MOLECULAR AND CELLULAR BIOLOGY, Nov. 1991, p. 5446–5453
0270-7306/91/115446-08$02.00/0

Vol. 11, No. 11

Expression of *CD44* Is Repressed in Neuroblastoma Cells

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Received 25 June 1991/Accepted 31 July 1991

We have used cDNA subtractive cloning to identify a group of human genes that are expressed in diverse differentiated derivatives of neural crest origin but not in neuroblastoma cell lines. One of these genes was identified as *CD44*, which encodes an integral membrane glycoprotein that serves as the principal receptor for hyaluronate and participates in specific cell-cell and cell-extracellular matrix interactions. The repression of *CD44* expression in neuroblastoma cell lines might be relevant to their high metastatic potential. We have cloned full-length cDNAs corresponding to *CD44* transcripts and identified a novel splice variant of *CD44* lacking 31 amino acids of the extracellular domain. As a first step toward analysis of *CD44* downregulation in neuroblastoma cells, we have mapped the *CD44* RNA initiation site and analyzed the structure of the upstream regulatory region. We constructed a series of plasmids containing different amounts of *CD44* upstream regulatory region linked to the bacterial chloramphenicol acetyltransferase gene and then analyzed their ability to promote transcription in neuroblastoma and melanoma cells. We found that a DNA segment including about 150 bp of the *CD44* upstream region and the 5′ end of the gene itself was sufficient to induce substantial transcription of the chloramphenicol acetyltransferase gene in both neuroblastoma and melanoma cells. Several upstream *cis*-acting elements contribute to the downregulation of *CD44* in neuroblastoma cells, the most prominent being a 120-bp DNA fragment located 450 bp upstream to the RNA initiation site. Our data suggest that multiple factors might be involved in downregulation of *CD44* in neuroblastoma cells.

Neuroblastoma is a rare malignancy that occurs primarily in children under the age of 2 and represents the most primitive neoplasm originating from the neural crest (37). The normal counterparts of neuroblastoma cells appear to be migratory cells of the neural crest that are destined to populate the medullary aspect of the fetal adrenal gland and thus are in the chromaffin lineage. Neuroblastomas apparently arise when the differentiation of these migratory cells is blocked (17).

The phenotypes of neuroblastomas reflect the pluripotentiality of the early neural crest. Some lines of neuroblastoma cells display varied assortments of markers for the chromaffin lineage (8) and can be grouped accordingly (8), but other cell lines express markers specific to melanocytes or Schwann cells (36). Moreover, neuroblastomas occasionally differentiate spontaneously in vivo to benign tumors composed of neuronal cells (ganglioneuroma). Differentiation of neuroblastomas to a neuronal phenotype also occurs in vitro when responsive cell lines are treated with nerve growth factor, retinoic acid, cyclic AMP, or a phorbol ester (28, 30, 31).

Loss of gene function is a common disorder in many types of human cancer (5). The loss may arise from direct damage to a gene or from mechanisms that act in *trans* to repress gene expression. Neuroblastomas exemplify both events: they frequently contain a deletion that affects the short arm of chromosome 1 (6, 10), with the crucial locus apparently lying at 1p36.1 (38), and they may also be characterized by the reduced expression of genes encoding two sorts of cell surface proteins, neural cell adhesion molecule (NCAM) (1) and major histocompatibility complex class I antigens (4). In an effort to explore further the role of repressed gene expression in the genesis of neuroblastomas, we have searched for genes that are expressed in diverse derivatives of the neural crest but not in neuroblastoma. Here we describe the cloning and screening strategy used to identify genes transcriptionally suppressed in neuroblastoma and report the characterization of the regulatory sequences of one of this genes, which encodes the cell surface transmembrane receptor for hyaluronate, CD44.

MATERIALS AND METHODS

cDNA libraries were constructed in λgt10 and λZAPII according to published protocols (18). Hybridization of single-stranded cDNA to an excess of RNA and selection of the unhybridized fraction were performed as described previously (16). RNA blot analysis was done with oligo(dT)-selected RNA electrophoresed through 1% agarose gels and transferred to nylon filters. Filters were hybridized with ^{32}P-labelled PM67 probe. Sequencing of DNA was done from M13 subclones, using the Sequenase sequencing kit (U.S. Biochemical). RNase protection and primer extension were performed according to published procedures (25).

Transient transfection of the melanoma cell line C32r and neuroblastoma cell line NMB was performed by the calcium phosphate precipitation method as described previously (25). Plasmid pCAT-basic (Promega) was used to construct all of the plasmid clones containing different fragments of the *CD44* regulatory region placed upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene. For transfection experiments, about 10^6 cells on a 10-cm dish were transfected with equimolar amounts of pCAT constructs corresponding to 15 μg of pCAT-basic. The efficiency of transfection was monitored by cotransfecting plasmids containing the bacterial β-galactosidase gene under control of the simian virus 40 promoter sequences (for the melanoma C32r) or the Rous sarcoma virus long terminal repeat (for the

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neuroblastoma NMB). Cells were harvested 50 to 52 h after transfection. Cytoplasmic extracts were prepared according to published methods and used to measure the levels of the exogenous β-galactosidase and the CAT gene product. The latter was done by measuring the diffusion of acetylated radioactive chloramphenicol into an organic phase (25).

