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Direct isolation of genes encoded within a homogeneously staining region by chromosome microdissection

YAN A. SU, JEFFREY M. TRENT, XIN-YAN GUAN, AND PAUL S. MELTZER*

Laboratory of Cancer Genetics, National Center for Human Genome Research, National Institutes of Health, Building 49, Room 4A10, 9000 Rockville Pike, Bethesda, MD 20892

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ABSTRACT **Identification of genes involved in recurring chromosome rearrangements has provided significant insight into the molecular basis of malignancy. We describe here a strategy combining chromosome microdissection and hybrid selection for the direct isolation of chromosome region-specific genes. We modeled this strategy by using sequences recovered from the microdissection of a homogeneously staining region to allow isolation of genes that were overexpressed and present at high copy number within the homogeneously staining region, including the direct isolation of two genes encoded within a 12q homogeneously staining region found in the osteosarcoma cell line OsA-CL. Although first applied to amplified genes, this strategy should be applicable to the isolation of cDNAs from any chromosomal region.**

Molecular examination of chromosome rearrangements can greatly facilitate the identification of human disease genes, particularly those associated with malignancy (1). We have recently developed a technique (termed micro-FISH, where FISH is fluorescent *in situ* hybridization) (2) that combines chromosome microdissection and FISH and can rapidly generate chromosome-region-specific probes. We now demonstrate that hybrid selection can be used in combination with chromosome microdissection to isolate transcribed sequences associated with chromosomal rearrangements.

A variety of different techniques have been developed to identify genes in mammalian genomes including: (*i*) restriction enzyme mapping of CpG islands (3); (*ii*) the use of interspecies sequence homology (3–5); (*iii*) detection of genomic clones with radiolabeled cDNAs (6, 7); (*iv*) cDNA library screening with yeast artificial chromosome (YAC) DNA (8, 9); (*v*) isolation of expressed species-specific sequences from somatic cell hybrids (10); and (*vi*) exon trapping and amplification from cells transfected with shuttle vectors carrying human genomic fragments (11–13). Additional strategies have utilized YAC or cosmid DNA attached to a membrane or to streptavidin-coated magnetic beads for hybridization to linkered cDNA. Hybrid-selected cDNAs are then recovered by PCR (14–17), leading to a 10^2- to 10^4-fold enrichment of YAC or cosmid-insert-specific cDNAs (15, 16). Although hybrid selection provides a significant advance (especially in enrichment), it requires the necessary chromosome-region-specific YACs or cosmids.

We now describe a technique that combines chromosome microdissection and cDNA hybrid selection to eliminate the requirement for YAC or cosmid pools, while allowing the direct identification of region-specific genes. To model this approach, we selected a region of chromosome 12 that frequently undergoes amplification in human sarcomas (18–23). The osteosarcoma cell line OsA-CL carries a homogeneously staining region (HSR) encoding 10–15 copies of sequences derived from 12q13–15 (20). Several genes are known to map to this region, and it is likely that additional undescribed genes fall within it (21). An HSR was selected for our initial studies because the sequence complexity of the microdissected region is reduced relative to a normal chromosome. Additionally, biologically relevant genes encoded within the amplification unit were likely to be highly represented in an OsA-CL cDNA library. Using genomic sequences recovered by microdissection of the OsA-CL HSR for cDNA hybrid selection, we generated a cDNA sublibrary enriched for transcripts derived from the HSR and identified two uncharacterized genes.†

MATERIALS AND METHODS

Cell Culture and Isolation of RNA and DNA. Cell culture for cytogenetics was as described (24). The human osteosarcoma cell line OsA-CL (20) (kindly provided by Tom Look, St. Jude Children's Research Hospital, Memphis, TN) and the neuroblastoma cell line NGP-127 (25) (kindly provided by Garrett Brodeur, Children's Hospital of Philadelphia) were cultured in RPMI 1640 medium (GIBCO/BRL) supplemented with 10% (vol/vol) fetal bovine serum (HyClone) and 2 mM L-glutamine (GIBCO/BRL). DNA and RNA were isolated by standard methods (26, 37). Somatic cell hybrids NA10868 and NA10658 were obtained from the Coriell Institute (Camden, NJ).

