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Characterisation of the Transcription Factor, SIX5, Using a New Panel of Monoclonal Antibodies

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Abstract SIX5 is a member of the human SIX family of transcription factors, many of which are involved in eye development. However, SIX5 transcripts are known to be present at very low levels in cells and no study has yet convincingly demonstrated detection of endogenous SIX5 protein by Western blotting or immunolocalisation. We have produced a new panel of 18 monoclonal antibodies (mAbs) that recognise at least four different epitopes in order to identify authentic human SIX5 protein in cells and tissues. Phage-displayed peptide libraries were used to identify individual amino-acids important for antibody binding within each epitope. Endogenous SIX5 migrated in SDS–PAGE with an apparent M_r of 100 kDa and was present at similar levels in all foetal tissues and cell lines tested. In HeLa cells, it was located in the nucleoplasm with a granular distribution. An mRNA for a shorter splicing isoform of SIX5, with an altered carboxy-terminus, has been described, but further mAbs specific for this isoform did not detect any endogenous protein. We conclude that the full-length isoform is the major functional protein in vivo while the putative shorter protein is undetectable and may not be expressed at all. J. Cell. Biochem. 95: 990–1001, 2005.

Key words: homeobox; transcription factor; myotonic dystrophy; Six4; epitope mapping; phage-display; heart; skeletal muscle; myogenesis

The *SIX5* gene, previously known as myotonic dystrophy associated homeodomain protein, DMAHP, is situated downstream of the dystrophia myotonica protein kinase (*DMPK*) gene at band 13.3 on the long arm of human chromosome 19 [Boucher et al., 1995]. It has three exons that encode a putative homeodomain protein. RT-PCR and sequencing demonstrated an alternatively-spliced transcript that omits the second exon and uses a different reading frame in the third exon. However, in the absence of conclusive Northern blotting data or protein studies, the functional significance of this transcript remained unclear [Boucher et al., 1995].

The members of the *Six* gene family were identified as homologues of Drosophila *sine oculis (so)*. In Drosophila, the *so* gene is essential for the development of the entire visual system and has relatively restricted tissue expression [Cheyette et al., 1994]. Members of the Six5 family encode proteins characterised by the Six domain (SD, 110–115 amino acids) and the Six-type homeodomain (HD, 60 amino

In this paper "Six" is used for the mouse gene/protein and more generally, while "SIX" is used specifically for the human gene/protein.

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acids), both of which are essential for specific DNA binding and mediating protein–protein interactions. The Six family can be classified into three major subgroups based on the amino acid sequence similarities of the conserved homeodomains and Six domains [Seo et al., 1999]. In the mouse, these subgroups were designated as the *Six1/2*, *Six3*/6 and Six4/5 subfamilies. Uniquely, the Six4/5 subfamily proteins have large, additional C-terminal sequences containing a transactivation domain. The transactivation domain of Six4 has been mapped to the C-terminal 150 amino acids and retains its function when fused to a GAL4 DNA binding domain [Kawakami et al., 1996a]. Homeodomain proteins are transcription factors that regulate gene expression by binding to DNA. Harris et al. [2000] have shown that, in vitro, the homeodomain of human SIX5 acts as a sequence-specific DNA binding domain and that the SIX domain is necessary for the formation of a stable putative homodimer complex that enhances this binding activity. Both the Six-domain and homeodomain of Six2, Six4 and Six5 are necessary for specific binding of these proteins to the ARE, a transcription regulatory element of the Na,K-ATPase 1 subunit gene (Atp1a1) [Kawakami et al., 1996a,b]. The Six domain is important for protein interactions with the Eya-Dach regulatory networks in Drosophila [Pignoni et al., 1997; Ohto et al., 1999] and mouse [Heanue et al., 1999]. Six1, Six2, Six4 and Six5 have similar binding specificity to ARE and myogenic enhancing factor 3 (MEF3) sites [Kawakami et al., 1996a,b; Spitz et al., 1998; Ohto et al., 1999]. Therefore, potential targets of these Six proteins could be Atp1a1 and muscle-specific genes with flanking MEF3 sites, such as the myogenin, aldolase A and muscle creatine kinase genes. Expression of Six1, Six4 and Six5 in skeletal muscle suggests that muscle-specific genes may be one of the targets of these proteins in vivo [Fougerousse et al., 2002]. Six4 binds directly to the Trex element in the muscle creatine kinase enhancer region and is now thought to be a key regulator of muscle development [Himeda et al., 2004]. In order to "knockout" Six5 function, Klesert et al. [2000] replaced the first exon of Six5 with a beta-galactosidase reporter gene, whereas Sarkar et al. [2000] deleted the entire Six5 gene and replaced it with a neo cassette. The latter observed reduced male fertility and recently suggested that SIX5 defects may be linked to testicular problems in DM patients [Sarkar et al., 2004]. Both observed increases in cataract formation in the eyes. The Six4 knockout mouse also failed to display any dramatically-abnormal phenotype [Ozaki et al., 2001] and it is possible that Six4 and Six5 can each compensate for loss of function of the other to some extent. Recently, a C. elegans homologue of Six4/5 was identified and was shown to be necessary for correct migration and differentiation of a subset of mesodermal and ectodermal cells [Yanowitz et al., 2004].

