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ScienceDirect
Gene 411 (2008) 10–18
GENE
www.elsevier.com/locate/gene

Identification of the minimal promoter region of the mouse NKX5-3, a transcription factor implicated in eye development

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Received 19 September 2007; received in revised form 12 December 2007; accepted 12 December 2007
Available online 2 January 2008
Received by S. Yokoyama

Abstract

Early ocular development is controlled by a complex network of transcription factors, cell cycle regulators, and diffusible signalling molecules. Together, these molecules regulate cell proliferation and apoptosis, and specify retinal fate. NKX5-3 is a homeobox transcription factor implicated in eye development. The analysis of the 5'-flanking region of the mouse *Nkx5-3* gene revealed a predicted TATA-less promoter sequence between −416 and −166 of the translation start site. To functionally characterise *Nkx5-3* promoter activity, serial deletions of the promoter sequence were introduced in pGL-3 basic vector and promoter activity of these 5'- and 3'-deleted constructions was tested in HeLa and CHO cells. Transactivation assays identified a region between −350 and −296 exhibiting promoter-like activity. Combined analysis by deletions and point mutations showed that this sequence, containing multiple Sp1 binding sites was necessary to promote transcriptional activity. Binding of Sp1 to this region was confirmed by electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation, using an antibody specific for Sp1. Altogether, these results demonstrated that the immediate upstream region of *Nkx5-3* gene possessed a strong intrinsic promoter activity *in vitro*, suggesting a potential role in *Nkx5-3* transcription *in vivo*.

Keywords: TATA-less promoter; Homeobox family; Sp1 transcription factor; Transactivation assay

1. Introduction

The development of the vertebrate eye is controlled by a sequence of morphogenetic events comprising tissue–tissue interactions and necessitates the contribution of many regulators of cell cycle, diffusible signalling molecules, and transcription factors like BMP4, BMP7, PAX2, PAX6, RX, CHX10, OTX1, OTX2 and many others (Graw, 1996; Jean et al., 1998). Proper interaction of these factors during development of the eye is crucial for cell proliferation and cell fate specification of retinal cells.

Nkx5-3, (also called *Hmx1*), *Nkx5-2* (*Hmx2*) and *Nkx5-1* (*Hmx3*) constitute the *Hmx* family of genes characterised by the presence in each of them of a specific homeodomain (Stadler et al., 1995). This family contains a fourth member called *Soho-1*, only described in chicken (*Gallus gallus*) (Deitcher et al., 1994) and in the medaka fish (*Oryzias latipes*) (Adamska et al., 2001). *GH6*, the *Nkx5-3* homologous chicken gene, was first characterised during eye development and found to be expressed in the neural retina, the lens epithelium and the optic nerve (Stadler and Solursh, 1994), whereas the murine homolog was shown to be expressed at the optic vesicle

Abbreviations: EMSA, electrophoretic mobility shift assay; cDNA, DNA complementary to RNA; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; AP-2, activator protein 2; Sp1, specificity protein 1; SV40, simian virus 40; UTR, untranslated region; bp, base pair; Chr, chromosome; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; nt, nucleotide; oligo, oligodeoxyribonucleotide; RT, room temperature, CHO, Chinese hamster ovary; TFBS, transcription factor binding site; wt, wild type; mut, mutated; EST, expressed sequence tag; TSS, transcription start site; TBS, Tris-buffered saline; ChIP, chromatin immunoprecipitation.

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doi:10.1016/j.gene.2007.12.020

constriction stage and during lens formation (Yoshiura et al., 1998; Wang et al., 2000). *Nkx5-2* and *Nkx5-1* are highly expressed in the otic vesicle and in the neuroectodermal cells of the central nervous system (Rinkwitz-Brandt et al., 1995). Similarly, it was suggested that NKX5-2 and NKX5-1 might also be implicated in the development of sensory organs, especially the inner ear (Hadrys et al., 1998; Yoshiura et al., 1998; Wang et al., 2000).

The distribution of *Nkx5-3* and *Soho-1* transcripts into the retina is polarised, with a higher expression in the nasal and posterior parts (Yoshiura et al., 1998; Schulte and Cepko, 2000). Moreover, NKX5-3 and SOHO-1 are implicated in the control of the expression of axon guidance activity in the chick retina by down-regulation of *EPHA3* in the region where *NKX5-3* and *SOHO-1* are expressed. This Ephrin receptor is critical for controlling the ganglion cell axons (Schulte and Cepko, 2000).

