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Retinoic Acid and cAMP Differentially Regulate Human Chromogranin A Promoter Activity During Differentiation of Neuroblastoma Cells

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We report the first evidence that differential transcriptional regulation of human chromogranin A (*CHGA*) gene expression occurs during *in vitro* treatment of tumorigenic neuroblastoma (NB) cells with retinoic acid (5 μM) and/or dibutyryl-cAMP (1 mM). The *CHGA* gene encodes a tissue specific protein restricted to cells of the diffuse neuroendocrine system, but also widely expressed among NB tumours. We previously reported that *CHGA* as well as other neuroendocrine markers are modulated during NB differentiation *in vitro*. To investigate, at the molecular level, the mechanisms leading to NB tumour cell differentiation during the treatment with biologically active compounds, we sequenced and functionally characterised 2169 bp of a genomic DNA clone encompassing the 5′ flanking region of the human *CHGA* gene. Computer-assisted analysis of the sequence revealed the presence of a cAMP responsive element at positions −56 to −49, and Sp1 binding sites at positions −181 to −176 and −216 to −210. Two novel 9 bp motifs, located at position −462 to −454 and −91 to −83 of the *CHGA* promoter were identified in the regulatory regions of two other neuroendocrine genes encoding for tyrosine hydroxylase and neuropeptide Y. In addition, in the first 1000 bp of the untranslated 5′ region, we found the presence of several putative DNA binding sites of bHLH molecules, a protein family regulating tissue specific differentiation. Transient transfection experiments of chloramphenicol acetyltransferase (CAT) deletion constructs, showed the presence of an active promoter within the first 455 bp upstream from the start site. This region conferred tissue specific expression to a *CAT* reporter gene. In addition, the transcriptional activity of this fragment was modulated during the induction of differentiation of NB cells treated by retinoic acid and/or dibutyryl-cAMP. These observations provide preliminary data regarding *CHGA* transcriptional regulation in NB cells, and indicate that retinoic acid and cAMP activate distinct, apparently competitive, transcriptional pathways during NB cell differentiation. The molecular characterisation of the mechanisms regulating *CHGA* expression in tumour and normal neuroendocrine tissue could lead to the identification of novel molecules potentially relevant for future gene therapy of NB tumours.

Key words: chromogranin, neuroblastoma, promoter, e-box, differentiation, neuroendocrine, chromaffin
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INTRODUCTION

CELLULAR differentiation depends on specific signals present in the extracellular environment that alter intracellular gene expression affecting cell metabolism and growth. Neuroblastomas (NB) offer a flexible system to investigate at cellular and molecular levels the effect of several biological response modifiers. NB presumably arises from neural crest precursors that are altered in their normal development. However, many NB cell lines retain some histopathological feature of their normal precursors and the expression of a panel of neuroendocrine markers that seem to resemble the normal adrenal medulla histogenesis [1]. A large body of literature describes the effects of compounds, such as retinoic acid (RA) or cAMP elevating agents, which are able to cause differentiation of NB cells *in vitro* and *in vivo* and reverse their tumorigenicity [2]. Accordingly, we reported the activation of distinct molecular and cellular phenotypes in NB cells after treatment with RA and/or dibutyryl-cAMP (db-cAMP) [3]. We found, in fact, that a panel of chromaffin related genes, chromogranin A (*CHGA*) [4], neuropeptide Y (*NPY*) [5] and *DLK/PG2* [6], were upregulated at mRNA level during the *in vitro* treatment of NB cells with db-cAMP, while exposure to RA resulted in the suppression of the chromaffin phenotype in favour of the expression of neuronal-like markers [3]. To investigate, at the molecular level, the mechanisms controlling these processes in NB cells, we

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attempted to characterise the regulation of the neuroendocrine specific marker *CHGA* at the level of transcription.

