Characterization of the double stranded RNA dependent RNase activity associated with recombinant reverse transcriptases

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ABSTRACT

An *in situ* gel assay was applied to the study of double stranded RNA dependent RNase activity associated with reverse transcriptase (RT) of HIV-1 and murine leukemia virus. Polyacrylamide gels containing [³²P] RNA/RNA substrate were used for electrophoresis of proteins under denaturing conditions. The proteins were renatured and *in situ* enzymatic degradation of ³²P-RNA/RNA was followed. *E.coli* RNaseIII, but not *E.coli* RNaseH, was active in this *in situ* gel assay, indicating specificity of the assay to RNA/RNA dependent nucleases. Analysis of purified preparations of HIV-1 RT p66/p51 expressed in *E.coli* demonstrated an RNA/RNA dependent RNase activity comigrating with the large subunit (p66) of the enzyme. In addition, this activity of the RT was often accompanied by a contaminating RNA/RNA dependent RNase, with a molecular weight ~30,000 dalton identical to that of *E.coli* RNaseIII. As the p51 small subunit of HIV-1 RT and a mutant of RT p66/p51, at Glutamic acid #478, did not exhibit RNA/RNA dependent RNase activity, at least part of the active site of the RNA/RNA dependent RNase activity appeared to reside at the carboxy end of the molecule. As these RT proteins are also deficient of RNaseH, our results suggest overlapping or identical catalytic sites for degradation of the substrates RNA/DNA and RNA/RNA.

INTRODUCTION

The two enzymatic activities of reverse transcriptase (RT), DNA polymerase and RNaseH, have been localized to distinct domains at the amino and carboxy termini of the polypeptide, respectively. In the native RTs of viruses such as Moloney murine leukemia virus (MMuLV) the two enzymatic active sites appear to function independently (1). Thus, recombinant polypeptides derived from the amino and carboxy end of MMuLV RT exhibit either DNA polymerase or RNaseH activities (2). Mutation analysis of the HIV RT has indicated that the two domains are interdependent and many mutations which affect one activity are also detrimental to the other active site. Nevertheless, the arrangement of these activities in the HIV-1 enzyme is similar to that of MMuLV (3–7).

In the last several years the substrate specificity of the DNA polymerase active site of HIV RT has been extensively studied as it is a prime target for antiviral drugs development. Relatively little is known, however, about the substrate specificity of the RNaseH site. We have recently demonstrated a double stranded RNA dependent RNase activity associated with purified preparations of HIV-1 RT expressed in *E.coli* (8). This distinct activity comigrates with the native RT (p66/p51) through several chromatographic steps, which separate native proteins based on size, charge or hydrophobicity. These chromatographic techniques, however, did not yield information as to which of the RT two subunits p66/p51 manifests the RNA/RNA dependent RNase activity. To further characterize this activity of RT we have applied in the present work an *in situ* RNA/RNA gel assay similar to the DNA/RNA gel assay used extensively to characterize the RNaseH activity of RT (2,5,6).

MATERIALS AND METHODS

RNA/RNA gel assay: A cDNA pF12 (9) of 1300bp, cloned in the EcoRI site of pBluescript (Stratagene Co.), was used as a template for the RNA preparations. pF12 DNA was linearized at the polylinker site with NotI endonuclease and transcribed from the T7 promoter with T7 polymerase to produce antisense RNA. pF12 linearized with ClaI endonuclease was transcribed from the T3 promoter with T3 polymerase for sense RNA. Transcription kit was purchased from Promega Corp. and utilized as recommended with α [³²P]-CTP (Amersham Corp). Specific activity of synthesized RNA was 10⁵cpm/ μ g. The reaction mixtures were treated with 10 units of DNaseI (Promega Corp) for 15 min at 37°C, extracted twice with phenol/chloroform and ethanol precipitated. Sense and antisense [³²P]-RNAs were hybridized at 2:1 ratio in a buffer of 30% formamide, 40mM Tris HCl pH 7.4, 1mM EDTA and 0.3M NaCl. Hybridization was initiated by 10 min incubation without salt at 65°C and proceeded with NaCl at 42°C for 2hr. RNA/RNA was purified from excess single stranded RNA by electrophoresis on 1% agarose gel (10), or used without gel purification.

