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Machines for automated evolution experiments *in vitro* based on the serial-transfer concept

Günther Strunk, Tobias Ederhof

Max-Planck-Institute für biophysikalische Chemie, Evolutionsgruppe, Am Fassberg 11, D-37077 Göttingen, Germany

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Abstract

Two machine setups for automated evolution experiments *in vitro* are described. Both machines enable the monitoring of growing populations of RNA or DNA molecules in real time using high-sensitivity glass fiber laser fluorimeters and an automated sample handling facility for volumes in the microliter range. Growth conditions are kept constant by means of the serial-transfer technique, that is, the successive transfer of a small fraction of a growing population into a fresh solution containing no individuals prior to the transfer. The serial transfer technique was modified to work with large populations and constant growth conditions. In the single-channel evolution machine isothermal amplification reactions ($Q\beta$-system, 3SR, NASBA, SDA) are monitored successively in single test tubes. This machine is particularly well suited for the investigation of optimal adaptation to altered environmental conditions, as is experimentally demonstrated in the evolution of an RNA quasi-species using ribonuclease A as the selection pressure. The new variant of RNA appeared very rapidly (within approximately 80 generations) without stable intermediates, and it was selected by steadily increasing the RNaseA concentration during the serial-transfer experiment.

The other machine, which is described in the second part of this article, is a consequent extension of the single-channel machine, and was designed to allow the multichannel detection of up to 960 samples simultaneously. Thus, high-throughput screening can be applied to evolution experiments. In addition to monitoring isothermal amplification reactions, it is also possible to follow PCR amplifications through thin plastic foils. Initial experiments have demonstrated the suitability of the apparatus for uniformly processing samples and for performing thermocycling. © 1997 Published by Elsevier Science B.V.

Keywords: *serial-transfer experiment; Qβ-replicase; self-replicating systems; on-line detection; selection pressure; RNase A*

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1. Introduction

A new approach in modern biotechnology is the development of evolutionary strategies for the synthesis of biomolecules with novel functions or improved properties. These new molecules can then be used for technological and medical applications. The basis of such an evolutionary biotechnology is the application of selection to populations of replicating molecules, viruses, microorganisms or cell populations according to externally controlled criteria [1]. Although this technology is still in its infancy, and most of the experiments that have been carried out so far do not address particular practical problems, there is an increasing interest in molecular mechanism of evolution and in methods to perform evolution experiments under controlled conditions.

One of the best-known systems for studying evolutionary phenomena is the Qß-system that is based on the ability of the enzyme, RNA-dependent-RNA-polymerase, to amplify specific RNA templates autocatalytically under *in vitro* conditions [2,3,4,5,6]. The first extracellular Darwinian experiments with the Qß-system were carried out by Sol Spiegelman and coworkers [3]. In these experiments, the system consisted of a reaction mixture of appropriate buffer and electrolyte concentrations containing the four nucleoside triphosphates ATP, UTP, CTP and GTP as energy-rich substrates and Qβ-replicase as the polymerizing enzyme. RNA synthesis is initiated by incubation with a fixed amount of template RNA. After a certain incubation time an aliquot is transferred to a fresh solution containing no RNA prior to the transfer, thereby triggering the start of a new transfer cycle. This procedure is iterated indefinitely keeping the maximum RNA concentration at a constant stationary level, and thereby allowing for the unlimited evolution of optimally reproducing RNA species under any given environmental constraint. Several selection pressures were used: an increasing dilution factor [7], low concentrations of each of the NTPs, substituting the NTPs by base analogs [8], and adding mutagens, such as ethidium bromide [9,10]. Although new variants occurred in all these experiments, it was found that even better adapted species were produced if suitable experimental conditions were applied. The crucial question is: What are the suitable experimental conditions? The answer to this question is given by Manfred Eigen's theory on the evolution of self-replicating macromolecules [11], which is essentially a physical interpretation of the Darwinian evolution principal at the molecular level. According to this theory, molecular evolution will take place provided that certain prerequisites are fulfilled.

