fresh medium to 90% confluent cultures of G418-resistant cells, collecting the medium 16–20 h later, filtering it through a 0.45 μm filter, using it immediately for infection, or freezing it at −80°C. Retrovirus supernatants were titered for G418-resistant colony forming units (cfu)/ml on NIH3T3 cells. For virus infection of Balb/MK-2 keratinocytes, cells were seeded overnight at a density of 5×10^5 cells per 100 mm dish in growth medium containing 8 μg ml^{-1} polybrene. Virus supernatants, containing 1–5 × 10^5 cfu were added for overnight incubation, with virus containing medium then removed and replaced with fresh growth medium without EGF. The appearance of EGF-independent foci of transformed cells was monitored for up to five weeks. For infection of PC12 cells, 1–5 × 10^5 cfu were added to 5×10^5 cells/60 mm dish of cells in TMEM medium containing 8 μg ml^{-1} polybrene. After 16–20 h, the virus was removed, and fresh growth medium was added to the cultures. The appearance of neurite outgrowths were monitored for two weeks.

Colony formation in soft agar

G418-selected NIH3T3 cell populations were used for this analysis. To assay for growth in soft agar (Der & Stanbridge, 1978), an agar base layer containing growth nutrients was poured into 60 mm petri dishes and allowed to solidify. Cell suspensions in 0.3% agar medium were then layered over the base layer to a final cell density of 10^2 cells per dish. The appearance of colonies was monitored for up to three weeks.

Tumorigenicity analysis

Congenitally athymic nu/nu (nude) mice were used to determine the tumorigenic growth properties of each transfected cell line (Stanbridge & Wilkinson, 1978). Cells were harvested by trypsinization and suspended in serum-free TMEM. 0.2 ml suspensions of cells containing 1 × 10^5 cells were inoculated subcutaneously into the ventral midline. All animals were examined for the presence of tumors at regular intervals. Negative animals were maintained for up to three months.

Acknowledgements

We are grateful to C. Van Beveren, J. Buss and A. Diamond for helpful discussions on this manuscript. We thank J. Schaeffer for excellent technical assistance, W. Stallcup for the PC12 line and T. Clevenger for expert preparation of this manuscript. This investigation was supported by grants from the National Cancer Institute to C.J.D. (CA 42978) and to B.W. (CA 36936).

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Oncogene (1988), **3**, 113–122

Different regulation of oncogene expression in tumor and embryonal cells of *Xiphophorus*

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Melanoma formation in the poeciliid fish *Xiphophorus* is mediated primarily by a cellular oncogene, designated *Tu*. Elimination of *Tu*-specific genes releases the transforming function of *Tu* and leads to melanoma formation. Southern blot analyses revealed a tight linkage of a v-*erb* B related gene to the *Tu*-locus and Northern blot analyses of RNA of solid melanomas indicated a coordinated deregulation and/or mutational activation of several oncogenes. In order to get a better insight into the regulation of oncogene expression in normal and transformed cells of *Xiphophorus*, we studied the expression of X*src*, X*ras*, X*myc*, X*erb* A, X*sis*, and the v-*erb* B related gene in a melanoma derived cell line (PSM) and an embryonic cell line (A2) under conditions of low growth factor supply. Both cell lines express the X*src*, X*myc*, and X*ras* genes, while PSM cells in addition express the v-*erb* B related gene and A2 cells the X*sis* gene. In PSM cells serum deprivation leads to an accumulation of most of the oncogene mRNAs analysed. This is most apparent for a 5.0 kb transcript of the v-*erb* B related gene, probably due to an increase in transcript stability. The levels of these mRNAs returned to normal within 2 h after stimulation with 10% fetal calf serum. At the protein level we observed an initial decrease followed by an increase of the $pp60^{c-src}$ kinase (the protein product of the X*src* gene) activity in cells deprived of serum. Serum stimulation restored a normal $pp60^{c-src}$ kinase activity. In contrast serum deprivation of A2 cells reduced the transcript amounts of each of the oncogenes analysed. The same holds true for one β-tubulin transcript, while the level of a second β-tubulin transcript was unaffected. Serum stimulation led to a reactivation of X*ras* and X*src* after a delay of approximately 48 h. The $pp60^{c-src}$ kinase activity was found to be 6–10 times lower as compared to the PSM cells and did not differ between serum deprived and serum stimulated cells. Enzyme activities and isoenzyme patterns of several glycolytic enzymes were found to be not affected by serum deprivation and stimulation in both cell lines.

Introduction

One of the most prominent features of tumor cells is their autonomous growth due to a loss of constraint by exogenous proliferation signals. *In vitro* this is evidenced by an independence from growth factors, i.e. the tumor cells are able to propagate with low amounts or even without serum being the major source for growth stimulating factors in the tissue culture medium. It was proposed that autocrine stimulation (Sporn & Todaro, 1980) is the mechanism which enables the malignant cells to maintain their neoplastic phenotype (DeLarco *et al.*, 1978; Kaplan *et al.*, 1982; Ozanne *et al.*, 1980) by producing, secreting and utilizing their own growth factors. Several of these growth factors (so-called transforming growth factors, TGFs) were able to render normal indicator cell lines morphologically transformed and anchorage independent (Kaplan *et al.*, 1981; Moses *et al.*, 1981). In subsequent studies it was shown that cells transformed by introduction of viral oncogenes lose the growth control previously exercised by serum or platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) or other mitogenic factors (Powers *et al.*, 1984; Zhan & Goldfarb, 1986). Structural analysis of some oncogenes has uncovered a functional relationship between these genes and cellular growth factors. For example the v-*erb* B gene encodes for a truncated form of the epidermal growth factor receptor which itself is encoded by the c-*erb* B gene (Downward *et al.*, 1984), c-*fms* encodes for the mononuclear phagocyte growth factor (CSF-1) receptor (Sherr *et al.*, 1985) and the v-*sis* gene encodes the B chain of PDGF (Waterfield *et al.*, 1983), indicating that both viral and cellular oncogenes might be involved in the mechanisms leading to growth autonomy. On the other hand, not every cell becomes fully transformed after transfection with one oncogene, and often cotransfection experiments using different combinations of these oncogenes are necessary to obtain the full neoplastic phenotype of a cell (Land *et al.*, 1983a; Land *et al.*, 1983b; Newbold & Overell, 1983; Ruley, 1983). In addition it was demonstrated that tumor cell cultures obtained from solid tumors express a set of different oncogenes (O'Hara *et al.*, 1986; Perucho *et al.*, 1981; Pulciani *et al.*, 1982; Spandidos *et al.*, 1985) indicating that coordinated deregulation and/or mutational activation of several oncogenes is possibly a prerequisite for maintenance of the neoplastic phenotype in the *in vivo* situation.

