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Candidate Sertoli cell specific promoter element for a TGFβ family member (Amh) and a 3′ UTR enhancer/repressor for the same gene

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Abstract

Comparison between 400 nt of mouse DNA sequence immediately upstream (5′) of the coding sequence of the Sertoli cell expressed genes, *Amh* and *Tsx*, identified a 33 nt sequence with a significant identity: this was considered to be a candidate Sertoli specific regulatory element (SSRE). Another highly conserved sequence has been identified immediately downstream (3′) of the Amh polyadenylation signal (DSRE). The action and specificity of these two putative regulatory elements, inserted into a reporter vector (pd2EGFP), has been investigated by transient transfection of mouse fibroblast and Sertoli cells. When combined together or alone, with a minimal thymidine kinase (Tk) promoter, SSRE showed a weak incremental action on the expression of the reporter gene, regardless of the cell type: in contrast there was a 2- to 3-fold decrease when DSRE was present. However, in the absence of Tk there was evidence for a strong synergy between SSRE and DSRE, which was significantly greater in the Sertoli as compared with the fibroblast cells. These results support the view that SSRE exhibits a degree of Sertoli specificity and acts synergistically with DSRE in controlling the expression of Amh.

Keywords: Mouse; Fibroblasts; Sertoli cells; AMH promoter elements; Flowcytometry

1. Introduction

The TGFβ (transforming growth factor beta) family member AMH/Amh (Anti-Müllerian hormone) (Josso et al., 1993) plays an important role in the embryogenesis of maleness, at least in mammals and lies closely (developmentally) downstream of *Sry* (Hacker et al., 1995), the key Y-linked male determining gene (Koopman et al., 1990). *Amh* is expressed at very high levels in immature Sertoli cells from 12.5 dpc in the mouse (Münsterberg and Lovell-Badge, 1991; Behringer et al., 1994): the rate of expression decelerates, rapidly at birth, before terminating completely in the male at puberty under the direction of the transcription factor GATA-1 (Beau et al., 2000). In contrast in the female, expression starts at a low level in granulosa cells (same lineage as Sertoli cells) at puberty and continues at this level for life (Jost et al., 1975; Bezard et al., 1987; Münsterberg and Lovell-Badge, 1991; Weenen et al., 2004). High level Amh production is confined to cells of the Sertoli lineage and to a well defined time window.

Due to the presence of a spliceosome protein gene (*Sf3a2*), terminating only 430 bp upstream of the start of translation of Amh (Dresser et al., 1995, 2001), it seems that there is likely to be restricted space for a promoter for Amh, a fact which could be advantageous for purposes of experimentation. Experiments with mice transgenic for the Amh promoter itself and for Sf3a2 intronic DNA suggested that promoter activity is confined to this 430 nt region (Beau et al., 2001). Another possibility, that there are relevant elements lying downstream (3′) of the coding region of Amh, has been investigated and the results of some preliminary experiments are reported here.

Several short sequences (elements) in the promoter region of Amh which are recognized by specific transcription factors, have already been reported (Oreal et al., 2002; Rey et al., 2003). For example, these are as illustrated in Fig. 1, Sox8/9, SF1,

Abbreviations: AMH, anti-Müllerian hormone; d2EGFP, two hour half-life green fluorescent protein reporter; SSRE, potential Sertoli specific response element; DSRE, potential 3′ UTR (downstream) response element; 3T3, mouse fibroblast cell line; TM4, mouse Sertoli cell derived line.

