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Effect of Ku80 Depletion on the Preintegrative Steps of HIV-1 Replication in Human Cells

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To gain new insights regarding the role of Ku, the DNA-PK DNA-binding component, during lentiviral DNA integration, we have investigated the HIV-1 replication in Ku80-depleted human cells. CEM4fx cells underexpressing the Ku80 factor were selected after transduction by a retroviral vector expressing the Ku80 full-length antisense sequence. *De novo* infection experiment with NL4.3 HIV-1 strain led to the observation that the viral replication was delayed in the Ku80-depleted cells. Early events of the replicative cycle, including nuclear import of the viral DNA, were not affected. In contrast, the formation of the 2-LTR circles was impaired, thus demonstrating the implication of Ku in HIV-1 DNA circularization, for the first time in human cells. Furthermore, the detection of integrated proviruses by an Alu-LTR-nested PCR amplification method was affected in cells underexpressing Ku80. These results suggest that this factor may also be involved in the mechanisms leading to the stable establishment of HIV-1 provirus.

Key Words: HIV-1; Ku; DNA-PK; integration; RNA antisense.

INTRODUCTION

During the early steps of the HIV-1 replication cycle, the RNA viral genome is successively reverse transcribed into a linear double-stranded cDNA copy, transported to the nucleus, and covalently integrated into the cellular genome (*1*). These events take place in context of successive nucleoproteins which culminate in the preintegration complex that contains the viral DNA, the viral enzyme integrase, and other viral and cellular factors necessary to carry out the integration reaction (*2–5*). Despite many attempts to find preferred DNA sites for integration, virtually no sequence specificity has been found *in vivo*, clearly showing that no strong sequence homology is required between donor and acceptor DNA (*6*). Therefore, retroviral DNA integration can be viewed as a nonhomologous recombination event related to V(D)J recombination in terms of both its mechanism and the involvement of cofactors such as HMG proteins that stimulate both processes (*4, 7–9*). DNA-PK, a major component of the cellular DNA double-strand break repair pathway, which is strictly required for V(D)J recombination, was suggested to be involved in retroviral integration (*10*). The DNA-PK is a trimeric nuclear protein kinase consisting of a large catalytic subunit and the Ku70/80 heterodimer that regulates kinase activity by its association with DNA (*11*). Ku, which was first thought to bind preferentially to double-stranded DNA extremities, is also capable of binding to nicked dsDNA and to ssDNA (*12, 13*). DNA intermediates generated during the retroviral integration process resemble branched DNA structures that may constitute favorable DNA substrates for Ku binding during DSB repair process (*12, 14, 15*). From this viewpoint, Ku has been shown to be involved in mobile DNA transposition in *Drosophila* (*16*) and Ty retrotransposition in yeast (*17*).

A direct involvement of DNA-PK during the integration process remains controversial (*18, 19*). Nevertheless, there is an agreement that infection of rodent Ku-deficient cells with high titers of HIV-derived lentiviral vectors triggers a preapoptotic signal that eventually leads to cell death (*18, 20*). A first hypothesis stipulates an implication of Ku/DNA-PK in the repair of the single-strand gaps remaining at the 5′ ends of the provirus, which is required to complete integration. According to this model, the presence of as few as one gapped integration intermediate in Ku-deficient cells constitutes the preapoptotic signal (*21, 22*). A second model proposed that the apoptotic signal is provided by the accumulation of linear viral DNA. This model originates in the observation that Ku is responsible for the circularization of unintegrated linear DNA (*18*).

To date, such a putative role for Ku during lentiviral DNA integration was investigated by the roundabout way of rodent cells transduction by lentiviral vector (*10, 18, 20*). However, major differences may exist between rodent and human cells regarding DNA repair pathways (*23*). This possibility is illustrated by the fact that KARP, a second protein coded by Ku80 gene which is also implicated in the DNA-PK activity regulation, is present uniquely in primates (*9, 24*). Since no Ku null mutant cell

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line of human origin has been isolated to date, we used an antisense strategy to investigate the effect of Ku depletion in human cells. It was previously demonstrated that antisense sequences directed against Ku80 were capable of impairing the expression of the protein in human cell lines, notably in colon carcinoma cells HCT116, in fibroblast cells MRC5V1, and in chronically HIV-1-infected monocytic U1 cells (*25–27*). Antisense sequences directed against Ku70 were also used to deplete human T cell lymphoma MT2 cells (*28*). A 50% depletion was sufficient to affect both Ku and DNA-PK functions significantly in human cells (*27–29*).

We recently demonstrated that Ku affects the postintegration steps of HIV-1 replication by repressing the LTR-driven transcription (*27*). Here, we investigated a possible role of Ku during the early steps of HIV-1 replication. We used the antisense strategy to obtain CEM4fx cells displaying a selective decrease in Ku80 expression. We observed that the replication of HIV-1 was delayed in Ku80-depleted cells, although the early steps, including reverse transcription and nuclear import of the viral genome, were not affected. We demonstrate that Ku80 depletion resulted in an impairment of 2-LTR circles formation, thus showing that Ku was involved in the circularization of linear viral DNA in human cells. Furthermore, the amount of integrated events that were detected by an Alu-LTR PCR-amplification method was decreased in Ku-depleted cells. This result suggests that Ku may play another role during the integration process.

