Cytometry Part A 61A:26–34 (2004)

Whole Genome Amplification for CGH Analysis: Linker-Adapter PCR as the Method of Choice for Difficult and Limited Samples

Christine Pirker,[1] Maria Raidl,[2] Elisabeth Steiner,[1] Leonilla Elbling,[1] Klaus Holzmann,[1] Sabine Spiegl-Kreinecker,[3] Michaela Aubele,[4] Bettina Grasl-Kraupp,[2] Christine Marosi,[5] Michael Micksche,[1] and Walter Berger[1]*

[1]Division of Applied & Experimental Oncology, Institute of Cancer Research, Vienna University, Vienna, Austria
[2]Division of Toxicology, Institute of Cancer Research, Vienna University, Vienna, Austria
[3]Department of Neurosurgery, Landesnervenklinik Wagner-Jauregg Hospital, Linz, Austria
[4]GSF-National Research Center for Environment and Health, Institute of Pathology, Neuherberg, Germany
[5]Department of Medicine I, University Hospital Vienna, Vienna, Austria

Received 17 November 2003; Revision Received 17 March 2004; Accepted 19 March 2004

Background: Comparative genomic hybridization (CGH) is a powerful method to investigate chromosomal imbalances in tumor cells. However, DNA quantity and quality can be limiting factors for successful CGH analysis. The aim of this study was to investigate the applicability of degenerate oligonucleotide-primed PCR (DOP-PCR) and a recently developed linker-adapter-mediated PCR (LA-PCR) for whole genome amplification for use in CGH, especially for difficult source material.

Methods: We comparatively analyzed DNA of variable quality derived from different cell/tissue types. Additionally, dilution experiments down to the DNA content of a single cell were performed. FISH and/or classical cytogenetic analyses were used as controls.

Results: In the case of high quality DNA samples, both methods were equally suitable for CGH. When analyzing very small amounts of these DNA samples (equivalent to one or a few human diploid cells), DOP-PCR-CGH, but not LA-PCR-CGH, frequently produced false-positive signals (e.g., gains in 1p and 16p, and losses in chromosome 4q). In case of formalin-fixed paraffin-embedded tissues, success rates by LA-PCR-CGH were significantly higher as compared to DOP-PCR-CGH. DNA of minor quality frequently could be analyzed correctly by LA-PCR-CGH, but was prone to give false-positive and/or false-negative results by DOP-PCR-CGH.

Conclusions: LA-PCR is superior to DOP-PCR for amplification of DNA for CGH analysis, especially in the case of very limited or partly degraded source material. © 2004 Wiley-Liss, Inc.

Key terms: comparative genomic hybridization; CGH; degenerate oligonucleotide-primed PCR; DOP-PCR; linker-adapter-mediated PCR; whole genome amplification; paraffin-embedded tissue; degraded DNA

Besides mutations, under- and overrepresentations of tumor-suppressor genes and oncogenes (1), respectively, due to loss or amplification of chromosomal material, might be of decisive importance and a driving force for malignant progression. DNA amplification, especially, is a key mechanism that allows cancer cells to increase expression of critical genes responsible for cell growth and cell cycle progression (2). Comparative genomic hybridization (CGH), first described in 1992 (3), is a powerful technique that enables the detection of imbalanced genomic alterations throughout the genome. CGH experiments require 0.2–2.0 μg of labeled tumor and reference DNA for successful hybridization (4). Therefore, when only small amounts of sample DNA are available, whole genome amplification prior to labeling is required. Especially when CGH is performed in a routine setting, it might be necessary to investigate DNA samples of differing quality and purity, prepared from different institutions, by different methods, from different tissues, which all leads to high demands on reliability and reproducibility of the DNA amplification method.

Contract grant sponsor: Oberösterreichische Krebshilfe; Contract grant sponsor: Jubiläumsfonds der Österreichischen Nationalbank; Contract grant number: 7258.

*Correspondence to: Walter Berger, Institute of Cancer Research, Div. Applied & Experimental Oncology, Vienna University, Borschkegasse 8a; A-1090 Vienna.
E-mail: walter.berger@univie.ac.at

Published online 29 June 2004 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cyto.a.20060

Degenerate oligonucleotide-primed PCR (DOP-PCR), which was first described in 1992 (4), is currently the most widely used method to amplify whole genome DNA for CGH analysis. DOP-PCR is based on the use of a partially degenerated oligonucleotide primer and PCR amplification under increasingly stringent annealing conditions. Due to technical problems with regard to application for CGH analysis, this method has been frequently modified (5). DNA derived from paraffin-embedded tissues, especially, often causes problems in CGH analysis. This is partly due to DNA degradation per se, but also because the procedures of formalin-fixation and paraffin-embedding might interfere with the PCR reaction. Inhibition of the PCR reaction has also been observed for DNA containing melanin (6,7), a problem that can be overcome by adding bovine serum albumin to the reaction mix. More recently, an optimized strategy for global DNA amplification based on a linker-adapter-mediated PCR method (LA-PCR, also termed SCOMP) has been developed and validated for CGH analysis (8,9). In this method, DNA is first digested by the restriction enzyme MseI, resulting in a fragment size range required for successful CGH. Following digestion, DNA is amplified and labeled via a linker-adapter-PCR, using only a single primer.

In this study, we compared the applicability and quality of DOP-PCR and LA-PCR to amplify whole genome DNA for CGH analyses of difficult, i.e., partly degraded or very limited, DNA samples derived from different sources and prepared by different methods. In summary, we demonstrate that both DOP-PCR and LA-PCR are applicable for DNA amplification with respect to CGH analysis; however, we also demonstrate that the latter method is less prone to give false results in case of very low amounts or minor quality of the DNA samples that are to be analyzed.

