Behring Inst. Mitt., No. 81, 110–119 (1987)

Cellular Oncogenes and Lymphocyte Activation

J. SCHNEIDER-SCHAULIES, R. KNAUER, A. SCHIMPL, E. WECKER

Institut für Virologie und Immunbiologie der Universität Würzburg,
Versbacher Straße 7, D-8700 Würzburg, W.-Germany

The discovery that the oncogenes of acutely transforming retroviruses have their counterparts in normal cells and have actually arisen from these cellular genes has not only greatly influenced current views on the mechanisms of cell transformation, but has also had great impact on our thinking about the regulation of growth and differentiation. Oncogenes have turned out to code for molecules with a wide spectrum of different functions. As listed in Table 1 viral (reviewed in 1) and cellular oncogenes have been found to be analogous of a)

Table 1 Viral and cellular oncogenes

v/c oncogene	Size of protein product	Proposed location of product	Proposed function	Homology to cellular genes/proteins
v/c-sis	p^{28}	secreted; cytoplasm?	growth factor	PDGF-2/B
c-int-1	370 aa		growth factor?	
c-int-2				
c-neu	p^{185}	plasma membrane	protein tyrosine kinase	EGF-R
v/c-erb B	p^{72}	plasma membrane, ER, Golgi apparatus	protein tyrosine kinase	EGF-R
v/c-abl	p^{150} p^{145}	plasma membrane	protein tyrosine kinase	
v/c-src	pp^{60}	plasma membrane	protein tyrosine kinase	
v/c-fes/fps	$p^{85\,gag\text{-}fes}$	plasma membrane	protein tyrosine kinase	
v/c-yes	$p^{90gag\text{-}yes}$		protein tyrosine kinase	
v/c-fgr	$p^{70\,gag\text{-}fgr}$		protein tyrosine kinase	
v/c-fms	p^{120}	plasma membrane, ER, Golgi apparatus	protein tyrosine kinase	CSF-1-R
v/c-ros	$p^{62\,gag\text{-}ros}$		protein tyrosine kinase	
v/c-ras^{Ha}	p^{21}	plasma membrane	GTPase and regulates adenylate cyclase	rho-gene family
v/c-ras^{Ki}	p^{21}	plasma membrane	GTPase	rho-gene family
c-ras^{N}	p^{21}	plasma membrane	GTPase	rho-gene family
v/c-mil/raf1 raf2	$p^{100\,gag\text{-}mil}$	cytoplasm	serine/threonine kinase (pseudogene)	
v/c-mos	p^{40}	cytoplasm	serine/threonine kinase	proEGF
v/c-rel		cytoplasm		src-gene family
v/c-erb A	p^{46}	cytoplasm/nucleus	regulates transcription	thyroid/glucocorticoid R
p^{53}	p^{53}	nucleus	binds DNA, regulates transcription?	
v/c-myc	p^{60-64}	nucleus	binds DNA, regulates transcription	
N-myc		nucleus		
L-myc		nucleus		
v/c-fos	pp^{55}	nucleus	regulates transcription	
v/c-myb	p^{75}	nucleus		
v/c-ski		nucleus		
v/c-ets	p^{54}	nucleus, cytoplasm (fused with p-v-myb)		

growth factors (e. g. sis [2]), b) growth factor receptors (erb B[3], fms[4]), c) molecules involved in signal transduction from receptors into the interior of the cell (probably ras[5]) and d) proteins with nuclear localization. The last group seems to include proteins (e. g. erb A[6]) binding to regulatory elements within the DNA which could thus be responsible for turning on or off one or a number of genes whose products then, in turn, could be responsible for regulation of growth and differentiation.

One of the most easily accessible systems to study these parameters *in vitro* are cells of the immune system. Over the last twenty years we have learned to manipulate T- and B-lymphocytes *in vitro*. It was therefore attractive to use *in vitro* lymphocytes growth and differentiation for the analysis of cellular oncogene expression. 1983 *Kelly* et al. showed that murine spleen cells stimulated with T cell and B cell specific mitogens, i. e. Concanavalin A (ConA) and Lipopolysaccharide (LPS), respectively, transiently expressed c-myc[7]. This finding has since been confirmed and extended by various groups, including our own[8]. We were particularly interested in the expression of c-myc and c-fos, another nuclear protein (see Table 1 and ref. 1). Lymphocytes were either stimulated in a way which would lead to their transition through a full cell cycle or under conditions which would only make them growth competent, e. i., transition through the cell cycle would depend on an additional signal.

