A molecular predator and its prey: coupled isothermal amplification of nucleic acids

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Background: A novel approach to the study of *in vitro* evolution is provided by the investigation of continuous, functionally coupled, amplifying systems. To date, *in vitro* evolution experiments have focused on issues of mutation and selection. Our work contributes to the new field of *in vitro* molecular ecology studies in which detailed information about the relationship between sequence changes and molecular interactions is obtained. Predator–prey systems are interesting in this context both in terms of evolutionary limits and in terms of the potential kinetic properties of oscillation and spatial pattern formation. Such molecular predator–prey models can be extended to a further negative-interaction mode, viral–host molecular evolution.

Results: A simple, nonfunctional predator–prey system based on the self-sustained sequence replication reaction is proposed. Coupling within the system is achieved using the single-stranded DNA intermediate of one cycle, the prey cycle, as primer for the second one, the predator cycle. Hybridization by complementary base pairing is the second order reaction step underlying the predation. Single steps of the whole reaction system have been investigated by radiolabeling. Each isolated subsystem operates according to the proposed reaction scheme, and evidence for an efficient coupling of both subsystems according to the proposed mechanism was found.

Conclusions: Simple, interacting model systems based on nucleic acids can be designed and constructed for the study of coevolution. The results of studies such as the one described here will provide a basis for the construction of coupled systems of ribozymes, from which point the engineering of catalytic units for applications in biotechnology is feasible.

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Introduction

Applied molecular evolution is a new field of research developed on the basis of two major discoveries. First, Spiegelmann's finding [1] that RNA can be replicated *in vitro* opened the door to Darwinian chemical selection. Second, the discovery of catalytically active RNA led to a wider understanding that nucleic acids can constitute both genotype and phenotype at the same time [2,3]. *In vitro* systems are good models for the investigation of evolutionary strategies because large population numbers, short generation times and biochemical and physical characterization are achievable. Properties of evolving systems such as replication, mutation and selection have their equivalents in the physico-chemical properties of autocatalytic polymerization, incorporation of monomers during polymerization and steady state conditions [4].

An increasing number of examples of the successful application of *in vitro* selection techniques can be found in the literature (see [5] for a review). The procedures are all based on the generation of a pool of sequence variants, followed by selection using an appropriate screen and the subsequent amplification of the selected sequences. This process is carried through for several iterations. Because these *in vitro* selection schemes are time-consuming and only a limited number of generations can be achieved, the development of continuously operating procedures, providing faster and more efficient selection, would be beneficial. The development of complex functions can only proceed when the appropriate mechanisms of information exchange are present, so in systems of noninteracting species optimization is only possible for individual molecules. Specifically, the continuous optimization of catalytically active species can only proceed in coupled systems [6].

The coupled evolution, or coevolution, of distinct species is a major focus of ecological studies [7,8]. The interactions between two species (or two amplifying systems) can be classified into three main types, according to the dependence of growth rates on the density (concentration) of the other species: competition with decreasing growth rates for both populations; the predator–prey situation, in which the growth rate of one population is decreasing when the other is increasing; and cooperation (mutualism)

in which both populations show enhanced growth rates. These statements have been derived for and applied to macroscopic living systems. Our study helps to provide a basis for testing our understanding of evolutionary ecology at the molecular level.

Since the publication of the Lotka–Volterra model [9,10], predator–prey models have become among the best studied interacting systems in theoretical biology [11]. Oscillations in the concentrations of the participating species over time, is a key feature of this family of models. Although Lotka coached his theory in the framework of chemical reaction kinetics, chemical systems showing oscillatory behavior such as the Belousov–Zhabotinsky reaction [12] became established only much later. Such inorganic chemical systems, however, are not able to evolve. It is possible that the ideas of chemical oscillation implicit in Luther's early work [13] also influenced Lotka. In an attempt to resolve the structural instability of Lotka and Volterra's original model [14], concepts of prey and predator saturation akin to the Michaelis–Menten model of enzyme kinetics were introduced [15]. To our knowledge no truly molecular predator–prey system has yet been described.

The ability to develop oscillations is a condition for pattern formation in spatially distributed systems under diffusion control [14]. Pattern formation provides conditions necessary for the stabilization of interactions on the molecular level in long-time experiments on coevolution in functionally coupled systems. Spatial Lotka–Volterra dynamics have also not yet been studied *in vitro* in molecular evolution experiments. At this level, one can expect a wealth of new detail concerning the relationship between sequence changes, species interactions and pattern formation to emerge.

In this article, we present a coupled system based on an isothermal amplification scheme. The system is designed according to a predator–prey model which can serve as a platform for investigating optimization in other scenarios. The isothermal amplification method on which we build is the self-sustained sequence replication (3SR) reaction or nucleic acid sequence-based amplification (NASBA) [16,17]. The reaction is performed by the concerted action of an RNA polymerase and a reverse transcriptase. During multiple cycles of transcription and reverse transcription, RNA and DNA are amplified together.

The design of a model system for coupled amplification requires that the species involved have a function which is directly linked to their ability to replicate. The coupling of function and replication provides the selection screen for the experimental design. After performing this function and running through the replication cycle, the species have to return exactly to the initial state, otherwise the selection screen would work only for the first generation of species. Continuous conditions are provided by the isothermal reaction scheme in a flow reactor [18]. This is not possible with the conventional *in vitro* amplification tool, the polymerase chain reaction (PCR), because the need to run temperature cycles would impose an external frequency on the system. In this article, the coupling scheme of a predator–prey system based on the 3SR reaction is introduced. Single reaction steps of the isolated subsystems are investigated and evidence for an efficient coupling of the subsystems according to the reaction scheme is presented.