MATERIALS AND METHODS

Cell Cultures

Primary cell cultures were established from 48 histopathologically-confirmed human melanomas (10), three glioblastomas (11), and four soft tissue sarcomas. The SK-Mel-28, Calu-6, and HepG2 cell lines were obtained from the American Type Culture Collection (Rockville, MD), and the F2000 embryonic fibroblasts were obtained from Flow, Scotland. All primary cell cultures were established and grown in RPMI 1640 culture medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and glutamine (Sigma). Cell cultures were frequently checked for *Mycoplasma* contamination.

Paraffin-Embedded and Frozen Material

Samples were obtained from snap-frozen or from formalin-fixed, paraffin-embedded pathology specimens, prepared from resections done at the General Hospital of Vienna and the Landesnervenklinik Wagner-Jauregg Hospital, Linz. Frozen samples were stored in liquid nitrogen. Paraffin-embedded samples were archived at the Departments of Pathology and Dermatology, University Hospital of Vienna, as well as at Wagner-Jauregg Hospital.

Genomic DNA Isolation Procedures

DNA from all cell cultures and blood samples, except that from soft tissue sarcomas, was isolated with the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, and stored at –20°C. When comparatively tested against several other kits and methods, in our hands this kit gave the most reliable high-quality DNA extraction results from cultured cells (data not shown). From soft tissue sarcoma primary cell cultures, DNA was extracted using a standard phenol-chloroform-isoamylalcohol method. Briefly, 500 μl lysis buffer (50 mmol Tris, 10 mmol EDTA, 0.5% Na-lauryl-sarcosine) were added to 1.0–1.5 ml of a single-cell suspension. Incubation with 100 μg proteinase K (Sigma) was done at 37–56°C overnight. Then DNA was extracted twice by phenol-chloroform-isoamylalcohol, precipitated by sodium acetate/ethanol, dissolved in aqua bidest (Fresenius Kabi, Graz, Austria), and stored at –20°C. DNA from frozen material was isolated after histopathological examination from ≈50 mg of unstained tissue using the Aqua Pure Genomic Tissue Kit (BioRad, Munich, Germany) according to the manufacturer's instructions. In case of two soft tissue sarcomas, DNA was extracted by phenol-chloroform-isoamylalcohol, as described above. For DNA preparation from paraffin-embedded material of hepatocellular carcinomas and melanomas, three to five sequential sections were mounted onto separate microscope slides. The first one and the last one were stained with hematoxylin and eosin (H/E); the other sections remained unstained. Unstained sections were deparaffinized, and areas of interest were microdissected either with a sterile 20-gauge needle under a microscope or by laser-capture microdissection (PixCell II; Arcturus GmbH, Moerfelden-Walldorf, Germany). In some cases, unstained sections were stained very shortly with hematoxylin for better identification of tumor cells. DNA was then extracted using the Aqua Pure Genomic Tissue Kit following the manufacturer's instructions. In some cases, digestion with proteinase K was prolonged up to three days. For melanomas, the proteinase K digest was done in aqua bidest, and after enzyme inactivation by heat treatment, the sample was directly used for MseI digestion and PCR. For breast carcinomas, DNA was prepared as follows: one section (10 μm) was deparaffinized, the pellet dissolved in Tris-buffer (50 mmol; pH 8.3), digested by proteinase K at 50°C overnight, and the reaction stopped at 95°C for 10 min.

Amplification of DNA by DOP-PCR and LA-PCR

DOP-PCR was used for amplification and labeling of DNA samples and normal reference DNA (Promega, Mannheim, Germany), as described previously (10). In brief, for amplification, 10–100 ng of both tumor and reference DNA was used in a 25 μl reaction mixture: 250 μmol each dATP, dCTP, dGTP, and dTTP, 1% W1-detergent (Sigma, St. Louis, MO), 2 μmol degenerate primer 5′-CCGACTCGAGNNNNNNATGTGG-3′ (VBC, Vienna, Austria), and 0.1

U/μl Super Taq DNA Polymerase (ViennaLab, Vienna, Austria; HT Biotechnology, UK) in Buffer D (Invitrogen, Groningen, The Netherlands). Cycling conditions were as published (10). For the labeling PCR, 1–2 μl of the DOP-PCR product was used for labeling with Dig-11-dUTP (tumor DNA) or Bio-16-dUTP (reference DNA) (Roche, Mannheim, Germany). Labeling was carried out in a 50 μl reaction mixture: 200 μmol each dATP, dCTP, and dGTP, 160 μmol dTTP, 40 μmol Dig-11-dUTP or Bio-16-dUTP, 1% W1-detergent, 2 μmol degenerate primer, and 0.1 U/μl Super Taq DNA Polymerase in TAPS buffer (25 mmol TAPS, 50 mmol KCl, 2 mmol $MgCl_2$, 2.88 μmol β-mercaptoethanol). Labeling PCR conditions were as published (10).

LA-PCR and CGH analysis was originally described by Klein et al. (8), and later termed "SCOMP," for single cell CGH (8,9). In brief, tumor or reference DNA was MseI-digested for 3 h at 37°C in a volume of 5 μl of buffer containing 0.5 μl of 10× One-Phor-All buffer (Amersham, Freiburg, Germany) and 10 units of MseI-enzyme (New England Biolabs, Beverly, MA). Following that, the adapter MseLig12 (5′-TAACTAGCATGC-3′; Biometra, Göttingen, Germany) was ligated to the 5′-TA overhangs, thus providing the binding site for the primer MseLig21 (5′-ATGGGGATTCCGCATGCTAGT-3′). Amplification and labeling PCR were performed exactly as described (10), except that 2 μl of the primary PCR product was used for the labeling PCR. Finally, the linker and the adapter were removed by a second MseI digest and subsequent purification with Micro-bio spin 30 columns (BioRad). All DOP-PCR and LA-PCR products were analyzed by electrophoretic separation on 1% agarose gels.

