Transformation of baby hamster kidney fibroblasts by the Rous sarcoma virus causes a significant increase in the GlcNAc(b1,6)Man-branched oligosaccharides by elevating the activity and mRNA transcript levels encoding N-acetylgalactosaminyltransferase V (GlcNAc-T V). Elevated activity and mRNA levels could be inhibited by blocking cell proliferation with herbimycin A, demonstrating that Src kinase activity can regulate GlcNAc-T V expression. 5' RACE analysis was used to identify a 3-kilobase 5'-untranslated region from GlcNAc-T V mRNA and locate a transcriptional start site in a 25-kilobase pair GlcNAc-T V human genomic clone. A 6-kilobase pair fragment of the 5' region of the gene contained AP-1 and PEA3/Ets binding elements and, when co-transfected with a src expression plasmid into HepG2 cells, conferred src-stimulated transcriptional enhancement upon a luciferase reporter gene. This stimulation by src could be antagonized by co-transfection with a dominant-negative mutant of the Raf kinase, suggesting the involvement of Ets transcription factors in the regulation of GlcNAc-T V gene expression. The src-responsive element was localized by 5' deletion analysis to a 250-base pair region containing two overlapping Ets sites. src stimulation of transcription from this region was inhibited by co-transfection with a dominant-negative mutant of Ets-2, demonstrating that the effects of the src kinase on GlcNAc-T V expression are dependent on Ets.

The glycosylation of cell surface glycoproteins is a dynamic process that can be regulated by agents that cause differentiation, such as retinoic acid (1) or transforming growth factor-β (2), or by those that induce cellular proliferation, for example, interleukin-1 or tumor necrosis factor-α (3). In many instances, alterations of the oligosaccharides on cell surface glycoproteins cause significant changes in the adhesive or migratory behavior of a cell (4, 5). An induced alteration in the glycosylation of cell surface glycoproteins that has been documented for many years concerns the significant increase in oligosaccharide size caused by oncogenic transformation using a variety of agents (6–14). This increase in size was found to result mainly from an increase in the levels of asparagine-linked oligosaccharides containing N-acetylgalactosamine linked β1,6 to the α(1,6)-linked mannose in the trimannosyl core, (GlcNAcβ(1,6)Man), and in many cases these oligosaccharides express polyglucosamines that can be sialylated (15–18). The (GlcNAcβ(1,6)Man) branch is synthesized by N-acetylgalactosaminyltransferase V (GlcNAc-T V), 1 the enzyme whose activity is significantly and selectively increased after transformation by tumor viruses or isolated oncogenes (16, 19–22). Moreover, decreased expression of the GlcNAcβ(1,6)Man branch has been correlated with decreased metastatic potential (23, 24), whereas the increased expression of this branch appears in some instances to correlate with the progression of invasive malignancies (25).

The transformation of baby hamster kidney (BHK) fibroblasts by the src oncogene causes an increase in N-linked oligosaccharide (GlcNAcβ(1,6)Man) branching, and the mechanism by which this increase occurs has been under investigation in our laboratories. To elucidate this mechanism, we examined GlcNAc-T V enzyme activity and mRNA levels in BHK cells and their Rous sarcoma virus-transformed counterparts (RSV-BHK) in the presence of the Src kinase inhibitor, herbimycin A. The results from these experiments led us to examine the 5' region of the human gene encoding GlcNAc-T V and its increased expression caused by Src activity. Our results indicate that the N-acetylgalactosaminyltransferase V gene can be transcriptionally activated by Src tyrosine kinase activity, and this control is dependent on both the Raf-1 kinase and an Ets family transcriptional activator.

