Pax3 is a transcription factor that is required for the development of embryonic neural tube, neural crest, and somatic derivatives. Our previous study (Mayanil, C. S. K., George, D., Mania-Farnell, B., Bremer, C. L., McLone, D. G., and Bremer, E. G. (2000) J. Biol. Chem. 275, 23259–23266) reveals that overexpression of Pax3 in a human medulloblastoma cell line, DAOY, resulted in an up-regulation in α-2,8-polysialyltransferase (STX) gene expression and an increase in polysialic acid on neural cell adhesion molecule. This finding suggests that STX might be a previously undescribed downstream target of Pax3. Because Pax3 is important in diverse cellular functions during development, we are interested in the identification of additional downstream targets of Pax3. We utilized oligonucleotide arrays and RNA isolated from stable Pax3 transfectants to identify potential target genes. A total of 270 genes were altered in the Pax3 transfectants as compared with the vector control and parental cell line. An independent analysis by cDNA expression array and real-time quantitative polymerase chain reaction of several genes confirmed the changes observed in the oligonucleotide microarray data. Of the genes that displayed significant changes in expression, several contain paired and homeodomain binding motifs of Pax3 in their promoter regions. Using promoter-luciferase reporter transfection assays and electromobility shift assays, we showed at least one previously undescribed downstream target, STX, to be a biological downstream target of Pax3. Thus we report several previously undescribed candidate genes to be potential downstream targets of Pax3.

Regulation of genes during development is tightly modulated by specific transcription factor families, which include the homeobox, T-box, Hox, and Pax families. Pax3 is one such member of a paired homeobox family of an evolutionary conserved transcription factor (1) that plays a pivotal role during neural tube closure. Pax3 is expressed in a spatio-temporally restricted manner during embryogenesis (2), specifically at embryonic day 8.5 in the developing murine spinal cord and brain. After neural tube closure, Pax3 expression is maintained in the dorsal half of the neural tube (2). Deficient Pax3 expression, such as in Splotch mice, results in neural tube defects and an array of neural crest cell related abnormalities (3). Cell adhesion and migration are fundamental processes that contribute to many aspects of neural tube and neural crest dorsoventral patterning and morphogenesis (4–11). Because Pax3 is implicated in the invasive and metastatic potential of pediatric tumors, it is probable that it does so by modulating cell adhesion and migration (1, 12–14).

Up-regulation of specific genes and delayed expression of others is expected of a cell that is committed to adhesion or migration (15). Our previous study (16) showed that overexpression of Pax3 in a human medulloblastoma cell line, DAOY, had a direct impact on the cell adhesion properties. Pax3 transfectants were found to have up-regulated α-2,8-polysialyltransferase (STX)1 gene expression, which resulted in increased polysialylation of neural cell adhesion molecule (NCAM). Pax3 transfectants expressing high polysialic acid (PSA) on NCAM showed much less NCAM-dependent aggregation than those with less PSA. In addition, these Pax3 transfectants with high PSA-NCAM showed heterophilic adhesion to heparan sulfate proteoglycan (HSPG) and agrin. These experiments suggested that Pax3 might regulate the expression of additional genes involved in cell adhesion and migration. To search for additional downstream targets of Pax3, we employed a microarray approach. Several Pax3 downstream targets such as NCAM (17), MyoD (18), c-MET (19, 20), Dep-1 (21), myelin basic protein (MBP) (22), MITF (23, 24), and Tprp(1–24) have been reported. Even though these Pax3 downstream targets have been described, an unambiguous Pax3 consensus binding motif is lacking (25). As a result, the progress of identifying specific downstream targets of Pax3 has been considerably slowed down. Therefore, a more complete picture of the network of genes altered by Pax3 is required to fully understand

- The abbreviations used are: STX, α-2,8-polysialyltransferase; NCAM, neural cell adhesion molecule; PSA, polysialic acid; HSPG, heparan sulfate proteoglycan; RT-PCR, reverse transcriptase-polymerase chain reaction; FAM, 6-carboxyl-fluorescein; TAMRA, 6-carboxyl-tetramethyl-rhodamine; C₅, threshold cycle; FKHR, fork head in rhabdomyosarcoma; MES, 4-morpholineethanesulfonic acid; GST, glutathione S-transferase; electromobility shift assay; TGF, transforming growth factor; PDGF, platelet-derived growth factor; IGF, insulin-like growth factor-binding protein; MARCKS, myristoylated alanine-rich c kinase substrate; MITF, microphthalmia-associated transcription factor; PGEN, proliferating cell nuclear antigen.
its physiological significance. To identify Pax3 downstream targets in this study, we compared the gene expression of three stable Pax3-transfected clones (16) to vector-alone transfected and parental cell lines using oligonucleotide microarrays.

**EXPERIMENTAL PROCEDURES**

**Transfection of Pax3 cDNA into DAOY Cells and Selection of Transfectants**

Stable transfection of mouse Pax3 in the human medulloblastoma-derived cell line, DAOY, was done as described earlier (16). Briefly, a 2.3-kilobase murine Pax3 cDNA was inserted into the pcDNA expression vector (Invitrogen) at the EcoRI sites. The 2.3-kilobase Pax3 cDNA was prepared from pH3.2, which was kindly provided by Dr. Peter Gruss, Max Planck Institute, Götttingen, Federal Republic of Germany (19). pH3.2 was digested with EcoRI (Amersham Pharmacia Biotech) to remove the 2.3-kilobase (19). pBH3.2 was digested with EcoRI and then ligated into pcDNA3 at the EcoRI site. Restriction digest confirmed orientation of the cDNA insert. The pcDNA3/Pax3 construct or pcDNA3 alone as the vector control was then transfected into the cells using a cationic liposome system, DOTAP (N-[1-(2-3-diolaeoyloxy)propyl]-N,N,N-trimethylammonium salts; Roche Molecular Biochemicals). Transfectants were selected in the presence of DOTAP (16). Vector control was then transfected into the cells using a cationic lipid protocol (16). The manufacturer’s instructions. The in vitro transcription product was purified in RNeasy spin columns (Qiagen) according to the manufacturer’s protocol. This was followed by an overnight ethanol precipitation and resuspended in 15 µl of diethyl pyrocarbonate-treated water (Ambion Inc). The cRNA was quantified using a Beckman DU530 Life Science UV-visible spectrophotometer. 20 µg of cRNA was fragmented (0.5 µg/µl) according to Affymetrix instructions. The 5× fragmentation buffer includes 200 mM Tris acetate, pH 8.1, 500 mM potassium acetate and 150 mM magnesium acetate. Individual reagents were received from Sigma.

