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c-*myc* Reverses *neu*-Induced Transformed Morphology by Transcriptional Repression

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Amplification or overexpression or both of either the c-*myc* or the human *neu* (C-*erbB*-2) gene are common events in many primary human tumors. Coamplification or overexpression or both of both genes have been reported in some breast cancers. The possibility of cooperation between the c-*myc* and the normal rat *neu* (c-*neu*) genes in transforming cells was examined. Surprisingly, the expression of c-*myc* in B104-1-1 cells, an activated rat *neu* oncogene (*neu)-transformed NIH 3T3 line, resulted in morphologic reversion. This reversion was found to be a consequence of a transcription-repressive action of c-*myc* on the *neu* gene via a 140-bp fragment on the *neu* gene promoter. The effective concentration of a positive factor(s) interacting with this fragment seemed to be lowered by the expression of c-*myc*. Our findings lend support to arguments concerning the long-suspected function of c-*myc* as a transcriptional modulator. They also imply that an oncogene such as c-*myc*, or possibly the rapidly explored class that encodes transcription factors, under certain conditions may act to reverse a transformed phenotype that is induced by another oncogene instead of contributing positively towards the transformation process. Therefore, the activity of an oncogene may depend on the environment in which it is expressed. In addition, we may have identified the *neu* gene as a cellular target gene of negative regulation by c-*myc*.**

The *neu* oncogene was first identified and cloned from ethylnitrosourea-induced rat neuro- and glioblastomas (26, 53). Its human counterpart, referred to as c-*erbB*-2 or HER2, was identified as a homolog of the human epidermal growth factor receptor (EGF-r) (6, 15, 36, 52, 60). The *neu* gene-encoded protein p185 (2, 54) belongs to the class containing growth factor receptor tyrosine kinase (for a review, see reference 61). It may also be included in the class of cytoplasmic oncogenes (59). Compared with its normal allele (7), the activated *neu* oncogene (*neu**) contains a single point mutation in the transmembrane domain. The mutated protein product exhibits increased tyrosine kinase activity and transforming potential (7, 8). Transgenic mice that expressed *neu** developed mainly mammary carcinomas (10, 47), while a variety of tumors were induced by both normal and in vitro-mutated c-*erbB*-2 (55). However, by transfection assay, only c-*erbB*-2, or HER2 (17, 25), but not c-*neu* (26), was able to induce focus formation in NIH 3T3 cells.

Another oncogene, c-*myc* (for a review, see reference 13), which belongs to the class of nuclear oncogenes (59), also induced multiple forms of tumors in transgenic mice (44). However, similar to the situation with c-*neu*, morphologic transformation of NIH 3T3 cells upon transfection was not detected (34). Expression of c-*myc* has been shown to be stimulated by some growth factors and mitogenic signals (14, 35). It is possible that the still-to-be-identified ligand for putative growth factor receptor c-*neu* (62) also stimulates c-*myc* expression.

Both c-*neu* and c-*myc* have been shown to be associated with many primary human tumors (3, 51). In fact, coamplification or overexpression of both genes in some breast cancers (1, 21, 24, 56) and in breast cancer cell line SK-BR-3 (39, 40) has been reported. Enhanced transforming ability was recently shown for c-*neu* and EGF-r (38). Since c-*myc* has been known for its ability to cooperate with some other oncogenes to transform primary embryo fibroblasts (42), we investigated the possibility of cooperation or interaction between c-*neu* and c-*myc* in inducing transformation in NIH 3T3 cells. Unexpectedly, not only was c-*myc* unable to enhance the transforming potential of c-*neu*, but it also exerted an opposite effect in terms of reversing the transformed morphology that was induced by *neu**. Further study suggested that c-*myc* was acting as a transcriptional repressor on *neu*. This activity was mediated through a 140-bp DNA fragment on the *neu* gene promoter.

MATERIALS AND METHODS

Cell lines and transfection. Both cell lines used in the stable transfection experiments, B 104-1-1 and DHFR-G8, have been described before (26). NIH 3T3 cells were used as recipient cells in transient transfection and chloramphenicol acetyltransferase (CAT) assays. All the cell lines were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 Ham extract (DMEM-F12) containing 10% calf serum (GIBCO). HeLa cells were passaged in DMEM-F12 supplemented with 10% fetal calf serum and were used in the preparation of nuclear extract. Transfection was done by the modified calcium phosphate precipitation method (11). Recipient cells (5×10^5) were plated in a 100-mm tissue culture dish and grown overnight. The drug selection plasmid pSV_2neo (0.5 μg) was cotransfected with 10 μg of either pSVc-myc-1 or pSVHu-c-myc-1, which contain the functional mouse and human c-*myc* genes, respectively, under the transcriptional control of the simian virus 40 (SV40) early promoter (41). Plasmid pSV_2E was constructed by deleting the CAT gene containing the *Eco*RI-*Eco*RI fragment from pSV_2CAT (23) and was used in control transfection. The DNA precipitate was left on the cells for 10 to 12 h at 37°C in a 3% CO_2 incubator. After several washes with serum-free medium, fresh DMEM-F12 was added, and the cells were

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moved to a 5% CO_2 incubator and grown for 36 to 48 h. Each transfected plate was then trypsinized and the cells were split onto 10 plates, each with medium containing 400 μg of G418 (GIBCO) per ml for selection of drug-resistant colonies. The cells were then grown for at least 2 weeks. On the basis of morphology and refractivity, most colonies appeared nontransformed. Individual colonies were picked with a cloning cylinder and cultured in medium containing G418 until individual clones were established.

Microscopy. Cells were photographed in phase contrast with a Nikon Diaphotomicroscope (40×).

Southern and Northern (RNA) blot analysis. Both Southern and Northern blots were essentially done according to published procedures (46). For Southern blots, 10 μg of duplicate samples of DNAs were restricted with *Hin*dIII, electrophoresed in a 0.7% agarose gel, transferred onto nitrocellulose paper, and then hybridized to the respective probe. Hybridized filters were washed finally at 65°C in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate, air dried, and then exposed to Kodak XAR-5 film at −70°C. For Northern analysis, 10 μg of total RNA was electrophoresed in a 1.1% formaldehyde-agarose gel, transferred onto nitrocellulose, and then hybridized to the respective probe. Washes were done as for Southern blots except that the final wash was done at 60°C. DNA probes were the *neu*-specific probe, 0.4- and 0.8-kb *Bam*HI fragments from pSV_2neu (26); the *myc*-specific probe, a 1.2-kb *Sst*I fragment from pSVHu-c-myc-1 containing the highly conserved exon 2 of the human c-*myc* gene (41); and the glyceraldehydre-3-phosphate dehydrogenase (GAPDH) probe, a 1.2-kb *Pst*I fragment from pGAD-28 (19).