**EXPERIMENTAL PROCEDURES**

**Glycosyltransferase Activity Assays**—Cells were grown to confluency and harvested in 50 mM MES 6.5, 150 mM NaCl, and lysed by addition of Triton X-100 to 1%. Lysates were assayed according to the method of Palic et al. (22). Briefly, 10⁶ cpm of UDP-[3H]GlcNAc (25 cpm/pmol) and 10 nmol of synthetic trisaccharide acceptor for GlcNAc-T V (octyl (2-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranosyl)-β-D-glucopyranoside) were dried under vacuum in a 1.5-ml microcentrifuge tube. Extracts of various protein concentrations were prepared, and 10 μl were added to the assay tube. Assays were incubated at 37 °C for 4 h and quenched by the addition of 500 μl of water. Radiolabeled product was isolated on a C₁₈ Sep-Pak (Waters) column, eluted in 2 ml of methanol, and counted in a scintillation counter. Assays were performed in duplicate or triplicate, at two or three protein concentrations, and specific activity was calculated by linear least squares regression analysis of the data.

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1 The abbreviations used are: GlcNAc-T V, N-acetylgalactosaminyltransferase V; BHK, baby hamster kidney; RSV, Rous sarcoma virus; PEA, polyoma enhancer activator; GAPDH, glyceraldehyde phosphate dehydrogenase; RACE, rapid amplification of cDNA ends; GlcNAc-T I, N-acetylgalactosaminyltransferase I; MES, 4-morpholineethanesulfonic acid; kb, kilobase pair(s); PCR, polymerase chain reaction; bp, base pair(s); UTR, untranslated region.
...were directly subcloned into the TA cloning vector (Invitrogen). The PCR product was generated using the primers 5′GGATGGGAAAGGAAAGGc 3′ (Bio-Rad) and was probed with an anti-phosphotyrosine antiserum (a kind gift from Dr. Bart Selton) followed by a goat anti-mouse horseradish peroxidase conjugate. Bands were detected using the ECL reagents (Amerham Corp.) and quantitated by scanning densitometry.

**5′ RACE Analysis—** Marathon Ready cDNA from human whole brain (CLONTECH) was used as a template in a 5′ RACE PCR according to the manufacturer's instructions. A 3′ PCR primer (303, CCTGGAATTCTTACAGGCTGTGGT) and a genomic clone template. PCR products were separated on a 1% Tris-acetic acid-EDTA-agarose gel and blotted to nitrocellulose. A 1-kb fragment of a genomic clone was band-purified (Sephaglass, Pharmacia Biotech Inc.) and subcloned into the TA vector. TA clones of the correct orientation were cut with XhoI sites of the pGL2-Basic vector (Promega). For pGL2-T2V through pGL2-T4V, PCR products were generated using the genomic clone as a template and subcloned into the TA vector. TA clones of the correct orientation were cut with XhoI-SacI sites of the pGL2-Basic vector (Promega).

**Promoter Activity Determinations—** SV40-β-galactosidase (2 μg) and reporter construct (2 μg) = effector plasmids (2 μg) were transfected by the calcium-phosphate precipitation method (29) into 50% confluent HepG2 cells grown in 6-well culture plates. 40 h post-transfection, cell lysates were prepared and assayed for β-galactosidase and luciferase (Promega). Luciferase activity was normalized to vector-dependent β-galactosidase activity. 

**Plasmids—** The plasmids encoding the Raf-1 kinase and its dominant negative form (30) were kind gifts from Dr. Ulf Rapp. Plasmids encoding Ets-2 and its dominant negative form (31) were kind gifts from Dr. K. E. Boulukos. The v-src expression plasmid was a kind gift from Dr. Tony Hunter.

