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Analysis of gene amplification in human tumor cell lines

(oncogenes/drug resistance/in-gel renaturation/*MYC*)

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ABSTRACT **Oncogene amplification has been observed in various primary tumors and tumor-derived cell lines. In several types of cancer, amplification of specific oncogenes is correlated with the stage of tumor progression. To estimate the frequency of gene amplification in other tumor types and to determine whether the ability to grow *in vivo* is associated with gene amplification in tumor cell lines, we have developed a modified version of the in-gel renaturation assay that detects human DNA sequences of unknown nature amplified as little as 7- to 8-fold. This assay was used to screen 16 cell lines derived from various solid tumors and leukemias. Amplified DNA sequences were detected in only one cell line, Calu-3 lung adenocarcinoma. This cell line was found to contain co-amplified *NGL* (formerly termed *neu*) and *ERBA1* oncogenes. However, when one of the amplification-negative cell lines, PC-3 prostatic carcinoma, was selected for *in vivo* growth in nude mice, amplified DNA sequences became detectable in these cells. The amplified sequences included the *MYC* oncogene, which showed no amplification in the parental cell line but was amplified 10- to 12-fold in the *in vivo*-selected cells. *MYC* amplification may, therefore, provide tumor cells with a selective advantage specific for *in vivo* growth.**

Gene amplification is one of the most common types of genetic changes occurring in human neoplasia. In most cases, this phenomenon was studied by Southern hybridization with cloned probes corresponding to known cellular oncogenes, at least a dozen of which were found to be amplified in various human tumors (1–11). Oncogene amplification was observed in tumor-derived cell lines as well as in primary tumor tissues, suggesting that increased function of these genes provides tumor cells with a selective advantage for growth *in vivo* and *in vitro*. For several tumors such as neuroblastomas, breast carcinomas, and small cell lung carcinomas, consistently occurring amplification of specific oncogenes was shown to correlate with the stage of tumor progression, suggesting that oncogene amplification may be used as a prognostic marker (5–11). However, for most types of tumors no consistent association has yet been found with amplification of a particular oncogene. To evaluate the role of gene amplification in the development of such tumors, hybridization of tumor DNA samples with almost 40 oncogene probes does not seem feasible. Furthermore, such assays would miss amplification of any presently unknown genes that may also be amplified in human tumors.

The method of in-gel DNA renaturation was developed to detect amplified DNA sequences of unknown nature (12, 13). In this procedure, genomic DNA is digested with a restriction enzyme, labeled with T4 DNA polymerase, and separated by electrophoresis in an agarose gel. DNA in the gel is then enriched for restriction fragments present in multiple copies by two rounds of in-gel denaturation, renaturation, and digestion with single-strand-specific S1 nuclease. Repeated fragments that are normally present in genomic DNA, as well as the fragments derived from selectively amplified DNA regions (amplicons), are then detected by autoradiography. This technique has been used to detect and clone amplified genes associated with drug resistance in mammalian and insect cells (14–16) and to analyze amplified DNA in human tumor cells (17–20). Kinzler *et al.* (21) used this approach to identify and clone a gene (*GLI*) that was amplified and expressed in a malignant glioma.

The applicability of in-gel DNA renaturation as a general screening procedure for gene amplification has been limited by the sensitivity of the technique, which in our hands cannot detect fewer than 20–25 copies of an amplified DNA fragment per haploid human genome or 40–50 copies per mouse genome. To increase the sensitivity of detection of amplified DNA in mouse cells, we have developed a modified procedure, in which unlabeled repeated fragments remaining in the gel after in-gel renaturation are transferred onto a Southern blot and hybridized to a short interspersed repeated sequence, the SINE probe. The SINE probe is selected so that it will hybridize to a subset of fragments derived from any amplicon but not to most normal repeated fragments, which are derived from tandemly repeated (satellite-type) or long interspersed repeated sequences (LINEs). When B2 repeated element was used as a SINE probe for mouse DNA, this procedure allowed us to detect DNA sequences amplified 10- to 15-fold (22).

