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Tissue-specific Regulation of the Ecto-5′-nucleotidase Promoter

ROLE OF THE cAMP RESPONSE ELEMENT SITE IN MEDIATING REPRESSION BY THE UPSTREAM REGULATORY REGION*

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We have isolated the 5′ region of the ecto-5′-nucleotidase (low K_m 5′-NT) gene and established that a 969-base pair (bp) fragment confers cell-specific expression of a CAT reporter gene that correlates with the expression of endogenous ecto-5′-NT mRNA and enzymatic activity. A 768-bp upstream negative regulatory region has been identified that conferred lymphocyte-specific negative regulation in a heterologous system with a 244-bp deoxycytidine kinase core promoter. DNase I footprinting identified several protected areas including Sp1, Sp1/AP-2, and cAMP response element (CRE) binding sites within the 201-bp core promoter region and Sp1, NRE-2a, TCF-1/LEF-1, and Sp1/NF-AT binding sites in the upstream regulatory region. Whereas the CRE site was essential in mediating the negative activity of the upstream regulatory region in Jurkat but not in HeLa cells, mutation of the Sp1/AP-2 site decreased promoter activity in both cell lines. Electrophoretic mobility shift assay analysis of proteins binding to the CRE site identified both ATF-1 and ATF-2 in Jurkat cells. Finally, phorbol 12-myristate 13-acetate increased the activity of both the core and the 969-bp promoter fragments, and this increase was abrogated by mutations at the CRE site. In summary, we have identified a tissue-specific regulatory region 5′ of the ecto-5′-NT core promoter that requires the presence of a functional CRE site within the basal promoter for its suppressive activity.

Ecto-5′-nucleotidase (low K_m 5′-NT,[1] ecto-5′-NT, EC 3.1.3.5) is an extracellular enzyme that is anchored to the cell membrane through a glycosyl phosphatidylinositol linkage. The enzyme dephosphorylates purine and pyrimidine nucleoside monophosphates to the corresponding nucleosides and generates adenosine from extracellular AMP. Adenosine may then interact with a family of cognate receptors to produce a wide range of physiological effects that include the regulation of cardiovascular and cerebral blood flow (1), cytoprotection in myocardial and cerebrovascular ischemia (2), and inhibition of many aspects of immune function (3–6). Adenosine, which may accumulate to high concentrations in solid tumors, has also been postulated to be an important factor in stimulating angiogenesis (7), cancer growth through the increased expression of A_1 adenosine receptors (8), and in inhibiting the immune response toward the malignant tissue (9). Therefore, investigating the regulation of adenosine-metabolizing enzymes may have important impact on the understanding of tumor biology.

The activity of ecto-5′-nucleotidase, although variable, is frequently decreased as a result of neoplastic transformation of cells of hematopoietic origin (10). In contrast, elevated activity of ecto-5′-NT has been found in breast carcinoma (11), gastric cancer (12), pancreatic cancer (13), chronic myelogenous leukemia (10, 14), cutaneous T-cell lymphoma (15), and Walker 256 carcinoma (16). Human renal carcinoma cell lines induced to differentiate by butyric acid have demonstrated both an increase (Cur cell line) and a decrease (Caki cell line) in ecto-5′-NT activity (17), demonstrating a complex, and perhaps context-dependent, pattern of ecto-5′-NT expression in differentiation and malignant transformation.

The expression of ecto-5′-NT in thymocytes, lymphocytes, granulocytes, and undifferentiated myeloid cells is very low. However, differentiation of myeloid precursors along the monocytic lineage into macrophages results in a sharp increase in activity (18–20). The enzyme activity is lower than normal or undetectable in lymphocytes from patients with chronic lymphocytic leukemia and infectious mononucleosis (21) and in $CD8^+$ lymphocytes from AIDS patients (22). Given the strong immunosuppressive activity of adenosine, it seems likely that the variability in expression of this enzymatic activity among lymphoid cell populations has functional significance. In order to characterize the regulatory elements that contribute to the transcriptional regulation and tissue-specific expression of ecto-5′-NT, we have characterized the core promoter and upstream regulatory regions of the human gene.

EXPERIMENTAL PROCEDURES

Materials—Fetal calf serum was obtained from HiClone and Sigma. TRI Reagent, a high efficiency hybridization system, and Northern/Southern transfer solution were obtained from Molecular Research Center Inc. (Cincinnati, OH). Restriction enzymes, Klenow fragment of DNA polymerase, *Taq* polymerase, and polynucleotide kinase were from Promega (Madison, WI). Reverse transcriptase was from Life Technologies, Inc. D-threo[dichloroacetyl-1-^{14}C]chloramphenicol (56 mCi/mmol) was from Amersham Pharmacia Biotech.

Cells—The T-lymphoblast cell line K-T1 (23) was obtained from Dr. D. Sorscher (University of North Carolina). The K562 erythroleukemia cell line (ATCC CCL-243), Jurkat T-cell line (T-ALL origin), U937 cell line, PC-12 pheochromocytoma, HeLa S3 cervical carcinoma, and WI-38 fibroblasts were obtained from the ATCC (Manassas, VA). Jurkat, K-T1, K562, and U937 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. WI-38, PC-12, and HeLa S3 cell lines were maintained in Eagle's minimal essential medium sup-

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[1] The abbreviations used are: NT, nucleotidase; MOPS, 4-morpholinepropanesulfonic acid; bp, base pair(s); CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; CRE, cAMP response element; CREB, CRE-binding protein; CREM, CRE modulator; DTT, dithiothreitol; dCK, deoxycytidine kinase; EMSA, electrophoretic mobility shift assay.

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plemented with 10% fetal calf serum, nonessential amino acids, sodium pyruvate, penicillin, and streptomycin.

Northern Blot Analysis—Total cellular RNA (20 μg) purified by the acid guanidinium thiocyanate/phenol/chloroform method (24) was electrophoresed in 1% agarose with 50 mM MOPS, pH 7.0, and 1 M formaldehyde buffer. The RNA was transferred to Nytran nylon membranes overnight, fixed in a vacuum oven at 80 °C for 30 min, and probed with a randomly primed 1660-bp *Ava*I/*Hin*dIII cDNA fragment extending from bp 8 to 1668 of the ecto-5′-NT coding region (25). Hybridization was performed in high efficiency hybridization solution with 50% formamide, with a nylon membrane sandwiched between two Whatman 3MM papers, in a rotating incubator at 42 °C for 24 h. Blots were washed twice in 1× SSC, 0.2% SDS at room temperature and once at 60 °C and exposed overnight at −80 °C.

