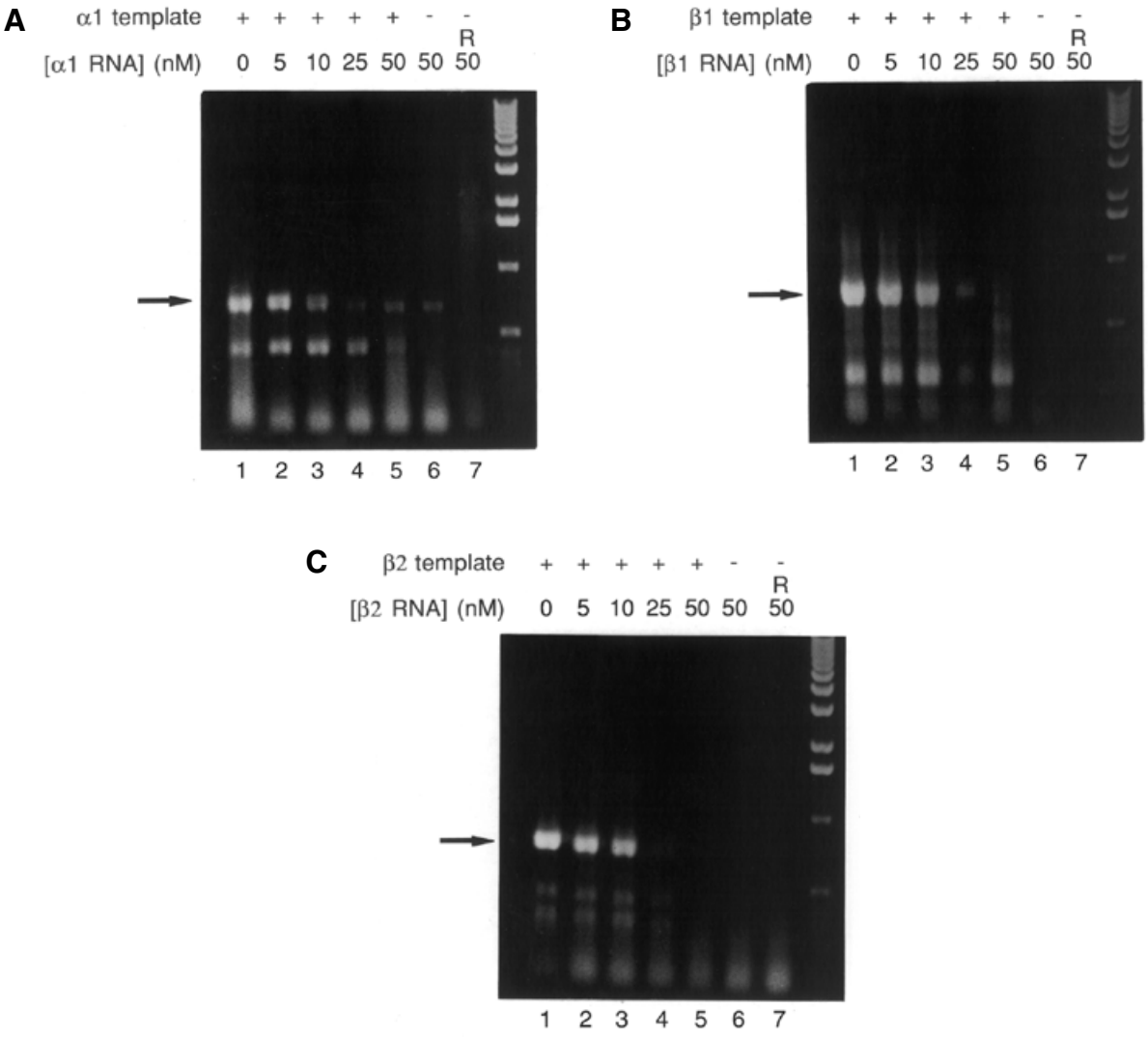

Figure 2. (**A**) Increasing concentrations of α1 RNA inhibit the amplification of α1 cDNA template. PCR was performed with 0.5 μM sense and 0.5 μM antisense degenerate primers, 0.5 pM α1 cDNA template and 0 (lane 1), 5 (lane 2), 10 (lane 3), 25 (lane 4), 50 nM (lane 5) α1 RNA. PCR was performed with 0.5 μM sense and 0.5 μM antisense degenerate primers without template in the presence of 50 nM α1 RNA (lanes 6 and 7). After PCR the sample (lane 7) was incubated for 15 min with 1 Kunitz U RNase A at 37°C. After electrophoresis, ethidium bromide staining was visualized. The arrow indicates the bands corresponding to the expected 750 bp PCR product. (**B**) Increasing concentrations of β1 RNA inhibit the amplification of β1 cDNA template. PCR was performed with 0.5 μM sense and 0.5 μM antisense degenerate primers, 50 pM β1 cDNA template and 0 (lane 1), 5 (lane 2), 10 (lane 3), 25 (lane 4), 50 nM (lane 5) β1 RNA. PCR was performed with 0.5 μM sense and 0.5 μM antisense degenerate primers without template in the presence of 50 nM β1 RNA (lanes 6 and 7). After PCR the sample (lane 7) was incubated for 15 min with 1 Kunitz U RNase A at 37°C. The arrow indicates the bands corresponding to the expected 750 bp PCR product. (**C**) Increasing concentrations of β2 RNA inhibit the amplification of β2 cDNA template. PCR was performed with 0.5 μM sense and 0.5 μM antisense degenerate primers, 0.5 pM β2 cDNA template and 0 (lane 1), 5 (lane 2), 10 (lane 3), 25 (lane 4), 50 