14. Genetics and molecular biology of tumour formation in Xiphophorus

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Genes and cancer

The fact that genes cause cancer has never been appreciated accordingly. Although as early as 1914 Theodor Boveri had postulated that 'cancer cells have lost certain features of their normal phenotype' and that 'this loss is the consequence of an abnormal chromosomal constitution' (Boveri 1914), his chromosome theory of cancer could not be verified for quite some time because neither the technical tools nor the experimental systems were available. In 1928, K. H. Bauer took up some of Boveri's ideas and postulated that cancer is due to mutations (Bauer 1928). It was the pioneering work of Henry Harris which led to the development of an experimental system that has made it possible to define and characterize the targets of carcinogenic mutations. Using somatic cell hybrids of cancer cells fused with normal cells, genes that are functionally impaired in the tumour cell and that suppress the neoplastic phenotype in the normal cell were detected (Harris *et al.* 1969). Such genes were then also recognized in certain hereditary forms of human cancer like retinoblastoma (Knudson 1971) and Wilm's tumour (Koufos *et al.* 1985). Some of these paradigmatic tumour suppressor genes have been characterized at the molecular level (e.g. Friend 1986; Gessler *et al.* 1990; Malkin *et al.* 1990). Besides the tumour suppressor genes, which are negative regulators of growth and behave as recessive genes by definition, another group of cancer genes has been detected from a totally different line of evidence. These genes are said to act dominantly because they acquire the potential to transform a cell neoplastically following their mutational activation. Such dominant oncogenes were first noticed in acutely transforming retroviruses and many such activated oncogenes have since been found in different animal and human tumours (Bishop 1987). Careful evaluation of the existing data has led to the generally accepted view that most cancers that become apparent as a disease arise not from a single genetic change but from the accumulation of multiple genetic changes in a cell (see Weinberg 1989) including dominant activation of oncogenes as well as inactivation of tumour suppressor genes in the same sequence of events (Fearon and Vogelstein 1990). The main difficulty in analyzing such a complex situation is that in most cases only the *endpoint* of the multistep process can be investigated. The biochemical and molecular biological changes in a tumour cell compared with whatever is regarded as its normal counterpart are numerous. In

most cases it appears impossible to decide which of these differences are due to the first genetic change instrumental in the causation of neoplastic transformation and which are required for tumour progression and metastasis. The sequence of the secondary events is difficult to determine. Finally, some of the changes observed in the cancer cell may be totally irrelevant for establishing and maintaining the neoplastic phenotype: they may simply reflect features of the chaotic molecular biology of the cancer cell. Genetic model systems have the advantage that at least the gene(s) that cause the primary event of neoplastic transformation are clearly defined by classical genetics. Such systems include tumour formation in the fruit-fly Drosophila (see Chapter 13), hereditary tumours in hybrids of Xiphophorus fish, and—at a lower level of genetic resolution—hereditary kidney tumours of the rat (Eker and Mossige 1961).

Genetic control of spontaneous melanoma formation in Xiphophorus

Fish of the genus Xiphophorus inhabit fresh water biotopes of the Atlantic drainage of Mexico, Honduras, and Guatemala. In several species some individuals (from 1 to 40 per cent of a given population) exhibit spot patterns composed of large, intensely black pigment cells. These cells have been termed macromelanophores to distinguish them from micromelanophores, the normal sized black pigment cells that make up the uniform greyish body colouration (Gordon 1927). More than 60 years ago it was discovered independently by Gordon, Kosswig, and Häussler that certain hybrids of platyfish carrying macromelanophore patterns (*Xiphophorus maculatus*) and of unspotted swordtail (*X. helleri*) spontaneously develop malignant melanoma (Gordon 1927, Häussler 1928; Kosswig 1928) (Fig. 14.1).

Shortly thereafter it was recognized that the occurrence of tumours in these hybrids is due to a single locus (the macromelanophore locus) of *X. maculatus* that 'interacted' with the *X. helleri* genome (Kosswig 1929; Gordon 1931). This interaction was later defined as the effect of modifying genes. It has been debated over the decades whether these modifying genes are 'intensifiers' contributed by *X. helleri* to the hybrid offspring genome or if they are 'suppressors', originally present in the *X. maculatus* genome, but are eliminated by the selective breeding process through substitution of the corresponding platyfish chromosomes by those from the swordtail (Gordon 1958; Atz 1962; Kosswig 1965; Zander 1969; Kallman 1970). Supposing multiple modifier genes and extrapolating Boveri's ideas, Breider explained melanoma formation in the hybrids by the loss of 'inhibitory' genes that suppress species-specifically the macromelanophore genes (Breider 1952). The currently generally accepted explanation for the observed phenomenon of hereditary melanoma by Anders and co-workers formalizes such considerations on the basis of numerous genetic experiments (Anders 1990).

