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Expression and Frameshifting but Extremely Inefficient Proteolytic Processing of the HIV-1 gag and pol Gene Products in Stably Transfected Rodent Cell Lines

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Expression, ribosomal frameshifting, and proteolytic processing of HIV-1 GAG and POL proteins were investigated in heterologous mammalian cells in order to elucidate the influence of the cellular background on these events. DNA fragments encoded by the gag and pol region were expressed in two rodent cell lines, LTK$^-$ and BHK. Both stably transfected cell lines continuously produce recombinant proteins which react with HIV-specific antisera. The GAG precursor and a 39-kDa proteolytic fragment thereof were the major recombinant proteins detected. Expression of the gag–pol region leads to the production of the GAG–POL precursor. Ribosomal frameshifting at the HIV-1 shifty sequence to a typical extent could be positively demonstrated by an enzyme assay. Despite the presence of the viral protease within the GAG–POL precursors, proteolytic processing of the HIV-derived polyproteins was extremely inefficient. The efficiency could not be enhanced by overexpression of the HIV-1 protease encoding region.

INTRODUCTION

Molecular analysis revealed that the human immunodeficiency virus type 1 (HIV-1) has a typical retroviral genome organization consisting of the major structural genes gag, pol, and env flanked by regulatory sequences in the long terminal repeats (LTR) (Muesing *et al.*, 1985; Ratner *et al.*, 1985; Sanchez-Pescador *et al.*, 1985; Wain-Hobson *et al.*, 1985). In addition, the HIV genome encodes for several regulatory gene products. Some of these influence viral replication or infectivity (Haseltine, 1988; Peterlin and Luciw, 1988). As is known for all retroviruses, the primary translation products of HIV-1 gag and pol are a GAG polyprotein and a GAG–POL polyprotein, respectively (Robey *et al.*, 1985; Veronese *et al.*, 1988; Jacks *et al.*, 1988a). Both precursor proteins of HIV-1 are proteolytically processed with participation of the virus encoded protease. The GAG polyprotein gives rise to at least three components p18, p24, and p15 (Mervis *et al.*, 1988). The maturation of the GAG–POL polyprotein results in the release of the protease, the reverse transcriptase, and the integrase (Farmerie *et al.*, 1987; Lightfoote *et al.*, 1986; Mous *et al.*, 1988). The HIV-1 protease which is encoded in the 5′ region of the pol gene is an 11-kDa protein generated by autocatalytic cleavage from a larger precursor protein (Debouck *et al.*, 1987; Mous *et al.*, 1988). The requirement of the viral protease for the HIV-specific maturation processes has been demonstrated by independent experimental approaches. (1) Expression of HIV-1 genome regions encoding GAG–protease, POL, or GAG–POL in certain heterologous expression systems resulted in the production of proteins with the same antigenic specificities and molecular weights as those found in mature HIV-1 particles (Kramer *et al.*, 1986; Farmerie *et al.*, 1987; Le Grice *et al.*, 1987; Madisen *et al.*, 1987; Mous *et al.*, 1988; Leuthardt and Le Grice, 1988; Overton *et al.*, 1989). (2) A protein chemically synthesized according to the protease coding sequence of HIV-1 revealed the same substrate specificity as the viral protease (Schneider *et al.*, 1988). (3) Mutations within the catalytic site of the HIV protease prevented GAG–POL processing in heterologous expression systems (Le Grice *et al.*, 1988; Seelmeier *et al.*, 1988) as well as in the authentic viral system (Kohl *et al.*, 1988). The HIV-1 protease belongs to the class of aspartic proteases (Toh *et al.*, 1985; Katoh *et al.*, 1987; Navia *et al.*, 1989). Biochemical and physical studies revealed a dimer structure of the active protease (Meek *et al.*, 1989; Navia *et al.*, 1989).

In the molecular clone BH-10 of HIV-1 the gag and pol genes overlap by 241 bp with the pol gene in the -1 translational phase with respect to the gag gene (Ratner *et al.*, 1985). It has been shown that the biosynthesis of the GAG–POL polyprotein of HIV-1 involves a ribosomal frameshift event. By site-directed mutagenesis and amino acid sequencing the frameshift site

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was localized to the sequence UUUUUUA in the gag–pol overlapping region (Jacks *et al.*, 1988a; Wilson *et al.*, 1988). Related sequences are shown to be responsible for ribosomal frameshifting during replication of RSV (Jacks *et al.*, 1988b) and MMTV (Jacks *et al.*, 1987).

Ribosomal frameshifting and the proteolytic processing mediated by the HIV protease are virus-specific events which are considered to be essential for virus replication and therefore potential targets for therapeutic intervention in the HIV replication cycle. The development of specific antiviral drugs requires a detailed understanding of the viral and cellular processes involved in virus replication.

To investigate the species and tissue specificity of the expression, translation, and processing of the HIV-1 gag and pol encoded proteins, we stably transfected rodent cells with the gag–pol region. We describe here for the first time mammalian cell lines in which the retroviral ribosomal frameshifting continuously occurs independent of other viral functions. We show that a transfected BHK cell line produces the primary translation products of the HIV-1 gag and pol genes. Our studies further revealed that expression of the HIV protease domain is not sufficient to exert efficient protease activity in transfected rodent cell lines. In contrast, primate cell lines allow efficient HIV protease activity when transfected with the HIV gag–pol region.