CHGA codes for a kDa 71 acidic protein that is a major constituent of neurosecretory granules [4, 7]. Expression of the gene encoding this protein appears to be restricted to cells of the diffuse neuroendocrine system (DNE), which is also known as the amine precursors uptake and decarboxylation (APUD) system [8]. Cells that comprise the DNE share features common to neurons and endocrine cells including the ability to produce and store biogenic amines. The DNE includes chromaffin cells of the adrenal medulla and smaller paraganglionic cells, parafollicular C cells of the thyroid gland, gut enterochromaffin cells, anterior pituitary cells, and islet cells of the pancreas. Although the precise function of CHGA is unknown, many lines of investigation suggest that this protein may play a role in the storage and processing of biogenic amines and peptide hormones in neurosecretory vesicles, which are a common structural feature of neuroendocrine-chromaffin cells [4, 7].

The adrenal medulla cell precursors do not express *CHGA* until after their arrival in the adrenal cortex. *CHGA* mRNA and protein can be detected in isolated cells of the adrenal gland at approximately 8 weeks of human development, long before the adrenal gland is morphologically recognisable [9]. These findings are in agreement with the hypothesis that local environmental factors contribute to the development of different cell types among migrating cells originating in the neural crest, although the precise identity of such factors is still unknown [10]. Since most of the NB cell lines tested were found to be *CHGA* positive [1], we reasoned that those events concurring in neuroblast transformation may have taken place after the early commitment to chromaffin differentiation of the neural crest precursors [11]. Therefore, an understanding of the molecular mechanisms by which the expression of genes, such as *CHGA*, is regulated may provide important insights into how the diversity of cells that comprise the DNE arise during development, and will contribute to elucidation of the molecular mechanism of those biological response modifiers active on NB cells.

MATERIALS AND METHODS

Cell culture and transfection

SK-N-AS [12], SK-N-BE [2, 13], COS-1 [14], MCF7 [15], RD [16] and NIH-3T3 (gift of Franco Tato', Department of Cell and Development Biology, University of Rome "La Sapienza")), C1300-N18 [17] cell lines were cultured in 60 mm plates (Becton Dickinson, San Jose, California, U.S.A.) in RPMI 1640 or Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Gibco-BRL, Paisley, U.K.) in a 7.5% CO_2 atmosphere.

DNA transfections were performed using the calcium phosphate precipitation technique [18]. Proliferating cells were transfected with an aliquot of a precipitate prepared in duplicate. For each 60 mm plate (7×10^4 cells), the precipitates contained: 10 μg pCHGA-CAT4, pCHGA-CAT5, pCHGA-CAT6, pRSVCAT, pSV40CAT or pA10CAT3M plasmid; and 0.2 μg of cytomegalovirus-β-galactosidase (CMVβ-gal) plasmid as an internal control for transfection efficiency. Stably transfected mouse neuroblastoma N18 cells [17] were generated by liposome-mediated delivery (DOTAP, Boeheringer Mannheim Gmbh, Manneheim, Germany) of pCHGA-CAT4 reporter plasmid and a vector containing the neomycin resistance gene under the control of retroviral long terminal repeats (LTRs) (pRSVneo). Transfection was performed according to the manufacturer's instructions. After 12 h, the culture medium was replaced and after an additional 48 h the cells were exposed to selection medium (DMEM containing 500 μg/ml) of the antibiotic G418 (Geneticin, Gibco-BRL). After 3 weeks in culture, mixed populations were assayed for endogenous chloramphenicol acetyltransferase (CAT) activity as previously described [19]. Differentiation treatments were performed exposing CAT positive N18 cells to 5 μM RA, 1 mM db-cAMP, or the two in combination, for at least 72 h.

DNA sequencing

The CHGA 5′ flanking region was sequenced on both strands by the standard dideoxy method [18], using the following internal synthetic primers:

(1) 5′-GGGGCCGGGCACCTAGGC-3′;
(2) 5′-CCCGCTTATATACCCCHGACCC-3′;
(3) 5′-AACCTCACCCCTCGCTACCTC-3′;
(4) 5′-CTGTGGCTGATCCTCCACCCC-3′;
(5) 5′-TCATGGCTCTCTCCAATATCT-3′;
(6) 5′-CAGGTAACTGATGAATGTATC-3′;
(7) 5′-AGCTTAAGCCACTGTTGGACA-3′;
(8) 5′-TTCTTGCCACTGGCAGCCCAT-3′;
(9) 5′-AGCTCTTTGGGCTCCAGCTCT-3′;
(10) 5′-AAATGAGAACCTGGTGGGAGC-3′;
(11) 5′-ACAGTTTGTCAGCACGGAAGG-3′;
(12) 5′-AATACAAAATTAGCTGGGCAT-3′;
(13) 5′-GTGTTTCATGGGTACAGAGTT-3′;
(14) 5′-AGCTTCTGTCCTCCAGGGAGG-3′;
(15) 5′-CTGGTTCTAGCCTGGAGGATA-3′;
(16) 5′-CAGCTCCTTCCGGTGGGCGCG-3.

