Comparison of *Escherichia coli* and rabbit reticulocyte ribosome display systems

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Abstract Ribosome display is a technology for library selection and simultaneous molecular evolution in vitro. We present here a comparison between an *optimized Escherichia coli* system and different rabbit reticulocyte ribosome display systems, optimized in a number of parameters, as a coupled eukaryotic system had been suggested to result in high enrichment factors [He and Taussig (1997) Nucleic Acids Res. 25, 5132–5134]. With all systems, antibody scFv fragments, complexed to the ribosomes and the corresponding mRNA, were enriched by binding to their cognate antigen and enrichment was always dependent on the absence of a stop codon and the presence of cognate antigen. However, the efficiency of the *E. coli* ribosome display system was 100-fold higher than an optimized uncoupled rabbit reticulocyte ribosome display system, with separate in vitro transcription and translation, which was in turn several-fold more efficient than the reported coupled system. Neither the *E. coli* nor the rabbit reticulocyte ribosome display system was dependent on the orientation of the domains of an antibody scFv fragment or on the spacer sequence. In summary, we could not detect any intrinsic advantage of using a eukaryotic translation system for ribosome display.

Key words: Ribosome display; In vitro translation; Library; Directed evolution

1. Introduction

Ribosome display has been shown to be a powerful technique for the screening and evolution of peptides and proteins (reviewed in [1,2]). It works by translating a library of peptides [3] or proteins [1,4–6] in vitro such that neither the polypeptide nor the mRNA is released from the translating ribosome. This is achieved by the absence of a stop codon and a buffer composition which stabilizes the ribosomal complexes. The translated polypeptide of interest is extended with a C-terminal tether, an encoded peptide sequence which allows the protein to emerge from the ribosomal tunnel and thus to fold independently, without clashing with the ribosome. The folded protein can thus be captured with a cognate ligand, and the attached corresponding genetic information (the mRNA) for this protein variant is thereby enriched in vitro.

Recently, we optimized an *Escherichia coli* ribosome display system for the display and selection of folded proteins [4] and successfully applied this technology to screen an scFv antibody library prepared from immunized mice [5]. A protocol for using ribosome display of folded proteins with a commercial rabbit reticulocyte lysate system, which employs coupled transcription/translation, was also presented [6] and had been suggested by the authors to give higher enrichment yields. A eukaryotic ribosome display system might at first seem attractive, since it is at least conceivable that its lower rate of translation, its lower RNase activity and the different molecular chaperones present might alter or improve the translation or folding efficiency of some proteins. However, the *E. coli* and rabbit reticulocyte ribosome display systems have not yet been compared side by side.

For this direct comparison, we developed a rabbit reticulocyte ribosome display system based on separate reactions for transcription and translation and further optimized it. Such an optimized system was compared to the *E. coli* ribosome display system and also to the coupled rabbit reticulocyte ribosome display system. It was found that for all constructs tested and under all conditions examined the *E. coli* system is the most efficient one.

2. Materials and methods

2.1. Model protein

As a model protein we used in all experiments a high-affinity fluorescein-binding scFv antibody fragment, called scFv12 (Fig. 1A), previously isolated from a murine library by using ribosome display with the same procedure as outlined recently [4]. This scFv fragment was cloned in the plasmid pAK200 [7] in V_L-linker-V_H orientation and designated pAK200scFv12. In some experiments we also used the hemagglutinin binding scFv antibody fragment 17/9 [8], designated scFvhag [4].

2.2. Preparation of scFv12 plasmids

To change the orientation of the domains, V_L and V_H were amplified separately from the pAK200scFv12 plasmid with *Taq* DNA polymerase (Gibco BRL). V_L was amplified either with oligonucleotides VLfor (5′-ATATATTCCGGAGGTGGTGGTTCTGGTGGTGGTGGTTCTGGCGG CGGCGGCTCCGGCGGTGGTGGATCCGACATCGTGATGAC-3′) and VLrev-Sfi (5′-ATATATGAATTCGGCCCCCGAGGCCGAACGTTTCAGCTCC-3′) or with VLfor and VLrev-BssH (5′-ATATATGCGCGCTTCAGCTCCAGCT-3′), and V_H with oligonucleotides VHfor (5′-ATATATCCATGGACTACAAAGAGGTGCAGCTGC-3′) and VHrev (5′-ATATATTCCGGAGGAGAC TGTGAG-3′). After digestion of the V_L- and the V_H-fragments with *Bsp*EI the two domains were ligated to yield two scFv12-V_H-linker-V_L fragments with either *Nco*I-*Sfi*I or *Nco*I-*Bsp*HII as flanking sites. Both fragments were gel-purified with QIAquick (Qiagen).

