MOLECULAR AND CELLULAR BIOLOGY, Feb. 1985, p. 414–418
0270-7306/85/020414-05$02.00/0

Vol. 5, No. 2

Amplification and Expression of a Cellular Oncogene (c-*myc*) in Human Gastric Adenocarcinoma Cells

MASABUMI SHIBUYA,* JUN YOKOTA,† AND YOSHITO UEYAMA‡

Department of Genetics, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108 Japan

Received 18 July 1984/Accepted 2 November 1984

Three of 16 human gastric adenocarcinoma samples, maintained as solid tumors in nude mice, were found to carry amplified c-*myc* genes. In two samples with a high degree of c-*myc* DNA amplification (15- to 30-fold), double minute chromosomes were observed in karyotype analysis. The level of c-*myc* RNA was markedly elevated in a rapidly growing and poorly differentiated tumor, whereas it was only slightly elevated in a slowly growing and more differentiated tumor.

Recent studies on cellular oncogene (c-*onc*) in a variety of naturally occurring tumors in humans have revealed that c-*onc* genes appear to be involved in tumorigenesis via qualitative (20, 27, 33) and quantitative (1, 6, 11, 15, 23, 24) mechanisms. c-*myc* gene, the cellular homolog of viral transforming gene of avian myelocytomatosis virus MC29, has been found to be activated in human lymphatic and hematopoietic malignancies. c-*myc* gene is rearranged or transcriptionally activated by chromosomal translocation in Burkitt lymphomas (8, 18, 28), and is amplified in a promyelocytic leukemia cell line called HL60 (6, 9). Furthermore, amplification of the c-*myc* gene has recently been reported in a human colon cancer cell line with neuroendocrine properties called COLO320 (1) and in several cell lines of human small cell lung cancer (15), demonstrating that the c-*myc* activation is not limited to leukemias and lymphomas.

To study a role(s) of c-*onc* genes in carcinogenesis of humans, we surveyed abnormalities of c-*onc* genes in tumor cell DNAs by Southern hybridization analysis. We chose the gastric cancer system for analysis because gastric cancer, adenocarcinoma derived from stomach epithelial cells, is one of the most frequent malignancies in humans, but little is known about activation of c-*onc* genes in this tumor. Here we show that some samples of gastric cancer bear amplified c-*myc* genes. The expression of the amplified c-*myc* gene was markedly enhanced in a rapidly growing and poorly differentiated tumor. Our findings further show that the c-*myc* gene may be involved in carcinogenesis in a wide variety of human tissues.

All of the gastric carcinoma samples used in this study were maintained by passaging carcinoma cells in nude mice as solid tumors. Most of the tumors maintained in nude mice were kindly provided by K. Maruo and T. Nomura (Central Institute for Experimental Animals, Kawasaki, Japan), and TYS and OSS tumors were supplied from the Cancer Institute, University of Kanazawa, Kanazawa, Japan. The total cellular DNA was prepared from these cells by proteinase K digestion and phenol extraction, digested with various restriction enzymes as described below, and analyzed by the Southern blot method (26). The probe used was nick-translated, 0.8-kb *Taq*I-*Eco*RI DNA containing almost the entire sequence of 3'-exon in chicken c-*myc* gene.

Figure 1A shows one of the results of Southern blot analysis for c-*onc* genes in gastric carcinoma. Cellular DNAs extracted from these tumor cells were digested with restriction endonuclease *Sac*I, separated on a 0.8% agarose gel, and transferred to a nitrocellulose sheet as described by Southern (26). The DNAs were hybridized with chicken c-*myc* DNA probe under a condition of low stringency (30% formamide, 1 M NaCl, 10 mM Tris-hydrochloride; pH 7.5, 37°C) (16). Among 16 samples tested (11 samples shown in Fig. 1A and 5 additional samples), DNAs of three tumors called Shiraishi, SC-2, and NS-3 showed a higher intensity of human c-*myc* DNA bands of 2.8 kilobases (kb). By quantitative comparison of c-*myc* DNA bands between human placenta DNA and serially diluted amounts of cell DNA of Shiraishi and of HL60 as a control for gene amplification, we concluded that the c-*myc* gene was amplified ca. 30-fold in Shiraishi tumor (Fig. 1B). Using a similar method, we detected a 15-fold increase of c-*myc* DNA in SC-2 and a 6-fold increase in NS-3 (data not shown). Shiraishi and SC-2, which showed higher levels of amplification, were further studied.

