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

Gauko A. Kuiken, Peter J.A. Bertens, Josi Peterson-Maduro, Gert Jan C. Veenstra, Johanna G. Koster and Olivier H.J. Destrée*
Hubrecht Laboratory, Uppsalaalaan 8, NL-3584 CT Utrecht, The Netherlands

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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 EcoRI/BamHI 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 BamHI fragment of 4.2kb hybridized with the 170bp EcoRI/SorI fragment located in the 5' region of the Xwnt-5C cDNA. Probes were labeled with α32P-dATP (NEN) using a random priming labeling kit (Amersham). The 4.2kb BamHI fragment was cloned into the BamHI 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 α32P-dCTP and a T7 DNA polymerase based kit

*To whom correspondence should be addressed
(Promega) or by employing an ABI 373A automated DNA sequencer (Applied Biosystems). Primers were the T7 or SP6 promoter primers, or the XmnI-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 NdeI (position 2062) and Apal (position 5692) and ligated into the NdeI and Apal sites of pGEM5Zf(−) (Promega) to generate a new luciferase reporter gene vector, called pGeluK. These 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 XmnI-5C genomic subclone pGEM5B.2 by digestion with SsII, after which the sticky ends were repaired with T4 DNA polymerase, and subsequent digestion with HindIII, which is in the polylinker region of pGEM5B.2. This fragment was ligated into HindIII/HindII-digested pBlueScriptSF(−) (Stratagene) to generate pSKBS. From this plasmid the 2.2kb fragment was cut out with HindIII and XhoI and ligated into the HindIII and SstII sites of pGeluK, this construct was called pBSgeluk. The 0.1kb SmaI/XhoI fragment was cut out from pSKBS and ligated into HindIII/XhoI-digested pBlueScriptSF(−) to generate pSKSS. The 0.1kb HindIII/KpnI fragment was cut out and subsequently ligated into HindIII/KpnI-digested pGeluK to generate pSSgeluk. pSKBS was cut with AwaII, after which the sticky ends were filled in with Klenow enzyme, and subsequently with XhoI. pSKBS was cut with AwaI, after which the sticky ends were repaired with T4 DNA polymerase, and with XhoI. The 0.3kb AwaII(blunt)/XhoI fragment and the 0.2kb AwaI(blunt)/XhoI fragment each were ligated into HindIII/XhoI-digested pBlueScriptSF(−) to generate pSKAS and pSKAIS respectively. From these plasmids, fragments were cut out with HindIII and KpnI and ligated into HindIII/KpnI-digested pGeluK to generate pASgeluk and pAISgeluk respectively. pmutAP-2geluk and pmutOCTgeluk were constructed by isolation of the 0.2kb HindIII/KpnI fragments from pSELmutAP-2 and pSELmutOCT (see below), respectively, and subsequent ligation of these fragments into HindIII/KpnI-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 performed employing the Altered Sites kit (Promega) according to the manufacturer’s protocol. In short, the AwaI/NatII fragment was cut as a HindIII/KpnI fragment from pSKAIS and ligated into the cognate sites of pSelect to yield pSelAIS. 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 Clonea Plus DNA Synthesizer (Milligen/Biosearch). 2 to 5 μg single strapped 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 AwaI/NatI fragment (positions −224 and −119 respectively) was used as a probe for gel shift assays. The AwaI/NatI fragment was cut out from pSKAIS with EcoRI and Smal and purified by agarose gel electrophoresis. The fragment was labeled by filling in 5’ sticky ends with Klenow enzyme in the presence of α32P-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 dithiothreitol, 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 Octl protein was used to identify Octl in gel mobility shift assays. The generation and characterization of this Octl 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 MgCl2, 125 mM EDTA, 5% Ficoll, 50 μg/ml BSA, 1.25 mM dithiothreitol, 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 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 Ava//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 x 10° cpm probe in hybridization buffer (40mM piparzine-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 copurificpated with 10µg yeast tRNA in ethanol, prior to separation in 5% polyacrylamide 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 Phosphorlmager (Molecular Dynamics).

RESULTS

Isolation of Xwnt-SC 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-SC in the adult.

We screened a Xenopus genomic library with Xwnt-SC cDNA probes (6) to isolate promoter sequences of the Xwnt-SC 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 positive recombinant phages, one of which was used for further analysis. This phage contained an insert of approximately 18.4kb positive recombinant phages, one of which was used for further analysis. This phage contained an insert of approximately 18.4kb positive recombinant phages, one of which was used for further analysis. This phage contained an insert of approximately 18.4kb positive recombinant phages, one of which was used for further analysis.

From these findings, we conclude that a fragment of 0.2kb of the Xwnt-SC gene constitutes a minimal promoter, sufficient for 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.

Xwnt-SC upstream sequences promote expression of a reporter gene

To determine whether the Xwnt-SC upstream DNA exhibits promoter activity, a BamHI/SstII 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 Xwt-1 promoter construct at similar conditions is much less active, whereas a histone H1c promoter construct shows much 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//SstII fragment, and pAlSgeluk, containing the 0.2kb AlwNI/SstII fragment, retained the activity to promote luciferase synthesis to levels comparable to that of pBSgeluk or higher (Figure 3A). However, pSSgeluk, containing only the 0.1kb Smal/SstII fragment, produced very low levels of luciferase, approximately 4% of that of pBSgeluk (Figure 3A).

Figure 1. Restriction map of the 4.2kb BamHI fragment of the Xwnt-SC genomic clone. The presumptive first exon of the Xwnt-SC 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: AlwNI; Av: AvaI; B: BamHI; H: HindIII; Hc: HincII; K: Kpnl; P: PstI; Sm: Smal and Ss: SstII.

Figure 2. Nucleotide sequence of Xwnt-SC 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-SC cDNA. Two transcription start-sites are indicated as >1 and >2. Putative binding sites for trans-acting factors are indicated underlined.
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, a consensus AP-2 motif (21) and an octamer (22) are notably present between the AlwNI and Smal 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 AlwNI/StfII 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, pAILgeluk, while the luciferase activity of pmutOCTgeluk was only 20% of the activity of pAILgeluk (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 AlwNI/Smal 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/Smal fragment change during early development (results not shown),
complex and total extract of stage 12 embryos, contains consensus elements for AP-2 and Octamer binding factors, both of which contribute to the expression of a luciferase region representing the minimal promoter. The minimal promoter activity in Xenopus embryos was determined by experiments in which was used a competitor in gel mobility shift assays. While the OCT oligonucleotide could compete for three Oct 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 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, CCCAGGC 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 CCCCCAGG (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 AlwNI/Smal probe is not competed 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.

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-SC promoter during early development of Xenopus embryos.

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