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Advances in Brief

Inducible Expression of Calcyclin, a Gene with Strong Homology to S-100 Protein, during Neuroblastoma Cell Differentiation and Its Prevalent Expression in Schwann-like Cell Lines[1]

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Abstract

Calcyclin gene expression was evaluated in different neuroblastoma cell lines and during neuronal differentiation induced by retinoic acid. Calcyclin gene expression was more frequently detected in epithelial-type or Schwann-like cells rather than in neuroblastic cells. This result indicates an increase of G_1 cell fraction, which may explain the limited growth potential usually observed for these cells. LAN-5 cell (neuronal type) differentiation experiments showed that calcyclin gene is detectable after 4 days of retinoic acid treatment, which induces G_1 phase accumulation (as detected by cytofluorometric analysis), and cell growth arrest. Otherwise, neither block of cell proliferation by 0.5% fetal calf serum medium nor addition of 15% fresh fetal calf serum after cell arrest induce calcyclin expression. The increase of calcyclin mRNA levels during cell differentiation shows that calcyclin gene expression is associated with neuronal differentiation. This bivalent role of the calcyclin gene, which is normally expressed in the G_1 phase of the cell cycle but also expressed during retinoic acid-induced neuroblastoma cell differentiation, suggests that (at least in neuroblastoma cells) the gene is subject to a complex transcriptional regulation.

Introduction

Calcyclin gene is a cell cycle-dependent gene mainly expressed in the G_1 cell cycle phase (1, 2). This gene is differentially expressed in human leukemia cells indicating a deregulation of calcyclin gene expression in malignant cells (2, 3). Calabretta *et al.* (4) demonstrated that the calcyclin gene has a 55% homology to the coding region of the β-subunits of S-100, a calcium-binding protein which is considered a tumor marker for malignant melanoma and neoplasm derived from Schwann cells (4–7). S-100 protein expression also increases during neuroblastoma differentiation, suggesting a role of the protein in neuronal maturation (8). Subclones of morphologically heterogeneous neuroblastoma cell lines show neuroblastic bipolar N cells[3] or larger and flatter cells resembling epithelial or S-cells with distinct biochemical characteristics (9, 10). *In vitro* morphological differentiation can be observed in many human NB cells after RA treatment (11, 12).

The present report is the first one describing calcyclin gene expression in NB cells with different steady state mRNA levels in N- and S-type cells. We also show an increase in calcyclin gene expression during morphological differentiation. The results indicate that calcyclin is more frequently expressed in epithelial- or Schwann-like than in N cells and that mRNA levels increase in NB cells after treatment with RA. The molecular monitoring of calcyclin gene expression may provide a useful approach to the study of NB cell growth and differentiation and the role of this gene in neuroectodermal cell lineage.

Materials and Methods

Cell Cultures. The human NB cell lines were grown exponentially and cultured in complete RPMI 1640 as described elsewhere (13, 14). Three cell lines were previously established in our laboratory, GI-LI-N (15), GI-ME-N (16), and GI-CA-N (15, 17); while SK-N-BE(2)C cells were a gift of Dr. V. Ciccarone (National Cancer Institute, Frederick, MD); and IMR-5, IMR-32, LAN-1, LAN-5, and SK-N-SH cell lines were kindly provided by Dr. R. Seeger (Children's Hospital of Los Angeles, Los Angeles, CA).

RA-induced Neuroblastoma Cell Differentiation. LAN-5 cell differentiation experiments were performed as described previously (18, 19). Briefly, cells were treated for 0, 48, and 96 h with 10^{-5} M RA dissolved in ethanol. The medium containing RA or the carrier used for RA (control cells) was replaced every 2 days. Cell morphology was observed daily and the cells were photographed using a phase contrast microscope.

0.5% Serum Cell Culture. LAN-5 cells were cultured for 48 h in RPMI 1640 containing 0.5% FCS. After 48 h of culture, at cell proliferation arrest (checked by [*methyl*-^{3}H]thymidine incorporation) the 0.5% FCS medium was replaced by a medium containing 15% FCS, and the cells were left growing for 96 h.

Assay for [*methyl*-^{3}H]Thymidine Incorporation. DNA synthesis was monitored daily by [*methyl*-^{3}H]thymidine incorporation plating 5×10^3 cells/well in 96-well plates as described elsewhere (19).