MATERIALS AND METHODS

Cells

The following cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum: BHK-21 (baby hamster kidney cells; ATCC CCL-10), LTK^- (mouse connective tissue cells) (Kit *et al.*, 1963), COS (SV40 transformed African Green Monkey kidney cells; ATCC CRL 1650), and 293 (transformed primary human embryonal kidney cells; ATCC CRL 1573).

Plasmids and plasmid constructions

The molecular DNA clone pBH-10-R3 of HIV-1 was the source for the expressed HIV genes. This construct consists of vector pSP64 and the *Sst*I fragment (nucleotide position 680–9606) of the HIV-1 clone BH-10 (Ratner *et al.*, 1985). The nucleotides are numbered according to the EMBL databank. The eukaryotic expression vector pMPSV (Artelt *et al.*, 1988) was used to express HIV genes in mammalian cells. This vector contains the LTR of the myeloproliferative sarcoma virus (MPSV) which is a strong transcriptional promoter for foreign genes in rodent cells, a pUC9 polylinker for DNA insertion, and the SV40 late sequences for transcript termination and polyadenylation. The latter sequence also encodes for translational stop codons in all reading frames to ensure translation termination of the expressed genes.

Five different regions of the gag and pol genes of pBH-10-R3 were inserted into the expression vector pMPSV (Fig 1). The plasmid p.gag–pol I containing the complete gag and pol genes was constructed by insertion of the *Tha*I–*Sal*I fragment (nucleotide positions 711 to 5819) of pBH-10-R3 into the blunt ended *Bam*HI site and *Sal*I site of pMPSV. The plasmid p.gag–pol II contains the HIV sequence from nucleotide position 680 to 4681. For its construction the plasmid pBH-10-R3 was cut in the *Eco*RI site located in the polylinker (upstream of the 5′ end of the inserted HIV sequence) and in the *Eco*RI site in position 4681. The fragment coding for the gag–pol region was isolated and ligated with the *Eco*RI cut pMPSV. The plasmid p.gag is a derivative of p.gag–pol II and encodes, with the exception of a terminal deletion on the 3′ end, the complete gag gene (nucleotide positions 680–2093). p.gag–pol II was cut in the *Bgl*II site (nucleotide position 2093) and in the *Bam*HI site located in the polylinker adjacent to the 3′ end of the HIV sequence. The shortened plasmid was isolated and religated. In the plasmid p.gag–pol III, which is another derivative of p.gag–pol II, the gag and pol genes are aligned in the same reading frame. The plasmid p.gag–pol II was cut in the *Bgl*II site (nucleotide position 2093), repaired with Klenow enzyme, and religated. Four nucleotides (GATC) were thereby added to the gag–pol region. The retroviral ribosomal frameshifting occurs six bases upstream of the *Bgl*II site. Thus the amino acid sequence of the recombinant GAG–POL protein (Leu*GlyLysIle*Asp) differs in three amino acids in the GAG–POL fusion region from the natural GAG–POL protein (Leu*ArgGlu*Asp, amino acid positions 434–438). The plasmid p.prot contains the 5′ end of the pol gene (nucleotide position 2093–2654) encoding the viral protease. For translation initiation of the protease reading frame, a chemically synthesized oligonucleotide containing the ATG-codon was added between the *Eco*RI site of the polylinker and the *Bgl*II (nucleotide position 2093) site at the 5′ end of the pol fragment:

5′-AATTCACC**ATG**GGA-3′

3′-GTGG**TAC**CCTCTAG-5′

p.gag–pol IV is a derivative of p.gag–pol I in which a fragment encoding the rev-responsive element (RRE, nucleotide position 7651–9081) is inserted into the *Sal*I site (nucleotide position 5819) downstream of gag–pol region. For rev expression p.crev a friendly gift of B. R. Cullen was used (Malim *et al.*, 1988).

The expression plasmids A, B, and C (Fig. 4), encoding for the HIV–luciferase fusion protein under the transcriptional control of the SV40 early promoter, are derivatives of pBFSLuc-1, which is described in detail by Reil and Hauser (1990). Plasmids B and C contain the shifty sequence from the gag–pol overlap region of HIV-1 (2071–2096). They are derived by elimination of the *Bgl*II–*Bam*HI fragment of pBFSLuc-1 and ligation before (C) or after a fill-in reaction (B). In plasmid A the *Hin*dIII–*Bam*HI fragment was replaced by the following oligonucleotide:

5′-AGCTTACCATGGGTCGACAGGCTAACTTCCTCAGGGAAGATC-3′

3′-ATGGTACCCAGCTGTCCGATTGAAGGAGTCCCTTCTAGCTAG-5′

In this construct the HIV sequence is altered so that no frameshifting can occur.

Transfection

DNA was transfected into BHK, LTK$^-$, COS, and 293 cells by the calcium phosphate precipitation technique (Graham and van der Eb, 1973) as described by Wigler *et al.* (1977). The calcium phosphate precipitate (0.5 ml) for 3–5 $\times$ 10^5 cells in 5 ml of medium contained 10 μg of the indicated constructs, 1 μg of pAG60 encoding for the neomycin resistence gene (Colbère-Garapin *et al.*, 1981), and 10 μg of high molecular weight DNA as carrier. For transient expression experiments cells were harvested 48 hr later and analyzed for HIV gene products or luciferase and β-galactosidase activity. To select for stable transfectants 48 hr after transfection, the cells were supplied with medium supplemented with 1000 μg G418/ml for BHK cells or 800 μg G418/ml for LTK$^-$ cells. Clones of the stably transfected cells (>100) were pooled and analyzed for the presence of HIV-1 gene products or luciferase and β-galactosidase.

