

# The expression of the non-receptor tyrosine kinases Arg and c-abl is differently modulated in B lymphoid cells at different stages of differentiation

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Received 29 May 2002; revised 30 July 2002; accepted 8 August 2002

First published online 19 August 2002

Edited by Giulio Superti-Furga

**Abstract** The products of the human ARG gene and the human ABL gene characterize the Abelson family of non-receptor tyrosine protein kinases. Both genes are ubiquitously expressed. The interactions of these two similar protein kinases are still not well known, although it has been suggested that they could cooperate, with redundant actions, to provide intracellular signals in the cells. Lymphopenia occurs in mice with homozygous disruption of c-abl, indicating that in certain tissues Arg is unable to substitute c-abl functions. In B and T lymphoid cell lines at different stages of differentiation, we studied, by a reverse transcriptase-competitive polymerase chain reaction and Western blotting, Arg and c-abl in order to evaluate whether the expression pattern of the two genes could give insight as to why they do not exhibit overlapping roles in lymphocytes and whether the product levels of the two genes are related to lymphoid differentiation. The data showed that their expression is differently modified in lymphoid B cell lines. The highest Arg transcript and protein levels are in the mature B cells. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** Arg; c-abl; Non-receptor tyrosine kinase; Competitive polymerase chain reaction; Lymphoid cell line; Differentiation

## 1. Introduction

The products of the human and mouse ARG and ABL genes and the *Drosophila* and nematode ABL genes characterize the Abelson family of non-receptor tyrosine protein kinase (TPK) [1–3]. The human Arg and c-abl proteins [3] have a high degree of amino acid sequence identity (90–94%) in the tyrosine kinase, SH2 and SH3 domains [3], and have two isoforms named IA and IB [3–6]. The long C-terminal domain of Arg is fairly divergent from that of c-abl although both contain three proline-rich sequences that bind to the SH3 domains of adaptor proteins [7,8]. Furthermore, the actin-

binding sequences present in c-abl are partially conserved in the C-terminal domain of Arg [9] in which a further F-actin-binding domain is present [10]. The Arg product is located in the cytoplasm [5], instead c-abl has a nuclear and cytoplasmic localization [11]. Arg and c-abl are ubiquitously expressed and Arg has a higher expression in nervous tissues [12]. Arg mRNA increases during granulocytic and macrophage-like differentiation of HL-60 cells [13]. The involvement of Arg in human pathology has been reported in acute leukemia [14,15] and in colon cancer [16]. It has been suggested that these two non-receptor TPKs in the cells in which they are coexpressed could cooperate with redundant actions that are still not well known. Both genes could cooperate in DNA repair or in cell cycle progression mechanisms [1,17], furthermore, interacting with actin filaments they play a role in neurulation [18], at the leading edge of growing axons [19] and in cell migration [20]. Like c-abl, Arg seems to have a role in genotoxic apoptosis, mediated in Arg by phosphorylation of the apoptotic Siva1 protein [21]. The development of mice harboring homozygous disruption of c-abl (c-abl<sup>-/-</sup>) or Arg [18,22,23] is likely permitted by the redundant actions. In c-abl<sup>-/-</sup> mice only relatively limited pathologies occur, i.e. lymphopenia [24,25], indicating that in lymphoid tissues Arg is unable to substitute c-abl functions. In B and T lymphoid cell lines at different stages of differentiation, we studied, by a reverse transcriptase (RT)-competitive polymerase chain reaction (PCR), Arg and c-abl transcripts and by Western blotting the respective proteins in order to evaluate whether the expression pattern of the two genes could give insight as to why they do not exhibit overlapping roles in lymphocytes and whether the expression of the two genes is related to lymphoid differentiation. The data showed that the expression of the two transcripts is differently modified in lymphoid B cell lines. The highest Arg transcript and protein levels are in the mature B cells.

