HIV-1 Rev Expressed in Recombinant *Escherichia coli*: Purification, Polymerization, and Conformational Properties

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ABSTRACT: The high-level expression of HIV-1 Rev in *Escherichia coli* is described. Protein in crude bacterial extracts was dissociated from bound nucleic acid with urea. A simple purification and renaturation protocol, monitored by circular dichroism, is described which results in high yields of pure protein. The purified protein binds with high affinity to the Rev-responsive element mRNA and has nativelike spectroscopic properties. The protein exhibits concentration-dependent self-association as judged by analytical ultracentrifugation and gel filtration measurements. Purified Rev showed reversible heat-induced aggregation over the temperature range 0–30 °C. This hydrophobic-driven and nonspecific protein association was inhibited by low concentrations of sulfate ions. Rev solutions at >80 μg/mL, incubated at 0–4 °C, slowly polymerized to form long hollow fibers of 20-nm diameter. Filament formation occurs at a lower protein concentration and more rapidly in the presence of Rev-responsive mRNA. The nucleic acid containing filaments are about 8 nm in diameter and up to 0.4 μm in length. On the basis of physical properties of the purified protein, we have suggested that in the nucleus of infected cells, Rev binding to the Rev-responsive region of *env* mRNA may be followed by helical polymerization of the protein which results in coating of the nucleic acid. Coated nucleic acid could be protected from splicing in the nucleus and exported to the cytoplasm.

Human immunodeficiency virus 1 (HIV-1)[1] contains two trans-activating nuclear proteins, namely, Tat and Rev, which regulate gene expression by binding to specific regions of viral mRNA (Dingwall et al, 1989; Daly et al., 1989; Green & Zapp, 1989). HIV Rev is a small (M_r = 13000) basic protein that is located in the nucleus (Cullen et al., 1988) and is essential for the expression of the viral structural genes, namely, *gag pol*, and *env* (Sodroski et al., 1986; Feinberg et al., 1986; Knight et al., 1987). Rev appears to facilitate the export of unspliced *env* mRNA to the cytoplasm (Emerman et al., 1989; Felber et al., 1989; Hammarkjold et al., 1989; Malim et al., 1989). In the absence of Rev, the *env* mRNA is retained in the nucleus and undergoes splicing. This results in increased levels of mRNAs for the nonstructural genes, including *tat*, *nef*, and *rev*.

Rev purified from recombinant *Escherichia coli* has been shown to bind specifically to a 224-nucleotide sequence referred to as the Rev-responsive element (RRE) of the *env* mRNA, and which is located at the junction encoding the gp120 and gp41 domains (Zapp & Green, 1989; Daly et al., 1989; Holland et al., 1990). Mutations in the RRE RNA in proviral DNA or in Rev-dependent gene expression systems result in loss of the Rev response (Rosen et al., 1988; Malim et al., 1989; Olsen et al., 1989; Holland et al., 1990). It is, therefore, believed that Rev function in vivo is dependent on the direct interaction of Rev with viral mRNA. How this specific RNA–protein interaction leads to the nuclear export of unprocessed *env* mRNA is not known.

Toward obtaining structural information on Rev and Rev–RNA complexes, we have investigated the conformational and physical properties of purified recombinant protein as well as its propensity to polymerize in vitro both in the absence and in the presence of RRE RNA.

Materials and Methods

Bacterial Strains and Plasmids. The *E. coli* strains used were MM294 (F-*end* A1, *hsd*r, *hsd*m, *sup*E44, *thi* 1–), DH1 (F *rec* A1, *end* A1, *gyr*A96, *thi* 1, *hsd* R17, *sup* E44, *hsd*M, –), and DH5, as DH1 but additionally (*rel*A1).

The Rev protein of the HIV-1 strain BH10 was expressed by using the pL-*ner* plasmid described by Allet et al. (1988). The region of this plasmid coding for the Ner protein was replaced with that encoding Rev. DNA encoding the first 30 amino acids of Rev was synthesized with a Model 380B DNA synthesizer (Applied Biosystems). DNA encoding the remaining 86 amino acids of Rev was supplied by the *Ava*I–*Rsa*I restriction fragment from nucleotides 7970–8297 of a proviral clone of BH10 (Ratner et al., 1985).

Protein Expression and Fermentations. Culture media, growth conditions, transformations, and fermentations at the 1.5-L scale were performed essentially as described by Allet et al. (1988). Temperature induction of cell cultures was at 42 °C for 3 h. A cell yield of 50–60 g wet weight/L was obtained.

Strain Selection. The plasmid pSS137 was transformed at 30 °C into a number of common strains of *E. coli*, but ex-

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[1] Abbreviations: CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DDT, dithiothreitol; HIV, human immunodeficiency virus; Rev, HIV antirepression transactivator protein; RRE, Rev-responsive element; TMV, Tobacco mosaic virus.

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pression was detected by Coomassie blue staining of SDS/PAGE analyses of whole cell extracts only for the strain DH5. MM294 is one of the strains used in the construction of the DH series of strains and was severalfold more efficient than either DH5 or its parent DH1 in the production of Rev. It was found that the introduction of the *gyrA* mutation from strain DH1 into MM294 resulted in a lower level of Rev production, similar to that found in either DH1 or DH5. The levels of Rev expression were 5–10% of the total cell protein for MM294 and about 3-fold lower for the *gyrA*-minus derivative. Strain MM294 was used as the production strain.

Purification of HIV Rev. The buffers used for protein preparation were as follows: buffer A, 100 mM Tris-HCl containing 5 mM dithiothreitol (DTT), 5 mM EDTA, 5 mM benzamidine, and 1 mM PMSF, adjusted to pH 8.0 with HCl; buffer B, 50 mM Tris-HCl/1 mM sodium azide, pH 8.0; buffer C, 20 mM sodium phosphate, pH 6.5, containing 2 M urea, 1 mM DTT, and 1 mM EDTA; buffer D, 50 mM sodium phosphate, pH 6.5, containing 0.6 M $(NH_4)_2SO_4$, 1 mM DTT, and 1 mM EDTA; buffer E, 50 mM sodium phosphate, pH 6.5, containing 0.15 M NaCl, 10 mM K_2SO_4, 1 mM DTT, and 1 mM EDTA. The temperature of all operations was 0–4 °C, and Rev in column fractions etc. was monitored by SDS/PAGE.

