Negative Regulation of the Neu Promoter by the SV40 Large T Antigen

Angabib Matin and Mien-Chie Hung
Department of Tumor Biology, The University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract
The neu gene is amplified and its protein product is overexpressed in certain human tumors. The adenovirus 5 E1a gene product and c-myc repress neu transcription. Moreover, expression of E1a in neu-transformed cells leads to decrease in transformation phenotype and metastatic potential. The simian virus 40 large T antigen (LT) shares structural and functional homology with E1a and c-myc, and all three proteins bind to the retinoblastoma gene product, Rb. We found that LT also represses neu expression at the transcriptional level. However, LT represses neu promoter by a different mechanism compared to E1a and c-myc, because the region of the neu promoter mediating repression by LT (−172 to −79) is downstream from the region responding to E1a and c-myc (−312 to −172). In addition, a LT mutant (K1) unable to complex Rb still represses neu promoter activity, indicating that the Rb binding domain of LT is not required for repression of neu. Since K1, unlike LT, does not transform rat-1 cells but, like LT, represses the neu promoter, we tested whether K1 functions as a transformation suppressor of activated neu oncogene. Focus-forming assays showed that K1 indeed suppresses the strong cell-transforming activity of activated neu.

Introduction
The neu protooncogene encodes a receptor-like tyrosine kinase, p185 (1), structurally homologous to the epidermal growth factor receptor (2, 3). A specific ligand that stimulates the tyrosine kinase activity of neu has been isolated, and its cDNA has been cloned (4–6). The human neu (c-erbB2 or HER-2) gene is amplified, and neu mRNA and protein are overexpressed in breast (7–10), ovarian (10–13), lung (14–16), and gastric cancers (17, 18). Overexpression of neu in breast and ovarian tumors correlates with higher probability of disease relapse and decreased survival of patients (7, 10, 12). This suggests that aberrant up-regulation of neu may play an important role in tumorigenesis. Indeed, suppression of neu expression by introduction of E1a into neu-overexpressing ovarian carcinoma cells leads to a decrease in their malignancy and tumorigenicity (19).

The adenovirus 5 E1a gene product (20, 21) and c-myc (22) inhibit neu through a 140-base pair (−312 to −172) region of the neu promoter. Another viral oncoprotein, the SV40 LT, shares structural and functional homology to E1a and c-myc (23). LT, E1a, and c-myc have transforming domains which share amino acid sequence homology and similar secondary structure (23). All three proteins complex with the tumor suppressor, Rb gene product (24–26), and the Rb binding domains of LT and E1a coincide with their transforming domains, suggesting that LT and E1a transform cells by binding cellular Rb and abrogating its tumor suppressor function. LT, E1a, and c-myc are also grouped as immortalization oncogenes as determined by the oncogene cooperation assay using rat embryo fibroblasts (27).

We investigated whether LT behaves like c-myc and E1a to repress neu-encoded p185. Our studies show that introduction of LT in cells leads to a significant decrease in the expression of neu-encoded p185. LT, like E1a and c-myc, represses the upstream-regulatory sequences of neu. However, LT represses a different region of the neu-regulatory sequences compared to E1a and c-myc, suggesting that LT affects neu expression through a different pathway. Previous studies have shown that the tumor suppressor Rb represses the activity of the neu promoter (28). Since Rb is known to complex LT, we investigated whether LT-Rb complex might affect the LT-mediated neu repression. We found that the Rb binding domain of LT is not required for its function in repressing neu promoter, indicating that LT can repress neu expression without binding Rb. Moreover, a mutant of LT that is nontransforming is able to repress the transforming activity of neu. This type of nontransforming mutant LT may have therapeutic implications for neu-overexpressing tumors.

Results
To test the effect of LT in cells that overexpress neu-encoded p185, we cotransfected plasmids encoding LT, pZ189 (driven by the SV40 promoter), together with pSV2neo (plasmids encoding the gene for neomycin resistance) into B104-1-1 cells. B104-1-1 cells are derived from NIH 3T3 cells transformed by the mutation-activated genomic rat neu oncogene (29, 30). B104-1-1 cells express high levels of activated neu-encoded p185, are phenotypically transformed (1, 29), are highly tumorigenic (31, 32), and have increased metastatic potential (31, 33). The LT-transfected and G418-resistant B104-1-1 cells were cloned after 3 weeks. Cell lines were expanded from the clones, and cell lysates were analyzed for expression of LT and p185. The results show are of two such cell lines, named B1n14 and B1n16 cells. Immunoblotting of cell lysates for LT using anti-LT antibody (SV40 T-Ag, Ab-2; Oncogene Science) showed two bands of molecular weights less than 111,000, indicating expression of LT in B1n14 and B1n16 cell lines (Fig. 1B, Lanes 1 and

