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Expression of proto-oncogenes in embryonic, adult, and transformed tissue of *Xiphophorus* (Teleostei: Poeciliidae)

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In *Xiphophorus* the causative, primary cellular oncogene for melanoma formation has been assigned by classical genetics to a sex-chromosomal locus, designated *Tu*. Activation of *Tu* was proposed to be the result of the elimination of *Tu*-specific regulatory genes which normally suppress the transforming function in the non-tumorous state. In order to understand the role which known proto-oncogenes might play in this process, we have analysed the expression of *src*, *erb* A, *erb* B, *ras*, *abl*, *sis* and *mil* related genes from *Xiphophorus* during embryogenesis, in non-tumorous organs and in melanoma cells. For *src*, *ras*, *erb* B and *sis* a differential expression during embryogenesis and/or in normal organs was detected, with preferential expression of *src* in neural tissues, a high abundance of *sis* transcripts in an embryonal epitheloid cell line and of *erb*B transcripts in the head nephros. In melanoma cells *ras*, *src* and a v-*erb* B related gene were found to be expressed. The *src* gene most likely is more involved in secondary processes during tumor progression, while the expression of the v-*erb* B related gene might be transformation-specific because recently such a sequence was found to map to the close vicinity of the *Tu*-locus.

Introduction

The question of the relevance of genetic factors to the process of tumor development has become increasingly important during the last decade through findings in clinical oncology. Genetic analysis of 'cancer-families' led to the assumption that genetic risk factors contribute considerably to the etiology of cancer. For instance in the case of human malignant melanoma this has clearly been demonstrated for the dysplastic nevus syndrome (FAMMM-syndrome, see Lynch *et al.*, 1985; Rhodes *et al.*, 1983). So far, there is no information on the molecular nature and the biological function of these genetic factors. As candidate genes for such factors several dominant acting cellular genes (proto-oncogenes) are discussed which, following a process of activation by qualitative or quantitative changes of the respective gene product, display in certain experimental systems the potential to initiate and maintain the neoplastic phenotype of a cell (Bishop, 1986; Müller, 1986; Weinberg, 1986). In human melanoma, e.g. activated c-ras^{Ha} 1 (Albino *et al.*, 1984; Sekiya *et al.*, 1985), c-ras^{N} (Padua *et al.*, 1985), c-*sis* (Westermark *et al.*, 1986) and c-*src* (Barnekow *et al.*, 1987) have been found. However, in the majority of tumors and tumor-derived cell lines investigated no activated oncogene could be detected. Moreover, in many cases the functional proof for the causal involvement of the activated oncogenes was impossible to obtain. Besides the possibility that a large variety of so far undiscovered potential dominant oncogenes are present in the genome, a totally different class of genes might be involved in tumor formation. A candidate class is that of the recessive oncogenes (sometimes also termed 'anti-oncogenes', Knudson, 1985), the disfunction of which in the homozygous condition would be responsible for tumor formation. Two such recessive oncogenes have been cloned recently, namely the gene for human retinoblastoma (Friend *et al.*, 1986; Friend *et al.*, 1987), and the lethal giant larvae gene of *Drosophila* (Mechler *et al.*, 1985; Jacob *et al.*, 1987). The melanoma system of the teleost fish *Xiphophorus* offers the unique opportunity to study the function of both oncogenes and anti-oncogenes in tumor development in one experimental system.

In *Xiphophorus*, certain hybrid genotypes develop spontaneously malignant melanoma. Melanoma formation has been attributed by classical genetic findings to the overexpression of a dominant acting cellular oncogene, termed *Tu*. In non-tumorous fish, *Tu* was proposed to be negatively controlled by cellular regulatory genes, termed *R*, which act as 'anti-oncogenes' (for review see Anders & Anders, 1978; Anders *et al.*, 1984). In a typical crossing experiment a female *Xiphophorus maculatus* (platyfish) containing a specific *Tu*-locus and its corresponding *R* gene, which are 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 *X. helleri* results, in effect, in the progressive replacement of *R* gene bearing chromosomes originating from *X. maculatus* by chromosomes of *X. helleri*. The homozygous elimination of regulatory genes allows increased expression of *Tu*, resulting in the development of malignant melanoma in the hybrids (see Figure 1).

We have shown previously that the c-*src* proto-oncogene of *Xiphophorus* (X*src*) is activated with respect to elevated kinase activity in the malignant melanomas and that X*src* expression correlates with the expression of *Tu* (Schartl *et al.*, 1982; Schartl *et al.*, 1985). To investigate, if and how known proto-oncogenes might be involved in melanoma formation of *Xiphophorus* and if there exists a functional relationship between these genes and the activity of *Tu*, we studied at first the expression of X*src*, X*erb* A, X*erb* B, X*ras*, X*sis*, X*abl* and X*mil* on the mRNA level of tumors of adult fish and in a tumor derived cell line (PSM). Reasoning that an

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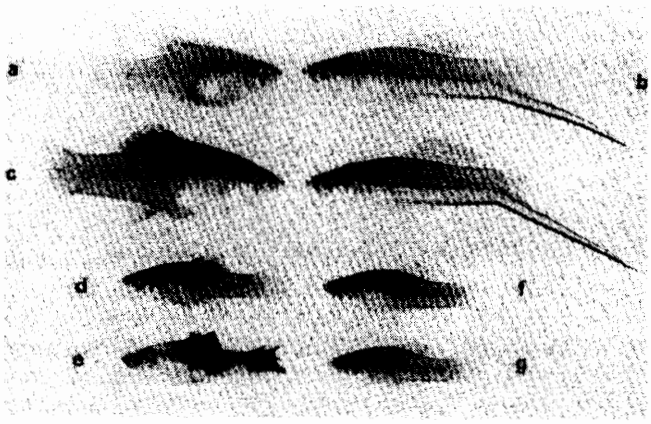

Figure 1 Crossing scheme of the platyfish (**a**) and the swordtail (**b**); (**c**) F_1-hybrids and (**d**) BC_1-hybrids developing benign melanoma; (**e**) BC-hybrid developing malignant melanoma; (**f**, **g**) melanoma free BC_1-segregants; for explanation see text

