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Isolation of Qβ polymerase complexes containing mutant species of elongation factor Tu

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Abstract

The RNA genome of coliphage Qβ is replicated by a complex of four proteins, one of them being the translation elongation factor Tu. The role of EF-Tu in this RNA polymerase complex is still unclear, but the obligate presence of translationally functional EF-Tu in the cell hampers the use of conventional mutational analysis. Therefore, we designed a system based on affinity chromatography and could separate two types of complexes by placing an affinity tag on mutated EF-Tu species. Thus, we were able to show a direct link between the vital tRNA binding property of EF-Tu and polymerase activity.

Keywords: Bacteriophage Qβ; RNA polymerase; Elongation factor-Tu

1. Introduction

Bacteriophage Qβ is a plus-stranded RNA virus belonging to the family of the *leviviridae* and infecting *Escherichia coli*. The easy culturing with short Qβ life-cycles and the availability of techniques for altering the genetic content of the *E. coli* host in this system is favourable for studying the replication processes of plus-stranded RNA viruses. Since these RNA viruses show many similarities in their RNA and polymerase structures [1], results obtained in the Qβ polymerase system may also contribute to the understanding of viral infection mechanisms afflicting animals or plants.

The single-stranded RNA genome of bacteriophage Qβ is replicated by an RNA-dependent RNA polymerase consisting of one virally encoded replicase subunit (65 kDa) and three host factors: ribosomal protein S1 (61 kDa) and the protein synthesis elongation factors Tu (43 kDa) and Ts (31 kDa) [2]. The role of EF-Tu in this RNA polymerase is still unclear and is the central topic of our present research.

During translation, EF-Tu interacts with tRNA; since many viral and other replicatable RNAs have tRNA-like structures at their 3′ end, EF-Tu may play a role in template recognition. Some replicatable RNAs were even shown to bind directly to EF-Tu or eEF1A [3–6], but so far no direct correlation between the tRNA-binding site of EF-Tu or eEF1A and its contribution to viral polymerase activity has been found.

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The necessity of having at least one translationally functional copy of the EF-Tu encoding gene (*tuf*) present in the cell dramatically interferes with the use of conventional mutational analysis. Therefore, we designed a system in which two different *tuf* genes are present in the cell. The wild-type (wt) gene, located on the chromosome, sustains translation and another *tuf* gene, located on a plasmid, contains the mutation of interest and the sequence for a C-terminal His tag. A second plasmid carrying a gene for the viral replicase subunit (pREP [7]) completes the system. We thus have a tool for separating the two possible types of replicase complexes by Ni–NTA affinity chromatography.

To establish whether it is possible to purify active polymerase complexes through a His tag on EF-Tu, we first used a strain developed earlier in our laboratory which carries the DNA sequence for the His tag fused to the chromosomal *tufA* gene [8]. With this strain transformed with the pREP plasmid, we set up a working protocol for isolating active Qβ polymerase complexes with Ni–NTA affinity chromatography. We showed this approach to be of more general applicability by successfully isolating active polymerase complexes of two leviviruses of different species, GA and MS2. A further purification of the Qβ polymerase preparation was obtained by removing free His-tagged EF-Tu that is not part of the polymerase complex by using gel-permeation chromatography.

With this method we went back to our initial goal and transformed a wt strain with two plasmids, one encoding EF-Tu with a His tag and another encoding the Qβ viral replicase subunit. After confirmation that this system also yielded active Qβ polymerase complexes, we introduced a set of mutations interfering with the tRNA binding properties of EF-Tu [9–11]. With this set of mutations, we were able to show a successful separation of the two possible types of complexes and we found a direct link between the tRNA binding capacities of these mutant EF-Tu species and the in vitro activities of the corresponding Qβ polymerase complexes.

In principal, our method may be useful in any situation where protein complexes with a mutant subunit must be isolated from cells for which the presence of the same subunit in its non-mutated form is of vital importance.

