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Mixed reconstitution of mutated subunits of HIV-1 reverse transcriptase coexpressed in *Escherichia coli* – two tags tie it up

Gottfried Maier[1], Ursula Dietrich[2], Barbara Panhans[2], Britta Schröder[2], Helga Rübsamen-Waigmann[2], Luciano Cellai[3], Thomas Hermann[1] and Hermann Heumann[1]

[1]*Max-Planck-Institut für Biochemie, Martinsried, Germany;* [2]*Georg-Speyer-Haus, Frankfurt, Germany;* [3]*Istituto di Strutturistica Chimica, CNR, Rome, Italy*

The active form of HIV-1 reverse transcriptase (RT) is a p66/p51 heterodimer, in which the p51 subunit is generated by C-terminal proteolytic cleavage of p66. A well-known problem of p66 recombinant expression is partial cleavage of a 15-kDa peptide from the C-terminus by host proteases that can not be completely suppressed. In order to analyse the contribution of specific residues to a particular function in one distinct subunit, an expression and purification system is required that selects for the combination of the two individual subunits with the desired substitutions. We reconstituted the p66/p51 heterodimer from subunits coexpressed in *Escherichia coli* as an N-terminal fusion protein of glutathione S-transferase (GST) with p51 and a C-terminally His-tagged p66, respectively. The two-plasmid coexpression system ensures convenience for gene manipulation while degradation is reduced to a minimum, as dimerization protects the protein from further proteolysis. The combination of glutathione-agarose, phenyl-superose and Ni/nitrilotriacetate affinity chromatography allows rapid and selective purification of the desired subunit combination. Truncated forms of p51 are efficiently removed. Mobility-shift assay revealed that the preparations are free of p66 homodimer. In a successful test of the novel expression system, mixed reconstituted RTs with p51 selectively mutated in a putative nucleic acid binding motif (the so called helix clamp) show reduced binding of dsDNA in mobility-shift assays. This indicates the p51 subunit has an active role in DNA binding

Keywords: HIV-1 reverse transcriptase; heterologous expression; GST-fusion; coexpression; helix-clamp motif.

The reverse transcriptase (RT) of HIV-1, the causative agent of AIDS, catalyzes the synthesis of the proviral dsDNA, a crucial step in viral replication [1,2]. Replication starts with the synthesis of minus-strand DNA initiated from the 3′-end of the host cellular $tRNA^{Lys3}$, which is complementary to a sequence of the viral ssRNA. The synthesis of proviral dsDNA therefore requires an RNA-dependent polymerase activity as well as an RNase H activity to degrade the copied genomic RNA template. Initation of plus-strand DNA synthesis from the so-called polypurine tract requires a DNA-dependent polymerase activity. Thus, during replication the RT needs to bind diverse primer/template substrates such as dsRNA, RNA/DNA, DNA/RNA and dsDNA.

RT consists of two subunits, where the smaller p51 subunit is generated from C-terminal proteolytic cleavage of the larger p66 subunit by viral protease [3]. Although both subunits harbour the same N-terminus, the crystal structure reveals an asymmetric heterodimer in which p66 comprises the polymerase as well as the RNase H active sites [4,5]. It has been suggested that a helix-turn-helix structure, termed the helix clamp, is involved in nucleic acid binding in both subunits [6].

The aim of this work is to establish a system for obtaining mixed reconstituted RT which consists of selectively mutated subunits in order to analyse the function of the helix clamp in the p66 and p51 subunits individually.

In recombinant expression of HIV-1 RT in *Escherichia coli* [7], degradation of p66 is a side reaction yielding p51 [8]. Mixed reconstitution of, for example, wild-type p66 with a mutated p51 results therefore in a mixed population of RTs containing a fraction of wild-type p66/p51. Moreover, the C-terminus of p51 can be degraded by bacterial proteases with an average loss of seven residues [8]. Deletion of 13 residues at the C-terminus of p51 was reported to impair RT functions dramatically [9].

Among the existing reconstitution strategies, the most straightforward ones are selection of N-terminally His-tagged p51 against the p51 derived from p66 degradation [10,11] and the use of coexpression systems in which *in vivo* heterodimer formation protects p66 from degradation [12,13].

We constructed a new coexpression system, where the two subunits are expressed in the same cells from two compatible commercially available plasmids. The system described here incorporates the advantages of the best described techniques, while introducing the GST-fusion approach for selection of desired products and a procedure that allows the removal of excess of p51, including its truncated forms, by hydrophobic chromatography. The N-terminal fusion of p51 to GST and the C-terminal fusion of p66 to a polyHis-tag allows selection for a stable heterodimer of the desired subunits.

MATERIALS AND METHODS

Plasmids, strains and nucleic acids

Plasmids pQE-12 and pREP4 as well as *E. coli* strains M15 and SG13009 were purchased from Qiagen. *E. coli* strain JM109

Correspondence to H. Heumann, Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, D-82152 Martinsried, Germany. Fax: +49 89 8578 2822, Tel.: +49 89 8578 2216, E-mail: heumann@biochem.mpg.de

Abbreviations: RNase H, ribonuclease H; GST, glutathione S-transferase.

Enzymes: reverse transcriptase, deoxynucleoside-triphosphate : DNA deoxynucleotidyltransferase, RNA-directed (EC2.7.7.49); RNase H (EC3.1.26.4); glutathione S-transferase (EC2.5.1.18)

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Table 1. Oligonucleotides used in this study.

