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Protein–Ribosome–mRNA Display: Affinity Isolation of Enzyme–Ribosome–mRNA Complexes and cDNA Cloning in a Single-Tube Reaction

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An enzyme–ribosome–mRNA complex was specifically purified by binding to the immobilized enzyme substrate and the cDNA was cloned in a single-tube reaction by one-step reverse transcription–PCR. The ganglioside GM3, used by sialyltransferase II (ST-II) as a substrate, was coated on a 96-well microtiter plate and ST-II was *in vitro* transcribed and translated from a cDNA library. The isolation of an enzyme-specific protein–ribosome (PRIME) complex was achieved with as little as 0.1 ng ST-II-specific cDNA in 5 μg of a total plasmid preparation or with the cDNA prepared from sublibraries previously inoculated at a density of 2000 clones/culture well. The affinity purification of the PRIME complex was highly specific for GM3 and did not result in cDNA amplification when a different ganglioside (GM1) was used for coating of the microtiter plate. The amplified cDNA was used for cloning or a second round of ribosome display, providing a fast analysis of enzyme affinity to multiple substrates. PRIME display can be used for host-free cDNA cloning from mRNA or cDNA libraries and for binding site mapping of the *in vitro* translated protein. The use of a single-tube reaction in ligand-coated microtiter plates indicates the versatility of PRIME display for cDNA cloning by automated procedures.

Key Words: **ribosome display; cDNA cloning; functional genomics; glycosyltransferase; ganglioside.**

Several methods have been established to isolate and clone a specific cDNA by binding of the encoded protein to an antibody after expression on the cell surface of viruses, bacteria, or eukaryotic cells (e.g., phage display, yeast surface display) (1, 2). These methods, however, require intensive cell cultivation, panning procedures for the isolation of a specific host cell, and cDNA preparation from the isolated cells for the analysis of the clone. Recently, immunoaffinity purification of a specific expression product was applied to peptides or proteins translated in a cell-free ribosomal system (3–5). The mRNA was recovered as ternary complex with the antibody and ribosome during *in vitro* translation, enabling reverse transcription and cDNA cloning of the isolated mRNA. So far, however, there is no study published reporting an application of ribosome display for isolation of proteins without the use of a specific antibody. In the present study, we describe a single-tube reaction for the isolation of a specific cDNA by functional binding of the encoded enzyme to its substrate during expression in a cell-free transcription/translation system. In this model system the cDNA of sialyltransferase II (ST-II),[1] an enzyme catalyzing the synthesis of the ganglioside GD3, was *in vitro* transcribed and translated, and the nascent enzyme was bound to its substrate GM3 previously coated onto the surface of the wells of a microtiter plate. Gangliosides are sialylated glycolipids of the cell membrane and critically involved in neuronal cell development (6). Accordingly, cloning of a variety of ganglioside-binding proteins is crucial for functional genomics of the nervous system.

MATERIALS AND METHODS

In Vitro Transcription/Translation on Coated Microtiter Plates

A series of wells of a microtiter plate (Immulon, Dynex Technology, Chantilly, VA) were coated with

[1] Abbreviations used: ST-II, sialyltransferase II; PBS, phosphate-buffered saline; PRIME, protein–ribosome–mRNA; RT-PCR, reverse transcription–PCR.

