RNA (1996), 2:982–994. Cambridge University Press. Printed in the USA.

In vitro selection of a novel catalytic RNA: Characterization of a sulfur alkylation reaction and interaction with a small peptide

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ABSTRACT

An in vitro RNA selection for catalytic activity was used to co-select for binding activity to a small peptide. 5′-phosphorothioate-modified RNA (GMPS-RNA) sequences were selected from a randomized pool of oligoribonucleotides for their ability to accelerate a halide substitution reaction with *N*-bromoacetyl-bradykinin (BrBK). One RNA selected shows a 2,420-fold increase in rate of reaction with BrBK relative to the starting pool. This reaction is specifically inhibited by free bradykinin (K_i 230 μM), indicating that interactions with bradykinin contribute to the rate enhancement. Inhibition of the reaction by the peptide requires both the amino- and carboxy-terminal arginine residues of the peptide for optimal inhibition activity. Reaction with *N*-bromoacetamide is not enhanced, indicating that the intrinsic reactivity of the 5′ phosphorothioate is not increased in the selected RNA. Through 3′-end boundary analysis, much of the catalytic activity of the selected GMPS-RNA is shown to reside in a hairpin structure in the selected region of the molecule. This hairpin structure is also implicated in the recognition of the peptide substrate.

Keywords: bradykinin; GMPS-RNA; in vitro evolution; SELEX

INTRODUCTION

SELEX (**S**ystematic **E**volution of **L**igands by **EX**ponential enrichment) (Tuerk & Gold, 1990), an in vitro selection technique, has enriched the study of interactions between nucleic acids and proteins, peptides, and other small molecules (Jenison et al., 1994; Nieuwlandt et al., 1995; Gold et al., 1995). RNA has been selected in vitro to catalyze the isomerization of a biphenyl (Prudent et al., 1994), to carry out cleavage of DNA (Beaudry & Joyce, 1992), self-cleavage (Pan & Uhlenbeck, 1992) or ligation (Bartel & Szostak, 1993), to perform phosphorylation (Lorsch & Szostak, 1994), and to catalyze carbon–nitrogen bond formation (Wilson & Szostak, 1995) and the aminoacylation of its 2′(3′) terminus when provided with phenylalanyl-adenosine monophosphate (Illangasekare et al., 1995). Additionally, 5-iodouracil-modified RNA molecules have been selected to crosslink to HIV-1 Rev both in the presence and absence of photo-induction (Jensen et al., 1995).

Catalytic RNAs, like other enzymes, employ binding interactions with their substrates to accelerate reactions. We reasoned that, by selecting for enhanced reactivity between an RNA pool and a substrate, we would concomitantly select RNAs that bind the substrate specifically. Here we report the selection and analysis of a 5′ guanosine monophosphorothioate-substituted RNA (GMPS-RNA), which recognizes *N*-bromoacetylated-bradykinin (BrBK) specifically and accelerates the formation of a thioether bond between the RNA and the peptide. Bradykinin (BK) is a 9-amino acid peptide that has no primary structural ground state in an aqueous environment (London et al., 1979). Even so, the selected GMPS-RNA binds specifically to BK and carries out a reaction with BrBK similar to that conducted by glutathione S-transferases, which detoxify alkyl halides in higher organisms by thiol substitution of the halide (Armstrong, 1991).

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RESULTS

Reaction of bulk 30N1 GMPS-RNA with BrBK

We have directed this selection (Fig. 1) to evolve only those ligands that react with their 5′ thioate group; unreacted ligands are partitioned away from reacted spe-

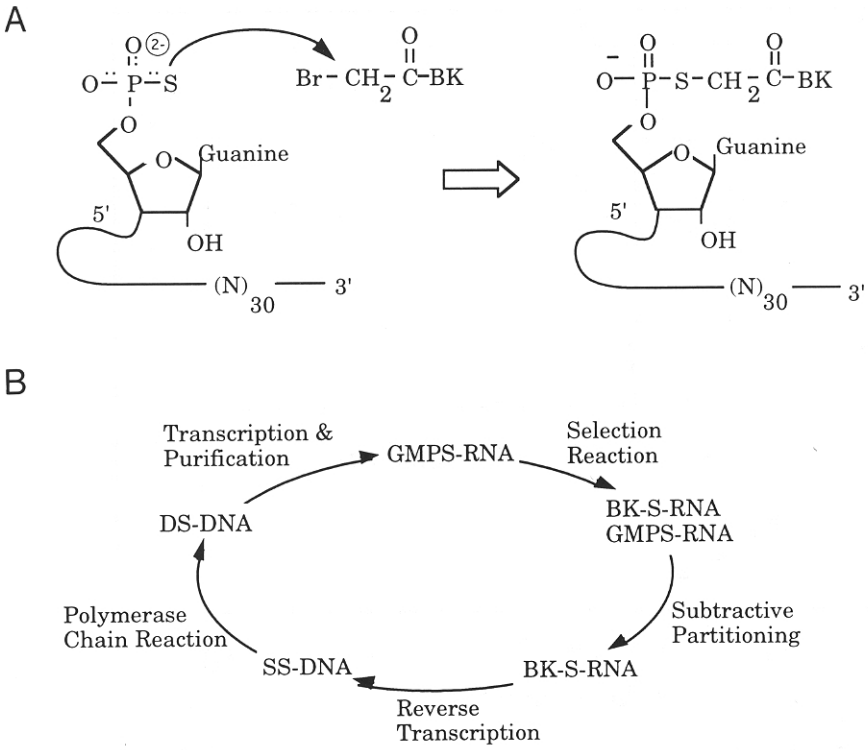


FIGURE 1. A: Reaction between GMPS-RNA and BrBK, forming the product BK-S-RNA. **B:** SELEX cycle. GMPS-RNA is selected for the ability to rapidly substitute the thioate group of the RNA for the bromine group of BrBK. The product, BK-S-RNA, is then partitioned subtractively from the remaining unreacted GMPS-RNA and re-amplified prior to continuing with another selection cycle. Transcription and purification: GMPS-RNA was transcribed and purified by microcon-30 spin separation to remove excess GMPS. GMPS-RNA is purified away from non-GMPS RNA using thiopropyl sepharose 6B, eluted from the matrix with DTT, and purified from the DTT by microcon-30 spin separation. Selection reaction: after the reaction of GMPS-RNA with BrBK, the reaction is quenched with 235 mM sodium thiophosphate or sodium thiosulfate and the excess quenching reagent is removed by microcon-30 spin separation. Subtractive partitioning: nonreacted GMPS-RNA was separated away from BK-S-RNA either by subtraction of the GMPS-RNA upon thiopropyl sepharose 6B, or by separation of the two species on an APM polyacrylamide gel. Reverse transcription and PCR were performed as reported (Schneider et al., 1993).

cies based on subtractive partitioning of the free thioate using either [(B-acryloylamino)phenyl]mercuric chloride polyacrylamide gel electrophoresis (APM-PAGE) or thiopropyl sepharose. This partitioning scheme ensures that only those RNAs whose thioate is blocked by the reaction will be separated. Reactions at other functional groups (e.g., Wilson & Szostak, 1995) will not cause a mobility shift.

To design the selection, the properties of the reaction of BrBK with the initial library of GMPS-RNA containing a 30-base randomized region (30N1 GMPS-RNA) were investigated. The reaction of 30N1 GMPS-RNA with BrBK at 37 °C goes to 95% completion in a log-linear fashion, indicating no observable reverse reaction (see Fig. 2). The reaction saturates with BrBK, with K_m values of 630 μM BrBK at 0 °C (see inset, Fig. 2) and 370 μM at 37 °C (not shown). The k_{cat} value of the reaction at 37 °C ($3.7 \pm 0.01 \times 10^{-3}$ s^{-1}) is some 100-fold faster than at 0 °C ($3.9 \pm 0.2 \times 10^{-5}$ s^{-1}).

Selection for an increase in k_{obs}

Selection was performed for 12 rounds as given in Table 1. Reaction profiles for RNA pools from rounds 0, 2, 4, 6, 8, 10, and 12 were compared with the reaction rates being determined from the linear range of these data as pseudo-first-order reaction constants. The k_{obs} value of the GMPS-RNA pool increased 100-fold between rounds 4 and 6, increasing only twofold with further rounds. Fifty-six independent clones were sequenced from round 10 and round 12 pools, resulting in 29 different sequences. Approximately one third of the total sequences have the core consensus 5′-UCCCC(C)G-3′ positioned freely along the length of the randomized region. Computer modeling (Jaeger et al., 1989, 1990; Zuker, 1989a, 1989b) of sequences containing this motif invariably had this consensus region base paired with the 5′ terminal GGGA of the nonrandomized region. Conceivably, such base pairing fixes the terminal GMPS nucleotide, coordinating the thioate group for reaction with the acetyl α-carbon of BrBK. Reactant species that did not contain the 5′-UCCCC(C)G-3′ motif, such as reactant 12.1 (Fig. 3), did not usually have the 5′ GMPS base paired in computer-generated structures. Sixteen reactants were compared with the 30N1 bulk pool for reactivity with BrBK; all tested reactants show a 10- to 100-fold increase in k_{obs} relative to the original pool (see Table 2). Reactant 12.1 was chosen for further kinetic analysis based on three criteria: (1) In a preliminary study of reaction inhibition with competing bradykinin, it had the lowest K_i for bradykinin. (2) It was the molecule represented most frequently in the round 12 population. (3) It had the second fastest k_{obs} of the reactants tested.