## 2. Materials and methods

### 2.1. Cells

Molt-4 (common thymocyte phenotype, T cells), Jurkat (post-thymic phenotype, T cells), Raji (mature B cells) and TOM-1 (Philadelphia chromosome positive cells) cells were obtained from the American Type Culture Collection. Nalm-6 (early pre-B cells), and LP-1 (plasmacytoma) were obtained from the German Collection of Micro-

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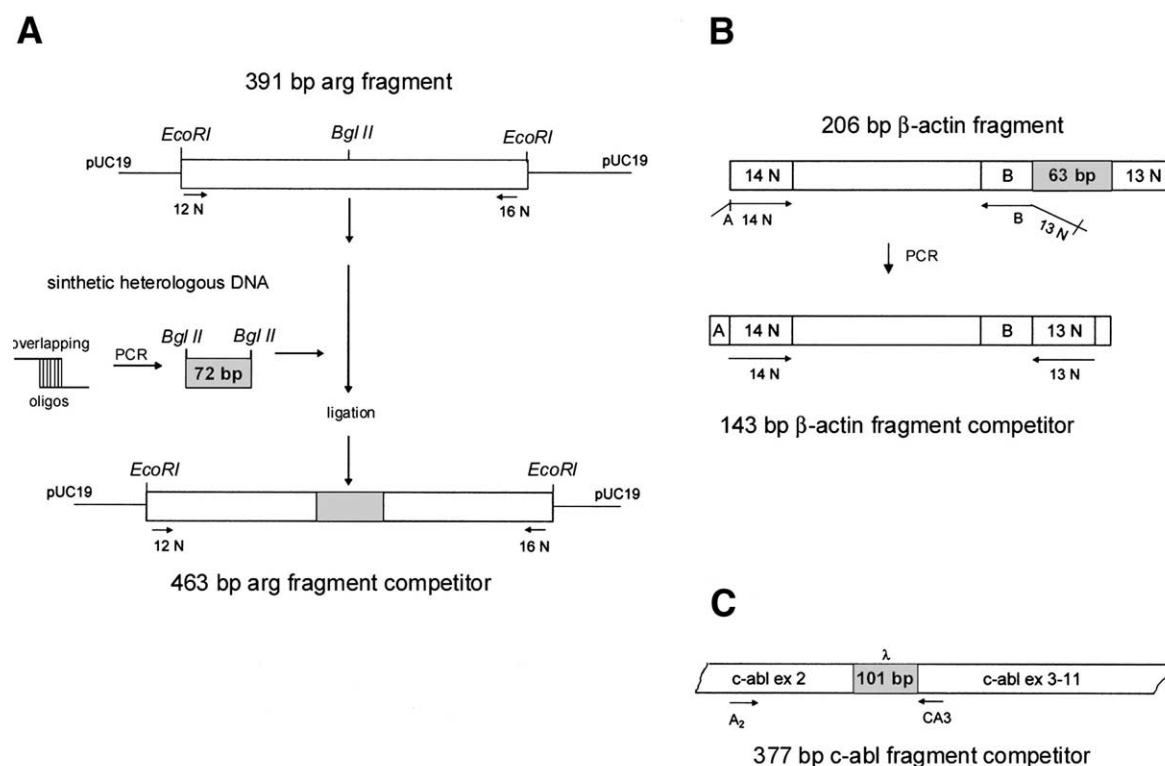


Fig. 1. A: Scheme of Arg competitor construction. B:  $\beta$ -Actin competitor construction. C: c-abl competitor (a kind gift from N.C.P. Cross, Royal Postgraduate Medical School, London, UK).

organism and Cell Cultures. AllPo (early pre-B cells) [26] was a kind gift from Dr. A. Biondi (Monza, Italy). Cells were cultured with RPMI 1640 medium supplemented with 10% fetal calf serum. The growth rate of the cells was assessed by daily count and viability was determined by trypan blue exclusion. The growing Raji and AllPo cells were treated with actinomycin D (10  $\mu$ g/ml) to study mRNA stability at various times after drug exposure. Differentiation of Nalm-6 from early to late pre-B cells was induced by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) ( $1 \times 10^{-7}$  mol/l), and the modifications of the cellular surface antigenic phenotype were analyzed with monoclonal antibodies 12, 36 and 72 h after induction [27].

## 2.2. Immunophenotype and cell cycle analysis

The flow cytometric cell surface immunophenotype (Table 1) was determined by specific monoclonal antibodies (Becton Dickinson, San José, CA, USA) and the standard direct immunofluorescence procedure. Biparametric bromodeoxyuridine (BrdUrd)/DNA flow cytometric analysis was performed on at least 20 000 exponentially growing cells by adding 20  $\mu$ M BrdUrd for 15 min, 24 or 48 h. The cells were then directly fixed in 70% ethanol before BrdUrd immunodetection [28] by the FacsCalibur instrument (Becton Dickinson). The percentage of the cell cycle phase distribution was calculated by the method of Krishan and Frei [29].