The $(CTG)_n$ repeat expansion associated with myotonic dystrophy type 1 (DM1) was originally thought to cause the disease by disruption of nearby transcription units, including SIX5 and a protein kinase gene DMPK. A DMPK knockout mouse with myopathy and cardiac abnormalities [Jansen et al., 1996; Reddy et al., 1996; Pall et al., 2003] and SIX5 knockout mice with cataracts [Klesert et al., 2000; Sarkar et al., 2000] gave some support to this view. However, a transgenic mouse expressing CUG repeats in nuclear RNA unrelated to DMPK or SIX5 displayed many features of DM [Mankodi et al., 2000]. The recent discovery that myotonic dystrophy type 2 (DM2) is caused by the expansion of a CCTG repeat located in intron 1 of the zinc finger protein 9 (ZNF9) gene appears to confirm conclusively that both DM1 and DM2 mutations are pathogenic at the RNA level and that most features of the DM phenotype are unrelated to reduced levels of DMPK, SIX5 or ZNF9. The current hypothesis is that the CUG repeat expansion in RNA accumulates as nuclear foci and interferes with the normal functions of CUG-binding proteins, such as muscleblind [Mankodi et al., 2001; Ho et al., 2004]. The severe congenital form of DM1 does not occur in DM2 families, so reduced DMPK or SIX5 levels might contribute to this more severe phenotype [Furling et al., 2003].

In the present study, we have used a novel panel of monoclonal antibodies (mAbs) against human SIX5 to characterise its endogenous expression. We show that a 100 kD nucleoplasmic protein is the only significant product of the SIX5 gene and that this SIX5 protein is widely-expressed in tissues, unlike the restricted expression pattern of many other SIX proteins. Proteins, like SIX5, that are produced only at very low levels in the cell are particularly prone to confusion with more abundant

proteins, due to antibody cross-reactions [Lam et al., 2000]. The availability of a panel of well-characterised antibodies against many different SIX5 epitopes will enable future studies of this transcription factor to be performed with confidence.

MATERIALS AND METHODS

Cloning and Expression of SIX5 Isoforms

SIX5A and SIX5B cDNAs were amplified as single exons from genomic cosmids. The primers were: (i) for SIX5A, forward 5′-GC**GAATTC**AACATGGCTACCTTGCCTGC and reverse 5′-CG**AAGCTT**ACCAGTTGCTGACCTGCGT. (ii) for SIX5B, forward 5′-**GAATTC**GAGTCTGATGGGAATCCCAC and reverse 5′-**AAGCTT**ATTCCTGTCCCTGCGTCTT. SIX5AC cDNA was amplified from a SIX5AC/pUNI10 construct (Harris et al., 2000) using the same SIX5A forward primer with 5′-**AAGCTT**GTTCGGAAGCGGCCT as reverse primer. The forward and reverse primers have *Eco*RI and *Hind*III sites (boldface) respectively for cloning into pET32a expression plasmids (Novagen) for expression in *Escherichia coli* BL21(DE3) as thioredoxin fusion proteins. DNA sequencing confirmed that the cloned sequences matched the GenBank human SIX5 sequence. Transformed bacteria were induced with 1 mM isopropyl-thio-β-D-galactoside (IPTG) for 16 h at 37°C. Expressed fusion proteins were purified from inclusion bodies by His-tag affinity chromatography according to the supplier's instructions (Novagen). For immunisation, they were precipitated with 50% ethanol and redissolved in 6M urea in PBS.

Production of Antibodies

Monoclonal antibodies were produced by immunisation of BALB/c mice with the purified recombinant fusion proteins from pET32 plasmids. Spleen cells were fused with Sp2/0 myeloma cells as previously described [Nguyen thi and Morris, 1996]. The mouse sera and the hybridoma culture supernatants were screened by ELISA, Western blotting and immunofluorescence microscopy. Hybridoma cell lines were cloned to homogeneity by limiting dilution. Ig subclass was determined using an isotyping kit (Zymed Laboratories, Inc., San Francisco, CA 94080). Polyclonal antibodies against SIX5 were produced by immunisation of New Zealand White rabbits with SIX5A and SIX5B recombinant proteins.

SDS–PAGE and Western Blotting

SDS–PAGE and Western blotting were carried out as described elsewhere [Nguyen thi et al., 1991]. The respective protein bands were visualised following development with a biotin/avidin/diaminobenzidine detection system for mouse mAbs (Vectastain; Vector Laboratories) or with peroxidase-conjugated rabbit anti-(mouse Ig) (DAKO) and a chemiluminescent system (SuperSignal; Pierce, Rockford, IL).

Eukaryotic Cell Culture and Transfections

For mammalian transfection, SIX5ABC and SIX5AC cDNAs were sub-cloned from pUNI10 plasmids into pcDNA4/HisMaxA (Invitrogen) using NdeI and SalI restriction enzymes. This plasmid contains an N-terminal tag for detection of fusion protein with anti-Xpress antibody (Invitrogen). Plasmids were purified using Endo-Free Plasmid Maxi Kits (Qiagen) and quantified by UV spectrophotometry. HeLa cells, human foetal muscle cells, mouse embryonic fibroblasts and normal human fibroblasts were grown in DMEM (Gibco) with 20% decomplemented horse serum (Gibco), 2 mM L-glutamine and antibiotics for transfection by electroporation [Espinos et al., 2001; Holt et al., 2003].