E. coli cells (100 g wet mass) were suspended into 200 mL of buffer A using a Polytron homogenizer (Kinematica, Basel) and broken by two passes through an Aminco French pressure cell (Kontron, Basel) operated at 124 mPa (18 000 lb/in.^{2}). The suspension was sonicated briefly to reduce the viscosity and centrifuged at 10000 *g* for 10 min. The supernatant was recentrifuged at 60000*g* for 60 min and the clarified solution (240 mL) diluted 3-fold with buffer B. The diluted supernatant (750 mL) was applied at 200 mL/h to a column (5-cm diameter × 20 cm) of DEAE-Sepharose (Pharmacia) equilibrated in buffer B. Rev was located in the flow-through fractions, which were pooled (700 mL), and solid urea was added to a final concentration of 6 M (900 mL). The solution was diluted 2-fold with buffer C, and the pH was adjusted with H_3PO_4 to 6.5 (conductivity = 2.2 mS^{-1}). The solution (1.8 L), which contained 4 M urea, was applied at 200 mL/h to a column (5-cm diameter × 20 cm) of Fast S (Pharmacia) equilibrated with buffer C. After the column was washed with 400 mL of column buffer, a 2-L linear gradient of NaCl (0–1.0 M) in buffer C was applied at 200 mL/h. Rev was eluted from the column with about 0.5 M NaCl. At this stage, the protein (400 mL at 1 mg/mL) contained 2 M urea and was >90% pure as indicated by SDS/PAGE. If further purification was required, the protein was concentrated by ultrafiltration using Diaflo PM10 membranes (Amicon) and applied to a column (2.5-cm diameter × 95 cm) of Ultrogel AcA54 (IBF) equilibrated with buffer C. Purified protein was either frozen and stored at −80 °C or directly applied to the protein refolding protocol described below.

Protein Refolding. Rev was concentrated to 1.5–2.0 mg/mL by ultrafiltration and solid $(NH_4)_2SO_4$ added to 0.6 M. The protein (100-mL batches) was applied to a column (5-cm diameter × 50 cm) of Sephadex G25 (Pharmacia) equilibrated in buffer D which contained 0.6 M $(NH_4)_2SO_4$. The protein peaks from separate chromatographic runs were pooled (420 mL) and dialyzed against two changes of 20 L of buffer E. The renatured protein was concentrated by ultrafiltration to 1–2 mg/mL and any precipitated or aggregated protein removed by centrifugation at 10000*g* for 30 min. The clear solution was sterile-filtered with 0.22-μm pore-size Millex-GV filter units (Millipore) and stored at −80 °C.

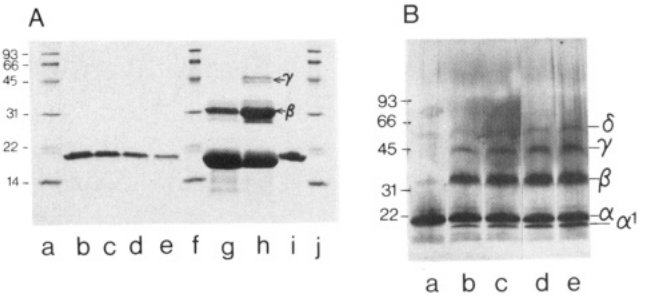

FIGURE 1: SDS/PAGE of HIV Rev. (A) Lanes a, f, and j, molecular weight standards. The values indicated on the left-hand side of the figure are $\times 10^{-3}$. Lanes b–e and i, purified Rev which contained little or no polymer, disaggregated with 10% (w/v) SDS and 20 mM DTT for 5 min at 90 °C; lane g, Rev with high polymer content treated the same as in (b) except heated for 10 min; lane h, as (g) except incubated at 25 °C for 30 min. The positions of dimeric (β) and trimeric (γ) proteins are indicated. The proteins were detected by staining with Coomassie blue. (B) Rev (0.20 mg/mL) cross-linked with dimethyl suberimidate. Lane a, control untreated protein; lanes b–e, protein incubated for 1.5 h at 22 °C with 0.5, 1.0, 2.0, and 3.0 mg/mL reagent, respectively. The positions of monomeric (α), dimeric (β), trimeric (γ), and tetrameric (δ) protein are indicated. The monomeric species α1 is probably derived from protein containing an intramolecular cross-link(s). Protein was visualized by $AgNO_3$ staining.

The yield of refolded protein was >80% based on the amount of purified and unfolded protein present after cation-exchange chromatography. To obtain these high yields, it was found important to perform the refolding stages below 10 °C (preferably at 4 °C). If the removal of urea by gel filtration was carried out, for example, at room temperature, protein aggregation and precipitation occurred during chromatography.

When the purification method and refolding scheme described above were used, about 3.5 mg of Rev was obtained per gram wet weight of *E. coli* cells. This represents an overall recovery of about 35% (based on a protein expression level of 10%). SDS/PAGE of the purified protein is shown in Figure 1A (lanes b–e).

Protein Determination. The concentration of purified protein was determined by measuring the absorbance at 280 nm. An approximate correction for light scattering was made by using a base-line value at 280 nm established by linear extrapolation of the absorbance between 350 and 320 nm. A calculated molar absorption coefficient (ε) of 8.34 mM cm^{-1} was used (based on a theoretical molecular mass of 13 036).

Analytical Separation Methods. SDS/PAGE and electrophoresis under native conditions were carried out using a PhastSystem (Pharmacia) according to manufacturer's instructions. Proteins were visualized by Coomassie blue or $AgNO_3$ staining. Gel filtrations of Rev (250 μL at 0.1–2.0 mg/mL) were performed on a Superose 12 column (30 cm × 1-cm diameter) connected to an FPLC system (Pharmacia). The flow rate was 1.0 mL/min, and buffer E (see above) was used as the eluent.