1. Expression of c-myc in Mouse T Cells

1.1 Nylon wool (NW) purified mouse T cells

NW enriched mouse T cells were stimulated with either ConA (2.5 µg/ml) or by leukoagglutinin (LA, 0.5 µg/ml). As previously described[8] and shown in Fig. 1, ConA leads to significant ^{3}H-thymidine incorporation in such cells while LA does so only in the presence of rIL-2. ConA stimulation resulted in a maximal expression of c-myc mRNA as early as 3 h after activation, at 20 h and 48 h post stimulation c-myc mRNA levels were significantly decreased. In contrast, in T cells stimulated with LA in the absence of IL-2 and thus in the absence of actual growth, c-myc gene expression continuously increased from 3 h until 48 h post stimulation. Addition of IL-2 did not lead to a change in kinetics, but the signals of c-myc RNA observed at later times were stronger.

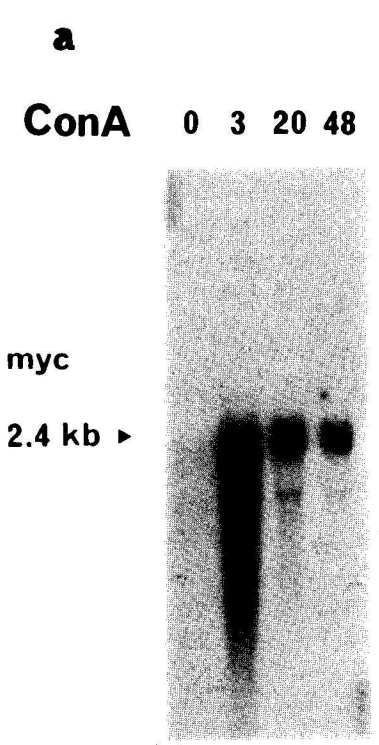
1.2 Lyt2$^+$ (CD8$^+$) T cells

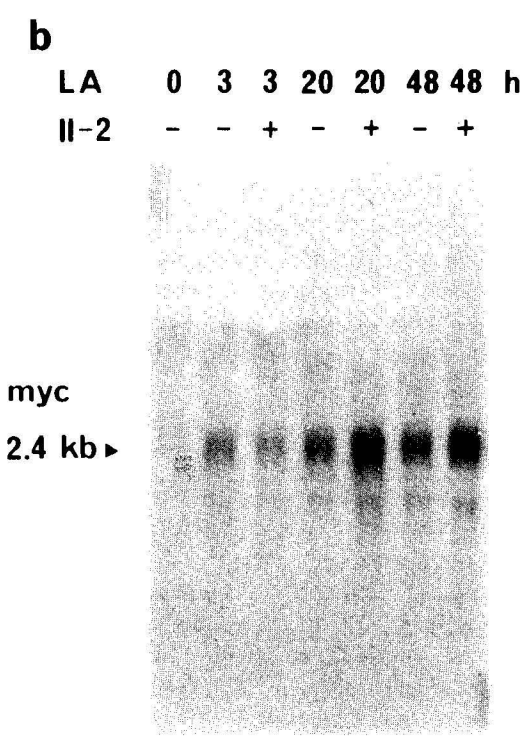
Isolated Lyt2$^+$ T cells activated by ConA or LA showed increased DNA synthesis only in the presence of IL-2 (ref. 8 and Fig. 2). When IL-2 was added, however, ^{3}H-thymidine incorporation was identical in both cases. The kinetics of c-myc expression in ConA activated Lyt2$^+$ cells was different from those observed with unseparated T cells, the maxima being now shifted to 20 h post stimulation with or without Il-2, similar to the kinetics observed upon LA stimulation of unseparated T cells. While ConA still led to some demonstrable c-myc expression at 3 h post stimulation, LA failed to do so although the proliferative capacity of both cultures upon IL-2 addition was identical.

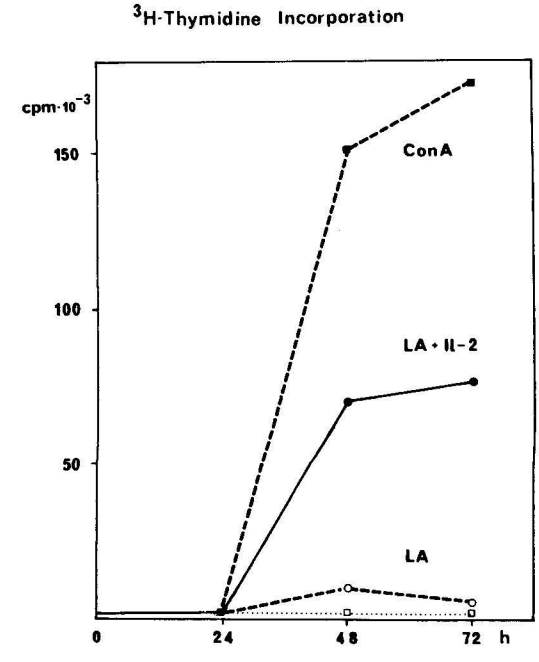
1.3 L3T4$^+$ (CD4$^+$) T cells

A KLH specific and H-2^d restricted L3T4$^+$ T helper cell line was maintained by regular restimulation. 6 h after restimulation by antigen and appropriate accessory cells, the cells expressed c-myc mRNA at maximal levels (Fig. 3). Thereafter, c-myc specific mRNA decreased markedly. Helper type T cells thus displayed kinetics of c-myc gene expression similar to ConA stimulated unseparated splenic T cells.