Computer analysis

Nucleotide sequence comparisons and alignments of the human CHGA 5′ flanking region were obtained using the SignalScan and the CGC-Wisconsin software packages available through the Human Genome Mapping Project database (Cambridge, U.K.).

Transfection and CAT assays

CHGA-CAT fusion genes were made according to standard recombinant techniques [18]. The numbers refer to the regions of the *CHGA* gene relative to the mRNA transcription start point. −726 CHGACAT5 contains a *Hind*III–*Pvu*I *CHGA* genomic DNA fragment that encompasses nucleotides from position −726 to +181 cloned into polylinker of the pA10CAT3M vector. This vector, a conventional vector for testing putative promoter sequences, contains the *CAT* gene segment from pSV2CAT fused to a polylinker upstream from the *CAT* transcription initiation site. −455 CHGACAT4 contains an *Xba*I–*Pvu*II DNA fragment from position −455 to +181 cloned into the plasmid pA10CAT3M. The −2091 CHGACAT6, containing the entire 5′ flanking region, has been cloned in *BamH*I *Pvu*II into the same vector. Transient transfections were performed by the calcium phosphate method according to standard procedures. 8 h after transfection, cells were washed with phosphate-buffered saline (PBS) and the medium was replaced. 36 h later cells were harvested. CAT activity was assayed in whole cell extracts by liquid scintillation counting [19] and standardised to β-galactosidase activity present in the same extract. CAT activity of stably transfected CAT-positive N18 cells was assayed after 72 h of exposure to various differentiation treatments. Measurements of whole cell extracts were standardised by protein content.

RESULTS

Sequence of the human chromogranin A 5′ flanking region

A genomic DNA fragment of approximately 5 kb was isolated and subcloned in the pGEM3Z vector (clone p5′HCHGA, L.J. Helman, unpublished data). The insert contained more than 2 kb spanning the 5′ flanking region of the human *CHGA* gene. Sequence analysis was performed by the dideoxy-chain termination method on both strands (Figure 1a). The sequenced region comprised 1979 nucleotides 5′ to the transcription initiation site and 183 bp of the transcription unit. As it did not contain any valid open reading frame, control elements encountered in this region may be relevant to *CHGA* gene regulation. By primer extension, the codon initiating transcription was identified 205 bp upstream of ATG (L.J. Helman, personal data) indicating the presence of a relatively long 5′ flanking untranslated region.

Computer analysis

A canonical TATA box sequence, but no evident CAAT box, was found −33 to −27 bp from the start site. A cAMP responsive element (CRE) consensus sequence (TGACGTCA) was identified at positions −56 to −49. Two consensus sequences for the binding of the transcriptional factor Sp1 were found at positions −181 to −176 and −216 to −210. In the first 250 bp upstream from the codon initiating transcription, the overall content of guanidine and cytosine residues was more than 67% as typically occurs in 5′ flanking regions devoted to the transcriptional control of housekeeping genes' expression [20]. The comparison among the 5′ flanking sequences of *CHGA*, *NPY* [5] and tyrosine hydroxylases (*TH*) genes [21], highly expressed in adrenal chromaffin cells, revealed the presence of two conserved 9 bp sequences designated I and II (see Figure 1b). The DNA sequence (AGGCCCCTC) located at −462 to −454 was identical in all 9 bases to the 5′ upstream sequences in both the *NPY* and *TH* genes. In addition, the *CHGA* sequence from −91 to −83 (TGGGGAGTG), was identical to sequences in the 5′ regulatory region of *TH* and matched 8 of 9 bp in the 5′ flanking region of the *NPY* gene. Since all three of these genes are specifically expressed at high levels within the neural crest derived adrenal medulla, these regions may represent putative binding sites for regulators of lineage specific transcription. Finally, several e-box consensus (CANNTG) were also found in the first 1000 bp of the sequenced region. They are approximately equally spaced (average distance 240 bp) and represent the potential binding sites for molecules of the helix-loop-helix protein family [22] (Figure 1c).