The structure of the human c-*myc* gene has recently been analyzed in great detail (5, 30, 31). The 2.8-kb *Sac*I fragment of human c-*myc* gene (Fig. 1A) represents the second intron and third exon. To investigate whether the whole sequences of the c-*myc* gene are amplified without DNA rearrangement in these tumors, 10 μg of DNA from Shiraishi, SC-2, HL60, and human placenta were digested with restriction endonuclease *Eco*RI, *Hin*dIII, or *Pst*I and examined by Southern blot hybridization. The sizes of restriction fragments derived from amplified c-*myc* genes in Shiraishi, SC-2, and HL60 were essentially the same as those detected in human placenta DNA, although the intensity of these restriction fragments was markedly enhanced (Fig. 1C). These results suggest that the amplified c-*myc* genes were not rearranged within the 13-kb *Eco*RI DNA fragment containing the entire c-*myc* gene. Another c-*onc* gene, c-*mos*, located close to c-*myc* on chromosome 8 (18), was not amplified in these cells (data not shown).

Cells with gene amplification usually indicate two types of abnormalities at the chromosomal level: HSRs (homogeneously staining regions) or DMs (double minutes) (7, 22). In HL60 and COLO320-HSR cell lines, amplified c-*myc* genes have recently been demonstrated to locate on HSRs (1, 19). On the other hand, a considerable body of evidence suggests

* Corresponding author.
† Present address: Department of Medicine, Center for Health Science, University of California, Los Angeles, CA 90024.
‡ Present address: Department of Pathology, Tokai University, Isehara 259-11, and Central Institute for Experimental Animals, Miyamae-ku, Kawasaki 213 Japan.