Name	Sequence (5′→ 3′)
P1	CTGGTTCCGCGTGGATCCGAATTCCAAGCTTAA
P2	CACTGGATCCATGCCATTAGTCCTATTGAAACTGTACCAGT
P3	TTCCGGATCCTCATAGTACTTTCCTGATTCCAGCACTGACT
P4	ATCTGTTAGATCTCCCATTAGTCCTATTGAAACTGTACC
P5	TTCCCGGGGATCCTATTTTCCTGATTCCAGCACTGA
P6	ATCTGACGTCAGATCAAAAGCCATTGACTC
P7	GATCAAGCTTGATTCAGTCAGGCGTC
P8	GTCAATGACATACAGGCGTTATGGGAGCATTAAATTGGGC
P9	GCAATTATGTGCACTCCTTGCGGGAACCAAAGCAC
P10	GAAAAAGACAGCTGGACTGTCAATGACATACAGAAGTTAGTGGGAAAATTAAATTGGGCAAGTCAGATTTATCCAGGGATTA AAGTAGCGCAATTATGTGCACTCCTTGCGGGAGCACTAAC
P11	CTTTCAGGTCCCTGTTCGGGCGCCA
P12	AGAGCTCCCAGGCTCAGATC
T1	GCCCGGAACCCTTCTGGTTCCCCTTTGGCGCCCGAACAGGGACCTGAAAGCGAAAG
T2	GGGUCUCUCUGGUUAGACCAGAUCUGAGCCUGGGAGCUCU

[14] incorporating plasmid pDY [15] was kindly provided by Y. Gursky and R. Beabealashvilli. *E. coli* M15 bearing plasmids pDM1.I and pRT6H-PROT [16] were kindly provided by S. F. J. Le Grice.

The RT analyzed was derived from the HIV-1 isolate D148 [17], which is a primary isolate of HIV-1 subtype B. The RT sequence deviates from isolate BH10 [18] as follows: V35I, E122K, S162C, K172R, I178M, Q207G, R211K, L214F, V245L, R277K, I293V, V317A, S322T, I326V, R356K, T376S, V381I, K390R, K451R, L452I, N460D, K461R, N471D, Y483H, K512Q, N519S, I559V. The complete nucleic acid sequence of the RT is deposited in the EMBL database (accession number AJ011836).

DNA oligonucleotides (HPLC purified) were purchased from Interactiva (Ulm, Germany). RNA template T2 (Table 1) was synthesized on an Applied Biosystems 392/2 synthesizer using the standard phosphoramidite method. Oligonucleotides used in RT activity or band-shift assays were purified on 12% polyacrylamide-7-M urea gels containing 50 mM Tris/borate pH 8, 0.1 mM EDTA. 5′-end oligonucleotide labeling was performed with [γ-^{32}P]ATP and T4 polynucleotide kinase (Boehringer Mannheim) according to the manufacturer's recommendation. Products were electrophoretically purified to obtain homogeneously labeled nucleic acids.

Electrophoretic methods

SDS/PAGE was performed as described by Laemmli [19] with a modified loading buffer (100 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 4% SDS, 0.01% bromphenol blue, 5% 2-mercaptoethanol, 0.1 M $MgCl_2$) with $MgCl_2$ added to remove chromosomal DNA [20]. Cannon-Carlson and Tang [21] reported the occurrance of cleavage of aspartyl-prolyl peptide bonds in sample buffers containing Tris/HCl pH 6.8. Although such a bond is present in RT, no artificial bands could be visualized. Furthermore, the suggested phosphate buffer was not compatible with the addition of $MgCl_2$. Staining of proteins in gels was generally achieved by the combined use of Coomassie brilliant blue R-250 and Bismarck brown R, following the protocol of Choi *et al*. [22]. Where used, rapid staining was performed with ethidium bromide according to Shevelev *et al*. [23], silver stain as devised by Blum *et al*. [24].

Agarose gels were prepared according to Sambrook *et al*. [25]. To circumvent the problem of UV-damage of DNA during preparative agarose gel electrophoresis for cloning procedures we tried zinc-imidazole staining described by Hardy *et al* [26], but reproducibility was found to be very poor. However, protection against UV-induced damage by addition of 1-mM guanosine to the electrophoresis buffer as described by Gründemann & Schömig [27] was successful, leading to a fivefold increase in the number of transformed cells after ligation. DNA purification from agarose gels was carried out as described by Boyle *et al*. [28].

Native polyacrylamide gels (4%, 20-cm long) for band shift assays were run at room temperature for 2 h (80 V). Buffer conditions were 20 mM Tris/HCl pH 8, 5 mM NaCl, 0.1 mM EDTA. For gel densitometric analysis BioRad Imaging Densitometer GS-690 with MOLECULAR ANALYST™ software was used.