Transient transfection studies

Attempting to identify sequences important for human *CHGA* expression, a series of 5′ deletion constructs driving *CAT* gene expression were produced (Figure 2a) according to standard recombinant techniques [18]. The numbers refer to the regions of the *CHGA* gene relative to the mRNA transcription start point. The three constructs contain the nucleotides from position −2091 (CHGA-CAT6), −726 (CHGA-CAT5), −455 (CHGA-CAT4) to position +183 cloned into the pSV2CAT derived pA10CAT3M vector (gift of L.J. Helman).

Transient transfection experiments were performed by a standard calcium phosphate precipitation technique [18], using 10 μg of plasmid DNA, and each transfection was repeated three times in duplicate. Cell lines from different tissues were tested to evaluate *in vitro* the presence of an active *CHGA* promoter and tissue specificity. The *CAT* reporter constructs CHGA-CAT4 and CHGA-CAT5 and to a lesser extent the CHGA-CAT6, were found to be active in the chromogranin-positive neuroblastoma cell lines SK-N-AS [12] and SK-N-BE [13] (Figure 2a) and in the rhabdomyosarcoma cell line RD [16], while they were found totally inactive in the non-chromaffin cell lines MCF-7 [15], COS-1 cells [14] and NIH-3T3 (Figure 2b). In our experiments, the CHGA-CAT4 construct functioned as the stronger promoter-driven CAT vector in the neuroblastoma cell lines SK-N-AS and SK-N-BE (5 and 30-fold activation, respectively). The decreased expression of the CAT gene in the CHGA-CAT5 and CHGA-CAT6 constructs suggests the presence of negative regulatory elements present in the 5′ sequence upstream from the −455/+183 promoter region.

Analysis of CHGA promoter activity during differentiation of N18 cells

Conversely, the presence of RA in the culture medium produced a reduction of approximately 70% in CAT activity in this experiment, although the average reduction from 3 independent experiments was 60%. The presence of both compounds (RA+db-cAMP) only slightly affected CAT activity. Only a 15% inhibition of CAT activity was found in the experiment illustrated in Figure 3, while in the other two independent experiments (data not shown), CAT was inhibited by 5 and 12.5%. Treatment with RA reduced the number of substrate-adherent (S phenotype) cells (≤ 20%), while the number of cells with neuronal-like morphology (N phenotype) was enhanced (≥ 80%). However, during db-cAMP treatment alterations in the content of S type versus N type cells were not observed (data not shown).

DISCUSSION

We postulated that uncharacterised molecular factors could be involved in the development of the neuroendocrine system. The identification of these factors could be of interest in defining targets for future therapeutic strategies applicable to human NB tumours. In this sense, *CHGA* a gene expressed in neuroendocrine cells which contain neurosecretory granules, represents a unique system in which to analyse the molecules involved in the process of neoplastic and normal tissue differentiation. In order to investigate the mechanism which leads to the development of the chromaffin pathway, we structurally and functionally evaluated human *CHGA* transcriptional regulation at the promoter level in chromaffin and non-chromaffin cell lines. We tested different deletion constructs of the *CHGA* promoter, and found that the CHGACAT4 vector, encompassing 455 bp of the *CHGA* promoter region and 183 bp of the first exon, conferred relative tissue specific expression to the *CAT* reporter gene. This construct was active in the chromaffin cells and inactive in all the non-chromaffin cells tested, with the exception of the rhabdomyosarcoma cell line, RD. RD has been reported to express other genes normally present in non-muscle cells such *MYCN* or *PAX* [23, 24]. Thus, RD cells seem to retain a certain degree of lineage promiscuity which could account for the expression of neuroectodermal genes. Alternatively, it is possible that the transformed RD cell line could have lost some of the mechanisms determining tissue specificity. Searching for DNA motifs common to the 5′ flanking region of other human neuroendocrine genes, we found two 9 bp sequences in the upstream regulatory region of the human *CHGA* gene that are also present within the 5′ regulatory regions of both the human *TH* and *NPY* promoters. Since these genes are specifically expressed at high levels within neural crest-derived adrenal cells,

