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Biochemical characterization of the p51 sub-unit of human immunodeficiency virus reverse transcriptase in homo- and heterodimeric recombinant forms of the enzyme

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The biochemical properties of the p51 subunit of HIV-1 reverse transcriptase (RT) were studied in order to understand its role in the heterodimeric form p66/p51 found in virions. A recombinant form of RT, p51/p51, expressed in yeast, was purified and characterized. The enzyme was affinity labeled using a 5′ modified oligonucleotide primer, covalently linked, that was further elongated in the presence of a radioactive dNTP precursor. We found that the p51 subunit was labeled in the p51/p51 form, thus reflecting its activity, while this subunit was catalytically silent in the heterodimer, since only the p66 subunit was labeled in the latter recombinant form. Processivity studies showed long-sized products synthesized by p51/p51, as in the case of the other RT forms. The effect of primer $tRNA^{Lys}$ on the p51/p51 activity showed a strong inhibitory effect in the absence of KCl, similar to that observed with the p66/p51 form, while the same p51/p51 enzyme was strongly stimulated by $tRNA^{Lys}$, like RT p66/p66, when KCl was present in the incubation mixture.

Reverse transcriptase; Recombinant form; p51 subunit; Affinity labeling; Primer tRNA interaction; HIV-1

1. INTRODUCTION

Human immunodeficiency virus type-1 reverse transcriptase (HIV-1 RT) is an enzyme playing a crucial role in the replication of this human retrovirus (for a recent review see [1]). As with other retroviral RTs, this DNA polymerase is able to catalyze three reactions, all essential, to transform the single-stranded retroviral RNA genome into double-stranded proviral DNA: polymerization of a DNA strand complementary to the retroviral genomic RNA template, hydrolysis of the RNA moiety of the DNA–RNA hybrid (RNAse H activity) and synthesis of the second DNA strand. In virions, HIV-1 RT is thought to occur as a dimer containing polypeptides of M_r 66,000 and 51,000 (p66 and p51, respectively) at a molar ratio of 1:1. The two polypeptides are identical except that p51 is deprived at its C-end of a fragment of M_r 15,000 corresponding to the RNAse H domain of the enzyme. Very little is known either about the protein–protein interactions that govern dimer formation, or the role of p51 in the heterodimeric enzyme. The idea that p66, which contains the DNA polymerase and RNAse H domains of the enzyme, constitutes the catalytic subunit has been accepted, while no function has been proposed for p51. Although this subunit has the DNA polymerase domain several reports have indicated that purified p51/p51 was either inactive or had very low levels of DNA polymerase activity [2–6]. Other reports, however, have shown its activity through activated gel analysis of the viral enzyme [7] or in recombinant RT [8–10]. More recently, Le Grice et al. showed that wild-type p51 RT failed to restore activity to a heterodimer whose p66 subunit was mutated [11]. Thus, if the catalytic ability of p66 seems clear, the role of the p51 subunit has not been established.

Our aim is to study the mechanism of action of HIV-1 RT with a special emphasis on the initiation step of cDNA synthesis, as well as the search of specific inhibitors of this enzyme. For this purpose we started working on the interactions of RT with its specific natural primer, $tRNA^{Lys}$. Using different approaches we showed complex formation between a recombinant form of HIV-1 RT and bovine $tRNA^{Lys}$ [12], as well as the significant structural changes in the enzyme conformation induced by complex formation with primer tRNA [13]. While testing the initiation of cDNA synthesis from $tRNA^{Lys}$ annealed to a fragment of viral RNA carrying the primer binding site (PBS), we observed that the p66/p51 form of the enzyme was strongly inhibited by the slightest excess of free $tRNA^{Lys}$ [14]. More recently we showed that the p66/p66 form of the enzyme

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produced in a protease deficient yeast strain was stimulated by tRNA under conditions where the heterodimeric enzyme p66/p51 was shown previously to be strongly inhibited. Both activities, DNA polymerase and RNAse H, carried by the p66 form were affected similarly (Andreola et al., submitted for publication).