Cloning

Plasmid pGEX-p66 ([17], designated pGEX-RT86.X) carries an insertion of the complete RT coding sequence of isolate D148 cloned into vector pGEX-2TH. It is a derivative of pGEX-2TK (Pharmacia, [29,30]) with linker sequence P1 (Table 1) at the cloning site. RT sequence was amplified using primers P2 and P3, the PCR-product cleaved with *Bam*HI using the sites introduced with the primers [31] and ligated into *Bam*HI-cleaved pGEX-2TH. Analogous plasmid pGEX-p51 was constructed by deleting a *Kpn*I-*Hin*dIII fragment from pGEX-p66 and subsequent insertion of a linker reconstituting a C-terminus comprising p51 wild-type without the last amino acid position (440). For C-terminal addition of a His-tag, p66 was subcloned into pQE-12 (QIAGEN) cleaved with *Bgl*II. p66 was amplified using primers P4 and P5 which introduced a *Bgl*II site at the upstream end and a *Bam*HI site at the downstream end. Additionally, the downstream primer changed the penultimate residue from valine to isoleucine (Fig. 1A). To create a coexpression system, GST-p51 coding sequence was subcloned into pREP4 (Qiagen). A *Nar*I-*Aat*II fragment was treated with Klenow fragment and ligated into pREP4 which was digested with *Cla*I and blunted with Klenow fragment. This construct was designated pGST-p51. *Dna*Y was introduced into pGST-p51 by PCR amplification [31] of a 357-bp fragment described to have *dna*Y$^+$ complementing activity [32,33]. Plasmid pDY [15] was used as a template and P6 and P7 as primers. The amplification product was treated with Klenow fragment and was phosphorylated using T4 polynucleotide kinase and inserted into *Sma*I-cleaved pGST-p51. The resulting plasmid was designated pGST-p51/Arg. Both orientations of *dna*Y containing insert were obtained and checked for influence on expression level of RT subunits. Insertion of *dna*Y in pGST-p51 was proven by restriction analysis (*Aat*II and *Hin*dIII sites were introduced via the primer ends) and by PCR using the same primers as for amplification prior to cloning.

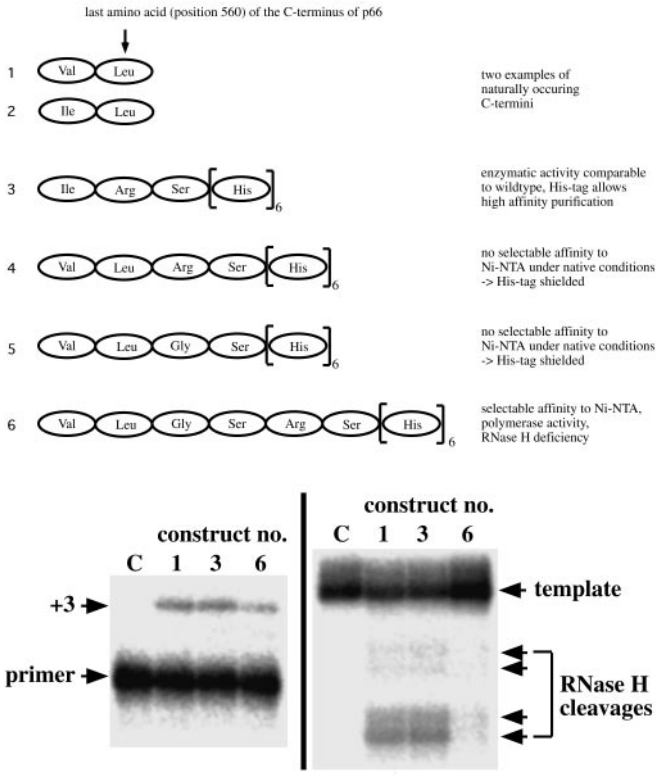

Fig. 1 Addition of a His-tag to the C-terminus of p66 was found to be nontrivial. (A) The figure gives an performance overview of several His-tag constructs. Residues of the aligned C-termini are given starting with position 559. The sequences shown are (1) that of the wild-type we used (isolate D148) (2) the end of the wild-type sequence of isolate BH10 [18] (3) the C-terminus of our construct p66-His as well as that of Le Grice and Grüninger-Leitch ([16]; the plasmid designated pRT6H-PROT), and (4)–(6) constructs that were either not purifyable under nondenaturing conditions (4 and 5) or defective in RNase H activity (6). (B) Analysis of polymerase and RNase H activities of His-tagged RT constructs 1, 3 and 6 described in Fig. 1A. Constructs 1 (no His-tag, wild-type C-terminus, expressed from pGEX-p66/pGEX-p51), 3 (the finally chosen approach for His-tagging, expressed from p66His/pGST-p51/Arg) and 6 are compared in respect to polymerase (left panel) and RNase H activity (right panel), respectively. Control reactions, C, are without enzyme. The extension of 5′-labeled DNA primer into arrested registers (left panel) and cleavage of 5′-end-labeled RNA template was monitored using denaturing gel electrophoresis and subsequent PhosphorImaging. Quantification after background subtraction: 15, 16 and 12% primer extension for Nos 1, 3 and 6, respectively; 24, 24 and 3% RNA cleavage for Nos 1, 3 and 6, respectively.

Site-directed mutagenesis was performed using the QuikChange™ Kit from Stratagene according to the manufacturers instructions. To change lysine residues at positions 259 and 263 to alanine, the primer P8 together with its complementary counterpart were used. For change of lysine at position 281 and arginine at position 284 the primer P9 and its complement were designed. Mutations K277A and K287A were performed with primer P10 and its reverse form. Mutagenesis was confirmed by DNA sequencing.

Protein expression and purification

For expression, plasmids were transformed into JM109 [14]. Transformed cells were grown in LB medium containing 10 g tryptone, 5 g yeast extract and 10 g NaCl per litre. For induction 1-mM isopropyl thio-β-D-galactoside was added to cultures (0.5 OD at 600 nm).