Immunofluorescence Microscopy

Cells were fixed and permeabilised by treatment with 50% methanol/50% acetone and incubated with mAb culture supernatant (diluted 1 in 4 with PBS) for 30 min at room temperature, followed by FITC-conjugated horse-anti-(mouse Ig) (Vector Laboratories, Peterborough, UK) diluted 1:50 in IMF buffer (PBS containing 1% horse serum, 1% foetal bovine serum and 0.1% bovine serum albumin) for 30 min at room temperature. Ethidium bromide or DAPI (4′-6′-diamidino-2-phenylindole) was added at 200 ng/ml in PBS for the final 5 min of the incubation to counterstain the nuclei. Coverslips were then washed four times with PBS and mounted in Hydromount (BDH Merck). Cells were examined with a Leica DMLB epifluorescence microscope (Leica, Milton Keynes, UK) with a 63 × objective (numerical aperture 1.40) and images captured with a JVC video camera (KY-F55B) and Neotech Image Grabber.

Epitope Mapping

Epitope mapping using phage-displayed random peptide libraries in filamentous phage was performed as previously described [Pereboev and Morris, 1996], using a modification of an earlier method [Smith, 1992].

A panel of SIX5 mAbs were diluted 1:50 with Tris-buffered saline (TBS) and immobilised onto sterile 35 mm Petri dishes coated directly with 1 ml of 1:200 dilution of rabbit-anti-[mouse Ig] in TBS (Dako, Denmark). Biopanning was performed using a 15-mer peptide library in phage f88-4 maintained in the K91Kan strain of *Escherichia coli*, generously supplied by G.P. Smith (University of Missouri). Any remaining binding sites on the dishes were blocked using 4% BSA in sterile TBS. The phage library was pre-incubated in dishes coated with the rabbit-anti-mouse antibodies alone to ensure any binding was specific for the SIX5 mAbs. Following the first round of biopanning, the bound phage were eluted and amplified by infection of K91Kan *E. coli* cells. Three rounds of biopanning were performed. Colonies of the phage infected cells after the third round were grown on nitrocellulose membrane and screened with mAbs to reveal positive clones. Phage DNA was purified and sequenced using a primer with 5′–3′ sequence: AGTAGCAGAAGCCTGAAGA.

RESULTS

Production and Epitope Mapping of Antibodies Against SIX5 Protein

Recombinant thioredoxin fusion proteins containing SIX5 sequences encoded by exon A, exon B or exon AC2 (Fig. 1) were purified by His-Tag affinity chromatography and used to immunise Balb/c mice for hybridoma production. We also subcloned full-length (ABC) and short (AC2) isoforms of SIX5 cDNA into the mammalian expression vector, pcDNA4-HisMax, and transfected the constructs into HeLa cells. This enabled us to use transfected cells to screen hybridoma supernatants for co-localisation of new mAb staining with the Xpress epitope tag encoded by the plasmid.

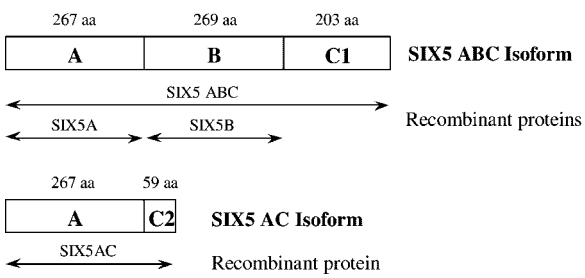

Fig. 1. Exon structure of the human *SIX5* gene and the recombinant protein fragments used in this study. The *SIX5* gene contains three exons, A, B and C. Alternative splicing produces both a full-length isoform, ABC and a shorter form, AC, with exon C in a different reading frame (C2 protein sequence instead of C1 in ABC). The number of amino-acids contributed by each exon and the 4 recombinant proteins generated in this study are shown.

Figure 2a shows that the localisation of transfected recombinant SIX5 is almost exclusively in the nucleoplasm, avoiding the nucleoli. Two mAbs from exon A and exon B fusions are shown in Figure 2a and a mAb against the Xpress-tag in the recombinant proteins gave a similar pattern (Fig. 2b). Transfection with isoform AC2 also gave an indistinguishable staining pattern (not shown) and this is not surprising since the nuclear localisation signal and DNA-binding sequences (Six domain and homeodomain) are encoded by exon A. Figure 2c shows that endogenous SIX5 in HeLa is also nucleoplasmic, as with transfected SIX5 in Figure 2a, but some cytoplasmic staining is also evident. Some, though perhaps not all, of this cytoplasmic staining can be attributed to non-specific staining by the secondary antibodies (Fig. 2d shows a control image without primary antibody captured under comparable conditions). There is also a granular appearance to the endogenous nucleoplasmic staining (see Fig. 2e for higher magnification), though whether these correspond to nuclear speckles associated with transcription has not yet been established. Exon C2 mAbs gave only background staining (not shown).

Figure 3 shows that a Western blot of the SIX5-transfected HeLa cells (lane 1) gives a major band at around 100 kDa with some lower M_r bands that may be degradation products. An endogenous protein of similar size was recognised in untransfected HeLa by the same mAb (lane 2). Co-migration of the recombinant SIX5 with the endogenous protein supports the authenticity of the latter, although it migrates slower on SDS–PAGE than predicted from the amino-acid sequence (85 kDa). The criteria adopted for screening hybridoma culture supernatants were that they should have (a) strong nuclear staining with transfected SIX5 in HeLa cells, (b) a band at the correct size on blots of recombinant fusion protein, (c) a band at about