Plasmid/Promoter Constructs—The chloramphenicol acetyltransferase (CAT) vector pCAT-Basic (Promega, Madison, WI) was used in reporter gene assays to examine ecto-5′-NT promoter activity. The corresponding promoter fragments were generated by restriction digests or by PCR as described previously (26). PCR was performed using *Pfu* polymerase (Stratagene, La Jolla, CA) followed by five cycles with *Taq* polymerase (Roche Molecular Biochemicals). The PCR products were cloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced. The heterologous ecto-5′-NT/deoxycytidine kinase (dCK) promoter construct was produced by replacing a *Pst*I/*Nde*I fragment encompassing the 5′-NT proximal promoter fragment and part of the CAT gene from pCAT-Basic with the corresponding dCK-244 *Pst*I/*Nde*I fragment containing the 244-bp dCK promoter (27). Plasmid DNA used for transfections was purified using Quiagen Maxi columns, and isopropyl alcohol-precipitated plasmid DNA preparations were further deproteinated by the sequential treatment with equal volumes of phenol/chloroform/isoamyl alcohol (50:49:1) and twice with chloroform. Final plasmid DNA preparations were precipitated in the presence of 0.1 M sodium acetate with 70% ethanol, washed with 75% ethanol, and suspended in H_2O at 2 mg/ml. The quality of DNA was tested on agarose gel electrophoresis with and without treatment by *Hin*dIII and *Xba*I.

Cell Transfections—Cells were transfected by electroporation using a Bio-Rad Gene-Pulser. Approximately 6.0×10^6 Jurkat, K-T1, K562, or U937 cells; 3.0×10^6 HeLa cells; and 1.0×10^6 fibroblasts were transfected at room temperature in RPMI 1640 medium with equimolar amounts of the promoter constructs corresponding to 20 μg of control pCAT-Basic plasmid. Herring sperm DNA was added to a total quantity of 50 μg of DNA/electroporation. Electroporation efficiency was determined by transfecting separate aliquots of cells with 20 μg of β-galactosidase plasmids driven by β-actin promoter and 30 μg of herring sperm DNA. When the effect of PMA was tested on ecto-5′-NT promoter activity, co-transfection of β-galactosidase and CAT constructs was performed to account for variable cell growth rates in the presence of PMA. Electroporations were performed at 300 V and 960 microfarads (330 V and 960 microfarads for HeLa cells), and cells were subsequently suspended in 12 ml of RPMI 1640 medium containing 20% horse serum. After 44–48 h, cells were harvested, washed with cold PBS, and freeze-thawed three times with 80 μl of 0.25 M Tris, pH 8.0, containing 0.1 mM phenylmethylsulfonyl fluoride and 10 μg/ml pepstatin and leupeptin. CAT activity assays were performed on supernatants following treatment of extracts at 62 °C for 10 min.

CAT and β-Galactosidase Assays—Chloramphenicol acetyltransferase activity was measured at 37 °C in a reaction medium containing 1 mM dibutyryl CoA (25 μg/assay), 10 μM D-threo[dichloroacetyl-1-^{14}C]chloramphenicol (56 mCi/mmol, 0.25 μCi/assay) in 0.25 M Tris, pH 8.0, buffer. The incubation was initiated by the addition of 2–50 μl of cell extract in a total volume of 125 μl and terminated after 1–6 h with the addition of 300 μl of xylenes. Following three extractions, reaction products in the organic phase were counted in a scintillation counter. Reaction velocity was expressed as cpm converted/h/mg of protein. Reaction time and extract amount were adjusted so that no more than 30% of substrate was utilized. The final results are expressed as the -fold increase or decrease over values obtained with the pCAT Basic vector alone. To normalize for transfection efficiency, the CAT activity was divided by the β-galactosidase activity of simultaneous transfections. β-Galactosidase activity was assayed spectrophotometrically at 564 nm in 25 mM phosphate buffer, pH 7.5, 5 mM $MgCl_2$, and 3 mg/ml chlorophenol red-β-D-galactopyranoside at 37 °C. The reaction was initiated by the addition of 5–25 μl of cell extract and analyzed for 1 min. The linear portion of the curve was used to compute 0 order reaction rate and expressed as μmol of substrate used/min/mg of protein. The β-galactosidase activity varied by less than 20% in duplicate cell samples. Protein concentration was assayed by the Bradford method (28).

Preparation of Nuclear Extracts—Extracts were made from logarithmically growing Jurkat T cells according to the method of Dignam *et al.* (29) with modifications as described by Blake *et al.* (30). Cells were homogenized in buffer A (10 mM HEPES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 100 nM ocadaic acid), and the nuclei were recovered by centrifugation at 30,000 × *g* for 30 s. Nuclear factors were extracted in buffer C (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 20% glycerol, 0.15 mM spermine, 0.75 mM spermidine, 1 mM phenylmethylsulfonyl fluoride, 0.4 M NaCl, 100 nM ocadaic acid), followed by centrifugation at 30,000 × *g* for 45 min. The supernatant was dialyzed in buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 12.5 mM $MgCl_2$), aliquoted, and stored at −70 °C (31).