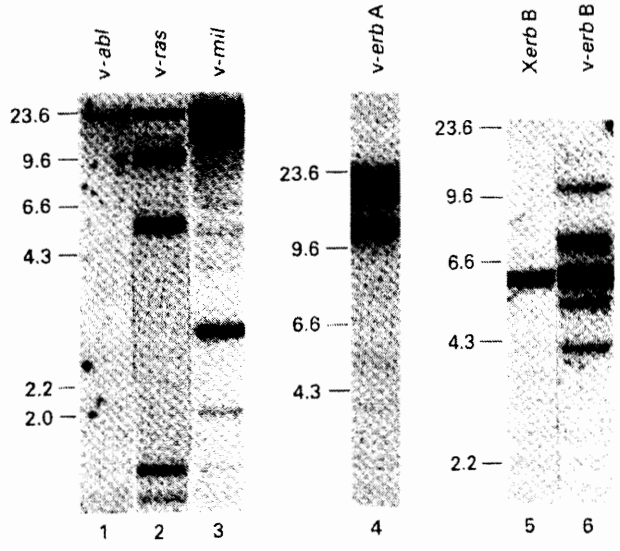

Figure 2 Southern blot analysis of *Xiphophorus* genomic DNA for the presence of sequences homologous to viral oncogenes. EcoRI (1,2,3,5,6) and BamHI (4) digested DNA from *X. helleri* (1,3,5), *X. maculatus* (2,6) and the A2 cell line (4) probed with v-*abl* (1), v-*ras* (2), v-*mil* (3), v-*erb* A (4), the *Xiphophorus* X*erb* B (5) gene (probe: 4.5 kb EcoRI fragment of clone λ 44), and v-*erb* B (6)

understanding of normal cellular functions of proto-oncogenes would give an indication of the role of the activated oncogenes in tumor cells, we have also examined the expression of these genes in non-transformed tissues of adult fish and during normal embryogenesis. Such experiments seemed to be additionally justified because to date our knowledge on the expression pattern of proto-oncogene transcripts in a defined experimental system in the *in vivo* situation is still enigmatic. Comparative studies analysing the expression of several c-*onc* genes during embryogenesis of chicken and mouse and in different normal adult chicken, mouse and human tissues were carried out by Gonda *et al.* (1982), Müller (for review see Müller & Verma, 1984), Sheiness *et al.* (1980), Vennström & Bishop (1982), Wang & Baltimore (1983) and Westin *et al.* (1982). It was demonstrated, that in general these proto-oncogenes are differentially expressed in different organs of the adults and during different stages of embryonic development.

We report here that in the lower vertebrate *Xiphophorus* the proto-oncogenes show an expression pattern comparable to that of higher vertebrates, both in normal organs and during embryogenesis. We also show that multiple transcriptional and/or translational activation of proto-oncogenes accompanies the process of tumor formation and/or progression, but – at least for some of the genes – most likely is not the primary event leading to melanoma development.

Results

Sequences in the genome of Xiphophorus *similar to retroviral oncogenes*

The presence of the X*src* gene in *Xiphophorus* (exhibiting 90% similarity on the amino acid level to the chicken c-*src* gene) has been demonstrated by gene cloning and sequence analysis (Robertson *et al.*, submitted, Schartl *et al.*, 1987), and probes derived from that gene were used for the expression studies. Sequences related to the *sis* and to the *erb* B gene were also cloned recently from *Xiphophorus* (Zechel, Schlehenbecker, manuscripts in preparation). In hybridizing a probe for the X*erb* B gene to *Xiphophorus* genomic DNA we obtained a banding pattern (Figure 2) which is consistent with the presence of this sequence as single copy genes. However, the v-*erb* B probe detects several sequences besides the actual *erb* B gene of *Xiphophorus* (represented by a 6 kb-band in EcoRI digests), suggesting the presence of additional *erb* B-related genes. To detect genes which are homologous to the *abl*, *ras*, *mil* and *erb* A oncogenes, we used the viral oncogenes as probes under conditions of moderate stringency. The clear and strong signals obtained with each of the probes (Figure 2) indicate the presence of sequences similar to these oncogenes in the *Xiphophorus* genome. Weaker hybridizing bands might represent more distantly related sequences cross-hybridizing with the probe.

Detection of proto-oncogene transcripts

For X*src* we detected two transcripts designated X*src* 1 (3.7 kb) and 2 (3.4 kb) (Figure 4), and one X*src*-related transcript of about 3.0 kb (the signal of this transcript is reduced when washed at very high stringencies; 75°C, 0.1 × SSC; data not shown). For X*sis* we detected two transcripts designated X*sis* 1 (3.4 kb) and 2 (2.5 kb, see Figure 5) and an additional high molecular weight band of about 10 to 15 kb which is possibly a precursor RNA. In the case of X*erb* B we found two transcripts numbered X*erb* B 1 (5.0 kb) and 2 (3.2 kb) (see Figure 7). For X*ras*, three related transcripts called X*ras* 1 (3.4 kb), 2 (3.0 kb) and 3 (1.7 kb) were detected (Figure 6). Transcripts of the proto-oncogenes X*erb* A, X*mil* and X*abl* were not detectable during embryogenesis, in melanoma cells or in any tissue from adult fish investigated so far.

Expression of proto-oncogenes during embryonic development

In order to find out whether the expression of the proto-oncogenes investigated correlates with distinct processes during embryogenesis and early postnatal development, total RNA isolated from whole embryos of different morphologically defined stages (Tavolga,