Kinetic analysis of reactant 12.1

The relative reactivity of selected reactant 12.1 versus the starting 30N1 GMPS-RNA pool is shown in Figure 4. Reactant 12.1 reacts in a biphasic manner with BrBK, with 20–40% of the total GMPS-RNA reacting approximately 2,000-fold faster than that of the initial

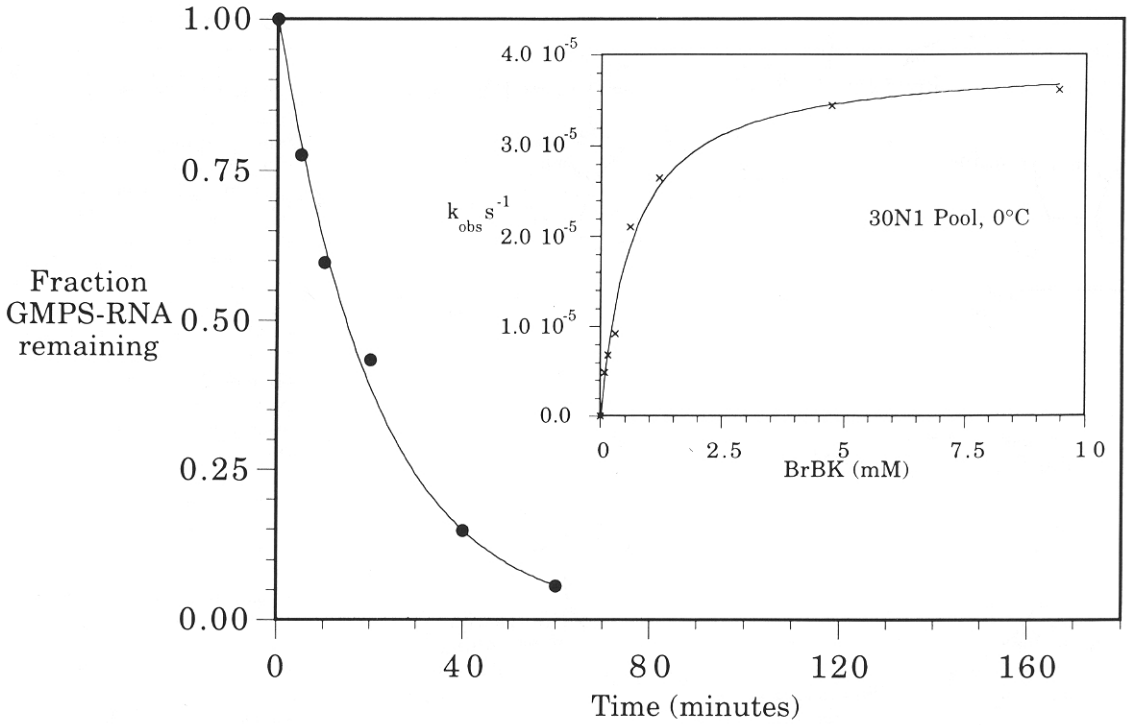


FIGURE 2. Reaction of 30N1 GMPS-RNA with BrBK essentially goes to completion. Reaction conditions: 2 μM GMPS-RNA, 500 μM BrBK in reaction buffer at 37 °C. Inset: k_{obs} versus [BrBK] for the reaction of 30N1 GMPS-RNA with BrBK at 0 °C. Values of K_m and k_{cat} were obtained from the data by fitting the equation $k_{obs} = k_{cat} * [\text{BrBK}]/(K_m + [\text{BrBK}])$ using Kaleidograph software (Synergy Software).

30N1 GMPS-RNA pool, after which the reaction rate is approximately the same as that of the initial 30N1 GMPS-RNA pool (see Fig. 5). This biphasic behavior may be due to some side reactivity of the 5′ thioate such that it is not readily available for reaction with BrBK, but is retarded by thiol-sensitive partitioning methods such as APM-PAGE and thiopropyl sepharose chromatography. Hence, the thioate is apparently still available for reaction. The biphasic behavior is probably not due to reactant 12.1 being present in multiple conformations: the unreacted GMPS-RNA shows only a slow reaction with BrBK when reheated and rereacted with new BrBK (data not shown).

The inset of Figure 5 shows a plot of reactant 12.1 GMPS-RNA in reaction with BrBK, where k_{obs} values are derived from only the fast phase of the reaction.

TABLE 1. SELEX progress profile for RNA reactants to BrBK.[a]

	Round number												
	0	1	2	3	4	5	6	7	8	9	10	11	12
Temperature (°C)	–	37	30	30	24	24	20	0	0	0	0	0	0
Reaction time (s)	–	60	60	30	60	30	30	60	60	120	60	30	60
[RNA] (μM)	–	40	40	40	40	40	40	20	20	20	20	20	20
[BrBK] (μM)	–	650	650	650	650	650	650	650	650	650	650	650	650
% RNA reacted	–	1.3	0.7	0.8	0.7	1.9	2.5	1.2	3.4	4.5	5.0	2.5	2.8
Background ratio	–	3.2	3.2	3.4	1.7	2.5	3.9	3.1	4.0	4.9	10.1	9.0	4.5
k_{obs} ($s^{-1} \times 10^{-6}$)	1.9	–	2.0	–	2.7	–	320	–	420	–	690	–	630

[a] Reactions to determine k_{obs} values were performed at 0 °C at 2 μM GMPS-RNA and 130 μM BrBK, with monitoring at 0, 1, 3, 10, 30, and 90 min. Reactions were quenched on ice with 235 mM sodium thiosulfate and run on a denaturing APM-PAGE gel. k_{obs} values were determined as the negative slope of the linear range of data points from plots relating the concentration of unreacted GMPS-RNA versus time. Rounds of selection were performed in reaction buffer with the indicated GMPS-RNA and BrBK concentrations for the given times and temperatures. % RNA reacted refers to the percent of the total GMPS-RNA present as BK-S-RNA from acrylamide gel partitioning, or, as freely eluting BK-S-RNA in affinity column partitioning. Background was subtracted from the recovered RNA in both of these cases; background refers to the amount of RNA recovered from a control treatment where the reaction was quenched prior to the addition of the BrBK. The background ratio is the ratio of reacted RNA to that present as background. We attempted to keep this ratio between 2 and 10 throughout the rounds of SELEX by adjusting the reaction time.