Nucleotide sequence accession number. The nucleotide sequence shown in Fig. 4 has been submitted to GenBank and assigned accession number M69215.

RESULTS

Construction of subtracted library and screening strategy. The aim of this study was the identification and analysis of cellular genes subject to transcriptional suppression in neuroblastoma cell lines. To this end, we have constructed a subtracted cDNA library enriched for sequences transcribed in pheochromocytoma but not represented or found at reduced levels in RNA from a neuroblastoma cell line. Polyadenylated RNAs were prepared from a surgical specimen of a benign pheochromocytoma and from the neuroblastoma cell line NMB. Single-stranded cDNA was synthesized by using the pheochromocytoma RNA as template. It was subjected to hybridization with a 10-fold excess of neuroblastoma RNA, and the unhybridized single-stranded cDNA fraction was selected on a hydroxyapatite column as described previously (16). Approximately 8% of the initial amount of cDNA was recovered from the hydroxyapatite column in a single-stranded form, indicating about a 10-fold enrichment for the pheochromocytoma-specific transcripts. The cDNA was converted into double-stranded DNA and cloned into λgt10 by standard techniques. The subtracted cDNA library consisted of 30×10^3 clones.

Screening of the cDNA library was designed in such a way as to eliminate from further analysis transcripts specific to the highly specialized phenotype of the chromaffin cells, such as elements of the synthetic and secretory pathways for neurotransmitters. The library was screened with three radioactive cDNA probes prepared with polyadenylated RNAs from the original pheochromocytoma, the neuroblastoma cell line NMB, and the melanoma cell line HT144. Only those clones that hybridized to both pheochromocytoma and melanoma cDNA probes, not to the neuroblastoma cDNA, were selected for further analysis. The use of melanoma cDNA in the screening allowed us to identify cDNA clones derived from transcripts common to both pheochromocytoma and melanoma but absent from neuroblastoma.

Screening of the subtracted cDNA library resulted in identification of 110 clones that were shown by cross-hybridization analysis to represent 45 individual RNAs. The faithfulness of the selection strategy was verified through Northern (RNA) blot analysis of the cDNA clones; 80% of the clones were confirmed to be transcriptionally suppressed in at least three neuroblastoma cell lines compared with melanoma cell lines (data not shown).

Identification of two genes whose expression is reduced in neuroblastoma cells. We next examined whether any of the cDNA clones obtained were derived from the human chromosome 1p or 11, both of which have been implicated by cytogenetic and molecular analysis in development of human neural crest-derived malignancies (10, 39). The initial analysis was done by using DNA from somatic cell hybrids containing fragments of chromosome 1 or 11 as the only human DNA components in the rodent background. This preliminary analysis showed that none of 45 cDNA probes was derived from chromosome 1. Twenty-four of the clones were also tested with hybrids containing portions of chromosome 11. None mapped to 11q. Instead, four of the clones were found to represent genes located on the short arm of chromosome 11.

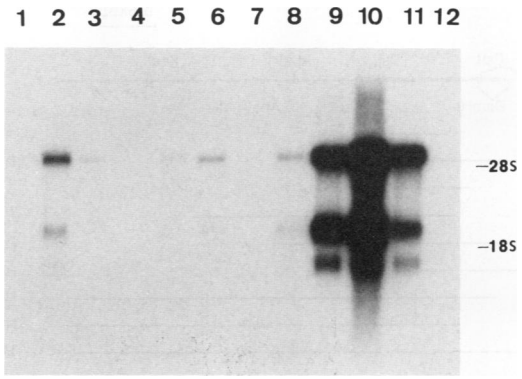

FIG. 1. RNA blot analysis of *CD44* expression in human cell lines. Lanes: 1 to 6, neuroblastomas NMB, LAN-1, LAN-5, NGP, NLF, and SK-N-SH, respectively; 7, neuroepithelioma SK-N-MC; 8, small cell lung carcinoma N417; 9, melanoma HT144; 10, osteosarcoma MG63; 11, promyelocytic leukemia HL-60; 12, Burkitt's lymphoma Daudi.

Two of the cDNAs localized to chromosome 11 (PM67 and PM227) showed a remarkable degree of conservation in rodent DNAs (data not shown). We have examined the expression of RNAs represented by PM67 and PM227 in human cell lines of diverse tissue origins and found that the two genes exhibit remarkably similar transcription patterns (data not shown). Sequence analysis of PM227 and PM67 indicated that the former represented a novel gene whereas the latter showed a 100% sequence identity to the previously cloned cDNA of *CD44* (12, 19, 32). Here we report the analysis of expression of *CD44* in neuroblastoma cell lines and the structural and functional analysis of its upstream regulatory region. A similar analysis of the gene represented by clone PM227 will be reported elsewhere.