To analyse such a coordinate deregulation, studies on both solid tumors and the corresponding *in vitro* cultured cells are necessary. The *Xiphophorus* melanoma system offers the unique possibility to study the expression of oncogenes in hereditary solid melanoma as well as in a melanoma derived cell line *in vitro*. In the poeciliid fish *Xiphophorus*, certain hybrid genotypes spontaneously develop malignant melanoma. Melanoma formation has been attributed by classical genetic studies to the overexpression of a dominant cellular oncogene, termed *Tu*. In non-tumorous fish, *Tu* was

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Received 19 April 1988; accepted 6 May 1988

proposed to be negatively controlled by cellular regulatory genes, termed *R*, which act as 'anti-oncogenes' (for review see Anders *et al.*, 1984). In a typical crossing experiment a female *Xiphophorus maculatus* (platyfish) containing a specific *Tu*-locus and its corresponding *R* gene located on different chromosomes, is crossed with a male *Xiphophorus helleri* (swordtail) which is thought to contain neither this particular *Tu*-locus nor its corresponding regulatory gene. Backcrossing of the *Tu*-containing F_1-hybrids to *Xiphophorus helleri* results, in effect, in the progressive replacement of *R* gene bearing chromosomes originating from *Xiphophorus maculatus* by chromosomes of *Xiphophorus helleri*. The homozygous elimination of regulatory genes allows increased expression of *Tu*, resulting in the development of malignant melanoma in the hybrids.

Studies on the molecular biology of melanoma formation in *Xiphophorus* have revealed the expression in melanomas of a set of oncogenes (X*src*, X*ras*, X*erb* B, a v-*erb* B related gene; see ref. Mäueler *et al.*, 1988) and that the expression of two particular genes might be of relevance for the establishment of the neoplastic phenotype: (1) The X*src* gene (*Xiphophorus* c-*src* gene) shows with respect to the tyrosine kinase activity of its gene product ($pp60^{c\text{-}src}$) a correlation in expression to *Tu* (monitored by the severity of the tumor) (Schartl *et al.*, 1982; Schartl *et al.*, 1985); (2) a v-*erb* B related gene, which is tightly linked to or even part of the *Tu*-locus (Schartl, 1988), exhibiting a 5.0 kb and a 3.2 kb transcript, is highly expressed in melanoma cells. These transcripts could not be detected in any normal tissue analysed so far (Mäueler *et al.*, 1988). The data also indicate that a coordinated deregulation of oncogenes in hereditary fish melanoma might be responsible for the neoplastic phenotype. Studies in well defined *in vitro* systems are required to get a better insight into the functional role played by these two genes in the process of tumor formation. The PSM-1a cell line derived from a hereditary *Xiphophorus* melanoma (Wakamatsu *et al.*, 1984) offers such a system. It has been shown that this cell line shows an expression pattern of oncogenes similar to the melanoma *in situ* (Mäueler *et al.*, 1988).

To investigate the relation of the expression of the v-*erb* B related and the X*src* genes to growth autonomy as a unique phenotypic feature of transformed cells, we determined whether serum insufficiency in *in vitro* cultured nontransformed and transformed cells results in changes in expression of these oncogenes. In addition we studied the steady state mRNA level and the transcriptional activity of the X*ras*, X*sis*, and X*myc* genes. For comparison, house keeping genes were studied by measuring the level of β-tubulin transcripts and the activities and isoenzyme pattern of several glycolytic enzymes. The expression pattern obtained from the tumor cells was compared to that observed in a cell line derived from non-tumorous embryos (Kuhn *et al.*, 1979).

Results

Characterization of the cell lines

The two cell lines used in this study were derived from a spontaneous malignant melanoma of a *Xiphophorus* F_1 hybrid (PSM-1a) (Wakamatsu *et al.*, 1984) and from normal late stage embryos of parental wildtype *Xiphophorus* (A2 cells). The PSM-1a cell line consists of large spindle-shaped, unpigmented melanoma cells that tend to differentiate following growth up to very high cell densities. This process is marked by synthesis of melanin, a flat and round cell-morphology and the appearance of multiple nuclei in a single cell. All these morphological characters are reminiscent of findings *in vivo* on melanoma cell differentiation. A2 cells are epithelial-like cells. Both cell lines show several common features: both are immortalized, have a similar mean doubling time during log-phase growth (PSM-1a = 58 h, A2 = 55 h) and form large foci after reaching confluency. They differ by several parameters: The PSM-1a cells express a cell surface onco-fetal antigen of apparent Mw 130000 Dalton (Claus *et al.* submitted), while the A2 cells – like all non-melanoma cells in adult *Xiphophorus* – do not exhibit such a protein (data not shown). PSM-1a cells have a high plating efficiency of $74.7 \pm 11.3\%$. The plating efficiency of A2 cells is only $3.6 \pm 1.6\%$, however, this value is only obtained if A2 cells are seeded with at least 10^3 cells/dish. If single cells are seeded onto 96 well plates, PSM-1a cells form colonies with 65.5% while the A2 cells do not form colonies. The same holds true for colony formation in soft-agar. PSM-1a cells continue to grow in medium containing only minimal amounts of serum (0.5%). A2 cells are unable to proliferate in low serum and die after an exposure of 8 days to medium containing 0.5% serum. As shown by cytofluorimetric analyses (Figure 1), neither the PSM-1a nor the A2 cells were arrested in specific phases of the cell cycle through serum deprivation (0.5% serum for 24 h).

Both cell lines contain the set of oncogenes which was detected in germline DNA from *Xiphophorus* fish (Figure 2). Some oncogenes show a considerable restriction fragment length polymorphism between the cell lines. No evidence for amplification of any of these sequences was found. According to a sex-chromosomal restriction fragment length marker, detected by the v-*erb* B probe (Schartl, 1988), the PSM-1a cells contain a Y-chromosome of the *Xiphophorus maculatus* parent which harbors an allele of the *Tu*-locus.

Effect of serum deprivation and serum stimulation on the expression of oncogenes

To determine whether the expression of X*src*, X*sis*, X*ras*, X*myc*, X*erb* A, and the v-*erb* B related gene is influenced by serum factors, cells were cultured for 24 h in medium containing 0.5% fetal calf serum (FCS) and were then serum stimulated by addition of 10% FCS. The amount of specific mRNAs was determined by Northern blot analysis. X*erb* A was found not to be expressed in either cell line. PSM cells harvested 24 h after serum deprivation show a ten-fold increase in the amount of the 5.0 kb v-*erb* B related transcript (Figure 3). Two hours after serum stimulation the amount of this transcript declined considerably and within 48 h returned to the level detected in normal exponentially growing cells. The second v-*erb* B related transcript of 3.2 kb in PSM cells was found to be expressed at stable levels and may therefore serve as an internal control during this time course experiment (Figure 3). Expression of the v-*erb* B related gene in A2