We used two different C-terminal spacers (tethers). The first, designated geneIII spacer, was derived from geneIII of filamentous phage

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Abbreviations: scFv, single-chain Fv fragment of an antibody in which the V_L domain is linked to the V_H domain by a linker; V_L, variable domain of the light chain; V_H, variable domain of the heavy chain

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M13 mp19 [4,5] and the second, designated kappa spacer, was derived from the kappa-C_L domain of an antibody [6]. The geneIII spacer was cut out from the pAK200scFv12 plasmid with *Sfi*I/*Hin*dIII, and the appropriate scFv12-V_H-linker-V_L fragment was digested with *Nco*I/*Sfi*I. The purified fragments were ligated to the *Nco*I/*Hin*dIII digested plasmid pTFT74 [9] yielding plasmid pTFT74scFv12V_H-linker-V_L-geneIII. The kappa spacer was PCR-amplified from plasmid pComb VH/κ, containing the anti-progesterone antibody DB3 (a kind gift of Dr. M. Taussig, Babraham Institute, Cambridge, UK) with the oligonucleotides KAPPAfor (5′-ATATATGCGCGCTGATGCTGCAC-3′) and D1 [6]. The resulting PCR product was digested with *Bsp*HII/*Bam*HI and the appropriate scFv12-V_H-linker-V_L fragment was cut with *Nco*I/*Bsp*HII. The purified fragments were ligated to the *Nco*I/*Bam*HI digested plasmid pTFT74 [9] yielding plasmid pTFT74scFv12V_H-linker-V_L-kappa. All constructs were verified by sequencing.

2.3. Other methods

Northern blot analysis and SDS-PAGE were performed as described [4].

2.4. Ribosome display based on E. coli S30 extracts

2.4.1. DNA constructs. Ribosome display constructs were prepared by PCR in two steps as described [5], using plasmids pAK200scFv12, PTFT74scFv12V_H-linker-V_L-geneIII, pTFT74scFv12V_H-linker-V_L-kappa or an fd phage displaying the scFv 17/9 [8,10] as templates. For the constructs with the geneIII-derived spacer primer T3te [5] was replaced by primer T5te (5′-CCGCAC-ACCAGTAAGGTGTGCGGTATCACCAGTAGCACC-3′) which anneals further downstream in geneIII, and for the constructs with the kappa-derived spacer primer T3te was replaced by primer D1 [6], which anneals at the 3′-end of the kappa domain. PCR products were directly used for in vitro transcription.

2.4.2. In vitro translation and selection. *E. coli* S-30 extracts were prepared as in [11]. Translations were carried out as described [5], always in the absence of DTT, with the following modifications. Vanadyl ribonucleoside complexes and ammonium acetate were omitted in the translation reaction and 100 mM potassium acetate was replaced by 200 mM potassium glutamate as described in Section 3.2. Affinity selections were carried out with immobilized ligand on a polystyrene surface or in solution, followed by capturing with magnetic beads. Surface panning was carried as described previously [5] with the following modifications. Microtiter plates were coated with transferrin-FITC overnight and then blocked for 2 h with 4% skimmed milk in PBS. For controls, plates were only blocked with milk without any prior coating. Selection in solution was performed by adding fluorescein-biotin (Sigma) (final concentration of 50 nM) to the translation mixture, after it had been diluted 5-fold with washing buffer (50 mM Tris-Ac pH 7.5, 150 mM NaCl, 50 mM $Mg(Ac)_2$, 2.5 mg/ml heparin and 0.1% Tween-20). After 1 h of end-over-end rotation on ice 20 μl of streptavidin-coated magnetic beads (Boehringer Mannheim), previously washed three times in washing buffer, were added and the mixture was end-over-end rotated for 10 min on ice. Washing and elution steps were identical to surface selection. After affinity selection, RNA was eluted with elution buffer (20 mM EDTA, 50 mM Tris-Ac pH 7.4, 150 mM NaCl) and isolated with the RNA purification kit (Boehringer Mannheim), including a 10 min DNase digestion of each RNA sample.

2.5. Ribosome display based on rabbit reticulocyte lysate

2.5.1. DNA constructs. Ribosome display constructs were prepared by two subsequent PCR reactions. First, we used the sense oligonucleotide EN (5′-AGACCACAACGGTTTCCCTTGCTTGT-TCTTTTTGCAGAAGCTCAGAATAAACGCTCAACTTTGGCA-GATCTACCATGGACTACAAAGA-3′), containing the translational enhancer of the *Xenopus laevis* β-globin gene and an optimal Kozak sequence [12] and either T5te or D1 [6] as an antisense primer (depending on the spacer sequence used). The second PCR introduced the T7 promoter at the 5′ end with oligo T7B [5], while using the same antisense primers. PCR products were directly used for either in vitro transcription or coupled transcription/translation.

