Technical Report

Nucleofection, an Efficient Nonviral Method to Transfer Genes into Human Hematopoietic Stem and Progenitor Cells

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ABSTRACT

The targeted manipulation of the genetic program of single cells as well as of complete organisms has strongly enhanced our understanding of cellular and developmental processes and should also help to increase our knowledge of primary human stem cells, e.g., hematopoietic stem cells (HSCs), within the next few years. An essential requirement for such genetic approaches is the existence of a reliable and efficient method to introduce genetic elements into living cells. Retro- and lentiviral techniques are efficient in transducing primary human HSCs, but remain labor and time consuming and require special safety conditions, which do not exist in many laboratories. In our study, we have optimized the nucleofection technology, a modified electroporation strategy, to introduce plasmid DNA into freshly isolated human HSC-enriched CD34⁺ cells. Using enhanced green fluorescent protein (eGFP)-encoding plasmids, we obtained transfection efficiencies of approximately 80% and a mean survival rate of 50%. Performing functional assays using GFU-GEMM and long-term culture initiating cells (LTC-IC), we demonstrate that apart from a reduction in the survival rate the nucleofection method itself does not recognizably change the short- or long-term cell fate of primitive hematopoietic cells. Therefore, we conclude, the nucleofection method is a reliable and efficient method to manipulate primitive hematopoietic cells genetically.

INTRODUCTION

FOR MORE THAN 30 YEARS, hematopoietic stem cells (HSCs) have been successfully used in a various number of clinical applications (1). However, the current understanding about the molecular and cellular mechanisms governing the biology of these cells remains limited. In a variety of different organisms (e.g., *Drosophila* and *Caenorhabditis elegans*) or cellular systems, the understanding of different cellular processes was fundamentally improved by genetic approaches (2). In this regard over- or ectopic-expression studies, as well as inhibition of expression by RNA interference, revealed important results, sim-

ilar to naturally occurring mutations. Therefore, an effective method to manipulate human HSCs or hematopoietic progenitor cells (HPCs) genetically should help to increase the current understanding of the basic mechanisms governing the biology of human HSCs and HPCs. Additionally, an effective transfection method might help to improve HSC/HPC applications in clinical trials.

In principle, there are different strategies to manipulate human HSCs/HPCs genetically, i.e., techniques based on viral or nonviral gene transfer. Although viral strategies are highly efficient to transfer genes into primary cells (up to 90%) (3), they are time consuming and require special safety precautions to minimize the risk of

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exposure to biohazards (4), most nonviral techniques, e.g. electroporation, require a prestimulation of the corresponding cells and result in a maximal, regularly non-permanent transfection efficiency of 30% (5,6).

Recently, a new and highly efficient nonviral method called nucleofection technology was described. This modified electroporation strategy, which delivers the transfected nucleic acids directly into the nuclei of most cell types investigated so far, permits transient transfection of primary cells without any prestimulation (7–10). Indeed, we and others have successfully applied this technique to transfect freshly isolated human CD34⁺ cells and have obtained transfection efficiency rates of up to 80% when using green fluorescent protein (GFP)-encoding plasmids (11,12). Because the survival rate of transfected cells dropped significantly with decreasing cell numbers used in a single nucleofection reaction, we now have optimized the technology to transfect as little as 2×10^5 freshly isolated CD34⁺ cells.

For many experiments, especially those involving immature cell types, it is required that the cell fate of treated cells is not significantly altered by the transfection method itself. Therefore, we have also assessed whether the optimized nucleofection technology has any influence on the developmental potential of human HSCs/HPCs.

MATERIALS AND METHODS

Cell preparation

Umbilical cord blood (CB) samples were obtained from donors after informed consent according to the Declaration of Helsinki. Mononuclear cells were isolated from individual samples by Ficoll (Biocoll Separating Solution, Biochrom AG, Berlin Germany) density gradient centrifugation. Remaining red blood cells were lysed at 4°C in 0.83% ammonium chloride with 0.1% potassium hydrogen carbonate, followed by a phosphate-buffered saline (PBS) washing step. CD34⁺ cells were isolated by magnetic cell separation using the MidiMac technique according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany), yielding CD34⁺ cells of 80.2 ± 8.6 % purity ($n = 19$).