Both, the population size and the mutation rate during the reproduction process are crucial parameters for optimization during evolution. Conditions for optimal evolution require populations of quasi-species [12,13,14]; the quasi-species include a broad spectrum of mutants with variable mutation rates, varying from high to low properties for producing new mutants that enable the selection of advantageous mutants which may have existed at only very low levels at the beginning of an experiment. The fact that viruses reproduce close to these optimal evolutionary conditions is one reason why they adapt very efficiently to any new host environment. Therefore, viruses and virus-like systems are particularly interesting systems for investigation. A detailed treatment of Eigen's evolution theory is described elsewhere [11,12,13,14]. It should be mentioned that in order to obtain reproducible results from evolution experiments, it is important to work with sufficiently large and controlled population sizes and within certain limits in order to retain the same selection criteria during the course of the experiment. For example, it is known that selection criteria change during the transition from the exponential growth phase, where Qβ-replicase is in large excess over the RNA concentration, to the linear growth phase where the RNA and the enzyme are present in equimolar concentrations [5,6] (Fig.1). Whereas in the exponential growth phase the species with the highest turnover numbers are selected, in the linear phase, there is a competition for the limited amounts of enzyme, and this leads to selection of species that bind most rapidly to the enzyme. However, this is not necessarily the mutant with the highest replication rate. Thus, in order to get reproducible results it is important to control the RNA concentration in relation to the Qβ-replicase concentration in the reaction mixture, which determines the threshold for the transition between both growth phases. It is particularly

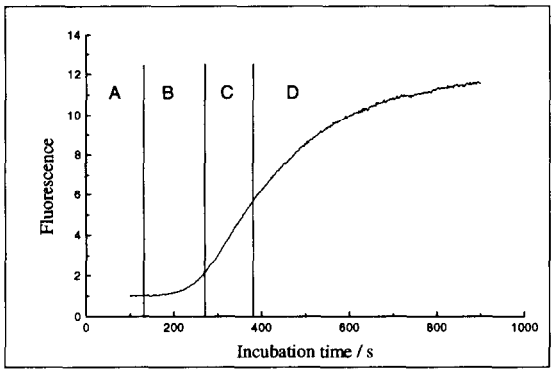

Fig. 1: RNA amplification with Qß-replicase in vitro. A: RNA concentration below the detection limit. B: exponential growth phase C: linear growth phase and D: saturation due to product inhibition. Reaction conditions: 50mM Tris-Cl, pH=7.5, 10 mM MgCl_2, 10 mM DTT, 100 g/l Glycerol, 0.5 mM each of ATP, CTP, GTP and TTP, 100 mM NaCl, 15 µmol/l EtBr and 1 µmol/l Qβ-replicase. Fluorimeter: LS5B (Perkin Elmer) at T=30°C.

interesting to perform evolution experiments in the exponential growth phase because under these conditions selection values are identical with the overall replication rates [11]. In contrast to earlier serial-transfer experiments, which were performed either with high dilution factors [7,8], or with extended incubation times disregarding the concentration conditions in the mixtures [3,9], the serial transfer machine makes it possible to follow RNA growth in the exponential growth phase, where the number of molecules is always kept large enough to prevent the dilution of advantageous mutants.

The single-channel machine is a facility for *on-line* measurement of RNA concentration by means of a glass fiber laser fluorimeter and an automatic sample-handling device [15,16]. The RNA population is monitored by means of fluorescence enhancement of ethidium bromide through intercalation into double-stranded regions of nucleic acids [17]. The fluorimeter was especially constructed for measuring fluorescence in small sample volumes (20 µl), thereby minimizing required amounts of rare and expensive materials needed for growth through many generations. In addition to controlling the population size, this technique enables the *on-line* detection of a faster growing RNA quasi-species during evolution experiments. Hence, it is possible to control experiments *on-line*. The single-channel serial-transfer machine is particularly well suited for the investigation of evolutionary dynamics in the presence of externally applied selection constraints. Because the experiments are reproducible, it is possible to test different strategies for applying selection pressures in order to find optimal solutions.

