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Efficient Production of Human Immunodeficiency Virus Proteins in Transgenic Mice

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Transgenic mice containing the complete human immunodeficiency virus (HIV) coding sequences fused to the mouse mammary tumor virus long terminal repeat were generated. They were found to produce high levels of authentic *gag* and *env* HIV proteins in several tissues known to support mouse mammary tumor virus-driven transcription. HIV proteins were also detected in serum and in body fluids (milk and epididymal secretions) known to be natural sites of retrovirus, and specifically of HIV, production. These results indicate that primary mouse cells from different tissues have the capacity to produce HIV proteins. These mice represent a novel animal model for HIV infection.

Human immunodeficiency virus (HIV) has been shown to replicate in chimpanzees (11), in rabbits (10, 22), and in mice reconstituted with human hematopoietic cells (30, 31). However, these models may not always meet some important criteria, such as easy accessibility or high levels of viral protein synthesis or viral titers. A small, inexpensive animal that is well defined genetically, such as the mouse, appears suitable as a model for HIV studies. Transgenic mice harboring various reporter genes (17, 24, 36) or some HIV genes (7, 41) under the control of the HIV long terminal repeat (LTR) have already been produced. The HIV LTR/*tat* mice were found to develop Kaposi-like sarcoma (41), while those carrying the 3'-end HIV genes developed nephropathy (7). In addition, transgenic mice harboring the whole HIV genome have previously been generated (25). The F_1 progeny of one of these founders appeared to have developed some pathological changes resembling those found in some HIV-infected patients. However, no transgenic mouse lines expressing high levels of all HIV structural proteins in several tissues have yet been produced. If such proteins were expressed in some specific mouse cell types, one might hypothesize that high HIV protein levels would induce some of the manifestations of HIV disease observed in humans, as already suggested by a study of HIV LTR/*tat* transgenic mice (41).

However, previous results with various established mouse cell lines in vitro have shown that these cells cannot support the production of HIV proteins because of a defective *rev* function (40). If these results reflected a species difference, as suggested by Trono and Baltimore (40), the construction of transgenic mice with the HIV genome would become impractical.

Therefore, to ascertain the feasibility of developing transgenic mice as an animal model of AIDS, we first tested whether mouse cells in vivo could sustain the synthesis of HIV proteins, in contrast to established cell lines in vitro. We constructed transgenic mice expressing the whole HIV genome under the transcriptional control of a strong promoter, the mouse mammary tumor virus (MMTV) LTR. This promoter was chosen because it is known to be a very efficient promoter in various epithelial and hematopoietic tissues (including T-lymphoid cells) (3, 23, 37–39), some of which are known to naturally support lentivirus (and especially HIV) replication.

Transgenic mice were generated by microinjection, into one-cell (C57BL/6 × C3H)F_2 embryos (3, 16, 39), of the 12-kbp *Xba*I-*Aat*II chimeric DNA fragment containing the complete HIV coding sequence under the transcriptional control of the MMTV LTR (Fig. 1). The 5' LTR and part of the untranslated 5' leader sequences of the HIV genome were deleted to transcribe it under the control of a surrogate promoter. A portion of the 3'-end LTR was also deleted. However, all the HIV genes were intact in this construct. The deletions were made for safety reasons, to avoid the production of infectious HIV particles. Indeed, cells harboring such a transgene should be able to produce noninfectious HIV particles at best or murine leukemia virus (MuLV)-HIV pseudotypes. Four transgenic founders were produced. In each of these founders, the MMTV/HIV sequences appeared intact and localized at a unique integration site (data not shown). Three founders transmitted the transgene to their progeny, apparently in a Mendelian fashion, and the transgene has now been transmitted to several generations. Mouse lines (MMTV/HIV-R3, MMTV/HIV-R4, and MMTV/HIV-R10) were established by mating founder mice with C3H mice (Charles River Laboratories, St-Constant, Québec, Canada).

No abnormal phenotype was apparent in transgenic mice, some of which were sacrificed when they were as old as 17 months. In appearance and behavior, they are indistinguishable from age-matched nontransgenic mice. At autopsy, the macroscopic examination appeared normal. Histological examination of tissues known to support expression from the MMTV LTR in transgenic mice (mammary glands, epididymis, salivary glands, harderian glands, and spleen) (3, 38, 39) revealed no lesions (data not shown).

