Reverse transcriptase (RT) isolated from Rous sarcoma virus (RSV) consists of heterodimeric RTαβ, RTα, and RTβ. The α subunit (63 kDa) contains an N-terminal polymerase and a C-terminal RNase H domain. The N terminus of β (95 kDa) corresponds to α with the inte- grase domain attached to the C terminus (32 kDa). We have constructed baculoviruses expressing the genes for α or β or the entire pol (99 kDa). Infection of insect cells with recombinant virus yielded highly active and soluble RSV RT enzymes that could be purified to >90% homogeneity. HPLC gel filtration showed that α is a dimeric enzyme that can be partially monomerized upon the addition of 45% Me2SO. DNA synthesis on DNA-DNA and DNA-RNA primer-templates in the presence of competitor substrates revealed that αβ and β as well as α are processive polymerases. However, the affinity of β and αβ for primer-template substrates appears to be higher than that of α. All RSV enzymes investigated have the potential to displace RNA-RNA duplexes more efficiently than human immunodeficiency virus type 1 RT. Unlike human immunodeficiency virus type 1 RT, RSV RTs can catalyze an initial RNase H endonucleolytic cleavage of the RNA template but not a 3′ → 5′ directed processing activity.

Reverse transcriptase (RT) is the key enzyme required for retroviral replication. Retroviral RTs are encoded by the pol gene and are expressed as large precursor proteins together with the gene products of the gag gene. The Gag-Pol precursor is processed into individual proteins by the viral protease. RTs from closely related lentiviruses like human immunodeficiency virus (HIV-1 and HIV-2), simian immunodeficiency virus and equine infectious anemia virus show similar RT organization (1–4). The RTs of these viruses are heterodimeric with a large ~66-kDa subunit harboring the N-terminal polymerase and the C-terminal RNase H domains, and a small ~51–58-kDa subunit lacking the RNase H domain. The RT from mouse leukemia virus is a monomeric ~80-kDa polypeptide that was suggested to dimerize only upon binding to nucleic acid (5). Like HIV-1 RT, it contains an N-terminal polymerase domain and an RNase H domain at the C terminus.

The situation in the group of avian sarcoma and leukemia viruses (ASLV) including Rous sarcoma virus (RSV) is quite different. Pol is composed of the polymerase, RNase H, and integrase domains. The C terminus of Pol harbors a short 4.1-kDa protein, which is removed by the viral protease during processing of the Pol precursor protein (6). It has been shown to be dispensable for the formation of virions (7). The reverse transcriptase of RSV consists of an α and a β subunit. The α subunit with a molecular weight of 63 kDa comprises the N-terminal polymerase and the C-terminal RNase H domain. The β subunit (95 kDa) contains the N-terminal polymerase and RNase H domains and the integrase domain at the C terminus (8–11). The integrase domain is also present as an independent protein with a molecular mass of 32 kDa (10, 12). Three forms of RT have been isolated from ASLVs: homodimeric RT β and heterodimeric αβ and α (8, 13, 14). The α protein isolated form virions has been suggested to exist as either a monomer or dimer (8, 14). Glycerol gradient centrifugation analysis of recombinant RSV α isolated from Escherichia coli indicated that the enzyme is active as a homodimer. It sedimented in a single peak corresponding to a molecular mass of about 105 kDa. The recombinant α protein was shown to exhibit considerable polymerase activity with long DNA products being synthesized (15).

The presence of the three different Pol cleavage products, α, β, and αβ, in virions suggests that they might have different roles during viral replication. Interestingly, recent findings with the RT of human T-cell leukemia virus type 1, the etiologic agent of human adult T-cell leukemia, expressed in an in vitro transcription/translation system, indicate a subunit organization similar to RSV RT, with the larger subunit harboring the integrase domain (16).

Because of the different subunit organization of RSV RT, we are interested in comparative studies to obtain more information on structure-function relationships of retroviral RTs. Previous analyses of RSV RT functions have caused problems, since the pol-derived proteins do not normally exist in a soluble and active form in infected cells. Furthermore, isolation of RSV RT from virions does not allow the introduction of mutations that are lethal to the virus. Efforts to express the α and β RT subunits of ASLV RT in E. coli proved to be difficult, because the major fraction of the recombinant proteins (~90%) was found in inclusion bodies (15).