The STM is not exclusively designed for Qß experiments. Recently, other enzymatic isothermal amplification techniques have been introduced: SDA (strand displacement amplification reaction) [18], 3SR (self-sustained sequence amplification reaction) [19] and NASBA (nucleic acid sequence-based amplification) [20]. SDA [21], 3SR [22] and NASBA [23] have been successfully tested in the single-channel evolution machine.

In order to follow the quasi-species nature of a self-replicating system more precisely, an amplification procedure requires compartmentation and evaluation of the subpopulation fitness in those compartments. Therefore, as an extension of the STM, a 960-channel machine has been constructed that is capable of processing, monitoring and evaluating up to 960 samples simultaneously [24, 25, 26]. The fluorescence is monitored in this multichannel machine by the same principle used to record a reporter dye in a glass fiber laser fluorimeter. In the STM, the experimentator imposes the selection pressure. The self-replicating system then responds by selection of the fittest mutant, together with its quasi-species. A higher degree of experimental control is achieved when the experimentator evaluates a broad spectrum of those responses by reamplifying certain subpopulations and discarding others. This active selection of desired subpopulations can be performed in addition to the imposed selection pressures. A possible selection strategy would then be to pool several subpopulations that are performing well according to the desired requirements before redistributing them in the next serial-transfer step. Theoretical concepts and aspects have been discussed in [27].

Other advantages of large-scale automation are: better reproducible conditions for large arrays of samples, reduced handling requirements and higher statistical significance of the conclusions. In the context of current development towards high-throughput screening with the help of automation, in particular for drug development by combinatorial

chemistry and evolutionary biotechnology (Koltermann & Kettling, this issue), the multichannel machine described here will have a number of additional applications.

2. The single-channel serial-transfer machine

The single-channel serial-transfer machine (STM) is a computer controlled apparatus with laser optics and a glass fiber fluorimeter, supplemented by the corresponding mechanics.

2.1. Optical Part

In order to measure fluorescence-based RNA concentrations in small sample volumes on-line with RNA growth, a sensitive glass fiber laser fluorimeter was constructed (Fig. 2). The 514 nm line of an 0.5mW argon-ion laser is coupled into a glass fiber with a diameter of 1 mm and a length of 1 m which leads the light beam to the measurement part of the machine. The fluorescence is collected in a front-surface-arrangement with a second glass fiber of the same kind. Because of this technical restriction applied by usage of so-called Y-shaped fibers, the signal in the emission branch contains a large fraction of reflected excitation light. Therefore, the light is passed through a nitrogen-cooled, double-banded interference filter (DAL-595.4 nm, Schott) with a high rejection at 514 nm and a high transmission at 600 nm before the fluorescence is measured by a photodiode. The signal is then visualized on a Keithley digital multimeter, and transmitted to a Digital PDP11/23 computer, where the data are collected with a data collection rate of *1,7 sec* and compared with a predefined threshold value corresponding to a known RNA concentration. After the concentration threshold has been reached, two electronic shutters block the light pathway, thereby stopping the measurement. Because fast-growing populations reach the threshold value in shorter times than the slow-growing ones, the system is self-adaptive with respect to the incubation times. This feature is of particular importance for the reproducibility of population size in different sample carriers.

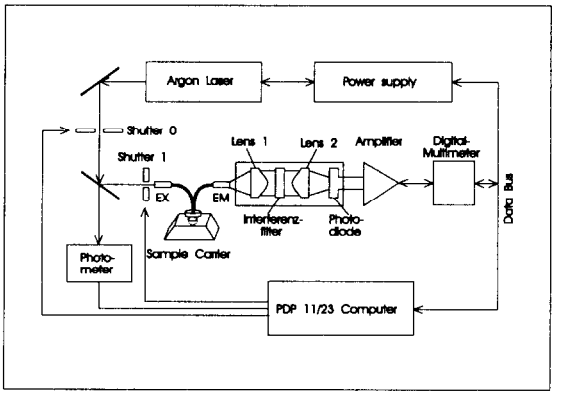

Fig. 2: Optical system employed for fluorescence-based on line detection of the RNA concentration.