Expression of the transgene was measured by the agarose gel transfer (Northern blot) procedure with total RNA from various organs of transgenic mice. The three main species of HIV RNA, usually detected in cells productively infected with HIV, were detected in several organs known to support transcription from the MMTV LTR, such as the mammary, harderian, and salivary glands, epididymis, thymus, and

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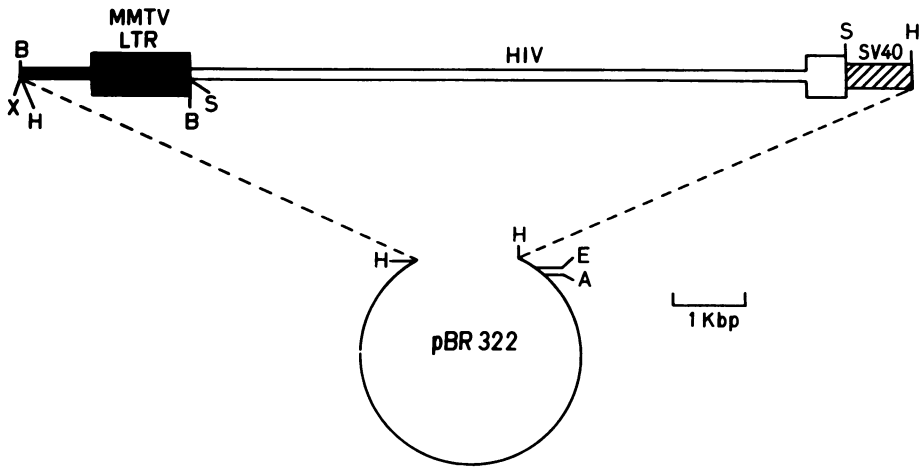

FIG. 1. Schematic representation of the MMTV/HIV fusion gene. The transgene consisted of three fragments: the MMTV LTR *Hin*dIII-*Sac*I fragment, the HIV genome *Sac*I fragment, and the simian virus 40 (SV40) *Sac*I-*Hin*dIII fragment. These were ligated together in *Hin*dIII-digested vector pBR322 in an orientation which placed the 3'-end SV40 sequences close to the *Cla*I site of pBR322. The 2.3-kbp MMTV *Bam*HI fragment of the pA9 plasmid (39) was first subcloned in pUC18 in an orientation which placed the end of the 3'-end LTR close to the *Eco*RI site. It was then prepared as a *Hin*dIII-*Sac*I fragment by *Hin*dIII digestion and partial *Sac*I digestion. The 8.9-kbp *Sac*I HIV fragment was obtained by digesting the BH10R3 plasmid (33, 35) with *Sac*I. The SV40 poly(A) addition signal was obtained from the pSV2neo vector. The 885-bp *Bam*HI-*Pst*I fragment was first subcloned in pUC18. The SV40 fragment was prepared from this subclone by digestion with *Sac*I and *Hin*dIII. The 12-kbp fragment to be microinjected was obtained by cleaving the plasmid DNA with *Xba*I (from the pUC18 polylinker) and *Aat*II (present in the pBR322 plasmid). The fragment was isolated by preparative agarose gel electrophoresis and further purified on a CsCl gradient essentially as described before (3, 26). Restriction sites: A, *Aat*II; B, *Bam*HI, E, *Eco*RI, H, *Hin*dIII; S, *Sac*I; X, *Xba*I.

spleen in the three transgenic lines tested (data not shown). In other organs (in line MMTV/HIV-R10) which are known to support MMTV LTR-driven transcription poorly, such as the kidneys, heart, testes, liver, and brain, the level of transgene expression was very low or negative, as expected.

Expression of HIV structural proteins in organs of transgenic mice. To determine whether the transgene HIV RNA was translated in various organs, we measured the levels of HIV structural proteins by Western blotting (immunoblotting) (14) using antisera specific to HIV proteins. Briefly, tissues were solubilized in a mixture of 100 mM Tris (pH 6.8), 2 mM EDTA, and 1% sodium dodecyl sulfate (SDS). Protein samples (150 μg) were diluted in 2× sample buffer (100 mM Tris [pH 6.8], 200 mM dithiothreitol, 0.2% bromophenol blue, 20% glycerol), separated by electrophoresis on 5 to 10% or 5 to 15% polyacrylamide–SDS gels, and transferred to Immobilon P membranes (Millipore) as described previously (14). The membranes were blocked with 5% (wt/vol) nonfat dry milk in TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.2% Tween 20). Next, the membranes were incubated with primary goat anti-gp160 (ERC-188) (1:7,500) or rabbit anti-p24 (ERC-384) (1:7,500) antibody (kindly provided through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases) in TBST containing 1% nonfat dry milk for 1 h. The membranes were then washed in TBST containing 1% nonfat dry milk and incubated with the alkaline phosphatase-linked second antibody (anti-goat or anti-rabbit immunoglobulin G) (Sigma).