Analytical Ultracentrifugation. Measurements were made by using a Beckman LB-70 preparative ultracentrifuge equipped with a Prep UV scanner. For sedimentation equilibrium analysis, Rev (0.4–0.8 mg/mL) in buffer E was centrifuged at 12 000 rpm at 15 °C. Measurements were taken after 15 h, and a corrected base line was established by further centrifugation at 50 000 rpm for 4 h. For sedimentation velocity analysis, Rev (0.15–1.6 mg/mL in buffer E) was centrifuged at 40 000 rpm at 20 °C. Measurements were automatically recorded every 10 min for 100 min. Molecular masses and sedimentation coefficients were calculated from

the least-squares fits of plots of ln A_{280} vs r^2 and ln r vs t, respectively, where r is the distance from the axis of rotation and t is the time in seconds. Sedimentation coefficients were corrected to standard conditions ($s_{20,w}$). For molecular mass determinations, a partial specific volume ($\bar{v}$) of 0.712 mg/mL was used, calculated from the amino acid composition.

Chemical Analysis. Amino acid analysis, N-terminal sequence analysis, and sulfhydryl analyses were carried out as previously described (Wingfield et al., 1986).

Protein Cross-Linking. Rev (0.20 mg/mL) in 50 mM sodium phosphate, pH 7.8, 0.1 M K_2SO_4, 1 mM DTT, and 1 mM EDTA was treated with freshly prepared dimethyl suberimidate (Pierce) for 1.5 h at room temperature. The reaction was stopped by boiling in SDS.

Circular Dichroic Spectra. Spectra were recorded on a Jasco J-600 spectropolarimeter. Measurements in the far-ultraviolet region were made using a 0.01-cm path-length cell and 2-nm bandwidth. Measurements in the near-ultraviolet region were made using a 1.0-cm path-length cell and 1-nm bandwidth. All spectra are the averages of four to eight scans with the base lines subtracted. A mean residue molecular mass of 112.4 was used. Samples were filtered with 0.22-μm pore-size GV-Millex filter units before use.

Fluorescence Measurements. A Perkin-Elmer LS5 luminescence spectrometer was used. The excitation wavelength was 295 nm with 2.5-nm excitation and emission bandwidths. Spectra were recorded at 22 °C.

Light-Scattering Measurements. Measurements were made by using a Hewlett Packard HP 84850A diode array spectrophotometer equipped with an HP 89100A temperature control accessory. Rev (1 mL at 0.5 mg/mL in 50 mM sodium phosphate, pH 6.8, 0.1 M NaCl, and 1 mM DTT) was heated in a 1-cm path-length semimicro cuvette from 5 to 20 °C at 0.5 °C/min. The temperature was maintained at 20 °C until the absorbance reached a plateau; then the solution was cooled back to 5 °C at 0.5 °C/min. Measurements at 320 nm were recorded every 10 s. The protein solution was not stirred.

Electron Microscopy. Polymer-containing Rev solutions were prepared for electron microscopy by negative staining. Solutions were adjusted to 0.1–0.3 mg/mL protein in buffer E (see above) at 0–4 °C, and drops (5 μL) were applied to nitrocellulose-backed carbon films rendered hydrophilic by glow-discharge. Alternatively, polymers were collected by centrifugation, and the pellet was overlaid with a small volume of cold (4 °C) buffer and allowed to resuspend overnight at 4 °C. Drops of this resuspended material were diluted 5–20× in cold buffer E and applied to electron microscopy substrates as described above. After adsorption for 30–60 s, grids were washed twice with cold buffer, or buffer diluted 1:5 with water, and stained with a 1% (w/v) aqueous solution of uranyl acetate. Specimens were observed in a Zeiss EM902 electron microscope (Carl Zeiss, Thornwood, NY), operating at 80 keV, with a liquid nitrogen anitcontaminator in routine use.

RNA Binding Measurements. RNA corresponding to the 244-nucleotide RRE sequence in the proviral plasmid pNL432 (positions 7749–7992) (Adachi et al., 1986) was prepared as previously described (Holland et al., 1990). RNA gel mobility retardation and nitrocellulose filter binding assays were performed as described by Holland et al. (1990).

Results

Protein Purification and Refolding. In *E. coli* cell extracts, Rev was not located in the pellet fractions following low-speed centrifugation (10000g for 30 min) and, hence, was not highly aggregated as is typical for recombinant proteins forming inclusion bodies (Marston, 1986). The protein was, however, moderately aggregated as it partitioned between the supernatant and pellet fractions following centrifugation at 60000–80000g for 2 h.

The aggregated protein was associated with substantial amounts of low molecular weight nucleic acid (possibly tRNA), and the following approach was used to prepare physically defined and nucleic acid free protein: first, the high-speed supernatant from bacterial extracts was applied to an anion-exchange column; Rev did not bind to the column. The column flow-through fractions were pooled, and solid urea was added to 6 M (urea at this concentration unfolds Rev and dissociates any bound nucleic acid). Second, Rev was purified to near-homogeneity by using a strong cation exchanger equilibrated in 2 M urea. Third, the purified and unfolded protein (in 2 M urea) was renatured by the addition 0.6 M $(NH_4)_2SO_4$. The urea and salt were then sequentially removed by gel filtration and dialysis, respectively. The renaturation of the protein was monitored by near- and far-ultraviolet circular dichroism (CD).

Rev in (2 M) urea at a protein concentration of 1 mg/mL, or less, showed negligible ellipticity in the near-ultraviolet region (Figure 2A, a). The far-ultraviolet spectrum (Figure 2D, b) indicated that only a small residual amount of secondary structure was present. The addition of 6 M urea completely unfolded the protein (Figure 2D, a). The addition of 0.6 M $(NH_4)_2SO_4$ to protein in 2 M urea resulted in a large increase in negative ellipticity in the near-ultraviolet region (Figure 2A, e). The increased ellipticity is evidence of acquired asymmetry of tyrosyl and tryptophanyl residues associated with protein folding. Similarly, in the far-ultraviolet region, addition of $(NH_4)_2SO_4$ resulted in increased negative ellipticity at 220 nm (Figure 2C), indicating α-helix formation. The use of $(NH_4)_2SO_4$ as a renaturant for protein unfolded by urea has been previously described by Mitchinson and Pain (1985, and references therein). For β-lactamase unfolded with urea, $(NH_4)_2SO_4$ was shown to increase the thermodynamic stability of the native state relative to the unfolded one (Mitchinson & Pain, 1985).