Micro-FISH Technique. Chromosome microdissection, PCR amplification, and FISH were performed as described (4, 27). Twenty copies of the chromosome 12q13 HSR from OsA-CL were dissected and PCR-amplified. PCR amplification of the microdissected DNA was performed with the SN1 primer (5′-CGGGAGATCCGACTCGAG-3′). The reaction was cycled 5 times at 95°C for 1 min, 37°C for 1 min, and 72°C for 2 min and then 30 times at 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min, with the final extension at 72°C for 3 min.

cDNA Library Construction. An OsA-CL oligo(dT)-primed cDNA library was constructed in bacteriophage λ ZAPII as reported (28). To isolate phage DNA, a 100-μl phage suspension [5×10^6 plaque-forming units (pfu)/ml] was digested with proteinase K (1.0 mg/ml) at 50°C for 2 h and sequentially extracted with phenol, phenol/chloroform, and chloroform (38). DNA was precipitated from 0.3 M sodium acetate, pH 7.0/66% ethanol and dissolved in 20 μl of TE (10 mM Tris·HCl/1 mM EDTA, pH 8.0) prior to PCR amplification as described below.

PCR Amplification of the cDNA Library. To minimize preferential amplification of small inserts and to maximize cDNA sequence complexity, the cDNA library was amplified by an unbalanced PCR. A 100-μl PCR mixture contained 1 μg of phage DNA, 1.25 μM T3 primer (5′-ATTAACCCTCAC-

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Abbreviations: HSR, homogeneously staining region; FISH, fluorescence *in situ* hybridization; YAC, yeast artificial chromosome; pfu, plaque-forming unit(s).

*To whom reprint requests should be addressed.

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TAAAG-3'), all four dNTPs (each at 200 μM), 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris·HCl (pH 8.3), gelatin (0.1 mg/ml), and 5 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus). The reaction was heated at 94°C for 5 min and then incubated for 20 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 3 min with final extension at 72°C for 5 min. After phenol/chloroform extraction and ethanol precipitation, 2 μl (one-fifth) of the PCR product was amplified for 50 cycles as above with the addition of 2 μM T7 primer (5'-AATACGACTA-CATATAG-3'). In a separate, otherwise identical PCR, the reaction was carried out with the T7 primer first. The products of both unbalanced cDNA PCRs were pooled. The sizes of the amplified cDNA fragments ranged from 0.2 kb to 2 kb, with fragments of ≈0.5 kb predominating.

cDNA Selection. Sixty micrograms of amplified microdissected DNA in 600 μl of 20× SSC was boiled for 5 min and immediately chilled on ice. Three hundred microliters of this was evenly applied to one side of a nylon membrane (4.5 cm^2) moistened with 20× SSC. The membrane was then UV-irradiated, and the remaining 300 μl was applied to the other side. The membrane was hybridized at 68°C, first with 2 ml of hybridization solution (25 mM potassium phosphate, pH 7.4/5× SSC/5× Denhardt's solution) containing 20 μg of human denatured Cot-1 DNA (GIBCO/BRL) for 4 h and then with another 2 ml of the hybridization solution containing 350 μg of total OsA-CL RNA and 100 μg of denatured Cot-1 DNA for 4 h. Finally, the membrane was incubated at 68°C for 3 h or overnight in a fresh 2 ml of the hybridization solution containing 300 μg of denatured PCR-amplified OsA-CL cDNA and 100 μg of denatured Cot-1 DNA. At 20°C, the filter was washed in 200 ml of 2× SSC/0.1% SDS for three 10-min periods and in 200 ml of 0.1× SSC/0.1% SDS for two 10-min periods. At 42°C, the filter was washed in 200 ml of 0.1× SSC/0.1% SDS for 30 min. The filter was then placed in 8 ml of TE and boiled for 5–10 min. The solution was adjusted to a final concentration of 0.3 M sodium acetate/70% ethanol and left at −20°C overnight. The DNA was pelleted by centrifugation at 4°C for 15 min and resuspended in 4 μl of water for unbalanced PCR.