To circumvent these problems, we expressed the α and β subunits of RSV RT as well as Pol by means of recombinant baculoviruses constructs in insect cells. It has been shown recently that various constructs of recombinant baculoviruses harboring gag-pol or pol sequences of avian leukemia virus
expressed in insect cells exhibit RT activity (17). An optimized purification procedure of the His-tagged recombinant proteins now allows us to obtain pure and soluble proteins in sufficient amounts for further analysis. Here we show that the purified recombinant proteins α, β, αβ, Pol, and αPol possess polymerase as well as RNase H activity. We describe the enzymatic characterization of the purified proteins, which yields valuable information about structure-function relationships of RSV RTs in comparison with RTs from other retroviruses.

**EXPERIMENTAL PROCEDURES**

**Buffers**

Annealing buffer consisted of 20 mM Tris-HCl, pH 7.5, and 50 mM NaCl. RT buffer contained 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 50 mM KCl, 5 mM DTT, 0.1% Triton X-100. Formamide loading buffer was prepared as described (18). Lysis buffer contained 20 mM Tris-HCl, pH 7.5, 25% glycerol, 1 mM NaCl, 3 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.01% IGEPA CA 630 (Sigma) and 1 mM MgCl₂. 2X SDS loading buffer consisted of 8.5% SDS, 35% glycerol, 410 mM monothioglycerol, 0.05% bromphenol blue in 120 mM Tris, pH 8.5. Enzyme storage buffer contained 20 mM Tris-HCl, pH 7.0, 250 mM NaCl, 50% glycerol, 0.1% IGEPA CA 630, and 2 mM DTT. Enzyme dilutions were performed in enzyme storage buffer.

**Construction of Baculovirus Transfer Vectors**

For cloning of the different RT genes and pol into the baculovirus vector, a subclone of the RCASBP (A) vector of RSV was used (19). This subclone contains the entire pol gene and short flanking regions from nucleotide 5970 (PsrI site) to nucleotide 9089 (PvuII site) of RCASBP (A) in the vector pUC 119. Using a combination of polymerase chain reaction amplification and restriction site cloning, the genes encoding the α subunit and pol were cloned into a baculoviral vector in order to obtain a 5’ BamHI and a 3’ stop codon followed by a HindIII site. The desired DNA fragments were then cloned into the BamHI/HindIII-restricted baculovirus transfer vector pBlueBacHis2A (Invitrogen). The transfer vector encoding the β protein was produced by cleaving the pol containing transfer vector with KpnI and SalI, thus removing the 3’-terminal region of pol coding for the 4.1-kDa protein. A shortened 3’ fragment containing the correct end of the β gene and a TAA stop codon was produced via polymerase chain reaction and cloned into the KpnI/SalI-restricted pol-containing transfer vector. All of the recombinant baculoviruses constructs express RSV RTs that contain an additional 34 amino acids at the N terminus of the proteins derived from the transfer vector. This 34-amino acid extension includes a His tag, 11 amino acids of gene 10 from phage T7, and the enterokinase cleavage site vector (Invitrogen). In addition, β was also expressed in a similar construct lacking the N-terminal extension. This construct was used to produce heterodimeric αβ RT in which only the α subunit possesses the His tag for nickel-chelate affinity chromatography. No significant differences from the heterodimer containing His extensions on both subunits were observed.

**Isolation of Recombinant Baculoviruses**

Recombinant baculoviruses were produced by co-transfecting SF 21 insect cells with viral DNA from the wild type baculovirus AcMNPV (Bac-N-Blue-DNA, Invitrogen) together with recombinant transfer vector DNA. Homologous recombination between the transfer vector DNA and the polyhedrin gene of wild type virus DNA in vivo leads to expression of the recombinant gene under the control of the polyhedrin promoter. Transfection was performed using the Bac-N-Blue transfection kit (Invitrogen) according to the manufacturer’s protocol with minor modifications as follows. After the addition of the transfection mixture, the cells were incubated at 27 °C. After 4 h, 1 ml of TC 100 medium (Life Technologies, Inc.) plus 20% fetal calf serum (FCS) was added, and the cells were incubated further at 27 °C. 72 h later, the supernatant was removed, and 2 ml of fresh TC 100 plus 10% FCS was added. Cultured supernatants were harvested 5 days after transfection and tested for recombinant virus by plaque assays according to the manufacturer’s protocol. The supernatants were stored at 4 °C.