2.5.2. In vitro translation and affinity selection. Translation was either carried out as a separate step ('uncoupled') or coupled to transcription. For the uncoupled system we used the Flexi rabbit reticulocyte kit (Promega). We first optimized Mg^{2+} and K^+ concentrations with respect to translation yield. After in vitro translation with [^{35}S]methionine, aliquots of the translation reactions were separated by SDS-PAGE and the amount of synthesized protein was quantified by autoradiography. For ribosome display experiments, a 50 μl translation reaction contained as final amounts or concentrations 5 μg of mRNA, 20 μM of each amino acid, 0.8 mM MgAc, 40 mM KCl and 33 μl rabbit reticulocyte lysate. DTT was added to 2 mM final concentration only where indicated. After a 20 min incubation at 30°C the reaction was rapidly chilled by 8-fold dilution into ice-cold PBST with 5 mM $MgCl_2$. Selections were carried out as described above for the *E. coli* system, except that elution was achieved with PBS containing 20 mM EDTA.

For coupled transcription/translation reactions we used the TNT T7 kit (Promega), which contains 2 mM DTT. Here, a 50 μl reaction contained 40 μl master mix, 20 μM methionine and 1 μg of PCR product. Reactions were incubated at 30°C for 60 min and then treated as described for the uncoupled system.

3. Results and discussion

3.1. Construction of scFv fragments used for ribosome display

As our model system, we used a fluorescein binding scFv antibody fragment (scFv12) previously isolated by *E. coli* ri-

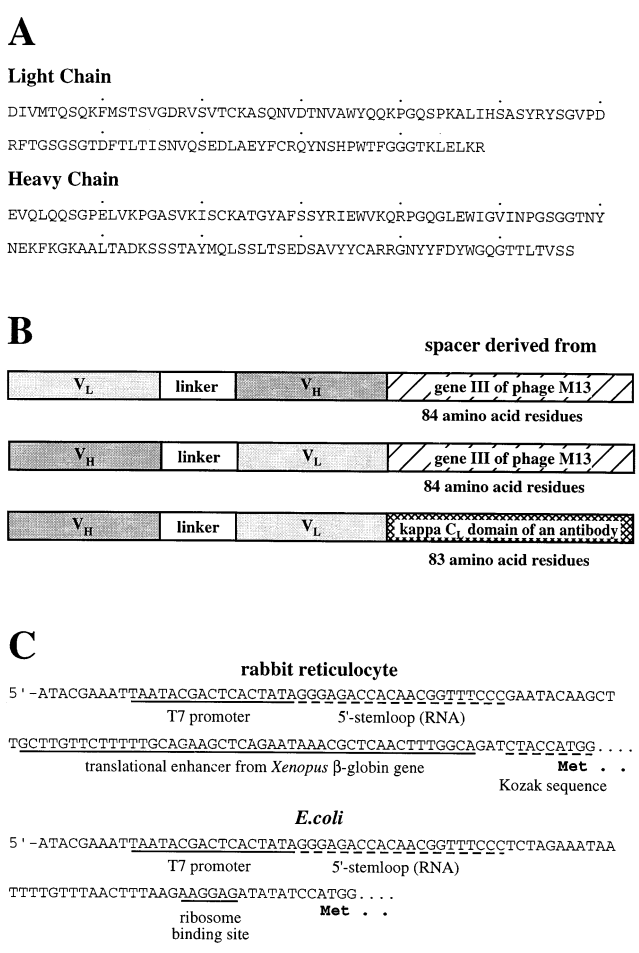

Fig. 1. A: Amino acid sequence of the scFv12 fragment. B: Schematic drawing of the scFv constructs used for both *E. coli* and rabbit reticulocyte ribosome display. 'V_L' and 'V_H' denote the variable domains of the light chain and the heavy chain of the scFv fragment, 'linker' the $(Gly_4Ser)_4$ linker and 'spacer' the part of the protein construct connecting the folded scFv to the tRNA in the ribosome. The number of amino acid residues refers to the spacer length. C: DNA upstream sequence used for the rabbit reticulocyte and the *E. coli* ribosome display constructs.

bosome display from a library prepared from immunized mice (J. Hanes and A. Plückthun, unpublished results). We constructed scFv fragments either in the V_L-linker-V_H or V_H-linker-V_L orientation, where the linker consisted of $(Gly_4Ser)_4$. In order for the scFv fragments to fold outside the putative ribosomal tunnel, we fused them to a spacer, a polypeptide tether connecting the scFv to the tRNA in the ribosome. Two different spacers of similar length were used. One was derived from geneIII of filamentous phage M13 (84 aa [7]) and the other one from the constant domain of the kappa light chain of an antibody (83 aa [6]). The constructs are schematically shown in Fig. 1B. On the DNA level the ribosome display constructs contained a T7 promoter and either a prokaryotic ribosome binding site for the *E. coli* system [4] or a translational enhancer from the *Xenopus laevis* β-globin gene and an optimal Kozak sequence for the rabbit reticulocyte system [12] (Fig. 1C).

