リインフルエンザなどグローバル化する感染症アウトブレークの制圧に取り組んでいる。2004 年 6 月より現職。緊急に対応を必要とする感染症アウトブレークおよび国際的健康危機に対する WHO の活動の総括責任者であり、最先端コミュニケーション技術を駆使した WHO のオペレーション中枢である Strategic Health Operations Centre をその管理下に置く。

Global Outbreak Alert and Response Network (GOARN)
－緊急時の国際協力－

進藤　奈邦子
WHO アウトブレーク対策および警戒事務局

多様化、広域化する新興・再興感染症アウトブレークに対応するには、地球規模の技術的協力が必要である。WHO の呼びかけに応じ、世界各国から代表的研究機関、国際組織、感染症情報ネットワークの代表者などが集まり、2000 年 4 月に Global Outbreak Alert & Response Network（GOARN）が発足した。GOARN が結ぶものは単に個々の技術機関だけではなく、すでに存在する情報・技術協力ネットワークや NGO、国際援助団体など多岐にわたる。GOARN を'Network of networks'とよぶ所以がここにある。

GOARN は迅速なアウトブレークの探知、状況把握、制圧を可能とするために、既存の組織をつないで知的資源や人材をプールする技術協力ネットワークである。GOARN は国際健康危機に対し、以下のように貢献する。

- アウトブレークの国際的波及を食い止める
- 時を逃さず被災国に適切な技術援助を行う
- 長期計画の導入を助けることにより、疾病の恒常的制圧に寄与する。

WHO は事務局としてネットワークを支援し、GOARN の代表者からなる運営委員会が活動方針を決定する。また、WHO はレスポンスのコーディネーションを担当し、さらにパートナー間の協力をスムーズにするため、ネットワークの構造、コミュニケーション、オペレーションのプロトコールを提供する。迅速なレスポンスを可能にするための諸作業も WHO が担当する。アウトブレークの発生が確認された場合、直ちに地域事務局あるいは国事務所を通じて当該国保健省に連絡をとり、具体的な対応策について交渉を開始する。当該国から速やかに調査、あるいは支援の要請が出た場合には、要請を受けてから 24 時間以内に専門家を現地に派遣することが可能な準備が整っている。

GOARN の主たる機能として専門家のプールとしての役割があり、すでに数百人の専門家がデータベースに登録されている。これら専門家は臨床医学、疫学、人類学、獣医学、民俗学、統計学、実験医学など、さまざまな分野をカバーしている。WHO には装備配置の専門家が配属されており、衛星通信施設、WHO プロパーなコンピューター装備、医薬品、サンプリングキット、個人防御装備、簡易実験室備品などが常に搬出可能な状態になっている。現地での資材の輸送、専門家の移動などの手配もこの人たちによって行われる。他の国連機関（UNHCR、UNICEF など）、赤十字、あるいは国境なき医師団など NGO の協力を得て実際の活動を行うこともある。GOARN はアウトブレーク制圧のために寄与できる組織に広く開かれたネットワークである。

メモ

前田　光哉
（まえだ　みつや）

学歴

平成　４年	神戸大学医学部卒業

職歴

平成　４年	厚生省入省（保健医療局精神保健課）
平成　６年	秋田県福祉保健部保健衛生課　主査
平成　８年	厚生省児童家庭局母子保健課　主査
平成　９年	厚生省保健医療局国立病院部経営指導課　課長補佐
平成１１年	厚生省大臣官房厚生科学課　課長補佐
平成１２年	厚生省大臣官房政策課　課長補佐
平成１３年	厚生労働省政策統括官付政策評価官室　室長補佐
平成１３年	山口県健康福祉部健康増進課　課長
平成１６年	厚生労働省健康局結核感染症課　課長補佐

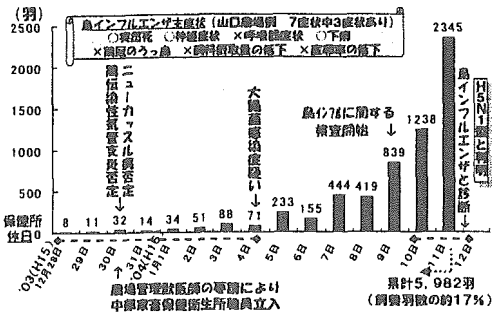
国際健康危機管理ネットワーク強化研究推進事業シンポジウム

アウトブレイク時の行政の対応
～高病原性鳥インフルエンザ発生～
保健所はどう対応したか？

厚生労働省健康局結核感染症課
前田光哉

経時的死亡鶏数

最終場ウインウインファーム山口農場 約35,000羽



初期対応の流れ （Ⅰ）

平成16年1月12日（月・祭日、早朝）
・県健康増進課から「鳥インフルエンザ」の発生報告
→保健所健康増進課長宅へ連絡→保健所感染症担当者へ連絡
↓
↓ 打ち合わせのため県健康増進課へ
保健所長宅へ→県健康増進課長から「鳥インフルエンザに関する資料」をFAX
・現地保健所対策会議を開催
メンバー 所長（山口環境保健所長＝保健環境部々長）
10名 健康増進課：課長、感染症担当（主幹、主宣、班員）
生活衛生課：課長、食品衛生班主宣
試験検査課：課長（＝主幹）
阿東支所 ：地域保健課長
（保健所事務次長と保健環境部課長へは状況報告）
主対策 ①：当該養鶏場の従業員とその家族の健康調査
（問診・採血・咽頭ぬぐい液採取）
②：当該養鶏場からの畜産対応
（12月28日からの出荷鶏卵の自主回収を指示）

初期対応の流れ （Ⅱ）

1月12日（祭日）
・地域住民への情報提供（電話等相談対応）：
保健対応→感染症等担当主幹
食品衛生対応→生活衛生課長
・関連機関への連絡 阿東町役場健康福祉課長
阿武医師会長
（鳥インフルエンザ資料をFAX）

1月13日（火）
・地域住民への情報提供（電話等相談対応）
→ 以後継続的に実施
・阿東町現地対策本部への協力（資料提供と説明）
・阿武医師会長へのFAX 資料確認
・当該養鶏場従業員の家族の追加健康調査

経時的対応一覧

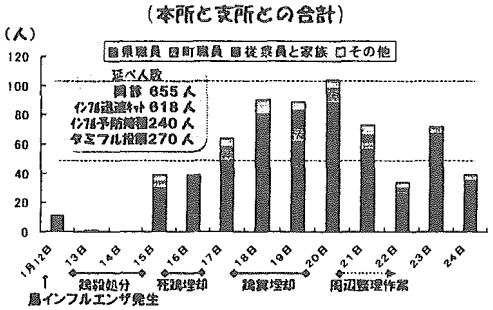
（ワクチン 1回限り、投票 5日分処方）

経過日目	月日 1月	対象者	問診 同診	迅速 キット	採血	ウイルス分離	インフル ワクチン	投薬 (タミフル)	作業工程
1	/12	従業員	○	○	○	○	×	×	鳥インフル発生
2	/13	とその家族	○	○	○	○	×	×	殺処分開始
3	/14			国立感染症研究所で検査					
4	/15	全鶏舎従事者	○	○	×	×	×	×	殺処分終了・採卵
5	/16	全鶏舎従事者	○	○			○	△	従業：A型陽性者
		従業員の家族	○	○			×	×	死鶏埋却開始
6	/17	全鶏舎従事者	○	○			○	○	死鶏埋却終了
7	/18	全鶏舎従事者	○	○			○	○	鶏舎埋却開始
8	/19	全鶏舎従事者	○	○			○	○	健康観察開始
		従業員の家族	○	○			×	×	保健師家庭訪問
9	/20	全鶏舎従事者	○	○			○	○	鶏舎埋却終了・消毒
10	/21	全鶏舎従事者	○	○			○	○	周辺整理作業終了
11	/22	全鶏舎従事者	○	○			○	○	
12	/23	全鶏舎従事者	○	○			○	○	
13	/24	全鶏舎従事者	○	○			○	○	
		従業員の2	全員いづれも「異常なし」						健康調査終了