The denaturation of Rev with urea was concentration-dependent. Protein at >2 mg/mL was only partially unfolded in 2 M urea (Figure 2A,b). This is most likely due to the stabilizing effect of protein–protein interactions. Rev was refolded at a concentration of 1 mg/mL or less, and it made no difference to the yields whether the protein, in 2 M urea, was directly chromatographed on Sephadex G-25 [equilibrated in $(NH_4)_2SO_4$] or preincubated with $(NH_4)_2SO_4$ prior to chromatography. The near-ultraviolet CD spectra of preparations obtained by both methods are shown in Figure 2A (c, d). The removal of $(NH_4)_2SO_4$ from renatured protein had no effect on the near- or far-ultraviolet CD spectra.

Rev was also refolded by a different method to check that the physical properties of the protein were independent of the method of preparation. Rev was denatured, with 6 M guanidine hydrochloride and the denaturant removed by dilution and equilibrium dialysis. The physicochemical properties of protein refolded in this manner were identical with those obtained from the urea-based method, although the yields were not as high.

Chemical Characterization. The amino acid composition and N-terminal sequence (20 residues) were as predicted from the gene sequence (data not shown). No N-terminal methionine was detected after the first cycle of protein sequencing, indicating complete processing of the initiating methionine. The two cysteine residues of Rev were titrated rapidly with DTNB in nondenaturing solvents, indicating they were sur-

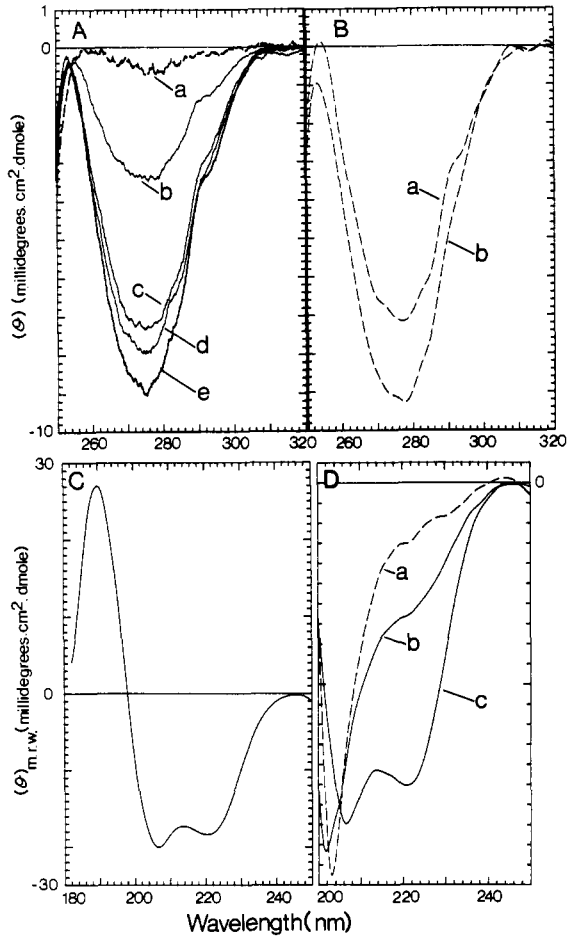

FIGURE 2: Circular dichroic spectra of HIV Rev. Panel A: (a) Rev (1.0 mg/mL) in 2 M urea; (b) Rev (2.1 mg/mL) in 2 M urea; (c) Rev (1.0 mg/mL) in 0.6 M $(NH_4)_2SO_4$ [solid $(NH_4)_2SO_4$ (0.6 M) was added to protein in 2 M urea followed by removal of urea by gel filtration using 0.6 M $(NH_4)_2SO_4$ as the eluent]; (d) as (c) except $(NH_4)_2SO_4$ was not added prior to chromatography; (e) Rev (1.2 mg/mL) in 2 M urea plus 0.6 M $(NH_4)_2SO_4$. Spectra were recorded by using a 1-cm path-length cell. Panel B: (a) Rev (0.5 mg/mL) and (b) Rev (12.0 mg/mL) recorded with 1- and 0.05-cm path-length cells, respectively. Panel C: Rev (2.0 mg/mL) centrifuged of 100000*g* for 2 h prior to measurement. A 0.01-cm path-length cell was used. Panel D: (a–c) refer to Rev in 6, 2, and 0 M urea, respectively. The concentration of Rev was 0.5 mg/mL, and a 0.01-cm path-length cell was used. Unless specified otherwise, all samples contained buffer E (see Materials and Methods). The spectra were recorded at 22–23 °C.

face-exposed and did not form a disulfide bridge. Complete S-carboxymethylation of the protein sulfhydryls with iodoacetamide had no effect on the near- and far-ultraviolet CD spectra (data not shown), indicating that the conformation was similar to the unmodified protein. In addition, alkylated protein had the same hydrodynamic properties as unmodified protein (see below). Rev did not contain nucleic acid that could be detected either by ultraviolet absorbance spectroscopy (Figure 3A) or by ethidium bromide staining of native protein separated by gel electrophoresis.