3.2. Further improvement of the E. coli ribosome display system

Generally, a ribosome display system can be characterized by the efficiency of the system or by the enrichment factor. As the efficiency we define the amount of mRNA isolated from the ribosomal complexes bound to the cognate antigen, and as the enrichment factor we define the ratio of mRNA isolated from the ribosomal complexes bound to the cognate and to a non-cognate surface. If such mRNA is reverse transcribed and amplified by PCR, the efficiency of the system or the enrichment factor can be estimated from the ratio of intensities of the PCR bands.

To achieve the maximal efficiency of the *E. coli* ribosome display system, in vitro translation has to be optimized for the concentration of magnesium and potassium ions, the concentration of extract itself and the translation time for each batch of *E. coli* extract. As a source of potassium ions for *E. coli* in vitro translation systems potassium acetate has been used primarily (e.g. [4,11,13]). However, potassium glutamate was also used [14,15]. Recently, it was shown that the replacement of potassium acetate by potassium glutamate in a *Salmonella typhimurium* in vitro translation system increased the translation efficiency up to 8-fold [16]. Glutamate, which is required to maintain the steady-state K^+ pool in many Gram-negative organisms (e.g. [17,18]) and which also accumulates

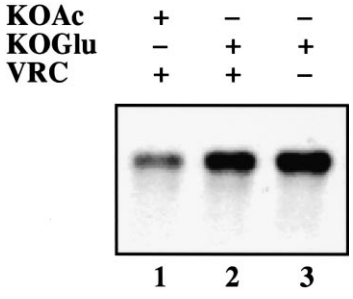

Fig. 2. Effect of additives on the efficiency of the *E. coli* ribosome display. The mRNA of the scFv12-V_L-linker-V_H-geneIII construct was used for one cycle of ribosome display as described in the experimental protocol in the absence of DTT. When indicated, 100 mM potassium acetate (KOAc), 200 mM potassium glutamate (KOGlu) or 0.5 mg/ml vanadyl ribonucleoside complexes (VRC) were included in the translation. After affinity selection of the ribosome complexes, mRNAs were isolated and analyzed by Northern hybridization.

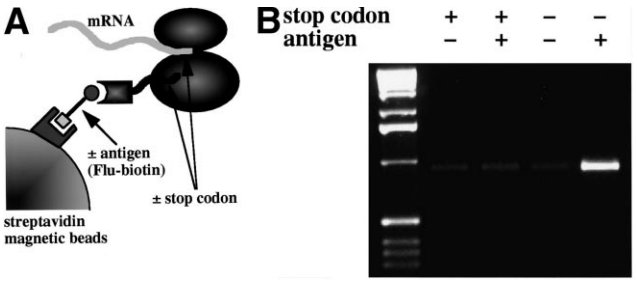

Fig. 3. Affinity selection in rabbit reticulocyte ribosome display is antigen-dependent and requires the absence of a stop codon. Two almost identical scFv12-V_L-linker-V_H-geneIII constructs (Fig. 1B) differing only in the presence (+) or absence (-) of a stop codon after the V_H domain were used in this experiment. A: Schematic drawing of the experiment. 'Flu-biotin' denotes a fluorescein-biotin conjugate (Sigma). B: After in vitro translation in the absence of DTT, the samples were diluted 4-fold with ice-cold PBST containing 5 mM $MgCl_2$. Affinity selection was carried out in solution in the presence (+) or absence (−) of the antigen fluorescein-biotin. After affinity selection, mRNA was isolated, reverse transcribed, amplified by PCR and analyzed by agarose electrophoresis.

in response to osmotic stress [19], has been considered to be the physiologically most relevant anion. We investigated the influence of potassium glutamate in the *E. coli* ribosome display system. We found that its optimal concentration was 200 mM, and it improved the efficiency of the system several-fold (Fig. 2, lanes 1 and 2).

