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Multiple Displacement Amplification Enables Large-Scale Clonal Analysis following Retroviral Gene Therapy▽

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Analysis of the fate of retrovirally transduced cells after transplantation is often hampered by the scarcity of available DNA. We evaluated a promising method for whole-genome amplification, called multiple displacement amplification (MDA), with respect to even and accurate representation of retrovirally transduced genomic DNA. We proved that MDA is a suitable method to subsequently quantify engraftment efficiencies by quantitative real-time PCR by analyzing retrovirally transduced DNA in a background of untransduced DNA and retroviral integrations found in primary material from a retroviral transplantation model. The portion of these retroviral integrations in the amplified samples was 1.02-fold (range 0.2, to 2.1-fold) the portion determined in the original genomic DNA. Integration site analysis by ligation-mediated PCR (LM-PCR) is essential for the detection of retroviral integrations. The combination of MDA and LM-PCR showed an increase in the sensitivity of integration site analysis, as a specific integration site could be detected in a background of untransduced DNA, while the transduced DNA made up only 0.001%. These results show for the first time that MDA enables large-scale sensitive detection and reliable quantification of retrovirally transduced human genomic DNA and therefore facilitates follow-up analysis in gene therapy studies even from the smallest amounts of starting material.

Limitations in molecular analysis are often set by the availability of only small amounts of genomic DNA. It is desirable to amplify the available DNA to a large extent and with good quality, thus conserving valuable DNA samples. In the past, several PCR-based methods have been developed to perform whole-genome amplification; among them are degenerate oligonucleotide-primed PCR (17) and primer extension preamplification (20). However, they proved to be relatively inefficient for genetic analysis. Large variation in the degree of amplification with incomplete coverage of loci and nonspecific amplification artifacts could be observed (19). The recently developed method called multiple displacement amplification (MDA) is a more promising approach to amplify the whole genome evenly across the target DNA (2). MDA is able to amplify highly uniform genomic DNA in an isothermal reaction using the ϕ29 DNA polymerase and random exonuclease-resistant primers (2). The resulting DNA products have an average length of more than 10 kb and accurately represent the original genomic DNA due to the efficient proofreading activity of the ϕ29 DNA polymerase (5). The easiness of the method and the small amounts of required input material of only about 10 ng make MDA an attractive approach for whole-genome amplification. Its usefulness for single-nucleotide polymorphism genotyping has already been demonstrated on a large scale (2, 5, 10, 18), but few attempts to evaluate in detail the performance of MDA in regard to balanced amplification have been made. The representation of different genomic loci has been examined using a TaqMan quantitative PCR assay (5), which showed a consistent representation of all genes of between 0.5- and 3.0-fold in amplified samples compared to the copy number in unamplified samples. However, information on retrovirally transduced DNA has been lacking.

Currently efforts are being made to optimize strategies for retrovirus-mediated gene therapy of hematopoietic stem cells, as this attempt has shown considerable preclinical and clinical progress in the past. However, cases of leukemia caused by insertional mutagenesis have been reported in mice (9), nonhuman primates (16), and human patients (3) and point to the need to further analyze retroviral integration patterns and clonal distribution to hematopoiesis (1) at early time points, when dominant clones are still at the beginning of the expansion process. Particularly with regard to future clinical monitoring of patients in gene therapy trials, considerable amounts of genomic DNA samples are needed for these analyses.

We analyzed genomic DNA samples obtained in vitro from a retrovirally transduced cell line and in vivo from a nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse transplantation model. In this model human retrovirally transduced peripheral blood progenitor cells can be studied after transplantation into immunodeficient mice and engraftment into the bone marrow (BM) (11, 12). For the first time, we evaluated the performance of MDA in the context of gene transfer studies with regard to its application before integration site analysis, determination of transduction efficiencies,