To obtain high expressing cell populations the combined selection method as described by Wirth *et al.* (1988) was applied by cotransfection of the puromycin resistence gene pSV-2pac (Vara *et al.*, 1986) as an additional selection marker followed by respective puromycin selection.

Immunoblot analysis

Cells (2 $\times$ 10^7) were harvested, rinsed twice with ice-cold phosphate-buffered saline (120 m*M* NaCl, 17 m*M* Na_2HPO_4, 3 m*M* KH_2PO_4, pH 7.2), resuspended in 300 μl extraction buffer (140 m*M* NaCl, 10 m*M* Tris–HCl, 2 m*M* $MgCl_2$, 1 m*M* DTT, 2 m*M* phenylmethylsulfonylfluoride, 0.5% (v:v) Nonidet-P40), and kept on ice for 10 min. After centrifugation (14,000 *g* for 15 min at 4°) of the lysate, the proteins of the resulting supernatant (soluble fraction) and pellet (membrane-associated fraction) were separated by SDS–PAGE. The pellet was resuspended in 300 μl of sample buffer (25 m*M* Tris–HCl, pH 6.8, 1.1% (w:v) SDS, 4 *M* urea, 0.25 *M* 2-mercaptoethanol) by sonication. Extract of 2–3 $\times$ 10^5 cells was applied per track in the SDS–PAGE. The proteins in the SDS–polyacrylamide gels were electrically transferred to nitrocellulose filters (Towbin *et al.*, 1979). Filters were incubated with serum from an HIV-positive individual or with rabbit hyperimmune sera directed against HIV protease (Le Grice *et al.*, 1988), p18, or p24 (Schneider *et al.*, 1986).

The sera were used at dilutions of 1:100 to 1:500. The immobilized antibodies were detected with a second alkaline phosphatase-conjugated antibody (Bio-Rad). The colorimetric detection of the antigen/antibody complexes was accomplished by an alkaline phosphatase assay system from Bio-Rad.

Luciferase assay

Cellular extracts from pools of transfectants were prepared by freezing and thawing. After removal of the cell debris in a microcentrifuge for 5 min at 4°, the luciferase activity was detected in one-tenth of the extract according to de Wet *et al.* (1987) in a luminometer. Luciferase activity was corrected concerning the transfection efficiency by measurement of β-galactosidase activity from a cotransfected β-galactosidase expression plasmid (Reil and Hauser, 1990). The amount of light units from construct A in BHK cells is 3 $\times$ 10^5 and 2 $\times$ 10^5 from 293 cells. One hundred light units are obtained from mock-transfected cells.

RESULTS

Expression of HIV-1 gag and pol genes in BHK and LTK$^-$ cells

We intended to analyze the expression and processing of the HIV gag and pol encoded proteins in mammalian cells independently of other viral components. We were also interested in investigating the influence of the cellular background on the realization of these HIV-1 gene products. Two cell lines, mouse LTK$^-$ and hamster BHK, which originate from different species and tissues, were transfected with expression constructs covering HIV-1 gag and pol regions (Fig. 1). Pools of stable transfectants were analyzed by immunoblot analysis using serum from an HIV-1-positive individual.

The expression construct p.gag–pol I contains the complete HIV-1 gag and pol coding region under the transcriptional control of the strong constitutive MPSV promoter. In the soluble fraction of extracts from p.gag–pol I-transfected BHK cells two dominant recom-

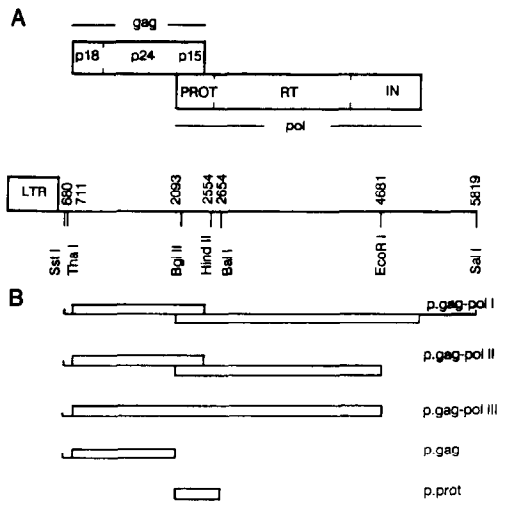


FIG. 1. Schematic diagram of the regions from the gag and pol open reading frame which were expressed with the various expression constructs. (A) The diagram shows part of the cloned HIV-1 genome BH-10 in which the 5′ LTR, gag, pol, and various restriction nuclease sites are indicated. The abbreviations PROT, RT, IN represent the protease, the reverse transcriptase, and the integrase encoded in pol. (B) The diagram indicates by single lanes the different region of BH-10 which were inserted into the expression vectors. The theoretically translated regions of the gag and pol reading frame are represented by open boxes.

binant proteins of 55 and 39 kDa, as well as a minor amount of a protein of 160 kDa were identified (Fig. 2A). Traces of a 24-kDa protein were also detected in this cell fraction when more extract was loaded onto the gel used for immunoblot analysis (data not shown). Likewise, in the membrane-associated fraction of p.gag–pol I-transfected cells proteins of 55, 39, and 24 kDa, but not 160 kDa, were identified (Fig. 2B). In p.gag–pol I-transfected LTK$^-$ cells dominant recombinant proteins of 39 and 55 kDa were also found (Fig. 3). The recombinant proteins detected corresponding to HIV proteins were named according to their molecular weights rp160, rp55, and rp24. A GAG–POL(p160) and a GAG(p55) polyprotein, of 160 and 55 kDa, respectively, are the primary translation products of the expression of the gag–pol region during the replication of HIV-1. The biosynthesis of the 160-kDa GAG–POL polyprotein requires a frameshift event to align the different reading frames of gag and pol. The GAG–POL precursor contains the viral protease which should lead to the processing of p55 and p160 itself. Both events, ribosomal frameshifting and proteolytic processing, have been investigated in more detail.