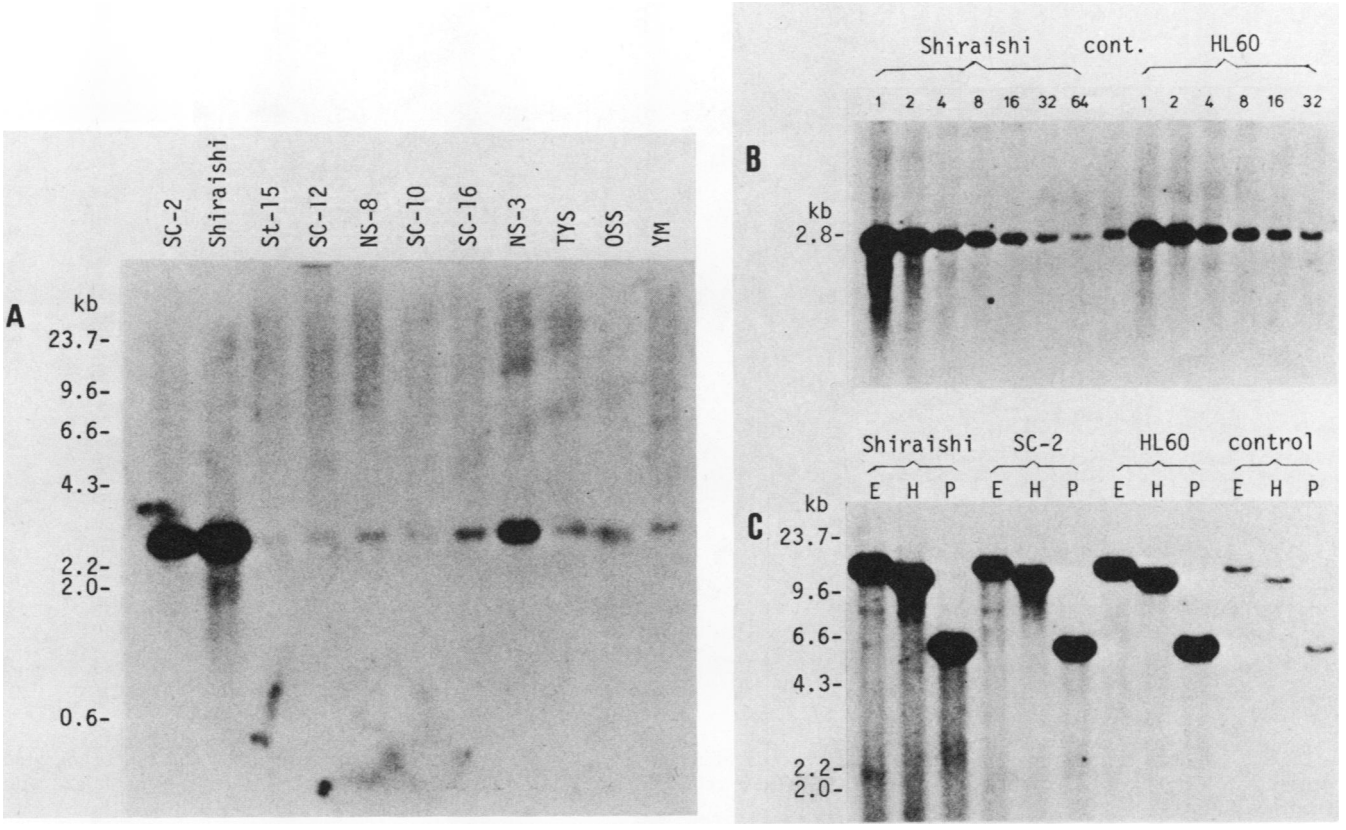

FIG. 1. Southern blot analysis of the cellular DNAs obtained from human gastric carcinoma cells. (A) Portions (5 μg) of DNA from various gastric cancer cells were digested with restriction endonuclease *Sac*I. (B) DNA (10 μg) from Shiraishi or HL60 was serially diluted with a solution containing carrier salmon DNA, and the intensity of the *Sac*I-digested, 2.8-kb c-*myc* bands in these samples was compared with that in 10 μg of human placental DNA (middle lane). The reciprocal of the dilution factor is shown above the lanes. (C) DNA (10 μg) from Shiraishi, SC-2, HL60, and human placenta was digested with restriction endonucleases *Eco*RI (E), *Hin*dIII (H), or *Pst*I (P). Chicken c-*myc* plasmid clone was obtained from W. S. Hayward (Memorial Sloan-Kettering Cancer Center, New York), and the *Taq*I-*Eco*RI 0.8-kb DNA fragment was purified by agarose gel electrophoresis. The marker DNA fragments indicated at the left side of the gels were λ DNA digested with *Hin*dIII.

that amplified genes are also present in the sequence of DMs (1–3, 10, 12, 17). We have made an attempt to examine karyotypes of c-*myc*-amplified gastric cancer cells. To yield enough mitotic cells, the solid tumors were cut into small pieces, and suspended cells were incubated in Dulbecco minimum essential medium with 6% fetal calf serum at 37°C for 2 days before karyotype analysis. In the Shiraishi tumor whose chromosomal number was ca. 120, we observed typical DMs (Fig. 2A). The number of DMs in this tumor was ca. 26 per cell. The SC-2 tumor whose chromosomal number was 57 contained ca. 31 DMs per cell; however, the size of DMs was much smaller than those in Shiraishi cells (Fig. 2B and C). NS-3 did not contain detectable DMs or HSRs.

Shiraishi and SC-2 tumors which bear almost the same copy number of c-*myc* genes (15- to 30-fold) possess different histological features (Fig. 3). Shiraishi cells are poorly differentiated and contain round and fine nuclei with clear nucleoli (Fig. 3A). On the other hand, SC-2 cells are a mixture of undifferentiated cells and differentiated, nonproliferative mucous epithelial cells (Fig. 3B). Shiraishi tumor grows faster than SC-2 tumor does in nude mice. These morphological properties of Shiraishi and SC-2 are consistent with those of the original gastric carcinomas, suggesting that at least some basic characteristics of these tumors may not be changed during maintenance of the tumors in nude mice. However, we were unable to conclude that the gene amplification took place before transplantation of the tumors into nude mice, since the original tumor tissues were not stored.