## 2.3. RNA extraction, cDNA synthesis and PCR

RNA extraction from exponentially growing cells and DNase treatment have been described previously [13]. An aliquot of 8  $\mu$ g of DNA-free RNA was reverse-transcribed in a 40  $\mu$ l reaction, and 1  $\mu$ l cDNA was amplified as described [13].

## 2.4. Construction of competitor templates

A 391 bp fragment of Arg cDNA amplified with 12N and 16N primers was cloned into the pUC19 *EcoRI* site. The Arg insert was cut with *BglII*<sup>o</sup> and ligated to a 72 bp heterologous synthetic DNA fragment, generated by PCR amplification of two 46 bp oligonucleotides overlapping for 20 bp. The resultant 3.15 kb plasmid was linearized with *NdeI*<sup>o</sup> and quantitated (Fig. 1).

$\beta$ -Actin competitor (Fig. 1) was a truncated recombinant cDNA generated by PCR amplification of a 206 bp  $\beta$ -actin cDNA fragment, obtained as previously described [13]. In a 100  $\mu$ l PCR reaction mixture containing 25 pmol each of the antisense B13N and the sense A14N primers, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTP, and 2.5 U AmpliTaq (Applera Italia, Monza, Italy), 1 ng of  $\beta$ -actin cDNA fragment was amplified with the following program: denaturation at 94°C/30 s; annealing at 55°C/30 s for two cycles, at 60°C/30 s for three cycles, at 65°C/30 s for 35 cycles; elongation at 72°C/1 min. The antisense B13N primer is composed of the juxtaposed B and 13N sequences,

Table 1  
Phenotype of human B and T lymphoid cell lines

Lines	Stage	HLA DR	CD34	CD10	CD19	CD20	CD22	CD23	CD37	CD38	CyIg	Smlg
Nalm-6	Pre-B	++	–	++	++	–	±	±	±	+++	±	–
AllPo	Pre-B	++	–	–	+++	–	±	–	–	+++	–	–
Raji	Mature B	+++	–	+++	+++	+++	+++	±	++	+++	±	+++
LP-1	Plasma cell	±	–	–	–	–	±	+	–	++	+	–
		CD1a	CD2	CD3	CD4	CD5	CD7	CD8	CD38			
Molt-4	Thymic	+++	+++	–	–	+++	+++	+++	+++			
Jurkat	Post-thymic	+++	+++	++	–	–	+	–	±			

+++ , >75% positive cells; ++, 50–75%; +, 25–50%; ±, 5–25%; –, 0–5%. CD, cluster of differentiation; CyIg, cytoplasmic Ig; Smlg, surface Ig.

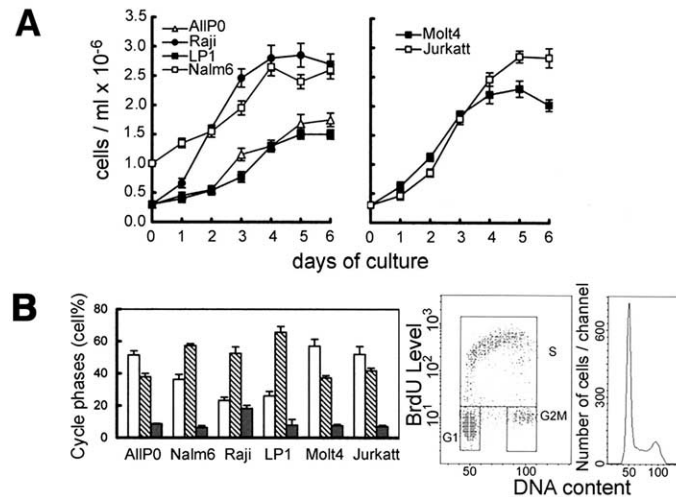


Fig. 2. A: Growth rate of lymphoid cell lines. Exponentially growing cells were plated at a density of  $3 \times 10^5$ /ml (Nalm-6 cells,  $1 \times 10^6$ /ml). The total number of cells determined in a Thoma chamber is expressed as means  $\pm$  S.E.M. of four separate experiments. Cell mortality during the exponential growth assessed by trypan blue exclusion was in the range of 5–7%. B: Percentage of the cell cycle phase distribution ( $\square$  G<sub>1</sub>; S;  $\blacksquare$  G<sub>2</sub>/M) during exponential growth (day 2) as determined by biparametric BrdUrd/DNA flow cytometric analysis (left). Representative flow cytometric analysis in Raji cells treated for 15 min with BrdUrd (right).