DNase I Footprinting—Two DNA probes encompassing the 378-bp fragment (from −48 to −426 from the ATG site, cluster I and II, Fig. 4) and 406-bp fragment (from −425 to −832, cluster III) were generated by PCR with *Pfu* DNA polymerase. Resulting fragments were incubated with the *Taq* polymerase, subcloned into the pCR 2.1 vector, and digested with the appropriate restriction enzymes to create a 5′-overhang on the noncoding strand at the 3′-end. The 3′- ends of the coding strands were filled in with radiolabeled dCTP using DNA polymerase large Klenow fragment. The resulting probes were digested at the opposite end to ensure one-strand labeling, gel-purified on a 6% nondenaturing polyacrylamide gel, and eluted by the "crush and soak" method (29). DNase I footprinting was performed according to Blake *et al.*(30). Ten ng of ^{32}P-labeled DNA were incubated with 120 and 240 μg of Jurkat and HeLa cell nuclear extracts in the presence of 15 μg of poly(dI-dC), 6.1% glycerol, 0.07 mM EDTA, 0.07 mM EGTA, 7.2 mM HEPES, pH 7.9, 39 mM KCl, 7.5 mM $MgCl_2$, and 0.7 mM DTT. The binding reactions were performed at room temperature for 30 min, after which $CaCl_2$ (2 mM final concentration) was added, and the probe was digested with DNase I (Worthington) at room temperature for 3 min. Digestions were terminated by the addition of 2 volumes of 100 mM Tris, pH 8.0, 20 mM EDTA, 0.1% SDS, 100 μg/ml proteinase K, and 100 μg/ml glycogen. After an incubation at 37 °C for 20 min, the samples were extracted with phenol/chloroform, precipitated with ethanol, resuspended in formamide loading dye, and analyzed on an 8 M urea, 6% polyacrylamide sequencing gel.

Electrophoretic Mobility Shift Assay (EMSA)—Wild-type and mutant double-stranded oligonucleotides were generated by annealing complementary single-stranded oligonucleotides yielding 5′-overhangs; 100 ng was labeled by fill-in using Klenow fragment of DNA polymerase. Labeled probes were purified on 1 ml of Sephadex-G50 (Sigma) columns. The flow-through was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol, washed with 75% ethanol, and resuspended at 20,000 cpm/μl. For competition experiments, unlabeled wild-type and mutant oligonucleotides were prepared by using unlabeled deoxynucleotides for fill-in and precipitated as described above. One μl of probe (approximately 0.1 ng of DNA) was incubated with 8 μg of nuclear extract in the presence of 20 mM HEPES, pH 7.5, 0.5 mM DTT, 1 mM EDTA, 2 mM $MgCl_2$, 50 mM KCl, 12% glycerol, and 2 μg of poly(dI-dC) (Sigma). Reactions were preincubated on ice for 10 min in the presence of competitor or for 30–60 min in the presence of antibodies. Probe was added to the mixture, and the reaction was incubated at room temperature for 20 min. The protein-DNA complexes were re-

TABLE I
Sequences of mutated CRE, Sp1, and Sp1/AP-2 sites and of oligonucleotides used in EMSA

Site	Wild type		Mutated[a]	For EMSA (wild type)
CRE	TGACGTCG	TGAACTCG	__	GGTCGGATCGGGTGACGTCGCGAACTTGCGCCTGG
Sp1/AP-2	CCCGCCCCGGCCCCAACCTTGC		__ __	
Sp1	CCCGCCCC	CAACCAAC	__ __	
TCF/LEF	TCTTTGAA	TATTCCAA	_ __	

[a] Mutated nucleotides are underlined.

solved on native 4% polyacrylamide (30:1 acrylamide/bisacrylamide), 0.5× Tris-borate-EDTA minigels at 100 V, dried, and autoradiographed. EMSA supershift experiments were performed with 1 μg of each of the following antibodies: ATF-1 antibody (sc-241X), ATF-2 (sc-242X), CREB-1 (sc-186X), CREB-2 (sc-200), CREM-1 (sc-440X), c-Jun (sc-1694X), and c-Fos (sc-413) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

5′-Nucleotidase Assay—Ecto-5′-NT activity in cell extracts was measured in 50 mM Tris, pH 7.5, buffer containing 20 mM $MgCl_2$, 5 mM β-glycerophosphate, 0.1 mM erythro-9(2-hydroxy-3-nonyl)adenine (EHNA; inhibitor of adenosine deaminase), and 20 μM [8-^{14}C]AMP. Reaction products were separated on plastic-supported cellulose (Kodachrome; Eastman Kodak Co.) in ammonia/butanol/methanol/H_20 (1:20:20:60), and corresponding spots were visualized under UV light, excised, and counted in a scintillation counter.

RESULTS

Cell-specific Expression of Ecto-5′-nucleotidase mRNA—To determine the level of ecto-5′-NT mRNA in human cell lines, Northern blot analysis was performed (Fig. 1). A high level of expression of a single 4.1-kilobase mRNA was observed in cultured human fibroblasts (WI38), whereas HeLa cells expressed an intermediate level, and PC-12, U937, Jurkat, K562, and K-T1 cells had low or undetectable levels. The corresponding ecto-5′-NT enzyme activities in cell homogenates were 319.3 ± 20.6, 29.9 ± 6.1, 6.3 ± 2.4, 2.40 ± 0.62, 0.37 ± 0.17, 0.12 ± 0.14, and 0.40 ± 0.22 nmol/min/mg of protein, respectively (±S.D., 3–5 independent measurements). There was a 863-fold difference in 5′-nucleotidase activity between fibroblasts and the Jurkat T-cell line, and relative enzyme activities in different cell lines corresponded with differences in the levels of mRNA expression.

Cell-specific Activity of the Ecto-5′-nucleotidase Promoter—In order to determine the relative levels of ecto-5′-NT promoter activity, we transiently transfected several cell lines with the pCAT-Basic reporter construct containing 969, 371, or 201 bp of the promoter. As shown in Fig. 2, the full-length 969-bp promoter fragment yielded CAT activity that was consistently lower than the marginal activity of the pCAT-Basic vector in Jurkat, K-T1, and K562 cell lines, whereas relative CAT activity in U937, HeLa, and WI-38 cells was increased by 10–25-fold. Two further deletions of 5′-sequences, producing 371- and 201-bp promoter fragments, caused moderate increases in promoter activity in Jurkat T-cells and K562 cells, no significant changes in U937 and HeLa cells, and a significant

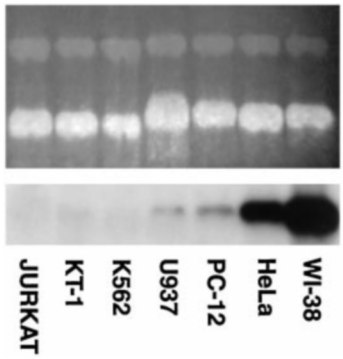

FIG. 1. **Northern blot analysis of ecto-5′-nucleotidase mRNA levels in human cell lines.** Twenty μg of total RNA were loaded in each lane. *Upper panel*, 4.1-kb ecto-5′-NT mRNA; *lower panel*, 18 and 28 S rRNA stained with ethidium bromide.