Ribosomal frameshifting is equally efficient in primate and rodent cells

The observation of a 160-kDa protein suggests that the ribosomal frameshifting leading to a GAG–POL fusion protein occurs in the transfected BHK cells. Evidence that the 160-kDa protein is encoded by the transfected gag–pol region is provided by the analysis of p.gag–pol II-transfected BHK cells (Fig. 2A). p.gag–pol II, a derivative of p.gag–pol I which codes for a C-terminally truncated GAG–POL fusion protein, resulted in the expression of a polypeptide with the expected size of 145 kDa (Fig. 2A). It has to be pointed out that the GAG–POL protein bands shown here have been reproduced with sera from different HIV-positive individuals (data not shown). For technical reasons we could not clearly identify a protein corresponding to the GAG–POL precursor in p.gag–pol I-transfected LTK$^-$ cells by immunoblotting.

A more sensitive enzymatic detection system (Reil and Hauser, 1990) was used to determine the frameshift efficiency and to delimit the frameshift locus. This system is based on the expression of an N-terminally extended firefly luciferase gene which requires frameshifting in order to be translated as a functional enzyme (Fig. 4). This was done by replacement of the translational start codon by an HIV-1 nucleic acid sequence extending from nucleotide position 2071 to 2096 (BH-10) including the frameshift site (construct B, Fig. 4). To determine the enzymatic activity of the N-terminally ex-

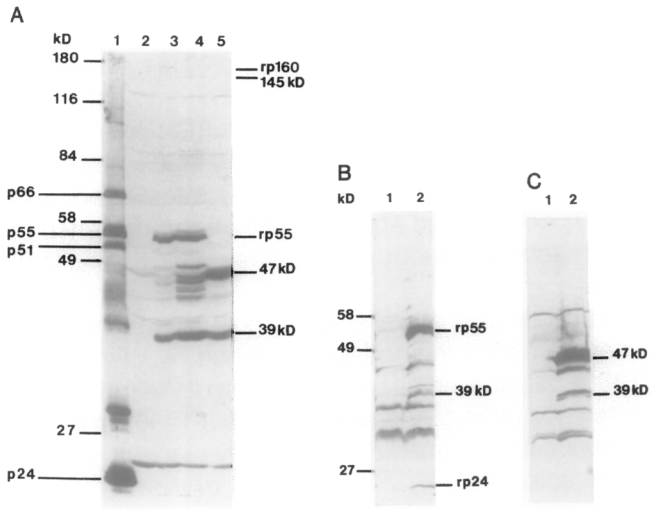


FIG. 2. Immunoblot analysis of the gag and pol gene products produced by transfected BHK cells. Proteins in the soluble (A) as well as in the membrane-associated (B and C) fractions of the extracts from the transfected BHK cells were analyzed with SDS–PAGE followed by the immunoblotting reaction. Serum from an HIV-positive individual was used to identify the HIV-1 gene products. Lanes in A: HIV-1 lysate (1), soluble fraction of extract from cells transfected with pMPSV (2), p.gag–pol I (3), p.gag–pol II (4), or p.gag (5). Lanes in B: membrane-associated fraction of extracts from cells transfected with pMPSV (1) or p.gag–pol I (2). Lanes in C: membrane-associated fraction of extracts from cells transfected with pMPSV (1) or p.gag (2). The positions of the viral and recombinant proteins as well as of the molecular weight markers run in a parallel lane are indicated.

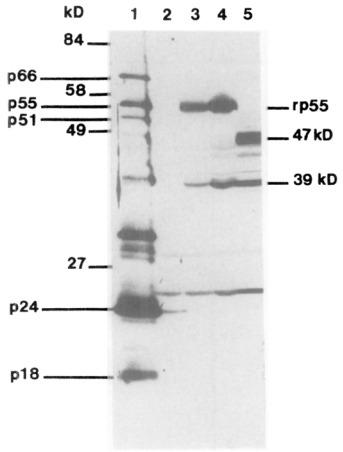


FIG. 3. Immunoblot analysis of the gag gene products produced by transfected LTK⁻ cells. Proteins in the soluble fraction of the extract from transfected LTK⁻ cells were analyzed with SDS–PAGE followed by immunoblotting reaction. Serum from an HIV-positive individual was used to identify the HIV gene products. Lanes: HIV-1 lysate (1); extract from cells transfected with pMPSV (2); p.gag–pol I (3); p.gag–pol II (4); or p.gag (5).

tended luciferase, an inframe fusion gene was used (construct A, Fig. 4). To confirm the −1 frameshifting from construct B we have transfected construct C in which the luciferase coding region is fused in the -2 frame with regard to the translation initiation codon. The design of constructs B and C was done in a way to exclude the possibility that internal translational initiation or RNA splicing would have led to active luciferase. Figure 4 shows the frameshifting efficiency in BHK and 293 cells as determined by transient expression experiments. Identical results were obtained in stable transfectants from BHK and LTK⁻ cells (Reil and Hauser, 1990). The fact that the frameshifting efficiency is independent of the status of the transfected DNA excludes internal DNA rearrangements which could affect ribosomal frameshifting in stable transfectants. Analysis of luciferase activity in the transfected cells showed that the HIV-1-derived sequence is responsible for frameshifting. The efficiency of frameshifting was determined to be 1.5–4% in hamster BHK and human 293 cells.