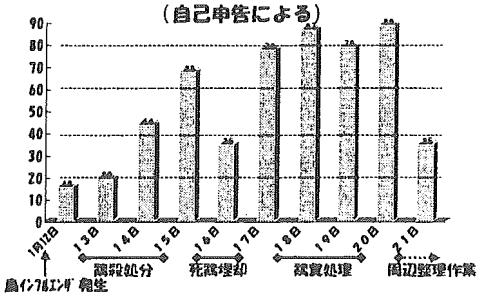
○ 実施 × 非実施

経日的健康調査実施人数

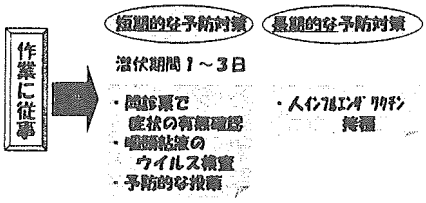
（本所と支所との合計）



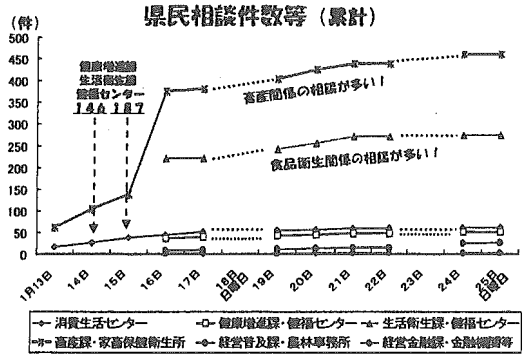
健康調査票にみる 当日の作業従事者数 （自己申告による）



今回の 作業従事者の感染予防策の 考え方

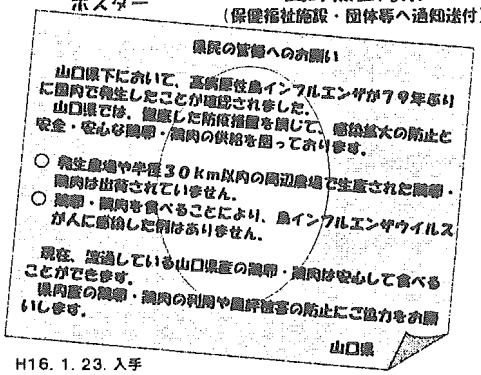


県民相談件数等（累計）



風評被害対策

ポスター （保健福祉施設・団体等へ通知送付）



H16. 1. 23. 入手

まとめ

- 本邦で７９年ぶりに鳥インフルエンザが阿東町生雲地区の養鶏場（山口環境保健所阿東支所管内）で発生した。
- 発生報告日は休日であったが、山口環境保健所本所にて感染症対策会議を開催し、円滑な対策作業に入れた。
- 当該養鶏場からの感染拡大防止対策（鶏殺処理→死鶏埋却処理→鶏糞埋却処理、消毒作業など）の従事者の健康調査（問診、咽頭ぬぐい液ウイルス検査）を実施したが、感染を思わせる者は認められなかった。
  （養鶏場従業員とその家族を含めて･･･）
- また、作業従事者全員にインフルエンザワクチン接種と、症状の有無にかかわらず抗ウイルス剤を投与した。
- 当該養鶏場周辺の住民に対して、保健師による全戸訪問を実施し、不安解消に努めた。
- 作業従事者の大部分は山口県職員（農林部所属）であり、本症の潜伏期間１～３日間を踏まえたフォローが非常に円滑に行えた。

Opening Remarks

In recent years, the dangers looming over a good part of the world have become more serious, and the lives and health of the people are at risk in many countries due to reemerging infectious diseases including the familiar influenza virus and tuberculosis which are said to cause immense health hazards and greatly impact social activities around the globe. In addition to these, there are newly emerging infectious diseases such as bovine spongiform encephalopathy (BSE), severe acute respiratory syndrome (SARS), avian influenza and others, as well as bioterrorism which all pose a threat to our lives and wellbeing. Faced with such circumstances, people are voicing the need to enhance the health crisis management system in order to minimize the health hazards suffered by people through such methods as establishment of preventative measures against the occurrence of health crises, measures for recovery from the spread of the disease and preventative measures against the spread of disease and others.

The Health and Labour Sciences Research administered by the Ministry of Health, Labour and Welfare (MHLW) is a government-funded project conducted for the purpose of ensuring the scientific advancement of administrative policies on healthcare, welfare, environmental health, occupational safety and health, etc. of the people and also strives for improvement in its technical standards. The research project to enhance the International Health Crisis Management Network, which is a part of this government-funded project, aims to enhance the healthcare system in Japan in order to reduce the concerns and worries of the people regarding their health. It will also contribute to ensuring the safety and comfort of living in society by conducting studies on a monitoring system of trends in the occurrence of bioterrorism, infectious diseases and other issues of concern, studies on the construction of networks for a system of treatment cooperation and others, studies on coordination and information sharing with international organizations, and studies on the assistance to and involvement in human development projects, such as for experts at WHO and other international organizations as well as in various countries.

For this symposium with its main theme "Health Crises-forthcoming to us –Advanced preventive measures obtained from our experiences and the recommendations for the future–" we have invited administrative officials on healthcare from WHO and Japanese researchers on medical technology to give lectures followed by panel discussion sessions requiring your participation.

Upon this occasion of the symposium, we wish to ask for your further understanding and support towards the research project to enhance the International Health Crisis Management Network conducted by the Health and Labour Sciences Research.

March 2005

Ichiro Kaneda
Chairman
Imperial Gift Foundation Boshi-Aiikukai
(Aiiku Association for Maternal/Child Health and Welfare)

1st International Symposium on International Health Crisis Management Networks

Theme: "Health Crises-forthcoming to us"
Cutting-edge preventive measures drawn from experience and a proposal for the future
Date: 10:00-16:00, March 11, 2005 (Friday)
Place: "Tenpyo" 3rd Floor, New Takanawa Prince Hotel

Program

10:00 Opening

10:10-12:00 Presentations

Facilitator: Hidehiko Tamashiro (Professor, Graduate School of Medicine, Hokkaido University)

10:10-11:00 Michael J. Ryan (Director, Office of Outbreak Alert and Response, Department of Communicable Disease Surveillance, WHO Headquarters)
"Global Outbreak, Global Response"

11:00-11:40 Hiroshi Kida (Graduate School of Veterinary Medicine, Hokkaido University)
"Experience of Avian Influenza Countermeasures (Tentative)"
Additional comment: Kazunori Ohishi (Associate Professor, Institute of Tropical Medicine of Nagasaki University)
"From the Experience of SARS Countermeasures" (approx. 10 minutes)

11:40-12:00 Miyako Yamamoto (Chief of Section, Division of Safety Information on Drug, Food and Chemicals, National Institute of Health Sciences)
"Chemical Disasters (Tentative)"

13:30-16:00 Symposium

Facilitator: Etsuko Kita (Professor, Nursing, The Japanese Red Cross Kyushu International College of Nursing)

13:30-14:00 Nobuhiko Okabe (Director, Infectious Disease Surveillance Center, National Institute of Infectious Diseases)
Raising issues "(Requires consultation)"

14:00-14:15 Nahoko Shindo (Department of Communicable Disease Surveillance, WHO Headquarters)
"The Global Outbreak Alert and Response Network(GOARN)"

14:15-14:30 Mitsuya Maeda (Deputy Director, Tuberculosis and Infectious Diseases Control Division, Health Service Bureau)
"Administrative Response in Times of Outbreak"

14:30-16:00 Panel Discussion

16:00 Closing

国際健康危機管理のための情報ネットワークのあり方に関する研究

初年度資料編

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 313 (2004) 1073–1078
BBRC
www.elsevier.com/locate/ybbrc

Topoisomerase I dissociates human immunodeficiency virus type 1 reverse transcriptase from genomic RNAs

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[a] *Department of Pathology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan*
[b] *Laboratory of Molecular and Cellular Pathology, Hokkaido University School of Medicine, Sapporo, Japan*
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[d] *CREST, JST, Japan*

Received 12 November 2003

Abstract

Both HIV-1 reverse transcriptase (RT) and topoisomerase I bind to structural RNAs and they cooperate to synthesize cDNA during the replication of HIV-1. In this study, we find that human topoisomerase I exclusively dissociated HIV-1 reverse transcriptase, which strongly binds to structural RNAs. Meanwhile, topoisomerase I did not dissociate either HIV-1 nucleocapsid proteins or murine leukemia virus RT which was bound to RNA. We propose that human topoisomerase I may regulate the binding of RT to RNAs and play a pivotal role in HIV-1 replication.

Keywords: HIV-1; Reverse transcriptase; Topoisomerase I; Dissociation; RNA

Human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is a multifunctional enzyme that has an RNA- and DNA-dependent polymerase activity for synthesis of the minus or plus strand DNA and ribonuclease H (RNase H) activity, which degrades RNA complementary to the newly synthesized DNA for plus strand DNA synthesis and second-strand primer formation [1]. The RT-associated RNase H activity causes cleavage of both double-stranded (ds) RNAs [2,3] and RNA templates of DNA–RNA hybrids, suggesting that RT has a high affinity for structured RNAs containing double-stranded regions with RNAs or DNAs.

