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DIFFERENTIAL EXPRESSION OF THE *NEU* ONCOGENE IN MOUSE LIVER AND PANCREATIC CELL LINES

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Summary: To study the tissue-specific expression of *neu* oncogene, we used two mouse tumor cell lines derived from liver, Hep1-a, and pancreatic, 266-6, tumors as a model system. The endogenous *neu* gene is expressed in Hep1-a but not in 266-6 cells. We demonstrate in this report that differential expression of the *neu* gene in these two cell lines is mainly regulated at transcriptional level. The *neu* promoter sequence responsible for the differential regulation is localized within a 90 bp region and it is possibly due to lack of a specific positive transcription factor(s) interacting with this region in 266-6 cells.

Neu gene expression in normal human and rodent tissues occurs preferentially in epithelial cell types including the gastrointestinal, respiratory, reproductive, and urinary tract as well as skin, breast, and placenta.(1,2). Studies of human adenocarcinomas of breast (3-7), ovary (8-10) lung (11,12), and kidney (7) as well as tumors of digestive tract tissues including the salivary gland (13) and stomach (7,14,15) frequently overexpress *neu* p185 protein. These finding suggest that the tissue-specific expression of the *neu* gene may be important in the process of tumorigenesis where the *neu* gene is generally expressed or overexpressed.

It is known that *neu*-encoded protein, p185, expresses in both human and rat liver tissues (16). In contrast, in normal adult human, the pancreatic ducts and acini have none or very low level of p185 protein (1). We have studied tissue-specific expression pattern of the *neu* gene by comparing the *neu* expression between a mouse liver (Hep1-a) (17) and a pancreatic (266-6) (18) cell lines as a model system. The *neu* gene

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expression is readily detectable in liver but not pancreatic cell lines. The mechanism for the differential expression between Hep1-a and 266-6 cell lines was further shown to be regulated at transcriptional level and is likely due to the lack of specific positive transcription factor(s) in the pancreatic, 266-6, cell line.

Materials and Methods

Cell lines. Hep1-a is a cell line derived from a spontaneous mouse liver tumor (17). 266-6 cells were obtained from a mouse pancreatic tumor immortalized by simian virus 40 (SV40) large T antigen (LT) (18). The hybrid cell line, H/26, is a result of cell fusion between Hep1-a and 266-6 cells (19). AR42J (20) is a rat pancreatic tumor cell line without SV40 LT immortalization.

DNAs. The *neu* promoter deletion CAT constructs have been described previously (21). The pXhoI-NarI plasmid was constructed by *Bam*HI digestion of pNeuXhoICAT to remove the CAT gene, followed by religation. For gel-shift assays, two synthetic oligonucleotides were made: GCF binding site(5' *TCGA*$^{-149}$GTGGAGCTGAGATTGCCCGCCGCTG^{-125} 3') and E4TFI binding site(5' *AGCT*$^{-173}$CTCGAGGAAGTGCGG^{-159} 3'). The protein binding consensus sequences are underlined (22,23), and the artificially added restriction enzyme sticky ends (*Sal*I and *Hind*III,respectively) are italicized. The positions are numbered relative to the first ATG.

Western blot, CAT, and gel-shift assays were performed as described previously (24,25).

Results and Discussion

To test the endogenous *neu* expression in liver and pancreatic cell lines, we examined expression of p185 protein by western blot analysis. Like the positive control, NIH3T3 cells, Hep1-a liver cell line expresses substantial amounts of p185 protein as compared with two pancreatic cell lines, 266-6 (Fig. 1) and AR42J (20) (data not shown) whose expression level is almost non-detectable. This result demonstrates the differential expression of p185 protein in these two types of cell lines.

To test if this differential expression of p185 protein occurs at the transcriptional level, a 2.2 kb rat *neu* promoter-CAT construct (pNeuEcoRICAT) (21) (Fig. 2A) was transfected into both Hep1-a and 266-6 cell lines. The results clearly show that the rat *neu* promoter is highly active in Hep1-a cells but is basically silent in 266-6 cells, in which the CAT activity is comparable to that of the negative control, the "promoterless"-CAT plasmid (pMTIC3) (Fig. 2B,C). The finding that a different promoter, the Rous sarcoma virus (RSV) long terminal repeat, is equally active in both cell lines indicates that the lower transcriptional activity of the *neu* gene is a specific phenomenon for 266-6 cells.

