TRANSDUCTION OF THE (Neo^r) MARKER GENE INTO HEMATOPOIETIC STROMAL PRECURSOR CELLS

N. I. Drize, V. L. Surin, O I. Gan, E. I. Deryugina, and I. L. Chertkov

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In recent years much progress has been made with transduction of genes into hematopoietic precursor cells in mammals of various species [9]. Attempts have been made to introduce foreign genes into stromal cells of the hematopoietic microenvironment – into endothelial cells [2] and fibroblasts [7]. The erythropoietin gene has been tranducted into cells of the supporting layer of long-term bone marrow cultures [4]; its expression induced erythroid differentiation of the hematopoietic cells in culture, whereas usually in cultures of this type only myelopoiesis is observed. It could not be determined in which cells of the hematopoietic microenvironment – stromal cells proper or macrophages, of hematopoietic origin – erythropoietin was synthesized, The problem of introducing foreign genetic material into stromal cells, capable of maintaining long-line hematopoiesis, remains unsolved.

This paper describes an attempt to introduce a foreign gene into stromal precursor cells, capable of transferring the hematopoietic microenvironment and of maintaining hematopoiesis in the long term in vitro and vivo.

EXPERIMENTAL METHOD

Female (DBA/2 × C57BL/6)F_1 hybrid mice (DBF_1) aged 12-18 weeks were used. Producer strains of retroviruses were obtained by transformation of cell cultures of the $\varphi 2$ line with $CaCl_2$ [8], by plasmid pPs3neo (Fig. 1), generously provided by P. M. Chumakov (Institute of Molecular Biology, Academy of Sciences of the USSR). Virus production in supernatants of the producer strains was determined by the standard method, by titration on NIH 3T3 cells with the antibiotic G418 ("Gibco," England) (concentration of the pure substance 0.5 mg/ml). Two producer strains were selected, namely $\varphi 2$neo3 and $\varphi 2$neo7, with titers of $1 \cdot 10^6$ and $2 \cdot 10^6$ virus particles per milliliter of supernatant respectively. The producer strains and NIH 3T3 cells were cultured on medium DMEM, enriched with 2 mM L-glutamine, antibiotics, and 10% fetal calf serum (FCS).

A long-term bone narrow culture was obtained by Dexter's method [6]. Bone marrow from one femur was explanted in 10 ml of complete nutrient medium [Fischer's medium with 2 mM L-glutamine, 7% FCS, 13% horse serum (all from Flow Laboratories, England), 10^{-6} M hydrocortisone hemisuccinate ("Sigma," USA), 100 U/ml penicillin, and 50 μg/ml streptomycin] in plastic flasks with a bottom measuring 25 cm^2. Culture was carried out at 33°C in 5% CO_2. A complete change of medium was made once a week. Primary long-term cultures were infected with retrovirus by the following scheme: on the 4th, 7th, 11th, and 14th days after explanation of the bone marrow the medium with cells was drawn off and 2 ml of supernatant of retrovirus producer strains with 8 μg/ml polybrene ("Sigma," USA) was poured over the newly formed supporting layer. After 2 h (33°C, 5% CO_2) the supernatant was replaced by complete nutrient medium with cells from the same cultures.

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The supporting layer of adherent cells from four-week long-term cultures was removed with a rubber policeman and implanted beneath the renal capsule of intact syngeneic mice, as described previously [3]. The size of the focus of ectopic hematopoiesis was determined from the number of nucleated cells in it.

Cells of the foci were explanted into secondary cultures of two types 1 month after implantation, without conversion into a single-cell suspension: Dexter [6] and Whitlock–Witte [11] [medium RPMI 1640 with 10% preselected FCS ("Flow," England) with antibiotics, but without hydrocortisone, incubation temperature 37°C]. Half the medium was changed every week in the secondary cultures. After culture for 3-4 weeks, DNA was isolated from the supporting layer of adherent cells [8]. The presence of the marker gene in cells of the stromal microenvironment was determined by the polymerase chain reaction (PCR) [10], in the course of which a 0.79 kbp region of the Neo^r gene was revealed. Primers, first described by Correll and co-workers [5], synthesized and generously provided by M. L. Markelov (Institute of Bioorganic Chemistry, Academy of Sciences of the USSR), were used. The reaction conditions were: 94°C – 1.0 min, 60°C – 2 min, 72°C – 3 min, 30 cycles. Thermostable DNA-polymerase from *Thermus thermophilus*, generously provided by O. K. Kaboev (Leningrad Institute of Nuclear Physics, Academy of Sciences of the USSR, Gatchina), was used. Southern blot hybridization was carried out as described previously [8]. The Pst 1 fragment of plasmid pSV2neo was used as the probe.