Transient transfection and CAT assays. The construction of the *neu* promoter CAT plasmids has been described elsewhere (55a; also see Fig. 4a). Plasmids pNeuEcoRICAT (construct 1 in Fig. 4a), pRSVCAT (23), and pERCAT9 (30) (4 μg each) were transfected with 10 μg of either control vector plasmid pGEM3 (Promega) or pSVc-myc-1. pSV_2E was also used in control transfection in the case of NeuCAT (for the pNeuEcoRICAT in Fig. 4a). For concentration-dependent experiments, 4 μg of pNeuEcoRICAT was cotransfected with 2, 5, 10, or 20 μg of pSVc-myc-1, and an appropriate quantity of pGEM3 was added to each transfection so that the total amount of DNA being added remained constant.

For *myc*-responsive-element cotransfection competition experiments, 2 μg of pNeuEcoRICAT was transfected with 25 μg of pGEM3 as the control or with 5 μg of pSVc-myc-1 plus 0, 5, 10, or 20 μg of $pSp64(StuI\text{-}XhoI)_2$, a plasmid which contains two copies of the presumably *myc*-responsive element cloned into vector pSp64; total DNA was again made constant by the addition of an appropriate amount of pGEM3 to each transfection.

Three micrograms of pRSVlacZ (20), a plasmid that contains the bacterial β-galactosidase gene under the control of the Rous sarcoma virus long terminal repeat, was included in each transient transfection to monitor the transfection efficiency. Transfection was done as in the stable transfection experiments. Cells were harvested 36 h after transfection, and cell extracts were obtained by several freeze-thaw cycles alternating between −70 and 37°C. Of the cell extract, 1/10 to 1/5 was used for the β-galactosidase assay (49). The results of the β-galactosidase assay were used to normalize the quantity of cell extracts required for the CAT assay (23). Experiments were repeated at least three times, and the standard error was less than 15%.

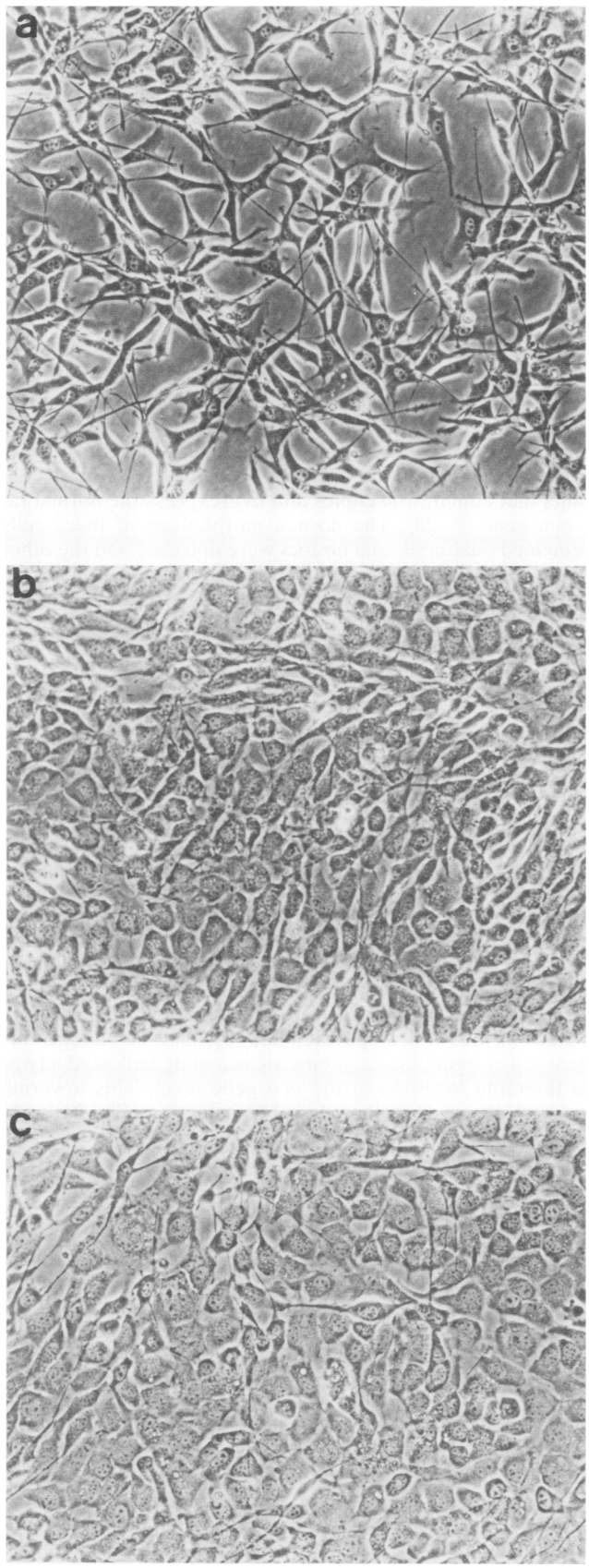
Gel retardation assay. Nuclear extract was isolated from HeLa cells by homogenization under hypotonic conditions as previously described (18). The 140-bp *Stu*I-*Xho*I fragment was isolated and 5′ end labeled with [γ-^{32}P]ATP by Klenow enzyme. Probe (1 ng, or 20,000 cpm) was incubated with the nuclear extract in the presence of 5 μg of poly(dI-dC) · poly(dI-dC) at room temperature for 20 min. Unlabeled competitive fragment, where appropriate, was added 10 min before the addition of labeled fragment. The reaction was stopped by the addition of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanole, 30% glycerol), and then the mixture was loaded onto a 4% polyacrylamide gel and the gel was run at 150 V for 2 to 3 h.

RESULTS

Morphologic reversion of *neu-transformed NIH 3T3 cells by stable transfection of c-*myc*.** To test if c-*myc* and c-*neu* could cooperate to induce transformation, a focus-forming assay was used. Plasmid pSVHu-c-myc-1 or pSV-c-myc-1 (41) was transfected into DHFR-G8 cells, NIH 3T3 transfectants that contain 100 copies and overexpress the normal rat c-*neu* gene (5, 26). The normal morphology of these cells remained unaltered, and no foci were detected. On the other hand, when these c-*myc*-encoding plasmids were transfected into B104-1-1 cells (Fig. 1a), NIH 3T3 transfectants that overexpress the transforming *neu**, the transformed morphology of these cells reverted to normal and the cells became contact inhibited and formed a monolayer (Fig. 1b and c). This morphologic reversion was not observed in the control transfection, in which the control plasmid pSV_2E, which contains only the SV40 early promoter, was used.

