Isolation of genes amplified in human cancers by microdissection mediated cDNA capture

Edgardo Gracia\textsuperscript{1,+}, Ulrike Fischer\textsuperscript{2,+}, Abdel ElKahloun\textsuperscript{1}, Jeffrey M. Trent\textsuperscript{1}, Eckart Meese\textsuperscript{2,*} and Paul S. Meltzer\textsuperscript{1}

\textsuperscript{1}Laboratory of Cancer Genetics, National Center for Human Genome Research, National Institutes of Health, Bethesda Maryland 20892, USA and \textsuperscript{2}Institut fur Humangenetik, Universitatskliniken des Saarlandes, Homburg/Saar, Germany

Received August 30, 1995; Revised and Accepted February 2, 1996

It has been increasingly recognized that homogeneously staining regions (hsr) in human cancers may be complex structures composed of large amplified DNA domains containing multiple genes. It is therefore important to devise strategies for the rapid isolation of cDNAs expressed from these structures. Using a procedure we term microdissection mediated cDNA capture, we recovered hsr specific cDNAs from two different human tumors. The glioblastoma cell line TX3868 and the human sarcoma cell line OsA-CL carry hsr regions containing amplified sequences from chromosome 12q13–15. We recovered 17 hsr specific cDNAs following microdissection of these hsr which had been previously hybridized in situ with linked cDNA. Northern blot analysis with these cDNAs revealed hybridization to distinct transcripts in OsA-CL RNA and TX3868 RNA. None of the OsA-CL cDNA clones showed cross hybridization with the TX3868 cDNAs suggesting that despite their coincident band localization on 12q, the OsA-CL and TX3868 amplification units do not completely overlap. These results significantly increase the number of amplified genes assigned to the 12q13–15 amplicon illustrating both the complexity of hsr regions derived from this region and the utility of microdissection mediated cDNA capture to gain rapid access to cDNAs transcribed from amplified genes.

INTRODUCTION

Human cancers frequently harbor large tracts of amplified DNA which are often cytologically evident as homogeneously staining regions (hsr) or double minutes (dmin). These anomalies are a manifestation of the intrinsic genomic instability of cancer cells, and it is presumed that overexpression of amplified genes confers a selective advantage on hsr bearing clones (1). Unfortunately, it is often difficult to identify the amplified genes in cells which contain hsr or dmin. This is because chromosome banding techniques provide no positional information for dmin, and intrachromosomal amplified DNA is frequently integrated in a site distant from the single copy locus (2). Comparative genomic hybridization (CGH) can be used to detect and map amplified sequences, but this approach is purely analytical and does not directly lead to gene isolation (3). Hybrid selection or exon trapping schemes using YACs or cosmids that map within an hsr can be used to clone amplified cDNAs (4,5). However, a relative disadvantage of these approaches is their reliance on the isolation of genomic clones corresponding to the amplified region.

We have previously demonstrated that chromosome microdissection is a powerful approach for the analysis of hsr and dmin (6–9). The microdissected material is amplified by degenerate oligonucleotide primed PCR (DOP-PCR) generating a probe which has had two major applications. First, the probe is utilized for fluorescent in situ hybridization (FISH) to the hsr (or dmin) and to normal chromosomes. This process confirms the presence of amplification and establishes the chromosomal origin(s) of the hsr. Second, the microdissection PCR product can be used to establish a microclone library which provides entry point clones for mapping the amplified region. Alternative strategies are suggested by the probability that transcribed sequences are included in the microdissection PCR product. Recently, we have reported a cDNA selection strategy based on hybridization of cDNA to microdissected hsr sequences immobilized on a solid support (10), and Sen et al. have reported a similar strategy using microdissected dmin (11). We have now developed a direct cDNA selection strategy based on the hybridization of linked cDNA directly to tumor metaphase chromosomes. After hybridization, the hsr is microdissected, and the hybridized cDNAs are recovered by PCR, a process we term microdissection mediated cDNA capture.

Amplification of the 12q13–15 region is frequent in sarcomas and brain tumors (12,13). Eight genes have been previously mapped to the amplified region including the α-2 macroglobulin receptor (A2MR/LRP), the zinc finger protein GLI, the transcription factor CHOP (GADD153/DDIT3), a membrane protein (SAS), a cyclin dependent kinase (CDK4), and a regulator of p53 (MDM2) (14–19). In addition, two transcripts (OS-9 and OS-4) which we cloned by microdissection-hybrid selection also map...
within this region (10). Because the 12q amplicon contains several well characterized genes, and because its expression map remains incomplete, the 12q13–15 hsr is an attractive model for the investigation of expression mapping techniques.