which in the 206 bp  $\beta$ -actin cDNA are separated by 63 bp, and of a 5' tail of 6 nt. The sense A14N primer was the 14N sequence plus a 5 nt tail at its 5' end. The 154 bp competitor obtained was purified on agarose gel and quantified. The 13N and 14N  $\beta$ -actin primers spanned exons 3–4 and 4–5 [30], amplified a 143 bp  $\beta$ -actin competitor and do not amplify genomic DNA.

The c-abl competitor (Fig. 1) provided as a kind gift by N.C.P. Cross (Royal Postgraduate Medical School, London, UK) has been described [31]. The Arg competitor, the  $\beta$ -actin competitor and the c-abl competitor were serially diluted in the range of  $10^9$ –10 molecules per 2.5  $\mu$ l with steps at every half order of magnitude on a logarithmic scale, i.e.  $10^9$ ,  $3.2 \times 10^8$ ,  $10^8$ , etc. The same set of dilutions for each competitor was used for all the experiments.

Sequence of primers used for generating competitors: B13N: 5'-AGCAGGGAGGAGCAATGATCTTGATCTTCATGGTGCCGC-CAGACAGCACT-3' antisense [30]; A14N: 5'-CGAATTTCC-TTCCTGGGCATGGAGTCTGT-3' sense [30]; and for competitive PCR reactions: 12N: 5'-ATGAATTCCTTTAGGTGTGTACT-3' sense [3]; 16N: 5'-ATGAATCCAAAAGCCCAGACGTCA-3' antisense [3]; 13N: 5'-GAGGAGCAATGATCTTGATCTTCA-3' antisense [30]; 14N: 5'-TTCCTTCTGGGCATGGAGTCTGTG-3' sense [30]; A2: 5'-TTCAGCGGCCAGTAGCATCTGACTT-3' sense [31]; CA3: 5'-TGTTGACTGGCGTGATGTAGTTGCTTGG-3' antisense [31]. The fragment sizes from competitors and transcripts are reported in Figs. 1 and 3.

### 2.5. Competitive PCR

A 2.5  $\mu$ l cDNA sample (1  $\mu$ l for  $\beta$ -actin) plus 2.5  $\mu$ l of competitor dilution were amplified in a 50  $\mu$ l mixture containing 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M dNTP, 0.5  $\mu$ M of each primer, and 1.5 U of AmpliTaq. The amplification program used for Arg and c-abl was 95°C/30 s, 60°C/30 s (for c-abl 64°C/30 s), 72°C/30 s and 1 s/cycle of autoextension for 30 cycles. For  $\beta$ -actin it was 95°C/30 s, 55°C/30 s, 72°C/1 min for 35 cycles. The quantity of target mRNA present in the sample was defined by the regression curve obtained from densitometric analysis of electrophoresed bands by plotting log (optical density (OD) target band/OD competitor band) against log competitor molecules added in PCR reactions [32]. The value at the equivalence point between the target molecules and the competitor molecules multiplied by the ratio between the competitor dimension and the target dimension (1.18 for Arg; 1.37 for c-abl; 0.69 for  $\beta$ -actin) gives the number of target molecules in the sample, expressed as molecules per  $\mu$ g of RNA. The RNA integrity and the variations of RNA or cDNA among samples were checked using as a reference the  $\beta$ -actin mRNA level of each cell line, established as a mean of four independent experiments performed in duplicate. The Arg and c-abl transcript levels in the same sample were normalized by multiplying by the ratio between

the mean level of  $\beta$ -actin of the respective cell line and the  $\beta$ -actin level of the specific sample.