Integration of exogenous c-*myc* in revertants. To ensure that the exogenous c-*myc* gene actually integrated into the genome of these revertants and that the *neu* gene was not lost or rearranged, DNAs were isolated from these revertant lines and hybridized with the c-*myc*- and c-*neu*-specific probes, respectively. Figure 2a shows that multiple copies of the exogenous c-*myc* (left panel, lanes 4 and 5) had integrated into the genome of these revertants. The acquisition of c-*myc* did not seem to alter or rearrange the *neu* gene at the DNA level. However, an apparently lower level of *neu* gene was detected in the revertant lines. Quantitation of the signals of the rat *neu*-specific bands against the endogenous mouse *neu* signal by densitometric scanning indicated a two- to threefold lowering of the *neu* gene level. This lowering was not observed in the control transfection (Fig. 2a, right panel, lane 3).

c-*myc* revertant lines expressed significantly lower level of *neu* RNA. Since the transformed morphology of the B104-1-1 cells was induced by the expression of the *neu** oncogene, the observed morphologic reversion could be due to suppression of *neu** expression or to interference with the signal transduction pathway of *neu** by c-*myc*. To distinguish between these two possibilities, the RNA levels of *neu** in these revertants were examined by Northern blot analysis. The c-*myc* transfectants expressed different-sized transcripts in addition to the endogenous 2.4-kb c-*myc* (Fig. 2b, left panel, lanes 4 and 5). As expected, *neu** was undetectable in the NIH 3T3 cells (Fig. 2b, middle panel, lane 1), and a transfection of pSV_2E and the neomycin-resistant genes alone did not alter the level of *neu** (compare lanes 2 and 3, middle panel of Fig. 2b). In contrast, the previously described 9.0-, 5.0-, and 4.5-kb *neu* transcripts (6) were significantly reduced in the c-*myc* revertants (Fig. 2b, middle panel, lanes 4 and 5). The loading of RNAs was examined with a control probe of GAPDH (Fig. 2b, right panel).



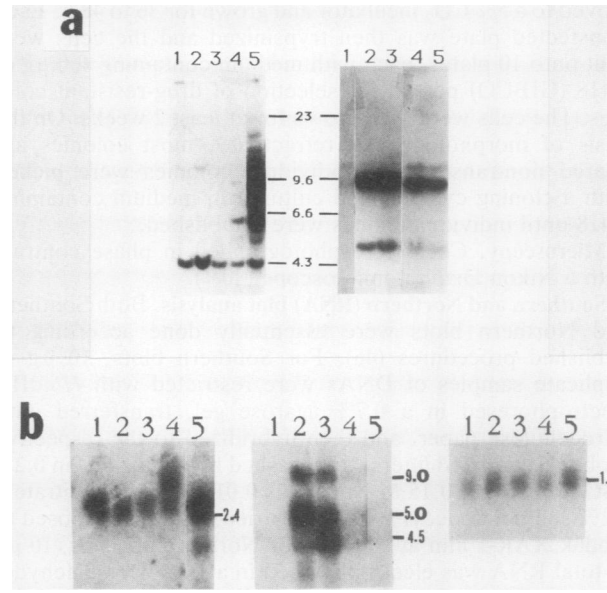


FIG. 2. (a) Southern blot analysis of B104-1-1 cells and their c-*myc* transfectants. Left panel, *myc* gene-specific probe; right panel, rat *neu*-specific probe. Lane 1, NIH 3T3 cells; lane 2, B104-1-1 cells; lane 3, B104-neo1 (B104-1-1 cells transfected with control plasmid pSV_2E); lane 4, B104-MoMyc3; lane 5, B104-HuMyc7. Size markers shown in kilobases are λ phage DNA restricted with *Hin*dIII. (b) Northern blot analysis of total RNA from B104-1-1 cells and their c-*myc* transfectants. Left panel, *myc*-specific probe. The 2.4-kb transcript is the endogenous c-*myc*. Middle panel, rat *neu*-specific probe. The 9.0-, 5.0-, and 4.5-kb transcripts are *neu* specific and have been described before (6). Right panel, GAPDH. The 1.3-kb transcript is the specific transcript. Lane assignments are the same as in panel a.

Quantitation by densitometric scanning showed a 10- to 12-fold decrease of the *neu*-specific transcripts in the *myc* revertants.

c-*myc* also suppressed transcriptional activity of *neu* in a transient assay. Although a decrease of *neu* gene at the DNA level (2- to 3-fold) was observed in the revertants, it could not account for the decrease that was seen at the RNA level (10- to 12-fold). This suggests that c-*myc* might have an effect on transcription of the *neu* gene. We therefore studied the possibility that c-*myc* interacts with the promoter of the *neu* gene to cause suppression of transcription. We have recently defined and characterized the promoter of the *neu* gene (55a). The functional 2.2-kb *neu* promoter CAT construct (pNeuEcoRICAT, construct 1 in Fig. 4a) was cotransfected with the c-*myc* plasmid, and the result is shown in Fig. 3a. c-*myc* was found to inhibit NeuCAT activity to 30% (Fig. 3a, right panel, lane +), compared with transfection of the control plasmid vector pGEM3 (Fig. 3a, right panel, lane −). This suppressing activity was not observed on the EGF-r promoter or the Rous sarcoma virus long terminal repeat

FIG. 1. Morphology of B104-1-1 cells and their c-*myc* transfectants. (a) Parental B104-1-1 cells. (b) B104-MoMyc3. These are B104-1-1 cells transfected with plasmid pSVc-myc-1, which carries a functional mouse c-*myc* gene. (c) B104-HuMyc7. These are B104-1-1 cells transfected with plasmid pSVHu-c-myc-1, which carries the functional human c-*myc* gene.

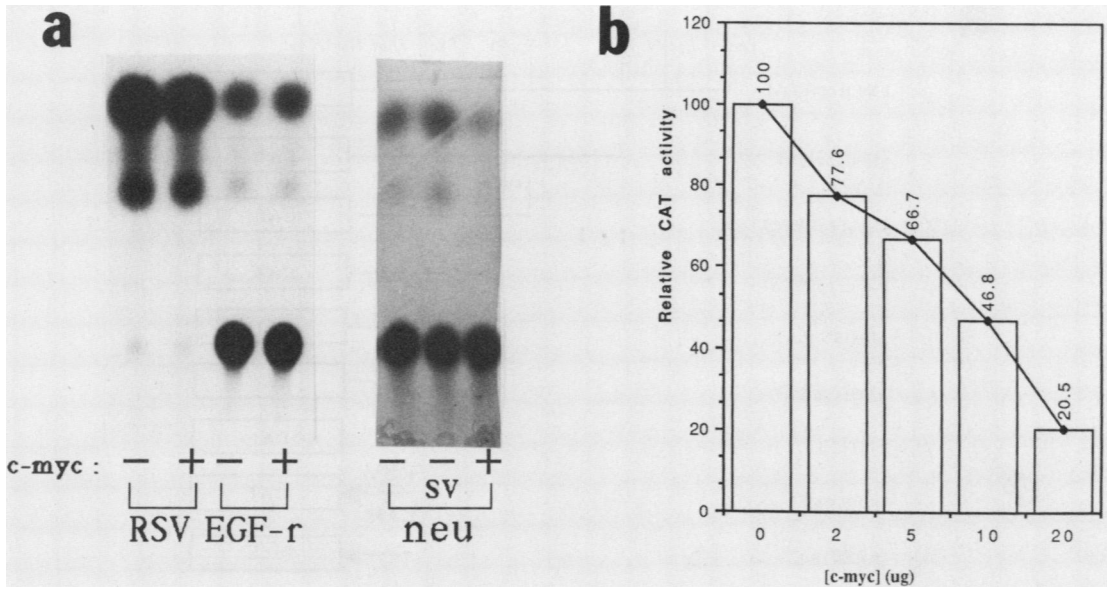


FIG. 3. (a) Effect of c-*myc* on the transcriptional activity of *neu* in a transient CAT assay. Left panel, Effect of c-*myc* on the Rous sarcoma virus long terminal repeat and the EGF-r promoter. Lanes −, Control cotransfection with pGEM3; lanes +, cotransfection with pSVc-myc-1. Right panel, Effect of c-*myc* on the *neu* gene promoter. Lane sv, Cotransfection with plasmid pSV_2E. (b) Concentration-dependent reduction in *neu*-CAT activity by c-*myc*.