In the present study we have modified the above technique to make it applicable to the analysis of gene amplification in human DNA. The modified procedure allows for detection of human DNA sequences amplified as little as 7- to 8-fold. This technique was used to screen cell lines derived from various human tumors and leukemias for the presence of amplified DNA. Only 1 out of 16 *in vitro*-grown cell lines was positive for gene amplification. This cell line, Calu-3 lung adenocarcinoma, was found to contain co-amplified *NGL* (formerly termed *neu*) and *ERBA1* genes. However, when one of the amplification-negative cell lines, PC-3 prostatic carcinoma, was selected for the ability to grow in nude mice, the *in vivo*-selected cells were found to contain amplified DNA sequences that included the *MYC* gene. This gene was not amplified in the unselected PC-3 cells, suggesting that *MYC* amplification may provide tumor cells with a selective advantage specific for *in vivo* growth.

MATERIALS AND METHODS

Cell Lines and DNA Clones. KB-3-1 and multidrug-resistant KB-C4 cells were obtained from M. M. Gottesman and I. Pastan (National Cancer Institute, Bethesda, MD); CEM, YT, Jurkat, and Daudi cell lines were a gift of K. Teshigawara (Dartmouth College, Hanover, NH); HL-60, B-II, and ML-2 cell lines were obtained from S. Murao (Argonne National Laboratories, Argonne, IL); all the other cell lines were received from American Type Culture Collection. The cell

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lines were propagated under the conditions recommended by the suppliers.

For *in vivo* selection, PC-3 prostatic carcinoma cells were inoculated into nude mice subcutaneously and passaged by *in vivo* transplantation. The cell line 431-P was established after 17 generations of direct subcutaneous passage and maintained in culture for 15 or 16 passages. After 9 generations of subcutaneous passage, a spontaneous metastasis to the right axillary lymph node was obtained. The metastatic tissue was minced and again transplanted subcutaneously. For the next two passages, spontaneous mediastinal lymph node metastases were observed, and the metastatic tissues were used for subcutaneous transplantation. In the third passage, a left axillary lymph node metastasis was obtained and a cell line, designated C505-N, was established from this metastasis. C505-N cells were maintained in culture for five passages prior to analysis.

Blur-8 (23) was used as an *Alu* repeat clone. *Kpn* and *O* repeat clones (24) were obtained from C. W. Schmid (University of California, Davis, CA). A 0.45-kilobase (kb) *Pst* I fragment of the pAE plasmid (25) was used as the v-erbA probe. A 0.42-kb *Bam*HI fragment of neuc(t)/sp6400, derived from neuc(t)34 (26) was used as the NGL probe. The 1.6-kb *Cla* I–*Eco*RI fragment of the plasmid pHSR-1, containing the third exon of the human *MYC* gene (27), was used as the MYC probe. pAT4.6 (28) was used as an α_1-antitrypsin probe. Inserts of all clones were gel-purified prior to labeling.

Detection of Amplified DNA. Cellular DNA was extracted by standard procedures (29). After digestion with *Hin*dIII, DNA was purified by phenol extraction and ethanol precipitation. Digested DNA (15 μg) was electrophoresed in a 1% agarose gel (size, 20 × 40 × 0.4 cm; 0.9-cm wide wells) and subjected to two cycles of in-gel renaturation and digestion with S1 nuclease, as described (13). After the second S1 nuclease digestion, the gel was washed twice with 25 mM NaH_2PO_4 (pH 6.5; phosphate buffer) for 30 min per wash. The gel was then placed upside down in a casting tray, and melted 1.5% (wt/vol) agarose in phosphate buffer was poured along the edges of the gel to make the gel adhere to the surface of the tray. Once agarose at the edges of the gel solidified, an additional volume of 1.5% agarose in phosphate buffer was cast on top of the original gel to form an extra 6-mm gel layer. The gel "sandwich" was then trimmed to the size of 25 cm × 15 cm to fit in the electroblotting chamber (model TE42, Hoefer, San Francisco). The chamber was filled with 5 liters of phosphate buffer, and the gel sandwich was placed into the chamber vertically without a supporting cassette. DNA was then electrophoresed across the gel sandwich for 150 min at 1.1 A (Fig. 1). The required duration of electrophoresis may vary depending on the type of electroblotting apparatus. This duration can be determined in preliminary experiments by using a standard set of markers; the optimal time corresponds to the point when the 1.0-kb size marker is completely removed from the gel sandwich. DNA from the gel was then transferred onto a nylon membrane (Biotrans, ICN) by capillary blotting (30). The direction of blotting was opposite to the direction of second electrophoresis. Amplified fragments containing *Alu* sequences were detected by hybridization with 0.5–1.5 × 10^8 dpm of the gel-purified 300-base-pair (bp) insert of the Blur-8 clone, as described (22).