Transfection of CD34⁺ cells

The pEGFP-N1 vector (BD Clontech, Heidelberg, Germany) was amplified in *Escherichia coli* strain DH12S (Invitrogen GmbH, Karlsruhe, Germany) and purified using an Endofree-Plasmid-Maxi-preparation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

CD34⁺-enriched cells were divided into aliquots of equal size ($2\text{--}5 \times 10^5$ cells per aliquot) and were either not trans-

fected, transfected without DNA (mock), or transfected with 5 μ g of the vector pEGFP-N1 as described in the text. By rinsing the transfection cuvettes two times with 1 ml of I20 [Iscove's modified Dulbecco's medium (Invitrogen GmbH) supplemented with 20% fetal calf serum (FCS; Biochrom, Berlin, Germany)] transfected cells were transferred into a 15-ml plastic tube and washed in a total volume of 10 ml of I20. Pelleted cells were incubated for 15 min in a humidified atmosphere at 37°C and 5% CO₂, then they were resuspended in 1 ml of Myleocult H5100 (Stem Cell Technologies Inc, Vancouver, Canada) supplemented with early-acting cytokines (fetal liver tyrosine kinase-3 ligand [FLT3L], stem cell factor [SCF], thrombopoietin [TPO], each at 10 ng/ml final concentration [all PeproTech, Inc., Rocky Hill, NJ]). Cells were cultured at a density of $\approx 1 \times 10^5$ cells/ml, either in the presence or absence of the general caspase inhibitor Z-VAD-FMK (BD Biosciences, Heidelberg, Germany) in a humidified atmosphere at 37°C and 5% CO₂.

Flow cytometry and cell sorting

After 2 days, cultivated mock- and GFP-transfected cells, as well as nontransfected cells, were stained with an anti-CD34-PeCy5 antibody (clone 581; BD Pharmingen, Heidelberg, Germany). CD34⁺ cells of the controls and successfully transfected CD34⁺GFP⁺ cells were highly purified using a Coulter EPICS Elite ESP fluorescence cell-sorting system equipped with the Expo32 software (Beckman Coulter, Krefeld, Germany). For functional assays, defined numbers of appropriate cells were immediately sorted into corresponding media. Flow cytometric analyses were performed on a Cytomics FC 500 flowcytometer equipped with the RXP software (Beckman Coulter).

Functional assays

Primitive hematopoietic progenitors were assessed as long-term culture initiating cells (LTC-IC) as described before (13). Briefly, 5,000 cells were sorted into LTBMCM medium consisting of Iscove's modified Dulbecco medium (IMDM; Invitrogen GmbH) containing 12.5% horse serum, 12.5% FCS for human myeloid LTC (both Stem Cell Technologies Inc), 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (all Invitrogen GmbH). The cells were transferred into 96-well tissue culture plates (Costar, Corning Incorporated, New York) containing irradiated stroma cells of the murine fetal liver cell line AFT024 (14) in limiting dilutions (LDA; 22 replicates per concentration: 150, 50, 15, 5 cells/well). Half-medium exchanges were performed once a week. After 5 weeks of culture in a humidified atmosphere at 37°C and 5% CO₂, all medium was replaced by secondary clonogenic methylcellulose medium consisting of 1,12% methylcellulose (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in IMDM (Invitro-

gen GmbH) containing 30% FCS for human myeloid CFC (Stem Cell Technologies, Inc.), 2 U/ml erythropoietin (EPO; NeoRecormon; Roche Diagnostics GmbH, Mannheim, Germany), and 10% supernatant of the human bladder carcinoma cell line 5637 (13,15). Cells were cultured for another 2 weeks before individual wells were scored for the presence or absence of secondary colony-forming units (CFU). Using Poisson statistics and the weighted mean method, the frequencies of LTC-IC were calculated (15,16).

To perform CFU-GEMM assays, 250 cells were sorted into MethoCult GF H4434 containing recombinant human SCF, interleukin-3 (IL-3), granulocyte/macrophage colony-stimulating factor (GM-CSF), and EPO (Stem Cell Technologies Inc.). The cells were incubated in a humidified atmosphere at 37°C and 5% CO₂. Hematopoietic colonies were scored after 10–14 days as described before (13,15).