2.2. Mechanical part and experimental procedure

In a typical serial-transfer experiment, several sample carriers, containing 20 µl of a solution that includes all components for the reaction except for the replicating RNA, are placed at the starting position of the machine (Fig. 3). They are frozen in order to prevent uncontrolled reactions. At the beginning of an experiment, the first carrier is inoculated with an RNA quasi-species containing about 10^{11} molecules. Two motors, DCM(2) and DCM(3/6), move the inoculated carrier to the turnable disc which is

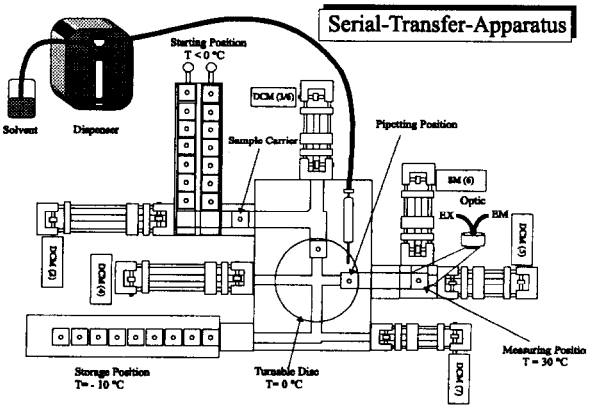

Fig. 3: Schematic diagram of the machine part of the Serial-Transfer-Machine.

thermostatted at T=0 °C in order to melt the solution. The carrier is then moved with the help of DCM(4) to the measuring position where RNA growth is initiated by a fast temperature transition to T~30°C, the optimal temperature for replicase activity. This process is supported by stepping motor, SM(6), that presses the carrier tightly to the walls of the apparatus. After a certain RNA concentration has been reached, motor DCM(5) moves the sample carrier back to the disc, where the reaction is stopped due to the transition to T=0°C. An automatic pipette, provided with a Hamilton micro-lab, transfers a 2 µl aliquot to the next sample carrier which has been positioned on the disc in the meantime. DCM(6) and DCM(7) move the old mixture to storage at T= −10°C, where the samples are available for post-experimental analysis. The fresh mixture that has been inoculated is incubated until the concentration threshold is again reached, triggering the beginning of a new transfer cycle. This technique provides constant growth conditions and indefinite numbers of generations. Because of the small transfer dilutions, the temporal behavior is close to that of a stirred tank reactor, which cannot be used with volumes as small as 20 µl. Evolutionary constraints can be introduced by adding selection pressure, i.e. mutagens, to the reaction mixture at the starting position. The adaptation to a new environment can be followed by analyzing the stored transfer mixtures. Different precautions are used to minimize the errors during an experiment. An argon atmosphere saturated with water steam which is used at the measuring position, prevents volume effects due to the evaporation of the sample solution and photo-decomposition of the ethidium bromide during the incubation at T=30°C. Furthermore, dry argon is used at positions where the temperature is low. This avoids water condensation. Because enzymatic activity is controlled by different temperatures, a rapid temperature transition at the appropriate positions is required. Therefore, all sample carriers are made of silver and the reaction vessels are made of gold (Fig. 4). The computer is electrically insulated from the rest of the machine using optocoupler devices. This prevents interference from the motors during the experiments. A computer program permits the convenient development of individual machine protocols; the programming is similar to writing a flux diagram. Because it is not required to compile a new program, this technique reduces the risk of erroneous programming.

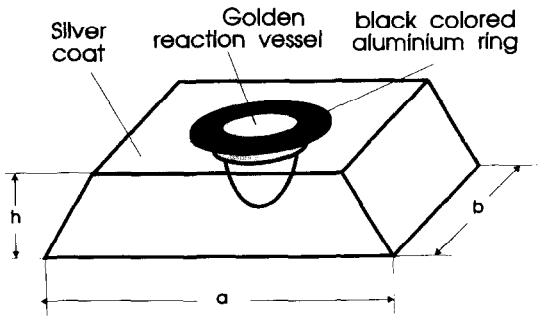

Fig. 4: Sample carrier for 20 µl reactions.