**Hybridization**—Quantification of cRNA from total RNA was adjusted to reflect the relative amount of RNA with an equation given by Affymetrix: adjusted cRNA yield = cRNA measured after in vitro transcription (µg) – (starting amount of total RNA) (fraction of cDNA reaction used in in vitro transcription). 15 µg of adjusted fragmented cRNA was added to a 300-µl volume hybridization mixture that included final concentrations of 0.1 mg/ml herring sperm DNA (Promega/Fisher), 0.5 mg/ml acetylated bovine serum albumin (Life Technologies Inc.), and 2× MESS hybridization buffer (Sigma). The mixture also contained the following hybridization controls: 50 µg oligonucleotide B2 (Genset Corp.) and 1.5, 5, 25, and 100 µg cRNA BioB, BioC, BioD, and Cre, respectively (ATCC). 200 µl of this mixture was hybridized to the chips with 24-µm x 24-µm probe cells for 16 h according to Affymetrix procedures. The 50-µm x 50-µm test chips are hybridized with 80 µl of target for 16 h.

**Washing, Staining, and Scanning**—The probe arrays were washed with stringent (100 mM MES, 0.1 M Na+, 0.01% Tween 20) and non-stringent (6× SSPE, 0.01% Tween 20, 0.005% antifade) buffers in the Affymetrix GeneChip Fluidics station using pre-programmed Affymetrix protocols. The probe arrays were then stained with streptavidin phycoerythrin, and the signal was amplified using an antibody soybean agglutinin (Vector Laboratories). The antibody amplification solution contained 2× stain buffer (Sigma), 1× concentration (100 mM MES, 1 M Na+, 0.05% Tween 20, 0.005% antifade, 2 µg/µl acetylated bovine serum albumin, and 10 µg/ml streptavidin (Molecular Probes)). The antibody amplification solution contained 2× stain buffer, 2 µg/µl acetylated bovine serum albumin (Life Technologies Inc.), 0.1 mg/ml normal goat IgG (Sigma), and 3 µg/ml biotinylated antibody (Vector Laboratories). Staining was done in the GeneChip Fluidics station using pre-programmed Affymetrix protocols. The probe arrays were scanned in the Affymetrix GeneChip scanner.

**Data Analysis**—Six data files (DAOY, PcDNA-1, PcDNA-2, B9, E7, and H6) were uploaded into Affymetrix MicroDB 1.0 software. This data base file was then sorted and studied with Affymetrix data mining tool 1.2. Using DAOY as a base line, genes showing fold changes between −1.5 and 1.5 in the vector controls were organized into a list. The data from the transfected cell lines were then queried using this gene list, an absolute call of “Present,” and a difference call of “Increased” or “Decreased.” The resulting list of genes was studied, and further information on genes of interest was compiled.

**Hybridization of cDNA Probe to the CLONTECH Neurobiology Atlas cDNA Expression Arrays**

**Preparation of cDNA Probes**—Preparation of cDNA probes was done with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in the presence of gene-specific primers (CLONTECH) and 1γ-32P-PdATP according to manufacturer’s protocol. For cDNA synthesis, an equal amount (0.5 µg) of total RNA from control DAOY and B9 clone. The labeled cDNA was purified on ChromaSpin 200 diethyl pyrocarbonate-H2O columns (CLONTECH), and fractions 2–3 corresponding to cDNA were pooled and counted in a Beckman LS liquid scintillation spectrophotometer.

**Hybridization**—We use 20 × 106 cpm of 32P-labeled probes/membrane. After the test hybridization, the pre-hybridization and the hybridization of the Atlas human cDNA expression arrays-neurobiology...
The 7700 sequence detector system chemistry is described in our previous paper (16). Each 50 µl of RT-PCR reaction (PerkinElmer Life Sciences) volume included 25 ng of total RNA, 5 µl 1× (Molecular Dynamics) buffer A (composed of 500 mM KCl, 100 mM Tris-HCl, 0.1 M EDTA, 600 mM passive reference dye, pH 8.3, at room temperature), 10 µl of 25 mM MgCl₂, 1.5 µl of each dNTP (2 mM each) and 20 mM dUTP, 0.5 µl of forward and reverse primers (10 µM), 1 µl of the corresponding TaqMan probe (50 nM), 0.25 µl of AmpliTaq Gold supplied at 5 units/µl, and 0.25 µl of murine leukemia virus reverse transcriptase. RT-PCR cycle parameters were 48°C for 30 min and 95°C for 15 min followed by 40 cycles at 95°C for 15 s and 59°C for 1 min. The primers and the probes used in the study were designed using Primer Express software (PerkinElmer Life Sciences) and synthesized by Life Technologies Inc. For murine Pax3, the forward and reverse primers were 5′-CCA ACC ATA TCC GCC ACA A-3′ and 5′-TCT TAG AGA CGC AAC CAT GGC-3′, respectively, and the TaqMan probe was 6-FAM-ATG GCA TTC GCT GGT GCC TGA TTT-CMT. For human β-actin, the forward and the reverse primers were 5′-TCA CCC ACA CTG TGC CCA TCA TCT ACG A-3′ and 5′-CAG CGG ACC CGC CTA TG-3′, respectively, and the TaqMan probe was 6-FAM-ATG GCA TTC GCT GGT GCC TGA TTT-CMT. For PEAD, the forward and the reverse primers were 5′-TAG GAG AGG CGG AGA ACT GAA G-3′ and 5′-GAA GGC TGC TGC GGT GAA G-3′, respectively, and the TaqMan probe was 6-FAM-AGG CGA AGC ACAG CAC GAG GTG TGT GCC TCA TTA-3′.