[³²P] RNA/RNA (~10⁴–10⁵ cpm per 4ml gel) was immobilized in a 10% SDS polyacrylamide gel (10). Proteins in 3% SDS sample buffer (with reducing agent, 2mM DTT) were heated for 1 min at 95°C, unless otherwise indicated. Following

electrophoresis the gels were soaked for 2 days in 100 ml of a solution containing 50mM Tris HCl pH 8.0, 50mM NaCl, 5mM DTT, 2mM MnCl₂ at room temperature with several changes of the buffer. SDS is removed during this incubation, permitting renaturation/activation of enzymes. The degraded [³²P] RNA diffuses from the gel and clearing zones are detected after autoradiography (B). Gels were stained with Coomassie brilliant blue before exposure to x-ray films (A). Enzymes: purified *E. coli* RNaseIII was a gift from Ofri Pines of the Hebrew University, Jerusalem. *E. coli* RNaseH was purchased from Promega Co. BH10 HIV RT p66/p51 was expressed in *E. coli* and purified as described (11). Briefly, the RT in *E. coli* extract was fractionated by ammonium sulfate followed by chromatography on four resins, phenyl-Sepharose, Q-Sepharose, phosphocellulose, and Superose gel. Specific activity of the purified RT (5,000 units/mg) was determined by a standard RT assay using poly(A)/oligo(dT) as a template/primer (12). SDS polyacrylamide gel analysis indicated that the enzyme is over 95% pure. HIV-1 RT enzymes p66/p51, p66/p66, p51 and RT Glu⁴⁷⁸→Gln, which contained six histidine residues at the amino terminus, were expressed in *E. coli* and purified by metal chelate Ni+2 affinity chromatography as described before (13,17). Purified MMuLV RT expressed in *E. coli* was purchased from USB Co.

Figure 1. Analysis of nuclease specificities with the RNA/RNA gel assay. RNA/RNA gel assay was carried out as described in Materials and Methods. *E. coli* extract was prepared by solubilizing cells in sample buffer containing 2% SDS. Purified *E. coli* RNaseIII and RNaseH are described in the text. S.M. protein standard markers of molecular weights 94, 67, 43 30 20 & 14 kD. (A) Coomassie stain, (B) Autoradiography.

RESULTS

In order to further characterize the RNA/RNA dependent RNase activity associated with HIV RT, we have adapted an *in situ* gel assay that is commonly used in the studies of RNaseH (2,5,6). Sense and antisense RNAs were produced *in vitro*, the template DNA was removed by DNaseI digestion and the two

Figure 2. HIV-1 RT activity in the RNA/RNA gel assay. HIV-1 RT (p66/p51) (3–15µg) was applied on the gel as described in Material and Methods. (A) Coomassie stain, (B) Autoradiography.

Figure 3. MMuLV RT activity in the RNA/RNA gel assay. MMuLV RT (p80) 1–4µg was applied on the gel as described in Material and Methods. (A) Coomassie stain, (B) Autoradiography.

complementary RNAs were hybridized. The double stranded RNA was separated on an agarose gel and the RNA/RNA structure was validated by resistance to digestion by S1 nuclease at high salt (10). Uniformly [32 P]-labelled RNA/RNA was incorporated into an SDS polyacrylamide gel and preparations of denatured proteins were applied for electrophoresis. The proteins were renatured *in situ* in a buffer containing Mn^{++} to facilitate RNase activity. Clearing zones detected upon exposure of such a gel to x-ray film represent degradation of RNA/RNA to small oligonucleotides.