Comparative Genomic Hybridization (CGH) and Image Analysis

CGH was performed as recently described (10). Increases and decreases in DNA sequence copy numbers were defined by tumor to reference ratios of >1.2 and <0.8. These reference values were established before the study by CGH analysis of normal DNA samples from different sources as specificity control, as well as different mixtures of male and female normal DNA and analysis of X-chromosome material as sensitivity control. Chromosomes 19, 22, and 1p32-pter (3) have not been included in the analyses. To exclude DNA contamination, control PCR reactions were performed without template DNA as a negative control.

Preparation of Metaphase Spreads, FISH, and CDD-Banding

Tumor and normal human metaphase chromosomes were prepared according to standard protocols and FISH analyses with whole chromosome and single locus paints were performed as described (10). BACs for selected gene loci were searched from ENSEMBL (http://www.ensembl.org/) and obtained from the Sanger Center, Cambridge, UK. Chromomycin/Distamycin/DAPI-staining of metaphase chromosomes (CDD-Banding) allowed the simultaneous production of reverse bands and DAPI-bands (12).

Table 1
Success Rates Achieved by DOP-PCR-CGH and LA-PCR-CGH

Sample source	DOP-PCR-CGH experiments, successful/total (%)	LA-PCR-CGH experiments, successful/total (%)
Cell cultures		
Melanoma	54/54 (100)	15/15 (100)
Soft tissue sarcoma	4/4 (100)	4/4 (100)
Glioblastoma	3/3 (100)	3/3 (100)
Hepatocellular carcinoma	1/1 (100)	1/1 (100)
NSCLC	1/1 (100)	1/1 (100)
Embryonic fibroblasts	1/1 (100)	1/1 (100)
Frozen tissues		
Liver	19/19 (100)	3/3 (100)
Soft tissue sarcoma	2/2 (100)	2/2 (100)
Paraffin-embedded tissues		
Liver	8/48 (17)	16/36 (44)
Melanoma	4/12 (33)	21/24 (87.5)
Breast carcinoma	4/4 (100)	4/4 (100)

RESULTS

CGH Success Rates Using DOP-PCR and LA-PCR

CGH experiments that resulted in an adequate hybridization signal of the amplified and labeled DNA samples compatible with the standard settings of the evaluation software (Leica, QFISH) were defined as "successful CGH experiments." Success rates for DOP-PCR- and LA-PCR-CGH experiments for DNA samples derived from living cells, frozen tissues, and paraffin-embedded tissues are summarized in Table 1. All cell culture- and frozen-tissue-derived DNA samples hybridized well to the metaphase spreads and resulted in successful CGH experiments, irrespective of tissue type, DNA extraction method (compare Material and Methods), and amplification by DOP-PCR or LA-PCR. Correspondingly, in all cases DNA was proven to display no signs of degradation in gel electrophoresis. Amplification and labeling by both methods resulted in an adequate range of DNA fragment lengths for CGH (Fig. 1). DOP-PCR-CGH experiments, as well as LA-PCR-CGH experiments, displayed high hybridization intensities and excellent signal-to-noise ratios. In general, LA-PCR-CGH experiments more often showed a smoother hybridization with very low granularity, as compared to DOP-PCR-amplified samples (example shown in Fig. 2). In summary, the data indicate equal quality of the two amplification methods for high quality DNA derived from living cells and frozen sections.

However, differences between the two methods tested became obvious when analyzing DNA from paraffin-embedded tumor tissues. In case of liver samples ($n = 80$) obtained retrospectively from routinely prepared paraffin blocks, only eight of 48 microdissected samples (17%) resulted in DOP-PCR products that were also successfully applied to CGH experiments. In all other samples, the DOP-PCR product was undetectable or the fragment size was too small to be suitable for CGH (Fig. 1), corresponding to intense DNA degradation in the extract. On the contrary, LA-PCR products of most of these degraded DNA samples showed the same fragment size as seen in case of

