Characterization of a Repressor Element and a Juxtaposed Tissue-restricted Activator Element Located on the Distal neu Gene Promoter*

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The proto-oncogene neu (HER2 or c-erbB2) is overexpressed with or without gene amplification in 20–30% of breast cancers. In patients, neu amplification or overexpression in breast and ovarian cancer correlates with poor prognosis and tumor resistance to chemotherapy. neu-induced transformation can be reversed by the suppression of neu gene transcription. To further understand how neu gene transcription is regulated and to identify a possible transcriptional repressor(s) of neu, we identified a negative regulatory element known previously to be located within a 1-kilobase (kb) DNA fragment of an unknown sequence, upstream of the proximal neu gene promoter. One of several DNA fragments subcloned from this region suppressed transcriptional activity of the proximal neu gene promoter. Sequencing of the 1-kb fragment confirmed the location of the repressor element to be between an AluI and a RsaI sites, around 1.4 kb upstream to the translation start site. Various deletions were introduced into the AluI-RsaI fragment and subcloned into both the native neu promoter and a heterologous thymidine kinase promoter. Subsequent transfections and reporter gene assays in cell lines of various tissues of origin confirmed and narrowed the repressor activity to a 120-base pair NsiIV-MspI fragment located between −1385 and −1266. Importantly, specific protein binding activity to this element could be detected with nuclear extracts isolated from these cell lines. In contrast, a 28-base pair MspI-RsaI fragment (−1265 to −1238), located immediately 3′ of the putative repressor element, was found to form protein-DNA complexes with only nuclear extracts isolated from a colon carcinoma cell line. This specific protein binding activity correlated with a previously unknown transcriptional stimulatory activity only in this cell line.

neu was originally identified as the transforming gene in ethylnitrosourea-induced neuroblastomas in BDIX rats (1, 2). Its human ortholog was isolated and found to be homologous to the epidermal growth factor receptor (EGFr, HER1, or erbB1, where v-erb is the retrovirally transduced and truncated EGFr (3–7) and was therefore named as HER2, or erbB2. In this report, neu will be used to refer specifically to the rat gene, whereas HER2 will be reserved for the human neu gene, and erbB will be used as a general term across species. There are now four members of the erbB gene family: erbB1, erbB2, erbB3, and erbB4 (8, 9). They are all located at the cell surface and function as growth factor-activated membrane tyrosine kinase receptors (9). Many ligands for the erbB family have been identified including epidermal growth factor, transforming growth factor-α, heregulin, neu differentiation factors, amphiheregulin, and the expanding family of neuregulins (10–12). Despite intense efforts over the years, no direct ligand for the erbB2 protein has been found, and its very existence has recently been questioned (13). Nevertheless, erbB2 appears to play a central role in the signal transduction pathway for other family members, as it is a preferred dimerization partner (13–15).

HER2 is one of the most commonly altered proto-oncogenes in human cancers (9, 16, 17) and almost always involves amplification and overexpression. Recent studies have suggested that certain subtle deletions of the erbB2 gene product might accompany overexpression (18), resulting in an activation of its signal transduction pathway. As many as 20–30% of human breast and ovarian cancers are found to exhibit HER2 gene amplification or overexpression (19, 20, and reviewed in Ref. 16), which correlates with reduced survival (19, 20). A correlation between tumor erbB2 status and resistance to therapy has also been demonstrated (16, 21), although others have suggested that this effect might be cell line- or tumor-specific (22).

It has been clearly demonstrated that overexpression of erbB2 causes cell transformation (23–25) and mammary cancer in transgenic mice (26–28). Several proteins, including c-myc (29), adenoviral E1A (30), SV40 large T antigen (31), and Rb (32), were found to suppress neu gene expression through the neu gene promoter (33). Although the underlying mechanisms of how these genes suppress neu are not well understood, the E1A gene has now proceeded to early clinical testing in breast cancer patients with tumors overexpressing HER2 (34, 35). However, E1A and the other genes as mentioned above, are known to participate in a wide spectrum of important cellular processes and are likely to have undesired effects if given clinically. Thus, a more specific repressor of neu gene transcription would be most desirable.