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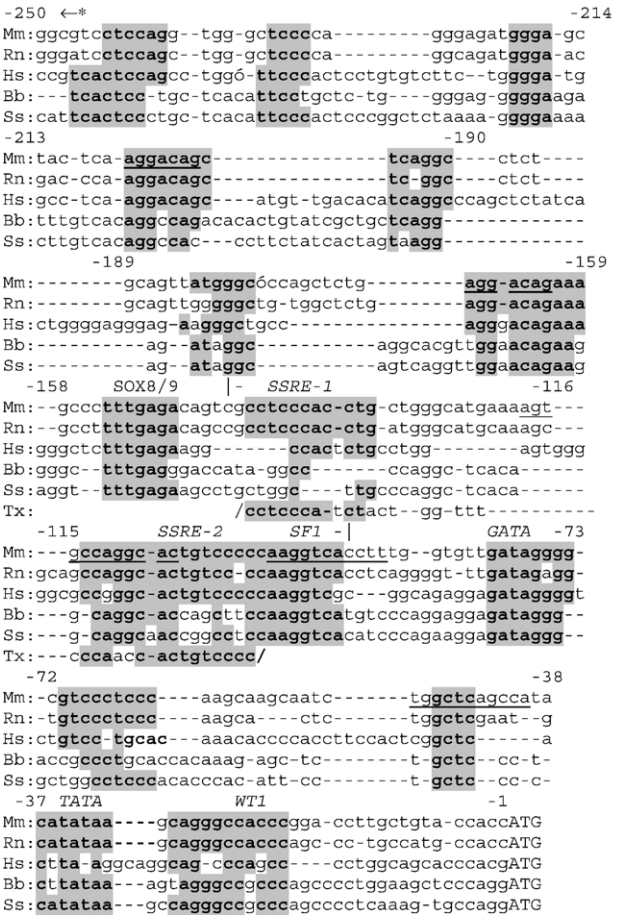

Fig. 1. Comparison of the promoter region of Amh in the genome of five mammalian species. Areas of significant similarity are highlighted. ←* Potential GATA site at −283; PA (ATTAAA) of Sf3a2 at −338; potential stem-loop at −225 to −255 in Mm. Numbering refers to mouse (Mm) sequence, with which the other species are compared. Gaps have been inserted to maximize similarity. Rn is rat; Hs is human; Bb is bovine; Ss is porcine; Tx is a testis specific antigen (Tsx) coded on the X chromosome (Simmler et al., 1996); this elements corresponds to SSRE ("Sertoli-1" plus "Sertoli-2") in the mouse sequence. Estrogen receptor element-like (ERE) palindromic sequences and half-elements, in the mouse sequence are underlined. Other elements include Sox8/9 (Koopman, 1999); GATA; TATA and WT1 (de Santa Barbara et al., 2000; Hossain and Saunders, 2003).

GATA, TATA and WT, which incidentally are in the same order in all 5 species for which currently there is information: this supports the generally accepted view that a promoter acts by ordering the assembly of transcripto–spliceosome components for optimum transcription, and fine tuning, as well as being involved in the initiation and basic mechanism of the process (Ohler and Niemann, 2001), although more complex situations can be envisaged, such as direct chromosomal interactions (Kioussis, 2005). However none of these elements have been shown to have a role in localizing expression to cells of the Sertoli lineage. It is therefore of some interest that a potential Sertoli element has been identified on the basis of sequence identity with an element in the 5′ UTR of Tsx, an X-linked testis specific protein secreted by Sertoli cells (Simmler et al., 1996).

In vitro experiments have been carried out using a promotorless green fluorescent protein vector (pd2EGFP) into which potentially Sertoli specific regulatory elements have been inserted in the MCS 5′ of the reporter gene. Elements 3′ of the Amh coding region have been inserted 3′ of the EGFP reporter. These elements have also been added to vectors carrying a minimal thymidine kinase (Tk) promoter to test the possibility that expression due to Tk can be modified. The efficacy of these constructs has been tested by transient transgenesis in tissue culture lines of mouse fibroblast cells and (mature) mouse Sertoli cells. These experiments have provided circumstantial evidence for a degree of Sertoli specificity.

2. Materials and methods

2.1. Preparation of constructs

Originally DNA constructs were prepared using pEGFP-1 (Clontech), however when it was subsequently found necessary to insert DNA elements at a position 3′ of the reporter gene, the appropriately positioned *AflII* site was found difficult to work with, so it was changed to *MluI*. Further changes had to be made when it was found that persistence of expressed green protein masked the process of expression. Fortunately the two hour half-life pd2EGFP reporter became available and this was easily exchanged with the original using the *AgeI* and *NotI* sites.

DNA constructs were electroporated into *E. coli* (DH10B) using a BioRad GenePulser II (1 mm cuvette, 2.4 kv, 100 ohms, 25 μF). Immediately after electroporation the bacteria were suspended in 0.5 ml cold DOC medium (Sigma) and incubated in a shaking incubator at 37° for 1 h. Bacteria containing plasmid were selected on LB agar plates containing 50 μg/ml Kanamycin and minipreps from individual colonies grown in LB containing the same antibiotic were then extracted for circular plasmid DNA using the appropriate Qiagen kit. Large preparations of selected DNA were made using Qiagen Maxi kit procedures after the parent bacteria had been recloned. In some experiments DNA was linearized using *SacII*, followed by extraction and precipitation using phenol and ethanol. DNA for use in cultures was adjusted to a concentration in the range 200–500 ng/μl and filtered through a 0.22 μm membrane filter (4 or 13 mm Millipore Millex).