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GM3 by incubation of each well with 10 μg of GM3 in 50 μl of methanol. After the solvent was evaporated, the wells were washed three times with phosphate-buffered saline (PBS) to remove the excess GM3. Unspecific binding sites on the solid surface were blocked by incubation with 2% bovine serum albumin in PBS for 2 h. As a negative control, several wells were coated with GM1 which was not used by ST-II as a substrate. The wells were then washed three times with *in vitro* translation buffer (50 mM Tris/HCl, pH 8.0, 2 mM $MgCl_2$) and loaded with 50 μl of the reticulocyte (TNT)–*in vitro* transcription/translation incubation mixture prepared according to the manufacturer's instructions (Promega, Madison, WI). For analysis of the translation product the incubation mixture was supplemented with 2 μCi of [^{35}S]methionine (1200 Ci/mmol, ICN Biochemicals, Costa Mesa, CA). The transcription/translation reaction was initiated by addition of 0.1–1.0 ng of a plasmid vector (pCMV-Sport 2) containing the full-length cDNA of ST-II (1148 bp) under the control of a T7 promoter (7). For immunodetection of the translation product the cDNA was endowed with a C-terminal tag encoding the FLAG epitope. Optionally, the template was supplemented with 5 μg of plasmid DNA from a 15.5-day-old mouse embryo cDNA library constructed in the pCMV-Sport 2 vector (Gibco BRL, Gaithersburg, MD). In another experiment the cDNA library was first subdivided into twelve 96-well flat tissue culture plates at an inoculation density of 2000 clones/well. A plasmid preparation (5 μg DNA) of each sublibrary was directly used for *in vitro* transcription/translation without adding exogenous ST-II cDNA. The *in vitro* transcription/translation reaction proceeded for 60 min at 30°C. A 2-μl aliquot was taken from the reaction mixture in order to analyze the size of the translation product by SDS–gel electrophoresis and autoradiography or immunoblotting.

Affinity Purification of the Protein–Ribosome–mRNA (PRIME) Complex and First-Strand cDNA Synthesis

The affinity purification of the specific PRIME complex was achieved by removal of unbound translation product from the ternary complex containing the enzyme bound to its immobilized substrate. The wells were washed five times with 200 μl of *in vitro* translation buffer and the mRNA retained in the PRIME complex was used for first-strand cDNA synthesis. The PRIME complexes were incubated for 10 min at 70°C with 50 μl of first-strand synthesis buffer provided by the manufacturer of the cDNA synthesis kit (Gibco BRL) ensuring the dissociation of the mRNA. The reaction mixture was supplemented with 500 U Superscript II RT, 1 μl RNase inhibitor (1 U), and 10 pmol of an oligonucleotide primer complementary to the FLAG epitope sequence (5′tagtcgacttgtcatcgtcgtccttgtaatc3′) or a vector (pCMV-Sport 2)-specific sequence (5′ccgggtcgacccacgcgtcc3′ or 5′tcgaggatcctctagagc3′). The first-strand cDNA synthesis was carried out for 50 min at 42°C. The reaction was then stopped by heating at 70°C for 15 min and an aliquot of 10 μl was taken for protein and DNA analysis. Five microliters of the reaction mixture was applied to SDS–PAGE and the radiolabeled translation product was visualized by autoradiography.

PCR Amplification and cDNA Cloning

The presence of specific first-strand cDNA was verified by PCR amplification with two oligonucleotide primers located at positions −14 bp (5′acaccgagctgcgatgag3′) and 495 bp (5′tttgacggccacagccac3′) of the ST-II sequence using 4 μl of the first-strand reaction mixture as source for the template. Twenty microliters of the mixture was diluted 1:2 with deionized water and 10 pmol of the sense oligonucleotide primer located at position −14 bp and 10 pmol of the FLAG-specific antisense primer at position 1148 bp was added for PCR amplification of ST-II-specific full-length cDNA. Alternatively, first-strand cDNA synthesis and PCR amplification were achieved with a buffer system conditioned for both reverse transcriptase (Superscript II) and *Taq* polymerase (Platinum, Gibco BRL) to perform one-step reverse transcription (RT)-PCR. Optionally, the sense primer was endowed with the sequence of the T7-promoter region and an *Eco*RV restriction site (5′tactgcagtaatacgactcactatagggacaccgagctgcgatgag3′) for subsequent cloning and *in vitro* transcription of the PCR product. First-strand cDNA obtained from affinity-purified PRIME complex previously generated with a plasmid preparation from a sublibrary was amplified with a vector (pCMV-Sport 2)-specific primer (5′gggtcgacccacgcgtcc3′ or 5′gagggatcctctagagcgg3′) and the ST-II-specific sense primer endowed with the T7-promoter sequence. The PCR amplification reaction was performed using the following cycle conditions: 95°C for 1 min, followed by 35 cycles at 95°C for 30 s, 58°C for 45 s, and 68°C for 60 s; final extension was achieved at 72°C for 10 min. The amplified ST-II cDNA was used as a template for a second round of *in vitro* transcription/translation or for cloning into the pSVSport2 vector using the *Sma*I and *Sal*I restriction sites. A second round of ribosome display was achieved as described for the initial *in vitro* transcription/translation reaction using either the SP6- (PCR product inserted into pSVSport2) or the T7-promoter (PCR product itself or inserted into pSVSport2 or a T/A cloning vector (pGEM-T Easy, Promega)).