We reported that the presence of the RNase transition state inhibitor vanadyl ribonucleoside complexes (VRC) during in vitro translation improved the efficiency of the *E. coli* ribosome display system, although the efficiency of translation itself was slightly decreased [4]. It is known that *E. coli* extract contains a high RNase activity, and therefore this improvement was presumably due to the inhibition of RNases present in the system. However, we observed that VRC could be omitted, if the translation mixture was rapidly chilled with ice-cold washing buffer immediately after the translation, the whole affinity selection was performed on ice, and all materials used for the affinity selection (e.g. pipette tips, microtiter plates) were also kept ice-cold. Omitting VRC while meticulously keeping all materials ice-cold during affinity selection resulted in a slightly improved efficiency of the *E. coli* ribosome display system (Fig. 2, lanes 2 and 3).

3.3. Optimization of the uncoupled rabbit reticulocyte system and its comparison to the coupled one

We developed a rabbit reticulocyte ribosome display system using the Flexi rabbit reticulocyte kit (Promega), where ion concentrations can be individually optimized and DTT can be omitted, which interferes with disulfide bond formation of proteins. First, we prepared RNA by in vitro transcription, and after its purification this RNA was used for in vitro translation in the Flexi system. By systematic experiments, the optimal conditions for the in vitro translation were found to be 20 min at 30°C (data not shown) and the optimal Mg^{2+} and K^+ concentrations were established (Section 2.5). In the *E. coli* ribosome display system, after translation, the mixture is rapidly chilled and diluted with ice-cold washing buffer containing 50 mM magnesium acetate [4], which presumably stabilizes ribosomal complexes. However, we found that for the rabbit reticulocyte system a more suitable 'washing buffer'

is PBST containing only 5 mM $MgCl_2$. We observed that unspecific binding significantly increases when using buffers containing 50 mM Mg^{2+} (see below). Furthermore, we investigated the optimal amount of input mRNA and found that 0.1 μg mRNA/μl of in vitro translation gave both the best enrichment factor and the highest efficiency. If lower amounts of mRNA were used [6] for the translation (10–10 000-fold lower), the amounts of affinity-selected ribosomal complexes decreased proportionally to the amount of input mRNA used, but unspecific binding decreased much less, resulting in lower enrichment factors (data not shown).

To demonstrate specificity of enrichment in the rabbit reticulocyte ribosome display system we translated in vitro almost identical mRNAs of scFv12, differing only in the absence or the presence of a stop codon situated directly behind the scFv coding sequence. After one round of ribosome display, where the affinity selection was performed in the presence of 5 mM Mg^{2+}, we analyzed the PCR products (Fig. 3). The enrichment was antigen-dependent, demonstrating correct protein folding, and required the absence of a stop codon, demonstrating the integrity of the ribosomal complexes containing mRNA and the synthesized protein.

We directly compared enrichments in the rabbit reticulocyte ribosome display system using affinity selections carried out either in solution or with immobilized antigen, performed in the presence of 5 or 50 mM Mg^{2+} (data not shown). From these experiments we could conclude that the efficiency of the system and the enrichment factor was independent of whether the selection was carried out in solution or on a microtiter well surface in the presence of 5 mM Mg^{2+}. If affinity selection was performed in the presence of 50 mM Mg^{2+} the rabbit reticulocyte ribosome display was no longer dependent on the absence of a stop codon (data not shown), indicating nonspecific binding when surface panning was used. This was not observed for the *E. coli* system [4]. If magnesium was completely omitted during affinity selection [6], the results were irreproducible, and the efficiency of the system was definitely not higher than in the presence of 5 mM Mg^{2+} (data

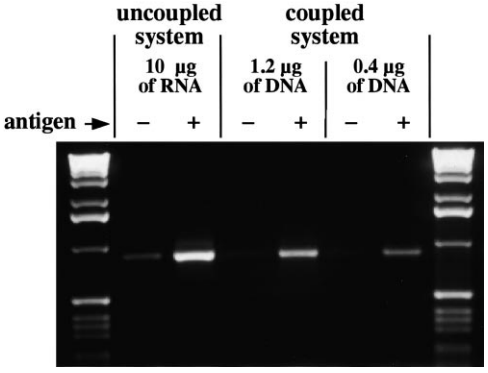

Fig. 4. Comparison of the coupled and the uncoupled rabbit reticulocyte system. The scFv12-V_L-linker-V_H-geneIII construct was used in all experiments. Both experiments, the in vitro translation using the Flexi rabbit reticulocyte kit and the coupled in vitro transcription-translation using the TNT T7 kit, were performed in the presence of 2 mM DTT. After in vitro translation, the samples were diluted 4-fold with ice-cold PBST containing 5 mM $MgCl_2$ and applied for affinity selection of scFv12 ribosomal complexes using either transferrin-FITC (+) or milk (−) coated microtiter plate wells. After affinity selection, mRNA was isolated, reverse transcribed, amplified by PCR and analyzed by agarose electrophoresis.