2.3. Experiments with RNase A as selection pressure

One of the first results with the single-channel serial-transfer machine was the selection of an RNA quasi-species that is far more resistant to ribonuclease A (RNaseA) than the starting quasi-species; the original RNA does not survive in the presence of high concentrations of RNaseA [15]. RNaseA is an enzyme that cleaves RNA molecules specifically next to unpaired pyrimidines (U,C).

Initial experiments were carried out to investigate the effect of RNaseA on the growth behavior of the short-chained, 87 nucleotides long RNA, MNV-11[1]. A distinct inhibition of the RNA replication was detectable at a concentration of *1.0 ng/µl* RNaseA, whereas higher concentrations (*2.0 ng/µl* and above) are definitely lethal.

The adaptation of MNV-11 when RNaseA is used as a selection pressure was investigated by serial-transfer experiments using steadily increasing concentrations of RNaseA, starting with mild (non-lethal) conditions. During the experiment, the growth rate first dropped significantly after each transfer into a solution with an increased concentration of RNaseA; but very quickly the rate of growth in-

[1] The sequence of MNV-11 that were used as starting RNA in these experiments contained some point mutations compared to the one reported in [28].

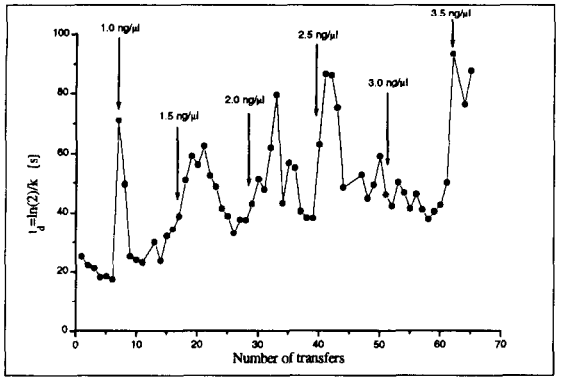

Fig. 5: Results from a serial-transfer experiment with the RNA MNV-11 and RNaseA as selection pressure: Presentation of the time that is required to double the RNA concentration during the exponential growth phase for each transfer. Reaction conditions: 20 µl reaction mix containing 50mM Tris-Cl, pH=7.5, 10 mM MgCl_2, 10 mM DTT, 100 g/l Glycerol, 0.5 mM each of ATP, CTP, GTP and TTP, 100 mM NaCl, 15 µmol/l EtBr and 1 µmol/l Qβ-replicase. RNA growth was followed in the STM at T=30 0C. At an RNA concentration of 5 × 10-7 M 2µl of the solution corresponding to a dilution factor of 11 or 3.5 generations respectively, were transferred to fresh solution. The RNaseA concentration was as indicated by the arrows and remained unchanged until to the next step.

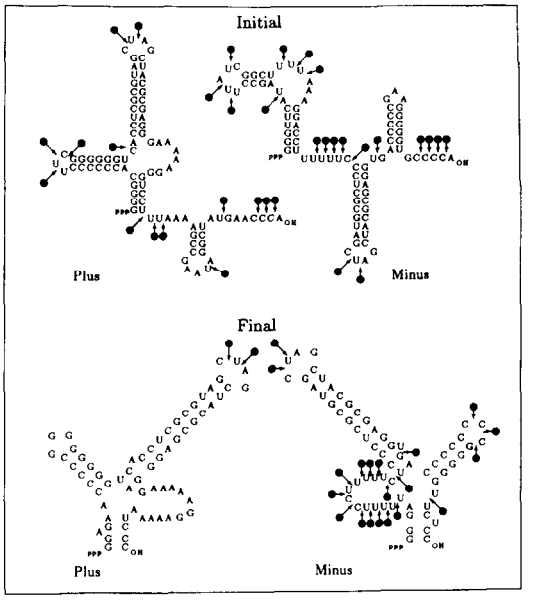

Fig. 6: Secondary structures of plus and minus strands of the initial RNA and the selected (final) product after 80 generations of exponential growth during an automated evolution experiment in vitro in the presence of ribonuclease A as selection pressure. The dots indicate cleavage sites for RNaseA. The palindromic sequence motive CUAG in the loop region of all strands is required for the recognition by Qβ-replicase.

creased, triggering the transfer to the next increased concentration of RNaseA (Fig. 5).

