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Molecular Cloning of the cDNA for a Growth Factor-inducible Gene with Strong Homology to S-100, a Calcium-binding Protein*

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Bruno Calabretta‡, Renata Battini‡, Leszek Kaczmarek‡, Jon K. de Riel§, and Renato Baserga‡

From the ‡Department of Pathology and the §Fels Research Institute, Temple University Medical School, Philadelphia, Pennsylvania 19140

We have identified a cDNA whose sequence is preferentially expressed when quiescent fibroblasts are stimulated to proliferate. The steady-state levels of the mRNA corresponding to this clone, called 2A9, are increased by serum, platelet-derived growth factor, and epidermal growth factor, but not by insulin or platelet-poor plasma. mRNA levels of 2A9 are also increased in human acute myeloid leukemia. The 2A9 cDNA has been molecularly cloned from an Okayama-Berg library, and its complete nucleotide sequence has been determined. It has an open reading frame of 270 nucleotides, which has a 55% homology with the coding sequence of the β-subunit of the S-100 protein, a calcium-*binding* protein that belongs (like calmodulin and the vitamin D-dependent intestinal calcium-binding protein) to the family of calcium-modulated proteins and is found in abundance in several human tumors, including melanoma. The S-100 protein and the deduced aminoacid sequence of 2A9 are also partially homologous to the small subunit of a protein complex that serves as a cellular substrate to tyrosine kinase. The partial homology of 2A9 (whose RNA is inducible by growth factors and is overexpressed in human acute myeloid leukemias) to the S-100 protein, other calcium-modulated proteins, and the subunit of a substrate for tyrosine kinase, is particularly interesting in view of the role attributed to calcium and tyrosine kinases in the regulation of cell proliferation.

We have previously isolated by differential screening of a cDNA library five cDNA clones representing sequences that were inducible by serum (1). Specifically, steady-state levels of the cognate RNAs were very low in G_0 cells, but markedly increased between 6 and 16 h after stimulation with serum. One of the five cDNA clones, called 2A9, was found to be overexpressed in human acute myeloid leukemias (2, 3).

The cDNA clones were originally isolated from a cDNA library of ts13 cells, a G_1-specific temperature-sensitive mutant derived from Syrian baby hamster kidney cells (4). We report in this paper the nucleotide sequence of the full length human cDNA corresponding to 2A9, as well as some of the biological characteristics of this clone. The human 2A9 cDNA was isolated from an Okayama-Berg cDNA library (5); it has 55% homology with the β-subunit of a calcium-binding protein, the S-100 protein (6). The S-100 protein is part of a family of calcium binding proteins, which Van Eldik *et al.* (7) call calcium-modulated proteins and which include calmodulin (8). The homology between 2A9 and the β-subunit of S-100 is particularly striking in two regions, which in the S-100 protein, code for the putative binding sites for calcium. The S-100 protein is considered a marker for several human tumors, especially malignant melanoma and neoplasms derived from Schwann cells (9, 10). The S-100 protein was also reported to have homologies with the p11 (or p10) subunit of a protein complex (11, 12) which is a major cellular substrate for tyrosine kinase (13). Although, we have not isolated yet the protein encoded by the 2A9 gene, the homologies at the nucleotide level are of striking interest in terms of the role that calcium and tyrosine kinases are thought to play in the control of cell proliferation (see "Discussion"). For this reason, we think it worthwhile to report our findings at this stage.

EXPERIMENTAL PROCEDURES

Molecular Cloning of 2A9—The 82-bp[1] *Bam*HI insert derived from the hamster p2A9 (1) was subcloned in the *Bam*HI site of the riboprobe vector pSP64 from Promega Biotec (Madison, WI). An RNA probe of high specific activity (4×10^8 cpm/μg) complementary to the 82-bp *Bam*HI insert was synthesized according to the procedure described by Melton *et al.* (14). Five hundred thousand recombinants of the Okayama-Berg library derived from SV40-transformed human fibroblasts (5), were screened at high density according to the procedure described by Hanahan and Meselson (15). Eighty positive clones were identified in duplicate filters of the primary screening. Nine cDNA clones were isolated after secondary and tertiary screening, and six of them with the longest inserts were grown in large scale for further analysis.