The cDNA synthesis of HIV-1 at the post-entry steps is regulated either by the cellular factor(s) incorporated into HIV-1 virions from producer cells or by the factor present in target cells. Topoisomerase I is incorporated into retroviral particles from the producer cells during replication [4] and thus is a candidate for the cellular regulatory factor. HIV-1 [5,6] and Rous sarcoma virus RNAs [7] have been reported to be tightly bound with topoisomerase I. We reported that topoisomerase I bound to stem-loop RNAs and appeared to be incorporated into HIV-1 virions with structured gnomic RNAs [5,6], and also reported that topoisomerase I enhances HIV-1 cDNA synthesis in vitro and in vivo [8,9]. In addition, expression of topoisomerase I mutant lacking ligation activity in the producer cells remarkably reduced the infectivity of virions originating from the producer cells to target cells, and purified topoisoemerase I mutant inhibited viral cDNA synthesis [5]. Similarly as with RT, topoisomerase I can recognize structured genomic RNA and play important roles in cDNA synthesis.

Based on these findings we hypothesized that RT and topoisomerase I compete for the binding to structured RNAs. Here, we show that topoisomerase I dissociates RT from structured RNAs in a specific manner.

Materials and methods

Cloning and expression of His_6-tagged RT proteins and topoisomerase I. For expression of RT proteins, the primers 66F

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(5′-TGCACTTTAGGATCCCCCATTAGTCCTA-3′), 66R (5′-ATCTA TTCCAAGCTTTTATAGTACTTTCCT-3′), and 51R (5′-GGCTG CCCCAAGCTTTTAGAAAGTTTCTGC-3′) (underlines denote *Bam*HI and *Hin*dIII recognition sites) were used to produce fragment DNAs by polymerase chain reaction (PCR) using pNL43 [10]. The primers 66F/66R were used for P66 cDNA (2550–4229) and 66F/51R for P51 cDNA (2550–3869). Each PCR fragment was subcloned into the bacterial expression vector pQE-9 (Qiagen) at the downstream region of the His_6-tag sequence between *Bam*HI and *Hin*dIII sites, and transformed in XL-1 Blue (Strategene). *Escherichia coli* pellets containing P66 and P51 were suspended in the buffer containing 20 mM sodium phosphate (pH 7.0), 50 mM NaCl, and a protease inhibitor cocktail without EDTA (Roche) following a double pass through a French press at approximately 1.5 kgf/cm^2. The samples were ultracentrifuged in an SW41 rotor at 25,000*g* for 30 min at 4 °C and the supernatants were applied to an HiTrap chelating column (Amersham–Pharmacia). The concentration of NaCl was adjusted to 50 mM in imidazole-eluted fractions and the fractions were applied to a (1.0 × 2.0 cm) Hitrap SP column (Amersham–Pharmacia) equilibrated with 20 mM sodium phosphate (pH 7.0) and 50 mM NaCl. The RT protein was eluted from the column using a linear gradient of NaCl at concentrations ranging from 0.05 to 0.4 M (total volume 18 ml). The purity of purified proteins was 90% as determined by Coomassie brilliant blue staining of the sodium dodecyl sulfate (SDS)–polyacrylamide gels. The peak fractions were mixed with 20 mM sodium phosphate (pH 7.0), 1 mM dithiothreitol (DTT), 0.1 M NaCl, and 50% glycerol. Expression and purification of GST-gag proteins was previously described [5,8]. Murine leukemia virus RT was purchased from Roche Diagnostics.

Human topoisomerase I cDNA was amplified by PCR, using the primers, TF (5′-CGT CCC TCC GAA TTC ATG AGT GGG GAC CA-3′) and TR (5′-GCC TCT TGA GCG GCC GCT AAA ACT CAT AGT CA-3′) (underlined sequences correspond to *Eco*RI and *Not*I recognition sites, respectively). Wild type (wt) human topoisomerase I cDNAs were subcloned into baculovirus transfer vectors with six His-tag-sequences at the N-terminus (pFASTBAC HTa, Invitrogen). Recombinant baculovirus was prepared according to the manufacturer's instructions. The nuclei of infected insect cells were resuspended with phosphate buffer [20 mM phosphate (pH 7.5), 1 M NaCl, 10 mM imidazole, and 1 tablet of protease inhibitor cocktail without EDTA (Roche)]. Following precipitation of polynucleotides with 6% polyethylene glycol 6000, supernatants were applied to HiTrap chelating columns. Imidazole-eluted fractions were applied to 1.0 ml UNO S-1 (Bio-Rad), cation exchange columns. Wt topoisomerase I was eluted from the column by a high concentration of NaCl and separated by SDS–polyacrylamide gel electrophoresis (PAGE). After staining with SYPRO (Molecular Probes), the polyacrylamide gel was visualized with FLA 2000 (Fuji film).

Synthesis of SL1 RNA. To analyze the binding of HIV-1 RT and topoisomerase I to structured RNAs, we used a stem loop RNA (SL1) (59 nt) derived from HIV-1 dimer initiation site (DIS) and longer RNAs (180 nt) from plasmids. SL1F (5′-TAATACGACTCACTATAGGGAGATCTCTCGACGCAGGACT-3′) and SL1R (5′-CCCCTCGCCTCTTGCCGTGCGCGCTT-3′) were used to synthesize SL1 RNA (corresponding to 677–734 nt of pNL43) as previously described [11]. Two 180 mer RNAs containing 100 nucleotides (nt) of complementary regions (Fig. 1D) were transcribed from pSP72 and pSP73 (Promega) by T7 RNA polymerase (Ambion). RNA from pSP72 was internally labeled with [α-^{32}P]CTP. Both RNAs were hybridized in 50 mM Tris–HCl (pH 8.0) and 0.2 M NaCl, and the hybridized RNA or single-stranded RNA was purified from 6% TBE (90 mM Tris-borate, 2.5 mM EDTA) acrylamide gel.

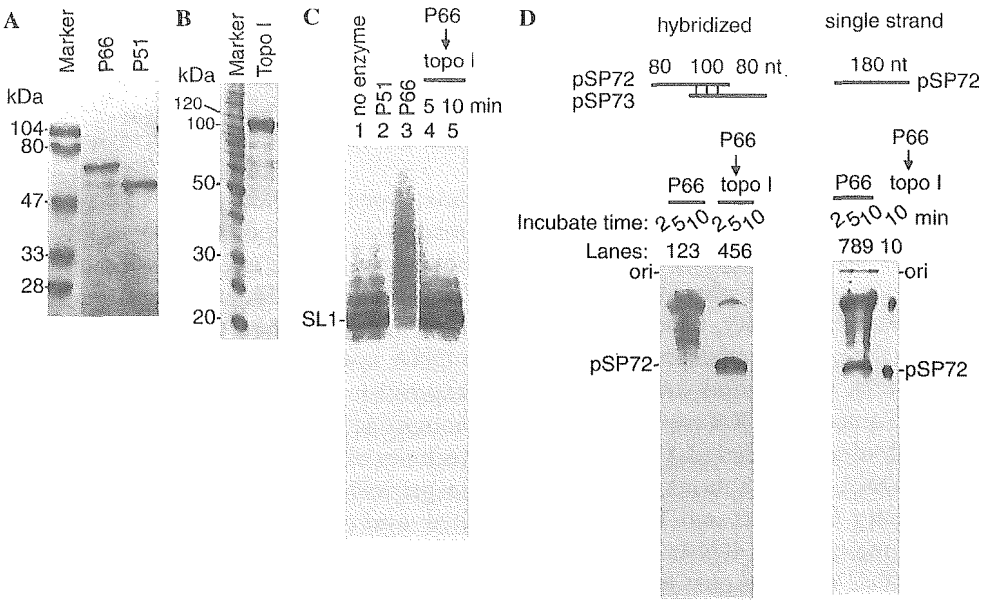

Fig. 1. Analysis of the interaction of HIV-1 RT and RNAs, and the dissociation effect of topoisomerase I to RT from the RT–RNA complex. (A,B) Electrophoresis of purified HIV-1 RT subunits, P66 and P51 (A) or human topoisomerase I (Topo I, B). The purified proteins were analyzed by SDS–PAGE and visualized by Coomassie brilliant blue staining (A) or SYPRO staining (B). The molecular size markers are indicated on the left side. (C) Electrophoresis of RT and [α-^{32}P]CTP-labeled SL1 RNA mixture samples in the presence and absence of topoisomerase I. Binding reaction mixtures containing only SL1 RNA substrates (lane 1), and additionally including P51 (lane 2) or p66 (lane 3) were incubated for 10 min at 37 °C. Samples were further incubated with topoisomerase I for 5 min (lane 4) or 10 min (lane 5). After incubation the reactions were extracted by phenol and chloroform treatment, precipitated with ethanol, and dissolved in water. Heated samples were loaded on a denaturing acrylamide gel. (D) Similar binding reactions as (C) except using longer substrates (partially double-stranded RNA composed of a 100-bp double-stranded region and an 80-mer single-stranded region or a 180-mer single-stranded RNA). The samples were incubated with P66 in the absence of topoisomerase I for 2, 5 or 10 min (lanes 1, 2, 3, 7, 8, and 9), or incubated with P66 for 5 min following incubation with topoisomerase I for 2, 5 or 10 min (lanes 4–6), or for 10 min (lane 10).