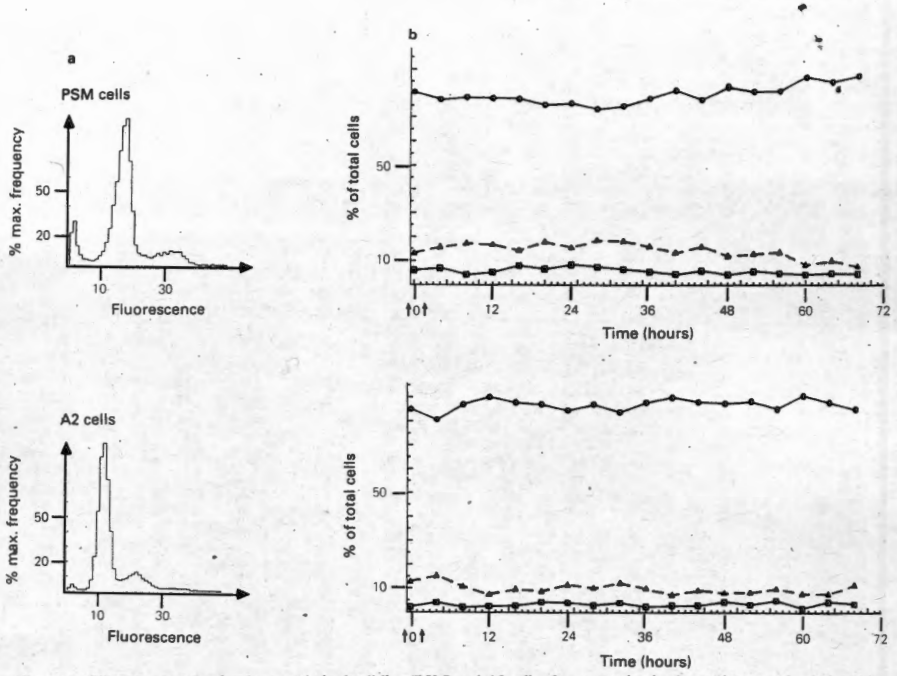


Figure 1 Cell cycle analysis of melanoma derived cell line (PSM) and A2 cells after serum deprivation and serum stimulation. (a) Distribution of cells in different phases of the cell cycle after 24 h exposure to 0.5% fetal calf serum (FCS); peak 1 cell debris, peak 2 cells in Go/G1, peak 3 cells in G2. Fluorescence in arbitrary units. Similar observations were obtained for cells exposed 48 or 72 h to 0.5% FCS (data not shown). (b) Relative distribution of the cells in Go/G1 ●—●, S ■—■, and G2 phase ▲—▲ after serum deprivation (0) and after serum stimulation (1–72 h)

cells was not observed. For the X*src* gene we detected a 3.4 kb transcript in PSM and a 3.7 kb transcript in A2 cells (Figure 3). In PSM cells exposed to 0.5% FCS for 24 h the 3.4 kb transcript was found to be accumulated, but to much lower extent than the 5.0 kb v-*erb* B related transcript. Within 2 h after serum stimulation it decreased to the level found in normal exponentially growing cells. Surprisingly, in A2 cells the 3.7 kb c-*src* transcript declined following serum deprivation to barely detectable levels without reconstitution of the normal level of expression within 72 h after serum stimulation. This phenomenon in A2 cells was also observed for the two transcripts of the X*sis* gene (X*sis* 1, 3.4 kb; X*sis* 2, 2.5 kb) which were not detected in PSM cells, (Figure 3) and for the two X*ras* transcripts (X*ras* 2, 3.0 kb and X*ras* 3, 1.7 kb; Figure 4), except that the transcripts reappeared at the normal level at 72 h after serum stimulation. In the PSM cells the level of X*ras* 2 and 3 transcripts followed the pattern observed for the X*src* transcripts with elevation of the transcript numbers after serum deprivation and normalization after serum stimulation (Figure 4). For X*myc* we detected a 2.4 kb transcript. In PSM cells the level of X*myc* transcript increased after serum deprivation and remained at this higher level until 2 h after serum stimulation. Then the level declined within 48 h to the normal level. In A2 cells only traces of the transcript could be detected.

To determine whether the protein product of the X*src* gene, the $pp60^{c\text{-}src}$ kinase shows an activity pattern comparable to the X*src* RNA pattern observed in the two cell lines, we have determined $pp60^{c\text{-}src}$ kinase activity by performing a kinase assay. Both cell lines contain a tyrosine specific protein kinase activity reactive with anti-pp60 antibodies (Figure 5b), but at different specific activities. PSM cells 24 h after serum deprivation showed a 2-fold decreased (600 cpm/mg protein) $pp60^{c\text{-}src}$ kinase activity, compared to normal exponentially growing cells (1200 cpm/mg; Figure 5). Two hours after serum stimulation the kinase activity increased to 2000 cpm/mg and turned back to normal activity within 4 h after serum stimulation indicating a temporarily delayed effect to the effect observed for the mRNA. In contrast, no difference in the $pp60^{c\text{-}src}$ kinase activity (400 cpm/mg) of A2 cells prior or after serum deprivation and serum stimulation (followed up to 72 h after serum stimulation) was detectable. In order to evaluate the significance of the differences in kinase activity we determined the RNA content and X*src* copy numbers per cell in both cell lines. Normal exponentially growing cells contain $15\,pg \pm 3\,pg$ (PSM) and $8\,pg \pm 1.5\,pg$ (A2) total RNA per cell. The copy number of X*src* in these cells was determined as approximately 10 copies per cell for both cell lines by RNAase protection assay (data not shown). As PSM cells contain approximately two times as much protein as A2 cells (soluble in the kinase

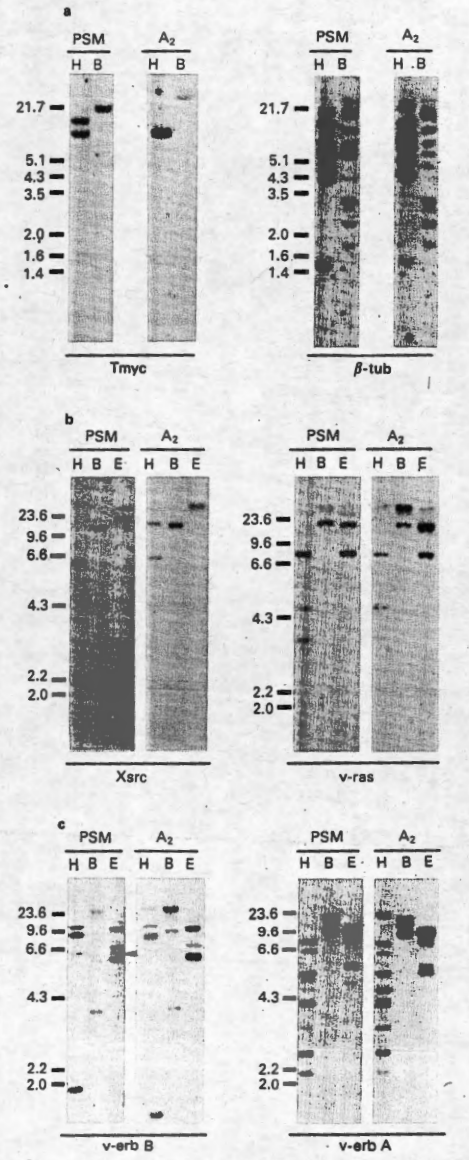


Figure 2 Southern blot analyses of melanoma derived cell line (PSM) and A2 DNA digested with HindIII (H), BamHI (B) and EcoRI (E) probed with the trout c-*myc* gene (*Tmyc*), the *Drosophila* β-tubulin gene (*β-tub*), the *Xiphophorus* c-*src* gene (*Xsrc*), and the viral *ras*^{H}, *erb* B and *erb* A genes. Arrow in c marks a Y-chromosome specific RFLP which is indicative for the presence of a sex-chromosomal *Tu* locus

extraction buffer), the pp60 kinase activity is increased up to 10 fold in melanoma cells if calculated on the single cell level.