The thymidine kinase (Tk) minimal promoter was prepared by excision from the vector pBLCAT2 (GenBank X64410, 435–603) using *BglII* and *BamHI* and inserting the fragment into the *BamHI* site of pEGFP. The Amh promoter elements for insertion into pEGFP were prepared by PCR using oligonucleotide primers with appropriate restriction enzyme tails: these oligos were designed from the sequence of the mouse Amh (U3, GenBank X83733), the U3 DNA was used as the template for these syntheses. Initial selection of mini-prep DNA was by restriction enzyme digestion followed by agarose gel electrophoresis. All selected DNAs were sequenced to check for orientation of insertion and accuracy of PCR synthesis. Amh promoter elements and the intact promoter were inserted at the *XmaI* site.

Fig. 2. Comparison of conserved sequences 3′ of the PA site of Amh in three species. The numbering refers to the mouse (Mm) sequence with which the other species are compared.

2.2. Cell cultures

Cultures of a mouse fibroblast line (nih3T3) and a mouse Sertoli cell line (TM4; ATCC) were grown initially in DMEM but latterly and more successfully in DMEM/F12 medium (Invitrogen) containing 10% fetal calf serum (FCS) and antibiotics (pen/strep) in a humidified atmosphere of 5% CO_2 at 37°. When required aliquots of cells were stored in liquid nitrogen in 90% FCS plus 10% DMSO. In preparation for a transfection experiment cells were grown to 85–95% confluence in 90 mm dia. tissue culture grade Petri dishes or equivalent sized flasks. The adherent cells were harvested by first rinsing the dish with D-PBS (Ca, Mg free), treating the cell layer with Trypsin–EDTA (X1, Sigma) for 3 to 5 min at 37°, followed by suspension of the cells in 0.5 ml of medium containing FCS but no antibiotic, in the wells of a 24-well Costar plate (Corning) at a concentration of $1.2–1.3 \times 10^5$ TM4 or $0.8–0.9 \times 10^5$ 3T3/well. They were grown for about 24 h until 85–95% confluent. In a few experiments 6-well plates were used with a pro rata increase in cell number. Immediately before addition of a complex of DNA and LipofectAmine 2000 (Invitrogen), the medium containing FCS was removed and replaced by medium with neither FCS nor antibiotics. The optimum amount of DNA and lipofectAmine was found by experiment to be as follows — for 3T3 cells 800 ng DNA and 2 μl lipofectAmine per well of a 24-well plate and for TM4 cells 1200 ng DNA and 4–5 μl lipofectAmine. After a minimum of 5 h culture at 37°, 0.5 ml of medium containing 20% FCS (but still no antibiotic) was added to each well. Cultures were harvested for flowcytometric analysis 48 h post transfection: it had been found there was less and quite variable expression after only 24 h and slightly less after 72 h culture compared with the expression after 48 h.

3. Results

3.1. Conserved promoter elements

The promoter region of mouse Amh when compared with similar regions of four other mammals, identified several regions of strong similarity which are illustrated in Fig. 1. Several of these promoter "elements" such as those for Sox-8/9 (Koopman, 1999; Schepers et al., 2003), SF1 (Jeyasuria et al., 2004), GATA (Beau et al., 2000; Tremblay et al., 2001), and WT (Hossain and Saunders, 2003) recognition have been identified and their function described in the literature by several workers (de Santa Barbara et al., 2000; Rey et al., 2003). The putative Sertoli specific regulatory element was identified after a comparison of the Amh promoter sequence with a likely promoter region of the gene for a testis specific protein situated on the X-chromosome (Tsx) (Simmler et al., 1996). The similarity between the Tsx sequence and Amh is illustrated in Fig. 1. It is clear that the order of identified elements is identical for all five species compared although the gaps between elements is variable. Comparisons were also made with the 3′ UTR post polyadenylation (AATAAA) sequence of mouse with two other available Amh 3′ UTR sequences, those of rat and human. A striking degree of similarity was observed which is illustrated in Fig. 2. It was on the basis of these preliminary explorations that SSRE (Sertoli specific) and DSRE (Downstream; 3′ UTR) elements were designed and synthesised by PCR, with tails with appropriate restriction enzyme sites, for incorporation into the MCS of green fluorescent protein expression vector (pd2EGFP), for use in transfection experiments.