RESULTS

Assay for DNA Amplification in Human Cells. As a model system for detection of amplified sequences in human DNA, we used KB-3-1, an epidermoid carcinoma cell line, and its multidrug-resistant derivative KB-C4 (31), which contains the amplified *MDR1* gene (32). The *MDR1* gene copy number in these cells was determined by slot blot hybridization with the pMDR1 probe (32) by using an α_1-antitrypsin probe as a single-copy control (data not shown). The level of *MDR1* amplification in KB-C4 was determined to be 30-fold, in agreement with our reported estimates (32). In the initial experiments with this model system, *Hin*dIII-digested genomic DNA was transferred onto a Southern blot immediately after in-gel renaturation and then hybridized to clones containing interspersed repeated sequences *Alu* (33) or *Kpn* or *O* (24). However, both Alu and Kpn probes produced a very high background smear. The O probe, on the other hand, gave no background, but it hybridized to only one band in KB-C4 DNA (data not shown). Since the frequency of *O* repeats has been estimated at only 4500 copies per human genome (24), it seemed likely that many amplicons would not contain *O* repeats and, therefore, would remain undetectable with that probe.

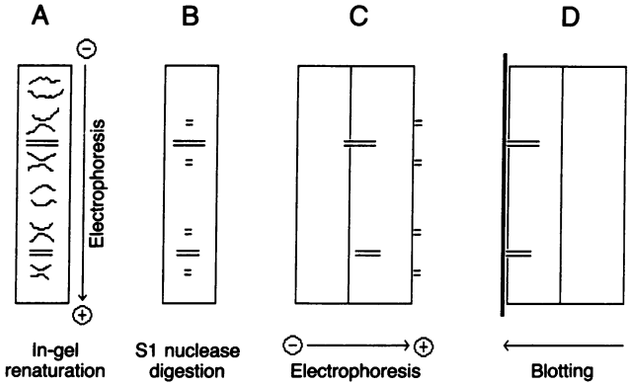

FIG. 1. Schematic representation of the procedure for detection of amplified DNA. A lengthwise cross-section of an agarose gel slab is shown. (*A*) After electrophoresis in a 1.0% agarose gel, restriction fragments of genomic DNA are denatured and then renatured in the gel. Repeated and amplified fragments, present in the gel at a higher local concentration than single-copy fragments, reanneal preferentially. Some reannealing also occurs between different single-copy fragments containing highly repeated *Alu* sequences. (*B*) Digestion with S1 nuclease destroys single-stranded DNA sequences, while the reannealed restriction fragments and short (≈300 bp) *Alu* duplexes remain in the gel. (*C*) After two rounds of in-gel renaturation and S1 nuclease digestion, an additional layer of 1.5% agarose is cast on top of the original gel. The gel sandwich is placed vertically into an electroblotting apparatus, and DNA is electrophoresed across the sandwich until the *Alu* duplexes are eluted from the gel. (*D*) Repeated and amplified fragments are transferred onto a nylon membrane by capillary blotting, and *Alu*-containing fragments are detected by hybridization with a cloned *Alu* repeat.