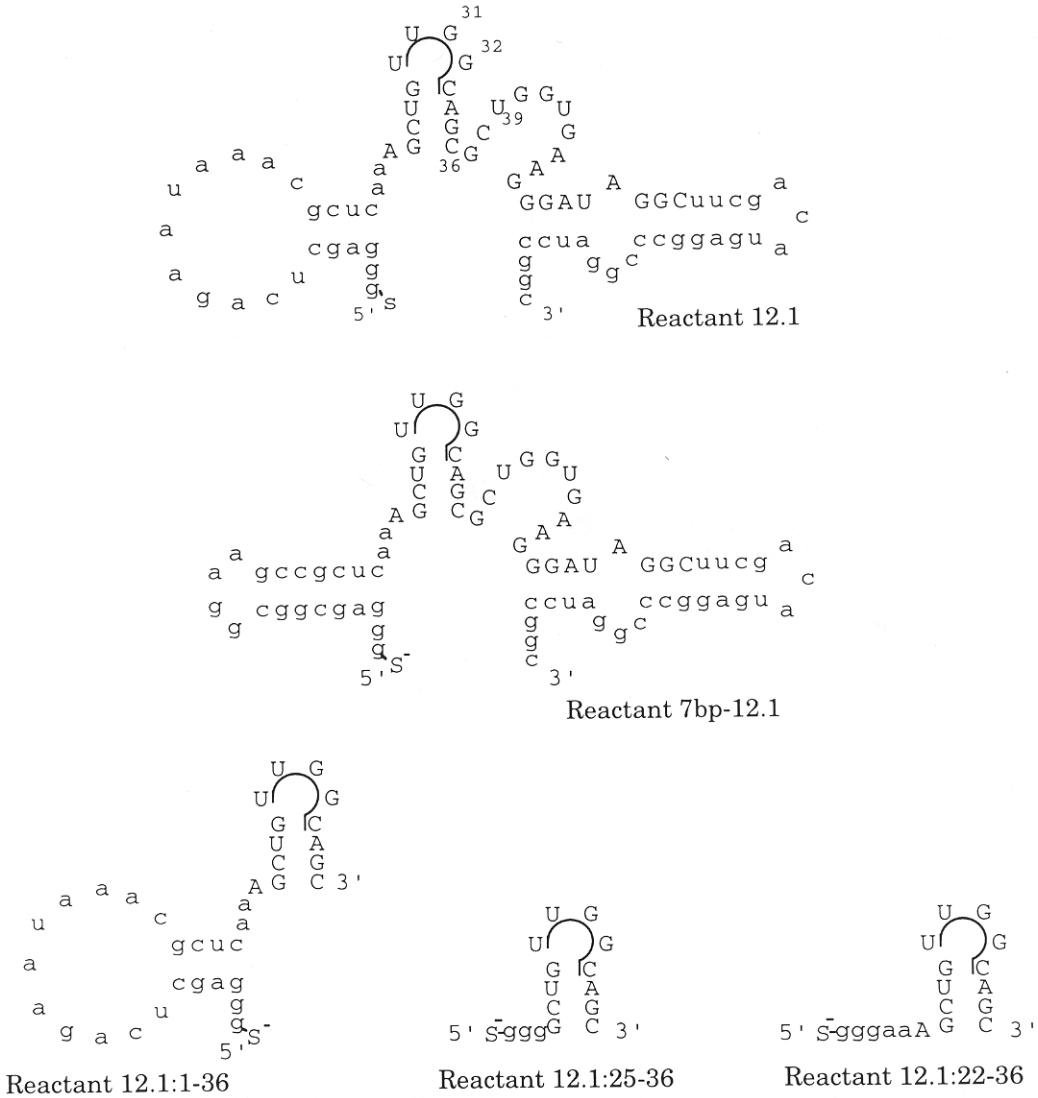


FIGURE 3. Predicted structure of reactants 12.1 and its truncates as modeled by computer algorithm. Lowercase letters represent fixed sequences. g-s$^-$ indicates the 5′ incorporated GMPS. Underlined uppercase letters indicate conserved sequence elements.

The apparent second-order rate constant for this reactant is 150 M^{-1} s^{-1}, or 2,400-fold faster than that of the random 30N1 population (see Table 3). Due to the speed and biphasic nature of the reaction, measurements of pseudo-first-order reaction rates at high (>100 μM) BrBK concentrations were not practical. Thus, it was not possible to determine whether the reaction of reactant 12.1 with BrBK would saturate at higher BrBK concentrations.

Specific inhibition by BK

The interaction of reactant 12.1 with BrBK was studied by inhibition analysis, using native BK (BK), des-Arg^9-BK (a BK analogue lacking the carboxy-terminal arginine), des-Arg^1-BK (a BK analogue lacking the amino-terminal arginine), and arginine alone. Additionally, the reaction of 30N1 GMPS-RNA with BrBK was tested for inhibition by BK. Inhibition of the reaction in the presence of the inhibitors is shown in Figure 6, with K_i values derived from these data given in Table 4. The K_i value of BK is 2.3 ± 0.8 × 10^{-4} M, whereas K_i for des-Arg^9-BK is 1.8 ± 0.4 × 10^{-3} M. Neither arginine alone, nor des-Arg^1-BK inhibit the reaction within the range of inhibitor tested, indicating that such inhibition is on the order of 10 mM or greater. In addition, BK does not measurably inhibit the reaction of 30N1 GMPS-RNA with BrBK (data not shown).

RNA protection by BK

Chemical modification of reactant 12.1 GMPS-RNA and GMPS-BK using kethoxal in the presence of varying concentrations of free BK is shown in Figure 7.

TABLE 2. Representative ligands from the selection.[a]

Reactant	30N1 region	Frequency	Reaction rate (s^{-1})
	UCCCC(C)G Motif		
10.28	CGUUUAGGACUCCCCCCGUUCGUCGAGCGAA	2	1.8×10^{-3}
12.14	ACGUCAUUCCGAGUCGGGUUCGUUCCCCGC	1	1.7×10^{-3}
12.16	CUCCCCGUUAGCGCCUCACUGACGUGUCGA	4	1.3×10^{-3}
12.47	UGUGUGAGUGGAUCCGUUCCCCGCCUGGUG	1	1.5×10^{-3}
12.3	CAUCACAACUUGUUGUCCUGGUCGAUGUCC	3	7.5×10^{-4}
12.31	UCGACACAACUCGAUCUCCGUGGCUGUCAC	2	1.2×10^{-3}
12.40	GUCUCACAACUGGCUUAUCCGGUGCGCACG	1	1.6×10^{-3}
12.46	UGGACACAACUCCAUUCAUCCCGGGACCGCGUG	1	6.7×10^{-4}
12.4	CGAAUCAAUGCGCGGAUCUCAGGAUAUUCG	5	1.7×10^{-3}
12.6	GCGGUAACAUGCUGGAUCUCAGGAAACCGC	3	2.2×10^{-3}
12.45	GCGGUAACAUGCUGGAUCUCAGGAAACCGU	1	5.1×10^{-3}
12.1	AGCUGUUGGCAGCGCUGGUGAAGGGAUAGGC	6	2.8×10^{-3}
12.9	GUGGAGCUUCGUGACUUGGUCGGAGCCGUG	1	1.3×10^{-3}
12.22	UGCCACUUUUGUUCGGAUCUUAGGAAGGCA	1	1.2×10^{-3}
12.24	CGUCAGCGGAUCUCCAUUGCGUUAUACGGG	1	1.4×10^{-3}
12.30	UCCGUGUUGCCACUCCAGUUACUGGACGCC	1	7.4×10^{-4}

[a] Sequences given are from the randomized region only. The sequences 5′ and 3′ of the sequences are GGGAGCUCA GAAUAAACGCUCAA and UUCGACAUGAGGCCCGGAUCCGGC, respectively. Underlined bases indicate consensus elements between sequences. The number to the right of the sequence is the number of identical sequences isolated. Approximate observed pseudo-first-order reaction rates for individual sequences are given on the far right and were determined at concentrations of 300 μM BrBK and 2 μM GMPS-RNA. The rate of the initial 30N1 pool GMPS-RNA under these conditions was 5.3×10^{-5} s^{-1}.

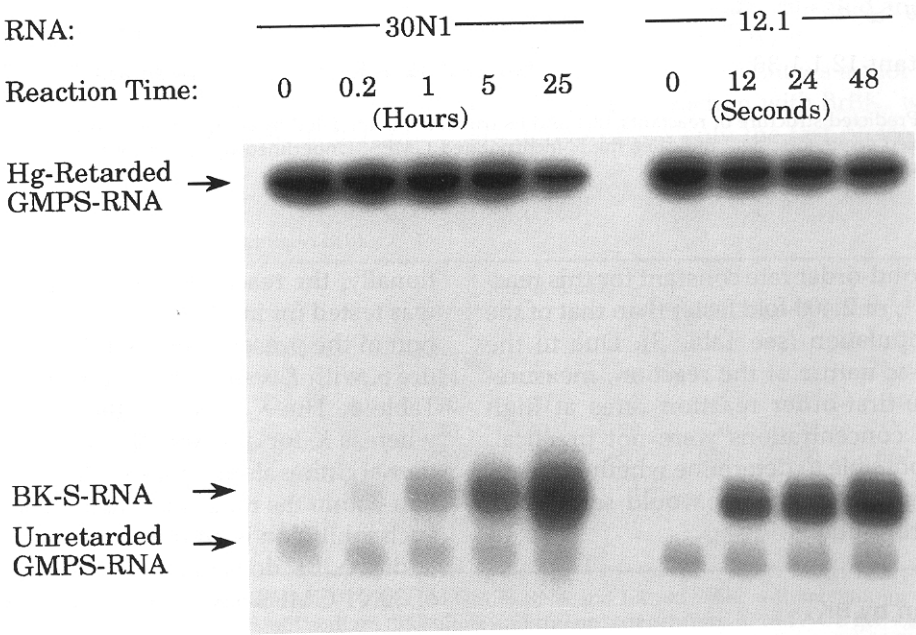


FIGURE 4. Comparison of the activity of reactant 12.1 and the 30N1 pool RNA for BrBK. Reactions were conducted at 0 °C in reaction buffer at 2 μM RNA and 0.3 mM BrBK. Reactions were quenched on ice with 235 mM sodium thiophosphate and run on a two-phase gel. The first phase (not shown here) consisted of 10 cm of a 7 M urea 8% denaturing polyacrylamide gel. The second phase of the gel was identical to that of the first, except that it included APM. APM acts to retard free GMPS-RNA at the interface between the two gel phases, but does not retard the migration of BK-S-RNA. Approximately 3% of the GMPS-RNA is in a form that runs freely through APM-acrylamide.