To determine the specificity of the RNA/RNA activity gel, crude *E. coli* extract as well as purified preparations of *E. coli* RNaseH and RNaseIII were tested (Fig. 1). Only one clearing zone, at a molecular weight of $\sim 30,000$, could be observed in the *E. coli* extract (B). The molecular weight of this activity corresponds to that of a purified preparation of *E. coli* RNaseIII. As the many other *E. coli* proteins observed in the Coomassie blue stained gel (A) do not produce clearing zones in the gel, the specificity of the *in situ* assay appears to be high. RNaseIII appears to be the only enzyme in extracts from *E. coli* that selectively degrades RNA/RNA structures (19). *E. coli* RNaseH which is highly specific to RNA/DNA structures (14) shows no activity in the RNA/RNA gel assay. In hybrid RNA/DNA activity gel, *E. coli* RNaseH produces a clearing zone corresponding to a protein of $\sim 20,000$ dalton (2,5). Since specific activities of purified preparations of both *E. coli* RNaseIII and RNaseH were very high, only small amounts of these proteins were applied to the gel and, therefore, no stained proteins could be detected in the corresponding lanes (Fig. 1 A).

Figure 4. Analysis of HIV RT polypeptides in the RNA/RNA activity gel. HIV RT preparations (2 and 4 μ g), w.t. p66/p51 homodimer p66/p66, p51 and p66/p51 Glu⁻ mutant were loaded on the gel and analyzed as described in Material and Methods. (A) Coomassie stain (B) Autoradiography.

These results demonstrate the applicability of the RNA/RNA activity gel for the detection of new RNA/RNA dependent nucleases in extracts. Moreover, in combination with Coomassie blue staining this assay may serve to identify correlation of subunit proteins with RNA/RNA dependent RNase activity in partially purified enzyme preparations.

When a purified preparation of HIV-1 RT was analyzed in the RNA/RNA gel, an RNase activity corresponding to a molecular weight of about 67000 was observed (Fig. 2B). This activity comigrated with the large subunit (p66) of RT, as evident from the Coomassie blue stained gel (Fig. 2A). No RNase activity could be detected at a position corresponding to the small subunit (p51) of RT. Increasing amounts of RT showed larger clearing zones in the activity gel (compare lanes 3–15 μ g). Exposure of the gel to X-ray film immediately following the electrophoresis (zero time) showed no clearing zones, indicating that this zones do not represent electrophoresis artifacts.

We next examined whether purified RT of Moloney murine leukemia virus (MMuLV) also exhibited similar RNase specificity. RNase activity is clearly observed in the RNA/RNA gel assay (Fig. 3B), corresponding to the MMuLV RT protein with a molecular weight of $\sim 80,000$ (Fig. 3A). Increasing amounts of MMuLV RT again produced larger clearing zones in the activity gel. The results presented in Figures 2 and 3 suggest correspondence between the RT proteins and the RNA/RNA dependent RNase activity.

In an attempt to further investigate this RNase activity by genetic means, we have analyzed four preparations of HIV RT, which were purified in parallel by metal chelate affinity chromatography technique (13,15,17). The p66/p66 homodimer exhibited both DNA polymerase and RNaseH activities (15). The p51 preparation was deleted of 120 amino acids from the carboxyl terminus of RT and it showed low DNA polymerase and no RNaseH (15). The Glu⁻ RT is a heterodimer (p66/p51) with a point mutation Glu⁴⁷⁸→Gln. This mutation at the active site of

Figure 5. Detection of *E. coli* RNaseIII in purified preparations of HIV-1 RT expressed in *E. coli*. Recombinant HIV RT preparations were analyzed in the RNA/RNA gel assay, without boiling of the proteins before loading onto the gel. RT I (4 μ g) was purified according to Le Grice et al. (17). This enzyme preparation was also used in the experiments shown in Fig. 2,3 and 4. RT II and RT III preparations (6 μ g each) were purified according to Fisher et al. (11). *E. coli* extract (40 μ g) was analyzed for RNaseIII. (A) Coomassie stain, (B) Autoradiography.

