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The promoter of the *Xwnt*-5C gene contains octamer and AP-2 motifs functional in *Xenopus* embryos

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ABSTRACT

The *Xwnt*-5C gene is expressed in *Xenopus* embryos from the early gastrula stage onwards. The transcription of *Xwnt*-5C is regulated differentially with respect to transcript size, timing and localization. To gain insight into the generation of the *Xwnt*-5C expression pattern, we started to analyze the transcriptional regulation of this gene. We isolated *Xwnt*-5C genomic DNA sequences. By microinjection of chimaeric reporter constructs into *Xenopus* embryos we demonstrate that the upstream region contains a promoter functional *in vivo*. Of the several putative binding sites for *trans*-acting factors, present in a minimal promoter fragment, some have been studied in more detail. Mutations in an octamer motif and in an AP-2 consensus sequence interfere with the activity of the *Xwnt*-5C minimal promoter. *In vitro* binding assays with extracts from gastrula stage *Xenopus* embryos show that the octamer motif of the *Xwnt*-5C promoter can bind several Octamer binding factors, one of which is Oct1.

INTRODUCTION

Wnt proteins mediate intercellular signaling and they form a family of secreted factors found throughout the animal kingdom in all species analyzed (1, 2). In *Xenopus* at least fourteen different nucleotide sequences, representing either complete or partial *Wnt* coding regions have been identified, each having a specific expression pattern (3, 4, 5, 6). So far, the knowledge about the molecular mechanisms that establish the expression patterns of *Wnt* genes has remained limited. It is not known, for instance, which developmental regulators like peptide growth factors or transcription factors are involved in the regulation of *Wnt* gene expression. Promoter studies have thus far only been carried out for *Wnt*-1 genes. The mouse *Wnt*-1 promoter has been analyzed in stably transfected P19EC cells, which express the gene after neural differentiation by retinoic acid (7, 8). The transcription of *Wnt*-1 in these cells is regulated by a transcription factor called WiF, which binds to a GC-rich sequence located in the upstream region and is indispensable for activation by RA (9, 10). In *Xenopus*, the transcriptional regulation of *Xwnt*-1 in the context of the embryo is mediated by a sequence motif resembling the GT-I and GT-II protein binding elements of the SV40 enhancer (11). Upstream sequences of *Wnt*-1 genes of zebrafish (12) and man (13) have been described, but functional DNA elements or regulating transcription factors have yet to be determined for these species.

The expression profile during development suggests that *Xwnt*-5C may play a role in the establishment of the anteroposterior axis of the embryo (6) Here, we describe the initial analysis of the transcriptional regulation of the *Xwnt*-5C gene. To that aim we isolated *Xwnt*-5C genomic DNA. We defined a minimal promoter region by means of microinjecting chimaeric promoter–reporter constructs into *Xenopus* embryos. By mutational analysis we determined that an octamer motif as well as an AP-2 motif contribute to the activity of the *Xwnt*-5C promoter. Gel mobility shift assays confirmed that Octamer binding factors, including Oct1, indeed can bind to the proximal promoter region.

MATERIALS AND METHODS

Library screening, hybridization and sequencing

From a tadpole stage *Xenopus laevis* genomic library in EMBL-3, which was a gift of Dr L.DuPasquier, 900.000 plaques were screened with the 0.9kb *Eco*RI/*Bam*HI fragment of the *Xwnt*-5C cDNA (6), according to standard procedures (14). Positive phages were selected and rescreened resulting in the isolation of two single positive recombinants, one of which was further analyzed by restriction mapping. The total insert length of this phage appeared to be 18.4kb, of which one *Bam*HI fragment of 4.2kb hybridized with the 170bp *Eco*RI/*Sst*I fragment located in the 5′ region of the *Xwnt*-5C cDNA. Probes were labeled with α^{32}P-dATP (NEN) using a random priming labeling kit (Amersham). The 4.2kb *Bam*HI fragment was cloned into the *Bam*HI site of pGEM3Zf(−) (Promega) to generate pGEMB4.2 and subclones of this fragment in pGEM3Zf(−) (not shown) were constructed for sequencing. Sequencing was carried out either by using α^{32}P-dCTP and a T7 DNA polymerase based kit

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(Promega) or by employing an ABI 373A automated DNA sequencer (Applied Biosystems). Primers were the T7 or SP6 promoter primers, or the *Xwnt*-5C specific primers 5C0 and 5C1 (see Table 1). All nucleotide sequences were read at least twice. The sequence was submitted to the EMBL nucleotide sequence database and is available under accession number X76190.