Table 1 Average values of enolase (EN), pyruvate kinase (PK), phosphoglycerate mutase (PGM), phosphoglycerate kinase (PGK), lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) activities from PSM and A2 cells, expressed in U/mg protein

	PSM cells	*A2 cells*
EN	0.39 ± 0.08	0.25 ± 0.05
PK	0.64 ± 0.06	0.31 ± 0.06
PGM	2.94 ± 0.29	2.32 ± 0.43
PGK	0.69 ± 0.09	2.79 ± 0.52
LDH	2.55 ± 0.57	2.01 ± 0.22
MDH	0.07 ± 0.01	0.07 ± 0.01

Effect of serum deprivation and serum stimulation on the expression of house-keeping genes

To investigate whether or not the phenomenon observed for oncogene transcripts also holds true for the expression of house keeping genes, the filters were rehybridized with a β-tubulin cDNA probe. In both cell lines we detected two transcripts of about 2.8 kb (*β-tub* 1) and 1.6 kb (*β-tub* 2; Figure 4). In PSM cells both transcripts were found at relatively stable levels following serum stimulation, however a slight increase of both transcripts may be seen after serum deprivation. In A2 cells after serum deprivation the level of the *β-tub* 1 transcripts declined to undetectable levels as was observed for the oncogene transcripts. In contrast, the levels of *β-tub* 2 transcripts were found to be relatively stable after serum deprivation.

In order to find out whether the protein products of other house keeping genes show similar features to that observed for the oncogene product pp60$^{c\text{-}src}$ we analysed the specific activity of enolase (EN), pyruvate kinase (PK), phosphoglycerate mutase (PGM), phosphoglycerate kinase (PGK), lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) as well as the isoenzyme pattern of EN, PK and LDH prior and after the serum deprivation. After serum stimulation no significant deviations in the total activities of the different enzymes (followed up to 48 h) from the values shown in Table 1 were detectable. Comparison of the isoenzyme pattern revealed that both cell lines contained the same set of isoenzymes of LDH, EN and PK (Figure 6). In no case was any significant change observed in isoenzyme pattern of the considered enzymes prior to or after serum deprivation and stimulation followed up till 48 h after serum stimulation.

Effect of serum deprivation and serum stimulation on transcription

In order to find out whether the observed phenomenon of increasing mRNA amounts in the PSM cells following serum deprivation is due to regulation on the transcriptional or post-transcriptional level, nuclei from exponentially growing cells as well as from cells grown for 24 h at 0.5% FCS and from stimulated cells were isolated and used for run on transcription assays. The isolated labeled RNA was hybridized against oncogene and house keeping gene containing plasmids immobilized on slot blot filters. With the oncogene probes we detected a transcriptional activity of the corresponding cellular sequences which was only very low. No significant difference in transcriptional activity of the X*src*, X*sis*, X*ras*, X*myc*, the v-*erb* B related, EN and LDH genes between nuclei from normal PSM cells, or PSM

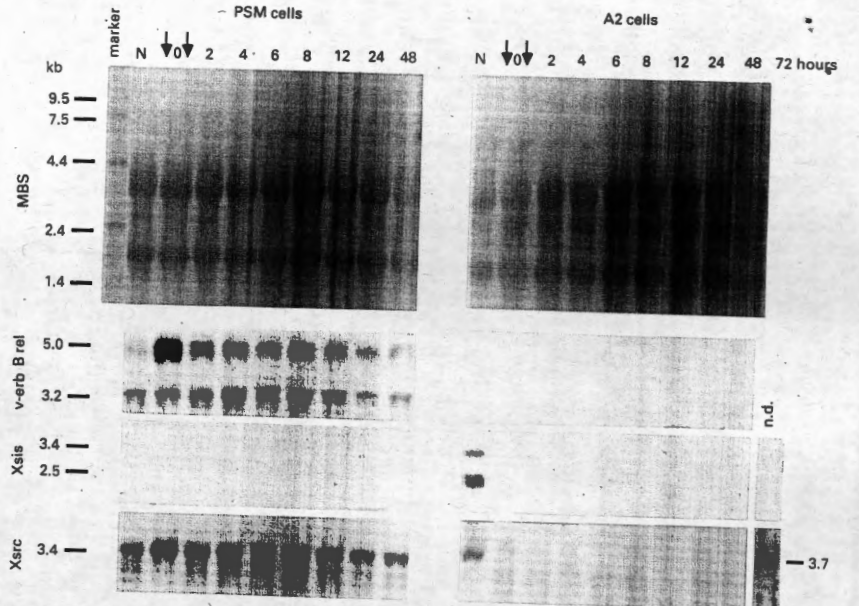


Figure 3 Expression of oncogenes in the cells prior (N) and after serum deprivation (0) and serum stimulation (2–72 h). MBS; methylene blue staining of the filters; marker 9.5–1.4 kb RNA marker purchased from BRL; n.d. not done. Arrows indicate serum depletion and serum stimulation, respectively.

cells exposed 24 h to 0.5% FCS, or PSM cells 2 h after serum stimulation were observed (Figure 7).

Discussion

In this study we have shown that the regulation of expression of the oncogenes analysed is different in the tumor and embryo derived cell lines used. The same holds true for one β-tubulin gene transcripts, while no differences between the two cell lines were found for other house keeping genes (analysed on protein level).

Northern blot analysis revealed in general a distinct Expression pattern of oncogenes in PSM cells as compared to A2 cells. A2 cells express X*src*, X*ras*, X*sis*, and

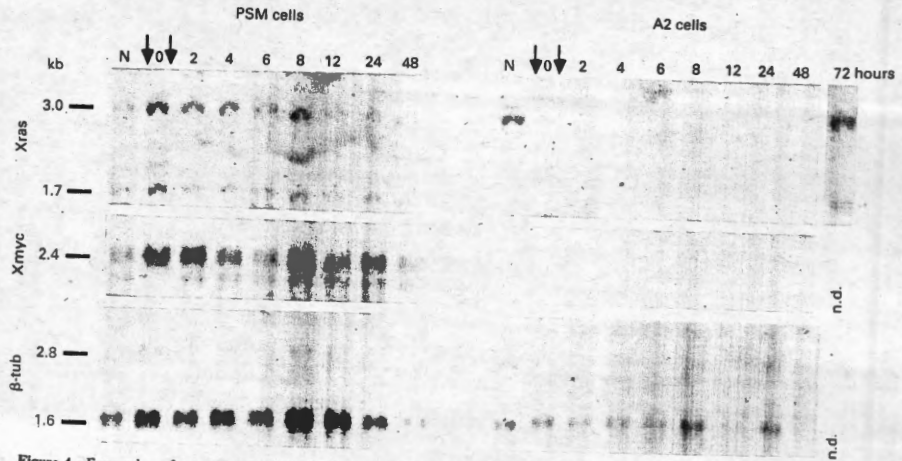


Figure 4 Expression of oncogenes and the *β-tub* gene in the cells prior (N) and after serum deprivation (0) and serum stimulation (2–72 h); n.d. not done

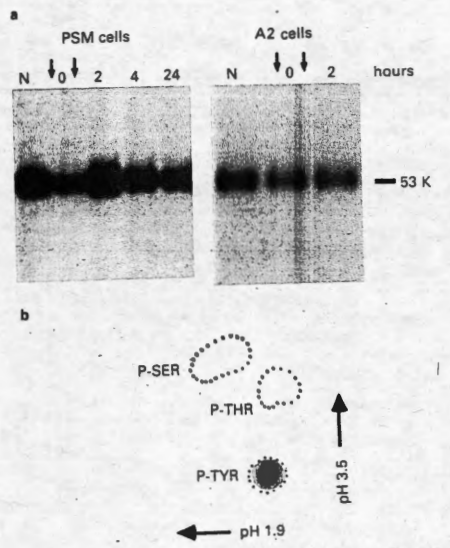
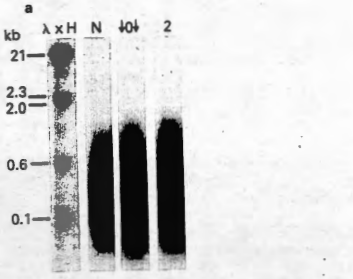


