Tightly Bound Zinc in Human Immunodeficiency Virus Type 1, Human T-Cell Leukemia Virus Type I, and Other Retroviruses

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Human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus type I (HTLV-I) were purified by sucrose density gradient centrifugation in the presence of 1 mM EDTA. Pelleted gradient fractions were analyzed for total protein, total Gag capsid protein, and total zinc. Zinc was found to copurify and concentrate with the virus particles. Through successive cycles of resuspending in buffer containing EDTA and repelleting, the zinc content remained constant at about 1.7 mol of zinc per mol of Gag protein. Proteins from purified virus (HIV-1 and HTLV-I) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted to polyvinylidene fluoride paper, and probed with ⁶⁵ZnCl₂. Viral nucleocapsid (NC) proteins (HIV-1 p7^{NC} and HTLV-I p15^{NC}) bound ⁶⁵Zn²⁺. Other retroviruses, including simian immunodeficiency virus, equine infectious anemia virus, bovine leukemia virus, Moloney murine leukemia virus, mouse mammary tumor virus, and Mason-Pfizer monkey virus, were found to contain amounts of zinc per milligram of total protein similar to those found in HIV-1 and HTLV-I. Collectively, these data support the hypothesis that retroviral NC proteins function as zinc finger proteins in mature viruses.

Retroviruses are enveloped, single-stranded RNA viruses that replicate via a DNA intermediate synthesized by a viral reverse transcriptase. The viral genome contains a number of genes that code for structural, enzymatic, and regulatory proteins necessary for viral replication. The structural proteins are encoded by the *gag* gene, and the primary translational product of this gene is called the Gag precursor. During viral assembly, approximately 2,500 to 3,000 Gag precursors participate in self-associations and specifically bind two copies of viral genomic RNA to form an immature core of a budding virion (7, 8, 21). After assembly, the Gag precursors are acted upon by a virus-encoded protease and are cleaved to generate structural proteins of the mature infectious virus (27, 29-31, 36, 38). The number of mature Gag proteins varies among the various classes of retroviruses, but all retroviruses have at least three mature Gag proteins generically referred to as the matrix (MA) protein, the capsid (CA) protein, and the nucleocapsid (NC) protein (26). In mature virions, the MA protein is associated with the inner side of the lipid envelope, the CA protein forms the outer shell of the nucleocapsid, and the NC protein complexes with RNA inside the nucleocapsid.

NC proteins of all retroviruses contain one or two copies of an amino acid sequence that can be represented as Cys-X₂-Cys-X₄-His-X₄-Cys (6, 22, 35), where X represents the amino acid residues that can vary from one virus to another. This group of amino acids is referred to as a cysteine array. Retroviral cysteine arrays are required for specific viral RNA packaging by the Gag precursor during viral assembly (1, 9, 10, 32-34, 51) and have also been implicated in the function of the NC protein in the mature infectious virus (32, 33, 40). These arrays closely resemble zinc fingers found in various other nucleic acid-binding proteins (3, 23), but it is not clear whether they function as

zinc coordination sites in either the Gag precursor or the NC protein or both. On one hand, it has been shown that NC proteins and synthetic peptides containing retroviral cysteine arrays can coordinate zinc ions (11, 12, 42, 46-48). These coordination complexes have been obtained by adding zinc ions to the purified proteins or peptides and occur by ligation of cysteine sulfurs and histidine imidazole nitrogens to zinc, forming classical zinc fingers. However, retroviruses in general are not known to contain zinc, and it has been specifically reported that avian myeloblastosis virus (AMV) does not contain significant amounts of zinc (19). In this study, we investigated this question by examining the zinc content of several purified viruses, including human immunodeficiency virus type 1 (HIV-1), human T-cell leukemia virus type I (HTLV-I), and other prototype viruses representing the major classes of the retrovirus family (type B, type C, type D, and lentivirus). In contrast to the previous finding for AMV, we found that stably bound zinc copurifies with HIV-1 and HTLV-I in molar amounts in excess of their respective NC proteins and that the other viruses investigated contain similar amounts of zinc. Additionally, we demonstrated that the NC proteins in HIV-1 and HTLV-I preparations are capable of binding zinc and concluded that retroviral NC proteins are zinc finger proteins.