3.2. Transient transfections

With one exception the transfection experiments described here were made with circular plasmid DNA, the expression of the reporter (d2EGFP) being mostly recorded after 48 h of culture of cells of the mouse fibroblast line nih3T3 and the mouse mature Sertoli cell line TM4. Many preliminary experiments were carried out to determine optimum conditions for the transfection and length of culture post transfection procedure. Several transfection reagents such as calcium phosphate and GeneJuice (Novagen) have been tried, however LipofectAmine 2000 (Invitrogen) has been found to give the

Table 1
Transient green expression by a d2EGFP plasmid vector containing a minimal (Tk) promoter with or without various Amh elements

#	Promoter	3T3 index (SEM)	TM4 index (SEM)
1	No DNA	0.55 (0.18)	0.02 (0)
2	CMV	27922 (1177)	1796 (94.9)
3	Tk	4010 (61.4)	8.9 (1.30)
4	(SSRE,Tk,DSRE)	2013 (80.9)	2.8 (0.18)
5	(SSRE,Tk)	6126 (280)	17.5 (1.33)
6	(Tk,DSRE)	2173 (391)	3.6 (0.43)
7	SSRE	532 (296)	1.6 (0.26)
8	DSRE	685 (226)	1.4 (0.21)
9	(SSRE,DSRE)	5604 (2175)	66.1 (4.50)
10	Amh	321 (92)	0.34 (0.09)
11	(Amh,DSRE)	214 (35)	0.21 (0.11)
12	Vector only	607 (78)	1.1 (0.05)

Quadruplicate 48 hr cultures in 24-well plates. Mean index (±SEM). Index = % cells gated (see Fig. 3) × Geo.Mean green fluorescence (FACScan). Transient transgenesis induced with circular DNA by LipofectAmine 2000. CMV = cytomegalovirus promoter (vector d2EGFP-N1); Tk = Thymidine kinase minimal promoter from pBLCAT2 (GenBank X64410, 435–603); SSRE = putative Sertoli element; DSRE = putative 3′ UTR conserved element. The parentheses enclose elements on the same DNA construct.

best and most consistent results, the conditions for its use are described in Material and methods. Table 1 summarises the result of a typical transient transfection with circular DNA in both kinds of cells. Expression of EGFP is measured using a flow cytometer (FACScan), the result are presented as a response index so that the data can be presented in a compact form:

$$\text{Index} = (\%\ \text{of cells expressing green} \times \text{geometric mean intensity})$$

(see Fig. 3 for details). It is obvious that the response driven by a cytomegalo virus (CMV) promoter is far higher than for any other promoter for both 3T3 and TM4 cells. It is also clear that the transient TM4 response is far lower than that seen in the 3T3 cells. In both kinds of cell the presence of the 3′ UTR element (DSRE) has a small (2- to 3-fold) suppressive effect on the expression driven by the thymidine kinase promoter (Tk) alone or in combination with the putative Sertoli specific regulatory element (SSRE). The thymidine kinase promoter (Tk) is highly directional, if inserted in reverse orientation expression is reduced at least ten fold. When compared with the effect of the vector alone the presence of the complete Amh promoter is inhibitory. When SSRE and DSRE are present separately and without the Tk promoter, the expression does not differ from that seen with the vector alone, but combined in the same vector SSRE+DSRE promote a highly significant augmentation of expression in both 3T3 and TM4 cells. The results of 9 transient transfection experiments, each similar to that illustrated in Table 1, are summarized in Table 3 where it can be seen that the ratios involving Tk with SSRE and DSRE in various combinations do not differ significantly between the two kinds of cells, but