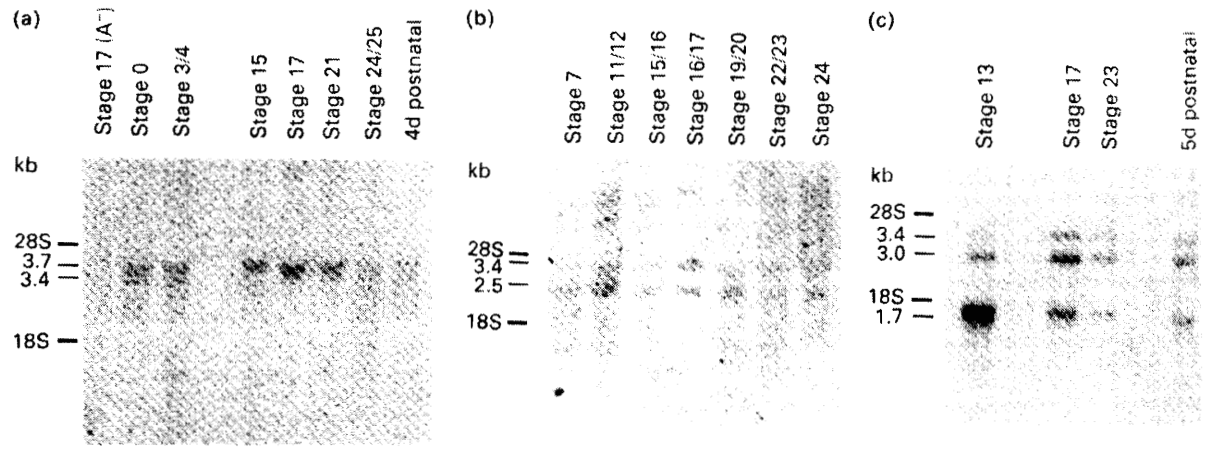

Figure 3 Expression of X*src* (**a**), X*sis* (**b**), and X*ras* (**c**) during embryogenesis of *Xiphophorus*; 20 μg of total RNA of each embryonic stage were hybridized to the 1.2 kb X*src* riboprobe (**a**), the 300 bp nicktranslated X*sis* probe (**b**), and the v-*ras* riboprobe (**c**)

1949) and young fish were analysed by Northern blot hybridization.

For X*src* both transcripts could be detected during embryogenesis in a differential manner (Figure 3a). In unfertilized mature ova (stage 0) and in early stages of embryogenesis, e.g. late blastula (0.5–1 day after fertilization, stage 3/4), approximately similar amounts of X*src* 1 and X*src* 2 were found. During late organogenesis (5–7 days after fertilization, stage 14–17) a considerable elevation of X*src* 1 transcript was apparent forming a peak around stage 17, while the X*src* 2 transcript decreased in comparison to early stages. Later in embryogenesis, in neonate and young fish both X*src* transcripts were present at basal levels.

X*sis* mRNAs were first detectable at very low levels in two day old embryos during late neurula and early organogenesis (stage 7, Figure 3b). Both transcripts were found slightly elevated during late organogenesis. Some stages showed also the additional transcript of about 10–15 kb.

For X*ras* a clear differential expression of the three transcripts was found (Figure 3c). X*ras* 1 and 2 peaked about 8 days after fertilization (stage 17), while very high amounts of X*ras* 3 mRNA were already detected in five day old embryos (stage 13). These levels decreased to the basal levels observed during late embryogenesis and in neonates.

Neither for X*erb* B nor for the v-*erb* B-related genes could detectable levels of mRNA be found during embryogenesis or in postnatal fish (data not shown).

Expression of proto-oncogenes in adult and transformed tissue of Xiphophorus

To investigate the expression pattern of proto-oncogenes in adult and transformed tissue of *Xiphophorus* total and poly(A)$^+$ enriched RNA from these tissues were analysed by Northern blot hybridization.

Both transcripts of X*src* showed a tissue specific distribution (Figure 4). Brain, eyes and melanoma exhibited the two transcripts, while the other tissues showed only one X*src* transcript. The highest level of X*src* RNAs was found in A2 cells*. Lower amounts were detected in PSM cells, brain, eyes and melanoma. In head nephros, heart and spleen low amounts of X*src* transcripts, while in muscle, liver and testes only barely detectable amounts were observed. The X*src* related transcript was detected at comparable low levels in heart, spleen and a higher level in head nephros. Hybridizations using the viral *src*-gene as a probe yielded a similar expression pattern (data not shown).

All transcripts of X*sis* were detected at an extraordinary high level in A2 cells (Figure 5). Testes, head nephros, eyes, brain, melanoma and muscle showed barely detectable levels. In liver and PSM cells X*sis* mRNA was not detectable. Experiments using the v-*sis* gene for hybridization gave no specific signals (data not shown).

The distribution of the different *ras* related transcripts is also tissue specific (Figure 6). Eyes, brain and melanoma exhibited all three transcripts, while heart, muscle, liver and PSM cells only showed X*ras* 2 and 3 RNA. In spleen no X*ras* expression was observable. The highest level of X*ras* 1 RNA was found in brain, while eyes and melanoma showed lower amounts of this message. High levels of X*ras* 2 RNA was found in eyes, brain and

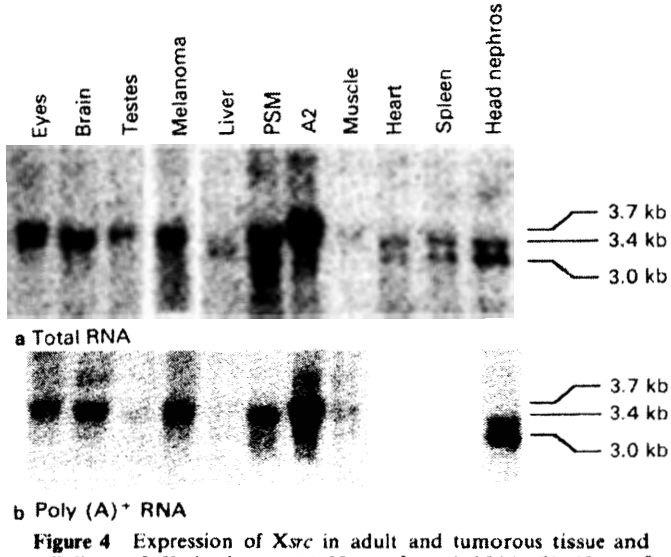

Figure 4 Expression of X*src* in adult and tumorous tissue and cell lines of *Xiphophorus*; (**a**) 20 μg of total RNA, (**b**) 10 μg of poly(A)$^+$ enriched RNA of each sample, heart and spleen not done; filters were hybridized with the 1.2 kb X*src* riboprobe

* If the amounts of transcripts detected in Northern blots are corrected for the RNA content per cell, a similar abundance of X*src* mRNA in A2 and PSM cells is obtained.