Labeling of Selected cDNA. Because each cDNA contained ≈100 bp of vector sequence, they were removed to optimize the signal-to-noise ratio during library screening. Selected cDNAs were amplified by PCR with 5'-biotinylated T3 and T7 primers. The biotinylated cDNAs (2 μg) were purified in a Centricon 30 (Amicon) and digested with *Eco*RI to release the inserts. The biotinylated vector sequences (and any undigested DNAs) were separated from the insert DNAs by using streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin, Dynal, Great Neck, NY). Ten microliters (100 μg) of magnetic beads were added to 2 μg of the *Eco*RI-digested/biotinylated DNAs in a 1.5-ml microcentrifuge tube containing 100 μl of BW buffer (10 mM Tris·HCl, pH 7.5/1 mM EDTA/1.0 M NaCl). The tube was rotated at room temperature for 30 min and then placed into a magnetic device (Dynal) for 1 min. The supernatant was transferred into a Centricon-30, and the beads were washed twice each with 100 μl of BW buffer. The washes were combined with the supernatant, concentrated, and desalted in the Centricon-30. To screen the cDNA library, the cDNA inserts (240 ng) were labeled by random priming.

Probes and Library Screening. Specific gene probes were as follows: for *CDK4*, a PCR product, pCDK4-PCR, prepared according to Khatib *et al.* (23); for *CHOP*, pBH1 (kindly provided by Nikki Holbrook, National Institute on Aging, Baltimore); for *MDM2*, pMDMc14-2 (kindly provided by Bert Vogelstein, Johns Hopkins University, Baltimore); for *GAPDH*, pHcGAP; for *MYF6*, Myf-6; for *A2MR*, LRP06 (pHcGAP, Myf-6, and LRP06 were obtained from the American Type Culture Collection). For screens with the mixed-hybrid-selected probes, probe concentration was 5 ng/ml. Filters were washed to a stringency of 0.5× SSC at 65°C.

Southern and Northern Blot Hybridization. Southern and Northern blot hybridizations were performed by standard methods (29). Probes were labeled by the random priming method (30) or PCR amplification (31). Filters were washed to a stringency of 0.1× SSC at 42°C.

Nucleotide Sequence Analysis. Phage cDNA inserts amplified by PCR with vector-specific primers were sequenced by the DNA Sequencing Core of The University of Michigan Human Genome Center (Ann Arbor). The current nucleotide databases were searched by the BLASTN and FASTA programs (32, 33) (April 26, 1994).