nM (lane 5) β2 RNA. PCR was performed with 0.5 μM sense and 0.5 μM antisense degenerate primers without template in the presence of 50 nM β2 RNA (lanes 6 and 7). After PCR the sample (lane 7) was incubated for 15 min with 1 Kunitz U RNase A at 37°C. The arrow indicates the bands corresponding to the expected 750 bp PCR product.

respective non-specific bands (Fig. 2A and C), which is consistent with mispriming of the sense primer; in contrast, β1 RNA did not inhibit amplification of the non-specific band, which may reflect a different source for non-specific amplification.

After demonstrating the effectiveness of α1, β1 and β2 RNA as inhibitors for the amplification of the corresponding templates, we tested the specificity of each RNA inhibitor. Each RNA was used at 50 nM, a concentration that was sufficient to cause virtually complete inhibition. When α1 was used as a template, α1 RNA inhibited amplification, and there was no discernible inhibition by β1 or β2 RNA (Fig. 3A). When β1 template was amplified, β1 RNA was a specific inhibitor, as α1 or β2 RNA did not inhibit β1 amplification (Fig. 3B). Inhibition of β2 amplification was also specific for β2 RNA, with no inhibition by α1 or β1 RNA. Therefore, each RNA effectively and specifically inhibits its corresponding template under ideal conditions, when pure template is used.

We tested whether each RNA, alone or in combination, could also produce specific and robust inhibition in a complex mixture, as we had observed with pure templates. For a