We report here the sequence of a 1-kb DNA fragment, located upstream to the proximal 500-bp neu gene promoter (36), which possesses such repressor activity. Subcloning different fragments from this 1-kb DNA into reporter gene constructs, using successive deletions from the 5′-end, and analyzing in an

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank¶¶/EBI Data Bank with accession number(s) AF208052.

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¶¶ The abbreviations used are: kb, kilobase pair(s); bp, base pair; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; TK thymidine kinase.

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heterologous promoter allowed us to narrow the repressor activity to a 148-bp fragment. Detailed functional analyses and protein binding electrophoretic mobility shift assays (EMSSAs) correlated protein binding activity to a 120-bp NtAlv-MsiI fragment, with transcriptional repressor activity detected in various cell lines. Interestingly, the immediate 3′ 28-bp MsiI-RsaI fragment was able to function as a transcriptional activator in a colon carcinoma cell line. The functional stimulatory effect of this 28-bp fragment correlated with the formation of specific protein-DNA complexes, detectable only with nuclear extract isolated from the same colon carcinoma cell line. As HER2 gene overexpression has been demonstrated in colon cancer, this cis-acting activator element and its interacting proteins might play a role in colon cancer. The characterization and localization of the repressor element provides a good starting point for subsequent isolation of the interacting protein(s). In the future, it may be possible to use such proteins as anti-cancer therapy in patients with tumors that overexpress HER2.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents—Restriction enzymes and other DNA modifying enzymes including Klenow fragment, T4 polymerase, T4 ligase, and calf intestinal phosphatase were purchased from Life Technologies Inc., New England Biolabs (Mississauga, Ontario), Roche Molecular Biochemicals, or Amersham Pharmacia Biotech. All isocrypts were obtained from Amersham Pharmacia Biotech. Chemicals used for the chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were purchased from Sigma-Aldrich. Thin layer chromatography (TLC) plates were the products of Eastman Kodak Co. Cell culture media and reagents were obtained from Life Technologies Inc.

Plasmids—The plasmid pBluescript (IKKS) (Stratagene, La Jolla, CA) was used for general subcloning purposes. pMTIC3 is a plasmid containing multiple cloning sites that was placed upstream of the CAT gene (36). Most of the neu promoter DNA restriction fragments were cloned into pBluescript (IKKS) and were then shuffled into the matching unique restriction sites on the polylinker of pMTIC3. DNA fragments were blunt-ended with Klenow fragment or T4 polymerase when no appropriate restriction enzymes could be used for directional cloning. The pNeuEcoRCAT construct containing the proximal 500-bp neu gene promoter linked to the CAT gene has been previously described (36). This construct was used as a reporter for analyzing the effects of various subcloned DNA fragments on the transcriptional activity of the native neu gene promoter. The plasmid pBLCAT2 (37), containing the herpes simplex virus thymidine kinase (TK) promoter linked upstream to the CAT gene, was used for analyzing the effects of various subcloned fragments on the transcriptional activity of a heterologous promoter. Successive deletions from the 5′-end of neu promoter were generated with appropriate restriction enzyme sites. pCMVβ (CLONTECH, Palo Alto, CA) is a plasmid that contains the human β-globin gene driven by the hamster ovary cell line; HeLa, a human cervical carcinoma cell line; Caco2, a human colon carcinoma cell line; MCF7 and MDA-MB-453, human mammary carcinoma cell lines; and C2C12, a mouse myoblast cell line. All cell lines were cultured in Dulbecco’s modified Eagle’s F12 medium (Life Technologies Inc.), supplemented with 10% fetal calf serum and kept in a humidified, 37 °C, 5% CO2 incubator.