Figure 5 (a) Activity of the pp60$^{c\text{-}src}$ kinase in cells prior (N) and after serum deprivation (0) and serum stimulation (2–24 h). (b) Phosphoamino acid analysis of the IgG heavy chain from pp60$^{c\text{-}src}$ immunocomplexes following the kinase reaction; P-TYR phospho tyrosine, P-THR phospho threonine, P-SER phospho serine



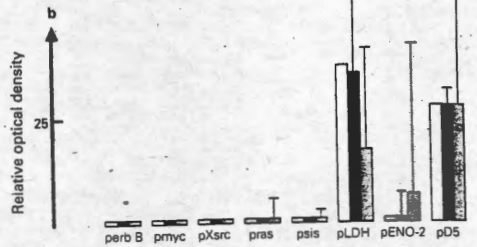


Figure 7 Transcriptional activity of nuclei from melanoma derived cell line (PSM) cells prior □ (N) and after serum deprivation ■ (0) and serum stimulation ▩ (2). (a) Size of the transcripts after optimal incubation time (25 min at 26°C); labelled transcripts were electrophoretically separated a 2% agarose gels containing 2.2 M formaldehyde, gels were dried and autoradiographed; (b) semiquantitation of the signals obtained from slot blot filters hybridized with the different nuclear run on transcripts of PSM cells, signals are expressed relative to the signals obtained from pD5 (= 100%), I standard deviation. All values were corrected for the background hybridization signals obtained with vector sequences alone

only barely detectable amounts of X*myc*, while the PSM cells express the X*src*, X*ras*, X*myc* genes and a v-*erb* B related gene, which recently was found to be expressed

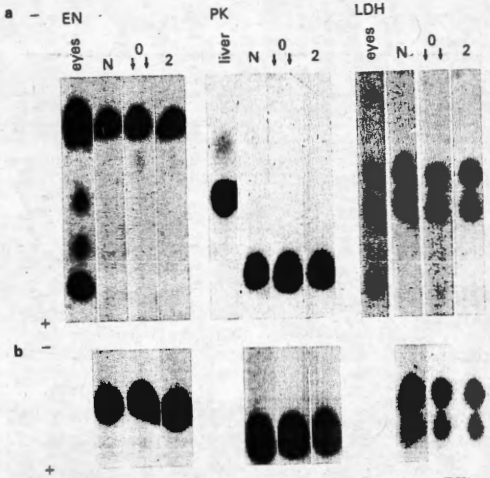


Figure 6 Isoenzyme pattern of enolase (EN), pyruvate kinase (PK) and lactate dehydrogenase (LDH) prior (N) and after serum deprivation (0) and serum stimulation (2); (a) A2 cells; (b) PSM cells; marker tissue; eyes from *Xiphophorus*, liver from rat. Equal amounts of proteins were electrophoretically separated on cellulose acetate strips (Schleicher & Schüll)

specifically in melanoma cells (Mäueler *et al.*, 1988). Since this gene belongs to a subgroup of a superfamily of growth factor receptor genes which is well characterized by an unsplit tyrosine kinase domain (Kraus *et al.*, 1987; Yarden *et al.*, 1986) we are tempted to assume that its expression plays an important role for the development and progression of fish melanomas and the maintenance of the neoplastic phenotype of the cells. In addition it was shown that this gene is closely linked or even part of the melanoma inducing locus *Tu* (Adam *et al.*, 1988; Schartl, 1988). In the case of X*src*, there are indications that expression of this gene is a general feature of neoplastically transformed cells including pigment cells: (1) *In situ* hybridization studies on healthy adults and embryos of *Xiphophorus* gave no indication of X*src* transcripts in normal pigment cells (Raulf *et al.*, submitted). (2) Enhanced kinase activity of pp60$^{c\text{-}src}$, the gene product of the X*src* gene, as compared to the corresponding normal tissue has been reported also for a variety of non-melanomatous tumors of neurogenic or mesenchymal origin in *Xiphophorus* (Schartl *et al.*, 1985). (3) The melanoma derived cell line (PSM) expresses a smaller X*src* transcript than the non-tumorous A2 cell line at the same abundance and shows a nearly 6–10 fold elevated specific kinase

activity per cell than A2 cells (corrected for mRNA and protein content per cell). This might indicate a qualitative difference in *src* expression of PSM and A2 cells in addition to the quantitative variations. The significance of other oncogene expression is unclear at the moment. With respect to a possible tumor specific function of the v-*erb* B related gene and the *Xiphophorus src* gene it is interesting to note that it was shown recently that pp60$^{c\text{-}src}$ might directly interact with the mitogenic signal transduction pathway of EGF (Luttrell *et al.*, 1988).

The most striking observation was that PSM cells exposed to 0.5% FCS contain higher amounts of most of the oncogene transcripts and that the levels decline to normal within 2 h after serum stimulation. This phenomenon was most obvious for the v-*erb* B related gene. The large 5.0 kb transcript is about 10-fold enriched in serum deprived cells while the amount of the smaller 3.2 kb transcript is unchanged, indicating that this phenomenon is not due to different amounts of RNA on the filter. As shown by nuclear run on transcription this effect is not due to a transcriptional activation of the genes. Thus most of the mRNAs investigated might accumulate reversibly by prolongation of their half life time. This is a potentially important, but unclear mechanism of response to serum deprivation. As shown for the pp60$^{c\text{-}src}$ kinase, serum deprived cells exhibit a decreased activity which 2 h after stimulation increased to a 2-fold higher level than observed in normal exponentially growing cells. A similar elevation of the kinase activity was observed when the cells were exposed to 0.5% serum for 48 h (data not shown) indicating that serum deprivation directly causes first a decrease and later an increase in the kinase activity. Until now we do not know if this phenomenon is due to a temporarily delayed enhancement of translational activity as a response to an elevated mRNA supply and/or if the enzyme itself becomes regulated through unknown mediators. But these are two additional ways in which the cells may compensate the decreased supply for growth factors. It will be interesting to characterize the possibly altered features of pp60$^{c\text{-}src}$ in the PSM cells. Recently it was shown that the cellular *src* gene is involved in the regulation of junctional cell-to-cell communication and that altered *src* genes displaying an activated kinase activity (like v-*src*) suppress the cell-to-cell communication (Azarnia *et al.*, 1988). Interestingly both β-tubulin transcripts were also accumulated after serum deprivation, a phenomenon which is still not well understood.