Stromal feeders for LTC-IC assays

The murine fetal liver cell line AFT024 was used as a stromal feeder layer to support hematopoietic growth in LTC-IC assays. The cell line was maintained at 33°C in Dulbecco's modified Eagle's medium (DMEM; Invitrogen GmbH) containing 20% FCS (Biochrom AG) and 160 μ M 2-mercaptoethanol (Invitrogen GmbH). The 96-well plates (Costar, Corning Incorporated) used for the LTC-IC assays were coated with 0.1% gelatin (Stem Cell Technologies, Inc.) and seeded with AFT024 cells. Cells were grown to confluence and irradiated with 2,000 rad. One day after irradiation, all medium was replaced by LTBM medium.

Immunofluorescence and microscopy

Analyses of living cells were performed at 37°C in a humidified, 5% CO₂ atmosphere using a DMIRB inverse fluorescence microscope (Leica, Bensheim, Germany). Pictures were taken with an AxioCam digital camera and processed using Axiovision Software (Carl Zeiss, Goettingen, Germany).

Statistics

Experimental results from different experiments were reported as mean $\pm$ standard deviation of the mean (SD). Significance analyses were performed with the paired Student's *t*-test.

RESULTS

Optimization of the nucleofection technology to transfect primitive hematopoietic cells

As described recently, we have successfully applied the nucleofection technology to transfect freshly isolated

CD34⁺ cells with different GFP-encoding plasmids (12). When following the manufacturer's instructions (especially using the nucleofection program U08) and using more than 5×10^5 CD34⁺-enriched cells, we obtained satisfying transfection and survival rates. However, when using less than 5×10^5 CD34⁺-enriched cells in a single transfection reaction, the survival rate decreased dramatically, and transfection buffer-derived floe-like structures arose within the concomitant cell culture. To overcome these difficulties when transfecting as little as 2×10^5 CD34⁺-enriched cells, we tested different nucleofection programs and obtained the best results using the nucleofection program U01. However, even a large proportion of the transfected cells were still viable at day 1 post transfection (p.t.), most of them died within two days p.t. (data not shown). In accordance with the original protocol, we initially cultured transfected CD34⁺ cells in the presence of the transfection buffer. When the transfection buffer was removed immediately after the transfection procedure, the survival rate of cultivated, transfected cells was obviously increased (data not shown).

Electroporation processes create minipores in cell membranes that selectively enable the passage of small ions, resulting in a colloidal osmotic swelling that kills cells (18,19). Because the postpulse pelleting procedure, i.e., the immediate precipitation and incubation of pelleted, transfected cells for 15 min at 37°C, was reported to suppress this swelling in human primary hematopoietic stem cells (19,20), we adapted this procedure to our protocol. Using our improved protocol and 5 μ g of Endo-free-prepared plasmid DNA (pEGFP-N1), we now obtain survival rates at day 2 p.t. of $52.9\% \pm 18.9\%$ ($n = 19$) with a mean transfection efficiency of $79.8\% \pm 14.1\%$ ($n = 19$). Upon usage of higher amounts of plasmid DNA (10 μ g), the viability of transfected cells dropped down (data not shown).

Effects of apoptosis inhibitor Z-VAD-FMK on cell survival

Recently, it was shown that the electrotransfection-induced DNA uptake resulted in the induction of large-scale apoptosis in CD34⁺ cells isolated from peripheral blood, and that this apoptotic effect was clearly reduced when caspase inhibitors, e.g., Z-VAD-FMK, were added to the postpulse culture media (20). To analyze whether inhibition of caspases also increase the survival rate of nucleofected CD34⁺ cells, we compared the survival rate of nontransfected, mock-transfected, and GFP-transfected CD34⁺ cells that were cultured in the presence or absence of Z-VAD-FMK (20 μ M or 120 μ M) for 2 days ($n = 6$). As depicted in Table 1, we did not find any significant impact of Z-VAD-FMK on the survival rate of GFP transfected CD34⁺ cells; a slight increase was observed when added at a final concentration of 120 μ M (Table 1).