We now report the application of this microdissection based approach to the isolation of hsr specific cDNAs from two cell lines, a sarcoma (OsA-CL) and a glioblastoma (TX3868), both of which carry hsrs derived from 12q13–15 (10). Using microdissection mediated cDNA capture, we isolated 17 amplified cDNAs including three previously identified genes and 14 novel cDNAs. Our results significantly extend the expression map of this amplicon and suggest that this microdissection based technology will be useful for the expression mapping of other amplified regions in human cancers.

RESULTS

cDNA in situ hybridization and microdissection

In order to establish appropriate hybridization and washing conditions, we used FISH to confirm that cDNAs which hybridized to the hsr were present in our library and retained on the chromosome following stringency washes. Biotinylated and non-biotinylated cDNAs from either OsA-CL or TX3868 were hybridized in parallel to metaphase chromosomes from the same source as the cDNA (Fig. 1). A weak diffuse FISH signal was seen on all chromosomes except for the hsrs which exhibited intense fluorescence confirming preferential retention of cDNA in this region. To recover the bound cDNA, several copies of the entire hsr (ten copies for OsA-CL and five copies for TX3868) were then microdissected from slides hybridized with non-biotinylated cDNA, and the bound cDNAs were recovered by PCR with linker primers.

After microdissection and PCR, the captured cDNAs were analyzed by gel electrophoresis in comparison to the starting cDNA and several controls (Fig. 2). The bulk of the PCR product was 400–500 bp in size. In contrast, the results of mock dissection (done by touching a microneedle to an area of the slide devoid of chromatin) typically demonstrate one or two dominant bands superimposed on a much fainter smear.

Characterization of captured cDNAs

After electrophoretic evaluation, the captured cDNAs were cloned in a plasmid vector, and characterized by hybridization to Southern blots of cell lines known to carry 12q13–15 amplification and grouped by cross-hybridization. In the OsA-CL captured cDNA library 64/89 (72%) clones hybridized to amplified restriction fragments in OsA-CL (Fig. 3). Cross-hybridization placed these in 11 distinct cDNA groups (designated osteosarcoma expressed sequence [OSES]). The cDNAs derived from OsA-CL were also tested for amplification against two additional cell lines known to contain amplification of sequences from 12q13–15 [rhabdomyosarcoma cell line RMS-13 (20) and neuroblastoma cell line NGP-127 (21)] (Fig. 3). Several of the cDNAs which were amplified in OsA-CL were also amplified in one or both of these additional cell lines suggesting that they fall...
in regions of overlap between these amplicons (Table 1). In the captured cDNA library from TX3868, 8/18 (44%) clones analyzed recognized amplified restriction fragments in TX3868 (Fig. 3). Six non-crosshybridizing clones [designated glioma amplified sequence (GAS)] were analyzed in detail. All six (GAS89, GAS27, GAS16, GAS41, GAS56, and GAS64) identified amplified DNA fragments in an additional glioblastoma cell line [T3564 (22)] (Fig. 3, Table 1).

Table 1. Characteristics of captured cDNAs

<table>
<thead>
<tr>
<th>Designation</th>
<th>Number of clones</th>
<th>Amplification status</th>
<th>mRNA size (kb)</th>
<th>Insert size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OsA</td>
<td>NGP</td>
<td>RMS</td>
</tr>
<tr>
<td>OSES-1</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>(MDM2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSES-2</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OSES-3</td>
<td>24</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OSES-4</td>
<td>14</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OSES-5</td>
<td>6</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>OSES-6</td>
<td>2</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>OSES-7</td>
<td>5</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>OSES-8</td>
<td>1</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>OSES-9</td>
<td>1</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>OSES-10</td>
<td>1</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>OSES-11</td>
<td>3</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>GAS16</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GAS27</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GAS41</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GAS56</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GAS64</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GAS89</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a Amplification status in OsA-CL, NGP-127, and RMS-13 (OSES cDNAs) or in TX3868 and T3564 (GAS cDNAs). + denotes amplification; − denotes single copy.
b mRNA size is that observed in either OsA-CL RNA (for OSES cDNAs) or TX3868 (for GAS cDNAs). N.D. indicates that mRNA was not detected on Northern blot analysis. c Size of the insert sequenced and used for hybridization in Figures 3, 4 and 5. GenBank accession numbers for the sequences of these clones are OSES-4, U47340; OSES-5, U47341; OSES-6, U47342; OSES-7, U47343; OSES-8, U47344; OSES-9, U47345; OSES-10, U47346; OSES-11, U47347; GAS16, U46847; GAS56, U46846; and GAS89, U46845.
Northern blot analysis demonstrates expression of captured cDNAs in OsA-CL and TX3868. Ten µg of total RNA from the indicated source was loaded in each lane.