All purification steps were carried out at pH 7.2–8 in order to ensure heterodimer stability [34]. Cells were resuspended in lysis buffer (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 40 mM $MgCl_2$, 0.05% NP-40, 1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin A, 1 μM leupeptin) and lysed by lysozyme treatment (0.5 $mg \cdot mL^{-1}$) and subsequent french press passage (two times, 1000 psi; Aminco). The supernatant was filtered through a glass microfibre filter (Whatman), a Millex-GV filter (Millipore, 0.22 μm) and loaded on glutathione agarose column equilibrated with lysis buffer. Proteins were eluted with buffer containing 100 mM Tris/HCl, pH 8, 500 mM NaCL and 10 mM glutathione (reduced). Subsequent thrombin cleavage (2–3 h, room temperature; 5 $U \cdot mg^{-1}$ of RT) was carried out while concentrating the solution in a stirred cell (Amicon 8050) with an ultrafilter (Filtron, 10 kDa exclusion size). To the concentrated protein solution, 1.5 M $(NH_4)_2SO_4$ and 50 mM phosphate, pH 7.8, was added to a concentration of 1 M $(NH_4)_2SO_4$. The sample was then filtered through a Millex-GV filter (Millipore, 0.22 μm) and applied via a superloop on either Phenyl-Superose™ or Phenyl Sepharose™ CL-4B column for separation on FPLC™ (Pharmacia). The column was equlibrated to 1 M $(NH_4)_2SO_4$, 50 mM phosphate, pH 7.8, 5 mM 2-mercaptoethanol and eluted with a 1–0.45 M gradient of $(NH_4)_2SO_4$ in a volume of 20 mL, then the gradient was stopped for heterodimer elution until absorbance returned to baseline level (usually 10 mL, when approximately 2 mg of heterodimer was bound using 1 mL bed volume) and 0.45–0 M $(NH_4)_2SO_4$ in a volume of 15 mL; flow rate was 0.25 $mL \cdot min^{-1}$. After this procedure, RT preparations were found free of RNases as tested in an analogous preparation of the RNase H deficient mutant enzyme depicted in Fig. 1A, no. 6. The final purification step was affinity chromatography using Ni/nitrilotriacetate agarose column equilibrated to 50 mM phosphate pH 7.8, 300 mM NaCl. Pooled fractions of phenyl-superose runs were directly loaded on this column and eluted with an 0–0.25 M imidazole gradient at a flow rate of 1 $mL \cdot min^{-1}$. Pooled fractions were set to 50 mM phosphate pH 7.8, 300 mM NaCl, 1 mM dithiothreitol and 0.1 mM EDTA using Centricon-30 concentrators (Amicon) and stored at −20 °C. Homodimer p66-His was expressed from *E. coli* strain JM109 carrying p66His and purification started on a Ni/nitrilotriacetate column using conditions as described above. Subsequently, a phenyl-superose column was run as above, followed by a sizing column (Superose 12, Pharmacia) using 50 mM phosphate buffer pH 7.8, 500 mM NaCl, 5% glycerol, 1 mM dithiothreitol.

Band-shift and RT assays

DNA primer/template was prehybridized before incubation with RT. A mixture containing the template strand (120 nM) and ^{32}P-labeled primer (100 nM) in a buffer containing 50 mM Tris/HCl, pH 7.8, 50 mM NaCl was heated to 95 °C for 2 min followed by incubation at 72 °C for 10 min and cooling for 20 min to room temperature. Complete hybridization was confirmed on native polyacrylamide gels.

Annealed primer/template substrate (100 nM) was preincubated for 5 min at 37 °C with HIV-RT (50 nM or 100 nM) in a buffer containing 50 mM Tris/HCl, pH 7.8 and 50 mM NaCl. Prior to the band-shift analysis, the first nucleotide was given as ddATP to stabilize the complex. Primer was P11 and template was T1. For band-shift analysis 0.5 μL of loading buffer was added per 10 μL of sample and 5 μL applied on a denaturing gel (see above). Loading buffer contained 20 mM Tris/HCl pH 8, 0.02% bromophenol blue and 40% sucrose.