EXPERIMENTAL RESULTS

During retrovirus transfer of genes integration of the foreign material takes place only in the genome of dividing cells. Under normal conditions stromal cells do not proliferate, whereas after explanation of bone marrow into culture, during the first 3 weeks a stromal supporting layer is formed, and during this period stromal precursor cells embark upon the cell cycle [3]. In this connection,integration of the retrovirus vector into the genome of the stromal cells is possible in principle. During the first 2 weeks of culture, cells from the supporting layer were incubated 4 times with supernatant of the retrovirus producer strains, carrying the bacterial gene of resistance to neomycin. After 4 weeks of culture the supporting layer of the adherent cells of the experimental and control (not exposed to retrovirus infection) cultures were implanted beneath the renal capsule of intact syngeneic mice. The supporting layer of adherent cells from long-term bone marrow cultures is able to transfer the hematopoietic microenvironment [3]; the stroma of the focus is of donor origin, whereas the hematopoietic cells belong mainly to the recipient [1]. Supporting layer cells of cultures transduced by the retrovirus vector can construct a completely normal focus of ectopic hematopoiesis (Table 1). The cell composition of the foci constructed by supporting layers of the control and experimental cultures was approximately the same. An increase in mass of the bone was noted in implants from the experimental cultures. It is generally considered that the Neo^r gene is only a marker for eukaryotic cells. Integration even of a defective neutral retrovirus may perhaps somehow stimulate proliferation of osteogenic precursors. However, this hypothesis is premature, for control cultures were not treated in this investigation by supernatant of the $\varphi 2$ strain, not producing the retrovirus. The procedure of treating the stromal supporting layers with supernatants from any fibroblastoid strains may itself stimulate osteogenic precursors.

To detect the integrated provirus foci of ectopic hematopoiesis were explanted into the genome of stromal cells in secondary long-term cultures of two types: Dexter and Whitlock—Witte. Hematopoiesis was controlled in the culture mainly by the supporting layer of adherent cells. Depending on the conditions of culture, supporting layers of these two types of cultures differed functionally. The presence of a marker gene in the stromal cells of the two types of cultures (Dexter and Whitlock—Witte) was determined by the polymerase chain reaction method (Fig. 2), The 0.79-kbp fragment of the Neo^r gene was amplified by means of the PCR system described previously [5]. As electrophoresis showed (Fig. 2a), a fragment of the expected length was found both in the positive control test (DNA from cells of the producer strain – track 1), and in the experimental DNA samples (tracks 2, 3, and 6), To determine the characteristics of the amplified fragment more precisely, restriction analysis of the PCR-product and Southern blot hybridization with the Pst 1 fragment of plasmid pSV2neo were carried out (Fig. 2b, c). The results of restriction analysis are in full agreement with the known map of the Neo^r gene and also reflect the identity of the PCR products of the control and experimental samples (Fig. 2b). Similar information was obtained by blot hybridization analysis (Fig. 2c), which also revealed the sequences of the Neo^r gene in a number of samples in which previously the corresponding PCR product had not been detected by staining the gels with ethidium bromide (Table 2).

TABLE 1. Dimensions of Foci of Ectopic Hematopoiesis Formed by Stromal Precursors from Supporting Layer of Adherent Cells of Long-Term Bone Marrow Cultures Infected with Retroviruses

Source of retrovirus	Number of foci including		Mean mass of bone (mg)	Cell composition of focus, $\times 10^{-6}$ (M±m)
	Experiment 1			
—	5	5	1,8	2,6±0,3
φ2neo7	5	5	13,6	2,8±0,5
φ2neo3	5	5	10,8	2,9±0,6
	Experiment 2			
—	5	5	3,0	5,1±1,3
φ2neo3	5	5	6,2	3,3±0,9

TABLE 2. Effect of Transduced Neo^r in Supporting Layer of Adherent Cells of Secondary Long-Term Cultures from Foci on Ectopic Hematopoiesis

Expt. No.	Source of retrovirus	Number of foci including with bone	Presence of Neo^r gene	
			staining with ethidium bromide	southern blot hybridization
1	φ2neo7	Dexter	+	+
		Whitlock–Witte	ND	ND
2	φ2neo7	Dexter	+	+
		Whitlock–Witte	—	+
3	φ2neo3	Dexter	—	+
		Whitlock–Witte	+	+
4	φ2neo3	Dexter	ND	ND
		Whitlock–Witte	—	+

Legend. ND) Not determined.