In a typical crossing experiment a female *X. maculatus* which carries the X-chromosomal macromelanophore locus *Sd* (spotted dorsal, small spots in the dorsal fin) is mated to *X. helleri*, which does not carry the corresponding locus. The F_1

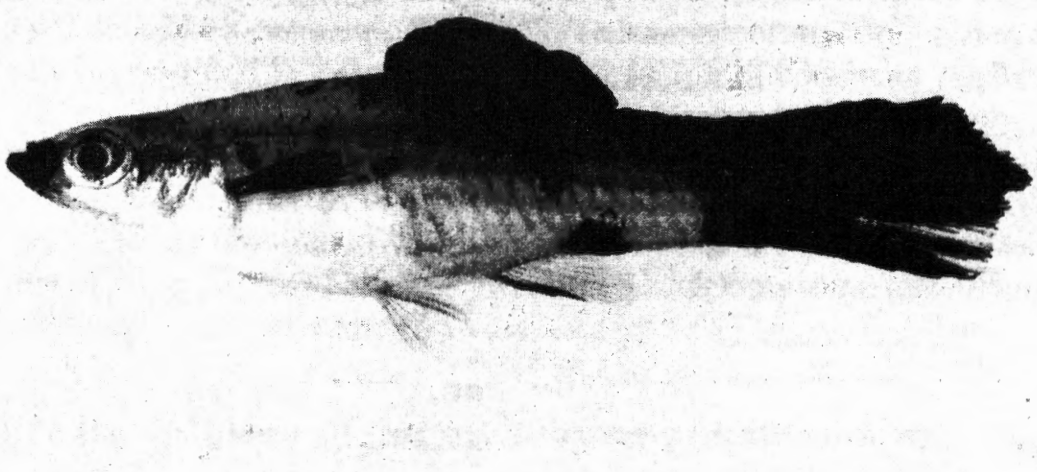

Fig. 14.1. Melanoma-bearing hybrid of Xiphophorus (genotype: *Tu-Sd*/–; –/–).

hybrid shows enhancement of the *Sd* phenotype. Backcrossing of the F_1 hybrid to *X. helleri* results in offspring that segregate, giving 50 per cent which have not inherited the *Sd* locus and are phenotypically like the *X. helleri* parental strain and 50 per cent which carry the macromelanophore locus and develop melanoma. The severity of melanoma ranges from very benign in some individuals (phenotype like the F_1 hybrids) to highly malignant in others. Highly malignant melanomas of such fish become invasive and exophytic and are fatal to the individual. They even grow progressively following transplantation to thymusaplastic (nude) mice (Schartl and Peter 1988).

Based on a variety of such classical crossing experiments a genetic model has been developed to explain tumour formation in Xiphophorus (Ahuja and Anders 1976) (see Fig. 14.2). The macromelanophore locus was formally equated to a sex chromosomal melanoma oncogene locus, whose critical constituent was designated 'tumour gene' (*Tu*). Melanoma formation was then attributed to the uncontrolled activity of *Tu*. In non-tumourous fish *Tu* activity was proposed to be negatively controlled by cellular regulatory genes or tumour suppressor genes (*R* genes, corresponding to the repressing modifying genes mentioned above). For the crossing experiment outlined above this means that *X. maculatus* contains the *Tu-Sd* locus on the X-chromosome and the corresponding major *R* on an autosome, while *X. helleri* is proposed not to contain this particular *Tu* locus and its corresponding *R*. According to the model, backcrossing of the *Tu*-containing hybrids to *X. helleri* results, in effect, in the progressive replacement of *R*-bearing chromosomes from *X. maculatus* by *R*-free chromosomes of *X. helleri*. The stepwise elimination of regulatory genes is thought to allow expression of the *Tu* phenotype, leading to benign melanoma if one functional allele of *R* is still present or malignant melanoma if *R* is absent.

