Nuclear Import of Moloney Murine Leukemia Virus DNA Mediated by Adenovirus Preterminal Protein Is Not Sufficient for Efficient Retroviral Transduction in Nondiving Cells

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Moloney murine leukemia virus (MoMLV)-derived vectors require cell division for efficient transduction, which may be related to an inability of the viral DNA-protein complex to cross the nuclear membrane. In contrast, adenoviruses (Ad) can efficiently infect nondiving cells. This property may be due to the presence of multiple nuclear translocation signals in a number of Ad proteins, which are associated with the incoming viral genomes. Of particular interest is the Ad preterminal protein (pTP), which binds alone or in complex with the Ad polymerase to specific sequences in the Ad inverted terminal repeat. The goal of this study was to test whether coexpression of pTP with retroviral DNA carrying pTP-binding sites would facilitate nuclear import of the viral preintegration complex and transduction of quiescent cells. In preliminary experiments, we demonstrated that the karyophytic pTP can coimport plasmid DNA into the nuclei of growth-arrested cells. Retroviral transduction studies were performed with G418/350-LTA cells or stationary-phase human primary fibroblasts. These studies demonstrated that pTP or pTP-Ad polymerase conferred nuclear import of retroviral DNA upon arrested cells when the retrovirus vector contained the corresponding binding motifs. However, pTP-mediated nuclear translocation of MoMLV DNA in nondiving cells was not sufficient for stable transduction. Additional cellular factors activated during S phase or DNA repair synthesis were required for efficient retroviral integration.

Integration of viral DNA into the host chromosome is an essential step in the retroviral life cycle. With their remarkably efficient ability to integrate, retrovirus vectors are an important tool in obtaining stable gene expression in vitro and in vivo (37, 65). However, the most commonly used and as yet best-characterized vectors based on mammalian C-type retroviruses cannot transduce quiescent cells, which often represent the targets for gene therapy approaches (20, 38, 43, 63).

After entry of the retroviral core into the cytoplasm, the retroviral genome is reverse transcribed by using enzymatic activities associated with the incoming virion. The resulting double-stranded linear viral DNA is associated with viral proteins, forming a large nucleoprotein complex. To complete the retroviral life cycle, this preintegration complex must be translocated to the nucleus. The mechanisms of nuclear transport appear to differ among different retrovirus subfamilies and host cells (for a review, see reference 8). For Moloney murine leukemia virus (MoMLV)-derived vectors, it is believed that breakdown of the nuclear membrane during mitosis is necessary to allow access of the preintegration complex to chromosomal DNA. This is supported by two lines of evidence: (i) inhibitors, which delay the onset of mitosis, delay integration as well (3, 33, 38); and (ii) MoMLV proviruses segregate into only one daughter cell during the first mitotic division after infection, implying that integration occurs after DNA replication (19, 43). The MoMLV preintegration complex contains linear DNA associated with the CA, IN, and possibly RT and NC proteins (8). This complex with a sedimentation constant of ~160S is too large to pass through the nuclear membrane by simple diffusion. To enter the nucleus, larger proteins (40 to 60 kDa) require nuclear localization signals (NLS) (for a review, see reference 55). NLS are currently classified into several classes, including those consisting of a single stretch of basic residues, bipartite NLS composed of two clusters of basic amino acids separated by a spacer of 10 to 12 amino acids, and those resembling the NLS of the yeast homeodomain protein Mata2 (55). These NLS interact with cytoplasmic receptors variously named importin α/β, importin 58/97, or karyopherin α/β, initiating an energy-dependent multistep translocation into the nucleus. Importantly, the rate of active nuclear import of proteins is independent of cell proliferation and is similar between arrested and dividing cells (14). Karyophytic proteins containing NLS can function as a nuclear import shuttle for other proteins, RNA, or DNA (11, 39, 51).

In contrast to MoMLV-based retrovirus vectors, adenovirus type 5-derived vectors (Ad) can efficiently transduce nondiving cells in vitro and in vivo (for a review, see reference 24). The efficiency of Ad infection relies to a large degree on efficient targeting of the Ad genome to the host cell nucleus. Ad DNA is packaged together with viral core proteins and pTP/TP, the terminal protein, into virions. After entry into the host cell, the virion is uncoated and the Ad DNA is transported into the nucleus where its replication occurs. It is generally thought that the NLS in the pTP/TP and the core protein V play a crucial role in directing this complex to the nucleus. Both the 80-kDa precursor to the terminal protein (pTP) and the 55-kDa terminal protein (TP) contain an NLS (RLPVR RRRRVP, corresponding to residues 362 and 373 within the TP), which is well conserved among all Ad serotypes (48). It was shown that this motif can mediate nuclear import when transplanted into other proteins (42). Once in the nucleus, one of the first early viral proteins synthesized is the pTP, which binds with its N terminus to bp 9 to 18 of the origin for Ad
replication found at each end of the linear DNA genome within the inverted terminal repeats (62, 68). During lytic infection, pTP forms a stable heterodimer with the Ad polymerase (Pol), which is translocated to the nucleus by using the NLS of pTP. The pTP-Pol complex binds with increased affinity and specificity to the origin. This binding is further enhanced by interaction of the heterodimer with the cellular factors NFI and OCT-1, which bind at nucleotides nt 25 to 50 of the origin (60). pTP functions as a protein primer for Ad DNA replication. After binding, pTP is covalently linked to dCTP, providing a 3'-p terminal group to begin the synthesis of a daughter DNA strand. Furthermore, pTP serves as the site of primary attachment of the viral DNA to a specific protein(s) in the nuclear matrix, forming replicative complexes (5, 17). Late in infection, pTP is proteolytically cleaved by the viral protease, generating the 55-kDa terminal protein.