In contrast to the PSM cells A2 cells express the X*sis* gene, exhibit a X*src* mRNA which is at least 300 nucleotides longer than that of the PSM cells, express the X*myc* gene only at barely detectable amounts and do not express the v-*erb* B related gene. Interestingly, serum deprived A2 cells reduced the expression of any oncogene analysed so far to a nearly detectable level. Reduced expression of several oncogenes (including *sis*, *ras* and *myc*) and other growth factor dependent genes was observed also in serum deprived A 31 (Campisi *et al.*, 1984; Kelly *et al.*, 1983; Ran *et al.*, 1986) and AKR 2B cells (Leof *et al.*, 1986), which are also immortalized non-transformed cells like the A2 cells. Most of these genes were inducible within 15 min–24 h after serum or growth factor stimulation (Dean *et al.*, 1986; Ran *et al.*, 1986), e.g. c-*myc* transcripts increase in early G1-, c-*ras*^{Ki} in mid S- (Campisi *et al.*, 1984) and c-*sis* in very early G1-phase of the cell cycle (Leof *et al.*, 1986). As shown by cytofluorimetric analysis A2 cells were not arrested in specific phases of the cell cycle after serum deprivation. Since the relative distribution of the cells in the different cell cycle phases is not changed after serum deprivation and serum stimulation we believe that any deviation in gene expression observed is not due to different proliferating cell populations which go from Go/G1 to S-phase, or from S-phase to G2/M-phase. In addition growth of these cells is dependent on cell contact when seeded at low cell densities and on mitogenic growth factors in the serum independent of the cell density. A2 cells died within 8 days of exposure to 0.5% serum suggesting to us that the cells are extremely dependent on one or more mitogenic growth factors in the serum so that deprivation of these factors possibly leads to a rapid stop of proliferation without an accumulation of the cells in Go/G1 or in G2/M. The oncogene RNA reappearing first was X*ras* at 72 h after serum stimulation. At this point the X*src*, but not X*sis* or X*myc* expression, also begins to return. A similar phenomenon was observed for one β-tubulin transcript, while another transcript was only scarcely influenced, indicating that this is not a general characteristic of mRNAs in these cells. Obviously, serum deprivation is an extreme stress factor for these cells which results in a decline of the expression of all oncogenes analysed so far while long term serum stimulation leads to a late sequential reactivation of these genes.

We also examined the pp60$^{c\text{-}src}$ kinase activity in A2 cells. Although the kinase activity is lower in the PSM cells, it is not altered by serum deprivation and stays constant during the whole time course after serum stimulation indicating that the pp60$^{c\text{-}src}$ kinase in the A2 cells has a very long half life time. It seems conceivable however that prolonged serum deprivation in these cells will lead to a total loss of such gene products resulting in cell death.

To see whether key enzymes of cellular metabolic pathways are also influenced by growth factor withdrawal we determined the activity of 6 enzymes of intermediary metabolism, the isoenzyme pattern of EN, PK and LDH and the transcriptional activity of the EN and LDH genes in both cell lines. A specific enhancement of EN, PK, LDH and MDH of PSM cells dependent on the cell density was reported earlier (Mäueler *et al.*, 1987). We could show that serum deprivation neither activates the transcription of the genes, nor leads to an altered activity of the enzymes or to a changed composition of isoenzymes. This indicates that the intermediary metabolism of the cells on the level of enzyme activity is not altered directly through this treatment. However this does not exclude an altered turnover rate for substrates like glucose or glutamine, as has been observed for 3T3 cells (Sumi *et al.*, 1984).

The PSM cell line and the A2 cell line, the former being a fully transformed cell line, differ in their genetic make up only by the absence of a regulatory locus for the melanoma oncogene locus in the PSM genome. This has been shown to be the primary event leading to tumor formation in *Xiphophorus*. How this lack of regulation of the *Tu* locus could be related to the different regulation of gene expression in the two cell lines investigated is unclear at the moment. The ability of melanoma cells to respond to an insufficiency of growth factors

by the accumulation of mRNAs whose protein products are implicated in the regulation of cell growth may be an essential feature of the neoplastic phenotype of these cells. The fact that this response is most marked for one transcript of a gene which is related to several members of a growth factor receptor family, and is structurally associated with the *Tu* locus, points to a functional link between the activation of the melanoma oncogene locus and the neoplastic phenotype of the pigment cells.

Material and methods

Cell lines

Cell lines derived either from spontaneous hereditary melanoma of *Xiphophorus* hybrids (PSM cells) (Wakamatsu *et al.*, 1984), or from non-tumorous embryos of wildtype *Xiphophorus xiphidium* (A2 cells) (Kuhn *et al.*, 1979) were used. Cells were cultured either in F12 or in Dulbecco's modified Eagle medium (DMEM) [Biochrom KG, Seromed, Berlin] containing 10% fetal calf serum (FCS) and 1.25 g $NaHCO_3$/l at 28°C under 5% CO_2.

Plating efficiency

10, 50, 500, 1000, 5000, and 10000 cells were seeded onto 50 mm plastic plates in F12 medium supplemented with 10% FCS. Triplicate cultures were set up for each cell population density. After two weeks, cells were fixed in 10% buffered formalin (pH 6.8) and stained with methylene blue. The number of colonies were counted under a dissecting microscope.

Soft agar colony formation

5×10^4, 1×10^5, 2×10^5, and 5×10^5 cells were suspended in 5 ml of culture medium (F12, 10% FCS) containing 0.36% agar. The cell suspension was placed on 2 ml medium containing 0.9% agar in 50 mm plastic plates. Triplicate cultures were set up for each population density. Colonies were counted under a dissecting microscope.

Analysis of the cell cycle

The cells were fixed with 70% ethanol and rehydrated with HEPES buffered saline pH 7.35 (HBS: 150 mM NaCl, 0.75, 5 mM HEPES) and stained with 50 $\mu g\,ml^{-1}$ mitramycin (Pfitzer, Karlsruhe, FRG)), 20 $\mu g\,ml^{-1}$ ethidium bromide, 10 mM $MgCl_2$ in HBS. The cytofluorimetric measurements were made using a FLUVO-METRICELL equipped with a Hg lamp (excitation 300–400 nm, emission 500–700 nm) and evaluated on a Vax 782 with FORTRAN IV programs (Valet, 1980).

Hybridization probes

All probes used for nick-translation were separated from vector sequences and highly GC-rich sequences of the insert after appropriate restriction enzyme digestion, low-melting point agarose gel electrophoresis and further purification through NACS-columns (BRL, Eggenstein, FRG). The following heterologous probes were used: (1) 600 bp BamHI fragment D of pAEII (Vennström *et al.*, 1980) representing the central part of the cytoplasmic domain of the v-*erb* B gene of Avian erythroblastosis virus; (2) 800 bp PvuII/SacI fragment B of the BamHI fragment B of pAEII (Vennström *et al.*, 1980), representing the v-*erb* A gene of avian erythroblastosis virus; (3) 700 bp BglII/PstI fragment D of pHB-II (Ellis *et al.*, 1980) of the v-*ras* gene of Harvey murine sarcoma virus; (4) the 1.5 kb EcoRI/PstI fragment of the trout c-*myc* clone C-181, containing sequences homologous to exon 2 and 3 of the chicken c-*myc* gene (VanBeneden *et al.*, 1986); (5) the 1.6 kb EcoRI c-DNA fragment of the β1-tubulin (β1-*tub*) gene of *Drosophila* from pc 56-A (Gasch *et al.*, 1988). The following *Xiphophorus* specific fragments were used: (6) 1.2 kb PstI fragment of pX*src* 19-4 (Robertson *et al.*, submitted), sharing 92% similarity on amino acid level to the kinase domain of the human c-*src* gene; (7) 300 bp BamHI/BglII X*sis* fragment (generous gift from U. Schlehenbecker). For *in vitro* transcription the 1.2 kb PstI X*src* specific fragment was subcloned into pGEM1 (Promega Biotec, Madison). Prior to *in vitro* transcription, the vector containing the insert in the correct orientation (producing antisense RNA) was linearised by restriction enzyme digestion.