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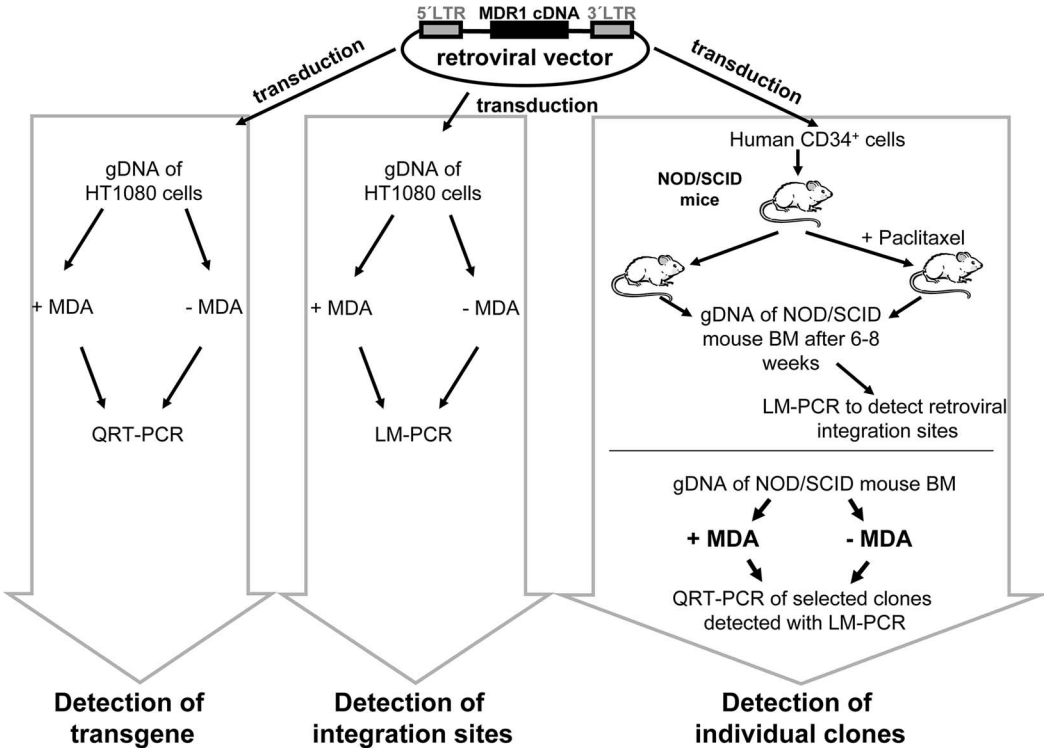


FIG. 1. Experimental design. LTR, long terminal repeat of retroviral vector; gDNA, genomic DNA; +MDA, with MDA; −MDA, without MDA.

and even clonal analysis of individual marrow-repopulating cells.

MATERIALS AND METHODS

Transduction of HT1080 cell line and selection of clones. The retroviral packaging cell line and the human fibrosarcoma cell line HT1080 were maintained as described before (14). Transduction of HT1080 cells with the Friend-mink cell focus-forming/murine embryonic stem cell virus-based retroviral vector SF1m containing multidrug resistance gene 1 (MDR1) was performed as previously described (14). Individual SF1m-transduced HT1080 cells were selected by single-cell deposition as described previously (7). The cell line clones 1 and 2, carrying up to three integration sites, and untransduced HT1080 cells were used here. DNA was prepared according to the QiaAmp protocol (Qiagen, Hilden, Germany).

Human xenografts in NOD/SCID transplantation model. Human $CD34^+$ cells were isolated, retrovirally transduced with the SF91m3 vector (4, 6), and transplanted into NOD/SCID mice (Fig. 1). One cohort was left untreated, and the other was treated with paclitaxel chemotherapy for 5 days (13). BM was collected from two untreated mice (E15M1 and E15M5) and from four chemotherapy-treated mice (E17M27, E17M28, E17M29, and E17M30) as described previously (8), and genomic DNA was prepared using the QiaAmp blood kit (Qiagen, Hilden, Germany).

Detection of retroviral integration sites. For detection of retroviral integration sites, ligation-mediated PCR (LM-PCR) was performed as described before (7). Four clones (E15M1K9, E15M1K11, E15M5K4, and E15M5K6) of the untreated mice (11) and six clones (E17M27K2, E17M27K7, E17M28K4, E17M29K1, E17M30K1, and E17M30K4) of the chemotherapy-treated mice (this paper) were measured by quantitative real-time PCR (QRT-PCR). By this means, a library of the different integration sites detected by LM-PCR and cloned into plasmid vectors was created. These plasmid vectors can be used as a reference standard curve to quantify the different retroviral integration sites in the original DNA samples as described below. Cells bearing a certain integration site make up one clone. The size of one clone is defined as the proportion of this individual clone (in percentage) in relation to all retrovirally transduced cells. Different clones can differ in their size.

Validation of amplification of retrovirally transduced cell line clones. Two dilution series were generated for analysis by either LM-PCR or QRT-PCR. For LM-PCR analysis, the SF1m-transduced cell line clone 1 was mixed with untransduced HT1080 carrier DNA to a total of 100 ng by decreasing the composition of the transduced DNA from 10% to 1%, 0.1%, 0.01%, and 0.001%. These samples and 100 ng untransduced HT1080 control DNA were amplified by MDA using the Repli-g kit (Qiagen, Hilden, Germany). The cells were cultured for several weeks after transduction prior to isolation of genomic DNA. Therefore, the possible existence of unintegrated viral DNA can be neglected. Purification of genomic DNA was performed with the Repli-g kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Subsequently, for MDA, 2.5 μg template DNA from each amplified sample was denatured for 3 minutes at room temperature with a denaturation solution containing KOH and EDTA. After neutralization, ϕ29 DNA polymerase and reaction buffer were added to a total reaction volume of 40 μl and incubated for 8 h at 30°C. Concentrations before and after MDA were determined by optical density measurement.