SYBR Green RT-PCR

SYBR Green allows us to perform real-time RT-PCR using a 7700 sequence detector system (PerkinElmer Life Sciences). Measuring SYBR Green fluorescent dye that intercalates into the minor groove of the double-stranded DNA determines the amount of PCR product. Each 50 µl of RT-PCR reaction (PerkinElmer Life Sciences) volume included 25 ng of total RNA, 5 µl of 10× SYBR Green PCR buffer (includes passive reference 1), 7 µl of 25 mM MgCl₂, 6 µl of each dNTP (2.5 mM dATP, dCTP, dGTP, and 5 mM dUTP), 0.5 µl of forward and reverse primers (5 µM), 0.25 µl of AmpliTaq Gold supplied at 5 units/µl, and 0.25 µl of murine leukemia virus reverse transcriptase. RT-PCR cycle parameters were 48°C for 30 min and 95°C for 15 min followed by 40 cycles at 95°C for 15 s and 59°C for 1 min. SYBR Green RT-PCR was performed with Versicin V1, V2, and V3 splice variants. The forward and reverse primers for the Versicin V1 splice variant was 5′-CCC AGC GTT GAG GAG GTG TCT TAC T-3′ and 5′-GCC TCA AAT CAC TTC TTC GAC GTT-3′, respectively. For the Versicin V2 splice variant, the forward primer was 5′-GCA AGT GTC ATC TGG AAA GTG-3′, the reverse primer was 5′-TGG ATC AAG AGG AAC TGT CAG TAG GA-3′, and for the Versicin V3 splice variant, the forward primer was 5′-CCC TCC CCC TGA TAG CAG AT-3′, and the reverse primer was 5′-GGC AGG GTG TCA TTT GTC-3′.

Analysis of STX Promoter Activity

The plasmids pB01-NhN3.5 and pB01-SN0.45, containing STX promoter and luciferase reporter gene, and pGBlI, containing only the luciferase gene (37), were kindly provided by Dr. S. Tsujii (Institute of Physical and Chemical Research, RIKEN, Saitama, Japan). The DAOY cells, the pcDNA3 vector, and the Pax3-transfected stable cell line B9 were seeded at 5 × 10⁴ cells/60-mm-diameter dish in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum 24 h before transfection. The luciferase plasmids (6.5 µg) used as the reporter, and Renilla luciferase plasmid (0.5 µg) (Promega dual luciferase system), used as an internal control for transfection efficiency, were transfected into the cells with DOTAP ([N-1-[2,3-dioleyloxy]propyl]-N,N,N-trimethylammonium salts). After 42 h, the cells were washed three times with phosphate-buffered saline and then lysed with passive lysis buffer. The luciferase activity was measured using a Lumat LB 9501 luminesimeter (EG&G Berthold, Germany).

Preparation of Nuclear Extracts

For EMSA experiments, 2 µg of nuclear extracts from B9 or DAOY cells were incubated in a 15-µl reaction mixture containing 15 mM Tris, pH 7.5, 6.5% glycerol, 90 mM KCl, 0.7 mM EDTA, 0.2 mM dithiothreitol, 1 mM MgCl₂, 50 µg/ml bovine serum albumin, 20 mM dithiothreitol, 100 µM DTT, and 500 nM 32P-labeled double-stranded oligonucleotide probes. Double-stranded oligonucleotide probes were digested with micrococcal nuclease and purified on classical nucleic acid paper. The probe concentration was 5000 nM. The unbound probes were washed with phosphate-buffered saline and eluted with 10 µl reduced glutathione in 50 mM Tris-HCl, pH 8.0. The eluted protein was concentrated using Centricon 10 (Amicon) as per the manufacturer’s instructions.

Electromobility Shift Assays (EMSA)

EMSA results were used to determine the specific DNA binding of Pax3 to the STX promoter. The purified nuclear extracts were incubated with 100 or 300 nM probes for 20 min on ice. Samples were loaded onto a 4% polyacrylamide gel for separation of free DNA and DNA-protein complexes. The electrophoresis was performed in cold buffer at 150 mV and 30 mA for 4 h. The bands were detected using phosphorimager analysis to view the shifted bands.

RESULTS

Oligonucleotide Microarray—A human medulloblastoma cell line, DAOY, was stably transfected with murine Pax3 and pcDNA vector controls. Of the stable Pax3 transfectants, B9, E7, and H6 were chosen for the oligonucleotide microarray because they showed the same relative Pax3 expression (16). Biotin-labeled cRNA from DAOY cells, two vector-transfected controls (pcDNA-1 and pcDNA-2), and three Pax3 transfectants (B9, E7, and H6) were prepared, and microarray analysis was carried out as described under “Experimental Procedures.”
After scanning, the six data files (DAOY, pcDNA-1, pcDNA-2, B9, E7, H6) were uploaded into Affymetrix MicroDB 1.0 software (Supplemental Table I). This data base file was then sorted and analyzed with Affymetrix Data Mining Tool 1.2. Using DAOY as a base line, genes showing fold changes greater than $\frac{1}{1.5}$ and $\frac{1}{1.5}$ in the vector controls were excluded from further analysis. These represented genes that were possibly altered by the transfection and selection process. The remaining genes that varied by less than 1.5-fold were organized into a list and compared with the Pax3 transfectants. The expression of 1126 genes of a total of 7000 genes analyzed appeared to be altered by vector transfection (pcDNA-1 and pcDNA-2) and excluded from further analysis. The data from each of the three transfected cell lines (B9, E7, and H6) were then queried against the "unchanged" gene list. Genes that had an absolute call of Present or Absent call had to be modified in our further calculations because known target genes such as MyoD and genes related to myogenesis were not identified. This appeared to be due to the method of "binning" to an absolute call of Present. For instance, MyoD and myogenic repressor 1 (MF1) were changed in the Pax3 transfectants but were missed because they were called Absent in DAOY or in transfectants. Thus, the genes that were Absent in DAOY were also included in subsequent analysis.