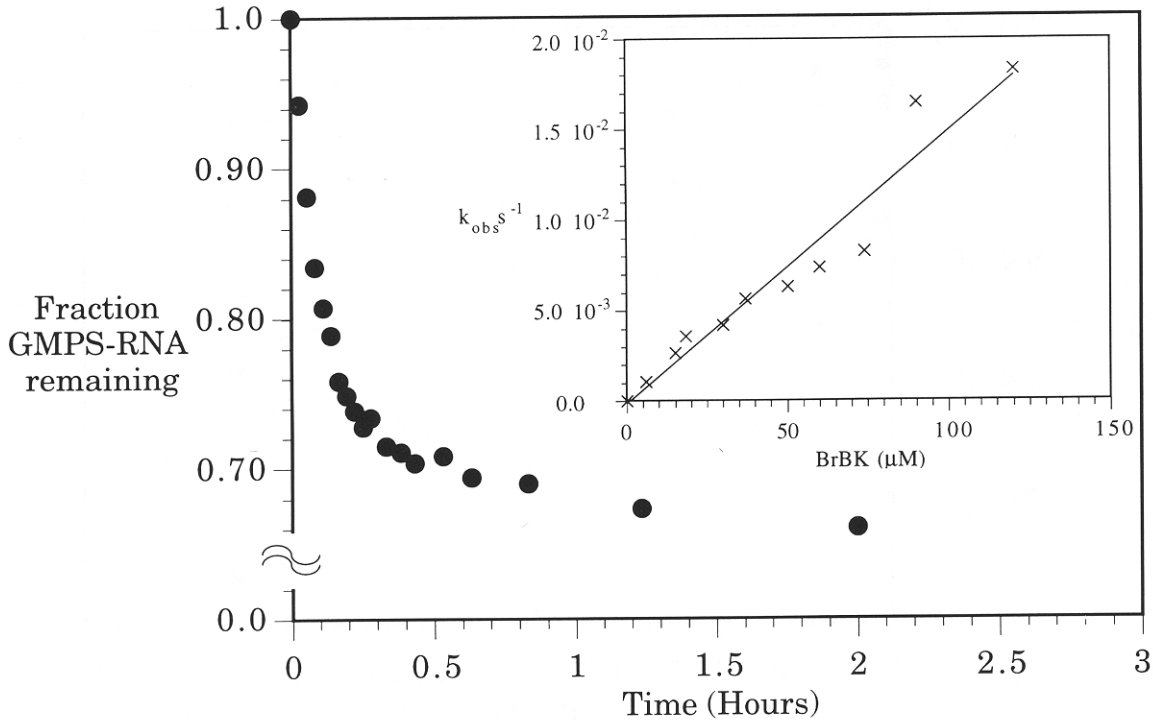


FIGURE 5. Reaction of reactant 12.1 GMPS-RNA with BrBK is biphasic. Reaction conditions: 1 μM GMPS-RNA, 15 μM BrBK, 0 °C in reaction buffer. The ordinate is broken for clarity. Inset: k_{obs} rates of the fast phase of the reaction of reactant 12.1 GMPS-RNA with varying BrBK concentrations at 0 °C.

From these data, it is apparent that reactant 12.1 GMPS-RNA, when reacted with BrBK prior to modification, shows protection at positions G31 and G32. Protection at these bases also occurs for reactant 12.1 GMPS-RNA when it is pre-incubated with free BK and kept in the presence of free BK during the modification reaction. When bands at G31 and G32 are quantified by densitometric analysis, the half-maximum of this protection is on the order of 1 mM.

Intrinsic reactivity of the 5′ thioate

One way the selected RNAs could increase their reaction rate with BrBK is by increasing the reactivity of the 5′ thioate. We tested this possibility by comparing the reactions of selected and starting RNAs with a minimal substrate, *N*-bromoacetamide ($BrAcNH_2$). The reactions of the 30N1 random GMPS-RNA pool and reactant 12.1 with $BrAcNH_2$ were identical at all $BrAcNH_2$ concentrations tested (250 μM to 60 mM), indicating that the selected rate increase is not due primarily to increased accessibility or reactivity of the 5′ thioate.

Truncate analysis of reactant 12.1

Effects of deletions from the 3′ end of reactant 12.1 upon its reaction rate with BrBK are shown in Figure 8. The bands in this figure represent differing lengths of reactant 12.1, whose 5′ ends are intact and thus retain the 5′ GMPS group. These RNA species are derived from alkaline hydrolysis of the parent mol-

TABLE 3. Summary of kinetic parameters for reactant 12.1 and its truncates.

GMPS substrate	k_{obs}/[S] (M^{-1} s^{-1})	k_{obs}/[S] Ratio to 30N1
30N1	0.062	1
12.1:22–36	0.11	1.7
12.1:25–36	0.10	1.6
12.1	150	2419
7bp-12.1	181	2919
12.1:1–36	3.7	60

TABLE 4. Effects of four potential inhibitors upon the reaction of BrBK with the initial pool GMPS-RNA or reactant 12.1 GMPS-RNA.

GMPS-RNA reactant	Inhibitor	K_i (mM)
12.1	BK	0.23 ± 0.08
12.1	des-Arg^9-BK	1.8 ± 0.4
12.1	des-Arg^1-BK	>10
12.1	Arginine	>10
30N1	BK	>10

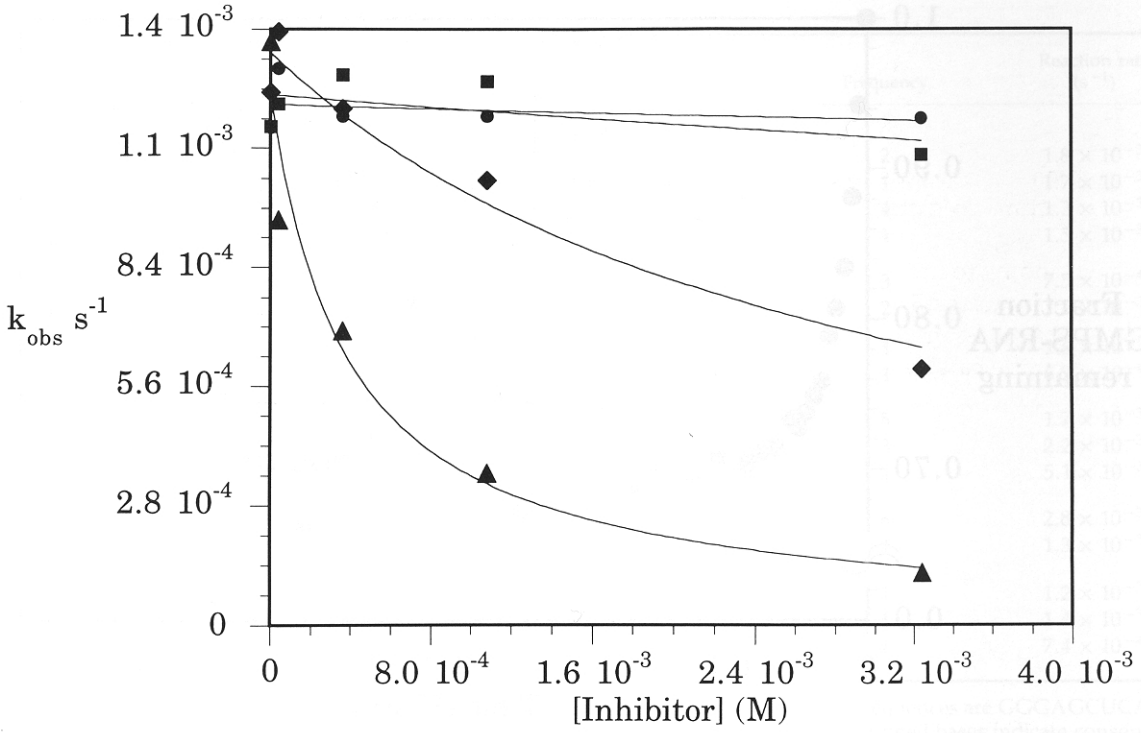


FIGURE 6. Inhibition of the reaction between reactant 12.1 and BrBK with varying concentrations of four potential inhibitors: BK (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) (▲), des-Arg^9-BK (◆), des-Arg^1-BK (■), and arginine (●). Reaction performed at 0 °C in reaction buffer; 2 μM GMPS-RNA was pre-incubated with inhibitor in reaction buffer for 10 min at 0 °C prior to the addition of 30 μM BrBK.