For QRT-PCR analysis, the proportions of cell line clone 2 transduced DNA in the dilution series were 50%, 25%, 10%, 1%, and 0.1% in a background of untransduced HT1080 DNA (e.g., 50% transduced DNA and 50% untransduced background DNA). DNA from each sample was amplified using the Repli-g kit.

QRT-PCR on amplified cell line clones and murine hematopoietic clones. Absolute quantitation of input DNA was done by generating a standard curve with serial dilutions of the plasmid-cloned target DNA and then comparing the signals obtained from samples with unknown amounts of vector or clone-specific DNA to the standard curve (ABI PRISM 7700 sequence detection system user's manual; PE Applied Biosystems). The concentration in each plasmid solution was determined as the mean of three A_{260} photometric measurements and converted to the number of copies using the molecular weight of the plasmid. We made serial dilutions of the plasmids in the range of 10^0 to 10^5. To minimize the amount of plasmid DNA absorbed to the wall of the tube, a constant amount of carrier DNA (salmon sperm DNA, 10 ng/μl) was added to the diluted plasmids. All real-time reactions were performed using new aliquots, to minimize degradation of DNA because of repeated freeze/thaw cycles. A dilution series was considered acceptable only if the correlation coefficient of the standard curve was above 0.98 and the threshold cycle values increased by approximately 3 for each

10-fold dilution, as recommended by Applied Biosystems (ABI PRISM 7700 sequence detection system user's manual; PE Applied Biosystems). The threshold cycle value is defined as the cycle number at which a significant increase in the fluorescence signal is first detected. The PCR efficiency was deduced from each standard curve by ABI Prism 7700 software and expressed as $10^{-1/s} - 1$, where s is the curve slope.

The total copy number of MDR1 transduced DNA and the absolute amount of human genomic DNA in the samples were assessed by generating reference standard curves by diluting plasmid DNA containing either the human erythropoietin receptor gene (hEpoR) (in pCR4-TOPO) as a housekeeping gene or the cDNA of MDR1 (in pUC18), respectively.

To assess the copy number of a specific clone, standard curves were constructed by diluting plasmids containing the LM-PCR product of this clone (11). The mean PCR efficiency for all 10 clones was 88.37% ± 5.05% (range, 81.04% to 97.73%); the deviation of about 10% remains in the scope of the standard errors of the means. Thus, the PCR efficiencies allow a direct comparison of the copy numbers. Reduction of the PCR efficiency due to nonspecific retroviral common primer annealing was excluded using a clone-specific reverse primer in combination with a common retroviral specific forward primer and probe (see "Primers and probes" below).

Primers and probes. All primers and TaqMan 5′-6-carboxyfluorescein/3′-6-carboxytetramethylrhodamine probes were designed using Primer Express software version 1.5 (PE Applied Biosystems). The following primer-probe sets were used as described before (12, 14): MDR1 primers, 5′-CAGGTGTGCTCGGAGCCA-3′ and 5′-ACAGTGGTCCAGCTCCTGGAG-3′; MDR1 probe, 5′-CCATCAAGCAGCACTTTCCCTGCCA-3′; hEpoR primers, 5′-CTGCTGCCAGCTTTGAGTACACTA-3′ and 5′-GAGATGCCAGAGTCAGATACCACAA-3′; hEpoR probe, 5′-ACCCCAGCTCCCAGCTCTTGCGT-3′.

Ten unique reverse primers binding to the flanking human DNA of the different LM-PCR products were designed to quantify the clones detected by LM-PCR, whereas the retrovirus-specific primer (SF-LTR forward primer, 5′-TGATCTGAACTTCTCTATTCTTGGTTTG-3′) and fluorogenic probe (SF-LTR probe, 5′-CATGCCTTGCAAAATGGCGTTACTGC-3′) were identical for all integration sites, as described before (11).

QRT-PCR was performed on an ABI Prism 7700 sequence detection system instrument (PE Applied Biosystems) as described before (11). The reactions were performed two times in triplicates.

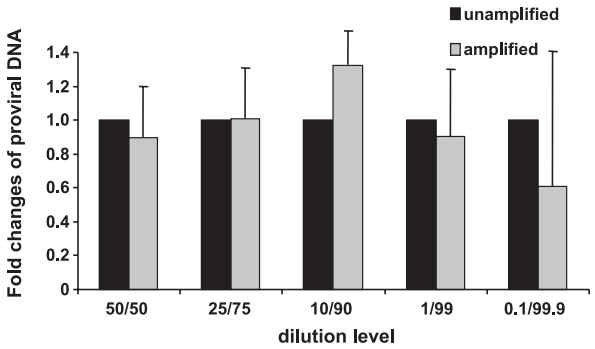
To measure the specificity of each clone-specific PCR primer for amplifying only the appropriate clone sequence, we performed PCRs with the corresponding plasmid and control plasmid. Only if those reactions containing the primer designed for a given clone were positive and those with primers designed for other clones were negative were these unique primers considered for further application, which enables the high specificity of the QRT-PCR and eliminates false positives. Negative controls were mock mouse DNA (untransduced), serving as a "no-amplification" control, and water, serving as a "no-template" control.