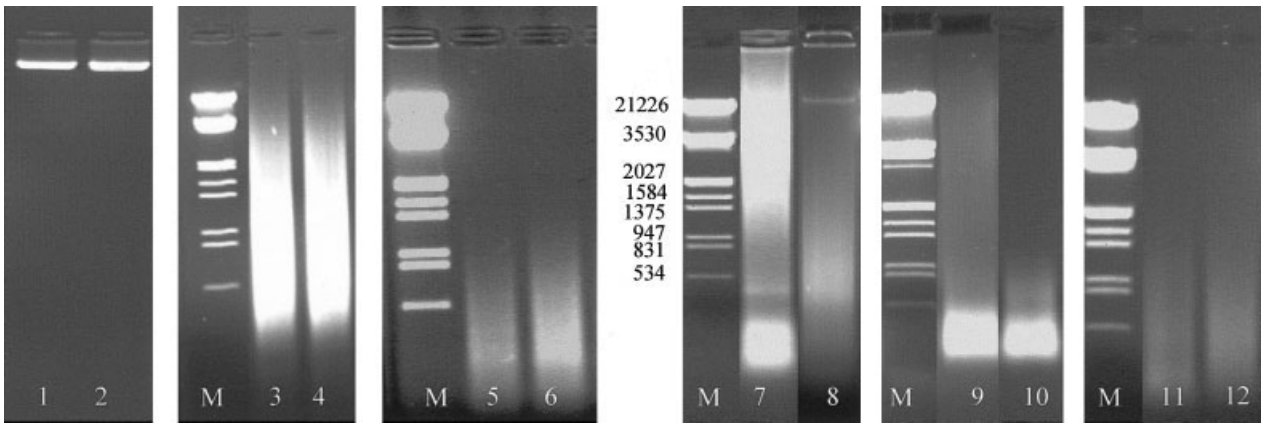

FIG. 1. Impact of DNA quality on DOP-PCR amplification and LA-PCR amplification. Two high quality genomic DNA samples (from frozen tissues of human liver cancer; lanes 1 and 2) and two partly degraded samples (from formalin-fixed, paraffin-embedded tissues of a melanoma and a liver cancer; lane 7 and 8, respectively) are opposed to the respective DOP-PCR amplification products (lanes 3 and 4, as well as 9 and 10, respectively), and LA-PCR amplification products (lane 5 and 6, as well as 11 and 12, respectively). DNA samples and PCR products were separated by 1% agarose gel electrophoresis and stained by ethidium bromide. Notably, the fragment length of the LA-PCR amplification products derived from both degraded and high quality DNA lies within the same range. Molecular weight markers (M) are given in base pairs.

high quality samples (Fig. 1), and 16 of 36 (44%) LA-PCR-CGH experiments were successful with regard to the guidelines for hybridization intensity, signal-to-noise ratio, low background staining, and/or granularity. In case of melanoma tissue sections (n = 26), out of 12 paraffin-embedded tissues, only four (33%, all derived from one pathology department) could be applied to successful DOP-PCR-CGH analysis. In the other eight samples (all from another pathology department), the extracted DNA was too degraded or inaccessible for successful amplification by DOP-PCR. On the contrary, when applying LA-PCR, 21 of 24 experiments (87.5%) led to successful CGH and even allowed comparison of several tumor regions with reliable results (data not shown). In the remaining three LA-PCR-CGH experiments, hybridization signal intensities were too low and background noise too high to score the CGH experiment "successful."

Comparison of DOP-PCR-CGH and LA-PCR-CGH of the Same DNA Sample

A total of 36 DNA samples was comparably analyzed by both methods. In the case of high quality DNA from cell cultures and frozen material prepared by the kit method, both DOP-PCR-CGH and LA-PCR-CGH experiments showed comparable quality and intensity of hybridization. Both methods resulted in identical CGH profiles in all cases analyzed, independent of tumor type and origin (from cell cultures or frozen tissues). All changes checked by FISH analyses with whole chromosome and locus-specific probes were verified (example in Fig. 2). Surprisingly, DOP-PCR-CGH and LA-PCR-CGH led to inconsistent results for four soft tissue sarcomas extracted by a phenol-chloroform-isoamylalcohol method, despite a high molecular size of all DNA samples and a comparable hybridization quality and intensity with both methods. When compared to LA-PCR-CGH and CDD-banding, DOP-PCR-CGH resulted in multiple false-positive signals, whereas false-negative signals were almost absent (Fig. 3).

The tendency toward false results from DOP-PCR-amplified probes, despite CGH experiments scored as successful, was also detected when analyzing a variety of samples from paraffin-embedded tissues. The DNA extracted from several of these samples showed strong degradation (Fig. 1). Hybridization intensity of DOP-PCR-CGH and LA-PCR-CGH was comparable in most of these cases, however, the hybridization quality of DOP-PCR-CGH showed a rather high degree of granularity as well as high background noise when compared to LA-PCR-CGH. For 11 DNA samples derived from paraffin-embedded tissues that displayed distinct signs of DNA degradation, the false results derived from DOP-PCR-CGH, which were avoided by LA-PCR-CGH, mainly concerned chromosomes 4 (10/11; 91%), 17 (10/11; 91%), 13 (7/11; 64%), 1p (6/11; 54%), 5 (5/11; 45%), 9p (5/11; 45%), 12 (4/11; 36%), and 16p (4/11; 36%).

Aged DNA Samples

Two DNA samples derived from the blood of healthy donors and 1 DNA sample derived from a melanoma cell culture were analyzed. All samples had been stored for 3.2 years at –20°C, and had been repeatedly thawed and refrozen during this time. Gel electrophoresis showed in all cases some high molecular weight DNA and a smear of degraded fragments (not shown). LA-PCR-CGH correctly detected the normal karyotypes of the two blood-derived DNA samples of healthy donors (Fig. 4). The aged DNA derived from the melanoma cell culture showed identical results to those obtained by the first CGH analysis three years before. In contrast, DOP-PCR-CGH of all tested DNA samples revealed a tendency towards false gains and losses. Affected loci were almost equal to the ones prone

