Comparative Biochemistry and Physiology, Part C 178 (2015) 116–127

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Transcriptional control analyses of the *Xiphophorus* melanoma oncogene☆

Janine Regneri [a], Jean-Nicolas Volff [b], Manfred Schartl [a,c,*]

[a] Physiological Chemistry, Biocenter, University of Würzburg, Am Hubland, Würzburg, Germany
[b] Institut de Génomique Fonctionelle de Lyon, Ecole Normale Supérieure de Lyon, 46, allée d'Italie, 69364 Lyon cedex 07, France
[c] Comprehensive Cancer Center Mainfranken, University Clinic Würzburg, Würzburg, Germany

ARTICLE INFO

Article history:
Received 30 June 2015
Received in revised form 25 August 2015
Accepted 1 September 2015
Available online 5 September 2015

Keywords:
Melanoma
Xiphophorus
xmrk oncogene
Transcriptional control
Pigment cell
EGF receptor
Tumor suppressor
Cis-regulatory element

ABSTRACT

Melanoma development in interspecific hybrids of *Xiphophorus* is induced by the overexpression of the mutationally activated receptor tyrosine kinase Xmrk in pigment cells. Based on the melanocyte specificity of the transcriptional upregulation, a pigment cell-specific promoter region was postulated for *xmrk*, the activity of which is controlled in healthy purebred fish by the molecularly still unidentified regulator locus *R*. However, as yet the *xmrk* promoter region is still poorly characterized. In order to contribute to a better understanding of *xmrk* expression regulation, we performed a functional analysis of the entire putative gene regulatory region of the oncogene using conventional plasmid-based reporter systems as well as a newly established method employing BAC-derived luciferase reporter constructs in melanoma and non-melanoma cell lines. Using the melanocyte-specific *mitfa* promoter as control, we could demonstrate that our *in vitro* system is able to reliably monitor regulation of transcription through cell type-specific regulatory sequences. We found that sequences within 200 kb flanking the *xmrk* oncogene do not lead to any specific transcriptional activation in melanoma compared to control cells. Hence, *xmrk* reporter constructs fail to faithfully reproduce the endogenous transcriptional regulation of the oncogene. Our data therefore strongly indicate that the melanocyte-specific transcription of *xmrk* is not the consequence of pigment cell-specific *cis*-regulatory elements in the promoter region. This hints at additional regulatory mechanisms involved in transcriptional control of the oncogene, thereby suggesting a key role for epigenetic mechanisms in oncogenic *xmrk* overexpression and thereby in tumor development in *Xiphophorus*.

1. Introduction

Fish model systems have been used extensively in biomedical research to study human diseases, including a variety of tumors, such as hematological and liver cancers, sarcomas and melanoma (Bailey et al., 1996; Amatruda and Patton, 2008; Patton et al., 2010; Jing and Zon, 2011; Schartl, 2014). In contrast to most other fish cancer models, which are genetically engineered and based on transgenes often of human origin, the melanoma models in several species of the teleost genus *Xiphophorus* are based on a naturally occurring system of oncogenes and tumor suppressor genes. In these fish, carcinogen and UV induced as well as hereditary melanoma formation has been described and analyzed (Walter and Kazianis, 2001; Meierjohann and Schartl, 2006). The development of melanoma in *Xiphophorus* is always initiated by standardized crossing procedures and thus clearly defined genetic events are underlying melanoma initiation. In the case of hereditary melanoma, this guarantees the development of highly uniform tumors with respect to molecular and pathological features, making *Xiphophorus* a valuable tool to study the molecular processes of melanomagenesis.

The development of hereditary melanomas in certain interspecific backcross hybrids of platyfish (*Xiphophorus maculatus*) and swordtails (*Xiphophorus hellerii*), which was discovered already in the 1920s (Gordon, 1927; Häussler, 1928; Kosswig, 1928), has been attributed to the uncontrolled activity of a dominantly acting, pigment cell-specific oncogene locus called *Tu*. According to the genetic model developed to explain melanoma development in *Xiphophorus* hybrids (Ahuja and Anders, 1976), the tumor-inducing potential of *Tu* is suppressed in purebred species (e.g., *X. maculatus*) by an unlinked regulatory locus *R* (also termed *Diff*; Vielkind, 1976; Walter and Kazianis, 2001), which is supposed to act as a tumor suppressor. This suppression is progressively eliminated upon crossing when using a species as recurrent parent that contains neither *Tu* nor *R* (e.g., *X. hellerii*). In hybrid offspring

☆ This paper is based on a presentation given at the 7th Aquatic Animal Models of Human Disease Conference, hosted by Texas State University (December 13–December 19, 2014).