Based on a comparison with the known Pax3 targets, we selected candidates that were altered in all three (B9, E7, and H6) clones or, alternatively, in two of three clones. The resulting list of genes was examined and compared with known genes modulated by Pax3.

Upon analysis of this resulting list of genes that changed, it became apparent that the Present or Absent call had to be modified in our further calculations because known target genes such as MyoD and genes related to myogenesis were not identified. This appeared to be due to the method of "binning" to an absolute call of Present. For instance, MyoD and myogenic repressor 1 (MF1) were altered in the Pax3 transfectants but were missed because they were called Absent in DAOY or in transfectants. Thus, the genes that were Absent in DAOY were also included in subsequent analysis.

Based on a comparison with the known Pax3 targets, we selected candidates that were altered in all three (B9, E7, and H6) clones or, alternatively, in two of three clones. The modi-

---

**Table I**

Relative levels of gene expression in Pax3 transfectants as compared to DAOY cells

| Gene | Description | Expression |
|------|-------------|------------|
| MyoD | Myogenic repressor | Increased |
| MF1  | Myogenic repressor | Decreased |
| PKC  | Protein kinase C | Present |
| MyHC | Myosin heavy chain | Present |
| MyS | Myosin light chain | Absent |
| MyHSP | Myosin binding protein | Present |
| Myl | Myosin light chain | Absent |

---

*Note: The full table is not provided here. The table describes the expression levels of various genes in Pax3 transfectants as compared to DAOY cells.*
fied criterion yielded a cluster of genes that included most of the known downstream targets of Pax3. In addition to the known downstream targets, we observed a total of 270 genes that were altered by Pax3 transfection. Of these 270 altered genes, 202 genes showed an increase, and 68 genes showed a decrease in their expression. More specifically, a direct comparison of the data sets for B9, E7, and H6 clones revealed that 38 genes displayed consistent changes (26 up-regulated and 12 down-regulated). In B9 and E7 clones, the expression of an additional 36 genes were altered, of which the expression of 27 genes were up-regulated, and 9 genes were down-regulated. E7 and H6 clones showed alterations in the expression of an additional 38 genes, of which 15 genes were up-regulated and 23 genes were down-regulated. Similarly, B9 and H6 showed changes in the expression of 56 genes, of which 38 were up-regulated and 18 were down-regulated. Finally the expression of 102 genes was altered in any one of three clones. Of this, 96 genes were up-regulated, and 6 genes were down-regulated. The data and the relative expression levels of different selected genes in at least two of the three Pax3 transfectants as compared with DAOY controls are summarized and categorized according to their respective biological function(s) in Table I.

**Validation of Microarray Results**—To confirm whether the changes observed by Pax3 were authentic, two different independent methods were used. We performed a repeat hybridization experiment using a CLONTECH neurobiology cDNA expression array, which does not require any amplification step in contrast with the T7 amplification in the Affymetrix microarray protocol. Furthermore, real-time quantitative RT-PCR was used to corroborate both sets of array data.

cDNA Expression Arrays—The B9 clone was selected for CLONTECH neurobiology cDNA expression array analysis because it showed the highest Pax3 expression (16). Fig. 1 shows the autoradiographic image of 33P-labeled cDNA obtained from control DAOY cells and the Pax3-transfected B9 clone. The spot intensities were quantitated using ImageQuant software (Molecular Dynamics) For each gene, DAOY was compared with B9, and the intensity ratio of B9/DAOY was calculated. Ratios of 1 indicate equal intensities and, therefore, no change in gene expression between the two cell lines. Ratios below 1 indicate down-regulation of gene expression in B9; on the other hand, ratios above 1 indicate an up-regulation of gene expression in B9 (Supplemental Table II). Using this criterion, we found that 494 genes of 588 genes were expressed both in DAOY and B9. Of the 7000 genes on the Affymetrix chip and 588 genes on CLONTECH array, 107 genes were common to both. Of these 107 genes, 81 genes were eliminated from our Affymetrix data due to changes observed in the pcDNA3 vector transfection. This left 27 genes for direct comparison between the Affymetrix and CLONTECH arrays. Of the remaining 27 genes, 21 dis-
played the same relative expression change in both the Affymetrix and CLONTECH neurobiology arrays. Of the six that were not the same, three were at/or below our limits of detection. The three remaining, namely Versican, Tenascin, and protein-tyrosine phosphatase were called different.

**Real-time Quantitative RT-PCR**—In efforts to provide further experimental evidence that the gene changes observed on both arrays were valid, real-time quantitative RT-PCR was used to analyze a selection of representative genes. Throughout the real-time quantitative RT-PCR experiments, RNA was extracted from DAOY, vector, and the B9 clone only. First, we selected genes where there was a disagreement between Affymetrix and CLONTECH methods. On the other hand, Tenascin was not changed in the Affymetrix arrays but was decreased on the CLONTECH. In this case, real-time quantitative RT-PCR confirmed that there was a decrease. Interestingly, Versican was slightly increased in Affymetrix data and decreased in CLONTECH.