(Fig. 3a, left panel), which suggests that the transcriptional repression on *neu* is not a general effect of c-*myc*. Since, the c-*myc* plasmid is driven by the SV40 early promoter, the effect of the SV40 early promoter itself on NeuCAT activity was examined. Cotransfection of the NeuCAT construct with pSV_2E actually resulted consistently in a 10% increase in CAT activity (Fig. 3a, right panel, lane sv). This demonstrates that the reduction in NeuCAT activity was not due to competition of a positive *trans*-acting factor(s) with the SV40 early promoter for binding to the *neu* gene promoter. A concentration-dependent inhibition of NeuCAT activity by c-*myc* is also shown in Fig. 3b, providing more support to rule out the possibility of c-*myc* being an artifact. Furthermore, a similar suppressive effect of c-*myc* on NeuCAT activity was observed in Rat-1 fibroblasts (data not shown).

Localization of c-*myc*-responsive element on *neu* gene promoter. To localize the DNA element that mediates this repression by c-*myc*, a series of *neu* promoter deletion CAT constructs (Fig. 4a) was used in cotransfection experiments. As shown in Fig. 4b, CAT activity of all the deletion constructs was suppressed to about 30 to 50% in the presence of c-*myc*, with the exception of construct 6. This suggests that the c-*myc*-responsive element is located between the *Stu*I and *Xho*I restriction sites. Since it is still possible that the transcriptional repression is due to sequences on the c-*myc* exon and intron acting in *cis*, all the deletion constructs were tested by cotransfection with pfsmyc-20 (37), a plasmid identical to pSVc-myc-1 except that a 4-bp deletion has been introduced into exon 2 of the c-*myc*-coding sequence, resulting in a frameshift and thus a truncated *MYC* protein product. No inhibition of NeuCAT activity was observed in any of the deletion constructs (Fig. 5, compare lanes + and fs). This result suggests that the *MYC* protein is the mediator of the transcription inhibition. To further study how the transcription repression was mediated through the DNA element on the *neu* gene promoter, a plasmid containing two copies of the presumed c-*myc*-responsive element (the *Stu*I-*Xho*I fragment) cloned into pSp64 was cotransfected with the NeuCAT construct, either in the presence or absence of c-*myc*. As shown in Fig. 6, the plasmid was able to further lower the CAT activity (columns 3 to 5) to a level which was already suppressed by c-*myc* (column 2). This plasmid, however, did not alter the NeuCAT activity by itself in the absence of c-*myc* (data not shown; 55a). Therefore, the c-*myc*-responsive element is able to show a competitive effect only in the presence of c-*myc*.

Sequence and characterization of c-*myc*-responsive element. The sequence of the c-*myc*-responsive element is shown in Fig. 7. It is located between nucleotides −312 and −173 upstream of the *neu* gene translation start site. A CCAAT box is found between −211 and −207, while a consensus GC box or Sp1-binding site is located between −203 and −198. A repetitive sequence, GGAGGAGGA, is observed from −199 to −191 and from −187 to −176. This sequence has also been found in the EGF-r promoter, albeit in the opposite orientation as TCCTCCTCC (29, 30). Both Sp1 and an EGF-r-specific factor, named TC factor, from HeLa cells were shown to bind to this sequence and stimulate transcription (31–33). A gel retardation assay was performed to see if there were factors bound to this c-*myc*-responsive element. The *Stu*I-*Xho*I fragment was 5′ end labeled with [γ-^{32}P]ATP and incubated with nuclear extract from HeLa cells in the presence or absence of an excessive quantity of unlabeled fragment (Fig. 8, left panel). The specificity of the protein-DNA complex (indicated in Fig. 8 with a C) was determined by the competitive effect of the same fragment but not of the nonspecific pSp64 DNA. No competition was detected either with a fragment from the EGF-r promoter or with SV40 promoter, which contain the GGAGGAGGA repeats and six GC boxes, respectively. However, the specific complex was competed away by a fragment that contains the SV40 72-bp enhancer. This result was confirmed by a concentration-dependent competition shown in the right panel of Fig. 8.