Taken together, these results suggested that kinetics of c-myc expression in mouse T cells are strongly influenced by the mitogen used and/or by the subpopulations investigated. They also indicated that expression of c-myc







only reflects competence for growth, but per se does not signify entrance into S-phase.

2. Expression of c-myc in Mouse B Cells

LPS activated B cells are able to proliferate and to mature to Ig secretion while cells activated with high doses of anti μ $F(ab')_2$ only proliferate. Low doses of anti μ $F(ab')_2$ or intact anti μ antibodies activate B cells neither to prolifera-

Fig. 1: C-myc mRNA expression in unseparated splenic T cells.

a) Nylon wool purified splenic T cells were stimulated with ConA (2,5 μg/ml) for 3, 20 and 48 h. Total cellular RNA was blotted and hybridized to a c-myc specific probe.

b) Nylon wool purified splenic T cells were stimulated with LA (0,5 μg/ml) without and with rIL-2 (30 U/ml). Total cellular RNA was blotted and hybridized to a c-myc specific probe.

c) ^{3}H-thymidine incorporation of ConA (■ - - - ■); LA + IL-2 (●——●); LA (○ - - - ○) and serum alone (□ - - - □) stimulated nylon wool purified splenic T cells.

For all detailed experimental procedures see ref. 8.

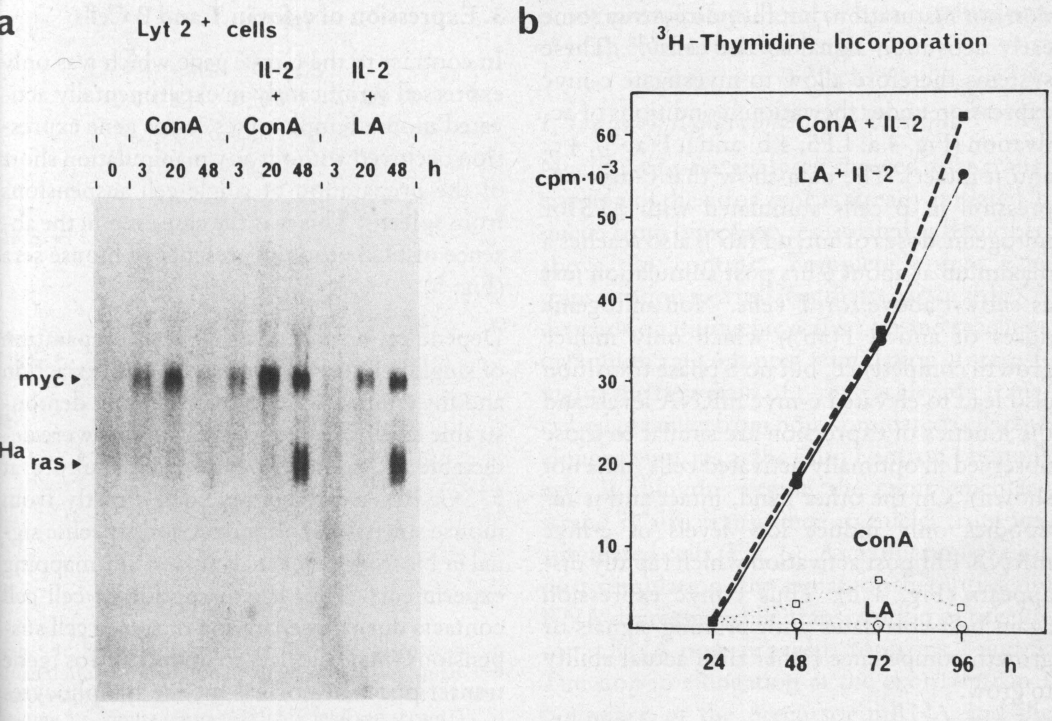


Fig. 2: c-myc mRNA expression in Lyt2$^+$ splenic T cells.

a) Lyt2$^+$ cells were stimulated with ConA (2,5 µg/ml), ConA + IL-2 (30 U/ml) or LA (0,5 µg/ml) + IL-2 for 3, 20 and 48 h. Total cellular RNA was blotted and hybridized to c-myc and c-Ha-ras specific probes.

b) ^{3}H-thymidine incorporation of Lyt2$^+$ cells stimulated by ConA + IL-2 (■ - - - ■); LA + IL-2 (●——●); ConA (□ - - - □) and LA (○ - - - ○).