Apparently the system continually selected mutants that survived under the given conditions. After 24 transfers, corresponding to about 80 generations, the RNaseA concentration reached a level that is lethal for the wild-type. In our experiments, which covered about 80 transfers (280 generations), we observed reproducible RNA replication up to *3.5 ng/µl* RNaseA. An improved fluorescence-based technique for direct RNA sequencing [29] was used to investigate the molecular events underlying the adaptation process. The result of this analysis is shown in Fig. 6. What we expected to find was a shortening of the length of the initial RNA in order to accelerate the rate of synthesis, and this was exactly what we found. The length of the initial RNA was shortened from 87 bases to a final product of 65 bases. However, in addition to this, many pyrimidines were substituted by purines in one of the single strands, the so-called plus-strand. This was a surprise because the RNA amplification is plus-minus-based; that means that one strand codes for the complementary strand, and vice versa. Consequently, the substitution of a pyrimidine by a purine in one strand inevitably causes the appearance of a pyrimidine in the complementary minus-strand. However, the plus-strand had lost almost all cleavage sites except two positions; these two sites are known to be indispensable for the recognition of these types of RNA molecules by the replicase. The reason for this cannot be explained by different folding patterns for plus- and minus-strand because both strands have very similar structures. It should be mentioned that 24 nucleotides of the RNaseA-resistant variant form a palindromic stem; hence, 36% of the sequence are identical in both strands. The final result was an asymmetric enrichment in favor of the (more resistant) plus-strand. Kinetic studies on isolated single strands using radioactive labeling techniques, re-

vealed about a hundred times higher template activity for the minus-strand. Two major advantages arise from this evolutionary strategy. First, the resistant plus-strand is present at much higher concentrations than the ribonuclease-sensitive minus-strand. And second, the minus-strand ($M_r \approx 22$ kdaltons) is permanently protected by the polymerase ($M_r \approx 215$ kdaltons) which avoids the degradation of the strand by the ribonuclease ($M_r \approx 65$ kdaltons).

The serial-transfer machine represents a powerful tool for studying evolutionary dynamics. It provides a possibility to control experimental conditions and to perform evolution experiments in a reproducible way. Future experiments with variable error rates for the broadening of the mutant distribution of a quasispecies are needed in order to find optimal conditions for accelerated evolution [14]. These investigations will give insight into the principles of molecular evolution and will help us to develop evolutionary strategies.

3 The multichannel machine

3.1. Experimental setup

The multichannel machine was constructed to perform amplification experiments with a large array of samples. This machine has a transportation unit capable of moving a sample carrier to different temperature stations (Fig. 7). All temperature stations are equipped with lids to enclose the sample carrier in a homogeneous temperature bath. Due to the negligible thickness of the sample carrier (5mm) in comparison to its extension (443mm x 259mm), temperature inhomogeneities at the edges are avoided [24,25]. For non-isothermal amplification reactions, i.e. PCR [30,31,32,33,34], all temperature stations can be adjusted according to the experimentator's requirements. A glass fiber matrix is integrated into the lid of one of the three thermoblocks permitting optical communication with all 960 samples. For every sample, one fiber leads to the excitation light source, whereas another fiber is connected to the detector. An argon-ion laser beam (Lexel Laser) with an output power of 0.5W at 514 nm and a diameter of 1.3

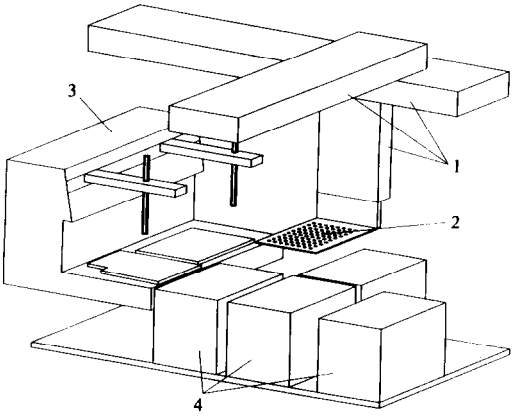

Fig. 7: Schematic of the 960-channel machine without the detection unit; the transportation unit (1) moves the sample carrier (2) between the pipetting (3) and the temperature stations (4).