**Preparation of High Titer Viral Stocks**

Isolation of viral DNA from the culture supernatants (see above), and polymerase chain reaction analyses were performed according to the protocol from Invitrogen. Recombinant virus that was free of wild type virus DNA as determined by polymerase chain reaction was used for preparing high titer viral stocks according to the protocol from Invitrogen with the exception that TC100 plus 10% FCS was used for cell growth. Viral stocks were kept at 4 °C. Virus titers were determined by end point dilution. The virus titer was calculated as described by Gruenwald and Heita (20). Virus titers determined were in the range of 7 to 107 to 3 x 109 infectious units/ml.

**Characterization of RSV RT Enzymes**

Small amounts of SF 21 cells were grown in TC 100 plus 10% FCS as monolayers in 75-cm² culture bottles. Large amounts of cells were grown as shaker cultures in 1.8-liter Fernbach flasks (360 ml/ flask). 0.1% Phorunic F68 was added to the growth medium (TC100 plus 10% FCS) to prevent aggregation of the cells. After growth of the cells at 27 °C to a density of 2.5–3 x 10⁵ cells/ml, cells were concentrated to a titer of 8 x 10⁶/ml and infected with recombinant virus with a multiplicity of infection of 10. For the expression of the heterodimeric proteins αPol and αβ, cells were coinfected with the two corresponding viruses with a multiplicity of infection of 5 for each virus. After 1 h at 27 °C, cells were diluted with fresh medium to their original concentration and shaken further at 27 °C. Cells expressing β or Pol were harvested 60 h postinfection, and all other infected cells were harvested 72 h postinfection. Cells were centrifuged for 10 min at 2500 x g and 4 °C, and the cell pellets were stored at −20 °C.

**Optimization of the Purification of RSV RT Proteins**

2–4.5 liter of cells were grown as shaker cultures and infected and harvested as described above. After centrifugation, the cell pellets were resuspended in lysis buffer, and the cells were lysed by sonication and centrifugation. The supernatant was dialyzed against 20 mM Tris-HCl, pH 7.5, 10% glycerol, 500 mM NaCl, 1 mM DTT, and 0.01% IGEPA CA 630 (Sigma), the solution was diluted in the same buffer lacking NaCl to reach a concentration of 75 mM NaCl for α or of 150 mM NaCl for all other RSV RT enzymes. The solution was loaded onto a nickel-nitrilotriacetic acid-Sepharose column (Qiagen). RT was eluted by applying a gradient of 15–300 mM imidazole. After dialysis of the eluate in a buffer containing 20 mM Tris-HCl, pH 7.5, 10% glycerol, 500 mM NaCl, 1 mM DTT, and 0.01% IGEPA CA 630 (Sigma), the solution was diluted in the same buffer lacking NaCl to reach a concentration of 75 mM NaCl for α or of 150 mM NaCl for all other RSV RT enzymes. The solution was loaded onto a Sephacryl 100 column (High-Trap, Amersham Pharmacia Biotech), and with 50 volumes of the corresponding loading buffer. RSV α was eluted by applying an NaCl gradient from 0.075 to 1.2 x NaCl. For all other RSV RTs, a gradient from 0.15 to 1.2 x NaCl was used. The eluted RTs were about 90–95% homogeneous. The eluted enzymes were dialyzed against enzyme storage buffer and could be stored at −20 °C under these conditions for several months without losing activity. The concentration of the enzymes was 45 μg/ml for β and in the range of 1–2.5 mg/ml for all other RSV RTs.

**HPLC Gel Filtration Analysis**

HPLC size exclusion chromatography was performed as described previously (21) at a flow rate of 0.5 ml/min in a buffer containing 50 mM Tris-HCl and 500 mM NaCl. The molecular mass standard kit for HPLC from U.S. Biochemical Corp. was used for calibration. The standard proteins were run under the same conditions as the RSV enzymes. The retention times of the marker proteins were used to obtain a calibration curve for determination of the molecular mass of RSV α.

**Quantitative RT Activity Assay**

DNA-dependent DNA polymerase activity was quantitated on a poly(A) oligo(dT)12-18 substrate in a standard assay (10-μl reaction volume with 5–15 ng of enzyme in RT buffer). Under these conditions, 1 unit of RT activity catalyzes the incorporation of 1 nmol of dTTP into poly(rA)·oligo(dT)12-18 in 10 min at 37 °C.