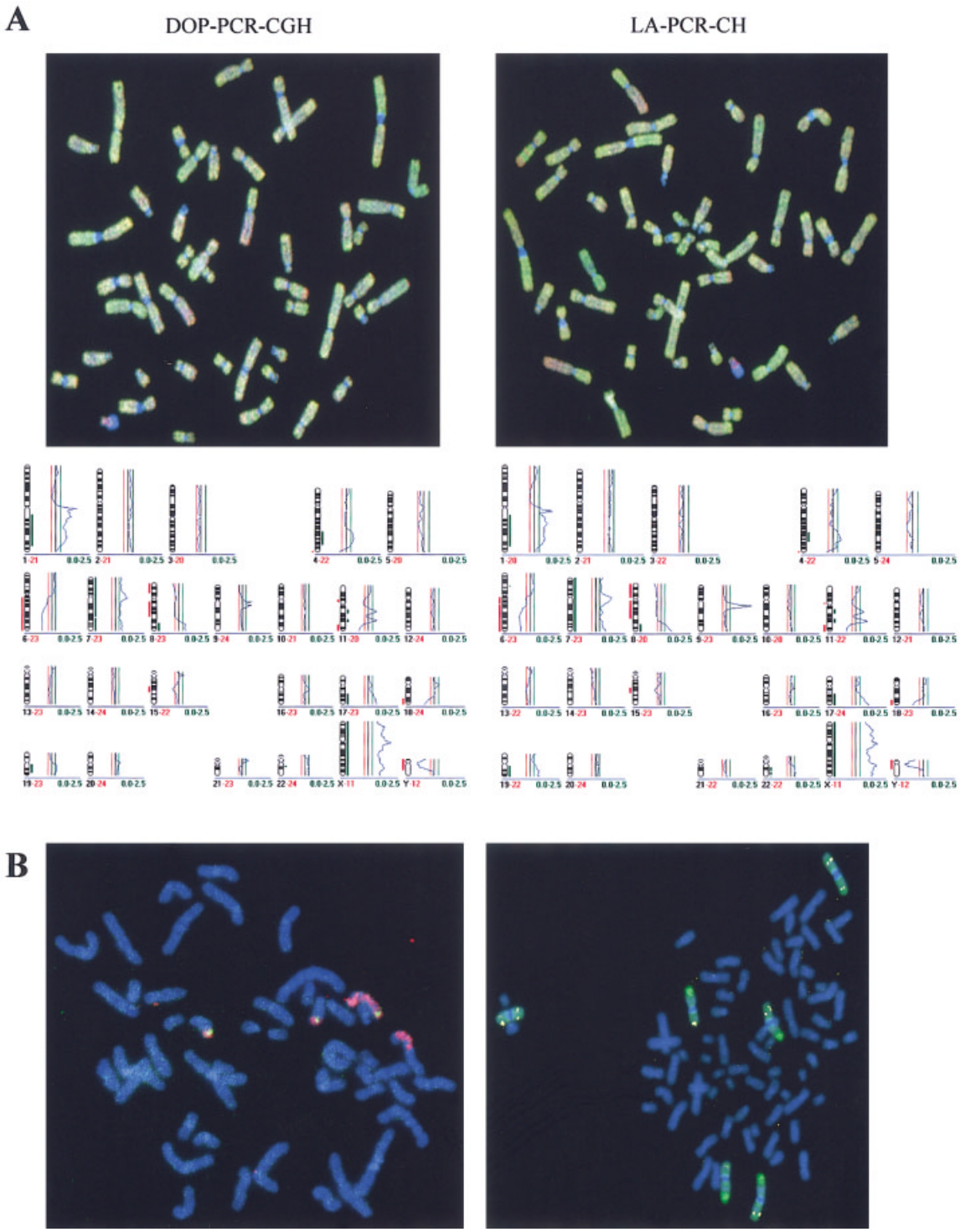

FIG. 2. Comparison of CGH results using a high quality DNA sample. DNA was extracted from a melanoma brain metastasis cell culture. **A:** Analysis was done by DOP-PCR-CGH and LA-PCR-CGH as indicated. To illustrate hybridization quality, a representative metaphase for DOP-PCR-CGH (left) and LA-PCR-CGH (right) is shown. Mean CGH profiles were calculated from 12 metaphases each. **B:** FISH analysis of selected tumor metaphase chromosomes was used as control. Left: Chromosome 8 (red) with the gene locus for c-myc on 8q24 (green). Right: Chromosome 1 (green) with the gene locus for "glioma amplified on chromosome 1 protein" (GAC1) on 1q32.1 (yellow).