Transfections and CAT Assays—A calcium phosphate precipitation method (39) was used for transfection as modified and described previously (29). Briefly, cells were split at a predetermined ratio into 100-mm tissue culture dishes (Falcon, Becton Dickinson) the day before transfection. Unless otherwise indicated, 1 µg of pCMVβ and 10 µg of a CAT reporter DNA were co-precipitated in the buffer at room temperature for 25 min before they were directly added to the cells. Precipitate was incubated with the cells for 16–20 h, after which the cells were washed three times with phosphate-buffered saline, re-fed with fresh medium, and returned to the 37 °C incubator. Cells were washed and harvested after 20–24 h, and the freeze/thaw/vortex cycle method was used to lyse the cells. One-fifth of the cell lysate was used for the β-galactosidase assay using O-nitrophenyl-β-d-galactopyranoside as substrate, and the results were used to adjust the amount of lysate for the CAT assay. The TLC method of CAT assays was performed as described previously (36), except that the standard [(14C)]chloramphenicol was replaced with 1-(dichloroacetyl)-1-phenyl-2-naphthylchloramphenicol (Amersham Pharmacia Biotech) (40). The single acetylated product improves the quantitative assay of the CAT activity.

EMSSAs—EMSSAs were performed as described previously (40). Nuclear extract was isolated from the different cell lines with the Dignam method (41). The DNA fragments were isolated by digesting a plasmid subclone with appropriate restriction enzymes, gel-purified, and labeled with [32P]dATP or [32P]dCTP (depending on the restriction site) by a Klenow fragment. A final volume of 30 µl of reaction mixture was prepared in the order of H2O, 10× binding buffer (1×: 10 mM Tris, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 1 mM MgCl2, 5% glycerol), 3 µg of poly(dI-dC)-poly(dI-dC), 10 µg of nuclear extract, an appropriate amount of unlabeled competitor if desired, and last, 20,000 cpm of the DNA fragment. The mixture was incubated at room temperature for 25 min, after which it was loaded onto a 6% native polyacrylamide gel. The gel was dried under vacuum in a gel dryer and exposed to a Kodak XAR-5 film at −80 °C.

RESULTS

Sequence of the 1-kb Putative Repressor Element—The relative position of the 1-kb XbaI -EcoRV repressor fragment in relation to the transcription start sites of neu is shown at the top of Fig. 1. As described previously, the nucleotide A at the translation start site (ATG) of neu gene was assigned +1, and nucleotides upstream of +1 were assigned negative numbers (36). Complete sequencing revealed a 1,046-bp DNA fragment. Multiple E-boxes (CANNTG, marked by striped boxes underneath the nucleotides), representing canonical binding sites for the helix-loop-helix family of transcription factors (42), were found. Two GT boxes (GGGTGG, on the opposite strand, marked by striped boxes above the sequences), which represent consensus recognition sequences for the Sp1 family of transcription factors (43), were observed. Numerous TC-rich (or GA-rich on the opposite strand) sequences of various lengths (marked by open boxes beneath the nucleotides), which are potential binding sites for the Sp1 (43) and eukaryotic transcription factors, were detected throughout the 1-kb fragment. An A-rich (55 out of 45 nucleotides) sequence could be detected between −715 and −759. Extensive discussion of consensus transcription factor binding sites is not meaningful unless functional relevance is demonstrated. Our discussion will therefore be limited to several sites that were located within the minimal functional repressor element as noted in a later section.

Localization of Repressor Activity to an Alu-I-Real Fragment—It has been shown previously that the sequence between the XbaI (−1543) and EcoRV (−502) sites contains a transcriptional repressor element (36). Before the sequence information was obtained, blunt end restriction fragments from the 1-kb

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Orientation—Orientation of the neu gene promoter was confirmed when deletion mutants and individual subcloned fragments were sequenced.

Cell Culture—Several cell lines that represent different tissues of
DNA were randomly cloned upstream of the proximal neu gene promoter-CAT reporter construct, pNeuEcoRVCAT (36). These constructs were transfected into Chinese hamster ovary cells, and their activities were compared with that of pNeuEcoRVCAT (assigned an activity of 100 as a reference) (Fig. 2A). A 600-bp Alu1-Alu1 fragment (construct pNeuAA2/EcoRVCAT) was found to lower CAT activity by 3-fold, whereas another smaller Alu1-Alu1 fragment (pNeuAA2/EcoRVCAT) did not alter CAT activity significantly. Repressor activity could be further localized to a 215-bp Alu1-RsaI fragment (constructs pNeuAR/EcoRVCAT and pNeuAR/EcoRVCAT). These constructs showed repressor activity, were sequenced, and their positions were mapped when the complete XbaI-EcoRV sequence was obtained. However, during the analysis of the random fragments from the 215-bp Alu1-RsaI region as in Fig. 2A, a series of deletions from the 5′-end were generated (Fig. 2B). The activities of these deletion constructs were evaluated in two more cell lines, HeLa and C2C12 as shown. Consistent with the data from the random subcloning experiments, the 215-bp Alu1-RsaI fragment as seen in the random subcloning experiments.