**Analysis of Polymerization Products by High Resolution Gel Electrophoresis**

DNA-dependent DNA Polymerase—Products of DNA-dependent DNA synthesis were determined using single-stranded bacteriophage M13 DNA to which a 32P-end-labeled 17-mer primer was hybridized (23). 0.1 pmol of primer-M13 DNA substrate and 0.1 pmol of the desired RSV RT were preincubated in RT buffer at 37 °C. Polymerization was started by the addition of 250 μM dNTPs and incubated further at 37 °C for 10 min in a total reaction volume of 10 μl. Reactions were stopped with 10 μl of formamide buffer and heating to 95 °C for 3 min and
Characterization of RSV RT Enzymes

loaded onto a 10% denaturing polyacrylamide gel containing 7 M urea. To analyze processivity and substrate affinity, either poly(rA)/oligo(dT)_{12–18} or an 18/36-mer DNA-DNA p-t was incubated with 0.1 pmol of RT in RT buffer in the absence of dNTPs for 10 min or 40 min in a total volume of 10 μl. The reactions were stopped by the addition of 10 μl of formamide buffer, and the hydrolysis products were analyzed on 10% denaturing gels as described above.

The same substrate (0.1 pmol) was used to determine RNase H activity during DNA polymerization. In this case, stepwise polymerization from the 3'-OH end of the primer was accomplished using a mixture of dNTPs (50 μM) containing one chain-terminating ddNTP (250 μM). Depending on the ddNTP chosen, elongation of the primer by 4 nucleotides (dATP plus ddGTP), 10 nucleotides (dATP, dGTP, ddTTP), or 19 nucleotides (dATP, dGTP, ddTTP, ddCTP) was achieved. The addition of four dNTPs leads to full extension of the primer. Reactions were started by the addition of 0.1 pmol of RT in a total volume of 10 μl, incubated for 10 min at 37 °C, and further analyzed as described above. When DNA polymerization products were examined with this substrate, similar assays were performed, however with the 36-mer DNA primer labeled at the 5’ end.

RESULTS

Purification of RSV pol Gene Products—The availability of RSV RT as a soluble recombinant protein that can be purified in sufficient amounts is an important prerequisite for the analysis of enzyme function. Since it has been shown previously that expression of the α and β subunits of RSV RT in E. coli leads to mainly insoluble proteins (15), we decided to make use of an E. coli expression system. We constructed recombinant baculoviruses expressing RSV α, β or Pol and infected Sf21 insect cells with these viruses. Co-infection of Sf21 cells with two different types of viruses yielded heterodimeric RSV αβ and αPol. Pol and αPol were included in some of our studies to analyze the influence of the C-terminal 4.1-kDa extension of Pol on enzyme activity. The 4.1 kDa polypeptide is cleaved off during virus maturation to yield β and αβ.

In order to get the different forms of RSV RT into the soluble fraction of the cell lysate and purify them to homogeneity, we modified the purification protocol described for partial purification of pol gene products from insect cells by Stewart and Vogt (17). Since the affinity of the pol gene products to DNA appears to be very high, 1 M NaCl was added to the lysis buffer. 25% glycerol and 0.01% of detergent (IGEPAL CA 630) were added to avoid aggregation of the proteins. Fig. 1 shows a Coomassie stain of the enzymes after purification over nickel-nitritotriacetic acid-Sepharose and heparin columns. The preparation of Pol contains an additional band with an approximate molecular mass of 95 kDa. The same band is present in the αPol preparation. We assume that this protein corresponds to the Pol gene product shortened by the C-terminal 4.1-kDa protein by cellular proteases and thus corresponds to RSV β.

Quantitative Analysis of Polymerase Activity—In a quantitative RT assay, the RNA-dependent DNA polymerase activities of the purified enzymes were determined on the homopolymeric substrate poly(rA)/oligo(dT)_{12–18} and compared with commercially available AMV RT purified from virions and with HIV-1 RT purified in our laboratory (21). The specific activities are summarized in Table I. For better comparison, enzyme activities were also calculated as units/pmol of enzyme. Our results demonstrate that all of the RSV RTs purified are highly active and that their activity is comparable with that of commercially available AMV RT purified from virions. The activities of α and αβ are higher than those of the other RSV RT enzymes. The heterodimeric αPol enzyme appears to be less active than the mature RT αβ. The low activity of β might be due to the low concentration of the stock solution of RT β (45 μg/ml), which can lead to protein instability. However, we were not able to increase the expression level of the β protein. In general, higher activities were found for α and αβ in different polymerization assays (see below).