In this article we have attempted to study the role of the p51 subunit by characterizing the DNA polymerase activity of the homodimeric form p51/p51. We show that significant activity is present in this recombinant protein. Moreover, we describe the effect of $tRNA^{Lys}$ on the DNA polymerase activity of p51/p51, as well as the ability of p51/p51, p66/p66 and p66/p51 to initiate DNA synthesis from a primer covalently attached to the corresponding polypeptides by affinity labeling.

2. MATERIALS AND METHODS

2.1. *Materials*

Unlabeled nucleotides, oligonucleotides or polynucleotides were obtained from Sigma or Pharmacia. [^{3}H]dTTP was purchased from Commissariat à l'Energie Atomique-Saclay (CEA-France) or Amersham (UK). DEAE-Trisacryl M was from IBF, phosphocellulose P-11 from Whatman, Mono S HR 5/5 from Pharmacia, heparin–agarose from Sigma. Aprotinin, leupeptin, pepstatin A were from Sigma. Phenyl methane sulphonyl fluoride (PMSF) was from Boehringer and benzamidine from Fluka. The oligonucleotide $(pA)_{14}$ was obtained by hydrolysis of poly rA using the endonuclease from snake venom of *Naja naja oxiana* [15].

2.2. *Plasmid construction*

The parent yeast expression plasmid, pBS24, and the yeast alcohol dehydrogenase-2/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP) hybrid promoter have been described [16]. For construction of the RT5 gene encoding amino acids Pro^{156} to Leu^{715} of the HIV-1 *pol* open reading frame, the previously described RT4 gene [17] was modified using synthetic oligonucleotides. The 78mer duplex included the restriction enzyme *Asp*718 target site at amino acid Trp^{690} and a *Sal*I site immediately following the termination codon [5]. Oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystems 380A DNA synthesizer.

2.3. *HIV-1 RT purification*

The HIV-1 RT p66/p51 was obtained as described before [12]. RT p66/p66 and p51/p51 were purified as described for the heterodimeric form but using a protease-deficient yeast strain (JSC 302, derivative of AB 116 : Mat a, leu 2, trp-1, ura 3-52, prB1-1122, pep 4-3, prC1-407 [cir°] transformed either with the expression vector pBS24RT5 or pBS24RT6, respectively) [5]. During the extraction and the purification of the enzyme the following proteinase inhibitors were used: 1 mM PMSF, 1 μg/ml each of aprotinin, leupeptin and pepstatin A and 0.5 mM benzamidine.

2.4. *Purification of bovine tRNAs*

tRNAs from beef liver were purified as described before [14].

2.5. *Reverse transcriptase assay*

The reaction mixture contained in a final volume of 0.05 ml, 50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, 10 mM dithiothreitol, 0.48 OD_{260}/ml poly(rA)-oligo(dT) (5:1), 0.5–1 μCi [^{3}H]dTTP (56 Ci/mmol), 50 μM dTTP and enzyme as indicated. 80 mM KCl was used for p66/p51 and p66/p66, while no KCl was present in the assay of p51/p51. Incubation was carried out at 37°C for various times. Reactions were stopped by the addition of 1 ml of cold 10% trichloroacetic acid plus 0.1 M sodium pyrophosphate. The precipitates were filtered through nitrocellulose membranes, washed with 2% trichloroacetic acid, dried and counted in a PPO/POPOP/toluene scintillation mixture.