DNA Sequencing—DNA sequencing was carried out according to the method of Sanger *et al.* (16) following cloning in M13 (17), M13mp8, M13mp9 (18), and M13mp19 (19) with the use of a universal M13 sequence primer TCCAGTCACGACGT (New England BioLabs, Beverly, MA). Sequence gels were 6% acrylamide and 8M urea in Tris-borate buffer. The same clones were sequenced (in both directions) by the method of Maxam and Gilbert (20). The Data Bank from Palo Alto (CA) was searched for sequence homologies.

Cell Cultures—WI-38 cells, a strain of human diploid fibroblasts, at the 32nd population doubling level, were cultured as previously described (21). The methods for making them quiescent, for serum stimulation, or for synchronizing them are described in the legend to Fig. 4. Labeling with [^{3}H]thymidine and autoradiography were carried out by standard procedures.

BALB/c/3T3 fibroblasts were plated in Dulbecco's modified minimal essential medium, supplemented with 10% fetal calf serum, L-glutamine, and antibiotics (high serum medium). When cells were semiconfluent, the high serum medium was washed out and low serum medium (containing 1% calf serum instead of fetal calf serum) was added to the cultures. Five days later, the cells were quiescent (less than 1% of cells incorporated [^{3}H]thymidine following a 24-h incubation) and were then treated with fresh high serum medium, PDGF

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[1] The abbreviations used are: bp, base pairs; kb, kilobase pairs; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; PPP, platelet-poor plasma.

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(Collaborative Research), EGF (Collaborative Research), insulin (Sigma), or PPP, prepared from human blood as described by Kaczmarek *et al.* (22). Except for fetal calf serum, growth factors were added to the conditioned low serum medium. Eight h after the addition of growth factors, cells were washed with ice-cold phosphate-buffered saline and harvested with a rubber policeman, and total cytoplasmic RNA was extracted.

RNA Extraction and Blotting—Total cytoplasmic RNA was extracted as previously described (1). Blotting was carried out as described by Thomas (23).

Hybridization—Hybridization and autoradiography were carried out by standard procedures (23). The human 2A9 clone was nick-translated (24) to a specific activity of $4–5 \times 10^8$ cpm/μg. The amount of RNA blotted on each filter was constantly monitored by hybridizing the filters to probes for genes that are not expressed in a cell cycle-dependent manner, and/or a probe for histone H3, as previously described (1–3).

Materials—PDGF and EGF (receptor grade) were purchased from Collaborative Research.

RESULTS

Nucleotide Sequence of the Human 2A9 cDNA Clone—Fig. 1 shows the complete nucleotide sequence of the insert from the p2A9 plasmid isolated from the Okayama-Berg library. The total number of nucleotides is 434. There is an open reading frame of 270 nucleotides from nucleotide 66 to nucleotide 335 coding for a putative polypeptide of 90 amino acids (including the initial methionine). Below the nucleotide sequence of 2A9 is the coding sequence of the β-subunit of the rat S-100 protein (6); the extent of homology is 55%. The RNA for the β-subunit of S-100, though, is much longer than that for 2A9: 1488 bp, which include 120 bp of the 5′-noncoding region, 276 bp of the coding region, and 1092 bp of the 3′-noncoding region (6). The 2A9 clone hybridizes, on Northern blots, to a band which is about 0.6 kb (see Fig. 4). There is also extensive homology between the α- and β-subunits of the 2A9 (25), but the nucleotide sequence of the α-subunit is not yet available. The homology between 2A9 and the β-subunit of S-100 is particularly striking around the regions which, in the S-100 sequence, code for putative calcium-binding sites (6, 7, 25). The regions coding in the S-100 protein for the calcium-binding sites extend, in Fig. 1, between nucleotides 126–164 and 240–284. Note, though, that in Fig. 1, the numbers refer to the nucleotide sequence of 2A9. The distance between the putative calcium-binding sites is conserved in 2A9 in respect to the S-100 protein. This distance, which may have an important role in the structure and function of these proteins, is highly conserved in the so-called calcium-modulated proteins, which include both subunits of the S-100 protein, calmodulin and the vitamin D-dependent intestinal calcium-binding protein (for a review, see Ref. 7).