RNA Purification and Northern Blot Analysis. Total RNA was extracted from the cells according to the guanidinium thiocyanate acid-phenol-chloroform method (20). Northern blot analysis was performed as described by Sambrook *et al.* (21). Filters were hybridized first with a [^{32}P]calcyclin probe and, after stripping of the probe, hybridized again with a ^{32}P-pLLRep3 probe to check the amount of RNA, as described previously (22). The amount of RNA loaded on the gel was also estimated by staining the nucleic acid with ethidium bromide.

Plasmid Isolation. The calcyclin (hp2A9) probe was isolated by screening the Okayama-Berg human complementary DNA library with G_1-specific hamster complementary DNA (23). The O-B pc D-X vector-hp2A9 was provided by Dr. S. Ferrari (Temple University, Philadelphia, PA). The plasmid was digested with *Bam*HI restriction enzyme and a 0.7-kilobase fragment was separated by 1% agarose and electroeluted according to the method of Sambrook *et al.* (21). A 0.8-kilobase fragment was isolated from pLLRep3 plasmid (provided by Dr. D. Heller, Medical School, Boston, MA) in the same condition described above. The probes were labeled with [α-^{32}P]dCTP (3000 mCi/mmol) by random prime technique (24) using a multiprime DNA-labeling system (Amersham, United Kingdom).

Results

Expression of Calcyclin Gene in Neuroblastoma Cell Lines. We analyzed the steady state mRNA levels of calcyclin gene in 9 human NB cell lines exponentially growing in 15% FCS

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[3] The abbreviations used are: N, neuroblastic; S, Schwann-like; NB, neuroblastoma; RA, retinoic acid; FCS, fetal calf serum; I, intermediate.

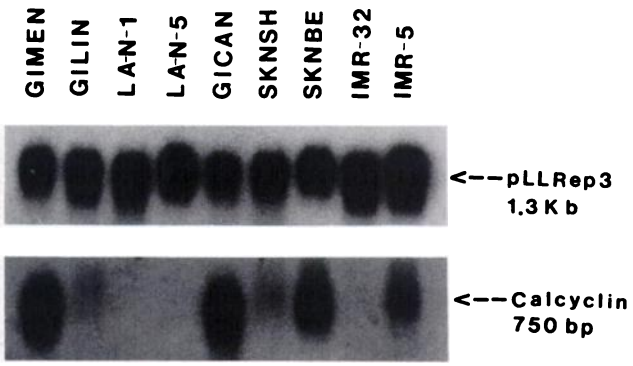

Fig. 1. Northern blot analysis of calcyclin gene expression in human neuroblastoma cell lines. *kb*, kilobases; *bp*, base pairs.

medium. The Northern blot analysis of calcyclin mRNA is shown in Fig. 1. GI-ME-N, GI-CA-N, SK-N-BE(2)C, and IMR-5 cells show high levels of calcyclin gene mRNA, whereas low levels were detected in GI-LI-N and SK-N-SH cells. No calcyclin expression was detected in the other cell lines studied. Among cell lines expressing calcyclin, GI-ME-N displays S-type morphology with flat cells and very few neurites (Fig. 2, *1*). The SK-N-BE(2)C cell line intermediate type (I) was subcloned from the SK-N-BE(2) line which showed spontaneous interconversion from N- to S-type (9). As shown in Fig. 2, SK-N-BE(2)C cells are larger with some neurite processes. GI-CA-N and SK-N-SH consist of two cell types, N and S. However, GI-CA-N shows predominantly S-type cells, whereas the SK-N-SH line is composed of 50% S- and 50% N-type cells (Fig. 2, *2* and *4*, respectively). The IMR-5 and GI-LI-N cell lines expressing calcyclin show N-type morphology, *i.e.*, small with a round body and several neurite-like structures (Fig. 2, *5* and *6*). Finally, IMR-32, LAN-1, and LAN-5, cells in which calcyclin mRNA was undetectable all have neuroblastic morphology (Fig. 2, *7*, *8*, and *9*).