We then modified the procedure so as to decrease the background observed with the Alu probe. The principle of this modification is illustrated in Fig. 1. We have reasoned that the background results from partial renaturation of *Alu* sequences that are contained within single-copy rather than amplified restriction fragments. The frequency of *Alu* repeats was estimated to be 9 × 10^5 per human genome, or one per every 3 kb of DNA (34). At this frequency, the concentration of *Alu* sequences in the gel would be high enough to provide for their partial reannealing even when the rest of *Alu*-containing restriction fragments is composed of unique sequences that remain single-stranded (Fig. 1*A*). After digestion with S1 nuclease, such *Alu* sequences would form short (≈300 bp) double-stranded fragments (Fig. 1*B*). These short S1 nuclease-resistant duplexes would hybridize with the Alu probe after Southern transfer, resulting in the observed high background. It is possible, however, to separate the short *Alu* duplexes from reannealed restriction fragments that are >1.5 kb in size, as shown in Fig. 1*C*. For this purpose, after in-gel renaturation and S1 nuclease digestion, an additional layer of 1.5% (wt/vol) agarose is cast on top of the original gel. The gel sandwich is then placed vertically into an electroblotting

apparatus, and DNA is electrophoresed across the gel sandwich at 90° to the original direction of electrophoresis (Fig. 1*C*). Electrophoresis is continued until the short *Alu* duplexes are completely removed from the gel without concurrent losses of restriction fragments that are at least 1.5 kb long. After electrophoresis, the full-length repeated and amplified fragments that remain in the gel are transferred onto a nylon membrane by capillary blotting (Fig. 1*D*), and the fragments that contain *Alu* sequences are then detected by hybridization with an Alu probe.

When this procedure was applied to DNA from multidrug-resistant KB cells, a large number of amplified fragments could be readily visualized in KB-C4 cells (Fig. 2*A*, lane 5), but not in the parental KB-3-1 cells, where only a few bands were detectable (Fig. 2*A*, lane 1). Band patterns indistinguishable from KB-3-1 were also observed in DNA from leukocytes of four unrelated individuals (Fig. 2*A*, lanes 6–9). This result was at marked contrast with a high frequency of polymorphisms observed in human repeated fragments detected by the original in-gel renaturation technique (13) or in mouse repeated fragments containing B2 SINE element (22). This lack of polymorphism should simplify screening of tumor samples for the presence of amplified DNA by making it unnecessary to use normal tissues from the same individual as a control.

To estimate the sensitivity of this method for detection of amplified DNA, we have prepared mixtures of KB-C4 and KB-3-1 DNA, corresponding to 7.5, 10, and 15 copies of the *MDR1* gene per haploid genome (Fig. 2*A*, lanes 2–4). Some of the amplified bands could be detected even in the mixture corresponding to 7.5 copies of *MDR1* (Fig. 2*A*, lane 2), indicating that the technique is sensitive enough to detect amplified DNA in cells containing as few as 7 or 8 copies of the amplified gene, and possibly even fewer. Since the bands detected with the Alu probe were not necessarily derived from the *MDR1* gene, there is a theoretical possibility that the bands detected with the Alu probe have a higher level of amplification than *MDR1*. This is unlikely, however, since a *MDR1* cDNA probe hybridized to the same blot as well or better than the Alu probe (data not shown).

Analysis of Gene Amplification in Tumor Cell Lines. The above procedure was used to analyze gene amplification in 10 solid tumor-derived cell lines, including Calu-3 and SK-LU-1 lung adenocarcinomas, SK-N-SH neuroepithelioma, SK-CO-1 colon adenocarcinoma, HT-144 malignant melanoma, Capan-1 pancreatic adenocarcinoma, DU145 and PC-3 prostatic carcinomas, Saos-2 osteosarcoma, and SK-LMS-1 leiomyosarcoma. We have also analyzed six leukemia-derived cell lines, including CEM, YT, and Jurkat acute lymphoblastic leukemias, Daudi Burkitt lymphoma, B-II acute promyelocytic leukemia, and ML2 acute myeloblastic leukemia. To the best of our knowledge, none of these cell lines have been reported to contain amplified genes, and none of them belonged to those tumor types where amplification of specific oncogenes is known to occur at high frequency. As a positive control, we used HL-60 leukemia cells, which are known to contain 8–16 copies of the amplified *MYC* oncogene (35, 36). Gene amplification in HL-60 cells was readily detectable by our technique (Fig. 2*B*, lane 1). DNA from one of the other cell lines, Calu-3 lung adenocarcinoma, also contained multiple amplified fragments (Fig. 2*B*, lane 2). No amplified bands were detectable in any of the other cell lines (data not shown). To determine if amplified DNA sequences in Calu-3 cells included any of the known oncogenes, Calu-3 DNA was analyzed by Southern hybridization with *MYC*, *NMYC*, *HRAS*, v-Ki-*ras*, *NRAS*, v-*erbA*, v-*erbB*, and *NGL* (or *ERBB2*) gene probes. By this assay, *NGL* and *ERBA1* oncogenes were found to be amplified in Calu-3 cells (Fig. 3). The degree of *NGL* amplification in Calu-3 was estimated to be ≈40 copies per haploid genome, and the *ERBA1* gene was amplified ≈16-fold.