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TABLE 1. MEAN SURVIVAL RATE OF CULTURED NONTRANSFECTED AND TRANSFECTED CELLS 2 DAYS POST TRANSFECTION

	Untreated	20 μ M Z-VAD-FMK	120 μ M Z-VAD-FMK
Control	84.5% $\pm$ 4.8%	76.3% $\pm$ 12.4%	78.9% $\pm$ 11.0%
		$p = 0.061$	$p = 0.171$
Mock	76.7% $\pm$ 10.5%	76.3% $\pm$ 10.7%	76.1% $\pm$ 6.2%
		$p = 0.246$	$p = 0.045$
GFP	49.5% $\pm$ 19.0%	52.8% $\pm$ 16.3%	60.4% $\pm$ 7.6%
		$p = 0.130$	$p = 0.044$

Content of CD34⁺ cells, 78.8% $\pm$ 11.6%; GFP transfection rate, 85.1% $\pm$ 5.0%; $n = 6$; p Values are given in relation to corresponding untreated cell fractions.

Effects of the nucleofection procedure on the cell growth of CD34⁺ cells

As we have described recently, upon cultivation CD34⁺ cells increase in size and acquire a dynamic, polarized cell morphology, forming a leading edge at the front and an uropod at the rear pole (12). To analyze whether the nucleofection procedure has any morphological effect on CD34⁺ cells, we compared GFP-transfected, mock-transfected, and nontransfected cells. We realized that most cultivated mock- or GFP-transfected CD34⁺ cells acquire a polarized cell shape similar to that of non-

transfected cells (Fig. 1). However, whereas nontransfected CD34⁺ cells reach their final size at culture day 2, transfected CD34⁺ cells seem to be delayed in their cell growth. This difference vanishes largely until day 3 post-transfection (Fig. 2A). It should be mentioned that we observed an additional reduction in cell growth upon treating transfected cells with Z-VAD-FMK (data not shown).

To analyze whether the transfection procedure has any obvious influence on the expression of stem cell-associated surface marker, we compared the content of CD34 and CD133 expression of nontransfected as well as of GFP-transfected cells over time. Under the con-

FIG. 1. Upon cultivation, nontransfected as well as transfected CD34⁺ cells acquire a polarized cell shape. (A) Nontransfected CD34⁺ cells. (B) Mock-transfected CD34⁺ cells. (C,C') GFP-transfected CD34⁺ cells. All depicted cells were derived from the same CD34⁺ preparation. Apart from the nucleofection reaction, cells were treated the same. Photos were taken at day 2 post transfection. GFP-transfected cells are clearly smaller than non- or mock-transfected cells (compare C to A or B, respectively). Note that most cells, even the GFP-transfected cells, acquired a polarized cell shape.

FIG. 2. Flow cytometric analysis of nontransfected and GFP-transfected cells. **(A)** Comparison of the size (FSC-height) and granularity (side scatter) of freshly isolated CD34⁺ (day 0) and GFP-transfected cells (GFP) versus nontransfected cells CD34⁺ cells at day 2, day 3, or day 9 past transfection. Upon cultivation, cultured cells increase in size and granularity. While cultivated control cells reach their final size after 2 days, the growth of GFP-transfected cells is delayed approximately 1 day. The “live” gate includes the living and excludes the dead cells. **(B)** Expression of CD34 and CD133 in GFP-transfected or nontransfected control cells that were cultured for 2 or 9 days in the presence of early-acting cytokines. Note that GFP-transfected cells express comparable levels of CD34 and CD133 expression than non-transfected cells. Only cells within the live gate (shown in **A**) are plotted.

ditions used, we observed similar dynamics of CD133 and CD34 expression in cultivated nontransfected or GFP-transfected CD34⁺ cells, respectively (Fig. 2B). Starting from culture day 3, CD133 and CD34 expression declines over time leaving approximately 20% CD133⁺CD34⁺ cells at day 9 p.t. (Fig. 2B). In this context, it is worth mentioning that although the strength of GFP expression decreases more than 20-fold, approximately 50% of the cultured offspring of originally GFP-transfected cells still express GFP after 9 days (Fig. 2B).

