Ovine IGF Antisense RNAs?

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ABSTRACT

Antisense RNA is RNA synthesized in the opposite direction to normal transcription, across part or all of the same gene. However, it could also be any RNA with sufficient homology to hybridize to a given mRNA. In mammals antisense RNA has been observed in total RNA from several growth-related genes, notably cellular oncogenes and insulin like growth factor (IGF) genes. At present the function - if any - of this RNA is unknown. When riboprobes from both human and ovine IGF-I cDNAs were used as *in situ* probes for ovine tissues, an antisense signal was observed for the ovine probe but not for the human probe. Probed Northern blots of total sheep liver RNA with ovine IGF-I riboprobes at normal stringencies also revealed both sense and antisense IGF RNA. In one series of low stringency *in situ* hybridization experiments, both sense and antisense riboprobes from both human and ovine cDNAs bound strongly to ovarian follicles. However, the pattern of sense RNA detected was markedly different (granulosa ≈ thecal/interstitial) compared with the antisense RNA detected (thecal/interstitial > granulosa). We discuss two possible causes for the detected antisense RNA, the nature of which is currently being resolved. Ovine IGF-II sequences may be involved, since IGF-I and IGF-II mRNAs show cross homology and since ovine IGF-II cDNA contains a 700 base pair antisense open reading frame.

Keywords: antisense, RNA, IGF.

INTRODUCTION

Insulin-like growth factors (IGFs) are considered necessary for normal and growth hormone (GH) enhanced growth in livestock. The endocrine activity of the two known IGFs, IGF-I and IGF-II, is regulated at many levels not only during synthesis and secretion but also during transport and by receptor and post-receptor mechanisms (reviewed by Rutter, 1991). It is not unreasonable to expect more subtle autocrine and paracrine controls over their biological activity, particularly at the RNA (transcript or mRNA) level of synthesis (Sussenbach *et al*, 1991, 1992; Lund *et al*, 1991).

Studies of RNA regulation using Northern hybridization analysis (Thomas, 1983) to allow estimation of RNA size and abundance have identified several major IGF transcripts from both IGF-I and IGF-II genes. These are somewhat similar among species, with human RNAs being 7.5, 1.3 and 1.1 kilobases (Kb) for IGF-I, and 6, 4.8 and 1.8 Kb for IGF-II (Sussenbach *et al*, 1991, 1992). However, few studies of IGF-I mRNAs in sheep using Northern hybridization of total RNA have been reported. Some of the problems have been technical. Controls for cross hybridization between IGF-I and -II RNAs are rarely included, and detected IGF transcripts can be confused with hybridization to ribosomal RNAs. Other observations are more difficult to explain. For example, Wong *et al*, (1989) showed that the amount of ovine (o)IGF-I mRNA from liver (a major site of IGF-I synthesis) detected from 2.5 ug of polyadenylated (poly(A)) ovine mRNA by Northern hybridization was less than that detected from 10 ug of total ovine RNA. Since the isolation of poly-A mRNA is expected to enrich poly-A mRNAs by up to 100-fold, this result is contrary to expectation unless a considerable amount of the transcript was not polyadenylated. Furthermore, Northern hybridizations of ovine IGF-I RNA can yield smears rather than discrete bands due to the existence of multiple transcripts (Wong *et al*, 1989).

We have been studying the expression of oIGF-I RNA during development in fed and fasted sheep tissues. In several RNA hybridization experiments, antisense riboprobes synthesized from an oIGF-I cDNA used to detect oIGF-I mRNAs yielded lower signals on tissue sections than the antiparallel sense riboprobes used as a negative control. Here we describe this 'antisense' RNA and discuss two alternative reasons for its detection.

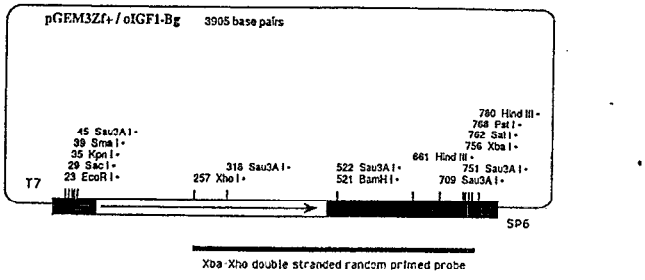
MATERIALS AND METHODS

Liver tissues for *in situ* hybridization and RNA were isolated from adult Coopworth females out of several sires (Hua *et al*, 1992). Ovarian tissues for *in situ* hybridization were fixed by a 15 minute paraformaldehyde infusion via the ovarian artery. Ovaries were then removed and stored as segments in the fixative before embedding and sectioning.