Sp1, one of the first identified eukaryotic transcription factors, was originally cloned as a factor that binds to the SV40 early promoter (Kadonaga et al., 1987). Sp1 is the founder member of the Sp1-like transcription factor family. Members of this family share similar DNA binding affinity and some common structural features, but may play distinct roles in the early development of vertebrates' embryos (Zhao and Meng, 2005). The Sp1 protein binds selectively to a GC-rich decanucleotide sequence known as the GC box via three Cys_2His_2 zinc-finger motifs (Kadonaga et al., 1987). This ubiquitously expressed nuclear protein has been implicated in the activation or repression of many genes and was shown to drive a large number of cellular processes such as cell cycle regulation, apoptosis and chromatin remodeling (Saffer et al., 1991; Black et al., 1999; Kavurma et al., 2001; Milavetz, 2002). Sp1 is important for normal mouse embryogenesis, since Sp1 knockout mice died at approximately day 11 of gestation (Marin et al., 1997). Moreover, Sp1 is expressed during mouse eye development (Nakamura et al., 2005), when *Nkx5-3* is activated, i.e. during optic vesicle constriction and lens formation (Yoshiura et al., 1998; Wang et al., 2000).

To better define the function of NKX5-3, we identified and characterised the promoter region of the mouse *Nkx5-3* gene. We showed that the 5'-flanking region of *Nkx5-3* was composed of enhancer and repressive parts. We identified a region with a promoter-like activity, from −416 to −166 from translation start site and localised several binding sites for Sp1 transcription factor. By transactivation assay, electrophoretic mobility shift assay (EMSA) experiments and chromatin immunoprecipitation, we demonstrated that Sp1 was implicated in the transactivation of the *Nkx5-3* promoter *in vitro*.

2. Materials and methods

2.1. Bio-informatic analysis of the promoter region and putative transcription factor binding sites

A BLAST homology search against the murine genomic sequences of the public Genbank database was performed for mouse *Nkx5-3* sequence. Two kb of the 5'-upstream sequences of the mouse *Nkx5-3* cDNA (NM_010445) and of the human *NKX5-3* cDNA (NM_018942.2) were aligned with the publicly available web-based tool, mVista using the Shuffle-LAGAN glocal alignment algorithm (http://genome.lbl.gov/vista/) to identify conserved sequences (Frazer et al., 2004). The web-tool rVista was used to identify potential transcription factor binding sites (TFBS) and to determine the conservation of these sites between species (Loots and Ovcharenko, 2004). The rVista software used TRANSFAC Transcription Factor Binding Sites Database (Wingender et al., 2000) (http://www.gene-regulation.com) for TFBS search. The rVista analysis was performed on aligned sequences among the 2-kb sequences used for mVista analysis. The same 2-kb genomic region was analysed for putative promoter sequence with MatInspector (Quandt et al., 1995) (http://www.genomatix.de/cgi-bin/eldorado/main.pl) and PROSCAN softwares (Prestridge, 1995) (http://thr.cit.nih.gov/molbio/proscan).

2.2. Generation of reporter plasmids

For murine *Nkx5-3* promoter deletion analysis, 15 different PCR fragments (named pGL3 −1884/−1, pGL3 −502/−1, pGL3 −350/−1, pGL3 −296/−1, pGL3 −260/−1, pGL3 −164/−1, pGL3 −1884/−502, pGL3 −1884/−350, pGL3 −1884/−296, pGL3 −1884/−260, pGL3 −1884/−164, pGL3 −502/−350, pGL3 −502/−296, pGL3 −502/−260, pGL3 −502/−164) spanning from −1884 to −1 from translation start site were generated using mouse genomic DNA as template. The PCR reaction conditions varied depending on the size and G+C content of the amplified genomic sequences. The corresponding primers used for PCR amplification are listed in Table 1. The forward and reverse primers were designed to introduce internal *SacI* and *BglII* restriction sites, respectively, and the resulting *SacI*/*BglII*-digested PCR products were ligated into the pGL3-basic luciferase reporter gene vector (Promega).

2.3. Site-directed mutagenesis

Deletion and point mutations in the Sp1-binding sites (GC box) were performed by using the QuickChange II mutagenesis kit (Stratagene). The forward oligonucleotides (PromDel F, PromMut F) are shown in Table 1, the sequence of the reverse primers is complementary to that of the corresponding forward primer. Briefly, pGL3 −350/−1 mut and pGL3 −350/−1 del constructs were generated by PCR by using pGL3 −350/−1 as template, the complementary mutated oligonucleotides and the *Pfu* DNA polymerase (Stratagene). The PCR product was digested with *DpnI* to remove the DNA template.

2.4. Cell culture and transient transfection conditions

HeLa cells were cultured in RPMI medium (Invitrogen) containing 10% FBS (foetal bovine serum) in a humidified incubator at 37 °C under 5% CO_2. CHO cells were grown in Dulbecco's modified Eagle's medium with GlutaMAX™ I (4500 mg/l D-glucose, 25 mM HEPES), without sodium pyruvate. Twenty-four hours before transfection, cells were plated at a density of 10,000 cells/well in 96-well plate and allowed to grow to 90% confluency.