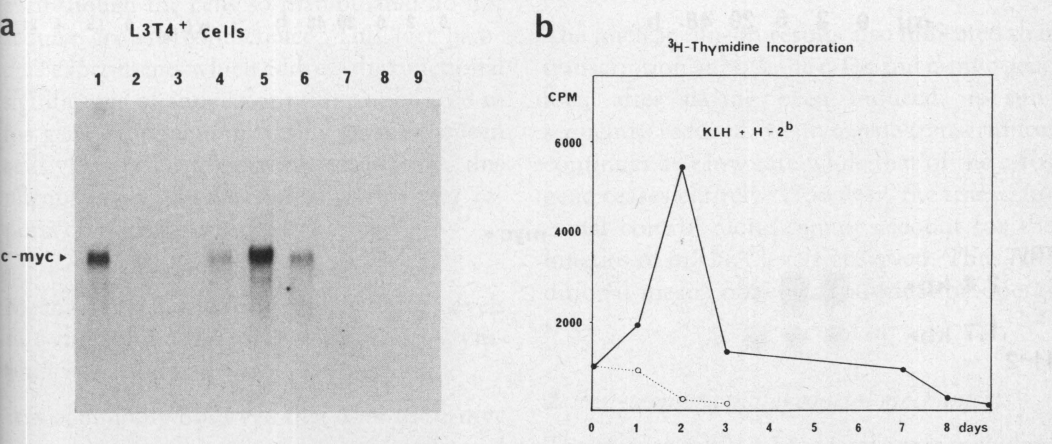


Fig. 3: C-myc mRNA expression in L3T4$^+$ cells (helper cell line).

a) Northern blot of total cellular RNA hybridized to a c-myc specific probe. Lane 1 contains RNA from 12 h ConA + 1 h CHI treated splenic cells. Lane 2 contains RNA from 1 h CHI treated quiescent L3T4$^+$ cells. Lanes 3 to 8 contain RNA from L3T4$^+$ cells stimulated for 0 (10 days), 3, 5, 12, 24 and 72 h by KLH (10 µg/ml) + H-2^b accessory cells, respectively. Lane 9 contains RNA from accessory cells alone.

b) ^{3}H-thymidine incorporation of L3T4$^+$ cells stimulated by KLH + H-2^b accessory cells (●——●) and L3T4$^+$ cells in medium without antigen and accessory cells (○ - - - ○).

tion nor maturation, but they do convey some early activation signal to the cells[9,10]. These systems therefore allow to investigate c-myc expression under the various conditions of activation (Fig. 4 a: LPS, 4 b: anti μ $F(ab')_2$, 4 c: anti μ intact). The data show that c-myc expression in B cells stimulated with LPS or mitogenic doses of anti μ $F(ab')_2$ also reaches a maximum at about 6 hrs post stimulation just as shown above for T cells. Non mitogenic doses of anti μ $F(ab')_2$ which only induce growth competence, but no S phase transition also lead to elevated c-myc mRNA levels and the kinetics of expression are similar to those observed in optimally activated cells (data not shown). On the other hand, intact anti μ antibodies only induce low levels of c-myc mRNA 1 hr post activation which rapidly disappears (Fig. 4 c). Thus c-myc expression again best correlates with priming signals or growth competence rather than actual ability to grow.

3. Expression of c-fos in T and B Cells

In contrast to the c-myc gene which was only expressed significantly in experimentally activated mouse lymphocytes, c-fos gene expression occurred without any manipulation short of the preparation of single cell suspensions from spleens. This was the case even in the absence of FCS or in the presence of mouse sera (Fig. 5).

Depending on the time between preparation of single cell suspensions and RNA extraction and the temperature during isolation, demonstrable levels of c-fos specific mRNA were detectable at around 30 min of cell culture at 37° C. RNA extracts obtained directly from mouse spleens did not give a c-fos specific signal in Northern blot analysis and SP6 mapping experiments. Thus the disruption of cell-cell contacts during preparation of spleen cell suspensions may suffice to induce c-fos gene transcription in normal mouse lymphocytes