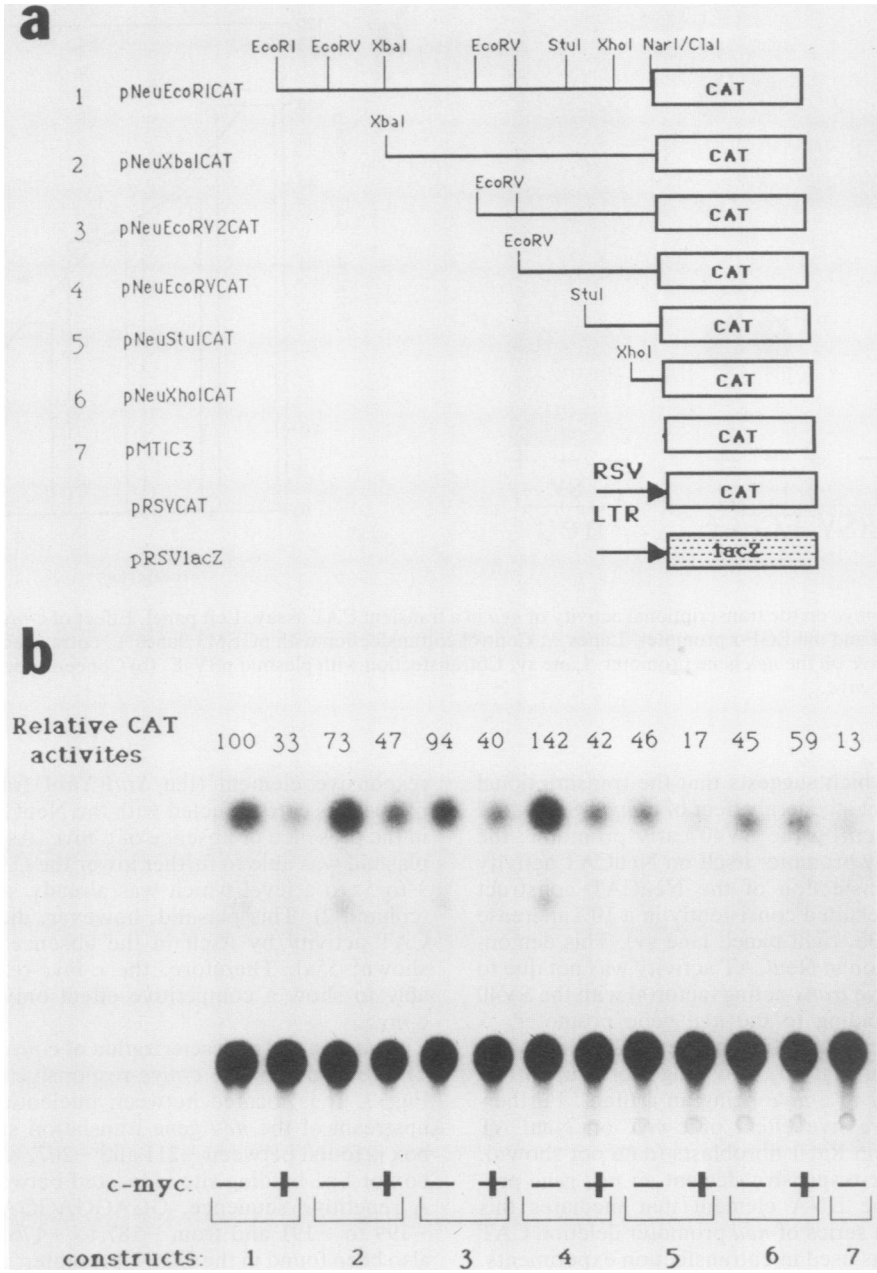


FIG. 4. (a) *neu* promoter CAT deletion constructs are numbered from 1 to 6. Number 7 represents the negative-control CAT vector plasmid; pRSVCAT and pRSVlacZ, used respectively for control and for monitoring transfection efficiency, are also shown. (b) Localization of the c-*myc*-responsive element. The effect of c-*myc* cotransfection on the series of CAT deletion constructs is shown. Numbers 1 to 7 correspond to the constructs shown in panel a. + and −, Cotransfection with plasmids pSVc-myc-1 and pGEM3, respectively. CAT activities were expressed relative to the activity of construct 1 cotransfected with pGEM3. The experiment was repeated four times, and the standard error was less than 10%.

Therefore, the protein complex that binds to the c-*myc*-responsive element is likely to be a common factor that also binds to the SV40 enhancer.

DISCUSSION

In an attempt to study the possible interaction between the proto-oncogenes c-*neu* and c-*myc* in a rather simple focus-forming assay, we discovered an interesting and exciting phenomenon: c-*myc* is able to reverse the transformed morphology that is induced by the oncogene *neu**. Our results imply that an oncogene, particularly the class of oncogene that encodes transcription factors (57), does not necessarily contribute positively towards a transformed phenotype, as one might predict. The activity of an oncogene may be dependent on the environment in which it is expressed.

The decrease at the DNA level of the *neu* gene in the *myc*

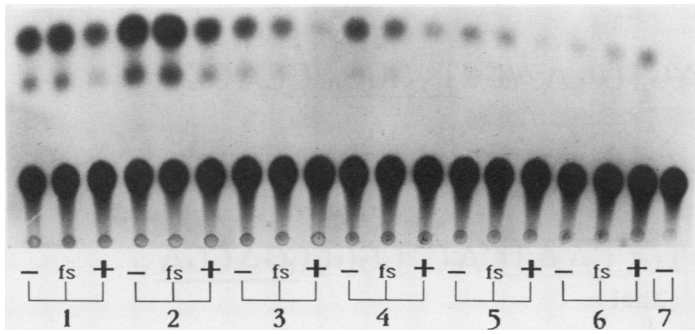


FIG. 5. The frameshift *myc* plasmid has no effect on CAT activity. The experiment was done as for Fig. 4b. Numbers 1 to 7 refer to the CAT constructs in Fig. 4a. Lanes fs, Cotransfection of the CAT construct with the frameshift *myc* plasmid pfsmyc20; lanes + and –, cotransfection with pSVc-myc-1 and pGEM3, respectively.

revertants could be due to negative selection against the *neu** oncogene, since colonies that exhibited nontransformed morphology were selected. Nevertheless, the much-reduced RNA level of the *neu* gene could not be explained by the lowered DNA level alone. Therefore, a mechanism at the level of transcription might be involved. The transient expression assays we used support strongly the possibility that c-*myc* is able to act as a transcriptional repressor. *myc* may be involved in DNA replication (27). The *MYC* protein is known to bind DNA nonspecifically (16, 58), and certain regions of the *MYC* protein are similar to some enhancer-binding proteins (43, 48). Some have suggested that the *myc* family of proteins negatively regulates transcription of c-*myc* (12, 50). More recently, c-*myc* was shown to bind directly to a 7-bp sequence located upstream of the first exon of the c-*myc* gene which is indispensable for both DNA replication and enhancer activities (4, 28). The expression of *Drosophila melanogaster* heat shock protein 70 has also been shown to be stimulated by c-*myc* (37). Other than these findings, little has been reported on the transcriptional modulatory effects of c-*myc*. Therefore, we may have identified a mammalian target gene, besides c-*myc* itself, that is negatively regulated by c-*myc*. The fact that the frameshift-mutated *myc* plasmid did not suppress the NeuCAT activity of the deletion constructs provides evidence that the *MYC* protein, but not the DNA or RNA of the c-*myc* gene, is the mediator of transcription inhibition of the *neu* gene. When the stable transfection experiments with B104-1-1 cells were repeated with the frameshift c-*myc* plasmid, no morphologic reversion was observed, as expected (data not shown). In addition, c-*myc* is known as one of the early response gene for some growth factors and mitogenic signals (14, 35). It is possible that c-*myc* is a normal negative-feedback control factor on the signal transduction pathway of the *neu* gene, a putative growth factor receptor.

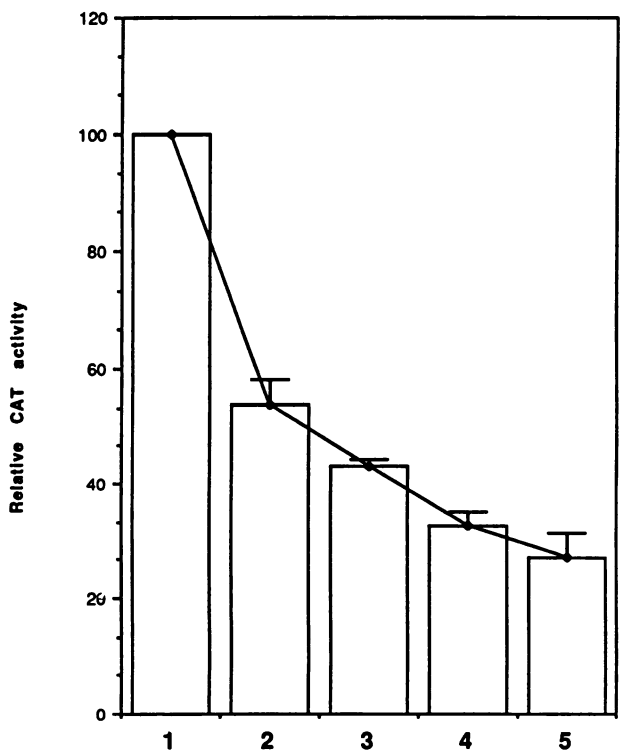


FIG. 6. Effect of c-*myc*-responsive element on CAT activity in the presence of c-*myc*. Activities of CAT plasmid 1 in Fig. 4a cotransfected with pGEM3 (column 1), pSVc-myc-1 alone (column 2), or pSVc-myc-1 plus 5 μg (column 3), 10 μg (column 4), or 20 μg (column 5) of pSp64(StuI-XhoI)$_2$. For experimental details, see Materials and Methods.