Product analysis: the reaction mixture, incubated at 30°C, contained 50 mM Tris–HCl, pH 8.0, 10 mM dithiothreitol, 5 mM $MgCl_2$, 80 mM KCl (p66/p51 and p66/p66) and no KCl for p51/p51, 0.48 OD_{260}/ml poly(rA)·$(dT)_{10}$ and 10 μCi α-[^{32}P]dTTP (3,000 Ci/mmol). After a pulse of 5 min, 100 μM dTTP was added and the reaction proceeded for different lengths of time. The reaction was stopped by the addition of 12 mM EDTA and 0.1% SDS. The products were phenol-extracted and precipitated with ethanol. Samples were resuspended in sample buffer: TBE (100 mM Tris–HCl, pH 8.3, 0.083 M borate and 2 mM EDTA) plus 10% glycerol, 80% deionized formamide, 0.1% xylene cyanol and 0.1% Bromophenol blue. Migration buffer was TBE. Electrophoresis was performed on a 14% polyacrylamide gel in the presence of 8 M urea for 3.5 h at 20 V/cm. The gel was then submitted to autoradiography on 3MR films.

2.6. *Affinity labeling of HIV RT with modified primers*

The reaction mixture containing in a final volume of 15–25 μl, 125 mM Tris–HCl, pH 8.0, 10 mM Mg acetate, 1 μM RT, $p(A)_{14}$ as template and an oligothymidylate analog modified at its 5′ end as primer (the detailed procedure of synthesis and affinity labeling with this analog will be described elsewhere) was incubated for 2 h at 30°C. The covalent binding of primer to enzyme was stabilized by addition of an excess of $NaBH_4$ (5 mM) and dithiothreitol (0.75 mM). After 20 min at 0°C, followed by an incubation of 20 min at 30°C the polymerization reaction was started by addition of 10 μCi of α-[^{32}P]dTTP. Primer elongation was done for 90 min at 30°C. Samples were then submitted to SDS-PAGE (12% acrylamide). After electrophoretic migration the gel was stained with Coomassie brilliant blue, dried and submitted to autoradiography on 3MR films.

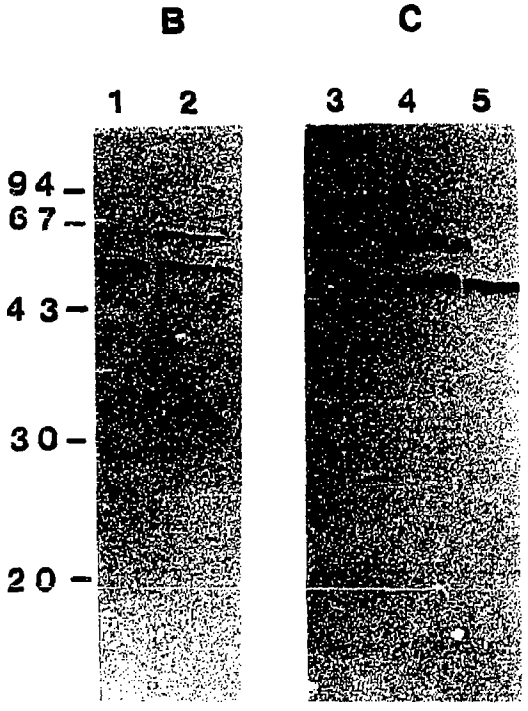

Fig. 1. (A) Purification of the three forms of HIV-1 RT. One unit of enzyme activity is defined as that amount catalyzing the incorporation of 1 nmol of [^{3}H]dTMP/h at 37°C. Assays were performed as described in section 2. (B) SDS-PAGE of purified RT p51/p51 (lane 1) and p66/p51 (lane 2). Proteins were stained with Coomassie blue. (C) Western blot analysis of the three forms of HIV-1 RT. Immunoblotting was performed as described in [14]. Lane 3, p66/p66; lane 4, p66/p51; and lane 5, p51/p51.