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2 To whom requests for reprints should be addressed, at Department of Tumor Biology, University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030.
3 The abbreviations used are: cDNA, complementary DNA; LT, large T antigen; Rb, retinoblastoma; kb, kilobase(s); CAT, chloramphenicol acetyltransferase; bp, base pairs.
...gene, we analyzed the level of rat neu DNA in these cells by Southern blot analysis. The levels of genomic rat neu oncogene in the BTN14 and BTN16 cell lines (Fig. 1C, Lanes 1 and 2) were equivalent to that in BEN5 cell line (Fig. 1C, Lane 3). NIH 3T3 cells, used as negative control, do not have rat neu DNA. These experiments show that when LT is stably expressed in cells that originally express high levels of neu-encoded p185, there is a resulting decrease in the level of p185, indicating that LT, similar to c-myc and E1a, can repress neu expression.

To determine whether the LT antigen inhibited rat neu expression at the transcriptional level, we examined the effect of LT on the upstream regulatory sequences of neu using transient transfection assays. Plasmids encoding LT antigen (pVu-0 or pZ189) (35) were cotransfected with plasmids encoding 2.2-kb rat neu upstream regulatory sequences linked to a reporter CAT gene (pNeuEcoRI-CAT) (36) into NIH 3T3 cells. The cotransfected plasmids, pSV2neo, were used as a filler plasmid to adjust concentrations in cotransfections. About 80% inhibition of the 2.2-kb neu promoter activity was achieved by a 10-fold excess of LT plasmid (Fig. 2A, Lanes 1 and 2). The inhibitory activity of LT was specific to neu, since the activity of the epidermal growth factor receptor-regulatory sequence (pEGFrCAT) (37) was unaffected by a similar amount of LT (Fig. 2A, Lanes 3 and 4). In addition, LT had a dose-dependent effect on the activity of the regulatory sequences of neu as increasing amounts of LT led to decreased CAT activity of pNeuEcoRI-CAT (Fig. 2B). Thus, LT specifically inhibits the activity of the rat neu promoter.

We next mapped the region of the 2.2-kb neu-regulatory sequences that responds to LT. To this end, a series of deletion constructs of the neu-regulatory sequence-CAT (Fig. 3A) (36) were cotransfected with plasmid encoding LT into NIH 3T3 cells. Fig. 3B shows the CAT activity of each of the neu deletion constructs and the inhibition of this activity in the presence of LT (pVu-0 or pZ189). There was a 70 to 80% inhibition of the CAT activity of most of the neu deletion constructs except for pNeuXba1CAT and pNeuEcoRV2CAT. In repeated experiments, we found less repression by LT of these two constructs. Overall, the activity of all the deletion constructs, including pNeuXho1CAT, was repressed by LT. This indicates that repression of neu by LT is mediated through the 94-base pair Xhol-NarI region (−172 to −79, relative to first ATG) of the rat neu promoter.

Stimulation experiments have identified four transcription initiation sites in the rat neu promoter. Three of them, including the two major sites (at −158 and −147) are within 30 bp downstream of the Xhol site (36). We made further minor deletions of nucleotides downstream of the Xhol site using XbaI digestion (38). However, this led to a dramatic reduction of activity of the neu promoter (data not shown). Thus, the Xhol-NarI region of neu encompasses the minimum promoter of the rat neu gene, and LT inhibits the activity of the minimum promoter of neu.

Gel shift assays indicated that the 94-base pair Xhol-NarI DNA fragment specifically complexes with proteins in the nuclear extract of NIH 3T3 cells (Fig. 4, Lane 1). The complex, A, is detected using gels with large pore size (4.5% gels, acrylamide:bisacrylamide = 80:1) which have previously been shown to detect large DNA-protein complexes involved in transcription initiation (39), but not with gels with smaller pore size (acrylamide:bisacrylamide = 29:1) (data not shown). This suggests that A is a large DNA-protein complex that may involve factors in the initiation or elongation phase of transcription.
complex for neu transcription. However, nuclear extracts from cells that express LT, BTn 14 cell line, also gave a similar DNA-protein complex profile in such gel shift assays (Fig. 4, Lane 3). Thus, the presence of LT in the nuclear extract did not affect the mobility of complex A. One explanation of this is that complex A is already so large that the presence of LT (in nuclear extracts from BTn14 cells) does not create an observable difference in the shift. Indeed, complex A is found very near the top of the gel and is a broad band, suggesting the presence of multiple types of DNA-protein complexes. Another possibility is that HT has indirect or subtle effects on complex A at the XhoI-Nar fragment such as a change in phosphorylation of protein factors or a change in conformation of some factors that can not be detected by gel shift assays.