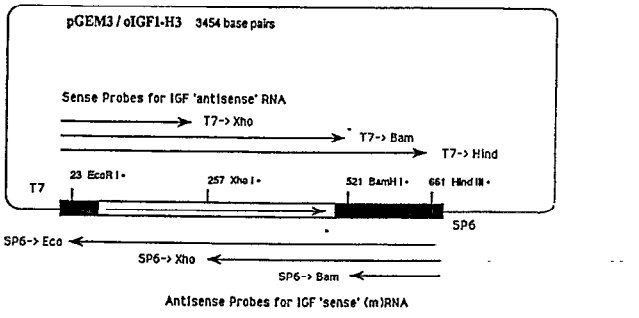
Probes were generated from an ovine IGF-I cDNA clone (Wong *et al*, 1989; clone A21) subcloned in pGEM (Promega) vectors (Figure 1). (The subclones were confirmed by restriction mapping and sequencing of ≥ 100 base pairs on both ends). A 500 base-pair, 32P-dATP random primed IGF-I probe was synthesized from the region shown in Figure 1A. For IGF-I riboprobes, ^{35}S-UTP was incorporated into RNA transcribed from various ovine (o)IGF-I cDNA fragments from the plasmid described in Figure 1B. Anti-sense probes for oIGF mRNA were transcribed with SP6 RNA polymerase from the pGEM SP6 promoter, while the control sense probe for antisense RNA was transcribed by T7 RNA polymerase from the pGEM T7 promoter. Also used was a human IGF-I

FIGURE 1: Ovine IGF-I cDNA fragments used for riboprobes. The closed box includes the cDNA insert, cloning sites and 'SP6' and 'T7' promoter sites for riboprobes. The arrow in the cDNA insert indicates the direction and ends of translation. A. Plasmid pGEM3Zf+/ oIGF1-Bg contains a BglII fragment of ovine cDNA (Wong et al, 1990; clone A21) cloned into the BamHI polylinker site of pGEM3Zf+ (Promega; defined here by the Sau3A sites at each end). The XbaI-XhoI fragment was used as a substrate for ^{32}P-dATP random primer probe labelling. B. Plasmid pGEM3/oIGF1-H3, bears a HindIII-EcoRI fragment from the first plasmid. ^{35}S-UTP labelled antisense RNA riboprobes for IGF-I mRNA, and sense riboprobes for detecting 'antisense RNA' are shown by arrows.

A



B



cDNA fragment (Jansen *et al*, 1983) cloned in the polylinker of a pGEM3 vector (A. Molenaar, pers. comm.). In this case the antisense probe was transcribed from the T7 promoter.

In situ hybridization was based on the procedure of Wilkins *et al*, (1989), as modified by Molenaar *et al*, (1992). Approximately 300,000 to 500,000 cpm was applied per section over roughly equal areas, with sense and antisense probes being within 5000 cpm/ug of one another.

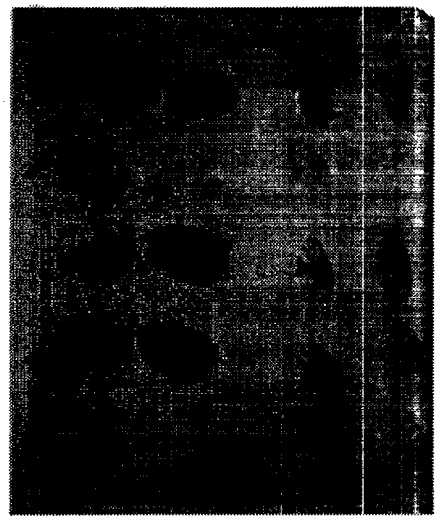
For total RNA analyses, the Guanidium RNA isolation procedure described by Chomczynski and Sacchi (1987), was followed. DNAase I treatment was necessary to remove DNA from guanidium preparations. RNA samples were stored in aliquots at -70°C in water. For Northern analysis, formaldehyde gel electrophoresis followed a modified protocol of Fourney *et al*, (1988). Glyoxal gel electrophoresis followed the procedure of Thomas (1983) with minor modifications. RNA dry gels were probed as described by Ahmad *et al*, 1991. Northern blotting was carried out in accordance with directions supplied by Amersham Australia for Hybond N+ (Figure 3A) or Hybond-C+. Hybridization conditions are described in the Figure legends.

Images of bound probe from both *in situ* and Northern analyses were generated from developed x-ray (Kodak XAR-5) films scanned using a Molecular Dynamics Personal (laser) densitometer and Macintosh II computers as described by Ord, Hodges, Nixon, Molenaar, Dobbie and Kirk (1993).