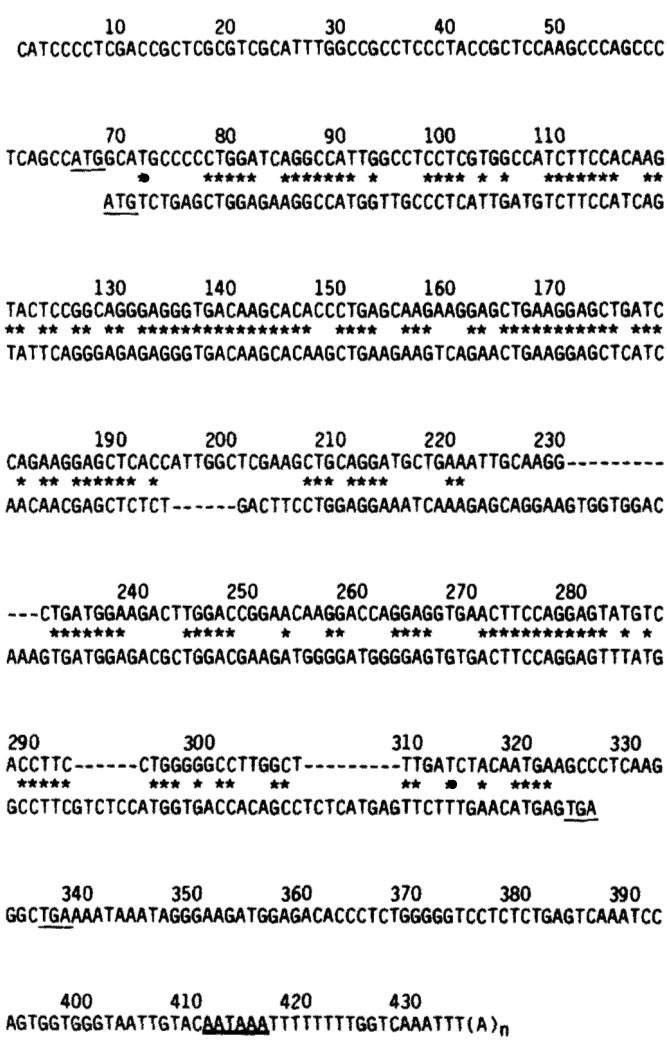

FIG. 1. **Complete nucleotide sequence of the insert in human p2A9.** The sequence of 2A9 (*upper row*) is compared with the coding sequence of the cDNA for the β-subunit of the S-100 protein (*6th row*). *Asterisks* identify homologies. AUG and stop codons are *underlined*. The *double line* indicates the putative poly(A) signal for 2A9. The *numbers* refer to the 2A9 sequence. The calcium-binding sequences of S-100 correspond to the 2A9 sequences extending from nucleotide 126 to nucleotide 164, and from nucleotide 240 to nucleotide 284.

Comparison of Amino Acid Sequences—The amino acid sequence of the α- and β-subunits of S-100 and the deduced amino acid sequence of 2A9, are shown in Fig. 2. The homology again is especially remarkable in the two sequences that in the S-100 and other calcium-binding proteins are thought to constitute the calcium-binding sites (6, 7, 25, 26). In the first site, the homology between both subunits of S-100 and 2A9 is 88%. At the second site, the homology of the deduced

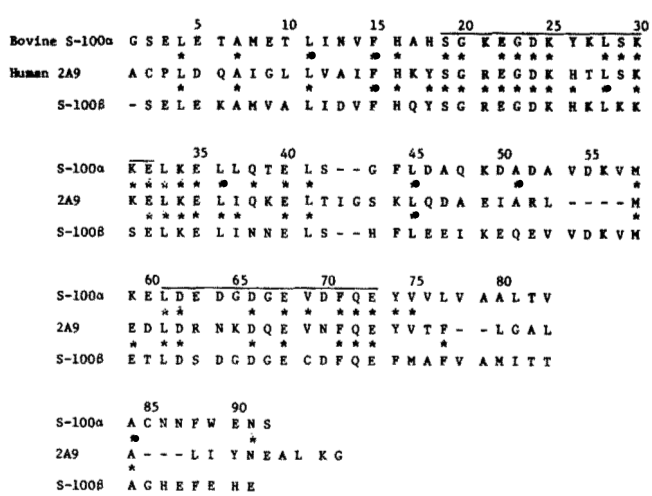

FIG. 2. **Comparison of the amino acid sequences of the α- and β-subunits of the S-100 protein with the amino acid sequence deduced from the nucleotide sequence of 2A9.** The *lines above the first row* delimit the putative calcium-binding sequences. *Asterisks* denote homologies.

amino acid sequence of 2A9 to that of the α-subunit of S-100 is 61%, to the β-subunit, 54%. Notice also that where there is substantial divergence between the deduced amino acid sequence of 2A9 and the sequences of the S-100 subunits, the two subunits are also less homologous to each other.