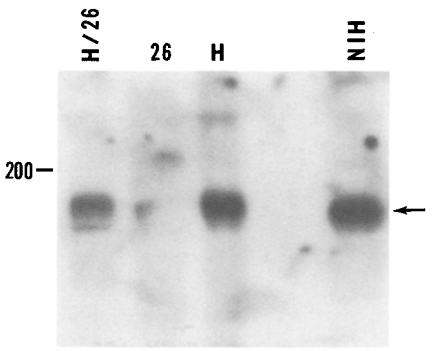

FIG. 1. The expression of p185 protein in Hep1-a, 266-6 and H/26 cells. One hundred micrograms of cell lysate from each cell line were subjected to a 6% SDS-polyacrylamide gel electrophoresis followed by a western transfer to a nitrocellulose filter. The filter was incubated with a p185 specific antibody, c-*neu* Ab-3 (Oncogene Sciences) and a goat anti-mouse antibody-peroxidase conjugate was used as a secondary antibody. ECL Western blotting detection system (Amersham) was then used to detect the p185-antibody complex on the filter by exposing to X-ray film. H/26, Hep1-a and 266-6 hybrid cell line; 26, 266-6; H, Hep1-a; NIH, NIH3T3. 200 KDa protein size marker is shown. The arrow indicates the p185 protein.

To localize the *neu* promoter region responsible for this differential transcription, a series of *neu* promoter deletion-CAT constructs (Fig. 2A) were transfected into both cell lines. All of the deletions maintained the differential transcriptional activity between Hep1-a and 266-6 cells, suggesting that the promoter element responsible for the differential expression must be downstream from the *Xho*I site of the pNeuXhoICAT, i.e. *Xho*I-*Nar*I element (-77 to -173 from the initiator ATG) (Fig. 2B,C).

To elucidate the mechanism by which *neu* gene transcription is differentially regulated in these two cell lines, an *in vivo* competition assay was employed by transfecting the reporter plasmid, pNeuXhoICAT, and the competitor plasmid, pXhoI-NarI (see Materials and Methods), into both cell lines. As shown in Fig. 3, pXhoI-NarI could readily inhibit the CAT activity of pNeuXhoICAT in Hep1-a cells in a concentration dependent manner, suggesting the presence of a positive rate-limiting transcription factor(s) interacting with *Xho*I-*Nar*I fragment in Hep1-a cells. In contrast, the CAT activity of pNeuXhoICAT in 266-6 cells is insensitive to the pXhoI-NarI competition. Since the success of the *in vivo* competition assay relies only on the assumption that the examined specific factor(s) exists in a limited amount, and thus the loss of its transcriptional function could be observed after competition by the specific binding elements. This result therefore suggests at least two possibilities regarding the lack of the *neu* expression in 266-6 cells: first, 266-6 cells may lack a positive factor(s) which is essential for the *neu* gene