There are several possibilities for how c-*myc*-induced repression might occur through interaction with the c-*myc*-responsive element: (i) c-*myc* might bind directly to the fragment and suppress transcription; (ii) c-*myc* might increase the effective concentration of a negative factor(s) that interacts with this fragment; or (iii) c-*myc* might decrease the effective concentration of a positive factor(s) that interacts with this fragment. If either one of the first two possibilities was true, we would expect to see a derepressive effect on CAT activity when the amount of the c-*myc*-responsive element in the CAT competition experiments was increased. However, what we observed was exactly the opposite: the c-*myc*-responsive element further decreased the CAT activity to a level lower than that caused by c-*myc* (Fig. 6). Furthermore, the recently described c-*myc*-specific binding sequence (4) was not detected in this DNA element. We therefore favor the third possibility as the mechanism of c-*myc*-induced repression on the *neu* gene transcription. Moreover, our recent characterization of the *neu* gene promoter by deletion and competition studies also suggested the presence of positive factor binding to this fragment (55a). This factor is likely to exist in abundance, since the fragment itself is unable to lower the CAT activity by competition. However, the fragment is able to show a competitive effect in the presence of c-*myc*, possibly because the effective concentration of the positive factor(s) in the cell has already been lowered by c-*myc*. This kind of enhancer repression has been demonstrated in N-*myc*-induced suppression of the transcription factor H2TF1, resulting in a lowering in major histocompatibility complex class I antigen expression (9, 45). Whether the same or more-complicated mechanisms are responsible for the c-*myc*-induced suppression of *neu* observed in this study is now under investigation.

The c-*myc*-responsive element has been sequenced (Fig. 7) and characterized by gel retardation assay (Fig. 8). Since the GGAGGAGGA repeat has also been found in the EGF-r promoter and since *neu* and EGF-r show high homology, the factor that was shown to bind to EGF-r promoter may represent a common regulatory factor between the two genes. However, this is unlikely, since the EGF-r promoter

FIG. 7. Sequence of the 140-bp c-*myc*-responsive element. This sequence is located between −312 and −173 upstream of the translation start site of the *neu* gene. The CCAAT box and GC box (Sp1-binding site) are boxed in solid and broken lines, respectively. The GGAGGAGGA repeats are underlined. A palindromic sequence, half of which resembles the TC-I or TC-II motif of the SV40 enhancer, is underlined with a pair of inverted arrows. The consensus E12-MyoD (CANNTG)-binding site is marked by bold dots. The sequence with a wavy overline also resembles a κE2 site.

did not compete against the formation of protein-DNA complex with the c-*myc*-responsive element. A fragment from the SV40 early promoter which contains six GC boxes also could not compete for the protein-DNA complex, suggesting that the complex we detected is not Sp1. However, the formation of complex is inhibited in a concentration-dependent manner by the SV40 enhancer (Fig. 8). We therefore searched for homologous sequences in the c-*myc*-responsive element and the SV40 enhancer. A sequence located between −285 and −277, GCTGGGAGC, matches very well with the TC-I or TC-II motif of the SV40 enhancer (63), with the exception of four Gs in the SV40 enhancer. Another sequence, located between −194 and −186 (GGAG GTGG) but not found in the SV40 enhancer, must be noted. This sequence resembles the κE2 site (GGCAGGTGG) found in the immunoglobulin kappa-chain enhancer (48). This is particularly interesting since protein factors E12 and E47, which were shown to bind to this site, share DNA-

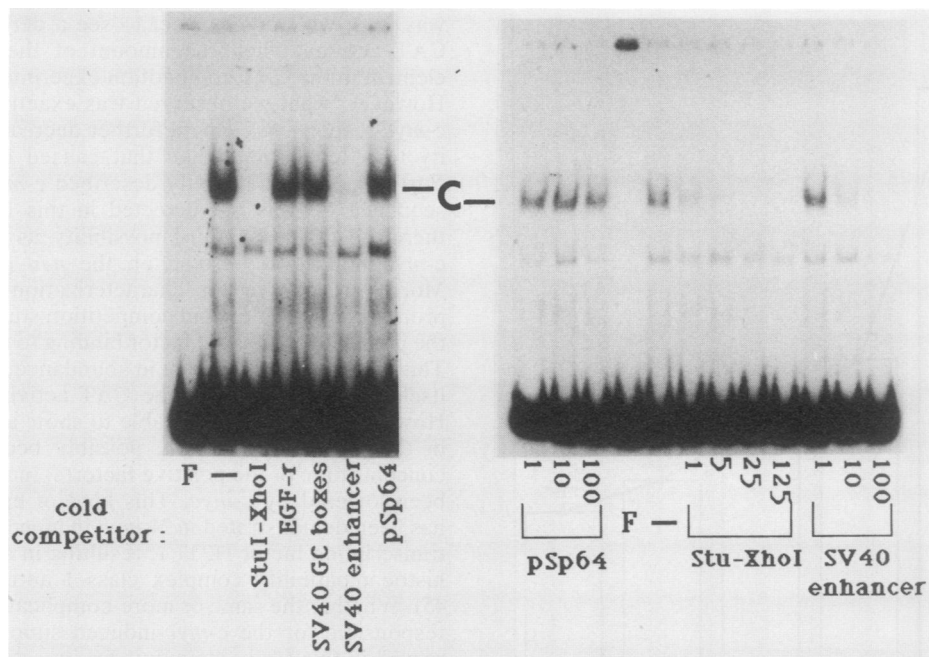


FIG. 8. Detection of protein-DNA complex on the c-*myc*-responsive element (*StuI-XhoI*) from 10 μg of HeLa cell nuclear extract incubated with 5 μg of poly(dI-dC). Left panel, All lanes except lane F contain the probe plus nuclear extract from HeLa cells. Unlabeled competitors added are shown at the bottom of each lane except lane −, to which no competitor was added. Right panel, Concentration-dependent competition of specific complex. The number at the bottom of each lane indicates the fold excess of unlabeled competitor. The specific protein-DNA complex is indicated by C. Competitors are *StuI-XhoI*, the c-*myc*-responsive element itself; EGF-r, a DNA fragment from the promoter of EGF-r which contains the TCCTCCTCC repeats as well as GC boxes; SV40 GC boxes, the promoter region of SV40 which contains six GC boxes in tandem; SV40 enhancer, a fragment containing only the SV40 72-bp enhancer; and pSp64, a nonspecific competitor from plasmid vector pSp64.

binding and dimerization motifs with the *myc* proteins (48). At the same time, a CAGTTG sequence between −252 and −247 also fits the consensus of the recognition sequence for the E12-MyoD helix-loop-helix family. At this moment, we do not know whether any of the described factors or a novel transcription factor is responsible for c-*myc* suppression. Further deletion and subcloning into a heterologous promoter will be necessary for narrowing down the location of the c-*myc*-responsive element to a smaller region. These studies are now under way.