mm is widened into a diameter of 57 mm and its gaussian intensity profile is rendered homogeneous before the light is coupled into the excitation branch with the 960 glass fibers. The fibers of the branch then diverge into a matrix to illuminate the array of samples. A second branch of fibers (emission branch) is likewise connected to the sample array in order to lead the fluorescence signal to a CCD camera (Photometrics). The emitted light passes through a combination of an interference filter and a cut-off filter (550nm) to block the excitation line. The signal recorded by the CCD camera is stored in a VME data bus system (Eltec Electronic) which also controls the processing of samples. To avoid tedious pipetting procedures, an automatic pipetting station is integrated, which can likewise be addressed by the central command structure of the VME data bus system. In any large-scale amplification experiment cross-contamination must be completely avoided. Moreover, PCR requires extreme processing temperatures up to 95°C. Therefore, the samples must be contained in chambers which stay sealed throughout the experiment. They must be resistant to extreme temperatures and must be optically transparent to allow sensitive detection of fluorescence. In addition, the chamber material must not influence the biochemical solution. The requirements were met by using a special plastic material made of polycarbonate which can be extruded and sealed in a device

developed in the workshop of our Max-Planck-Institute. Every foil contains 96 reaction chambers for experimental purpose. Experiments showed that cross-contamination of the samples is completely avoided.

3.2. Experimental procedure

Different self-replicating systems were used for investigating the multichannel apparatus: RNA replication by Qß replicase, the self-sustained sequence-replication reaction (3SR) and PCR. In all experimental procedures every aliquot contained 30 µl of solution enclosed in a plastic reaction chamber.

Conditions for Qß amplification were chosen as follows: The conditions of the reaction mixture and the buffer were as described in [16]. 15 µM EtBr was used to detect the RNA. The initial concentration of the template RNA MNV-11 was approximately 10^7 molecules per 100µl. Template and Qß replicase were added at 4°C to the reaction mixture. Filling the 192 aliquots was done at the same temperature. The reaction was initiated by moving the sample carrier to the 37°C position for amplification and on-line detection. An image was taken every 15.04 s. For 150 images the total recording time was 37min 36s.

The self-sustained sequence replication reaction (3SR) [19,35] was carried out as a second self-replicating system and detected on-line. A 76 nt RNA oligomer template, together with the appropriate primers, was used. Components and reaction conditions of this two-enzyme system (HIV-1 reverse transcriptase and T7 RNA polymerase) have been given in [22,23]. 3µM EtBr were added to the reaction solution for on-line detection. The reaction mixture was heated to 65°C, cooled to room temperature and then loaded into eight reaction vessels of the sample carrier for incubation at 37°C and on-line detection. Every 15.04s an image was taken. The recording time for 280 images was 70min 11s.

Amplification products of all aliquots were analyzed by polyacrylamide gel electrophoresis (PAGE).

3.3. Results

A linear regression analysis of the signal of different EtBr concentrations showed that the signal was linear for EtBr concentrations between 3×10^{-7} M and 10^{-4}M for all 960 channels.

In this parallel amplification experiment in the multichannel machine (Fig. 8), the doubling times for MNV-11 determined by fits of the exponential growth phase were between 20-25s in the different