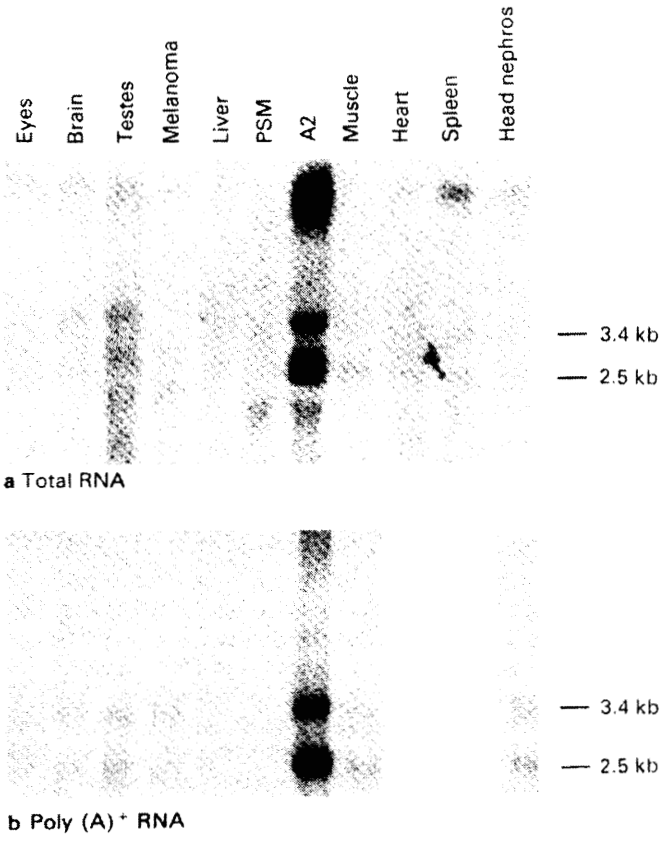

Figure 5 Expression of X*sis* in adult and tumorous tissue and cell lines of *Xiphophorus*; (**a**) 20 μg of total RNA, (**b**) 10 μg of poly (A)$^+$ enriched RNA of each sample, heart and spleen not done; filters were hybridized with the 300 bp nicktranslated X*sis* probe

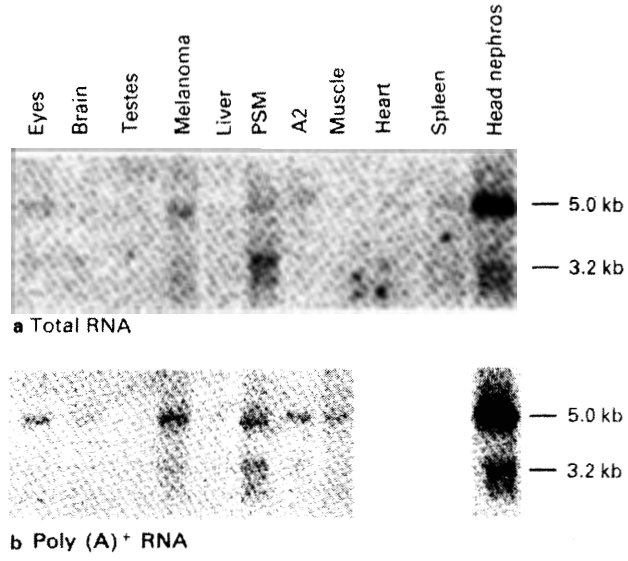

Figure 7 Expression of X*erb* B in adult and tumorous tissue and cell lines of *Xiphophorus*; (**a**) 20 μg of total RNA, (**b**) 10 μg of poly(A)$^+$ enriched RNA of each sample, heart and spleen not done; filters were hybridized with the nicktranslated X*erb* B 60-221. Hybridization with the X*erb* B 60-222 probe revealed the same result

melanoma. Lower amounts were detected in heart and PSM cells, while muscle and liver showed only barely detectable amounts. The level of X*ras* 3 RNA was low in all the tissue and cells analysed so far.

Hybridization with both the X*erb* B probes or with the v-*erb* B fragment revealed a different pattern of transcripts in the different tissues. A very high amount of X*erb* B 1 transcript was found in RNA of the head nephros (Figure 7, a and b). Eyes, brain, melanoma, PSM cells, A2 cells, muscle and spleen were found to contain the X*erb* B 1 transcript at low levels. X*erb* B 2 transcripts were observed in head nephros and PSM cells. In RNA from testes and liver no X*erb* B transcript was detectable. Using the same hybridization stringency for the v-*erb* B fragment as a probe two transcripts of similar size compared to those detected with the two X*erb* B fragments (Figure 8) were found. In contrary to the X*erb* B hybridization the v-*erb* B probe did detect low levels of the X*erb* B transcripts in head nephros and no X*erb* B transcript in eyes, brain, muscle, heart and spleen (Figure 7b and 8b). However, in RNA from the melanoma cell line (PSM) extraordinary high amounts of a 5.0 kb and a 3.2 kb transcript were detected by the v-*erb* B fragment (Figure 8, a and b) indicating that the cells contain two additional mRNA species which seem to share only limited similarity to the X*erb* B probes. Hybridization with the v-*erb* B probe of RNA from melanoma biopsies from the fish revealed also signals from the 5.0 and 3.2 kb transcripts (Figure 8a and 8b).

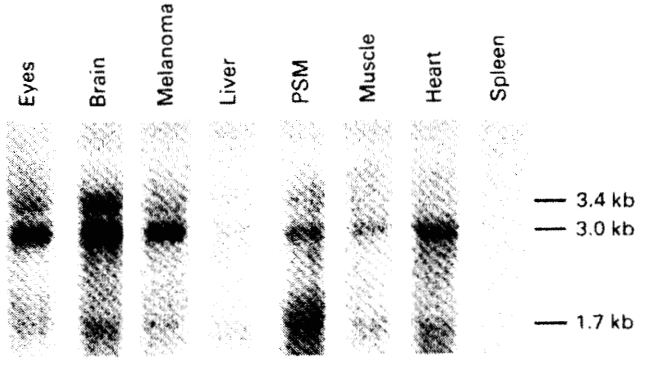

Figure 6 Expression of X*ras* in adult and tumorous tissue and cell lines of *Xiphophorus*; 20 μg of total RNA of each sample; filters were hybridized with the nicktranslated v-*ras*^{Ha} fragment