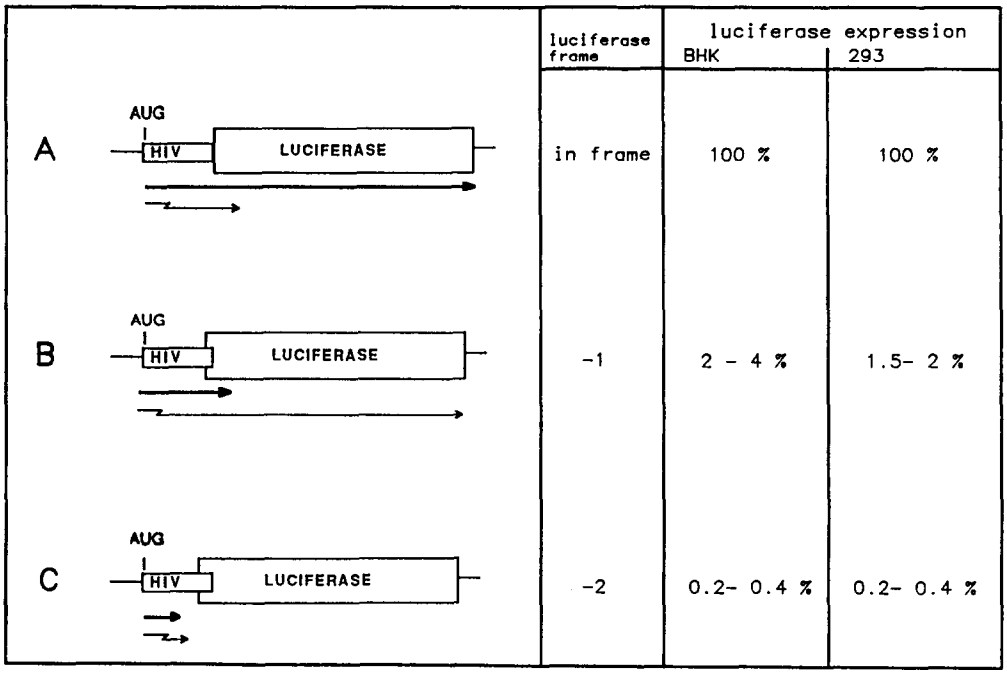
HIV-1 protease-specific processing is inefficient in rodent cells transfected with the HIV-1 gag–pol region

The major precursor to be expressed and processed in the HIV-infected cells is p55 (Mervis *et al.*, 1988). To show that the detected rp55 is identical to the viral GAG precursor, we have carried out further immunoblot analysis with rabbit hyperimmune sera to p18 and p24 with extracts from p.gag–pol I-transfected cells. Again, a 55- and a 39-kDa protein were detected in the soluble fraction of p.gag–pol I-transfected BHK (Fig. 5) and LTK⁻ cells (data not shown). Its molecular weight and antigenic specificity imply that the 55-kDa protein is indeed the GAG precursor protein.

The demonstration of the 24-kDa protein (rp24) reacting with serum from an HIV-positive individual indicates the specific activity of the HIV protease in BHK cells transfected with p.gag–pol I (Fig. 2B). We thereby assume that the 24-kDa protein is a proteolytic cleavage product of rp55 and is identical to the viral p24. This assumption is further supported by the absence of a protein of this size in extracts of p.gag-transfected cells (Fig. 2C). The plasmid p.gag encodes for a C-terminally truncated GAG precursor protein of 47 kDa but not for the viral protease. Indeed, a 47-kDa protein was detected in p.gag-transfected BHK and LTK⁻ cells (Figs. 2A, 2C, and 3). Since we could not detect mature GAG proteins in p.gag–pol I-transfected LTK⁻ cells, there is no evidence for HIV protease-specific processing in these cells.

BHK and LTK⁻ cells transfected with p.gag–pol I, p.gag–pol II, and p.gag produced among others a recombinant protein of 39 kDa reacting with serum from an HIV-positive individual (Figs. 2A, 2B, and 3) and with rabbit hyperimmune sera to p18 and p24 (example shown for p.gag–pol I-transfected BHK cells in Figs. 5A and 5B). Since p.gag does not code for the viral protease domain we conclude that the 39-kDa protein is generated by proteolytic processing of rp55 independent of the HIV protease. From the immunoblot analysis with hyperimmune sera (Fig. 5) we further conclude that the 39 kDa protein consists of p18 and p24 components. Since the expression of p.gag–pol II encoding a C-terminally shortened GAG precursor does not affect the size of the 39-kDa protein it must be encoded upstream of the *Bgl*II site (nucleotide position 2093) of the gag gene.