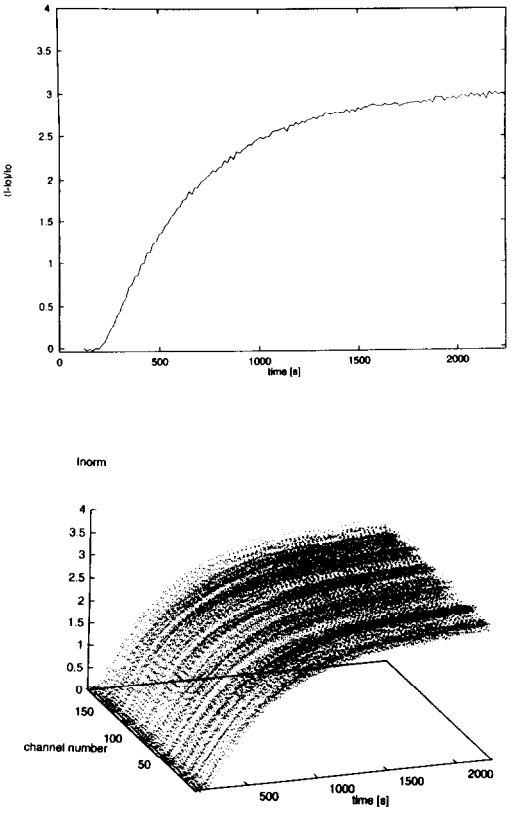

Fig. 8A: Normalized intensity (I-Io)/Io for one channel. The fluorescence intensity of the signal during the late lag time is the normalization constant Io: at that time (t = 2min) the temperature no longer influences the noise, and the specific amplification signal does not emerge from the background until t = 3min. The time course of the signal can be divided into the lag time (background signal only), the exponential growth phase, the linear phase and the saturation phase.

Fig. 8B: A 192-channel amplification of MNV-11 in the 960-channel machine. The normalized fluorescence intensities (I-Io)/Io for all 192 channels are as described in Figure 8A. The error between the signals from different channels for the last picture was 5.7 %. In the saturation phase, the error between the different channels did not exceed 7% for any single picture.

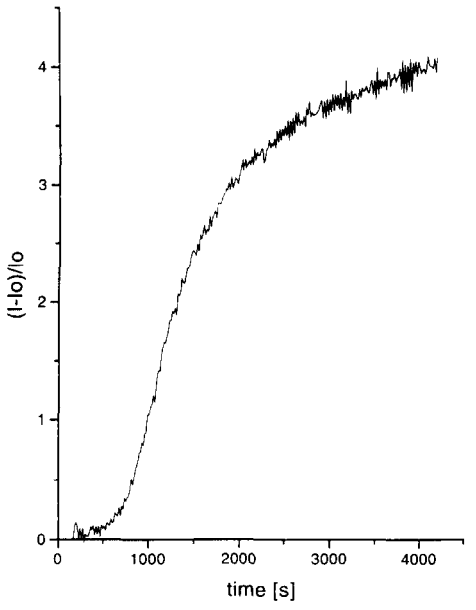

Fig. 9: 3SR amplification in the multichannel machine. Time development of the normalized fluorescence signal of one channel. The doubling times could be reproducibly determined for different channels because the exponential growth phase was long enough to gather a sufficient number of images; the doubling time in the exponential growth phase was 2min 40s.

channels; this is in good agreement with those determined for MNV-11 in the STM (cf. part 1) and by radioactive measurements (doubling time: 25s). As shown by PAGE analysis, the amplification had been performed properly. A successful 960-channel amplification has also been performed and monitored [26]. Furthermore, 3SR-amplification of all eight aliquots containing the reaction mixture could be monitored successfully on-line in the multichannel machine (Fig. 9). It is known that especially with 3SR one faces the problem that selfish RNA molecules emerge [36]. According to PAGE analysis this was not the case in the experiment performed under conditions of hermetically sealed reaction chambers. Successful results were also obtained when the 3SR amplification system was subjected to a serial-transfer experiment in the STM [23].

The non-isothermal amplification system PCR could also be processed successfully in the multichannel apparatus [26]. The technique for sealing the reaction chambers proved valuable in order to avoid evaporation completely. The time course of the fluorescence signal throughout the entire PCR-process could be monitored successfully. The results show that the presented machines are able to process and monitor on-line successfully different amplification systems for experimental purposes.

4. Conclusion

It is widely accepted that evolution experiments based on the serial-transfer principle are an indispensable tool for studying evolutionary dynamics at the molecular level. Initial studies described here in detail show that experiments of this kind can be performed successfully in an automated environment. We believe that employing instruments for large-scale amplification techniques and on-line detection for high-throughput screening stimulated by the serial-transfer principle will be applicable to a large variety of problems in molecular biology, such as creating molecules with novel properties (i.e. novel drugs or catalysts) by means of evolutionary biotechnology, be it new pharmacological effects or catalytic.

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