Molecular Weight and Hydrodynamic Properties. Typical gel filtration elution peaks for Rev chromatographed at various protein concentrations (see Materials and Methods) showed advancing edges which were much sharper than the trailing edges, indicating rapidly reversible associating systems (Winzor & Scheraga, 1963; Ackers, 1970). Sedimentation equilibrium measurements (made at starting concentrations of 0.6–1.0 mg/mL) also indicated concentration-dependent association of protein as plots of ln c vs r^2 showed upward curvature. By

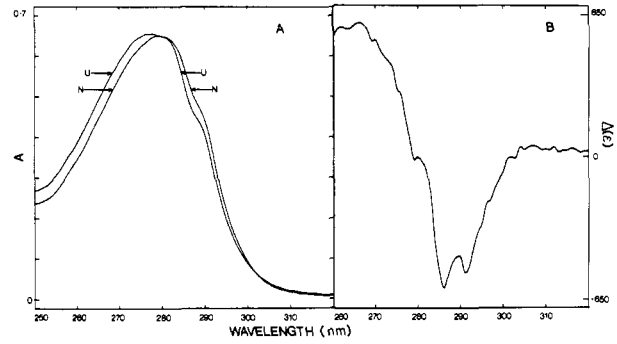

FIGURE 3: Ultraviolet absorbance and difference spectrum. Panel A: Ultraviolet absorbance spectra of Rev in the absence (N) and presence of 2 M urea (U). Panel B: Difference spectrum obtained by electronic subtraction of the spectra shown in panel A. The molar absorption difference coefficient is indicated by $\Delta\epsilon$.

approximation of the curve to a straight line, a molecular weight of 56 000 was estimated. The molecular weights, estimated near the bottom and top of the analytical cell, were 47 000 and 74 000, respectively (the molecular weight of the monomer predicted from the cDNA sequence is 13 030).

The sedimentation coefficient(s) increased markedly with increase in protein concentration. An s value of 3.2–3.5 S was estimated by extrapolation to zero protein concentration. Assuming a spherical shape and a hydration value of 0.30 g of H_2O/g of protein, this value is close to that predicted for a dimer (s = 3.1 S). Dimeric protein and lesser amounts of higher multimers were detected by SDS/PAGE after chemical cross-linking of protein (0.20 mg/mL) with the chemical cross-linking reagent dimethyl suberimidate (Figure 1B).

The above findings indicate that Rev is a self-associating protein, being predominantly dimeric at, and probably below, 0.1 mg/mL and forming tetramers and higher multimers to an increasing extent at >1 mg/mL. It must be emphasized that the studies described above were performed on freshly refolded protein or protein preparations centrifuged at 100000*g* for 2 h prior to analysis. Protein incubated at 4 °C slowly polymerized into very high molecular weight structures (see below).

The effect of urea on the sedimentation coefficient was studied in order to correlate hydrodynamic properties with the CD measurements described above. Rev (1 mg/mL) incubated in 0, 2, and 6 M urea had the following respective $s_{20,w}$ values: 5.0, 1.0, and 0.8 S. This indicated that urea at concentrations greater than 2 M both monomerizes and unfolds Rev (a hydrated sphere of the same molecular mass as monomeric Rev has a predicted s value of 1.91 S). The above interpretation is consistent with the CD spectra (Figure 2D) which indicated loss of tertiary and secondary structure in protein treated with >2 M urea.

RNA Binding Studies. The binding of purified Rev to RRE RNA was evaluated by RNA gel mobility retardation and nitrocellulose filter binding assays. The RRE transcript was bound by Rev at a stoichiometric ratio of 1:8 with an apparent K_d of 5×10^{-9} M. Under the same conditions, an antisense RRE transcript and an unrelated RNA were bound inefficiently or not at all. At high ratios of Rev to RRE RNA, multiple protein–nucleic acid complexes were visualized by gel electrophoresis (data not shown). These complexes may be derived either from the direct binding of oligomeric forms of Rev to RRE RNA or by the binding of, for example, monomers or dimers followed by protein–protein association.

Protein Aggregation and Polymerization. Rev solutions became cloudy at room temperature (20–23 °C) but cleared again once the temperature was reduced to below 4 °C. This

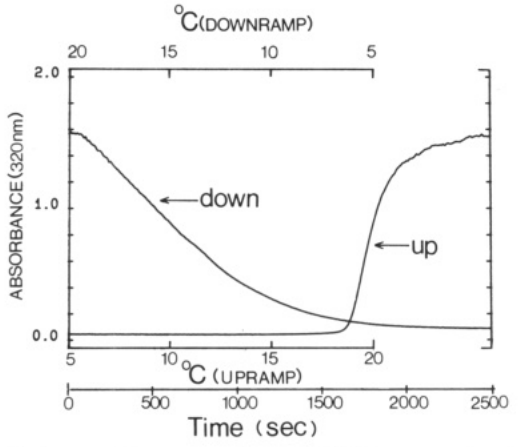

FIGURE 4: Reversible aggregation of HIV Rev as a function of temperature. The absorbance of protein heated from 5 to 20 °C and held at 20 °C until the absorbance is constant is indicated by the curve labeled "up". The absorbance of the same sample cooled to 5 °C and held at 5 °C for the same time period as used in the up-ramp measurement is indicated by the curve labeled "down". The time scale drawn on the abscissa is the same for both the up-ramp and down-ramp measurements.

phenomenon is illustrated in Figure 4, where the reversible aggregation of a dilute protein preparation (0.5 mg/mL) is monitored by light scattering. Interestingly, if solutions containing protein aggregated at 20 °C were held at this temperature, the absorbance at 320 nm slowly decreased in a linear manner over a period of 10–12 h to a base-line value. The addition of >2 mM K_2SO_4 to the solution completely prevented the temperature-induced aggregation (data not shown).

Dialysis of Rev against relatively low ionic strength buffers, for example, 20 mM sodium phosphate, pH 6.5, resulted in precipitation of the protein. This was prevented by the addition of >0.1 M NaCl. Hence, protein was usually stored in buffer (Rev buffer) which contained 50 mM sodium phosphate, pH 6.5, 0.15 M NaCl, 10 mM K_2SO_4, and 1 mM DTT (the DTT was included to prevent oxidation of the surface-accessible cysteine residues).