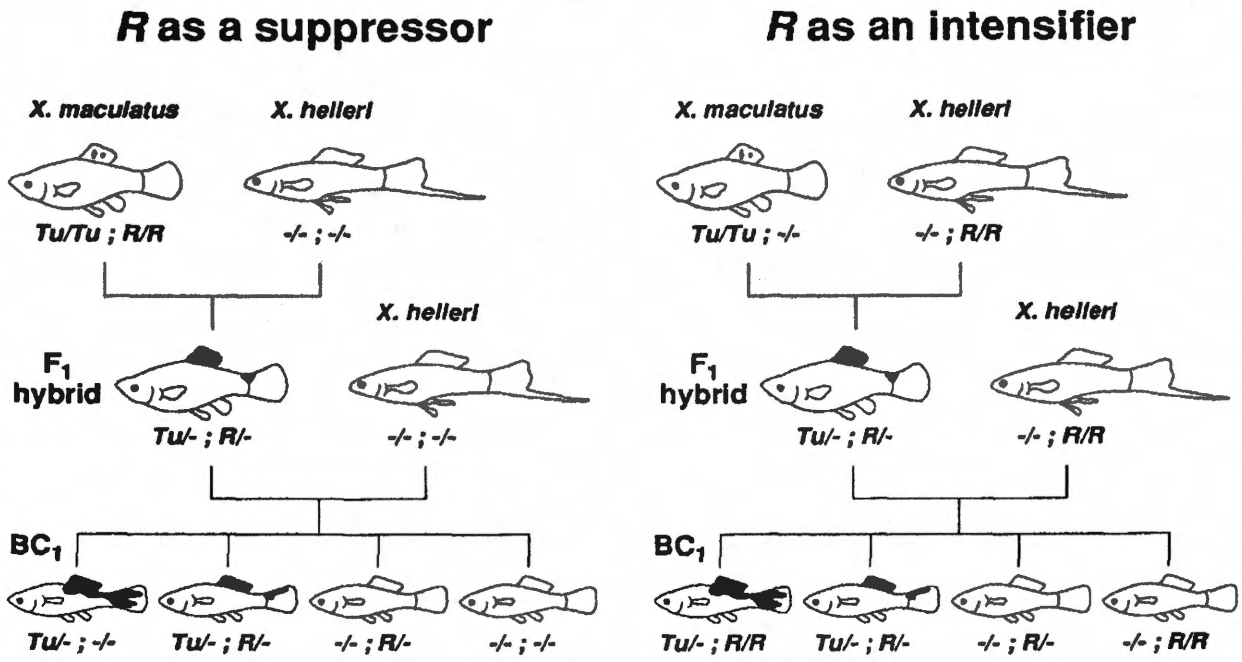

Fig. 14.2. Models to explain the classical crossing experiment leading to hybrids that develop melanoma.

It should be noted, however, that there is another scenario that is also compatible with the crossing data, namely that *Tu* activity is due to the presence of intensifying genes contributed by *X. helleri* chromosomes to the hybrid genome (see Fig. 14.2). To our knowledge, there is no crossing experiment that would help to decide between these two possibilities. Reintroduction of suppressor genes or diluting out activating genes, respectively, by crossing hybrids bearing malignant melanomas to parental *X. maculatus* was shown to lead to a reversion of the malignant phenotype resulting in totally tumour-free fish in the succeeding backcross generations using *X. maculatus* as the recurrent parent (Anders *et al.* 1984). This demonstrates that the melanoma oncogene *Tu* itself remains structurally unaltered during the process of activation via hybridization.

Depending on the macromelanophore pattern of the parental platyfish, the melanomas of the hybrids are localized in different body compartments. For example, in crosses where the *Sd* locus was introduced into the hybrid genome, melanomas spread from the dorsal fin, while in fish with an *Sp* (spotted) locus, which causes macromelanophore spots on the flanks of the parental platyfish, melanomas of the corresponding hybrids originate exactly from this region. This pattern information and the neoplastic transformation locus *Tu* are extremely closely linked and only a few mutants exist which affect the original macromelanophore pattern and, consequently, the melanoma compartment (Anders *et al.* 1973; Kallman 1975). According to Kallman's work the different patterns are due to a series of alleles of macromelanophore genes each harbouring the capacity for melanoma formation (Kallman 1975) while Anders *et al.* proposed that a single *Tu* locus is closely linked to a series of non-allelic, compartment-specific genes that suppress the appearance of macromelanophores in the various body regions. The different patterns would

then be due to mutational impairment of one of the *cis*-acting compartment genes (Anders 1991). The implication from both explanations is that the gene that determines the macromelanophore phenotype is identical to the dominant oncogene.

Siciliano and Perlmutter (1972) noted a considerable maternal effect on development of melanoma in *Tu-Sd*-bearing hybrids. The nature of this effect seen early in the life of the hybrid fish is to augment the expression of enhanced pigmentation, melanosis, or malignant melanoma. The maternal effect was observed only in those hybrids whose female parent had a closer relationship to the swordtail than hybrids of the reciprocal cross. Whether this phenomenon represents genomic imprinting of 'modifier genes' or a hormonal effect (see below) due to the different combinations of platyfish and swordtail sex chromosomes and sex chromosome homologues (see Kallman 1984) has to be elucidated.