Levels of Calcyclin Gene Expression in Differentiating LAN-5 Cells. Because the calcyclin gene was not expressed in cycling LAN-5 cells we treated the cells with 10^{-5} M RA to induce growth arrest (25). RA also induces change in cell shape together with neurite-like processes indicating neuronal maturation. LAN-5 cells induced to differentiate by RA showed reduction of [*methyl*-^{3}H]thymidine uptake after 3 days of culture (Fig. 3, *top*). As reported previously (18), treatment of LAN-5 cells with 10^{-5} M RA dramatically changed their morphology showing neurite outgrowth from the cell body and elongation after 7 days of culture (Fig. 4). Cell viability ranged from 80 to 90% at all time points (Fig. 3, *bottom*). No morphological alteration was observed during the first day and calcyclin gene expression was undetectable (Fig. 5). The maximum levels of calcyclin mRNA were detected after 4 days of culture (Fig. 5) when the cells clearly displayed neurite formation. At that time [*methyl*-^{3}H]thymidine incorporation was reduced by 78%. To

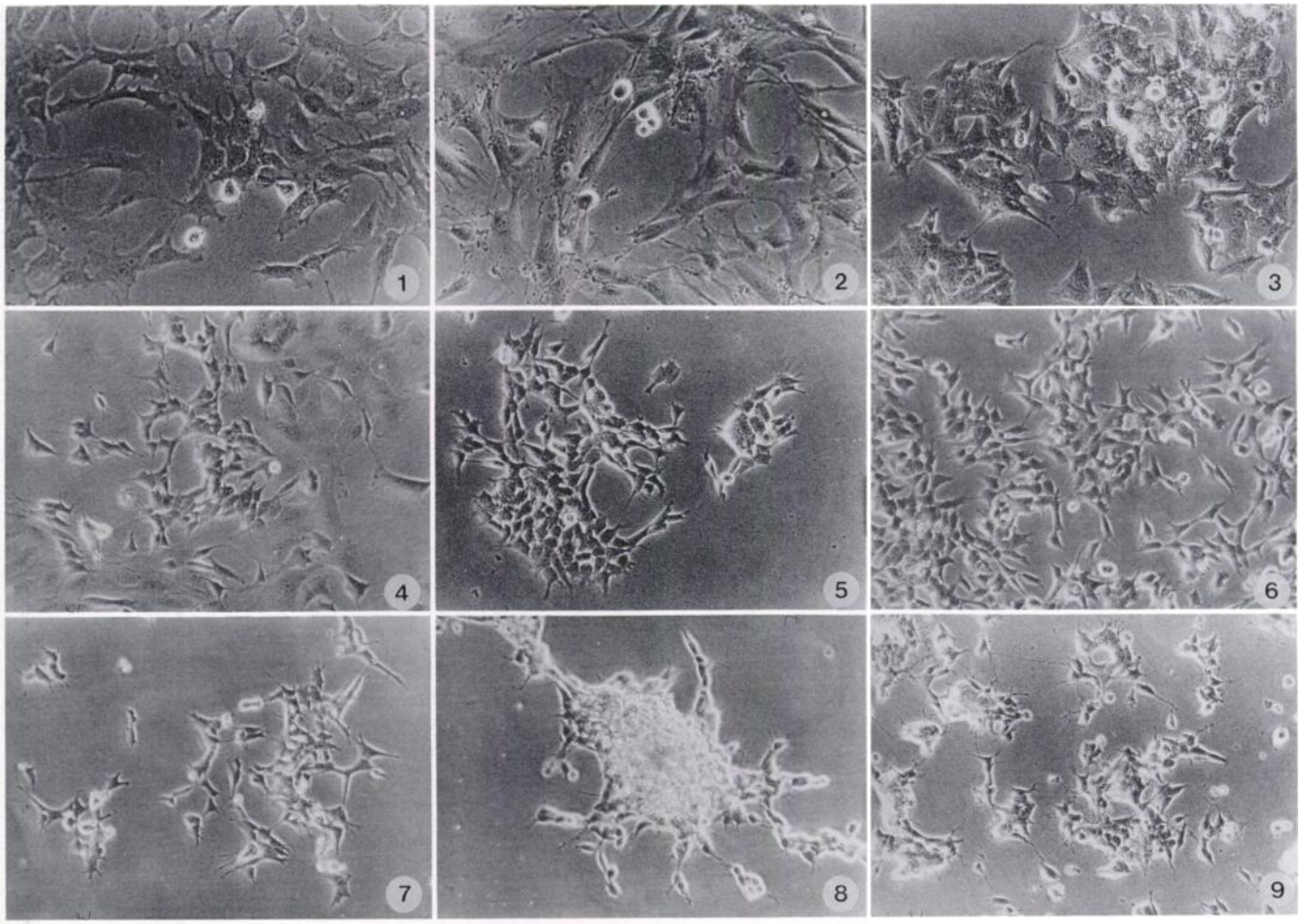

Fig. 2. Morphology of human neuroblastoma cell lines. *S*, epithelial-like; *N*, neuroblastic; *I*, intermediate. *1*, GI-ME-N: S; *2*, GI-CA-N: prevalently S with few cells N (7–10%); *3*, SK-N-BE2(C): I; *4*, SK-N-SH: mixed N/S; *5*, GI-LI-N: N; *6*, IMR 5: N; *7*, IMR 32: N; *8*, LAN-1: N; *9*, LAN-5: N. Phase contrast, ×160.

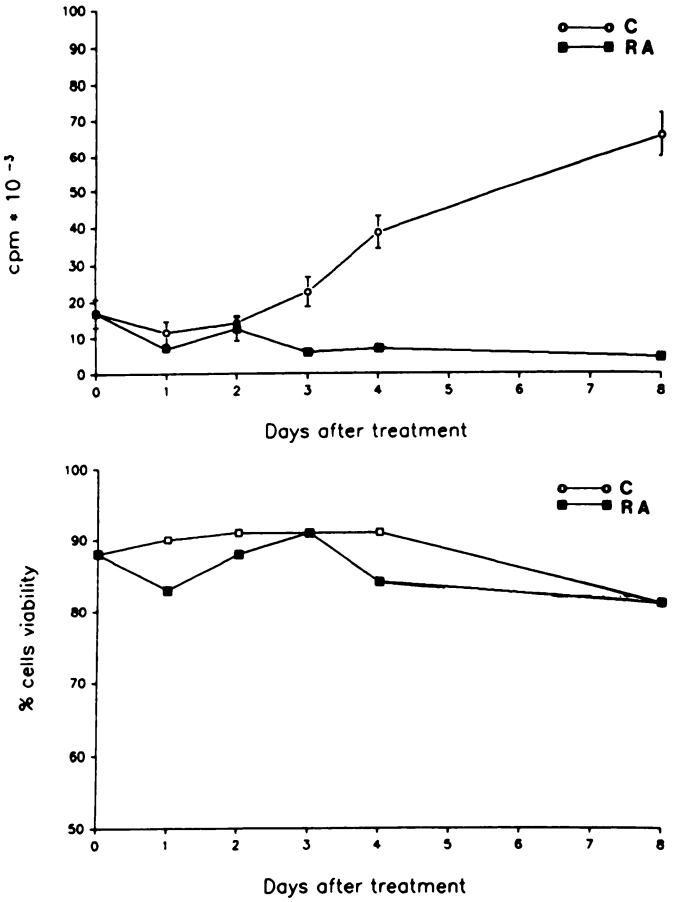

Fig. 3. Time course experiment of [*methyl*-^{3}H]thymidine incorporation and cell viability in RA-treated LAN-5 neuroblastoma cells. *C*, control.