The *env* proteins gp160 and gp120 were detected at very high or moderate levels in the mammary glands of animals of three transgenic lines; at lower levels in the salivary and harderian glands; in the liver, seminal vesicles, epididymis, and testes (only gp160) of mice from line MMTV/HIV-R10; and in the epididymis, spleen, or liver (only gp160) of mice from line MMTV/HIV-R4 (Fig. 2; Table 1). These proteins appeared to comigrate with authentic HIV *env* proteins from HIV-infected cells, except that transgenic gp120 migrated slightly faster.

The $p55^{gag}$ precursor protein was detected at high levels in mammary glands of animals of the three transgenic lines (Fig. 3). The $p55^{gag}$ or $p41^{gag}$ precursor or both were also detected in salivary and harderian glands; in the spleen, seminal vesicles, and epididymis of mice from line MMTV/HIV-R10 (Fig. 3); and in the spleen and epididymis of mice from line MMTV/HIV-R4 (data not shown). The final cleavage product of *gag*, the $p24^{gag}$ protein, was detected in mammary glands of mice from lines MMTV/HIV-R3 and MMTV/HIV-R10 and in salivary glands of animals from line MMTV/HIV-R10. Both $p55^{gag}$ and $p41^{gag}$ precursors and p24 protein from transgenic mice migrated with authentic HIV *gag* proteins from HIV-infected cells (Fig. 3, lane 1), indicating proper synthesis and cleavage in some transgenic mouse organs. The $p160^{gag\text{-}pol}$ precursor was only occasionally detected at very low levels in mammary glands of mice from the MMTV/HIV-R10 line, suggesting efficient processing in these mouse cells.

Since HIV proteins were detected in several glands or organs, some of which support the production of specialized body fluids, it was of interest to determine whether some of these HIV proteins were released in the blood circulation or in various secretion fluids. In serum, HIV proteins reactive with HIV-specific antibodies were detected in mice from two lines (Table 1). Higher levels of HIV serum proteins were detected in lactating female mice. HIV *env* (gp160 and gp120) (Fig. 2, lanes 19 and 20) and *gag* (p55, p41, and p24) (Fig. 3, lane 19) proteins were also detected at very high levels in milk from lactating female transgenic mice. In addition, HIV proteins were detected, at a lower level, in secretions of the male genital tract (Table 1). This pattern of expression was expected, since mammary glands as well as epididymis were positive for HIV proteins. These results