MATERIALS AND METHODS

Virus production. HIV-1(MN) was grown in chronically infected H9 cells (45), and virus production was boosted by periodic additions of uninfected H9 cells. HTLV-I was produced on C10-MJ2 cells (39). Both were grown in suspension culture with 850-cm² roller bottles in a Biosafety Level-3 laboratory. Cultures were maintained on RPMI 1640 (JRH Biosciences) supplemented with 5% heat-inactivated fetal bovine serum (Flow Laboratories, Inc.), 2 mM L-glutamine, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. Each roller culture contained 400 ml of medium

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FIG. 1. Virus purification. Virus was purified from cell culture supernatants by a single ultracentrifugation (single banded) or two successive ultracentrifugations (double banded) in sucrose density gradients as described in Materials and Methods. The first centrifugation (K-RUN) was performed in an Electronucleonics model KII ultracentrifuge. The second ultracentrifugation (B-RUN), if done, was performed in a Beckman model L5-50 ultracentrifuge.

and was incubated at 37°C while rotating at 0.75 rpm on roller racks. Viruses were harvested by decanting 200 ml of culture and adding 200 ml of fresh medium [or uninfected H9 cell culture in the case of HIV-1(MN) grown on H9 cells] at 3- or 4-day intervals. Viable-cell counts were performed on each harvest by the trypan blue dye exclusion method. Harvested material was stored at 4°C overnight to allow cells to settle. The supernatant was decanted and stored at 4°C prior to virus purification.

Virus purification. Virus was purified by a single ultracentrifugation or two successive ultracentrifugations in sucrose density gradients (49) as outlined in Fig. 1. Approximately 70- to 120-liter batches of culture supernatants were subjected to continuous-flow ultracentrifugation (Electronucleonics model KII with a K-6 rotor containing an integral preclarifier) using a 20 to 60% (wt/vol) sucrose gradient in TNE buffer (0.01 M Tris-HCl [pH 7.2], 0.1 M NaCl, and 1 mM EDTA in deionized water). The supernatant was pumped at 15 liters/h with the centrifuge running at 35,000 rpm, and centrifugation was continued for 45 min after all of the supernatant was applied to allow banding of the virus. After the rotor decelerated to rest, the sucrose gradient was pumped at approximately 50 ml/min through a dual-wavelength UV monitor (ISCO model UA-5 with a model 1133 multiplexer-expander) set at 254 and 280 nm (two absorbance units, full scale) to detect nucleic acids and proteins, respectively. Fractions (50 or 100 ml each) were collected and analyzed for sucrose density with a refractometer (Bausch and Lomb). The virus-containing fractions, as determined by the UV profile and sucrose density, were stored at 4°C overnight.

On the next day, these fractions were pooled, diluted to <20% sucrose with TNE buffer, and further purified by rate zonal ultracentrifugation (Beckman model L5-40 ultracentrifuge with a type Ti-15 rotor with a standard core) using a 25

to 50% (wt/vol) sucrose gradient in TNE buffer. After loading, centrifugation was continued at 30,000 rpm for 3 h before deceleration to 3,000 rpm for unloading. The sucrose gradient was pumped through a UV monitor and collected in 50-ml fractions, which were analyzed for sucrose density with a refractometer.

Virus-containing fractions were pooled, diluted approximately 1:3 with TNE buffer, and centrifuged at 30,000 rpm (Beckman model L5-50 ultracentrifuge with a type 45 Ti rotor) for 1 h to pellet the virus. The resulting viral pellet was resuspended in TNE buffer at a final concentration of 1,000× or 5,000× relative to the cell culture supernatant. These resuspended pellets are referred to as double-banded virus to indicate that they were purified by two successive centrifugations in sucrose density gradients. Single-banded virus was prepared by omitting the second sucrose density gradient centrifugation step.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (24).

Total protein determinations. Protein concentrations were determined by the method of Lowry et al. (28). Samples were disrupted before assay by adding an equal volume of 0.4% (wt/vol) Sarkosyl in deionized water. Bovine serum albumin (Sigma) was used as the standard.