3. RESULTS AND DISCUSSION

As stated above, virions of HIV-1 contain approximately equal amounts of the two reverse transcriptase polypeptides, p66 and p51. The presence of these two peptides in equimolar amounts has led to the assumption that the enzyme found in the virions is a heterodimer. Interestingly, in avian retroviruses, reverse transcriptase is also a heterodimer complex; however, in this case the catalytic subunit is the smaller polypeptide (α subunit) which possesses both DNA polymerase and RNAse H activities, while subunit β, which still possesses the integrase domain, has very low catalytic activity and seems to be involved in the recognition of the specific primer tRNA [18]. In the case of HIV-1 the polypeptide possessing the integrase activity is found in the virions as an independent protein of M_r 32,000.

Recombinant proteins have been extensively used for the study of this viral polymerase given the safety risks of manipulating highly pathogenic agents and the very low yield of reverse transcriptase when purification is attempted from virions. Although some of the expression systems used are able to produce the heterodimer, most of the work reported has been performed with expression systems that produce the homodimer p66/p66. Data obtained with the p66/p66 form are very useful to study the catalytical properties of HIV-1 RT, because the DNA polymerase domain is essentially identical in the p66/p66 and in the p66/p51 forms. However, the nearest the chosen target is (in this case HIV-1 RT) to the heterodimeric enzyme found in virions, the best equivalences will be obtained between in vitro and in vivo experiments. As stated above, different groups have reported that the p51 chain was either inactive in DNA polymerization, had very low levels of activity or was weakly active as a loosely associated dimer. Thus, the question remains open concerning the role of the p51 subunit in the mature retroviral polymerase.

In order to answer this question we have tried to further characterize the p51 subunit of HIV-1 RT. We expressed this protein by transforming protease deficient yeast cells with a plasmid containing the reverse transcriptase gene deleted of its C-end in the domain attributed to the RNAse H activity. Purification of p66/p51, p66/p66 and p51/p51 recombinant forms was performed through several chromatographic steps as described before [12,14]. The summary of the purification procedure for the three recombinant proteins is shown in Fig. 1A. The two homodimers showed a lower, but important, specific activity when compared with that of the p66/p51 form. The degree of purification to apparent homogeneity of these enzymatic forms was very similar (Fig. 1A). Immunoblotting using antibodies raised against p66/p51, obtained as described before [12–14], showed a positive reaction with polypeptides identical to those revealed by staining (Fig. 1B). It is worth mentioning that to measure the activity of RT