Gene amplification appears to affect cell metabolism through an increase in the amount of mRNA and gene product (7, 22). Expression of the c-*myc* gene in HL60, COLO320, and human lung cancer cell lines which carry amplified c-*myc* genes has been shown to be elevated under growing culture conditions (1, 21, 32). It has recently been reported that the level of c-*myc* mRNA in HL60 becomes much lower after differentiation into granulocytes or monocytes (21, 32). Furthermore, Kelly et al. (13) and Campisi et al. (4) have reported that the c-*myc* gene is inducible in lymphocytes and fibroblasts in a cell cycle-dependent manner. These results suggest that expression of the c-*myc* gene is correlated to the stage of the cell cycle or cell differentiation. Since the histological features of Shiraishi and SC-2 were different from each other, the expression of the amplified c-*myc* genes in these tumors was examined.

Total cellular RNA was extracted from samples of gastric cancer and separated on a 1% agarose gel containing 1.1 M formaldehyde (14). The relative level of c-*myc* RNA in a constant amount of cellular RNA was examined by Northern

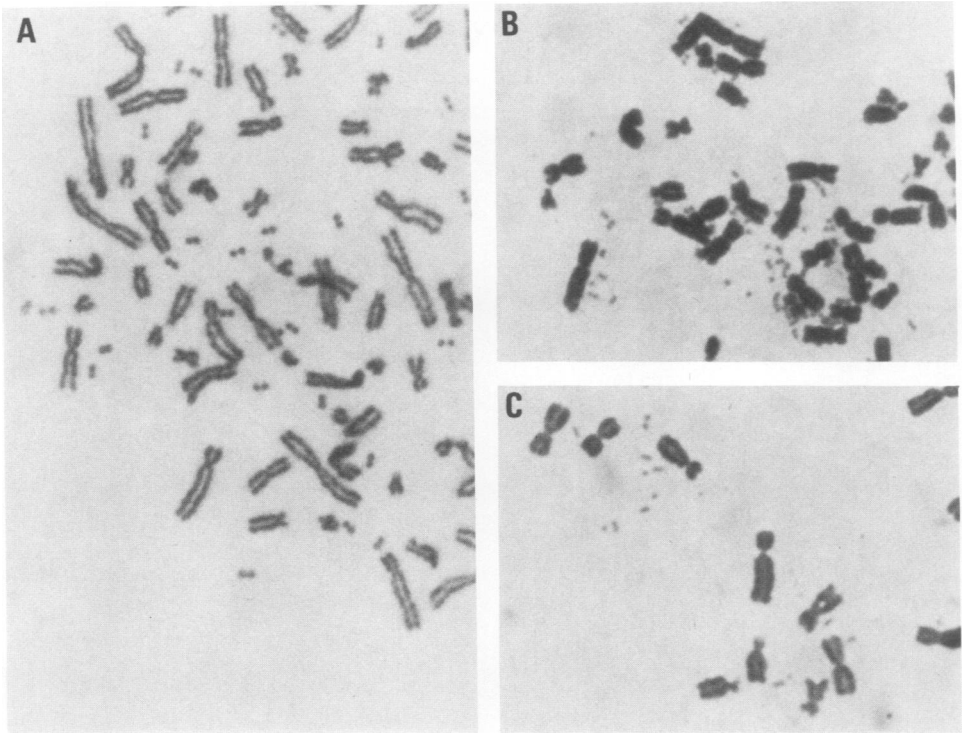

FIG. 2. Double minute chromosomes in gastric cancer cells. Shiraishi cells (A) or SC-2 cells (B and C) were treated with 0.2 μg of colcemid per ml for 2 h and fixed with methanol-acetic acid (3:1). These cells were spread on a glass slide, and the chromosomes were stained with Giemsa solution.

