Lentivirus Vector Can Integrate in the Genome and Exist and Replicate in the Cell as an Episome

M. A. Ershler, N. I. Drize, I. N. Nifontova, A. V. Terskikh, and I. L. Chertkov

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Lethally irradiated mice were reconstituted with few purified primitive hemopoietic stem cells containing sequences of a gene encoding green fluorescent protein. The gene was transferred using a lentivirus vector. The presence of the marker gene in splenocyte colonies derived from the bone marrow of reconstituted mice and in cells of other hemopoietic and non-hemopoietic organs was studied during the life. It was shown that the lentivirus vector can persist for a long time and replicate in hemopoietic cells as an episome.

Key Words: *lentivirus vector; gene transfer; stem hemopoietic cell; episome; green fluorescent protein*

Lentivirus vectors (LVV) are widely used as potential carriers of genes in genetic diseases, because their structure allows transfer of large genes, for example hemoglobin, blood clotting factors VIII, IX genes, *etc.* [1]. The major advantage of LVV over other retroviruses is the possibility of their integration into the genome of nonproliferating cells. This is particularly important for gene transfer into primitive stem hemopoietic cells (pSHC), because the majority of these cells is not involved in the cell cycle, and recruitment into the cycle considerably modifies the proliferative potential and the fate of SHC after transplantation [7].

We investigated the integration of LVV carrying green fluorescent protein (GFP) gene in the genome.

MATERIALS AND METHODS

Lethally irradiated C57Bl/6 mice from the laboratory headed by Dr. I. Weissman (Stanford University, USA) were used in the study. pSHC were selected from the bone marrow of inbred male mice [5] and LVV was transduced *in vitro*. The vector was constructed and described previously [4]. Hemopoiesis of lethally irradiated females was reconstituted with labeled pSHC in doses of 300 and 3000 cells.

Bone marrow was aspirated from the femur of narcotized reconstituted mice 6 and 8 months after cell transplantation. After the second irradiation on an IPK device (10 Gy, 2 fractions with 3-h interval) females were intravenously injected with $2\text{-}3\times10^5$ bone marrow cells. After 10 days the mice were sacrificed, splenic colonies were isolated under a magnifying glass, and the cells were used for isolation of genome and episome DNA and for evaluation of GFP expression.

Genome [6] and episome [2] DNA was isolated by standard methods. Green protein was evaluated in cell suspensions under a fluorescent microscope (FITC filter). PCR analysis of GFP sequences was carried out using primers specific for GFP cDNA (3"ATGGTGAGCAAGGGCGAGGA and 5"AGA CGTTGTGGCTGTTGTAG) amplifying a DNA fragment of 453 b. p. The donor origin of the colonies was identified by PCR on the y-chromosome sequence [3]. Southern blot hybridization was carried out by the standard method [6] with DNA fragment amplified from pEGFP-C1 plasmid (Clontech Laboratories) for the probe.

Hematological Research Center, Russian Academy of Medical Sciences, Moscow. ***Address for correspondence:*** ndrize@blood.ru. N. I. Drize

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RESULTS

Eight weeks after reconstitution multilinear expression of GFP, detected on a flow fluorometer, was observed in 5-6% peripheral blood cells in mice reconstituted with 300 cells and in 10-12% cells of mice reconstituted with 3000 cells (Table 1).

The integration of LVV in splenic cells was studied in 110 splenic colonies from 7 mice reconstituted with 300 cells and in 92 colonies from 6 mice reconstituted with 3000 cells. In all mice hemopoiesis was realized by transplanted highly purified pSHC: virtually all (98-100%) splenic CFU were of donor origin, which was shown by y-chromosome PCR. The efficiency of labeling was very high. The mice reconstituted with 300 cells had 88.8±5.1% GFP-positive colonies, those reconstituted by 3000 cells had 98.8±1.5% GFP positive colonies.