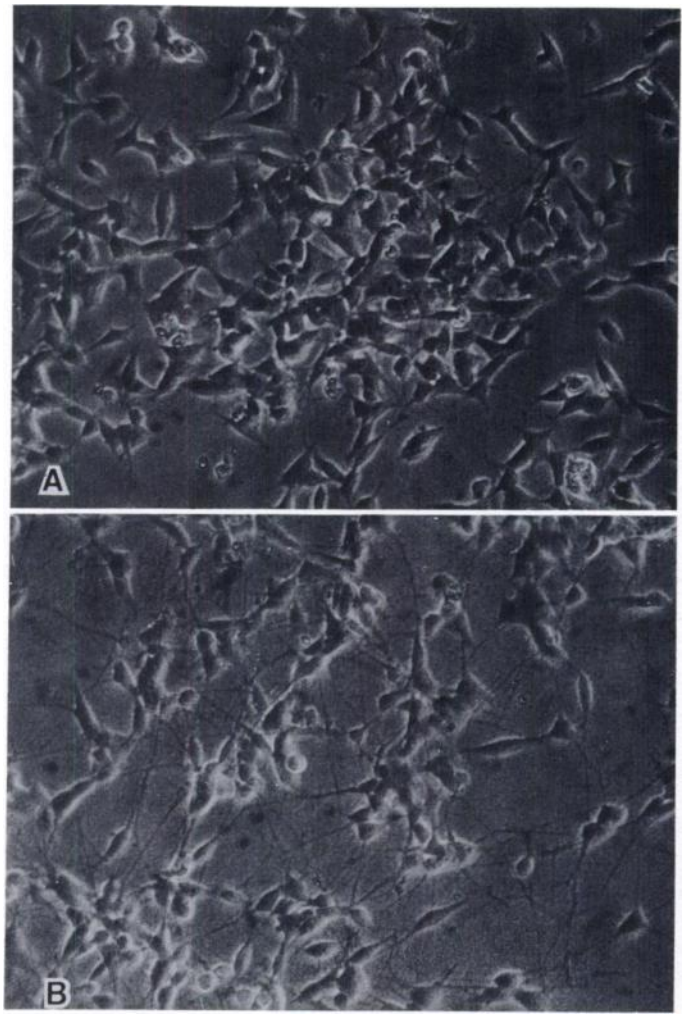

Fig. 4. Morphology of LAN-5 neuroblastoma cells. *A*, untreated; *B*, after 7 days of RA treatment. RA induces dramatic change of cell morphology and neurite outgrowth from the cell body forming interconnections. Phase contrast, ×160.

estimate the amount of RNA loaded on the gel nucleic acids were stained with ethidium bromide and hybridized with the pLLRep3 probe, a non-Alu family of interspersed repetitive DNA sequences, abundant in mammalian cells and encoding a housekeeping mRNA (26, 27).

Calcyclin Expression in LAN-5 Cells Cultured in 0.5% FCS Medium. As reported above, treatment of LAN-5 cell with RA results not only in the induction of cell differentiation but also in cell growth arrest. To ascertain that increase of calcyclin expression was not due to cell proliferation arrest induced by RA treatment, we cultured the cells in the presence of a 0.5% FCS medium for 48 h. Cell proliferation block was evaluated by [*methyl*-^{3}H]thymidine incorporation (C_{t_0} = 33,908 ± 3,992; $C_{t_{48}}$ = 74,332 ± 9,170; 0.5% $FCS_{t_{48}}$ = 29,529 ± 5,763). Calcyclin mRNA was not detected in these cultures. Because calcyclin gene is a Ca^{2+}-dependent gene, FCS concentration was maintained at 0.5% to ensure that a sufficient amount of calcium was provided in the medium. After 48 h cells were pulsed with 15% FCS medium for 96 h. Calcyclin gene expression remained undetectable and was not induced by the replacement of fresh serum (Fig. 6).

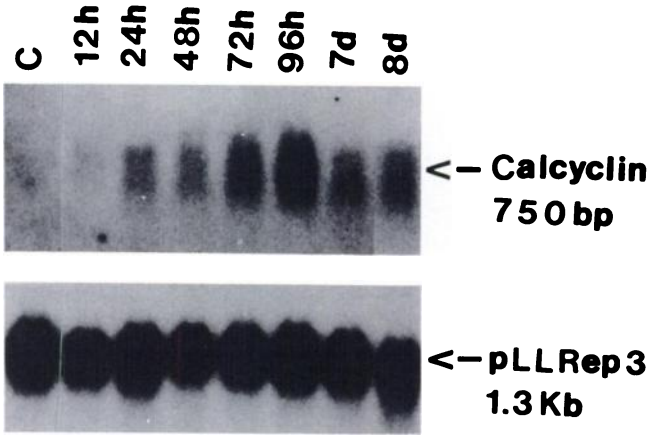

Fig. 5. Northern blot analysis of calcyclin gene expression during LAN-5 neuroblastoma cell differentiation induced by RA. *bp*, base pairs; *kb*, kilobases.

Discussion

We have shown that calcyclin gene expression is present in cells of neuroectodermal origin. In our experience, calcyclin gene expression was frequently detected in epithelial-like cells whereas steady state mRNA levels were low or below detection levels in neuroblastic cells. Moreover, our data show that the calcyclin gene is expressed in neuroectodermal-derived cells in addition to human fibroblast and leukemic cells as reported previously (1, 23, 28). Our results further demonstrate that calcyclin expression is induced during differentiation of neuroblastoma LAN-5 cells treated with RA.