Amino acid analysis. Approximately 1 µg of purified protein was lyophilized and subsequently hydrolyzed in 200 µl of 5 M HCl containing 0.1% phenol. Reactions were conducted in evacuated glass tubes at 110°C for 24 h. The samples were then dried in vacuo and analyzed on a Durrum model D5000 automated amino acid analyzer as described previously (37). The quantity of protein in the sample was computed to be the sum of the masses of the amino acids detected.

Quantitation of HTLV-I and HIV-1 CA proteins. CA protein concentrations were determined by radioimmunoassay (RIA). To develop these RIAs, viral CA proteins were first purified by a series of column chromatographic steps (20). Briefly, purified virus was disrupted by the addition of Triton X-100 to a final concentration of 0.5% in the presence of 0.8 M NaCl. Solubilized material was freed of nucleic acids by passage through a DEAE-cellulose column, and the CA protein was isolated by ion-exchange chromatography on a cellulose phosphate column eluted with a salt gradient. Fractions containing CA protein were pooled, concentrated by ultrafiltration, and purified to homogeneity by size exclusion chromatography. HIV-1 CA protein was prepared in a similar manner, except that the DEAE-cellulose chromatography step was omitted.

Purified HTLV-I and HIV-1 CA proteins were iodinated by the chloramine T method (13) by mixing 20 µg of protein with 10 µl of chloramine T (BDH Chemicals; 2.5 mg/ml in phosphate-buffered saline [PBS]) and 1 mCi of Na¹²⁵I (Amersham) for 1 min at room temperature. The reaction was stopped by the addition of 25 µl of sodium metabisulfite (Fisher Scientific Co.; 2.5 mg/ml in PBS). Free and bound ¹²⁵I were separated by a minicolumn method (50) with fine-grade Sephadex G-25 (Pharmacia Fine Chemicals, Inc.).

Polyvalent antisera prepared in rabbits immunized with multiple doses of disrupted HTLV-I or HIV-1 were titrated against approximately 10,000 trichloroacetic acid-precipitable cpm of the appropriate ¹²⁵I-labeled CA protein. The dilution precipitating approximately 50% of the radiolabeled protein was selected for use in assays. The homologous purified CA protein, quantitated by amino acid analysis, was used to generate a standard curve for each assay.

Samples were disrupted in 1% SDS and diluted 1:10 in RIA buffer (0.05 M Tris-HCl [pH 7.8], 0.15 M NaCl, 0.4% Triton X-100, 0.5% bovine serum albumin, 300 mg of phenylmethylsulfonyl fluoride per liter, 0.1% sodium azide) prior to assay. Standard and samples were serially diluted three-fold in RIA buffer with an automated pipetting device (Cetus Propette) and 96-well plates (Nunc). One hundred microliters of each dilution was transferred to macrowell tubes (Skatron, Inc.) and incubated for 1 h at 37°C with 50 µl of antisera diluted in RIA buffer. Approximately 10,000 trichloroacetic acid-precipitable cpm of ¹²⁵I-CA protein in 50 µl was added and incubated first for 1 h at 37°C and then at 4°C overnight. Formalin-fixed *Staphylococcus aureus* (20 µl; 5% [wt/vol]) was then added to each tube, and after 15 min at room temperature, 500 µl of rinse buffer (0.01 M Tris-HCl [pH 7.8], 0.1 M NaCl, 1.0 mM EDTA, 0.1% Triton X-100) was also added to each tube. The tubes were centrifuged at 1,500 × *g* for 15 min at 4°C, and the pellets were washed once with 500 µl of rinse buffer. Radioactivity in the pellets was determined with a gamma counter (LKB model 1272), and the results were evaluated with a log-logit transformation followed by linear regression (43).

Atomic absorption spectroscopy. Zinc was determined by atomic absorption spectroscopy using a Perkin Elmer Corporation model 5000 spectrophotometer equipped with background correction, an electrodeless discharge lamp (operated at 6 W), and an electrodeless discharge lamp power supply. A lean air-acetylene flame was used (10-cm-wide burner), and the $A_{213.9}$ of zinc was measured. Virus samples were analyzed after disruption in 1% SDS. A standard zinc stock solution (1 mg/ml; Fisher Scientific) was serially diluted with distilled deionized water to obtain a calibration curve which was used to determine the zinc concentration in samples.