Results

The predator–prey model system

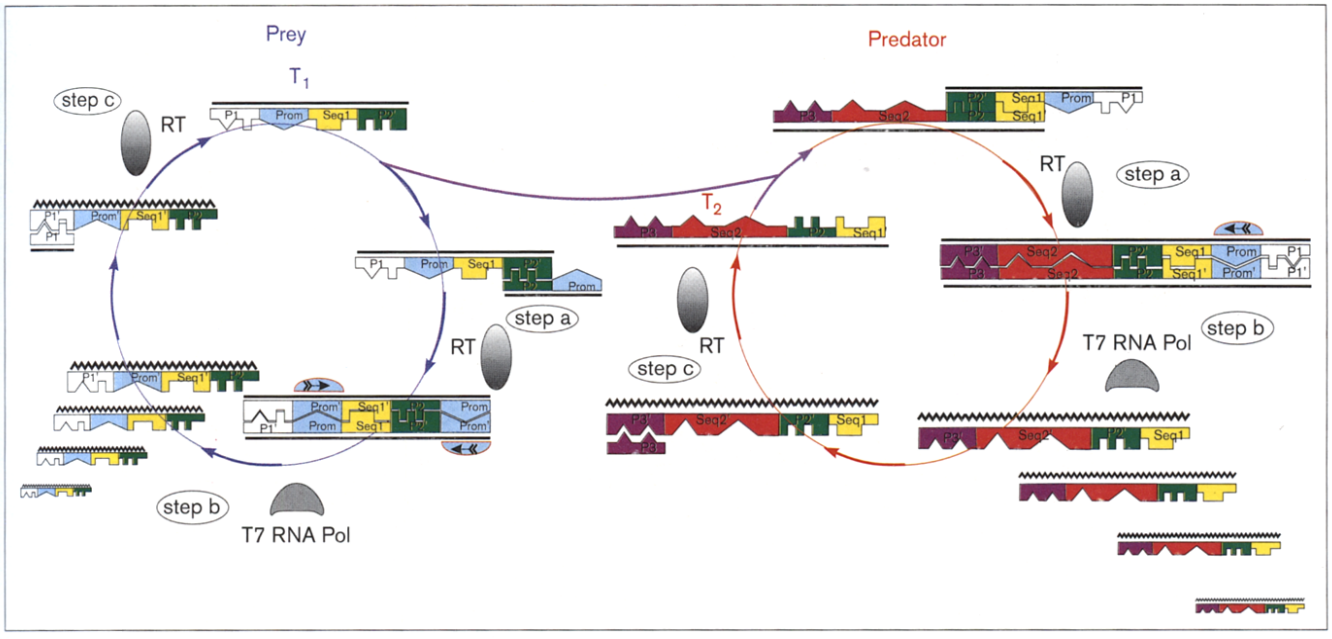
The simplest way to design the coupling of two amplification systems according to a predator–prey model is to use an essential intermediate of one replication cycle as a primer for the second one. In this way, the second replication cycle becomes the predator of the first. This coupling scheme is shown in Figure 1.

The prey cycle (Fig. 1, left) operates as follows. The reaction can be started from a synthetic, single-stranded DNA template, T_1. Annealing of a DNA primer, P_2, containing the promoter sequence for the T7 RNA polymerase (T7 RNA Pol) and the subsequent DNA-dependent DNA polymerization by the action of M-MLV reverse transcriptase (RT) (step a) generates a fully double-stranded species which is the substrate for transcription *in vitro* by T7 RNA Pol (step b). Multiple copies of antisense RNA are synthesized. Another DNA primer, P_1, initiates reverse transcription. Simultaneous degradation of the RNA strand by RNAse activity of RT or by an additional RNAse H (step c) produces the single-stranded DNA template, T_1, anew. All compounds necessary for the amplification are provided in the reaction mixture, in particular both primers, P_1 and P_2, for the prey cycle. The predator cycle, however, is provided with only the primer initiating reverse transcription, P_3. The second primer (T_1) is the single stranded template of the prey cycle. The sequence of T_1 is designed so that the predator template T_2 and the prey template T_1 share a complementary sequence element at their 3′ ends. The consensus sequence of the promoter of T7 RNA Pol is located upstream of this complementary sequence element on the prey template. After annealing the predator and prey templates, RT (step a) can synthesize the double-stranded substrate for T7 RNA Pol, which can then initiate the transcription of antisense predator RNA (step b). Subsequent reverse transcription with primer P_3 (step c) closes the cycle. The internal promoter sequence of template T_1 does not lead to interfering side products.

General features of the reaction system

The specificity and efficiency of single steps of the predator–prey reaction system have been investigated by radiolabeling the 5′ ends of the primers P_1, P_2, P_3 and the

Figure 1



Coupling scheme of the predator–prey system. Waved lines refer to RNA, straight lines to DNA. Sequence modules are: P_1, P_2, P_3, primers; Prom, T7 promoter; Seq1, Seq2, specific sequences of prey and predator; RT, M-MLV reverse transcriptase; T7 RNA Pol, T7 RNA polymerase. Complementary sequence elements are indicated by a prime following the module name.

templates T_1 and T_2 with $\gamma[^{32}P]$-ATP using T4 polynucleotide kinase. Parallel experiments were carried out with the isolated reaction steps and the complete reaction cycle (3SR) by observing the transformation of a single labeled component. The 3SR reactions were carried out with primer concentrations of around 10^{-7}M and template input of around 10^{-10}M (a detailed description of the experiments is found in the Materials and methods section). The sequences of the oligonucleotides used are listed in Table 1.