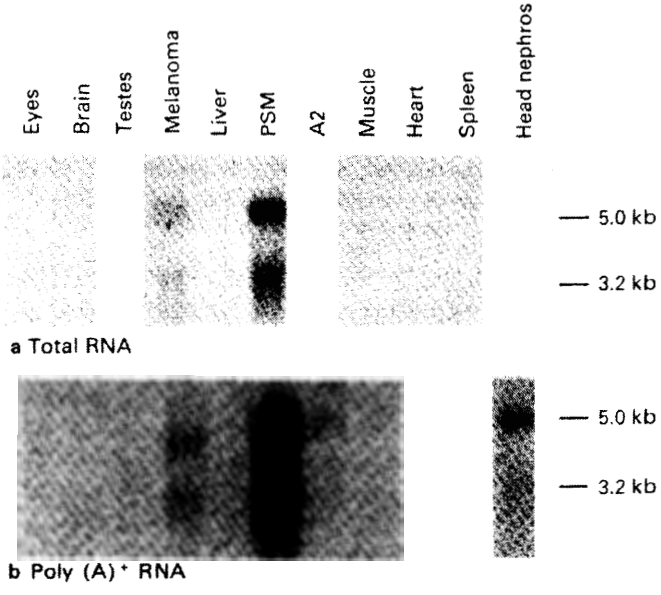

Figure 8 Expression of v-*erb* B related gene in adult and tumorous tissue and cell lines of *Xiphophorus*; (**a**) 20 μg of total RNA, testes, A_2 and head nephros not done; (**b**) 10 μg of poly(A)$^+$ enriched RNA of each sample, heart and spleen not done; filters were hybridized with the nicktranslated v-*erb* B fragment

Discussion

In this paper we have demonstrated differential expression of several proto-oncogenes in normal organs of adult fish and during embryogenesis. In melanoma cells we found several proto-oncogenes to be expressed, with an obvious tumor specificity of an *erb* B related transcript.

Transcripts of X*abl*, X*mil* and X*erb* A were not detectable. This may be due to either an abundance below our detection limit or no transcription of these genes at all. A possible low homology with the heterologous v-*onc* probes used could have had an additional effect.

For X*src* two transcripts of different size were observed. These transcripts might be considered as the consequence of differential splicing or of the presence of multiple transcription start and/or termination sites in the X*src* gene, which has been shown to be a single copy gene in the genome of *Xiphophorus* (Robertson *et al.*, submitted). It is interesting to note that the *Drosophila* c-*src* gene (D*src*) shows three transcripts (Lev & Segev, 1986; Simon *et al.*, 1985), while the chicken and the human c-*src* gene show a single transcript or two transcripts of nearly identical size (Gessler & Barnekow, 1984; Gonda *et al.*, 1982; Tatosyan *et al.*, 1985). This indicates possible changes in elements responsible for RNA-splicing etc., during evolution. The possibility that the two X*src* transcripts are the result of a differential splicing in a cell type specific manner, giving rise to a neuron specific and a fibroblast specific form of $pp60^{c\text{-}src}$ which have been shown in the chicken (Brugge *et al.*, 1987; Martinez *et al.*, 1987), needs further investigations. To date we are not able to decide if both transcripts observed are translated and whether the resulting proteins show the same properties, e.g. the same specific kinase activity. The X*src*-related transcript possibly originates from the X*yes* gene which has been cloned recently from *Xiphophorus* (Robertson *et al.*, submitted). This assumption is supported by the fact that the *yes* gene displays the highest similarity to the *src* gene as compared to the other members of the *src* gene-family (Kitamura *et al.*, 1982; Sefton, 1985) and by the finding that the highest amounts of the X*src*-related transcript was found in RNA of the head nephros (the posterior part of the fish kidney). The highest level of c-*yes* RNA in chicken was also observed in normal kidney (Müller & Verma, 1984).

During embryonic development the maximal accumulation of X*src* transcripts occurred around stage 17. This corroborates earlier findings on the enzyme activity level (Schartl & Barnekow, 1984), and also strengthened the hypothesis that c-*src* may play an important role in differentiating processes of neuroectodermal cells. The peak in X*src* 1 expression correlates with the appearance of stellate epineural and cutaneous melanophores, and among other events predominantly with the development of the mesencephalon. An interesting discrepancy between mRNA and kinase activity data was established for the unfertilized egg. Our data revealed that in the unfertilized egg and the following early stages of development a reasonable amount of X*src* mRNA is present. However, no or only a minimal amount of protein kinase activity could be detected (Schartl & Barnekow, 1984). This would indicate that the maternal X*src* message does not give rise to an enzymatically active protein. The RNA data are in agreement with results from the *Drosophila* c-*src*, where maternal message was also found (Simon *et al.*, 1985). Unfortunately there exist no kinase activity data from *Drosophila* eggs and embryos. The X*src* expression pattern of normal adult tissue of *Xiphophorus* revealed preferential expression of the gene in tissues of neural origin. This result confirms earlier data on $pp60^{c\text{-}src}$ kinase activity in fish (Barnekow *et al.*, 1982) and studies using Northern blot analysis, *in situ* hybridization and immunohistochemical methods as tools for determination of c-*src* expression in higher vertebrates and in *Drosophila* (Cotton & Brugge, 1983; Fults *et al.*, 1985; Simon *et al.*, 1985; Vardimon *et al.*, 1986). This indicates that the X*src* gene in adult tissue, like the *Drosophilac-src* gene, or the chicken c-*src* gene, has functions more related to differentiation of neuronal tissue, than to cell proliferation in general. However, RNA obtained from the highly proliferative melanoma contained similar high amounts of both X*src* transcripts as compared to brain. Moreover highly malignant melanomas have been found to contain up to fivefold increased $pp60^{c\text{-}src}$ kinase activity (Schartl *et al.*, 1985). This indicates that the melanoma cells either contain a kinase which is more active than the normal kinase, or that due to different regulatory mechanisms on the post-transcriptional and/or -translational level, more of the protein product is present in the melanoma cells.

In the case of X*sis* we detected two low molecular weight and one high molecular weight transcripts. However, to date we are not able to distinguish if the probe used detects two *Xiphophorus PDGF* A transcripts of different size or a *PDGF* A and a *PDGF* B transcript. The significance of the appearance of the high molecular weight transcript in RNA of several tissues and cells remains unclear at present. The observation that this transcript is drastically reduced in $poly(A)^+$ enriched RNA, and that rehybridization of the total RNA containing filters with other c-*onc* genes revealed no high molecular weight bands, led us to the assumption that this transcript is a non-polyadenylated, unprocessed precursor of the low molecular weight X*sis* transcripts. More detailed experiments are needed to confirm this hypothesis.