Zinc blotting. Sucrose density gradient-purified retroviruses, H9 cell extract, and ¹⁴C-labeled protein standards were electrophoretically transferred to a polyvinylidene fluoride membrane (Immobilon-P membrane; Millipore) as described elsewhere (25). Blots were processed as described elsewhere (2, 44), with minor modifications. Briefly, the blots were equilibrated in 100 mM Tris-HCl (pH 6.8)–50 mM NaCl–10 mM β-mercaptoethanol three times for 10 min each time. They were then rinsed three times with deoxygenated 100 mM Tris-HCl (pH 6.8) containing 50 mM NaCl and incubated for 20 min at 37°C with gentle rocking in 5 ml of this buffer containing 40 µM ⁶⁵ZnCl₂. The blots were then rinsed two times with 100 mM Tris-HCl (pH 6.8)–50 mM NaCl–1 mM β-mercaptoethanol and then soaked for 1 h with three changes of buffer. Finally, the blots were air dried for 30 min, placed in plastic wrap, and autoradiographed overnight.

RESULTS

Preparation of samples for zinc analysis. Some organic materials require prior hydrolysis to obtain accurate zinc determinations by atomic absorption analysis (4). To determine whether virus samples require hydrolysis prior to assay, the concentration of zinc was determined for a double-banded mouse mammary tumor virus (MMTV) preparation with samples prepared (i) by disruption in 1% SDS, (ii) by hydrolysis at 110°C for 1 h in an equal volume of concentrated nitric acid, or (iii) without treatment. No differences in zinc concentrations were found in these samples. Additionally, untreated MMTV was added to a known concentration of zinc, and no interference was observed.

TABLE 1. Zinc analysis of representative retrovirus and mock virus preparations^a

Virus or mock virus	No. of lots tested	µg of Zn/mg of protein (avg)
HIV-1 (MN)	10	0.28
SIV _{Mne}	5	0.16
EIAV	5	0.35
HTLV-I	6	0.64
BLV	3	0.82
Mo-MuLV	5	0.40
MMTV	5	0.56
MPMV	5	0.22
H9 mock virus	7	0.05

^a Preparations of sucrose density gradient-purified retroviruses and H9 mock virus (prepared from uninfected H9 cell supernatant) were analyzed for protein (Lowry method) and zinc content. Mo-MuLV and SIV were single-banded preparations; all others were double-banded preparations.

Accordingly, all subsequent samples were only disrupted in 1% SDS to inactivate the viruses prior to assay.

Zinc content of retroviral stock preparations. A number of production lots representative of the wide variety of retroviruses in the Program Resources, Incorporated-Frederick Cancer Research and Development Center repository were analyzed for total protein and zinc content, and the results are given in Table 1. In all cases, viruses were produced in cell cultures and isolated by continuous-flow sucrose density-gradient centrifugation (single banded) in buffers containing EDTA; in most cases, the viruses were purified again by a second density gradient centrifugation step (double banded) as indicated in Table 1 footnote *a*. EDTA, a chelating agent, minimized the potential incorporation of zinc into virions during purification. Also shown in Table 1 are the results for cellular material shed from uninfected H9 cells that bands at the same density as virus (H9 mock virus). The determined amount of zinc per unit amount of protein in the viruses ranged from 0.16 µg/mg for simian immunodeficiency virus strain Mne (SIV_{Mne}) to 0.82 µg/mg for bovine leukemia virus (BLV) and in all cases was greater than the value of 0.05 µg/mg for H9 mock virus. Each virus lot was examined by SDS-PAGE to assess the relative proportions of the viral and cellular proteins in the preparation (data not shown). In general, visual inspection of these gels suggested that virus lots showing a higher proportion of viral proteins yielded higher ratios of zinc to total protein. For example, the lots of BLV and HTLV-I yielded the highest ratios of zinc to total protein and were judged most free of cellular protein by SDS-PAGE. These results suggested that the virions have tightly bound zinc that remains bound through purification in the presence of EDTA.