Construction of chimaeric promoter reporter gene vectors

First, a high copy number version of p19LUC (15) was constructed to allow high yields of plasmid DNA. The entire functional reporter unit from p19LUC was cut out with *Nde*I (position 2062) and *Apa*I (position 5692) and ligated into the *Nde*I and *Apa*I sites of pGEM5Zf(−) (Promega) to generate a new luciferase reporter gene vector, called pGeluk. The amounts of luciferase produced by this vector were similar to those of p19LUC, as was tested by microinjection and transient transfection experiments, in which the same or no promoter inserts were used in both vectors (data not shown). All promoter reporter constructs were generated with the same strategy as described before (11), except that the constructs described here, were derivatives of pGeluk. A 2.2kb fragment was isolated from the *Xwnt*-5C genomic subclone pGEMB4.2 by digestion with *Sst*II, after which the sticky ends were repaired by T4 DNA polymerase, and subsequent digestion with *Hin*dIII, which is in the polylinker region of pGEMB4.2. This fragment was ligated into *Hin*dIII/*Hin*cII-digested pBluescriptSK(−) (Stratagene) to generate pSKBS. From this plasmid the 2.2kb fragment was cut out with *Hin*dIII and *Xho*I and ligated into the *Hin*dIII and *Sal*I sites of pGeluk, this construct was called pBSgeluk. The 0.1kb *Sma*I/*Xho*I fragment was cut out from pSKBS and ligated into *Hin*cII/*Xho*I-digested pBluescriptSK(−) to generate pSKSS. The 0.1kb *Hin*dIII/*Kpn*I fragment was cut out and subsequently ligated into *Hin*dIII/*Kpn*I-digested pGeluk to generate pSSgeluk. pSKBS was cut with *Ava*II, after which the sticky ends were filled in with Klenow enzyme, and subsequently with *Xho*I. pSKBS was cut with *Alw*NI, after which the sticky ends were repaired by T4 DNA polymerase, and with *Xho*I. The 0.3kb *Ava*II(blunt)/*Xho*I fragment and the 0.2kb *Alw*NI(blunt)/*Xho*I fragment each were ligated into *Hin*cII/*Xho*I-digested pBluescriptSK(−) to generate pSKAS and pSKAlS respectively. From these plasmids, fragments were cut out with *Hin*dIII and *Kpn*I and ligated into *Hin*dIII/*Kpn*I-digested pGeluk to generate pASgeluk and pAlSgeluk respectively. pmutAP-2geluk and pmutOCTgeluk were constructed by isolation of the 0.2kb *Hin*dIII/*Kpn*I fragments from pSELmutAP-2 and pSELmutOCT (see below), respectively, and subsequent ligation of these fragments into *Hin*dIII/*Kpn*I-digested pGeluk. All the reporter constructs were checked by sequencing either the whole insert or the junctions between the insert and the vector.

Site-directed mutagenesis

Site-directed mutagenesis was carried out employing the Altered Sites kit (Promega) according to the manufacturer's protocol. In short, the *Alw*NI/*Sst*II fragment was cut as a *Hin*dIII/*Kpn*I fragment from pSKAlS and ligated into the cognate sites of pSelect to yield pSelAlS. Single-stranded DNA was isolated using R408 as a helper phage. Two mutagenesis reactions were carried out using the lower strands of either the mutAP-2 or the mutOCT oligonucleotides (see Table 1) together with the ampicillin resistance repair oligonucleotide. After two rounds of transformation and plasmid DNA isolation the mutant plasmids pSELmutAP-2 and pSELmutOCT were selected by restriction enzyme digestion of sites, which had been introduced into the mutant plasmids. The mutations were checked by sequencing.