Real-time quantitative RT-PCR was attractive method for validation because it could detect different isoform changes that were not obvious in the Affymetrix chip or CLONTECH cDNA array experiments. For example, the Versican gene can be expressed in four known splice variants, V0, V1, V2, and V3, which share significant homology. The discrepancy between array data may be due to the sequences being detected. Real-time quantitative PCR made this distinction possible in that the expressions of splice variant V2 was increased 26.8-fold, and V3 was decreased more than 2-fold (B9/DAOY ratio of 0.4335), whereas V1 was not changed (Fig. 3 and Table II). In summary, there was very good agreement between data obtained from both arrays and real-time quantitative RT-PCR (Table II).

**Comparison of Promoter Regions**—After validation of the array data, the mechanisms by which the transcriptional regulation by Pax3 is brought about can now be explored. We hypothesized that there may be some similarity in the promoter regions of the altered genes. The promoter sequences for several known genes altered by Pax3 or PAX3-FKHR were examined for potential Pax3 binding motifs. The Pax3 downstream targets are MITF (23), MyoD (27), NCAM (28), c-RET (29), and Tyrp-1 (30), and PAX3-FKHR downstream targets...
include \textit{ATF3} (31), \textit{c-MET} (32), PDGF-\alpha receptor (33), IGFBP-5 (34), and PCNA (35). Despite numerous Pax3 targets described in the literature, a single consensus binding motif has not emerged (25). We therefore, scanned for known paired domain and homeobox domain binding motifs in the promoter region for those genes. Commonly described motifs for paired and homeodomain binding are ATTA(N)GTTCC or GTTAT. Similarly the paired domain recognition sites GTTCC, GTTAT, GTTAC or the respective inverted sequences are reported Pax3 binding sites. The luciferase activity due to each reporter plasmid was normalized as to the \beta-actin expression by reporter plasmids containing putative Pax3 binding sequences (25, 41). An additional Pax3 binding site, GTGTGTA, has been described for the melanocyte-specific tyrosinase-related protein-1 promoter (30). This consensus sequence is also present in MITF (23) and \textit{c-RET} (29). The presence of the complimentary sequences TAAT or CAAGG was observed in the \textit{STX} by searching for these Pax3 binding motifs, we assigned a score based on the number of times a Pax3 binding motif was present in the promoter region of a gene. Thus we came up with a score of 16, 40, 12, 23, and 9 for \textit{Dep-1}, \textit{NCAM}, \textit{c-RET}, and \textit{Tyrp1}, respectively (Table IIIA). The same criteria was applied to genes implied as Pax3 downstream targets. We reasoned that the authenticity of our prediction as putative Pax3 downstream target based on microarray results and similarities in the promoter region, we identified 17 previously unidentified potential Pax3 downstream targets. Because there is no clear consensus binding motif for Pax3 (25), we wanted to demonstrate that our selection criteria could identify downstream targets. One candidate gene, \textit{STX}, was selected to validate the selection criteria. This gene was chosen because the increased expression had been previously shown to alter the phenotype and aggregation properties of the DAOY cells (16). In addition, the motif score for \textit{STX} was 11 and near the lower cutoff score of our putative Pax3 downstream targets. We reasoned that the authenticity of our prediction as putative Pax3 downstream target based on the motif score was best tested with a motif score that was toward the lower end of our cutoff score.}

\begin{table}[h!]
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
Accession no. & Affymetrix & Clontech & Taqman & Description \\
& B9/DAOY ratio & B9/DAOY ratio & B9/DAOY ratio & \\
\hline
U79299 & 0.4995 & 0.6811 & ND & Neuronal olfactomedin-related endoplasmic reticulum-localized protein \\
L02321 & 0.7047 & 0.4563 & ND & Glutathione S-transferase \\
M64572 & 0.2171 & 0.7013 & ND & Protein-tyrosine phosphatase \\
U54977 & 0.4400 & 0.7509 & ND & Low-M, GTP-binding protein (RAB31) \\
D2893 & 0.0656 & 0.8906 & ND & Clathrin-like protein \\
L36983 & 0.5877 & 0.7288 & ND & Dynamin \\
U23315 & 0.5343 & 0.7891 & ND & Syntaxin 3 \\
D14838 & 0.0498 & 0.7789 & ND & Fibroblast growth factor-9 \\
D2340 & 0.6613 & 0.6876 & ND & Atrogin-1 \\
M97252 & 0.0219 & 0.4201 & ND & Kallmann syndrome protein (KAL); adhesion molecule \\
U90579 & 0.5305 & 0.7659 & ND & Melanoma differentiation-associated protein (mda-6) \\
S76965 & 0.3754 & 0.7381 & ND & Protein kinase inhibitor \\
X05908 & 0.5487 & 0.4641 & ND & Annexin 1; lipocortin 1 \\
X79870 & 0.3588 & 0.4563 & ND & YPT3 human homolog \\
L20688 & 0.2300 & 0.2672 & 0.1076 & rhoGDP-dissociation inhibitor protein (Ly-GDI) \\
X86809 & 1.0682 & 1.1810 & 1.1178 & Astrocytic phosphoprotein PEA-15 \\
U08002 & 0.0822 & 0.4476 & 0.5123 & Drebrin E2 \\
X78565 & 1.0705 & 0.4545 & 0.1879 & Tenascin C \\
U16306 & 1.3691 & 0.5833 & ND & Versican V0 \\
U16306 & ND & ND & 0.8945 & Versican V1 splice variant \\
U16306 & ND & ND & 26.7792 & Versican V2 splice variant \\
U16306 & ND & ND & 0.4335 & Versican V3 splice variant \\
D17517 & ND & ND & 0.6403 & Protein-tyrosine kinase, SKY \\
\hline
\end{tabular}
\caption{Comparison of Affymetrix, Clontech, and real-time quantitative PCR results for selected genes}
\end{table}