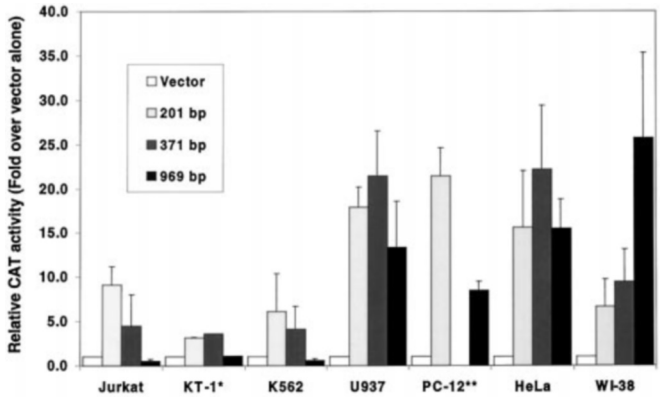

FIG. 2. **Cell specificity of ecto-5′-nucleotidase promoter fragment activity in different human cell lines.** Promoter constructs were generated as described under "Experimental Procedures." Values represent the mean ± S.D. of 3–5 independent determinations done in duplicate. *, mean values from two independent experiments. **, in PC-12 cells, only the 201- and 969-bp constructs were tested.

Cluster IV

```
          GATA                    CAAT box
-817   TCCTGATAGC TGCCGGATTT TACCCCAATC GTGTTTCTGC TTCCAGGAGC TGTCCGCGCG
                                                AP-1       PU.1
-757   TCGCCTCTGT TTTATCACTA AGGTTTTAAT TGTGAATAAA CGAGGAACTG GATTCGAATG
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Cluster III

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          Ets-1                                                unknown
-639   CCAACAGGAA GCGTCACATC TCAGTGAGCT TCGGCTCTCA CAGCCCGAAA CTGCAAGGAG
                   ⇓⇓                 NRE-2a                          ⇓⇓
-579   CTCCTCTGCC CGGCCTCTCT TTACTCCTCC TCTCTGCCCC TCAGCTCGCT CATCTTTCTT
          Sp1/NF-AT          ⇓       LEF-1/TCF-1
-519   CCCGCCCCTC TCTTTTCCTT CTTTGGTTCT TTGAAGTGAT GAGCTAGCGC
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Cluster II

```
          Sp-1          CAT Box  PU.1
-397   GCGCGGGGCG GACCCCTCCA ATTCCTTCCT CGCGCCCCCG AAAGAGCGGC -348
```

Cluster I