Plasmids used for nuclear run on transcription assay

The following nonlinearized plasmids were blotted with a slot-blot manifold (Schleicher & Schüll, Dassel) onto GeneScreen following the protocol of the suppliers (New England Nuclear Chemicals, Dreieich): (1) pGEM*myc*, containing the 1 kb SalI/BamHI fragment C of pMC29 (Alitalo *et al.*, 1983); (2) pSP65*erb* B (see above); (3) pSP64*ras* (see above); (4) pSP64*sis*, containing the 900 bp XbaI/PstI fragment of pC60*sis* (Gelmann *et al.*, 1981); (5) pGEMX*src* (see above); (6) pLDH; (7) pENO-2 and pD5 (Matrisian *et al.*, 1985) containing DNA clones of the LDH and the enolase genes of the rat and an unknown fragment of a gene (D5) which is constitutively expressed in different tissues of the rat (Matrisian *et al.*, 1985).

Southern blot analysis

For Southern blot analysis DNA from exponentially growing cells was isolated. 10 μg of each sample were digested to completion with restriction enzymes and subjected to electrophoresis in 0.8% agarose gels. DNA was transferred to GeneScreen plus membranes by the capillary blot method of Southern (1975) using alkaline transfer (Reed & Mann, 1985). Filters were hybridized in 1 ml of a mix containing either 40% formamide (heterologous probes), or 50% formamide (homologous probes), 5 × Denhardt's, 1% SDS, 5 × SSC (1 × SSC is 0.15 mM NaCl, 0.015 M sodium citrate, pH 7.0), 250 $\mu g\,ml^{-1}$ calf thymus DNA and approximately 10^7 dpm of nick-translated [^{32}P]-labeled probe at 42°C for at least 24 h. Filters were washed at 63°C, 0.5 × SSC (for heterologous probes), or at 68°C, 0.1 × SSC (homologous probes) for 1 h and exposed to a Kodak XAR 15 X-ray film. Nick-translations were performed as described by Maniatis *et al.* (1982), using a kit from Amersham Buchler (Braunschweig, FRG).

Northern blot analysis

Total cellular RNA was isolated following the LiCl procedure of Auffray & Rougeon (1980) using ultraturrax N8 (Janke & Kunkel, Staufen, FRG) for homogenization. 20 μg of total RNA were denatured with formamide/formaldehyde and electrophoresed in 1.2% agarose gels containing 2.2 M formaldehyde (Lehrach *et al.*, 1977). RNA was then electroblotted to GeneScreen membranes according to the protocol of the suppliers (New England Nuclear Chemicals, Dreieich). For exact quantitation of the RNA amount present on the hybridization membrane, each filter was stained with methylene blue (Khandjian, 1986) and the relative amounts of RNA were determined densitometrically. As shown in Figure 3 only minor variations (±10%) in the amount of the different samples was observed. Filters were hybridized with 10^7 dpm ml^{-1} of labeled probe. Hybridization with nick-translated probes was carried out under the conditions described for Southern blots, excepting the addition of yeast total RNA to 500 $\mu g\,ml^{-1}$ to the hybridization mix. Filters were then washed 55°C, 0.5 × SSC, 1% SDS for 1 h. Hybridization with *in vitro* transcribed probes was carried out in the same mix at 59°C and filters were washed at 68°C, 0.1 × SSC. Filters were then exposed to Kodak XAR 15 X-ray film. *In vitro* transcriptions with SP6 RNA-polymerase using [α-^{32}P] UTP were carried out according to the suppliers recommendation (Genofit, Heidelberg).

Isolation of nuclei, nuclear run on transcription

PSM cells were suspended in lysis buffer containing 60 mM KCl, 15 mM NaCl, 15 mM 2-mercaptoethanol, 0.5 mM EGTA, 2 mM EDTA and 15 mM HEPES, pH 7.5 (Schibler *et al.*, 1983). After addition of 0.05% NP40 lysis was performed by passing the cells 40 times through a 10 ml plastic pipette. The lysate was centrifuged (800*g*, 15 min), and washed 2 times in lysis buffer without NP40. Nuclei were counted and recentrifuged. Nuclei were resuspended to $10^8\,ml^{-1}$ in storage buffer containing 60 mM KCl, 15 mM 2-mercaptoethanol 15 mM EDTA, 15 mM HEPES 25% (v/v) glycerol, pH 7.5, and then were immediately frozen in liquid nitrogen prior to storage at −70°C. For one reaction 10^7 nuclei were incubated for 25 min at 26°C in a mix containing 250 μCi [α-^{32}P]UTP (2.5 μM), 400 μM rNTPs, 60 U RNasin, 70 mM KCl, 5 mM $MgCl_2$, 2.5 mM DTT and 0.1 mM EDTA. Synthesized RNA was then purified as described by Groudine *et al.* (1981), and then used for hybridization with slot-blot filters. The incorporation of [α-^{32}P]UTP into RNA of the nuclei normally was about 3–6 × 10^5 cpm/μg nuclear DNA and typical average length of the transcripts were about 400 bp (compare Figure 7). Slot blot filters were hybridized with 10^7 dpm ml^{-1} of labeled RNA as described for Northern blots and washed at 55°C, 1 × SSC for 1 h followed by RNAase digestion (10 μg RNAase A ml^{-1} in 2 × SSC, 30 min, 37°C) and again washed at 55°C, 1 × SSC for 30 min. Each experiment was performed 3 times, always with comparable results.

Antisera

Sera from tumor bearing rabbits (TBR-sera) were prepared by the simultaneous injection of two different strains of Rous sarcoma virus (SR-RSV-D and PR-RSV-C) into newborn rabbits in a modification (Ziemiecki & Friis, 1980) of the procedure described by Brugge & Erikson (Brugge & Erikson, 1977).

Preparation of cell extracts and immunoprecipitation

2×10^7 cells were homogenized in extraction buffer (10 mM sodium phosphate, 10 mM EDTA, 40 mM sodium fluoride, 5% Trasylol, 1% Triton). The lysates were clarified as described previously (Barnekow & Bauer, 1984). An aliquot of soluble protein (0.2 or 0.4 mg) was incubated with antiserum for at least 12 h at 4°C and precipitated with the protein A containing *Staphylococcus aureus* bacteria. The bacterial bound immunocomplexes were washed and subjected to the kinase assay (labeling for 5 min., 4°C). The protein kinase assay was carried out by a modification (Barnekow & Bauer, 1984) of the method of Collet & Erikson (1978). The reaction products were separated on 11% polyacrylamide gels. The labeled proteins were detected by autoradiography after staining, destaining and drying of the gels. For the quantitative analysis, the [^{32}P]-labeled 53K protein band was cut out of the gel, eluted from the gel sample and the sample counted in a liquid scintillation counter. All immunoprecipitations were performed at least with 2 different TBR-sera, always with the same result.

Protein determination

Determination of protein content in cell extracts was carried out on trichloroacetic acid (TCA)-precipitated aliquotes according to the method described by Lowry *et al.* (1951).