CD44 was first recognised as a lymphocyte antigen, but was later shown to be expressed on diverse cells and tissues. It is known now that various cell surface antigens such as phagocytic glycoprotein Pgp-1, a major 80- to 90-kDa glycoprotein from mouse and human fibroblasts and from erythrocytes, the extracellular matrix receptor type III, and the Hermes antigen are in reality various guises of the same gene product (for review, see reference 15). Moreover, CD44 was demonstrated to be the principal receptor for hyaluronate (2, 9). Expression studies on the CD44 protein demonstrated that it is found also on the cells of nonneuronal derivatives of the neural crest (24). CD44 has been implicated in diverse processes involving specific cell and cell-matrix interactions (15). The absence of CD44 from the surface of transformed neuroblasts might be relevant to the highly metastatic properties of the neuroblastomas from which the cell lines were derived.

To confirm that the absence of *CD44* RNA in neuroblastoma is not restricted to the neuroblastoma cell line used in the cDNA cloning, we extended our analysis to additional cell lines (Fig. 1). Northern blot analysis showed that *CD44* either is not expressed or is expressed at greatly reduced levels in human neuroblastoma lines compared with melanoma, pheochromocytoma (not shown), or other diverse cell lines that express *CD44*. We found that *CD44* is transcribed

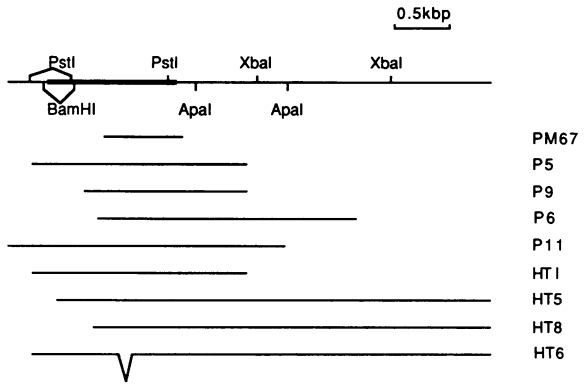

FIG. 2. Schematic representation of *CD44* cDNA clones. Clones were isolated from a subtracted cDNA library (PM67), placental cDNA (P5, P6, P9, and P11), and melanoma HT144 cDNA (HT1, HT5, HT8, and HT6). The upper bar represents the composite restriction map of *CD44* cDNAs. The thickened portion of the bar shows the open reading frame of *CD44*.

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   GAT GAT GAC GTG AGC AGC GGC TCC TCC AGT GAA AGG AGC AGC ACT
175 D   D   D   V   S   S   G   S   S   S   E   R   S   S   T

   TCA GGA GGT TAC ATC TTT TAC ACC TTT TCT ACT GTA CAC CCC ATC
190 S   G   G   Y   I   F   Y   T   F   S   T   V   H   P   I

   CCA GAC GAA GAC AGT CCC TGG ATC ACC GAC AGC ACA GAC AGA ATC
205 P   D   E   D   S   P   W   I   T   D   S   T   D   R   I

   CCT GCT ACC AGA GAC CAA GAC ACA TTC CAC CCC AGT GGG GGG TCC
220 P   A   T   R   D   Q   D   T   F   H   P   S   G   G   S
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FIG. 3. Sequence of the *CD44* cDNA region spanning the deletion in clone HT6. The nucleotides and amino acid residues deleted in HT6 are underlined. The numbering of amino acids is as in reference 33.

as three RNAs of 4.4, 2.1, and 1.5 kb, in accordance with previously reported data (12, 19, 32).

Cloning and analysis of full-length *CD44* cDNAs. To study the molecular mechanisms leading to the downregulation of CD44 in neuroblastoma, we had to clone and analyze the upstream regulatory region of *CD44*. As a first step, we cloned the full-length *CD44* cDNAs from a human placental cDNA library and a cDNA library constructed from RNA of the melanoma cell line HT144. Analysis of several cDNA clones by restriction enzyme mapping and partial sequencing revealed that they could be subdivided into two groups, apparently corresponding to the two longer mRNA species. Thus, clones P5, P9, and HT1 (Fig. 2) contained a short poly(A) stretch at a position 693 nucleotides downstream of the translation stop codon (data not shown). cDNA clones HT5 and HT8 extended for an additional 2.3 kbp in the 3′ direction but were otherwise identical to the shorter cDNA clones. We did not obtain cDNA clones representing the 1.5-kbp *CD44* transcript because the cDNA libraries used for the cloning were constructed with size-selected cDNA to enrich for clones longer that 1.5 kbp. We conclude that the three classes of mRNA are due to the existence and alternative usage of polyadenylation signals located 0.13, 0.69, and 3.0 kbp downstream of the translation stop codon (not shown).