Statistical analysis. Data were analyzed using SPSS software (SPSS Inc., Chicago, IL).

RESULTS

As the amount of genomic DNA available for analysis is often limited in current gene therapy trials, our aim was to show the feasibility of MDA as a preamplification method in various settings. We amplified genomic DNA obtained from retrovirally transduced HT1080 cell line clones and from a NOD/SCID mouse model, in which the immunodeficient mice were transplanted with retrovirally transduced human $CD34^+$ cells. We analyzed the amplified DNA in terms of detection of the transgene MDR1 by QRT-PCR and identification of retroviral integration sites by LM-PCR. In addition, individual hematopoietic clones in these mice bearing a unique integration site were quantified by QRT-PCR, and the sizes in amplified and unamplified samples were compared (Fig. 1).

Detection of the MDR1 transgene. At first, we compared the portion of retrovirally transduced DNA in a background of untransduced DNA before and after amplification by MDA technology (Fig. 1). DNA of each sample of the dilution series, which included 50%, 25%, 10%, 1%, and 0.1% of the HT1080 cell line clone 2 transduced DNA in a background of untransduced HT1080 DNA, was amplified. Subsequently, QRT-PCR was performed on unamplified and amplified DNA, and these were compared. The copy number of the MDR1 transgene was used to determine the amount of transduced cells and the copy number of the housekeeping gene hEpoR was used to determine the amount of all cells. The ratio of the mean copy number of the MDR1 transgene and the mean copy number of the endogenous hEpoR gene gives an estimate of the contribution of the transduced cells in the sample (Fig. 2). When comparing the percentages of MDR1-transduced cells in the dilution series without and with amplification, there was no significant difference between the two groups. The Spearman rank order correlation is 1.000, which implies a significant correlation ($\alpha = 0.01$). Furthermore, regression lines were generated for every quantification reaction, and amplified and unamplified values were compared. As calculated by regression analysis, the slopes of the lines for the amplified (0.438) and unamplified (0.471) samples were not significantly different (data not shown). The average portion of the whole retroviral DNA in the amplified samples was 0.9-fold of the portion determined in the original unamplified genomic DNA. These findings imply that retrovirally transduced DNA was amplified in the same way as the host genomic DNA and that transduced DNA can be detected after application of MDA even at the smallest dilution step. In all dilutions, even in the samples with



unamplified	45.97% +/- 14.61%	18.37% +/- 2.24%	5.90% +/- 0.51%	0.50% +/- 0.02%	0.05% +/- 0.01%
amplified	41.23% +/- 16.80%	18.53% +/- 4.87%	7.82% +/- 0.99%	0.45% +/- 0.19%	0.03% +/- 0.04%

FIG. 2. Proportion of MDR1 transgene for comparison of fold changes of amplified to unamplified retrovirally transduced DNA. The ratio of proviral MDR1 to the endogenous hEpoR gene was determined in unamplified and amplified genomic DNA by QRT-PCR. The hEpoR gene was used to measure the total amount of DNA in the samples of the dilution series. Each gray bar represents the relative proportion of MDR1 in amplified DNA in comparison to the unamplified DNA template (black bars). This value was normalized to 1. The percentages of proviral MDR1 in relation to the hEpoR gene are tabulated for the same unamplified and amplified genomic DNA samples. For dilution series, transduced genomic DNA of cell line clone 2 was mixed with different amounts of untransduced HT1080 DNA from 50% to 0.1% (e.g., 25/75 means 25% transduced DNA and 75% untransduced background DNA), followed by amplification using the Repli-g kit. For all reactions, water served as a "no-template" control and untransduced HT1080 DNA as a "no-amplification" control. Error bars indicate standard deviations.

the highest dilution and DNA of only 0.1% transduced cells, the MDR1/hEpoR copy numbers ratios were similar for unamplified and amplified DNA (Fig. 2). They correctly reflected the actual DNA dilution, which is equivalent to the amount of transduced cells. For example, in the 50/50 dilution, where we expected 50% transduced cells, we estimated 45.97% ± 14.61% transduced cells without MDA (unamplified) and 41.23% ± 16.8% transduced cells with MDA (amplified); with the highest dilution of 0.1/99.9, where we expected only 0.1% transduced cells, we estimated 0.05% ± 0.01% transduced cells without MDA and 0.03% ± 0.04% transduced cells with MDA. This proves that determination of the copy number of MDR1 with or without MDA can indeed be used for estimation of the proportion of transduced cells. Therefore, we are now able to determine transduction efficiencies in gene transfer models after preamplification of low genomic DNA amounts.