RESULTS

Depletion of Ku80 in CEM4fx cells

A retroviral expression vector coding for an antisense RNA containing the complete coding sequence of Ku80 was constructed. The amphotropic virus-packaging murine cell line, GPAm112, was transfected either with this construct or with the empty vector pLNCX. Cellular clones were selected for their resistance to geneticin and used as a source of recombinant amphotropic virus. Transduction of CEM4fx cells with the retroviral vector containing the Ku80-antisense RNA was followed by clonal selection. Clonal selection of cells was necessary due to the prolonged half-life of the Ku protein (>5 days). Ku content was estimated by Western blot in three different clones in which the presence of the antisense was first verified by standard RT-PCR procedure (data not shown). As shown in Fig. 1A, the three clones (clones G3, P5, and F5) displayed a strong decrease of Ku80 expression compared to the parental CEM4fx cells. To confirm that the depletion of Ku80 content actually led to a diminution of Ku activity in cells, we investigated the double-strand DNA end-binding (DEB) activity of Ku-depleted cells. Indeed, Ku is the major factor responsible for the DEB activity in human cells which can be detected using an electrophoretic mobility shift assay (*26*). The gel-shift

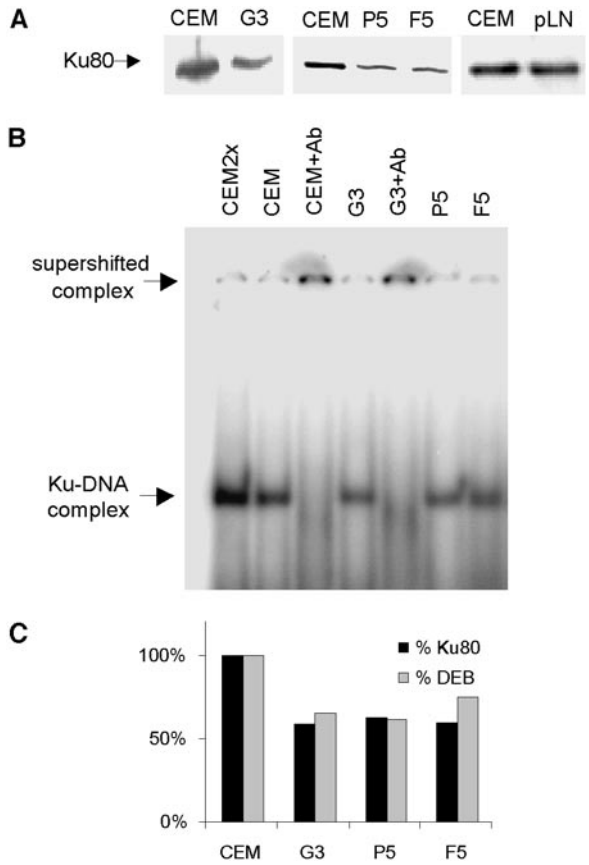

FIG. 1. Determination of Ku content and activity in three Ku80-depleted clones G3, P5, and F5. (A) Immunoblot analysis of 10 μg protein extracts from G3, p5, and F5 clones stably transfected with human Ku80 cDNA. Ten micrograms of either human cells (CEM4fx) proteins or of the empty PLNCX vector-containing cells (pLN) were used as a control for human Ku80 detection. CEM 2× refers to a load of twice as much protein as in the CEM lane. The same CEM cells extract was used as a control in all experiments to avoid any bias due to an eventual variation of the Ku content. Ku was revealed using an antibody raised against human Ku80. Homogenous protein transfer was assessed by Ponceau red staining. (B) Ku DNA end-binding (DEB) activities of nuclear extracts from CEM4fx, G3, P5, and F5 cells. Ten micrograms of extracts were incubated with radiolabeled double-stranded oligonucleotide probe and separated on 5% polyacrylamide gel. Ten micrograms of either CEM or G3 nuclear extracts were further incubated with anti-Ku80 antibody to confirm the presence of Ku80 in the complex by inducing a supershift of this one. (C) Quantification of Ku80 content and Ku DEB activity using densitometry analysis. Values were normalized to CEM4fx cells.

assay was performed with cellular extracts of either parent CEM4fx or Ku80-depleted cells. Results of the experiment are shown in Fig. 1B. The amount of shifted probe was significantly lower in the presence of G3, P5, and F5 extracts than in CEM4fx extracts. The presence of Ku in the shifted complex was confirmed by supershifting with a Ku80 antibody. The complex was efficiently displaced, thus confirming that Ku80 was its major component. These results indicated that the Ku depletion was functionally significant. Quantification of Ku80 amount and DEB activity are summarized in Fig. 1C. The three