RNaseH does not affect the DNA polymerase activity (13). In the RNA/RNA gel assay, both RT p66/p66 and wild type RT p66/p51 showed RNase activity, while p51 and RT Glu⁻ were inactive (Fig. 4). BSA, which was used as a control protein showed no RNA/RNA dependent nuclease activity. These results further indicated that the RNA/RNA dependent RNase is inherent to the RT protein. Furthermore, at least part of the site responsible for this activity appears to be located within the carboxy end of RT, which also corresponds to the RNaseH site.

The application of the RNA/RNA activity gel in these studies demonstrated that many of the available purified preparations of RT expressed in *E. coli* were contaminated with *E. coli* derived RNA/RNA dependent RNase activities. The pattern of the RNA/RNA dependent RNases in some of these recombinant RT preparations is shown in Fig. 5. In addition to the RNase activity which corresponds to the p66 subunit of HIV RT, one of the HIV RT preparations, (RT III), manifested RNA/RNA dependent RNase activities which migrated as ~30,000 and ~52,000 dalton proteins. These two activities were also evident in *E. coli* extracts and may, therefore, represent monomer and dimer of RNaseIII (16). In the experiment presented in Fig. 5, the protein samples were not heated prior to electrophoresis; the RNase activity at 52,000 molecular weight could be chased, however, by heating of the protein samples as shown in Fig. 1. Since active RNaseIII of *E. coli* is found as both monomer and homo dimer (16), boiling of the samples (Fig. 1) dissociated all the dimer form of RNaseIII into monomers.

DISCUSSION

We demonstrated in the present work, with the use of the RNA/RNA gel assay, double stranded RNA dependent RNase activity associated with both HIV-1 and MMuLV RTs. Several lines of evidence indicate that the clearing zones observed in the gel represent enzymatic degradation of the [³²P] RNA/RNA substrate. (i) The plasmid that was used as a DNA template for the transcription of [³²P] RNA was digested by DNaseI. Furthermore, following hybridization of sense and anti-sense RNAs, the RNA/RNA substrate was purified on agarose gel and was shown to be resistant to S1 nuclease and RNaseA. (ii) Purified *E. coli* RNaseIII but not *E. coli* RNaseH showed activity in the RNA/RNA gel assay, as expected for their known substrate specificities (14). (iii) The reaction was dependent on divalent ion (Mn⁺⁺). Although the gel assay is semi quantitative (6), increasing amounts of RT produced larger clearing zones. However, as it is impossible to estimate the amount of RT which undergoes renaturation in the gel assay, enzyme kinetic studies on RNA/RNA substrates, will have to be carried out in solution. It should be noted that most, if not all, available purified preparation of RT expressed in *E. coli* are contaminated with *E. coli* RNaseIII. The later enzyme appears to cochromatograph with native RT p66/p51 during the purification procedure. We reason that even recombinant RTs which in the RNA/RNA gel assay do not show RNaseIII may exhibit this activity in a more sensitive assay solution. Thus, we are aware that the purified recombinant HIV RT used before (8) contained traces of *E. coli* RNaseIII. Moreover, it should be noted that in the presence of Mg⁺⁺ ions, no RNA/RNA nuclease activity could be detected in the gel assay; it is possible that ion preference is dependent on the substrate used (18). We conclude, therefore, that the activity reported previously in the presence of Mg⁺⁺ (8) was related to RNaseIII.

Analysis of RT p66/p66 and p51 localized the RNA/RNA dependent RNase active site to the carboxyl end of the molecule. The deficiency of RT Glu⁴⁷⁸→Gln for both RNA/DNA and RNA/RNA substrates suggests that the two activities are manifested by the same or by overlapping active sites in the RNaseH domain. Furthermore, the two RNase activities appears to degrade heteropolymeric RNAs. As the DNA polymerase site can utilize both RNA and DNA template/primers, it is possible that the RNaseH site in the presence of Mn⁺⁺ is permissive for RNA/DNA and RNA/RNA substrates. It may differ in this respect from *E. coli* RNaseH which shows selectivity for RNA/DNA hybrids (14).

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