Demonstration that zinc copurifies with HIV-1(MN) and HTLV-I. To determine whether zinc cosediments with virions during the initial virus purification, 50-ml fractions were collected from sucrose density gradient centrifugation production runs of HIV-1(MN) and HTLV-I and analyzed as follows. Samples (10 ml) from each fraction were diluted 1:3 with TNE buffer and centrifuged at 100,000 × *g* for 1 h. The resulting pellets were resuspended in TNE buffer and analyzed for total protein by Lowry assay, CA protein by RIA, and zinc by atomic absorption spectroscopy. Figure 2 summarizes these results for HIV-1 (Fig. 2A), HTLV-I (Fig. 2C), and a mock virus preparation of H9 cellular protein (Fig. 2B). For the two virus preparations (Fig. 2A and 2C), the peaks of purified virions in the sucrose density gradient fractions were coincident with the peaks of zinc concentra-

FIG. 2. Analysis of HIV-1(MN) (A), H9 mock virus (B), and HTLV-I (C) single-banded fractions (from K-runs [see legend to Fig. 1]). Cell culture supernatants were processed as described in Materials and Methods, except that 10 liters of RPMI 1640 was loaded after all of the cell-culture supernatant was processed to assist in flushing residual serum-associated zinc from the feed and collection lines. Fifty-milliliter fractions were collected from the sucrose density gradients after centrifugation. The percent sucrose was determined for each fraction. A 10-ml sample from each fraction was centrifuged at $100,000 \times g$ to pellet any virus that may have been present. The pellets were resuspended and analyzed for total protein (Lowry method), CA protein, and zinc content.

TABLE 2. Zinc analysis of HIV-1 and HTLV-I lots after multiple centrifugations^a

Virus	Lot no.	No. of additional centrifugations	Concn (mM) of:		Molar ratio (Zn:CA)
			Zn	CA	
HIV-1 (MN)	P3086	0	0.109	0.067	1.6:1
		1	0.102	0.072	1.4:1
		2	0.091	0.054	1.7:1
		3	0.083	0.045	1.8:1
HIV-1 (MN)	P3268	0	0.018	0.014	1.3:1
		1	0.018	0.014	1.3:1
		2	0.015	0.011	1.4:1
		3	0.013	0.009	1.5:1
HTLV-I	P3194	0	0.033	0.022	1.5:1
		1	0.030	0.017	1.7:1
		2	0.026	0.013	2.0:1
		3	0.022	0.012	1.8:1
HTLV-I	P3224	0	0.011	0.007	1.5:1
		1	0.012	0.008	1.4:1
		2	0.013	0.007	1.7:1
		3	0.010	0.007	1.5:1

^a Viruses were purified by two successive centrifugations in sucrose density gradients containing EDTA. The virus in the peak fractions was pelleted, resuspended in TNE buffer, and frozen at -70°C . Samples were thawed and centrifuged at $100,000 \times g$ for 45 min each as needed to prepare aliquots which were pelleted either one, two, or three times after dilution into 50 ml of TNE buffer. A control sample (no additional centrifugations) from each lot was analyzed.

tion. The corresponding fractions from the H9 mock virus purification contained much smaller amounts of zinc and did not show a peak of zinc concentration (Fig. 2B). These data clearly show that zinc copurifies with HIV-1 and HTLV-I virions during sucrose density gradient centrifugation and subsequent pelleting.

In these single-banded preparations (Fig. 2), the zinc concentrations in the peak fractions of HIV-1(MN) and HTLV-I were 0.24 and 0.48 $\mu\text{g}/\text{ml}$, respectively, and the concentration of CA protein in the HIV-1(MN) peak fractions was 6.75 $\mu\text{g}/\text{ml}$, while that of HTLV-I was 109.8 $\mu\text{g}/\text{ml}$. Thus, the molar ratio of zinc to CA protein in peak HIV-1(MN) fractions was 13.1:1 and that of HTLV-I was 1.6:1. Virus-containing fractions prepared in the experiments whose results are shown in Fig. 2 were stored overnight at 4°C in buffer containing 1 mM EDTA and subjected to a second centrifugation through a sucrose density gradient as described in Materials and Methods for double-banded virus preparations. The molar ratio of zinc to CA protein in these double-banded preparations was approximately 2.3:1 for HIV-1 and 1.5:1 for HTLV-I. This is only a minimal reduction in the zinc-to-CA protein ratio of HTLV-I but a large decrease for HIV-1(MN).