The 3SR reaction can be started at any phase of the reaction cycle (Fig. 1). The experiments in this study were initiated either by the single-stranded templates T_1 (prey) and T_2 (predator), which were synthesized by standard phosphoramidite chemistry, or by the fully double-stranded species of the prey or the predator cycles which were generated by PCR. Starting the reaction from the double strand proved to be favorable in most cases because the initial reaction step involves amplification. Because of the limited lifetimes of the enzymes, especially M-MLV RT, a high reaction rate in the initial phase leads to a better yield. In the following sections, the products of the main reaction steps and of the complete 3SR reactions of the prey and predator subsystems are illustrated. An experiment establishing the coupled reaction system is then presented and finally, the necessity of modifying the prey subsystem is discussed; the data show that this modification does not alter the efficiency of predator–prey coupling.

Table 1

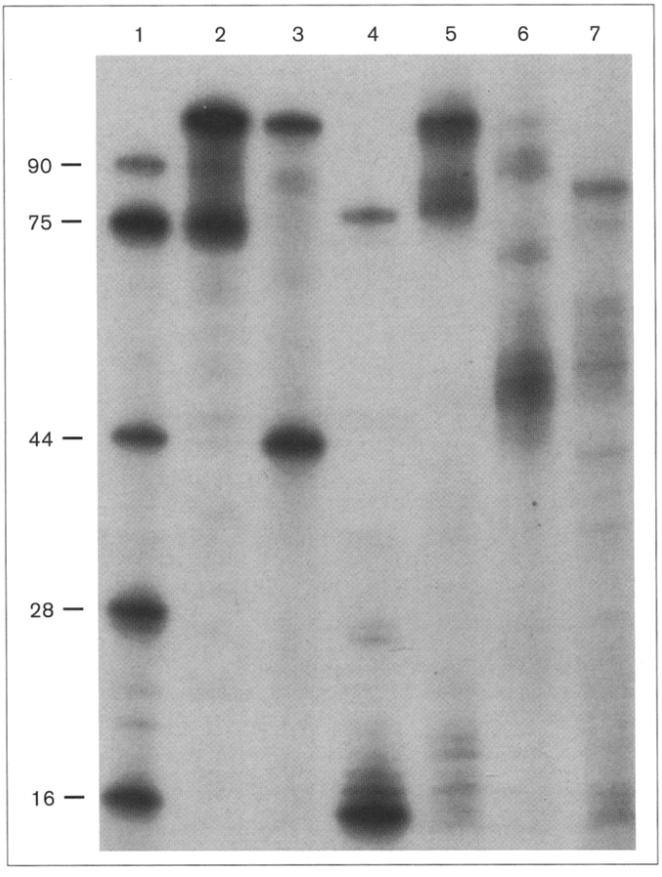
Oligonucleotides used in this study.

Name	Sequence
P_1	GAA TGC ATG GTT AGC G
PrP_1	GTT AGC GTA ATA CGA CTC ACT ATA GGG A
P_2	TAG AAT TTA ATA CGA CTC ACT ATA GGG ACT TCT GAT ACT TCA CA
T_1	GAA TGC ATG GTT AGC GTA ATA CGA CTC ACT ATA GGG ATT CTG TAT GAT GTT ACA CGT AGT GTG AAG TAT CAG AAG
P_3	CTA GCA TGA ATC TCA C
T_2	CTA GCA TGA ATC TCA CGA CAA TCT TGT ATA TCG TAT ACA TTA CTT AAG ACT AGG GAC TTC TGA TAC TTC ACA CTA CGT GTA ACA TCA TAC

The prey subsystem

The reaction products formed during the amplification cycle of the isolated prey subsystem are shown in Figure 2. First, the isolated reaction step of DNA-dependent DNA polymerization by RT was investigated in parallel experiments with either labeled T_1 or labeled P_2 (step a in Fig. 1). In the annealing complex of T_1 and P_2, there are two recessed 3′ ends to fill in. The elongation of template T_1 to the full-length 103-base pair product, generating the functional promoter region, is shown in lane 2. A single band was obtained, which ran slower than the 90-mer T_2 in the marker lane. The result of the elongation of primer P_2 is shown in lane 3. The single product band has the same length as the one in lane 2. This finding is consistent with the expected full-length 103-mer product.

Figure 2



Autoradiogram of a 10% denaturing polyacrylamide gel showing the results of experiments with the prey subsystem. Lane 1, marker lane; lane 2, DNA-dependent DNA polymerization converting template T_1 into full-length product (step a in Fig. 1); lane 3, DNA-dependent DNA polymerization converting primer P_2 into full-length product (step a in Fig. 1); lane 4, reaction sequence of transcription and reverse transcription converting primer P_1 into template T_1 (steps b and c in Fig. 1); lane 5, 3SR reaction of the prey subsystem monitored by labeled P_1; lane 6, 3SR-reaction of the prey subsystem monitored by labeled P_2; lane 7, RNA produced during the 3SR reaction of the prey subsystem, monitored by the incorporation of $\alpha[^{32}P]$-UTP. The experimental details are described in the Materials and methods section.

The reaction sequence of transcription and reverse transcription (steps b and c in Fig. 1) results in the conversion of primer P_1 into template T_1. The result of these two reaction steps monitored using labeled primer P_1 is shown in lane 4. A single product band was obtained which runs slightly slower than the synthetic 75-mer T_1 in the marker lane. The product template is indeed expected to be four bases longer because of the transcription initiation sequence at the 3′ end introduced by *in vitro* transcription after the first round of amplification. The reaction sequence was initiated using the double-stranded 103-mer obtained by PCR. In the lower part of the lane are some truncated products of reverse transcription.