**RESULTS**

Earlier experiments utilizing BHK and RSV-BHK cells metabolically radiolabeled with [2-3H]mannose indicated at least a 2-fold increase in the total amount of (2,6)-substituted mannose in the RSV-BHK cells, normalized to total mannose-labeled glycopeptide. Although the specific activity of GlcNAc-T V was increased over 6-fold in the RSV-transformed cells, no significant differences in the kinetic properties of GlcNAc-T V in the transformed cells could be detected. These results suggested that the increases in GlcNAcβ1,6Man levels after transformation were most likely not due to post-translational effects on the enzyme (22). The specific activity of GlcNAc-T V and its mRNA levels were measured, therefore, to determine if the increase in GlcNAc-T V activity in the transformed cells could result from differences in mRNA levels. GlcNAc-T V activity was assayed under optimal conditions in BHK and RSV-BHK cells using a synthetic trisaccharide acceptor. The transformed BHK cells demonstrated a GlcNAc-T V enzyme specific activity 6-fold higher than the untransformed BHK cells. By contrast, no difference was seen in the specific activity of another N-acetylgalactosaminyltransferase that functions in the synthesis of N-linked oligosaccharides, GlcNAc-T I (Fig. 1), indicating the specificity of Rous sarcoma virus transformation on GlcNAc-T V activity. To investigate the possibility that the difference in GlcNAc-T V specific activity is associated with a difference in steady-state mRNA levels, Northern blots were performed using a fragment of a cDNA encoding GlcNAc-T V. Compared with BHK cells, RSV-transformed cells were found to have a 6-fold increase in the expression levels of both 8.7 and 9.3 kb GlcNAc-T V transcripts, but no change in either GAPDH or GlcNAc-T I transcripts (Fig. 2). Although not apparent in Fig. 2, PhosphorImager quantitation demonstrated an equivalent increase in both GlcNAc-T V mRNA transcripts. These results demonstrated that the elevation of enzyme activity in the RSV-transformed cells was a result of either transcriptional activation or increased mRNA stability and argue against posttranslational modifications of the enzyme causing a significant increase in its catalytic activity.

To obtain convincing evidence that the differences in GlcNAc-T V expression result from src tyrosine kinase activity, we made use of a src-selective tyrosine kinase inhibitor, herbimycin A, a metabolite produced by Streptomyces sp. MH237-CF8. This inhibitor was first identified for its ability to reverse the transformed morphology of Rous sarcoma virus-infected rat kidney cells (32), and this reversion of morphology was associated with a reduction in total cellular phosphotyrosine levels (33). Herbimycin A was unable, however, to reverse the transformed morphology induced by the ras,raf, or myc oncogenes, demonstrating its specificity for the src family of tyrosine kinase oncogenes (34). Herbimycin A is also able to reverse src-stimulated expression of the glucose transporter gene (35) and to cause G1 arrest in src-transformed normal rat kidney cells (36). We utilized herbimycin A, therefore, to test the hypothesis that the expression of GlcNAc-T V is positively regulated by the src tyrosine kinase.

First, to monitor the effects of src kinase and demonstrate its inhibition by herbimycin A, we measured total cellular phosphotyrosine levels by performing Western blots with an α-phos-
phototyrosine antibody on extracts made from cells treated for 24 h with various concentrations of the drug. These results demonstrate that, as expected, herbimycin A caused a dose-dependent decrease in cellular phosphotyrosine levels (Fig. 3). At a concentration of 1 μg/ml, herbimycin A caused a reversal of the RSV-transformed morphology and a complete inhibition of cell division (data not shown). Consistent with the drug’s effect of inhibiting the src kinase (33) and blocking cell division in $G_0$ (36), herbimycin A caused a dose-dependent decrease in GlcNAc-T V enzyme specific activity in RSV-transformed BHK cells (Fig. 4). Interestingly, although the drug blocked cell division and caused a modest decrease in phosphotyrosine levels in the untransformed BHK cells (data not shown), it had little effect on the expression level of GlcNAc-T V enzyme activity in RSV-transformed BHK cells (Fig. 4). Interestingly, although the drug blocked cell division and caused a modest decrease in phosphotyrosine levels in the untransformed BHK cells (data not shown), it had little effect on the expression level of GlcNAc-T V enzyme activity in RSV-transformed BHK cells. This result suggests that regulation of GlcNAc-T V expression is complex, with both src-dependent and src-independent factors. Herbimycin A had no effect on the specific activity of GlcNAc-T I (data not shown), arguing against nonspecific toxic effects on the transformed cells and confirming that GlcNAc-T I is not regulated by src. To determine if the inhibition of expression of GlcNAc-T V enzyme activity by herbimycin A was a result of inhibiting the expression of the mRNA encoding the enzyme, Northern blots were performed on RNA samples prepared from RSV-transformed cells treated with various concentrations of the drug. Similar to its effects on GlcNAc-T V enzyme specific activity, herbimycin A caused a decrease in GlcNAc-T V message levels in the RSV-BHK cells in a dose-dependent manner (Fig. 5). Taken together, these results indicate that expression of the GlcNAc-T V mRNA in the src-transformed cells is under the control of the src tyrosine kinase.