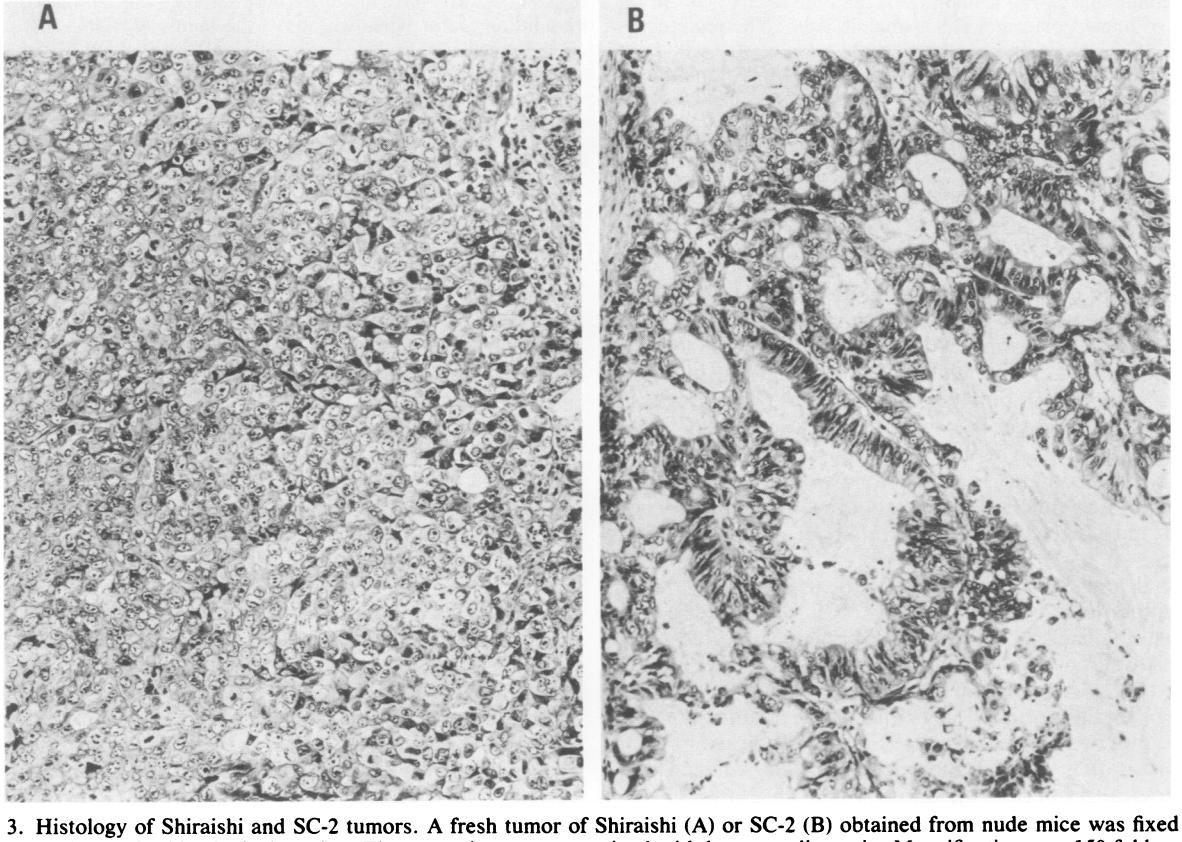

FIG. 3. Histology of Shiraishi and SC-2 tumors. A fresh tumor of Shiraishi (A) or SC-2 (B) obtained from nude mice was fixed in 10% chloroform and used for histological section. These specimens were stained with hematoxylin-eosin. Magnification was 150-fold.