Detection of retroviral integration sites after MDA. LM-PCR is a method for the detection of retroviral integration sites, in other words, the genomic DNA flanking the provirus. It enables analysis of integration patterns, which is important for safety studies of retroviral gene therapy. However, in every single LM-PCR only a subset of all existing integrations in one sample can be found, as described before by Nagy et al. (11). This indicates the necessity of repeated LM-PCRs in order to obtain several dozen integrations per DNA sample. This multiplicity of PCRs is often strongly limited by the small amount of DNA, especially for clinical samples. Therefore, to allow application of MDA as a preamplification method before integration site analysis, we first validated the LM-PCR method after preamplification of a transduced cell line clone (Fig. 1). We mixed clone 1 in different proportions (10%, 1%, 0.1%, 0.01%, and 0.001%) in a total of 100 ng with untransduced HT1080 background DNA to test the ability to detect this clone after amplification in a complex sample and to specify the sensitivity of the LM-PCR method. We were able to detect even 0.001% transduced DNA in a background of 100 ng untransduced DNA (Fig. 3). However, if LM-PCR was performed using the same dilution steps but without MDA, the result was that no bands were detected (results not shown). To verify that the band of the same size in all three dilutions actually corresponds to the same clone (Fig. 3, EB), we sequenced the LM-PCR product. In all dilution steps we found the same specific integration site. The second band (Fig. 3, IB) corresponds to the internal band which originates from the 3′ long terminal repeat serving as a general control in LM-PCRs.

Further bands in lane with 0.1% clone 1 are a result of a further integration site, which was proven by subcloning and subsequent sequencing, since clone 1 contains more than one integration site. The weak external band in the lane with 0.01% and the absence of the internal band in the lane with 0.001% can be explained by specific characteristics of the LM-PCR method. The enrichment of fragments using paramagnetic beads is not equally efficient in different reactions. Therefore, the subsequent LM-PCR, which is not a quantitative reaction, does not result in proportional amplifications. To obtain quantitative results for certain clones identified by LM-PCR, subsequent QRT-PCR is necessary. Furthermore, previous experiments even showed that the number of bands visible in the LM-PCR underestimates the actual number of integration sites (11).

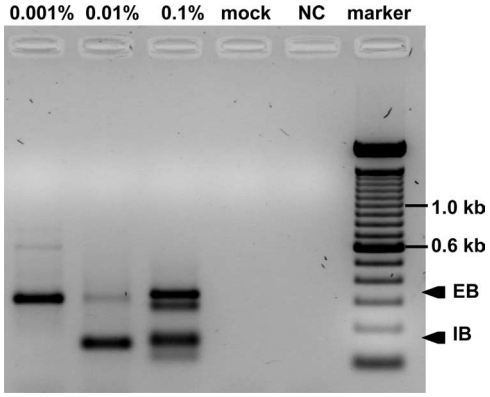


FIG. 3. Detection of highly diluted transduced DNA after amplification followed by LM-PCR. The upper arrow indicates the specific integration site (external band [EB]; cell line clone 1). Mock, LM-PCR of untransduced DNA; NC (negative control), LM-PCR performed with water as template. The internal band (IB) originates from the 3′ long terminal repeat serving as a general control in LM-PCRs, which is the same for all SF1m vector-transduced cells. The disproportion between dilution factor and band intensity is not due to insufficient MDA but must be explained by specific characteristics of the LM-PCR method.

Improvement of sensitivity of integration analyses by MDA. After validating MDA as an amplification step prior to LM-PCR for integration site analyses in transduced cell lines, we wanted also to establish this method for DNA samples of primary cells. For this reason, for MDA we used 100 ng of genomic DNA of BM cells of a NOD/SCID mouse which received human hematopoietic stem cells transduced with the MDR1-containing virus SF1m. From the amplified material, three LM-PCRs were set up with 2.5 μg starting material each, in comparison with one LM-PCR with 2.5 μg of untransduced material. The results of all reactions are shown in Fig. 4A and B. With the LM-PCR of the unamplified DNA, five distinct bands can clearly be detected. In contrast to this, in the three LM-PCRs with amplified material, at least 10 additional bands are visible. Excluding the two bands which are only seen in lane 1, eight additional bands could now be used for subcloning and sequencing in order to identify the specific integration site. This experiment obviously demonstrated the advantage of performing MDA prior to LM-PCR to obtain amplification of the genomic DNA. Thus, multiple LM-PCRs are possible, resulting in detection of more integration sites and therefore rendering the integration site analyses more sensitive.

Quantification of individual clones. In the NOD/SCID mouse model we used LM-PCR for the detection of retroviral integrations. In a second step, we quantified different clones, each with one specific integration found by LM-PCR before. For this quantification we evaluated the feasibility of the MDA as a method to amplify the genomic DNA in order to get enough material for the quantification process.