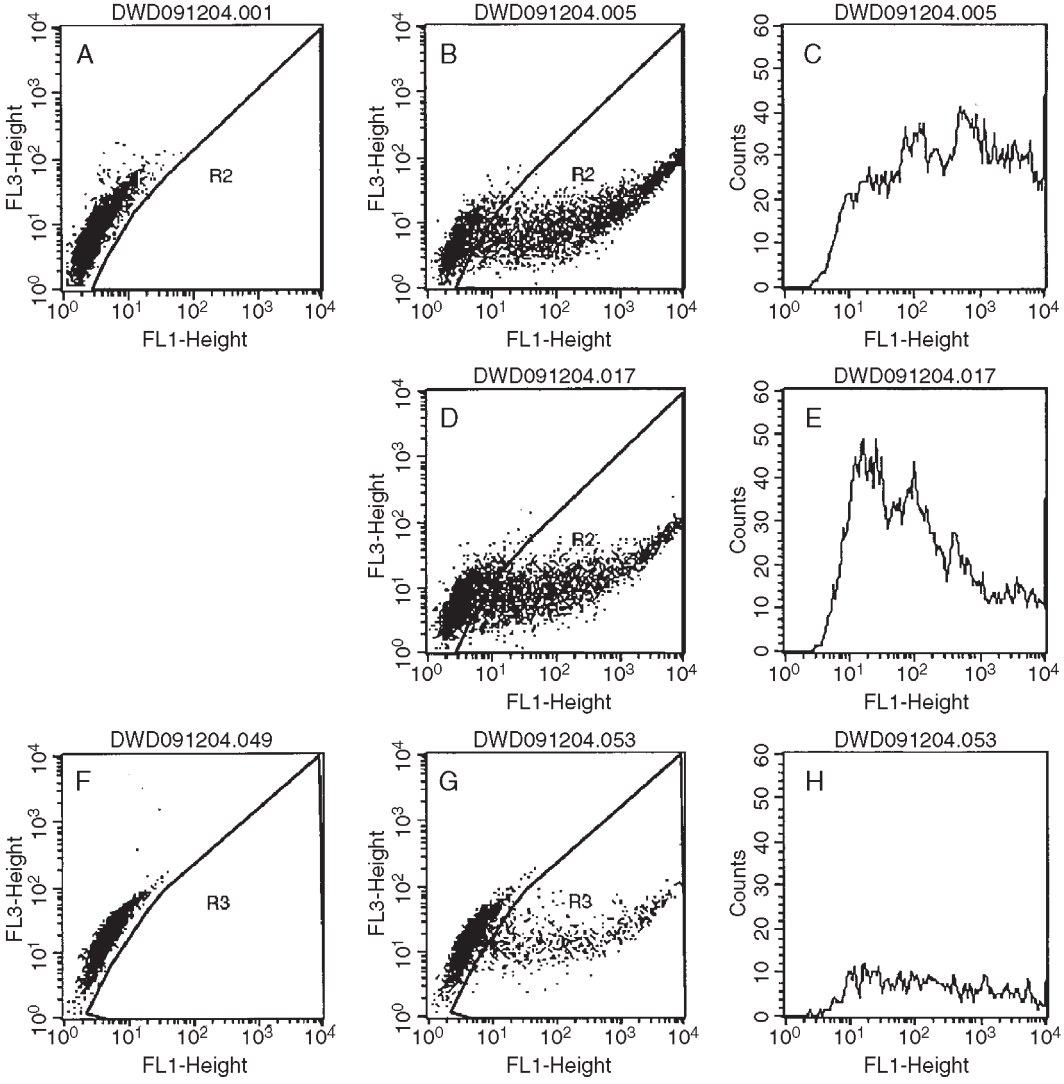

Fig. 3. Examples of FACScan (BD) analyses of cells from 48 h cultures. A–E are 3T3 cells; F–H are TM4 cells. A and F are dot plots (Fl1 green v. Fl3 red) represent the autofluorescence of cells treated with LipofectAmine 2000 but which have not received pd2EGFP DNA. B and G are similar dot plots of results obtained with cells which received DNA with a CMV promoter and D which received DNA with a Tk+SSRE promoter (for details of promoter elements see Table 1): a window (R2 for 3T3 and R3 for TM4 cells) has been drawn to exclude autofluorescence but include all green emitting cells, the distributions of which are shown in histograms C, E and H, respectively. The hint of a cyclic distribution of green intensity is seen in all 3T3 responses. The curved distribution of d2EGFP expression shown in B, D and G is due to an extension of green expression into the red channel: it was not possible to compensate for this.