The goal of this study was to incorporate the pTP-based, Ad, nuclear import machinery into MoMLV vectors and to test whether this would allow retroviral transduction of nondividing cells. To approach this hypothesis, we first analyzed whether pTP could mediate the nuclear import of plasmid DNA carrying pTP-binding sites into arrested cells. Based on these preliminary results, MoMLV-based vectors containing pTP-binding sites were generated and tested for the ability to transduce nondividing cells expressing pTP.

**MATERIALS AND METHODS**

**Plasmids.** The pTP coding sequence (Ad5 bp 8533 to 10589), including a small exon around Ad map unit 39, which contained the initiation codon, was provided by Jerry Schauf, University of Colorado, Denver, Colo. The pTP gene was cloned under the control of different promoters into pEL31sp1A derivatives (Microbiex, Toronto, Canada). To generate pRSV-pTP or pPGK-pTP, the pTP gene was inserted as a 1.2-kb HindIII-EcoRI fragment into pAd.RSV or pAd.PGK (26). To generate pCMV-pTP, a cytomegalovirus (CMV) promoter-pTP gene bovine growth hormone polyadenylation signal (bpa) containing the 4.1-kb Nink-NruI fragment (provided by J. Schauk) was inserted into pEL31sp1Sp. To generate pMT-pTP, the pTP gene was first cloned into pMRENeo (53) and then transferred as a NotI-Xhol fragment into pEL31sp1A. The Ad Pol expression plasmid pCMVpol was also provided by J. Schauk. The Pol cDNA (Ad bp 5187 to 8357) contains a mutation at the C terminus to create a SpI site that does not impair enzymatic activity.

To generate the test plasmid for pTP-mediated nuclear transport (pITR-hAAT), a 0.8-kb fragment of pFG140 from bp –382 to +1452 (Microbiex) containing two head-to-head joined Ad inverted terminal repeats (provided by Jim Nelson, University of Manitoba) was inserted as a pTP-binding motif into the XhoI site of pBS-RSV.hAAT (26) in front of the human α-antitrypsin (hAAT) expression cassette.

Plasmid DNA was purified by ultracentrifugation in two CsCl gradients.

**Ad vectors.** All Ad vectors were generated by homologous recombination using pEL1M (Microbiex) in 293 cells. The shuttle vectors containing pTP expression cassettes based on pEL31sp1A (Microbiex) were cotransfected with pM17 into Ad5-low-passage 293 cells by calcium phosphate coprecipitation as previously described (35). The plaque titers of all viruses were determined on 293 cells. The plaque titers of all viruses were determined on 293 cells. The presence of replication-competent Ad and contamination with endotoxin in virus preparations was excluded by tests described previously (35). Viruses with a titer of 5 × 105 CFU/ml were stored at −80°C in 10 mM Tris-Cl (pH 8.0)–1 mM MgCl₂–10% glycerol. Attempts to generate an Ad vector expressing Ad Pol under a CMV promoter were not successful.

**Retrovirus vectors.** All retrovirus vectors were based on MSCVneoEB (21). The murine stem cell virus (MSCV)-series vectors were derived from LN and MESV vectors. They contain the extended packaging signal from LN vectors for Ad vectors.

**Materials.** pTP functions as a protein primer for Ad DNA replication. After binding, pTP is covalently linked to dCTP, providing a 3'-p terminal group to begin the synthesis of a daughter DNA strand. Furthermore, pTP serves as the site of primary attachment of the viral DNA to a specific protein(s) in the nuclear matrix, forming replicative complexes (5, 17). Late in infection, pTP is proteolytically cleaved by the viral protease, generating the 55-kDa terminal protein.

The goal of this study was to incorporate the pTP-based, Ad, nuclear import machinery into MoMLV vectors and to test whether this would allow retroviral transduction of nondividing cells. To approach this hypothesis, we first analyzed whether pTP could mediate the nuclear import of plasmid DNA carrying pTP-binding sites into arrested cells. Based on these preliminary results, MoMLV-based vectors containing pTP-binding sites were generated and tested for the ability to transduce nondividing cells expressing pTP.

**Western blot analysis.** After pTP plasmid transfection or Ad pTP gene transfer, cell pellets were lysed on ice for 30 min in 20 mM HEPES (pH 7.5)–2 mM EDTA–10% glycerol–1% Triton X-100–0.1 M dithiothreitol–and protease inhibitors. After 5 min of boiling, 80 μg of total protein in 1× Laemmli buffer with 4% β-mercaptoethanol was separated on a sodium dodecyl sulfate–10% polyacrylamide gel. After electrophoresis and blotting, the filters were incubated with monoclonal antibodies against pTP (gift from Sarah Jones, University of St. Andrews, St. Andrews, United Kingdom) at a dilution of 1:40 and then incubated with peroxidase-labeled anti-mouse immunoglobulin antibodies (1:1,000). The filters were developed with the ECL detection kit (Amersham, Buckingham, United Kingdom).