2. Experimental

2.1. Cloning procedures

Bacteria were grown in LC broth (10 g/l bactotrypton, 5 g/l yeast extract (Duchefa), 8 g/l NaCl and 1 ml Tris–HCl pH 7.0 per liter). The bacterial strain JM109 was used for cloning procedures and protein expression except where stated otherwise. *E. coli* strain AZ46 was previously developed in our laboratory and contains a single chromosomal *tuf*A gene which is provided with an extra sequence encoding six C-terminal His residues called a His tag [8]. PCR, digestions and ligations were performed in buffers and according to protocols provided by the manufacturers of the enzymes used. Restriction endonucleases were from New England Biolabs or Fermentas. Clean-up and gel-extraction of DNA fragments was done with Qiagen columns. pREP contains the coding sequence of the viral Qβ replicase under transcriptional control of P_R (gift from V. Berzins, Riga University). The repressor gene for this promotor, cl857 is also provided on pREP together with the *bla* gene conferring ampicillin resistance. The propagation of pREP is under control of the pMB1 replicon [7]. pIQtacR carries the coding sequence for the MS2 viral replicase subunit behind a P*tac* promotor and a *spec* marker gene (gift from M.H. de Smit, Leiden University). pGA2-10 expresses the GA viral replicase subunit from its P*trp* promotor and has *bla* and *tet* marker genes (gift from V. Berzins). Both plasmids pIQtacR and pGA2-10 use the pMB1 replicon. pKECAHis [12] contains behind the IPTG-inducible P*tac* promotor the *tufB* sequence for EF-Tu provided with a C-terminal His tag. Since pKECAHis also uses the pMB1 replicon and the *bla* resistance marker gene, it is not compatible with pREP and we constructed a new plasmid. With *Bam*HI and *Hin*dIII, this coding sequence together with the P*tac* promotor was excised from pKECAHis, as a 1.6-kb fragment and ligated (Promega) into pIJ2925 [13] digested with *Bam*HI and *Hin*dIII. The resulting plasmid pSIEN has convenient *Bgl*II and *Pst*I sites, which were used to clone the 1.6-kb fragment into pACYC177 (New England Biolabs) digested with *Bam*HI and *Pst*I. *Bam*HI and *Bgl*II generate compatible cohesive ends, which after

ligation result in a sequence no longer recognised by either enzyme.

The resulting plasmid was named pSM1 and carries a kanamycin-resistance gene and the p15A replicon, making it possible to maintain this plasmid in the same cell as pREP.

Some EF-Tu mutants are known to have a reduced solubility upon overexpression in the cell, which can be improved by co-overexpressing EF-Ts [14]. For this reason and to prevent EF-Ts becoming a limiting factor for complex formation after overexpression of EF-Tu and the viral subunit, we amplified the EF-Ts-encoding region by PCR from pTuTs [14] using the oligos *Pst* I SD *tsf* (AAATTCTGCAGGAGGTA-GAATGGCTGAAATTACC) and *tsfBgl*I (AACTC-GCCGGAAGGGCTTAAGACTGCTTGGACATC). The resulting fragment was digested with *Pst*I and *Bgl*I and ligated into pSM1 digested with the same enzymes. This places the EF-Ts-encoding region behind the EF-Tu-encoding region under transcriptional control of the P*tac* promotor, and translation is enabled from the Shine–Dalgarno sequence provided in *Pst* I SD *tsf*.

The resulting plasmid, pSM2, is now a versatile vector for the introduction of a variety of mutations. The point mutations R58E, E259Y and R288E, originally made in pGEXtufA [9–11], were transferred to pSM2 by exchanging the corresponding *Hpa*I/*Blp*I fragments between the vectors. The presence of the point mutations was confirmed by restriction and sequence analysis.

2.2. Protein expression

Cultures (20 or 500 ml) were inoculated 1:100 with fresh overnight cultures with the appropriate antibiotics (0.1 mg/ml ampicillin, 0.05 mg/ml kanamycin, 0.1 mg/ml spectinomycin) and grown at 37 °C. pSM2 was induced at an A_{650} of 0.2 by adding 0.25 m*M* IPTG.

Strains harbouring pREP were grown at 28 °C and induced at an A_{650} of 0.6 by shifting the culture to 42 °C. Cells carrying pGA2-10 are grown in presence of 10 mg/l Trp to block transcription from the *trp* promotor. Induction is achieved by centrifuging the cells at 4300 *g* for 20 min and then resuspending them in LC without Trp added. pIQtacR was induced by adding 0.5 m*M* IPTG to cells that reached an A_{650} of 0.6. After induction of the viral replicase subunits, cells are grown for 30 min and harvested by centrifugation (4300 *g*, 20 min).