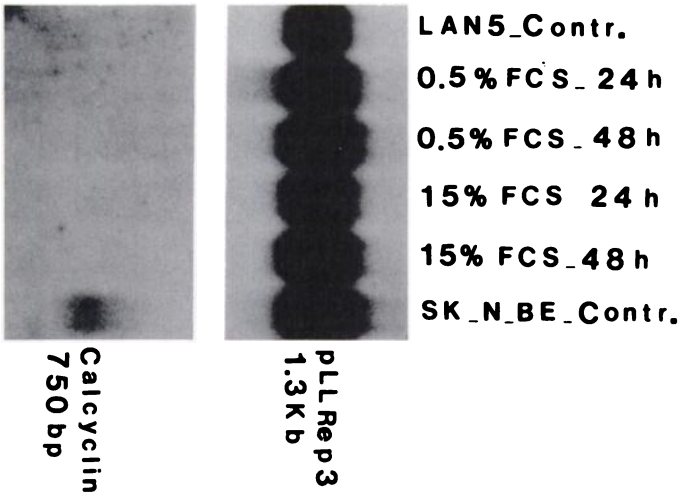

Fig. 6. Northern blot analysis of the calcyclin gene in LAN-5 cells cultured in 0.5% FCS medium for 24 and 48 h and then pulsed with 15% FCS medium for 96 h. LAN-5 cells cultured in 15% FCS medium for 0 to 96 h were used as control. *kb*, kilobases; *bp*, base pairs.

The calcyclin gene was expressed in 4 of 4 cell lines with S or I or mixed N/S cell morphology. Among the N-type cell lines, only 2 of 5 expressed the calcyclin gene; however, these cells can occasionally transdifferentiate into S-type cells (9, 10). The I morphology is associated with an intermediate stage in which the cell moves from the N to the S morphological type (9). The transdifferentiation process from N to S cells can be observed spontaneously *in vitro* in the IMR-5 cell line (29). This may explain the elevated calcyclin mRNA in IMR-5, an N-type cell line. However, no morphological change was detected in this cell line during continuous cell culture. Rettig *et al.* (9) and Ciccarone *et al.* (10) have shown that the three different cell types have different surface antigens and biochemical properties. It has been suggested that N, I, and S cells are multipotent cells able to differentiate one from the other. In any case, they are clearly different and may represent a different stage in which the transformation occurs. Thus, the study of the calcyclin expression may indicate that the S-type cells have a deregulated cell cycle compared with the N-type. It remains to be clarified if this difference is a consequence or plays a key role in malignant transformation. Because calcyclin is a G_1-specific gene, it is possible that Schwann-like cell lines have a higher cell distribution in the G_1 phase. As a consequence, S cells should proliferate less with reduced malignancy potential, as is also reported by Biedler *et al.* (29).

Calcyclin is highly homologous to the β-subunit of S-100 protein, a protein mainly expressed in glial and Schwann cells. Like S-100, calcyclin is expressed in Schwann-like cells and may become an additional marker of NB.

In order to analyze the pattern of calcyclin expression during neuroblastoma cell differentiation we treated LAN-5 cells with RA. NB cells are extremely sensitive to RA which is thought to promote a maturational change of the cell (11). After treatment with RA, the cells extend neurite-like processes forming a complex network. In our experiments calcyclin reached its maximum expression when more than 70% of LAN-5 cells displayed neurite outgrowth (4 days after RA treatment). At that time [*methyl*-^{3}H]thymidine incorporation decreased to 12% of the initial value. Cells were arrested irreversibly in the $G_0 + G_1$ phases of the cell cycle as detected by flow cytometric analysis (data not shown). Morphological differentiation of neuroblastoma cell lines is associated with several biochemical and molecular changes (12, 19); in particular human NB GOTO cells treated with 5-bromodeoxyuridine show induction of S-100 protein expression (8). Altogether, these results show that increase of calcyclin expression, as in the case of the S-100 protein, is associated with morphological differentiation of neuroblastoma cells and is not related to induced cell growth arrest. In fact, when LAN-5 cells cultured in 0.5% FCS medium stopped proliferating, calcyclin mRNA expression was not observed. Since Ca^{2+} is present in FCS the failed expression of calcyclin in resting LAN-5 cells may depend on loss of transcriptional activating factor(s) that regulate gene expression in cell type. The addition to the cells of 15% fresh FCS medium after cell arrest did not induce calcyclin expression, thus indicating a role for calcyclin in neuroblastoma cell differentiation. The differential expression of calcyclin in proliferation and differentiation state suggests a bifunctional role of the gene as other genes such as c-*src*, vimentin, and c-*fos* (30, 31).

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