(a)

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                    10        20        30        40
-2091 TTTTTATTGTGATAAAATATCCCTAACATAACATTTACCATCTTAACCAT
-2041 TTTTAGGGGCATAATTCAGTGGCATTAAGTCCAATTATGTTGCTGTGCAG
-1991 CCATCATCACTATCCATTTCCAGAACTTTTTCATCTGCCCAAACTCTGTA
-1941 CCCATGAAACACTAACTGTTTCCTCCTCCCCTCAGCCCCTGAAAGGAGGT
-1891 CAATCCTATTTCCCACTTCTGTTGTTGTTGTTGTTTGTTTGTTTGTTT
-1841 TGAGACAGAGTTTCACTCTTGTTGCCCAGGCTGGAGTGCAGTGGCATGAT
-1791 CTTGGCTCACACTGTACTCTGTCCTCCACCTCTTGGGTTCAAGCGATTCC
-1641 CCTGCCTCAGCCTTCCAAGTAGCTGGGATTACAGATGCGCCACCATGCCC
-1591 AGCTAATTTTTGTATTTTTAGTAGAGATGGGATTTCACCATGTTGGTCAGG
-1541 CTGGTCTCGAACTCCTGACCTCAGGTGATCTGCCCGCCTCAGCCTCCCAA
-1491 ACTGCTGGGATTACAGGTGTAAACCACTGCGCCCAGCTGACCCACCCTAT
-1441 TTAAGTATAACCCCACCCTGTCTCAGCACAGCCGTCCCCTTCCGTGCTGA
-1391 CAAACTGTGGGTGTTACTCTTATTCATTGTCTGTCTTCACCATCTAGAAT
-1341 GTAGGGCTTTCTGTCTGTTCTGTTTAATGCTGTACCCTCAACTGTGTACT
-1291 GTTCTAGGCTGTTCTAAGCACATAGTAGGTGCTTAGCAAAGGTCAAATAT
-1241 GTAAATTCATGAATGTGGCCATTTCAGTCTCCTTTGAGCTCCCACCAGGT
-1191 TCTCATTTAGGGACAGGCGTGAGCACAGGTGTGGGAAAGGAAGTATTACT
-1141 AGAAAACAGCAGCCGTCAGATGGAGAGGGGTGGGCAGGAAGGAGGGGCTC
-1091 TGGACGATGCCAGCCAATGACAACCGTCACCCAGATCCCAGTTCCCTGTG
                E-BOX
-1041 ACAGTGGGTCAGGTGGCAAAGAGCTTCCAACACGGCCCCCAGAGCTGGGA

-0991 GCCCAAAGAGCTGGAGCATCAGGGGCCCCAGAAATAAGGGAAGCCAACCA

-0941 GGAAACTGACCTTTTACACAAAATATCCAGGTTTTGAGAATGCTCAGGAA
                          E-BOX
-0891 GAGGTGAGAGCTGTTCTCAAATTCAGATGGGCTGCCAGTGGCAAGAAGGG

-0841 TGATGCTAAGGTGCACTAGGCTATGGGTAGGTGTTGCAAGGACTCCTATT

-0791 TTGGCGGATGTAATAAGAACTGGCCCCACTCCTCCCTCTACCTAACAAGA

-0741 GGTGTCCAACAGTGGCTTAAGCTTCCTGGTGAAAGTGAGCCCCTGGTTCC

-0691 CGTGTGTATGCAGAGCCCAGGATTCACTCACCCATGGCTTGAACCTTTAA
                          E-BOX
-0641 TGGATACATTCATCAGTTACCTGTCAAGTGCGTTTCCTCTGTGCCAGATT

-0591 TGCGTGGCGCTCAGCACTGTGAAAGTAATGATGACTAAACATTATTCAAA

-0541 TAAATACACATTCAACAACAAAGTGTGAAGGCACAAAACTAGCTTGCCAG
                                    R1
-0491 AGAGAGAGAAGCCTCACTCAGACAGGGGATCAAAGGCCCCTCTAGAGCCT