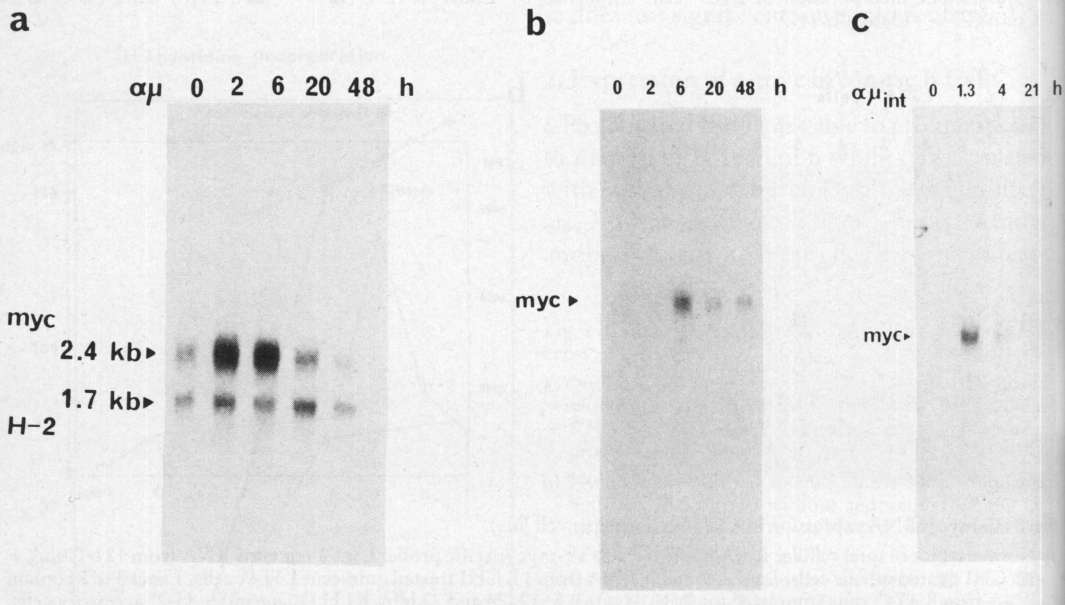


Fig. 4: Cells were stimulated with a) anti μ $F(ab')_2$ at mitogenic concentrations (12,5 μg/ml, resting B cells), b) LPS (10 μg/ml, B cells) or c) intact rabbit anti μ (resting lymphocytes). At the times indicated RNA was isolated, separated, blotted and hybridized to a c-myc specific probe (b, c) and to a c-myc specific and an H-2 Cl I specific probe (a).

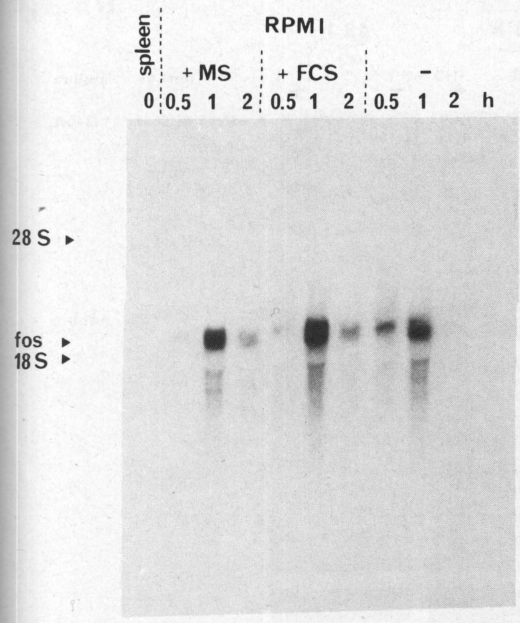


Fig. 5: Spleens were either directly dissociated in guanidinium/isothiocyanate or processed for single cell suspensions. The latter were then cultured in RPMI containing 5% mouse serum (MS), 5% fetal calf serum (FCS) or no serum. At the times indicated cells were harvested, RNA was isolated, separated, blotted and hybridized to a c-fos specific probe.

even though the cells so manipulated do not acquire growth competence. This fact hampers experiments which address the functional significance of antigen or mitogen induced c-fos gene expression in freshly isolated spleen cells. It may be worthwhile to consider this phenomenon also in context with other reports on c-fos gene expression.

Mechanisms Regulating Steady State Levels of c-myc and c-fos mRNA in Mouse Lymphocytes

It is commonly observed that both the c-myc and c-fos genes are only transiently expressed in normal cells, the respective mRNA levels reaching a maximum (c-fos ca. 1 h, c-myc 6–12 h post stimulation) and decreasing thereafter[7,8]. This regulation of gene expression may occur at the transcriptional, the post-transcriptional level or by a combination of both control mechanisms.

1. Transcriptional control mechanisms

Nuclear run-on analyses showed that transcription of the c-fos gene is already initiated in nuclei from lymphocytes isolated as described above. In contrast, complete c-myc gene *transcription to a full length precursor mRNA* depends on mitogenic activation and reaches a maximum rate 6 h post stimulation. Interestingly, the first exon of c-myc is already transcribed in nuclei from non-stimulated cells, but elongation stops at the exon 1/intron 1 boundary. Within this region the complementary strand is also transcribed in nuclei from unstimulated cells (Fig. 6). As a function of time post stimulation, the signal for this anti-sense RNA decreases while transcription of precursor mRNA now proceeds to exons 2 and 3. The stop in elongation at the exon1/intron 1 boundary of the precursor mRNA and the concomitant anti-sense transcription in non-activated cells may be mechanistically linked. Transcription from the anti-sense strand of *myc have also been described for tumor cells*[11,12].