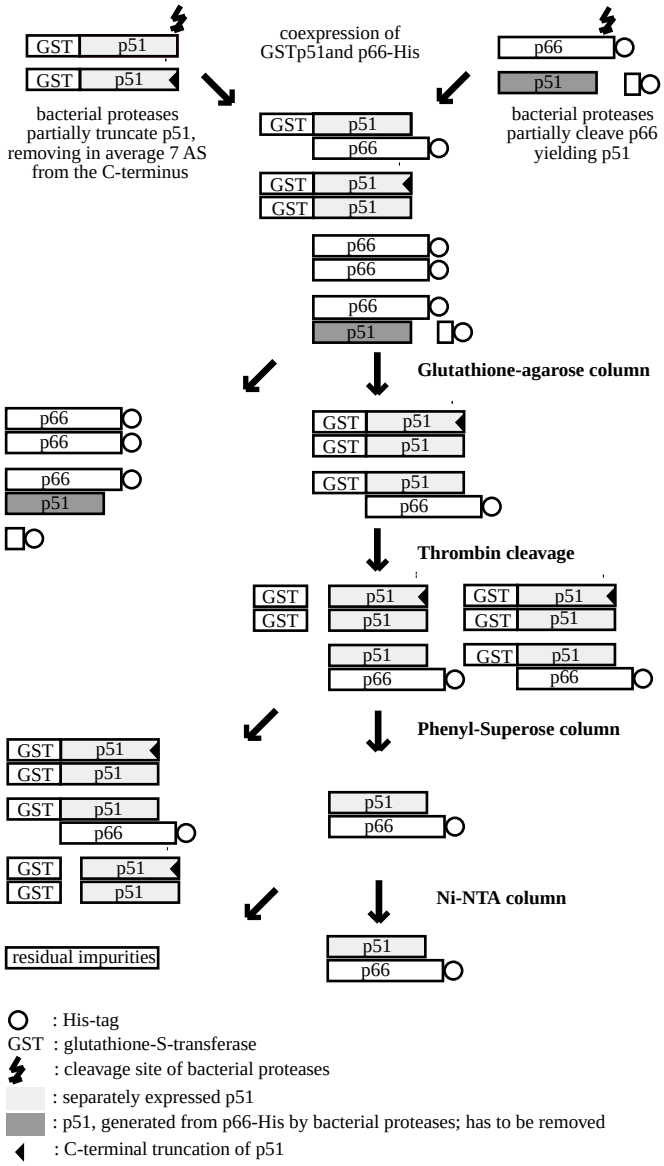

Fig. 2. Mixed reconstitution and purification of p66 and p51. The scheme shows the purification protocol (for details see Materials and methods). The following possible subunit combinations and monomeric forms were not considered: truncated GST-p51/p66-His heterodimers, monomeric GST-p51 and homodimeric forms of GST-p51 with both or none of the subunits truncated. This was due to the fact that all forms of p51 not being hybridized to p66-His are quantitatively removed using the phenyl-superose column, independent of whether they were truncated or not (see Results and Fig. 4). Truncated GST-p51 hybridized to p66-His is not detectable in the heterodimeric fraction after phenyl-superose run (Figs 3 and 4).

RNase H activity was performed using DNA primer P12 and RNA template T2 (Table 1), hybridization of primer/template and preincubation as described above for band shifts. RT concentration was 50 nM. Reaction was started by addition of $MgCl_2$ (6 mM final concentration) and stopped after 5 min by adding 10 μL of 95% formamide containing 40 mM EDTA. Reaction products were analyzed on 15% polyacrylamide-7 M urea gels using PhosphorImaging. RT polymerase activity was analogously monitored adding dATP and ddGTP (100 μM each) and using the same dsDNA as for band shifts. Primer extension was thus arrested in register +3 allowing easy quantification (PhosphorImaging).

RESULTS

Construction of the coexpression system

In order to express the two subunits of HIV-1 RT in *E. coli* simultaneously we constructed a coexpression system described in detail in Materials and methods. Plasmid p66His for overexpression of C-terminally His-tagged p66 is based on commercially available plasmid pQE-12 (Qiagen). Addition of a His-tag to the C-terminus of p66 was found to be a critical step for obtaining functional enzyme. Changes in this region can affect conformation and activity (constructs shown in Fig. 1A), leading to RNase H deficient enzyme (Fig. 1B) or shielding of the His-tag in the native state. The latter was observed for a few constructs (data not shown). Le Grice & Grüninger-Leitch [16] reported about a C-terminal His-tagged p66 construct, designated pRT6H-PROT, yielding fully active RT which bound to Ni/nitrilotriacetate with high affinity. Only when we used the same C-terminal sequence (Fig. 1A, lane 3) were we able to purify and obtain fully active enzyme (Fig. 1B), indicating that this region is sensitive towards changes of the amino acid sequence. The C-terminal sequence of each construct was confirmed by DNA sequencing and the presence of the His-tag at the C-terminus was proven by purification on Ni/nitrilotriacetate columns. With the exception of the penultimate residue (position 559), sequences of the C-termini of isolates D148 (this work) and BH10 [18] used by Le Grice & Grüninger-Leitch [16]

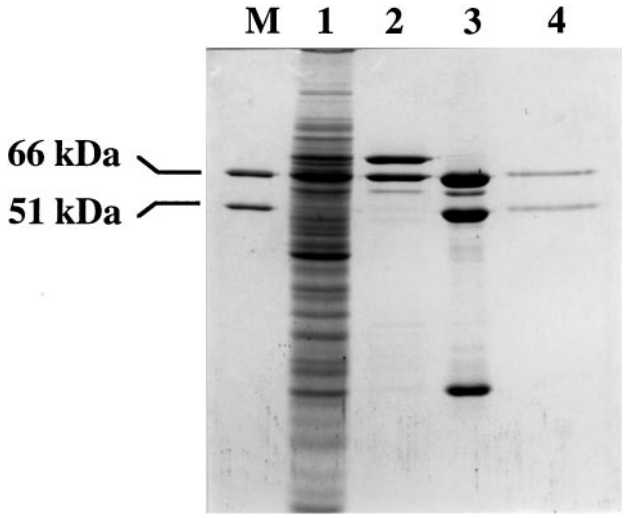

Fig. 3. Purification of GST-p51 and p66-His using glutathione-agarose, phenyl-superose and Ni/nitrilotriacetate columns. Coomassie-stained SDS-gel of purified RT (lane M) following the protocol depicted in Fig. 2. Lane 1 shows the crude cell lysate, the eluate from the glutathione-agarose column is shown before (lane 2) and after thrombin cleavage (lane 3). Lane 4 shows eluate from phenyl-superose (aliquot of a pool of 10 mL eluted at 0.45 M $(NH_4)_2SO_4$).

are identical within a stretch of the last 40 residues. Therefore, it seems very likely that the observed dramatic differences in enzymatic activity and Ni/nitrilotriacetate binding affinity of the C-terminal variants are solely influenced by their slight differences of the His-tag constructs depicted in Fig. 1A.

Construction of plasmid pGST-p51 for coexpression of GST-p51 is described in detail in Materials and methods.