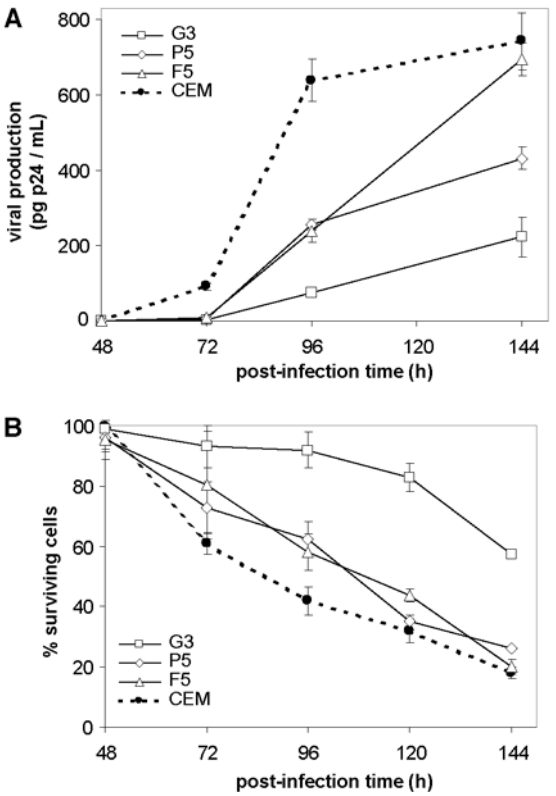

FIG. 2. HIV-1 replication in Ku80 depleted cells. (A) Viral production was determined by p24 ELISA as a function of time following infection with the HIV-1 NL43 strain. (B) Determination of cell viability using the MTT assay. Errors bars are standard deviation from three experiments. Values were normalized to noninfected CEM4fx.

independent clones showed 40 to 50% depletion in Ku80 amount and function, whereas CEM4fx cells selected after infection with a retroviral vector carrying an empty pLNCX construct showed Ku levels comparable to control cells. No difference could be detected. Taken together, these results demonstrate that a depletion of Ku80 can be achieved in the lymphoid human cell line CEM4fx by a full-length RNA antisense strategy.

Effect of Ku80 depletion on HIV-1 replication

To address the consequences of the Ku80 depletion on HIV-1 replication in CEM4fx cells, the Ku80-depleted clones G3, P5, and F5 were infected *de novo* with the NL4–3 HIV-1 strain. Both the virion release and the cell mortality were monitored over a 5-day period using standard p24 antigen ELISA and MTT assays. Results are shown in Fig. 2. In parental CEM4fx cells, release of virions was detectable 72 h after infection. In contrast, the viral production was delayed in the Ku80-depleted clones (see Fig. 2A). The virus release reached a plateau 96 h after infection in parent cells, whereas the amount of viruses was still low in the supernatants of the three depleted clones. Since the viral production can be followed by the cytopathogenicity effect of virus release, the viability of infected cells was monitored using a standard MTT assay over the same period of time. As shown in Fig. 2B, the cell loss paralleled the viral production. A delay similar to the one obtained during viral production was observed for the three Ku80-depleted cell lines compared to the parent cells. In conclusion, the depletion of Ku80 led to a slowing down of the HIV-1 replication rate in CEM cells. This effect was not due to a bias related to variation of the cell metabolism as the growth rates of Ku80-deficient clones were not distinguishable from that of control cells with a doubling time close to 24 h for each clone (data not shown).

Early steps of HIV-1 replicative cycle in Ku80-depleted cells

To gain additional information concerning the nature of the replicative step that was affected in Ku80-depleted cells, we first evaluated the amount of the different viral DNA species, namely the linear, circular, and integrated DNAs, depicted in Fig. 3, by PCR amplification in either control cells or Ku80-depleted cells after infection with the NL4–3 HIV strain. First, late reverse transcripts were detected using appropriate primers as described previously (*31*). Detection was performed 24 h after infection to remain in conditions of a single-round infection. PCR amplification with these primers located within the LTR (see Fig. 3) allows the detection a short viral sequence present in all viral species, the amount of which being consequently an estimation of total viral DNA in cells. The linearity of the PCR responses was assessed by a control dose-response curve. Results are shown in Fig. 4A. As expected, no viral DNA could be detected either in noninfected cells or in mock-infected cells (see Fig. 4D). In contrast, viral DNA was detected in CEM4fx-, G3-, P5-, and F5-infected cells. The amplification of total viral DNA yielded comparable amounts of products in either Ku-proficient or in Ku-deficient cells, indicating thereby that the depletion of Ku80 did not interfere either with the viral entry or with the reverse transcription steps.