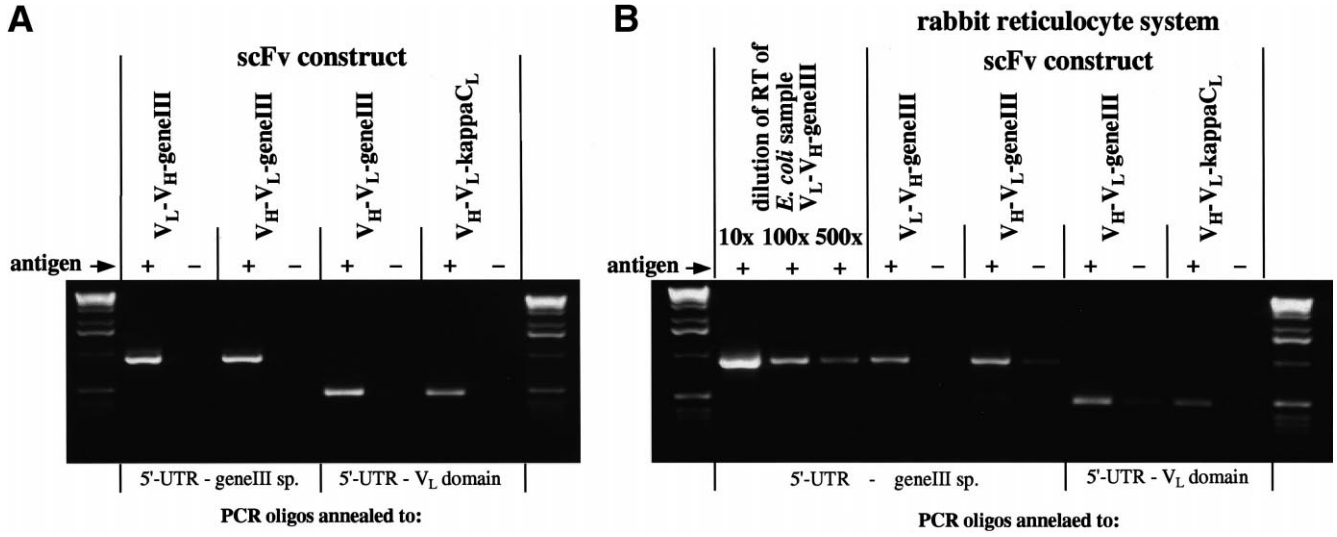

Fig. 5. Comparison of the *E. coli* and the rabbit reticulocyte ribosome display systems. A: Influence of the construct in the *E. coli* ribosome display system. Enrichments of the ribosomal complexes translated from different scFv12 mRNA constructs are compared. mRNA of a defined molecule (scFv12) was used for ribosome display in each experiment. After in vitro translation in the absence of DTT, the samples were diluted 4-fold with ice-cold washing buffer containing 50 mM $Mg(Ac)_2$ [4] and centrifuged for 5 min at 4°C at 10 000×*g*. The supernatants were used for affinity selection of scFv12 ribosomal complexes using either transferrin-FITC (+) or milk (−) coated microtiter plate wells. After affinity selection, mRNA was isolated, reverse transcribed, amplified by PCR and analyzed by agarose electrophoresis. B: Influence of the construct in the rabbit reticulocyte ribosome display system. Enrichments of the ribosomal complexes translated from different scFv12 mRNA constructs by the rabbit reticulocyte system are compared to the *E. coli* system. mRNA of a defined molecule (scFv12) was used for ribosome display in each experiment. After in vitro translation in the absence of DTT, the samples were diluted 4-fold with ice-cold PBST containing 5 mM $MgCl_2$ (rabbit reticulocyte system) or ice-cold washing buffer containing 50 mM $Mg(Ac)_2$ (*E. coli* system) and centrifuged for 5 min at 4°C at 10 000×*g*. The supernatants were applied for affinity selection of scFv12 ribosomal complexes using either transferrin-FITC (+) or milk (−) coated microtiter plate wells. After affinity selection, mRNA was isolated, reverse transcribed, amplified by PCR and analyzed by agarose electrophoresis.

not shown). Thus, we carried out all other experiments with antigen immobilized to microtiter wells and affinity selections were performed in 5 mM Mg^{2+}.