### 2.6. Western blotting

Cells were lysed with 1% Triton X-100, 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 20  $\mu$ g/ml leupeptin, 5  $\mu$ g/ml aprotinin, 1 mM PMSF. The protein concentration was determined by Bio-Rad microassay (Hercules, CA, USA). The lysates (100  $\mu$ g) separated on a 7.5% SDS-PAGE gel were blotted to nitrocellulose membrane. The Ponceau S stain of the blot was obtained to show equal loading of lanes. Western blotting was performed with antibodies specific to mouse Arg [18] directed against the SH2 and SH3 domains (a kind gift of A. Koleske, Yale University, CT, USA). These antibodies proved to be able to recognize, with Western blot, human Arg expressed in bacteria and were unable to cross-react with human Abl, when tested with a BCR/ABL positive TOM-1 human cell line. The anti-Abl 8E9 antibodies (Pharmingen, San Diego, CA, USA) and anti-actin antibodies (Sigma-Aldrich, Saint Louis, MO, USA) were used to detect c-abl and  $\beta$ -actin proteins respectively. The detection has been performed with secondary antibodies coupled to horseradish peroxidase and with SuperSignal Detection System (Pierce, Rockford, IL, USA).

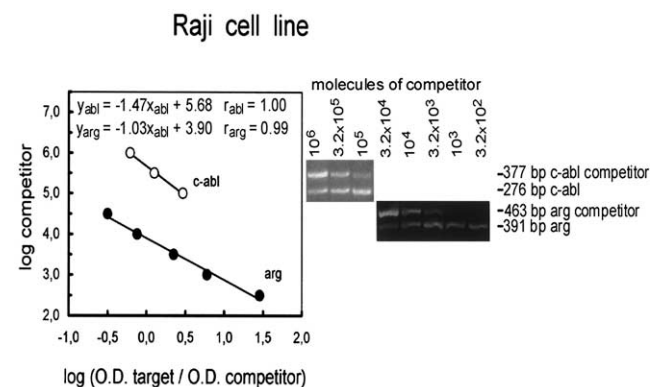


Fig. 3. Quantification with competitive PCR of Arg and c-abl transcripts. Representative experiment in the Raji B cell line. The regression curve obtained by plotting log (OD sample band/OD competitor band) against log competitor molecules gave the equivalence point between the number of molecules of sample transcript and competitor. The value multiplied by a factor of 1.18 for Arg and 1.37 for c-abl gave the number of target molecules that will be expressed as molecules per  $\mu$ g of RNA.

3. Results

3.1. Cell line growth rate, cell cycle and phenotype

The growth rate of different cell lines showed that whereas Jurkat and Molt-4 cells had substantially the same growth

rate, the cell lines AIIPO, Nalm-6 and LP-1 grew more slowly and Raji cells had the highest growth rate (Fig. 2).

Cell cycle analysis showed that the highest percentage of Nalm-6, Raji and LP-1 cells was in the S phase, whereas the remaining cell lines were more represented in the G<sub>1</sub> phase.