Sequence Analysis of the 215-bp Putative Alu1-RsaI Repressor Element—An interesting feature of the 215-bp Alu1-RsaI fragment is the existence of numerous pairs of direct or inverted repeats (Fig. 3, shown as arrows above or below sequences with numbers, indicating the matching pair of repeats). Two GT boxes (GGGTGG), representing the consensus binding sites for the Sp1 family (43), could be found on the bottom strand at −1232 and −1307 (striped boxes above nucleotides). An E-box (CANNTG), which represents a consensus binding site for the helix-loop-helix family of transcription factors, is marked with striped boxes above the nucleotides. Canonical binding sites for the helix-loop-helix family of transcription factors are marked with open boxes underneath the sequences. Sp1 family of transcription factor recognition sites are marked with striped boxes above the nucleotides. Canonical binding sites for the helix-loop-helix family of transcription factors are marked with stippled boxes beneath the sequences. The sequence shown here has been deposited in the GenBank (accession number AF208052).

To confirm if repressor function could be mapped to the same (Alu1-RsaI) region as in Fig. 2A, a series of deletions from the 5′-end were generated (Fig. 2B). The activities of these deletion constructs were evaluated in two more cell lines, HeLa and C2C12 as shown. Consistent with the data from the random subcloned fragments, the pNeu5′AluCAT and pNeuAccI CAT, when compared with pNeuEcoRVCAT, was observed only in C2C12 cells. These data suggested that repressor activity could be found between the Alu1 and Sp1 sites, which encompasses the 215-bp Alu1-RsaI fragment as seen in the random subcloning experiments.
Transcriptional Repressor Element of the neu Gene Promoter

**FIG. 2.** Localization of transcriptional repressor activity to a 215-bp Alu-I-RsaI fragment within the 1-kb DNA. A, restriction enzymes that generate blunt ends (AluI, EcoRV, HaeIII, and RsaI) were used to cut the 1-kb XbaI-EcoRV fragment, and the subfragments generated were cloned upstream of the proximal neu gene promoter CAT constructs (pNeuEcoRVCAT). After transfection into Chinese hamster ovary (CHO) cells, their effects on the transcriptional activity of the proximal neu promoter were compared with that of the complete 1-kb fragment (pNeuXbaICAT). Activities are shown as a relative number to that of the proximal promoter (assigned as 100). The positions of the different fragments were their effects on the transcriptional activity of the proximal neu promoter were compared with that of the complete 1-kb fragment (pNeuXbaICAT). Activities are shown as a relative number to that of the proximal promoter (assigned as 100). The positions of the different fragments were confirmed only after the complete 1-kb sequence was obtained. The closed box at the bottom marks the position of a 106-bp Alu-I-EcoRV fragment, which was not analyzed in this experiment. B, successive deletions from the 5'-end were made between the XbaI site and the proximal promoter (EcoRV site at ~502). The position of the Alu-I-RsaI fragment mapped in A is shown as a striped box. The activities of these constructs were evaluated in both the HeLa and C2C12 cells and shown as a relative number to that of the pNeuEcoRVCAT construct (assigned as 100). Cross-comparison between the cell lines should not be made with individual constructs, since transfection was not normalized across the cell lines in this experiment. Relative CAT activities shown are the means of three experiments, and the standard deviation was less than 10%.