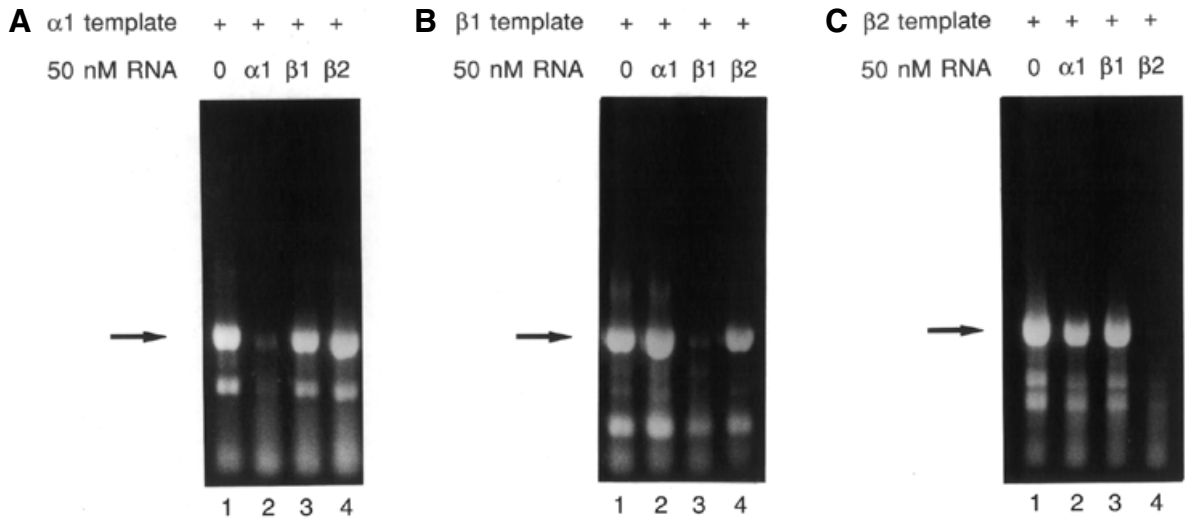

Figure 3. (**A**) α1 RNA specifically inhibits the amplification of the α1 cDNA template. PCR was performed with 0.5 μM sense and 0.5 μM antisense degenerate primers, 0.5 pM α1 cDNA template, without RNA (lane 1), with 50 nM α1 RNA (lane 2), with 50 nM β1 RNA (lane 3) or with 50 nM (lane 4) β2 RNA. The arrow indicates the bands corresponding to the expected 750 bp PCR product. (**B**) β1 RNA specifically inhibits the amplification of the β1 cDNA template. PCR was performed with 0.5 μM sense and 0.5 μM antisense degenerate primers, 50 pM β1 cDNA template, without RNA (lane 1), with 50 nM α1 RNA (lane 2), with 50 nM β1 RNA (lane 3) or with 50 nM (lane 4) β2 RNA. The arrow indicates the bands corresponding to the expected 750 bp PCR product. (**C**) β2 RNA specifically inhibits the amplification of the β2 cDNA template. PCR was performed with 0.5 μM sense and 0.5 μM antisense degenerate primers, 0.5 pM β2 cDNA template, without RNA (lane 1), with 50 nM α1 RNA (lane 2), with 50 nM β1 RNA (lane 3) or with 50 nM (lane 4) β2 RNA. The arrow indicates the bands corresponding to the expected 750 bp PCR product.

template we chose rat kidney cDNA, which contains α1, β1 and β2 (23). The α- and β-based degenerate primer pair amplified a mixture of α1, β1 and β2, which were detected individually. Aliquots were subjected to electrophoresis, transferred to three membranes by Southern blot and hybridized with α1, β1 or β2 probe (Fig. 4A, B and C, respectively). Amplification of α1 was inhibited by α1 RNA alone, in combination with β1 RNA, in combination with β2 RNA or in combination with both β1 and β2 RNAs (Fig. 4A, lanes 2, 5, 6 and 8, respectively). Accordingly, the presence of β1 and/or β2 RNA did not diminish the effectiveness of α1 RNA as an inhibitor. α1 RNA was a specific inhibitor because amplification of α1 was not inhibited by β1 or β2 RNA alone (Fig. 4A, lanes 3 and 4, respectively) or in combination (Fig. 4A, lane 7). When β1 PCR product was examined, a comparable specificity was detected. Inhibition of β1 amplification occurred only in the presence of β1 RNA, either alone, combined with α1 RNA, combined with β2 RNA or combined with α1 and β2 RNA (Fig. 4B, lanes 3, 5, 7 and 8, respectively). Specificity was also established as α1 RNA, β2 RNA or a combination of α1 and β2 RNA (Fig. 4B, lanes 2, 4 and 6, respectively) failed to inhibit amplification of β1. Similarly, we found β2 RNA to be a specific inhibitor of β2. Whether alone, with α1 RNA, with β1 RNA or with α1 and β1 RNAs (Fig. 4C, lanes 4, 6, 7 and 8, respectively), β2 RNA inhibited the amplification of β2 cDNA. In the presence of α1 and/or β1 RNA, amplification of β2 was unaffected (Fig. 4C, lanes 2, 3 and 5).

We attempted to identify a novel α- or β-subunit with the KGQMI/MPRYCLF degenerate primer pair, by using the combination of α1, β1 and β2 RNA when amplifying rat kidney cDNA, as well as cDNA from several other tissues. We failed to amplify a band by ethidium bromide staining, except with extensive re-amplification. Subsequent subcloning and sequencing resulted in only α1, β1 and β2, as well as some non-specific products. In contrast, PCR products were clearly visible when only two of the three RNAs were used (data not shown). Our inability to identify new α- or β-subunits most likely results from an absence of novel family members, but it is also possible that new family members lack conservation of one or both degenerate primers.