```
                    ⇓    ⇓  ATF-1/CREB                ⇓    AP-2
-207   GGGTACCAGG GTCGGATCGG GTGACGTCGC GAACTTGCGC CTGGCCGCCA AGCCGGCCTC
                ⇓  ⇓⇓   Sp1 /AP-2                ⇓    Sp1
-147   CAGGCTGAAG AAGGACCCGC CCCGGCCTTG ACCCGGGCCC CGCCCCTCCA GCCGGGGCAC
```

FIG. 3. **Sequence of the ecto-5′-nucleotidase promoter with putative transcription factor binding sites.** Four clusters of binding sites were identified within the proximal 969-bp fragment. Bases are numbered in relation to the ATG translation start site in the first exon. Consensus transcription factor binding sites are in *boldface type* and *underlined*. Clusters I/II and III were analyzed with a DNase I protection assay, and protected areas are *double underlined*. Hypersensitive sites are indicated with *arrows*.

decrease in promoter activity in WI38 fibroblasts (Fig. 2). These data suggest that the distal 768-bp fragment contains tissue-specific regulatory elements that function to repress the core promoter activity in lymphoid (Jurkat and K-T1) and erythroid (K562) cell lines while enhancing the activity in WI-38 fibroblast. In general, the promoter activity in transient transfection assays correlated well with the levels of mRNA and ecto-5′-NT enzymatic activity in a majority of cell lines.

In Vitro DNase I Footprinting—To identify regulatory elements that interact with cognate transcription factors, we performed *in vitro* DNase I footprinting analysis using Jurkat T-cell and HeLa nuclear extracts. Four clusters of putative transcription factor binding sites were identified within the proximal 770 bp of the ecto-5′-NT promoter (Fig. 3). We have generated two DNA probes that encompass cluster I/II and cluster III and used them in DNase I footprinting analysis with Jurkat T-cell and HeLa nuclear extracts. Results presented in Fig. 4 demonstrate that within the core promoter region (cluster I/II) both Jurkat (*A*) and HeLa (*B*) nuclear extracts produce similar protected areas that coincide with Sp1, Sp1/AP-2, and CRE transcription factor binding sites. However, within cluster III, a strong protected area coincident with a TCF-1/LEF-1 consensus binding site (32–34) is present with extract from Jurkat cells but is absent with HeLa nuclear extracts. Further upstream, an extensive 22-bp protected area that coincides with adjacent NF-AT and Sp1 binding sites in Jurkat corresponds to a more restricted 16-bp protected area representing a single Sp1 site in HeLa extracts. An additional protected area further upstream coincides with a NRE-2a/2b transcription factor binding site (35) and is also more pronounced with the Jurkat T-cell nuclear extract. A protected area that does not correspond to a known consensus transcription factor binding site is present at bp −579 to −599 only with Jurkat T-cell nuclear extract.

Cell-specific Activity of the Upstream Regulatory Region in a Heterologous System—To determine whether the silencing/enhancing activity of the upstream regulatory region is specific for the ecto-5′-NT promoter or could be reproduced in a heterologous system, we generated a chimeric promoter construct containing the distal 768 bp of the regulatory region ligated to the 244-bp core promoter of the human dCK gene (27) (Fig. 5*A*). As shown in Fig. 5*B*, the 5′-NT upstream promoter fragment decreased CAT expression mediated by the dCK promoter alone in Jurkat and K-T1 cells by 78%, whereas it had a lesser suppressive effect on dCK promoter-mediated CAT expression in HeLa cells (decrease by 40%) and no effect in WI38 fibroblasts. The lack of enhancer activity in fibroblasts, as seen with the native ecto-5′-NT core promoter (Fig. 2), suggests that either the dCK core promoter activity was already maximal or that the function of the enhancer is promoter-specific.

Mutational Analysis of the Ecto-5′-NT Core Promoter—To further investigate the importance of the Sp1, AP-2, and CRE transcription factor binding sites within the core promoter region, we mutated these sites as illustrated in Table I and tested the respective mutants individually in transient transfection assays. Data presented in Fig. 6 show that none of the mutations in the context of the 201-bp core promoter region had any significant effect in either Jurkat or HeLa cells. However, within the context of the 969-bp promoter fragment, mutation of the CRE site at bp 184–185 produced a dramatic increase of the promoter activity to the level found with the wild-type 201-bp core promoter region alone in Jurkat cells, while resulting in a 50% decrease in HeLa cells (Fig. 6). Mutations at the Sp1 and especially at the Sp1/AP-2 site brought about significant decreases in promoter activity in both Jurkat and HeLa cells. In Jurkat T-cells, the wild-type 969-bp promoter activity was strongly suppressed to a level of 40 ± 10% of pCAT-Basic vector alone (Fig. 2) and decreased further to 15 ± 10% (± S.D. from four experiments) of pCAT-Basic with mutation at the Sp1/AP-2 site. These data suggest that tran-

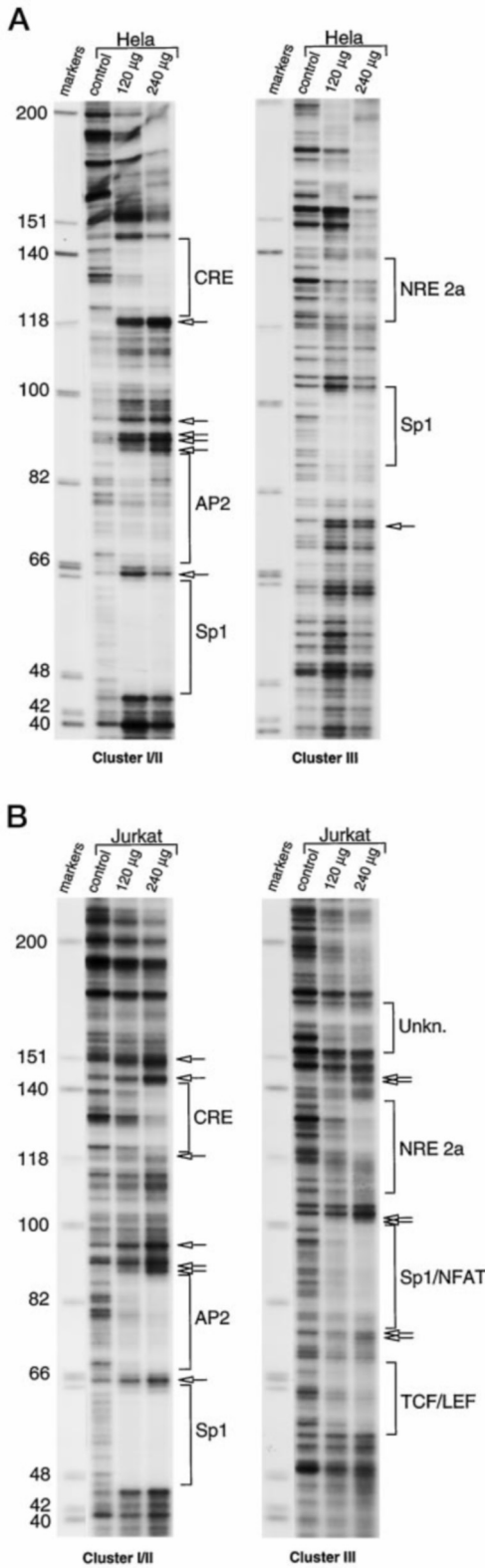

FIG. 4. **DNase I protection assay of the ecto-5′-nucleotidase promoter fragments corresponding to clusters I/II and III.** Nuclear extracts from Jurkat T-cells (*A*) and from HeLa cells (*B*) were prepared and processed as described under "Experimental Procedures." DNA markers are shown on the *right*. AP-2 footprint represents a combined Sp1/AP-2 transcription factor binding site.