2.3. Protein purification

All steps described below were performed at 4 °C or on ice. Small-scale isolations were performed with 20-ml cultures by resuspending cells in 0.5 ml replicase buffer (50 m*M* Tris–HCl pH 7.8, 10 m*M* $MgCl_2$, 1 *M* NH_4Cl, 10% glycerol, 0.02% NaN_3) and lysing them by sonication; 5 min, 3 s on, 2 s off. After clarification in Eppendorf vials by centrifugation (13 000 *g*, 30 min) 50 μl of Ni–NTA (Qiagen) equilibrated with replicase buffer was resuspended in the resulting lysates. Protein binding was allowed by shaking the suspension for 10 min, after which the Ni–NTA was pelleted by centrifugation (13 000 *g*, 5 min). The Ni–NTA with bound protein was washed with 1 ml replicase buffer by resuspending the pellet and shaking for 10 min. The bound protein was eluted by another cycle of resuspending and shaking with 0.2 ml replicase buffer containing 100 m*M* imidazole. After centrifugation the resulting pellet was carefully removed and the supernatant tested for protein content and activity.

For larger-scale purifications, cell pellets from 500-ml cultures were resuspended in 5 ml replicase buffer and sonificated with ten 20-s bouts with 30-s cooling intervals. After adding 4 μg/ml phenylmethylsulfonyl fluoride and 0.2% Triton X100, cell-free lysates were prepared by centrifugation at 30 000 *g* for 20 min. The obtained lysate was applied to a Ni–NTA (13×10 mm) column in replicase buffer, and washed with this buffer at 0.5 ml/min until the A_{280} of the effluent was the same as that of replicase buffer. Bound protein was subsequently eluted with a gradient of 0–500 m*M* imidazole in 5 ml replicase buffer. Collected fractions were analysed by SDS–PAGE and tested for activity as described below.

For gel-permeation chromatography active fractions from the Ni–NTA column were pooled and concentrated with an Amicon Centriprep (30×10^3 cut-off) down to 0.5 ml. Of this preparation, 0.2 ml was injected on a Biorad TSK 250 (600×7.5 mm)

column equilibrated with replicase buffer. This column was operated on a Beckman SystemGold HPLC running at 0.4 ml/min. Fractions were collected at 1.5-min intervals.

2.4. In vitro activity tests

In vitro activity tests were based upon the protocols as described [2,15]. Polycytidylic acid (polyC, Amersham) was used as a template and purified from salts and nucleotides using P30 Biospin columns (Biorad). Incubation mixtures consisted of 18 μl of freshly prepared mix immediately added to 10 μl of polymerase sample and contained (final concentrations): 75 m*M* Tris–HCl pH 7.5, 9.4 m*M* $Mg(acetate)_2$, 4 m*M* $MnCl_2$, 360 m*M* NH_4Cl, 0.9 m*M* EDTA, 0.09 m*M* dithiothreitol, 0.7 μg polyC, 0.01 U/μl RNAguard (porcine, Amersham), 1 m*M* phosphoenol pyruvate, 3.5 μg/ml pyruvate kinase, 1.8 μg/ml rifampicin, 0.014 mCi/ml [8-^{3}H]GTP (Amersham), 0.27 m*M* GTP, 0.1 m*M* UTP, 0.11 m*M* ATP, 0.19 m*M* CTP (Ultrapure NTPs, Amersham). After an incubation at 30 °C for 20 min, 25 μl of the assay was spotted on a GF/C filter (Whatman) which was immediately submerged and rinsed in ice-cold 10% (w/v) trichloretic acid (TCA), 1 *M* NaCl, 10 m*M* $Na_2P_2O_7$, 10 μ*M* GDP. The filter was then washed twice in ice-cold 5% (w/v) TCA, 1 *M* NaCl, 10 m*M* $Na_2P_2O_7$, 10 μ*M* GDP and once in ice-cold 96% (v/v) ethanol. Filters were air-dried at 40 °C and incorporated ^{3}H was measured by scintillation counting with 3.5 ml Filtercount in a Beckman LS-5000TD. Blank values obtained from samples of 18 μl of mix and 10 μl of replicase buffer without polymerase material were subtracted for all values shown.

3. Results and discussion

3.1. Isolation of polymerase complexes with a chromosomally encoded His-tagged EF-Tu

The isolation method was first tested with replicase subunit expression in *E. coli* strain AZ46 containing His-tagged EF-Tu (EF-TuHis) as the sole chromosomally encoded EF-Tu species. As shown in Fig. 1, the optimal induction time for pREP is 30 min, whereas longer induction times result in lower rather than higher amounts of Qβ polymerase activity due to toxic effects of the Qβ replicase subunits on cells. This toxicity is also demonstrated by a drop in cell division speed after induction of replicase genes (data not shown).