Binding and dissociation of P66 proteins. For the analysis of RT binding to RNAs, reaction mixtures (20 μl) containing 50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 4 mM DTT, 10 ng of internally labeled substrates, and 20 ng P66, P51 were incubated at 37 °C. For the dissociation by topoisomerase I, mixtures (20 μl) containing 50 mM Tris–HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 10 μl of the binding reaction products, and 50 ng of topoisomerase I were allowed to react for the indicated times at 37 °C. Formamide was added to the samples for a final concentration of 20% and the samples were heated for 4 min at 85 °C. Reaction products were then analyzed by electrophoresis on 6–12% polyacrylamide sequence gels containing 7 M urea in TBE. Following electrophoresis, the gels were dried for phosphorimage analysis (FLA2000, Fuji Film).

The experiments of binding and dissociation of P66 proteins were performed on a BIAcore 3000 biosensor (Biacore). All experiments were performed at 25 °C using a Hepes buffer (pH 7.4) with 20 mM NaCl and 5 mM $MgCl_2$. The buffer flow was 2 μl/min. Sensor chip SA (Biacore) was pre-coated with streptavidin. The 3′-biotinylated oligonucleotide (5′-AAATGCGTCGAGAGATCTCC AAAAAA-3′) and SL1 RNA were prehybridized in solution (25 mM Tris–HCl, pH 8.0, 0.2 M NaCl). Sixteen to thirty microliters of the hybridized substrates was injected (2 pM/μl) and captured on the streptavidin pre-coated sensor chip (SA). After binding of P66 with the SA, the captured oligonucleotide and RNA were regenerated by injection of 0.05% SDS.

Results

Topoisomerase I dissociated P66 from RNAs

It has been reported that HIV-1 RT is a hetero- and/or homo-dimer consisting of two 66 and 51 kDa polypeptides (P66 and P51, respectively) [1]. Polymerase-active sites of RT were associated with the amino terminus of both the P66 and P51, whereas its RNA hydrolysis activity was exclusively associated with the carboxyl-terminal domain of the P66 [1]. We initially examined the binding activity of P66 and P51 which were separately synthesized and purified. The SDS–PAGE analysis revealed that each recombinant protein had a clear single band around 70 and 55 kDa in size, corresponding to the expected molecular weights (Fig. 1A). Synthesized and purified wild-type (wt) human topoisomerase I was electrophoresed on SDS–containing polyacrylamide gels (Fig. 1B). The recombinant protein containing a His-tag peptide at its N-terminus had a single band on the gel around 110 kDa in size, corresponding to the expected molecular weight. After incubation of [α-^{32}P]CTP-labeled SL1 RNAs with either P66 or P51, the smear band was detected only in the lane of the SL1 RNA in the presence of P66 (lane 3 in Fig. 1C). As this smear band was not observed in the absence of recombinant protein or in the presence of P51 (lanes 1 and 2 in Fig. 1C), it seems likely that multiple P66 randomly bound with SL1 RNA. The SL1 RNA–P66 complex was not formed in the presence of 0.5% SDS, suggesting that the binding was not covalent (data not shown).

We next examined whether topoisomerase I which binds with the stem loop RNA competes with P66 for the binding to structured RNAs. After incubation of P66-bound SL1 RNA with topoisomerase I, the smear band disappeared and the SL1 RNA migrated in a similar manner as those in the absence of P66 (lanes 4 and 5 in Fig. 1C).

In order to confirm the results, we prepared longer RNAs as substrates (180 mer) composed of a 100-bp double-stranded region and an 80-mer single-stranded region or a 180-mer single-stranded RNA (Fig. 1D). We detected the smear bands of the RNAs composed of a 100-bp double-stranded region and an 80-mer single-stranded region after incubation with P66 for 2, 5, and 10 min (lanes 1–3 in Fig. 1D); however, the smear bands disappeared after incubation with topoisomerase I for 2, 5, and 10 min (lanes 4–6 in Fig. 1D), in a manner similar to the SL RNA in Fig. 1B. In contrast, when the single-stranded 180 mer RNA was incubated with P66 for 2, 5, and 10 min, the smear formation was not as intense as that of the partially double-stranded RNA, and a single band was detected (lanes 7–9 in Fig. 1D). The smear band disappeared after incubation with topoisomerase I for 10 min (lane 10 in Fig. 1D). These results suggest that P66 bound with stem-looped, partially double-stranded, and single-stranded RNAs and topoisomerase I resulted in the dissociation of P66 from these structured RNAs.

Kinetic analysis of the function of topoisomerase I to the P66–RNA complex

Next, we examined the function of topoisomerase I in the P66–RNA complex using the BIAcore system that can measure changes in surface plasmon resonance representing the changes in the mass of molecular species bound to the surface [12]. The measured differences of the mass reflected the kinetic events including association and dissociation of complexes that were composed of bound molecules and other molecules brought into contact with them in the flow cell. Initially, the 3′-biotinylated oligonucleotide which was hybridized with SL1 RNA was immobilized on a streptavidin-coated chip (Fig. 2A). The sensorgram from the biosensor analysis revealed the association of P66 to the immobilized SL1 RNA and the formation of a P66–SL1 RNA complex shown as a plateau curve (Fig. 2B). After topoisomerase I treatment, P66 was gradually dissociated from the complex and the regeneration level decreased compared to that before the treatment with topoisomerase I. To analyze the phenomenon, we separate the reaction and compared the dissociation of P66 in the absence (Fig. 2C) and the presence (Fig. 2D) of topoisomerase I. Although the condition how much RNA could be trapped to the sensor chip affected the binding and dissociation of P66, experiments repeated more than

three times showed topoisomerase I clearly enhanced the dissociation of P66.

For synthesis of DNA in the 5′–3′ direction by the polymerase, P66 recognizes the free 3′-end of DNA and/or RNA duplex. We examined whether P66 bound with SL1 RNA in the polymerase-dependent manner with the substrates hybridized with 3′- and 5′-biotinylated oligonucleotides. As both substrates demonstrated the same results (data not shown), suggesting that P66 did not require the free 3′-end of DNA/RNA helix for its binding, we represented the data using 3′-biotinylated oligonucleotides.

To prove that topoisomerase I did not directly bind to SL1 RNA, P66 was continuously applied to the system following topoisomerase I (Fig. 3A). The plateau curve of the sensorgram during the topoisomerase I treatment indicated that topoisomerase I did not bind to SL1 RNA in the sensor chip. In contrast, P66 after topoisomerase I treatment bound to the sensor chip in a manner similar to that shown in Figs. 2B–D, indicating that topoisomerase I did not affect the binding of P66 to SL1 RNA. Next, we examined whether P66 could bind to SL1 RNA in the presence of topoisomerase I. As shown in Fig. 3B, P66 could combine with SL1 RNA even in the presence of topoisomerase I (Fig. 3B). Taken together, topoisomerase I did not inhibit the binding of P66; however, the bound P66 was dissociated from SL1 RNA by topoisomerase I.

We determined the dissociation rate by continuous injection of topoisomerase I. The dissociation rate constant (k_d) of P66 by topoisomerase I was calculated from curves of sensorgrams with different concentrations of topoisomerase I using the following kinetics evaluation software: a (500 pg/μl of topoisomerase I), $k_d = 9.45 \pm 0.36 \times 10^{-3}\,s^{-1}$; b (50 pg/μl of topoisomerase I), $3.55 \pm 0.04 \times 10^{-3}\,s^{-1}$; and c (buffer only), $3.08 \pm 0.03 \times 10^{-3}\,s^{-1}$ (Fig. 3C). We found that the k_d value of P66 in the presence of 500 pg/μl topoisomerase I was about 3 times higher than that in the absence of topoisomerase I, suggesting that P66 strongly bound to SL1 RNA.