Coexpression level of GST-p51 and p66-His is not influenced by concomitant overexpression of $tRNA^{Arg4}$

Codon usage limitations in the expression of eukaryotic genes expressed heterologously in *E. coli* are described repeatedly (e.g. [35,36]). The least used codons in *E. coli* are AGA and AGG [37], which are decoded by $tRNA^{Arg4}$, the gene product of *dnaY*. Overproduction of $tRNA^{Arg4}$ in *E. coli* was reported to increase expression level of several eukaryotic genes with high content of these rare codons [35,36]. Because, in the case of isolate D148, all arginine codons of the p66 coding sequence are of the rare species we addressed the question whether expression of RT subunits was limited by the cellular level of $tRNA^{Arg4}$. In order to check possible codon usage limitations of the overexpression caused by rare arginine codons we inserted a copy of *dnaY* encoding $tRNA^{Arg4}$. We found there was no effect of overproduction of $tRNA^{Arg4}$ on expression level of the two coexpressed RT subunits detectable.

Purification by affinity and hydrophobic chromatography

A purification protocol was established, schematically outlined in Fig. 2. Cells coexpressing GST-p51 and p66-His were lysed and the supernatant was loaded on a glutathione-agarose column. After elution with glutathione the GST-fusion protein was cleaved by thrombin. Subsequently the preparation was loaded on phenyl-superose column followed by Ni/nitrilotriacetate affinity chromatography.

Figure 3 shows the SDS-gel analysis of an RT purification following the protocol depicted in Fig. 2. Figure 3, lane 3, shows that the ratio of GST-p51 to p66-His is close to 1 : 1 after elution from glutathione-agarose. Apart from GST that is present as thrombin cleavage product (Fig. 3, lane 3), RT is pure to at least 90% after this first purification step.

It is interesting to note that elution from glutathione-agarose was performed with 10 mM glutathione at high salt conditions (100 mM Tris/HCL, ph 7.8, 500 mM NaCl). Elution in the presence of 50 mM Tris/HCl according to the manufacturer's recommendation released only 25% of the protein from the column. The subsequent thrombin cleavage was not significantly impaired by the high salt and could be performed directly in the eluate.

After the second purification step on phenyl-superose (Fig. 3, lane 4) RT was sufficiently pure for activity measurements. The assay revealed that the RT preparation was free of any contaminating nuclease and polymerase activity indicating a homogeneity $> 95\%$.

E. coli chaperone GroEL (60 kDa) which has been reported to co-purify with GST fusion proteins ([38], Fig. 3, lane 3) was quantitatively removed in the subsequent purification step using a phenyl-superose column (Fig. 4, lanes 1, 8 and 9). Moreover, this phenyl-superose step permits quantitative removal of the cleaved GST (Fig. 4, lanes 2–7). Reducing conditions during and prior to the chromatography are essential in order to avoid disulphide bridge formation between GST and RT (data not shown). Therefore, glutathione (c = 10 mM) was supplemented by 5 mM 2-mercaptoethanol. Dithiothreitol is not recommended as a reducing agent, as it interferes with the following Ni/nitrilotriacetate chromatography step.

Phenyl-superose chromatography facilitates not only removal of uncleaved GST-p51 (Fig. 4, lane 1 in comparison to lanes 3–7) but also excess of p51 (Fig. 4, lane 9). It is interesting to note that a truncated form of p51 can also be removed that only

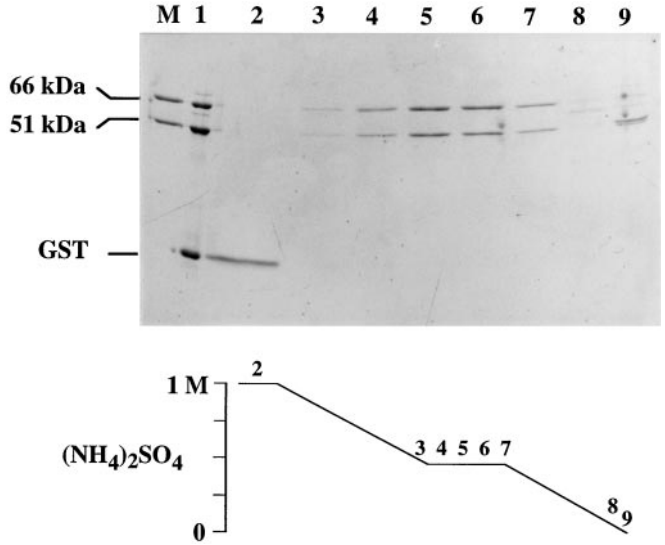

Fig. 4. Separation of p66-His/p51 from cleaved GST and excess of p51 as well as degraded p51 by running a phenyl-superose column. Coexpressed RT heterodimer is loaded on phenyl-superose column after elution from glutathione-agarose and subsequent thrombin cleavage. Lane 1 shows an aliquot of the loaded sample, lane 2 shows the run-through during loading of the sample; lanes 3–7 show the elution of RT heterodimer at 0.45 M $(NH_4)_2SO_4$, lanes 8 and 9 show the elution of excess of p51 (including a truncated fraction), contaminating GroEL, and residual p66-His at 0.05–0 M $(NH_4)_2SO_4$ (gradient profile shown below the gel).

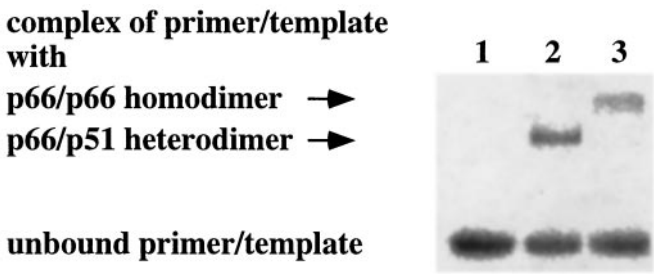

Fig. 5. Analysis of RT preparations for p66 content. Primer/Template with the primer 5′-labeled was loaded on a nondenaturing gel, not preincubated with RT (lane 1), preincubated in the presence of equimolar amount of reconstituted RT heterodimer (lane 2) and preincubated with RT $(p66)_2$ homodimer (lane 3).

eluates along with free p51. It is not incorporated into the heterodimeric RT (Fig. 4, lanes 3–7).