Therefore, in this experiment, individual transduced clones contributing to hematopoiesis in the NOD/SCID mouse model were quantified by QRT-PCR with amplified and unamplified DNA in order to evaluate the possibility of applying MDA to transduced genomic DNA and subsequently quantifying spe-

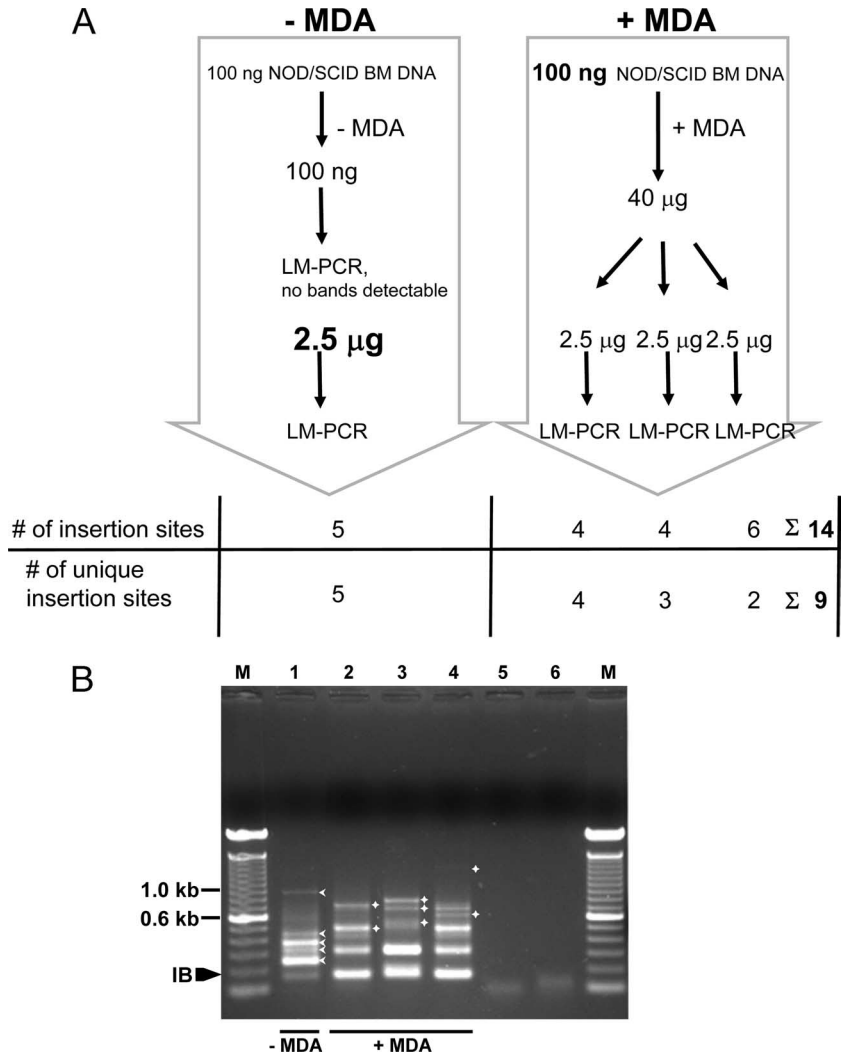


FIG. 4. Improvement of sensitivity of integration analyses by MDA in the NOD/SCID mouse. (A) As shown in the scheme, 100 ng of genomic DNA of BM cells from a NOD/SCID mouse was used for MDA (right side) followed by three LM-PCRs. If the LM-PCR were performed by using 100 ng, no integration sites would be detectable, since this amount is not sufficient for performing LM-PCR with the NOD/SCID mouse BM samples used. Therefore, 2.5 μg starting material in the reaction without MDA was compared with 100 ng starting material in the reaction with MDA. The table shows the numbers of insertion sites and unique insertion sites without MDA and with MDA. (B) LM-PCR was performed with 2.5 μg of unamplified material (lane 1) and 2.5 μg of amplified material (starting material used for MDA, 100 ng) (three different reactions in lanes 2 to 4). The five distinct insertion sites of the LM-PCR detected in the unamplified material are marked by arrowheads, and the additional insertion sites after LM-PCR with amplified material are marked by diamonds (only once per band even if can be detected in different lanes). The internal band (IB) is indicated. In lanes 5 and 6 controls were loaded with water as template. The diffuse bands seen here are the primers. The data demonstrate that a multiplicity of LM-PCRs is superior to only one LM-PCR in terms of integration sites found.