DISCUSSION

We demonstrated that, when using degenerate primers, RNA corresponding to known gene family members can effectively and specifically inhibit the amplification of the cDNA for that family member. This study represents only a 'proof of principle' and a number of issues surrounding our concept were not explored, including a number of parameters that may affect the application of this general method to other templates and degenerate primers. For example, we do not know the minimum length of RNA that is required for inhibition. We presume that higher concentrations of a shorter RNA would be needed in order to inhibit amplification. We also do not know if a gap between the 3′-end of the degenerate primer and the 5′-end of the specific RNA can be tolerated, and how long that gap could be. If a large gap can be tolerated, then a number of degenerate primers could be tested, and only one RNA per known gene family member would need to be synthesized. Each gene family can potentially have very similar and very dissimilar members. The homology between one gene family member and another may also vary, depending on what region of the

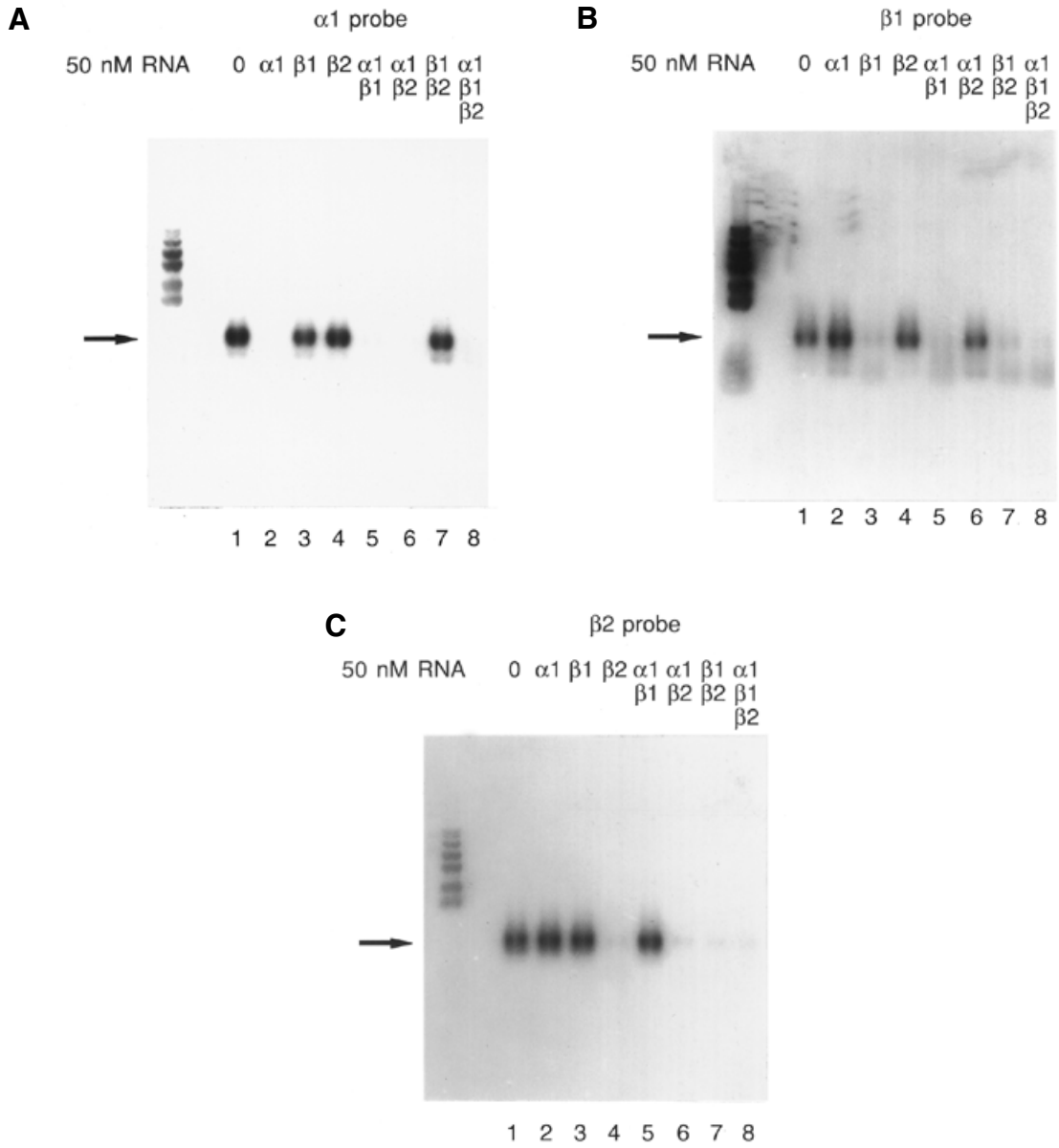

Figure 4. (**A**) α1 RNA specifically inhibits the amplification of α1 from a rat kidney cDNA template. PCR was performed with 1 μM sense and 1 μM antisense degenerate primers and rat kidney cDNA; without RNA (lane 1), with 50 nM α1 RNA (lane 2), with 50 nM β1 RNA (lane 3), with 50 nM β2 RNA (lane 4), with 50 nM α1 RNA and 50 nM β1 RNA (lane 5), with 50 nM α1 RNA and 50 nM β2 RNA (lane 6), with 50 nM β1 RNA and 50 nM β2 RNA (lane 7) or with 50 nM α1 RNA, 50 nM β1 RNA and 50 nM β2 RNA (lane 8). After electrophoresis and Southern blotting, the blot was hybridized with a radiolabeled probe specific for α1, washed at high stringency and subjected to autoradiography. A ladder of molecular weight markers is on the left and the arrow indicates the bands corresponding to the expected 750 bp PCR product. (**B**) β1 RNA specifically inhibits the amplification of β1 from a rat kidney cDNA template. PCR was performed with 1 μM sense and 1 μM antisense degenerate primers and rat kidney cDNA; without RNA (lane 1), with 50 nM α1 RNA (lane 2), with 50 nM β1 RNA (lane 3), with 50 nM β2 RNA (lane 4), with 50 nM α1 RNA and 50 nM β1 RNA (lane 5), with 50 nM α1 RNA and 50 nM β2 RNA (lane 6), with 50 nM β1 RNA and 50 nM β2 RNA (lane 7) or with 50 nM α1 RNA, 50 nM β1 RNA and 50 nM β2 RNA (lane 8). After electrophoresis and Southern