Since LT and Rb are known to form a protein complex (25) and Rb also modulates neu expression (28), we wanted to test whether a LT-Rb complex might be involved in repression of neu. To examine this, we utilized an available mutant of LT (K1) that has a single amino acid change within the region required for Rb binding (amino acids 105 to 114 of LT) (Fig. 5A) (35). K1 expresses mutant LT protein which is unable to complex Rb (25), and K1 is defective for transformation as assayed by focus-forming assay in Rat-1 cells (35, 40). We cotransfected pNeuXhoICAT together with plasmids encoding wild-type (pVU-0) or mutant LT (K1) into NIH 3T3 cells. Interestingly, K1 represses neu as effectively as wild-type LT (Fig. 5B). This suggests that complex formation between LT and Rb is not required for LT-mediated repression.

K1, unlike wild-type LT, is unable to transform Rat-1 cells in focus-forming assays (35). Therefore, the above results raise an interesting question as to whether K1 may function as a transformation suppressor of activated neu in Rat-1 cells. To test this possibility, we carried out focus-forming assays to determine the effect of stably transfecting K1 with activated genomic neu. The cosmids cNeu-104 encodes the activated genomic neu, which has a single point mutation in the transmembrane domain and is driven by 2.2 kb of neu upstream regulatory sequences (30). Upon introduction of cNeu-104 into normal Rat-1 fibroblasts, those cells that stably express activated neu are transformed and 3 to 4 weeks later form visible foci on a background of normal monolayer cells. When K1 was cotransfected with cNeu-104 into Rat-1 cells, it led to 50% reduction in the number of foci formed by cNeu-104 (Fig. 5C). Transfection of K1 alone does not induce any foci. Suppression of transforming activity of wild-type LT (pVU-0) is complicated by the fact that wild-type LT itself forms transformed foci in Rat-1 cells (data not shown), which makes it impossible to analyze the data. Therefore, mutant LT, unable to complex Rb, acts as a transformation suppressor of activated neu.
Discussion

Our results show that the function of the rat neu promoter is suppressed by the transforming viral oncogene SV40 LT antigen. The effect of LT is specific for the neu promoter since the activity of the promoter of the related epidermal growth factor receptor is not affected by LT. The inhibitory activity of LT on the neu promoter is similar to that observed for the adenovirus 5 E1a and the c-myc oncoproteins, with which LT shares structural and functional similarities. The inhibitory activity of LT is apparent in the LT-transfected stable cell lines, which showed an inverse correlation of neu p185 to LT protein expression. Thus, expression of LT in cells leads to reduced expression of neu-encoded p185 in cells.

LT inhibits the activity of the upstream regulatory sequences of neu. Series deletion analysis of the regulatory sequences of neu showed that repression by LT is mediated through the 94-bp XhoI-NarI region of the neu gene, which contains the minimum promoter 30 bp downstream of the XhoI site. However, in repeated experiments, we found that LT repressed the activity of two of the neu deletion CAT constructs, pNeuXbaI CAT and pNeuEcoRV2CAT to a lesser extent (Fig. 3B). One explanation is that these neu deletion constructs contain sequences with strong enhancer-like activity which become obvious when 5' flanking sequences are deleted (41). Thus, the CAT activities of these constructs are not significantly inhibited by LT. When the enhancer-like sequences are deleted in subsequent smaller constructs, the inhibitory effect of LT is clearly visible. Overall, the activities of all of the deletion constructs, including pNeuXho1CAT, were repressed by LT. This indicates that repression of neu by LT is mediated through the 94-base pair XhoI-NarI region (−172 to −79, relative to first ATG) of the rat neu promoter. This result is unlike that of c-myc and E1a, since these repress neu through an upstream region of the regulatory sequences of neu. Thus, LT mediates repression of neu through a different pathway compared to c-myc and E1a. Therefore, these structurally related oncogenes repress the activity of the neu promoter by acting through different regions of the regulatory sequences of neu. Although the promoter of the epidermal growth factor receptor and the promoter of neu share some common features (36, 37), LT did not inhibit the activity of the promoter of epidermal growth factor receptor. Thus, LT specifically affects the promoters of certain growth factor receptors.