We also examined in which way Mg^{2+} in the lysis buffer affects heterodimer formation. Two approaches were compared. Cells expressing p51 and p66 separately were mixed for preparing RT. Alternatively, both subunits were coexpressed in the same strain. Mg^{2+} up to 40 mM improved dimerization and diminished proteolysis of p51. While this Mg^{2+} effect was strongly pronounced when the subunits were separately expressed, the effect was moderate with coexpressed subunits (data not shown).

A final affinity chromatography using Ni/nitrilotriacetate completes the protocol (Fig. 3, RT used as marker) and selects for p66 full length products. This purification might lead to trace amounts of Ni-ions in the eluate. The activity assay in absence of Mg^{2+} however, revealed that the preparations did not contain metal ions that could substitute for Mg^{2+} neither at the polymerase nor at the RNase H active site. To prevent oxidation of cysteine residues due to trace amounts of Ni-ions the storage buffer contained 100 μM EDTA [39].

Band-shift assays to analyze RT preparations for their content of p66 homodimer

Principally, our purification protocol rules out the possibility that RT is contaminated by p66 homodimer provided the heterodimer is sufficiently stable during the last purification step. As it is reported that heterodimer stability dramatically decreases below pH 7.2 [34], elution from the Ni/nitrilotriacetate column was performed without lowering pH, in contrast to previous protocols [40]. However, SDS/PAGE analysis of RT eluted from the phenyl-superose column showed that the intensity of p66-His was stronger than that of p51, suggesting an excess of p66. We attribute this effect to differences in the staining efficiency of both subunits, as purification of RT via the p66 His-tag on Ni/nitrilotriacetate agarose did not affect the intensity ratio.

Moreover, we checked whether the heterodimer fraction was contaminated by p66 homodimers. For that purpose we applied a band-shift assay which permits differentiation between the two forms of RT. RT was incubated with DNA primer/template. The 5′-labeled primer was elongated by one nucleotide enhancing the stability of the RT-DNA complex. Homodimer and heterodimer can be differentiated by their different electrophoretic mobility if bound to the primer/template. Figure 5 shows that an RT preparation following the described protocol does not contain a homodimer contamination.

RTs with mutations in the helix-clamp motif of p51 show reduced binding of dsDNA

It has been suggested that a helix-turn-helix motif [^{259}KLVGKL $(X)_{16}$ KLLR^{284}], termed the helix clamp, might be involved in nucleic acid binding in both subunits of RT [6]. Residues with basic side chains in the helices αH and αI of the helix clamp are proposed to interact with the nucleic acid backbone. A number of mutations in the helix-clamp motif of subunit p51 were introduced in order to selectively analyze the contribution of the motif in p51 to nucleic acid binding and activity of RT. Four mutated p51 subunits were investigated (Fig. 6). Because a detailed description of these and additional mutants will be presented elsewhere, we confine ourselves here to the characterization of four mutants by a band-shift experiment, performed as described above. Differences in the nucleic acid binding capacity were analysed using reconstituted RT mixed from wild-type p66 and p51, carrying mutations in the helix-clamp motif as indicated in Fig. 6. This figure reveals that the binding activity of reconstituted RTs decreases with progressive neutralization of basic residues in the motif. This effect can not be attributed to reduced dimer stability as the purification procedure strongly selects for stable heterodimers and no significant effect on yield of the purified mutant proteins was observed. Homodimer p66 is not detectable in mutant preparations as well as in wild-type RT.

DISCUSSION

Cleavage of HIV-1 RT subunit p66 by virus encoded protease yields a p51 subunit comprising 440 amino acid residues [41]. Recombinantly expressed p66 is partially cleaved by bacterial proteases giving rise to p51 subunits of 426–446 residues [8,42].

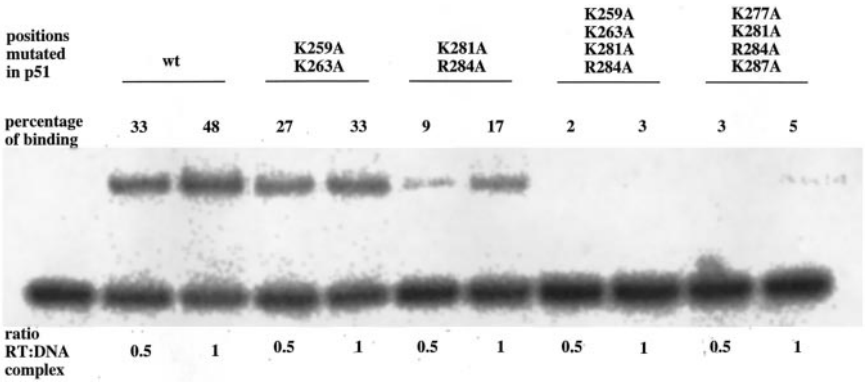

Fig. 6. Mixed reconstituted RTs with mutations in the helix-clamp motif of p51 show reduced binding of dsDNA. Band-shift analysis comparing binding of wild-type RT to dsDNA with that of four reconstituted RTs with mutations in the helix-clamp motif of p51. Each enzyme was incubated in a ratio of 0.5 : 1 and 1 : 1, respectively, to primer/template complex (100 nM). Primer was 5′-endlabeled (see Materials and methods). Percentage of binding was quantified using PhosphorImaging. The first lane shows primer/template without RT. Conditions were the same as in Fig. 5.

