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Genetics

Cell-cycle-specific genes differentially expressed in human leukemias

(cell cycle/gene expression/oncogenes)

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ABSTRACT **Three cDNA clones isolated from Syrian hamster cells (p4F1, p2F1, and p2A9) contain sequences that are preferentially expressed in the G_1 phase of the cell cycle. The expression of these sequences was investigated in human peripheral blood cells from normal individuals and from patients with leukemia. The expression of p4F1 and p2F1 is clearly dependent on the cell cycle in peripheral blood mononuclear cells stimulated to proliferate with phytohemagglutinin; the p2A9 sequences cannot be clearly detected in human lymphocytes but are expressed in a cell-cycle-dependent manner in human diploid fibroblasts (WI-38). These genes also show different levels of expression in lymphoid and myeloid leukemias. The highest level of expression for p2A9 is found in patients with chronic myelogenous leukemia, and the lowest in patients with chronic lymphocytic leukemia. For p2F1 and p4F1, the highest levels of expression are found in chronic and acute myelogenous leukemia. At least two other cell-cycle genes are not expressed at detectable levels in human leukemias. These findings suggest that the activation of cell-division-cycle genes might contribute, like cellular oncogenes, to the phenotype of human malignancies and that, perhaps, new oncogenes could be found by identifying and isolating genes whose expression is dependent on the cell cycle.**

We have identified and initially characterized five cDNA clones whose levels of expression specifically increase during the G_1 phase of the cellular cycle (1). We have selected these cDNA clones from a cDNA library prepared from poly(A)$^+$ mRNA derived from Syrian hamster ts13 cells (2) 6 hr after serum stimulation.

A number of oncogenes have been shown to be expressed in a cell-cycle-dependent manner (3–7), and partial homologies have been detected between the "start" gene of yeasts and certain oncogenes (8). Cellular oncogenes are often activated or overexpressed in transformed cells and in certain types of human neoplasia (9, 10). Therefore, we investigated the expression of three of our cDNA clones in human leukemias. First, though, we have asked whether these sequences are expressed also in a cell-cycle-dependent manner in human cells. For brevity in this paper, we shall call genes that are expressed in a cell-cycle-dependent manner simply "cell-cycle-dependent genes."

MATERIALS AND METHODS

Culture Conditions of Peripheral Blood Mononuclear Cells and Human Diploid Fibroblasts WI38. Peripheral blood was obtained from normal human volunteers. Mononuclear cells were separated on Ficoll-Paque gradients and cultured as described by Maizel *et al.* (11). The cells were washed with Hanks' solution and then cultured in plastic Falcon tissue culture flasks for the desired periods of time at a concentration of 10^6 cells per ml of RPMI 1640 medium supplemented with penicillin, streptomycin, L-glutamine, 5% heat-inactivated fetal calf serum, and phytohemagglutinin (PHA; Sigma) at 10 μg/ml. Each culture was monitored for the level of stimulation by determining autoradiographically the percentage of cells that incorporated [^{3}H]thymidine after 60 hr in culture. After culture, cells were harvested by centrifugation and washed twice in 0.9% NaCl, and total cellular RNA was then extracted. Human WI-38 fibroblasts (12) were cultured in plastic Petri dishes in medium containing 10% fetal calf serum until they almost reached confluency. The serum concentration was then shifted down to 1% calf serum. After 3 days in low serum, the cells were stimulated to enter the cell cycle with fresh medium containing 10% fetal calf serum. At 0, 6, and 16 hours after stimulation, the cells were washed with phosphate-buffered saline and collected with a rubber policeman, and total RNA was extracted.

Leukemic Cells. Leukemic cells were obtained from the M. D. Anderson Hospital in Houston, TX, where the diagnosis was established.

Nucleic Acid Isolation. Total cellular RNA was extracted as described by Frazier *et al.* (13). Recombinant plasmid DNAs were isolated and purified by CsCl centrifugation and phenol/proteinase K extraction after standard procedures (14).

RNA Blot Analysis. Total cellular RNA was denatured with 6.3% formaldehyde and 50% formamide at 65°C and then size-fractionated on a 1% agarose gel containing 6.6% formaldehyde (14). Blotting of RNA to nitrocellulose was done as described by Thomas (15). Nick-translation (16, 17) of the plasmid DNA at high specific activity was performed as described by Lai *et al.* (18). Prehybridization, hybridization, and posthybridization washes for DNA and RNA blots were essentially as described by Wahl *et al.* (19). For autoradiography, the blots were exposed to Kodak XAR-5 film at −70°C in the presence of an intensifying screen.