Epigenetic modulation of the melanoma phenotype

Besides a genetic control through the action of regulatory genes like *R*, the malignancy of melanoma can also be influenced by a variety of epigenetic factors some of which are androgens. In certain genotypes melanoma formation starts earlier in males and leads to a higher malignancy (Siciliano *et al.* 1971) and in other genotypes only mature males develop melanoma (Anders *et al.* 1984). Treatment with testosterone has dramatic effects on melanoma formation depending on the genotype of the fish and the developmental stage of the tumour. Melanoma induction, enhancement of malignancy, and melanoma regression were observed using the same treatment protocol but different hybrid genotypes and *Tu* alleles. All the various effects could be explained by promotion of pigment cell differentiation by androgens (Schartl *et al.* 1982).

Another substance that has been found to modulate the malignancy of melanoma in Xiphophorus is dinitrochlorbenzene (DNCB). Application of this compound led to tumour regression (Scholz 1977). DNCB is known to stimulate the immune system and thus may enhance an immune response against the melanoma cells. The same mechanism may explain the observation that raising tumour-bearing backcross hybrids under hyperthermic conditions also results in suppression of melanoma formation (Perlmutter and Potter 1988). The high temperature in the environment most likely induces some kind of 'artificial fever' in the poikilothermic animals.

Besides steroids and the immune system many more factors may exist that modulate the neoplastic phenotype. Due to the fact that the genetic factors determining malignancy are defined and lead to tumours of high pathophysiological uniformity, melanoma formation in Xiphophorus provides a unique system for studies on epigenetic modulatory factors and their mechanism of action. This hopefully will also offer new perspectives for therapeutic approaches.

Hereditary ocular and thyroidal tumours in Xiphophorus

Gordon reported on a strain of *X. montezumae* that spontaneously developed thyroidal tumours at high incidence (Gorbman and Gordon 1951). A genetic

susceptibility was obvious; however, no further genetic analyses were performed. Ocular tumours appeared in two broods of hybrids of platyfish and swordtails (Gordon 1947) similar to the case for melanoma. An autosomal recessive gene (*oc*) was defined that contained the genetic information for tumour formation but 'tumour-modifier' genes seemed also to be involved. Unfortunately, all these strains were lost and could not be analysed further.

Genetic factors in the formation of carcinogen-induced tumours

Besides the above-mentioned tumours of hereditary aetiology, a large variety of tumours of neurogenic, epithelial, and mesenchymal origin arose after treatment of fish with chemical carcinogens or X-rays. The surprising finding was that purebred descendants of feral populations of five Xiphophorus species developed tumours only at very low rates. When the interspecies hybrids of F_1 and further filial generations as well as the backcross generations were similarly treated, the tumour rates were increased up to 10-fold (Anders *et al.* 1991). A similar observation was made for N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)-induced melanoma but not hepatoma in the medaka fish (*Oryzias latipes*) (Hyodo-Taguchi and Matsudaira 1987). The genetic loci possibly involved are so far ill-defined and there are no molecular data that would help towards an explanation of these phenomena.

Reverse genetic approaches towards isolation of *Tu* and *R*

In order to understand the molecular basis of hereditary melanoma, isolation and characterization of the genes involved was attempted. Until then no candidate gene product of *Tu* and *R* had been characterized precluding cloning by conventional recombinant DNA technology. We therefore applied a strategy that has been termed 'reverse genetics' (Orkin 1986) or more recently 'positional cloning' (Collins 1992) to isolate the melanoma-inducing gene of *X. maculatus* and gene(s) encoded by *R*. Our strategy included the following steps:

1. determination of the chromosomal location of the genes in question;
2. identification and cloning of a molecular marker sequence for the corresponding loci;
3. cloning of the *R*- and *Tu*-containing region by chromosome walking or jumping;
4. identification and isolation of a candidate gene;
5. verification that the candidate gene is indeed responsible for the *Tu* or *R* phenotype.

The *R* locus has so far resisted molecular cloning because of the lack of phenotypic markers, characterized mutants or alleles resulting in a paucity of information on its map position and genetic behaviour. A distantly linked (approximately 30 cM) isozyme marker (EST-1) (Siciliano *et al.* 1976; Ahuja *et al.* 1980; Förnzler *et al.*

1991) locates *R* to linkage group V of Xiphophorus (Morizot and Siciliano 1983). So far a large number of probes with polymorphism information content have been found that can now be tested for linkage to *R* in informative platyfish/swordtail backcross hybrids (Förnzler *et al.* 1991).

The situation was much more promising for the cloning of the *Tu*-encoded gene(s) thanks to the availability of many mutants, cross-overs in the vicinity, phenotypic markers, and many different *Tu* alleles. The chromosomal localization of *Tu* had been clearly defined by recombination and mutation analyses as residing within the distal portion of the sex chromosomes (Anders *et al.* 1973; Kallman 1975). The most critical step then was to identify a molecular marker sequence. One of several approaches (Schartl *et al.* 1993) was to use heterologous oncogene/proto-oncogene probes for Southern hybridizations. The rationale for this strategy was that most oncogenes/proto-oncogenes of higher vertebrates fall into one of several classes of multigene families. The members of such gene families share highly conserved regions, e.g. kinase domains and DNA-binding domains. Under conditions of reduced stringency in Southern hybridization, a molecular probe containing such a conserved region detects not only all members of the gene family of the same organism but also those from distantly related species (Mäueler *et al.* 1988*a*,*b*; Hannig *et al.* 1991). As many sequences are identified in such experiments with a single probe, these are very informative with respect to the detection of RFLPs. In addition, it appeared not totally fantastic to expect that the sought dominant melanoma oncogene of Xiphophorus might be a member of one of the known oncogene/proto-oncogene multigene families.