\section{Identification of \textit{STX} as a Pax3 Downstream Target—Based on microarray results and similarities in the promoter region, we identified 17 previously unidentified potential Pax3 downstream targets. Because there is no clear consensus binding motif for Pax3 (25), we wanted to demonstrate that our selection criteria could identify downstream targets. One candidate gene, \textit{STX}, was selected to validate the selection criteria. This gene was chosen because the increased expression had been previously shown to alter the phenotype and aggregation properties of the DAOY cells (16). In addition, the motif score for \textit{STX} was 11 and near the lower cutoff score of our putative Pax3 downstream targets. We reasoned that the authenticity of our prediction as putative Pax3 downstream target based on the motif score was best tested with a motif score that was toward the lower end of our cutoff score.}

\section{Analysis of Promoter Activity—To determine whether Pax3 could influence \textit{STX} promoter activity, we used luciferase reporter plasmid \textit{pB01-NH2.5} and \textit{pB01-SN0.45} containing the \textit{STX} promoter sequences (37). The promoterless luciferase gene in \textit{pPBII} was used as a control (37). The constructs were assayed for promoter activity by transient transfection into stable \textit{Pax3} transfectant B9, stable pcDNA3 vector transfected cells, and wild type DAOY cells. Fig. 4A shows the activation of luciferase expression by reporter plasmids containing putative Pax3 binding sites. The luciferase activity due to each reporter plasmid was normalized as to the \textit{Renilla} luciferase activity by...
cotransfecting an internal control plasmid pRL-null carrying cDNA encoding Renilla luciferase gene. The pB01-NhN3.5 and pPGBI control vector were essentially inactive. These data were consistent with the previous results of Yoshida et al. (37), indicating the pB01-SN0.45 construct to be minimal essential promoter for STX. This construct showed 10–12-fold increases in activity over the pB01-NhN3.5 and pPGBI control constructs. These data suggested that the minimal essential region of the STX promoter contained elements that are responsive to Pax3 binding.

**Identification of a Pax3 Binding Sequence**—The STX minimal essential promoter region did not appear to contain known consensus Pax3 binding motifs. We used EMSA with both nuclear extracts and purified GST/Pax3 fusion protein to identify sequences that may bind Pax3. Ten double-stranded oligonucleotides, 30 base pairs each, were synthesized. These covered the region from −298 to −118 of the STX promoter and overlapped each other by 10 base pairs at the 3′ end. Of the 10 oligonucleotides tested, only two showed specific binding that was increased in B9 compared with DA0Y (Fig. 4B). These were CSC2 and CSC3 covering the sequence between −151 to −118 (complete sequences of these oligonucleotides can be found under “Experimental Procedures”). Common to both of these oligonucleotides was the CAAGG motif suggested by our selection criteria described above and previously suggested as a Pax3 binding sequence (25, 41). An additional oligonucleotide, CSC1, also contained the CAAGG motif but did not exhibit increased binding in the Pax3 transfectant B9 (Fig. 4B). When the CAAGG motif was in the middle of the oligonucleotide (CSC2), the nuclear extract from B9 bound with the greatest intensity.

The increased binding of CSC2 and CSC3 to B9 nuclear extracts suggested that increased Pax3 might be responsible for the increased binding. A purified GST/Pax3 fusion protein was used to determine whether CSC2 and CSC3 could interact directly with Pax3. EMSA studies using the GST/Pax3 fusion protein (Fig. 4C) indicated that Pax3 could bind directly to CSC2 oligonucleotide, which had the CAAGG motif in the middle, and not to CSC1 or CSC3, which had CAAGG motif at either the 5′ end or 3′ end. Thus, our studies indicate that Pax3 could bind directly to a sequence in the STX promoter and suggest that STX may be a previously unidentified biological downstream target of Pax3.

**DISCUSSION**

Using oligonucleotide arrays and RNA from stable Pax3-transfected DAOY cells to identify potential Pax3 downstream targets, a total of 270 genes were altered by Pax3 transfection. Of these 270 altered genes, 202 genes showed an increase, whereas 68 genes showed a decrease in their expression. Before making any definitive conclusions about these changes, we determined how reproducible and representative these changes were. We employed two independent techniques to validate our Affymetrix data, namely the CLONTECH neurobiology cDNA expression array and real-time quantitative RT-PCR. The CLONTECH array was chosen to validate the Affymetrix results because it provided a repeat hybridization of 107 genes and did not require an amplification step. The consistency observed between the two types of arrays indicated that changes were reproducible and that the in vitro transcription step did not skew the message representation. Furthermore, the sensitivity of real-time quantitative RT-PCR helped define the limits of significant changes and resolved any apparent inconsistencies in the array data. Real-time quantitative RT-PCR and cDNA expression array analysis confirmed the changes of RhoGDI, MyoD, Drebrin, Tenascin, and glutathione S-transferase. On the other hand, certain gene changes observed in Affymetrix were inconsistent with those observed with the CLONTECH array. Notably, an increase in astrocytic phosphoprotein PEA-15 was observed with the CLONTECH array, whereas the Affymetrix microarray gave us a No Change call. Real-time quantitative RT-PCR was instrumental in resolving this discrepancy, indicating that a relatively small magnitude of changes like those for PEA-15 is below the cut-off sensitivity of the array method used. Similarly, real-time quantitative RT-PCR resolved the discrepancy between the Affymetrix and CLONTECH data for Versican. An increase in the expression of Versican (V0), a common region in all the splice variants (V1, V2, and V3) observed in Affymetrix microarray, was confirmed to be due to the splice variant V2. This result suggested that the observed changes in Versican gene expression probably arose from the oligonucleotide sequences selected by Affymetrix.

Quantitative real-time RT-PCR validated the gene expression changes observed by the array analysis, but it remains to be seen whether these genes are direct downstream targets of Pax3. Despite numerous downstream targets identified in the literature, a consensus sequence for Pax3 binding motif does
not exist (25). Analysis of known Pax3 binding motifs did, however, reveal similarities in their promoter regions. Of the genes that displayed significant alterations in expression, 17 previously undescribed downstream targets of Pax3 had promoter regions with similarity to known Pax3 targets. In addition, another 17 genes showed reproducibly large changes, but their promoter sequences are unknown. These genes may also be Pax3 downstream targets.