**Cell lines.** 293 cells (Microbiex), LITA cells (American Type Culture Collection, Rockville, Md.) (61), and neonatal primary human fibroblast (64) were used in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal calf serum (FCS). Stationary-phase fibroblasts were maintained in DMEM plus 5% FCS and 1 μM dexamethasone (46). For cell cycle arrest, LITA cells were synchronized by serum starvation and then incubated with either 10 mM hydroxyurea or 2 μg of aphidicolin per ml. Selection for G418 resistance was performed by transypanizing the cell cultures in 6-cm dishes and plating them as single-cell suspensions in 10-cm dishes in the presence of 600 μg of active G418 per ml. For studies with the ZnSO₄-inducible pMRE-pTP construct, FCS was replaced by a medium containing 0.1 copy per cell and subjected to PCR with hAAT-specific primers (59) as determined by X-Gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside) expression. Positive colonies were screened for hAAT expression. The clone with the highest transgene expression for each vector was amplified and subjected to titer determination on mouse LTA cells by counting the number of G418-resistant colonies. The titers of the corresponding aphidicolin viruses were as follows: RV.hAAT, 5 × 10⁴ CFU/ml; RV.18-hAAT, 5 × 10⁴ CFU/ml; RV.Δ16-hAAT, 8.5 × 10⁴ CFU/ml; and RV.90-hAAT, 4 × 10⁵ CFU/ml.

**Plasmid transfection and virus infection.** For standard transfections, 5 × 10⁵ LTA cells in 6-cm dishes were transfected by calcium phosphate coprecipitation with 10 μg of plasmid DNA. The transfection efficiency was between 30 and 35% as determined by X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) staining of a parallel transfection with 10 μg of test plasmid plus 1 μg of pSVneo. If the transfection efficiency was less than 30%, the preprepared and transfection was repeated with double CaCl₂-banded DNA. For Ad infections, cells in 6-cm dishes or 12-well plates were incubated overnight with virus in 2 or 0.5 ml of DMEM plus FCS, respectively. The multiplicity of infection was between 1,000 and 1,000,000. For Ad hAAT fibroblast as determined by infection with Ad.RSVβGal (26) and subsequent X-Gal staining. For retroviral infection, cells in 6-cm dishes or 12-well plates were incubated for 2 h at an MOI of 1 in a total volume of 2 or 0.5 ml, respectively. For superantigen (resolution of retroviral packaging), the pulse were through 0.45-μm-pore-size filters, diluted with DMEM plus FCS, supplemented with 4 μg of Polybrene per ml, and immediately used for infection. Test cells were incubated with retrovirus for 2 h and then extensively washed.

**Southern analysis.** Nuclei were isolated from transfected cells by Nomide P-40 cell lysis and centrifugation though a sucrose step gradient as described by Fitzgerald et al. (15). Extraction of genomic DNA and Southern analysis were performed as described previously (35). Loading differences were adjusted by rehybridization of the filters with a fragment of the mouse metallothionein gene. The following DNA fragments were used as labeled probes: a 1.4-kb fragment of the hAAT cDNA (EcoRI fragment of pAd.RSVhAAT [26]), and a 2-kb fragment of the mouse metallothionein gene (HindIII-EcoRI fragment of pmmMT [65]).

**PCR.** For semiquantitative PCR, a specific competitor plasmid was constructed by inserting a blunt-ended 2.3-kb HindIII λ-DNA fragment into the EcoRV site of the hAAT cDNA in pR5.SVhAAT (26). A 500-ng portion of genomic DNA from isolated nuclei was spiked with competitor plasmid DNA corresponding to 0.1 copy per cell and subjected to PCR with hAAT-specific primers (5'-ATGC GTCCTTCGTCGTCGCG-3' and 5'-GGACCCGGCTCCGAGAGGCTC-3'). PCR buffer containing 1.5 mM MgCl₂ and 2.5 μl of Taq polymerase (Perkin-Elmer) in a total volume of 100 μl. The PCR was run for 20 or 30 cycles (1 min at 95°C, 1 min at 60°C, and 1 min at 72°C). Then 10 μl of the reaction product was analyzed by electrophoresis in a 0.8% agarose gel.

**BrdU labeling.** Bromodeoxyuridine (BrdU) labeling reaction (Amersham) was adapted for the determination for RV.90-hAAT [see the figure legends]. After metabolic labeling, the cells were washed twice with phosphate-buffered saline and fixed with acetic alcohol for 30 min at room
temperature. Endogenous peroxidase activity was blocked by incubation with 0.03% methanol for 30 min at room temperature. The cells were incubated in 1.5 N HCl for 15 min at 37°C, blocked with 10% FCS, and incubated with anti-BrdU antibodies (DAKO; 1:50 diluted in 10% FCS). Specific antibody binding was enhanced and developed with Vectastain ABC kit (Vector Laboratories, Burlingame, Calif.). To quantify S-phase cells, the number of BrdU-positive cells per 1,000 cells was counted from random fields.