From all probes tested, the viral *erb*B (v-*erb*B) probe was most informative. It is derived from the B oncogene of avian erythoblastosis virus and represents an oncogenically activated version of the avian epidermal growth factor receptor (EGFR) gene (Ullrich *et al.* 1984). A probe that encompasses most of the highly conserved kinase domain detects in *Eco*RI digests, besides other strongly hybridizing bands, two weaker bands that are only present in the DNA of fish carrying a sex chromosomal *Tu* locus, one of 6.5 kb cosegregating with Y-chromosomal *Tu* loci and one of 5 kb, cosegregating with X-chromosomal *Tu* loci (Schartl 1988). In linkage analysis employing more than 500 individual fish no recombinant between this RFLP and the *Tu* locus was found (Schartl 1988, 1990; Zechel *et al.* 1988; Wittbrodt *et al.* 1989) indicating that this sequence is either intimately linked to *Tu* or even an integral part of the locus. The 5 kb band was cloned and found to detect besides the Y-chromosomal 6.5 kb band a third hybridizing sequence of 7 kb which was invariably present in DNA of all fish irrespective of the presence or absence of a *Tu* locus (Adam *et al.* 1988; Schartl 1988).

Cloning and characterization of the *Tu* locus encoded gene, *Xmrk*

The 5.0 kb X-chromosomal genomic fragment corresponding to the *Tu*-linked RFLP was used to isolate a cDNA clone from the Xiphophorus melanoma cell line PSM (Wittbrodt *et al.* 1989). The predicted protein has all the features of a typical

growth factor receptor with an extracellular ligand binding domain, a transmembrane region, and an intracellular domain containing all 11 structural motifs diagnostic for a protein tyrosine kinase moiety. In addition there are 12 putative glycosylation sites, all located in the potential extracellular domain and an N-terminal signal peptide of 25 amino acids.

Comparison of the primary structure of the protein with other structures in sequence databases revealed marked similarity with receptor tyrosine kinases (RTKs), most prominently with the human EGFR (Ullrich *et al.* 1984). Isolation of other EGFR-related sequences from Xiphophorus showed clearly the presence of an EGFR homologue distinct from the *Tu* gene in the fish genome demonstrating that this gene encodes a novel member of the subclass I RTK family. Based on these findings the *Tu* gene was designated X*mrk* for Xiphophorus melanoma receptor kinase.

X*mrk* proto-oncogene and oncogenic copies in the Xiphophorus genome

The initial RFLP analysis had revealed the presence of three different copies of X*mrk* in the Xiphophorus genome, which exist as independent loci (Schartl 1990). All of them were isolated and partially characterized. As far as investigated the three genes share a common exon/intron arrangement. The identical arrangement and exon sizes are also found in the EGFR, HER2, and *erb*B3 genes, again pointing towards the close evolutionary kinship of these genes (Adam *et al.* 1991).

All genotypes of Xiphophorus contain one copy of X*mrk* on each sex chromosome, termed INV. INV is also present in other poeciliid fish species and is not associated with the tumour phenotype. Hence, it represents the corresponding proto-oncogene of *Tu*. The remaining two copies (named X or Y according to their chromosomal location), which are only found in animals carrying the *Tu* locus are tied to the macromelanophore spot pattern loci that give rise to melanomas in appropriate crossings. Thus, they are regarded as oncogenic versions of the INV copy (Schartl and Adam 1992).

The nucleotide sequences of all three X*mrk* genes are highly conserved (Adam *et al.* 1991). Only a few sequence differences (< 1 per cent) are found between the proto-oncogene and the two oncogenes in a total of 18 kb of genomic sequences including introns. At present it is not clear whether the observed sequence differences or possible mutations in the so far not analysed extracellular, transmembrane and juxtamembrane domains in the X and Y oncogenes contribute to the process of neoplastic transformation.