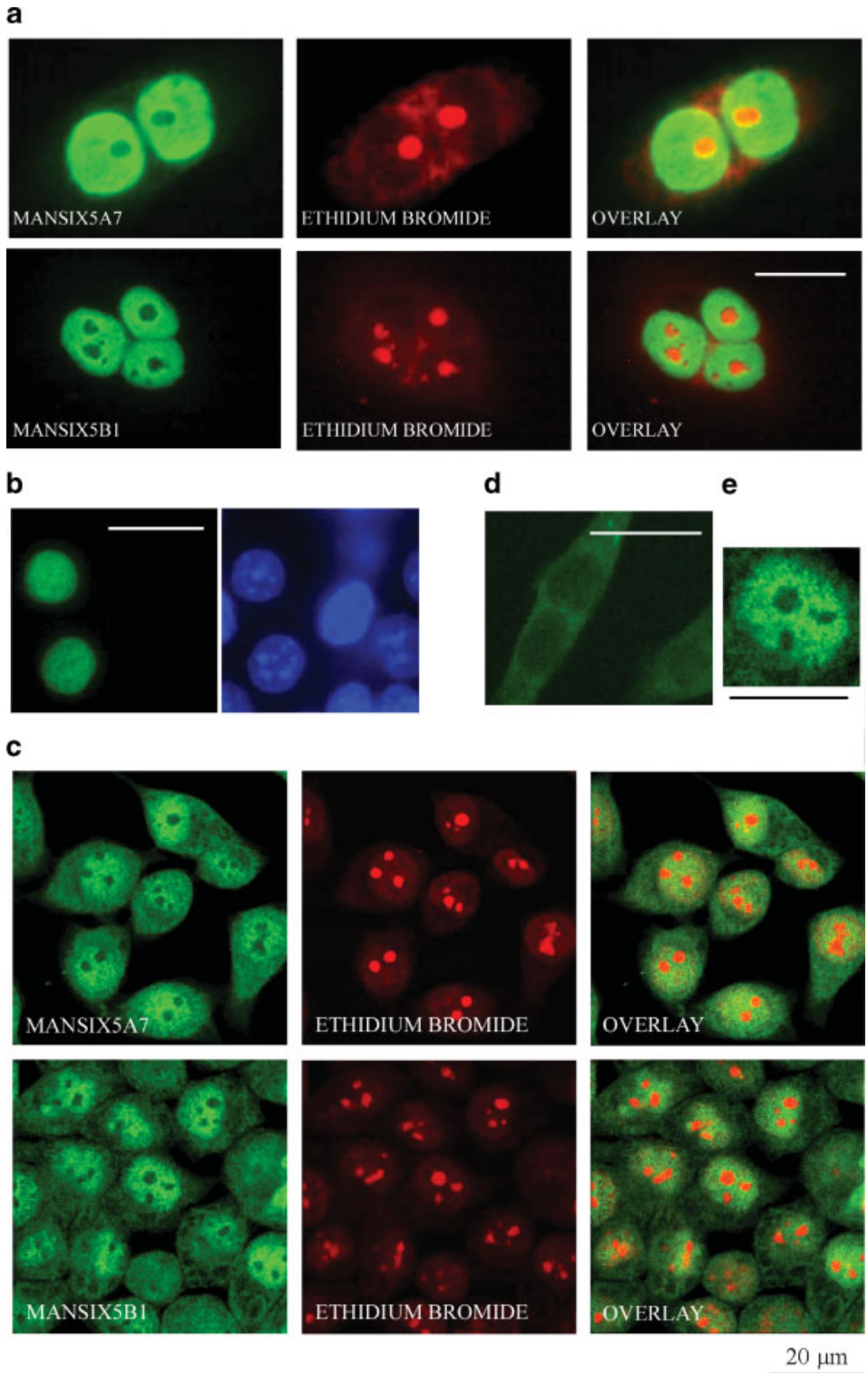

Fig. 2. **a**: Transfected recombinant SIX5 is localised in the nucleoplasm. Immunolocalisation of recombinant SIX5 in HeLa cells transfected with pcDNA4/SIX5ABC using mAbs MANSIX5A7 (exon A) or MANSIX5B1 (exon B). Bound mAb was detected with FITC-labelled anti-(mouse Ig) [green] and nuclei were counterstained with ethidium bromide [red]. **b**: The transfected SIX5 is also detected in the nucleoplasm by anti-Xpress tag mAb and FITC-anti-mouse Ig [green], while DAPI (blue) stains DNA in both transfected and untransfected HeLa nuclei. **c**: Localisation of endogenous SIX5 in HeLa cells. Binding of mAbs MANSIX5A7 (exon A) or MANSIX5B1 (exon B) was detected with FITC-labelled anti-(mouse Ig) [green] and nuclei were counterstained with ethidium bromide [red]. **d**: shows a control in which the primary mAb was omitted. **e**: is a close-up showing the granular nucleoplasmic staining pattern. All bar lines are 20 microns, except for 10 microns in (e).

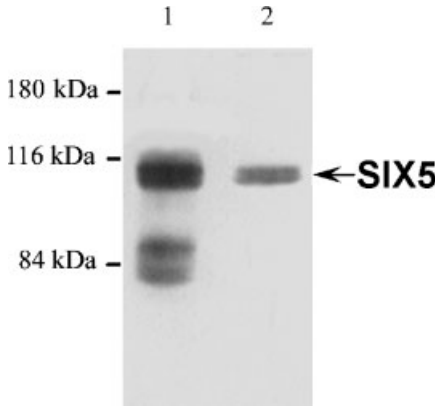

Fig. 3. Both transfected and endogenous SIX5 of HeLa cells migrate as a 100 kDa protein on SDS–PAGE. Western blot of extracts of (**lane 1**) SIX5ABC transfected HeLa cells and (**lane 2**) non-transfected HeLa cells were developed with mAb MANSIX5B3. Horseradish peroxidase-conjugated anti-(mouse Ig) and a chemiluminescent substrate were used. Positions of pre-stained M_r markers (Sigma) are shown.