ELISA. hAAAT concentrations were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (26). The detection limit of the assay was 500 pg/ml. Culture supernatants were used undiluted for hAAAT detection.

RESULTS

pTP-mediated nuclear import of transfected plasmid DNA into arrested cells. Transfection of plasmid DNA into cells represents a simple model system for studying nuclear import of DNA in nondividing cells. It is generally known that the efficiency of plasmid transfection by calcium phosphate coprecipitation depends on the proliferative stage of target cells and is inefficient in confluent or growth-arrested cells (18). A number of studies with synchronized cells or regenerating tissues in vivo have demonstrated that mitosis with nuclear membrane breakdown is a prerequisite for efficient transfection measured by reporter gene expression (58, 66, 71). A simple diffusion of plasmid DNA through the nuclear membrane is impossible due to the high molecular mass (1 kb = 618 kDa) and the large gyration radius (12, 45). Previously, it was hypothesized that karyophilic, DNA-binding proteins would promote plasmid transfection into nondividing cells (22, 29). To test this, we investigated whether pTP can mediate nuclear transport of plasmids carrying pTP-binding sites after transfection into nondividing cells.

To express pTP in test cells, we generated a number of constructs with the pTP gene under the control of promoters, which varied in their activity. This was done because a priori it was not clear which level of pTP expression would allow for nuclear import of DNA and would, at the same time, avoid cytotoxicity. Earlier attempts to establish stable, pTP-expressing cell lines indicated that at a certain expression level, pTP was capable of mediating nuclear import of DNA and would, at the same time, avoid cytotoxicity. To analyze cytotoxic effects associated with pTP expression, the percentage of TUNEL-positive cells was determined on day 3 after transfection or infection by using the in situ cell death detection kit from Boehringer Mannheim. SEM, standard error of the mean (n = 3).

Ad vectors with pTP expression cassettes were generated to facilitate pTP gene transfer in vitro into nondividing cells.

In preliminary experiments, we selected the murine fibroblast cell line LTA (61) as an in vitro test cell system because this cell line can be transfected by calcium phosphate coprecipitation with a relatively high efficiency (>30%) and can easily be cell cycle arrested. pTP was expressed in mouse LTA cells after transfection of pTP expression plasmids or Ad gene transfer. On day 3 after transfection or infection, the amount of expressed pTP was analyzed by Western blotting with pTP-specific antibodies (Fig. 1). To correlate pTP expression levels with cytotoxicity, the percentage of apoptotic cells measured by a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was determined in a parallel set of pTP-expressing test cells. Generally, pTP (77kDa) expression levels were higher after Ad gene transfer than after plasmid transfection. About half of the pTP expressed after Ad gene transfer was converted into TP (55 kDa), probably by the Ad protease expressed in transduced LTA cells. Notably, a low level of pTP expression was observed in LTA cells infected with a first-generation control Ad vector (Ad.CMV-bGal), indicating that the E2 promoter is active in these cells. RSV and CMV promoters yielded similarly high levels of pTP expression after transfection or infection. In comparison, pTP levels were about threefold lower when the PGK promoter was used. Expression of pTP was maintained for at least 7 days after transfection or infection (data not shown). Basal, noninduced expression from the transfected pMRE-pTP construct was barely detectable. HAAT expression was ~15-fold induced in the presence of ZnSO4 added 36 h before protein analysis. After removal of ZnSO4, pTP expression returned to baseline levels within 24 h (data not shown). In cells infected with the RRE promoter-containing Ad vector (Ad.RRE-pTP), there was a high level of basal expression, which was comparable to that from Ad.PGK-pTP (data not shown). We recently reported that the MRE promoter in Ad vectors is transactivated by viral enhancers (D. Steinwaerder and A. Lieber, submitted for publication). Because of this undesirable interference, Ad.MRE-pTP was excluded from further experiments.

High-level pTP expression in cells transfected with pCMV-pTP and pRSV-pTP or after infection with pTP-expressing Ad was associated with cytotoxicity (Fig. 1, bottom). Importantly, in cells transfected with pMRE-pTP, noninduced pTP expression and transient induction of pTP expression over 36 h had
FIG. 2. pTP-mediated nuclear import of transfected plasmid DNA. (A) Test plasmids to study pTP-mediated nuclear import of plasmid DNA. (B) Scheme of the experiment (see Results for a detailed description). Cells were transfected with pMRE-pTP in a proliferative stage. One set of cells was arrested in the cell cycle by adding 10 mM hydroxyurea (HU) to serum-starved cells 48 h before transfection of test plasmids. Nucleus-localized plasmid DNA was analyzed by Southern blotting 24 h after transfection. At 48 h after transfection, the hAAT concentrations in culture supernatants were measured.
ported observation that mitosis is a prerequisite for efficient DNA import. In nondividing cells, confirming the requirement of a functional nuclear import apparatus for efficient nuclear import of plasmid DNA. The level of transgene expression in this case was much lower, comparable to that from transfected proliferating cells. Taken together, this demonstrates that nuclear import of plasmid DNA associated with the outer nuclear membrane, without translocation. To measure the concentration of plasmid DNA present in the nucleus and subjected to Southern analysis with a 32P-labeled (1.4-kb) probe specific to the hAAT cDNA. For Southern analysis, 10 µg of genomic DNA was loaded on each gel. (D) At 48 h after transfection of the test plasmids, a separate set of transfected cells was analyzed by ELISA for hAAT expression. +pTP, pTP expression was induced by ZnSO4. Means and standard deviations of three determinations are given. 