RESULTS

Expression of ts13 G_1-Specific Genes in the Cell Cycle of Lymphocytes and in Leukemia. In these experiments, three cDNA clones were used that were originally described by Hirschhorn *et al.* (1) and that contain inserts representing sequences preferentially expressed in G_1. These sequences, p2F1, p4F1, and p2A9, were isolated from ts13 cells, which derive from BHK cells (2) and therefore are of Syrian hamster

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Abbreviations: PHA, phytohemagglutinin; CML, chronic myelogenous leukemia; AML, acute myelogenous leukemia; kb, kilobase(s); CLL, chronic lymphocytic leukemia; PDGF, platelet-derived growth factor.

origin. Although some of these sequences are short, they contain reading frames, and one of them, 2A9, has significant homologies with c-fes (unpublished data). Preliminary experiments showed that these sequences hybridized to human genomic DNA, giving (with three different restriction enzymes) band patterns similar to those reported (1) for Syrian hamster genomic DNA (data not shown).

We next asked whether the ts13 G_1-specific sequences we have identified are also expressed in human cells in a cell-cycle-dependent manner. For this purpose, peripheral blood mononuclear cells were stimulated to proliferate with PHA. The methodology we followed for the isolation and stimulation of human lymphocytes is the one described by Maizel *et al.* (11). In Fig. 1 human RNAs are blot-hybridized (15) to two cDNA clones, p4F1 and p2A9 (1). The same amount of total RNA was applied to each lane. p4F1 [which recognizes a band at about 1.6 kilobases (kb)] was induced early in PHA-stimulated human lymphocytes with a high level of expression at 6 hr (Fig. 1, upper band in lane c). By 48 hr after stimulation (lane d), the expression of p4F1 was approximately the same as in unstimulated lymphocytes (lane b). p4F1 also was expressed highly in two patients with chronic myelogenous leukemia (CML; lanes f and g). This will be discussed further below. But we have included here these samples to compare the levels of expression of p4F1 during the cell cycle and in leukemias. Notice also that at this level of autoradiographic exposure, p2A9 gave a distinct band (about 0.9 kb) with the RNAs from the two CML patients (lanes f and g), but it was not detectable in the RNA of resting or stimulated lymphocytes (lanes b, c, and d). At longer exposure times, p2A9 was barely detectable in PHA-stimulated lymphocytes (not shown).

Fig. 2 shows the cell-cycle dependence of p2F1 expression in human lymphocytes. There was a several-fold increase in the intensity of the band in lanes b and c (respectively 6 and 22 hr after PHA stimulation), although the same blot (reused) showed no differences among lanes a, b, and c when another cDNA clone (p3A5, whose expression is not cell cycle dependent during the initial 36 hr of T-lymphocyte stimulation) was used for hybridization (see below). Incidentally, the human RNAs corresponding to the inserts of the three cDNA clones have roughly the same size as their Syrian hamster counterparts (1)—i.e., 1500 bases for p2F1, about 1600 for p4F1, and 900 for p2A9.

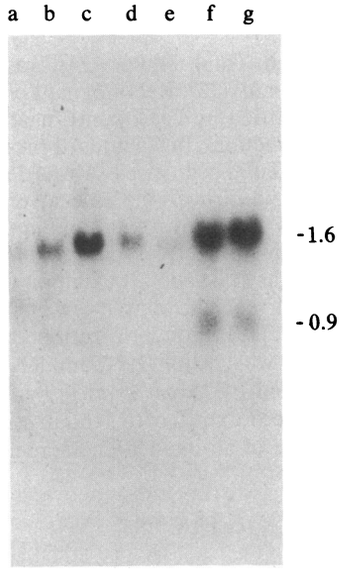

FIG. 1. Expression of p4F1 and p2A9 in PHA-stimulated lymphocytes and leukemic cells. Total RNA was isolated, and 15 μg per lane was electrophoresed on a 1% agarose/formaldehyde gel and subsequently transferred to nitrocellulose as described by Thomas (15). Hybridization was carried out with both plasmids, nick-translated to a specific activity of 4–5 $\times$ 10^8 cpm/μg. The p4F1 insert is 220 base pairs in length, and the p2A9 insert is 100 base pairs. The larger RNA band ($\approx$1.6 kb) corresponds to the p4F1 insert, and the smaller one (0.9 kb) corresponds to the p2A9 insert. The sources of the RNAs in the various lanes are as follows: a, CLL; b, c, and d, human peripheral blood mononuclear cells cultured with PHA for 0 hr (lane b), 6 hr (lane c), and 48 hr (lane d); e, AML; f and g, CML.