-0441 CCTAGATATTGGAGAGAGCCATGAGTGATGGGCATGCCTCCAGCCTGTGA
          AP2
-0391 GACCGGGGGAGCATACCCCAGGCAGAGGGCACTTAGGAAAAATAGCACGG
                          E-BOX
-0341 GGTCGTAGCGCTGCCAGGGACCTCAGTACACATGGAAGAAGGCAAGGGTC

-0291 CAGAGATCCGAGTGACTTGCCCAGGGACACAAGGCAAATCGGTGGAATCG
                          SP1
-0241 TCGAGTGGTGGAGGATCAGCCACAGCCCCCCGCCTCCCACGCCACCTCTT
          SP1
-0191 GGAAACCAGATACCCGCCTCGGCCAAGACCCACCAGCTCCAAGCGGCGGA

-0141 GGCCAGAGGTAGCGAGGGGTGAGGTTAGAGGTGGGGGCGAGCGGGGACTG
  E-BOX  R2                                   CRE
-0091 GACACCTGGGGAGTGGGGAAAGGGGAAGGGGGCACACCGCTGACGTCATT
                                              +1
-0041 TTCCGGGGTCGGGGTATATAAGCGGCGCGAGGGCGTGCTGCTGCCACCGC

+0011 TCCTGCCACTGCAGTGCTCGAGCCCCGTGCAGGGGAGCTTGCGGGAGGAT

+0061 CGACCCGCCGAGGCACTGCGCCCCCAGCCCCGCGCCGGTGCCACCGCAGC
                          SP1
+0111 CCGACCCGGCCGCCAGTCCAGCCGCCCCTCGCCCGGTGCCTAGGTGCCCG
          E-BOX
+0161 GCCCCACACCGCCAGCTGCTCGG +0183
```

(b)

R1

CHGA	5'-465	**CAAAGGCCCCTCTAG**	-451	3'
TH	5'-317	**GAAAGGCCCCTCCCT**	-303	3'
NPY	5'-63	**CCGAGGCCCCTCCTG**	-49	3'

R2

CHGA	5'-94	**ACCTGGGGAGTGGGG**	-80	3'
TH	5'-191	**GGCTGGGGAGTGAAG**	-177	3'
NPY	5'-179	**GGTTGGGGTGTGGGT**	-165	3'

(c)

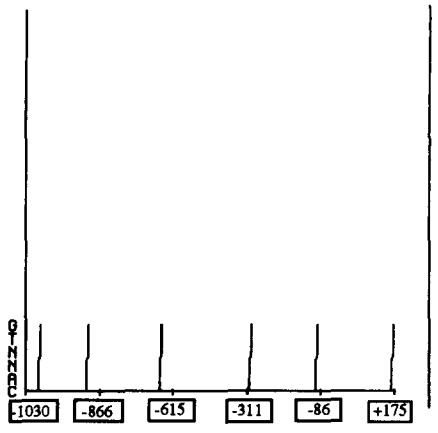

Figure 1. Sequence and computer analysis of human *CHGA* promoter. (a) Nucleotide sequence of 2169 bp 5′ flanking region of the human *CHGA* gene. Relevant consensus sequences are underlined and the relative *trans*-acting factor's name is overwritten in bold. Boxes outline those sequences also found present in the *TH* and *NPY* promoters. (b) Comparison of two 9 bp motifs present in the upstream regulatory regions of *CHGA*, *TH* and *NPY*. Nucleotide sequence is listed in the 5′ to 3′ direction and numbered as in (a) with negative numbers representing nucleotides 5′ to the transcription initiation site. (c) Graphic plot of the e-box consensus (*y*-axis) frequency in the *CHGA* promoter region (*x*-axis). The average number of nucleotides between each site is 240 bp.

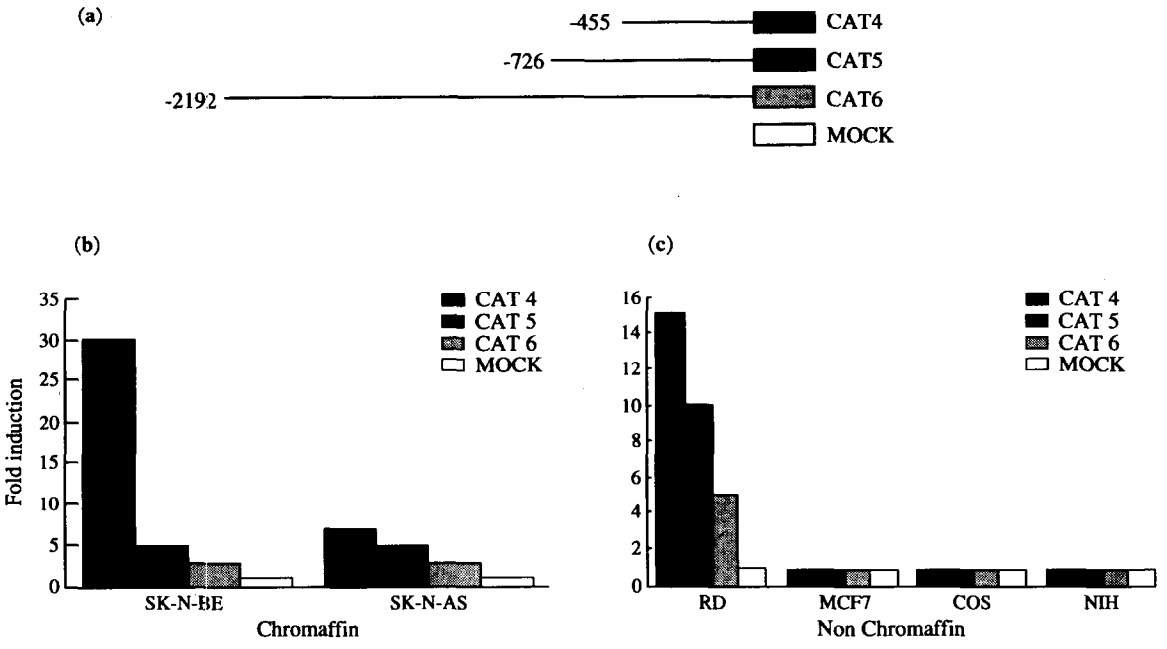

Figure 2. Schematic represention of *CHGA–CAT* constructs used for the transient transfection analysis of the human *CHGA* promoter region. (a) Schematic representation of *CHGA* promoter fragments linked to the *CAT* reporter gene present in pCAT3M vector. Transient transfection analysis of the *CHGA* promoter-dependent CAT activity in (b) chromaffin and (c) non-chromaffin cell lines. The constructs used are presented in a grey colour scale corresponding to that shown in (a). The graph represents a representative experiment performed in duplicate.