Embryos, microinjection and luciferase assays

Xenopus embryos were obtained and cultured as described previously (16). Embryonic stages were determined according to (17). Plasmid DNA for microinjection was purified by centrifugation through a CsCl gradient and concentrations were determined by spectrophotometry at 260 nm before and after dilution to the final concentration for microinjection, i.e. 10 ng/μl. Physical forms of plasmid DNA were determined by electrophoresis of aliquots of DNA in agarose gels containing ethidiumbromide. 100 pg covalently closed circular DNA was microinjected into a fertilized egg as described previously (11). Luciferase measurements of one embryo equivalent were carried out as before (11).

Oligonucleotides

Oligo-deoxyribonucleotides (Table 1) were synthesized on a Cyclone Plus DNA Synthesizer (Milligen/Biosearch). 2 to 5 μg single stranded oligonucleotide was annealed to its complementary counterpart in 50 μl buffer containing 50 mM Tris−HCl, pH 8.0, 50 mM NaCl, by heating to 85°C for 5 minutes, after which the mixture was allowed to cool down to room temperature over a time period of at least one hour.

Gel mobility shift assays

The *Alw*NI/*Sma*I fragment (positions −224 and −119 respectively) was used as a probe for gel shift assays. The *Alw*NI/*Sma*I fragment was cut out from pSKAlS with *Eco*RI and *Sma*I and purified by agarose gel electrophoresis. The fragment was labeled by filling in 5′ sticky ends with Klenow enzyme in the presence of α^{32}P-dATP. Whole cell extracts from whole *Xenopus* embryos were prepared by homogenizing 10 to 50 embryos in 3 μl/embryo ice cold WCE buffer (20 mM HEPES [pH 7.9], 400 mM KCl, 1 mM EDTA, 10% glycerol, 10 mM dithiotreitol, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Homogenates were centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatant was 10 times diluted in ice cold WCE buffer. Only freshly prepared embryo extracts were used for gel shift reactions. Oligonucleotides used as competitor are indicated in Table 1. 12F11, a monoclonal antibody raised against the C-terminus of the *Xenopus* Oct1 protein was used to identify Oct1 in gel mobility shift assays. The generation and characterization of this Oct1 specific antibody will be published elsewhere (18). In binding reactions, 4 μl of this antibody were first mixed with 3 μl of diluted extract and incubated for 1 hour on ice, after which 13 μl of a mixture containing the probe (see below) was added. In all other cases 3 μl of diluted extract was mixed simultaneously with 4 μl competitor DNA (or deionized water) and 13 μl of a mixture containing 0.1 ng labeled probe (approximately 5000 cpm), 4 μg poly(dI-dC) and 5 μl 4×retardation buffer (1×retardation buffer is 25 mM Tris−HCl [pH 8.0], 50 mM NaCl, 8 mM $MgCl_2$, 1,25 mM EDTA, 5% Ficoll, 50 μg/ml BSA, 1.25 mM dithiotreitol, 0.031% Nonidet P40). The reactions were allowed to continue for 15 minutes on ice. After adding 4 μl of 10% glycerol, containing bromophenolblue, the mixture was loaded onto a 5% non-denaturing polyacrylamide (39:1) gel in TGE buffer (25 mM Tris base, 190 mM glycine, 2 mM EDTA, pH 8.3). Gels were run for 4 to 6 hours at 10 V/cm at 4°C, dried under vacuum and subjected to autoradiography.

RNA isolation and RNase mapping

RNA isolation has been described previously (3). RNase protection assays were performed as described before by Melton *et al.* (36). A subclone containing the *AvaII/SstII* fragment (Figure 1) was used to generate template for riboprobe synthesis. Antisense RNA probes were made with the Promega Riboprobe kit using 1.0 μg of template. Reaction mixtures were treated with DNase I and probes were purified on 5% polyacrylamide gel obtaining 8M urea. Ten micrograms of total RNA were hybridized at 60°C for 16 hours with 1.5×10^5 cpm probe in hybridization buffer (40mM piperazine-N, N′ bis [2-ethanesulfonic acid], pH 6.4, 1mM EDTA, 400mM NaCl and 80% formamide). After hybridization, the mixture was incubated with 1μg/ml RNase T1 (Pharmacia) and 40μg/ml RNase A (GibcoBRL) at 37°C for 30 minutes in digestion buffer (300mM NaCl, 10mM Tris−HCl pH 7.4, 5mM EDTA). Protected fragments were purified by phenol/chloroform extraction and copricipitated with 10μg yeast tRNA in ethanol, prior to separation in 5% polycrylamide gel containing 8M urea. DNA sequencing ladders of pGEM 3 Zf(⁻) sequenced with T7 primer were used as size markers. Gels were dried under vacuum and protected fragments were visualized by scanning on a PhosphorImager (Molecular Dynamics).