blotting, the blot was hybridized with a radiolabeled probe specific for β1, washed at high stringency and subjected to autoradiography. A ladder of molecular weight markers is on the left and the arrow indicates the bands corresponding to the expected 750 bp PCR product. (**C**) β2 RNA specifically inhibits the amplification of β2 from a rat kidney cDNA template. PCR was performed with 1 μM sense and 1 μM antisense degenerate primers and rat kidney cDNA; without RNA (lane 1), with 50 nM α1 RNA (lane 2), with 50 nM β1 RNA (lane 3), with 50 nM β2 RNA (lane 4), with 50 nM α1 RNA and 50 nM β1 RNA (lane 5), with 50 nM α1 RNA and 50 nM β2 RNA (lane 6), with 50 nM β1 RNA and 50 nM β2 RNA (lane 7) or with 50 nM α1 RNA, 50 nM β1 RNA and 50 nM β2 RNA (lane 8). After electrophoresis and Southern blotting, the blot was hybridized with a radiolabeled probe specific for β2, washed at high stringency and subjected to autoradiography. A ladder of molecular weight markers is on the left and the arrow indicates the bands corresponding to the expected 750 bp PCR product.

sequence is examined. For any degenerate primer pair, the intervening sequence of a new family member may be similar enough to a known family member that amplification of this new family member is inhibited by the RNA of the known family member. We do not know how dissimilar the RNA would have to be in order to prevent non-specific inhibition,

but the α1, β1 and β2 RNA that we tested was specific for its corresponding template. α1 and β1 RNA inhibitors are 60% identical, β1 and β2 RNA inhibitors are 47% identical, and α1 and β2 RNA inhibitors are 52% identical. The number of RNA inhibitors that can be used in combination is theoretically unlimited, as long as all of them correspond to the same strand. This practical issue applies especially to large gene families, such as protein kinases, olfactory G protein-coupled receptors, zinc finger transcription factors, cytochrome P450, serine proteases or immunoglobulins (24). For such large families, synthesis of RNA corresponding to every known member would not be worthwhile. Rather, careful design of degenerate primers could allow examination of a subfamily. Whether an entire family or a subfamily is examined, analysis of the intervening sequences may reveal that an inhibitor RNA could prevent the amplification of multiple family members that have closely related sequences. If this were the case, a smaller number of RNA inhibitors would cover the large number of known family members.