The results on the developmental expression of X*sis* are reminiscent of findings in previous studies with a v-*sis* probe, showing only slightly modulated *PDGF* expression during mouse development (Slamon & Cline, 1984). In most of the normal adult tissues X*sis* was found to be expressed at low basal levels. Since melanoma contained barely detectable, and PSM cells no detectable amounts of X*sis* mRNAs, we assume that X*sis* is neither primarily nor secondarily involved in melanoma formation. Surprisingly A2 cells exhibited extraordinary high amounts of both X*sis* transcripts. It will be interesting to determine the significance of this high expression. *PDGF* A expression studies to date are only performed with some human tumor cell lines (Betsholtz *et al.*, 1986). In mammals, expression of c-*sis* (*PDGF* B) was observed in megakaryocytes, placental trophoblasts (Gorestin *et al.*, 1985), endothelial cells (Collins *et al.*, 1985), activated macrophages (Martinet *et al.*, 1986), and in some human tumor cells (Igarashi *et al.*, 1987). Expression studies on other normal tissues are still lacking.

For X*ras* we observed three different transcripts.

Since the heterologous v-*ras*^{Ha} fragment was used the transcripts detected with this probe may belong to different members of the *ras* gene family (*ras*^{Ha}, *ras*^{Ki}, *ras*^{N}; see Müller, 1983; Hall *et al.*, 1983). For c-*ras*^{Ki} often two transcripts (4.4 and 2.0 kb), and for c-*ras*^{Ha} one single transcript (1.4 kb; Müller, 1983) have been found.

During embryogenesis of *Xiphophorus* X*ras* transcripts were found in all stages but especially the level of X*ras* 3 was modulated in a distinct manner. A differential c-*ras* expression was also described during the development of *Drosophila* (Lev *et al.*, 1985) and the primitive eukaryotic organism *Dictyostelium discoideum* (Pawson *et al.*, 1985). In higher vertebrates only slightly varying levels of c-*ras*^{Ha} and c-*ras*^{Ki} transcripts during development of the mouse fetus were reported (Müller *et al.*, 1982), but unfortunately no data on embryonal c-*ras*^{N} expression are available. In normal adult tissue of *Xiphophorus* we detected a relatively ubiquitous distribution of all the three transcripts with a preferential expression of X*ras* 1 and 2 in neuronal tissue. These findings are in agreement with the results of Müller (1983) using RNA dot blot hybridization for detection of c-*ras* transcripts and of Furth *et al.* (1987), using Western blot analysis and immunohistochemical methods for detecting c-*ras* proteins (p21). In the melanoma and in PSM cells no overexpression of X*ras* was observed. In addition no amplification or rearrangements of *ras* sequences in the *Xiphophorus* melanoma were detected, and transfection of melanoma DNA to NIH3T3 cells, which provide a sensitive assay for activated *ras* genes, did not generate foci of transformed cells (Schäfer & Schartl, unpublished data). This points to the assumption that activation of *ras* is not involved in tumor formation of *Xiphophorus*. A similar interpretation has been drawn for the pigment cell tumors in goldfish (Nemoto *et al.*, 1987).

In the case of *erb* B expression we detected transcripts of the *Xiphophorus erb* B gene (X*erb* B) in RNA from normal tissue and two transcripts of a related gene also with similarity to the v-*erb* B sequence in RNA from melanoma and the melanoma derived cell line. Both probes from the X*erb* B and the v-*erb* B gene, respectively map to the tyrosine kinase-encoding region of a subgroup of a superfamily of growth factor receptor genes, which is well characterized by an unsplitted tyrosine kinase domain (Kraus *et al.*, 1987; Yarden *et al.*, 1986). This subgroup includes the human epidermal growth factor receptor gene (hu*EGFR* or c-*erb* B 1), the human homolog of the c-*neu* proto-oncogene of the rat (*HER* 2 or c-*erb* B 2), tumor growth factor receptor α (*TGFR* α) and the insulin receptor. For several of these genes, multiple transcripts were observed in a sometimes tissue- and cell type-specific and transformation related manner (Müller & Verma, 1984; Ullrich *et al.*, 1984, 1985). The fact that the v-*erb* B probe shares more than 82% similarities to the hu*EGFR* leads us to the assumption that the v-*erb* B related transcripts are encoded by a gene belonging to this subgroup and being closely related to the hu*EGFR*.

During embryogenesis neither X*erb* B nor v-*erb* B related transcripts were detectable. This is consistent with data on higher vertebrates reviewed by Müller and Verma (1984). Adult tissues mostly display low levels of X*erb* B 1 transcripts, only RNA of the head nephros contains very high amounts of X*erb* B RNAs. The head nephros of fish is a composite organ which consists of kidney tubules and lymphoid tissue and functions as a part of the immune system. Future studies using *in situ* hybridization methods should help to clarify any function of the X*erb* B gene in the cell differentiation processes that occur in this organ. Results from several human tissues obtained with immunohistochemical methods screening for the EGF-receptor (Gusterson *et al.*, 1984) are in several instances consistent with our RNA data. In accordance with our data thymic epithelium and kidney tubules were found to contain high amounts of EGF-receptor molecules, while testes and spleen were negative. In contrast to our data high amounts of EGF-receptor are detected in liver (liver was negative in the fish) while muscle was negative (positive in the fish). Expression studies in other systems on the RNA level involving the spectrum of tissue used in this paper to date are not available. The expression pattern of the v-*erb* B related gene might be considered to be tumor specific, if the signal obtained in head nephros is the result of cross-hybridization to the large amounts of X*erb* B transcripts in this organ. Experimental proof for this possibility can only be obtained, when probes from structurally divergent parts of the transcripts (e.g. the untranslated leader) will be available.

The transcription of proto-oncogenes in the melanoma cells could be either a feature of the pigment cell lineage in general or a tumor-specific phenomenon. These alternatives are not easily distinguished, because normal melanophores and melanocytes of *Xiphophorus* can not be obtained in sufficient purity and amounts to perform comparative biochemical analysis. However, at least in the case of X*src*, there are indications that expression of this gene is specific for neoplastically transformed 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 kinase activity of pp60$^{c\text{-}src}$ in melanoma cells seems to be enhanced as compared to pp60$^{c\text{-}src}$ from non-tumorous cells (see above). With respect to the significance of the expression of v-*erb* B-related gene in the melanoma and the melanoma derived cell line it is interesting to note that such a gene has been mapped to the close vicinity (less than 0.2 centimorgan) of the *Tu*-locus (Schartl, 1988). Recently it was shown that also in several human mammary tumor cell lines overexpression of a hu*EGFR* related proto-oncogene, the c-*erb* B 2 (*HER* 2), occurs (Kraus *et al.*, 1987).