The nuclear run-on results also indicated that transcription at both the c-fos and c-myc gene loci, after having been induced, is subsequently reduced. C-myc gene transcription continues at a low rate while that of the c-fos gene ceases entirely. However, the transcriptional control alone cannot account for the kinetics of mRNA levels observed. Thus, additional means of regulation must be operative.

2. Post-transcriptional control mechanisms

The mature mRNA of several genes including c-myc and c-fos have been found to be unusually unstable[13,14]. This was borne out by determining their half-lives (t ½) (Fig. 7a and b). In doing so, it turned out that the decay rates of both c-myc and c-fos mRNAs change as a

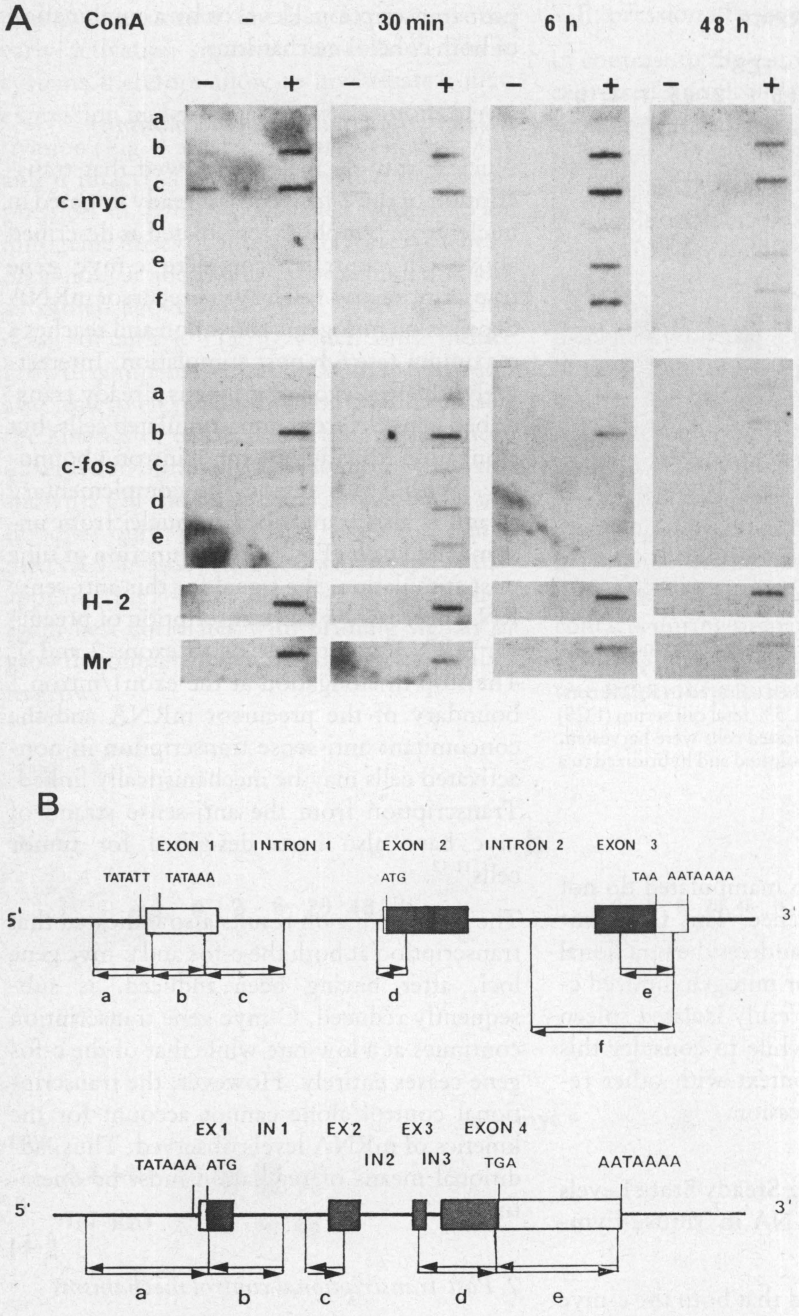
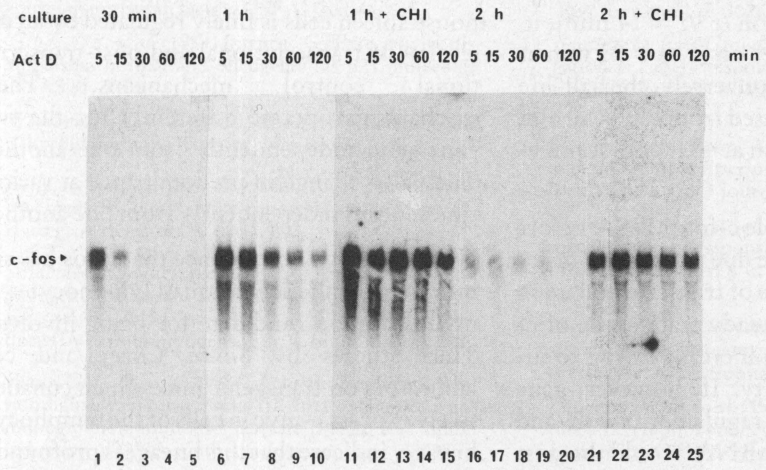