RESULTS

Isolation of *Xwnt*-5C promoter sequences and transcriptional start-site mapping

The position of transcriptional initiation was determined by RNase protection assay. Two protected fragments were detected using total RNA from stage 14 embryos. A start site was determined corresponding to a position of 164 bp upstream of the presumptive initiation codon, another start-site was determined at a position of 74 bp upstream (see Figure 2, starts 1 and 2). The same start-sites were found using RNA from heart and lung, tissues expressing Xwnt-5C in the adult.

We screened a *Xenopus* genomic library with *Xwnt*-5C cDNA probes (6) to isolate promoter sequences of the *Xwnt*-5C gene. After three rounds of plating and hybridization we obtained two positive recombinant phages, one of which was used for further analysis. This phage contained an insert of approximately 18.4kb as appeared from restriction fragment mapping (not shown). A *Bam*HI fragment of 4.2kb hybridizing to *Xwnt*-5C cDNA probes containing sequences comprising the putative first exon of the gene was subcloned and used for further analysis (Figure 1). Sequencing revealed a stretch of complete identity between this fragment and the most 5′ part of the *Xwnt*-5C cDNA, showing that it indeed contains the first exon of the *Xwnt*-5C gene and upstream DNA sequences. A relevant part of the sequence is shown in Figure 2. Analysis of the deduced amino acid sequence of the first exon revealed that the intron−exon boundary is conserved at the same amino acid residue (position 83 in figure 2) compared to *Wnt*-1 genes (12, 13, 19). In the proximal upstream DNA region we found putative binding sites for the *trans*-acting factors CREB (−284), AP-2 (−187), Octamer binding factors (−173) and HNF1 (−83) (20). No typical TATA box is present in the upstream DNA region.

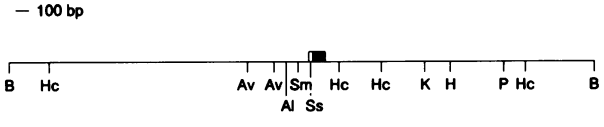

Figure 1. Restriction map of the 4.2kb *Bam*HI fragment of the *Xwnt*-5C genomic clone. The putative first exon of the *Xwnt*-5C gene, as it was deduced from comparison of the cDNA with the genomic DNA sequence, is depicted by a box, of which the striped part represents coding DNA. Restriction site abbreviations are: Al: *Alw*NI; Av: *Ava*II; B: *Bam*HI; H: *Hin*dIII; Hc: *Hin*cII; K: *Kpn*I; P: *Pst*I; Sm: *Sma*I and Ss: *Sst*II.

Xwnt-5C upstream sequences promote expression of a reporter gene

To determine whether the *Xwnt*-5C upstream DNA exhibits promoter activity, a *Bam*HI/*Sst*II fragment of 2.2kb was fused to a reporter gene encoding luciferase (see 'Materials and methods'). This construct, pBSgeluk, was microinjected into *Xenopus* fertilized eggs and the amount of luciferase was measured in embryos which were cultured to stage 20. The upstream fragment indeed promotes luciferase synthesis very efficiently (Figure 3A), whereas the control luciferase reporter vector pGeluk only produced residual levels of luciferase (not shown). For comparison, a Xwnt-1 promoter construct at similar conditions is much less active, whereas a histone H1c promoter construct shows mich higher activity (11). We deleted the 2.2kb promoter fragment from the 5′ end and the shorter constructs were tested in experiments as described above. Both pASgeluk, containing the 0.3kb *Ava*II/*Sst*II fragment, and pAlSgeluk, containing the 0.2kb *Alw*NI/*Sst*II fragment, retained the ability to promote luciferase synthesis to levels comparable to that of pBSgeluk or higher (Figure 3A). However, pSSgeluk, containing only the 0.1kb *Sma*I/*Sst*II fragment, produced very low levels of luciferase, approximately 4% of that of pBSgeluk (Figure 3A). From these findings, we conclude that a fragment of 0.2kb of the *Xwnt*-5C gene constitutes a minimal promoter, sufficient for