We also compared the ribosome display system based on coupled transcription-translation with the uncoupled ribosome display system, using scFv12 in the format V_L-linker-V_H-geneIII. This particular scFv folds well even in the presence of 2 mM DTT, which reduces the amount of functional protein only about 2-fold, as judged from the intensity of the PCR band after one round of ribosome display (data not shown). We thus performed in vitro translations, in identical volumes for both the coupled and the uncoupled systems in the presence of 2 mM DTT, according to the optimized protocols (uncoupled system) or the procedure recommended by the supplier (coupled system). For the coupled system we used 0.4 μg and 1.2 μg of purified PCR product for a 50 μl reaction, close to the maximal recommended template concentration for the TNT T7 kit. Affinity selections were performed in PBST with 5 mM $MgCl_2$. After one cycle of ribosome display, we analyzed the PCR products (Fig. 4). In both systems, we observed a clear enrichment dependent on the presence of antigen, demonstrating correct protein folding. However, judging from the intensity of the PCR bands, the enrichment yield was several-fold higher when using the uncoupled rabbit reticulocyte system.

3.4. Influence of domain orientation and spacer sequence on the efficiency of ribosome display

Next, we investigated the influence of the domain orientation of the scFv fragment for the *E. coli* and the rabbit reticulocyte ribosome display systems. We analyzed both the V_L-linker-V_H and the V_H-linker-V_L orientation, fused either to the geneIII-spacer or to the kappa constant domain of an antibody serving as a spacer. The latter spacer was used in a coupled transcription/translation with a rabbit reticulocyte ribosome display system [6]. In this paper, a very high enrichment factor was reported and, therefore, we were interested if this particular spacer or domain arrangement might have an effect on this. For the analysis, we used our optimized uncoupled system, as it had the highest efficiency. We observed that the orientation of the domains in the scFv fragment had no influence on the efficiency of either the *E. coli* or the rabbit reticulocyte ribosome display systems (Fig. 5). Furthermore, both the *E. coli* and the rabbit reticulocyte ribosome display systems showed slightly higher efficiency with the geneIII-derived spacer constructs than with the constant kappa domain (Fig. 5).

3.5. Comparison of enrichment factor and efficiency of E. coli and rabbit reticulocyte ribosome display systems

We never observed the reported enrichment factor of 10^4–10^5 per cycle [6], either for the uncoupled (Fig. 5B) or for the coupled rabbit reticulocyte system (Fig. 4) or for the *E. coli* system (Fig. 5A), even though we systematically evaluated a large number of experimental parameters. In our experiments we estimated the enrichment factor of the coupled and the uncoupled rabbit reticulocyte ribosome display system by comparing the intensity of the PCR products obtained from samples enriched on cognate antigen or control surface immobilized on microtiter wells. The enrichment factor of the rabbit reticulocyte system was lower than that observed for the *E. coli* system (Fig. 5).

We then compared the efficiency of the *E. coli* and rabbit reticulocyte ribosome display systems. One cycle of ribosome display in these two systems was performed according to the optimized protocols. We used scFv12-V_L-linker-V_H-geneIII mRNAs which differed only in the 5′-upstream region according to the translation system (Fig. 1C). In both systems we used the same volume and the same amount of mRNA for in vitro translations. After affinity selection mRNA from bound ribosomal complexes was isolated and reverse transcribed. The reverse transcript from the *E. coli* system was diluted 10-, 100-, and 500-fold and amplified with the same conditions as the undiluted reverse transcript from rabbit reticulocyte system by PCR, using the same oligonucleotides. From the comparison of intensities of the resulting PCR bands (Fig. 5B) we could conclude that the efficiency of the rabbit reticulocyte ribosome display system was about 100-fold lower than the efficiency of the *E. coli* system. This means that from the same amount of input mRNA in the rabbit reticulocyte ribosome display system 100-fold less ribosomal complexes, containing the full-length mRNA and the folded protein, are formed and/or recovered than in the *E. coli* system. As a consequence, the diversity of a library which can be efficiently screened by the rabbit reticulocyte ribosome display system would be about 100-fold lower than with the *E. coli* system.

In the next experiment, we estimated the enrichment factor of scFv12 ribosomal complexes from a binary mixture. scFv12-V_L-linker-V_H-geneIII mRNA was mixed with scFvhag-V_L-linker-V_H-geneIII mRNA in a ratio of 1:250 for the *E. coli* system or 1:1 and 1:20 for the rabbit reticulocyte system and selected in one round of optimized ribosome display, using uncoupled in vitro translations. After affinity selection, on either transferrin-FITC or milk coated microtiter plate wells, we analyzed the PCR products. The scFvhag construct is slightly larger than the scFv12 one, mainly because of a slightly longer geneIII spacer. Therefore, we could estimate the enrichment of ribosome display systems from agarose gel electrophoresis of the PCR products. By comparing the intensities of the lower band (scFv12) to the higher band (scFvhag) we could deduce the enrichment factor. If affinity selections