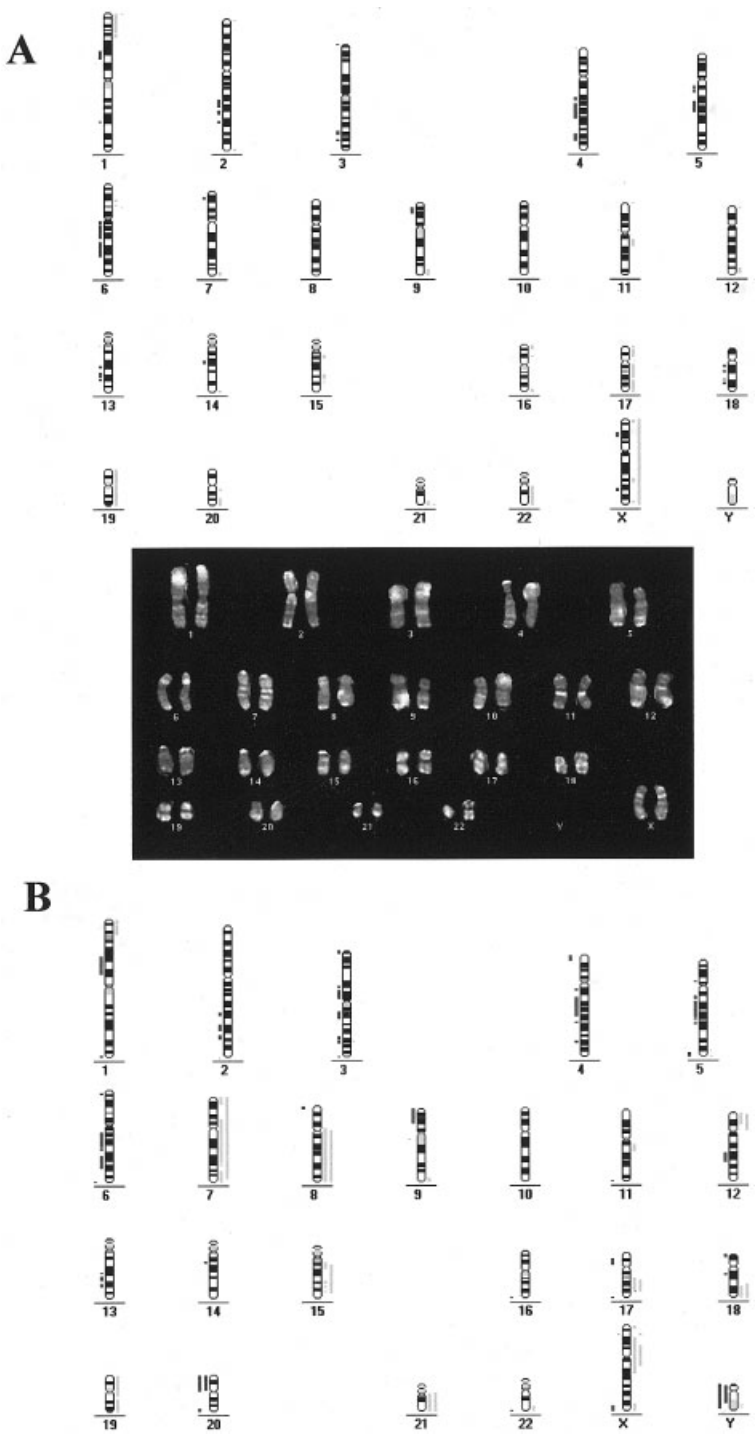

FIG. 3. Comparison of CGH results using DNA samples derived from two soft tissue sarcomas. **A:** In this case, CGH results (schematic illustration in the upper panel; gains are indicated by gray bars on the right of respective chromosomes, losses as black bars on the left) from DOP-PCR-CGH (inner bars) indicated a series of genomic changes (mainly losses), while LA-PCR-CGH (outer bars) only detected changes in the sex chromosome ratio. CDD-banding of the corresponding tumor metaphase chromosomes (lower panel) showed a normal female karyotype and thus confirmed the results obtained by LA-PCR-CGH. **B:** In this case, in addition to gains/losses detected by both methods, copy number changes by DOP-PCR-CGH (inner bars) were seen, e.g., on chromosomes 1p, 2q, 4q, 5q, 6q, 9p, and 13q, as well as on chromosome 19. CDD-banding of the corresponding tumor metaphase chromosomes revealed a highly complex karyotype and widely confirmed the results obtained by LA-PCR-CGH (data not shown).

to give false results in the degraded paraffin-embedded samples (Fig. 4).

Dilution Experiments

In order to test the efficiency of DOP-PCR and LA-PCR to amplify very small amounts of DNA corresponding to one single diploid cell (5–7 pg) and consequently produce reliable CGH results, we performed DNA dilution experiments. DNA amounts analyzed ranged from 500 ng to 2 pg. PCR products derived from DNA samples containing 20 pg or more, produced in both methods reliable and identical CGH profiles (Fig. 5A and B). In case of less than 20 pg of template DNA, DOP-PCR-CGH showed additional to sample-specific changes a series of false-positive and

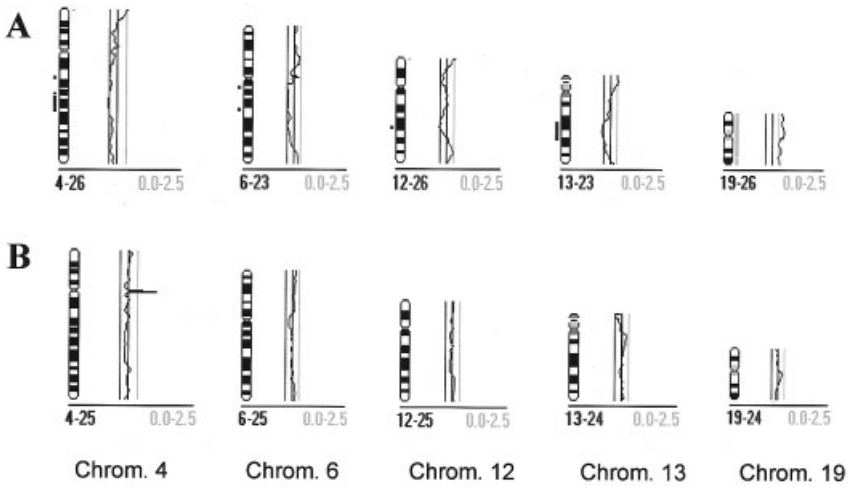

FIG. 4. Comparison of CGH results from an aged DNA sample. DNA was derived from blood of a healthy donor stored for 3.2 years at –20°C, and repeatedly thawed and refrozen during this time. CGH profiles for selected chromosomes as indicated are shown. **A:** DOP-PCR-CGH revealed several gains and losses that would have been avoided by redefining thresholds to >1.25 and <0.75 (not shown). **B:** LA-PCR-CGH correctly indicated the normal karyotype at the standard threshold >1.2 and <0.8.

false-negative results, as well as changes in the green to red ratio of certain chromosomal regions (Fig. 5C). These were mainly characterized by a decrease of gained regions and an increase of lost regions, as well as the excessive amplification of heterochromatic regions, e.g. at the short arms of acrocentric chromosomes. Also, those chromosomal regions described as critical in CGH (13) (1p32-pter, 16p, 19, and 22) displayed increased gains as compared to the original experiment. On the other hand, all LA-PCR-CGH experiments using only ≈5 pg DNA showed reliable results, with profiles identical to the profiles obtained from undiluted DNA samples (Fig. 5B and D). LA-PCR-CGH experiments from less than 5 pg of template DNA could not be evaluated, because the hybridization intensity was too weak (not shown).