There are also homologies between the deduced amino acid sequence of 2A9 and that of the vitamin D-dependent intestinal calcium-binding protein (not shown). Since Gerke and Weber (11) and Glenney and Tack (12) have reported homology between the subunits of the S-100 protein and the p11 (or p10) subunit of a protein complex that serves as a substrate for viral tyrosine kinase, we have compared the amino acid sequence of the latter with the putative sequence of 2A9. Out of 90 amino acids, 26 are the same (the homology between p11 and S-100 is better, 43 amino acids); in addition, there are six conservative changes, and the homology is particularly striking in the regions of the putative calcium-binding sites (Fig. 3). It should be stated here, though, that Gerke and Weber (11) could not detect any calcium-binding activity in the p11 subunit.

Response of 2A9 Gene to Growth Factors—Fig. 4 shows the cell cycle dependency of expression (in terms of steady-state RNA levels) of 2A9 in WI-38 cells, a strain of human diploid fibroblasts (27). The RNA (estimated size, 0.6 kb) is not detectable in G_0 cells, but is already apparent 1 h after serum stimulation and reaches a peak at 16–24 h. We could not

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                  20                  30        35        40
2A9   I F H K Y S G R E G D K H T L S K K E L K E L I Q K E L
        * * *         * * *     * o *   o * o   *     * *
p11   T F H K F A - - - G D K G Y L T K E D L R V L M E K E F

                  60        65        70
2A9   M E D L D R N K D Q E V N F Q E
        *   * * *     o *     *   * *
p11   M K D L D Q C R D G K V G F Q S
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FIG. 3. **A comparison of the amino acid sequence of the p11 subunit and the deduced amino acid sequence of 2A9 in the regions surrounding the calcium-binding sites.** The sequence of p11 is taken from the paper by Gerke and Weber (11). *Asterisks* denote homologies, *open circles* conservative changes.

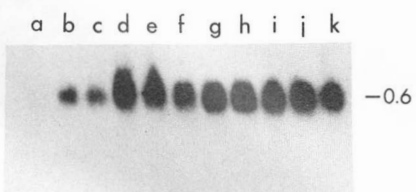

FIG. 4. **Cell cycle-dependent expression of p2A9 cDNA clone in human diploid fibroblasts.** Human diploid fibroblasts (WI-38) were plated at a density of 1×10^4 cells/cm^2 in plastic Petri dishes in Earle's minimal essential medium supplemented with 10% fetal calf serum, amino acids, vitamins, and antibiotics as described (21). After 14 days in culture, the cells were quiescent and were used for the experiments. Fibroblasts were either stimulated with fresh medium for 1, 6, 16, and 24 h or replated at a dilution of 1:20 and, after 3 days, synchronized in S phase with 2.5 mM hydroxyurea. After overnight exposure to hydroxyurea, the drug was removed by extensive washing and cells were harvested 4, 9, 13, 18, and 23 h later. Total cytoplasmic RNA was extracted from quiescent as well as from stimulated cells and cells released from the hydroxyurea block. RNA (15 μg/lane) was separated on formaldehyde containing agarose gels and transferred to nitrocellulose filter, as described by Thomas (23). The filter was then hybridized to a nick-translated p2A9 probe. *Lane a* represents hybridization to RNA from quiescent WI-38 fibroblasts; *lanes b, c, d,* and *e* represent hybridization to RNA obtained from cells stimulated with fresh medium for (respectively) 1, 6, 16, and 24 h; *lanes f, g, h, i,* and *j* represent hybridization to RNAs extracted from hydroxyurea-synchronized cells 4, 9, 13, 18, and 23 h (respectively) after release from the early-S phase block; *lane k* is RNA from exponentially growing cells.

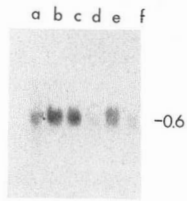

FIG. 5. **The full length human 2A9 clone was hybridized to RNAs obtained from BALB/c/3T3 mouse fibroblasts treated with different growth factors (see "Experimental Procedures").** *Lane a* represents hybridization to RNA obtained from quiescent cells; *b*, cells treated with fresh, high serum medium; *c*, PDGF (2 units/ml); *d*, PPP (5%); *e*, EGF (20 ng/ml); *f*, insulin (10 μg/ml). Other conditions were as in Fig. 4.