Our results imply that the expression of the gag–pol region in LTK⁻ cells results in no proteolytic activity and in only weak proteolytic activity in BHK cells, mediated by the HIV protease. Additional experiments were carried out to evaluate our findings on GAG–POL processing: First, we sequenced the protease encoding region within the expression construct p.gag–pol I. The results exclude the possibility that mutations in the protease region led to the production of inactive protease (data not shown). Second, since it is conceivable that protease expression leads to cytotoxic effects, the selection of stable transfectants encoding spontaneously mutated proteases is possible. We therefore performed transient expression experiments which exclude a counter selection against protease expression (Fig. 6A). In BHK cells transiently transfected with



luciferase frame	luciferase expression BHK	293
in frame	100 %	100 %
-1	2 - 4 %	1.5- 2 %
-2	0.2- 0.4 %	0.2- 0.4 %

FIG. 4. Enzymatic detection of frameshifting in BHK and 293 cells. Plasmids A, B, and C encode mRNA which give rise to HIV–luciferase fusion proteins. The short HIV sequence from the gag–pol overlap region (nucleotide position 2071–2096 of BH-10) including the shifty sequence (Wilson *et al.*, 1988) is fused with a short linker to the 5′ end of the coding region of the firefly luciferase. Expression of active luciferase from plasmid B requires frameshifting within the HIV sequence. The same effect could be achieved by translation reinitiation (-1 frame). Such a reinitiation upstream of the shifty sequence would lead to translational termination at the stop codon, which is in front of the shifty sequence. In order to exclude translational initiation, downstream the shifty sequence expression of construct C, which contains a 4-bp insertion 3′ proximal to the shifty sequence, was determined. The low expression of luciferase obtained from this construct indicates that significant translational initiation as well as −2 frameshifting does not occur. mRNA from plasmid A directs the translation of active HIV–luciferase fusion protein and gives rise to the specific enzyme activity of the fusion protein. The drawings represent mRNA from plasmids A, B, and C. The different reading frames of luciferase in relation to HIV-derived sequences are symbolized by different positions of the open boxes. Thick arrows indicate the resulting inframe proteins. Thin arrows depict the -1 transframe proteins. The expression of active luciferase from cells transfected with plasmid A, B, and C was determined. Luciferase expression is given as the percentage of the expression from plasmids B and C in relation to that from A.

p.gag–pol I, the same expression pattern as in analogous stable transfectants was observed. Third, to prove that expression of the gag–pol sequence used in our studies can result in a HIV-specific processing pattern in mammalian cells, this sequence was transfected into two primate cell lines (COS and 293). Smith *et al.* (1990) have shown with COS cells that cotransfection of the HIV gag–pol region including the RRE sequence together with a rev expression vector led to an efficient processing of p55 into the mature compounds. The expression of p.gag–pol IV, which corresponds to p.gag–pol I plus the RRE sequence, in both primate cell lines resulted in the production of the GAG precursor (rp55) and its mature cleavage products (rp24 and rp18), as well as a 39-kDa protein (Figs. 6B and 6C). Transient expression of p.gag–pol IV plus p.crev in BHK cells led to the same results as after transfection of p.gag–pol I (data not shown).

One possible explanation for the impaired HIV protease activity in the transfected rodent cells is that the amount of GAG–POL precursor protein is too low to exert detectable protease activity. For this reason we investigated the influence of overexpression of the protease coding region on the processing of the precursor proteins. Plasmid p.prot was constructed for the overexpression of the protease coding region of HIV-1. This construct encodes the 5′ region of the pol reading frame. Its expression should lead to a 20-kDa polypeptide, consisting of a protease precursor protein of about 18 kDa (Mous *et al.*, 1988; Le Grice *et al.*, 1988) fused C-terminally to 24 amino acids of the reverse transcriptase. LTK^- cells transfected with p.prot produced a protein of about 16 kDa which reacts with a rabbit hyperimmune serum specific to the HIV protease (Fig. 7). The reason for the difference between the calculated and the apparent molecular weight of this recombinant protein is unknown. In LTK^- cells cotransfected with p.prot and p.gag–pol II, no HIV-specific processing of the GAG precursor protein was observed by immunoblot analysis (data not shown).

As an alternative we intended to enhance the expression of a GAG–POL precursor to increase the

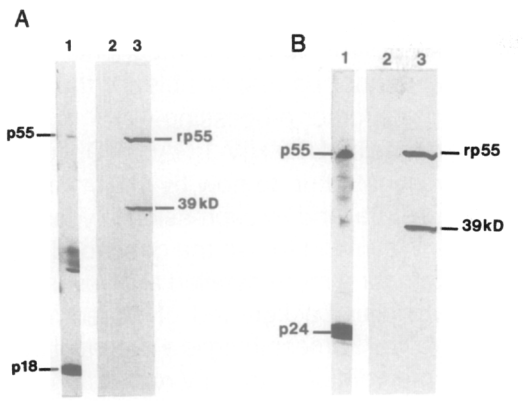


FIG. 5. Immunoblot analysis using specific antisera to HIV-1 p24 and HIV-1 p18 of recombinant GAG proteins produced by BHK cells transfected with p.gag–pol I. The soluble fraction from extracts of transfected cells was electrophoretically resolved, transferred onto nitrocellulose membrane, and analyzed using antisera to HIV-1 p18 (A) or HIV-1 p24 (B). Lanes in A and B: HIV-1 lysate (1); extracts from BHK cells transfected with pMPSV (2); or p.gag–pol I (3).