Southern blot hybridization detected labeled DNA fragment of the same size (about 4000 b. p.) in 90% colonies from different mice (Fig. 1). Only 8.1% labeled colonies had one or more fragments differing by size in individual colonies (Fig. 1, bands *6* and *15*), which was in line with the data on accidental integration of retrovirus vectors in SHC genome [8]. These data suggest that only such fragments characterize the GFP DNA really integrated in the genome. The vector contains the only restriction site EcoR1, and hence, the 4000 b. p. fragment cannot be cut from LVV. In order to identify the origin of this fragment, other restriction enzymes were used (Bam H1, Kpn 1, and Sal 1) having one restriction site in the vector used. The size of the fragment was the same for all studied colonies (Fig. 2, bands *2-7*). Moreover, we found a 6000 b. p. fragment, which was similar in all samples even in unrestricted DNA (Fig. 2, bands *9* and *12*). Presumably, it is a DNA fragment not integrated in cell genome, most likely this is episome DNA. Restriction transforms circular DNA into linear, which explains its higher mobility. In order to verify this hypothesis, we isolated episome DNA from 20 colonies. Episome fraction was GFP-positive in 16 of 19 GFP-positive colonies. Thus, we demonstrated long-term (about half of mouse life span) persistence of not integrated LVV. This vector can maintain long expression of foreign genes. We detected green fluorescence in some splenic colonies and in some granulocytic macrophagal colonies in semiliquid medium formed by long-living bone marrow cultures derived from reconstituted mice.

TABLE 1. GFP Expression by Labeled pSHC in Mouse Peripheral Blood Cells 8 Weeks after Reconstitutation (*M*±*m*)

Number of injected GFP$^+$ pSHC	Erythroid cells	Lymphoid cells	Myeloid cells
300	5.0±5.9	6.0±5.6	0.8±1.4
3000	10.5±4.0	12.8±3.9	1.6±0.9

The mechanism of provirus replication as an episome in hemopoietic cells (each colony is a clone consisting of several millions of cells, and hence, the provirus was replicated at least millions times) is not clear. However, long-term persistence of the provirus not integrated into the genome and capable of expression is potentially hazardous.

We evaluated the infective activity of the provirus by measuring its content in hemopoietic (bone marrow, thymus, and peripheral blood) (Fig. 2, bands *15*, *18*, *20*) and nonhemopoietic tissues (heart, liver, muscle, kidney, lung). The provirus was detected by Southern blot analysis only in hemopoietic tissues, which indicates the impossibility of horizontal transfer of the provirus. However we must interpret this conclusion with certain limitations, because DNA from nonhemopoietic tissues was GFP-positive in PCR analysis. Presumably, this can be due to the presence of

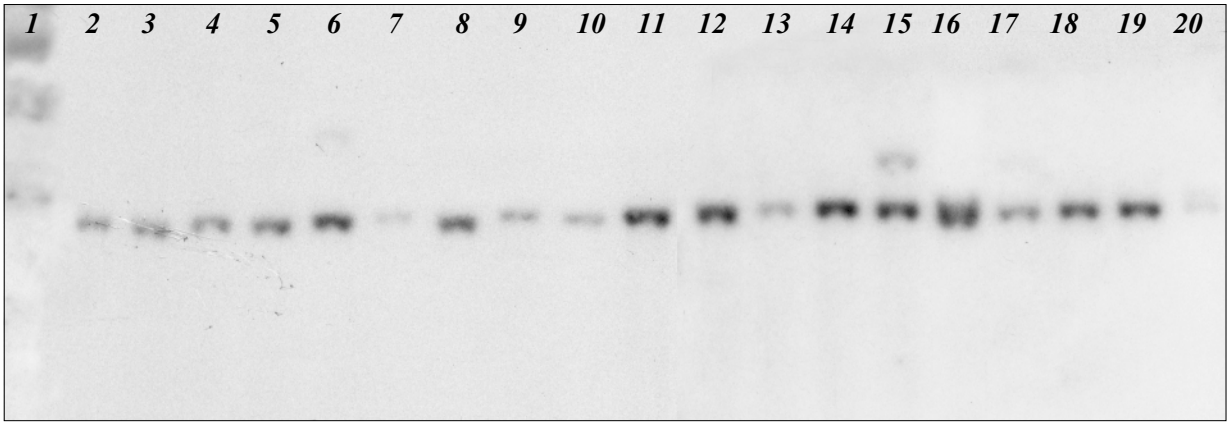

Fig. 1. Southern blot analysis of DNA from splenic colonies. *1*) marker (phage lambda DNA fragmented with HindIII enzyme); *2-20*) DNA of splenic colonies from the bone marrow of reconstituted mice, fragmented with EcoR1 fragment.