This unspecific cleavage results in a heterogeneous population of heterodimers, which particularly hinders functional studies involving wild-type and mutant forms of p51 and p66. The bacterial proteases that cause these cleavages are unknown, some 50 *E. coli* proteases having been described [43]. Because the use of protease inhibitors as well as that of protease deficient strains is limited [7,42], it is not possible to totally suppress the truncations.

Support of dimerization of p66 and p51 by coexpression of the two subunits was shown to protect from further cleavage [12,13]. Our findings are in accordance with this previous work showing that detectable amounts of truncated p51 are found only in the fractions of p51 which were removed by hydrophobic chromatography. No contaminating p51 truncated product was observed with the heterodimeric fraction (see Fig. 4). Additionally, Mg^{2+} reduces degradation of reconstituted subunits efficiently (Fig. 4, and unpublished results). The dramatically increased dimerization of the heterodimer in presence of 40 mM Mg^{2+} in the lysis buffer is in line with data of Divita *et al*. [44] showing a 100-fold acceleration of heterodimer association at 10 mM Mg^{2+}. In our study we have observed that dimerization is increased at Mg^{2+}-concentrations higher than that described by Divita *et al*. [44]. This might reflect the fact that part of the added Mg^{2+} was bound to the nucleic acid present in the cell lysate.

The amount of RT heterodimers obtained in our coexpression system was approximately 10 $mg \cdot L^{-1}$ of cell culture, thus making it comparable to most established RT expression systems in *E. coli* (e.g. [45,46]). The codon usage of HIV-1 genes differing from that in *E. coli* [37,47], however, suggested that expression rate in *E. coli* might be modulated by the expression level of $tRNA^{Arg4}$ (for an overview, see [48]). The reading frames of both subunits altogether contained 32 arginine codons rarely used in *E. coli*. For *p66-His* the content of AGA and AGG was 3%, a ratio that was described as critical in this respect [35,36]. Some authors claim that positioning near the translational start and the consecutive nature of these codons is more important than their abundance [49,50]. The latter applies to RT, as coexpression with $tRNA^{Arg4}$ did not improve the expression yield.

Coexpression itself does not prevent undesired truncations. Jonckheere *et al*. [13] presented mass spectra of coexpressed RT showing a high homogeneity of p51. However, the minor peaks of the spectrum coincide with the expected length of cleavage products generated by bacterial proteases ranging from 426 to 446 residues [8,42]. In the cases where p66 wild-type is combined with mutated p51, a truncation of p66 would be especially distorting for subunit-selective analysis. As a consequence, selectivity has to be achieved in the coexpression approach.

Fusion of p51 to GST allows the discrimination between native p51 and p51 generated by bacterial proteases (Fig. 2). It is improbable that this artificial p51 dimerizes as p51 mainly appears as monomer [12,51]. As p66-His could be purified only if associated to GST-p51, an excess of p66-His in the eluate from the glutathione-agarose column is prevented. Therefore, unfavourable dimerization of p66-His with artificial p51 is avoided.

Hottiger *et al*. [52] described a GST-p66 fusion with only low dimerization competence. The enhanced dimerization by the use of the coexpression system and 40 mM Mg^{2+} in the lysis buffer presented in this work could therefore be ascribed to particular properties of the GST-fused p51. At least Mg^{2+} addition and coexpression together allow an almost quantitative dimerization of GST-p51 with p66-His, which is expressed in excess relative to GST-p51.

Hydrophobic chromatography was shown not only to be a powerful tool in RT purification, allowing not only separation of heterodimer from $(p66)_2$ homodimer [42,53] and $(p51)_2$ homodimer (this study, Fig. 4) but also to be indicative of the folding state of proteins [54]. All reconstituted RTs examined here elute between 0.55 and 0.45 M $(NH_4)_2SO_4$, suggesting that mutational changes did not influence folding.

A polyhistidine-tag fused to RT was successfully used for affinity purification in numerous applications [40]. To our knowledge, the only case where problems occurred was described by Pekrun *et al*. [55] for an N-terminal fusion in baculoviral systems that are barely comparable to the *E. coli* systems. However, we describe here several constructs where a His-tag added to the C-terminus of RT caused severe problems in folding and activity (Fig. 1A,B). These constructs deviated only in a few amino acids and slightly in length from those successfully used by Le Grice and Grüninger-Leitch [16] and in this study, indicating a critical role of the C-terminus for structure and function of RT. Therefore, it is necessary to carefully analyze whether changes at the C-terminal amino acids affect RT function.

In all cases where His-tagging is undesired, the combination of GST-p51 with authentic p66 in a coexpression approach is the method of choice: the precise removal of GST via thrombin and a two-column purification using gutathione-agarose along with hydrophobicity chromatography yield a highly pure reconstituted heterodimer.

It should be emphasized that mutations in HIV-1 RT can yield RT subunits having reduced affinity. Therefore, it is difficult to fully suppress homodimer formation in mixed reconstituted RT. As a consequence, analysis of the homogeneity of the RT preparations is strongly recommended. Using the electrophoresis technique as diagnostic tool we could exclude that our mixed reconstituted RTs contained contamination of p66 homodimer.

The reconstitution technique described above was used to analyse the effect of mutations in the helix-clamp motif. We were able to show that mutations in the p51 subunit alone led to a reduced DNA binding affinity of mixed reconstituted RT and we could exclude the possibility that the result was impeded by p66 homodimeric RT. The reconstituted RTs are still under study at this time. Preliminary results show that the helix clamp of p51 significantly influences enzymatic functions.

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