Evidence that the additional X- or Y-chromosomal copies of X*mrk* are the crucial, i.e. melanoma-inducing, constituent of the *Tu* locus is provided by 'loss-of-function mutations' of *Tu*. Such mutations arise spontaneously with a very low frequency ($< 10^{-5}$) in the progeny of melanoma-bearing hybrids and are characterized by their inability to develop spontaneous melanoma. One such mutant was found to carry an insertion within an exon of the X-chromosomal X*mrk* copy, rendering the carriers incapable of developing hereditary melanomas (Wittbrodt

et al. 1989). This demonstrates clearly that the X*mrk* oncogene is absolutely required for tumorigenesis and thus is the critical constituent of the *Tu* locus.

Evaluation of the sequence differences of the three X*mrk* genes with respect to their phylogeny and linkage data (Schartl 1990) strongly supports a model involving a gene duplication which created a new copy of the INV gene. This copy was translocated 2 cM away from the original on the Y-chromosome during the duplication event and at a later stage transferred to the X-chromosome by a sex-chromosomal crossing over.

Transcriptional activation of the X*mrk* oncogene

The X*mrk* genes give rise to transcripts of different size, one of 5.8 kb and one of 4.7 kb; the longer one is derived from the proto-oncogenic INV copy (Wittbrodt *et al.* 1989; Adam *et al.* 1991). This transcript is highly abundant in the form of maternal mRNA in unfertilized eggs and is differentially expressed during organogenesis. In adult non-tumourous animals, expression of the INV gene is restricted to low levels in the skin, fins, and gills. Comparable amounts of RNA are observed in melanomas. Expression in the tumours is not influenced by the presence or the absence of the *R* locus, as the transcript is found at similar levels in melanomas of differing malignancy.

In contrast, expression of the X- and Y-copies of X*mrk* is restricted to one cell type and dependent on the presence or absence of the regulatory locus *R*. The 4.7 kb mRNA is exclusively detected in melanoma. It is important to note that the level of the oncogene transcript here corresponds to the malignancy of the tumours: the amount is low in benign and very high in malignant melanomas. This correlation shows clearly that low level expression of the oncogenic X*mrk* copies eventually carrying 'activating mutations' in the protein is not sufficient for the full-blown melanoma phenotype. It seems that high level expression is the primary cause of the neoplastic transformation.

Autophosphorylation of the X*mrk* protein

Ligand-induced autophosphorylation is thought to be a crucial step in the process of receptor-mediated signalling in the RTK family. Defects in this process lead to either a loss of biological activity, or constitutive activation, or loss of control.

The fact that as yet no ligand for the X*mrk* gene product is known has hampered the analysis of the biochemical properties and biological functions of the receptor. In an approach towards elucidating the role of the protein in normal and neoplastic tissue the Xmrk extracellular domain was replaced by the ligand-binding domain of the closely related human EGFR and the ligand-induced activity of the chimeric HER–Xmrk protein was examined in cells of different origin (Wittbrodt *et al.* 1992).

Ligand-dependent tyrosine kinase activity was observed *in vitro* and in living cells after transient or stable transfection of the chimera into mammalian and fish cells. In contrast, the wild-type Xmrk oncoprotein always displayed significant autophosphorylation in the transfected mammalian cells. The most likely explanation for the observed low level autophosphorylation of Xmrk is the presence of

(an) activating mutation(s) in the extracellular or transmembrane domain of the Xmrk oncoprotein both of which are substituted by HER sequences in the chimera. Similar phenomena have been observed in carcinogen-induced tumours of rats, where the product of the *neu* oncogene was found to bear an activating mutation in the transmembrane domain (Bargmann and Weinberg 1988; Stern *et al.* 1988) and in v-*fms*, where point mutations in the extracytoplasmic portion lead to an intracellular activation of the kinase (Roussel *et al.* 1988; Woolford *et al.* 1988). However, such potential 'activating mutations' in the oncogenic copies of X*mrk* would also be present in the wild-type fish, which never develop tumours. There these mutations cannot be effective. They could only elicit their effects in the hybrid fish where the X*mrk* oncogenes are activated through loss of a suppressor locus or gain of an activator locus. Thus, such mutations cannot be the primary cause of the appearance of the melanomas in the hybrids.

When analysed in Xiphophorus cell lines or biopsies from various melanoma and tissues the Xmrk protein has so far been detected only in melanoma cells, where it is the most abundant tyrosine-phosphorylated protein (Wittbrodt *et al.* 1992). This points towards a highly active receptor, whose targets in the intracellular signal transduction are so far unknown.

Cellular substrates for the X*mrk* protein

The X*mrk* gene codes for a tyrosine kinase type cell surface receptor putting it into a family of proteins which upon ligand-dependent activation induce similar primary responses within cells (for review see Ullrich and Schlessinger (1990)). They activate their substrates by phosphorylating them on tyrosine residues. To identify intracellular targets of the Xmrk protein and compare them with substrates of the closely related EGFR, 293 cells were transiently transfected with plasmids containing the HER–X*mrk* or HER gene and assayed for tyrosine-phosphorylated proteins by western blotting using anti-phosphotyrosine antibodies (Wittbrodt *et al.* 1992).