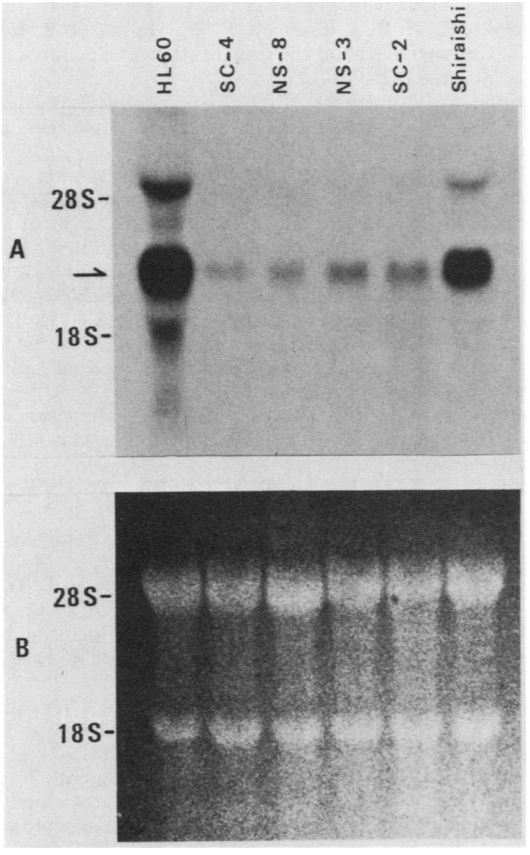

FIG. 4. Expression of c-*myc* RNA in gastric cancer cells. Tumors obtained from nude mice were immediately frozen in liquid nitrogen and stored at −70°C. Frozen tissues were homogenized in a solution of 4 M guanidium-thiocyanate, 0.1 M sodium acetate (pH 5.0), and 5 mM EDTA, and sedimented through a CsCl layer by centrifugation. RNA was further purified by phenol extraction. (A) Portions (20 μg) of total cellular RNA from gastric cancer cells and of HL60 were separated on a 1% agarose gel (14), transferred to nitrocellulose sheet (29), and hybridized with nick-translated human c-*myc* 3′-exon DNA. The arrow indicates the 2.3-kb human c-*myc* RNA. (B) The same amounts (20 μg) of cellular RNA were separated on a 1% agarose gel and stained with ethidium bromide.

blot analysis (29). Hybridization was carried out under conditions of moderate stringency (25), except for the addition of 0.1% sodium dodecyl sulfate. The probe used was the *Cla*I-*Eco*RI 1.5-kb fragment of the human c-*myc* 3′-exon DNA which was molecularly cloned from Shiraishi cell DNA. The details for molecular cloning and characterization of amplified c-*myc* DNA will be described elsewhere.

The levels of c-*myc* RNA in these cells were quite different from each other (Fig. 4A): c-*myc* RNAs in Shiraishi and HL60 were 10- to 20-fold more abundant compared with those in gastric cancer cells SC-4 and NS-8 which contained a normal copy number of the c-*myc* gene. NS-3 also showed a two- to threefold elevation of c-*myc* RNA. The level of c-*myc* RNA in SC-2 was much lower than that expected from a 15-fold amplification of *myc* gene DNA. Essentially the same results were obtained when chicken c-*myc* DNA was used as a probe (data not shown). Degradation of RNA in this SC-2 tumor seems unlikely, because its 28S and 18S ribosomal RNAs were not degraded significantly. The amount of 28S and 18S RNA was almost equal to those in other samples, as shown by staining with ethidium bromide (Fig. 4B).

There appear to be two possibilities for the low level of c-*myc* RNA in SC-2: (i) all SC-2 cells express very low levels of c-*myc* RNA, or (ii) actively dividing immature cells express high levels of c-*myc* RNA, but differentiated, mucous cell-type tumor cells contain very small amounts of c-*myc* RNA. Since we have not yet examined the levels of c-*myc* RNA in immature cells and in differentiated cells in SC-2 tumor separately, we cannot distinguish between these two possibilities at this time. A higher expression of amplified c-*myc* genes in actively growing HL60 and its strong suppression in differentiated HL60 might support the second possibility. However, further studies with more samples of gastric cancer will be necessary to clarify the relationship between expression of amplified c-*myc* genes and differentiation of tumor cells.

We thank K. Maruo and T. Nomura for the supply of samples of human gastric cancer. We are also grateful to W. S. Hayward and M. Oskarsson for c-*onc* DNA probe, to T. Odaka and H. Hanafusa for helpful discussions, and to D. Foster for reading the manuscript. The excellent technical assistance of S. Yamaguchi is greatly appreciated.

This project was supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture (58015040) in Japan.

ADDENDUM IN PROOF

After this manuscript was submitted for publication, Nakasato et al. (GANN **75**:737–742, 1984) reported amplification of the c-*myc* oncogene in human stomach cancers. However, the levels of c-*myc* RNA and the karyotypes of these tumors have not yet been examined.