FIGURE 2: In situ hybridization of sheep tissue sections with the ovine sense and antisense IGF-I riboprobes given in Figure 1 (Magnification ≈ 1X). Column A: liver sections probed for sense (m)RNA. Column B: liver sections probed for 'antisense' RNA. Column C: ovary sections probed for sense (m)RNA. Column D: ovary sections probed for 'antisense' RNA.

Row 1: human ribosomal RNA riboprobes. Row 2: human IGF-I riboprobes. Row 3: ovine SP6->Bam and T7->Bam IGF-I riboprobes. Row 4: ovine SP6->Eco and T7->H3 IGF-I riboprobes. Row 5: ovine SP6->Xho and T7->Xho IGF-I riboprobes.

Hybridization was at 50°C in a 50% formamide/SSC buffer. Non-specific probe was digested for 50 minutes with RNAses A and T1, then washed extensively. The lowest non-specific binding was determined by the human (h)IGFI antisense probe (section A2), which corresponds to an area at the 3' end of the coding region of all known IGF-I mRNAs. The human antisense riboprobe should have only slight homology with IGFII mRNA sequences (Tm for oIGF-II RNA and hIGFI RNA is ≈ 40°-45°C).



RESULTS

High levels of 'antisense' RNA in ovine tissue sections after *in situ* hybridization

Sense and antisense riboprobes from ovine cDNAs (Figure 1) were used to detect IGF mRNA in ovine tissues (Figure 2). As expected sense signal from a human IGF-I control probe was higher than antisense signal (Figure 2, row 2). However, ovine sections probed with IGF-I sense RNA yielded an 'antisense' signal which was greater than the sense mRNA signal, for two of three different ovine probes (Figure 2, rows 3,4 and 5).

Northern and 'dry gel' hybridization to ovine probes

Northern blots were also examined for the presence of IGF-I RNA. Fractionated, DNAase-treated, ovine liver total RNA probed with the human IGF-I ^{35}S-UTP riboprobe at high stringency yielded several predicted IGF-I RNA bands after prolonged exposure, especially at 5-7 Kb (Figure 3A). An expected difference between starved and fed animals (lane 1 versus lane 2; eg. Straus and Takemoto,1990) was also detected for 1 kb and 7 Kb bands. While some of the differences in the two lanes could be due to loading errors, the lowest bands in each lane did not differ in signal intensity, and there was no evidence of degradation. Hence the human probe appeared to be detecting ovine IGF-I RNA.

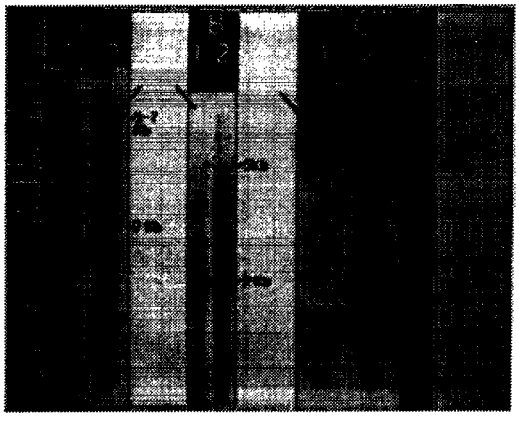
FIGURE 3: Fractionated total ovine liver RNAs probed for total IGF-I RNA. Arrows shows the putative position of 7 kb IGF-I mRNA.

A. Northern blot of ovine liver total RNAs from individual starved (lane 1) and fed (lane 2) sheep, fractionated on a 1% agarose/formaldehyde minigel and probed with ^{35}S-UTP RNA transcribed from a human IGF-I cDNA fragment.

Ethidium bromide staining showed that equivalent amounts of RNA were loaded, and that no substantial degradation had occurred. The gel was blotted to Hybond N+, prehybridized for 1 hour and hybridized at 43°C for 18 hours with 10^7 cpm of probe in NaPO4 buffer (Church and Gilbert, 1984) mixed 45:45:10 (V:V:W) with formamide and dextran sulfate, respectively. The blot was washed twice in 40 mM NaPO4/1% SDS, twice in the same buffer for 40 minutes at 65°C and once for 30 minutes at 65°C in 13 mM NaPO4/0.33% SDS, then exposed to x-ray film for 90 hours.

B. Northern blot of pooled samples of total liver RNA after DNAase treatment (see Figure 5), hybridized with a double-stranded, ^{32}P-dATP ovine IGF-I probe (Figure 1A). RNA was from starved (lane 1) and fed sheep (lane 2). Samples were fractionated using glyoxal, blotted to Hybond C-extra nitrocellulose, probed and washed as described in Current Protocols in Molecular Biology I:497 (1992).