cific genomic sections (Fig. 5). We used 10 clones detected by LM-PCR in BM of six mice and identified their proportions of all transduced cells in the appropriate mouse. Unique primers binding to the flanking human genomic DNA were designed, whereas the other primer and the fluorogenic probe were complementary to sequences of the long terminal repeat region of the retroviral vector and were therefore applicable for all clones. As shown above (Fig. 2), determination of the copy number of the MDR1 transgene is a method to estimate the number of all transduced cells. The individual transgenic clones analyzed contributed 0.06% to 7.51% of total human retrovirally transduced hematopoiesis, when quantified in the original unamplified samples. In the amplified genomic DNA, the sizes of the same clones ranged from 0.05% to 3.81%. There is no statistically relevant difference between the groups of clones measured in the unamplified or amplified samples (Fig. 5), with a mean value in the first group of 1.45% and a mean value in the second group of 1.19%. The Spearman rank order correlation is 0.927, which means that the correlation between the two groups is clearly significant ($\alpha = 0.01$). The 10 clones were represented in the amplified DNA at between 0.2- and 2.1-fold of the copy number in the original genomic DNA; the average representation was 1.02-fold. In total, when combining the results of this quantification of individual clones and the quantification of the MDR1 transgene in the dilution series, the average representation of retroviral DNA is 0.9-fold.

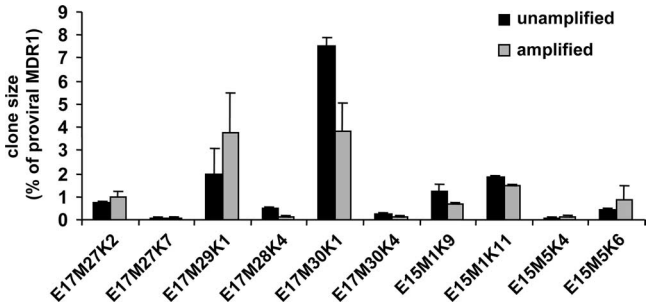


FIG. 5. Individual clone sizes of NOD/SCID repopulating retrovirally transduced $CD34^+$ mobilized peripheral blood progenitor cells quantified in unamplified and amplified genomic DNA. Clone sizes relate to the amount of transduced cells in the samples, as measured by the proviral MDR1 cDNA. The error bars, representing standard deviations, were generated from the mean values from two QRT-PCRs performed in triplicate.

Differences between the clone sizes in the chemotherapy-treated mice and the non-chemotherapy-treated mice can be found, as the mean value in the unamplified samples in the first group is 1.8% and that in the latter is 0.9%. In amplified samples the mean value in the group of chemotherapy-treated mice is 1.5% and that in the group of non-chemotherapy-treated mice is 0.8%. However, no conclusions can be drawn from these results concerning the clone sizes in mice with or without chemotherapy treatment because of the small number of quantified clones. In contrast, the values in unamplified and amplified samples are highly comparable.

DISCUSSION

The amount of genomic DNA in gene transfer studies is the most limiting factor for intensive insertional mutagenesis analysis. Preamplification of transduced genomic DNA would override this restriction. Here, we demonstrate for the first time that MDA is a suitable method for the amplification of genomic DNA obtained from gene transfer studies, allowing subsequent exhaustive insertional mutagenesis analysis. MDA is a robust, fast, and efficient method to amplify genomic DNA approximately 10,000-fold irrespective of the amount of template DNA (2). The amplification may even be performed directly from clinical samples, eliminating prior purification steps (5). However, the level of uniformity and correctness of the amplification are the important critical factors for the applicability of MDA for most molecular analyses. Here, we investigated these points for the first time in the context of retrovirally modified genomic DNA from different host cells. Our data show that transduced DNA is not amplified differently than the host genomic DNA.

For the understanding of retroviral integration patterns, especially in cases of insertional mutagenesis, several methods for the detection of integration sites are currently used. We performed LM-PCR on amplified retrovirally transduced cell line clones and were able to detect the specific clone in a large excess background of untransduced DNA. LM-PCR can detect retroviral integrations with high sensitivity and specificity but requires at least 100 ng (15) or 2.5 μg (7) genomic DNA. The difference in the required DNA amounts is explained by the different numbers of PCR cycles: Schmidt et al. (15) used 100 cycles with 100 ng, and Laufs et al. (7) used only 35 cycles with 2.5 μg.

For the combination of MDA and LM-PCR we used only 100 ng of genomic DNA to start; according to the manufacturer's instructions (Qiagen), a minimum of only 10 ng genomic DNA is required for the amplification reaction. It is possible to further reduce the amount of starting material, but it is questionable if in this case all possible integrations would be still available in the sample.

However, due to the large output of the MDA reaction, we are now able to increase the amount of genomic DNA up to 20 μg for one LM-PCR and by this means augment the sensitivity of integration analysis. Without putting an amplification step ahead of the LM-PCR it was not possible to start with less than 100 ng DNA per reaction. Schmidt et al., who used the so-called extension primer tag selection/LM-PCR method in order to increase the sensitivity of integration site analysis, were able to detect 1 ng of monoclonal DNA in a background of 100 ng DNA (15). We were now able to detect, in a background of 100 ng untransduced DNA, even 0.001% (1 pg) of retrovirally transduced DNA with LM-PCR after MDA. This represents a drastic increase in sensitivity for integration site analysis by combining MDA and LM-PCR.