Demonstration that zinc is stably associated with purified HIV-1(MN) and HTLV-I preparations. To determine whether further processing steps would cause additional decreases in the zinc-to-CA protein molar ratios, several lots of double-banded HIV-1 and HTLV-I were analyzed for zinc and CA protein concentrations after one to three additional centrifugations in TNE buffer. As can be seen in Table 2, zinc-to-CA protein ratios of double-banded preparations were extremely stable after multiple centrifugations. As an additional test of stability, double-banded HIV-1(MN) was incubated at 4°C in 200 volumes of TNE buffer for 1 to 3 days and then centrifuged to determine whether zinc could be removed by long incubations in EDTA. Analysis of zinc and CA protein (data not shown) revealed no notable differences

⁶⁵ZnCl₂ Probed WB of Viruses

FIG. 3. Western blots (WB) of sucrose density gradient centrifugation-purified HIV-1, H9 cell extract, and HTLV-I probed with ⁶⁵ZnCl₂. Molecular mass standards (not shown) consisted of the following ¹⁴C-labeled proteins: phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), lactoglobulin A (18.3 kDa), and cytochrome *c* (12.3 kDa).

in the zinc-to-CA protein ratios of these preparations. To determine whether zinc-to-CA protein ratios were stable in the presence of chelating agents other than EDTA, double-banded HIV-1(MN) was incubated for 16 h at 4°C in 150 volumes of 0.1 M potassium tartrate (pH 7.2), 0.1 M sodium citrate (pH 6.7), or 0.1 M diethyldithiocarbamate (pH 7.2). No marked differences in zinc-to-CA protein ratios were observed (data not shown) after centrifugation. These data demonstrate that zinc-to-CA protein ratios are stable elements of purified HIV-1 and HTLV-I preparations.

Identification of zinc-binding proteins in purified HTLV-I and HIV-1. Approximately 50 μg of sucrose density gradient-purified virus (HIV-1 or HTLV-I) or H9 cell extract was separated by SDS-PAGE and Western blotted to a polyvinylidene fluoride membrane. The immobilized protein was probed with ⁶⁵ZnCl₂ to determine which proteins would bind zinc. Only a single band with an apparent molecular mass of 7 kDa was detected in the HIV-1 lane, while a single 15-kDa band was detected in the HTLV-I lane (Fig. 3). These are the reported molecular masses of the respective NC proteins of these viruses (5, 16). No distinct bands were observed in the H9 cell extract lane. Additionally, blots were prepared with 5 μg of high-pressure liquid chromatography (HPLC)-purified (18) HTLV-I NC protein per lane. The identity of this protein was confirmed by amino acid sequencing. As seen in Fig. 4, the purified HTLV-I NC protein bound ⁶⁵Zn²⁺. We conclude from these data that the retroviral NC protein is capable of binding zinc and is the major zinc-binding protein detectable in the virus preparations.

⁶⁵ZnCl₂ Probed WB
of HTLV-I NC Protein

FIG. 4. Western blot (WB) of HTLV-I NC protein (p15) probed with ⁶⁵ZnCl₂. The p15 was purified by HPLC from sucrose density gradient centrifugation-purified HTLV-I. Molecular mass standards (not shown) consisted of the following ¹⁴C-labeled proteins: phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), lactoglobulin A (18.3 kDa), and cytochrome *c* (12.3 kDa).

DISCUSSION

The results presented here clearly demonstrate that zinc copurifies and concentrates with HIV-1(MN) and HTLV-I virions during sucrose-density gradient centrifugation and remains associated with the virus after being pelleted in the presence of EDTA. Molar ratios of zinc to CA protein in double-banded preparations were between 1.5:1 and 2.0:1 and were unchanged by additional centrifugations and resuspensions (Table 2) or incubation in the presence of various chelating agents, including EDTA. These data strongly suggest that the zinc is tightly associated with the virions in these preparations. In this study, the quantity of Gag protein was determined by quantitation of CA protein, and our results show that double-banded HIV-1(MN) and HTLV-I preparations contain on average about 1.7 zinc ions per CA protein molecule. Mature retroviral Gag proteins are normal cleavage products of the viral Gag precursor and are found in the mature viruses in equal molar concentrations (15, 17). Therefore, the data support the conclusion that HIV-1 and HTLV-I contain about 1.7 zinc ions per mature Gag protein molecule (either MA, CA, NC, or other cleavage products).