RESULTS AND DISCUSSION

ST-II was employed as a model protein since preliminary results demonstrated the activity of the *in vitro*

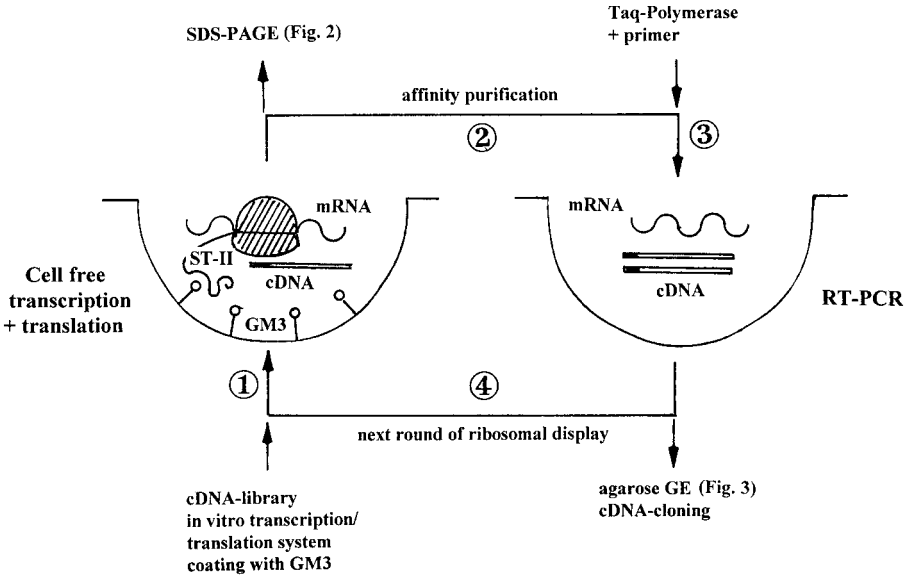

FIG. 1. Principle of the PRIME display cycle. The four-step procedure in a single-tube reaction is shown. Step 1, coupled *in vitro* transcription/translation reaction in a coated microtiter plate well; step 2, affinity purification of the translation product by binding to the immobilized ligand; step 3, one-step RT-PCR with the copurified mRNA used as a template; step 4, second round of PRIME display for binding to a different ligand.

translated enzyme toward its substrate GM3 (7). In addition, the application of GM3–agarose for affinity purification of ST-II from a rat brain homogenate and a low K_m value (50 μM) indicated the feasibility of GM3 affinity binding for isolation of the translation product (8, 9). The *in vitro* transcription/translation of ST-II (Fig. 1, step 1) was performed with various amounts of a plasmid containing the full-length cDNA of ST-II endowed with the sequence for the FLAG epitope. The sensitivity of the procedure was verified by the addition of an excess of cDNA prepared from a mouse library. The translation product was labeled with [^{35}S]methionine and analyzed by SDS–gel electrophoresis followed by autoradiography and immunostaining with an antibody against the FLAG epitope. As shown in Fig. 2, lane 1, the main product of the *in vitro* transcription/translation reaction was a polypeptide of 43 kDa corresponding to the molecular mass of ST-II. A comparison of the radiolabeled translation product (lane 1) with that immunostained with a FLAG-specific antibody (lane 5) revealed that full-length ST-II was synthesized as the major polypeptide. Figure 2, lane 2, shows that the translation product generated in the presence of a GM1 coating corresponded to that synthesized in the presence of GM3. However, it has been found previously that the activity of this enzyme is less than 30% of that obtained with GM3 coating (7). This observation is best explained by the assumption that only the ganglioside used as a substrate can stabilize the active protein conformation during the translation reaction.