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                                                    AvaII
-500   GTGAGTTTGT GGCATGGGAA AGACAGGGCA AAGGTGAGGA CCTGGTATAA

-450   CCCTCTAGTT ACCCCTTTTG CTAATATAGG TGCTGCTGCT CTATTTGCAC

-400   AGAGGGATAT TGTTTATTAT TTCCCATCTT TCTAACTACA CGTGGGGGGC

                                          AvaII
-350   TCCTTGTCCA CCTGTCTGTC TCTGTGGTCC GCCGACCCCC CTAGTTGCAG

                        CREB
-300   AGACGTGGGA AGAGAGACGT CAGAGTTCAA GAGCCCCTTT GGCAAATCTC

                                    AlwNI
-250   ATGTCTCCAA ACATCACCAA ATAATACAGT TGCTGCTGCC CCTGAACAAG

                  AP-2                OCT    >1
-200   CTCACACTCA CTTCCCCAGG CAATAAAATG CAAATTAGCG CAAGCACAAG

                                          SmaI
-150   GGGGGTTGAT GGGCAGAGAG TAACCTCCTC CCCCGGGATT CCGAGCCCCG

                                HNF1 >2
-100   CACACACTGC CAGGCGACTG CTTCTCATTG CGCTGCGACT CACCGTCTGT

                                                    SstII
 - 50  CTGTCTCACA GGGGCCCCAG TGCTGCCGGA GATCCGCGGA GAGGAGCAAC
                          *

   1   ATGACTCCAA TTCTGCGACT TCTCTTGCTC AGTTCTCTGC TGTGCTGTTG
       M  T  P  I   L  R  L   L  L  L   S  S  L  L   S  C  W

  51   GAAACAGTCT GTAGTTGGGG CAAACTCCTG GTGCTATAGT AGTGCAAGTT
        K  Q  S   V  V  G  A   N  S  W   W
```

Figure 2. Nucleotide sequence of *Xwnt*-5C genomic DNA containing the minimal promoter. Restriction sites are indicated over the sequence, amino acid residues are indicated below the sequence. The numbers at the left are relative to the A of the first codon. The asterisk indicates the first nucleotide of the region of complete identity with the *Xwnt*-5C cDNA. Two transcription start-sites are indicated as >1 and >2. Putative binding sites for *trans*-acting factors are indicated underlined.

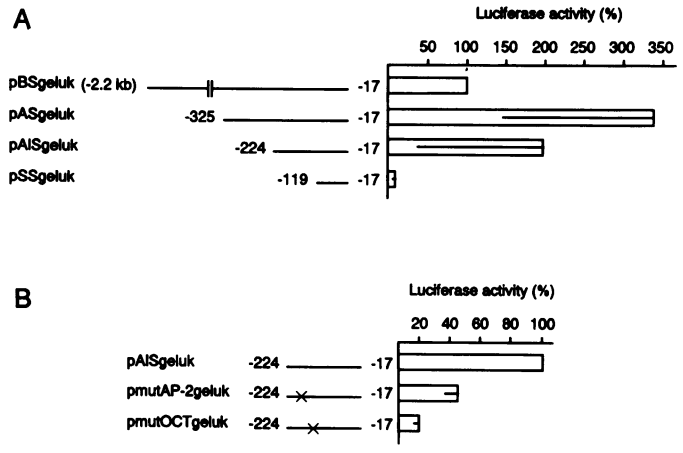

Figure 3. Relative activities of *Xwnt*-5C reporter gene constructs in *Xenopus* embryos. Genomic *Xwnt*-5C DNA fragments (compare with Figures 1 and 2) were fused to the luciferase reporter gene (see 'Materials and methods'). The constructs were injected into fertilized eggs and the amounts of luciferase were determined at neurula stages, by measuring the activity of one embryo equivalent of a homogenate from 5 to 10 embryos. The numbers indicate the coordinates of the cloned fragments relative to the ATG, except for the 5' end of the insert of pBSgeluk, which was not determined by sequencing, but was estimated from gel mobility. The relative luciferase amounts were determined by calculating the mean of the activities of several numbers of injected embryos (n) in several luciferase measurements (N). The error bar represents the standard error of the mean. **A.** Reporter constructs with different lengths of upstream DNA. For pBSgeluk: n = 54, N = 7; for pASgeluk: n = 15, N = 2; for pAlSgeluk: n = 12, N = 2; for pSSgeluk: n = 56, N = 7. Data collected from a single batch of embryos were calculated relative to the activity of pBSgeluk which was included in all experiments and its activity was set to 100% (absolute activity: 7.000–12.000 light units). **B.** Comparison of mutant and wild type reporter constructs. For pAlSgeluk: n = 106, N = 12; pmutAP-2geluk: n = 87, N = 11; for pmutOC-Tgeluk: n = 80, N = 11. pAlSgeluk was included in all experiments and its activity was set to 100%. Data collected in a single batch of embryos were calculated relative to the activity of this construct.

Table 1. Oligonucleotides

5C0	5'-GCAGAATTGGCGTCATG-3' (+16 - -4)
5C1	5'-GAGACAGACAGGTGGAC-3' (-329 - -345)
mutAP-2	5'-AAG-TCACACTCACTTggatccGCAATAAAATGCAAATT-3' 3'-TTCGAGTGTGAGTGAAcctaggCGTTATTTTACGTTTAAT-5'