All cDNAs were found to represent the version of CD44 having the cytoplasmic domain of 67 amino acids rather than the CD44 polypeptides that lack the intracellular domain (11). We found a single cDNA clone that was different in the protein-coding region from all the other clones in our study and from previously reported ones (11, 12, 19, 32, 33): clone pHT6 from the melanoma HT144 lacked 93 bp of the open reading frame coding for amino acid residues 192 to 223. We assume that the transcript represented by pHT6 was generated by alternative splicing, since the deletion of 93 nucleotides occurred in frame and encodes a CD44 polypeptide lacking 31 amino acid residues in the extracellular domain (Fig. 3). This part of the protein is the least conserved between human and rodent CD44 proteins (32, 40).

We conclude that the different-size *CD44* transcripts are derived through the alternative use of three polyadenylation signals in the 3′ end of *CD44* gene. Additional diversification of *CD44* transcripts and their protein products is achieved through alternative splicing of 93 nucleotides coding for 31 amino acid residues in the extracellular domain.

Structural analysis of the upstream regulatory sequences of *CD44*. The availability of the full-length cDNA clones for *CD44* made it possible for us to clone the genomic sequences located upstream of the RNA-coding region of *CD44*. We found through genomic Southern blot analysis that the first exon of *CD44* resides within a 5-kbp genomic *Eco*RI restriction fragment (data not shown). This fragment was cloned from normal human DNA into the λgt10 vector. The sequence of the first exon of *CD44* and about 1 kbp of DNA upstream of it is shown in Fig. 4. No TATA or CCAAT boxes were found in the *CD44* upstream sequences. The sequences directly upstream of the *Bam*HI site in the 5′ end of *CD44* cDNA are very GC rich; the hexanucleotide GGGCGG, which is the binding site for the transcription factor Sp1, occurs three times in the *CD44* upstream region (Fig. 4). The very 5′ end of the sequenced genomic fragment contains a degenerated *Fok* repeat of about 250 nucleotides with a core repeat unit GATG (nucleotides 1 to 330 in Fig. 4).

Comparison of the genomic and the cDNA sequences showed that the sequences of the 5′ ends of the cDNA clones P5, HT1, and HT6 (Fig. 2) are identical to the corresponding genomic sequences (Fig. 4). The 5′ ends of these cDNA clones as well as those of the previously published *CD44* cDNA clones (32) are within several nucleotides of one another (60 to 70 nucleotides 5′ to the *Bam*HI site in Fig. 4). Thus, these cDNA clones may contain the authentic 5′ end of *CD44* mRNA. However, a single placental cDNA clone, P11, was found to extend much farther in the 5′ direction. It had an additional 280 bp found also in the genomic sequence (Fig. 4).

To determine unequivocally the initiation site of *CD44* mRNAs, we performed primer extension and S1 nuclease protection experiments with *CD44*-specific probes (Fig. 5). For primer extension experiments, we chose an oligonucleotide primer that corresponded to the *CD44* cDNA sequence at positions from 42 to 72 nucleotides downstream of *Bam*HI site in Fig. 4. The extended products observed with RNA from cell lines A204 and HT144 were ca. 163 nucleotides long (Fig. 5A), which places the RNA initiation sites slightly upstream of the 5′ ends of *CD44* cDNA P5 (Fig. 4). We have not observed longer primer extension products with RNA isolated from several human cell lines (data not shown).

The S1 nuclease protection experiments were performed with the *Eco*RV-*Bam*HI single-stranded DNA fragment labelled at the *Bam*HI site (Fig. 4). The protected products ranged in size from 85 to 93 nucleotides (Fig. 5B), in good accord with the results of primer extension experiments.

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-1141 TGGTTTGTGGTTTTTATGAAGAGATGTGAAAAAGGAAGTGTGGAATGATGGGATGAGAAGTTGTATGGGGAAGATGAATAGAAGAATTAGGTGGTTGAAT

-1041 AAAATTAAAAGGTGTGTGGTTGGATGAATGAATGAGTGGGATGATAGATGGACCTAAGTGGTTAGTGGATGGACAGGAGGATGGATGGATGTGAGAGCCC

-941  CAGAAGGACATAAGGAAAGATGGGTGGATAGATGGATGGGCGGATGGAAGGATATTTAGGAGGATGAATGAGCATGTGTGTGGAGAGAGGTGCCCATTCA

-841  CACTGGCTTGAACACATGGGTTAGCTGAGCCAAATGCCAGCCCTATGACAGGCCATCAGTAGCTTTCCCTGAGCTGTTCTGCCAAGAAGCTAAAATTCAT
                                                  SacI
-741  TCAAGCCATGTGGACTTGTTATTGAGGGGAAAAAGAATGAGCTCTCCCTCTTTCCACTTGGAAGATTCACCAACTCCCCACCCCTCACTCCCCACTGTGG
                                                                                   EcoRV
-641  GCACGGAGGCACTGCGCCACCCAGGGCAAGACCTCGCCCTCTCTCCAGCTCCTCTCCCAGGATATCCAACATCCCTGTGAAACCAGAGATCTTGCTCCAG