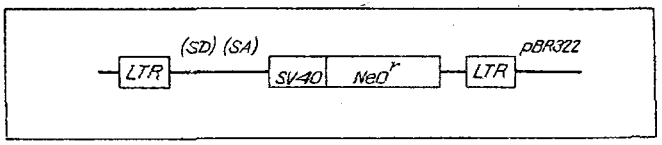

Fig. 1. Structure of retrovirus vector: LTR) Long terminal repeat of Moloney virus; SD) splicing donor site; SA) splicing acceptor site; SV40) promoter of virus SV40.

It follows from the results of direct PCR analysis and also of PCR analysis followed by blot hybridization, that the quantity of PCR product sought in the experimental samples varies strongly, possibly due to differences in the content of marked stromal cells in the supporting layer of adherent cells. In cultures of Dexter type, a more powerful supporting layer of adherent cells was formed. The supernatant of both producer strains of retroviruses effectively infected stromal precursor cells (Table 2).

The results provide the opportunity for transduction of foreign genes into stromal precursor cells that are able to construct a normal hematopoietic microenvironment. However, no final proof of introduction of foreign genes into stromal precursor cells was obtained. In ectopic foci formed by implantation of bone marrow not all the hematopoietic cells are known to belong to the recipient, but they account for on average 70% [1]. On implantation of the supporting layer of adherent cells from long-term bone marrow cultures beneath the renal capsule, hematopoietic cells in the foci belong entirely to the recipient, as has been shown karyologically. The essentially more sensitive method of PCR analysis may, in principle, reveal marked hematopoietic cells of donor origin during explanation of newly formed foci into secondary