With this information in hand, we can analyze the products of prey amplification. Lane 5 shows the result of a 3SR amplification of the prey subsystem monitored by the consumption of labeled primer P_1. Two main products were formed. The shorter product runs at the same level as the product of the reaction sequence of transcription and reverse transcription displayed in lane 4 (the 4-base elongated T_1). The slower-migrating band corresponds to the full-length 103-mer product, as presented in lane 2. This result is what is expected according to the above reaction scheme.

Labeling the second primer allows the partially complementary set of intermediates containing P_2 to be observed, distinguishing those species containing P_2 and not P_1 and *vice versa*. In lane 6 of Figure 2, the 3SR-reaction is monitored through the consumption of labeled primer P_2. The slowest-running product corresponds in length to the band resulting from the elongation of P_2, displayed in lane 3. Between the band of unreacted P_2 and the full-length product lie truncated products. The asymmetry in the yield of full-length product when comparing lanes 5 and 6 is remarkable. The reason for this asymmetry is a preference of the M-MLV RT for elongating the 3′ end of the template first, resulting in the formation of a double-stranded promoter region. In the presence of T7 RNA Pol, the elongation of the primer complementary to the template is hardly detectable. In the absence of T7 RNA Pol, both the elongation steps take place to a comparable extent (data not shown). This reaction is the bottleneck of the amplification mechanism.

In lane 7, the formation of RNA during the amplification cycle, monitored by incorporation of $\alpha[^{32}P]$-UTP, is shown. The main product corresponds approximately in length to T_1 in the marker lane. RNA is known to run about 10% slower than DNA of the same length, and a progression of truncated transcription products was also obtained.

The predator subsystem

We investigated the formation of products generated during the amplification cycle of the isolated predator subsystem, as for the isolated prey cycle. The results for the predator subsystem are shown in Figure 3. The isolated reaction step of DNA-dependent DNA polymerization (step a in Fig. 1) in the partially hybridized complex of T_1 and T_2 is monitored by labeled T_2 in lane 2. A single product was obtained under the action of RT. The result of the elongation of the 3′ end of labeled T_1 within the same complex is shown in lane 3. The product band corresponds in length to that in lane 2, consistent with the expected 131-base product.

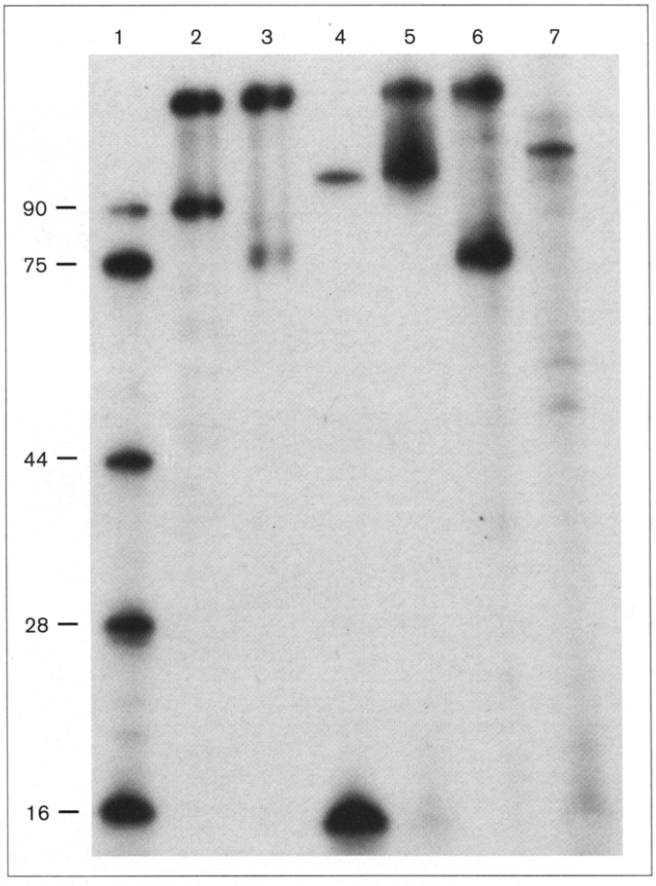
The result of the isolated reaction sequence of transcription and reverse transcription (steps b and c in Fig. 1), monitored by the consumption of labeled primer P_3, is shown in

lane 4. The primer was converted into a single product. As in the case of the prey cycle, the band ran slower than the synthetic T_2 in the marker lane because the four bases of the transcription-initiation sequence were added to the product template after the first round of replication.

In order to operate the isolated predator subsystem, template T_1 was added at a high concentration, equivalent to that of a primer. Lane 5 shows the complete 3SR amplification of the predator subsystem monitored by the consumption of labeled primer P_3. The main product of the reaction corresponds in length to the product in lane 4, the newly formed, four-base elongated T_2. The slowly migrating band corresponds to the full-length product of DNA-dependent elongation by RT as shown in lane 2. The amplification reaction monitored by the primer T_1 is shown in lane 6. The only product corresponds in length to the 131-mer product of the reaction displayed in lane 3 and to the slowest migrating band in lane 5. This band represents the product of DNA-dependent DNA polymerization by RT and the substrate for *in vitro* transcription. In the predator subsystem, the asymmetry in the turnover of primer P_3 and primer T_1 is not as distinct as in the prey system, but it is still detectable (data not shown).

The formation of RNA during the predator amplification cycle is monitored by the incorporation of $\alpha[^{32}P]$-UTP in lane 7. The main product corresponds roughly with T_2 in the marker lane, because RNA migrates somewhat slower than DNA of the same length. A progression of truncated transcription products was also obtained. The investigation of each isolated reaction step and the complete reaction cycle of the predator and prey subsystem establishes that each reaction leads to the expected products with high specificity.