100 kDa on normal human lung or HeLa blot (exon A and B mAbs only) and (d) high titres in ELISA. Using this approach, we obtained twelve mAbs against exon A, four against exon B and two against exon C2 (Table I).

To identify unequivocally Western blot bands or localisation patterns as authentic SIX5 protein, it is essential to have mAbs against different epitopes on SIX5, the argument being that any band or staining pattern common to all epitopes must be authentic protein while those produced by only one mAb may be cross-reactions. Although the use of separate exon immunogens (A and B for ABC, A and C2 for AC2 isoforms) ensures that at least two different epitopes are represented. We used phage-displayed peptide libraries to investigate further the epitope specificity of the 12 exon A mAbs. Figure 4A shows that four mAbs recognise an epitope near the N-terminus (Ser9-Ala20: MANSIX5A3, A4, A7, A9) and five mAbs recognise two adjacent epitopes a little further along (Ser72-Ser78: MANSIX5A2, A6, A8; Pro80-Arg85: MANSIX5A1, A5). MANSIXA11 recognised a peptide that could not be matched to the SIX5 sequence and may be a "mimotope" of a conformational epitope. Together with the four exon B mAbs, this shows that our mAb panel recognises at least five different SIX5 epitopes (Table I) and that the protein recognised by all these mAbs can be confidently identified as authentic SIX5. Using SIX5AC2 recombinant protein as immunogen, we produced two mAbs that required the C2 sequence (Fig. 1) for binding and one of them was mapped to a specific sequence within C2 (Fig. 4A). These mAbs should recognise the putative shorter isoform only. Attempts to map the exon B epitopes by this method were unsuccessful, possibly because their structures could not be mimicked by 15-mer peptides.

Although human SIX5 and mouse Six5 amino-acid sequences are very similar overall, they differ significantly in the region of the exon

TABLE I. Anti-SIX5 Monoclonal Antibodies, Characterisation and Epitope Mapping

Clone	mAb name and subclass	Western blot			IMF HeLa	Epitope mapping
		SIX5-r	HeLa	NHL		
SIX5A-2A6	MANSIX5A1 IgG1	+	+	+	+	P80-R85
SIX5A-3B4	MANSIX5A2 IgG1	+	+	+	+	S72-S78
SIX5A-3D9	MANSIX5A3 IgG1	+	+	+	+	S9-A20
SIX5A-3E11	MANSIX5A4 IgG1	+	+	+	+	S9-A20
SIX5A-10B12	MANSIX5A5 IgG1	+	+	+	+	P80-R85
SIX5A-1C11	MANSIX5A6 IgG1	+	+	+	+	S72-S78
SIX5A-3G10	MANSIX5A7 IgG1	+	+	+	+	S9-A20
SIX5A-7G4	MANSIX5A8 IgG2a	+	+	+	+	S72-S78
SIX5A-2G8	MANSIX5A9 IgG1	+	+	+	+	S9-A20
SIX5A-4H7	MANSIX5A10 IgG1	+	+	+	+	–
SIX5A-2H1	MANSIX5A11 IgG1	+	+	+	+	–
SIX5A-4E7	MANSIX5A12 IgG1	+	+	+	+	–
SIX5B-6E10	MANSIX5B1 IgG1	+	+	+	+	–
SIX5B-10B3	MANSIX5B2 IgG1	+	+	+	+	–
SIX5B-10D2	MANSIX5B3 IgG1	+	+	+	+	–
SIX5B-9G10	MANSIX5B4 IgG1	+	+	+	+	–
SIX5C2-6C1	MANSIX5C2a IgG2a	+	–	–	–	C2 (P31-A42)
SIX5C2-3A1	MANSIX5C2b IgG3	+	–	–	–	–

SIX5-r (recombinant SIX5); NHL (normal human lung); IMF HeLa (immunofluorescence microscopy on HeLa cells). Epitope mapping is based on data in Figure 4B. Amino-acids are numbered from the Met initiator, except for exon C2 where they are numbered from the start of the unique C2 sequence (see Fig. 1).

A

(1) MANSIXA1 AND A5

SIX5A protein sequence		66 SPGVPGSPPEAASE**PPTGLR**FSPEQVACVC	95
Phage peptide sequence	**1**	SELVLET**PP**S**GLR**IT	
	2	AV**PT**VFVRLVSASS	

(2) MANSIXA2, A6 AND A8

SIX5A protein sequence	63	PGSPGVPG**SP**PE**AAS**EPPTGLRFSPEQVAC	93
Phage peptide sequence	**1**	**AAS**PHMTLFHHTSQ	
	2	N**AS**WMSQDRFL	
	3	SSSLF**SP**AGQLLSTP	
	4	**AAS**PHMTLFHHTSQP	

(3) MANSIXA3, A4, A7 AND A9

SIX5A protein sequence	1	MATLPAEP**SA**G**P**A**A**GG**E**A**VA**AAAATEEEEE	30
Phage peptide sequence	**1**	**SA**A**P**FMSQSSDPSS	
	2	**SA**A**P**FMSQSSILRL	
	3	**E**G**VA**PRMRNLCDPHK	
	4	QCG**A**FY**E**S**V**FGSFVC	
	5	**E**G**VA**PRMRNLCIRI	