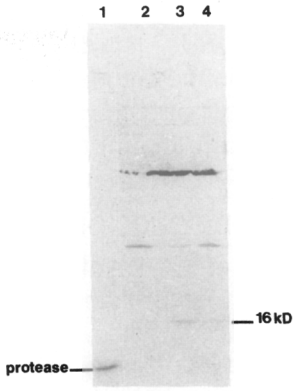


FIG. 7. Immunoblot analysis using an anti-protease (HIV-1) antiserum of LTK$^-$ cells transfected with p.prot. The soluble fraction from extracts of transfected cells was electrophoretically resolved, transferred onto nitrocellulose membrane, and analyzed using an antiserum to HIV-1 protease. Lanes: HIV-1 lysate (1); LTK$^-$ cells transfected with pMPSV (2); p.prot (3); or cotransfected with p.prot and p.gag–pol II (4).

amount of HIV protease in the transfected cells. For this purpose p.gag–pol III was constructed. This plasmid is a derivative of p.gag–pol II with the gag and pol genes aligned in the same reading frame, thereby coding for a 145-kDa GAG–POL fusion protein. Two major protein bands of 145 and 39 kDa were detected on immunoblots of extracts of BHK cells transfected with p.gag–pol III (Fig. 8A). Furthermore, a minor amount of a 24-kDa protein (Fig. 8B) was detected in the soluble fraction of the same cells when three times the amount of cell extract was applied for immunoblot analysis compared to the standard protocol. A protein of this size reacting with the same patient serum was also present in the membrane-associated fraction of the p.gag–pol III-transfected BHK cells (data not shown). The detection of a 24-kDa protein which we assume to be identical to the mature p24 provides evidence for

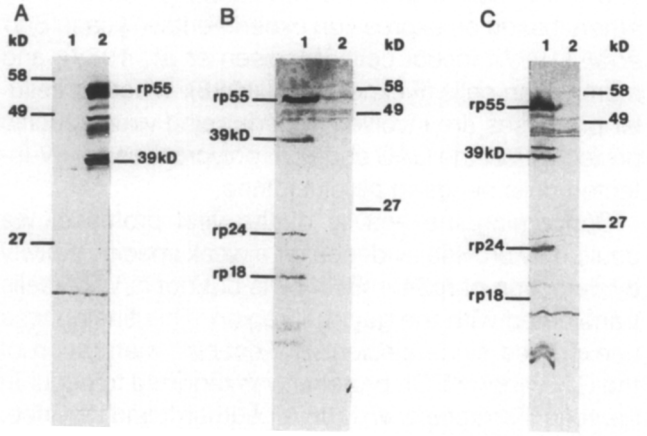


FIG. 6. Immunoblot analysis of transient expression of the gag–pol region in BHK, 293, and COS cells. Two days after transfection the cells were harvested and the proteins in the soluble fraction of the cell extracts were analyzed with SDS–PAGE followed by immunoblotting reaction. Serum from a HIV-positive individual was used to identify the HIV gene products. Lanes in A: extracts from BHK cells transfected with pMPSV (1); or p.gag–pol I. Lanes in B and C: extracts from COS cells (B) and 293 cells (C) cotransfected with p.gag–pol IV and p.crev (1); or transfected with pMPSV (2).

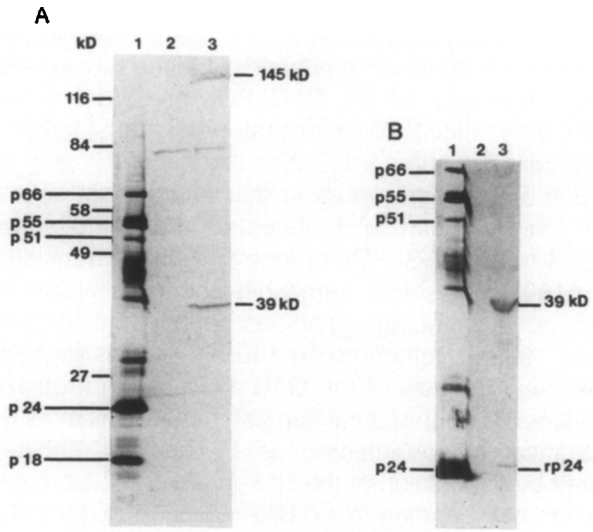


FIG. 8. Immunoblot analysis of a GAG–POL fusion protein produced in BHK cells transfected with p.gag–pol III. The soluble fraction from an extract of transfected BHK cells was analyzed with SDS–PAGE followed by immunoblotting reaction. Serum from an HIV-positive individual was used to identify the HIV gene products. Lanes in A and B: HIV-1 lysate (1); extract from cells transfected with pMPSV (2); or p.gag–pol III (3). Three times the amount of cell extract (lane 2 and 3) was applied in the immunoblot analysis in B compared to in A.

the specific activity of the HIV protease in p.gag–pol III-transfected BHK cells. However, although much more HIV protease in the precursor form is present in these cells, the extent of precursor processing is not increased compared to that of the p.gag–pol I transfectants.

DISCUSSION

To investigate the realization of gag and pol encoded proteins of HIV-1 in heterologous mammalian cells, BHK and LTK$^-$ cells were stably transfected with expression constructs containing different regions of HIV-1 gag and pol genes. The resulting cell lines continuously produce HIV-specific proteins reacting with serum from an HIV-positive individual and hyperimmune sera to HIV p18 and p24. Some authors reported that the rev function of HIV is essential for expression of the gag and pol genes in HIV-infected cells (Feinberg *et al.*, 1986; Sodroski *et al.*, 1986) as well as in eukaryotic expression systems which undergo nuclear transcription (Dayton *et al.*, 1988; Felber *et al.*, 1989; Hammarskjöld *et al.*, 1989; Smith *et al.*, 1990). Our data demonstrate that gag–pol expression does not depend on the rev function in stably transfected rodent cells.