Nuclear translocation in Ku80-deficient cells

In a second experiment, the nuclear translocation of postreverse transcription complexes was estimated from the amount of 1-LTR and 2-LTR circles that are exclusively detected in the nucleus of infected cells (see Fig. 3) (*32, 33*). Circularized DNA was amplified 48 h after infection with primers allowing the detection of both 1-LTR and 2-LTRs products. Results of this experiment are shown in Fig. 4B. 1-LTR circles represent the most abundant circularized DNA species in infected cells. Accordingly, they were readily detected using ethidium bromide staining (see Fig. 4B). In contrast, the less abundant 2-LTR circles products could be only detected using Southern blotting of amplified bands (Fig. 4B). No difference between parental and Ku80-depleted cells could be

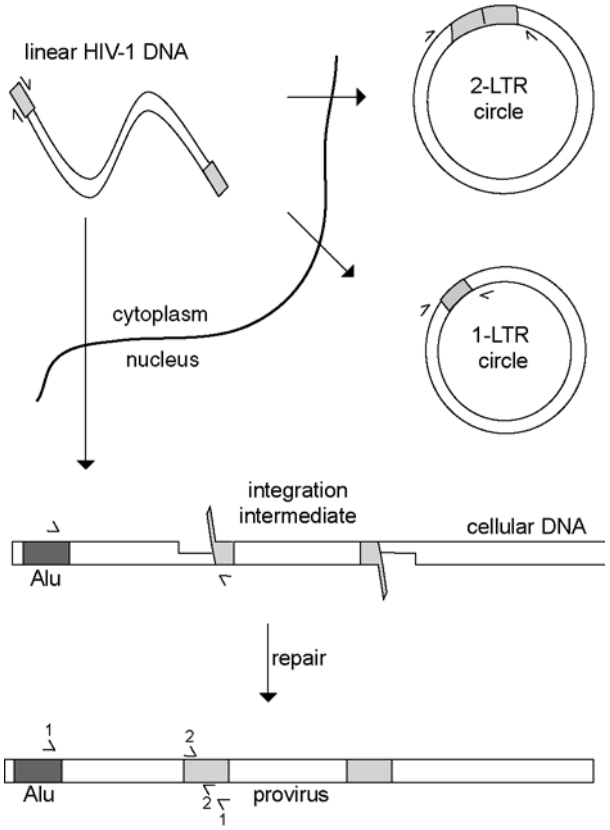

FIG. 3. PCR-amplification scheme for detecting viral DNA in infected cells. Total viral DNA was estimated by amplification using primers located within the LTR. 1-LTR and 2-LTRs circles were detected using primers located outside of the LTRs. Size of the amplified products allowed the distinction between 1-LTR and 2-LTRs species. Integrated provirus were detected using an Alu-LTR amplification scheme. Primers "1" were used for the first Alu-LTR amplification which yielded bands of variable lengths depending on the provirus location. Primers "2" were used for the nested LTR-LTR amplification that yielded one homogenous PCR-product for all integration events.

detected in the amplification of 1-LTR circles, thereby eliminating translocation to the nucleus as the step inhibited in Ku-depleted cells. On the contrary, 2-LTR circles were only poorly detectable in the F5 and the P5 clones and not visible at all in the G3 clone. This result showed that Ku80 depletion led to a defect in circularization of the unintegrated viral DNA.

Integration of HIV-1 proviral DNA

Finally, we used an Alu-LTR nested-PCR scheme to distinguish integrated from nonintegrated viral DNA in both infected Ku80-depleted cells and in parental CEM4fx cells (*34*). Total DNA was prepared from CEM4fx, G3, P5, and F5 cells 24 h after infection and submitted to an initial amplification step between genomic Alu and LTR sequences (see Fig. 3). Then, a nested amplification specific to the LTR viral sequence was carried out. The same amplification scheme was performed in absence of the Alu primer during the initial amplification step to discriminate between integrated and potentially nonintegrated viral DNA carried over from the cellular extracts (further referred to as the "carry-over" products). Mock infection using a heated virus was used as a control. Results are shown in Fig. 5. Proviruses were readily detected in CEM4fx cells with no carry-over products. No signal could be detected in mock-infected cells. In contrast, the amount of provirus was markedly lower in the three Ku80-depleted clones than in CEM4fx cells. Any bias due to variations in the initial amount of DNA was discarded by the result of the cellular globin gene amplification that was identical among all cell lines. Furthermore, the linearity of the response was assessed by a control dose-response curve. Altogether, these results indicate that an integration-related event was perturbed in Ku80-depleted cells.