the complete 215-bp Alu-I-RsaI fragment (insert 1). The 3' 84-bp HaeIII-RsaI fragment was not effective in suppressing transcriptional activity of the pNeuEcoRVCAT. These data suggested that the 64-bp NlaIV-HaeIII fragment was more effective than the 50-bp AluI-RsaI fragment (insert 5). Hormone receptor activity of the 264-bp fragment was therefore, weaker than the NlaIV-RsaI fragment. When this 64-bp fragment (insert 5) was analyzed in the same way, the repressor activity was 2-fold weaker than the NlaIV-RsaI fragment. The addition of sequences to the NlaIV-HaeIII fragment (insert 5) from the 5'-end (AluI-HaeIII, insert 3) also increased its repressor activity. Therefore, although the NlaIV-HaeIII fragment (insert 5) fragment was able to suppress the neu gene promoter by 2-fold, addition of sequences from either the 5'- or the 3'-end resulted in an enhancement of repressor activity. It is interesting that neither the 5' AluI-NlaIV fragment (insert 2) nor the 3' HaeIII-RsaI fragment was able to suppress the neu gene promoter by 2.5-fold, addition of sequences from either the 5'- or the 3'-end resulted in an enhancement of repressor activity. It is interesting that neither the 5' AluI-NlaIV fragment (insert 2) nor the 3' HaeIII-RsaI fragment (insert 6) fragments alone contain appreciable repressor activity. These results suggested that possible interactions among the fragments may contribute to the overall repressor activity. This is consistent with the progressive loss of repressor activity being observed with successive 5' deletions from the AluI site to the SphI site in Fig. 2B.

Repressor Activity Can Be Transferred to a Heterologous Promoter and May Be Tissue-specific—To confirm repressor activity and to test its functionality in the context of a heterologous promoter, the individual fragments (Fig. 3B, inserts 1-6) were cloned upstream of the TK promoter. A colon carcinoma cell line, Caco2, was also examined in these experiments to provide stronger evidence for the functional activity and test whether the observed activity was tissue-specific. As seen with the native neu gene promoter, the NlaIV-RsaI fragment (insert 4) was able to suppress the TK promoter (activity was assigned 100 as a reference) by 3-fold in the C2C12 cells (Table I, column 2), thereby confirming its function as a repressor element. All the other fragments except the entire 215-bp (insert 1, a 2.5-fold suppression) were not very effective in suppressing the TK promoter. Most interestingly, the 215-bp AluI-RsaI fragment (insert 1), although able to suppress both neu and the TK promoter in C2C12 cells, was found to have the opposite effect, i.e. transcription activation, in the Caco2 cells. This 3-fold transcriptional stimulatory effect could be mapped to the 3' 84-bp HaeIII-RsaI fragment (insert 6). Thus, the suppressive effect of the NlaIV-RsaI fragment (insert 4) fragment (on either the neu or TK promoter in C2C12 cells) was overcome by a transcriptional enhancing activity in the Caco2 cells, since it did not alter the activity of the TK promoter. Nevertheless, these data clearly showed 148-bp NlaIV-RsaI to be the minimal DNA fragment that was able to suppress the transcriptional activity of both the neu and the TK promoters in several cell lines. However, it was not clear whether the same repressor activity also existed in the colon carcinoma cell line or whether it was masked or nullified by an equally potent activator located within the 84-bp HaeIII-RsaI fragment (insert 6).

Segregation of the Repressor and Activator Activities into Individual Fragments—The 148-bp NlaIV-RsaI fragment contains many pairs of inverted and direct repeats, two inverted GT boxes, and an E-box (Fig. 3). Since the C2C12 cell line used in the experiments described above is a mouse myoblast cell line and many E-box-binding proteins are muscle-specific, the possible contribution of the E-box to functional activity was examined. The NlaIV-RsaI fragment was cut at the middle of the E-box, generating a 120-bp NlaIV-MalII and a 28-bp MalII-RsaI fragments. These were cloned upstream to the pNeuEcoR-
VCAT, and their effects on the neu gene promoter were compared with that of the complete 148-bp NlaIV-RsaI repressor fragment (Fig. 4). A breast cancer cell line MDA-MB453 was also tested in this experiment, which served two purposes. 1) It represents another cell line of a different tissue of origin, and 2) since it overexpresses HER2, would provide evidence as to whether the stimulatory effect in the colon carcinoma cell line was tissue-specific or was related to the level of expression of HER2. As seen in previous experiments in C2C12 cells, the NlaIV-RsaI fragment (lane 2) suppressed the proximal neu gene promoter (lane 1) by more than 5-fold. The shortened NlaIV-MslI fragment, which now harbored a truncated E-box (lane 3), suppressed the neu promoter as effectively as the NlaIV-RsaI repressor, thereby suggesting that the E-box was not necessary for repressor activity. The 3928-bp MslI-RsaI fragment in contrast did not have repressor activity (lane 4).