Furthermore, not all integration sites will be detected with a single LM-PCR. This has been shown before by Nagy et al. (11), who could detect several different integration site sequences in six LM-PCRs with the same starting material. The advantage of MDA in this context could now clearly be demonstrated by processing three parallel LM-PCRs with amplified material in comparison to an LM-PCR with unamplified DNA. These results show that several LM-PCRs are absolutely necessary for detection of multiple integration sites. For this reason, a large amount of starting material is required. MDA provides this and by this means allows extensive integration site analysis by multiple LM-PCRs. The combination of MDA and LM-PCR has already been applied to primary material by our group with great success, as integration sites could not be identified before by using only LM-PCR. In this clinical trial, a patient with transformed large-cell lymphoma was transplanted with retrovirally transduced hematopoietic progenitors and BM samples were analyzed by LM-PCR after amplification with MDA (P. S. Becker, Y. P. Yu, A. M. Ceredona, W. V. Walsh, J. L. O'Donnell, C. Baum, L. Kallander, P. A. Lowry, T. J. Fitzgerald, P. Westervelt, and M. Stewart, presented at the American Society of Hematology, 2004).

Transduction efficiencies can be easily determined in quantitative PCR assays (12). As these reactions consume a great deal of precious sample DNA, it is worthwhile to put an amplification step ahead that reduces the required input material. We could detect retrovirally transduced DNA in a dilution series with untransduced carrier DNA and additionally found no significant difference in the percentages in amplified and unamplified DNA (Fig. 2). Hosono et al. compared copy numbers of different genomic loci in amplified and unamplified DNA samples (5). They showed that genomic DNA was evenly amplified with complete coverage and consistent representation of all genes. They further proved that amplification of genomic DNA directly from cells is highly reproducible. Based on these results, we went a step further and determined the

proportion of a retrovirally transduced DNA as a specific genomic section which is not present in all cells in the sample. We measured the proportions of retrovirally transduced DNA mixed at different dilutions with untransduced background DNA. We could clearly show for all tested dilutions that the proportions with and without amplification were equal. This situation is more relevant for gene transfer studies, where the proportion of transduced DNA is often very small compared to the unmodified DNA, which does not carry the transgene. Additionally, we performed a purification step after the amplification reaction using the DNAMicroKit (Qiagen). We did not find any difference or even improvement (data not shown), so no further reaction cleanup for downstream application after MDA is needed.

Following infection, the viral DNA becomes integrated into the host cell genome. After integration, the provirus acts as a transcription template for the efficient synthesis of the gene(s) carried by the retroviral vector. Using the MDA technique invented by Hosono et al. (5), we showed for the first time that proviral DNA sequences were amplified proportionally to human host cell DNA sequences. Additionally, we could even apply this method for amplification of proviral sequences which made up only 0.001% of total genomic DNA.

The quantification of individual BM-repopulating cells is an attractive technique for the study of hematopoiesis in mice, canines, and nonhuman primates and for monitoring of patients after retroviral gene transfer. Retrovirally marked cells can be quantified after the detection of the specific integration site and put in relation to other cells with high sensitivity and specificity using QRT-PCR (11). However, this method requires large amounts of genomic DNA, and therefore large sample volumes are needed. This is not feasible in gene transfer studies, and therefore this important analysis has not yet been performed on a large scale. We were able to show in this study that MDA can be applied prior to clonal analysis, as the representation of clones in transduced genomic DNA samples is not changed by the whole-genome amplification. We quantified 10 clones detected by LM-PCR, which contributed to hematopoiesis in a NOD/SCID mouse model. The proportion of these clones with reference to all transduced cells ranged from 0.06% to 7.51%, which is a representative selection of regular clones in gene transfer models. The 10 clones were represented in the amplified DNA at between 0.2- and 2.1-fold of the copy number in the original genomic DNA. This is in line the findings of Hosono et al., who found a difference of 0.5- to 3.0-fold when comparing different genomic loci in amplified and unamplified DNA samples (5). However, we went a step further, as we have shown that retroviral integrations are amplified in the same way and at the same probability as all other parts of the genome. This proves that MDA can be used prior to clonal analysis in gene transfer studies and makes it possible to screen and monitor patients in future clinical gene therapy studies. Retrovirally marked cells can be tracked over time due to the specific integration site, and in this way hematopoiesis can be analyzed. A quantitative assessment of hematopoietic progenitors is possible after MDA, and therefore a deeper comprehension of this process will be obtained.

In summary, we describe here complete new applications of the MDA method in combination with LM-PCR for integration site analyses. It is not unusual that only some 100 ng of genomic DNA from clinical samples is provided, limiting integration and clonal analyses. Since amplification by MDA of such a small amount of starting material results in up to several micrograms of DNA, this bottleneck in integration analyses can be circumvented. Thus, our results show significant advances in the analysis of insertional mutagenesis at different time points using the smallest amounts of starting materials from human gene therapy trials.

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