Representative multiple tissue Northern blot analysis of captured cDNAs. The indicated probe was hybridized to a blot containing RNAs from the indicated tissues. Hybridization with a β-actin probe is illustrated as a control for loading error. This probe hybridizes to two actin isoforms in heart and skeletal muscle.

All 11 OSES cDNAs hybridized to DNA from a monoclonal somatic cell hybrid containing chromosome 12 as the sole human component confirming that they all map to chromosome 12 (Fig. 3). The GAS cDNAs also localized to chromosome 12 either by PCR or by Southern blot analysis (data not shown). None of the OSES clones detected a cross hybridization with the GAS cDNAs. In addition sequence comparison revealed no identity or near identity between sarcoma and glioma derived clones.

Amplified cDNA clones were used to probe Northern blots containing OsA-CL RNA, TX3868 RNA. All of the amplified cDNAs except OSES-6 hybridized to readily detectable transcripts in OsA-CL or TX3868 RNA (Fig. 4, Table 1). The pattern of expression of the OSES and GAS cDNAs was evaluated in eight adult human tissues by northern blot analysis (Fig. 5). Patterns of expression varied from ubiquitous (e.g. OSES-5 and GAS16) to highly tissue specific (e.g. GAS64 and OSES-4). Several cDNAs identified transcripts which were expressed most strongly in heart and skeletal muscle (GAS64, OSES-4, OSES-9, and OSES-10). All of the GAS cDNAs detected transcripts in brain, and OSES-7, OSES-8 and OSES-11 hybridized most intensely to brain RNA.

As indicated in Table 1, three of the OSES cDNAs identified genomic restriction fragments and transcripts corresponding to genes previously mapped to the hsr (MDM2, OS-4 and OS-9). This identity was confirmed by sequence analysis of these clones. Sequence comparison of the remaining OSES and GAS cDNAs with GenBank identified no significant identity with each other or known sequences (except for ESTs) indicating that 14 hsr specific cDNAs isolated by microdissection mediated cDNA capture represent novel partial cDNAs previously unassigned to the 12q13–15 amplification unit.

DISCUSSION

It is of considerable interest to identify the amplified genes found in hsr in order to assess the impact of their expression on tumor phenotype. However, cDNAs for amplified genes are difficult to clone without previously mapping the amplification unit at the genomic level. In this report, we demonstrate the utility of microdissection mediated cDNA capture for the isolation of transcribed sequences encoded in an hsr. A previous study demonstrated the feasibility of establishing regional cDNA libraries after in situ hybridization to normal human and mouse chromosomes (23). Using the strategy presented above, we have now been successful in using microdissection mediated cDNA capture to isolate transcripts from two different hsr. The cDNA libraries generated following microdissection were highly enriched for sequences derived from the amplification unit. Because this approach combines the use of cDNA from the hsr bearing cell line with exploitation of the genomic composition of the hsr itself, it avoids false positives such as retro-pseudogenes which might confound alternative strategies such as direct hybridization of cDNA libraries with microdissected hsr sequences. The sensitivity of this procedure is more difficult to assess. A relatively limited analysis of the OsA-CL library identified three of the seven genes previously known to be amplified in OsA-CL (10,14), certainly a useful level of sensitivity. Although we did not identify known genes in the TX3868 library or any overlap between the OSES and GAS cDNAs, this is probably accounted for by the size and complexity of the amplicons (21) and by differences between sarcoma and glioblastoma gene expression patterns. However, we recognize that not all amplified genes will prove equally tractable to this strategy. Success in recovering a given transcript is likely to depend on its size and level of expression as well as other factors which may affect its representation in the linkeded library.

It has become increasingly apparent that regions of DNA amplification in human cancers may encompass several genes. This is particularly evident in the case of 12q13–15 amplification where there are multiple genes which may potentially be included in the amplicon in various tumor types. It will be necessary to use a battery of several techniques to accomplish the important goal of completing the expression map of a given amplified region.
However, strategies for rapidly identifying a group of amplified cDNAs (such as microdissection mediated cDNA capture) are particularly useful. Besides providing candidate target genes for the amplification event, amplified cDNAs also provide landmarks for physical mapping of the amplification unit thereby facilitating the construction of an integrated physical and expression map. By using these mapped genes to analyze multiple tumors, it is then possible to develop a map of the consensus amplification unit core. Our results strongly suggest that microdissection mediated cDNA capture is a useful approach for the isolation of cDNAs encoded by amplified genes.