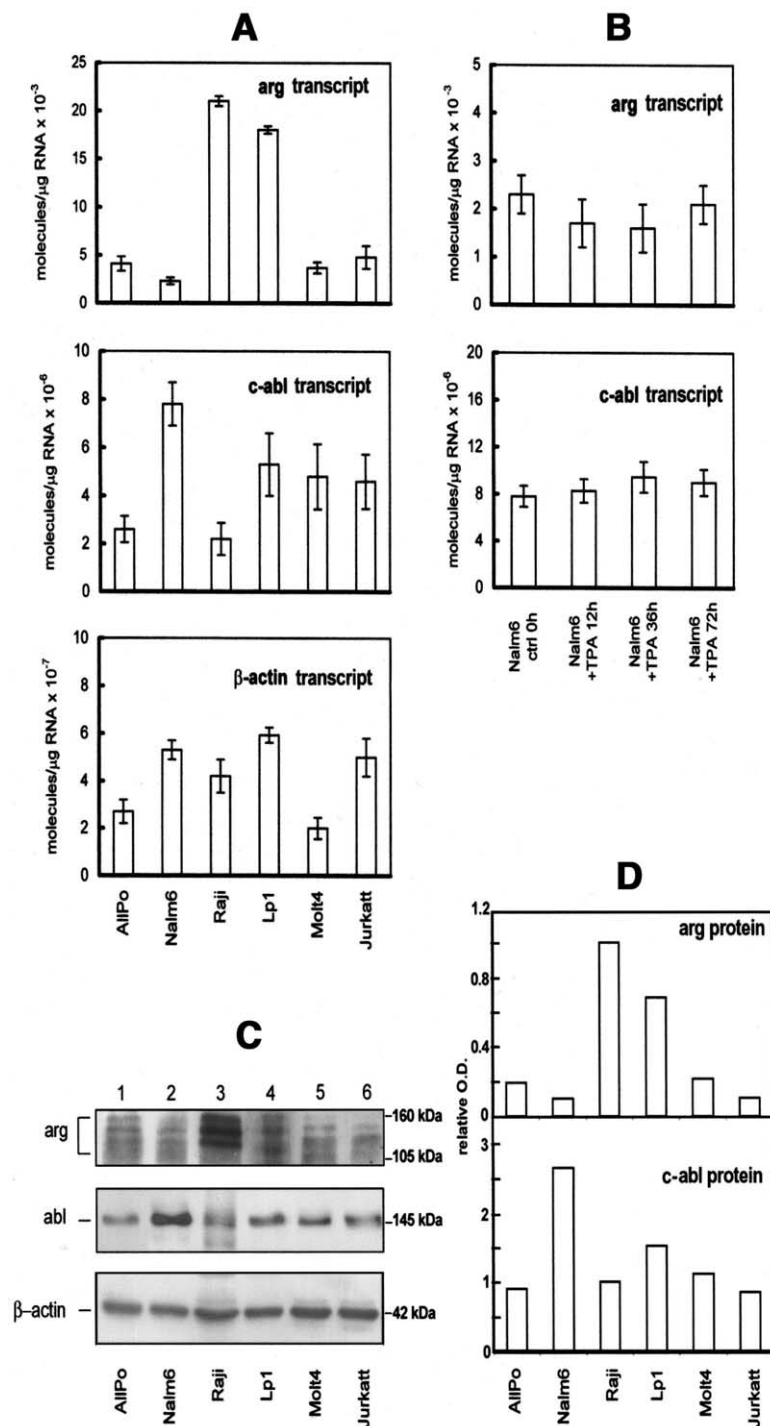


Fig. 4. A: Level of Arg, c-abl and  $\beta$ -actin transcripts in human B and T lymphoid cell lines as evaluated by competitive RT-PCR. The values, expressed as number of transcript molecules/ $\mu$ g total RNA, are means  $\pm$  S.E.M. of four independent experiments. B: Level of Arg and c-abl transcript levels in Nalm-6 cells treated with TPA for 12, 36 and 72 h, as evaluated by competitive PCR. Maturation of Nalm-6 from pre-B to late pre-B phenotype was established on the basis of the variation in surface markers: appearance of CD20, increment in CD22 and CD19, reduction of CD10. C: Western blot analyses with anti-Arg, anti-c-abl and anti- $\beta$ -actin antibodies of 100  $\mu$ g cell lysate from B and T cell lines. D: Level of Arg and c-abl proteins evaluated by densitometric analysis of Western blot. The values, expressed as relative OD, have been referred to Raji cell values considered equal to 1.

The percentage of cells in the G<sub>2</sub>/M phase was low for all cell lines except Raji cells, in which the value was two-fold (Fig. 2).

The phenotype of the studied cell lines on the basis of surface markers is shown in Table 1 and evidenced the AllPo and Nalm-6 as pre-B cells, Raji as mature B cells, LP-1 as plasma cells, Molt-4 as a thymic cell, and Jurkat as a post-thymic cell [33].