Figure 3



Autoradiogram of a 10% denaturing polyacrylamide gel showing the results of experiments with the predator subsystem. Lane 1, marker lane; lane 2, DNA-dependent DNA polymerization converting template T_2 into full-length product (step a in Fig. 1); lane 3, DNA-dependent DNA polymerization converting primer T_1 into full-length product (step a in Fig. 1); lane 4, reaction sequence of transcription and reverse transcription converting primer P_3 into template T_2 (step b and c in Fig. 1); lane 5, 3SR reaction of the predator subsystem monitored by labeled P_3; lane 6, 3SR reaction of the predator subsystem monitored by labeled T_1; lane 7, RNA produced during the 3SR reaction of the predator subsystem monitored by the incorporation of $\alpha[^{32}P]$-UTP. Experimental details are described in the Materials and methods section.

Coupling the subsystems according to the predator–prey reaction scheme

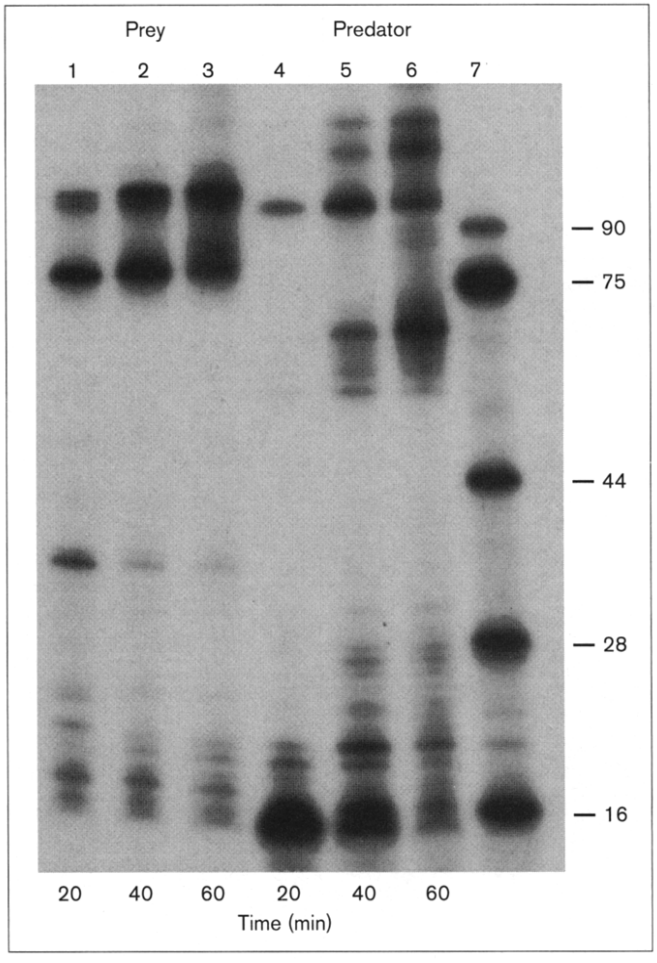
Following the verification of the independent operation of predator and prey cycles, the reaction system was investigated by a set of parallel experiments to establish the coupling of the two amplification cycles. The results are shown in Figure 4.

In the coupled system, primer P_2 and template T_2 compete for the common annealing site at the 3′ end of template T_1. Under batch conditions, the difference of the initial concentrations of the two components is several orders of magnitude, so the predator amplification, initiated by the annealing of T_2 and T_1, will, in the initial phase, be only a side reaction with less turnover than the prey cycle.

The prey subsystem is monitored by the consumption of primer P_1, in lanes 1–3, and the predator subsystem by primer P_3, in lanes 4–6. The input templates that start the prey and predator cycle are the relevant double-stranded species, which were prepared by PCR. Using radiolabeled primer P_1 for the prey system and primer P_3 for the predator system, each amplification cycle can be monitored independently. As discussed in the previous section, the transformation of these primers results first in reverse transcription to DNA templates and then in DNA-dependent DNA polymerization to the full-length double-stranded product. The occurrence of reverse transcription establishes implicitly the presence of RNA transcription products, so these primers can be used to indicate the progress of both amplifying subsystems.

In Figure 4, the course of the coupled reaction is viewed at three different time points. After 20 min, the prey

Figure 4



Autoradiogram of a 10% denaturing polyacrylamide gel showing the predator–prey reaction system at 20 min, 40 min and 60 min time points. Lanes 1–3, the prey subsystem of the coupled reaction monitored by the consumption of labeled primer P_1; lanes 4–6, the predator subsystem of the coupled reaction monitored by the consumption of labeled primer P_3; lane 7, marker lane. Experimental details are described in the Materials and methods section.

amplification had nearly run to completion; primer P_1 is already consumed. Later time points show only the production of more of the full-length 103-mer generated from the 75-mer T_1. The emergence of a faint, slower running band, corresponding in length to the full length 131-mer product of the predator reaction, is an indicator for the incorporation of the prey template into the predator amplification through strong coupling of both systems. In the predator cycle, only a small amount of 90-mer T_2 is produced in the initial 20 min. The absence of full-length 131-mer product at this time indicates that T_2 is the result of transcription and reverse transcription initiated by the initial input of double-stranded material.

After 40 min reaction time, full-length 131-mer was also produced in large amounts, showing that the predator amplification had gone through complete reaction cycles (serial transfer data not shown). A shorter product indicated the emergence of an independent, primer-consuming parasite. Almost all primer P_3 is consumed after 1 h reaction time. In the absence of prey template, no predator amplification was detectable. In conclusion, an efficient coupling of two amplification cycles occurs, as proposed in Figure 1.