To elucidate the mechanism by which src induces the expression of GlcNAc-T V, we isolated the 5′-flanking region of the gene and analyzed this region for promoter activity. The GlcNAc-T V message is approximately 9 kb in most rodent and human tissues, with brain having high expression levels. To locate a promoter for GlcNAc-T V, 5′ RACE PCR techniques were used to isolate and sequence the 5′ end of the message from human brain. RACE PCR products were first generated from the genomic clone using the 303 primer (designed against the 5′ end of the human GlcNAc-T V cDNA sequence) and then analyzed by Southern blotting. GlcNAc-T V-specific sequences were detected using a nested 501–305 PCR product as the hybridization probe. Multiple bands were detected, the longest of which was 2.9 kb (Fig. 6). PCR products were ligated into the TA cloning vector, and clones corresponding to the 600- and 1200-bp products were obtained and found to overlap and differ...
only in the length of their 5’ ends. To isolate a product that encompassed all of the 5’-untranslated region of the GlcNAc-T V message, a second round of RACE PCR was then performed using two nested primers designed near the 5’ end of the 0.6 kb clone, and the resulting products were subcloned and sequenced. Clones corresponding to the 1.8- and 2.9-kb bands were isolated from this second round of RACE PCR and sequenced. Clones corresponding to the 1.8- and 2.9-kb bands were found to differ only in the 3’-UTR sequences, which were analyzed. A third round of RACE PCR using a 3’-anchored primer (GCCTTGATGTACCTTTTGCCGAGGTCCAGG) and a 5’-most primer designed to the sequence near the 5’-end of the 2.9 kb clone produced a single product whose size was 70 bp. The 3’-most transcriptional start site utilized in transcription of the GlcNAc-T V mRNA in untreated cells was set at 100%.

To examine the 5’ region flanking the 5’-most transcriptional start site, a 6-kb SacI-XhoI genomic fragment containing 848 bp of the brain 5’-UTR and 5.5 kb of 5’-flanking sequence (depicted in Fig. 7), designated pGL2-TV1, was cloned into the luciferase expression vector pGL2-basic. The activity of this region as a promoter and its responsiveness to src were then examined in transiently transfected HepG2 cells. pGL2-TV1 was found to act as a weak promoter, shown in Fig. 8, consistent with the low levels of GlcNAc-T V transcript observed in HepG2 cells and most tissues. Moreover, this DNA fragment conferred transcriptional responsiveness to src when co-transfected with a src-containing expression plasmid (Fig. 8). The similarity between the increases in GlcNAc-T V expression in HepG2 cells and most tissues. Moreover, this DNA fragment conferred transcriptional responsiveness to src when co-transfected with a src-containing expression plasmid (Fig. 8). The similarity between the increases in GlcNAc-T V expression in HepG2 cells and most tissues. Moreover, this DNA fragment conferred transcriptional responsiveness to src when co-transfected with a src-containing expression plasmid (Fig. 8). The similarity between the increases in GlcNAc-T V expression in HepG2 cells and most tissues.