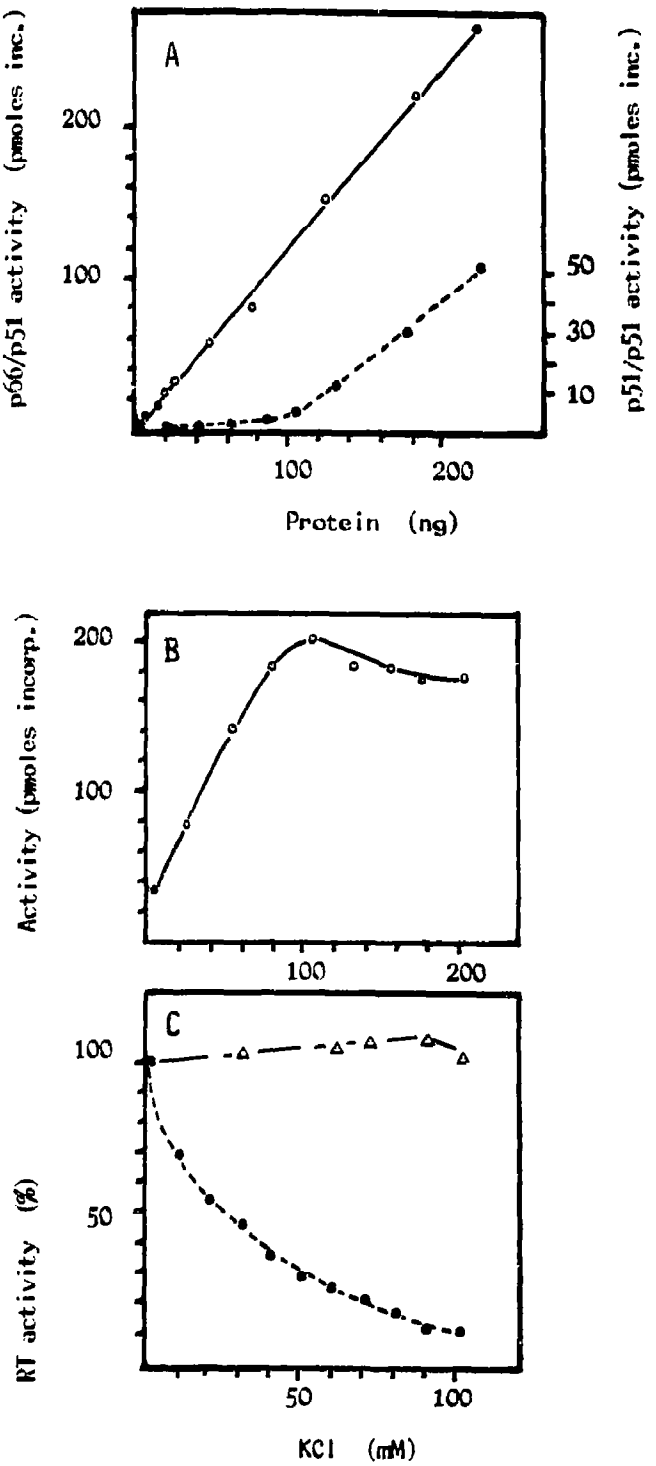

Fig. 2. (A) Activity of RT p66/p51 and p51/p51 as a function of protein concentration. Different concentrations of RT p66/p51 (○) or p51/p51 (●) were assayed as described in section 2. Incubation was for 10 min at 37°C. Effect of KCl on the activity of (B) RT p66/p51 (○) or (C) p66/p66 (△) and p51/p51 (●). The concentration of RT in each case was 34 nM p66/p51, 30 nM p66/p66 and 39 nM p51/p51.

p51/p51 different factors must be considered. First, the non-linearity in cDNA synthesis when important dilutions of the enzyme were used impaired an accurate

assay. As shown in Fig. 2A, RT p51/p51 must be rather concentrated in the enzymatic test (100 ng/assay) to obtain linearity as a function of protein concentration, while RT p66/p51 can be diluted in the same range without loosing its activity (1–2 ng/assay). Second, the strong inhibition of this enzyme by salt led us to eliminate KCl from all dialysis during the purification procedure and from the enzymatic test. In fact a striking difference between the p51/p51 and the two other recombinant forms of HIV-1 RT is the effect of KCl on these proteins. As seen in Fig. 2B the p66/p51 form is strongly stimulated by KCl, the p66/p66 homodimer is not affected by salt and the p51/p51 enzyme activity is extremely sensitive to KCl (Fig. 2C). This property of RT p51/p51 may explain the previous claims of other groups, who assayed the enzyme in the presence of salt, concerning the lack or very low activity of the p51/p51 recombinant form. Similar sensitivity of the p51/p51 form was observed with urea: the p66/p51 and p66/p66 forms were not affected by urea at final concentrations higher than 2 M, while the p51/p51 form was 50% inhibited at 0.5 M urea (data not shown). It has been reported that dimer association in the case of the p51/p51 is much weaker than in the case of the two other recombinant forms [19]. Thus, we explored the possibility that the inhibitory effect of KCl was due to the monomerization, and hence inactivation of the enzyme, since it has been reported that both p66 and p51 monomers are catalytically inert [9]. Not shown are results indicating that after molecular filtration on an AcA 44 ultrogel column in the presence of 0.1 M KCl, the p51/p51 form is completely inactivated, although more than 50% of the enzyme eluted as a dimer when followed by its reaction with the anti-RT IgG.