***MYC* Amplification Correlates with *in Vivo* Growth in PC-3 Cell Line.** We were also interested to determine if the ability of tumor cell lines to grow *in vivo* is associated with amplification of any specific genes. For this purpose, PC-3 prostatic carcinoma cells (37), which were negative for gene amplification in our assays, were selected for the ability to give rise to tumors in nude mice. Ohnuki *et al.* (38) observed by karyotypic analysis that growth in nude mice results in selection of a specific subpopulation of PC-3 cells, and Ware *et al.* (39) reported that an *in vivo*-grown subline of PC-3 acquired double minutes, cytogenetic markers of gene amplification (3, 4), that were absent in the original cell population. We established several cell lines from PC-3 cells after

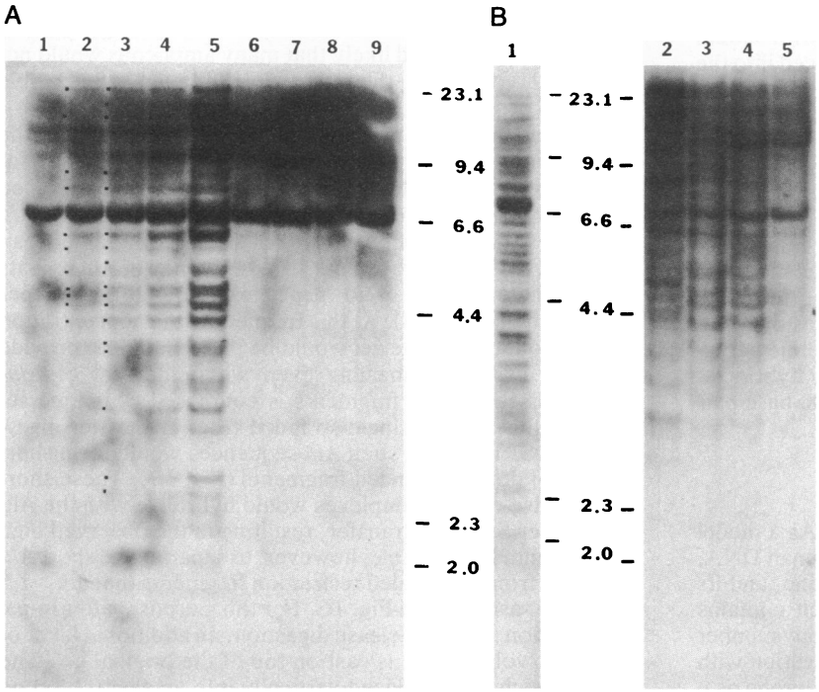

FIG. 2. Detection of amplified DNA in human cell lines by modified in-gel renaturation. Each lane contains 15 μg of *Hin*dIII-digested DNA from various cell lines. *Hin*dIII fragments of λ phage DNA were used as size standards. (*A*) Detection of amplified DNA in multidrug-resistant KB cells. DNA was extracted from the parental KB-3-1 cells (lane 1) and from multidrug-resistant KB-C4 cells containing 30-fold amplification of the *MDR1* gene (lane 5). KB-3-1 and KB-C4 DNAs were mixed to yield 7.5- (lane 2), 10- (lane 3), and 15-fold (lane 4) amplification of *MDR1*. Lanes 6–9 contain DNA from leukocytes of four unrelated normal individuals. Amplified fragments in lanes 2 and 3 are indicated with dots. (*B*) Detection of amplified DNA in tumor cell lines. DNA was extracted from HL-60 (lane 1), Calu-3 (lane 2), *in vivo*-selected PC-3 isolates C505-N (lane 3) and P-431 (lane 4), and from parental PC-3 cells maintained in culture (lane 5).