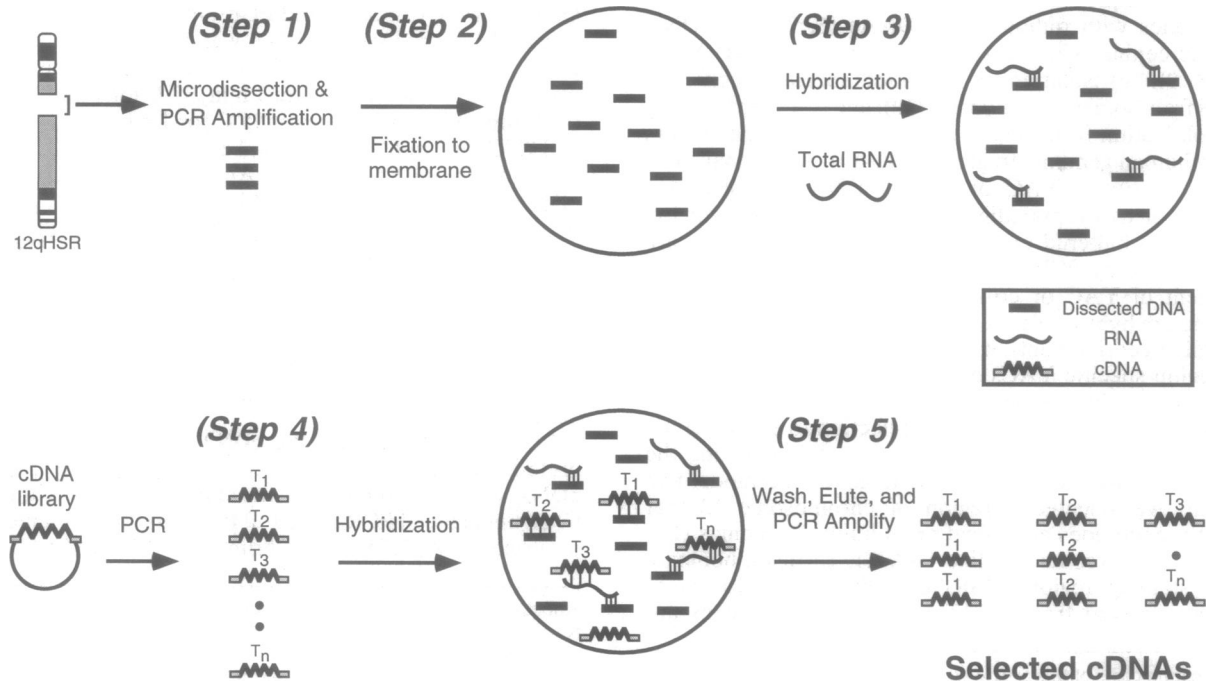

FIG. 1. Diagrammatic representation of the direct isolation of chromosome-region-specific cDNAs by microdissection (see text for details).

RESULTS

Experimental Strategy. Fig. 1 outlines the strategy for direct isolation of chromosome-region-specific cDNAs by chromosome microdissection and hybrid selection. In step 1 degenerate-oligonucleotide-primed PCR is used to amplify dissected DNA fragments. In step 2, the denatured amplified DNA is immobilized on a nylon membrane. In steps 3 and 4, the DNA-coated membrane is used to capture cDNAs by either DNA·cDNA hybridization or DNA·RNA·cDNA hybridization. The cDNA inserts are synthesized by PCR using primers directed against the vector sequences. During the hybridization (steps 3 and 4), human repetitive DNA is utilized to block nonspecific cross-hybridization. Finally, in step 5 after a stringency wash, the captured cDNA is amplified by PCR.

Isolation and Amplification of the HSR-Specific cDNAs. The process outlined in step 1 (Fig. 1) is illustrated in Fig. 2, where microdissection of the 12q HSR from OsA-CL metaphases (Fig. 2 *A* and *B*) is followed by PCR amplification of the dissected DNA (Fig. 2*C*). The PCR-amplified DNA from Fig. 2*C* was then labeled with biotin-16-dUTP in a secondary PCR and used for FISH against OsA-CL metaphase chromosomes and interphase nuclei (Fig. 2*D*). These results confirm that the dissected DNA fragments were derived from the 12q HSR. For hybrid selection, the HSR-specific microdissected DNA was immobilized to a nylon membrane and hybridized with total RNA and cDNA prepared from OsA-CL (Fig. 1). Selected cDNAs were eluted, amplified using an unbalanced PCR, and utilized to screen an OsA-CL cDNA library.