The purification of a ternary ST-II–ribosome–mRNA complex was achieved by binding to GM3 immobilized on the surface of the microtiter plate wells followed by removal of unbound translation product (Fig. 1, step 2). Figure 2, lane 3, shows that at least 20% of the translation product detected before affinity purification could be recovered in the first-strand cDNA synthesis mixture (Fig. 1, step 3). As little as 0.1 ng of ST-II-specific cDNA in 5 μg of a total cDNA library resulted in *in vitro* translation of ST-II detectable by autoradiography after affinity purification (Fig. 2, lane 3). In contrast, with GM1

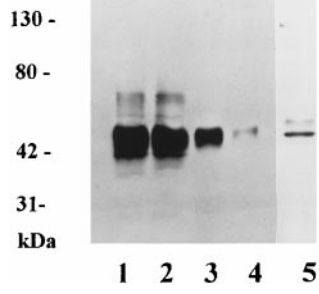

FIG. 2. Analysis of the *in vitro* translation product by SDS–PAGE. Aliquots were taken from the reaction mixtures in step 1 or 3 (Fig. 1) and the *in vitro* translated protein analyzed by SDS–PAGE followed by autoradiography or immunoblotting. Lanes 1–4, protein labeled with [^{35}S]methionine; lane 1, wells coated with GM3; lane 2, wells coated with GM1; lane 3, protein after affinity purification with immobilized GM3; lane 4, protein after affinity purification with immobilized GM1; lane 5, as in lane 3 but immunostaining with antibody against the FLAG epitope.

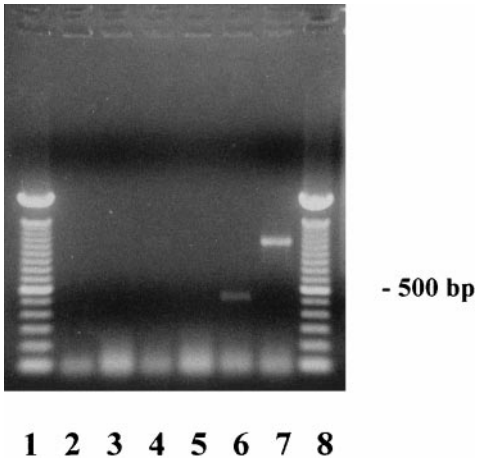

FIG. 3. Analysis of the RT-PCR amplification using purified PRIME complexes as substrates. Lanes 1 and 8, DNA standard; lanes 2–4, RT-PCRs with PRIME complexes isolated on GM1-coated wells; lane 2, PCR without previous reverse transcription (primer combination −14/495); lane 3, RT-PCR with primer combination −14/495; lane 4, RT-PCR with primer combination −14/1148; lanes 5–7, RT-PCRs with PRIME complexes isolated on GM3-coated wells; lane 5, PCR without previous reverse transcription (primer combination −14/495); lane 6, RT-PCR with primer combination −14/495; lane 7, RT-PCR with primer combination −14/1148.

used for affinity purification, only minute amounts of ST-II were detectable (lane 4), indicating the efficacy and specificity of the purification procedure. The presence of ST-II-specific first-strand cDNA was verified by PCR amplification with ST-II-specific oligonucleotides used as primers. As shown in Fig. 3, lanes 6 and 7, agarose gel electrophoresis of the PCR product followed by DNA staining with ethidium bromide revealed a single band at approximately 500 or 1150 bp, respectively, corresponding to the DNA fragment expected from the amplification of ST-II-specific cDNA. None or only trace amounts of amplification product could be detected using the first-strand synthesis mixture from the GM1-coated well (lanes 3 and 4), indicating that there was only negligible carryover or contamination with plasmid DNA during the affinity purification procedure. The amplification product of the first round of PRIME display endowed with a T7-promoter sequence was used for a second round of *in vitro* transcription/translation (Fig. 1, step 4), again yielding the 43-kDa ST-II polypeptide detectable by autoradiography or immunostaining.