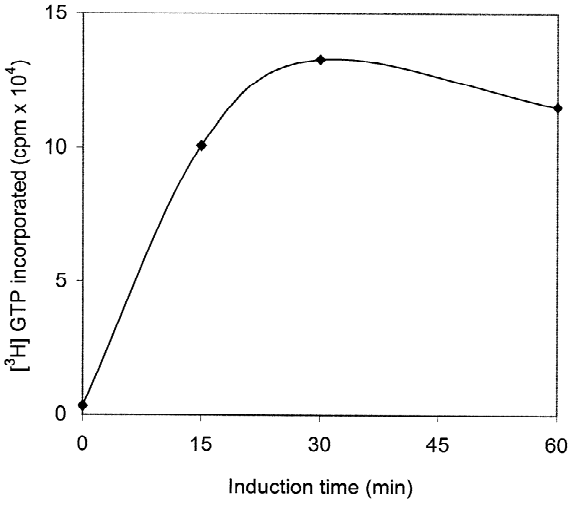

Fig. 1. Activity of Qβ polymerase complexes after varying induction times in lysates of pREP transformed *E. coli* AZ46 cells. With 1 ml of overnight preculture of AZ46 pREP, 100 ml of LC^{amp} was inoculated of which 4 portions of 20 ml were distributed in 100 ml flasks and incubated at 28 °C for the indicated times. Lysates were prepared as described. Qβ polymerase activity in lysates was determined by polyC-directed [^{3}H] GTP incorporation.

Increasing the concentration of ammonium chloride in the buffer results in a dramatic increase in the amount of Qβ polymerase complex eluted from the Ni–NTA beads. This is most likely due to a higher stability of the complex at higher salt concentrations [16] and points toward a predominantly hydrophobic nature of protein–protein interactions in the polymerase complex. Adding more than 500 m*M* ammonium chloride causes a loss of polymerase activity in both lysate and eluate samples (Fig. 2A). Apparently, this loss is due to the increased final NH_4Cl concentrations under assay conditions, as demonstrated in Fig. 2B. The observed higher activity of the eluate in comparison to that of the corresponding lysate at 500 m*M* NH_4Cl (Fig. 2A) may be caused by the presence of inhibitors in the lysate that are not