DISCUSSION

In this study, we have investigated the effect of Ku80 depletion on the HIV-1 replicative cycle in CEM4fx lymphoid human cells. An antisense strategy was used to decrease the cellular expression of Ku80, which forms, along with Ku70, the DNA-binding component of the DNA–PK complex. Full-length antisense RNA was constructed by cloning the Ku80 cDNA in reverse orientation into a retroviral expression vector. Western blot analysis and DNA-binding assay demonstrated depletion of Ku80 in CEM4fx cells that were transduced with the antisense retroviral construct. We have recently demonstrated that Ku represses the viral expression, most likely through a specific interaction with the HIV-1 LTR (*27*). Consequently, a significant enhancement of HIV-1 expression in the Ku80-deficient cells was expected. On the contrary, HIV-1 infection of three independent Ku80-depleted clones demonstrated that the replication efficiency of HIV-1 was reduced in these cells. This partial impairment of HIV-1 replication in Ku80-deficient cells suggested that Ku is involved in other steps of the replication. Therefore, we have extended our investigations to the preintegrative events. Determination of the viral DNA species by PCR amplification demonstrated that Ku affected one or more of these events in human cells. This possibility was first evoked by Daniel and colleagues who observed the unproductive infection of DNA-PK deficient rodent *scid* cells by lentiviral vectors (*10*). In our study, no differences could be detected between Ku80-depleted and control cells concerning the preintegrative steps except for the formation of 2-LTR circles which results from the circularization of unintegrated linear viral DNA. This is in agreement with a previous work from Li *et al.,* who have found that Ku along with putative other factors from the nonhomologous end joining pathway (NHEJ) were required for the formation of 2-LTR circles in rodent cells (*18*). Thus, our data confirm in human cells the importance of Ku for the circularization process of

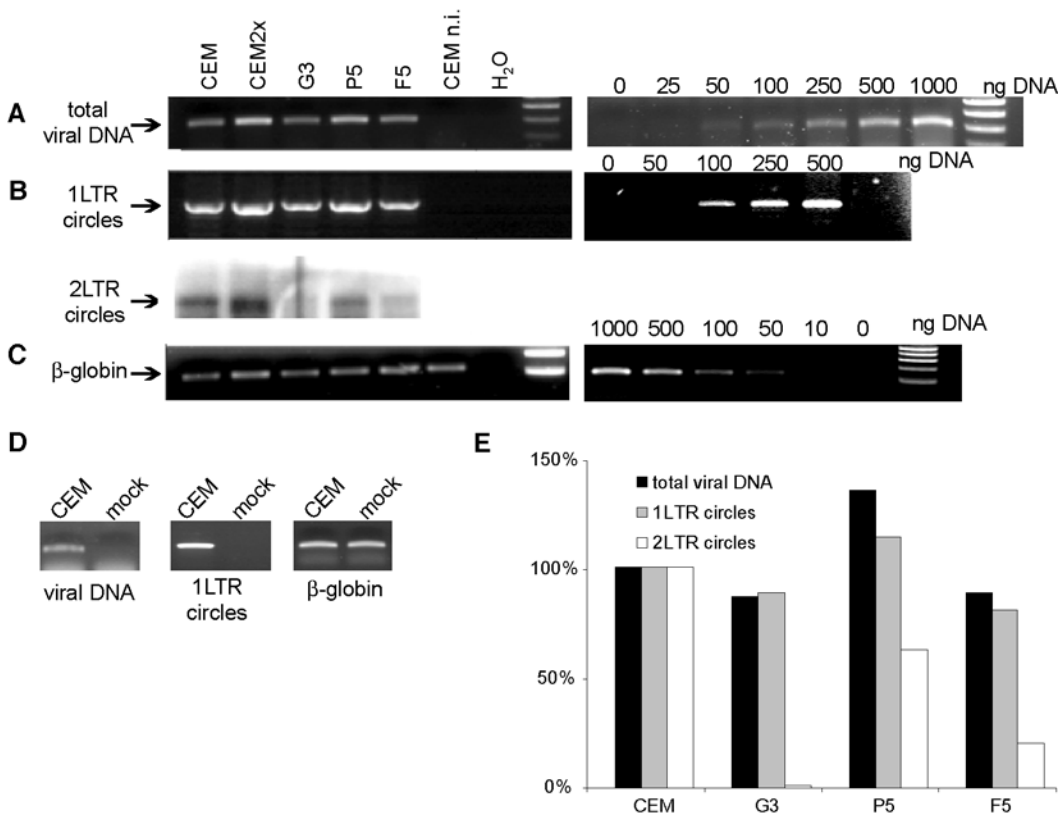

FIG. 4. Early replicative steps in Ku80-depleted cells. Semiquantitative PCR analysis of HIV-1 DNA species at 24 h (total viral DNA) or 48 h (circular DNAs) postinfection. (A) Total viral DNA, (B) 1LTR and 2LTR circles, aand (C) β-globin were amplified from 250 ng of total cell DNA using specific primers. Amplification products were stained with ethidium bromide on agarose gels except for 2LTR circles that were detected after Southern blotting and hybridization with an HIV-1 radiolabeled probe. For every DNA species, a calibration range of PCR amplification was performed using the infected CEM4fx DNA to determine the range of template amount yielding a linear response. CEM 2× refers to an amplification of twice as much DNA as that in CEM lane and CEM n.i. refers to extract from noninfected cells. (D) Control amplification of the different DNA species from CEM4fx cells infected either with heat-inactivated virus (mock) or with nontreated virus (CEM). (E) Quantification of viral DNA in the different cell populations by densitometry analysis. Values were normalized to signal obtained with CEM4fx DNA.

unintegrated retroviral DNA. This function is consistent with the fact that Ku is capable of aligning free DNA ends to facilitate their intermolecular ligation (*29, 35, 36*). In context of the replication of wild-type HIV-1, circularization of unintegrated DNA may be important to avoid accumulation of free DNA ends that can trigger cell apoptosis (*18*).