Zinc finger domains of HIV-1 gag proteins are known to bind with genomic RNAs to carry them into virions. We next analyzed whether topoisomerase I dissociated gag proteins from the gag protein–RNA complex using the same system. Although Gag proteins bound to SL1 RNA, topoisomerase I did not affect the gag protein–RNA complex at all (Fig. 4A). We next

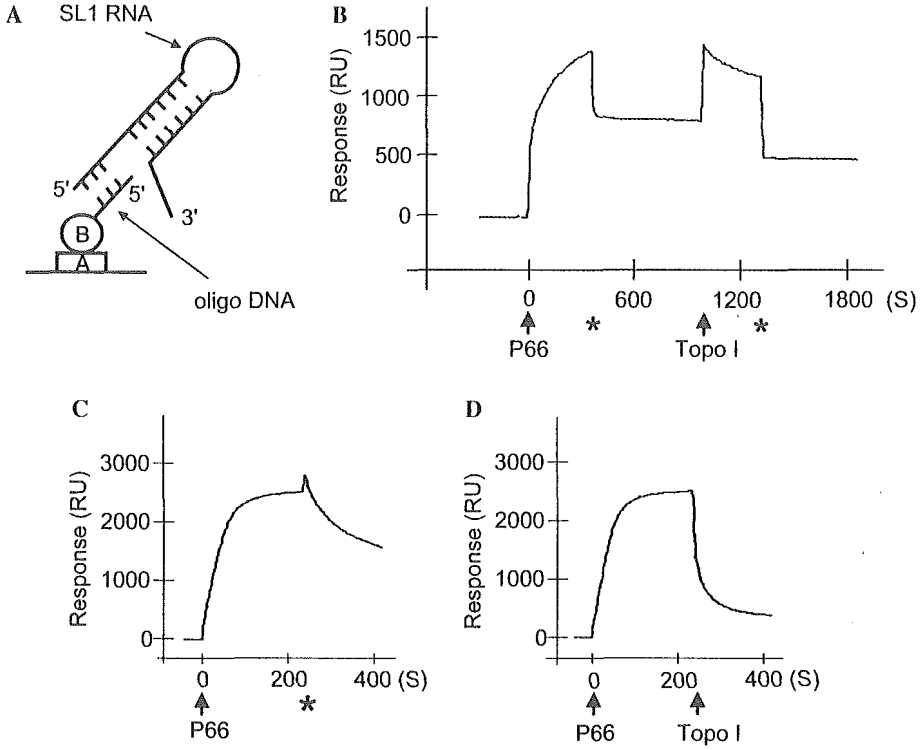

Fig. 2. Analysis of interaction among P66, SL1 RNA, and topoisomerase I using the surface plasmon resonance change. (A) A schematic figure of SL1 RNA substrate immobilized on the surface chip of the BIAcore system. Characters A and B represent avidin and biotin, respectively. Oligonucleotide was biotinylated at the 3′-end and prehybridized with SL1 RNA. (B) A representative sensorgram of three experiments illustrating the real-time binding of P66 (200 pg/μl) and dissociation of P66 by injection of topoisomerase I (500 pg/μl). Arrows indicate the initiation of injection of the purified P66 and topoisomerase I (Topo I). Asterisks indicate the end of the injection. The vertical and horizontal axes represent relative amounts of RT bound to immobilized RNA [Response, plasmon resonance units (RU)] and time [seconds (s)], respectively. (C,D) A representative sensorgram of three experiments illustrating the real-time binding and dissociation of P66 (200 pg/μl) (C) or further continuous injection of topoisomerase I (500 pg/μl) (D).

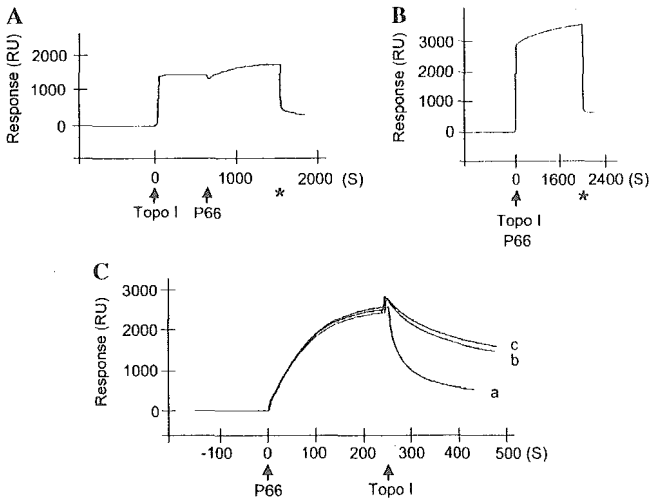

Fig. 3. Analysis of binding affinity of P66 to SL1 RNA in the presence of topoisomerase I using the surface plasmon resonance. (A) A representative sensorgram of two experiments illustrating the injection of P66 (200 pg/μl) after the injection of topoisomerase I (500 pg/μl). Arrows indicate the initiation of injection of the purified topoisomerase I (Topo I) and P66. Asterisks indicate the end of the injection. (B) A representative sensorgram of two experiments illustrating the coinjection of P66 (200 pg/μl) and topoisomerase I (500 pg/μl). (C) Representative overlay sensorgrams of three experiments illustrating the real-time binding and dissociation of P66 (200 pg/μl) in the presence of various concentrations of topoisomerase I (a, 500 pg/μl; b, 50 pg/μl; and c, 0 pg/μl).

applied murine leukemia virus (MuLV) RT and investigated the interaction between MuLV RT and human topoisomerase I. Although MuLV RT bound with SL1 RNA, topoisomerase I did not affect the binding of MuLV RT to SL1 RNA (Fig. 4B). Thus, it was suggested that topoisomerase I specifically dissociated the human P66 subunit of RT from the complex with SL1 RNA.

Discussion

In this study we demonstrated that topoisomerase I specifically dissociated P66, a subunit of HIV-1 RT, which strongly binds to structured RNAs. It has been reported that HIV-1 cDNA synthesis by RT pauses at the well-defined regions forming secondary structures, and efficient cDNA synthesis is limited to relatively short stretches of nucleotides lying in these sites [13]. Thus, cDNA synthesis during HIV-1 replication is considered to be much different from reverse transcription of heat-denatured RNA template. First, HIV-1 RNAs are fragmented in virions [11] and we found RNA ligase activity of cellular topoisomerase I played a pivotal role in cDNA synthesis [5]. Second, in addition to polymerase-dependent P66 at the 3′-end of primer, polymerase-independent P66 recognizing 5′-end or helix formation of structured RNA affects the reverse transcription of HIV-1 RNA. In RNA/DNA hybrids helix structure was reported to promote the binding of RT and RT measures the distance from the point of hybridization even in the case 3′-end of primer was unannealed [14,15]. Such tight binding of RT to the structured RNAs would inhibit polymerization of RNA. Therefore not only the RNA ligase activity of topoisomerase I but also the dissociation of polymerase-independent RT by topoisomerase I plays an important role in cDNA synthesis. Topoisomerase I is necessary for the adjustment of condition of RNA template during polymerization.

The specificity of dissociation of HIV-1 RT from SL1 RNA by topoisomerase I might partly explain the narrow host range of the HIV-1 tropism. Because topoisomerase I of African green monkey clearly suppressed cDNA synthesis of HIV-1 [9], the activity of human topoisomerase I including the dissociation of P66 from RNA template and

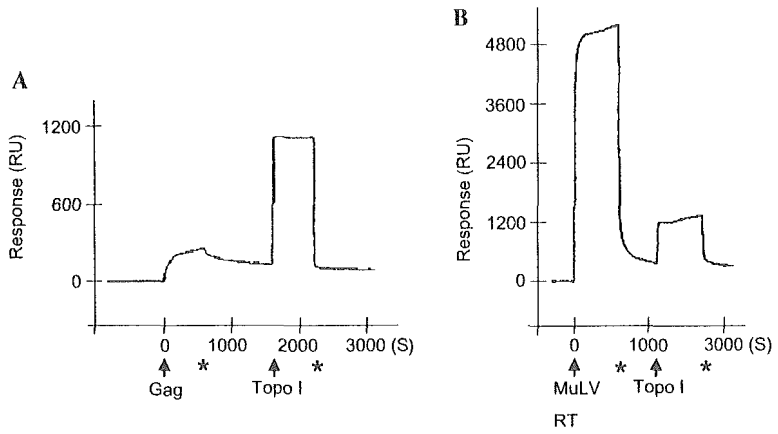

Fig. 4. Analysis of interaction of various RNA-binding proteins and topoisomerase I using the surface plasmon resonance. (A,B) Sensorgrams illustrating the real-time binding of GST-gag proteins (Gag) (50 pg/μl) (A) or MuLV RT (200 pg/μl) (B) in the presence of topoisomerase I (Topo I) (500 pg/μl). Arrows indicate the initiation of injection of Gag (A) or MuLV RT (B) and Topo I (A,B). Asterisks indicate the end of the injection. The vertical and horizontal axes represent relative amounts of Gag, MuLV RT or topoisomerase I bound to the immobilized RNA [Response, plasmon resonance units (RU)] and time (seconds (s)), respectively.

religation of nicked RNA genome would determine the effectivity of cDNA synthesis.

Thus, the binding of polymerase-independent RT to genomic RNA seems to be important for the inhibition of HIV-1 replication. It is expected that drugs which interfere with the association of RT and cellular topoisomerase I and do not inhibit topoisomerase I itself could be useful for the treatment of HIV-1 infection. Moreover, such drugs could be associated with the low frequency of the appearance of escape mutants. Future studies should determine the potential therapeutic role of such agents.