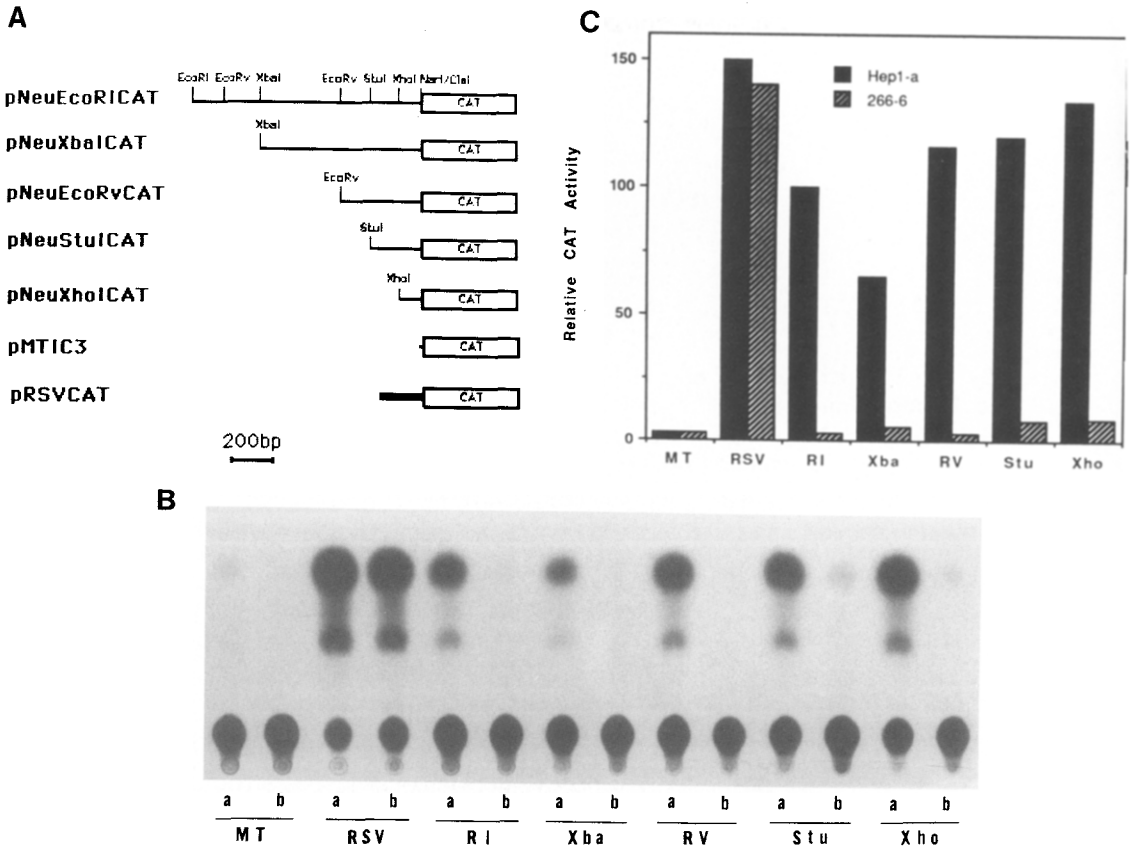

FIG. 2. Transcriptional down-regulation of the *neu* gene in 266-6 cells. (A) The diagram depicts the *neu* promoter deletion CAT constructs, "promoterless"-CAT(pMTIC3), and Rous sarcoma virus long terminal repeat-CAT(pRSVCAT). (B) Twenty micrograms of each CAT expression plasmid and three micrograms of RSV-LTR-lacZ plasmid were transfected into both Hep1-a (a) and 266-6 (b) cells. To normalize the transfection efficiency, the corrected amount of cell lysate, corresponding to the same β-galactosidase activity, was used for the subsequent CAT assay. Various promoters and the rat *neu* promoter deletion constructs were tested: MT (pMTIC3), RSV (pRSVCAT), RI (pNeuEcoRICAT), Xba (pNeuXbaICAT), RV (pNeuEcoRVCAT), Stu (pNeuStuICAT), and Xho (pNeuXhoICAT). (C) The CAT activities from (B) were quantitated by densitometry. The ^{14}C-chloramphenicol conversion rate of pNeuEcoRICAT in Hep1-a cells was set at 100 units.

transcription; second, 266-6 cells may possess a negative transcriptional factor(s) which is in excess to transcriptionally inhibit the multiple-copied *neu* gene expression in a transient transfection assay. In addition, these results also demonstrate that, in the liver Hep1-a cells, a specific and rate-limiting positive transcription factor(s) interacting with the *Xho*I-*Nar*I region of the *neu* promoter is required for efficient transcriptional activity of the *neu* gene.