Since LT specifically mediates repression of neu through the XhoI-NarI region, which contains only minimum sequence upstream of the two major transcription initiation sites, one possibility is that LT may modulate transcription initiation or elongation from the neu promoter. LT is known
to interact with cellular transcription factors such as AP-2 and to abrogate their function (42). However, examination of the 94-bp sequences within XhoI-NarI revealed no motif with significant homology to the AP-2 (36). Gel shift assays indicate that the XhoI-NarI sequence binds nuclear proteins to form a large DNA-protein complex. How LT affects the function of this complex is at present unclear. Moreover, whether the repression of neu is regulated at transcription initiation or elongation is an interesting issue yet to be determined.

Repression of neu by LT is independent of its ability to complex Rb and to transform cells. Therefore, although E1a, LT, and c-myc share a common domain for transformation (23) and Rb binding (24–26), this domain, at least in LT, is not required for repression of the neu promoter. This also supports the observation that LT represses neu via a different pathway compared to E1a and c-myc. Our results also show that a LT mutant defective for Rb binding and transformation function can be a transformation suppressor of the activated neu oncogene. This finding may provide an interesting approach to develop potential therapeutic agents that could down-modulate neu expression in human cancers.

**Materials and Methods**

**Cell Culture.** NIH 3T3, B104-1-1, and Rat-1 cells were maintained in 5% CO2 in Dulbecco’s modified Eagle’s medium-F-12 supplemented with 10% calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells transfected with the drug selection plasmid pSV2neo were grown in the above media containing 400 μg/ml G418.

**Plasmids.** The following plasmids have been described: neu deletion-CAT constructs (36), epidermal growth factor receptor-CAT construct, pERCAT-9 (37), plasmid encoding activated genomic neu, cNeu-104 (30), and control filler plasmid, pSV2E (36). Two LT-encoded plasmids were used, pZ189 or pVU-0, both of which showed similar results. Plasmids pVU-0 (43) and mutant LT-encoding plasmid, pK1 (35), were generous gifts from Dr. D. M. Livingston.

**Stable Transfections.** The drug selection plasmid pSV2neo was cotransfected with plasmids encoding LT into B104-1-1 cells. The transfected plates were trypsinized after 48 h, split into four plates, and subsequently maintained in media containing 400 μg/ml G418. After 3 weeks, colonies were isolated and established in media containing G418.

**Transient Transfections and CAT Assays.** Cells were transfected using the modified calcium phosphate precipitation technique (44). Cells were harvested 48 h after transfection, and cell extracts were obtained by freeze-thawing. For transfections involving LT, the protein concentration was determined using an aliquot of the extract. Aliquots of extracts containing equal amounts of protein were used for CAT assay (45). Transfections and CAT assays were repeated three to four times, and representative data are shown.

**Immunoblotting.** Immunoblotting was performed as described (46). Confluent cells grown in 10-cm dishes were washed and lysed with lysis buffer, and 100 μg protein were loaded for electrophoresis on sodium dodecyl sulfate-polyacrylamide gels followed by transfer to nitrocellulose. To detect expression of p185, blots were incubated with anti-neu antibody (c-neu, Ab-3; Oncogene Science, Manhasset, NY), and then reacted with secondary antibody, goat anti-mouse conjugated with horseradish peroxidase. The nitrocellulose was subsequently developed with horseradish peroxidase substrate, 4-chloro-1-naphthol and hydrogen peroxide. To analyze the expression of LT antigen, blots were probed with monoclonal antibody specific for LT (SV40 T-Ag, Ab-2; Oncogene Science). Blots were incubated with 1 μg/ml 125I-Protein A. After further washing, dried blots were exposed for autoradiography.

**Southern Blotting.** Genomic DNA was harvested from cells and digested with BamHI for Southern blotting as described (11). Blots were hybridized using 32P-labeled rat neu cDNA probe.

**GeI Shift Assay.** This assay was carried out as described (36).

**Focus-forming Assay.** Focus-forming assay was carried out as described (28). The cosmid clone cNeu-104 (30) contains 30 kb of activating genomic rat neu including 2.2 kb of the neu promoter. cNeu-104 (0.5 μg) was cotransfected into normal fibroblasts (Rat-1 cells) with 0.1 μg of the drug selection plasmid pSV2neo, and 5 × 106 μg plasmids encoding mutant LT (pK1) or control filler plasmid, pSV2E. Cells were trypsinized and split into four plates 48 h after transfection. Two plates were maintained in regular media, whereas the other two plates were maintained in media supplemented with G418. For cells kept in regular media for 3 weeks, foci of transformed cells appeared on a background monolayer of nontransformed cells. G418-resistant colonies appeared for plates maintained in G418 media. Foci and G418-resistant colonies were stained with 1% crystal violet and counted. To normalize for transfection efficiency, the number of foci formed for each transfection was divided by the number of G418 colonies obtained.

**References**