-541  CCGGATTCAGAGAAATTTAGCGGGAAAGGAGAGGCCAAAGGCTGAACCCAATGGTGCAAGGTTTTACGGTTCGGTCATCCTCTGTCCTGACGCCGCGGGG
                                                         ┌──P11
-441  CCAGCGGGAGAAGAAAGCCAGTGCGTCTCTGGGCGCAGGGGCCAGTGGGGCTCGGAGGCACAGGCACCCCGCGACACTCCAGGTTCCCCGACCCACGTCC
                                                                                  SmaI
-341  CTGGCAGCCCCGATTATTTACAGCCTCAGCAGAGCACGGGGCGGGGCAGAGGGGCCCGCCCGGGAGGGCTGCTACTTCTTAAAACCTCTGCGGGCTGCT
                                                                                                PstI
-241  TAGTCACAGCCCCCCTTGCTTGGGTGTGTCCTTCGCTCGCTCCCTCCCTCCGTCTTAGGTCACTGTTTTCAACCTCGAATAAAAACTGCAGCCAACTTCC
           *       *            ┌──P5                                                                    BamHI
-141  GAGGCAGCCTCATTGCCCAGCGGACCCCAGCCTCTGCCAGGTTCGGTCCGCCATCCTCGTCCCGTCCTCCGCCGGCCCCTGCCCCGCGCCCAGGGATCCT
                                            +1
-41   CCAGCTCCTTTCGCCCGCGCCCTCCGTTCGCTCCGGACACCATGGACAAGTTTTGGTGGCACGCAGCCTGGGGACTCTGCCTCGTGCCGCTGAGCCTGGC
      exonI───┬───intron
60    GCAGATCGGTGAGTGCCCGCCGCAGGCTGGGCAGCAAGATGGGTGCGGGGTGCTCAGCGCGGAC
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FIG. 4. Nucleotide sequence of the *CD44* upstream regulatory region. The first nucleotide of the ATG codon serving as the translation initiation codon of *CD44* is marked as +1. The sequence corresponding to the oligonucleotide used in the primer extension experiments is underlined. The two nucleotides at positions −136 and −128 serving as major RNA initiation sites are marked by asterisks. Positions of the 5′ ends of cDNA clones P11 and P5 and the 3′ boundary of the first *CD44* exon are shown.

Again, we did not observe longer protected species with RNA from several different human cell lines.

Thus, we were unable to detect initiation sites for *CD44* RNA at a more upstream position than 93 bp 5′ to the *Bam*HI site in the human cell lines that we have examined. Northern blot analysis of RNA from human cell lines of diverse origins failed to detect any RNA hybridizing to the

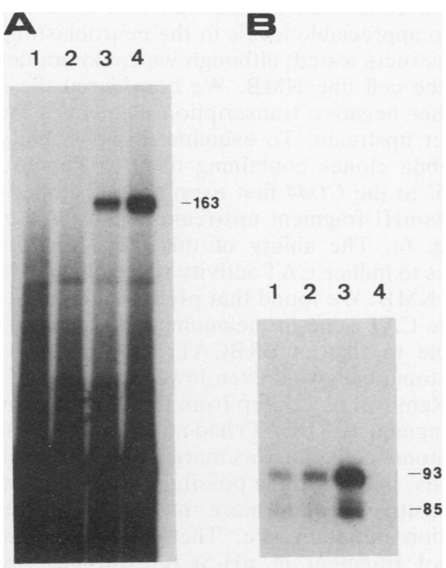

FIG. 5. Mapping of the transcription initiation sites of *CD44* RNA. (A) Primer extension analysis of 10 μg of polyadenylated RNAs from neuroblastoma NMB (lane 2), rhabdomyosarcoma A204 (lane 3), and melanoma HT144 (lane 4). Lane 1 contained 10 μg of yeast tRNA. See text for details. (B) S1 nuclease protection analysis of 20 μg of total RNA from colon carcinoma SW480 (lane 1), promyelocytic leukemia HL-60 (lane 2), melanoma HT144 (lane 3), and neuroblastoma NMB (lane 4). The genomic probe used was a 535-bp-long *Eco*RV-*Bam*HI fragment labelled at the 5′ end of the *Bam*HI site (Fig. 4). Sizes on the right are indicated in nucleotides.

sequences at the extreme 5′ end of *CD44* cDNA clone p11 (data not shown).