Interestingly, in Caco2 cells, the NlaIV-RsaI fragment, although unable to suppress the TK promoter (Table I, column 2, insert 4), was effective in suppressing the neu gene promoter by 3-fold (compare lane 2 to lane 1). The same suppression was observed with the shorter NlaIV-MslII fragment (lane 3), again

FIG. 3. Sequence of the 215-bp AluI-RsaI fragment and its sub-fragments. A, the sequence between the AluI (−1452) and RsaI (−1238) sites, which was demonstrated to harbor the repressor activity in Fig. 2. The consensus recognition sequences for the Sp1 family of transcription factors are marked with striped boxes above the nucleotides. A consensus E-box, representing a potential binding site for the helix-loop-helix family of transcription factors, is marked with a stippled box underneath the nucleotides. Direct or inverted repeats are marked by matching pairs of numbered arrows. The open circles and Xs on the arrows of repeats 1 and 5 mark the position of a mismatched nucleotide within the repeat. Repeats 3, 8, 9, and 10 all contain the tetranucleotides GTGT. B, subcloned fragments within the AluI-RsaI DNA. The complete AluI-RsaI fragment is shown schematically with all the features matching the sequence noted in A. All fragments were named with the bounding restriction sites as shown and were cloned into pBlueScript(IIKS) for EMSAs or into pNeuEcoRVCAT and pBLCAT2 for functional analyses.

![Sequence of the 215-bp AluI-RsaI fragment and its sub-fragments](image)

**Table 1**

| Row | Insert     | NeuEcoRVCAT (C2C12 cells) | TK-CAT C2C12 cells | Caco2 cells |
|-----|------------|---------------------------|-------------------|-------------|
| 1   | AluI-RsaI  | 37                        | 45                | 299         |
| 2   | AluI-NlaIV | 100                       | 57                | 89          |
| 3   | AluI-HaeIII| 26                        | 67                | 93          |
| 4   | NlaIV-RsaI| 19                        | 33                | 113         |
| 5   | NlaIV-HaeIII| 42                      | 80                | 115         |
| 6   | HaeIII-RsaI| 74                       | 77                | 336         |
ruling out the contribution of the E-box to repressor activity. Most interestingly, the 3′ 28-bp MslI-RsaI fragment functioned to activate the neu gene promoter by 2-fold (lane 4). These data were consistent with that observed with the TK promoter in this particular cell line. In the MDA-MB453 (HER2 overexpressing) breast cancer cell line, as in the C2C12 cells (low erbB2 expression), the functional activities of the fragments behaved similarly. These results suggested that repressor activity could be localized within the 120-bp NlaIV-MslI fragment and that this repressor activity might be universally found in cells of various tissues of origin. The activator effect, in contrast, was only found in the Caco2 cell line. Nevertheless, the repressor activity appeared to be dominant over the activator within the context of the native neu gene promoter.