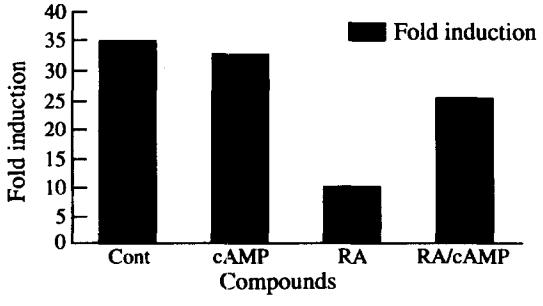

Figure 3. Analysis of CAT activity in stably transfected CHGA-N18 cells treated with differentiation agents. Cont, control cells; cAMP, dibutyryl-cyclic AMP. The graph represents one representative experiment performed in duplicate.

these regions may represent putative binding sites of regulators of chromaffin specific gene transcription. In fact, a growing body of literature (for review see ref. [25]) suggests that specific classes of molecules are involved in cell differentiation along distinct pathways. In this sense, the basic helix-loop-helix (bHLH) protein family seems to represent one of the most important group of regulators of tissue specific differentiation (for review see ref. [25]). They recognise a motif, CANNTG, named e-box, that represents the site of action of *trans*-acting complexes involved in the regulation of cell growth and gene expression [22]. In the first 1000 bp of the human chromogranin A promoter, we found at least 6 equally spaced e-box consensus sequences. These structural data could provide the basis for the identification of new molecules active during neuroblastoma cell differentiation. Further experiments are now in progress to address this.

Recently, Mouland and associates described the organisation of the complete genomic human *CHGA* and functionally characterised its promoter region [26]. They found that a 87 bp fragment of 5′ flanking DNA region is sufficient to confer tissue specificity to the *CHGA* promoter. This portion of the *CHGA* promoter encompasses a cAMP responsive element and the TATA box and seems sufficient for the regulation of *CHGA* promoter activity in neuroendocrine cells [26]. Our sequence (GeneBank accession number X77734) is identical to that presented by those authors (GeneBank accession number U03742), with the exception of an Sp1 site which we could not locate immediately upstream to the CRE consensus as they did (Figure 1a and ref. [26]). Most NB cell lines exhibit a chromaffin phenotype, defined by the expression of a series of molecular markers, which are present during chromaffin differentiation in human embryos [1]. This observation suggests that NB tumours may be affected by genetic alterations that prevent complete differentiation to the neuroendocrine phenotype. However, we previously reported that *in vitro* treatment of NB cells with biological response modifiers, such as RA or db-cAMP, elicits the appearance of molecularly distinct phenotypes. RA induced the expression of neuronal-like markers, negatively regulating *CHGA* expression, while the exposure of NB cells to