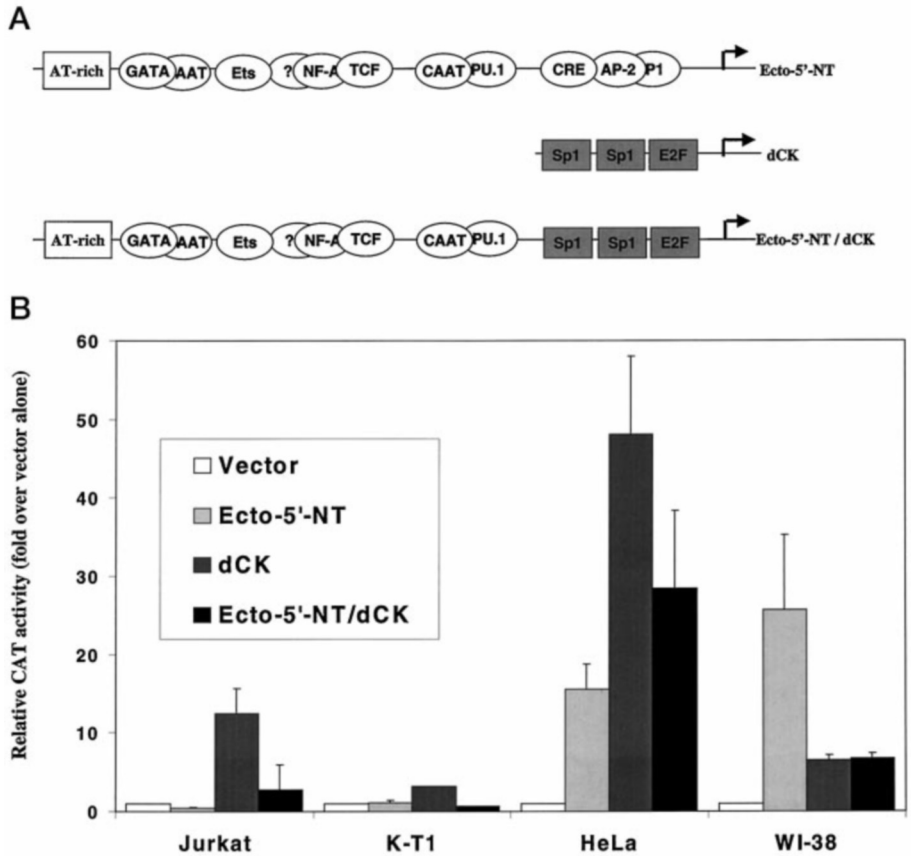

FIG. 5. *A*, scheme illustrating the chimeric dCK/ecto-5′-nucleotidase promoter. *B*, activity of the ecto-5′-nucleotidase upstream regulatory region in the context of dCK core promoter in several human cell lines. Values represent the mean ± S.D. of four independent determinations done in duplicate.

scription factors binding to the CRE and Sp1/AP-2 sites may influence the activity of the upstream regulatory region.

Identification of Transcription Factors Binding to the CRE Site—We next employed EMSAs with a probe encompassing the CRE site to identify cognate transcription factors (Table I). We used a panel of antibodies recognizing members of the CREB and ATF family of proteins and c-Fos and c-Jun transcription factors known to dimerize with members of the CREB family. Results shown in Fig. 7 demonstrate that antibodies recognizing CREB-1, CREB-2, CREM-1, c-Fos, and c-Jun were not able to supershift the DNA-protein complexes, whereas antibodies against ATF-1 and ATF-2 caused distinctly decreased intensities of the corresponding lower band coincident with the appearance of a more pronounced (ATF-1) or a new (ATF-2) upper band (Fig. 7, *A* and *D*). The intensity of the supershifted band with ATF-2-specific antibody was similar with nuclear extracts prepared from control and PMA-treated cells; however, there was a somewhat less pronounced decrease in ATF-2-specific lower complex intensity with PMA-treated nuclear extracts (Fig. 7*D*). Similar results were obtained with polyclonal antibody against ATF-2 (data not shown). Mutated CRE probe did not produce specific band shifts (Fig. 7*C*). These results suggest that ATF-1 and ATF-2 or closely related proteins specifically interact with the CRE site in the ecto-5′-NT core promoter in Jurkat T-cells. In contrast, in HeLa nuclear extract, despite the presence of strong gel shifts, none of the specific antibodies described above resulted in a supershifted complexes (data not shown).

Effect of PMA on Ecto-5′-NT Promoter Activity in Jurkat T-cells—We have previously shown that induction of promyelocytic HL60 cells to differentiate with PMA coincided with a dramatic increase in ecto-5′-NT mRNA level (20). Since the CRE site has been identified as a PMA-responsive element through the modulation of the ATF-2 transcription factor (36, 37), we tested the possibility that in Jurkat T-cells PMA might increase ecto-5′-NT promoter activity through activation of a transcription factor binding to this site. Results presented in Fig. 8 show that activity of the 201-bp promoter fragment was induced up to 3-fold by a 20-h treatment with 20 nM PMA, whereas the same promoter fragment with a mutated CRE site was not affected. Furthermore, the 969-bp promoter fragment was activated approximately 17-fold by PMA. Mutation of the CRE site in the 969-bp fragment increased the promoter activity as described previously in Fig. 6, and PMA did not further increase this activity.

Mutational Analysis of the TCF-1/LEF-1 Transcription Factor Binding Site—A limited number of promoters or enhancers have been shown to contain consensus sites for the developmentally important transcription factors TCF-1 and LEF-1. Their intrinsic ability to bend DNA has implicated these high mobility group (HMG) proteins in an architectural role of juxtaposing distantly located transcription factors. Recent data suggest that TCF-1 may act as a transcriptional repressor when associated with Groucho (Grg) proteins, and we hypothesized that this particular function could explain the strong negative and lymphocyte-specific activity of the upstream regulatory region within the ecto-5′-NT promoter (38). We have tested the activities of the wild type and TCF-1/LEF-1-mutated 969-bp ecto-5′-NT promoter in Jurkat T-cells that endogenously express both TCF-1 and LEF-1 proteins and in HeLa cells that lack both transcription factors. The results in Fig. 9 demonstrate that the strong negative activity of the upstream regulatory region in Jurkat T-cells is not significantly affected by TCF/LEF site mutagenesis. The same mutations, however, completely eliminated gel shifts in an EMSA (data not shown), suggesting either that the TCF-1/LEF-1 transcription factors do not contribute or that elimination of this site alone is not

sufficient to affect the negative activity of the upstream regulatory region.

DISCUSSION

The highly variable level of expression of ecto-5′-NT in human and animal tissues and cells suggests tissue-specific mechanisms controlling the expression of this enzyme. These mechanisms are unknown; therefore, an analysis of the transcriptional regulation of this gene was undertaken, and in this study we have analyzed the tissue-specific regulatory elements within the ecto-5′-NT promoter. Sequence analysis of the 5′-flanking region of the ecto-5′-NT gene reveals a number of potential transcription factor binding sites within the 820 bp upstream of the translation start site within the first exon (26, 39). As illustrated in Fig. 4, these sites were concentrated in four clusters that contain a number of lymphocyte- and macrophage-specific, developmentally important, as well as general transcription factor binding elements. The occupancy of several of these sites was confirmed by *in vitro* DNase I footprinting analysis. The number of potential regulatory elements within the ecto-5′-NT promoter suggests that there might be a high level of