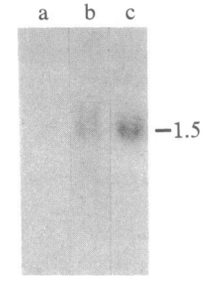

FIG. 2. p2F1 expression in PHA-stimulated human lymphocytes. Total RNA was isolated from peripheral blood mononuclear cells before stimulation (lane a) and 6 hr (lane b) or 22 hr (lane c) after PHA stimulation. The same amount of RNA (15 μg per lane) was electrophoresed on a 1% agarose/formaldehyde gel and transferred to nitrocellulose as described by Thomas (15). Nick-translation of the probe, hybridization, and washing conditions were as described. The whole plasmid (p2F1 has an insert of 420 base pairs) was used. Size is shown in kb.

Expression of p2A9 in Human Diploid Fibroblasts. Because the expression of p2A9 in PHA-stimulated lymphocytes was barely detectable, we investigated the expression of this sequence in WI-38 cells, a strain of human diploid fibroblasts (10). Quiescent WI-38 cells were serum-stimulated, and total RNA was prepared at different times after stimulation and blotted as before (15). The blot hybridization is shown in Fig. 3, which shows that the RNA sequence corresponding to p2A9 was expressed in human diploid fibroblasts in a cell-cycle-dependent manner similar to the expression in Syrian hamster fibroblasts (1). We also analyzed the cell-cycle dependence of p2F1 and p4F1 expression in human fibroblasts. The pattern of induction of p2F1 was similar to that of p2A9, whereas the induction of p4F1 reached its peak at 6 hr (not shown) as in human lymphocytes (Fig. 1).

Therefore, it seems that these three cDNA clones (p4F1, p2F1, p2A9) are all expressed in human cells, and all can be expressed in a cell-cycle-dependent manner, but only two of

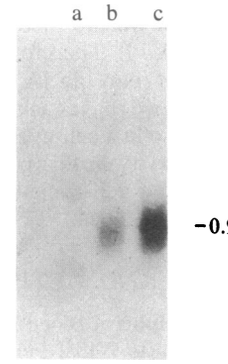

FIG. 3. Expression of p2A9 in serum-stimulated human diploid fibroblasts. Total RNA (15 μg per lane) was electrophoresed, blotted, and hybridized as described. WI-38 cellular RNA was isolated at 0 (lane a), 6 (lane b), and 16 (lane c) hr, respectively, after serum stimulation and was hybridized with nick-translated p2A9. Size is shown in kb.

them (p4F1 and p2F1) are clearly dependent on the cell-cycle in lymphocytes.

Expression of c-*myc* During the Cell Cycle of Lymphocytes. It has been shown recently (3) that c-*myc* is an inducible gene if mouse lymphocytes are activated by mitogens or mouse fibroblasts are stimulated with platelet-derived growth factor (PDGF). c-*myc* induction is an early event in the cell-cycle progression associated with the transition $G_0 \rightarrow G_1$. We used in our experiments human peripheral blood mononuclear cells stimulated to proliferate with PHA. The purpose was to use the levels of expression of c-*myc* for comparison to the cDNA clones isolated from ts13 cells. Fig. 4 shows the relative densitometer reading of a blot hybridization obtained with the RNA of PHA-induced lymphocytes and the human c-*myc* probe (20). The G_0 level of expression was made equal to 1. There was a several-fold induction of c-*myc* at 6 hr after stimulation. The level of induction approximated (not shown) the constitutive expression of c-*myc* in the human HL-60 cell line in which the c-*myc* locus is amplified (21, 22) and highly expressed (23). In Fig. 4 we also have included a densitometer reading of another blot hybridization in which the same RNAs were hybridized to the cDNA clone 3A5, which, as mentioned above, is not expressed in a cell-cycle-dependent manner in lymphocytes, at least up to 36 hr after stimulation.