high doses of db-cAMP enhanced the chromaffin phenotype, increasing the intracellular levels of *CHGA* mRNA [3]. These findings indicate that, at least under certain conditions, it is possible to bypass the genetic alterations which affect NBS. To verify whether the regulation of *CHGA* expression during NB differentiation occurred at the transcriptional level, we examined *CHGA* promoter activity of stably transfected N18 cells and found that while db-cAMP treatment did not alter CAT levels in these cells, RA treatment dramatically inhibited *CHGA* promoter-dependent transcription of the *CAT* gene. The presence of both db-cAMP and RA restored CAT activity to levels comparable with controls. Since *CHGA* is a molecular marker of chromaffin commitment and most of the NB cell lines are *CHGA* positive

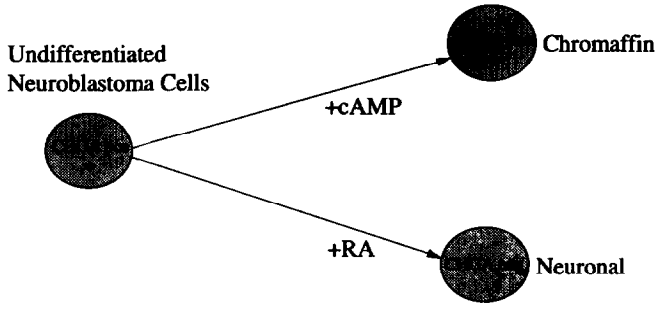

Figure 4. A model of NB differentiation based on data presented in this work. The db-cAMP does not alter the original chromaffin commitment of NB cells, while RA opens a distinct (neuronal) pathway in which *CHGA* is negatively regulated at the transcriptional level.

[1], the failure of db-cAMP to increase CAT activity above the basal level accounts for a constitutive, endogenous, high level of *CHGA* transcription in CHGA-N18 cells. In Figure 4, we have designed a schema of NB differentiation based on these observation. According to this model, our data may be interpreted as the activation of distinct molecular pathways in NB cells, depending on the biological response modifiers present in the extracellular environment. This hypothesis is also in agreement with our previous observations based on the evaluation of mRNA levels [3]. The experiments reported in this work suggest that, at least in part, the molecular mechanisms at the origin of NB differentiation occur at the transcriptional level. In addition, the absence of canonical RA responsive elements (RARE) in the *CHGA* promoter sequence raised the possibility that retinoids inhibit *CHGA* promoter activity by an indirect mechanism. The presence of multiple bHLH consensus in the *CHGA* promoter and the evidence that these molecules may be modulated by RA during NB cell differentiation [27] account for the possibility that one or more of these elements could be involved in the modulation of *CHGA* promoter function.

In conclusion, we believe that the identification of molecules able to drive gene expression and differentiation, even in transformed NB cells, will open the possibility of defining novel targets for a future NB gene therapy.

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