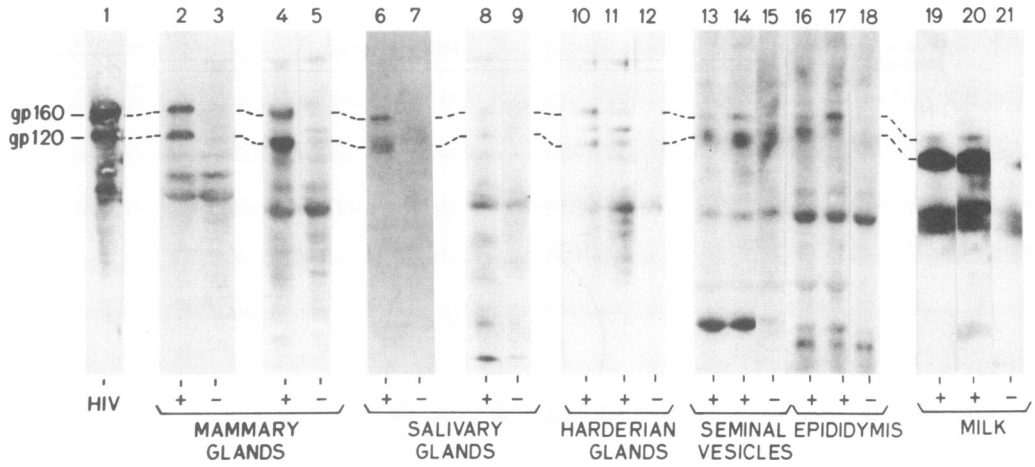

FIG. 2. Western blot analysis of gp160^{env} and gp120^{env} proteins in tissues and milk of transgenic mice of line MMTV/HIV-R10. The Western blot analysis was performed essentially as described in the text and reference 14. A 5 to 10% polyacrylamide gradient was used in lanes 1 to 11, and a 5 to 15% polyacrylamide gradient was used in lanes 12 to 21. The membranes were probed with a polyclonal goat antibody against gp160 (ERC-188) (1:7,500). Chemiluminescent detection was performed with Lumi-Phos 530 (Boehringer Mannheim, Montréal, Québec, Canada) as the substrate, except in lanes 6 and 7, where BCIP (5-bromo-4-chloro-3-indolylphosphate *p*-toluidine) and nitroblue tetrazolium chloride were used as substrates for the alkaline phosphatase reporter enzyme. Protein samples were from transgenic (+) or control nontransgenic (−) mice. Lane 1 contains a positive control (CEM cells infected with HIV [LAV strain]). The sources of proteins in the other lanes are listed at the bottom.

clearly indicated that some specialized mouse cell types are capable of releasing HIV proteins extracellularly either into the circulation or in secretions.

We searched for antibodies to HIV proteins in the sera of these mice, using an enzyme immunoassay technique (Genetic Systems). No antibodies were detected in any of 17 tested serum samples from 10 mice of line MMTV/HIV-R10 and 7 mice of line MMTV/HIV-R4 (data not shown).

An animal model for HIV infection. The results presented here clearly establish that several types of mouse cells have

TABLE 1. Detection of HIV proteins in MMTV/HIV transgenic mice

Organ or fluid	Proteins detected				
	Western blot[a] (*env* and *gag*) with transgenic line:		ELISA[b] (OD units/mg of protein)		
			Control	Transgenic line	
	MMTV/HIV-R4	MMTV/HIV-R10		MMTV/HIV-R4	MMTV/HIV-R10
Organs					
Mammary glands	+	++++	0.34 (1)	3.12 (1)	17.43 (2)
Salivary glands	−	++[c]	ND[d]	ND	ND
Harderian glands	−	+	ND	ND	ND
Spleen	+	−	0.30 (1)	4.48 (4)	0.38 (2)
Liver	+[e]	+[f]	ND	ND	ND
Kidneys	ND	−	ND	ND	ND
Seminal vesicles	−	+	0.04 (3)	0.09 (2)	0.71 (2)
Epididymis	++	+	0.22 (3)	11.61 (3)	2.34 (2)
Testes	−	+[e]	0.03 (3)	0.42 (3)	0.02 (2)
Fluids					
Milk	+++	++++	0.01 (2)	1.28 (3)	1.82 (5)
Saliva	−	−	2.07 (1)	1.63 (3)	0.95 (2)
Epididymal wash	ND	ND	0.06 (1)	1.82 (3)	1.76 (3)
Serum	ND	ND	0.006 (5)	ND	0.02 (11)
Serum (from lactating mice)	ND	ND	0.006 (1)	ND	0.26 (3)

[a] In most positive tissues, gp160^{env} and gp120^{env} as well as p55^{gag} and p41^{gag} were detected. p24^{gag} was detected mostly in mammary and salivary glands and in milk, especially when levels of *gag* proteins were high. The levels of proteins are arbitrarily graded from ++++ (equivalent to levels shown in Fig. 2, lane 2) to + (equivalent to levels shown in Fig. 2, lane 10). −, no HIV proteins detected. For transgenic mice from the MMTV/HIV-R3 line, only the mammary (++++), salivary (−), and harderian (−) glands were tested.

[b] The number of mice tested is given in parentheses. An Abbott HIVAG-1 enyme-linked immunosorbent assay (ELISA) kit was used. OD, optical density.

[c] p55^{gag} was not detected, but p41^{gag} and p24^{gag} were detected.

[d] ND, not determined.

[e] Only gp160^{env} was detected.

[f] Only gp160^{env} and gp120^{env} were detected; no *gag* proteins were detected.