Determination of the Quaternary Structure of RSV RT α—It has been shown previously that RT isolated from virions of ASLV consists of αβ heterodimers, β homodimers, and α proteins. RSV RT α has been suggested by different groups to be enzymatically active as either a monomer or dimer (8, 14, 15).

To determine whether RSV RT α isolated from insect cells is active as a monomer or dimer, we analyzed purified RSV RT α by HPLC gel filtration. Fig. 2A shows that RSV RT α yields a single peak with a retention time of 26.6 min. The corresponding molecular mass of 117 kDa was determined by comparison with molecular mass standard proteins under the same conditions (see “Experimental Procedures”).

Since the apparent molecular mass found for α was higher than that expected for the monomer (67 kDa), we assumed a homodimeric organization and attempted to monomerize the enzyme. The use of acetonitrile to monomerize RSV RT α as described for HIV-1 RT was unsuccessful. However, we were able to partially monomerize the α homodimer (3.9 μM) by the addition of 45% Me2SO (26). The enzyme was incubated for 15 min on ice in the presence of Me2SO in a buffer consisting of 20 mM Tris-HCl, 5% glycerol, 475 mM NaCl, 0.1% IGEPA CA 630, and 2 mM DTT. HPLC gel filtration analysis shows the appearance of a peak with a retention time of 30.35 min (peak 3), corresponding to a molecular mass of 41 kDa (Fig. 2B). To determine whether this peak corresponds to the monomerized α protein or to a protein degradation product, the Me2SO-treated enzyme was analyzed by SDS-polyacrylamide gel electrophoresis. In addition, all of the peak fractions were collected, precipitated with trichloroacetic acid, and also analyzed by SDS-polyacrylamide gel electrophoresis. Coomassie staining of the gel (Fig. 2C) shows that no degradation products are visible.
activities on a homopolymeric substrate

Activities are given in units/mg or units/pmol protein, where 1 unit catalyzes the incorporation of 1 nmol of dTTP in poly(rA)/oligo(dT)12–18 in 10 min at 37 °C. For calculation of the molarity, the enzymes were assumed to be present as dimers.

| Enzyme | units/mg protein | units/pmol protein |
|--------|-----------------|-------------------|
| α      | 40,400          | 4.5               |
| β      | 12,815          | 2.4               |
| Pol    | 12,510          | 2.2               |
| αβ     | 34,150          | 5.7               |
| αPol   | 17,170          | 2.6               |
| HIV-1 RT p66/51 | 59,730          | 7.0               |
| AMV RT | 28,260          | 4.5               |

activities similar to those of AMV RT. The other RSV RT α is responsible for the polymerase activity of the enzyme. No band is visible with the fractions corresponding to peak 1 (16.12 min), indicating that this peak is not due to protein.

To analyze the polymerase activity of the partially monomerized α, the sample was diluted 10-fold to reach a Me2SO concentration of 4.5% (final concentration of α was 390 nM). The RT polymerase activity of the Me2SO-treated sample was reduced to about 40% as compared with untreated α protein incubated in buffer with 4.5% Me2SO. This result suggests that the polymerase activity measured with the partially monomerized sample is probably due to remaining dimeric protein.

Qualitative Analysis of DNA-dependent DNA Polymerization Activities—For qualitative analysis of DNA-dependent DNA polymerase function, single-stranded M13 DNA was used to which a 5′-end-labeled 17-mer DNA primer was hybridized. For comparison, AMV RT and HIV-1 RT were included in the experiment. Fig. 3 shows that RSV RT α is highly active and yields long extension products similar to AMV RT. No short polymerization products are visible with α. The other RSV enzymes appear to be less active than α, yielding also short extension products with only a few nucleotides added to the primer and a major pause site at +8/+9 nucleotides. In addition, high molecular weight DNA products are synthesized. All RSV enzymes stall at similar sites; however, more of the high molecular weight DNA products are synthesized with α, αPol and αβ than with Pol and β. Similarly to the data obtained with the RT activity assay (Table I), Pol and β are less active than the other RSV enzymes (i.e. less DNA product is synthesized).