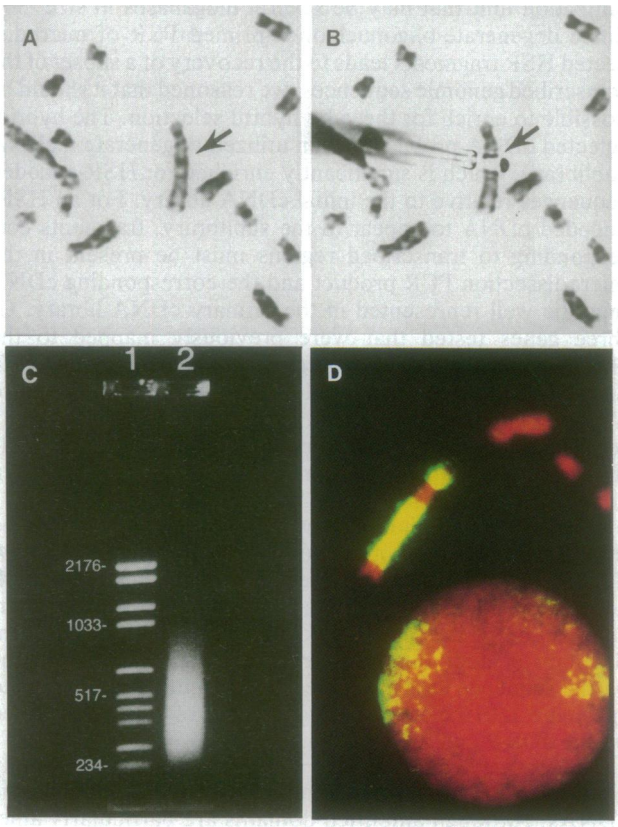

FIG. 2. Chromosome microdissection and PCR amplification of HSR DNA localized at 12q13 from an osteosarcoma cell line (OsA-CL). (*A*) G-banded partial metaphase spread indicating the HSR-bearing marker chromosome (arrow). (*B*) Microdissection of the HSR material (arrow) for PCR amplification. (*C*) PCR products from *B*, using the microdissected DNA as templates, and resulting in a smear of 200–1000 bp in lane 2. (*D*) A micro-FISH probe from *C* was generated and hybridized to metaphase and interphase nuclei from the OsA-CL cell line. The fluorescent signal is interrupted by a nonhybridizing region. This suggests the presence of a complex chromosome rearrangement in the genesis of this marker as confirmed with whole chromosome composite probes and hybridization of this micro-FISH probe to normal chromosomes (data not shown).

Generation of a Region-Specific cDNA Sublibrary. The hybrid-selected cDNAs were then used to generate a sublibrary enriched for sequences from the HSR. Approximately 900,000 plaques were screened. Three hundred seven positive plaques were picked, pooled, and replated. From this plating, ≈7500 well-separated plaques were screened again with the same probe yielding 735 positive plaques. These plaques were picked and pooled to constitute a sublibrary, which was then screened for both known and uncharacterized sequences from the 12q HSR.

Quantitative Comparison of the Initial Library and the Sublibrary. To assess the representation and enrichment of the sublibrary relative to the initial library, both libraries were screened with three genes known to be amplified in OsA-CL: *CDK4*, *CHOP*, and *MDM2*, as well as a closely linked but nonamplified control *A2MR* (α_2-macroglobulin receptor) (34). Probes for *CDK4* and *CHOP* hybridized to plaques in both the initial library and the sublibrary (Table 1). In the sublibrary, plaques hybridizing to *CDK4* and *CHOP* probes were present at 3.5 and 18.9%, respectively, in contrast to 0.029 and 0.022%, respectively, in the initial library. The *MDM2* probe, however, detected 0.009% plaques in the initial library but did not hybridize to any plaques in the sublibrary. The control gene *A2MR* was present at 0.011% in the initial library but was absent from the sublibrary. These results suggested that the sublibrary is enriched for a subset of the HSR-encoded genes.

Identification of Two HSR-Encoded Genes. We next evaluated the sublibrary for the presence of previously undescribed transcripts. Seventeen clones (designated λOS-*n*) were selected from the sublibrary because they did not hybridize to the known HSR-encoded genes. These 17 clones were divided into two groups by dot-blot hybridization. Hybridization to λOS-3 defined one group of 10 clones (λOS-1, 2, 3, 6, 7, 9, 11, 13, 22, and 23), and hybridization to λOS-4 defined a second group of 7 clones (λOS-4, 5, 10, 12, 17, 19, and 20). There was no cross-hybridization between these two groups. Further analysis revealed that λOS-9 (1.5 kb) and λOS-4 (1.1 kb) contain the largest inserts in each group and were subsequently used as probes to determine the relative abundance in the original library and the sublibrary. The sublibrary plaques hybridizing with λOS-9 and λOS-4 were present at 4.8 and 6.1%, respectively, whereas their frequencies in the initial library were 0.033 and 0.036%, respectively (Table 1).