(4) MANSIXA11

SIX5A protein sequence	No match
Phage peptide sequence	FEMPYTQLISQTPNN

(5) MANSIXC2a

SIX5C2 protein sequence	23	QDHPHRHL**P**H**Q**H**ARL**P**GP**A**A**SPRPGPATER	52
Phage peptide sequence	**1**	TMANILLM**RL**LLL	
	2	SKTEQSVL**A**E**L**L**G**F	
	3	TMAND**P**PYA**A**L**L**L	
	4	TSKT**P**S**A**IGWC	
	5	N**Q**S**A**PWLWIRRY	

B **Human and mouse six5 alignment near the N-terminus.**

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                    (3)_____________
Human       1 MATLPAEPSAGPAAGGEAVAAAAATEEEEEEARQLLQTLQAAEGEAAAAAGAGAGAAAAG
Mouse       1 MATSPAEPSAGPAARGE---AAAATEEQEEEARQLLQTLQAAEGEAAA---AGAGDAAAA

                  (2)_______ _______(1)
Human      61 AEGPGSPGVPGSPPEAASEPPTGLRFSPEQVACVCEALLQAGHAGRLSRFLGALPPAERL
Mouse      61 ADS-GSPSGPGSPRETVTEVPTGLRFSPEQVACVCEALLQAGHAGRLSRFLGALPPAERL
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Fig. 4. Epitope mapping of exon A and exon C2 mAbs using phage-displayed peptide libraries. **A**: For each of the four epitopes identified, we show (line 1) the names of the mAbs that recognise the epitope, (line 2) the sequence of the epitope (bold and underlined) within the SIX5 sequence and (subsequent lines) the sequences of the 15-mer peptides pulled out of the library with matching amino-acids (bold and underlined) aligned with the SIX5 sequence. The residue numbers at each end of the SIX5 protein sequence are indicated in the right or left hand columns. The peptide recognised by MANSIX5A11 is also shown, although it could not be matched to the SIX5 sequence. **B**: Alignment of human SIX5 (GenBank: NP787071) and mouse Six5 (Genbank: Q8N196) protein sequences. The first 120 amino-acids are shown. The three epitopes identified in (A) are in boldface on the human sequence and the differences within these epitopes in mouse are underlined in the mouse sequence.

A epitopes that we have mapped and the amino-acid sequences of all three mapped epitopes are different in the corresponding mouse sequence (Fig. 4B). This may explain why our anti-human mAbs were unable to detect the Six5 protein in mouse tissues (our unpublished data). For this reason, we were unable to use Six5 knockout mouse tissues as final confirmation of mAb specificity. The exon B mAbs could not be mapped by phage-display but the explanation for their lack of cross-reaction with mouse six5 may be similar.

SIX5 in HeLa Cells is a 100 kDa Nucleoplasmic Protein

Figure 5 shows that mAbs against exons A or B of SIX5 all recognise a 100 kDa protein on Western blots (white arrows). MANSIX5A10, 11 and 12 (lanes 10, 11, 12) recognise SIX5 only weakly, if at all, but they do recognise other cross-reacting proteins (asterisks in Fig. 5). The 100 kDa band is not recognised by C2 mAbs (lanes 16, 17) because the C2 amino-acid sequence is not present in full-length SIX5 protein. Bands in lanes 16 and 17 that are equally strong with all mAbs (black arrows) are cross-reactions of the Vectastain second antibody system and are also seen in the "no primary antibody" control (lane 18).

In support of the immunolocalisation data shown in Figure 2c–e, subcellular fractionation and Western blotting show that nearly all 100 kDa SIX5 is present in nuclear fractions with only faint traces in the cytoplasmic fractions (Fig. 6).

Levels of SIX5 Protein Are Similar in All Tissues Tested and the Shorter Alternatively-Spliced Isoform is not Detectable

Figure 7 shows that SIX5 levels in all foetal human tissues tested (lung, liver, kidney, stomach, eye, skin, brain and limb) were similar to those in HeLa cells. This was found with mAbs against either exon A (Fig. 7A) or exon B (Fig. 7B). No lower M_r proteins were detected, except in lung, where the lower M_r proteins appear to be degradation products rather than cross-reacting proteins since they are recognised by both mAbs. No bands close to, or slightly larger than, the expected size of the AC2 isoform (44 kDa) were seen with either exon A mAbs or exon C2 mAbs (Fig. 5). Indeed, no bands at all detectable by all exon A mAbs, but not by exon B mAbs (the criterion for the AC2 isoform), were seen (Fig. 5).

DISCUSSION

These studies have shown that SIX5 is a mainly nucleoplasmic protein that migrates on SDS–PAGE with an apparent M_r of 100 kDa. SIX5 levels varied little between different tissues or cell types and no shorter RNA splicing