Phosphoamino acid analysis

[^{32}P]-labeled IgG was cut out of the gel, eluted from the gel sample and processed for phosphoamino acid analysis according to the method described by Hunter & Sefton (1980). The hydrolysates were dissolved in a mixture of pH 1.9 buffer and phosphoamino acid marker (1 μg each) and resolved in two dimensions on thin-layer cellulose-coated glass plates by electrophoresis toward the anode at pH 1.9 for 180 min at 500 V in acetic acid/formic acid (88% (by vol.)/H_2O (78 : 25 : 897, v/v), followed by electrophoresis toward the anode at pH 3.5 for 100 min at 500 V in acetic/pyridine/H_2O (50 : 5 : 945, v/v). The markers were detected by staining with ninhydrin.

Analysis of enzyme activities and isoenzyme pattern

Enzyme activities of lactate dehydrogenase (LDH, EC 1.1.1.27), malate dehydrogenase (MDH, EC 1.1.1.37), enolase (EN, EC 4.2.1.11) and pyruvate kinase (PK, EC 2.7.1.40) were determined as described earlier (Büchner & Pfleiderer, 1955; Eigenbrodt *et al.*, 1983; Rübsamen *et al.*, 1982). Activities of phosphoglycerate mutase (PGM, EC 2.7.5.3) and phosphoglycerate kinase (PGK, EC 2.7.2.3) were determined using a kit from Boehringer (Mannheim) containing all substrates, cosubstrates and helper enzymes at optimal concentrations.

Isoenzyme analyses of EN, PK, and LDH were performed on cellulose acetate membranes (Schleicher & Schüll, Dassel) as described (Harris & Hopkinson, 1978; Reinacher *et al.*, 1986; Rigaut & Chalumeau, 1984). 1–5 μg protein or 1–3 mU of enzyme activity were electrophoretically separated. For visualization of the isoenzymes the membranes were covered with a 0.1% agarose gel according to Susor & Rutter (1971) containing all substrates, cosubstrates and helper enzymes in the same concentrations as used for determination of the enzyme activities and incubated for 30–45 min at 37°C. Membranes were then exposed to UV-light (366 nm) and photographed with a polaroid camera (filmtype 667). The isoenzymes appeared as dark blue spots on the membranes.

Acknowledgements

We thank Dr K. Bister, Dr E.P. Gelmann and Dr R. Gallo for providing the viral oncogenes, Dr L. Matrisan for the pLDH, pENO-2 and pD5 clones, S.M. Robertson for the pX*src* 19-4, U. Schlehenbecker for the pX*sis* probes, Dr R.J. VanBeneden for the trout c-*myc* clone C-181, Dr R. Renkawitz-Pohl for the β1 tubulin fragment and Dr M. Cross for critical reading of the manuscript. The technical assistance of S. Andexinger and S. Leib is gratefully acknowledged. This study was supported by the Bundesministerium für Forschung und Technologie, Schwerpunkt 'Grundlagen und Anwendungen der Gentechnologie' and Schwerpunkt 'Carcinogenese, Mutagenese, Teratogenese' (CMT 32 A), the Deutsche Forschungsgemeinschaft, Schwerpunkt 'Tumorzytogenetik' the Sonderforschungsbereich 47 'Virologie', and the Sonderforschungsbereich 324 Schwerpunkt 'Die maligne Zelle'. W.M. was a recipient of a scholarship of the Studienstiftung des Deutschen Volkes. This article contains parts of the Ph.D. theses of W. Mäueler and F. Raulf.

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High efficiency transfection of primary human lymphocytes and studies of gene expression

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Human peripheral blood lymphocytes are refractory to DNA transfection, yet an increasing number of molecular genetic applications require the introduction of biologically active DNA into primary cells. We have developed a technique employing electroporation for efficient introduction of recombinant DNA into primary lymphoid cells. Electroporation has previously been used for transfection of cultured cell lines. We define conditions which allow introduction of DNA into primary cells with efficiencies comparable to those obtained for lymphoid cell lines and by calcium phosphate transfection of fibroblast cell lines. We use the method to investigate relative activities of tissue-specific and non-specific promoters and *trans*-activation of human T-cell leukemia virus and human immunodeficiency virus promoters in their normal target cells, i.e., primary human T cells.

Introduction

The ability to introduce DNA into eukaryotic cells has contributed to many recent advances in molecular biology. Transformation assays using high molecular weight input DNA have resulted in the identification of cellular oncogene-related sequences, and subsequent studies have revealed some of the mutations responsible for activation of oncogenes (Bishop, 1983). The development of efficient eukaryotic expression vectors has contributed to understanding the function of many genes. Expression of cloned genes has also been used to screen cDNA libraries, and has facilitated the isolation of genes encoding novel proteins, particularly in the case of cellular growth factors (Clark & Kamen, 1987).

A variety of techniques have been developed to transfer DNA into cells from higher organisms, although the precise mechanisms by which many of these methods operate are not fully understood. Two of the most frequently utilized methods are calcium phosphate precipitation (Chu & Sharp, 1981; Graham & Van der Eb, 1973) and diethylaminoethyl (DEAE)-dextran transformation (McCutchan & Pagano, 1968). In the former technique, DNA in a solution of calcium chloride is mixed with phosphate-containing buffer, causing the formation of an insoluble calcium-phosphate precipitate which entraps the DNA. A major limitation of this technique is that it is most efficient when applied to fibroblasts and other adherent cell types. In contrast, DEAE-dextran is an ionic polymer, and does not cause precipitation of DNA. Therefore, this technique is applicable to both adherent cells as well as nonadherent cells, such as those of lymphoid origin. Another technique applicable to lymphoid cells is protoplast fusion, in which DNA is transferred into the cells by membrane fusion of bacterial protoplasts and target cells promoted by polyethylene glycol (Schaftner, 1980). However, all of the above techniques suffer from the disadvantage that, although efficient for established cell lines, they are unsuitable for the introduction of DNA into primary cells.

The technique of electroporation relies on the transfer of extracellular DNA molecules into the cell when an electric field is applied. This technique has recently begun to gain wider acceptance, and is now being used to transfer DNA into mammalian (Neumann *et al.*, 1982; Potter *et al.*, 1984), plant (Boston *et al.*, 1987; Fromm *et al.*, 1985), and even bacterial cells (Lian-ying & Wong, 1984; Shivarova *et al.*, 1983). However, almost without exception, the technique has been applied to cultured cell lines. We have modified the procedure for application to primary human lymphoid cells. Here we describe the procedure and report model applications involving foreign gene expression, relative promoter activities and *trans*-activation of human retrovirus LTRs in their normal target cell. The ability to study gene expression in primary lymphocytes, where the internal cellular environment differs from that of immortalized cells in many ways which are not fully understood, will be valuable in elucidation of mechanisms of genetic control.

Results

Transient expression of CAT in lymphocytes

High cell viability (>90%, as assayed by trypan-blue staining) prior to electroporation was found to be necessary for efficient expression of chloroamphenicol acetyltransferase (CAT) activity from recombinant constructs. (Peripheral blood lymphocytes (PBL) stimulated with phytohemaglutinin (PHA) for 3 days as described in Materials and methods were generally >90% viable). Although individual experiments showed variation from 50–90% viability 24 h after electroporation, cell viability was normally between 70–80% at this time. Transient expression of CAT activity was found to be highest 24 h after electroporation, gradually declining thereafter (not shown).

PBL used for electroporation were stimulated for three days with PHA and IL-2. No attempt was made to examine CAT gene expression in cells stimulated for

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Received 5 April 1988; accepted 26 April 1988