mutOCT	5'-CCCCAGGCAATAAAATctAgATTAGCGCAAGCAC-3' 3'-GGGGTCCGTTATTTTAgaTcTAATCGCGTTCGTGTT-5'
AP-2	5'-GATCAAAGTCCCCAGGCTCCCCAGCAGGCA -3' 3'- TTTCAGGGGTCCGAGGGGTCGTCCGTCTAG-5'
OCT	5'-GATCAATGGATGCAAATGATGGT -3' 3'- TTACCTACGTTTACTACCACCCTAG-5'
Rb	5'- CCTCTTATCAATTGGGATCTAGCAG-3' 3'- GGAGAATAGTTAACCCTAGATCGTC-5'
BR3	5'- TCTGATGGAAGGACAGCTC -3' 3'- ACTACCTTCCTGTCGAGGA-5'

transcriptional activity in *Xenopus* embryos. The DNA elements of the *Xwnt*-5C promoter, mediating this expression, are located between −119 and −224 relative to the translation start site.

An octamer and an AP-2 motif contribute to *Xwnt*-5C promoter activity

The nucleotide sequence of the upstream region of the *Xwnt*-5C promoter revealed that several known protein binding elements are present in the proximal promoter. Two of these elements,

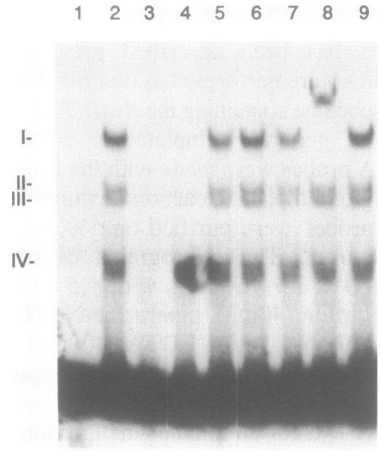

Figure 4. Gel mobility shift assay of a *Xwnt*-5C promoter fragment with *Xenopus* embryo extracts. The *Alw*NI/*Sma*I fragment (see Figures 1 and 2) was labeled as described in 'Materials and methods'. One tenth embryo equivalent of whole cell extract of stage 12 embryos was used per binding reaction. Competitor DNA was added in a 100 fold molar excess. Lanes: **1**: free probe, **2**: stage 12 extract, **3**–**9**: as **2** with: **3**: *Alw*NI/*Sma*I fragment, **4**: OCT oligonucleotide, **5**: mutOCT oligonucleotide, **6**: aspecific Rb oligonucleotide, **7**: aspecific BR3 oligonucleotide, **8**: 12F11, anti-Oct1 monoclonal antibody, **9**: culture medium without monoclonal antibody.

a consensus AP-2 motif (21) and an octamer (22) are notably present between the *Alw*NI and *Sma*I sites, enclosing the fragment that is necessary for full expression of the luciferase reporter gene (Figure 2). We considered the possibility that either one or both elements in the *Xwnt*-5C promoter might be functional. To test this, we generated constructs harboring the 0.2kb *Alw*NI/*Sst*II fragment with mutations in either the AP-2 (pmutAP-2geluk) or in the octamer motif (pmutOCTgeluk) (see Table 1). These constructs were microinjected into *Xenopus* zygotes and the luciferase activities were measured at stage 20. The luciferase activity of pmutAP-2geluk was approximately 40% of that of the wild type construct, pAlSgeluk, while the luciferase activity of pmutOCTgeluk was only 20% of the activity of pAlSgeluk (Figure 3B). From these results we conclude that the AP-2 and the octamer motifs of the *Xwnt*-5C minimal promoter both contribute to the transcriptional regulation of the luciferase reporter gene *in vivo*.

Multiple proteins from *Xenopus* extracts bind to the *Xwnt*-5C promoter *in vitro*

To determine which regulatory proteins may bind to the *Xwnt*-5C promoter, we performed gel mobility shift assays. The 0.1kb *Alw*NI/*Sma*I fragment, which is essential for full promoter activity in reporter gene constructs, was radiolabeled and used as a probe. As a protein source we used total cell extracts of stage 12 *Xenopus* embryos. Stage 12 is within the time interval, in which the *Xwnt*-5C transcripts accumulate during development (6) and thus we may expect essential activating factors to be present at this stage. Figure 4 (lane 2) shows that at least four retarded complexes are formed. When a hundred fold molar excess of the same unlabeled DNA fragment is added, all bands are competed for (Figure 4, lane 3), showing that these retarded bands are not due to aspecific binding. Analysis of different stages shows that octamer binding activities for the AlwNI/SmaI fragment change during early development (results not shown),

complex I being present from stage 7 onwards, all in accordance with observations by Hinkley *et al.* (37).