In all further experiments, we used aphidicolin instead of hydroxyurea to obtain a more complete cell cycle arrest. Aphidicolin is a more potent inhibitor of DNA polymerase α. A 6-h treatment with aphidicolin arrested most of the cells (>2% BrdU-positive cells) when LTA cells were presynchronized by serum starvation (Fig. 4). According to a previous report, cell cycle arrest by aphidicolin occurs at the G1/S border (43). Cell viability as measured by plating efficiency after trypsinization was decreased by less than 10% when cells were treated for 24 h with aphidicolin prior to trypsination (data not shown). This is in agreement with earlier reports demonstrating that aphidicolin incubation for 16 h (43) or 24 h (16, 56) does not significantly affect cell viability. For retroviral transduction experiments, it was important to determine the kinetics of cell cycle progression after removal of aphidicolin. Therefore, after removal of aphidicolin, the cells were pulse-labeled with BrdU for 1 h intervals (Fig. 4). The percentage of cells undergoing DNA synthesis peaked at approximately 4 h and declined by 6 h after aphidicolin removal, indicating that most cells had passed through S phase and had entered the G2/M phase by this time.

To determine how long retroviruses that have successfully
entered the target cell retained their ability to integrate, the intracellular stability of retroviruses was estimated for each test retrovirus vector in arrested target LTA cells. The ability to integrate and express the transgene is a function of the time between virus entry and the next mitosis (4). This period was extended from ~6 to ~30 h by varying the lengths of G1/S arrest mediated by aphidicolin (Fig. 5). The total lengths of these periods include the time of aphidicolin treatment plus the ~6 h required by LTA cells to enter the next mitosis after aphidicolin is removed. Retroviral transduction, as measured by neo gene expression resulting in G418-resistant colonies, decreased to ~1% when aphidicolin-mediated cell cycle arrest was extended for 4 h or more after completion of retrovirus infection. This implies that nearly all of the transduction-competent virus was inactivated or degraded when mitosis was delayed for more than 10 h. This appears to be in agreement with the results of an earlier study in which, by a different method, the intracellular half-life of retrovirus vectors was measured to be 6.4 h (4). The data shown for RV.18-hAAT/neo was representative of all four test retroviruses.

A number of important parameters obtained in these studies were used in our central experiments aimed to analyze the effect of coexpressed pTP on retrovirus transduction. These parameters include the following: (i) 2 h after the removal of aphidicolin, cells reentered the S phase of the cell cycle, and (ii) treatment with aphidicolin for 4 h or more after retrovirus infection prevented transduction, because all the virus was inactivated or degraded before the cells entered the next mitosis.

To test whether pTP can mediate the nuclear import of viral DNA carrying pTP-binding motifs and to analyze whether this effect would be more pronounced with pTP in complex with Ad Pol, proliferating LTA cells were transfected with pTP, pTP plus Pol, or control plasmids (Fig. 6A). After transfection, the cells were arrested by serum starvation and aphidicolin treatment. Retrovirus infection was performed at an MOI of 1 in arrested cells. Our first experimental design was based on an extended cell cycle arrest by incubation with aphidicolin that was shown earlier to prevent transduction because all viral preintegration complexes were inactivated or degraded before the cells entered the next mitosis. However, in this scheme, transduction-competent virus was still present when cells entered the S phase (Fig. 6A).

After retroviral infection of arrested LTA cells followed by aphidicolin treatment extended for 4 h, pTP-mediated nuclear import of viral DNA was analyzed by competitive PCR of genomic DNA isolated from purified nuclei (Fig. 6B). PCR
FIG. 5. Retroviral life span in arrested cells. Cells were arrested as described for Fig. 4. Arrested cells were infected with retrovirus (RV18.hAAT/neo) at an MOI of 1 for 2 h. After infection, cell cycle arrest by aphidicolin was continued for different periods, after which the cells were trypsinized and subjected to G418 selection. The selected periods of cell cycle arrest were based on the consideration that the lag phase between aphidicolin removal and next mitosis was assumed to be at least 6 h (Fig. 4). The period between virus entry and mitosis (0 h of incubation with RV) was performed with hAAT-specific primers. A strong vector-specific signal appeared in nuclear DNA from cells coexpressing pTP and viral DNA with pTP-binding sites. This signal was stronger in cells expressing both pTP and Pol and the RV.90-hAAT/neo DNA containing the extended binding motif for pTP, Pol, and NFI. This indicates that pTP can mediate the nuclear import of viral DNA carrying pTP-binding sites into arrested cells and that this process is enhanced by Ad Pol (probably due to increased pTP-binding affinity or possibly use of the NLS present in Ad Pol). Vector-specific background signals were visible in all control lanes. These signals probably originated from the transduction of the small percentage of nonarrested cells, which were present at the time of retroviral infection. Retroviral transduction was assessed based on transgene (hAAT and neo) expression (Fig. 6C). hAAT expression was analyzed 3 days after infection. The number of stably transduced cells was determined based on the number of G418-resistant colonies present after 3 weeks of selection. Significant hAAT expression was observed only in cells expressing pTP or pTP plus Pol and containing viral DNA with the corresponding binding motifs. Combined expression of pTP and Pol yielded higher transgene expression in cells infected with RV.90-hAAT/neo compared to cells containing the pTP-binding motif alone or cells infected with RV.90-hAAT/neo expressing Ad Pol. The number of G418-resistant colonies mirrored the transduction data obtained based on hAAT expression, suggesting that pTP-mediated transduction is associated with stable vector integration. For comparison, transduction of proliferating cells with the same MOI of RV.18-hAAT/neo yielded about 50 times more G418-resistant colonies than in arrested cells with pTP-supported import of retroviral DNA. Clearly, there was a significant stimulation of transduction supported by pTP; however, this mechanism was not as efficient as transduction during cell division.