Colony-forming potential of GFP-nucleofected CD34⁺ cells

To identify potential effects of the nucleofection procedure on the colony-forming potential of CD34⁺ cells,

we performed CFU-GEMM assays of either transfected or nontransfected CD34⁺ cells. To allow transfected cells to recover from the transfection procedure and to express the *trans*-gene, they were cultured for 2 days in the presence of early-acting cytokines. Then, viable GFP⁺ CD34⁺ cells or CD34⁺ cells of the controls were purified by cell sorting and transferred in discrete numbers into the CFU-GEMM assay.

According to our data, GFP transfected CD34⁺ cells form fewer colonies than non- and mock-transfected control cells do. However, we did not find any significant alteration in the ratio of erythroid to myeloid to mixed colonies in our assays (Table 2). The addition of the caspase inhibitor Z-VAD-FMK to the cultures of the 2-day lasting recovery phase did not reveal any positive effect on the frequency of colony formation of transfected or nontransfected cells (data not shown).

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TABLE 2. MEAN COLONY FORMATION OF NONTRANSFECTED AND TRANSFECTED CELLS AND RATE OF WHITE TO RED TO MIXED COLONIES OF COLONIES FORMED

	<i>Survival rate</i>	<i>Colonies per 250 CD34⁺ cells</i>	<i>Rate of red colonies</i>	<i>Rate of white colonies</i>	<i>Rate of mixed colonies</i>
Control	82.3% ± 6.0%	119.6% ± 64.7%	18.2% ± 11.3%	62.8% ± 14.2%	19.0% ± 11.6%
Mock	74.2% ± 9.7%	108.6 ± 56.0	15.3% ± 12.1%	64.0% ± 16.9%	20.8% ± 12.8%
	<i>p</i> = 0.007	<i>p</i> = 0.331	<i>p</i> = 0.206	<i>p</i> = 0.440	<i>p</i> = 0.386
GFP	50.5% ± 17.5%	86.3 ± 76.2	16.0% ± 10.5%	60.3% ± 15.8%	23.7% ± 20.8%
	<i>p</i> < 0.001	<i>p</i> = 0.097	<i>p</i> = 0.288	<i>p</i> = 0.245	<i>p</i> = 0.177

Content of CD34⁺ cells, 78.8% ± 10.0%; GFP transfection rate, 79.6% ± 15.6%, *n* = 10, *p* values are given in relation to corresponding control cell fractions.

LTC-IC content of GFP-nucleofected CD34⁺ cells

To test if more primitive hematopoietic cells within the CD34⁺ cell fraction can be transfected without losing their primitive cell fate, we performed LTC-IC assays of successfully transfected CD34⁺ cells in comparison to CD34⁺ cells of the controls. Again, to allow the cells to recover from the transfection procedure, we cultured them for 2 days in the presence of early-acting cytokines, before, either in the presence or in the absence of Z-VAD-FMK.

According to our results, we detected a slight reduction in the LTC-IC frequency of transfected versus non-transfected cells (Table 3). A beneficial effect of the caspase inhibitor was not observed (data not shown).

DISCUSSION

In addition to their clinical relevance, primary human HSCs provide an attractive and challenging system to study certain biological processes, e.g., the process regulating the decision whether a stem cell is maintained as a stem cell or becomes committed to differentiate. To analyze such processes genetically, it is extremely helpful to have a reliable and highly efficient method to genetically manipulate these cells without altering their cell fate just by the method itself. As we and others have published recently, the nucleofection technology is very efficient method to transfect human HSC-enriched CD34⁺ cells transiently (11,12). Herein we report the optimization of the nucleofection protocol to transfect transiently as little as 2×10^5 freshly isolated, human HSC-enriched CD34⁺ cells and the effects of the transfection procedure on the short-term as well as on the long-term cell fate of these cells.

Using our optimized conditions and an enhanced (e) GFP-encoding plasmid, we obtained transfection efficiencies of 80.2% ± 8.6% (*n* = 19) and survival rates of

52.9% ± 18.9% at day 2 post transfection. This rate is higher than that reported of the manufacturers, in which precultured human CD34⁺ cells were transfected with an efficiency of ~70% and a mean survival rate of less than 40% at day 2 post transfection (11). Optimized classical electroporation procedures of human CD34⁺ cells result in transfection efficiencies of around 30% and survival rates up to 77%, but require a precultivation of transfected cells (5,6), which frequently alters the cell fate of primitive hematopoietic cells (21,22). In several studies, freshly isolated CD34⁺ cells were electroporated with only low transfection efficiencies (6.9% and 12%) (5,23). Comparable to classical electroporation procedures, transfection rates obtained with liposome-based technologies remain far below the efficiency obtained with the nucleofection technology (24).