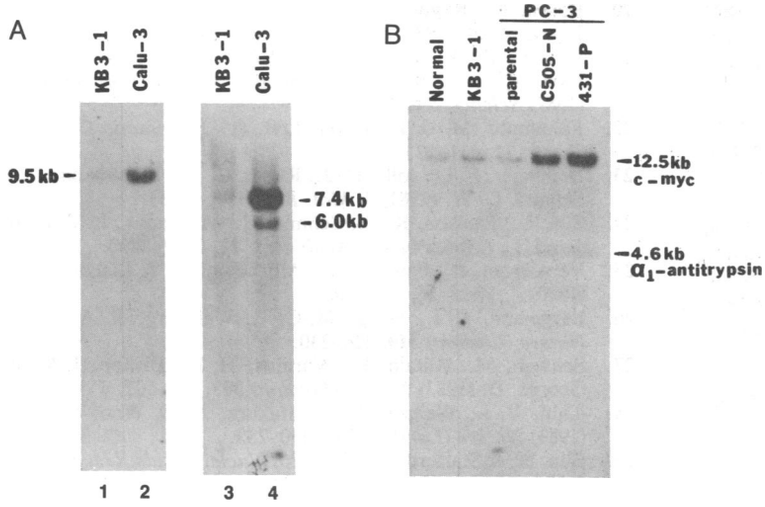

FIG. 3. Oncogene amplification in human tumor cell lines. Each lane contains 4 μg of DNA digested with *Eco*RI. An α_1-antitrypsin probe was added to the *MYC* probe as a single-copy control. The *NGL* and *ERBA* copy numbers in Calu-3 cells were estimated by densitometry and serial dilutions (data not shown). The 6.0-kb band hybridizing to the NGL probe in Calu-3 DNA may correspond to *NGL* gene sequences rearranged in the course of amplification; this band did not hybridize to the related v-*erbB* probe (data not shown). The copy number of *MYC* in various PC-3 isolates was determined by densitometry; to account for variations in loading and transfer, the intensity of the *MYC* band in each lane was normalized for the intensity of the control α_1-antitrypsin band in the same lane. (*A*) DNA was isolated from KB-3-1 (lanes 1 and 3) and Calu-3 (lanes 2 and 4) and hybridized with ERBA (lanes 1 and 2) and NGL (lanes 3 and 4) probes. (*B*) DNA was isolated from normal and KB-3-1 cells and various PC-3 isolates and probed with a MYC probe and an α_1-antitrypsin probe.

propagating these cells in nude mice. The cell line 431-P was established after multiple subcutaneous passages, and the line C505-N was isolated from a lymph node metastasis.

When DNA from various isolates of PC-3 cells was assayed for gene amplification, amplified bands were detected both in 431-P and in C505-N, but not in the original stock of unselected PC-3 cells (Fig. 2*B*). DNA from PC-3 isolates was then analyzed with various oncogene probes, and the *MYC* gene was found to be amplified (Fig. 3). The degree of *MYC* amplification was estimated as 10-fold in C505-N and 12-fold in 431-P cells, indicating no correlation between *MYC* amplification and lymph node metastasis. Amplification of *MYC*, however, appears to correlate with selection for *in vivo* growth, since no amplification was found in the unselected PC-3 cells.

