Varying Degrees of Amplification of the N-ras Oncogene in the Human Breast Cancer Cell Line MCF-7

Kathryn A. Graham, Carol L. Richardson, Mark D. Minden,1,2 Jeffrey M. Trent,3 and Ronald N. Buick1,4

Ontario Cancer Institute and the Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada M4X 1K9 [K. A. G., C. L. R., M. D. M., R. N. B.], and Cancer Center, University of Arizona, Tucson, Arizona [J. M. T.]

ABSTRACT

The oncogene N-ras has been found to be amplified (=20 copies) in the human breast carcinoma cell line MCF-7. The amplified sequences have been localized to a marker chromosome by in situ hybridization. Sublines of MCF-7, serially passaged in different laboratories, have marked variation in the degree of N-ras amplification. The differing degrees of amplification of N-ras are further evidence of heterogeneity within MCF-7 subclones. The phenomenon may not have general relevance for breast cancer, since other breast cancer cell lines and DNA from patient biopsies failed to show evidence of N-ras amplification.

INTRODUCTION

The MCF-7 cell line was derived by Soule et al. (25) from a malignant pleural effusion obtained from a patient with metastatic carcinoma of the breast. This line has since been passaged independently in several laboratories, and there have been reports that various sublines of MCF-7 now display marked heterogeneity for several characteristics. Variations have been demonstrated in the antigenic phenotype between MCF-7 strains from different laboratories (8). Hand ef al. (8) also describe clones derived from one MCF-7 cell line which bear different and unstable phenotypes. Katzenellenbogen et al. have reported differences in estrogen responsiveness (10) and in plasminogen activator activity (17) between two lines of MCF-7 obtained from different sources. In addition, a marked instability in the karyotype of MCF-7 has been reported (32) and may underlie clonal variation in growth properties (21).

It is now understood that, in certain circumstances, abnormal expression of cellular oncogene (c-onc) sequences (or expression of mutated c-onc sequences) is involved in the malignant process (13). A number of cases of c-onc gene amplification have been reported in human tumor cell lines (1, 11, 12, 16, 20), although it is not clear at this time whether such amplification is causally related to either tumor development or progression (19). No c-onc amplification has been reported in MCF-7, although an unidentified activated oncogene has been detected by DNA transfection to NIH/3T3 cells (14). We report here an analysis of N-ras amplification in sublines of MCF-7.

MATERIALS AND METHODS

Cell Lines. We received a sample of the MCF-7 cell line [MCF-7(KO)] from Dr. C. K. Osborne (San Antonio, TX) in 1982. This line has been cryopreserved in liquid nitrogen and has been thawed and passaged minimally for these experiments. We have also passaged this line continuously in our laboratory and have designated it MCF-7(OCI). A second sample was recently obtained from Dr. Osborne and designated MCF-7(KO-2). Analysis of this line was made immediately on receipt. We were also provided by Dr. Osborne with another sample of MCF-7, which had been passaged previously by Dr. S. Shaffe (NIH) [MCF-7(SSL)]. Additional samples of MCF-7 were provided by Dr. M. Lippman (NIH) [MCF-7(ML)] and Dr. J. Schiom (NIH) [MCF-7(US)]. Several other breast cancer cell lines were obtained from the American Type Culture Collection, including MCF-7 [MCF-7(ATCC)], BT-20 (15), ZR-75-1 (5), and SK-BR-3 (29). All cells were passaged in αMEM containing 10% (v/v) FCS, except for MCF-7(KO), which was passaged in αMEM containing 5% (v/v) FCS and 1.5 x 10^-4 unit insulin/ml and MCF-7(SSL), which was passaged in αMEM containing 5% FCS and 1.5 x 10^-3 units insulin/ml. The HL-60 promyelocytic leukemia cell line (3) was passaged in αMEM containing 10% (v/v) FCS.

Patient Samples. Samples of malignant pleural effusions were collected into heparinized (10 units/ml) containers by thoracentesis from patients with advanced breast cancer. Cells were harvested by centrifugation, and contaminating erythrocytes were removed by Ficoll-Hypaque density centrifugation. The samples selected for use in this study contained more than 90% tumor cells, as estimated by analysis of Wright-Giemsa-stained slides.

Isolation of DNA and RNA. DNA was prepared according to the technic of Gusella et al. (6). RNA was isolated using the guanidium-CsCl method of Chirgwin (2).

Gel Electrophoresis and Southern Transfer. Agarose gel (0.8%) electrophoresis was carried out in 89 mM Tris-borate: 89 mM boric acid:2 mM EDTA, pH 8.0. HindIII digested phage λ-DNA (Bethesda Research Laboratories) was used for molecular weight markers. Ten-μg samples of cellular DNA were digested with EcoRI restriction enzyme according to the recommendations of the supplier (Boehringer-Mannheim) and subjected to electrophoresis in a horizontal agarose slab gel. Gels were stained with ethidium bromide and photographed under a UV light to ensure that an equal amount of DNA was loaded in each lane. The DNA was then transferred to a nylon membrane (Zetabind from A. M. F. Cuno, Meridian, CN) essentially as described by Southern (26).