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REFERENCES

1. **Adnane, J., P. Gaudray, M.-P. Simon, J. Simony-Lafontaine, P. Jeanteur, and C. Theillet.** 1989. Proto-oncogene amplification and human breast tumor phenotype. Oncogene **4**:1389–1395.
2. **Akiyama, T., C. Sudo, H. Ogawara, K. Toyoshima, and T. Yamamoto.** 1986. The product of the human c-*erbB*-2 gene: a 185 kilodalton glycoprotein with tyrosine kinase activity. Science **232**:1644–1646.
3. **Alitalo, K., P. Koskinen, T. P. Makela, K. Saksela, L. Sistonen, and R. Winqvist.** 1987. *myc* oncogenes: activation and amplification. Biochim. Biophys. Acta **907**:1–32.
4. **Ariga, H., Y. Imamura, and S. M. M. Iguchi-Ariga.** 1989. DNA replication origin and transcriptional enhancer in c-*myc* gene share the c-*myc* protein binding sequence. EMBO J. **8**:4273–4279.
5. **Bargmann, C. I., M.-C. Hung, and R. A. Weinberg.** 1986. Multiple independent activations of the *neu* oncogene by a point mutation altering the transmembrane domain of p185. Cell **45**:649–657.
6. **Bargmann, C. I., M.-C. Hung, and R. A. Weinberg.** 1986. The *neu* oncogene encodes an epidermal growth factor receptor related protein. Nature (London) **319**:226–230.
7. **Bargmann, C. I., and R. A. Weinberg.** 1988. Oncogenic activation of the *neu* encoded receptor protein by point mutation and deletion. EMBO J. **7**:2043–2052.
8. **Bargmann, C. I., and R. A. Weinberg.** 1988. Increased tyrosine kinase activity associated with the protein encoded by the activated *neu* oncogene. Proc. Natl. Acad. Sci. USA **85**:5394–5398.
9. **Bernards, R., S. K. Dessain, and R. A. Weinberg.** 1986. N-*myc* amplification causes down-modulation of MHC class I antigen expression in neuroblastoma. Cell **57**:931–936.
10. **Bouchard, L., L. Lamarre, P. J. Tremblay, and P. Jolicoeur.** 1989. Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-*neu*. Cell **57**:931–936.
11. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**:2745–2752.
12. **Cleveland, J. L., M. Huleihei, P. Bressler, U. Siebenlist, L. Akiyama, R. N. Eisenman, and U. R. Rapp.** 1988. Negative regulation of c-*myc* transcription involves *myc* family proteins. Oncogene Res. **3**:357–375.
13. **Cole, M. D.** 1986. The *myc* oncogene: its role in transformation and differentiation. Annu. Rev. Genet. **20**:361–384.
14. **Coughlin, S. R., W. M. F. Lee, P. W. Williams, G. M. Giels, and L. T. Williams.** 1985. c-*myc* gene expression is stimulated by agents that activate protein kinase C and does not account for the mitogenic effect of PDGF. Cell **43**:243–251.
15. **Coussens, L., T. L. Yang-Feng, Y.-C. Liao, C. Chen, A. Gray, J. McGarth, P. H. Seeburg, T. A. Libermann, J. Schlessinger, U. Francke, A. Levinson, and A. Ullrich.** 1985. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science **230**:1132–1139.
16. **Dang, C. V., H. van Dam, M. Buckmire, and W. M. F. Lee.** 1989. DNA-binding domain of human c-*myc* produced in *Escherichia coli*. Mol. Cell. Biol. **9**:2477–2486.
17. **Di Fiore, P. P., J. H. Pierce, M. H. Kraus, O. Segatto, C. R. King, and S. A. Aaronson.** 1987. *erbB*-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science **237**:178–182.
18. **Dignam, J. D., R. M. Leibovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
19. **Dugaiczyk, A., J. A. Haron, E. M. Stone, E. Dennison, K. N. Rothblum, and R. J. Schwartz.** 1983. Cloning and sequencing of a deoxyribonucleic acid copy of glyceraldehyde-3-phosphate dehydrogenase messenger ribonucleic acid isolated from chicken muscle. Biochemistry **22**:1605–1613.
20. **Edlund, T., M. D. Walker, B. J. Barr, and W. J. Ratter.** 1985. Cell specific expression of the rat insulin gene: evidence for role of two distinct 5′ flanking elements. Science **230**:912–926.
21. **Garcia, G., P.-Y. Dietrich, M. Aapro, G. Vauthier, L. Vadas, and E. Engel.** 1987. Genetic alterations of c-*myc*, c-*erbB*-2, and c-Ha-*ras* proto-oncogenes and clinical associations in human breast carcinomas. Cancer Res. **49**:6675–6679.
22. **Gorman, C. M., G. T. Merlino, M. C. William, I. Pastan, and B. H. Howard.** 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA mediated transfection. Proc. Natl. Acad. Sci. USA **79**:6777–6781.
23. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase. Mol. Cell. Biol. **2**:1044–1051.
24. **Guerin, M., M. Baross, M.-J. Terrier, M. Spielmann, and G. Riou.** 1988. Overexpression of either c-*myc* or c-*erbB*-2/*neu* proto-oncogenes in human breast carcinomas: correlation with poor prognosis. Oncogene Res. **3**:21–31.
25. **Hudziak, R. M., J. Schlessinger, and A. Ullrich.** 1987. Increased expression of the putative growth factor receptor p185 HER2 causes transformation and tumorigenesis of NIH3T3 cells. Proc. Natl. Acad. Sci. USA **84**:7159–7163.
26. **Hung, M.-C., A. L. Schechter, P. M. Chevray, D. F. Stern, and R. A. Weinberg.** 1986. Molecular cloning of the *neu* gene: absence of gross structural alteration in oncogenic alleles. Proc. Natl. Acad. Sci. USA **83**:2611–2614.
27. **Iguchi-Ariga, S. M. M., T. Itani, Y. Kiji, and H. Ariga.** 1987. Possible function of the c-*myc* product: promotion of cellular DNA replication. EMBO J. **6**:2365–2371.
28. **Iguchi-Ariga, S. M. M., T. Okazaki, T. Itani, M. Ogata, Y. Sato, and H. Ariga.** 1988. An initiation site of DNA replication with transcriptional enhancer activity present upstream of the c-*myc* gene. EMBO J. **7**:3135–3142.
29. **Ishii, S., Y.-H. Su, R. H. Stratton, B. A. Roe, G. T. Merlino, and I. Pastan.** 1985. Characterization and sequence of the promoter region of the human epidermal growth factor receptor gene. Proc. Natl. Acad. Sci. USA **82**:4920–4924.
30. **Johnson, A. C., S. Ishii, Y. Jinno, I. Pastan, and G. T. Merlino.** 1988. Epidermal growth factor receptor gene promoter. Deletion analysis and identification of nuclear protein binding sites. J. Biol. Chem. **263**:5693–5699.