DISCUSSION

CGH is a helpful screening tool to detect DNA sequence copy number imbalances in malignant cells (3,14,15). However, investigations of early lesions, specific tumor regions, and small metastatic lesions led to the necessity to perform CGH from minute amounts of DNA. In order to solve this problem, several methods for whole genome DNA amplification have been developed. Due to its simplicity and success rate, DOP-PCR has become the method of choice (3). However, technical problems in case of DNA prepared from paraffin-embedded samples led to several re-evaluations of this technique (4–7). Recently, another method, LA-PCR, was introduced, suggesting that it would allow whole genome amplification down to the level of one metastatic cell (16,17). Our study aimed to investigate to what extent these two methods would be suitable for DNA sample preparation in a routine clinical setting, which might include inefficiently prepared paraffin-tissues, aged samples, and DNA prepared by completely different methods. Our data demonstrate that DOP-PCR as well as LA-PCR are highly efficient tools for whole genome amplification in order to perform CGH from target tissues down to the level of a few cells. However, especially in the case of partly degraded DNA extracted from archived, paraffin-embedded tissues, LA-PCR-CGH provided a significantly higher success rate than DOP-PCR-CGH. Moreover, in partly degraded, but also in aged DNA samples, in DNA samples extracted by certain precipitation methods, and in the case of amplification of very low amounts of DNA (equivalent to one to five diploid cells), LA-PCR-CGH delivered distinctly more reliable results than DOP-PCR-CGH. In summary, our data demonstrate, in accordance with a previous report (8), that LA-PCR is of superior quality and reliability as compared to DOP-PCR for routine amplification of DNA from difficult and limited sources in order to perform CGH.

The observed differences between the two methods with respect to both the success rates and the reliability of the CGH results are thought to be based on the principal differences between the two amplification methods. DOP-PCR uses a multitude (4^6) of different partially degenerated primers that anneal under increasingly stringent PCR conditions at binding sites that are supposed to be present about every 4 kb of genomic DNA (4). On the contrary, LA-PCR is preceded by a DNA-digestion-step by MseI that allows, via ligation of an adapter, the binding of one single primer, and the subsequent amplification of the whole genome using only one primer. The exactly defined MseI-restriction sites are randomly present about every 200–300 bp (8), which makes this method more suitable for the amplification of already smaller DNA fragments.

We focused on the analysis of formalin-fixed, paraffin-embedded tissues of two tumor entities, namely malignant melanoma and hepatocellular carcinoma, which are known to be critical for whole genome amplification. For primary melanomas, microdissectable parts are frequently very small because of small tumor mass due to tumor detection at early stages. Additionally, high amounts of melanin can hamper the PCR procedure (7). Despite these limitations, and despite the use of aged (up to eight years) melanoma paraffin blocks, LA-PCR raised the CGH success rate to almost 90% of melanoma samples, while only a limited proportion of these specimens (33%) could be analyzed using DOP-PCR. This success rate resembles the one published previously for