TABLE I

Biological characteristics of the 2A9 gene

1. Inducible by serum in cells of at least four different species (see text)
2. Inducible by PDGF and EGF, but not by platelet-poor plasma or insulin (this paper)
3. Inducible by serum in the presence of cycloheximide (30)
4. Overexpressed in human acute myeloid leukemias (2, 3)
5. Inducible by serum in senescent, nonproliferating WI-38 cells (21)
6. Inducible by serum in ts13 cells (a G_1-specific ts mutant of the cell cycle) even at the nonpermissive temperature (1)
7. Not inducible by adenovirus infection, under conditions in which cellular DNA synthesis is stimulated (37)
8. Not detectable in human lymphocytes (2)

detect differences in steady-state RNA levels between G_1, S, and G_2 cells (*lanes f–j*). The progress of the cells through the cell cycle was monitored both by [^{3}H]thymidine incorporation and by the levels of histone H3 mRNA in Northern blots. Cells at 16 and 24 h after serum stimulation were mostly in S phase; after release from the hydroxyurea block, cells were in S phase at 4 h, in G_2 at 9 h, in G_1 at 13 h, and back in S phase between 18 and 23 h (data not shown). The situation is similar to that described for c-myc, which is not expressed in G_0 cells; it increases in proliferating cells and shows little change in the other phases of the cycle (28, 29).

2A9 RNA is induced by serum in ts13 cells (1), 3T3 cells (30), WI-38 human diploid fibroblasts (Fig. 4), and rat 3Y1 cells.[2] It is not detectable in human peripheral blood mononuclear cells, either before or after stimulation with phytohemagglutinin (2). Fig. 5 shows an RNA blot of RNAs from BALB/c/3T3 cells, stimulated by different growth factors. BALB/c/3T3 mouse fibroblasts were chosen for these studies because of their well-defined requirements for growth factors (for a review, see Ref. 31). All RNAs were prepared from cells 8 h poststimulation, which is the peak of expression of 2A9 after serum stimulation. Notice that the steady-state RNA levels of 2A9 are increased when G_0 3T3 cells are exposed to PDGF, EGF, and serum, but not after exposure to PPP or insulin. The biological properties of 2A9 known thus far, largely in terms of steady-state RNA levels, are summarized in Table I.

DISCUSSION

We have isolated from an Okayama-Berg library (5) the human cDNA corresponding to the Syrian hamster cDNA clone that was originally isolated by Hirschhorn *et al.* (1) and called 2A9. The cDNA insert consists of 434 base pairs, which agrees with the size of the RNA on agarose gels. It has an

[2] B. Calabretta, R. Battini, L. Kaczmarek, J. K. de Riel, and R. Baserga, unpublished data.

open reading frame of 270 bp from which a polypeptide of 90 amino acids can be deduced. We think that the insert of the human 2A9 cDNA is full length for the following reasons: 1) its size in respect to the size of the RNA band in Northern blots (assuming a poly(A) tail of 150–200 bases); 2) the size of the open reading frame which, beginning from the first AUG, is almost exactly the same size as the related subunits of the S-100 protein; and 3) although many 2A9 clones were isolated from the Okayama-Berg library, none had an insert longer than those analyzed in this paper.

In terms of steady-state RNA levels, the RNA corresponding to the 2A9 cDNA is not detectable in G_0 cells, increases after serum stimulation, and remains high in cycling cells (Fig. 4). In this respect, it behaves like c-myc (28, 29). 2A9 is inducible in 3T3 cells by PDGF and EGF, but not by PPP or insulin (Fig. 5).

2A9 is inducible in a variety of cells of different types and from different species, but is not detectable in human peripheral blood mononuclear cells, either before or after mitogen stimulation (2). It is detectable in human bone marrow cells and in cells from acute and chronic myeloid leukemias (Calabretta *et al.* (2, 3). Its expression seems to be deregulated in some forms of human acute myeloid leukemia (2), and it is indeed this finding that focused our attention on this clone.

The fact that 2A9 is induced, even in the presence of cycloheximide (30), places it with other cell cycle-dependent genes whose inducibility is not affected by inhibition of protein synthesis. These include c-fos (32, 33), c-myc (34, 35), JE-3 and KC-1 (36), and 2F1, 4F1, and p53 (30). In contrast though to some of these other genes that can be induced by cycloheximide alone (without additional growth factors), 2A9 is not induced unless growth factors are added. Despite the fact that it is not inhibited by cycloheximide, 2A9 reaches its peak of expression in mid-G_1, between 4–16 h poststimulation. The fact that it is inducible in a G_1-specific ts mutant of the cell cycle even at the restrictive temperature (1), and that it is not inducible by infection with adenovirus 5 (37), indicates that it is not a late G_1 gene. Finally, its inducibility in senescent WI-38 cells (21) should be seen in the context of the finding that most cell cycle-dependent genes, including histone H3 and thymidine kinase, are still inducible by serum in senescent WI-38 cells (21).