These results indicate that HIV-1 RT p51/p51 possesses a DNA polymerase domain with a significant degree of activity provided that the assay is performed under the appropriate conditions. The effect of strong inhibitors of reverse trasncriptase, like AZTTP, ddTTP and TIBO (Pharmatex, NJ, USA), showed no significant differences between the p66/p51, p66/p66 and p51/p51, except in the case of ddTTP which inhibited the p51/p51 to a lesser extent than the two other recombinant enzymes (results not shown).

Next we tried to answer the question whether the catalytic ability of the p51 subunit can operate in both dimeric forms. For this purpose each one of the RT forms was covalently bound to an oxidized primer analog of oligo dT modified at its 5′ end. After reduction with $NaBH_4$ to stabilize the complex formed between the enzyme and this modified primer, α-[^{32}P]dTTP and the other components of the incubation mixture necessary for cDNA synthesis were added. Incorporation of the dTMP residue was evidenced after SDS-PAGE and autoradiography. As it can be seen in Fig. 3A the p66 band of the heterodimer is labeled, while the p51 subunit showed no elongated primer. As expected, radioac-

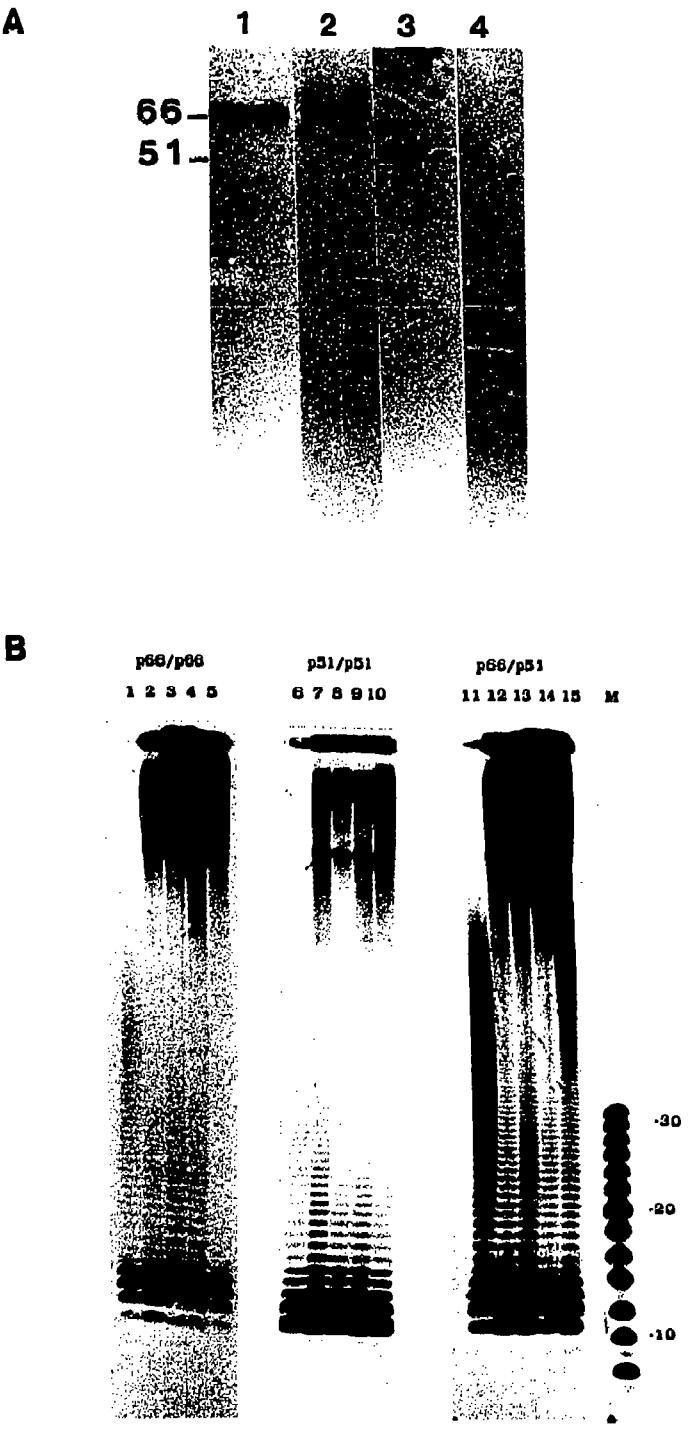