To assist in determining which Gag protein(s) bind zinc, we showed that HIV-1 and HTLV-I each contain a single zinc-binding protein detectable by Western blot analysis with ⁶⁵ZnCl₂ and that these proteins migrate with the same apparent molecular weight as their respective NC proteins. Additionally, HPLC-purified HTLV-I NC protein was shown to bind zinc in this analysis. These results are

consistent with a previous report that the AMV NC protein binds zinc in a Western blot assay (44). Collectively, these data indicate that zinc ions are stably associated with these viruses and are probably bound to the NC proteins.

The NC proteins of HIV-1 and HTLV-I each contain two cysteine arrays. If each array coordinated one zinc ion in a manner consistent with the zinc finger hypothesis (3, 11, 22, 46–48), the theoretical molar ratio of zinc to NC protein in the mature virus would be 2:1. The determined value of 1.7 zinc ions per NC protein molecule is an average based on the analysis of viral preparations after extensive exposure to EDTA and is remarkably close to the theoretical value of 2:1. In other experiments (14), it has been shown that the concentration of EDTA (1 mM) used in this study is sufficient to remove coordinated zinc from purified zinc-reconstituted HIV-1 NC protein. In the intact virus, the viral lipid envelope and possibly the nucleocapsid structure may prevent penetration of EDTA into the NC-RNA complexes. However, the virus preparations probably contain some partly disrupted virions that may be more susceptible to penetration by EDTA. Therefore, for the intact virions, the molar ratio of tightly bound zinc to NC protein may be closer to the theoretical value of 2:1 than the determined values given here.

A preliminary analysis of other virus preparations suggests that many other members of the retrovirus family, including equine infectious anemia virus (EIAV), BLV, Moloney murine leukemia virus (Mo-MuLV), MMTV, Mason-Pfizer monkey virus (MPMV), and SIV (Table 1), contain levels of zinc comparable to those found in HIV-1 and HTLV-I. The viruses examined in the experiment whose results are summarized in Table 2 were banded in the presence of EDTA and should be free of loosely bound zinc. A lack of quantitative RIAs to measure the CA protein concentration of these retroviruses prevented the determination of their zinc-to-NC protein ratios. However, their zinc-to-total protein values are close to the values for HIV-1 and HTLV-I and suggest that the other retroviruses contain similar levels of tightly bound zinc. The data suggest that the prototype members of the retrovirus family representing the type B (MMTV), type C (Mo-MuLV), type D (MPMV), lentivirus (EIAV, SIV, and HIV-1), and BLV/HTLV-I classes of retroviruses contain sufficient zinc to coordinate to cysteine arrays and form zinc finger structures in their respective NC proteins.

Although the data presented here support only the conclusion that mature retroviral NC proteins contain zinc finger structures, it is highly likely that zinc fingers are formed and present in Gag precursors at the time of virus assembly. This view is consistent with previous conclusions based on the results of site-directed mutagenesis. Three cysteine residues and a histidine residue are required for a single zinc finger. Mutation of any one of these residues in the cysteine arrays of Mo-MuLV, AMV, and HIV-1 results in the production of noninfectious virions that contain significantly reduced amounts of viral RNA (1, 9, 10, 32–34, 51). Thus, it appears that retroviral Gag precursors require zinc fingers in their NC domains for recognition and quantitative packaging of viral RNA and that these zinc fingers are necessary for an infectious retrovirus. The biological roles of zinc coordinated in the cysteine arrays of mature retroviruses are not known and will be the subject of continued research. In the mature virus, NC proteins are complexed with viral RNA, but it is not known whether NC zinc fingers participate in forming the nucleocapsid structure. Other possible roles for NC proteins have been suggested, includ-

ing involvement in viral RNA dimerization, tRNA primer hybridization, and unwinding during reverse transcription (32, 34, 40, 41). Understanding the functions of retroviral NC proteins and their zinc fingers presents a new and interesting challenge and may provide insights into vaccine and antiretroviral drug development.

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