The octamer motif of the *Xwnt*-5C promoter binds multiple proteins *in vitro*

Next, we tested whether the retarded complexes could be attributed to the binding of regulatory factors to the AP-2 or the octamer motif. These factors should be competed for by double stranded oligonucleotides containing these motifs. So far, experiments, performed with respect to the binding of factors to the AP-2 element were not conclusive. When a hundred to thousand fold molar excess of double stranded AP-2 oligonucleotides (see Table 1) was included in the reaction mixture of the labeled 0.1kb *Alw*NI/*Sma*I fragment and total extract of stage 12 embryos, no competition was observed of any of the retarded complexes. Initial experiments with the aim to supershift a putative AP-2 containing complex using an antibody specific for human AP-2 were also not successful (data not shown, see Discussion). Conversely, adding a hundred fold molar excess of a double stranded oligonucleotide containing the octamer motif (OCT: see Table 1) to the reaction mixture of the labeled 0.1kb *Alw*NI/*Sma*I fragment and total extract of stage 12 embryos, resulted in a decrease in intensity of the upper three bands (Figure 4, lane 4: complexes I–III). Two aspecific competitors (see Table 1) were also used in the same molar excess and showed no significant competition (Figure 4, lanes 6 and 7). We made mutations in the octamer motif and checked whether the ability of this mutant element to bind Oct factors was abolished. To that aim, the double stranded oligonucleotide mutOCT (see Table 1) was used as a competitor in gel mobility shift assays. While the OCT oligonucleotide could compete for three Oct complexes efficiently (Figure 4, lane 4), the mutOCT oligo was not able to compete for any of these complexes (Figure 4, lane 5). These results suggest that Octamer binding factors are able to bind to the *Xwnt*-5C promoter. This finding was further substantiated by experiments in which was determined, that specifically *Xenopus* Oct1 can bind to the octamer element as it is present in the *Xwnt*-5C promoter. A monoclonal antibody, raised against the C-terminus of the *Xenopus* Oct1 protein (18) was added to the reaction mixture after which the mobility of complex I was further retarded due to the binding of the antibody to the protein–DNA complex (Figure 4, lane 8). When, as a control, hybridoma culture medium without antibody was added, no supershift was observed (Figure 4, lane 9). These results show that at least Oct1 and, most likely, two other Octamer binding factors, present in stage 12 embryonic extracts, can bind to the *Xwnt*-5C promoter. Furthermore, the reduced activity in embryos of the *Xwnt*-5C promoter construct, containing the mutant octamer is most likely due to the inability of the mutated octamer to bind regulatory factors.

DISCUSSION

To investigate the transcriptional regulation of *Xwnt*-5C we have isolated upstream genomic sequences and tested their promoter activity in *Xenopus* embryos as well as the ability to bind putative regulatory factors *in vitro*. Two transcriptional initiation sites determined for the endogenous Xwnt-5C gene are present in a region representing the minimal promoter. The minimal promoter contains consensus elements for AP-2 and Octamer binding factors, both of which contribute to the expression of a luciferase reporter gene in embryos.