Transformation and some transcriptional activation by src occurs via the MAPK pathway in a Raf-1-dependent manner. For example, a dominant-negative Raf-1 mutant suppresses src transformation of BALB/c mouse fibroblasts (37) and is able to block src-stimulated transcriptional activation of the EGR gene (38). Furthermore, this mutant is able to block serum or Ras stimulation of the transcription of an AP-1/Ets-driven gene (39, 40). If transcriptional activation of GlcNAc-T V by src occurs at least in part via the MAPK pathway, we reasoned that the activation should be inhibited by the dominant-negative Raf-1 mutant. Consistent with this hypothesis, the transcriptional stimulation of the GlcNAc-T V promoter by src was significantly inhibited when co-transfected with a plasmid encoding a dominant-negative mutant Raf, RafC4B-DN (Fig. 8). Because proliferation often occurs in a Raf-dependent manner, this result is consistent with correlations noted between GlcNAc-T V expression and cellular proliferation in nontransformed cells (39, 40) and may predict in certain cell types a general association between GlcNAc-T V enzyme activity and cell proliferation.

To map more closely the region of the GlcNAc-T V promoter responsible for transcriptional activation by src, a series of 5’ deletions containing 70 bp of the brain 5’-UTR and different amounts of the 5’-flanking region were constructed by PCR
amplification from the genomic clone, the boundaries of which are depicted in Fig. 7. Promoter fragments were cloned into the pGL2-basic vector and tested for basal promoter activity and src responsiveness. Based on the results from several sets of experiments, both pGL2-TV2, which contained about 1.2 kb, and pGL2-TV3, which contained 739 bp, were both found to be weakly active as promoters and transcriptionally responsive to src (Fig. 9). The pGL2-TV4 construct containing 339 bp, however, was found to be inactive as a basic promoter and completely unresponsive to src. These results suggest a requirement for the two overlapping PEA-3 sites located near the transcriptional start site, contained in pGL2-TV3, in the src-mediated transcriptional activation of the GlcNAc-T V gene (Figs. 7 and 10).

PEA-3 sites are bound by the Ets family of transcriptional activators and mediate transcriptional activation in response to mitogenic signals from plasma membrane-associated tyrosine kinase oncogenes (41). To obtain more direct evidence for the involvement of an Ets family transcription factor in src-stimulated transcription of the GlcNAc-T V gene, we exploited the availability of an ets-2 expression plasmid along with an expression plasmid that encoded a truncated ets-2 with a dominant-negative activity, ets-2 D1–328 (31). Co-transfection of pGL2-TV3 with ets yielded stimulation of transcription, whereas co-transfection with ets D1–328 was able to block src-transcriptional stimulation (Fig. 11). This result demonstrates that this region is capable of being transcriptionally stimulated in response to ets-2 expression and suggests that src transcriptionally activates this region through a factor that binds to the PEA-3 site(s).

DISCUSSION

A major regulatory mechanism for controlling the expression of glycan structures is alteration in the activities of the enzymes involved in their synthesis. Changes in glycosyltransferase activity may occur by several different mechanisms, including post-translational interactions with specifier proteins (42) and translational control of glycosyltransferase transcripts. For example, the β(1,4)-galactosyltransferase is expressed at low to moderate levels in most tissues as a 4.1-kb transcript. However, high level expression in lactating mammary gland is accompanied by a switch from one transcriptional start site to another, which is more proximal to the translational start site, giving rise to the expression of a 3.9-kb transcript (43). The longer transcript is predicted to form a stable hairpin loop structure in the 5' untranslated region, which may reduce translation efficiency. The shorter transcript is predicted to be unable to form any significant 5' structure and therefore is thought to be translated with greater efficiency.
been described in several systems. Early indications of this regulatory mechanism were found in a functional analysis of a promoter of the α(2,6)-sialyltransferase gene. This transferase is highly expressed in liver, and its promoter was found to contain binding sites for liver-enriched transcription factors. These sites and their cognate transcription factors were found to be functional in luciferase reporter transactivation assays in Hep G2 cells but were not functional in Chinese hamster ovary cells. These transcription factors and their binding sites are thought to be responsible for the liver-restricted expression of this message (46, 47). Also, retinoic acid-induced differentiation of F9 teratocarcinoma cells has been documented to be associated with an increase in the activity of the murine α(1, 3)-galactosyltransferase and a switch in the expression of terminal linking structures from α(2, 3)-linked sialic acid to galactose. The increase in the activity of the transferase is associated with an elevation in the steady-state levels of the mRNA and a similar increase in the transcription rate of the gene (1).