These data indicate that pTP can mediate the nuclear import of viral DNA and that this is sufficient for integration. However, in experimental scheme I, transduction-competent virus was still present when cells entered the S phase. To analyze whether in addition to pTP-mediated nuclear import, events occurring during S phase are critical for vector integration, treatment with aphidicolin was continued for 24 h (scheme II) after retrovirus infection (Fig. 7A). During this period, all the virus was degraded or inactivated long before the infected cells could enter either the S or M phase according to data obtained earlier. Analysis of nucleus-localized viral DNA and retroviral transduction was performed as described for scheme I. No vector-specific signal was detectable by competitive PCR after 20 PCR cycles (data not shown). After 30 PCR cycles, vector-specific signals that were slightly stronger than background signals appeared in cells expressing pTP or pTP plus Pol after infection with RV.18-hAAT/neo or RV.90-hAAT/neo (Fig. 7B). This indicates that most viral DNA had not integrated and was degraded before analysis. It is thought that nonintegrated viral DNA is not protected from degradation by nucleases, which determines the short intracellular half-life of transduction-competent virus (4, 71). There was a low level of transduction (based on hAAT expression and formation of G418-resistant colonies) in cells coexpressing pTP or pTP plus Pol and viral DNA with the corresponding binding sites. However, in comparison, hAAT expression and formation of G418-resistant colonies was ~10-fold less efficient in scheme II (24 h of aphidicolin) than in scheme I (4 h of aphidicolin).

Ad-mediated pTP gene transfer allows the expression of pTP in 100% of test cells at a higher level than transfection with pTP expression plasmids. To test whether this property would change the outcome of retroviral transduction studies in arrested cells, LTA cells were infected with Ad.PGK-pTP or control virus (Ad.Co). Subsequently, arrested cells were infected with retrovirus, after which incubation with aphidicolin was continued for 4 or 24 h according to the experimental designs developed for Fig. 6 (scheme I) and Fig. 7 (scheme II). Retroviral transduction was evaluated based on hAAT expres-
FIG. 6. Effect of pTP expression on retroviral nuclear import and transduction (scheme I: treatment with aphidicolin extended for 4 h). (A) Scheme of the experiment. A total of \(8 \times 10^5\) LTA cells (70% confluent) were transfected with 10 \(\mu\)g of pcDNA3 (Invitrogen) as control plasmid (Co), 5 \(\mu\)g of pMRE-pTP plus 5 \(\mu\)g of pcDNA3 (pTP), or 5 \(\mu\)g of pMRE-pTP plus 5 \(\mu\)g of CMV-pol (pTP + Pol). After transfection, the cells were synchronized by serum starvation and arrested in the cell cycle by aphidicolin. Arrested cells were infected with retroviruses (RV.hAAT/neo, RV.D18-hAAT/neo, RV.18-hAAT/neo, or RV.90-hAAT/neo) at an MOI of 1 for 2 h. pTP expression was induced for the time of retrovirus infection by addition of ZnSO₄. Cell cycle arrest was continued for 4 h after retrovirus infection. At this time point, one set of infected cells was trypsinized and lysed, and genomic DNA was extracted from isolated nuclei (see Materials and Methods). Another set of dishes was analyzed for hAAT expression 3 days after infection and then subjected to G418 selection. The number of G418-resistant colonies was counted after 3
sion on day 3 postinfection (Fig. 8). Analysis of formation of G418-resistant colonies was not possible due to the cytotoxicity caused by the high level of pTP expression after Ad gene transfer. In agreement with the previously obtained data, in scheme I (4 h of aphidicolin) significant retroviral transduction in arrested cells was detected only in cells expressing pTP after Ad.PGK-pTP gene transfer, which were subsequently infected with vectors carrying pTP-binding sites (RV.18-

hAAT/neo, RV.90-hAAT/neo). Transduction rates were approximately 20-fold lower with these retrovirus vectors in cells after infection with the first-generation control vector (Ad.Co), which had yielded a low level of pTP expression in transduced LTA cells (Fig. 1). Transgene expression was not detectable in cells not infected with Ad or in cells infected with the control retroviruses (RV.hAAT/neo or RV.A18h-AAT/neo). 