Fig. 6: Transcription rates of c-myc and c-fos.

a) The transcription or elongation rates of the c-myc, c-fos, H-2 and ribosomal genes in lymphocytes were determined after various times by nuclear run-on analyses. Probes: 0,5 µg of single stranded M13 DNA were slot blotted on Hybond N filter; (−) indicates the probes detecting anti-sense RNA, (+) indicates the probes for positive sense RNA.

b) The upper part shows the location of probes a, b, c, d, e and f of the mouse c-myc gene, the lower part the location of probes a, b, c, d and e of the mouse c-fos gene.

a



b

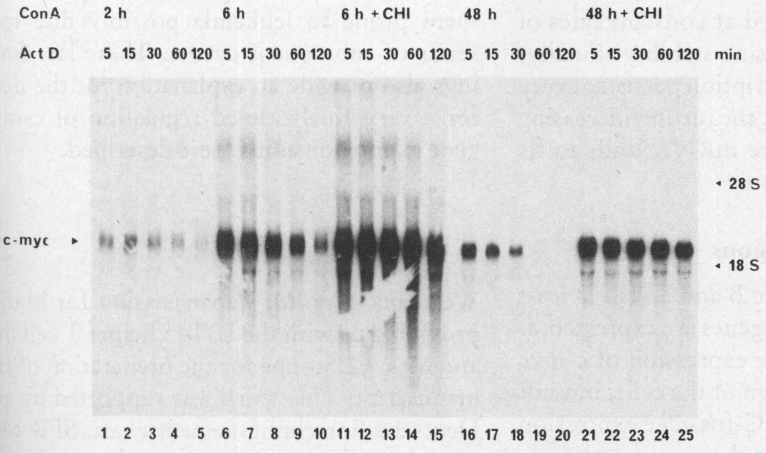


Fig. 7: Determination of the half-lives of c-fos mRNA.

a) Lymphocytes were cultured in RPMI 5% FCS with 2,5 µg/ml ConA. After 30 min (lanes 1–5) 1 h (lanes 6–15) and 2 h (lanes 16–25) transcription was blocked by addition of 5 µg/ml Act D and cells were harvested 5, 15, 30, 60 and 120 min later. CHI (20 µg/ml) was added 30 min before addition of Act D (lanes 11–15 and 21–25). The blot was hybridized with nick translated plasmid fos(M)780. The half-lives of c-fos mRNA were about 8 h for CHI treated lymphocytes, 70 min for 2 h ConA stimulated cells, about 25 min for 1 h and 14 min for 30 min stimulated lymphocytes.

b) Lymphocytes were cultured in RPMI 5% FCS with 2,5 µg/ml ConA. After 2 h (lanes 1–5) 6 h (lanes 6–15) and 48 h (lanes 16–25) transcription was blocked by addition of 5 µg/ml Act D and cells were harvested 5, 15, 30, 60 and 120 min later. CHI (20 µg/ml) was added 30 min before addition of Act D (lanes 11–15 and 21–25). The blot was hybridized with nick-translated plasmid containing the 1.0 kb XbaI/SstI fragment of the exon 2 of c-myc (mouse). The half-lives of c-myc mRNA were about 8 h for CHI treated lymphocytes, 50 min for 2 h and 6 h ConA stimulated cells, and 12 min for 48 h ConA stimulated lymphocytes.

function of time post stimulation. While the mRNA of c-fos displayed extreme instability at 30 min post stimulation ($t_{1/2}$ = 14 min), it became more stable later on ($t_{1/2}$ = 25 min at 1 h and 70 min at 2 h). Conversely, the half-life of c-myc mRNA decreased from $t_{1/2}$ = 50 min at 2–6 h to $t_{1/2}$ = 12 min at 48 h post stimulation.