Table 2
Green expression manifested by randomly integrated linear d2EGFP vector with or without a minimal (Tk) promoter and with or without additional Amh elements

#	Promoter	3T3 index (SEM)	TM4 index (SEM)
1	No DNA	3.4 (0.4)	0.9 (0.03)
3	Tk	1607 (1.0)	183 (50)
4	(SSRE,Tk,DSRE)	779 (42)	498 (118)
5	(SSRE,Tk)	2284 (75)	439 (104)
7	SSRE	178 (18)	281 (81)
8	DSRE	102 (3)	16 (8)
9	(SSRE,DSRE)	1488 (72)	317 (77)
12	Vector only	83 (6)	594 (67)

Triplicate cultures in 6-well plates, selected for NEO expression with G418 for 21 days but no selection for green expression. See footnote to Table 1 for further details of promoter elements. For transfection using LipofectAmine, DNA constructs were linearized with *SacII*.

support the contention that DSRE has a small inhibitory (repressor) effect.

Table 4 shows that synergy between SSRE and DSRE (as expressed by the ratio:

$$\frac{(\mathrm{SSRE,DSRE})}{(\mathrm{SSRE})+(\mathrm{DSRE})}$$

in the absence of Tk, is significantly greater in TM4 cells. Furthermore reversal of the elements ablates the synergy, with the one exception that in Sertoli (TM4) cells, where *both* SSRE and DSRE are reversed, there is a significantly reduced synergy compared with that obtained with *both* elements in a forward orientation.

3.3. Integrated transfection

The single exception to the experiments with transient *transfection* was with genomically integrated linear DNA in both kinds of cell, which were selected for 3 weeks in culture with G418 for Neo expression—the KAN/Neo gene being an integral part of the pEGFP plasmid vector. The results summarized in Table 2 are relatively similar to the transient experiment summarized in Table 1, with the difference that the overall response in the TM4 cells is much greater.

4. Discussion

A potential Sertoli specific regulatory element (SSRE) in the promoter of Amh was identified by comparison of approximately 400 nt of 5′ UTR sequence of the *Tsx* gene (Simmler et al., 1996) with the equivalent region of the Amh of mouse. A PCR version of this element (SSRE) was synthesised and when inserted into the MCS of the promoterless pd2EGFP vector, elicited a marginal response on its own when tested in a transient expression study in 3T3 and TM4 cells. When inserted in a vector already containing a minimal thymidine kinase (Tk) promoter, there was a small (2 or 3-fold) enhancing effect in both 3T3 (mouse fibroblast) and TM4 (mouse mature Sertoli) cells. However the absence of a relative difference between the response in fibroblast and Sertoli cell is not conclusive because the response in the Sertoli cells is very much weaker than in the fibroblasts: all that can be said with conviction is that relatively the response in the two cell lines is similar. Table 1 summarizes a typical example taken from a series of 9 experiments: there is considerable variation in index values from one experiment to the next but the relative values are consistent and are therefore summarized as ratios in Table 3.

Comparison of the DNA sequence immediately 3′ of the polyadenylation site of mouse, rat and human Amh, revealed a region of striking similarity (Fig. 2). On the principle that if a sequence is highly conserved, it is likely to have a specific function (Hardison, 2000), this sequence (DSRE) was synthesised and inserted into the EGFP vector at MluI (replacing AflII) 3′ of the coding region. The addition of this element alone barely changed the background level of expression in control transfections (see #12 Table 1). However when combined with Tk (or Tk+SSRE) there was a consistent if small decrease in expression across the whole range of experiments. This suggests that DSRE may have a mild repressor activity, in this particular hybrid DNA context, perhaps through post-translational control (Kuersten and Goodwin, 2003).

When SSRE *or* DSRE are inserted on their own into the vector, the responses in both cell types do not differ significantly from that obtained with the vector alone: the MCS of the vector may possibly be the source of a weak promoter activity, which seems to be suppressed when the complete Amh promoter is incorporated at the *XmaI* site at the 3′ end of the MCS (see #10,#11 Table 1). However when SSRE and DSRE are combined in the same vector there is a dramatic increase in expression indicating some form of synergy—this is expressed as a ratio in Table 4, where it can be seen that this synergism is significantly greater in the Sertoli cells (TM4). It is clear that in the absence of Tk, DSRE is not repressive but enhancing.