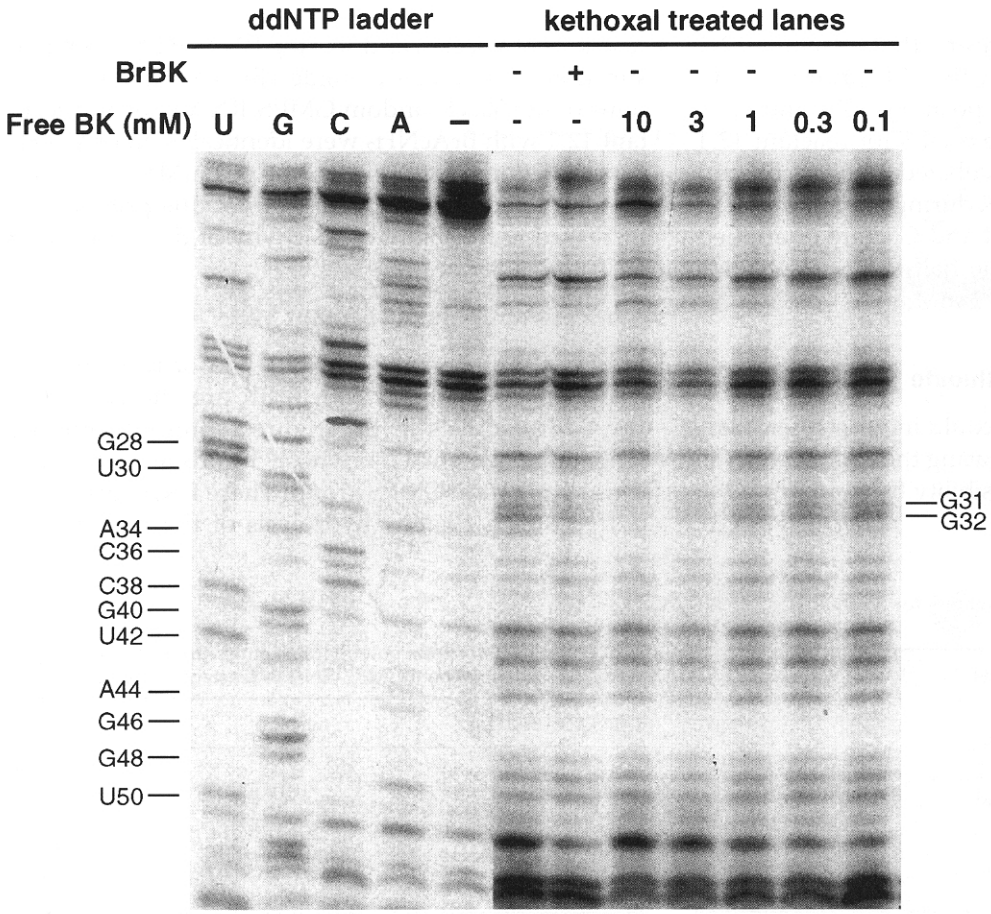


FIGURE 7. Chemical protection of reactant 12.1 GMPS-RNA: the effect of incubation of the GMPS-RNA with free BK, or of reaction with BrBK, prior to kethoxal modification.

ecule, purified for 5′ GMPS thioate activity, and reacted with BrBK. Only those species that have reacted with BrBK are visible, all other species are retained at the well of the gel by the presence of APM.

A major 3′ reaction boundary is located at C36 in the molecule, which corresponds to the base of the hairpin for the structure predicted by Zuker folding algorithms (refer to Fig. 3). At 640 and 1,280 μM BrBK, bands corresponding to the reaction with GMPS-RNA species that are truncated 5′ of C36 are evident, particularly at reaction times greater than 30 min. Such reactions represent the basal rate of reaction of the thioate group with BrBK.

Based on the 3′ boundary assay of reactant 12.1, several truncates were synthesized and tested for reactivity; second-order rate data for these truncates, along with data for the full molecule, are given in Table 3. With approximately one half of the selected randomized region removed from its parent reactant, reactant 12.1:36 retains a 60-fold rate enhancement over bulk 30N1 GMPS-RNA. Removal of the 5′-end stem-hairpin region from truncate 12.1:36 results in molecules (truncates 12.1:22–36 and 12.1:25–36) with a second-order reaction rate nearly identical to that of the bulk 30N1 pool. When the 5′ nonselected region of the molecule is modified with an extended stem structure and a tetraloop, the new molecule (7bp-12.1) retains the activity of reactant 12.1, and even shows a modest increase in second-order reaction rate.

DISCUSSION

Selection criteria

5′-GMPS modified RNAs were obtained that have the unique capacity to carry out a thioate-substitution reaction upon the acetyl carbon of BrBK. The entire selection was conducted at 650 μM BrBK, a concentration approximately twice the K_m of the initial pool of GMPS-RNA in reaction with BrBK at 37 °C. Because the reaction became too fast, as the selection progressed, to actually perform the selection at the initial temperature of 37 °C, the reaction temperature was decreased throughout the course of selection. Lowering the temperature to 0 °C increases the K_m in the reaction between bulk 30N1 GMPS-RNA and BrBK from 370 to 630 μM. Thus, the peptide was used in the selection at a concentration approximately equal to that of the K_m of the unselected pool. At this concentration, it seems unlikely that the selection would drive K_m at the expense of k_{cat}. Hence, selection may have been for reactants having faster reaction rates rather than better binding characteristics.

As a consequence of decreasing the selection temperature during the course of the selection, the selected ligands must be highly reactive over the range of temperatures used. As such, the final pool of reactants at 0 °C may have lost some complexity in that some very good reactants at 0 °C may not have been suitably reactive at higher temperatures to have been carried through the earlier rounds.

Specificity of the reaction

Through the course of this selection, the pseudo-first-order reaction rate at 300 μM BrBK increased some 360-fold within the pool of GMPS-RNA reactants. In reaction with BrBK, reactant 12.1 shows a 2,420-fold increase in the apparent second-order reaction rate over that of bulk 30N1 GMPS-RNA. It was not possible to determine whether the reaction of reactant 12.1 with BrBK saturated at high BrBK concentrations because the reaction was both much too fast at high BrBK concentrations, and showed biphasic behavior.

This biphasic behavior may be due to dimerization of reactant 12.1. In the case where one of the GMPS-RNA species of the dimer has reacted with BrBK, the covalently bound BK group is at an increased local concentration and would be more likely to inhibit the reaction of the second dimerized GMPS-RNA species with free BrBK. The biphasic behavior was shown not to be due to kinetic trapping of less-reactive conformations of reactant 12.1, was not due to an increase in temperature in the reaction during mixing, and was not due to degradation of the BrBK. Finally, because the GMPS-RNA monomer and GMPS-RNA-BK were the primary species detectable by PAGE analysis, loss of reactivity toward BrBK is not due to dimerization of GMPS-RNAs via a disulfide bridge.

Structural elements of BrBK recognized by reactant 12.1 were studied through inhibition of the reaction by BK analogues. Although inhibition by BK is not measurable in the reaction of bulk 30N1 GMPS-RNA with BrBK, BK has a K_i of 230 ± 80 μM for the reaction between reactant 12.1 and BrBK. Des-Arg^9-BK has a K_i of approximately 1.8 mM. Thus, the lack of the carboxy-terminal arginine decreases the binding between BK and reactant 12.1 approximately eightfold. Furthermore, des-Arg^1-BK does not show any measurable inhibition of the reaction between reactant 12.1 and BrBK, indicating that the amino-terminal arginine is absolutely required for the observed binding between reactant 12.1 and BrBK. Recognition of arginine must be in the context of the peptide, however, because free L-arginine alone does not measurably inhibit the reaction. Thus, the increase in affinity of reactant 12.1 over that of the bulk 30N1 GMPS-RNA for BrBK is in part attributable to reactant recognition of the amino-terminal arginine of BrBK, and to a lesser extent the carboxy-terminal arginine.