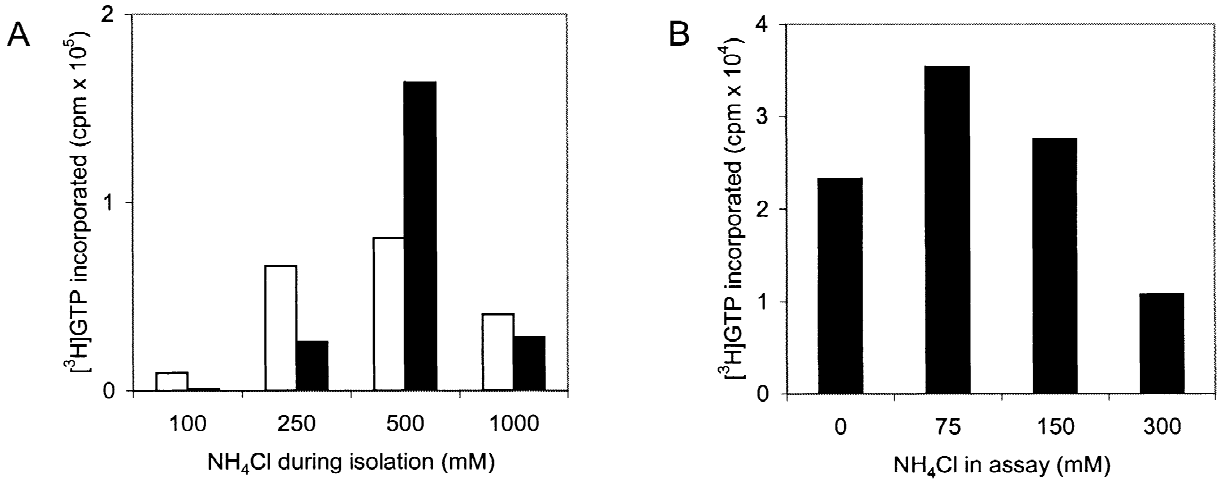

Fig. 2. Optimization of the NH_4Cl concentration for Qβ polymerase complex purification. (A) Effect of NH_4Cl on specific activity in lysates (□) and Ni–NTA eluates (■). Small-scale purifications were performed as described, with NH_4Cl concentrations as indicated. Under assay conditions, the latter NH_4Cl concentrations became 10:28 diluted. Obtained cpm values were corrected for protein concentrations as determined by Bradford analysis. (B) Effect of NH_4Cl concentrations in assay conditions for Qβ polymerase activity using replicase complexes purified by ion-exchange chromatography [19].

bound by the Ni–NTA. Likely candidates are RNases which are abundant in cell-free lysates and can break down the template RNA before the polymerase can use it.

For the other two viral replicases, from coliphages GA and MS2, complexes can also be readily isolated from the cell-free extract. (Fig. 3). GA polymerase was purified earlier using a laborious multi-step method [18] and here it is shown to behave similarly as Qβ polymerase. In contrast to Qβ polymerase, the yield for MS2 polymerase in the eluate is low in comparison to the activity in the lysate, which may

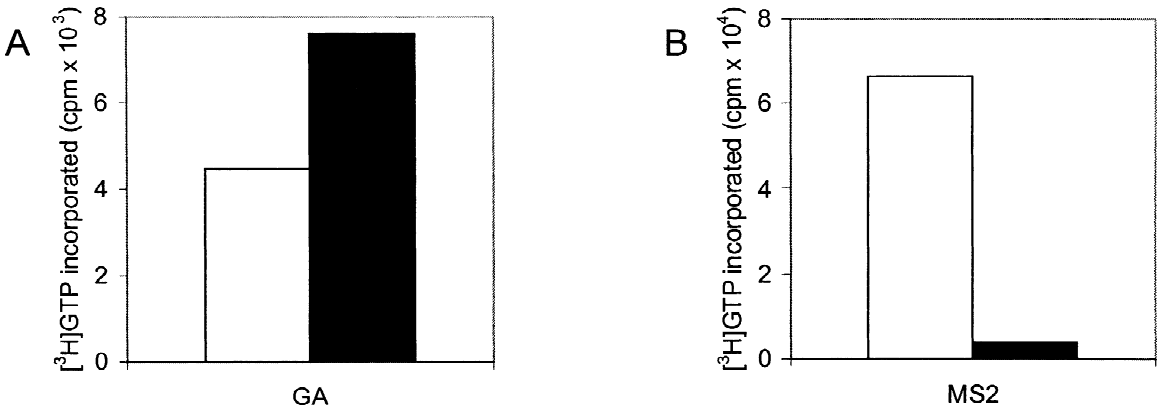

Fig. 3. Small-scale isolation of MS2 and GA polymerase complexes through His-tagged EF-Tu. Lysates (□) and Ni–NTA eluates (■) from cultures of AZ46 pGA2-10 or AZ46 pIQtacR were tested for their polymerase activity. The presented values are corrected for volumes of lysate and eluate, 0.5 and 0.2 ml, respectively.

reflect the lower stability reported earlier for group I polymerase complexes [17].

3.2. Isolation of polymerase complexes with a His-tagged EF-Tu encoded on a plasmid

Fig. 4 illustrates that our method also works for cells with chromosomally encoded wt EF-Tu that are cotransformed with pREP (replicase expression) and pSM2 (expression of wt or mutant EF-TuHis and EF-Ts). After expression of wt His-tagged EF-Tu from pSM2 again considerable amounts of Qβ polymerase could be isolated on Ni–NTA. Since EF-Tu is a very abundant protein in normal cells, a considerable part of the polymerase complexes in the lysate will not contain a His tag. This causes a slightly higher polymerase activity in the lysate, as compared to the eluate, in spite of the loss of inhibitors shown in Fig. 2A.