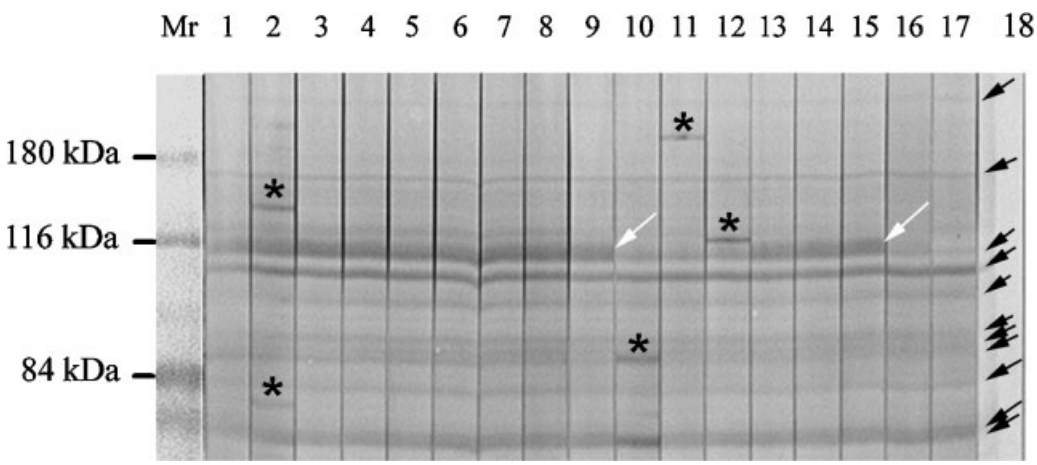

Fig. 5. The 100 kDa protein in HeLa cells is recognised by most mAbs against exons A and B but not by mAbs specific for the shorter isoform (exon C2). A Western blot of a HeLa extract was developed with anti-SIX5A, SIX5B and SIX5C2 mAb panel. The extract was subjected to 7% polyacrylamide SDS–PAGE as a strip. The lanes are as follows (M_r) Sigma protein markers, (**lanes 1–12**) MANSIX5A1-12 mAbs, (**lanes 13–15**) MANSIX5B1-3 mAbs, (**lanes 16, 17**) MANSIX5C2a,b mAbs, (**lane 18**) culture medium as a negative control. The Vectastain second antibody system and diaminobenzidine substrates were used. The position of the SIX5 band in lanes 1–9 and 13–15 is shown by the two white arrows. Several non-specific bands appearing in all lanes, including a faint one just below SIX5, are due to cross-reactions of the second antibody (black arrows). Cross-reactions shown by only one mAb are indicated by asterisks above the band.

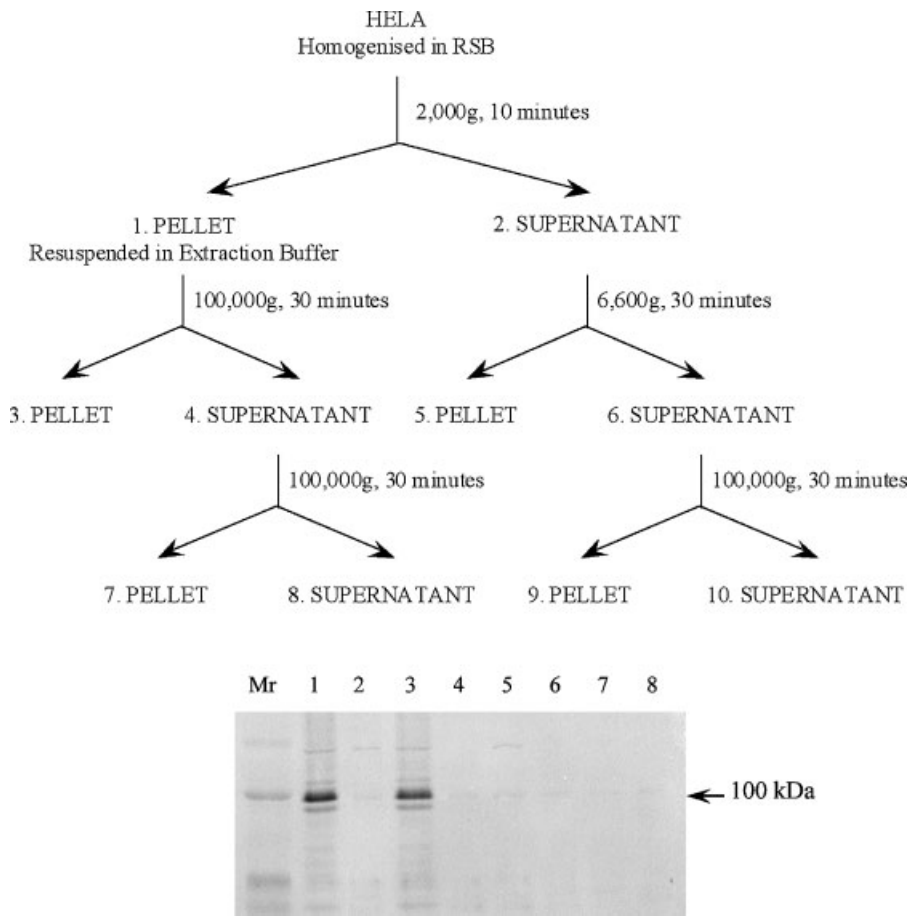

Fig. 6. The 100 kDa protein in HeLa cells is recovered in the nuclear fractions after subcellular fractionation. Hundred millilitres of HeLa suspension culture was grown for 5 days and then harvested by centrifugation at 2,000 g for 15 min at 4°C. The pellet was washed with 15 ml of ice-cold PBS and centrifuged at 2,000 g for 10 min at 4°C. The pellet was homogenised in 4 volumes of a buffer designed to keep nuclei intact (RSB: 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5) and separated by centrifugation into nuclear pellet (**lane 1**) and cytoplasmic supernatant (**lane 2**) fractions. The pellet was then washed by homogenisation with extraction buffer (10 mM Tris-HCl, pH 7.5 + 1% Triton X-100) to remove any unbroken cells and large membrane fragments into the next supernatant (**lane 4**) and leave a purer nuclear pellet (**lane 3**). All supernatants were subjected to ultracentrifugation to remove any microparticles of ribosome size and above (**lanes 5–8**). The blot was developed with mAb MANSIX5A3.

isoforms were found in significant amounts as protein.