The processivity of a polymerase is defined as the number of nucleotides incorporated before the enzyme dissociates from the template (27). To obtain some information on the processivity of the enzymes on DNA templates and on their affinities to the M13 substrate, competitor substrates were added to the assay. Since Pol and αPol revealed qualitative polymerase activities similar to those of β and αβ, respectively, they were not included in the assay. The results of these analyses are shown in Fig. 4. poly(rA)/oligo(dT)12–18 or an 18/36-mer DNA-DNA was added in a 100- or 1000-fold molar excess over the M13 substrate (see "Experimental Procedures"). Interestingly, already in the presence of a 100-fold excess of poly(rA)/oligo(dT)12–18, no extension products are visible with α, indicating a significantly lower affinity to the M13 DNA-DNA substrate than that of β and αβ. On the contrary, competition with poly(rA)/oligo(dT)12–18 or the 18/36-mer DNA-DNA still yields long extension products with β or αβ. Since determination of the molar excess of poly(rA)/oligo(dT)12–18 is not very precise (see "Experimental Procedures"), we cannot unequivocally conclude that the affinities of α or the other enzymes for DNA-RNA p-ts is higher than for DNA-DNA p-ts.

Our result indicates, however, that all enzymes tested are processive. In contrast to results published previously (14), we show here that α polymerizes in a processive mode, since long products are synthesized even after the addition of competitor.
Furthermore, α pauses at the same sites as αβ and β. The major difference between α and the other two enzymes appears to be the reduced affinity of RSV RT α for nucleic acid substrates.

Qualitative Analysis of RNA-dependent DNA Polymerization Activities—The results described above were confirmed on RNA templates. RNA-dependent DNA polymerization activity was analyzed on a homopolymeric poly(rA)/oligo(dT)12–18 substrate. oligo(dT)16 was radioactively labeled and hybridized in a 5-fold molar excess to poly(rA) with an average length of 357 nucleotides. Fig. 5A shows that all RSV enzymes tested are highly active on this substrate and capable of synthesizing high molecular weight products that are longer than the average template length of 357 nucleotides. This result indicates that α, β, and αβ are able to catalyze strand transfer reactions efficiently.

The behavior of α, αβ, and β was further analyzed in the presence of either a 100- or a 1000-fold excess of unlabeled poly(rA)/oligo(dT)12–18 or of 18/36-mer DNA-DNA p-t (Fig. 5B). Again, poly(rA)/oligo(dT)12–18 was more efficient as a trap. The experiment confirms that the affinity of α for nucleic acids is lower than that of the two other enzymes tested. Nevertheless, even after the addition of the competitor substrate, α produces high molecular weight DNA. Our results demonstrate that α, αβ, and β are able to synthesize in a processive mode on DNA as well as RNA templates.

Qualitative Evaluation of RNase H Activity—In contrast to the RTs from lentiviruses like HIV-1 RT, RSV RTs possess two RNase H domains in the homodimeric α, Pol, and β enzymes as well as in the αβ or αPol heterodimers. The heterodimeric HIV-1 RT p66/p51 harbors the RNase H domain only in the larger p66 subunit. To determine whether the different subunit composition has an impact on the properties of the RNase H activities, we performed qualitative RNase H assays with the various RSV RTs. A 5’-end-labeled 127-mer RNA was hybridized to a 36-mer DNA and incubated with RT. Fig. 6B shows that the RNase H activities of all RSV RTs investigated are similar with respect to the cleavage site in the DNA-RNA hybrid. However, in comparison with HIV-1 RT, there is a striking difference. It has been shown that retroviral RTs function as endonucleases (28–31). In addition, after the endonucleolytic cleavage by HIV-1 RT, the enzyme reveals a 3’ → 5’ directed processing activity (30–32) leading to a time-dependent shortening of the 5’ RNA fragment after cleavage of the hybrid. With the substrate used, HIV-1 RT performs an endonucleolytic cleavage at nucleotide 71 of the template strand, which is followed by 3’ → 5’ directed processing of the cleaved 5’ RNA strand to position 62 (Fig. 6, A and B) (33, 34). Contrary to the results observed with HIV-1 RT, even after prolonged incubation times of 40 min, no directed processing activity can be detected with any of the RSV RTs (Fig. 6B). Rather, only the endonucleolytic cleavage at positions 71 and 72 is performed. Furthermore, this result indicates that the distance between the active sites of polymerase and RNase H is similar to that in HIV-1 RT (31, 35, 36) and corresponds to about 18 nucleotides.