When the protein (1–2 mg/mL) in Rev buffer was incubated at 0 °C over several days, an increase in light scattering was observed, and the preparation appeared faintly opalescent. If the solution was centrifuged at 100000*g* for 2 h, a clear gelatinous pellet was obtained that was highly birefringent. Pelleted protein was resuspended and examined by electron microscopy. Polymers were observed which take the form of long uniform, unbranched, filaments, about 20 nm in outer diameter, as measured on specimens that were evenly spread and fully immersed in a layer of negative stain (Figure 5). The filaments are hollow, with a stain-penetrable axial channel of 5–7 nm in diameter. Preliminary image analysis of the micrographs indicates regular helical packing of the Rev subunits. The filaments have a tendency for lateral aggregation into bundles that is particularly evident in the concentrated suspensions that are obtained in a redispersed pellet (data not shown). The rapid temperature-induced association of Rev, referred to above, resulted in amorphous aggregates (data not shown).

Solutions of Rev can be conveniently concentrated by precipitation with $(NH_4)_2SO_4$ (35% saturation at 0 °C). Incubation at 4 °C for several weeks of solutions (2–3 mg/mL) derived from salt-precipitated protein resulted in the formation of macroscopic filaments about 10 μm wide and up to several millimeters in length. A filament in which helical periodicity can be observed is shown in Figure 6B. Structures containing

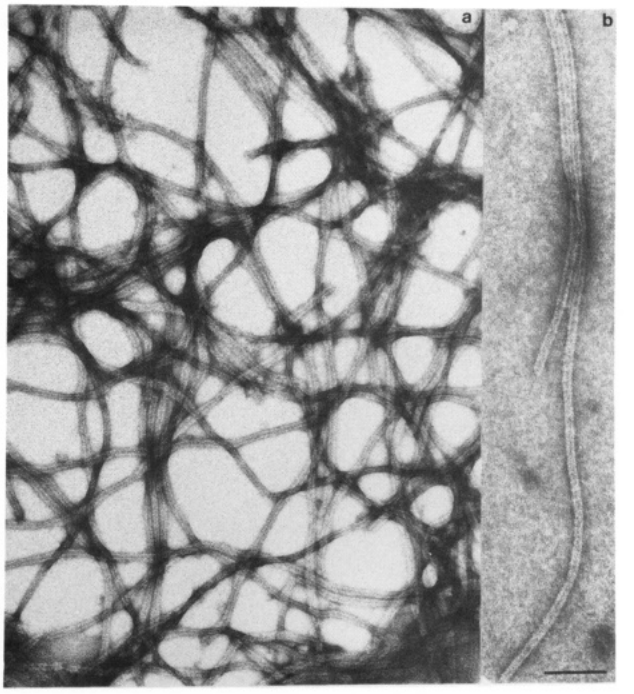

FIGURE 5: Electron micrographs of filamentous HIV Rev polymers. Samples were stained with 1% uranyl acetate. Panel a shows a meshwork of filaments in a resuspended pellet, with positive staining in places; panel b shows an example of filaments polymerized from solution. Bar = 100 nm.

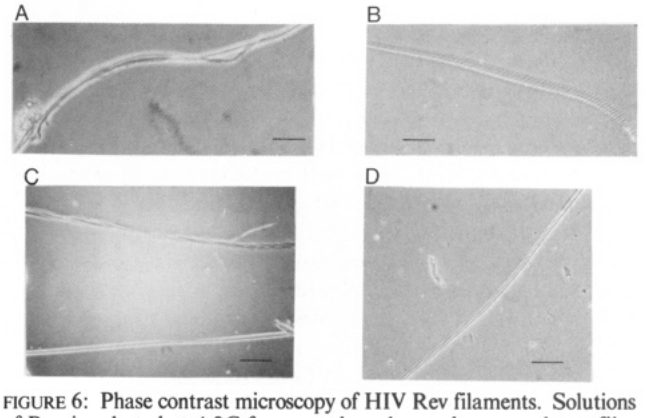

FIGURE 6: Phase contrast microscopy of HIV Rev filaments. Solutions of Rev incubated at 4 °C for several weeks produce very large filaments. Representative structures are shown in panels A–D. The bars in panels A and B = 10 μm and in panels C and D = 5 μm.

partially uncoiled (Figure 6C) and damaged frayed ends (Figure 6A) were also often observed. We suggest that these macroscopic filaments were formed by the lateral aggregation of individual 20-nm fibers into bundles, followed by higher order helical coiling of the bundles. The DNA binding protein RecA from *E. coli* has also been reported to assemble under certain conditions into large filaments about 2 μm in length (Brenner et al., 1988).

Analysis of Rev polymers by SDS/PAGE was carried out in order to observe whether proteolytic processing of protein had occurred before or after polymer formation. Polymers were pelleted by centrifugation and resuspended with SDS at 95 or at 23 °C. SDS/PAGE of both samples is shown in Figure 1, lanes g and h, respectively. The polymers were resistant to complete monomerization, as substantial amounts of dimeric protein were observed. This result suggests that Rev forms tightly associated dimers and is consistent with the sedimentation velocity data and the concentration dependence of urea denaturation (see above).

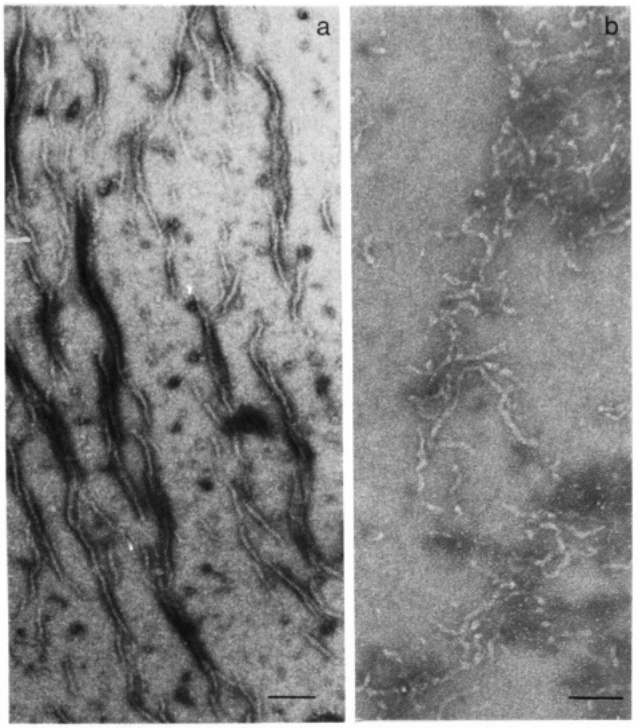

FIGURE 7: Electron micrographs of filamentous complexes of HIV Rev and RRE RNA. Samples were stained with 1% uranyl acetate. Panel a shows filaments formed by incubation of Rev (160 μg/mL) with RRE RNA at a molar ratio of 4:1. Panel b shows filaments formed by incubation of Rev (40 μg/mL) with RRE RNA at a molar ratio of 4:1. Bars = 100 nm.