We conclude that the major *CD44* RNA initiation sites are located 85 to 93 nucleotides upstream of the *Bam*HI site, i.e., 128 to 136 nucleotides from the translation initiation codon. The existence of a longer cDNA clone (P11) indicates that there might be a more upstream initiation site that we were unable to detect in the human cell lines analyzed. The possibility exists that this more distant start site is utilized in an as yet undefined tissue-specific manner.

Functional analysis of *CD44* downregulation in neuroblastoma cells. To confirm that we had cloned the authentic promoter region of *CD44* and to identify *cis*-acting elements in the regulation of *CD44* expression, we analyzed the effect of the *CD44* upstream region on the transcription of a heterologous gene. We constructed a series of 5′ and internal deletion variants of the *CD44* upstream region and subcloned them upstream of the bacterial CAT gene in plasmid pCAT-basic. These plasmids were cotransfected into melanoma and neuroblastoma cell lines along with a β-galactosidase-expressing plasmid, used to monitor the transfection efficiency. Figure 6 shows the constructs used in the transfection experiments and the relative levels of CAT activity generated in the transfected cells. For each cell line, the levels of CAT activity expressed from plasmid pRVBCAT were arbitrarily chosen as 100%, although the absolute CAT activity expressed from this plasmid in melanoma cells was about 10-fold higher than the activity in neuroblastoma cells (data not shown).

The starting pCAT plasmid was pRBCAT containing 1.9 kbp of genomic *CD44* sequences extending upstream from the *Bam*HI site in the first exon (Fig. 4). This genomic fragment induced substantial CAT expression in melanoma cell line C32r but had significantly lower activity in neuroblastoma cell line NMB. Progressively longer 5′ end deletions were made in pRBCAT and tested for induction of CAT activity in both cell lines. The deletions did not substantially influence CAT expression in C32r, the most significant change being an approximately 30% increase in

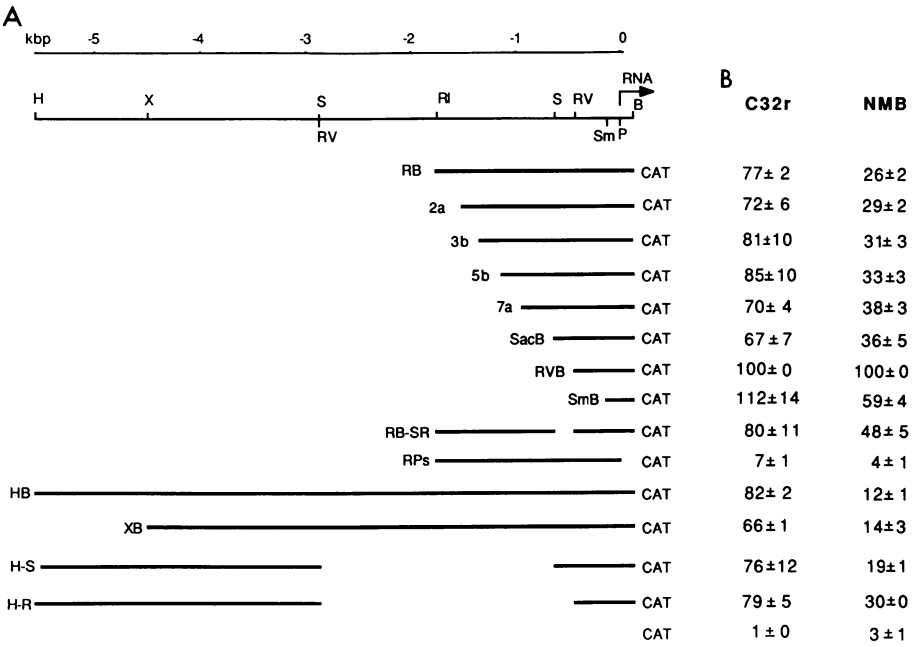

FIG. 6. Maps of CD44-CAT plasmid clones and transcriptional activities in CAT assays. (A) Constructs: Letters on the map of the *CD44* upstream region represent sites for restriction *Bam*HI (B), *Eco*RI (RI), *Eco*RV (RV), *Hin*dIII (H), *Pst*I (P), *Sac*I (S), *Sma*I (S), and *Xba*I (X). (B) Relative CAT activities and standard deviations for transfected melanoma cells (C32r) and neuroblastoma cells (NMB). Each plasmid was cotransfected with a β-galactosidase control plasmid in at least three independent experiments. The cell extracts were assayed for CAT and galactosidase activities as described elsewhere (25). CAT levels for all extracts were normalized to the galactosidase levels and are shown as percentages of pRVBCAT activity, which was arbitrarily chosen as 100%.