C. 'Dry-gel' hybridization of the double-stranded ^{32}P-dATP ovine IGF-I probe to the RNA described in Figure 3B, fractionated in glyoxal gels. Lane 1, RNA (10ug) from fasted sheep; lane 2, RNA (3 ug) from fed sheep); lane 3; size markers.



We then sought to probe ovine RNA using the ovine probe. When a Northern blot of pooled ovine liver RNAs was probed with ^{32}P-dATP double stranded DNA probe, the 7 Kb band was still faintly detectable above a smear from 4.4 to 0.5 Kb with a maximum at roughly 1 Kb, indicating that IGF-I mRNA was being detected (Figure 3B, arrows). In starved animals the overall hybridization to the probe was again reduced relative to fed animals. When a dried gel of the same RNA was similarly probed and compared to the Northern blot, a stronger 7 Kb band was evident from the fed liver RNA (Figure 3C), indicating that the faintness of this band in Northern blots of ovine RNA could be due to both inefficient blotting and degradation.

To compare our *in situ* hybridization results with the above data, Northern blots from glyoxal gels of the pooled RNA preparations were probed with ^{35}S-UTP labelled sense and antisense oIGF-I riboprobes. As shown in Figure 4, both sense and antisense riboprobes yielded results similar to that given in Figure 3B, and there appeared to be more antisense RNA, as was the case in Figure 2. However, no direct estimate can be made of levels of sense and antisense here because total RNA in the blot probed for 'IGF-I mRNA'

FIGURE 4: Northern hybridization of ^{35}S-UTP oIGF-I sense and antisense and riboprobes to Northern blots of the pooled samples of total liver RNA fractionated in formaldehyde gels and transferred to Hybond-C extra nitrocelluose membranes. Lanes 1 and 2: RNA from starved and fed sheep. respectively, probed for mRNA (SP6→Eco; Figure 1). Lanes 3 and 4: a second blot of the same RNA from starved and fed sheep, respectively, probed for antisense RNA (T7→Hind, Figure 1). Lanes 5 and 6 are lanes 1 and 2 overexposed to show the 7 Kb IGF-I mRNA band (arrow).

Hybridization was as described in Current Protocols in Molecular Biology I:497 (1992; except that dextran sulfate was omitted and 20mM dithiothreitol was included to reduce non-specific binding) using 2 X 10^7 cpm of probe at 43°C for 19 hours. The lower signal coming from the blot probed (with antisense probe) for mRNA may be partly due to poor transfer caused by ethidium bromide in the loading buffer.

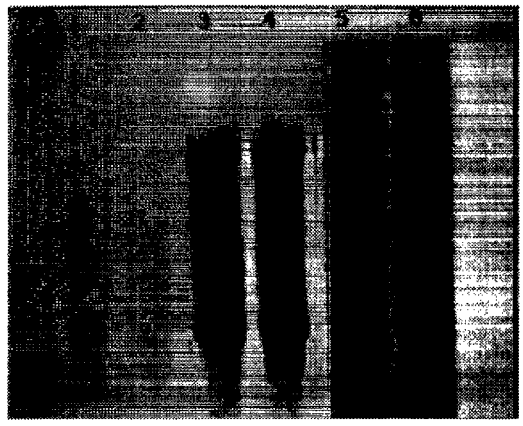
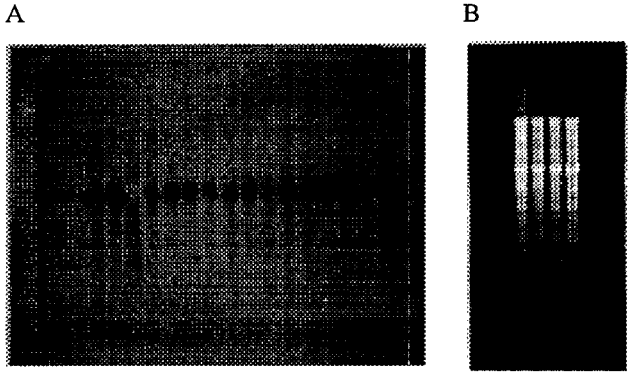