**Correlation of Protein Binding Activity with Respect to the Functional Activator or Repressor Element**

The data obtained indicated that the 148-bp NlaIV-RsaI fragment contains a universal repressor activity functional on different promoters in multiple cell lines tested and a tissue-restricted transcriptional activator activity. EMSAs were employed to determine if protein transcription factors might bind to this DNA fragment and whether binding activity could be localized to the functional repressor and activator DNA fragments, respectively. Nuclear extract was isolated from Caco2 cells and incubated with [32P]-labeled NlaIV-RsaI fragment (Fig. 5A, lanes 1–7). Multiple retarded bands representing protein-DNA complexes were detected in the presence of nuclear extract (lane 2), and their specificity was determined by the addition of a 100-fold excess of various unlabeled DNA as competitor (lanes 3–7). The intensity of several complexes (labeled C1–C3) was lowered in the presence of self-unlabeled fragment (lane 3), suggesting these bands were specific to the NlaIV-RsaI fragment. Progressively shorter fragments were used as competitors to localize the binding site for these complexes to a smaller region. The effective competition of formation of C3 observed with the NlaIV-MslI fragment (lane 4) but not the AccI-MslI fragment (lane 5) suggested that the sequence between the NlaIV and AccI sites was important for the formation of C3. The inability of the 3′ 28-bp MslI-RsaI fragment (lane 6) and a totally irrelevant DNA (lane 7, NS) to compete for the formation of C3 complex further confirmed its specificity. The competition profile for C1 and C2 suggested that the two complexes might not be as specific but might also reflect a more complex binding mechanism (see below). Since the NlaIV-MslI fragment appeared to be more effective in competing for the formation of C3 (Fig. 5A, lanes 1–7), the slower migrating bands seen with the NlaIV-RsaI fragment (including C1 and C2) were not detected, suggesting the 3′ MslI-RsaI fragment was required for their formation. C3, however, was clearly detected (lane 9) as well as the NlaIV-RsaI fragment (lane 2). Consistent with the binding activity to NlaIV-RsaI fragment, both NlaIV-RsaI (lane 10) and NlaIV-MslI (lane 11) were able to compete for C3, whereas the shorter AccI-MslI (lane 12) did not have this ability, again indicating the importance of the sequence between NlaIV-AccI. As expected, the MslI-RsaI (lane 13) and a nonspecific DNA (lane 14) did not compete for the formation of the C3 complex.

Although the competition for the more diffuse C1 and C2 bands suggested a low specificity (Fig. 5A, lanes 3–7), the deletion of the MslI-RsaI fragment (lanes 8–14) clearly showed that it was required for their formation. Most importantly, the MslI-RsaI fragment was found to harbor the transcriptional enhancing activity in the Caco2 cells (Fig. 4). The MslI-RsaI fragment was therefore labeled and subjected to similar EMSAs (Fig. 5B). As predicted from results shown in Fig. 5A, slow migrating complexes (positions indicated by arrows, labeled as M1–M3) were detected when Caco2 nuclear extract was incubated with the MslI-RsaI fragment (Fig. 5B, lane 2). These complexes were competed away by the unlabeled MslI-RsaI fragment.
lanes 1

the Caco2 nuclear extract and the three DNA fragments as indicated abolished protein binding activity. EMSAs were performed with (Nla with an arrow (49–61) has been well characterized. The presence of a repression activity of the 1-kb fragment was described as a net effect of the 1-kb region might modulate its activity depending on cell activity of the 1-kb fragment was described as a net effect of the

This result not only confirmed the existence of a putative cis-acting repressor activity but proved it worthwhile to proceed with sequencing the whole 1-kb fragment (Fig. 1). In retrospect, the strategy of analyzing randomly cloned fragments was satisfactory, as only a small 106-bp Alu-EcoRV fragment was missed. This minor drawback did not affect interpretation of the results, as the series of successive 5' deletion constructs (Fig. 2B) clearly complemented and confirmed the location of the repressor element to the 215-bp Alu-RsaI fragment.