A high expression of c-*myc* was detectable in a patient with acute myelogenous leukemia (AML) and two patients with CML, in agreement with previous reports (10, 24). It is worth noting that there was a complete lack of expression of c-*myc* in a patient with chronic lymphocytic leukemia (CLL). This finding applies to all of the CLL RNA samples we examined (five samples) and to all of the cell-cycle genes we used. This finding is in agreement with the fact that CLL cells have a low spontaneous mitotic index (25–27) and are poorly stimulated by mitogens (28).

Expression of ts13 Cell-Cycle Genes in Human Leukemias. We next asked if these cell-cycle-specific sequences are expressed in hematological malignancies and if there is an association between expression of cell-cycle-dependent genes and specific hematological malignancies. We have selected RNAs from patients with CLL, acute lymphocytic leukemia (ALL), CML, and AML. The cellular composition of the various hematological malignancies can be summarized as follows: (*i*) CML, 30–50% dividing immature cells (the RNA content in granulocytes is 1/10th that of immature forms; therefore, the contribution of granulocyte RNA to total CML RNA is minimal); (*ii*) AML, 80–95% were blast cells; (*iii*) ALL, 80–95% were blast cells; and (*iv*) CLL, 99% were small lymphocytes. Fig. 5 shows a blot-hybridization pattern of p4F1 against several leukemic RNAs. A single transcript of ≈1,600 nucleotides was detectable. This transcript was present in high abundance in total RNA obtained from leukocytes of patients with CML (Fig. 5, lanes f–i). A low level of expression of p4F1 was barely detectable in patients with ALL (Fig. 5, lanes a–c) and CLL (Fig. 5, lanes j and k). Intermediate levels of expression were detectable in patients with AML (lanes d and e).

Fig. 6 shows the hybridization pattern of p2F1 and p2A9 to different leukemic RNAs. p2F1 hybridized mostly with RNAs from patients with CML and AML (Fig. 6, upper band in lanes c–h). The expression of p2A9 was restricted to RNA obtained from cells of patients with CML (Fig. 6, lower band in lanes e–h). We analyzed several more leukemic RNAs for the expression of ts13 cell-cycle genes, and the pattern emerging is that the highest level of expression is present in CML patients, and the lowest, in CLL patients. Intermediate levels of expression are observed in AML.

Not All Cell-Cycle Genes Are Highly Expressed in Human Leukemias. Two other cDNA clones related to the cell cycle also were investigated. These are JE-3 and KC-1, which were isolated from mouse 3T3 cells (29) and are inducible by platelet-derived growth factor (PDGF). A filter previously hybridized to ts13 cDNA clones was, after removal of the previous probes, reused for hybridization with nick-translated probes prepared from JE-3 and KC-1. Fig. 7 shows the results obtained with KC-1. A band, roughly the same size as in 3T3 cells, was detectable in stimulated human lymphocytes that hybridized to the KC-1 probe. The RNA was expressed in a cell-cycle-dependent manner with the highest expression obtained after only 3 hr of stimulation with PHA (lane b), but it was not detectable (even after overexposure) in RNAs prepared from leukemic cells. Similar results were obtained with JE-3 (not shown).

Finally, none of the ts13 probes (p4F1, p2F1, and p2A9) or the 3T3 probes (KC-1 and JE-3) hybridized to RNA isolated from human granulocytes (for KC-1, lane k in Fig. 7), although all of the ts13 cDNAs hybridized to a different extent to RNA from normal bone marrow cells. We deliberately did not pursue further our study of bone marrow cells because of the difficulty of obtaining pure subfractions of

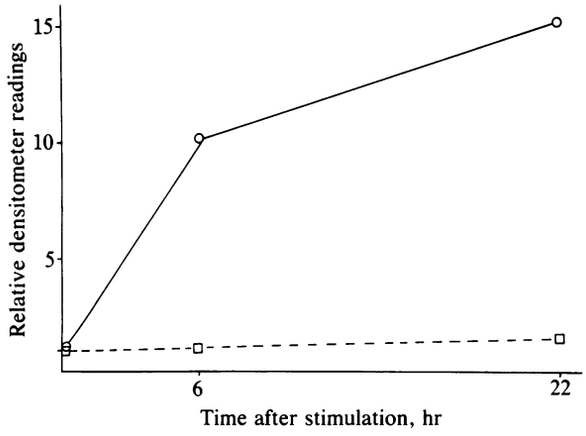

FIG. 4. Levels of expression of c-*myc* and 3A5 in PHA-stimulated lymphocytes. Blot hybridizations of RNA were made from lymphocytes as described in the previous figures and were hybridized to either a c-*myc* probe or a 3A5 probe. The bands were scanned with a laser densitometer. Levels at time 0 were made equal to 1, so that the ordinate gives the fold increase. It should be noted, though, that at time 0, the intensity of the 3A5 band was much higher than that of the c-*myc* band.