The intrinsic reactivity of reactant 12.1 was studied using $BrAcNH_2$ as a minimal bromoacetyl structure. The $k_{obs}/[S]$ values in the reactions of reactant 12.1 and the 30N1 RNA pool with $BrAcNH_2$ are approximately

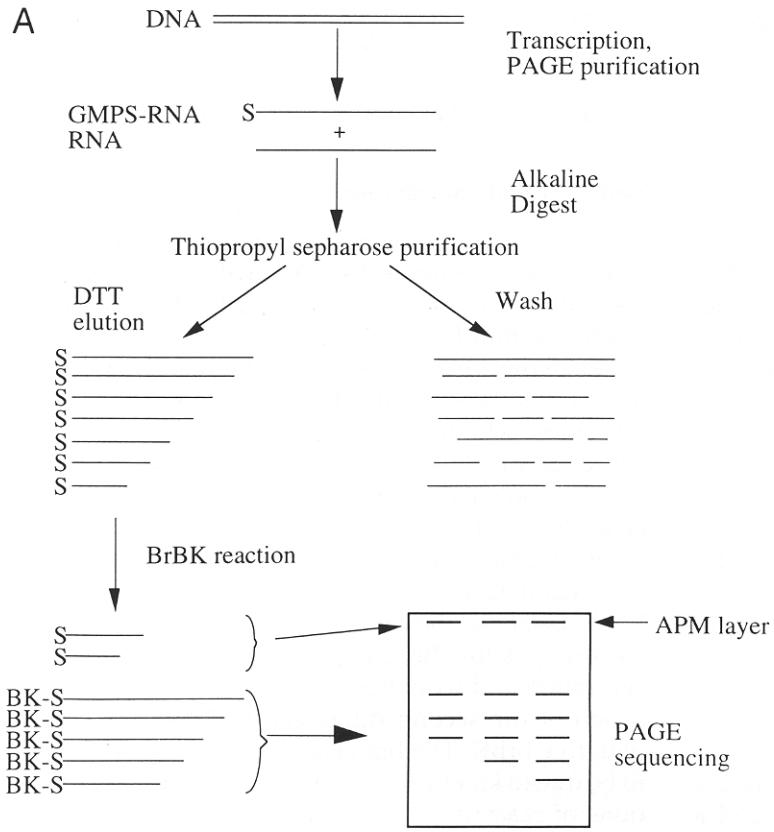


FIGURE 8. A: Schematic summary of the 3′ reaction boundary experiment. GMPS-RNA (S-) is transcribed, alkaline digested, and separated from RNA not containing a GMPS group. This GMPS-RNA is then reacted with BrBK. Unreacted GMPS species are retained at the well of an APM sequencing gel, whereas reacted BK-S-RNA is separated based on its size. **B:** Reaction boundary of reactant 12.1. The pool of variable-length GMPS-RNA was reacted for the specified time with the indicated concentration of BrBK prior to quenching and analysis using APM-PAGE. All unreacted species are retained at the well of the gel (not shown). T1 digest was made from full-length reactant 12.1 GMPS-RNA, which was digested and purified for those species still retaining their 5′ thioate. This preparation was then reacted for 30 min at 0 °C with 1 mM BrBK. All unreacted T1 species are retained at the well. (*Figure continues on facing page.*)

the same. Therefore, the enhanced reaction rate of reactant 12.1 with BrBK is probably a result of steric and/or entropic factors in the positioning of the two reactants rather than to increased nucleophilicity of the thioate group.

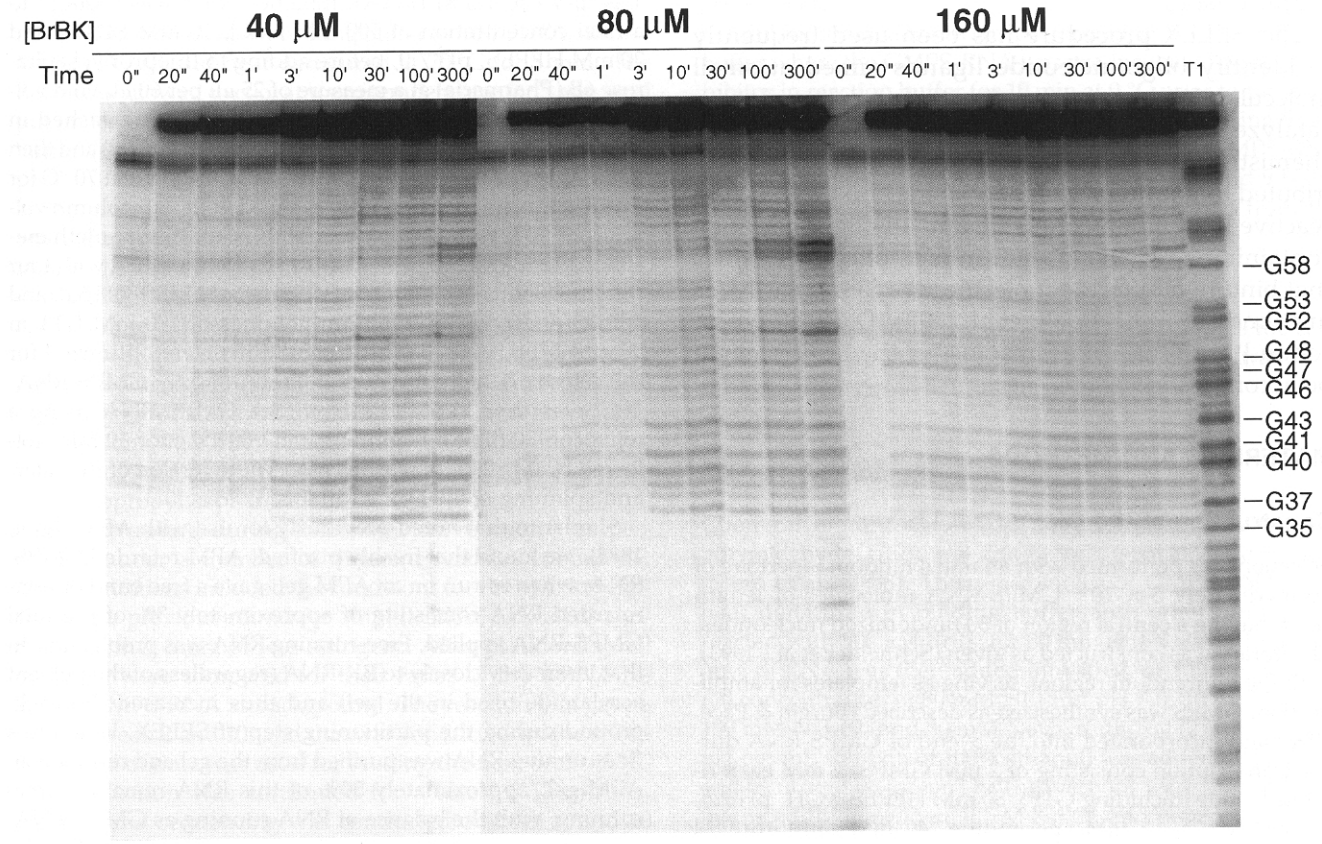
Elements of the RNA required for the reaction

The 3′ reaction boundary of Figure 8 suggests an expedient means of determining binding and rate characteristics for each length of reactant 12.1 GMPS-RNA extending from the 5′ end of the molecule. Alternatively, the experiment could have been designed to search the series of molecules from the 3′ end of the reactant by labeling the 3′ end of the molecule prior to alkaline hydrolysis, and kinasing the 5′ ends of the hydrolyzed pool with γ-S-nucleoside triphosphate. From such data, molecular species that emphasize binding, rate of catalysis, or both might be elucidated, offering a unique method of kinetic analysis of the reaction. Unfortunately, the biphasic reaction profile of the full-length reactant species precludes a clear understanding of the data of Figure 8B. Even so, a major 3′ reaction boundary is located at C36 in the molecule. As the BrBK concentration is lowered, the reaction product indicated at C36 diminishes more rapidly with diminished time of reaction than for the product indicated at U39. This suggests for the species ending at U39, that the reaction saturates at lower peptide concentration than for the species that ends at C36.

In chemical modification protection experiments, G31 and G32 of the selected UUGG hairpin-loop region appear to interact with BrBK, because they are protected from the modifying reagent kethoxal. Shown in Table 3 are the reaction data for reactant 12.1 and the various truncates. From the data of truncate 12.1:1–36, it is apparent that much of the reactivity of the RNA resides on the 5′ side of base G40. With truncates 12.1:22–36 and 12.1:25–36, reaction activity drops to that of the original starting pool, indicating that the selected region works in cohort with the constant 5′ region. In truncate 7bp-12.1, the 5′ end of reactant 12.1 was stabilized by the substitution of a seven-base paired stem with a four-base tetraloop. This construct, which retains the 5′ GMPS-GG and the four starting base pairs of the stem, but which has a very different loop structure, has much the same reactivity of the parent molecule. Taken together, these data are consistent with the hypothesis that the terminal 5′ stem loop of the molecule acts to present the thioate group to the acetyl carbon of a BrBK molecule, which is oriented, in part, by the selected UUGG stem loop structure.