RNase H Cleavage after Primer Elongation—The same DNA-DNA p-t substrate was used to investigate the RNase H activities of RSV RTs α, αβ, and β in the presence of dNTPs (Fig. 7). The polymerization process was stopped after the incorporation of 4, 10, or 19 nucleotides by the addition of the appropriate ddNTP. The addition of four dNTPs allows polymerization of the full-length DNA product. Previous enzymatic analyses of a p-t substrate possessing the same template overhang sequence by Ghosh et al. (34) revealed extensive secondary structures in the putative single-stranded region of the RNA template. In
fact, the entire region of the template overhang is involved in the formation of an extended intramolecular hairpin structure. Therefore, elongation of the DNA primer by RT requires strand displacement activity. Fig. 7B shows that at the enzyme concentrations used, HIV-1 RT performs RNA cleavages at sites close to the site used in the absence of dNTPs. This implies that due to the extensive secondary structures of the template overhang, primer extension is impaired with HIV-1 RT, thus allowing the RNase H to cleave the RNA before DNA synthesis can start. However, obviously each of the RSV RTs tested can unwind the RNA secondary structures very effectively and extend the primer to the end of the template. This is demonstrated by the presence of a short RNase H cleavage fragment of about 16 nucleotides in length at the bottom of the gel. These results imply that there is a qualitative difference in strand displacement activities of the RTs derived from RSV as compared with HIV-1 RT.

To prove that polymerization with such a template is less efficient with HIV-1 RT, we performed a control experiment with the same substrate but with 5' end-labeled primer DNA to visualize the polymerization products. Fig. 7C shows strong pause sites with HIV-1 RT after extension of 4 and 15 nucleotides in the presence of four dNTPs, allowing full extension of the primer. Again, RSV RT β appears to be less active than the other RSV RTs, as indicated by the larger amount of extended 36-mer primer DNA.

**DISCUSSION**

One purpose of this investigation was to establish an expression system that allows production of soluble RSV RT enzymes in sufficient amounts to purify them to homogeneity in order to obtain more reliable information on their enzymatic functions. In this study, we show that the baculovirus/Sf21 insect cell expression system fulfills this goal, and this allowed us to characterize the RTs of RSV more thoroughly than previously possible. Furthermore, since the expression system chosen is independent of RSV replication, it enables us to produce mutant RSV RTs and assess their function. This is of special interest when mutations are introduced that would be lethal to virus replication.

In this report, we describe the expression of five different products of RSV pol in Sf21 insect cells in a soluble form. Besides the three enzyme forms normally isolated from virions (α, β, and αβ), two additional proteins were expressed and purified; the full-length Pol and the heterodimeric αPol were included to study whether the 4.1-kDa extension of Pol has an impact on polymerase and RNase H activities. Quantitative analysis of the RT activities of the purified RSV RT enzymes shows that their activities are comparable with that of AMV RT isolated from virions, demonstrating that this expression system yields RSV RTs that are functional and can be used for further analysis.

One important question with respect to RT function in general is whether RTs are active as monomers, dimers, or even multimers. For HIV-1 RT p66/p51, it has been shown that the

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2 S. Werner and B. Wohrl, manuscript in preparation.
monomeric subunits do not possess enzymatic activities (37, 38). RSV RT αβ and β have been isolated from virions as dimeric proteins (14). However, at 20 °C and 4% glycerol the native active form of AMV RT appears to be a tetramer composed of two αβ heterodimers (αβ)2 (39).

Hizi and Joklik (14) suggested that the α subunit isolated from RSV exists and is active as a monomer. On the other hand, RSV RT α isolated from E. coli appears to be active as a dimer (15). Glycerol density centrifugation of RSV RT α isolated from virions yielded a molecular mass of about 90 kDa, which did not allow a precise determination of the quaternary structure of α (8). In this report, we show that the molecular mass of 117 kDa we determined corresponds to the dimeric form of α, since it can be partially monomerized upon the addition of 45% Me2SO. Our results are in agreement with those obtained by Soltis and Skalka (15), suggesting that the active form of RSV RT α is the homodimer.

Qualitative analysis of polymerization activities shows that all RSV RTs can synthesize long DNA products as well as RNA templates, thus being processive polymerases. This has been shown previously for β and αβ. Previous reports with α isolated from virions implied that α is a distributive polymerase (15). This was determined by Hizi et al. (40) by analyzing the amount of radioactively labeled nucleotide incorporated into a homopolymer in the presence or absence of a competitor substrate. However, this method does not allow measurement of the length of the synthesized DNA, which is an important parameter for determination of processivity.