Analysis of mRNA. Poly(A)+ RNA was purified using an oligo dT cellulose column (P. L. Biochemicals, Inc.). The poly(A)+ RNA was then denatured with glyoxal and dimethyl sulfoxide according to the technique of Thomas (28) and subjected to electrophoresis in a horizontal agarose slab gel. Gels were stained with ethidium bromide and photographed under a UV light to ensure that an equal amount of DNA was loaded in each lane. The DNA was then transferred to a nylon membrane (Zetabind from A. M. F. Cuno, Meridian, CN) essentially as described by Southern (26).

Hybridization Conditions. Hybridization was performed at 42°C for 24 h in a hybridization solution containing 5 x standard saline citrate (0.15 μM NaCl-0.15 μM sodium citrate, pH 7.0), 50% formamide, 1 x Denhardt's, 0.1% sodium dodecyl sulfate, and 200 μg/ml denatured salmon sperm DNA. The filters were washed successively: the final wash was 1 x standard saline citrate at 65°C. The filter was exposed at -70°C using a Cronex Lightening Plus intensifying screen and Kodak XAR-5 film, for varying lengths of time.

The abbreviations used are: α-MEM, α-minimal essential medium; FCS, fetal calf serum.
Hybridization Probes. Two hybridization probes were used in these experiments. pNP-1 (24) is a plasmid containing a Hindlll insert derived from an activated N-ras gene found in SK-N-SH (27) (kindly provided by M. Wigler). The second probe is a 0.6-kilobase PvuII piece isolated from pN-ras, a plasmid containing an active N-ras gene derived from HT1080 (7) (pN-ras was kindly provided by C. Marshall). Probes were labeled with either 32Pd-CTP or 3Hd-NTPs according to the method of Rigby et al. (18).

Karyotyping and in Situ Hybridization. Chromosome banding analysis was performed using methods described previously (30). In situ hybridization using 3H-labeled pNP-1 was also performed by methods described previously (31).

RESULTS

In an initial screen of human tumor cell lines for c-onc amplification, we noted elevated levels of N-ras hybridization to DNA from MCF-7(OCI) cell line. To investigate the origin of this N-ras amplification, we obtained samples of MCF-7 from a variety of sources, including the American Type Culture Collection (see "Materials and Methods"). Samples of these MCF-7 lines were analyzed for N-ras amplification using Southern blot analysis (Fig. 1). Digestion with EcoRI and hybridization with pNP-1 results in a 9.2-kilobase band in all lanes. Several of the MCF-7 lines are amplified for N-ras. The degree of hybridization is greatest in MCF-7(OCI) (Lane a), MCF-7(KO) (Lane c), and MCF-7(ML) (Lane f). The MCF-7(SS) line (Lane e) shows an intermediate level of amplification, while MCF-7(ATCC) (Lane d) and MCF-7(JS) (Lane g) demonstrate the lowest level of hybridization to the probe. A recent sample of Dr. Osborne's MCF-7 line MCF-7(KO-2) (Lane b) also showed amplification. Similar analyses of MCF-7 DNA for amplification of c-H-ras, c-myb, and c-myc failed to show elevated copy number (data not shown).

To estimate the degree of amplification of N-ras in MCF-7(OCI) in relation to MCF-7(ATCC), the intensity of hybridization was compared between a 10-µg sample of MCF-7(OCI) DNA and lesser amounts of MCF-7(ATCC) DNA. The results (Fig. 2) indicate that MCF-7(OCI) has a greater than 10-fold increase in copy number, relative to MCF-7(ATCC).

Other cell lines and patient samples were screened in order to assess the generality of N-ras amplification in human breast cancer. The breast carcinoma cell lines, BT-20 (Lane 3), SK-BR-3 (Lane 4), ZR-75-1 (Lane 5), and DNA from four breast tumors (Lanes 6 to 9) were compared to MCF-7(OCI) (Lanes 1 and 11) and MCF-7(ATCC) (Lanes 2 and 10) (Fig. 3). The degree of amplification seen in MCF-7(OCI) was not seen in other lines or patient samples. MCF-7(ATCC) consistently demonstrated a small degree of amplification. The apparent amplifications seen in DNA from patient 4 (Lane 9) and from BT-20 (Lane 3) were not consistently seen and can be attributed in this experiment to DNA loading differences (as assessed by ethidium bromide staining). We routinely observed three additional fragments (Lane 11) of between 4 and 6 kilobases in length; these may represent genes which are homologous to N-ras (22).

The size of the N-ras mRNA in MCF-7(OCI) was investigated by Northern blotting analysis and compared to the corresponding transcript from the HL-60 promyelocytic leukemia cell line (Fig. 4). The PvuII fragment from pN-ras detected 2 bands of approximately 4 and 2.3 kilobases in both cell lines. When pNP-1 was used as the probe, only the larger of the 2 bands was detected (data not shown).