DISCUSSION

We have described a modified in-gel renaturation technique that detects human DNA sequences of unknown nature amplified as little as 7- to 8-fold. The high frequency of *Alu* repeats in human genomic DNA makes it exceedingly unlikely that any amplicons, usually 10^2–10^3 kb long (3, 4), would be undetectable with the Alu probe. With the help of this method, we were able to detect gene amplification in several tumor cell lines that were subsequently found to contain 10- to 40-fold amplification of various oncogenes. Although the modified method would not detect very low levels of gene amplification, a high proportion of the reported cases of oncogene amplification in both primary tumor specimens and tumor-derived cell lines involve amplification levels that should be high enough for detection by our technique. For example, of the 53 cases of *NGL* gene amplification in breast cancer tissues, 27 had the gene amplified >5-fold (5), and out of 24 cases of *NMYC* amplification in neuroblastoma samples, only 3 had a <5-fold level of amplification (6). The method described in this article has been used to demonstrate gene amplification in primary biopsy samples of two ovarian carcinomas (M.F., R. Estensen, L. Sha, and I.B.R., unpublished data).

This technique was used to screen 16 human cell lines in which no gene amplification had been reported and that were derived from the types of tumors and leukemias that are not commonly associated with amplification of any specific genes. Gene amplification was detected in one of these cell lines, Calu-3 lung adenocarcinoma. The amplified DNA sequences in Calu-3 cells were found to include *NGL* and *ERBA1* oncogenes. Co-amplification of NGL and *ERBA1*, which are linked in the genome, was reported in several breast carcinomas (40); but, to the best of our knowledge, this is the first example of amplification of these genes in a lung adenocarcinoma. Interestingly, whereas *NGL* was amplified ≈40-fold in Calu-3 cells, only 16-fold amplification of *ERBA1* was detected in these cells. Differential amplification of DNA sequences within the same amplicon has been described in other systems, with the essential gene usually characterized by the highest degree of amplification (32, 41, 42). By analogy with these results, one could speculate that the *NGL* gene but not *ERBA1* may be essential in Calu-3 cells.

The low apparent frequency of gene amplification observed in the established tumor cell lines does not necessarily mean that gene amplification would be rare in primary tumor tissues. It is feasible that in some tumors the amplified genes provide tumor cells with a selective advantage for growth in the organism but not in tissue culture. Since gene amplification is frequently unstable (3, 4), such genes would likely be lost upon prolonged cultivation of tumor cells. In this regard our finding of *MYC* amplification associated with *in vivo* growth of PC-3 prostatic carcinoma cells is of particular interest. Increased amplification of the *MYC* gene in tumors induced in nude mice has been reported (43) for SW 613-8 breast carcinoma cell line, where the level of amplification increased from 5- to 10-fold in the unselected cells to the levels between 20-fold and 60- to 90-fold in cell lines derived from nude mouse tumors. Increased c-*myc* amplification may also be associated with *in vivo* growth in mouse SEWA osteosarcoma cells (44, 45). In the above examples, however, c-*myc* was significantly amplified prior to injection of cells into the nude mice, whereas in our case *MYC* amplification became apparent only in cells passaged *in vivo*. *In vivo* amplification of *MYC* in PC-3 cells could result from selection of a small number of preexisting cells carrying the amplified *MYC* gene, or it could reflect an amplification event that occurred during *in vivo* growth. In either case, it appears likely that *MYC* amplification provided the tumor cells with a selective advantage specific for *in vivo* growth. The mechanism by which *MYC* amplification provides a selective advantage *in vivo* remains to be determined.

The technique described in this article can be used as a general assay for the presence of amplified DNA sequences in human tumors. Once such sequences are detected, DNA from positive samples can be tested for amplification of known oncogenes. It is probable that other genes amplified in human tumors, including those that have not yet been identified, would be amenable to detection with this procedure. Established techniques can then be used to identify and clone the essential region of the amplicon (13). As a screening procedure, the modified method has several advantages over the earlier techniques. This method is about three times more sensitive than the original in-gel renaturation procedure. The

modified technique also avoids the laborious and artifact-prone step of labeling each individual DNA preparation with T4 DNA polymerase. Furthermore, since the Alu probe is specific for human DNA, this technique is proof to artifacts that may result from bacterial or mycoplasma contamination of cell lines or from contamination of human with mouse DNA when human tumor cells are grown in nude mice.

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