Compared to nontransfected control cells (82.3% ± 6.0%; *n* = 10), the cell survival rate of mock-transfected cells is only slightly decreased (74.2% ± 9.7%; *n* = 10) whereas many of the GFP-transfected cells die within the first 48 h post transfection (survival rate, 50.0% ± 17.5%; *n* = 10). These results clearly demonstrate that the lethality observed in our experiments is more related to the presence of the DNA or to the strong GFP expression than to the nucleofection procedure itself. In-

TABLE 3. MEAN LTC-IC RATE OF NONTRANSFECTED AND TRANSFECTED CELLS

	<i>Survival rate</i>	<i>LTC-IC in surviving cells</i>
Control	79.7% ± 7.5%	5.1% ± 1.5%
Mock	74.9% ± 14.3%	4.9% ± 0.5%
	<i>p</i> = 0.183	<i>p</i> = 0.353
GFP	53.0% ± 23.8%	3.4% ± 1.3%
	<i>p</i> = 0.028	<i>p</i> = 0.059

Content of CD34⁺ cells, 79.1% ± 3.3%; GFP transfection rate, 66.7% ± 18.7%, *n* = 4, *p* Values are given in relation to corresponding control cell fractions.

deed the occurrence of DNA-induced cell death in electroporation experiments and the induction of apoptosis by high levels of GFP expression have been reported before (19,20,25–27).

Consistent with these observations, we realized a reduced colony-forming frequency of isolated GFP⁺CD34⁺ cells in CFU-GEMM and LTC-IC assays compared to that of isolated CD34⁺ cells of the mock-transfected or non-transfected controls (Tables 2 and 3). Because the ratio of erythroid to myeloid to mixed colonies formed by GFP⁺CD34⁺ cells was largely the same as that of the CD34⁺ control cells, we assume that the DNA and/or GFP-induced cell death is rather unspecific than specific for any of the CD34⁺ cell subtypes. In summary, these results suggest that DNA transfer into freshly isolated human CD34⁺ cells by the nucleofection procedure reduces the overall survival rate but has no major impact on the cell fate of surviving cells. Therefore, the nucleofection method is indeed a highly efficient and reliable method to manipulate primitive hematopoietic cells genetically.

Although we observed that approximately 50% of the offspring of the initially transfected cells still express low levels of GFP after 9 days, it was not our aim to analyze whether these cells were stably transfected or whether GFP-encoding plasmids remained transiently in those cells. As mentioned before, culture conditions have a major impact on the fate of HSCs and HPCs; under most stroma-free culture conditions, the majority of primitive cells become committed within the first few days of culture (21,22).

Thus, the nucleofection method is a useful technique to manipulate and dissect the genetic programs that regulate the maintenance or early commitment of HSCs or HPCs. As shown here, by performing CFU-GEMM or LTC-IC assays, it can easily be analyzed whether expression of certain genes modifies the colony-forming frequency or the ratio of erythroid to myeloid to mixed colonies in progenitor assays. As the morphology of cultivated transfected CD34⁺ cells is not recognizably altered in comparison to cultivated control cells, this method is also very applicable to study the subcellular distribution of introduced GFP-fusion proteins (12). According to the high transfection efficiency, the method might also be helpful for in vivo applications of transfected HSCs/HPCs in scientific or clinical approaches.

ACKNOWLEDGMENTS

The authors thank Dr. Kay Giesen and Dr. Oliver Gresch for initial experimental support and Daniel Moik for a critical reading of the manuscript. This work was funded by grants from the Deutsche Forschungsgemeinschaft (SPP1109 GI 336/1–2 to B.G. and P.W.) as well as from the Forschungskommission of the HHU-Düsseldorf.

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Received November 3, 2005; accepted February 2, 2006.