The sequence of 2A9 reveals a 55% homology with the coding sequence of the β-subunit of the S-100 protein. This protein was originally described as a brain-specific protein (25, 38), but it has been more recently described in other cell types (10). The S-100 protein has been detected by immunofluorescence in several human tumors, especially malignant melanoma and tumors of neuroendocrine origin (9, 10), and is synthesized in the G_1 phase of cultured glioma cells (39). Both the bovine amino acid sequence (25) and the rat nucleotide sequence of the cloned cDNA for the β-subunit (6) have been published. The nucleotide sequence of the α-subunit is not available (for review, see Ref. 7); but at the amino acid level, the α- and β-subunits are strongly homologous to each other, and this homology extends to the putative amino acid sequence of 2A9. Indeed, the homologies between the deduced amino acid sequence of 2A9 and both subunits of S-100, particularly striking in certain regions, raises the question whether some reports of the presence of S-100 proteins, based on immunofluorescence studies, may be attributed to the product of 2A9 or other related genes.

The S-100 protein is a calcium-binding protein and has the two characteristic sequences that have been proposed as the calcium-binding sites and that are partially homologous to similar sequences in other calcium-binding proteins, like the intestinal calcium-binding protein, parvalbumin, troponin C, and calmodulin (8). The 2A9 clone has two sequences that are strongly homologous to the calcium-binding sequences of the S-100 protein (Figs. 1 and 2) and of another protein, the vitamin D-dependent calcium-binding protein of intestinal origin (for review, see Ref. 7). Obviously, no firm conclusion can be reached on the function of the protein encoded by the 2A9 gene, until the protein itself is isolated and purified. However, the homology at the nucleotide level is established, and this homology is particularly evident in those regions which, in the S-100 gene, code for the calcium-binding sites. We find this homology between 2A9 and other calcium-modulated proteins as particularly intriguing, because of the well-known role of calcium in cell proliferation. Calcium is required for growth of cells in culture, especially fibroblasts (40) in both early and late G_1 (41), and its requirement is decreased in transformed fibroblasts (42). Levels of intracellular free Ca^{2+} are markedly increased when quiescent fibroblasts are stimulated to proliferate by growth factors, especially PDGF and EGF, but they are not increased by insulin (43, 44). More recently, Praeger and Cristofalo[3] showed that calcium can actually replace EGF in the growth of WI-38 cells in serum-free media. 2A9 RNA levels are increased by EGF (Fig. 5). In addition, calmodulin mRNA is expressed in a cell cycle-dependent manner and has been proposed as playing a role in cell cycle regulation (45).

There is another protein that is homologous to the S-100 protein and to a lower, but significant, extent to the deduced sequence of 2A9, and that is the small subunit of a protein complex, which is a cellular substrate for tyrosine-specific protein kinases (13), and is called p11 by Gerke and Weber (11) and p10 by Glenney and Tack (12). Because tyrosine kinases are also involved in the response to growth factors (46, 47), these homologies can easily lead to speculations which, for the moment, we prefer to leave untouched except to mention that calmodulin has been reported to stimulate tyrosine phosphorylation (48). Also, on the speculative side is another role of calcium, *i.e.* the calcium mobilization that occurs when protein kinase C is activated (49 and review in Ref. 50), as it occurs, for instance, with the transforming gene of Rous sarcoma virus (51), polyoma middle T (52), and growth factors like Interleukin-2 (53) or EGF (54, 55). Finally, the possibility should be mentioned that the product of this gene, for which we would like to propose the name of calcyclin, may be related to the oncomodulin described by Whitfield *et al.* (56).

In conclusion, we have cloned a cDNA whose sequence is expressed when G_0 cells are stimulated to proliferate by different mitogens and is overexpressed in some forms of human acute myeloid leukemias. The fact that this cDNA clone, 2A9, has strong homologies with the coding sequence of the S-100 protein and homologies with other calcium-binding proteins is particularly intriguing in terms of its possible role in cell cycle regulation.

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