The Xmrk protein and HER seem to share some common substrates as a protein of 72 kDa can be detected in cells containing either of the two receptors. However, human (PLCγ) phospholipase C γ, a prominent target of HER, could not be classified as a major substrate for the Xmrk kinase as judged by these experiments. Several potential substrates are specific for the Xmrk tyrosine kinase; the two most prominent ones, which become phosphorylated after EGF treatment of HER–X*mrk*-expressing cells, have an apparent molecular weight of 105 and 140 kDa. Further analyses of these specific targets will provide important clues to the processes leading to tumorigenesis following activation of X*mrk*.

Transforming ability of X*mrk*

To investigate the transforming ability of the Xmrk protein, NIH3T3 cells were infected with replication-defective retroviruses carrying the cDNAs for HER–Xmrk and Xmrk (Wittbrodt *et al.* 1992). The chimera produced foci of transformed cells at

a rate of one focus per virus after stimulation with EGF. This focus formation assay clearly demonstrates that the Xiphophorus RTK can interact with the other components of the mammalian signal transduction apparatus and that the Xmrk protein has an extremely strong transforming potential even in a heterologous cell system.

In contrast to HER–Xmrk the native Xmrk protein did not show any transforming ability in this type of assay. This may indicate that the observed low level of constitutive tyrosine kinase phosphorylation at least in the heterologous cells is not sufficient to elicit a tumorigenic effect. It provides further evidence that 'activating mutations' in the protein alone are not sufficient for the oncogenic function of X*mrk* in the neoplastic transformation of pigment cells.

Other oncogenes possibly involved in the neoplastic phenotype

A generally accepted view is that cancer is the result of a multistep process. At first glance the situation in the Xiphophorus melanoma system appears to be simpler. It seems as if the activation of a single gene is sufficient to induce the neoplastic phenotype of the pigment cells. However, overexpression of X*mrk* leads to complex alterations in the cell, not all of which may be simply explained as physiological consequences of enhanced RTK activity. The so far analysed members of the family of *src*-related cytoplasmic protein tyrosine kinases in Xiphophorus, namely X*src*, X*yes*, and X*fyn*, are highly expressed in the melanomas. X*yes* and X*fyn* mRNAs are more abundant in tumours than in adult brain, which is the organ of preferential expression in non-tumorous fish (Hannig *et al.* 1991). The situation is even more intriguing with X*src*, where more details are known. Its expression is specific for the transformed state: non-transformed pigment cells do not contain detectable levels of the transcript (Raulf *et al.* 1989). The enzymatic activity of the X*src*-encoded protein, pp60^{Xsrc}, correlates with the degree of malignancy of the melanomas. As the X*src* gene is not associated structurally with the *Tu* locus, it might represent a 'secondarily' activated oncogene.

A new upstream region acquired by the X*mrk* oncogene

Where do the dramatic differences in the levels of the proto-oncogene and oncogene mRNA originate?

Differential northern analysis demonstrated that the observed size difference of 1.1 kb of the INV transcript on the one hand, and the X- and Y-transcripts on the other hand, could not be attributed to different 3′ end formation. Primer extension studies and comparison of the full-lengths cDNAs revealed intriguing differences in the 5′ regions of the X*mrk* genes.

In addition, colinearity of INV and the oncogenes is only found 3′ to codon 10 in exon 1, while 5′ of this point there is no similarity at all. Southern analysis with the divergent 5′ regions as probes confirmed the diversity of the proto-oncogene and oncogene in these parts of the genes. It turned out that the known upstream region of the oncogene is identical to that of another gene present in all Xiphophorus