When the sequence of the upstream DNA of *Xwnt*-5C is compared with the upstream sequences of *Wnt*-1 genes from other species (10, 12, 13) or from *Xenopus* (11), no regions of obvious sequence similarity are observed. This finding may be consistent with the different patterns of expression found for *Xwnt*-1, which is transiently expressed during neurula and tailbud stages in a highly restricted spatial pattern in the central nervous system (3, 23, 24, 25), and *Xwnt*-5C, which has a complex expression pattern from the early gastrula stage onwards (6). Thus, although the proteins encoded by these genes are homologous, the mechanisms for their transcriptional activation differ.

The activity data of serially 5′ deleted *Xwnt*-5C promoter constructs in embryos (Figure 3A), show that the promoter region located between positions −119 and −224 contains determinants important for the activation of transcription. We have shown that both the octamer and the AP-2 motifs contribute to the activity of the *Xwnt*-5C promoter within the embryo. The gel mobility assays provide evidence that octamer binding factors indeed can bind to the minimal promoter. Octamer binding factors are members of the POU family of transcription factors, which mediate processes important for mammalian embryonic development (22, 26, 27, 28) and development of the brain (29). POU factors have also been identified in *Xenopus* (see 22 and references therein) and in addition, a functional octamer motif is required for the transcriptional regulation of the histone H2B gene during early development (30). Binding of Oct1 and two other octamer binding proteins (complexes I–III, Figure 4) to the *Xwnt*-5C minimal promoter suggests that these octamer binding factors are involved in the transcriptional regulation of this gene. A transient presence of octamer binding factors as observed here for the Xwnt-5C promoter has been described in detail by Hinkley *et al.* (37). These authors have described a transient expression c.q. presence of multiple POU proteins in gastrula and neurula stages of early *Xenopus* development, which may be expressed in distinct cell types. It will be interesting to determine which of these POU proteins are important for Xwnt-5C regulation.

Whether AP-2 or related factors plays a role in *Xwnt*-5C regulation is less clear. Although the mutation of the AP-2 binding site reduces the activation of the luciferase reporter considerably, we could so far not show that a protein from embryonic extracts binds to this site. AP-2 is a transcription factor that binds to enhancer elements of several genes of mouse and man. A consensus sequence, CCCCAGGC has been determined, although individual binding elements may vary considerably (21, 31, 32). In *Xenopus*, KTF-1/XAP-2 is expressed during the late blastula stage and activates the epidermal keratin gene XK81A1, by binding to the sequence CCCTGAGG (33, 34, 35). Thus, although the element in the *Xwnt*-5C promoter completely matches the AP-2 consensus, it has not been proven that KTF-1/XAP-2 or other regulatory proteins from *Xenopus* can bind to it. The candidate AP-2 containing complex (IV, Figure 4) formed by binding to the *Alw*NI/*Sma*I probe is not competed for by an AP-2 consensus oligonucleotide, nor does it bind a monoclonal antibody, which can bind KTF-1/XAP-2 (35). These findings suggest that the consensus AP-2 site is not recognized by a *Xenopus* AP-2 factor, but possibly by another as yet unknown regulatory factor. Alternatively, binding to this element *in vitro* may require experimental conditions different from those used here.

Summarizing, the *Xwnt*-5C promoter is activated during *Xenopus* embryonic development and contains several elements,

which can be bound by transcription factors. Most likely, Octamer binding factors and possibly AP-2 related factors may play a role in the activation of the *Xwnt*-5C promoter during early development of *Xenopus* embryos.

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