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Nucleolin and the Packaging Signal, ψ, Promote the Budding of Human Immunodeficiency Virus Type-1 (HIV-1)

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Abstract: **Gag proteins of human immunodeficiency virus type 1 (HIV-1) play a pivotal role in the budding of the virion, in which the zinc finger motifs of the gag proteins recognize the packaging signal of genomic RNA. Nucleolin, an RNA-binding protein, is identified as a cellular protein that binds to murine leukemia virus (MuLV) gag proteins and regulates the viral budding, suggesting that HIV-1 gag proteins, the packaging signal, ψ and nucleolin affect the budding of HIV-1. Here we report that nucleolin enhances the release of HIV-1 virions which contain ψ. Furthermore, nucleolin and gag proteins form a complex incorporated into virions, and nucleolin promotes the infectivity of HIV-1. Our results suggest that an empty particle which contains neither nucleolin nor the genomic RNA is eliminated during the budding process, and this mechanism is beneficial for escape from the host immune response against HIV-1.**

Key words: **HIV-1, Nucleolin, Budding, Gag, Packaging signal**

The HIV-1 genome consists of three major genes: *gag*, *pol* and *env* (17). The *gag* gene is translated into a polyprotein that is sufficient to mediate the formation of virion-like particles (VLP) in the absence of other viral proteins (1). Gag precursor proteins ($Pr55^{gag}$) are subsequently cleaved into their structural component proteins including p17 matrix (MA), p24 capsid (CA), and p15 proteins. It has been reported that the p15 proteins are further cleaved into nucleocapsid (NC) p7 and p6 proteins. The NCp7 protein, which contains two zinc finger motifs thought to be important for packaging of the virus genomic RNA, binds to genomic RNA (10), mediates dimerization of the viral RNA, and promotes the annealing of a transfer RNA (tRNA) primer to the viral RNA genome. These zinc finger motifs are able to recognize the HIV-1 RNA packaging signal, forming a stem-loop structure which flanks the major subgenomic splice donor (12). Both the myristate and the basic motif signal at the N-terminal Gag sequence function as a targeting signal, which is essential for the proper assembly and budding of viral particles, to direct interaction with acidic phospholipids on the cytoplasmic leaflet of the plasma membrane (25).

As a host protein that binds to murine leukemia virus (MuLV) Gag, a nucleolin in which the carboxyl-terminal fragment interacts with the NC domain of MuLV Gag proteins has been identified by a yeast two-hybrid assay, and was shown to inhibit the assembly of viral virions (5). It has also been reported that a single point mutation adjacent to the capsid (CA)-nucleocapsid (NC) boundary of MuLV Gag diminished viral assembly, suggesting that the CA-NC junction is important for viral assembly.

Nucleolin, which contains four consensus RNA-binding domains, is one of the major non-ribosomal proteins in the nucleolus and is presumed to function in ribosomal DNA (rDNA) transcription, ribosomal RNA (rRNA) packaging, ribosome assembly and nucleo-cytoplasmic transport (18, 19). Structural and mutagenesis

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; rVV, recombinant vaccinia virus; VLP, virion-like particle.

studies have shown that nucleolin interacts specifically with a stem-loop RNA structure (3, 19). It has also been reported that nucleolin is expressed on the cellular surface as well as the intracellular pool within the nucleus and cytoplasm as a shuttle protein (21), and that cellular surface nucleolin and lipid rafts are implicated in early events in the HIV entry process (16).

In this study, we have examined whether nucleolin which are bound to HIV-1 gag proteins can regulate the budding of HIV-1. Nucleolin enhanced the release of both virion-like particles (VLPs) and HIV-1 virions in the presence of the RNA packaging signal, ψ, and form a complex with gag proteins incorporated into the virions. Furthermore, nucleolin promoted the infectivity of HIV-1, suggesting that an empty particle which contains neither nucleolin nor genomic RNA is eliminated during the budding process.

Materials and Methods

Cells and tissue culture. Human cervical carcinoma cells, HeLa cells (JCRB 9004), were provided from the Health Science Research Resources Bank (HSRRB, Japan). MAGIC5 cells which express human CD4 and human CCR5 and are derived from HeLa cells (14) were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 C in an atmosphere of 5% CO_2. Rabbit kidney cells, RK13 cells (CCL-37), were purchased from the American Type Culture Collection (ATCC, ML). RK13 cells and human osteosarcoma lacking thymidine kinase cells, 143TK− cells, were grown in modified essential medium (MEM) supplemented with 10% fetal bovine serum at 37 C in an atmosphere of 5% CO_2.

Plasmid constructions. It has been reported that the nucleocapsid (NC) domains of Gag bind to a packaging signal which is a ~110 nucleotide segment of the genome known as the ψ-site. The HIV-1 ψ-site contains four stem-loops (SL1 through SL4), all of which are important for genome packaging (4). In order to eliminate the influence of viral proteins on the budding of virions after packaging, mutant HIV-1 was synthesized by *in vitro* mutagenesis. HIV-1 infectious DNA, pNL43 (2), was truncated between *Apa*I and *BamH*I sites for elimination of all the HIV-1 proteins including Pol, Vif, Vpr, Vpu, Tat, Rev, Env and Nef, and the first ATG codon of Gag was replaced with TTG, designated as ψ (+) DNA (Fig. 1). Alternatively, we made another HIV-1 mutant lacking a packaging signal, the ψ-site. To remove the packaging signal, a further truncation of the sequence 5'-TAC GCC AAA AAT TTT GAC TAG CGG GAG GCT AGA AGG AGA G-3' (749–787 nt) was conducted by replacing the *BssH*II-*Sph*I fragment with a DNA fragment which was amplified from pNL43 with primers: 5'-GCT GAA GCG CGC ACG GCA AGA GGC GAG GGG CGG CGA CTG GTG AGA GTT GGG TGC GAG AGC GTC GGT ATT AAG CGG GGG AGA-3' (705–824 nt) (underlining denotes *BssH*II and mutated ATG site) and 5'-ATG TCA CTT CCC CTT GGT TCT CTC-3' (1473–1496 nt) and digested with *BssH*II and *Sph*I. The obtained vector was designated as ψ (−) DNA. Similarly, the *BssH*II-*Sph*I fragment of pNL43 was replaced with a *BssH*II-*Sph*I fragment containing the deleted packaging signal and the unmutated ATG codon, and was designated as ψ−. Mammalian expression vector encoding mouse Cyclin T1 mutated at 261-Tyrosine to Cystein, Y261C was obtained from Dr. Bieniasz (6).

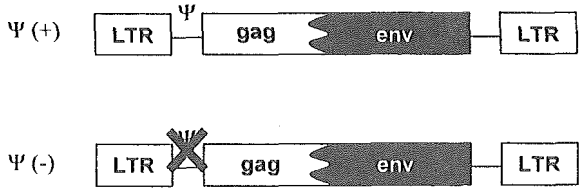

Fig. 1. Structure of plasmid producing HIV-1 truncated genomic RNA. ψ (+) and ψ (−) represent plasmids encoding HIV-1 LTR and provirus truncated between *gag* and *env*, with or without the packaging signal, respectively.

Human nucleolin cDNA (20) was amplified from HeLa cell cDNA (Clontech) by polymerase chain reaction (PCR) with primers NucF (5'-CAC TCC GCC GGA TCC ATG GTG AAG CTC GCG AA-3') and NucR (5'-GCA GAG GGA TCT AGA CTA TTC AAA CTT CGT-3') (underlining denotes *BamH*I and *Xba*I recognition sites). The PCR product digested by *BamH*I and *Xba*I was subcloned into a mammalian expression vector, pcDNA4/HisMaxC (Invitrogen) and designated as pchNuc.

Adenovirus and vaccinia virus vector. Recombinant adenovirus was synthesized following the manufacturer's instructions (Clontech). Briefly, the mammalian expressing vector pchNuc was digested by *Xba*I and *BamH*I and the digested fragment was subcloned into a shuttle vector, pTRE-Shuttle (Clontech), followed by digestion with I-*Ceu*I and *PI-Sce*I. The DNA fragment was ligated with linearized Adeno-X viral DNA (Clontech) and transformed into *E. coli*. The recombinant adenoviral DNA was extracted from *E. coli* and digested by *Pac*I followed by transfection to HEK293 cells by Fugene 6 (Roche). The adenovirus in the supernatant of the culture was amplified by recycling infection with HEK293 cells, and used infected to RK13 cells at 5 m.o.i. to express human nucleolin.

Recombinant vaccinia virus expressing $Pr55^{gag}$ (rVV-$Pr55^{gag}$) was constructed as described previously (22).