Filament Formation in the Presence of RRE RNA. Rev filament formation occurred at protein concentrations as low at 80 μg/mL. This protein concentration is in fact the solubility at equilibrium of nonfilamentous Rev at 4 °C (established by centrifugational analysis, data not shown). Filament-free Rev solution at 80 μg/mL was mixed with RRE RNA at a 4:1 molar ratio and then either diluted or rapidly concentrated to give solutions at 40 and 160 μg/mL with respect to protein concentration. In both cases, filament formation was rapid and observed by electron microscopy within a few minutes of incubation at 4 °C (Figure 7). At the higher protein concentration, filaments about 8 nm in diameter and 0.08–0.4 μm in length were observed (Figure 7, panel a). Filaments of about some diameter were formed at the lower concentration but were shorter (0.04–0.12 μm) and were less regular in appearance (Figure 7, panel b).

In contrast to the above, filaments were only observed in control Rev protein solution (160 μg/mL) after several days of incubation and were about 20 nm in diameter and similar in appearance to those which had been formed more rapidly from higher protein concentrations (Figure 5). In contrast, filaments were not observed in Rev solution at 40 μg/mL even after 7 days at incubation. The binding of RRE RNA to the Rev protein clearly stimulated the formation of filaments, and this was true at concentrations both above and below the solubility (80 μg/mL) of the Rev protein.

Spectroscopic Analysis. The far-ultraviolet CD spectrum of Rev, centrifuged prior to analysis to remove any polymeric material, was characterized by two pronounced negative peaks at 220 and 207 nm and one positive peak at 190 nm (Figure 2C). Estimation of the secondary structure by three methods (Greenfield & Fasman, 1969; Chang et al., 1978; Provencher & Gloeckner, 1981) indicated an average α-helical content of 48% with little or no β-sheet structure.

A preparation of Rev which contained polymeric material was centrifuged and the gelatinous pellet resuspended in a small volume of supernatant. The far-ultraviolet CD spectra of the supernatant (0.5 mg/mL) and the resuspended pellet (12.0 mg/mL) were both similar to that shown in Figure 2C, indicating that there is little change in the average secondary structure of the protein upon extensive polymerization. In the near-ultraviolet region, both of the above samples exhibited a peak of negative ellipticity at 276 nm derived from asymmetric tyrosyl residues. Although the ellipticity of the dilute sample was less intense than the concentrated one, it exhibited finer detail, with more clearly resolved shoulders at 280 and 291 nm. The shoulder at 291 nm may be a weak contribution from the L_b band of the single tryptophan residue (residue 15) (Strickland, 1974). The spectral differences may be due to increased immobilization of tyrosyl residues in polymeric protein. This interpretation is, however, complicated by the higher degree of light scattering by the concentrated solution. We are continuing to investigate these differences as part of our attempt to correlate polymer content with spectroscopic properties.

The ultraviolet absorbance spectrum of Rev in 0 and 2 M urea is shown in Figure 3A. The absorbance maximum at 280 nm is red-shifted 2.0 nm in urea-denatured protein. The characteristic broadening and shifting of the absorbance peak upon denaturation were found to be useful for monitoring protein conformation during refolding experiments. The difference absorbance spectrum (Figure 3B) indicates exposure to the solvent of tyrosyl and tryptophanyl residues upon denaturation (Donovan, 1969).

The fluoresence emission spectrum of Rev (10 μg/mL) excited at 295 nm showed a peak emission at 350 nm. Unfolding the protein with 4 M guanidine hydrochloride blue-shifted the emission peak 3–4 nm with little change in fluorescence intensity (data not shown). These results suggest that tryptophan-15, located in the arginine-rich region (residues 35–50), has a high degree of solvent exposure consistent with its weak contribution to the near-ultraviolet CD spectrum (Figure 2A,B).

Spectroscopic Analysis of Rev and RRE RNA. The circular dichroic spectrum in the far-ultraviolet region (180–240 nm) of Rev protein (40 μg/mL) mixed with RRE RNA in a 4:1 molar ratio was indistinguishable from that of protein alone (Figure 2C). This indicates that the gross secondary structure of the protein was unaltered by nucleic acid binding. The near-ultraviolet spectrum of the RRE RNA (240–340 nm) exhibited a single peak of positive ellipticity at 263 nm (the spectrum was indistinguishable from that of yeast tRNA). In the presence of a 3-fold molar excess of Rev, there was a 12% decrease in ellipticity at 263 nm (data not shown).

An electronically subtracted ultraviolet difference spectrum of a mixture of Rev and RRE RNA (4:1 molar ratio) minus RRE RNA and Rev protein gave a single peak of negative absorbance centered at 253 nm with a molar difference absorbance of −13 000 (data not shown). This hypochromatic shift may indicate an increase in structure of the nucleic acid upon binding to protein.

Discussion

We have described in detail methods that lead to the production of large quantities of chemically and physically defined HIV Rev protein. The purified protein binds with high affinity to the RRE RNA as previously reported by other groups [e.g., see Daly et al. (1989)].

The molecular weight of Rev under native conditions is concentration-dependent (see Results). At concentrations of less than 0.1 mg/mL, the protein appears to be mainly dimeric,

and at higher concentrations, the protein is in a rapid and reversible equilibrium with higher multimeric forms. A previous study indicated that Rev prepared by a method different from the one we have described was a stable tetramer (Nalin et al., 1990). This conclusion was reached solely on the basis of gel filtration measurements. However, the Rev elution profile shown by Nalin et al. is qualitatively very similar to that exhibited by the preparation described herein, thus, also indicating reversible protein association. The self-association of Rev is likely to be an inherent property of the protein and not due to a particular method of preparation. Most proteins which bind or associate with nucleic acid are multimeric, the classical example being the TMV coat protein (Butler, 1984; Shire et al., 1990), and more recent examples include some genetic regulatory proteins described by Jones (1990) and Bickel and Pirrotta (1990).