### 3.2. Level of Arg, c-abl and β-actin transcript in lymphoid cell lines as evaluated by competitive PCR

The principal condition to derive absolute quantitative information by competitive PCR is that the target and competitor sequences are amplified with equal efficiencies denoted by the regression curves with the slope close to -1, with  $r=1$  (Fig. 3) and the ratio of competitor band/sample band between 1.5 and 0.5 [32]. The amplified Arg transcript showed an almost identical level in the less mature pre-B cells (Nalm-6, AllPo), in the immature T cell line (Molt-4), and in the post-thymic cell line (Jurkat), whereas in the Raji cells (mature B cell) and in the LP-1 cells (plasma cell) it was much higher ( $21$  and  $18 \times 10^3$  molecules/μg RNA) (Fig. 4). The c-abl transcript level in the cell lines tested was two to three log higher than the Arg transcript of the corresponding cell lines (Fig. 4). In the two T cell lines, the c-abl transcript level was similar. Instead, in B cell lines the mature (LP-1) and mainly the immature (Nalm-6) cells showed a high level of c-abl mRNA, with respect to the other two cell lines, Raji (mature) and AllPo (immature), that had low levels of c-abl. The ratio Arg/Abl that can be derived from our data reflects the pattern of Arg expression. The six cell lines studied had their own characteristic level of β-actin mRNA. All the data, obtained with the same set of competitor dilutions, were checked with other sets of independent dilutions, and similar results were obtained.

### 3.3. Arg and c-abl transcript in the TPA-treated Nalm-6 cell line

Induction of differentiation of Nalm-6 cells from the pre-B to late pre-B phenotype by TPA, as proven by phenotypic variation of surface markers [27], showed that the Arg and c-abl mRNA level did not vary significantly after 12, 36 and 72 h of treatment (Fig. 4).

### 3.4. Characterization of Arg mRNA half-life

The Arg transcript half-life was established in Raji and AllPo cells. The cells were treated with actinomycin D (10 μg/ml) to obtain the maximal blockage of transcription. Two independent actinomycin treatment experiments per cell line were performed, and equivalent amounts of RNA were extracted from cells taken at various intervals of up to 3 h. Actinomycin D experiments might give variable values and transcripts may have different stabilities in different cells [34,35], nevertheless, our experiments gave comparable results and the estimated half-life of Arg mRNA was  $\cong 60$  min in Raji and AllPo cells (Fig. 5).

### 3.5. Level of Arg and c-abl protein in lymphoid cell lines as evaluated by Western blotting

Western blot analysis of lymphoid cell line lysates shows with specific anti-c-abl antibodies a discrete band of 145 kDa and with anti-mouse Arg antibodies able to recognize

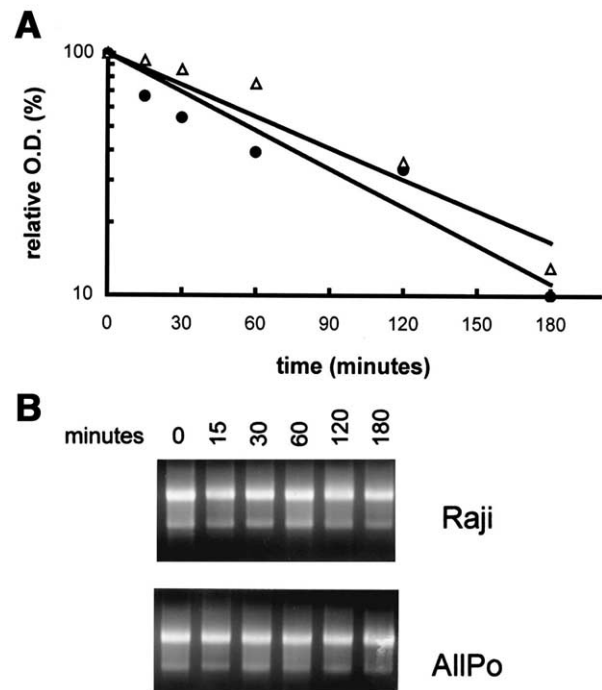


Fig. 5. Effect of actinomycin D on Arg expression in Raji (●) and AllPo (△) cells. A: Densitometric evaluation of a gel-electrophoresed Arg-amplified band plotted on a semilogarithmic scale. Equal amounts of RNA (8 μg) extracted from cells at various time points were reverse-transcribed and the amount corresponding to 0.2 μg was amplified with linear amplification for 35 cycles as described [13]. The control sample was defined as 100% amount of Arg mRNA. The half-life (50% relative OD point) was extrapolated from the best fit line obtained using non-linear regression analysis. B: Gel electrophoresis of an RNA aliquot corresponding to  $2 \times 10^5$  Raji and AllPo cells treated with actinomycin D at various time points.