* Corresponding author at: Physiological Chemistry, Biocenter, University of Würzburg, Am Hubland, 97074 Würzburg, Germany. Phone: +49 931 31 84148; fax: +49 931 31 84150.
E-mail address: phch1@biozentrum.uni-wuerzburg.de (M. Schartl).

http://dx.doi.org/10.1016/j.cbpc.2015.09.001
1532-0456/

heterozygous for *Tu*, but lacking the *R* locus, the *R*-mediated suppression of *Tu* is lost, resulting in the formation of fast growing and highly malignant melanoma in these fish. However, it should be noted that the crossing data can formally also be explained by *R* being a tumor inducer contributed by swordtail chromosomes to the hybrid genome (Schartl, 1995).

Molecular genetic studies identified an oncogene called *xmrk* (*Xiphophorus* melanoma receptor kinase) as the primary tumor-determining gene of the *Tu* locus (Wittbrodt et al., 1989). *Xmrk* was generated by a local gene duplication event from the preexisting proto-oncogene *egfrb* (Adam et al., 1993; Volff et al., 2003), which is one of the two fish co-orthologs of the human EGF receptor. The oncogenic properties of the *xmrk*-encoded protein result from two activating mutations in the extracellular domain of the growth factor receptor, which lead to ligand independent dimerization and thus constitutive activation of the receptor (Gomez et al., 2001; Meierjohann et al., 2006a).

Shortly after the identification of *xmrk*, expression studies provided evidence that linked melanoma development in *Xiphophorus* to a specific overexpression of the *xmrk* oncogene (Adam et al., 1991; Mäueler et al., 1993; Woolcock et al., 1994). Based on these data, it was hypothesized that besides activating mutations in the Xmrk receptor, the second precondition for melanoma development is transcriptional activation of the *xmrk* oncogene. A quantitative analysis of *xmrk* transcript levels in different tissues of hybrid and parental *Xiphophorus* genotypes confirmed a positive correlation between the abundance of *xmrk* transcripts and the development and progression of melanoma (Regneri and Schartl, 2012). Furthermore, the data clearly demonstrated that transcriptional activation of the *xmrk* oncogene is restricted to the black pigment cell lineage of *R*-free backcross hybrids. This led to the conclusion that the *xmrk* oncogene is controlled by a pigment cell-specific regulatory region, the activity of which is controlled by the *R* locus. Crossing-dependent loss of *R* would thus result in a release of the transcriptional control of *xmrk* in melanocytes, and the resulting overexpression of *xmrk* would consequently be the primary step that initiates tumor formation.

This hypothesis was further supported by comparing expression of a highly tumorigenic (mdl^{Sd}-$xmrk^{B}$) and a non-tumorigenic (mdl^{Sr}-$xmrk^{A}$) *xmrk* allele (Regneri and Schartl, 2012). Both alleles encode for the constitutively activated Xmrk protein. (For nomenclature of *xmrk* and *mdl* (macromelanophore-determining locus) alleles and a detailed description of *Xiphophorus* phenotypes and genotypes, see Schartl and Meierjohann, 2010). In contrast to the tumorigenic $xmrk^{B}$ allele, which is highly overexpressed in malignant melanomas compared to benign lesions and healthy skin, the transcription of the $xmrk^{A}$ allele in melanocytes is not influenced by elimination of the regulator locus *R*. Transcript levels of $xmrk^{A}$ remain at the same low level in *R*-free hybrids as in the wild-type platyfish, which possess two copies of *R*. This clearly demonstrates that transcriptional control of *xmrk* in pigment cells is causative for melanoma development in *Xiphophorus*.

Hence, all evidence indicates that the *R* locus controls tumor development in *Xiphophorus* on the transcriptional level by directly or indirectly downregulating *xmrk* transcription. However, as yet, the molecular identity of the *R* locus-encoded gene(s) has not been defined, and there is no direct experimental proof for its suggested mode of action.