Fig. 3. (A) Affinity labeling of the three forms of RT. The reaction of labeling was done as described in section 2, using 1 μM of RT. Lane 1, p66/p51; lane 2, p66/p66; lane 3, p51/p51; lane 4, same as lane 1, but no $(pA)_{14}$ template was present during the covalent binding of primer analog. (B) Analysis of the products synthesized by the three forms of RT. The enzymes were incubated in the conditions described in section 2. After a pulse of 5 min (lanes 1, 6 and 11), 100 μM dTTP was added and the reaction proceeded for 10 min (lanes 2, 7 and 12), 20 min (lanes 3, 8 and 13), 30 min (lanes 4, 9 and 14) and 60 min (lanes 5, 10 and 15). Lane M, oligonucleotide sizing markers of 8 to 32 bases (Pharmacia) labeled in 5′ with γ-[^{32}P]ATP (Pharmacia) were used.

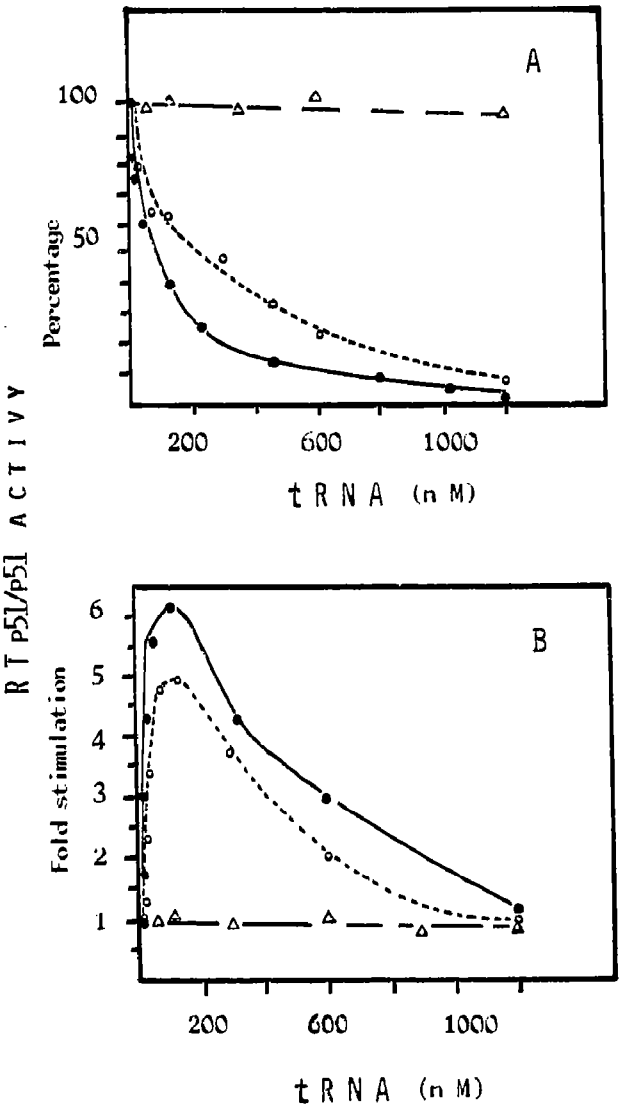

Fig. 4. Effect of tRNA on the activity of RT p51/p51. (A) 39 nM RT p51/p51 was pre-incubated for 15 min at 37°C in the presence of different concentrations of $tRNA^{Lys}$ (●), $tRNA^{Trp}$ (○) or $tRNA^{Val}$ (△). After the pre-incubation, the activity of p51/p51 was measured as described in section 2. (B) Same as in A, but KCl (80 mM) was present when measuring the activity of RT p51/p51.