31. **Johnson, A. C., Y. Jinno, and G. T. Merlino.** 1988. Modulation of epidermal growth factor receptor proto-oncogene transcription by a promoter site sensitive to S1 nuclease. Mol. Cell. Biol. **8**:4174–4184.
32. **Kageyama, R., G. T. Merlino, and I. Pastan.** 1988. Epidermal growth factor receptor gene transcription. Requirement for Sp1 and an EGF receptor-specific factor. J. Biol. Chem. **263**:6329–6336.
33. **Kageyama, R., G. T. Merlino, and I. Pastan.** 1988. A transcription factor active on the epidermal growth factor receptor gene. Proc. Natl. Acad. Sci. USA **85**:5016–5020.
34. **Keath, E. J., P. G. Caimi, and M. D. Cole.** 1984. Fibroblast lines expressing activated c-*myc* oncogenes are tumorigenic in nude mice and syngeneic animals. Cell **39**:339–348.
35. **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.
36. **King, C. R., M. H. Kraus, and S. A. Aaronson.** 1985. Amplification of a novel v-*erbB*-related gene in a human mammary carcinoma. Science **229**:974–976.
37. **Kingston, R. E., A. S. Baldwin, Jr., and P. A. Sharp.** 1984. Regulation of heat shock protein 70 gene expression by c-*myc*. Nature (London) **312**:280–282.
38. **Kokai, Y., J. N. Myers, T. Wada, V. I. Brown, C. M. LeVea, J. G. Davis, K. Dobashi, and M. I. Greene.** 1989. Synergistic interaction of p185c-*neu* and the EGF recepter leads to transformation of rodent fibroblasts. Cell **58**:287–292.
39. **Kozobor, D., and C. M. Croce.** 1984. Amplification of c-*myc* oncogene in one of five human breast carcinoma cell lines. Cancer Res. **44**:438–441.
40. **Kraus, M. H., N. C. Popescu, S. C. Amsbaugh, and C. R. King.** 1987. Overexpression of the EGF receptor-related proto-oncogene *erbB*-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J. **6**:605–610.
41. **Land, H., A. C. Chen, J. P. Morgenstern, L. F. Parada, and R. A. Weinberg.** 1986. Behavior of *myc* and *ras* oncogenes in transformation of rat embryo fibroblasts. Mol. Cell. Biol. **6**:1917–1925.
42. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (London) **304**:596–602.
43. **Lassar, A. B., J. N. Buskin, D. Lockshon, R. L. Davis, S. Apone, S. D. Hauschka, and H. Weintraub.** 1989. MyoD is a sequence-specific DNA binding protein requiring a region of *myc* homology to bind to muscle creatine kinase enhancer. Cell **58**:823–831.
44. **Leder, A., P. K. Pattengale, A. Kuo, T. A. Stewart, and P. Leder.** 1986. Consequences of widespread deregulation of the c-*myc* gene in transgenic mice: multiple neoplasms and normal development. Cell **45**:485–495.
45. **Lenardo, M., A. K. Rustgi, A. R. Schievella, and R. Bernards.** 1989. Suppression of MHC class I gene expression by N-*myc* through enhancer inactivation. EMBO J. **8**:3351–3355.
46. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
47. **Muller, W. J., E. Sinn, P. K. Pattengale, R. Wallace, and P. Leder.** 1988. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-*neu* oncogene. Cell **54**:105–115.
48. **Murre, C., P. S. McCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. Cell **56**:777–783.
49. **Norton, P. A., and J. M. Coffin.** 1985. Bacterial β-galactosidase as a marker gene of Rous sarcoma virus gene expression and replication. Mol. Cell. Biol. **5**:281–290.
50. **Penn, L. J. Z., M. W. Brooks, E. M. Laufer, and H. Land.** 1990. Negative autoregulation of c-*myc* transcription. EMBO J. **9**:1113–1121.
51. **Schwab, M., and L. C. Amler.** 1990. Amplification of cellular oncogenes: a predictor of clinical outcome in human cancer. Genes Chromosomes Cancer **1**:181–193.
52. **Semba, K., K. Kamata, and T. Yamamoto.** 1985. A v-*erbB*-related protooncogene, c-*erbB*-2 is distinct from the c-*erbB*-1/epidermal growth factor receptor gene and is amplified in human salivary gland adenocarcinoma. Proc. Natl. Acad. Sci. USA **82**:6497–6501.
53. **Shih, C., L. C. Padhy, M. Murray, and R. A. Weinberg.** 1981. Transformation gene of carcinomas and neuroblastomas introduced into mouse fibroblasts. Nature (London) **290**:261–264.
54. **Stern, D. F., P. A. Heffernan, and R. A. Weinberg.** 1986. p185, product of the *neu* proto-oncogene, is a receptorlike protein associated with tryosine kinase activity. Mol. Cell. Biol. **6**:1729–1740.
55. **Suda, Y., S. Aizawa, Y. Furuta, T. Yagi, Y. Ikawa, K. Saitoh, Y. Yamada, K. Toyoshima, and T. Yamamoto.** 1990. Indication of a variety of tumors by c-*erbB* 2 and clonal nature of lymphomas even with the mutated gene (Val^{659}-Glu^{659}). EMBO J. **9**:181–190.
55a. **Suen, T.-C., and M.-C. Hung.** 1990. Multiple *cis*- and *trans*-acting elements involved in the regulation of the *neu* gene. Mol. Cell. Biol. **10**:6306–6315.
56. **Varley, J. M., J. E. Swallow, W. J. Brammar, J. L. Whittaker, and R. A. Walker.** 1987. Alterations to either c-*erbB*-2 (*neu*) or c-*myc* proto-oncogenes in breast carcinomas correlate with poor short-term prognosis. Oncogene **1**:423–430.
57. **Varmus, H. E.** 1987. Oncogene and transcriptional control. Science **238**:1337–1339.
58. **Watt, R. A., A. R. Shatzman, and M. Rosenberg.** 1985. Expression and characterization of the human c-*myc* DNA binding protein. Mol. Cell. Biol. **5**:448–456.
59. **Weinberg, R. A.** 1985. The action of oncogenes in the cytoplasm and nucleus. Science **230**:770–776.
60. **Yamamoto, T., S. Ikawa, T. Akiyama, K. Semba, N. Nomura, N. Miyajima, T. Saito, and K. Toyoshima.** 1986. Similarity of protein encoded by the human c-*erbB*-2 gene to the epidermal growth factor receptor. Nature (London) **319**:230–234.
61. **Yarden, Y., and A. Ullrich.** 1988. Growth factor receptor tyrosine kinase. Annu. Rev. Biochem. **57**:443–478.
62. **Yarden, Y., and R. A. Weinberg.** 1989. Experimental approaches to hypothetical hormones: detection of a candidate ligand of the *neu* protooncogene. Proc. Natl. Acad. Sci. USA **86**:3179–3183.
63. **Zenke, M., T. Grundstrom, H. Matthews, M. Wimtzerith, C. Shatz, A. Wildermann, and P. Chambon.** 1986. Multiple sequence motifs are involved in SV40 enhancer function. EMBO J. **5**:387–397.