Briefly, a PCR fragment amplified from pNL43 with primers 55F (5'-AGA AGG AGA CCA TGG TGC GAG AGC GTC G-3') and 55R (5'-ATT GCC CCC GGA TCC TTA TTG TGA CGA GGG GTC-3') (underlining denotes *Nco*I and *BamH*I recognition sites) was digested with *Nco*I and *BamH*I, followed by a subcloning into pAK10 (9). Thymidine kinase-negative (TK−) viruses were isolated by plaque assay on 143TK− cells (9) in the presence of 5-bromo-2'-deoxyuridine at a concentration of 25 μg/ml, and were designated as pAKP55. The rVV-Pr55^{gag} was amplified by infection with RK cells, and used infected to RK13 cells at 5 m.o.i. to express Pr55^{gag} proteins.

Preparation of virion-like particles (VLPs) and HIV-1 virion. To prepare VLPs, RK13 cells were infected with the rVV-Pr55^{gag} with 5 m.o.i. at 37 C for 60 min. After washing with MEM-2% FBS, the cells were cultured in MEM-5% FBS. For HIV-1 virion stock, HeLa cells were transfected with HIV-1 provirus, pNL43 (2) using Fugene 6 (Roche). Either 24 hr after infection with rVV-Pr55^{gag} or 48 hr after transfection with pNL43, the culture supernatant was collected from each plate and centrifugated at 1,500×*g* for 10 min. After filtration to remove cell debris with a 0.45 μm filter, virion-like particles (VLPs) or viral virions were purified through a 25% sucrose cushion by centrifugation at 100,000×*g* for 2 hr and further isolated on 20 to 60% continuous sucrose gradients by centrifugation at 80,000×*g* at 4 C for 16 hr. The concentrations of VLPs or virions were estimated by measurement of p24 Gag antigen with an enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix, U.S.A.).

Each 0.5 ml of fraction was collected, and proteins in each fraction were precipitated with trichloroacetic acid in the presence of 1 mg of bovine serum albumin.

Immunoblotting. To estimate the efficiency of the HIV-1 budding from the cells, the amounts of Pr55^{gag} were examined by immunoblotting. For RK13 cells were infected with recombinant adenovirus encoding human nucleolin cDNA, and were thereafter transfected with the plasmids which transcribe a truncated HIV-1 genomic RNA with or without RNA packaging signal, designated as ψ (+) or ψ (−), respectively (Fig. 1). Forty-eight hr after transfection the cells were infected with the rVV-Pr55^{gag}. The amounts of gag proteins in the cells and supernatants were examined by immunoblotting 24 hr after infection. The virion-like particles (VLPs) in the supernatant was purified by ultracentrifugation at 50,000×*g* for 1 hr.

Collected samples were lysed with sodium dodecyl sulfate (SDS) sample buffer containing 2% (w/v) SDS, 63 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 30 mM bromophenol blue, and sonicated. The samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane, Immobilon (Millipore). For detection of exogenously expressed proteins, anti-p24 (1E4), anti-p17 (4D9) (9) or anti-nucleolin mouse monoclonal antibodies (Santa Cruz) were used as the primary antibodies, and horse-radish protein (HRP)-conjugated sheep anti-mouse IgG antibody as the second antibody. An ECL immunoblotting kit (Amersham Pharmacia Biotech) was used to visualize the detected proteins.

RT-PCR. From fractions separated from sucrose density gradient, RNAs were also extracted by an RNA extraction kit (Qiagen). After reverse transcriptase (RT) treatment, a real-time PCR was performed with primers NL-R 1F (5'-CTC TCT GGT TAG ACC AGA TCT GAG CCT GGG-3') (corresponding to 458–487 nt of pNL43, Gene Bank accession number, M19921) and NL-U51R (5'-ACT GCT AGA GAT TTT CCA CAC TGA CTA AAA-3') (607–636 nt). PCR was carried out in 20 μl total reaction volume with a LightCycler-RNA amplification kit (Roche), using a LightCycler (Roche). PCR conditions were as follows: 95 C for 5 min, followed by 50 cycles at 94 C for 15 sec, at 55 C for 20 sec, at 72 C for 15 sec, and a final extension at 72 C for 7 min. The relative amounts of genomic RNA were represented by the ratio of the amount against that of fraction 1, and were represented in a bar graph.

Immunoprecipitation of nucleolin-gag protein complex. RK13 cells (3×10^6) were transfected with 1 μg of pch-Nuc by using Fugene 6, 24 hr after infection with rVVpr55^{gag}. Twenty-four hr post transfection, cells were washed three times with cold PBS, swollen in 1 ml of hypotonic buffer (10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$), and disrupted by a 15 ml Dounce tissue grinder with a tight pestle (Wheaton, NJ). Cell extracts were subjected to centrifugation at 1,000×*g* at 4 C for 10 min to sediment nuclei, followed by centrifugation at 10,000×*g* at 4 C for 10 min to precipitate organelles and cytoskeleton components. The soluble fraction obtained (S10) was analyzed by immunoprecipitation followed by SDS-PAGE and immunoblotting with the antibodies described in the text.

Luciferase reporter virus assay. In order to prepare virus stock, 2×10^5 of RK13 cells were transfected with 1 μg of an HIV-1 clone DNA based on the NL-43, pNL-Luc-E+R+, in which the *env* and *vpr* genes were intact and the *nef* gene was replaced with the firefly luciferase gene (7, 23) and with 0.5 μg of human nucleolin (pch-Nuc) and mutant cyclin T1-expressing plasmids by Fugene 6, following the manufacturer's instructions. The culture supernatants were harvested 48 hr after transfection and normalized as the p24 antigen amount. Three hundred μl of the supernatant containing the recombinant luciferase reporter HIV-1 virus virions were

inoculated with 3×10^4 MAGIC5 cells, and after 48 hr the cells were lysed with 200 μl of a lysis buffer supplied in a luciferase assay system (Promega). The luciferase activity was determined with a Lumat LB 96V luminometer (Perkin Elmer).

Results

Promotion of the Budding of Both HIV-1 VLPs and HIV-1 Virion by Human Nucleolin and the RNA Packaging Signal, ψ

Initially, the effect of human nucleolin and the RNA packaging signal on the efficiency of budding of HIV-1 VLPs was examined by measurement of the amounts of $Pr55^{gag}$ in the cellular lysates and the supernatants of the RK13 cells. The cells were infected with recombinant adenovirus encoding human nucleolin cDNA or mock adenovirus, and transfected with the plasmids expressing a truncated HIV-1 genomic RNA lacking the coding regions of all the HIV-1 proteins with [ψ (+)] or without [ψ (−)] the RNA packaging signal, ψ (Fig. 1), followed with infection of recombinant vaccinia virus expressing HIV-1 $Pr55^{gag}$ (rVV-$Pr55^{gag}$). Exogenous human nucleolin did not affect the $Pr55^{gag}$ levels in the cell lysates in the presence or absence of ψ (Fig. 2A). On the contrary, nucleolin enhanced the amounts of $Pr55^{gag}$ in the supernatant (Fig. 2B). In addition, the amount of $Pr55^{gag}$ in the supernatant was also increased in the presence of ψ.

Next, we analyzed the effects of human nucleolin and ψ on the budding of native HIV-1 virion. RK-13 cells were cotransfected with pchNuc encoding human nucleolin cDNA or an empty vector and an HIV-1 clone DNA, pNL43 (ψ+) or without ψ (ψ−). The level of $Pr55^{gag}$ proteins in the supernatants from the cells was analyzed in a similar manner as that shown in Fig. 2. Neither human nucleolin nor ψ affected the cellular level of $Pr55^{gag}$ (Fig. 3A). In contrast, human nucleolin and ψ synergistically enhanced the release of $p24^{gag}$ in the supernatants (Fig. 3B). We also examined the effect of ψ in the human cell line HeLa cells, which express human nucleolin (Fig. 4, A and B). The protein expression levels of nucleolin were not significantly changed after transfection of the plasmid encoding human nucleolin cDNA in HeLa cells (data not shown). Although ψ did not affect the level of $Pr55^{gag}$ in the cells (Fig. 4A), the release level of p24 was enhanced in the presence of ψ in the supernatants (Fig. 4B). These results suggest that nucleolin enhanced the budding efficiency of VLPs and HIV-1 virions in the presence of ψ.