In addition, we also observed that a lower amount of integrated proviruses could be amplified using a nested PCR method in Ku-deficient cells in a single-round infection experiment. This apparent lack of provirus may be explained either by a loss of cells due to the infection by HIV-1 or by a bias during the PCR amplification. Regarding the first hypothesis, a loss of proviruses due to the cellular death should also result in an overall decrease of the total viral DNA in the cell population. This was not the case in our study since we observed no significant differences in the amount of total viral DNA in Ku-deficient cell lines compared to the parent cells. Moreover, the extent of cellular death was not distinguishable in Ku-proficient and Ku-deficient cell lines 48 h following infection. Thus, a bias in the Alu-LTR PCR amplification is more likely to account for this discrepancy.

Such a bias may originate from a change of the proviruses localization within the host genome. Since one possible role of *Alu* repeats which are frequently involved in nonhomologous recombination is the recruitment of recombinogenic proteins such as Ku (*37*), specific requirement for such factors during provirus integration may lead to integration near *Alu* sequences. Indeed, it was proposed that the vicinity of repetitive elements may be favorable for HIV provirus integration, although such specificity remains controversial (*37–39*). Consequently, a lack of Ku may result in a lower recruitment of proviruses in the vicinity of *Alu* sequences, thereby enriching the population of Alu-LTR fragments with longer DNA sequences. Since the amplification of the latter is less efficient compared to shorter ones, this effect is expected to give rise to an apparent decrease of the PCR yield. This model hints to the possibility that Ku may be involved in the localization of the provirus within the chromatin. Furthermore, the site of integration of the

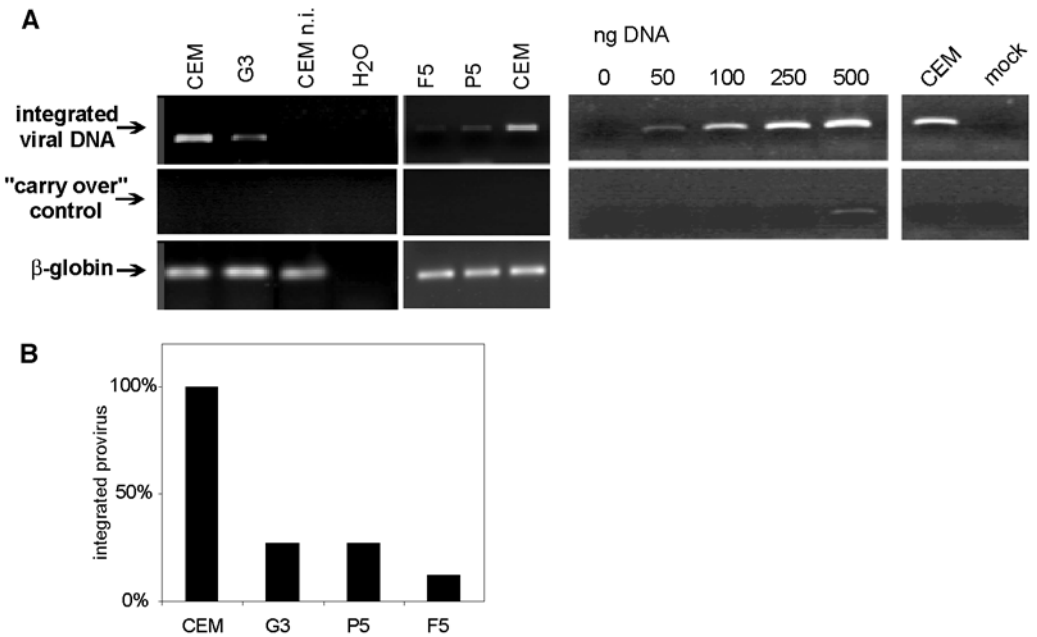

FIG. 5. Proviruses detection in Ku80 depleted cells. (A) Detection of provirus 24 h postinfection using a nested Alu-LTR PCR scheme. Total DNA was amplified using a human Alu sequence primer and an HIV-1 LTR primer. The resulting products were diluted and amplified using primers located in the HIV-1 LTR sequence. Amplification of DNA from either noninfected cells (CEM4fx n.i.) or cells infected with heat-inactivated virus (mock) were used as negative controls. Reaction in the absence of the Alu primer during the first amplification step was used as a control of amplification of nonintegrated viral DNA ("carry-over" control). (B) Quantitative analysis of provirus amplification by image densitometry. Values were normalized to signal obtained with CEM4fx DNA.

proviruses in the genome could have dramatic effects on its transcriptional activity, thus giving an explanation for the different rates of viral release observed for Ku-proficient and Ku-deficient cells (*40*).