Because there is no clear consensus binding motif for Pax3 (25), we used promoter-luciferase reporter transfection assays and EMSAs to demonstrate that our selection criteria could identify downstream targets. We chose a candidate gene STX to validate the selection criteria, because the increased expression of STX had been previously shown to alter the phenotype and aggregation properties of the DAOY cells (16). In addition, the motif score for STX was 11 and near the lower cutoff score of our putative Pax3 downstream targets. We reasoned that the authenticity of our selection criteria for predicting a gene as a potential Pax3 downstream target based on the motif score can be best tested with a motif score that was toward the lower end of our cutoff score. The demonstration that STX promoter activity was increased by Pax3 and the specific binding of Pax3 to STX promoter clearly suggests that the selection criteria we used for identifying downstream targets were appropriate and could be extended to identify common sequences in promoter regions of potential downstream targets of transcription factors. The Pax3 binding motif in the STX promoter ac-

**TABLE III**

| Accession no. | Gene accession no. | Promoter accession no. | Description | Score |
|---------------|--------------------|------------------------|-------------|-------|
| A. Known Pax3 downstream targets | X56677 | L34006 | MyoD | 16 |
| | XM109977 | AF034755 | MITF | 40 |
| | L19871 | U37542 | ATF3 | 9 |
| | M22092 | BE019907 | NCAM | 12 |
| | AF042830 | AF02124 | c-RET | 23 |
| | NM000245 | Z26936 | c-MET | 2 |
| | M84607 | AJ278993 | PDGF-α receptor | 17 |
| | X60855 | AF087673 | Typp-1c | 9 |
| | L27559 | U20271 | IGFBP5 | 11 |
| | J05614 | J05614 | PCNA | 17 |
| B. Literature-implied Pax3 downstream targets | U16306 | U15963 | Versican V0 | 7 |
| | NM009181 | X96645 | STX (STX8) | 11 |
| | AF032130 | AA362211 | Dep-1 | 4 |
| | XM008979 | M24410 | Myelin basic protein | 5 |
| | NM002356 | L76376 | MARCKS | 1 |
| C. Microarray-postulated Pax3 downstream targets | M60314 | M87843 | TGF-β2 | 39 |
| | M72985 | AA361340;AA358300 | GOS2 | 33 |
| | V00984 | X12751 | Amyloid A4 precursor | 27 |
| | Z74161 | Z74161 | Pre-pro(II) collagen | 26 |
| | X53331 | AF067176 | Matrix gla | 26 |
| | U35048 | AF156226 | TSC-22 | 25 |
| | U14394 | AF001361 | TIMP-3 | 21 |
| | U43203 | AF027332 | Thyroid transcription factor | 20 |
| | U20816 | U20816 | NF-KE | 19 |
| | M83363 | AK026443 | Calcium-pumping ATPase | 18 |
| | M60314 | S81957 | BMP-5 | 18 |
| | J09090 | AH005196;U03742 | Chromogranin A, alkaline phosphatase | 17 |
| | M94893 | BE225351;BE225423 | Testis-specific protein | 13 |
| | J05158 | BE225566 | Carboxypeptidase | 13 |
| | J05158 | Y18319 | Src-like adapter protein (SLAP) | 12 |
| | D89077 | J05614 | PCNA | 11 |
| | J05614 | J00073 | β-Actin | 4 |
| | M15465 | SEG AB01598S | Pyruvate kinase L | 1 |
| | D16227 | J05614 | ST8Sial | 1 |
| | M60314 | BE019307;BE019307 | GOS2 | 33 |
| | M53893 | L27559 | IGFBP5b | 1 |
| | J05614 | J05614 | TIMP-3 | 21 |
| | M60314 | M60314 | TGF-β1 | 17 |
| | M15465 | M15465 | BMP-5 | 18 |
| | J05614 | J05614 | TIMP-3 | 21 |
| | M60314 | M60314 | TGF-β1 | 17 |
| | M15465 | M15465 | BMP-5 | 18 |

a Determined from the average fold increase or decrease in expression of clones B9, E7, and H6 as compared to the parental line, DAOY.

b Identified as a Pax3-FKHR fusion protein downstream target.

c STX (STX8)
complimentary sequence, CAAGG, is present on the sense strand. Thus we showed at least one previously undescribed downstream target, STX, to be a biological downstream target of Pax3.

A limitation of the methodology used in this application is that the gene expression changes observed are dependent on many factors such as clonal selection and differential hybridization. In an effort to minimize the differences resulting from these factors, we used three Pax3-transfected clones (16) and performed repeat hybridizations. An additional possibility that may affect the expression of downstream target genes is the levels and the availability of Pax3. Chaleapakis et al. (8) show that the transcriptional regulation by Pax3 is concentration-dependent and biphasic. Furthermore, Phelan and Loeken (25) demonstrate that low levels of Pax3 in the nucleus activates only the high affinity responsive promoters. Conversely, at concentrations above the minimum threshold, only the low affinity responsive promoters may show transcriptional activation. Although we do not know if Pax3 is above or below a threshold in the Pax3 transfectants used in our study, we selected these three clones because they showed the same relative levels of Pax3 expression.