SELEX offers a unique approach to the study of catalytic activity by nucleic acids; with careful experimental design, selection might be biased to produce catalytic RNAs whose interaction with the target stress either k_{cat}/K_m or k_{cat} (Tsang & Joyce, 1994). We expect that a selection performed at target concentrations well below the K_m of the pool at each round of selection

B



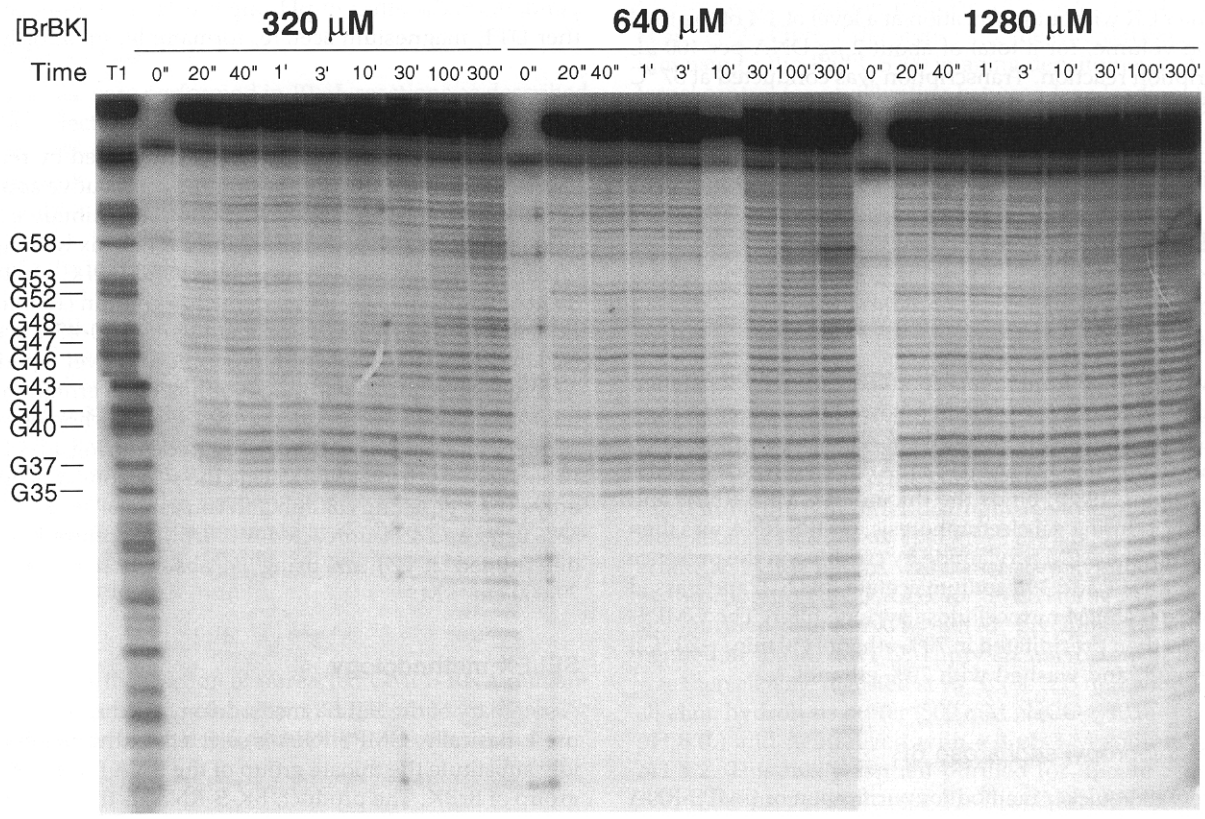


FIGURE 8. *Continued.*

will yield catalytic RNAs with improved binding characteristics.

The SELEX procedure has been used frequently to identify oligonucleotide ligands aimed at small molecules. We have now shown that such ligands may catalyze reactions involving nonspecific coupling chemistry: the rate enhancement observed can be attributed to nothing more than interaction between the reactive bradykinin conjugate and an oligonucleotide containing a reactive 5′ end. In fact, oligonucleotides that bind two different molecules are found easily (as in chimeric-SELEX [Gold et al., 1995; D. Burke, pers. comm.]), and thus more imaginative chemistries might be accomplished.

MATERIALS AND METHODS

Construction of the GMPS-RNA library

Selection was performed with an initial random repertoire of approximately 5×10^{13} GMPS-RNA molecules of length 76 nt, having a central region of 30 randomized nucleotides. The library was constructed as given (Schneider et al., 1993), with the nonrandom regions serving as templates for amplification. GMPS was synthesized as described (Burgin & Pace, 1990) and incorporated into the 5′ end of GMPS-RNA during transcription consisting of 2 mM GMPS, 2 mM each ribonucleotide (including GTP), 80 mM HEPES-KOH, pH 7.5, 12 mM $MgCl_2$, 2 mM spermidine, 40 mM dithiothreitol (DTT), 1 unit/μL RNasin (Promega), and 5 units/mL yeast inorganic pyrophosphatase (Sigma). DNA was added directly from the PCR without purification at a level of 1/4 of the total reaction volume, for a total of about 2 μg DNA per 100 μL transcription reaction. Transcription was conducted at 37 °C for 4–8 h. Under these conditions, GMPS was incorporated with an efficiency of approximately 80% as measured by precipitation analysis.

GMPS-RNA purification

Use of [(B-acryloylamino)phenyl]mercuric chloride (APM)

RNA was purified from unincorporated GMPS by separation on microcon-30 filters (Amicon) followed by separation from non-GMPS-RNA using denaturing APM-PAGE (7 M urea, 8% polyacrylamide, 25 μM APM). APM, synthesized as described (Igloi, 1988), binds the thioate of GMPS-RNA, and so retards it during gel electrophoresis. GMPS-RNA was then eluted from the APM acrylamide by crushing in the presence of 100 mM DTT and 3 M sodium acetate, pH 5.2, and passed through a 0.45-μM nitrocellulose syringe filter. The GMPS-RNA was then precipitated in 70% ethanol (10 min, −20 °C), centrifuged, and washed with 70% ethanol.

Use of thiopropyl sepharose 6B

An alternate, quicker method for purification of GMPS-RNA is as follows. The transcription reaction is size purified on a microcon-30 filter by centrifugation at 12,000 × *g* for 10 min, followed by two consecutive 200-μL washes with TE (10 mM Tris, pH 7.5, 1 mM EDTA). Retained RNA was brought to a final concentration of 500 mM NaCl, 10 mM EDTA, and 20 mM HEPES, pH 7.0, before adding to thiopropyl sepharose 6B (Pharmacia) at a measure of 25 μL per 60 μL void volume. Prior to use, the sepharose had been prewashed in column buffer (500 mM NaCl, 20 mM HEPES, pH 7.0) and then spun dry at 12,000 × *g*. The mix was then reacted at 70 °C for 5 min, spun at 12,000 × *g*, spin-washed with four column volumes of 90% formamide, 50 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), pH 5.0, at 70 °C, spin-washed with four column volumes of 500 mM NaCl in 50 mM MES, pH 5.0, and spin-eluted with four column volumes of 100 mM DTT in 50 mM MES, pH 5.0. These conditions were optimized for the retention and subsequent elution of only GMPS-RNA. DTT was then separated from the GMPS-RNA using a microcon-30 filter by spinning at 12,000 × *g* for 10 min, followed by two 200-μL washes with TE, inversion of the filter, and spinning at 3,000 × *g* for 30 s.

Similar to that noted previously for use with APM (Igloi, 1988), we found that freshly purified, APM-retarded GMPS-RNA, when re-run on an APM gel, gave a free band of nonretarded RNA consisting of approximately 3% of the total GMPS-RNA applied. Free-running RNA was problematic in that it ran very closely to BK-RNA (regardless of the percent acrylamide used in the gel) and thus increased the background during the partitioning step of SELEX. When this free-running RNA was purified from the gel and rerun on an APM gel, approximately 50% of this RNA remained free-running, with the balance of RNA running as GMPS-RNA. The amount of free-running RNA was proportional to the amount of time spent during precipitation, but was not dependent on the effect of pH, the presence or absence of either DTT, magnesium acetate, formamide, urea, or heat.

Synthesis of *N*-bromoacetyl-BK

BrBK used for kinetic analysis was synthesized by reacting 250 μL of 1 mM BK (Sigma), with three consecutive additions of 3.5 mg bromoacetic acid *N*-hydroxysuccinimide ester at 12-min intervals at room temperature. This mix was then filtered through a 0.2-μM syringe filter, and HPLC purified in 80-μL aliquots on a C_8 100-Å, 5-micron column (Rainin) at a flow rate of 1.0 mL/min using a gradient of 20–45% acetonitrile in ddH_2O and 0.1% trifluoroacetic acid over a period of 25 min. The peptide was then lyophilized overnight to dryness. Analysis of the product on HPLC gave a homogeneous product that did not noticeably degrade during 1 h of incubation at 37 °C. BrBK used during the selections was purified only over a desalting column and consisted of approximately 55% BrBK and 45% unreacted BK. BrBK concentrations were determined at 220 nm using an absorption coefficient of 39,000 cm^{-1} M^{-1}.