A second model may be summoned in which the lack of Ku would result in a temporary persistence of uncompleted integration event, affecting in turn their detection by the ALu-LTR amplification method and the replication efficiency. In this model, the 5′-gaps (see Fig. 3) would eventually become repaired, whether it is due to the residual Ku activity or to a different mechanism capable of coping with low dose of damages in a Ku80-independent manner. This latter effect is not unprecedented since ERCC6, which is implicated in the repair of damage occurring after high doses of UV in rodent cell lines, is not required for the processing of low levels of damage in CHO cells (*41*). Moreover, in the case of DSB repair, a DNA-PK-independent mechanism was previously proposed for *scid* cell (*42*). However, we do not favor this model as we noted that the persistence of the gapped intermediates should yield in theory a twofold decrease in the amplification assay (*43*). In our experiments, the yield of provirus recovery varied from 15 to 35% of the amount detected in the parental CEM4fx cell line. Thus, the presence of unrepaired gaps early after integration in Ku80-deficient cells DNA cannot explain the totality of the provirus lack.

Solving this question in a human cellular model would necessitate probing the boundaries of integrated proviruses early after integration in Ku-deficient cells to determine whether the lower detection actually arises from a defect in 5′-gaps repair that perturb the PCR amplification. This work is currently in progress.

We conclude that Ku is involved at several pre- and postintegrative stages of HIV-1 replication. Ku is the third cellular factor related to the modulation of chromatin structure and function to be potentially involved in an HIV DNA replicative step related to integration. PARP and Topoisomerase II are the two others (*30, 44, 45*). Thus, the role of Ku80 should be considered in the context that these proteins could cooperate to influence the interaction of the provirus with its insertion site. Ku and PARP were demonstrated to form a specific complex that binds synergistically to matrix-associated regions of chromosomes (MARs) (*9, 46*). Topoisomerase II is also directly involved in the attachment of MARs to the nuclear matrix or chromosome (*47*). Strikingly, another cellular protein, HMG-I (Y), which is part of the preintegration complex (PIC) and stimulates integration *in vitro*, binds specifically to AT-rich sequences that characterize MAR sequences (*4, 48*). Consequently, the possible interference of these cellular factors with some stage of the integration process may indicate that the spatial and/or functional organization of chromatin is involved in the regulation of HIV DNA integration.

MATERIALS AND METHODS

Cells and viruses

All culture media were supplemented with 10% fetal calf serum and penicillin/streptomycin. NIH3T3, GP+*env*Am 12, HeLa, and P4 (HeLa-CD4$^+$-βGal) cells were grown in DMEM. GP+*env*Am 12 (a gift of Dina Markowitz) is a packaging cell line providing the viral *gag*, *pol*, and *env* functions. CEM4fx were grown in RPMI 1640 medium. CEM4fx cells were derived from the human lymphoid cell line CEM (ATCC CCL119) and express high levels of CD4 antigen. The MoMLV-based pLNCX retroviral vector contains the retroviral LTRs and PSI sequences, the neomycin-resistance gene, and a multiple cloning site downstream of the human cytomegalovirus immediate-early promoter. Ku80 cDNA (a gift of Muriel Le Romancer), cloned in reverse orientation in pcDNA3 multi-cloning site (*25*), was excised by *Xho*I/*Bam*HI and cloned into *Xho*I/*Bgl*II previously digested pLNCX to generate the pLNCX-ASKu vector. Vector DNA was transfected into packaging cell line GP+*env*Am 12 by using Superfect reagent (Qiagen). Titers of produced retroviral vectors were determined after infection on NIH3T3 cells and G418 selection. Supernatant titer of GP+*env*Am 12 cells transfected with shuttle vector was 2×10^5 particles/ml. HIV-1 stocks, produced by transfection of HeLa cells with plasmid pNL4–3 using Superfect (Qiagen), were assayed by infection of P4 cells followed by cell fixation and X-Gal staining. For HIV-1 infection experiments, CEM4fx cells were infected with filtrated cell-free virus-containing supernatants in 96-well plates at 10,000 cells/well (p24 production) or in 75-cm^2 flasks for DNA preparation (2–4 $\times$ 10^6 cells/flask) at low m.o.i. (0.2 viral particle/cell). Viral supernatants were removed 2 h after infection. *De novo* viral production was estimated by ELISA-p24 assay (DuPont-NEN) every 24 h. Cell viability was estimated using a MTT assay (*30*). For mock controls, NL43 viral supernatant was incubated 45min at 65°C and used to infect CEM4fx cells.

Selection of Ku80-depleted cells

On the day of infection, 5×10^5 CEM4fx were centrifuged and resuspended in 2 ml medium containing pLNCX-ASKu viral particles (m.o.i. = 0.01). At 24 h, cells were diluted (10 cells/200 μl) and transferred into a 96-well plate in medium containing 800 μg/ml G418 for clonal selection. G418-resistant cells were analyzed 3 weeks later.