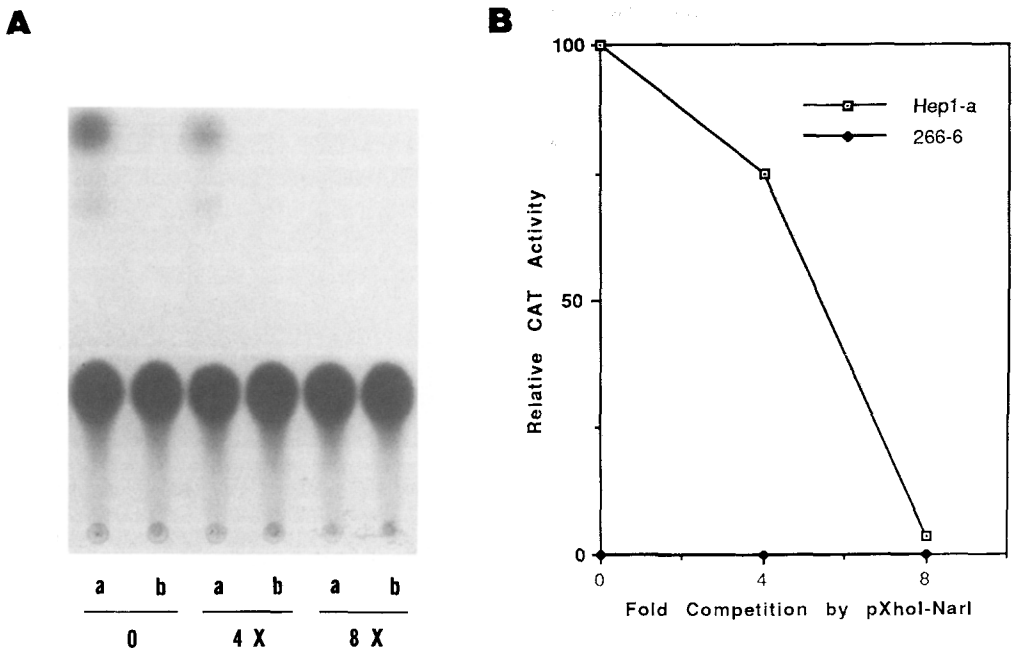

FIG. 3. In vivo competition of pNeuXholCAT by pXhol-NarI in Hep1-a and 266-6 cells. (A) pNeuXholCAT was cotransfected without (0) and with the competitor plasmid, pXhol-NarI, at 4- or 8-fold excess in molar ratio into both Hep1-a (a) and 266-6 (b) cells. Three micrograms of RSV-LTR lacZ plasmid was also transfected as an internal control. (B) Quantitative analysis of (A) by densitometry. The ^{14}C-chloramphenicol conversion rate of pNeuXholCAT in Hep1-a cells was again set at 100 units.

To distinguish the above mentioned two possible mechanisms for the lack of the *neu* expression in 266-6 cells, we examined the *neu* expression in a hybrid cell line, H/26, which is a fusion cell line between Hep1-a and 266-6 cells and has been well characterized to contain both liver and pancreatic specific markers (19). We reasoned if there is an excessive amount of the diffusible negative factor(s) present in 266-6 cells, the expression of the p185 protein should be greatly abolished in H/26 cells. However, the amount of p185 protein is not significantly repressed in H/26 cells as compared with that of Hep1-a (H) cells (Fig. 1). The slightly lower amount of p185 protein in H/26 compared with Hep1-a cells could be due to dilution of limited positive factor(s) present in Hep1-a cells shared by a larger size of the genomic DNA in H/26 cells. Therefore, this result supports the possibility that 266-6 cells may lack a positive transcription factor(s) which is required for efficient transcription of *neu* gene.

To examine the possible differential binding of specific *trans*-acting factors to *XhoI-NarI* region between Hep1-a and 266-6 cells, we employed a gel-shift assay in which the radioactively labeled *XhoI-NarI* fragment

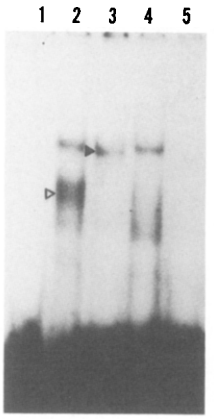

FIG. 4. Differential binding of *trans*-acting factors to the GCF binding site in Hep1-a and 266-6 cells. ^{32}P-labeled double-stranded GCF binding site was incubated with 5 μg of nuclear extract from Hep1-a cells (lanes 2 and 4) or 266-6 cells (lanes 3 and 5). The binding reactions were done either in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of 100-fold molar excess of cold GCF binding site as specific competitor. Lane 1 represents the GCF probe alone. The specific protein-DNA complexes are indicated by either an open or a closed triangle.

was incubated with crude nuclear extracts obtained from these two cell lines. The results revealed complex patterns with different DNA-protein complexes between Hep1-a and 266-6 cells (data not shown) which may be due to the binding of multiple specific and general transcriptional factors to the *Xho*I-*Nar*I region since the RNA initiation sites are located within this region (21). To simplify the DNA-protein binding patterns in the gel-shift assay, we searched for known specific protein binding sequences in the *Xho*I-*Nar*I region and identified two sequences perfectly matched to the consensus sequences of E4TFI, GGAAGTG, (-168 to -162) (23) and GCF, GCCCGCCGCT, (-135 to -126) (22) binding sites. No specific protein-DNA complexes were identified when the E4TFI binding site was used as a probe in the gel-shift assay (data not shown). However, when GCF binding sequence was used as a probe, we could detect specific protein-DNA complexes in both Hep1-a and 266-6 cell nuclear extracts. As shown in Fig. 4, the faster-migrating complex (indicated by an open triangle) in Hep1-a cells and the slower-migrating complex (indicated by a closed triangle) in 266-6 cells are specific since both of them can be efficiently competed away by the cold GCF binding site. The slower-migrating complex (lane 2), which also migrates slightly slower than the specific complex in 266-6 cell (lane 3; indicated by a closed triangle), is nonspecific since it can not be competed away by the cold GCF binding site (Fig. 4, lane 4). This result indicates that the protein factors interacting

with the GCF binding sequence behave differently in these two cell lines. These results are consistent with the idea that different or altered trans-acting factors in these two cell lines may contribute to their differential *neu* gene expression. Further investigation is required to determine if these transcriptional factors can directly mediate the differential expression of the *neu* gene in Hep1-a and 266-6 cells.

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