also human Arg [36] a set of bands as previously shown [18]. These bands located between the 105 and 160 kDa molecular weight markers (Fig. 4) have a different reciprocal intensity among the cell lines studied. The value of Arg and c-abl protein level has been obtained by densitometric analysis normalizing the respective OD with the respective β-actin OD in each cell line. Considering an equal protein loading of the lanes, as evidenced by the Ponceau S stain of the Western blots (data not shown), this procedure provides a reliable representation of the relative level of proteins. The normalized Arg and c-abl OD in each cell line has been related to the respective values of Raji cells considered equal to 1. The cumulative value of the different protein bands of Arg has been considered. Following these procedures the Arg protein level results to be about four to five-fold higher in Raji and LP-1 cell lines than in less mature B cells and in both T cell lines. The highest Abl protein level is in Nalm-6 cells. Overall these patterns of protein expression are in accordance with the Arg and Abl pattern of the correspondent transcript expression.

## 4. Discussion

Mice with homozygous inactivation of the c-abl locus display thymic and splenic atrophy with deficiencies of B and T lymphoid progenitors and a slow maturation of pro-B cells to pre-B cells [22–25]. Also v-abl and BCR/ABL, the transform-

ing variants of c-abl, have a role in B lymphoid tissue [37–39] that seems to be a sensitive target of c-abl functions. In lymphoid tissues the redundant functions of Arg and c-abl [18,22,23] seem not present since Arg is unable to replace the c-abl functions [24,25].

Our data showed that there is a difference in the expression pattern of Arg and c-abl transcripts and proteins in the six lymphoid cell lines studied. The level of Arg transcript and proteins increases in the more differentiated B lymphoid cells (Raji, LP-1). The increment of Arg is not related to cell growth rate or cell cycle phases as reported [21] and is in accordance with the observations that endogenous Arg and c-abl per se do not play a crucial role in normal cell cycle progression [18,40]. Contrasting results, instead, were obtained when c-abl was overexpressed in cells in which, however, a cytotoxic effect seems to be prevalent [1,17,41]. It should be noted that we found the highest level of c-abl mRNA in the two B cell lines with slowest growth rate (Nalm-6, LP-1). The level of c-abl transcripts in the six cell lines was not related to differentiation. Our data showing the increment of Arg level in the mature B lymphoid cells are in accordance also with previous observations made in granulocytes, monocytes [13] and neurons [18], and support the hypothesis that high levels of Arg are related to the mature state of specific cells. The hypothesis is not contradicted by the data showing no increment of Arg in Nalm-6 cells after TPA treatment. In fact, the differentiation obtained, from early pre-B to late pre-B stage, is very weak [27] and may not be enough to determine an increment in Arg mRNA, if this is a phenomenon elicited in more mature cells.

The actinomycin D experiments suggested that the increment in Arg mRNA in Raji cells with respect to AIIPO cells was due to transcription activation since the transcript half-lives are similar. All together, the data suggest that in B cell maturation, but not in T cells, Arg and c-abl have a different behavior concerning the expression. The different modification of expression may suggest that the two genes, in B cells, are involved in different pathways with independent functions without overlapping roles and the invoked redundant actions [18,22–25] might be lost. The lack of redundancy could be due to the fact that the poorly expressed Arg, being involved in an alternative pathway, is not enough to also support the redundant actions. Such a hypothesis is supported by the evidence that in homozygous c-abl<sup>-/-</sup> mice the pro-B and pre-B cells, with a low level of Arg, suffer more from the lack of c-abl than the mature B cells, which have a high level of Arg [22–25,40]. Future investigations should assess whether the increment in Arg is related to one of its two specific forms and evaluate whether the forms IA and IB of Arg have an eventual role in apoptosis, keeping into account that an anti-apoptotic role [42] has been assigned to the type IV myristoylated form of murine c-abl, which is more abundant in mature B cell lines than type I, which instead is the major form in the less differentiated pre-B cell lines [43]. In fact, although nuclear c-abl can activate the genotoxic apoptosis, it has been otherwise suggested that during cell adhesion and contact inhibition [44,45] there is an increased cytoplasmic localization of c-abl that is consistent with an attenuation of the apoptosis response.

*Acknowledgements:* Supported by AIRC, MURST (60%) and by

CNR. We thank Dr. G. Manfioletti (Trieste University) for providing some of the RNA analyzed and for valuable discussion.

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