Analysis of grain distribution following in situ hybridization with 3H-labeled N-ras probe DNA was performed on autoradiographic images of a total of 30 mitoses from the N-ras amplified MCF-7(OCI). Significant label was observed on the distal long arm of one submetacentric marker chromosome (designated Umar8). An example of the G-banding pattern of the Umar8 chromosome and examples of grain distribution on this chromosome from three different cells following in situ hybridization are presented in Figure 5. Of the cells examined, 60% showed evidence of one or more grains on the distal long arm of the Umar8 chromosome.

DISCUSSION

We have provided evidence of N-ras gene amplification in the human breast cancer cell line MCF-7. This has not been documented previously in MCF-7 or in any other breast carcinoma. However, various human tumor cell lines have elevated copy number of other c-onc genes. For example, c-myc is amplified in the breast cancer cell line, SK-BR-3 (12), in a number of small cell lung carcinoma cell lines (16), and in a neuroectodermal cell line derived from a colon carcinoma (1). It has also been reported that the c-H-ras gene is amplified in a human bladder tumor (9).
Fig. 3. Southern blotting of DNA from breast cancer cell lines and patient samples. Hybridization was performed to DNA on a zetabind filter with pNP-1 as described. Lanes 1 and 11, MCF-7(OCI); Lanes 2 and 10, MCF-7(ATCC); Lane 3, BT-20, Lane 4, SK-BR-3; Lane 5, ZR-75-1; Lanes 6 to 9, patient samples.

Fig. 4. Northern blotting analysis of cellular RNA from MCF-7(OCI) (Lane A) and HL-60 (Lane B), hybridized with the PvuII fragment as described.

However, this is the first description of N-ras amplification.

The N-ras oncogene was initially identified on the basis of DNA-mediated transformation of NIH/3T3 cells (7, 22, 23). These active N-ras genes have been shown to be mutated by a single base change to create an alteration at amino acid position 61 in the corresponding gene product (27). The molecular structure of the amplified N-ras reported in MCF-7(OCI) is under investigation.

The results of in situ hybridization were consistent with a chromosomal (rather than an extrachromosomal) location for the amplified sequences in the MCF-7(OCI) cell line. Accordingly, no evidence for double minutes was observed in >100 cells examined from this cell line. It is of interest that the Umar8 marker chromosome, which was directly demonstrated by in situ hybridization to contain the amplified N-ras sequences, did not reveal a cytologically distinguishable homogeneously staining region following G- or Q-banding analysis (Fig. 5). It is possible that the size of the amplification unit in the N-ras amplified MCF-7(OCI) cell line is relatively small, allowing recognition of the chromosomal locus of the amplified sequences only by the use of in situ hybridization techniques. The normal chromosomal locus for the N-ras oncogene has been reported previously on chromosome lp (4, 7). Unfortunately, the banding pattern of the Umar8 chromosome is highly abnormal and is not sufficiently similar to any normal chromosome to allow unequivocal determination of its exact constitution.

The Northern blot analysis of mRNA from MCF-7(OCI) indicates that the final transcript is not different from either the N-ras transcript in HL-60 (Fig. 4) or that reported in normal fibroblasts (7). Furthermore, this study demonstrates that the fragment pNP-1 hybridizes to the primary but not the final transcript. However, the PvuII fragment of pN-ras hybridizes to both primary and final transcripts and thus contains an exon.

The results reported here further emphasize the heterogeneity existing in MCF-7 sublines which have been passaged independently. Considerable data have been reported with respect to differences in antigen presentation, karyology, and hormone responsiveness of these sublines (8, 10, 17, 21, 32). N-ras amplification is another example of this heterogeneity.

The fact that N-ras amplification is seen in some sublines of MCF-7 but not in others suggests that amplification has occurred through laboratory passage. Indeed, a degree of amplification has occurred in the MCF-7(OCI) line subsequent to our receiving it from Dr. C. K. Osborne in 1982 [MCF-7(KO), Fig. 1]. In our screening of breast cancer cell lines or patient cells, we failed to demonstrate another example of N-ras amplification (Fig. 3). It therefore appears unlikely that N-ras gene amplification has broad generality for breast carcinoma cells, but those strains of MCF-7 having amplified copy number may represent useful models with which to investigate the function of the N-ras product.

The primary purpose of this paper is to demonstrate that amplification of the N-ras oncogene has occurred in the breast carcinoma cell line MCF-7. The heterogeneity of such amplification may have a biological basis or may be due to genetic differences between independently passaged lines. Both of these possibilities are under investigation.
that MCF-7 is amplified for N-ras.

REFERENCES


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Fig. 5. Karyotypic analysis of the MCF-7-OCI cell line using G-banding and in situ hybridization. A, G-banded cell from the MCF-7-OCI line demonstrating several marker chromosomes, including a submetacentric marker termed Umar8 (arrow). B, G-banded example of Umar8. C, Example of grain localization of the Umar8 chromosome from 3 different cell hybridized in situ with a $^3$H-labeled pNP-1 (see text).
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