The lack of demonstrable c-fos mRNA before 30 min in culture may be due to its initial instability. At a constant rate of transcription up to 1 h post stimulation, steady state levels of c-fos mRNA would then increase owing to increasing mRNA stability. If, however, gene transcription is down-regulated at 2 h and later, the remaining mRNA would be lost rather rapidly due to its still relatively short half-life. On the other hand, the appearance of c-myc specific mature mRNA in activated T cells is exclusively accounted for by increasing rates of transcription now occurring over the entire gene. Steady state levels of c-myc mRNA are then reduced at constant rates of transcription by decreasing stability. Finally, while c-myc gene transcription persists at very reduced rates up to 48 h, the further increasing instability of the mature mRNA leads to its further gradual reduction.

Summary and Conclusions

In normal mouse splenic B and T cells at least two cellular proto-oncogenes are expressed, i. e. c-myc and c-fos. The expression of c-myc depends on the activation of the cells, but not on subsequent growth. C-fos gene expression appears to be induced by the manipulation involved in preparation of single cell suspensions from spleens. In that respect, c-fos gene expression does not qualify as being significantly involved in transition from G 0 to S phase while expression of c-myc seems to be correlated with some early events of cell activation leading to growth competence. The kinetics and extent of c-myc gene expression vary with the mitogen used and the type of lymphocyte investigated as examplified by T cells and subpopulations thereof. The expression of both proto-oncogenes in normal mouse spleen cells is finely regulated by an interplay of transcriptional and post-transcriptional control mechanisms. These mechanisms operate differently for the two genes and independently from one another. They also change in predominance at various times, again independently from one another.

While we have no evidence that c-fos has significance for the activation of lymphocytes, c-myc is a good candidate for being involved. Thus studies by *Susan Corey* and collaborators on transgenic mice which constitutively express c-myc in cells of the lymphocyte lineage, indicate that this lineage is profoundly affected[15]. Among others, the effects concern the balance between proliferation and maturation and a constitutive high level of Ia expression, normally only observed in activated cells. Constitutive high expression of c-myc in B cells of these transgenic mice also makes them prone to leukemia possibly due to a series of subsequent events. These last findings also provide an explanation for the need for a very finely tuned regulation of c-myc gene expression as it is here described.

Acknowledgement

We thank Mrs. M. Papanastasiou for kindly providing us with the L3T4$^+$ helper T cell line and Mrs. C. Stoppe for the preparation of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft, SFB 165, Würzburg.

References

1 *Bishop, J. M.* (1985) Viral oncogenes. Cell *42*, 23.

2 *Waterfield et al.* (1983) Platelet derived growth factor is structurally related to the putative transforming protein p28^{sis} of simian sarcoma virus. Nature *304*, 35.

3 *Downward, J. et al.* (1984) Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature *307*, 521.

4 *Sherr, C. J. et al.* (1985) The c-fms proto oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell *41*, 665.

5 *Gibbs, J. B. et al.* (1984) Intrinsic GTPase activity distinguishes normal and oncogenic ras p21 molecules. PNAS *81*, 5704.

6 *Weinberger, C. et al.* (1985) The c erb A gene encodes a thyroid hormone receptor. Nature *318*, 670.

7 *Kelly, K. et al.* (1983) Cell specific regulation of the c-myc gene by lymphocyte mitogens and platelet derived growth factors. Cell *35*, 603.

8 *Schneider-Schaulies, J. et al.* (1986) Kinetics of cellular oncogene expression in mouse lymphocytes. I. Expression of c-myc and c-ras^{Ha} in T lymphocytes induced by various mitogens. Eur. J. Immunol. *16*, 312.

9 *Klaus, G. G. B. et al.* (1984) Activation and proliferation signals in mouse B cells III. Intact (IgG) anti-immunoglobulin antibodies activate B cells, but inhibit induction of DNA synthesis. Immunology *53*, 693.

10 *Howard, M.* & *Paul, W. E.* (1983) Regulation of B cell growth and differentiation by soluble factors. Ann. Rev. Immunol. *1*, 307.

11 *Bentley, D. L.* & *Groudine, M.* (1986) A block to elongation is largely responsible for decreased transcription of c-myc in differentiated HL60 cells. Nature *321*, 702.

12 *Nepveu, A.* & *Marcu, K. B.* (1986) Intragenic pausing and anti-sense transcription within the murine c-myc locus. The EMBO Journal *5*, 2859.

13 *Piechaczyk, M. et al.* (1985) Post-transcriptional mechanisms are responsible for accumulation of truncated c-myc RNA's in murine plasma cell tumors. Cell *42*, 589.

14 *Mitchell, R. L. et al.* (1986) c-fos expression is neither sufficient nor obligatory for differentiation of monomyelocytes to macrophages. Cell *45*, 497.

15 *Langdon, W. Y. et al.* (1986) The c-myc oncogene perturbs B lymphocyte development in E μ-myc transgenic mice. Cell *47*, 11.