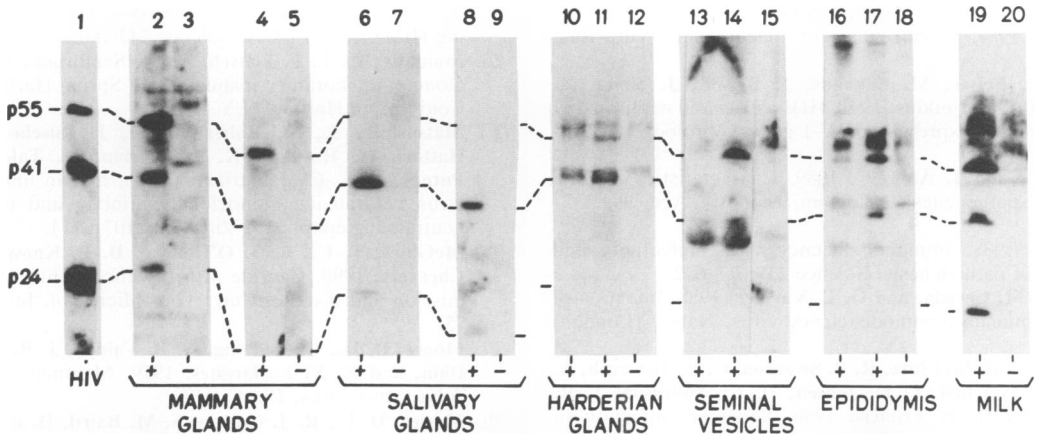

FIG. 3. Western blot analysis of *gag* proteins in tissues and milk of mice from line MMTV/HIV-R10. Protein samples from different tissues were separated by electrophoresis on 10% polyacrylamide–SDS gels and transferred to Immobilon P membranes. The membranes were probed with a polyclonal rabbit antibody against p24 (ERC-384) (1:7,500 dilution) and an alkaline phosphatase-linked second antibody against rabbit immunoglobulin (Sigma). Chemiluminescent detection was performed by using Lumi-Phos 530 as the substrate. Protein samples were from transgenic (+) or nontransgenic (−) mice. Lane 1 contains a positive control (CEM cells infected with HIV [LAV strain]). The sources of proteins in the other lanes are indicated at the bottom.

the capacity to express high levels of HIV proteins in vivo when the HIV genome is transcribed from a surrogate promoter. These results contrast significantly with those obtained with established mouse cells in vitro (40). In the mouse cell lines tested in vitro, the expression of HIV proteins was found to be very low, and this phenotype was attributed to species differences. Our results indicate that it does not reflect a species difference but more likely reflects cell type differences or differences related to the establishment of cells in culture.

The levels of nonstructural HIV proteins in the tissues of these transgenic mice have not yet been measured directly, but the presence of some of them can be ascertained indirectly. Indeed, the HIV protease must be present in an amount sufficient for protein cleavage, since the *gag* precursors were found to be properly cleaved. The *rev* protein is also likely to be synthesized in sufficient quantity and to function properly, since its absence or its malfunction would prevent the production of properly spliced HIV cytoplasmic RNAs and the synthesis of *gag* and *env* proteins (4).

Interestingly, the high levels of HIV *gag* and *env* proteins detected in these transgenic mice were found in some tissues known to be natural sites of retrovirus replication. The mammary glands have long been known to be a site of retrovirus replication. Transmission of several classes of retroviruses (and of the disease which they induce) through milk has been documented, e.g., for MMTV (29), MuLV (12), and human T-cell lymphotropic virus type I (1, 19, 20), as well as lentiviruses (maedi/visna virus [6, 13], caprine arthritis-encephalitis virus [28], and HIV [32, 44]). It remains to be determined, however, whether the HIV particles detected in human milk are produced from infected macrophages and lymphocytes or from mammary epithelial cells or both.

The presence of HIV proteins in the epididymis and epididymal fluid is also of interest, since this organ is known to be one of the preferential sites of MuLV replication (18) and since free MuLVs were found in epididymal fluid (18). Semen constitutes an important route of HIV transmission (8, 9, 15, 21, 42, 43), and HIV has been detected in cell-free seminal fluid (2). The fact that HIV proteins could be detected in the lumen of the epididymis of these transgenic mice may be relevant for the transmission of HIV. Moreover, HIV proteins have been detected in glandular epithelial cells of the prostate of a large proportion of patients with AIDS (5). These findings indicate that epithelial cells from humans, as well as from other species, have the capacity to produce retroviruses, and specifically to produce HIV.

The MMTV/HIV transgenic mice produced in this study therefore represent a novel and convenient animal model that could be instrumental in experiments directed at understanding and preventing HIV transmission. In addition, they could be useful to test anti-HIV drugs designed to block HIV replication at a postintegration step of the virus cycle.

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