tivity in the p66/p66 form was confined to the high molecular weight subunit, while the p51 subunit was labeled in the lane corresponding to the p51/p51 form. No labeling was obtained when the modifying reaction was done in the absence of template (Fig. 3A, lane 4). Thus, our results clearly indicate that the p51 subunit, although active as a homodimer, is catalytically silent in the mature form of HIV-1 RT. An interesting possibility opened by this approach is the fact that we have labeled the 'active' primer binding site of the enzyme. Further work is under progress to localize and sequence the peptide fragment of HIV-1 RT corresponding to this active site. This approach, in the absence of a crystallographic clear structure of the DNA polymerase domain of the enzyme, will help to get a clear picture of the topography of interactions between RT and its substrates.

In spite of the set of properties of p51/p51 described above the question about the role of the p51 subunit in the HIV-1 RT heterodimer is still unanswered. The processivity of DNA polymerases, i.e. the ability to synthesize long stretches of DNA without dissociating from the template–primer complex, is an important parameter governing the function of these enzymes. Results shown in Fig. 3B indicate that the processivity of HIV-1 RT during DNA synthesis using a poly(rA)·oligo dT template·primer system is essentially the same in the three recombinant forms. Thus, we have shown not only that the DNA polymerase activity of the p51/p51 form is significantly high, but that the size of the cDNA products synthesizing by this recombinant protein is similar to that obtained using the p66/p51 and p66/p66 forms.

An interesting possibility that deserves to be explored is a possible role of the p51 subunit on tRNA recognition, as it has been shown for the β subunit of the avian retroviral RTs [18]. All known reverse transcriptases use a host tRNA as the specific primer for the synthesis of the first strand of DNA. Evidence from our laboratory and others [20–23] has shown that reverse transcriptase is involved in the selection of the specific primer from the pool of total cellular tRNAs and it is, at least in part, involved in the annealing of the primer tRNA to the complementary region, PBS, in the retroviral genome [24,25]. In previous articles we have shown that the p66/p51 form of HIV RT can form a stable complex with bovine $tRNA^{Lys}$ and that the heterodimeric form of HIV-1 RT can be inhibited by its primer tRNA [12,13]. Nuclease digestion of $tRNA^{Lys}$ allowed us to localize the anticodon region as the probable origin of the inhibitory effect [14]. Recently we have shown that the homodimer p66/p66 was able to form a complex with $tRNA^{Lys}$; the interaction with primer tRNA was confirmed by the fact that the HIV-1 RT p66 form was strongly stimulated by $tRNA^{Lys}$ under conditions where the p66/p51 form was inhibited (Andreola et al., submitted for publication). A similar approach was used in this article with the p51/p51 recombinant form. The effect of $tRNA^{Lys}$ on RT p51/p51 was performed in the presence or absence of KCl. Results under these two conditions were diametrically opposed: $tRNA^{Lys}$ and $tRNA^{Trp}$ strongly inhibited the activity of the p51/p51 homodimer in the absence of KCl (Fig. 4A), while a stimulation of this recombinant enzyme form was obtained when DNA synthesis was assayed in the presence of salt (Fig. 4B). No effect was observed with $tRNA^{Val}$, as has been described before for the other recombinant forms.

Our results show that tRNA, by affecting the enzyme activity, is able to interact with this recombinant form. The effect of tRNA on the p51/p51 form is similar to

that observed previously concerning the interactions between p66/p66 and p66/p51 with $tRNA^{Lys}$. In the case of the latter recombinant enzymes important structural changes of the enzyme were induced by formation of a complex with the specific primer tRNA. Work is in progress to isolate a complex $tRNA^{Lys}$–RT p51/p51 and to study the topography of the interaction between $tRNA^{Lys}$ and p51/p51, as well as the other RT recombinant forms used in this work.

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