LITERATURE CITED

1. **Alitalo, K., M. Schwab, C. C. Lin, H. E. Varmus, and J. M. Bishop.** 1983. Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-*myc*) in colon carcinoma. Proc. Natl. Acad. Sci. U.S.A. **80**:1707–1711.
2. **Bostock, C. J., and C. Tyler-Smith.** 1981. Gene amplification in methotrexate-resistant mouse cells. II. Rearrangement and amplification of nondihydrofolate reductase gene sequences accompany chromosomal changes. J. Mol. Biol. **153**:219–238.
3. **Brown, P. C., S. M. Beverley, and R. T. Schimke.** 1981. Relationship of amplified dihydrofolate reductase genes to double minute chromosomes in unstably resistant mouse fibroblast cell lines. Mol. Cell. Biol. **1**:1077–1083.
4. **Campisi, J., H. E. Gray, A. B. Pardee, M. Dean, and G. E. Sonenshein.** 1984. Cell-cycle control of c-*myc* but not c-*ras* expression is lost following chemical transformation. Cell **36**:241–247.
5. **Colby, W. W., E. Y. Chen, D. H. Smith, and A. D. Levinson.** 1983. Identification and nucleotide sequence of a human locus homologous to the v-*myc* oncogene of avian myelocytomatosis virus MC29. Nature (London) **301**:722–725.
6. **Collins, S., and M. Groudine.** 1982. Amplification of endogenous *myc*-related DNA sequences in a human myeloid leukemia cell line. Nature (London) **298**:679–681.
7. **Cowell, J. K.** 1982. Double minutes and homogeneously staining regions: gene amplification in mammalian cells. Annu. Rev. Genet. **16**:21–59.
8. **Dalla-Favera, R., M. Bregni, J. Erikson, D. Patterson, R. Gallo, and C. M. Croce.** 1982. Human c-*myc onc* gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc. Natl. Acad. Sci. U.S.A. **79**:7824–7827.
9. **Dalla-Favera, R., F. Wong-Staal, and R. C. Gallo.** 1982. *onc* gene amplification in promyelocytic leukemia cell line HL60 and