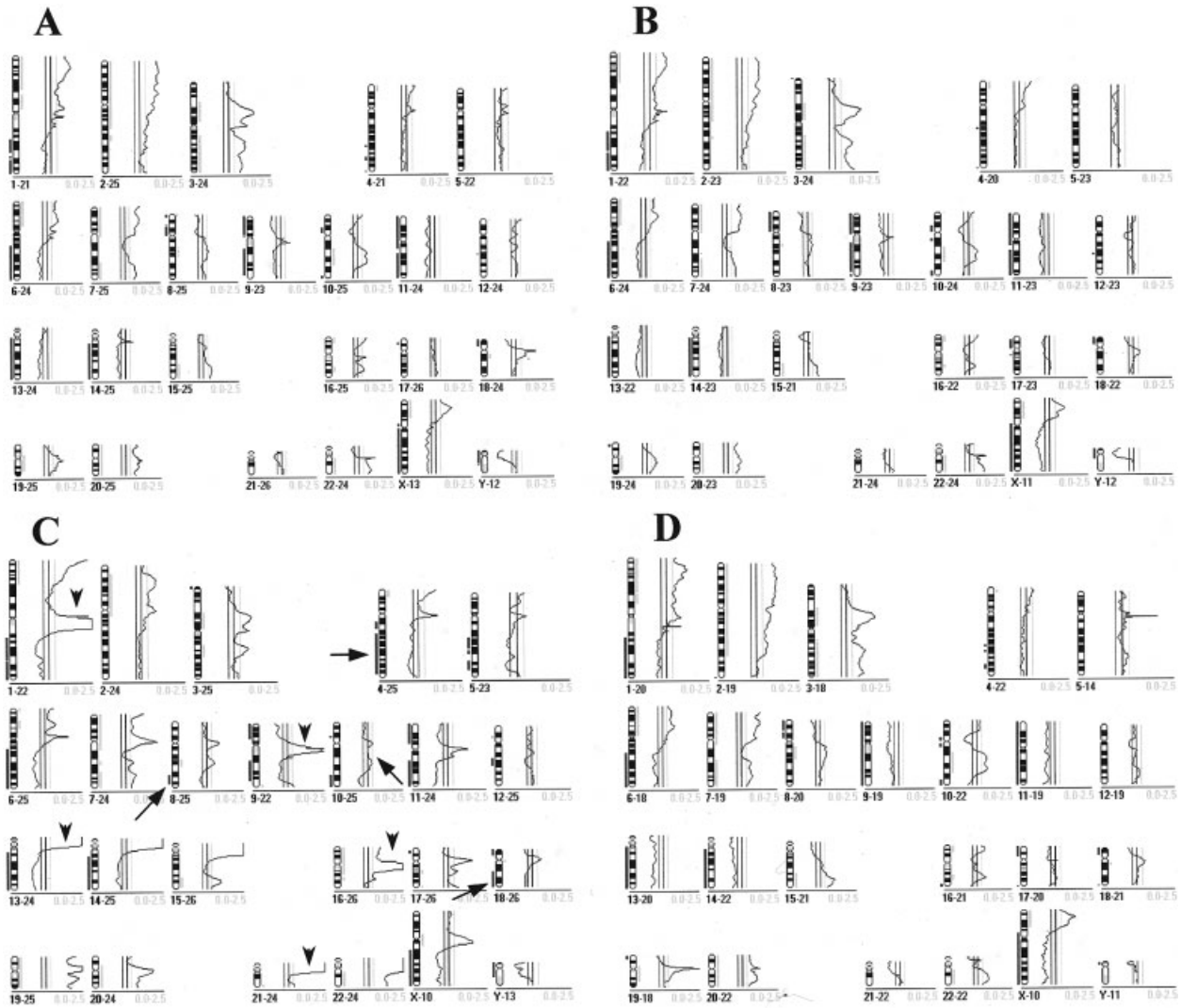

FIG. 5. Dilution experiments using DNA from a melanoma brain metastasis cell culture. The amount of DNA prior to amplification was 500 ng in the undiluted samples (**A**,**B**) and 5 pg in the diluted samples (**C**,**D**). DOP-PCR-CGH (A,C) and LA-PCR-CGH profiles (B,C) of the undiluted sample and the diluted sample are shown. Whereas LA-PCR-CGH of the diluted sample showed the identical results as obtained by both CGH experiments of the undiluted sample, DOP-PCR-CGH of the diluted sample (C) showed additional changes (examples indicated by arrows) as well as excessive gains at heterochromatic regions (arrowheads).

LA-PCR-CGH with respect to tumors of mixed origin (9). Also for formalin-fixed, paraffin-embedded liver samples, LA-PCR was clearly superior to DOP-PCR. However, as compared to the data above, success rates (44% versus 17% for LA-PCR and DOP-PCR, respectively) were relatively low. This might have been caused by the high amounts of degrading enzymes in the liver as well as enhanced DNA-degradation by formalin fixation (18,19). Stoecklein et al. (9) suggested the sample age of formalin-fixed, paraffin-embedded tissues as a decisive factor limiting successful amplification, especially by DOP-PCR. In contrast, we did not find any correlation between sample age and degradation of the extracted DNA in human liver samples. Our data, rather, suggest proper handling of the surgery specimen as a key requisite for DNA-amplification and CGH analysis. It is of utmost importance that liver samples are fixed immediately after surgery and that the fixation time is long enough, depending on the sample size (20). This is additionally corroborated by the fact that we monitored distinct differences in success rates between samples obtained from different pathology departments. In accordance with our findings, Dietmaier et al. (19) suggested that successful whole genome amplification for mutation analysis is critically dependent on a very quick sample procession (<30 min), and problematic when analyzing paraffin-embedded tissue samples. Thus, the use of LA-PCR is especially recommended in situations where CGH has to be routinely performed from samples derived from different tissues or from institutions with different fixation and embedding techniques.

Unexpectedly, comparable problems to those found with paraffin-embedded tissues were observed for DNA preparations from cell cultures using a precipitation

method in combination with analysis by DOP-PCR-CGH. Despite the presence of high molecular weight DNA without signs of protein contamination, DOP-PCR-CGH tended to give false-positive results that were absent in the case of LA-PCR-CGH. The observed problems suggest that incomplete precipitation and/or impaired solubility of DNA from specific chromosomal regions might be limiting for DNA amplification and CGH analysis. The resulting DNA aggregates might be inaccessible for DOP-PCR, causing false-positive results in several chromosomal regions. LA-PCR is preceded by an enzyme digestion-step and a following ligation reaction, which might both have a positive influence on the accessibility of the DNA.

Both, DOP-PCR and LA-PCR (also termed SCOMP) have been suggested to be suitable for analysis of very minor amounts of DNA, equivalent to one or a few cells (9,16,17,21–23). We compared the sensitivity and efficiency of both amplification procedures in a series of DNA dilution experiments. For either amplification method, DNA amounts from 300 ng to 20 pg resulted in reliable experiments, with identical results obtained from undiluted and diluted DNA samples. These data are in good agreement with previous studies in which the minimal DNA amounts for successful DOP-PCR-amplification and CGH were described to range between 12.5–50 pg (5,21,23). At lower amounts of target DNA, equivalent to one or a few diploid cells, we found LA-PCR to be clearly superior as compared to DOP-PCR. LA-PCR-CGH experiments using DNA amounts of only 5 pg for amplification showed good hybridization quality, as well as the identical profiles obtained by the corresponding undiluted sample. On the contrary, DOP-PCR-CGH experiments using less than 20 pg of template DNA showed a tendency to false results due to loss of specifically gained regions and increased losses of already underrepresented regions, as well as gains of heterochromatic regions and of chromosomal regions already described as critical in CGH (13). These results are in accordance with several studies demonstrating the applicability of LA-PCR for DNA analysis from one or a few cells (9), allowing detection of the genetic heterogeneity (16) and combined characterization of genetic alterations and gene expression (17).

In summary, our results demonstrate that LA-PCR leads to a higher number of successful CGH experiments and more reliable results when compared with DOP-PCR, especially if critical DNA samples derived from formalin-fixed, paraffin-embedded tissues, as well as very low amounts of DNA have to be investigated.

ACKNOWLEDGMENTS

We thank B. Stögermayer, M. Eisenbauer, and V. Bachinger for excellent technical assistance.

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