In this paper, we identified the products of DNA polymerization synthesized by α, β, and αβ. Determination of product lengths with DNA or RNA templates shows that RSV RT α can synthesize long DNA fragments similar to those synthesized by β or αβ, even after the addition of competitor substrate (Figs. 5 and 6). Therefore, we suggest that α is a processive polymerase. However, the affinity of the enzyme toward nucleic acid substrates is reduced as compared with β and αβ, presumably due to the lack of the integrase domain. The decrease in affinity does not make the enzyme a distributive polymerase, since long products are synthesized even in the presence of competitor substrate. The reduction in affinity can also explain the results observed by Hizi et al. (40) described above, since they did not examine the length of the DNA product but the incorporation of nucleotides into the competitor substrate. If the decrease in affinity was due to an increase in the dissociation rate, one would expect a distributive enzyme. This effect was found for homodimeric equine infectious anemia virus p51/p51 RT. In that case, deletion of the RNase H domain of the p66 subunit makes the enzyme distributive (24). Our results with RSV RT α suggest a different mechanism, i.e. faster incorporation of nucleotides as compared with homodimeric β or heterodimeric αβ. Alternatively, it is possible that the affinity of the enzyme increases in the presence of nucleotides or after the enzyme has changed from the initiation to the elongation mode of polymerization. Further experiments are necessary to elucidate these hypotheses.

Our results imply that one major function of the integrase domain in the β subunit is to increase the affinity to the substrate. All other enzyme functions analyzed do not differ significantly between RSV RT α and RSV RT β or αβ (see below). However, the differences in substrate affinities might lead to differences in fidelity or in the rates of nucleotide incorporation and polymerization. Experiments are being performed to test these possibilities.

We find that RSV RT α as well as RSV RT β and αβ are capable of performing template switching, indicated by polym-
Faras (44) showing that during reverse transcription of avian activity, which HIV-1 RT requires. Therefore, efficient strand capable of unwinding longer double-stranded regions than strand displacement activity of RSV RTs, these enzymes are processing activity. We suggest that due to the highly efficient relationships of RSV RTs.

Another striking difference we observed with RSV RTs in comparison with HIV-1 RT is the absence of a 3′ → 5′ directed processing activity during hydrolysis of RNA (Fig. 6B). This activity of HIV-1 RT further degrades the 5′ RNA fragment after the initial endonucleolytic cleavage has occurred in the RNA-DNA substrate (30–32). In contrast to HIV-1 RT, RSV RTs do not further degrade the 5′ RNA fragment after performing the initial cut at positions 71 and 72. It has been proved that the 3′ → 5′ directed processing activity of HIV-1 RT is necessary for catalyzing the strand transfer reactions during reverse transcription of the viral genome (43). During the production of minus strand strong stop DNA the RNA is degraded by the RNase H activity of HIV-1 RT. When the end of the template is reached, an initial 14-mer RNA fragment of the template is still annealed to the DNA. This 14-mer is further degraded by the polymerase independent 3′ → 5′ directed processing activity of the RNase H to give rise to a shorter RNA fragment of 8 nucleotides. Only then can an efficient strand transfer process occur. It requires the binding of the new template strand and displacement of the 8-mer RNA fragment (43). The length of the RNA fragment created when RSV RT enzymes reach the end of the template is a 16-mer, which apparently is not digested further (Fig. 7B). This result further indicates that RSV RTs do not possess a 3′ → 5′ RNase H processing activity. We suggest that due to the highly efficient strand displacement activity of RSV RTs, these enzymes are capable of unwinding longer double-stranded regions than HIV-1 RT and thus do not need a 3′ → 5′ RNase H processing activity, which HIV-1 RT requires. Therefore, efficient strand transfer reactions can be performed even in the presence of longer hybrid regions at the end of the template. In addition, this is in agreement with earlier findings by Omer and Faras (44) showing that during reverse transcription of avian retroviruses the tRNA primer is released intact. A 3′ → 5′ RNase H processing activity would lead to a tRNA shortened by several nucleotides.

Our results demonstrate that there are significant differences in the mechanism of polymerization and RNase H hydrolysis between RTs from different retroviruses and that the results obtained with one retroviral RT are not necessarily valid for RTs from other retroviruses. Additional studies will be required to test the results found here more thoroughly and to examine the organization of the different RSV RT enzymes with respect to their function during reverse transcription and integration. The stability and high activity of the recombinant RSV RTs we obtained now enables us to construct mutant enzymes to further analyze the structure-function relationships of RSV RTs.

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