Table 3
Ratio of expression promoted by a Tk minimal promoter together with different 5′ and 3′ UTR elements of Amh incorporated in a circular d2EGFP vector

Ratio*	3T3 cells, mean (±SEM) $n=9$	TM4 cells, mean (±SEM) $n=9$
$\frac{(\mathrm{SSRE,Tk})}{\mathrm{Tk}}$	1.13 (0.15)	0.89 (0.11)
$\frac{(\mathrm{Tk,DSRE})}{\mathrm{Tk}}$	0.52 (0.07)	0.58 (0.10)
$\frac{(\mathrm{SSRE,Tk,DSRE})}{\mathrm{Tk}}$	0.36 (0.10)	0.43 (0.12)
$\frac{(\mathrm{SSRE,Tk})}{(\mathrm{Tk,DSRE})}$	2.64 (0.46)	1.98 (0.41)

For explanation of promoter elements see Table 1. The parentheses enclose elements on the same DNA construct. n=number of experiments, each similar to that illustrated in Table 1.
* Mean ratio is based on mean index for each experiment.

Table 4
Synergic expression promoted by a potentially sertoli specific element and a conserved 3′ UTR element; including control experiments with elements in reverse orientation

Ratio	3T3 cells, mean (±SEM) $n=6$	TM4 cells, mean (±SEM) $n=8$
$\frac{(SSRE, DSRE)}{(SSRE), (DSRE)}$	2.70 (0.62)	13.95 (3.29)
	$n=3$	$n=3$
$\frac{(rSSRE, rDSRE)}{(rSSRE), (rDSRE)}$	0.49 (0.04)	8.8 (0.33)
$\frac{(rSSRE, DSRE)}{(rSSRE), (DSRE)}$	0.56 (0.17)	0.04 (0.02)

Micro dissection of the structure of SSRE and DSRE is required in the longer term and such studies are in progress, however, in the interim, experiments summarized in Table 4, using reversed elements, show that for synergy to be manifest, elements must be in forward orientation. It is interesting to note that in Sertoli cells but not in fibroblasts, when *both* elements are reverse orientation, there is a reduced synergy effect.

The possible nature of Sertoli specificity and of synergy of both SSRE and DSRE is intriguing. If further experimentation substantiates that SSRE is indeed Sertoli specific and acts to localize expression in these cells, then consideration will have to be given to its mechanism of action. For example, do cells of the Sertoli lineage, early in their differentiation activate a gene (perhaps as yet unknown; perhaps DaxI (Meeks et al., 2003), which codes for a SSRE-binding transcription factor, which in its turn plays a role in the assembly of the Amh transcriptosome? What is the repressor or enhancer role of DSRE, and is the enhancer/repressor role of this 3′-element confined to Sertoli cells? The undifferentiated or immature Sertoli cell settling down in the genital ridge of the 10 *dpc* male mouse, presumably receives position dependent signals through a membrane receptor system or by ligand uptake and interaction with a nuclear receptor, analogous to steroid signalling. An SSRE binding factor could therefore be seen in the male as part of a cascade starting with Sry (Grimes, 2004): exactly what is going on in the female largely remains a mystery (Gruijters et al., 2003; Themmen, 2005).

In a single experiment *SacII* linearized vector was introduced into both nih3T3 and TM4 cells, which were selected for neomycin resistance (Neo^r) in the presence of G418 for 21 days. It was assumed that since the genes encoding Neo^r and EGFP are part of the same vector, this procedure would result in integration of EGFP into the genome of the cells. Table 2 shows that the same relative pattern of response as was obtained from cells transfected with circular DNA (Table 1). There is one significant difference however—in absolute terme the response in TM4 cells is much higher when compared with the 3T3 response, than was the case with transient transfection. It can also be seen in Table 2 (#12) that the response in TM4 (Sertoli) from vector alone is very high. Several experiments have indicated that a likely explanation of this is that the DNA integrates randomly, some sites being significantly more favourable than others for the expression of Neo and EGFP, perhaps in a manner analogous to a promoter trap (unpublished data).

AMH gene transcriptional control has been intensively investigated during the previous decade. One reason for this is that AMH has been considered as the first functional marker of Sertoli cell differentiation triggered by *Sry*, the sex determining gene. Although numerous transcription factors (see introduction) have been shown to be involved in the regulation of Amh expression, so far none of them has been shown to be Sertoli cell specific. The present characterization of DNA motifs, conserved during evolution, in *Amh* gene regulatory regions, that seem to confer some sort of cell specific expression, may lead to the isolation of the proteins (transcription factors) which bind to them—the corresponding gene(s), as part of this cascade, will consequently become of prime interest.

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