Our RNA inhibitor strategy should be robust enough to be applied to other gene families, and it would be especially useful in those species without a sequenced genome. Some choices must be made when designing degenerate primers and RNA inhibitors, and we have outlined a few caveats above. Although these issues are unresolved, we offer the following guidelines to apply our method to other gene families. The first consideration is the selection of degenerate primers, which should be chosen to give the desired selectivity within a gene family with only one special consideration for the RNA inhibitors: the spacing of the primers. At some point, a degenerate primer pair would be too close together to allow specific RNA inhibition. If the primers were 10 bp apart, it would be doubtful that a specific, effective RNA inhibitor could be designed. We have demonstrated the utility of RNA inhibitors for degenerate primers that are 750 bp apart and it is reasonable to predict that RNA inhibitors would be equally successful for primers that are further apart. As degenerate primer pairs get closer together, a shorter RNA inhibitor would become proportionately less effective due to its decreasing affinity for the cDNA template of the corresponding known gene family member. In the case of closer degenerate primers (100 bp apart, for example), we propose that the RNA inhibitor be extended to an appropriate length that would ensure a high affinity for the template. This modification would extend the RNA beyond the opposing degenerate primer (antisense primer in Fig. 1) and allow binding of this primer to the RNA. Without any thermostable reverse transcriptase activity present, there should be no amplification of the inhibitor and the degenerate primer concentration should not be appreciably affected by its stoichiometric binding to the RNA inhibitor. Even so, the concentration of the degenerate primer could be adjusted accordingly. The other major consideration when designing RNA inhibitors is which of the two strands to choose. When using a cocktail of inhibitors, it is essential that the same strand is chosen for each inhibitor, so that inhibitors do not anneal to each other. Nucleic acid alignments of gene family members should be analyzed for the region between the degenerate primers, and the 5′-end of an RNA inhibitor should have less nucleic acid identity when compared to other family members.

It could be argued that RNA inhibition, with regard to the application for which it was intended, will eventually be made obsolete by whole genome sequencing. Even so, RNA inhibitors could be adapted to improve several methods. First, RNA inhibitors are not theoretically limited to degenerate primers; RNA should inhibit amplification when specific primers are used. In preliminary studies, RNA inhibition was effective and specific when specific PCR primers were used (P.S.T.Yuen, unpublished data). Whether degenerate or specific primers are used, RNA inhibition can be used to prevent unwanted amplification whenever the template has a mixed composition so that the amplified sequences are sufficiently different. For example, the differential display method compares a number of cDNA sequences simultaneously from two different cellular sources to detect differential expression levels of genes between different cell types or the same cell type in response to an external stimulus (25). Because arbitrary primers are used to amplify cDNA, the PCR product mixture is dominated by abundant and/or efficiently amplified cDNAs, some of which may obscure other PCR products by comigrating after electrophoresis. Therefore, an amplified band that is slightly more intense in one cellular sample may represent a background of several bands with unaffected abundance and a minor band that has a large difference between samples. Once an amplified cDNA is identified, an RNA inhibitor can be synthesized to prevent re-identification and to allow identification of other potentially regulated cDNAs. Similarly, the representational difference analysis (26) could be enhanced by using RNA inhibitors to prevent identification of previously identified PCR products. With the advent of genomic approaches such as microarrays, applications such as differential display and representational difference analysis may be limited to special circumstances. One example is the identification of rapidly evolving genes, including immunoglobulin genes or human immunodeficiency virus genes.

RNA inhibitors can also be used to detect the presence of a gene from one individual within a pooled source of DNA or RNA, especially if this individual contributes a small fraction of the total sample. Examples include detection of fetal DNA or RNA amidst a maternal background (27,28), detection of T cells to monitor graft versus host disease following allotransplantation or xenotransplantation, or even the presence of DNA or RNA following gene therapy.

Several methods have explored nucleic acids beyond the limited repertoire of a pre-existing genome. Libraries of randomized sequences represent a new type of genome, and these libraries are mostly amplified by PCR. The best example of this type of 'unbiased' strategy is the systematic evolution of ligands by exponential enrichment (SELEX) (29), where the essential components of evolution are recapitulated entirely *in vitro*. Each round of selection leaves DNA or RNA aptamers that have the desired properties, and they must be amplified by PCR or reverse transcription–PCR, respectively. RNA inhibitors can be designed and used as a negative selection step to prevent amplification of undesired aptamers such as previously identified aptamers that dominate the aptamer pool or aptamers that have been identified as non-specific.

There may be other applications of the RNA inhibitor strategy not mentioned here or yet to be developed. Our method can be applied to prevent any undesired amplification by PCR, as long as there is sufficient distinction between the intervening sequence of the desired and the undesired PCR product.

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