3.3. Characterisation of Qβ polymerase complexes containing defective mutant EF-TuHis species

As judged by SDS–PAGE followed by Coomassie staining or Western blotting with antibodies directed against EF-Tu, EF-Ts and S1, also comparable amounts of polymerase complexes could be purified for the three mutant EF-TuHis species. (not illustrated). Strikingly, the amount of activity on polyC for the R58E mutant is about half that of wt EF-TuHis. For E259Y and R288E no activity could be detected. This is in close agreement with the tRNA affinities reported earlier for each of these EF-Tu species. For R58E an affinity for tRNA half that of wt EF-Tu was reported [9], R288E hardly binds any tRNA [10] and E259Y does not bind tRNA at all [11]. Because other intrinsic EF-Tu properties, like those related to its GTPase-switch mechanism, are comparable between the mutant and wt EF-Tu species [9–11], we conclude from this correlation that the tRNA binding site on EF-Tu is of crucial importance for Qβ polymerase activity. Presumably, this interaction plays a role in the initial step of template recognition and may thus have been conserved during evolution, in view of the tRNA-like structures at the 3′ end of a number of viral template RNAs [20–22] and also the binding of eEF1A to some of these RNAs [3,6].

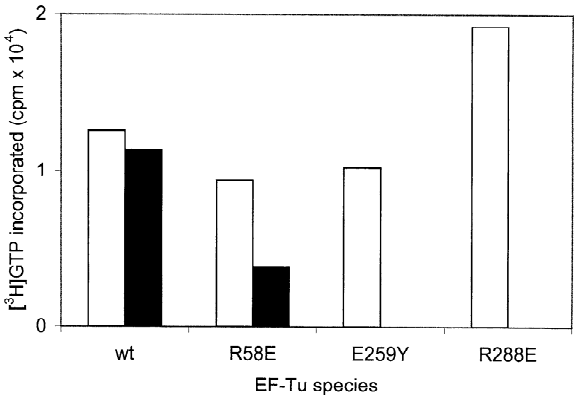

Fig. 4. Isolation of Qβ polymerase complexes containing wt or mutant EF-Tu species on Ni–NTA. Lysates (□) and Ni–NTA eluates (■) resulting from 20-ml cultures of JM109 pREP pSM2 wt, R58E, E259Y or R288E, were tested for their polymerase activity on polyC. Values are corrected for volumes of lysate and eluate, 0.5 and 0.2 ml respectively. The lysates contain both chromosomally encoded wt EF-Tu without His-tag and plasmid-encoded EF-Tu with His-tag in the Qâ polymerase complexes. The total Qβ polymerase activity in the lysates may also vary under influence of the toxic effects of varying levels of pRep expression as demonstrated in Fig. 1.

Although comparable amounts of EF-Tu are present in Ni–NTA eluates of the transformants with EF-TuHis species R288E and E259Y, no polymerase activity could be detected. Since our activity test is quite sensitive, this implies that all the EF-Tu present in these eluates is of the mutated species. We therefore conclude that this Ni-affinity method provides a very good separation of protein complexes with only one amino acid residue difference in one of the subunits.

3.4. Removal of excess EF-TuHis by gel-permeation chromatography

Obviously, excess of EF-TuHis over replicase subunits is always present in the Ni–NTA eluate. If desired, this excess can be removed by gel-permeation chromatography on a TSK 250 column. In the A_{280} profile of the TSK 250 run, one peak coincides with the single peak in the activity profile of the fractions (Fig. 5C). Comparison of the patterns obtained by SDS–PAGE before and after gel-per-