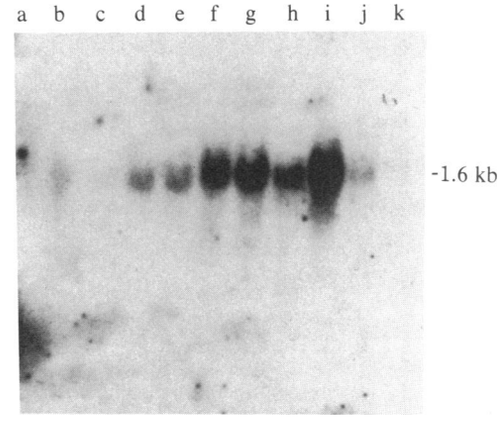

FIG. 5. p4F1 expression in human leukemias. Total RNA (15 μg per lane) was electrophoresed on a 1% agarose/formaldehyde gel and transferred to a nitrocellulose filter as described by Thomas (15). Nick-translation of p4F1, hybridization, and washing conditions were as described. The sources of leukemic cell RNAs were as follows: ALL (lanes a–c), AML (lanes d and e), CML (lanes f–i), and CLL (lanes j and k).

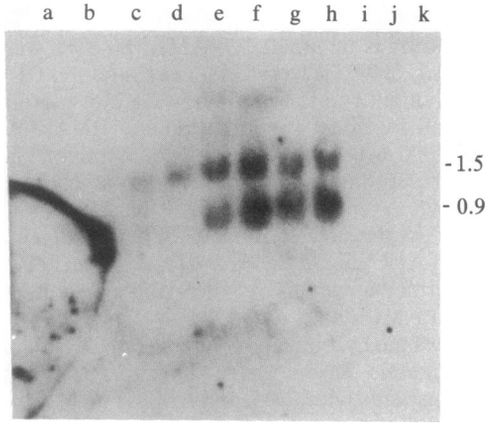

FIG. 6. Expression of p2F1 and p2A9 in human leukemias. Total RNA (15 μg per lane) was electrophoresed on a 1% agarose/formaldehyde gel and transferred to a nitrocellulose filter as described by Thomas (15). Nick-translation of p2F1 and p2A9 cDNA clones, hybridization, and washing conditions were as described. The sources of leukemic cell RNAs were as follows: ALL (lanes a and b), AML (lanes c and d), CML (lanes e–h), and CLL (lanes i–k). Sizes are shown in kb.

normal myeloid cells in sufficient amounts for the analysis of RNAs.

DISCUSSION

We have shown that the ts13 G_1-specific cell-cycle genes are closely related to human genes both in their sequence and in the cell-cycle dependence of their expression. The expression of p4F1 and p2F1 is clearly cell cycle dependent in human lymphocytes; p2A9 is expressed in a cell-cycle-dependent manner in human diploid fibroblasts. This raises the question of whether the expression of certain cell-cycle-dependent genes may be cell type specific. It should be noted at this point that the amount of RNA in each lane was carefully monitored. The same blot hybridizations used in these experiments were (after removal of the original probes) rehybridized to a probe (3A5) whose expression is not cell cycle dependent at least for the first 36 hr after PHA stimulation. The amount of expression of the 3A5 message was uniform across the lanes, indicating that the RNA amounts in each lane were comparable. This was especially important for G_0 lymphocytes, in which the extraction of undegraded RNA is particularly challenging. The time of induction of these genes is similar both in serum-stimulated hamster fibroblasts (1), in human fibroblasts, and in human lymphocytes after PHA induction. The induction of p2F1 and p4F1 resembles the induction of the c-*myc* gene in both mouse and human systems. The function of the c-*myc* or v-*myc* gene has been associated with a role in the establishment of transformation of cells in culture (30). It will be of interest to determine if the genes corresponding to the ts13 cDNA clones we have studied serve a similar function. Interestingly, the p53 gene was first shown to be expressed in a cell-cycle-dependent manner (31) and subsequently found to replace c-*myc* as a second oncogene (32).