primary leukemic cells of the same patient. Nature (London) **299**:61–63.
10. **George, D. L., and V. E. Powers.** 1982. Amplified DNA sequences in Y1 mouse adrenal tumor cells: association with double minutes and localization to a homogeneously staining chromosomal region. Proc. Natl. Acad. Sci. U.S.A. **79**: 1597–1601.
11. **Hayward, W. S., B. G. Neel, and S. M. Astrin.** 1981. Activation of cellular *onc* gene by promoter insertion in ALV-induced lymphoid leukosis. Nature (London) **290**:475–480.
12. **Kaufman, R. J., P. C. Brown, and R. T. Schimke.** 1979. Amplified dihydrofolate reductase genes in unstably methotrexate resistant cells are associated with double minute chromosomes. Proc. Natl. Acad. Sci. U.S.A. **76**:5669–5673.
13. **Kelly, K., B. H. Cochran, C. D. Stiles, and P. Leder.** 1983. Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35**:603–610.
14. **Lehrach, M., D. Diamond, J. W. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**:4743–4751.
15. **Little, C. D., M. M. Nau, D. N. Carney, A. F. Gazdar, and J. D. Minna.** 1983. Amplification and expression of the c-*myc* oncogene in human lung cancer cell lines. Nature (London) **306**:194–196.
16. **Martin, M. A., T. Bryan, T. F. McCutchan, and H. W. Chan.** 1981. Detection and cloning of murine leukemia virus-related sequences from African green monkey liver DNA. J. Virol. **39**:835–844.
17. **Montgomery, K. T., J. L. Biedler, B. A. Spengler, and P. W. Melera.** 1983. Specific DNA sequence amplification in human neuroblastoma cells. Proc. Natl. Acad. Sci. U.S.A. **80**: 5724–5728.
18. **Neel, B. G., S. C. Jhanwar, R. S. K. Chaganti, and W. S. Hayward.** 1982. Two human c-*onc* genes are located on the long arm of chromosome 8. Proc. Natl. Acad. Sci. U.S.A. **79**: 7842–7846.
19. **Nowell, P., J. Finan, R. Dalla-Favera, R. C. Gallo, A. ar-Rushdi, H. Romanczuk, J. R. Selden, B. S. Emanuell, G. Rovera, and C. M. Croce.** 1983. Association of amplified oncogene c-*myc* with an abnormally banded chromosome 8 in a human leukemia cell line. Nature (London) **306**:494–497.
20. **Reddy, E. P., R. K. Reynolds, E. Santos, and M. Barbacid.** 1982. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature (London) **300**:149–152.
21. **Reitsma, P. H., P. G. Rothberg, S. M. Astrin, J. Trial, Z. Bar-Shavit, A. Hall, S. L. Teitelbaum, and A. J. Kahn.** 1983. Regulation of *myc* gene expresion in HL60 leukemia cells by a vitamin D metabolite. Nature (London) **306**:492–494.
22. **Schimke, R. T., R. J. Kaufman, F. W. Alt, and R. E. Kellems.** 1978. Gene amplification and drug resistance in cultured murine cells. Science **202**:1051–1055.
23. **Schwab, M., K. Alitalo, K.-H. Klempnauer, H. E. Varmus, J. M. Bishop, F. Gilbert, G. Brodeur, M. Goldstein, and J. Trent.** 1983. Amplified DNA with limited homology to *myc* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature (London) **305**:245–248.
24. **Schwab, M., K. Alitalo, H. E. Varmus, J. M. Bishop, and D. George.** 1983. A cellular oncogene (c-Ki-*ras*) is amplified, overexpressed, and located within karyotypic abnormalities in mouse adrenocortical tumor cells. Nature (London) **303**:497–501.
25. **Shibuya, M., H. Hanafusa, and P. C. Balduzzi.** 1982. Cellular sequences related to three new *onc* genes of avian sarcoma virus (*fps*, *yes*, and *ros*) and their expression in normal and transformed cells. J. Virol. **42**:143–152.
26. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
27. **Tabin, C. J., S. M. Bradley, C. I. Bargmann, R. A. Weinberg, A. G. Papageorge, E. M. Scolnick, R. Dhar, D. R. Lowy, and E. H. Chang.** 1982. Mechanism of activation of a human oncogene. Nature (London) **300**:143–149.
28. **Taub, R., I. Kirsch, C. Morton, G. Lenoir, D. Swan, S. Tronick, S. Aaronson, and P. Leder.** 1982. Translocation of the c-*myc* gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc. Natl. Acad. Sci. U.S.A. **79**:7837–7841.
29. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. **77**:5201–5205.
30. **Watson, D. K., M. C. Psallidopoulos, K. P. Samuel, R. Dalla-Favera, and T. S. Papas.** 1983. Nucleotide sequence analysis of human c-*myc* locus, chicken homologue, and myelocytomatosis virus MC29 transforming gene reveals a highly conserved gene product. Proc. Natl. Acad. Sci. U.S.A. **80**:3642–3645.
31. **Watt, R., L. W. Stanton, K. B. Marcu, R. C. Gallo, C. M. Croce, and G. Rovera.** 1983. Nucleotide sequence of cloned cDNA of human c-*myc* oncogene. Nature (London) **303**:725–728.
32. **Westin, E. H., F. Wong-Staal, E. P. Gelmann, R. D. Favera, T. S. Papas, J. A. Lautenberger, A. Eva, E. P. Reddy, S. R. Tronick, S. A. Aaronson, and R. C. Gallo.** 1982. Expression of cellular homologues of retroviral *onc* genes in human hematopoietic cells. Proc. Natl. Acad. Sci. U.S.A. **79**:2490–2494.
33. **Yuasa, Y., S. K. Srivastava, C. Y. Dunn, J. S. Rhim, E. P. Reddy, and S. A. Aaronson.** 1983. Acquisition of transforming properties by alternative point mutations within c-*bas/has* human proto oncogene. Nature (London) **303**:775–779.