An interesting property of Rev protein is its ability to assemble into long hollow filamentous structures. The polymerization of Rev can occur in the absence of nucleic acid. Viral capsid protein (Harrison, 1990), the TMV coat protein (Butler, 1984), and the *E. coli* Rec A (Brenner et al., 1988), for example, may also self-assemble in the absence of nucleic acid. Furthermore, the structures and subunit association of the proteins in these assemblies are believed to be similar to those assumed in the nucleic acid containing complexes.

We have shown that RRE RNA stimulates the polymerization of Rev at concentrations where self-polymerization does not occur. The resultant polymers were about 8 nm in diameter (Figure 7). This size would be enough to accommodate a protein dimer (the calculated molecular diameter of a Rev monomer is 3.47 nm, assuming a typical hydration value of 0.3 g of water/g of protein and a spherical shape). The hydrodynamic measurements described (see Results) indicate that Rev may be dimeric at high dilution and the dimer may be the basic assembly unit of the polymer. The wider 20-nm-diameter filaments may be formed from intermediate 8-nm-diameter structures which rapidly associate in a lateral manner. Helical coiling of the lateral aggregates could produce the hollow filaments observed (Figure 5). The presence of nucleic acid in the 8-nm-diameter filaments appears to prevent this lateral association from occurring. The presence of nucleic acid also limits the length of the filaments. The growth of TMV coat protein is also reduced in the presence of nucleic acid [see Shire et al. (1990) and references cited therein].

Spectroscopic analysis of the Rev–RRE RNA complex indicates that nucleic acid has no, or little, effect on the gross overall conformation of Rev protein but that the nucleic acid may become more ordered upon binding. Further structural studies are required before more detailed conclusions can be reached.

Rev accumulates in the nuclei of infected cells and increases the levels of unspliced *env* mRNA present in the cytoplasm. On the basis of the physicochemical properties of the protein, we suggest that, following the initial binding of Rev to the targeted *env* mRNA, protein assembly occurs which results in coating in the viral nucleic acid. The protein coating may prevent nuclear RNA processing events. The findings of Pomerantz et al. (1990), that threshold levels of Rev are required for function are consistent with a concentration-dependent protein assembly mechanism. The nuclear export of the coated viral nucleic acid may proceed, for example, via the active transport system utilized by ribonuleoprotein particles (Silver & Goodson, 1989; Starr & Hanover, 1990). The uncoating of the nucleic acid in the cytoplasm may occur spontaneously or may require the assistance of, as yet, unidentified proteins. A cellular factor has been recently described as being essential for Rev function (Trono & Baltimore, 1990).

It is of interest to note that the overall secondary structures of Rev (see Results) and the TMV coat protein are very similar (Altschuh et al., 1987). Although there is little sequence similarity between these proteins (ca. 21%), there are many examples of proteins with dissimilar sequences having similar 3D folding. The analogous secondary structure and polymerization properties of Rev and the TMV coat protein raise the possibility that they also have similar tertiary structures.

We are currently optimizing the conditions of Rev polymerization and studying the mechanism of assembly both in the absence and in the presence of nucleic acid. Measurement of the kinetics of polymerization will give an indication of whether the aggregation is nucleation-controlled as, for example, the polymerization of sickle cell hemoglobin (Eaton & Hofrichter, 1990) and TMV coat protein (Butler, 1984). In order to clearly establish the biological significance of Rev filament formation, we are also attempting to identify these structures in vivo by electron microscopy of recombinant cell lines producing Rev.

Rev is essential for viral replication, and if its mode of action depends upon protein assembly, then inhibition of this process may lead to the development of antiviral compounds. In an attempt to gain structural information, we are investigating conditions for protein crystallization and fiber growth to obtain specimens suitable for X-ray analysis and electron microscopy coupled with image analysis.

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Induction of DNA Polymerase Activities in the Regenerating Rat Liver†

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ABSTRACT: The levels of DNA polymerase α, DNA polymerase δ, and its accessory protein, proliferating cell nuclear antigen (PCNA) were examined in the regenerating rat liver. The levels of DNA polymerase α and δ activities in regenerating liver extracts were determined by the use of the DNA polymerase α specific inhibitor, BuAdATP [2-(*p*-*n*-butylanilino)-9-(2-deoxy-β-D-ribofuranosyl)adenine 5′-triphosphate], and monoclonal antibodies. These reagents showed that the total DNA polymerase activities increased ca. 4-fold during regeneration and that the fraction of DNA polymerase δ activity at the peak was 40% of the total DNA polymerase activity. Immunoblots and inhibition studies using specific antibodies showed that DNA polymerase δ and ϵ and PCNA were concomitantly induced after partial hepatectomy. The levels of both DNA polymerase δ and ϵ and PCNA reached their maxima at 24-36 h post hepatectomy, i.e., at the same time that in vivo DNA synthesis reached its peak. Partial purification and characterization of DNA polymerases δ and ϵ from the regenerating rat liver were also performed. These observations suggest that the variation of DNA polymerase δ and ϵ and PCNA during liver regeneration is closely related to DNA synthesis and is consistent with their involvement in DNA replication.

DNA replication is a complex process that requires the cooperation of multiple enzymes and protein factors. DNA polymerases are central elements in this process, as they are directly responsible for the synthesis of the DNA chains. In eukaryotic cells, two major DNA polymerase activities are now considered to be involved in the replication process. The first is DNA polymerase α, which has long been recognized as having a role in DNA synthesis (Lehman & Kaguni, 1989). The second is DNA polymerase δ, first reported in 1976 as a new type of mammalian DNA polymerase that was apparently unique among mammalian polymerases in that it possessed an associated 3′ to 5′ exonuclease activity (Byrnes

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