**MATERIALS AND METHODS**

**Cell culture and metaphase preparations**

Cell culture and cytogenetic studies were performed as described previously (22,24). The human osteosarcoma cell line OsA-CL and rhabdomyosarcoma RMS-13 were kindly provided by Tom Look, St. Jude Children’s Research Hospital, Memphis, TN (20). Neuroblastoma cell line NGP-127 was kindly provided by Garrett Brodeur, Children’s Hospital of Philadelphia. Somatic cell hybrid GM10886 containing chromosome 12 as the sole human chromosome was obtained from the Coriell Medical Institute. Tumor samples were stored in liquid nitrogen immediately after surgical removal. Following xenografting of tumor cells from the glioblastoma cell line T3868 into nude mice, the glioblastoma cell line TX3868 was established.

**RNA isolation, cDNA synthesis, and linker addition (OsA-CL)**

RNA was isolated from cultured tumor cells by the single step guanidium thiocyanate method according to Chomczynski (25). Fifteen µg of oligo dT selected mRNA was used for random primed cDNA synthesis (Riboclone cDNA synthesis kit, Promega). Following second strand synthesis, the cDNA was phenol:chloroform extracted and ethanol precipitated. The cDNA was digested with 50 U XhoI for 3 h and then made blunt ended with T4 polymerase as indicated in the manufacturer’s protocol. The cDNA was modified for PCR amplification essentially according to the method of Kinzler (26). Two µg blunt ended cDNA were ligated to 15 µg of phosphorylated catch A (5’GAGTAGAATTCTAATATCTC 3’) and 15 µg of phosphorylated Catch B (5’GAGATAGAATTCTAATCTC 3’) linkers for 48 h at 16°C in a volume of 40 µl. After ligation, the cDNA was digested overnight with 100 U XhoI to remove linker concatemers and purified over two successive Sephadex G-50 columns. One fifth (10 µl) of the ligated, digested cDNA was amplified in a 50 µl reaction containing 7.5 µl Taq polymerase (Perkin-Elmer Cetus), 2.5 µM catch A primer, 200 µM of each dNTP, 50 mM KCl, 1.5 mM MgCl2, and 10 mM Tris–HCl, pH 8.4. The cDNA was denatured for 30 min at 94°C in the presence of primer and buffer. The dNTPs and Taq polymerase were added for 15 cycles of 94°C 1 min, 40°C 1 min, 72°C 1 min, and a final extension of 72°C 5 min. The cDNA was digested overnight with 50 U XhoI and purified over two successive Sephadex G-50 columns. Five µl (1/10) of the cDNA was amplified for 20 cycles in a 50 µl PCR as described above but with an annealing temperature of 52°C. The product of this reaction is the stock cDNA used for further PCR and for biotin labeling. All subsequent amplifications of cDNA destined for hybridizations used 1 µl of stock cDNA for a maximum of 20 cycles with 0.25 µl of catch A. Biotin labeling was done under the same conditions but with 130 µM dTTP and 150 µM biotin-16-dUTP.

**RNA isolation, cDNA synthesis, and linker addition (TX3868)**

RNA was isolated from cultured tumor cells according to Gough (27) and digested with RNase free DNase (Boehringer Mannheim) for 15 min at 37°C. After ethanol precipitation 9 µg RNA were used for cDNA synthesis. cDNA synthesis was primed with an oligo d(T)15 primer following the manufacturers protocol (Boehringer Mannheim). Two ng blunt ended cDNA were ligated to 2 pmole of Uni-amplTM Adaptor/Sall for 20 h at 16°C according to the Uni-amplTM Adaptor ligation protocol (Clontech) in a volume of 10 µl. One third of the ligated cDNA was amplified in a 50 µl reaction containing 2.5 µl Taq Polymerase, 25 µM Uni-Amp Primer (Clontech), 200 µM of each dNTP and 1.5 mM MgCl2. The reaction was first denatured for 3 min at 94°C following 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min and a final extension step at 72°C for 10 min. In a secondary PCR, 1 µl cDNA PCR product was labeled with biotin-16-dUTP. The PCR conditions were as described above except 1 µl of biotin-16-dUTP (1 mM) was added.