In conclusion, it appears that pTP can support the nuclear import of viral DNA carrying pTP-binding sites. Coexpression of Pol enhances this process. However, pTP-mediated nuclear import is not sufficient for transduction in long-term-arrested cells. Our data indicates that in addition to nuclear localization, events occurring during S phase are required for MoMLV integration and stable transduction.

So far, for convenience, we have used artificially arrested test cells for retroviral transduction studies. To exclude the possibility that aphidicolin nonspecifically affects cellular factors necessary for retroviral integration, another cell culture system was used as a model to test the pTP-mediated retroviral transduction of nondividing cells. Primary neonatal human foreskin fibroblast cultures contain only 2 to 4% of cells passing through the S phase each day when maintained as confluent monolayers at a reduced serum concentration (46, 47). However, transfection of these cells is extremely inefficient (data not shown). Therefore, for transduction studies, pTP expression was provided by infection with Ad.PGK-pTP at an MOI of 2,000, which allowed the transduction of all cells. Retrovirus vectors were applied to arrested cells (at an MOI of 1) 2 days after Ad infection, and hAAT expression was measured on day 3 after retrovirus infection to assess retroviral transduction. hAAT levels after infection of stationary human fibroblasts were detectable only in pTP-expressing cells after infection with retroviruses carrying pTP-binding sites (Fig. 9A). However, transgene expression was about 300-fold lower than retroviral infection of dividing fibroblasts (ranging from 145 to 167 ng/ml for RV.hAAT/neo, RV.18-hAAT/neo, RV.18-hAAT/neo, and RV.90-hAAT/neo). This underscores our conclusion that pTP-supported nuclear import is not sufficient to allow significant transduction of nondividing cells.

This model for nondividing cells allowed us to study whether in addition to pTP-mediated nuclear import, pretreatment with agents that stimulates DNA repair synthesis would increase retroviral transduction. Previous studies with recombinant adenovirus vectors have demonstrated that DNA synthesis inhibitors or DNA-damaging agents can induce unscheduled DNA synthesis or DNA repair pathways, resulting in an increased transduction of nondividing cells by rAAV vectors (2, 46). To test whether this had an effect on our system, stationary fibroblasts infected with Ad.pTP or Ad.Co were exposed to [3H]thymidine (10 μCi/ml), as a DNA-damaging agent (2), or the DNA synthesis inhibitors aphidicolin or distamycin A (72) prior to retrovirus infection. As shown in Fig. 9, pretreatment with these agents, which were able to induce cellular DNA repair enzymes, stimulated retroviral transduction; however, this occurred only in combination with pTP-mediated nuclear import of viral DNA. Notably, transduction rates as measured based on transient hAAT expression were still 20 to 30 times lower than infection of dividing cells.

**DISCUSSION**

Transduction with MoMLV-based retrovirus vectors does not occur in nondividing cells. It is thought that this phenomenon is related to an inability of the MoMLV preintegration complex to cross the nuclear membrane due to the absence of competent NLS in viral proteins associated with the incoming virion. In contrast to MoMLV, Ad efficiently infects nondividing cells, and this property is probably due to the presence of NLS in viral proteins, in particular the terminal protein. Furthermore, there is increasing evidence from other viral systems that NLS within viral proteins are crucial for nuclear translocation of viral genomes. For influenza virus, the nuclear entry of the viral genome is mediated by an NLS in the nucleoprotein component of the viral RNA-protein complex (10). A similar mechanism of nuclear localization of the viral genome seems to exist for simian virus 40, mediated by its small structural proteins Vp2 and Vp3 (7). Importantly, for both influenza virus and simian virus 40, quiescent cells are among their natural targets. Members of another retrovirus subfamily, the lentiviruses, do not require mitosis for transduction. It is thought that this property is based on active transport of the preintegration complex into the nucleus of an infected cell without the requirement for nuclear envelope breakdown during cell division (23, 40, 41).

pTP mediates nuclear import of plasmid DNA with pTP-binding sites in nondividing cells. The hypothesis underlying this study was that coexpression of the Ad pTP with viral MoMLV DNA carrying pTP-binding sites would facilitate nuclear import of the preintegration complex in nondividing cells and that this would be sufficient to allow retroviral transduction. To support this hypothesis, we demonstrated with arrested cells that pTP mediated the efficient nuclear import of transfected plasmid DNA carrying pTP-binding sites. This is in agreement with a study from 1984 demonstrating that transfection of Ad DNA attached to pTP or TP was several orders of magnitude more efficient than transfection of viral DNA without pTP or TP (22). It underscores the point that nuclear transport is a limiting step for transfection and that nuclear...
FIG. 7. Effect of pTP expression on retroviral nuclear import and transduction (scheme II: treatment with aphidicolin for 24 h). (A) Scheme of the experiment. Cells were transfected with pTP-Pol expression plasmids, arrested, and infected with retroviruses as described for Fig. 6. After retrovirus infection, treatment with aphidicolin was continued for 24 h. During this period, all virus was degraded or inactivated long before the infected cells could enter either the S or M phase. (B) Detection of nucleus-localized viral DNA by competitive PCR. PCR was performed for 30 cycles under the conditions described for Fig. 6B. (C) Transgene expression after pTP-mediated nuclear import of viral DNA in arrested cells. hAAT and long-term neo expression were analyzed as described for Fig. 6C.
entry of plasmid DNA could be improved by DNA-bound karyophilic proteins. Moreover, a recent report noted that plasmid DNA is rapidly degraded by cytosolic nucleases and that transfection is more efficient the faster the DNA is translocated to the nucleus (29).