ST-II was cloned from a subdivided cDNA library using affinity isolation of the respective PRIME complexes with no exogenous ST-II cDNA added prior to the *in vitro* transcription/translation reaction. In order to enhance the representation of endogenous ST-II mRNA the library was inoculated at a density of 2000 cDNA clones/well in twelve 96-well flat tissue culture dishes as described previously (7, 10). The plasmid preparation of each well was first screened with ST-II-specific primers (−14- and 1148-bp primers) and the DNA of 19 positive sublibraries containing full-length ST-II cDNA used for the cell-free protein synthesis reaction on GM3-coated microtiter plates. After isolation of the PRIME complexes RT-PCR was performed with an ST-II-specific sense primer endowed with a T7-promoter sequence and a vector-specific primer framing the cDNA insert. The main amplification product was identified as ST-II-specific full-length cDNA as verified by DNA sequencing. As shown in Fig. 4 these PCR products were used for *in vitro* transcription/translation and the ^{35}S-labeled polypeptides were analyzed by SDS gel electrophoresis and autoradiography. Full-length ST-II of 43 kDa was synthesized from 6 of 19 positive sublibraries (lanes 2–7) used for affinity isolation of the PRIME complexes. The smaller fragment found in lanes 6 and 7 may be a translation product initiated at an internal methionine used as start codon.

CONCLUSIONS

The method described here is an extension of the ribosome display technique recently introduced for the isolation of cDNA-encoding antibody species (3–5). The isolation of a variety of PRIME complexes depends on affinity binding of functional proteins. The cell-free synthesis of the active enzyme (ST-II) was achieved by *in vitro* translation in the presence of the coated substrate (GM3), an approach which is also expected to be applicable to other enzymes. Cloning of cDNA from a library can then be performed without any biological host and automated as a single-tube reaction by use of

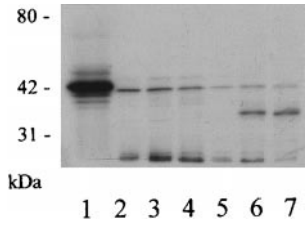

FIG. 4. *In vitro* translation of ST-II cloned from a subdivided cDNA library by PRIME display. A 15.5-day-old mouse embryo cDNA library was first divided into 1152 sublibraries on twelve 96-well flat tissue culture plates with 2000 clones/well. The plasmid DNA of each sublibrary was used for PCR with the primer combination −14/1148 for identification of ST-II-positive clones. The DNA of ST-II-positive sublibraries was then used for PRIME display with GM3-coated microtiter plates. RT-PCR was carried out with a vector-specific primer framing the insert DNA and an ST-II-specific sense primer introducing a T7-promoter sequence into the amplification product. The PCR product was used for *in vitro* translation in a reaction mixture supplemented with [^{35}S]methionine. Lane 1, PRIME display with control ST-II cDNA; lanes 2–7, PRIME display with six different ST-II-positive sublibraries.

microtiter plates with a substrate-coating for affinity purification of the PRIME complex followed by one-step RT-PCR.

Most recently, *in vitro* evolution of single-chain fragments of a particular antibody was achieved by selection of mutants in subsequent rounds of ribosome display in a stringent environment for antigen–antibody binding (4). The PRIME display described here may be used in a similar approach for selection of specific substrate binding sites from a library of *in vitro* generated mutants of enzymes or other binding proteins. It has been shown by computer-based sequence comparison that sialyltransferases contain two potential domains for binding to the ganglioside substrate (11). Coating of the microtiter plate wells with different gangliosides and PRIME display of a library constructed with cDNAs arising from *in vitro* mutagenesis of a particular sialyltransferase may thus facilitate the analysis of its binding site. We have shown the validity of this approach by successful display of wildtype ST-II. In future, we will use the PRIME display technique for cDNA cloning and functional genomics of sialyltransferases and other ganglioside binding proteins.

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