Moreover, the *xmrk* promoter region is still poorly characterized. Apart from a 460 bp fragment with more than 97% nucleotide identity (Volff et al., 2003), the putative promoter regions of *xmrk* and the proto-oncogene *egfrb* are completely different from each other. As result of the duplication event that has generated the *xmrk* oncogene, a single copy of a repetitive DNA element (called *D* locus) was integrated directly upstream of the transcription start site (TSS) of *xmrk* (Adam et al., 1993; Volff et al., 2003). Interestingly, the new *D* locus-derived upstream sequence contains TATA- and CAAT-like sequences at the expected distance from the TSS of the *xmrk* oncogene (Adam et al., 1993). The functional analysis of the most proximal 0.7 kb $xmrk^{B}$ promoter fragment (−675/+34) revealed the presence of an activating GC box element identical to the consensus binding site for the transcription factor Sp1 in close proximity to the TATA box of the oncogene. This element mediated high-level transcriptional activation in *Xiphophorus* cell lines, but the 0.7 kb fragment showed no melanoma cell specificity in reporter gene assays (Baudler et al., 1997), strongly suggesting the existence of additional *cis*-regulatory elements outside the region so far analyzed. To unravel the molecular mechanism by which *R* controls *xmrk* induced melanoma development in *Xiphophorus*, the identification of such regulatory elements and the respective transcription factors is a precondition.

Here we present a comprehensive functional analysis of 200 kb $xmrk^{B}$ flanking sequences representing the entire putative gene regulatory region of the tumorigenic $xmrk^{B}$ allele using conventional plasmid based as well as BAC (bacterial artificial chromosome)-derived luciferase reporter systems. Comparing transcriptional activity of $xmrk^{B}$ reporter constructs in cells of melanoma and non-melanoma origin, we found no sequence elements that mediated a pigment cell-specific transcriptional activation. Hence, we could not reproduce the endogenous *xmrk* expression pattern *in vitro* using $xmrk^{B}$ luciferase reporter constructs. Our data therefore strongly suggest that additional mechanisms are involved in controlling the melanocyte-specific transcriptional activation of *xmrk* and hint at a crucial role for DNA methylation changes in *xmrk* expression regulation and thereby in melanoma development in *Xiphophorus*.

2. Materials and methods

2.1. Cell culture

The *Xiphophorus* melanoma cell line PSM and the embryonic epithelial cell line A2 were cultured in F 12 medium (Gibco™, Life Technologies) supplemented with 15% fetal calf serum (FCS) and penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 28 °C. The PSM cell line was established from melanoma tissue of an adult F1 hybrid between a platyfish (*X. maculatus*) and an albino swordtail (*X. hellerii*) (Wakamatsu, 1981). PSM cells carry an $mdl^{Sp\text{-}type}$-$xmrk^{A}$ allele, which is highly tumorigenic and leads to melanoma development already in F1 hybrids between platyfish and swordtails, but its sex chromosomal origin is unknown (Schartl and Meierjohann, 2010). The differential tumorigenic potential of $mdl^{Sp\text{-}type}$-$xmrk^{A}$ (tumorigenic) and mdl^{Sr}-$xmrk^{A}$ (non-tumorigenic) has been attributed to sequence variations in the regulatory region as the open reading frames of both $xmrk^{A}$ alleles encode for the mutationally activated Xmrk protein (Regneri and Schartl, 2012). The A2 cell line (Kuhn et al., 1979) is derived from the southern swordtail species *X. hellerii* (Figs. S1 and S2). Embryonic epithelial cell lines derived from *X. maculatus* (SdSr24) and *X. hellerii* (XhIII, FOI8) (Altschmied et al., 2000) were maintained in DMEM medium (Gibco™, Life Technologies), 15% FCS in the presence of penicillin/streptomycin at 28 °C in a humidified atmosphere with 5% CO_2.

2.2. RNA isolation, reverse transcription and quantitative real-time PCR analysis

Total RNA was extracted from cell cultures using TRIzol® Reagent (Life Technologies) according to the supplier's recommendations. After DNase I treatment, reverse transcription was performed from 2 μg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Life Technologies) and random hexamer primers according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) analyses were performed on cDNA from 50 ng of total RNA. Amplification was monitored with Mastercycler® ep realplex (Eppendorf) using SYBR Green reagent. For quantification, data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and normalized to expression levels of the housekeeping gene elongation factor