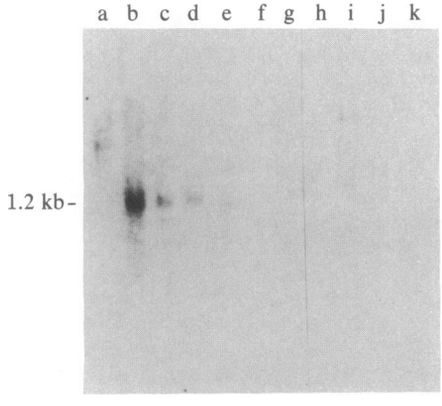

FIG. 7. pUC-KC-I expression in PHA-stimulated lymphocytes and leukemic RNAs. Total RNA (15 μg per lane) was electrophoresed on a 1% agarose/formaldehyde gel and transferred to nitrocellulose as described by Thomas (15). Nick-translation of the KC-I probe (25), hybridization, and washing conditions were as described. The sources of leukemic cell RNA were as follows: lymphocytes at time 0 (lane a), 3 hr (lane b), 6 hr (lane c), 12 hr (lane d), and 22 hr (lane e) after PHA stimulation; AML (lanes f and g); CML (lanes h and i); CLL (lane j); and normal granulocytes (lane k).

We also have shown that some of these cell-cycle-dependent genes are differentially expressed in human leukemias. A high expression of c-myc in human hematological malignancies has been reported (23, 24, 33). We have observed in approximately two-thirds of patients with acute leukemias significant but variable expression of c-*myc* messenger (unpublished results). Of the three ts13 cDNA clones, one (p2A9) is highly expressed in CML, and two (p2F1 and p4F1) are preferentially expressed in both CML and AML. Interestingly, two other cell-cycle-dependent cDNA clones (JE-3 and KC1), originally isolated from PDGF-stimulated 3T3 cells, were expressed in a cell-cycle-dependent manner in human lymphocytes but were not expressed (at least to a detectable level) in human leukemias. Finally, none of the cell-cycle genes we have tested (including c-*myc*) are expressed at a detectable level in nonproliferating granulocytes.

The findings we have described should be looked at in the light of recent observations that there is overlap between cell-cycle-dependent genes, oncogenes, growth factors, and receptors for growth factors. Apart from the well-known homologies between PDGF and v-*sis* (34, 35) and the epidermal growth factor receptor and v-*erbB* (36), partial homologies have been reported between the cellular oncogene B-*lym* and transferrin (37), between a serum-inducible cDNA clone and prolactin (38, 39), between p2A9 and c-*fes* (1), and between the "start" gene of yeast and several vertebrate oncogenes (8). Some of the cellular oncogenes are known to be expressed in a cell-cycle-dependent manner (3–7, 31) and to be overexpressed in cases of human malignancies (10, 20, 21). The finding that the ts13 G_1-specific cDNA clones are overexpressed in some forms of leukemia, especially CML, raises the question of whether the overlapping between oncogenes and cell-cycle dependent genes may be more common (and more important) than hitherto suspected. In this respect, the observation by Campisi *et al.* (5) that the cell-cycle control of c-myc expression (but not of c-ras) is lost after chemical transformation clearly supports the hypothesis that the regulation (or deregulation) of cell-cycle-dependent genes may contribute to the transformed phenotype. The fact that two of these genes (p2F1 and P4F1) are clearly detectable both in lymphocytes and in CML, while p4F1 is barely detectable in normal bone marrow cells, supports the concept of Till and McCulloch (40) that some forms of leukemias, and especially CML, may originate from a myeloid–lymphoid stem cell. Alternatively, the cell-type specificity of p2A9 raises the fundamental question of whether some cell-cycle genes might be cell type specific and, as postulated for the oncogenes, may become transforming when expressed in an inappropriate cell type (41).

In conclusion, these findings suggest that cell-cycle-dependent genes may represent an essential tool to our understanding of the molecular events associated with the impairment in proliferation control that characterizes the biological behavior of a neoplastic cell. It is indeed possible that other genes with the biological activity of cellular

oncogenes may be identified by identifying genes whose expression is cell cycle dependent.

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