Within the Alu-RsaI fragment, only the 148-bp NlaIV-RsaI fragment was able to suppress both the native neu gene and the heterologous TK promoter in various cell lines (Table I, Fig. 4, and data not shown). However, several notable results suggested that interactions among different regions of the Alu-RsaI fragment most likely contributed to its repressor activity. For instance, neither the AluI-NlaIV (insert 2) nor the HaeIII-RsaI (insert 6) fragment was effective in suppressing neu promoter activity (Table I, column 1), but their presence in the Alu-RsaI (insert 1) or NlaIV-RsaI (insert 4) fragment, respectively, resulted in an enhancement of the repressor activity of the NlaIV-HaeIII fragment (insert 5). For the repressor activity in Caco2 cells (Table I, column 2), neither the AluI-NlaIV (insert 2) nor the NlaIV-RsaI (insert 4) fragment had an effect on the TK promoter activity; however, the Alu-RsaI fragment (insert 1), which is equivalent to AluI-NlaIV and NlaIV-RsaI linked in tandem, was able to stimulate the TK promoter by 3-fold (Table I, column 2). Possible interactions among the fragments might also explain the apparent nonspecific competitive effect on C1 and C2 by various fragments in the EMSA (Fig. 5A). This possibility was further substantiated by the presence of multiple pairs of repeats within the fragment (Fig. 3), some of which share a common motif (GTGT in repeats 3, 8, 9, and 10). The stimulatory effect of the 215-bp Alu1-RsaI fragment on the TK promoter in the Caco2 cell line (Table I, column 2) could be due to a dominant repressor located within the HaeIII-RsaI fragment. This could also be accompanied by an absence of the repressor in this cell line, as suggested by the lack of effect of all the other fragments on transcription (inserts 2–4, column 2, Caco2 cells). However, when the effects of the same DNA fragments were tested in the context of the native neu gene promoter, a repressor activity clearly existed in the skin cells (Fig. 4). Thus, although both negative and positive factors appeared to interact with the same fragment in the Caco2 cells, the balance between the two effects could be shifted to either one or the other, depending on the context of the proximal promoter. This suggests that there might be interactions between the upstream regulatory element (the Alu1-RsaI fragment) and certain elements located in the proximal promoter (neu or TK) and that these interactions can determine whether the positive or the negative factor is dominant.

The C2C12 cell line used in many of our experiments are mouse myoblast cells, and muscle tissue contains many helix-loop-helix transcription factors, which potentially would recognize the E-box within the NlaIV-RsaI region. A possible contribution of the E-box to the repressor activity was ruled out, since its truncation had no effect on the repressor activity (Fig. 4). This result further narrowed the repressor activity to a minimal 120-bp NlaIV-MsiII fragment (Fig. 4). The C3 complex shown in Figs. 5 and 6 could ultimately be responsible for the repressor activity. Although our present data suggest that the sequence located at the AccI site was important for C3 binding,
complicated interactions among the various fragments might affect the protein binding and functional activity. Generating more detailed deletions and examining their effects on transcriptional represer binding site would help to clarify and confirm the location of the repressor binding site.

The stimulatory effect of the 28-bp Msl1-Ras1 fragment also correlated with protein-DNA complex formation (M1-M3), which was shown to be specific to the Caco2 cells. Further investigation is necessary to determine if and how the C1 and C2 complexes detected in Fig. 5A are related to the M1-M3 complexes in Fig. 5B. It is tempting to speculate that these proteins are colon-specific transcriptional activators of neu gene. HER2 has been found overexpressed in colon cancer, and a loss of balance between the repressor and the activator activities could certainly contribute to HER2 overexpression in colon cancer. Further studies on other colon cell lines that express various level of HER2 (62) would provide stronger evidence for the possible involvement of this regulatory element in colon cancer.

In summary, we have obtained sequence information on a 1-kb DNA fragment, known previously to harbor transcriptional repressor activity upstream to the proximal neu gene promoter. Cis-acting repressor activity has also been reported at the distal region of both the human (54) and mouse neu (63) promoter. Our work provides the first detailed characterization of such a distal neu promoter repressor activity. This transcriptional repressor activity has now been narrowed down to a 120-bp NtaIV-Msl1 fragment, located approximately 1.4 kb upstream of the neu translation start site. Protein factors that bind to this repressor element were detectable in multiple cell lines of different tissues of origin. Regulation of neu gene expression by this repressor element might therefore be a universal mechanism and might explain the generally low level of expression of erbB2 in most adult tissues (64). A mutation in such a repressor could be the underlying cause of HER2 overexpression in certain tumors. The identification of such a repressor could lead to another approach to turning off the HER2 gene in cancer. In addition, we have detected a 28-bp previously unknown activator sequence located immediately downstream of the repressor element. Its stimulatory effect on transcription was specific to a colon cancer cell line and correlated with protein binding activity, detectable only in the same cell line. This raises the possibility that there might be colon-specific activators that regulate erbB2 gene transcription in the colon. A disruption in the balance between this activator and the repressor could be an underlying alteration that leads to HER2 overexpression in colon cancer.

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Characterization of a Repressor Element and a Juxtaposed Tissue-restricted Activator Element Located on the Distal neu Gene Promoter
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