The expression of several proto-oncogenes in the *Xiphophorus* melanoma cells raises the question of the significance of this phenomenon for the process of tumor formation and/or progression. Multiple transcriptional activation of proto-oncogenes has also been reported in various other systems; e.g. HeLa cells (O'Hara *et al.*, 1986) and in human head and neck solid tumors (Spandidos *et al.*, 1985). In these systems it was not possible to decide whether expression of one of the oncogenes or the concerted action of several genes is the causative event leading to neoplastic transformation. For expression of X*src* in the hereditary melanoma of

Xiphophorus we have to exclude this possibility. It has been clearly demonstrated that the primary process is the elimination of the regulatory locus *R*, allowing enhanced expression of the *Tu* oncogene-locus (Anders & Anders, 1978; Anders *et al.*, 1984). We could show that X*src* which we have reported to be expressed in the melanoma is not identical to the *Tu* gene (Robertson *et al.*, submitted). Obviously the gene becomes activated directly or indirectly by the *Tu* gene product as a secondary step in tumorigenesis. At present we are not able to determine if expression of this proto-oncogene then is a functional requirement for further processes in melanoma formation and/or progression and what the function is. To evaluate the obvious tumor specific expression of the v-*erb* B-related gene which is different from the actual *erb* B gene of *Xiphophorus* studies on the tumor specific regulation of this gene is required. The fact that in all melanomas examined we reproducibly observed the same expression pattern of proto-oncogenes, tempts us to assume that they participate in the multistep process of tumorigenesis in *Xiphophorus*.

Material and methods

Experimental animals

The fish used in this study were bred under standard conditions (see Kallman, 1975) in the aquarium of the gene center at the Max-Planck-Institute for Biochemistry. Different hybrids between *Xiphophorus maculatus* (*X. mac*) and *Xiphophorus helleri* (*X. hell.*) from the following crossings were analysed (Figure 1): Crosses of *X. mac* (a) with *X. hell.* (b) lead to F_1 hybrids (c) developing benign melanoma in the dorsal fin. Backcrossing of these hybrids with *X. hell.* produces three different types of segregants. 25% of the offspring develop benign melanoma (d), 25% develop malignant melanoma (e) and 50% of the animals are tumor free (f, g) (for a detailed description of the crossing procedures, the genotypes and the phenotypes see Anders *et al.*, 1973, 1978, 1984). Brain, muscle, heart, spleen, head nephros, eyes, liver and testes of nearly 1000 adult nontumorous fish of genotype f and g as well as melanoma of several hundred melanoma bearing fish (genotype e) were surgically removed and immediately frozen and stored in liquid nitrogen prior to preparation of RNA. Tumor-free embryos of *Xiphophorus* hybrids (genotypes f and g) were staged according to Tavolga (1949) and also stored in liquid nitrogen prior to RNA preparation.

Cell lines

Cell lines used were derived either from hereditary melanoma of *Xiphophorus* hybrids comparable to e in Figure 1 (PSM cells, Wakamatsu *et al.*, 1984), or from non-tumorous embryos of wildtype *Xiphophorus xiphidium* (A2 cells, Kuhn *et al.*, 1979). Cells were cultured in F 12 medium (Biochrom KG, Seromed, Berlin) containing 10% fetal calf serum (FCS) and 1.25 g $NaHCO_3$/l at 28°C under 5% CO_2. After reaching confluency cells were harvested and used for preparation of RNA.

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 viral fragments were used: (1) 600 bp PstI fragment F of pSRA-2 (De Lorbe *et al.*, 1980) encompassing most of the conserved tyrosine-kinase domain of the viral *src* gene of Rous sarcoma virus; (2) 600 bp BamHI fragment D of pAE II (Vennström *et al.*, 1980) representing the central part

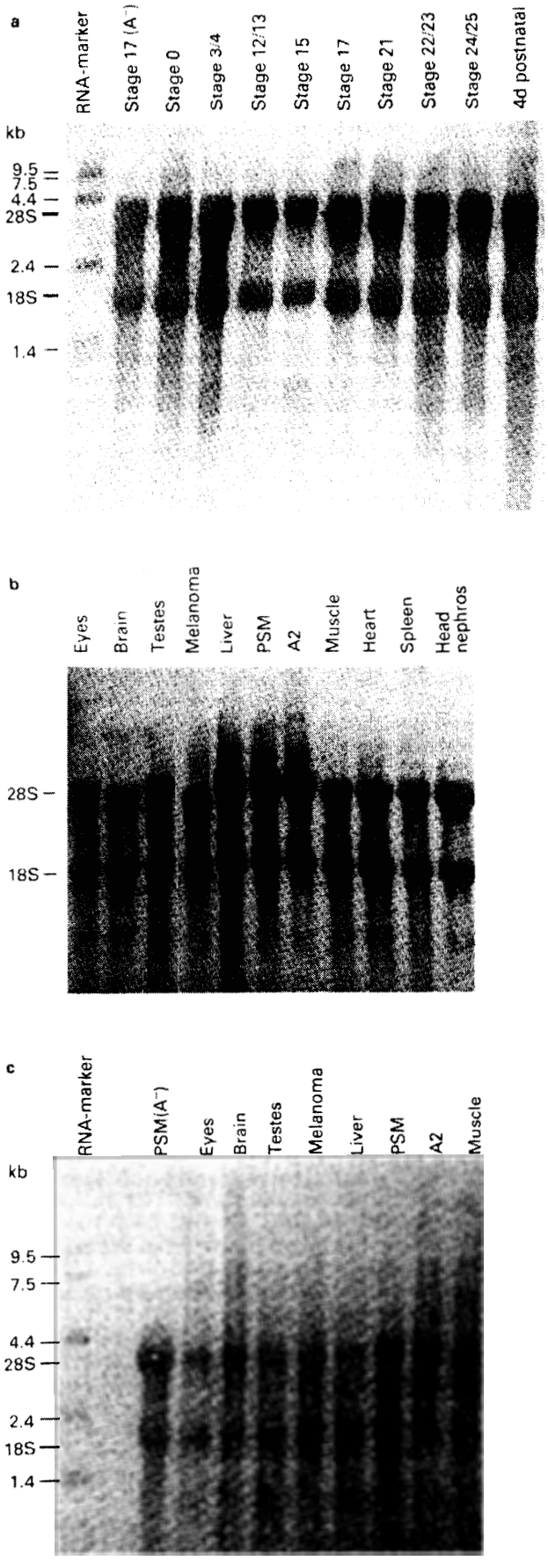

Figure 9. Quantitation of RNA blotted onto GeneScreen filter-membranes via methylene blue staining; (**a**) 20 μg of total RNA from embryos of different age; (**b**) 20 μg of total and (c) 10 μg of poly(A)$^+$ enriched RNA from different adult tissues, from melanoma and from two cell lines of *Xiphophorus* (PSM, A2)