complexity in the interactions between binding factors, especially between those localized immediately 5′ to the transcription start site and those further upstream. Deletional analysis of the 5′-upstream fragment of the ecto-5′-NT gene confirmed that the 969-bp promoter fragment mediates cell-specific regulation of expression. Deletion of 768 bp at the 5′-end of this fragment eliminated the cell-specific expression of the reporter gene and suggests that this 768-bp fragment contains regulatory elements responsible for both the decreased promoter activity in Jurkat cells as well as the up-regulation in fibroblasts. Further deletions within the 201-bp fragment decreased the promoter activity in Jurkat T-cells (data not shown); therefore, we designated this 201-bp region as the core promoter. The dual and cell-specific function of the 768-bp upstream regulatory fragment raises important questions with regard to whether transcription factors binding to this region confer this specificity as well as how the core promoter activity relates to the upstream regulatory region.

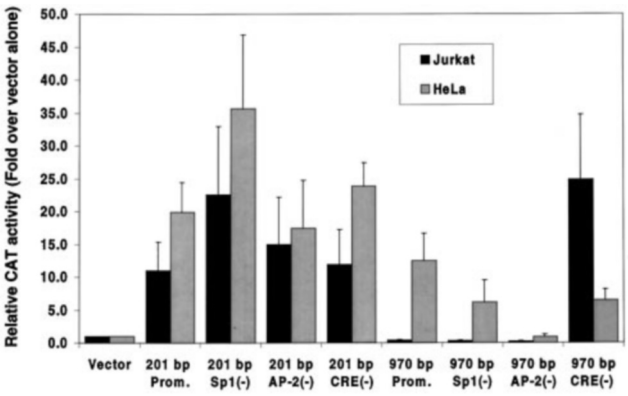

FIG. 6. **The effect of mutagenesis of transcription factor binding sites within the 201- and 969-bp promoters on ecto-5′-NT promoter activity.** Transcription factor binding sites were subjected to site-directed mutagenesis using the oligonucleotides outlined under "Experimental Procedures." Values represent the mean ± S.D. of three independent determinations done in duplicate.

The cell-specific regulation of the ecto-5′-NT promoter may be conferred by proteins binding to either the core promoter or the upstream regulatory region. Mutational analysis of the 201-bp core promoter region led to several important observations. First, while single mutations at the Sp1, Sp1/AP-2, and CRE binding sites did not alter the 201-bp core promoter activity, mutation at the CRE had a profound effect on the promoter activity when analyzed within the context of the 969-bp fragment. Elimination of the CRE site dramatically increased the promoter activity in Jurkat T-cells, suggesting a major role for factors binding at this site in mediating suppression of core promoter activity by the upstream regulatory region. In addition, mutation of the Sp1/AP-2 site caused a further decrease in the low basal activity of the 969-bp promoter fragment in Jurkat T-lymphocytes and a significant decrease of the promoter activity in HeLa cells. In combination, these observations suggest that the regulatory function of the upstream 768-bp fragment requires the presence of specific transcription factors within the core promoter region to either down- or up-regulate the rate of ecto-5′-NT transcription in a cell- or tissue-specific manner. The finding that the activity of the dCK core promoter, containing a Sp1 and E2F (half-site) transcription factor binding site, was also inhibited by the ecto-5′-NT upstream regulatory region, suggests that the CRE site, while essential for the negative regulation of the ecto-5′-NT promoter, may not be absolutely required for transcriptional repression mediated by this region. Nonetheless, the abrogation of PMA responsiveness by mutation of the CRE site strongly supports an important role for CRE-binding proteins in regulating ecto-5′-NT activity. The latter conclusion is consistent

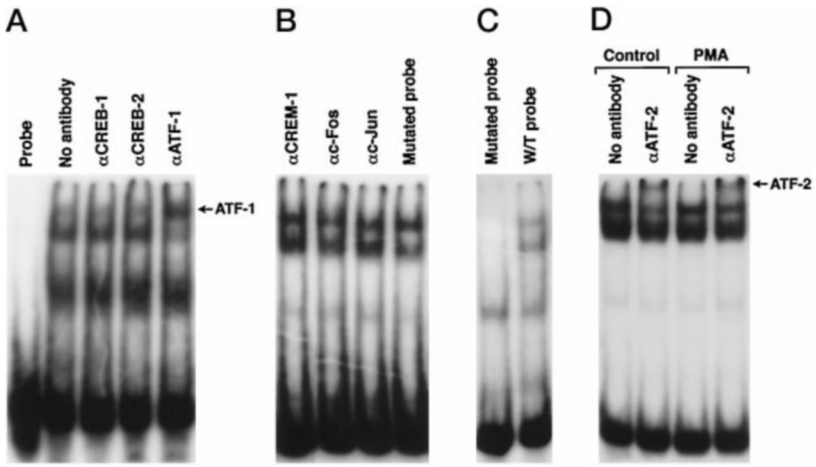

FIG. 7. **EMSA of the CRE binding site with Jurkat T-cell extract in the presence of antibodies against members of the CREB and ATF family of transcription factors.** *A*, incubations performed as described under "Experimental Procedures" (50 mM KCl). *B*, incubations performed in the presence of 70 mM KCl to reduce nonspecific interactions. Cold oligonucleotide containing the mutated CRE site was added as a competitor at a 1000-fold excess in the *last lane*. *C*, comparison of gel shift patterns of mutated (Table I) and wild-type CRE probes. *D*, the effect of ATF-2 antibody on the gel shift pattern in the absence and presence of PMA treatment (20 ng/ml for 24 h).

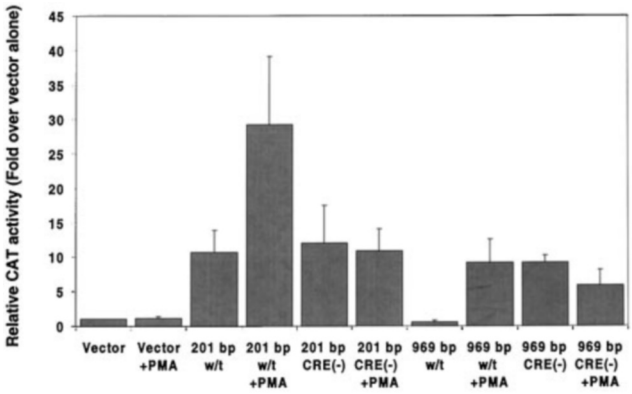

FIG. 8. **The effect of PMA on the native and CRE-mutated 201- and 969-bp promoter fragments in Jurkat T-cells.** Values represent the mean ± S.D. of three independent determinations done in duplicate.

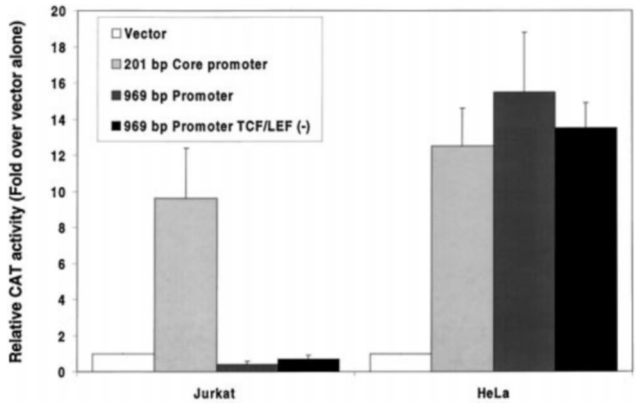

FIG. 9. **The effect of mutation of the TCF-1/LEF-1 transcription factor binding site on the activity of the upstream regulatory region.** Values represent the mean ± S.D. of three independent determinations done in duplicate.

with reports that describe the CRE site as a PMA-responsive element (TRE) (36, 37). The mechanism of PMA-induced ecto-5′-NT promoter activation is unknown. It is possible, however, that as reported previously (36), PMA may induce changes in the composition of protein complexes binding to the CRE site. Although we have not seen a significant change in the appearance of supershifts with ATF-2 antibodies in EMSA after pretreatment of cells with PMA (Fig. 7*D*), the intensity of the lower band did not decrease as strongly as in the control, suggesting the presence of an increased amount of other protein components in this complex. The identity of this component and its relevance to the increased promoter activity after treatment with PMA remain to be established.