The association between oncogenic transformation and increases in GlcNAc-T V enzyme activity and expression of GlcNAcβ(1,6)Man branches on glycoproteins raises the question concerning the mechanism by which oncogenes and proliferative signals in general up-regulate the synthesis of GlcNAcβ(1,6)Man branches. From our results it is reasonable to conclude that the increases in GlcNAc-T V enzyme activity seen in src-transformed cells are not due to an increase in the catalytic efficacy of the glycosyltransferase; rather, they are due to an increase in the expression of the GlcNAc-T-V mRNA and subsequently an increase in the number of GlcNAc-T-V enzyme molecules/cell. The elevation in steady-state mRNA levels could be a result of increased mRNA stability or an increase in the transcription rate of the gene. Our results demonstrate that a 5′-fragment of the GlcNAc-T V gene is able to confer src-transcriptional responsiveness upon a heterologous reporter gene, indicating that the mechanism by which src increases steady-state mRNA levels is most likely transcriptional activation. Also, the 5′-flanking region of the GlcNAc-T-V gene was found to contain AP-1 and PEA-3 binding sites, the presence of at least two being required for src-stimulated transcriptional enhancement. These data suggest that cooperation may be required between AP-1 and Ets transcription factors to render the gene fully responsive to oncogenic stimulation. Finally, transcriptional stimulation by src was found to be significantly dependent on the Ras-Raf pathway, again implicating transcription factors that are activated by the MAPK pathway and suggesting a general association, at least in some cell types, between GlcNAcβ(1,6)Man branching and cellular proliferation. Other studies have shown that particular glycosyltransferase activities are increased after ras-transformation; notably, α(2,6)-sialyltransferase activity in NIH3T3 cells is specifically increased by ras expression, and this increase is due to increased levels of the mRNA encoding this enzyme (49, 50).

The regulation of the expression of the GlcNAc-TV gene is quite complex, involving the use of multiple promoters and alternative splicing. The brain transcript described in this paper begins at a promoter about 3 kb upstream of the translation initiation site, generating a long 5′-untranslated sequence that is unspliced and colinear with the genomic sequence. This transcript and its promoter are different from those observed in HuCC-T1 human bile duct carcinoma cells (48). The GlcNAc-T V mRNAs in these cells appear to be initiated from multiple promoters, one of which is about 1.4 kb downstream of the brain promoter. These mRNAs are also differentially spliced in the 5′-UTR to generate messages with much shorter 5′-UTRs. There are also data to suggest that there are additional pro-
motors and alternative splice sites by A431 cells when expressing this gene.2 The precise identification of the cis-acting sequences that mediate the activation of GlcNAc-T V transcription from these alternate promoters will require further investigation. Other regulatory mechanisms may also be operative in some cell types, including translational control. The GlcNAc-T V transcript expressed in human brain and in HepG2 cells is approximately three times larger than the coding region, indicating the presence of extensive 5′- and 3′-untranslated sequences. The transcript from human brain was found to have a 5′-untranslated region of approximately 3 kb, the longest 5′-UTR of any glycosyltransferase observed to date. This 5′-UTR may function as a regulator of translation efficiency, as has been suggested in the case of the mammary-specific transcript for the a(2,6)-sialyltransferase.

Of what consequence is the increased expression of GlcNAc(1,6)Man branches during proliferation? One possibility is that these branches expressed on cell surface proteins that function in cell adhesion can significantly alter the adhesiveness of cells to the extracellular matrix or to each other, allowing them to become more migratory. Evidence in support of this hypothesis has been observed in the case of mink lung epithelial cells stably transfected with and overexpressing mouse GlcNAc-T V (51). The cells overexpressing GlcNAc-T V show an altered morphology show altered rates of migration in an in vitro assay. Moreover, these cells show less adhesion to laminin-coated surfaces, compared with the nontransfected controls, suggesting an effect of GlcNAc-T V expression on laminin adhesion.

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