SELEX methodology

A summary of the SELEX methodology used is shown in Figure 1. Basically, GMPS-RNA is selected for the ability to rapidly substitute the thioate group of the RNA for the bromine group of BrBK. The product, BK-S-RNA, is then partitioned subtractively from the remaining unreacted GMPS-RNA and re-amplified prior to continuing with another selection cycle.

Reaction conditions for each of the selection cycles is given in Table 1. Prior to reaction, 4.88 μL of GMPS-RNA was mixed with 1.63 μL of 4× heating buffer (4 mM EDTA, 40 mM MES, pH 5.0) and heat denatured at 70 °C for 3 min. The RNA was allowed to cool at room temperature for 5 min, and put on ice prior to the addition of 2.5 μL ice-cold 4× reaction buffer (600 mM NaCl, 22.6 mM $MgCl_2$, 50 mM HEPES, pH 8.0). This reaction mix was then allowed to preincubate at the given reaction temperature prior to the addition of BrBK. Final reaction conditions were 150 mM NaCl, 5.65 mM $MgCl_2$, 0.65 mM EDTA, 6.5 mM MES, and 12.5 mM HEPES, with a pH of 7.0. Reactions were quenched with a final concentration of either 235 mM sodium thiophosphate (rounds 1–8) or sodium thiosulfate (rounds 9–12) and subtractively partitioned using either denaturing APM-PAGE (rounds 1–6) or affinity chromatography (rounds 7–12). Upon quenching, the reactions were immediately run, without heating, in one volume of loading buffer on APM gels, or loaded directly onto thiopropyl sepharose 6B.

At each round during selection, the reaction was performed under a number of different reaction conditions. These conditions included the conditions for the reaction chosen in the previous round for amplification, as well as conditions either more or less stringent. After partitioning, all reactions were compared for their ratios of BK-S-RNA retained:GMPS-RNA as background. The reaction that gave a ratio between 2 and 10, and that was conducted for the shortest period of time was used for seeding the next cycle of SELEX. Selection was continued for a total of 12 rounds. Cloning and sequencing was conducted on both round 10 and round 12 pools as described previously (Schneider et al., 1993).

Kinetic analysis

All analyses were performed in 10-μL reactions as described for the selections themselves. The reaction plot for bulk 30N1 GMPS-RNA at 37 °C was determined at 4, 20, 100, and 500 μM BrBK at 6-, 10-, 20-, 40-, and 60-min time points. The reaction plot for bulk 30N1 GMPS-RNA at 0 °C was determined at 74, 148, 295, 590, 1,200, 4,700, 9,400 μM BrBK at 1-, 3-, and 6-h time points. The second-order reaction rate for reactant 12.1 was determined at 6, 15, 30, 50, 60, 90, and 120 μM BrBK at 10-s time points between 0 and 100 s, with additional time points of 3.5, 10, 20, 100, 200, and 400 min. The second-order reaction rate for truncate 12.1:1–36 was determined at 37, 74, 148, and 295 μM BrBK at 12, 24, 36, 60, and 180 s. The second-order reaction rate for truncate 7bp-12.1 was determined at 4, 16, 64, and 256 μM BrBK at 12, 24, 36 s. The second-order reaction rates for truncates 12.1:22–36 and 12.1:25–36 were determined at 37, 74, 148, and 295 μM BrBK at 6, 10, 30, and 100 min. Monophasic reactions were fit to the equation:

$$S_t/S_0 = e^{-k_{obs}t},$$

where S_t/S_0 is the fraction of unreacted GMPS-RNA remaining at time t, and k_{obs} is the observed rate constant. Biphasic reactions were fit with the equation:

$$S_t/S_0 = Fe^{(-k_1 t)} + (1 - F)e^{(-k_2 t)},$$

where S_t/S_0 is the fraction of unreacted GMPS-RNA remaining at time t, F is the fraction of fast-reacting GMPS-RNA, and k_1 and k_2 are the fast and slow rate constants, respectively.

Inhibition reactions were performed at 0 °C in reaction buffer where the GMPS-RNA was pre-incubated with the inhibitor in reaction buffer for 10 min at 0 °C prior to the addition of 30 μM BrBK. Inhibition was performed using 0, 40, 120, 360, 1,080, and 3,240 μM inhibitor, at 2 μM GMPS-RNA for 24, 60, and 120 s (for reactant 12.1) or 1, 3, and 9 h (for 30N1 GMPS-RNA). Values for K_i were obtained from the data by fitting the equation

$$k_{obs} = k_0/(1 + [I]/K_i),$$

where k_0 is the rate of the uninhibited reaction, I is the concentration of inhibitor used, and K_i is the concentration of inhibitor that yields half-maximal inhibition.

RNA protection experiments

Chemical modification using kethoxal (2-keto-3-ethoxy-*n*-butyraldehyde; USB), was conducted essentially as described (Stern et al., 1988). Thirty picomoles of GMPS-RNA were heat denatured in binding buffer and, where indicated, reacted with 500 μM BrBK for 30 min at 0 °C. The 10-μL aliquot of RNA was then added into 40 μL of a buffer for a final concentration of 80 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 100 mM NH_4Cl, and 30 mM NaCl. Kethoxal (2.5 μL of a 225 mg per mL solution of 100% ethanol) was added to the RNA and incubated on ice for 1 h. The reactions were then stopped by the addition of 15 μL 0.5 M K_2BO_4 and precipitated with 300 μL 100% ethanol. Samples were resuspended in 15 μL ddH_2O containing 20 mM K_2BO_4 and stored at −70 °C. Reverse transcription was then performed as described (Schneider et al., 1993). Optical densities were measured from the X-ray film using a Bio-Rad GS700 imaging densitometer. As an internal control for equilibrating between-lane loading, the G40 band for each lane was quantified and used to adjust the intensities of the other measured bands in other lanes. Bands G31 and G32 were combined for each lane to facilitate densitometric analysis. Intensity of bands G31 and G32 were adjusted for the relative amounts loaded for each particular lane by comparison to the control lane in which no BrBK or BK was added during the chemical modification step.

3′ reaction boundary assay

Reactant 12.1 has a GMPS group attached to its 5′ end, and thus cannot be 5′ end-labeled, as would occur in a typical 3′ boundary assay. Thus, the GMPS-RNA was treated as indicated in Figure 8 in order distinguish those molecules that, after alkaline hydrolysis, had intact 5′ ends. Radiolabeled reactant 12.1 GMPS-RNA was transcribed in a 100-μL reaction by incorporation of 50 μCi of α-^{32}P-GTP. The RNA was then size-purified using PAGE, eluted, precipitated, and resuspended in 100 μL ddH_2O. Thirty-six microliters of this RNA was then alkaline digested at 90 °C for 12.5 min with 4 μL 10× alkaline hydrolysis buffer (500 mM $NaCO_3$, 10 mM EDTA at pH 8.0,) and neutralized with 4.8 μL 3 M sodium acetate, pH 5.2. This mix was then purified for thioate activity on 300 μL thiopropyl sepharose 6B. The GMPS-RNAs were then eluted with 100 mM DTT, precipitated overnight at −20 °C, and resuspended in 36 μL ddH_2O. The mass of the GMPS-

RNA was established from its specific radioactivity; 4.88 μL of this RNA was reacted with BrBK in a 10-μL reaction, as described above, for a final RNA concentration of 120 μg/mL. BrBK concentrations of 80, 160, 320, and 640 μM were used for reaction times of 0, 20, and 40 s, and 1, 3, 10, 30, 100, and 300 min at 0 °C. Reactions were quenched and loaded on 10% PAGE sequencing gels for analysis. The gels contained a 1-cm layer of APM polyacrylamide at the top. This layer effectively removed unreacted GMPS-RNA, yet allowed the reacted BK-S-RNA fragments to migrate freely into the gel. APM was not used throughout the gel in order to limit the background retardation of BK-S-RNA caused by APM.

GMPS-RNA to be T1-digested as a standard was treated in much the same manner. In this case, 10 μL of PAGE-purified GMPS-RNA transcription was added to 50 μL 1× T1 buffer (20 mM sodium citrate, pH 5.0, 1 mM EDTA, pH 8.0, 7 M urea) with 6 μL of RNase T1 (Boehringer Mannheim) at 4 units/μL for 30 min at 50 °C. The reaction was then purified using thiopropyl sepharose 6B, as above. T1-digested GMPS-RNA was then reacted at a concentration of 5 μM with 1 mM BrBK for 30 min at 37 °C prior to loading.

ACKNOWLEDGMENTS

We thank Bruce Eaton, Steve Ringquist, Olke Uhlenbeck, and Mike Yarus for their thoughtful review of this work. This research was funded by a research grant from the NIH (GM 19963) and by funds from NeXstar Pharmaceuticals, Inc.

Received June 19, 1996; returned for revision July 15, 1996; revised manuscript received July 26, 1996

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