We next determined the chromosomal origin of each clone and its amplification status in OsA-CL and NGP-127. Fig. 3 *A* and *B* shows the result of Southern blot hybridization using λOS-9 and λOS-4 probes, respectively, against *Eco*RI-digested genomic DNA from human placenta (lane 1), OsA-CL (lane 2), NGP-127 (lane 3), NA10868 (a human–Chinese hamster hybrid containing human chromosome 12 only, lane 4), and NA10658 (Chinese hamster line RJK88, lane 5). The λOS-9 probe hybridized to 11-kb and 9-kb bands, which were more intense in OsA-CL and NGP-127 than in placental DNA. In addition, both bands were present in the monochromosomal hybrid NA10868 DNA clearly demonstrating their chromosome 12 origin. λOS-4 probe detected a 9-kb band in OsA-CL and NGP-127 DNAs (Fig. 3*B*, lane 3) and an 11-kb band in OsA-CL DNA (Fig. 3*B*, lane 2). Of these two bands, only the 9-kb band was detected in placental (Fig. 3*B*, lane 1) and NA10868 (Fig. 3*B*, lane 4) DNAs, where it was much less intense than in OsA-CL and NGP-127 DNAs. Both bands were absent in the hamster DNA NA10658 (Fig. 3 *A* and *B*, lanes 5). Although the *Eco*RI fragments hybridizing to λOS-9 and λOS-4 were similar in size, these probes hybrid-

Table 1. Quantitative analysis of the HSR-specific genes in the initial library and the sublibrary

Gene	Initial library Positive/total, pfu/pfu	%	Sub-library Positive/total, pfu/pfu	%	Fold enrichment of sublibrary relative to initial library
Known					
CDK4	59/200,000	0.029	35/990	3.5	121
CHOP	11/50,000	0.022	110/582	18.9	859
MDM2	9/100,000	0.009	0/582	0	—
A2MR	11/100,000	0.011	0/520	0	—
Uncharacterized					
λOS-9	33/100,000	0.033	30/628	4.8	145
λOS-4	36/100,000	0.036	33/538	6.1	169

Positive vs. total pfu screened with a probe for the corresponding gene are shown. *A2MR* is located on 12q but is not amplified in OsA-CL.

ized to easily distinguished fragments in *Hin*dIII and *Bam*HI digests (data not shown). These results demonstrate that λOS-9 and λOS-4 probes recognize distinct genomic fragments derived from chromosome 12, which are amplified in both OsA-CL and NGP-127 cell lines.

Fig. 3 *C* and *D* shows the hybridization of λOS-9 and λOS-4 probes to total cellular RNA from OsA-CL and NGP-127 and the normal lymphoblastoid cell line TL9912. The λOS-9 probe detected a 3-kb band only in OsA-CL RNA (Fig. 3*C*, lane 1), whereas the λOS-4 probe revealed 5- and 2.5-kb bands in OsA-CL RNA (Fig. 3*D*, lane 1) and a 5-kb band in NGP-127 RNA (Fig. 3*D*, lane 2). These bands are absent in TL9912 RNA (Fig. 3 *C* and *D*, lane 3). Thus, as expected, both transcripts were expressed in OsA-CL. However, only transcripts recognized by λOS-4 were also detected in NGP-127.

We further characterized these two transcripts by partial DNA sequence analysis and database searches using the BLASTN and FASTA programs (32, 33). No significant identity was identified with the current GenBank, EMBL, or EST databases. Therefore, these sequences appear to represent two unidentified genes within the OsA-CL 12q HSR isolated by our chromosome microdissection hybrid selection strategy.