CAT activity observed with plasmid pRVBCAT compared with pSacBCAT (Fig. 6). Analysis of the same deletion variants for their effects on CAT expression in the neuroblastoma line NMB revealed that removal of sequences 5′ to the *Eco*RV site had a strong upregulating effect on CAT transcription: we consistently observed a 60 to 70% difference in the CAT activity induced by plasmids pSacBCAT and pRVBCAT (Fig. 6). Thus, the 120-bp *Sac*I-*Eco*RV fragment has a negative effect on transcription which is pronounced in neuroblastoma but not in melanoma. To confirm the existence of a negative neuroblastoma-specific element within this 120-bp DNA fragment, we removed it from plasmid pRBCAT. We found that the resulting plasmid, pRB-SRCAT, consistently produced higher CAT activity than did pRBCAT in neuroblastoma but not in melanoma cells (Fig. 6). Nevertheless, deletion of the *Sac*I-*Eco*RV fragment from pRBCAT did not restore transcriptional activity to the level of the pRVBCAT activity, a result we attribute to the presence of the 1.3 kbp of the DNA 5′ to the deleted fragment. Thus, we conclude that the negative regulator contained within the 120-bp *Sac*I-*Eco*RV fragment must be only one of several *cis*-active elements that govern expression of *CD44* from upstream of the gene.

Plasmid pRPsCAT contains 1.8 kbp of the CD44 upstream region but lacks the sequences downstream of the *Pst*I site (Fig. 4), i.e., the RNA initiation site and the 5′ end of the *CD44* first exon, both of which are present in all of the other CAT constructs. This plasmid induces very low levels of CAT activity in both melanoma and neuroblastoma cell lines, comparable to the background levels observed with the pCAT-basic promoterless plasmid. This finding confirms the results of our previous identification of the *CD44* transcription initiation site: the presence of this region is essential for *CD44* transcription and cannot be replaced functionally by the more upstream sequences.

We were concerned by the fact that CAT activity was induced to appreciable levels in the neuroblastoma cell line by all constructs tested, although we could not detect *CD44* RNA in the cell line NMB. We considered the possibility that another negative transcription element is located at a site farther upstream. To examine this possibility, we isolated lambda clones containing over 15 kbp of DNA sequences 5′ to the *CD44* first exon and subcloned a 5.7-kbp *Hin*dIII-*Bam*HI fragment upstream of the CAT gene (pHB CAT; Fig. 6). The ability of this clone and its deletion derivatives to induce CAT activity was evaluated in cell lines C32r and NMB. We found that pHBCAT induces transcription of the CAT gene in melanoma cells with an efficiency comparable to that of pRBCAT, whereas its activity in neuroblastoma cells was even lower than that of pRBCAT (Fig. 6). Removal of 1.5 kbp from the 5′ end of the *Hin*dIII-*Bam*HI fragment (pXBCAT) had no effect on transcription in neuroblastoma cells but was marginally detrimental in melanoma cells, indicating the possibility of a weak melanoma-specific positive element more than 4 kbp upstream of the transcription initiation site. The internal deletion of the 3-kbp *Sac*I fragment in pH-SCAT did not result in an appreciable change of activity compared with that of the longest pHBCAT construct. The deletion from pHBCAT of the internal *Eco*RV fragment of 3 kbp (pH-RCAT), which includes the neuroblastoma-specific negative element identified above, had no effect on transcription in melanoma cells but enhanced transcription in neuroblastoma cells. We conclude that an additional neuroblastoma-specific negative element is localized between 1.8 and 4.2 kbp upstream of the *CD44* transcription initiation site.

DISCUSSION

Repression of *CD44* in human neuroblastoma cells. We have reported here that expression of the gene encoding the cell surface protein CD44 is repressed in neuroblastoma cell lines. CD44 is the principal receptor for hyaluronate (2, 9, 23) and has been implicated in diverse processes involving specific cell-cell and cell-extracellular matrix interactions and cell migration (3, 7, 33, 34). Among these are homing of lymphocytes to high endothelial venules (20), T-cell activation and signal transduction (27), and CD2-dependent adhesion (14). Recently a novel splice variant of CD44 was identified on a rat carcinoma cell line and was shown to confer to it a metastatic potential (13). It is conceivable that different regions of the CD44 protein are responsible for the variety of biological activities ascribed to this molecule.

The CD44 protein is expressed on all nonneuronal derivatives of the neural crest examined to date (24), as illustrated in this study by expression of CD44 in pheochromocytoma and melanoma cells. The significance of the downregulation of *CD44* in neuroblastoma cells is not apparent at this time. It might play some role in the high metastatic potential of the neuroblastoma tumors. In this respect, it will be of interest to determine whether CD44 is expressed in primary neuroblastomas of a lower malignancy which are not metastatic.

Our findings with CD44 are reminiscent of a previous report that expression of the adhesion protein NCAM is downregulated in rat neuroblastoma cells, apparently through the action of the proto-oncogene N-MYC (1). Expression of NCAM in cells of the embryonic neural crest is specifically repressed during the period when the cells are migratory, suggesting that NCAM may contribute to the suppression of migration (35). These findings in turn implicate the downregulation of NCAM in the migratory properties of neuroblastoma cells. Thus, analysis of the developing neural crest may also help to illuminate the role of CD44 in the migration of normal and neoplastic cells.