Very few antibody studies of Six proteins in general, and Six5 in particular, have been published previously and none in which antibodies against different epitopes on the protein were used to identify it unequivocally, as in the present study. Using a gel retardation assay with an anti-Six5 serum, murine Six5 was detected in nuclear extracts of adult rat kidney, liver and lung but not in the brain. Six5 was also detected in neonatal and embryonic rat lung nuclei and in HeLa, 3T3 mouse fibroblast and C2C12 myoblast cells. By Western blot analysis, this anti-Six5 antibody detected a 71 kDa protein in C2C12 myoblast cell nuclear extract [Ohto et al., 1998] and at this time an incomplete murine sequence suggested an M_r of 69 kDa. It is now known, however, that murine Six5 is only slightly shorter (719 amino-acids; 83 kDa) than the human protein. Human SIX5 has 739 amino-acids with a predicted molecular size of 85 kDa, but proteins can migrate with anomalously high apparent M_r on the SDS–PAGE system commonly used [Laemmli, 1970]. DMPK, for example, has a predicted M_r of 69 kDa but migrates at 80–85 kDa [Lam et al., 2000]. Spitz et al. [1998] reported an apparent M_r of 98 kDa for mouse Six4 protein which has a predicted M_r of 89 kDa, so there is a precedent

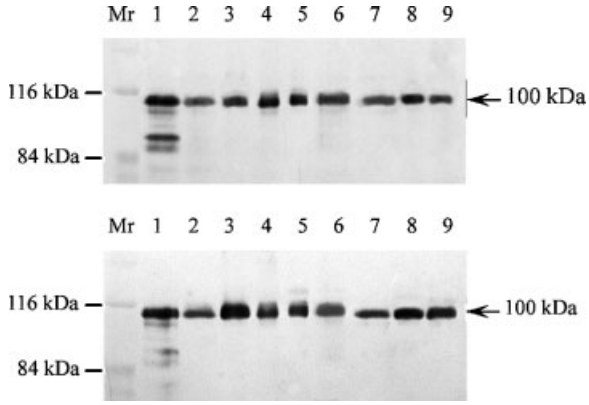

Fig. 7. Western blot analysis of SIX5 in human foetal tissues using (**A**) MANSIX5A3 mAb, (**B**) MANSIX5B1. The lanes were loaded as follows: (M_r) Sigma protein marker, (1) foetal lung, (2) foetal liver, (3) foetal kidney, (4) foetal stomach, (5) foetal eye, (6) foetal skin, (7) foetal brain, (8) foetal limb, (9) HeLa cell extract. The extracts were subjected to 7% polyacrylamide SDS–PAGE. Position of the 100 kDa protein is shown. Horseradish peroxidase-conjugated anti-(mouse Ig) and a chemiluminescent substrate were used.

for Six4/5 proteins migrating more slowly than predicted.

Published studies of SIX5 mRNA are broadly consistent with the uniform distribution of SIX5 protein among different tissues observed in our present antibody study of the Six5 protein. Human SIX5 mRNA was detected in a number of human tissues and cell lines, including skeletal muscle, heart, brain, fibroblasts and lymphocytes by RT-PCR [Boucher et al., 1995]. Murine Six5 mRNA was detected in a wide range of adult tissues including skeletal muscle, heart, testes, brain, smooth muscle, thymus, kidney and liver by RT-PCR. Similarly, expression was detected in heads, limbs, liver, kidney and heart samples from embryonic day 12.5 [Heath et al., 1997]. By Northern blotting, abundant expression of Six5 mRNA was observed in neonatal mouse heart and skeletal muscle and it was detected as early as embryonic day 7. In adult tissues, Six5 mRNA was expressed strongly in the heart, moderately in the lung, kidney and liver and weakly in skeletal muscle, spleen and testis [Murakami et al., 1998]. A quantitative PCR study also found a decrease in Six5 mRNA between neonate and adult in mouse skeletal muscle [Eriksson et al., 2000]. In the eye, Six5 mRNA was observed in the inner and outer nuclear layers, the ganglion cell layer and the pigment epithelium layer of mouse retina by in situ hybridisation [Kawakami et al., 1996b]. Human SIX5 mRNAs were also found in normal human adult corneal epithelium and endothelium, lens epithelium, ciliary body epithelia, cellular layers of the retina and the sclera by RT-PCR and in situ hybridisation [Winchester et al., 1999]. They were also present in normal epithelium throughout most of the female reproductive tract.

The Six family of transcription factors was originally associated with development of the eye [Kawakami et al., 1996a,b], but it is now clear that interactions of Six proteins with transcription factors related to eyes absent (Eya1) and dachshund (Dach) are involved in the development of many tissues. The Six4/5 subgroup has a significant role in muscle development, Six4 being involved in skeletal muscle development, while Six5 may be important for gene expression in the heart [Himeda et al., 2004]. High levels of Six5 mRNA expression have indeed been found in neonatal and adult mouse heart by Northern blotting [Murakami et al., 1998]. We found high levels of SIX5 protein in all foetal human tissues (Fig. 7), but were not able to study neonatal tissues or adult heart. Interaction of Six4/5 with Eya1 involves their N-terminal regions, since the interaction is also observed with Six1/2. However, our demonstration that the full-length isoform of SIX5 is by far the dominant, if not the only, form in foetal tissues reinforces the likely additional importance of exon C and its transactivation domain for specific SIX5 functions in development.

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