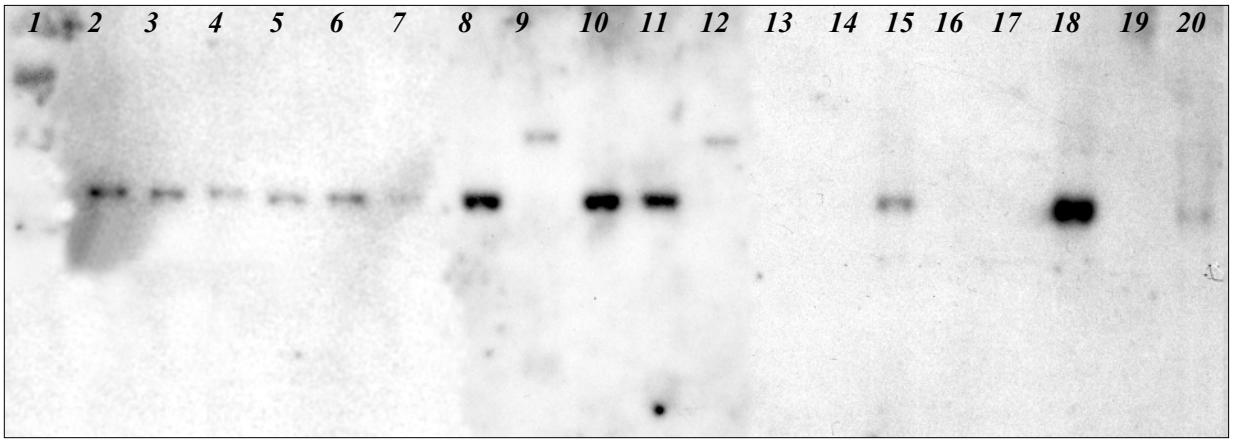

Fig. 2. Southern blot analysis of DNA from splenic colonies and organs of reconstituted mice. *1*) marker (phage lambda DNA fragmented with HindIII enzyme); *2*) DNA from colony No. 146 restricted by Bam H1 enzyme; *3*) DNA from colony No. 146 restricted by Kpn 1 enzyme; *4*) DNA from colony No. 146 restricted by Sal 1 enzyme; *5*) DNA from colony No. 152 restricted by Bam H1 enzyme; *6*) DNA from colony No. 152 restricted by Kpn 1 enzyme; *7*) DNA from colony No. 152 restricted by Sal 1 enzyme; *8*) DNA from colony No. 154 restricted by EcoR1 enzyme; *9*) DNA from colony No. 154 without enzyme treatment; *10*) DNA from colony No. 135 restricted by EcoR1 enzyme; *11*) DNA from colony No. 135 restricted by Bam H1 enzyme; *12*) DNA from colony No. 135 without enzyme treatment; *13*) DNA from the heart; *14*) DNA from the liver; *15*) DNA from the bone marrow; *16*) DNA from the muscle; *17*) DNA from the kidney; *18*) DNA from the thymus; *19*) DNA from the lung; *20*) DNA from the peripheral blood.

blood cells in all tissues, but still, we cannot rule out the minimum presence of the provirus transferred horizontally.

These data characterize the lentivirus system of gene transfer from an unexpected side. It is possible that LVV can integrate in the genome of only dividing cells, which is seen from the provirus integration in only 8% pSHC. A recent report indicates that LVV integrates mainly into G_1 phase cells, but not in the G_0 phase cells [1]. Presumably, later the episomes do not integrate either, as all the studied individual pSHC divided at least once during 6-8 months, and integration was observed in only 8% colonies.

These data indicate that LVV is unfit for labeling and identification of individual stem cells of hemopoietic and other tissues, and data on possible transdifferentiation of stem cells, obtained using LVV, are to be revised. On the other hand, persistence of expression during the entire life span and the absence of horizontal dissemination of the vector seem to allow its use for gene therapy. However, the use of LVV for gene therapy should be limited until complete elucidation of the mechanism of episome reproduction and verification of impossibility of their propagation through infection of other tissues. Though the vector used in our study is one of the most often used, it is possible that other LVV effectively integrate in the genome and are incapable of replication and persistence outside the genome DNA. Each of the known LVV is to be tested in this respect before clinical use.

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