of the cytoplasmic domain of the v-*erb* B gene of Avian erythroblastosis virus; (3) 0.8 kb PvuII/SacI fragment B of the BamHI fragment B of pAE II (Vennström *et al.*, 1980), representing the v-*erb* A gene of avian erythroblastosis virus; (4) 700 bp BglII/PstI fragment D of pHB-II (Ellis *et al.*, 1980) of the v-*ras* gene of Harvey murine sarcoma virus; (5) 1.2 kb SmaI/BglII fragment of pAB3sub3 (Goff *et al.*, 1980) containing a part of the v-*abl* of the Abelson murine sarcoma virus; (6) 924 bp BamHI/RsaI v-*mil* specific fragment B of pMH2BH* of avian leukemia and carcinoma inducing retrovirus MH2 (Jansen *et al.*, 1983); (7) 0.9 kb XbaI/PstI fragment of pC60sis (Gelmann *et al.*, 1981) from simian sarcoma virus, representing the v-*sis* gene.

The following *Xiphophorus* specific fragments were used: (8) 1.2 kb PstI fragment of pX*src* 19-4 (Robertson *et al.*, submitted), homologous to the kinase domain of the cellular *src*-gene of chicken and mammals; (9) 258 bp HindIII/BglII X*erb* B 60-221 and the 268 bp ClaI/BglII X*erb* B 60-222 fragment (both kindly provided by Ch. Zechel) and the 4.5 kb EcoRI fragment of clone λ 44, all containing sequences homologous to the central part of the v-*erb* B fragment; the two small probes were used for the RNA-analysis, while the large probe was used in Southern blot experiments; (10) 300 bp BamHI/BglII X*sis* fragment, (kindly provided by U. Schlehenbecker).

For *in vitro* transcription the 1.2 kb PstI X*src* specific fragment was subcloned into pGEM1 (Promega Biotec, Madison), the v-*erb*B, v-*ras*^{Ha}, and v-*sis* specific fragments into the pSP64 and pSP65 vectors (Melton *et al.*, 1984). Prior to *in vitro* transcription, pSP65, pSP64 and pGEM1 vectors containing the inserts in the correct orientation (producing antisense RNA) were linearised by restriction enzymes.

Sequence comparisons were carried out using the sequence analysis software package version 5 of the University of Wisconsin Genetics Computer Group.

Southern blot analysis

For Southern blot analysis DNA either from pooled liver, brain and testes of individual fish of the above mentioned genotypes or from exponentially growing cell cultures was used. 10 μg of each sample was digested to completion with restriction enzymes and subjected to electrophoresis in 0.8% agarose gels. DNA was transferred to nitrocellulose membranes by the capillary blot method of Southern (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 (0.1 g ficoll, 0.1 g polyvinylpyrrolidone, 0.1 g BSA per 100 ml H_2O), 1% SDS, 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 250 μg ml^{-1} calf thymus DNA and 10^7 dpm ml^{-1} of nick-translated [^{32}P]labeled probe at 42°C for at least 24 hrs. Filters were washed at 60°C, 1 × SSC (for heterologous probes), or at 68°C, 0.1 × SSC (homologous probes) for 1 hr and exposed to a Kodak XAR 15 film. Nick-translations were performed as described by Maniatis *et al.* (1982), using a kit from Amersham Buchler (Braunschweig).

Northern blot analysis

Total RNA was isolated following the LiCl/urea procedure of LeMeur *et al.* (1981) using an ika-ultraturrax N8 for homogenization (Janke & Kunkel, Staufen, FRG). Poly (A)$^+$ RNA was prepared by oligo(dT)-cellulose selection according to Maniatis *et al.* (1982). 20 μg of total RNA or 10 μg of poly (A)$^+$ RNA were denatured with formamide/formaldehyde and electrophoresed in 1.2% agarose/2.2 M formaldehyde gels (Lehrach *et al.*, 1977). RNA was then electroblotted onto GeneScreen membranes according to the protocol of the suppliers (New England Nuclear Chemicals, Dreieich). Filters were hybridized with 1×10^7 dpm ml^{-1} [^{32}P]labeled probe. Hybridizations with nick-translated probes were carried out under the conditions described for Southern blots, except for the mix containing in addition 500 μg ml^{-1} yeast total RNA. Filters were then washed for 1 hr at 50°C, 1 × SSC, 1% SDS for heterologous probes and 60°C, 1 × SSC, 1% SDS for homologous probes. Hybridizations with *in vitro* transcribed probes were 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 film. *In vitro* transcriptions with *SP6*-, *T3*- or *T7*- RNA-polymerases using [α-^{32}P]UTP were performed according to the supplier's recommendation (Genofit, Heidelberg).

Quantitation and size determination of proto-oncogene related mRNAs

To determine the actual amounts of RNA transferred to nylonmembranes, filters were stained with methylene blue (Khandjian, 1986; see Figure 9), and filter bound RNA was quantified densitometrically. This direct measurement reduces artefacts produced by RNA loss after ethanol precipitation following spectrophotometric quantitation and indicates the efficiency of transfer of RNA after the electroblotting procedure. Quantitation of specific mRNAs was carried out on total RNA, with respect to the amounts of RNA detected on the filters. Size determination of the mRNAs was performed on poly(A)$^+$ enriched RNA, since we observed in some cases that mRNAs of similar size to 28 S rRNA were changed to an apparent lower molecular weight by the large amounts of 28 S rRNA. In order to maximise the sensitivity of transcript detection, we alternatively used [^{32}P]labeled *in vitro* transcripts ('riboprobes') as hybridization probes.

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