Our results imply that BHK and LTK$^-$ cells transfected with the complete gag–pol region produce recombinant GAG and GAG–POL precursors. Both are indistinguishable from the primary translation products of the gag and pol genes in HIV-infected cells. Due to technical difficulties a gag–pol fusion protein could not be identified unequivocally in LTK$^-$ cells. However, our results on frameshifting provide evidence that in these cells translation of the GAG–POL precursor is very likely (see below). We infer that in the transfected rodent cells the same translation signals of the gag–pol region are recognized as in the natural host cells of HIV. This is of particular interest regarding the biosynthesis of the GAG–POL precursor. For the translation of p160 a ribosomal frameshift occurring within the gag–pol overlapping region has been identified (Jacks *et al.*, 1988a). Detection of rp160 in BHK cells suggests that the synthesis of the GAG–POL fusion protein is mediated by a ribosomal frameshift mechanism as it is assumed for HIV-infected cells. This was substantiated by expression of the HIV–luciferase fusion gene construct B, leading to a mRNA which requires a ribosomal frameshift event within the HIV-specific sequence to give rise to active luciferase. From the investigations of Jacks *et al.* (1988a) and Wilson *et al.* (1988) it is known that the ribosomal frameshift occurs with a frequency of 5–10%. A lower extent of frameshifting (1.5–4%) was calculated from the expression of the luciferase constructs from both rodent and primate cells. From these results we conclude that retroviral ribosomal frameshifting does not qualitatively depend on species or tissue-specific factors and that it occurs independently of virus-specific components. Therefore frameshifting cannot be responsible for the observed unequal Gag precursor processing.

The biosynthesis of the HIV-1 GAG–POL fusion protein was investigated up to now by *in vitro* translation (Jacks *et al.*, 1988a) and by expression in yeast (Wilson *et al.*, 1988). We intend to use the described mammalian cell system for a more detailed analysis of the molecular and cellular background of ribosomal frameshifting. Retroviral frameshifting is a potential target for therapeutic intervention in the HIV replication cycle, as such a mechanism in eukaryotic cells seems to be restricted to moveable elements and viruses (Mellor *et al.*, 1985; Brierley *et al.*, 1987). This system might be helpful for development and evaluation of potential antiviral compounds.

A further subject of our investigations was the HIV-specific proteolytic maturation of the GAG and GAG–POL precursors in transfected rodent cells. The 24-kDa protein (rp24) and a 39-kDa protein are obviously cleavage products of the GAG precursor. The 39-kDa protein, which consists of p18 and p24 components, was detected in transfected rodent as well as primate cells producing a GAG precursor. A protein consisting of p18 and p24 was also found as an intermediate product during the cleavage of HIV-derived p55 in human lymphocytes (Mervis *et al.*, 1988; Veronese *et al.*, 1988). Our results from rodent cells prove that the production of this protein does not depend on the HIV protease and probably involves a cellular protease. Cellular proteases involved in the maturation of gag and pol encoded proteins have been postulated by others based on expression experiments in yeast (Barr *et al.*, 1987), insect cells (Madisen *et al.*, 1987), and mammalian cells (Flexner *et al.*, 1988). Whether cellular proteases are involved in performing virus-specific proteolysis of the GAG and POL polyproteins in HIV-infected cells needs to be elucidated.

Concerning the activity of the viral protease, we could only provide evidence for a weak specific activity by detection of rp24 in BHK cells but not in LTK$^-$ cells transfected with the gag–pol region. This finding was unexpected since efficient HIV-specific maturation of the GAG and/or POL proteins was reported to occur in bacteria (Farmerie *et al.*, 1987; Leuthardt and Le Grice, 1988; Le Grice *et al.*, 1988; Mous *et al.*, 1988), yeast (Kramer *et al.*, 1986), insect cells (Madisen *et al.*, 1987; Overton *et al.*, 1989), and mammalian cells (Smith *et al.*, 1990). With two experimental approaches we could further demonstrate that overexpression of the HIV protease does not enhance the proteolytic activity in the transfected cells. Coexpression of the GAG precursor and the HIV protease domain from two independent DNA constructs in LTK$^-$ cells did not lead to de-

tectable HIV protease activity, although a recombinant protein reacting with HIV protease-specific antibodies could be demonstrated. In bacteria (Le Grice *et al.,* 1988) and insect cells (Overton *et al.,* 1989) an HIV protease provided *in trans* results in a proteolytic activity. In a second attempt we successfully enhanced expression of the viral protease domain by overexpression of an inframe GAG–POL fusion protein in BHK cells. In these cells we obtained evidence for a weak HIV protease activity comparable to that found in cells transfected with the native gag–pol region. The data suggest that the low or missing HIV protease activity in rodent cells transfected with the gag and pol genes is not due to insufficient expression of the HIV protease. We could further show that in primate cells, in contradiction to rodent cells, the expression of gag–pol leads to an efficient GAG–POL processing. Our results are consistent with recent reports which describe that the specific maturation of gag and pol encoded proteins does not occur with the same efficiency in different mammalian cell lines (Gowda *et al.,* 1989; Shioda and Shibuta, 1990). Currently, it is difficult to explain why proteolytic processing mediated by the HIV protease efficiently occurs in *Escherichia coli,* yeast, and baculovirus expression systems but only in some mammalian cell expression systems using certain cell lines. Concerning our data, it is conceivable that GAG and GAG–POL processing in natural HIV host cells involves cellular mechanisms and/or factors which are distinct from those in the transfected rodent cells.

Stably transfected mammalian cells expressing the gag–pol genes of HIV-1 constitute a novel model system to investigate retroviral ribosomal frameshifting and to identify cellular components required for HIV-specific maturation of the GAG and GAG–POL polyproteins in rodent cells versus in the natural HIV host cells.

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