Modified prey system

A preliminary experiment under serial transfer conditions showed a high mutagenic rate for the 3SR reaction. A modification was introduced in order to circumvent a fast decoupling of the predator–prey system by mutations within the internal promoter region of the prey sequence during long-term experiments under flow conditions. A new primer, PrP_1, was synthesized; it starts 10 bases downstream of the initial T_1 sequence and covers the whole promoter region. As a consequence, both DNA species of the altered prey cycle are 10 bases shorter than in the initial system.

Figure 5 shows a comparison of the initial and modified predator–prey systems. The progress of the prey amplification is monitored by the consumption of labeled primer P_1 for the initial system and of labeled primer PrP_1 for the modified coupled system; no modification is introduced in the predator subsystem. In each case, the predator reaction is monitored by labeled primer P_3. Lane 2 displays the unmodified prey amplification in the coupled reaction system. Newly synthesized template T_1 and full length 103-mer product are again generated. The slowest running band corresponds in length to the full-length 131-mer product of the predator reaction, indicating the coupling of the two systems. The predator amplification, obtained by a parallel reaction, shows the expected two main products. Again the shorter product indicates the emergence of an independent, system-specific parasite.

As expected, the modified prey reaction shown in lane 4 of Figure 5 displays the same product pattern as the initial system, although each product is 10 bases shorter. The predator part of the reaction produces a band in a position corresponding to the product template T_2. The full-length product is 10 bases shorter than in the unmodified predator–prey reaction as a result of the shorter *in situ* synthesized 'primer' T_1. A similar side product is formed to that in the coupled case shown in lane 3.

Discussion

The 3SR reaction was developed as a tool for the specific detection of HIV RNA in blood and plasma samples [16,17,19]. As it is largely independent of the sequence of the target of amplification, it is also a suitable tool for the amplification of RNA in the context of Darwinian evolution experiments [20,21]. A coupled reaction system based on an isothermal amplification scheme modeled according

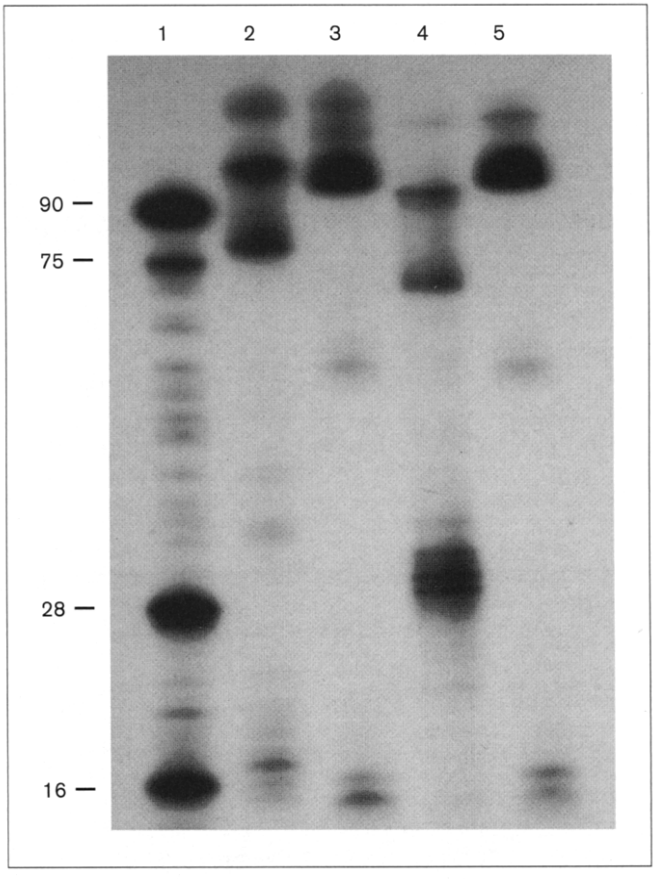
to predator–prey interactions is presented here. Coupling is achieved using a reaction intermediate of the prey cycle, the single-stranded template T_1, as primer for the predator amplification cycle. The system consists of two parallel amplification cycles with staggered initial phases, because one cycle is feeding the second one.

The reaction system was surveyed, step by step, by radioisotopically-labeling all components. DNA species were 5′ labeled with $\gamma[^{32}P]$-ATP using T4 polynucleotide kinase, and RNA species were labeled by the incorporation of $\alpha[^{32}P]$-UTP during transcription *in vitro*. In general, the reaction can be started at each point of the cycle, although it is initiated more readily from the double-stranded species. In this case, the first step involves amplification yielding a high initial rate. Investigation of single reaction steps and the isolated subsystems showed high specificity of the reactions in each case. Both systems react according to the scheme in Figure 1, as in the results of earlier analyses of the 3SR reaction [22]. In both predator and prey systems, the bottleneck for amplification has been shown to be the DNA-directed DNA polymerization by RT. This step is expected to be the major focus of evolutionary change. In the presence of T7 RNA Pol, an asymmetry of the filling-in of the recessed 3′ ends of the annealing product of template and promoter-containing primers is observed. Under optimized reaction conditions, RT prefers elongation of the template rather than of the primer, yielding a double-stranded promoter region for T7 RNA Pol.

In this type of coupled system, the outcome of the amplification reaction of each isolated system is best monitored by 5′ labeled primer P_1 for the prey reaction and primer P_3 for the predator reaction. P_1 and P_3 monitor directly two out of three enzymatic reaction steps in their respective cycles. The third enzymatic reaction of the amplification, transcription, is then monitored indirectly through the formation of the templates T_1 and T_2.