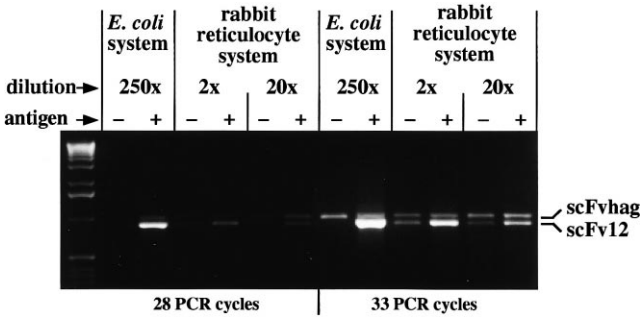

Fig. 6. Enrichment of scFv12 ribosomal complexes from a binary mixture by ribosome display. mRNA of scFv12-V_L-linker-V_H-geneIII was mixed with scFvhag-V_L-linker-V_H-geneIII mRNA in ratios of 1:250 for the *E. coli* system or 1:1 and 1:20 for the rabbit reticulocyte system and used for one cycle of ribosome display in the absence of DTT. The affinity selection of scFv12 ribosomal complexes was performed as described in Fig. 5 using either transferrin-FITC (+) or milk (−) coated microtiter plate wells. After affinity selection, mRNA was isolated, reverse transcribed, amplified by PCR and analyzed by agarose electrophoresis. 'Dilution' denotes the *n*-fold dilution of the scFv12 mRNA with scFvhag mRNA used for the ribosome display experiment.

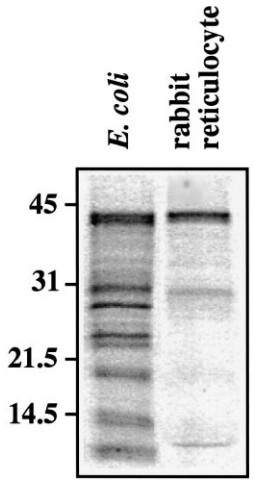

Fig. 7. Comparison of synthesized protein products in *E. coli* and rabbit reticulocyte translation systems. In vitro translation was performed in the absence of DTT using [^{14}C]leucine and scFv12-V_L-linker-V_H-geneIII mRNA. An autoradiograph of an SDS-PAGE of translation products is shown.

were carried out on control surfaces, no change from the input ratio of scFv12/scFvhag mRNAs was observed (Fig. 6, lanes '−'). If affinity selections were carried out with immobilized fluorescein, we observed a clear enrichment in both *E. coli* and rabbit reticulocyte systems (Fig. 6). The enrichment factor for the *E. coli* system was at least 10-fold higher than the one for the rabbit reticulocyte system. While enrichment of scFv12 ribosomal complexes was more than 250-fold after one cycle ribosome display in the *E. coli* system, in the rabbit reticulocyte system the scFv12 ribosomal complexes could be enriched only about 20-fold (Fig. 6).

Furthermore, we compared the amount of protein produced in the *E. coli* and the rabbit reticulocyte ribosome display systems. We performed in vitro translations using scFv12-V_L-linker-V_H-geneIII mRNA with ^{14}C-leucine, and protein products were analyzed by SDS-PAGE (Fig. 7) and quantified with a phosphorimager. From equal volumes of translation reaction, the amount of full-length proteins produced in the *E. coli* system was 30% higher, compared to the rabbit reticulocyte system which, however, does not explain by itself the difference in efficiency between these two systems. The 100-fold higher efficiency of the *E. coli* ribosome display system is most likely due to the higher stability of *E. coli* ribosomal complexes, in conjunction with their lower non-specific binding.

3.6. Conclusions

We conclude from the direct side-by-side comparison that there are no indications that the rabbit reticulocyte ribosome display system has any general advantage over the *E. coli* system, as had been suggested previously [6]. Rather, the poorer enrichment rate and the lower efficiency of the rabbit reticulocyte ribosome display constitute significant drawbacks of this system. In the commercial coupled rabbit reticulocyte system [6] the presence of DTT is the third limitation for disulfide containing proteins. The ability to fold in 2 mM DTT is protein-dependent, and we have found an antibody which folds almost as well in the presence of 2 mM DTT as in its absence presumably because of its higher stability. However, there are other antibodies which give a very low enrichment yield in the presence of 2 mM DTT (e.g. scFvhag, Jermutus et al., unpublished data), while they can be efficiently enriched in its absence. From the data presented here there is no evidence either for improved folding or for better translation yield of scFv fragments of an antibody in a rabbit reticulocyte translation system. However, it might be possible that the expression level and folding of other proteins could vary between the *E. coli* and eukaryotic ribosome display systems.

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