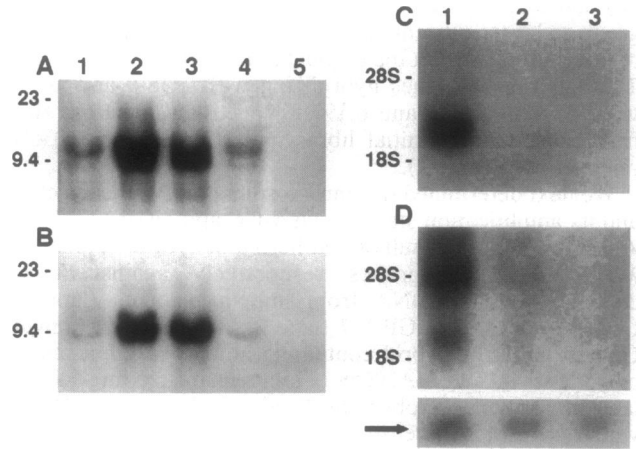

FIG. 3. Southern (*A* and *B*) and Northern (*C* and *D*) blot analyses with λOS-9 and λOS-4 probes. Distinct genomic amplified restriction fragments and transcripts are recognized by each probe. Lanes 1–5 contain 10 μg of *Eco*RI-digested DNA from human placenta, tumor cell lines OsA-CL and NGP-127, monochromosomal 12 hybrid NA10868, and hamster cell line NA10658, respectively. The blot was sequentially hybridized with λOS-9 (*A*) and λOS-4 (*B*). Northern blot analyses were with λOS-9 (*C*) and λOS-4 (*D*). Lanes 1–3 contain 10 μg of total RNA from OsA-CL, NGP-127, and lymphoblastoid line TL9912. The blot was reprobed for glyceraldehyde-3-phosphate dehydrogenase to control for loading error (arrow).

DISCUSSION

We have previously demonstrated the utility of chromosome microdissection for the characterization of DNA amplification in human tumors by FISH and for the construction of DNA microclone libraries (35). These libraries can serve as a source of entry point probes for the cloning and mapping of amplified regions. In this report we present a method for isolating cDNAs transcribed from an HSR that does not require the genomic cloning and characterization of an amplification unit that may be several megabases in size (36). Since degenerate-oligonucleotide-primed PCR of microdissected HSR fragments leads to the recovery of a subset of the transcribed genomic sequences, we reasoned that it should be possible to enrich for these by hybrid selection. The hybrid-selected cDNA probe was then utilized to generate a cDNA sublibrary, which is significantly enriched for HSR-encoded sequences relative to the initial cDNA library. For an HSR-encoded cDNA to appear in the sublibrary, fragments corresponding to transcribed regions must be present in the microdissection PCR product and the corresponding cDNA must be well represented in the primary cDNA library. Of three genes tested that were previously mapped to the chromosome 12q amplification unit in OsA-CL, two (*CDK4* and *CHOP*) were recovered in the sublibrary. A third (*MDM2*) was not present in the sublibrary but was also poorly represented in the primary library. In addition, it is possible that genomic sequences from *MDM2* may not have been adequately represented in the microdissected DNA.

Despite these limitations, two transcripts were identified from a limited screen of the sublibrary. Four genes (*CDK4*, *CHOP*, and the transcripts identified by λOS-9 and λOS-4) account for 33.4% of clones in the sublibrary. Further characterization of the remaining clones may result in the identification of additional HSR-encoded genes. Based on the partial sequence data available, it is currently not possible to speculate as to the biological significance of the genes defined by the λOS-9 and λOS-4 cDNAs.

The strategy reported here is a powerful tool for rapidly generating a collection of chromosome-region-specific cDNAs. Although amplified domains are particularly amenable to analysis because of their high copy number and level of gene expression, this approach is potentially applicable to other regions of chromosomal DNA isolated by microdissection. Hybrid-selection strategies using YACs or cosmids (20–23) may be preferable for identifying transcripts within regions that have been defined by specific markers. However, microdissection/hybrid selection may expedite the identification of transcripts associated with chromosome abnormalities in malignancy. The present study illustrates that transcripts can be isolated from a cytogenetically defined region of gene amplification without the intermediate steps of genomic cloning. This ap-

proach may be of particular value in the analysis of HSRs that are frequently highly complex and may include multiple chromosomal components, not all of which may contribute expressed sequences. Microdissection can also be applied to other tumor-associated aberrations, particularly translocations that are associated with high levels of gene expression.

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