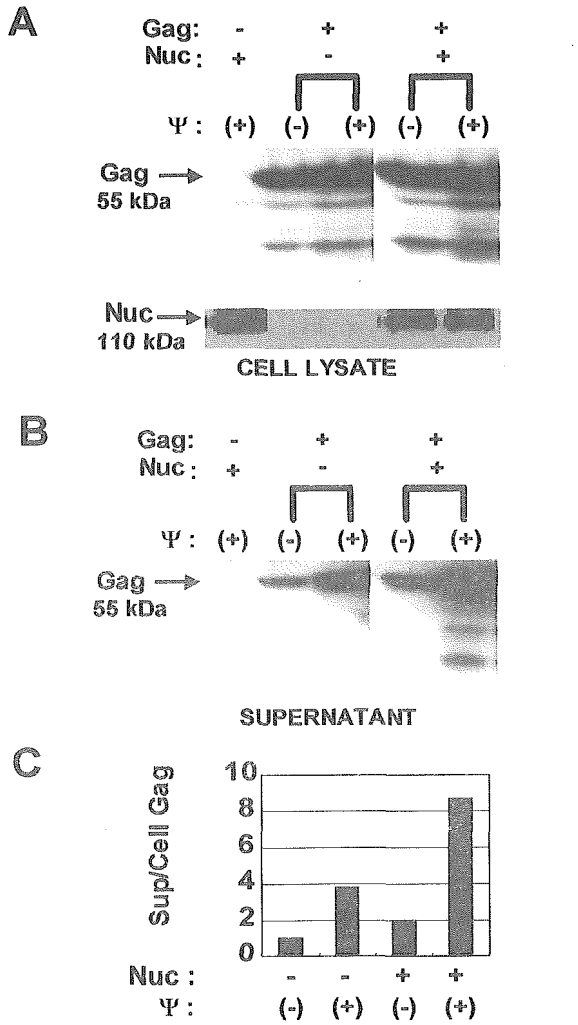

Fig. 2. Enhancement of the budding of VLPs by human nucleolin in the presence or absence of the RNA packaging signal, ψ, in RK13 cells. Cellular lysates (A) or supernatants (B) from RK13 cells expressing (Nuc+) or not expressing (Nuc−) human nucleolin which transiently expressed the truncated HIV-1 plasmid with [ψ (+)] or without [ψ (−)] RNA packaging signal, ψ, followed with (Gag+) or without (Gag−) infection of rVV-$Pr55^{gag}$, were analyzed by immunoblotting with both anti-gag and anti-nucleolin antibodies. Black arrows indicate the positions of $Pr55^{gag}$ (Gag) and nucleolin (Nuc). (C) The signal ratios of Gag proteins in supernatants to that in cell lysates were calculated and the fold increases (Sup/Cell Gag) to the lane without expression of both nucleolin and ψ are demonstrated as a column graph.

Enhancement of Incorporation of Nucleolin into the VLP with the Presence of ψ

Next, we analyzed whether nucleolin could be incorporated into VLPs. RK13 cells were transfected with pchNuc expressing human nucleolin, and a plasmid encoding ψ [ψ (+)] (Fig. 1) followed by infection with rVV-$Pr55^{gag}$. Because expression of Gag proteins by infection of rVV-$Pr55^{gag}$ produced a much higher amount in RK13 cells than transfection of pNL43, nucleolin or

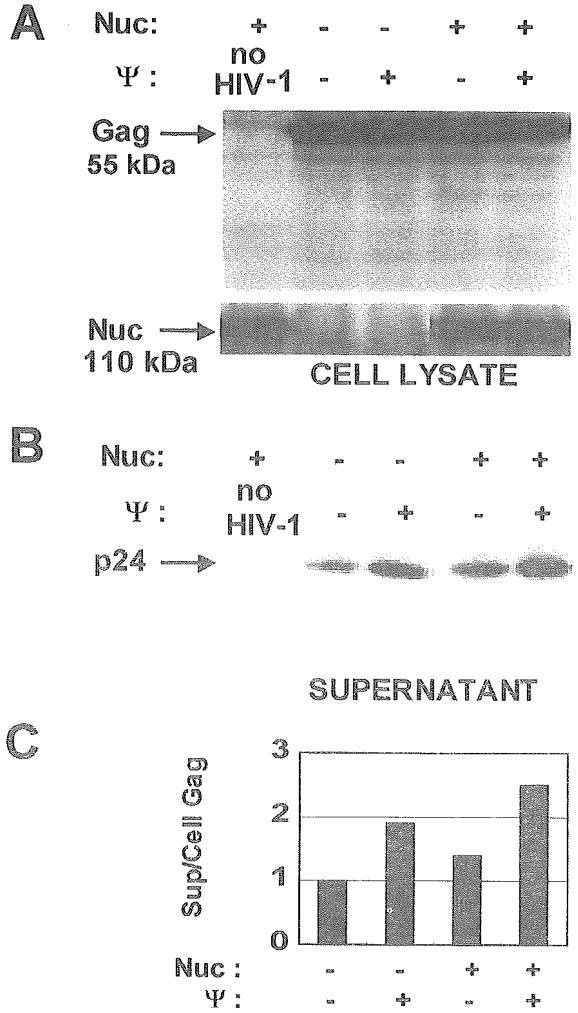

Fig. 3. Enhancement of the budding of HIV-1 virions by human nucleolin in the presence or absence of ψ in RK13 cells. Cellular lysates (A) or supernatants (B) from RK13 cells transfected with pchNuc expressing human nucleolin (Nuc+) or an empty vector (Nuc−) and an HIV-1 clone, pNL43 (ψ+) or pNL43 without ψ (ψ−) or without HIV clone (no HIV-1), were analyzed by immunoblotting with both anti-gag and anti-nucleolin antibodies. Black arrows indicate the positions of $Pr55^{gag}$ (Gag), nucleolin (Nuc) and p24. (C) The signal ratios of p24 proteins in supernatants to Gag proteins in cell lysates were calculated and the fold increases (Sup/Cell Gag) to the lane without expression of both nucleolin (Nuc−) and ψ (ψ−) are demonstrated as a column graph.

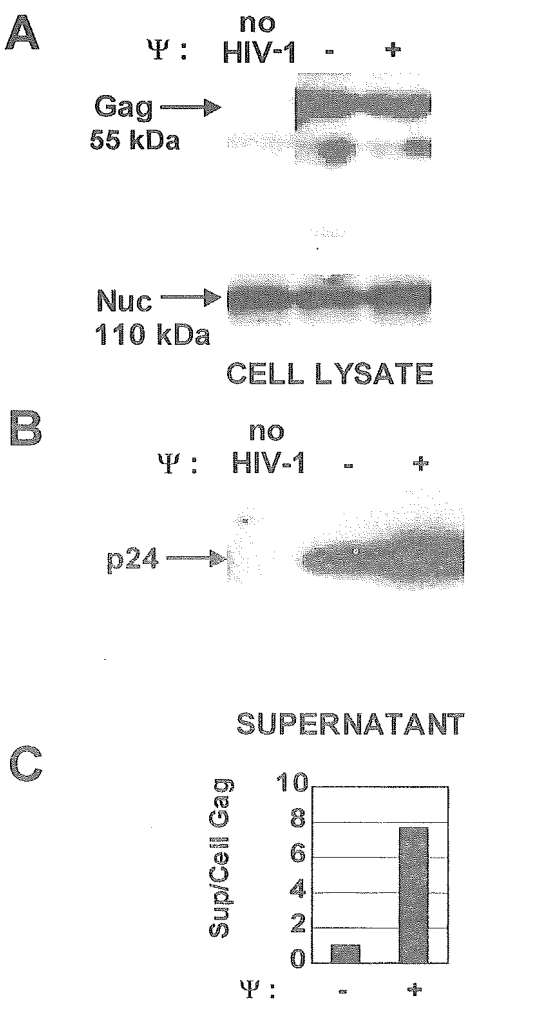

Fig. 4. Enhancement of the budding of HIV-1 virions in HeLa cells. Cellular lysates (A) or supernatants (B) from HeLa cells transfected with an HIV-1 clone, pNL43 (ψ+), pNL43 without ψ (ψ−) or not transfected (no HIV-1) were analyzed by immunoblotting with both anti-gag and anti-nucleolin antibodies. Black arrows indicate the positions of $Pr55^{gag}$ (Gag), nucleolin (Nuc) and p24. (C) The signal ratios of p24 proteins in supernatants to Gag proteins in cell lysates were calculated and the fold increases (Sup/Cell Gag) to the lane without expression of ψ (ψ−) are demonstrated as a column graph.

the packaging signal more effectively enhanced budding of virion when Gag proteins were expressed by rVV-$Pr55^{gag}$.

After fractionation of VLPs by sucrose density gradient, the amounts of gag proteins, nucleolin and genomic RNA in each fraction were examined. As shown in Fig. 5, a high expression level of human nucleolin was recognized in fractions 4, 5, and 6 (their sucrose concentrations being 35, 45, and 50, respectively), in which $Pr55^{gag}$ was also highly assembled. HIV-1 genomic RNA was highly assembled in fractions 5 and 6 (about a 5-fold increase in comparison to fraction 1) (Fig. 5).

To examine the role of ψ in the incorporation of human nucleolin into VLPs, RK13 cells were transfected with pchNuc expressing human nucleolin and a plasmid encoding [ψ (+)] or unencoding ψ [ψ (−)] (Fig. 1); thereafter, the cells were inoculated with rVV-$Pr55^{gag}$. Similarly as in Fig. 5, VLPs in the supernatants were separated by sucrose density gradient and 14 fractions were analyzed for gag proteins and human nucleolin. Gag proteins and human nucleolin were assembled in fractions 4,