Ku80 protein analysis

Cells were collected and washed in PBS and 5×10^6 cells/ml were lysed in lysis buffer (50 mM NaF, 20 mM HEPES pH 8, 450 mM NaCl, 25% glycerol, 0.5 mM DTT, 0.2 mM EDTA pH 8) in the presence of a protease inhibitor mix (Boehringer). After three freeze/thaw cycles, samples were centrifuged for 30 min at 12,000 rpm at 4°C. Protein concentration in supernatants was determined using a standard Bradford assay. An amount of 10 μg of total protein was electrophoresed on 8% polyacrylamide gel and electrotransferred onto nitrocellulose membranes. Membranes were washed in TBS, blocked in 5% dry nonfat milk/TBS, and then incubated with 0.2 μg anti-Ku80 antibody (Serotec) in TBS/0.05% Tween. Blots were washed in TBS-Tween and incubated with alkaline phosphatase conjugated secondary antibody at room temperature. Visualization was achieved using a chemiluminescence assay (Bio-Rad).

Nuclear extracts preparation

All buffers contained a complete protease inhibitor mix (Roche). Cells were washed with PBS and resuspended in 3 mL STM buffer (20 mM Tris pH 7.85, 250 mM sucrose, 1.1 mM $MgCl_2$) containing 0.2% Triton X-100 and incubated at 4°C for 4 min. The cell lysate was then centrifuged at 2000 rpm for 3 min at 4°C. The pellet, mostly containing the unbroken nuclei, was resuspended in 1 mL STM buffer and centrifuged at 2000 rpm for 2 min at 4°C. The nuclei were then resuspended in STM buffer containing 0.4 M KCl and incubated 10 min at 4°C. The extract was centrifuged at 14,000 rpm for 10 min at 4°C and the supernatant fraction was collected and used as nuclear extract.

Bandshift assay

Nuclear extract (10 μg) was incubated with a ^{32}P-labeled double-stranded oligonucleotide 5′- GGG CCA AGA ATC TTA GCA GTT TCG GG -3′ in binding buffer (20 mM Tris pH 8, 2 mM EDTA, 20% glycerol, 0.4 M NaCl, 200 μg/mL circular plasmid DNA) for 5 min at 4°C. For supershift, anti-Ku80 antibody was added and binding reaction was allowed to proceed 10 min longer. Ku–DNA complexes were separated by electrophoresis on a 5% non-denaturing polyacrylamide gel and visualized on a phosphorimager screen.

Analysis of viral DNA

Total DNA was prepared using QIAamp DNA Blood Mini Kit (Qiagen) either 24 h postinfection for total viral DNA and provirus DNA or 48 h postinfection for circular DNAs. PCR amplifications were performed in the presence of 5 units/ml Amplitaq polymerase (Cetus) in the buffer supplied by the manufacturer (*Taq* Gold buffer II) supplemented with 200 μM dNTPs and 1 μM of each primer. All PCR reactions were normalized by a control amplification of the β-globin gene with primers GloS and GloAS (*31*) on 250 ng of template DNA. Before any quantitative analysis, a calibration range of the PCR amplification was performed using the appropriate templates (total DNA from infected CEM4fx) to determine the range of template amount that did not lead to a saturated

signal. PCR for nonintegrated viral DNA amplification was performed as follows: 250 ng total DNA were amplified 30 cycles, 30 s at 94°C, 30 s at 58°C, 30 s at 72°C. The primers used were M667 and M661 (*31*). PCR products were resolved on an 1% agarose gel and stained with ethidium bromide.

PCR for 1-LTR and 2-LTR circles was performed as follows: 250 ng of total DNA was subjected to 35 amplification cycles (30 s at 94°C, 30 s at 64°C, and 2 min at 72°C) with the primers 1LTR2LTR (5′-CAC AAG AGG AGG AAG AGG TGG GT-3′) and gen3 (5′-CAC CAG TCG CCG CCC CTC GC-3′). This reaction resulted in a 0.8-kb amplification product for 1-LTR circles and in a 1.4-kb product for 2-LTR circles. Products were run on a 1% agarose gel. 1-LTR products were readily visible with ethidium bromide staining. To detect 2-LTR products, the upper part of the gel was Southern blotted. The membrane was hybridized with a random-primed HIV-1 LTR probe. 2-LTR products were then visualized on a phosphorimager screen.

To detect integrated viral DNA, 100 ng of total DNA was subjected to 22 amplification cycles: 30 s at 94°C, 30 s at 66°C, and 5 min at 72°C. The primers used were LTR5 (5′-AGG CAA GCT TTA TTG AGG CTT AAG-3′) and Alu3 (5′-TCC CAG CTA CTC GGG AGG CTG AGG-3′). Parallel amplification was performed without the Alu3 primer to detect carried over linear viral DNA. The amplification products were then diluted 400 times and submitted to 35 amplification cycles: 30 s at 94°C, 30 s at 63°C, and 1 min at 72°C. The primers used were NI1 (5′-CAC ACA CAA GGC TAC TTC CCT-3′) and NI2 (5′-GCC ACT CCC CAG TCC CGC CC-3′). Nested-PCR products were resolved on a 1% agarose gel containing ethidium bromide.

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