The effect of predation within the system is clearly evident from the set of experiments shown in Figure 4. Our study revealed that predator–prey amplification is taking place in two phases. In the first stage, the prey subsystem amplifies until the primers are consumed. The predator template, T_2 which is generated during this time, results solely from the initial input of double-stranded full-length template which leads to transcription and reverse transcription without running through the complete amplification cycle. This finding is confirmed by the lack of a band corresponding to the full-length 131-mer. From this point on, predator amplification proceeds cyclically in the second phase. The incorporation of prey template T_1 into the predator cycle is also indicated by the late emergence of a faint band corresponding in length to the full-length 131-mer product of the predator cycle in the lane representing the prey part of the coupled amplification system. Additional evidence for the coupling of the two systems can be obtained with partially coupled subsystems and different starting points for amplification (data not shown).

Figure 5



Autoradiogram of a 10% denaturing polyacrylamide gel showing a comparison of the coupled reaction systems with the unmodified and the modified prey subsystem. Lane 1, marker lane; lane 2, 3SR reaction of the prey subsystem within the unmodified, coupled system monitored by the consumption of labeled primer P_1; lane 3, 3SR reaction of the prey subsystem within the unmodified, coupled system monitored by the consumption of labeled primer P_3; lane 4, 3SR reaction of the prey subsystem within the modified, coupled system monitored by the consumption of labeled primer PrP_1; lane 5, 3SR reaction of the predator subsystem within the modified, coupled system monitored by the consumption of labeled primer P_3. Experimental details are described in the Materials and methods section.

The coupled amplification reaction gives rise to the emergence of a shorter parasite-like side product consuming P_3. This type of parasite is also known to occur in the isolated 3SR reaction — an example is RNA-Z [20] — and must be held in check by contamination-free conditions: appropriate concentrations to limit its replication rate and in extreme cases, a change in primer sequences. Part of the future significance of these studies should be in uncovering how coupled systems keep such parasites at bay.

Preliminary experiments under serial transfer conditions showed the isothermal reaction scheme to have a considerable mutation rate. The internal promoter sequence in template T_1 has no function within the prey cycle. On the other hand, a functional promoter sequence is essential for the predator reaction. To prevent a fast decoupling in long-term experiments under flow conditions, it is necessary to conserve this sequence element. Because the only conserved sequences in the reaction are the primer regions, the best means of conserving the promoter sequence is to use it as a primer-binding site. This modification was achieved by introducing a new primer PrP_1 into the prey cycle. The products of this modified prey cycle were 10 bases shorter than in the preceding system, but the reaction showed the same behavior as an isolated subsystem. The coupled reaction system preserved the desirable kinetic features of the original system.

Study of the predator–prey system under conventional batch conditions is a necessary precursor to investigating open systems, which involve continuous operation under flow conditions with a constant supply of educts and removal of reaction products. According to the Lotka–Volterra model, a predator–prey system can show oscillations in the concentrations of the reacting species. Numerical simulations of the reaction system in Figure 1 showed oscillations in a special range of parameters, and the conditions for oscillatory behavior were investigated (J. Ackermann, B.W. and J.S.M., unpublished observations). These theoretical studies provided valuable input for the experimental investigation of the multi-parameter system presented, as it is not possible to search the whole parameter space experimentally for suitable conditions for oscillations. Initial experiments in a homogeneous flow reactor showed that the isolated systems work under flow conditions. Subsequent studies will aim to observe and characterize oscillatory behavior.

Long-term time experiments under flow conditions will be investigated for evolutionary sequence changes by automated sequencing. Predator–prey coupling allows a qualitatively new level of dynamics in *in vitro* evolution. The monotonic optimization of sequence properties [23] is replaced by concentration-dependent selection through the joint action of predator and prey. In particular, theoretical models have demonstrated the efficacy of predator-driven coevolution in optimization [24], and the possibility of a continued 'chase' through sequence space rather than convergence to a stationary sequence population [25]. Detailed molecular studies of these phenomena may reveal additional insights into such molecular coevolution in viral-receptor recognition [26]. Finally, we anticipate that the investigation of pattern formation and sequence changes in spatially resolved flow reactors will uncover a new level of detailed understanding for coevolution in functionally coupled amplification systems.

Significance

The investigation of continuous, functionally coupled amplifying systems is a novel approach to studying evolution *in vitro*. To date, *in vitro* evolution experiments have focused on issues of mutation and selection. The work described here contributes to the new field of *in vitro* molecular ecology studies, in which detailed information about the relationship between sequence changes and molecular interactions is being obtained. Predator–prey systems are interesting in this context, both in terms of evolutionary limits and in terms of the potential kinetic properties of oscillation and pattern formation. Molecular predator–prey models can be extended to a further negative-interaction mode, that of viral–host molecular evolution. A simple predator–prey system designed using otherwise non-functional nucleic acid template sequences and based on the 3SR reaction was proposed. Coupling was established by a single-stranded intermediate of the prey cycle acting as a primer for the predator amplification cycle. After optimizing parameters, the coupled system was shown to operate according to the reaction scheme. The next step will be the investigation of oscillations in the concentrations of the participating species in a homogeneous flow reactor. The parameters derived for flow conditions will be useful in experiments in a two-dimensional flow reactor. In this environment, the full range of dynamic behavior can develop, including pattern formation. The results will provide a basis for the construction of coupled systems of ribozymes, and thence a starting point for the engineering of catalytic units for applications in biotechnology.