The ecto-5′-NT CRE element (TGACGTCG) differs from the consensus CRE palindrome by one base (underlined). It has previously been shown that single base changes in this cis-acting DNA element may dramatically influence the binding properties and function of this site (36, 40). We have shown that, despite the presence of several members of the CREB/ATF family of transcription factors in both Jurkat and HeLa cell lines, only antibodies against ATF-1 and ATF-2 interacted with protein complexes in EMSAs using Jurkat nuclear extracts. As a result of these interactions, two changes were observed: (*a*) decreased intensities of the initial ATF-1 and ATF-2 (lower band) complexes and (*b*) the appearance of the corresponding supershifts. The existence of two distinct gel shifts, each independently interacting with ATF-1 and ATF-2 antibodies, suggests the presence of two different species of protein complexes at the CRE site. The absence, as evidenced by the lack of supershifted bands of these and several other known CREB/ATF family members as well as c-Jun and c-Fos proteins, suggests that a different protein(s) bind to this site in HeLa nuclear extract. These observations, in conjunction with the higher basal promoter activity and endogenous ecto-5′-NT mRNA expression in HeLa cells, would suggest that ATF-1 and ATF-2 transcription factors mediate low promoter activity in lymphocytes. Whether both have the same potency in suppressing the ecto-5′-NT promoter activity remains to be established.

Mutation of the Sp1/AP-2 site increased the suppressive activity of the 969-bp promoter fragment in Jurkat cells and, interestingly, also uncovered the potential for the suppression in HeLa cells. This finding suggests that the intrinsic ability of the upstream regulatory region to inhibit core promoter activity may represent a more general property of this fragment and that the tissue- and cell-specific up- and down-regulation of the promoter activity may be more specifically mediated by factors binding to CRE and Sp1/AP-2 sites. In that respect, and due perhaps to the presence of NFAT, TCF/LEF, Ets, and an unknown transcription factor in Jurkat T-cells, the overall suppressive activity of the upstream regulatory region is inherently more potent in Jurkat than in HeLa cells. According to this model, the up-regulation of promoter activity in tissues that express low to intermediate levels of ecto-5′-NT would be achieved primarily by derepression. Thus, increased ATF-1 and/or ATF-2 transcription factors binding to the CRE site would facilitate repression of the promoter activity, whereas increased binding to the Sp1/AP-2 site would facilitate derepression.

The nature and specific mechanism of the negative activity of the upstream regulatory region will be a subject for future study. This region contains several regulatory elements that may potentially contribute to the repressive effect. One possible candidate is transcription factor binding to the NRE-2a/2b negative regulatory element (35, 41); however, the identity of the specific protein(s) binding to this site has yet to be established. Other candidates, specific for lymphocytes and of potential importance during development, are the TCF-1 and LEF-1 transcription factors. These proteins have been shown to interact with the transcriptional repressor Groucho (38) and to have an important architectural function in the context of the TCRα enhancer (42, 43). However, since mutation of the binding site for TCF/LEF did not produce a noticeable change in the function of this 768-bp fragment (Fig. 8), it is possible that several interacting transcription factors might form, in a cooperative manner, a functional higher order nucleoprotein complex in lymphocytes. This possibility is further supported by our finding that the 768-bp fragment was able to function independently in the heterologous promoter system with the 244-bp dCK promoter. Specific cooperation among Ets, LEF-1, AML, and CRE transcription factors has already been shown to be important for the function of the TCRα enhancer (42–44). In this regard, we have noted that the spatial alignment of GATA, Ets, TCF-1/LEF-1, and CRE transcription factor binding sites in the TCRα enhancer closely resembles their location in the ecto-5′-NT promoter (45). A similar arrangement of these transcription factors was also found in TCRβ and TCRδ, CD8, and human immunodeficiency virus enhancers (46–48). Whether this structural alignment, with an invariably central position of the relatively rare TCF/LEF site, carries out a specific function common to these enhancers and the ecto-5′-NT promoter remains to be established. TCF/LEF transcription factors have been identified as nuclear targets for Wnt signaling (49–51), and our current efforts are aimed at establishing whether the ecto-5′-NT promoter is a target for this evolutionarily conserved and developmentally important signaling pathway. Since only a single gene target for Wnt signaling has been

identified in mammals to date (52), the ecto-5′-NT promoter would offer an excellent model to study the mechanisms of induction of gene expression by this pathway. Thus, further delineation of the specific interactions involving a higher order nucleoprotein complex of the ecto-5′-NT promoter may not only enhance our understanding of how these signaling pathways modulate the transcription of ecto-5′-NT gene but will also lead to a better understanding of its function during development and transformation.

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