**cDNA in situ hybridization**

For OsA-CL hybridizations, cDNAs (5 µg) were added to a 10 µl hybridization mix that included 5 µg of Cot1 DNA (BRL), 19 µg of herring sperm DNA, 5% dextran sulfate, 1× SSC, 1× SSPE, and 1% Tween 20. The hybridization mix was added to 1 week old untreated slides, sealed with rubber cement and placed in a moist chamber for 20 min. The chromosomes and cDNAs were denatured in situ for 10 min at 100°C in a steam bath inside a covered beaker of boiling water. Hybridization was carried out for 18 h at 66°C in a moist chamber inside a water bath. For biotin labeled hybridizations slides were washed to a stringency of 2× SSC at 45°C. TX3868 hybridizations were done according to the method of Hozier (23). Fluorescent detection of the hybridized probe followed the method of Pinkel (28). Slides hybridized with unlabeled cDNA were washed to a stringency of 0.1× SSC, 0.05% Tween 20 at 65°C. These slides were stained with Giemsa for 10 min prior to microdissection.

**Microdissection**

Microdissection of the cDNA-hybridized hsr was performed using a glass needle controlled by a hydraulic micromanipulator (Narashige) as previously described (29). For amplification of genomic DNA, seven copies of an hsr were microdissected and transferred to a 5 µl collection drop containing 200 µM of each dNTP, 1 µM UNI primer (CCGACTCGAGNNNNNATGG) and 0.2 U topoisomerase I (Promega). After microdissection the collection drop was covered with a drop of mineral oil and incubated at 37°C for 30 min. cDNA-hybridized hsr were microdissected and transferred to a 5 µl collection drop containing 200 µM of each dNTP, 1 µM catch A primer (or 0.5 µM Uni-AmpTM Primer), 0.25 U Taq polymerase, 50 mM KCl, 1.5 mM MgCl2, and 10 mM Tris–HCl, pH 8.4. After microdissection the collection drop was covered with a drop of mineral oil prior to PCR.
Amplification of microdissected cDNAs

For OsA-Cl microdissections, the 5 μl collection drop with the microdissection material was pre-amplified for 15 cycles of 1 min 94 °C, 1 min 52 °C and 1 min 72 °C. The volume was increased to 60 μl with 1.25 U Taq polymerase, 200 μM of each dNTP, 0.25 μM catch A primer and amplified for 20 more cycles. A 10 μl aliquot from the first PCR was used in a 50 μl second round PCR for 30 more cycles as described but with 2.5 U Taq polymerase and 0.5 μM catch A. For TX3868 microdissections, the dissected hsr's and bound cDNAs were transferred to a PCR tube and amplified by 50 cycles of PCR (1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C) with 2.5 U Taq polymerase.

Cloning of PCR products

The microdissection selected cDNAs from OsA-Cl were prepared for UDG cloning. Four μl of cDNA were amplified for 20 cycles using CUA catch A primer (5′CUAUCUACUAGAGTAAGTTTCAATTCTCTC 3′) under the PCR conditions for stock cDNA amplification described above. The PCR products were purified with a Quiquick-spin column (Quiagen) and ethanol precipitated. Fifty ng of cDNA were mixed with 50 ng of UAGpUC19 (pUC19 PCR amplified for UDG cloning with UAG containing primers), incubated with uracil DNA glycosylase, and digested with SfiI, ligated into pBluescript and transformed in DH5α cells as described by Klein et al. (30).

Southern and Northern blot analysis

Isolation of high molecular weight DNA from cell cultures was performed according to standard protocols. Southern and Northern hybridization was carried out according to standard protocols. For the amplification and expression studies, 50 ng clone insert was gel-purified with a QUIquick-spin column (Quiagen) and ethanol precipitated. Fifty ng of cDNA were mixed with 50 ng of stock cDNA amplification described above. The PCR products were arrayed in 96 well plates for replica plating, long term storage at −80 °C and for insert isolation through 30 cycle PCR with catch A primer.

The selected cDNAs from TX3868 were purified with PrimerEraserQuick (Stratagene), phosphorylated, self-ligated, digested with SacII, ligated into pBluescript and transformed in DH5α cells as described by Klein et al. (30).

ACKNOWLEDGMENTS

The excellent assistance of D. Leja with preparation of the illustrations is gratefully acknowledged. This work was in part supported by a Grant from the Wilhelm Sander Stiftung (93.058.1) and by a Grant from the Deutsche Forschungsgemeinschaft (Me 917/2–2). Ulrike Fischer was a recipient of a grant from the Landesgraduierten Förderungs program.

REFERENCES