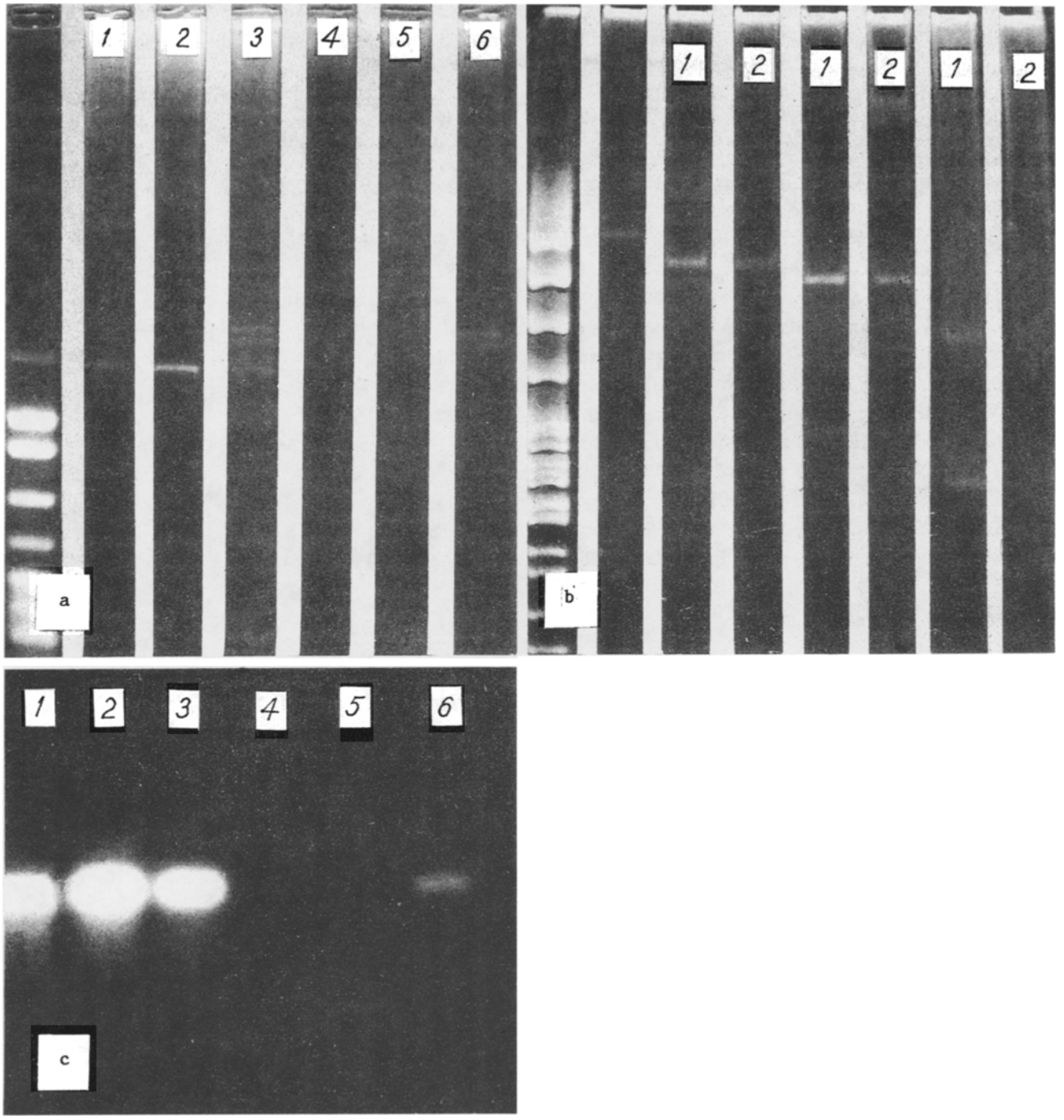

Fig. 2. Analysis of DNA from supporting layer of secondary long-term bone marrow cultures, by the polymerase chain reaction method. a) Electrophoresis of PCR-product of Neo^r gene in 6% polyacrylamide gel (stained with ethidium bromide); b) restriction analysis of amplified region of Neo^r gene; 1) DNA from cells of producer strain $\varphi_2$neo3; 2) DNA from supporting layer of secondary culture of Dexter type; c) Southern blot hybridization analysis of amplification products of region of Neo^r gene.

cultures. However, allowing for the fact that hematopoietic cells in long-term cultures of Dexter type differentiate in the myeloid direction, whereas in cultures of the Whitlock–Witte type mainly B cells differentiate, this hypothesis seems unlikely. Integration of the virus into hematopoietic cells of the stromal supporting layer (macrophages) may nevertheless not be completely ruled out at the present time.

An approach is thus suggested to the transfer of genetic material not only into hematopoietic, but also into stromal precursor cells. The suggested method offers the possibility of marking precursor cells of the hematopoietic microenvironment for their further study.

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MUTATIONS OF GENETIC REGION FIN OF F-LIKE PLASMID pAP18-1

N. I. Buyanova, V. P. Shipkov, and A. P. Pekhov

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Conjugative transfer of bacterial plasmids is controlled by their genetic region, under whose control a particular type of transfer inhibitor is synthesized in the cell [6, 9]. However, the genetic structure of this region has not yet been studied. It is likewise not known whether a change in the type of inhibitor synthesized under plasmid control takes place.

The aim of this investigation was to identify mutation changes in genes of the fin-region of F-like plasmid pAP18-1 (Tc, Col V), which is a carrier of the transfer inhibition system of the Fin V group [5].

EXPERIMENTAL METHOD

We used previously identified mutant pAP18-1 drd of plasmid pAP18-1, derepressed with respect to transfer functions, and also its transposon-containing derivatives pAP18-1 drd::Tn 5 and pAP18-1 drd::Tn9 [5], which cannot inhibit transfer of standard plasmid Flac (phenotype Fin^-). In conjugation crosses of the bacteria we used standard strains *E. coli* K-12 with chromosomal markers of antibiotic resistance (AP115 Nal, AP106 Str, HB101 Str), containing or not containing the test plasmids. Conjugation transfer of plasmids, sensitivity of bacteria to pilus-specific phage NS2, and ability of the

Department of Biology and General Genetics, Patrice Lumumba Peoples' Friendship University.

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