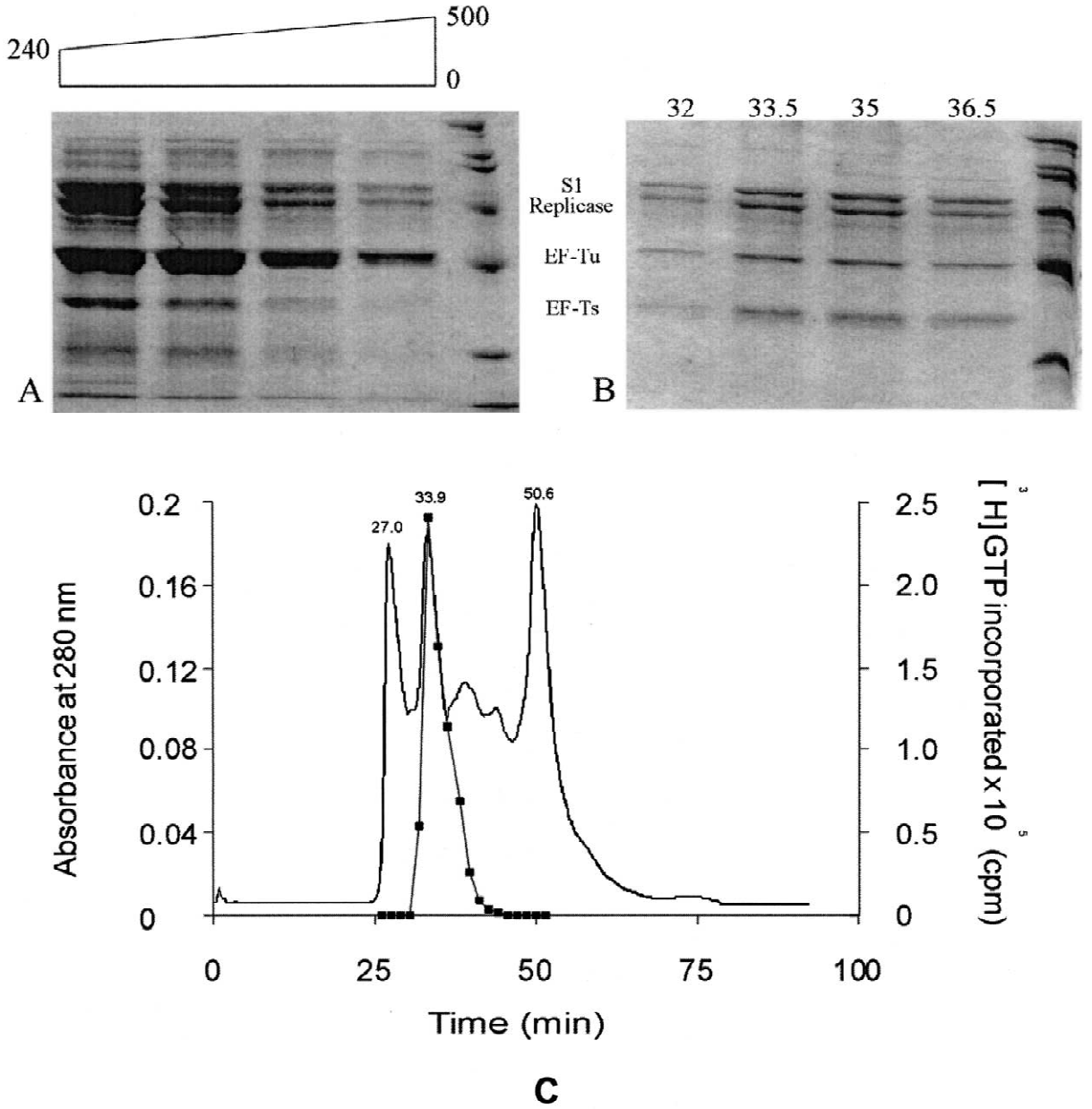

Fig. 5. Separation of Qβ polymerase complexes from free EF-Tu by gel-permeation chromatography. (A) SDS–PAGE analysis of fractions from Ni–NTA column with imidazole gradient. Molecular masses of marker proteins used: 200, 116, 97, 66, 45 and 31 kDa. The fractions were pooled and concentrated for application to a TSK 250 column. (B) SDS–PAGE analysis of peak fractions from the TSK 250 column run. Fractions were collected at 1.5-min intervals at the indicated times. (C) A_{280} profile and Qβ polymerase activity (■) of fractions from the TSK 250 column run.

meation chromatography (Fig. 5A, B) shows, that the band corresponding to EF-Tu becomes less intense and comparable with the intensity of the bands of the other three subunits. Furthermore, some minor Ni–NTA binding proteins, commonly present in Ni–NTA purified material from *E. coli*, are also

removed from the preparation. Therefore, this gel-permeation step is a valuable extra step in obtaining pure Qβ polymerase complex preparations after Ni-affinity chromatography.

3.5. Conclusions

The here described system enables us to use conventional mutational analysis for studying the role of EF-Tu in viral RNA-polymerase complexes after isolation of these complexes through an affinity tag on EF-Tu. We thus could show that the tRNA binding properties of EF-Tu are of vital importance for Qβ polymerase activity with a direct correlation between the affinity for tRNA of the EF-Tu species used and the in vitro activity of the resulting Qβ polymerase complex.

This method, which can be further refined by using gel filtration to remove an excess of non-complexed EF-Tu, may also be used for mutational analysis of totally different protein complexes even if the subunit of special interest is a protein essential for cell viability like EF-Tu.

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