pTP mediates nuclear import of viral MoMLV DNA containing pTP-binding sites. In arrested cells, pTP mediated the nuclear import of MLV DNA carrying pTP-binding sites. This allowed retroviral transduction in arrested cells that were later released into S phase while transduction-competent virus was still present. However, pTP-mediated transduction rates in arrested cells were still lower than in proliferating cells. There may be several explanations for this. For example, the affinity of pTP binding to viral DNA may be too low, which might limit the efficiency of nuclear translocation. This is supported by our observation that pTP-mediated nuclear import and transduction was increased by Ad Pol, which presumably increases the affinity of pTP binding to DNA. On the other hand, weak pTP binding may be advantageous by allowing rapid dissociation. Otherwise, this complex could be tightly associated with the nuclear matrix via pTP, which may decrease the efficiency of integration. Alternatively, pTP binding within the central region of viral DNA may affect the composition, stability, or activity of the preintegration complex, for example, resulting perhaps in the loss of viral integrase. However, it is thought that the preintegration complex, or the so-called intasome, is organized around several hundred base pairs at each end of the viral DNA (69, 70), which makes it unlikely that pTP bound in the middle of the viral DNA could critically affect integration. Finally, the concentration of nucleotides available for reverse transcription of retroviral genomes may be a limiting factor in nondividing cells.

Requirement of S phase or DNA repair for MoMLV integration. From the data obtained in this study, we concluded that nuclear translocation of MoMLV DNA is not sufficient for stable transduction and that additional cellular factors activated during S phase or DNA repair are required to mediate integration and stable transduction of nondividing cells. It is now generally accepted that cellular proteins are required for retroviral integration, and it is speculated that the activity of some of these accessory cellular proteins is regulated in a cell cycle-specific manner. Although the purified integrase protein is sufficient to carry out 3′ processing and DNA strand transfer in cell-free systems, viral integration is several orders of magnitude more efficient in cells, indicating that cellular factors are involved in the process of integration (70). Among the host proteins that are potentially involved in MoMLV integration are BAF (30), INI 1 (25), HMG 1 (1), HMGI(Y) (13), and cellular DNA repair enzymes. During the process of MoMLV integration, the 5′ ends of the viral DNA and the 3′ ends of the target DNA remain unjoined, and cellular repair enzymes are believed to be responsible for degradation of the unpaired nucleotides at the 5′ ends of the viral DNA, filling in the single-strand gaps, and for the subsequent ligation to complete the integration process (8). A recent study has demonstrated that retroviral integration intermediates are detected as DNA damage by the host cell that and completion of the integration process requires the DNA-dependent protein kinase-mediated DNA repair pathway (9). Importantly, DNA-dependent protein kinase is regulated in a cell cycle-dependent manner, with peaks of activity during the G1 and early S phases (31). In light of this, we speculate that viral DNA imported to the nucleus by pTP symport is rapidly degraded when not integrated and that
integration requires cellular factors, which are not activated in artificially arrested LTA cells or quiescent fibroblasts.

Alternatively, serum starvation or aphidicolin treatment may perturb cellular metabolism, which may affect retrovirus integration independently of the cell cycle requirements. Moreover, retroviral integration may require a specific chromatin structure, which is not present in arrested cells. There are a number of reports stating that MoMLV integration depends on the methylation or heterochromatinization state of chromosomal DNA (27, 44, 50, 64).

At this point, the question arises of how lentiviruses integrate in nondividing cells. It is thought that viral proteins can mediate the nuclear translocation of the human immunodeficiency virus type 1 preintegration complex. Vpr appears to play a central role in this process (40, 41). Interestingly, Vpr is known to interfere with cell cycle checkpoint control and is associated with the induction of chromosomal breaks and DNA repair synthesis (36, 54). The potential property of lentivirus proteins to induce cell cycle-dependent host proteins, together with a longer intracellular half-life for lentivirus
DNA, may be beneficial for integration in nondividing cells. In this context, a number of reports noted that although mitosis is not required for transduction by lentiviruses or lentivirus vectors, cell cycle progression through G1/S significantly increased transduction efficiencies (32, 52, 57).

Our study demonstrates that in arrested cells, the karyophilic, DNA-binding Ad protein pTP mediated the nuclear import of plasmid or MoMLV DNA carrying pTP-binding sites. This observation may provide a rationale for improving plasmid transfection techniques or nonviral gene transfer, particularly in nondividing cells. Instead of pTP, which exerts cytotoxic side effects, synthetic proteins, which contain strong, sequence-specific DNA-binding domains fused to strong NLS, may be a better alternative to include in nonviral delivery systems. Furthermore, our finding that nuclear import of MoMLV viral DNA is not sufficient for integration and appears to require cell cycle-dependent cellular factors contributes to a better understanding of retroviral transduction.

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