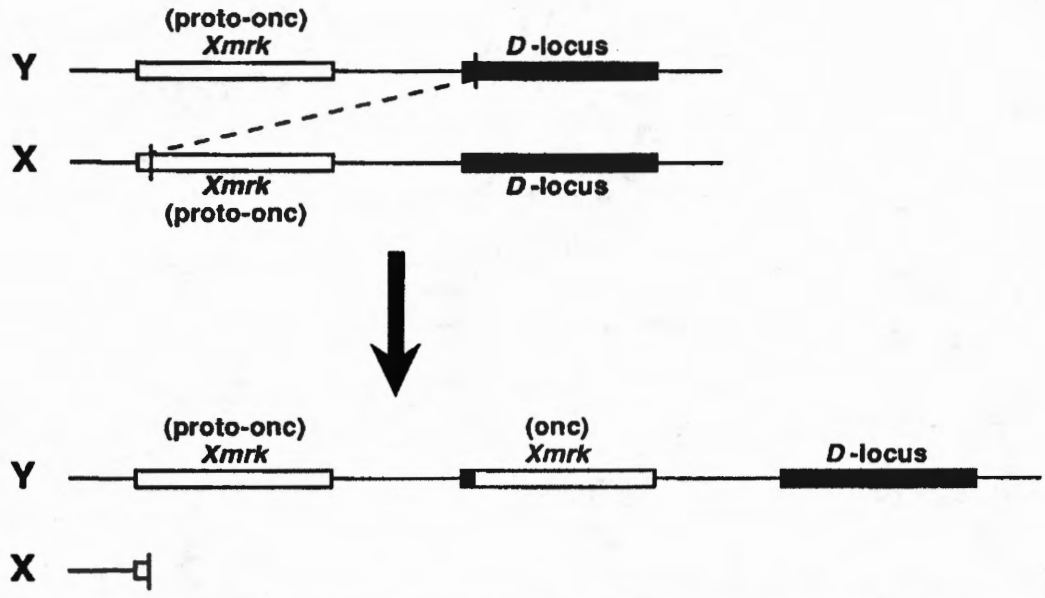

Fig. 14.3. Duplication/recombination model for the generation of the X*mrk* oncogene.

genotypes, irrespective of whether they contain a *Tu* locus or not. These findings indicate that the oncogene has acquired a new promoter as compared with the INV locus. This promoter contains TATA- and CAAT-like sequences (Adam *et al.* 1993) and thus represents the 'non-housekeeping gene' promoter type (Dynan 1986) in contrast to the GC-rich sequences driving transcription of the closely related RTKs like the human EGFR (Ishii *et al.* 1985) or the rat *HER2* gene (Suen and Hung 1990).

The composite structure of the X*mrk* oncogene can be explained simply by extending the previously introduced gene duplication model. A non-homologous recombination event must have taken place between the X*mrk* proto-oncogene and a locus providing the new promoter, therefore this locus was designated *D* (for donor) (see Fig. 14.3).

The breakpoint for the X*mrk* gene lies within exon 1 in the signal peptide which is cleaved off in the mature Xmrk receptor tyrosine kinase. Hence, the mature oncogene and proto-oncogene proteins could have been primarily identical, without excluding the possibility that the oncogene has acquired activating mutations in the course of evolution. Such mutations, however, would appear functional only in the hybrids where the X*mrk* oncogene is deregulated, as parental fish with an identical oncogene are melanoma-free.

This suggests that the genetically defined regulatory locus *R* is either directly or indirectly involved in the control of transcription from the new oncogene promoter and was pre-existing. It is the properties of this accidentally acquired promoter that lead to overexpression of X*mrk* in pigment cells of hybrid genotypes.

Perspective

The identity of the X*mrk* gene product as a RTK provokes further questions, the answers to which should help to understand the role of X*mrk* overexpression in the

process of tumour formation. It will be important to identify the ligand and the cellular substrates of Xmrk that are involved in the signal transduction as well as the genes that control X*mrk* activity and those that are activated following X*mrk* stimulation.

The cumulative evidence points towards a major role of the dramatically increased levels of the X*mrk* oncogene transcript in melanoma formation. It suggests that the elevated RNA level in melanomas is due to an increase in transcription caused by the newly acquired oncogene promoter. Structural and functional comparison of the X*mrk* oncogene promoter with that of the *D* locus from which it is obviously derived might point towards important regulatory elements involved in the control of X*mrk* transcription. The fact that the *D* locus is seemingly repetitive (D. Förnzler and M. Schartl, unpublished) poses a problem, since the possibility exists that different copies of this locus might contain different promoter sequences. In this case knowledge of the actual donor sequence involved in the recombination event leading to the generation of the X*mrk* oncogene is required.

So far it has not been possible to decide if the *R* locus is a tumour suppressor gene or an activator of *Tu*; the latter possibility cannot be excluded based on formal genetics. The finding that transcriptional control might be the mechanism through which *R* regulates X*mrk* emphasizes the importance of identifying the factors that control transcription of the oncogenic X*mrk* copies. These analyses will help to decide which model for the mode of action of *R* is correct and might lead to the characterization of the *R* gene. If the product of *R* turns out to be involved in the transcriptional control of X*mrk* it could be a transcription factor itself or interact with such a protein either directly or by altering transcription factor activity through post-translational modifications.

Although all evidence points towards transcriptional regulation of the X*mrk* oncogene probably directly through the *R* locus, there are other possibilities for the function of the *R* gene product. It could stabilize or destabilize the oncogene transcript and thereby lead to the increased mRNA level observed in melanomas, or *R* could code for a ligand, or affect the kinase activity of the receptor, or represent another molecule even further downstream in the signal transduction cascade.

The identification and characterization of the structural and functional components of the Xiphophorus melanoma system will provide essential information as to how a gene can lead to tumour formation in individuals where this gene becomes deregulated.

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