It has been suggested that when Pax3 interacts with other proteins through the paired domain, it behaves more like a repressor. Examples of such Pax3-binding proteins are Msx1, Ets, and hDaxx. On the other hand, if Pax3 interacts with proteins that leave the integrity of its homeodomain and paired domains intact, the resulting complex is a stronger transcriptional activator. An example of such strong transcriptional activator is a Pax3-FKHR fusion protein. Khan et al. (45, 46) use microarrays to identify downstream targets of a fusion protein, Pax3-FKHR. A comparison of our data with that of these investigators showed an overlap in some of the genes that may be downstream targets of Pax3. An interesting example is the PDGF-α receptor (47). Although it is implied to be a Pax3 downstream target, promoter studies show that Pax3 by itself cannot mediate transcriptional activation of the PDGF-α receptor promoter, whereas Pax3-FKHR can (47). This observation underscores the importance of Pax3 binding partners involved in transcriptional activation by Pax3. In addition to PDGF-α receptor, Khan et al. (45, 46) observe a significant increase in IGFBP5, ATF3, and PCNA expression levels in their microarray analysis, but we did not see any change in their expression levels by Pax3 alone. Even though these genes have high Pax3 binding motif score in their promoters, it appears that other factors in addition to Pax3 are essential in eliciting transcriptional activation.

Another way Pax3 may regulate downstream target genes is indirectly through regulation of transcription factor genes, which in turn regulate its downstream target genes. Among the genes identified as changed by Affymetrix analysis, 40 genes with known promoter sequences had a motif score of 9 or less. This implied that their promoter sequences were not that similar to known Pax3 targets and may be regulated by a transcription factor that may be a Pax3 target. c-MET was observed to be up-regulated by Pax3 in our Affymetrix analysis, although its Pax3 binding score was 2. It is very interesting to note that the c-MET promoter has a MyoD binding site. It is therefore possible that Pax3, which up-regulates MyoD (Pax3 binding score of 16), can up-regulate c-MET. In this respect c-MET is an indirect downstream target of Pax3 (Pax3 → MyoD → c-MET).

If we view our data from the perspective of early embryonic development regardless of the combinatorial nature of Pax3 activity (48), its biological effect is to ensure that neural crest cells do not execute a differentiation program that is inappropriate for their dorsoventral position in the neural tube. It is interesting to note that certain sets of genes that delay differentiation, namely Id-2, Id-4, and myogenic repressor-1, show an increase in expression in our Affymetrix analysis. Similarly several genes that are involved in cell migration show changes in expression in the Pax3 transfecteds. These include: Versican, Tenasin, RhodGDI, Drebrin E, (HSPG2), type IV collagenase, and tissue inhibitor of matrix metalloproteinase-3 (TIMP-3). Up-regulation of specific genes and the delay in others is expected of a cell that is committed to migration (15). These events prepare the extracellular matrix as well as the intracellular cytoskeletal elements for efficient migration. Our previous study (16) showed that overexpression of Pax3 up-regulated STX, which resulted in an increase in the polysialylation of NCAM (PSA-NCAM). PSA-NCAM prefers heparin sulfate proteoglycan, which is migration-permissive, whereas Versican, a large chondroitin sulfate proteoglycan (CSPG), is a migration non-permissive substrate and promotes NCAM-NCAM-mediated homophilic adhesion (16, 49–51). Migration of neural crest cells during early embryonic development would be facilitated if non-permissive substrates were down-regulated. Our data support this hypothesis. We observed that V2 splice variant of Versican is up-regulated and V3 splice variant is down-regulated. Henderson et al. (52) ob-
observe an overexpression of Versican in Splotch mouse and suggested that Pax3 may serve to negatively regulate Versican expression that is associated with defective neural crest migration in Pax3 mutant mouse (52). It is therefore possible to speculate that it could be the V3 Versican splice variant that is affected in Splotch mouse.

Migration involves not only cell surface interaction with extracellular matrix components but also of cell intracellular cytoskeletal elements that prepares the cell for migration. RhoGDI is one such gene that plays a critical role in the cytoskeletal-dependent cell functions (53). It is required for transcription of muscle-specific gene, myogenin through MEF-2. Down-regulation or inhibition of RhoGDI function suppresses myogenesis (42). A significant down-regulation of RhoGDI and an up-regulation of Id-2, Id-4 and myogenic repressor-1 (MF-1) in Pax3 transfectants supports the role of Pax3 in migration and delaying differentiation to take place. Our previous results and these observations suggest that Pax3 not only modifies NCAM through polysialylation but also regulates extracellular matrix and intracellular cytoskeletal genes.

Thus in summary, we report several Pax3 downstream targets using Affymetrix gene chip analysis. To confirm the observed gene changes as representative of the actual changes in the cell, we validated our Affymetrix data and compared it with a CLONTECH array data and found that there was a good agreement between the two sets of data. Validation of the criteria used to establish stringency conditions to arrive at a given result in both the Affymetrix as well as CLONTECH array data was established by performing real-time quantitative RT-PCR. Based on the Pax3 binding motif score in the array data was established by performing real-time quantitative RT-PCR. To confirm the observed gene changes as representative of the actual changes in the cell, we validated our Affymetrix data and compared it with a CLONTECH array data and found that there was a good agreement between the two sets of data. Validation of the criteria used to establish stringency conditions to arrive at a given result in both the Affymetrix as well as CLONTECH array data was established by performing real-time quantitative RT-PCR. Based on the Pax3 binding motif score in the array data was established by performing real-time quantitative RT-PCR. To confirm the observed gene changes as representative of the actual changes in the cell, we validated our Affymetrix data and compared it with a CLONTECH array data and found that there was a good agreement between the two sets of data. Validation of the criteria used to establish stringency conditions to arrive at a given result in both the Affymetrix as well as CLONTECH array data was established by performing real-time quantitative RT-PCR. Based on the Pax3 binding motif score in the array data was established by performing real-time quantitative RT-PCR.
Microarray Analysis Detects Novel Pax3 Downstream Target Genes
C. S. K. Mayanil, David George, Laura Freilich, Erik J. Miljan, Barbara Mania-Farnell, David G. McLone and Eric G. Bremer

J. Biol. Chem. 2001, 276:49299-49309.
doi: 10.1074/jbc.M107933200 originally published online October 5, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107933200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2002/01/18/276.52.49299.DC1

This article cites 53 references, 27 of which can be accessed free at
http://www.jbc.org/content/276/52/49299.full.html#ref-list-1