Materials and methods

Oligonucleotides

Oligonucleotides were synthesized on a Millipore DNA synthesizer and purified by polyacrylamide gel electrophoresis. The sequences of the oligonucleotides used in this study are given in Table 1.

Enzymes

M-MLV RT was purchased from USB (Cleveland, Ohio, USA). *E. coli* RNAse H and T7 RNA pol were purchased from MBI Fermentas (Lituani). RNAsin and T4 Polynucleotide kinase were purchased from Promega (Lyon, France). Thermostable Vent polymerase was purchased from New England Biolabs (Schwalbach, Germany).

Apparatus and reagents

The gel apparatus for polyacrylamide gel electrophoresis was purchased from Sigma (Deisenhofen, Germany). Radioactively labeled nucleotides were purchased from Amersham-Buchler Life Science (Braunschweig, Germany) and nucleotide triphosphates were purchased from Pharmacia (Freiburg, Germany). All standard chemicals were p.A. grade and were purchased from Sigma (Deisenhofen, Germany). Nensorb 20 columns for reversed-phase chromatography were purchased from DuPont (Bad Homburg, Germany).

5′ labeling of oligonucleotides with γ[^{32}P]-ATP

Reactions were carried out in a total volume of 25 µl containing 5 pmol oligonucleotide, 2.5 µCi γ[^{32}P]-ATP, 2.5 µl 10× kinase buffer and 10 units T4 polynucleotide kinase. The reaction mix was incubated for 30 min at 37°C. EDTA was added to a final concentration of

5 mM. Unreacted γ[^{32}P]-ATP was separated from the 5′-labeled oligonucleotide on Dupont Nensorb 20.

PCR

The double-stranded components of the predator and prey cycles were obtained by PCR over 25 cycles with thermostable Vent DNA polymerase; 100 fmol T_1 or T_2, 250 pmol P_1 and P_2 or P_3 and T_1, 0.2 mM dNTPs, 10 µl 10× PCR buffer and 4 mM $MgCl_2$ were brought to a final volume of 100 µl. Reaction conditions were 1 min 95°C, 1 min 50°C for T_1/1 min 42.5°C for T_2 and 1 min 72°C. After precipitation, the PCR products were gel-purified. The final products were used as templates for the reaction sequence of transcription and reverse transcription or as template input for the 3SR reaction.

Filling-in of recessed ends in annealing complexes by RT

For this and all subsequent experiments, the reaction was carried out in a total volume of 100 µl. The reaction mix contained 5 pmol T_1 and P_2 or T_1 and T_2, 50 mM HEPES pH 7.5, 50 mM NaCl, 5 mM dithiothreitol, 2 mM spermidine, 25 mM $MgCl_2$, 0.2 mM each dNTP and 50 units of M-MLV RT. For radioactive labeling, 10 000 cpm of the respective 5′-labeled oligonucleotide was added. The mix was heated to 95°C for 5 min prior to the addition of the enzyme and incubated at 37°C for 1 h. After precipitation, the products were analyzed by denaturing polyacrylamide gel electrophoresis.

Reaction sequence of transcription and reverse transcription

The reaction mix contained 25 pmol P_1 or P_3, 100 fmol PCR product obtained from T_1 or T_2, 50 mM HEPES pH 7.5, 50 mM NaCl, 5 mM dithiothreitol, 2 mM spermidine, 25 mM $MgCl_2$, 0.2 mM each dNTP and 2 mM each NTP. Enzyme concentrations were 75 units M-MLV-RT, 75 units T7 RNA Pol, 0.375 units *E. coli* RNAse H and 20 units RNAsin. The reactions were incubated for 1 h at 37°C. For radioactive labeling, 10 000 cpm of 5′-labeled oligonucleotide P_1 or P_3 was added. The reaction mixes were desalted on Dupont Nensorb 20 prior to loading on denaturing acrylamide gels.

Isothermal amplification of isolated subsystems

The reaction mix contained 1–25 pmol P_1 and P_2 or P_3 and T_1, 10–100 fmol PCR product obtained from T_1 or T_2, 50 mM HEPES pH 7.5, 50 mM NaCl, 5 mM dithiothreitol, 2 mM spermidine, 25 mM $MgCl_2$, 0.2 mM each dNTP and 2 mM each NTP. The reaction mixture was heated for 5 min at 95°C and cooled on ice before the enzymes were added. Enzyme concentrations were 50–150 units M-MLV RT, 50–150 units T7 RNA Pol, 0–1 unit *E. coli* RNAse H and 20 units RNAsin. The reactions were incubated for 30 min–1.5 h at 37°C. For radioactive labeling 10 000 cpm of 5′-labeled oligonucleotide or 1 µCi of labeled UTP was added. The reaction mixes were desalted on Dupont Nensorb 20 prior to loading on denaturing acrylamide gels.

Isothermal amplification of the coupled system

The reaction mix contained 1–25 pmol P_1, P_2 and P_3, 50–100 fmol PCR product obtained from T_1 and T_2, 50 mM HEPES pH 7.5, 50 mM NaCl, 5 mM dithiothreitol, 2 mM spermidine, 25 mM $MgCl_2$, 0.2 mM each dNTP and 2 mM each NTP. It was heated for 5 min at 95°C and cooled on ice before the enzymes were added. Enzyme concentrations were 100–150 units M-MLV RT, 100–150 units T7 RNA pol, 0–1 unit *E. coli* RNAse H and 20 units RNAsin. The reactions were incubated for 1–1.5 h at 37°C. For radioactive labeling, 10 000 cpm of 5′-labeled oligonucleotide was added. The reaction mixes were desalted on Dupont Nensorb 20 prior to loading on denaturing acrylamide gels.

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