Recent studies have uncovered the critical role played by CD44 in hemopoiesis within the bone marrow. High levels of CD44 were found on marrow progenitor cells (21). Antibodies to CD44 were found to block completely the production of lymphoid and myeloid cells in long-term bone marrow cultures (22). This effect was achieved most likely through specific inhibition of binding of hemopoietic precursor cells to stromal cells (22). These studies underscore the role of CD44-mediated cell recognition in cellular differentiation. We hypothesize that CD44 might play a similar role in the differentiation of neural crest progenitor cells into chromaffin cells.

Structure of *CD44* mRNAs. During the characterization of *CD44* cDNA clones, we found a previously undescribed splice variant expressed in a melanoma cell line. The alternative splicing removes a 31-amino-acid region from the extracellular domain of the CD44 protein at a position 47 amino acids N-terminal to the transmembrane domain. The same position also serves as a site for alternative splicing in *CD44* RNAs in epithelial cells (33). Three amino acids (221 to 223) are deleted in the epithelial form of CD44 and are substituted by an additional exon coding for 135 amino acids. The rat CD44 variant mentioned above has an insertion of 162 amino acids at the same site (13). This CD44 variant has been linked to metastatic properties of a rat carcinoma cell line (13).

We characterized the structure of the several *CD44* mRNA species and found that the variation in size is due largely to the existence of several polyadenylation signals in the last exon of *CD44*. In addition, we have identified a *CD44* cDNA molecule from human placenta that has a 5′ noncoding sequence significantly longer than those other *CD44* cDNAs characterized previously (11–13, 19, 32, 33) and in this study. We have failed to detect expression of the corresponding *CD44* RNA species in several human cell lines of diverse origins.

Control of *CD44* expression. Structural analysis of the *CD44* upstream region revealed the absence of TATA and CCAAT consensus sequences. Three copies of the hexanucleotide sequence GGGCGG were found upstream of the transcription initiation site. These sequences, referred to as GC boxes, have been found within promoters of many viral and cellular genes and typically bind the transcription factor Sp1 (for a review, see reference 26). Transfection of plasmids containing the *CD44* upstream region linked to the CAT gene into melanoma and neuroblastoma cell lines showed functional promoter activity. These plasmids were able to induce much higher CAT activity in melanoma than in neuroblastoma cells. Examination of a series of site-directed deletions showed that a plasmid containing only 150 bp of sequences upstream of the transcription initiation site induced substantial transcription. Removal of the sequence containing the RNA initiation site while leaving intact the more upstream region resulted in an almost total loss of activity (Fig. 6). It is interesting to note that the RNA initiation site defined here shares a sequence of seven nucleotides with the previously identified RNA initiator site (29).

Plasmids containing different amounts of *CD44* upstream sequences (from 0.12 to 5.7 kbp) induced similar CAT activities in melanoma cells. The most significant rise in activity was observed after the removal of sequences upstream of the *Eco*RV site 450 bp from the RNA initiation site. The difference in activity between constructs pSacB CAT and pRVBCAT in melanoma cells amounted to about 30%. The same two constructs exhibited a threefold difference in activity in neuroblastoma cells, indicating the presence of a cell-specific negative element within the 120-bp *Sac*RI-*Eco*RV fragment. The presence of sequences between 1.9 and 4.2 kbp upstream of the initiation site also had a negative effect on transcription in neuroblastoma cells. Further dissection of this distal region will be required to define the responsible negative regulator(s). We also suggest that other *CD44* sequence regions or possibly other mechanisms are involved in the downregulation of *CD44* RNA in neuroblastoma cells, since all of the CAT constructs that we examined possessed a higher than background activity in the neuroblastoma cell line NMB, even though this line lacks detectable levels of *CD44* RNA. One possibility is that an additional negative element is located farther upstream of the first exon or, alternatively, within an intron of *CD44*. The first intron of *CD44* is longer than 15 kbp (27a) and could conceivably be involved in transcriptional attenuation of *CD44* transcription.

In conclusion, we have identified *CD44* as one of the genes downregulated in neuroblastoma in comparison with other tissues of neural crest origin. We have shown that the repression of *CD44* in neuroblastoma cells can be attributed, at least in part, to a combination of *cis*-active elements that lie upstream of the gene. But there may be additional controls within or downstream of the gene as well. Further description of these controls may uncover a common mech-

anism for the repression of diverse genes during the genesis of neuroblastoma.

ACKNOWLEDGMENTS

We are grateful to M. Israel for the gift of pheochromocytoma tissue and to D. Cox, C. Jones, and V. van Heyningen for the DNA and cells of somatic cell hybrids. We thank D. Haas-Kogan for help in analysis of CD44 cDNA.

This work was supported by the National Institutes of Health (grant CA44338) and by funds from the G. W. Hooper Research Foundation.

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