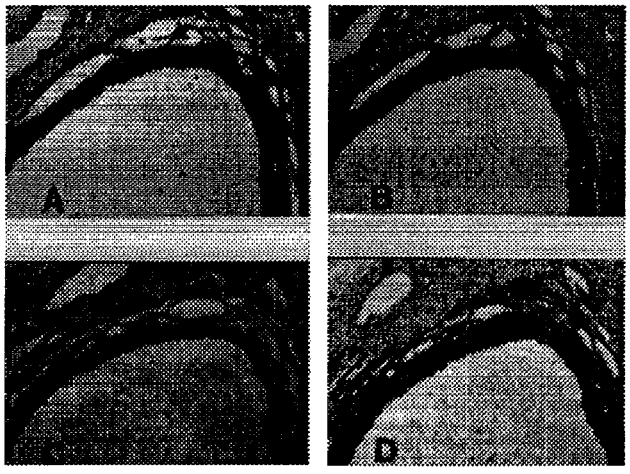
FIGURE 5: **A.** Northern hybridization of a 700 base, ^{35}S-UTP antisense riboprobe from human actin cDNA (Gunning et al, 1985; transcribed from the T7 promoter of a pGEM3 plasmid) to the individual total ovine liver RNA preparations pooled for experiments shown in Figures 3B,C and 4 (excepting the degraded sample). RNA (10 ug each) was fractionated as in Figure 3A. The arrow shows the putative position of 2kb ß-actin mRNA.

B. Ethidium-bromide stained formaldehyde gel containing the pooled and DNAased preparations of total liver RNAs probed in Figure 3B,C and 4. There was no loss of total RNA quality after this treatment, and the quality of the four RNA preparations was equivalent. Lane 1, pooled liver RNAs from fed sheep; lane 2, from GH-treated, fed sheep; lane 3, from fasted sheep; lane 4, from fasted sheep treated with growth hormone.



(Figure 4; lanes 1 and 2) contained ethidium bromide. Since the DNAase-treated RNA preparations were essentially intact prior to pooling as determined by hybridization with an actin probe (Figure 5A), and qualitatively similar after pooling as shown by ethidium bromide staining (Figure 5B), the 'messy' results observed here were not due to generalized degradation.

FIGURE 6: A large follicle in serial ovarian tissue sections (Magnification ≈ 100X) probed with: A. human IGF-I antisense ^{35}S-UTP riboprobe for mRNA; B. human IGF-I sense ^{35}S-UTP riboprobe for antisense RNA; C. ovine IGF-I antisense ^{35}S-UTP riboprobe for mRNA; D. ovine IGF-I sense ^{35}S-UTP riboprobe for antisense RNA, at a lower 'stringency' than for the in situ hybridization shown in Figure 2 (less extensive RNAasing) as confirmed by the high background levels detected with the human sense riboprobe.



Localization of 'antisense' ovine and human IGF-I RNA in ovarian follicles

Having demonstrated crudely that 'antisense' binding was occurring in both Northern and *in situ* hybridizations, we next looked for tissue specificity of the binding by microscopically examining sheep ovary sections from the same block as those shown in Figure 2, subjected to lower stringency *in situ* hybridization. The human antisense probe bound to both granulosa and interstitial zones surrounding a follicle (Figure 6A), whereas the sense probe appeared to be detecting antisense RNA mostly in the thecal/interstitial zone (Figure 6B). Like the human probe, the ovine antisense probe - where detectable - was found in both interstitial and granulosa cells (Figure 6C), and the antisense signal was again found primarily in the thecal/interstitial boundary throughout the follicular wall area (Figure 6D). Hence both ovine and human probes detected 'antisense' RNAs in the same locale.

DISCUSSION

Whether the 'antisense' oIGF RNA detected here is artifactual or *bona fide* is presently being determined. Antisense IGF-II RNAs have been detected and mapped in chicken embryos (Taylor *et al*, 1990). There are several reasons to believe that our probes may have detected IGF-II antisense RNA, albeit under 'low stringency' conditions.

First, the sense and antisense signals from the IGF-I probe localize differently in the follicle walls of sheep ovaries at low stringency (confirmed by high levels of background detected with the human sense probe; similar *in situ* hybridization performed at higher stringency resulted in very low antisense signal from the human probe; Figure 2). The homology between the oIGF-I and oIGF-II regions used for probing here is sufficient that high stringency would be required to avoid cross hybridization. Both IGF RNAs have been detected in the same tissue (eg. Han *et al*, 1987).

Second, there is a large antisense open reading frame (ORF) of 700 base pairs in at least one oIGF-II cDNA sequence (EMBL DNA database, Wong *et al*, 1991). Interestingly, in a BLAST (NIH) search of the EMBL and Genbank databases, 47 amino acids of the 230 amino acid (predicted) ORF sequence bore 38% identity and 61% homology with a 47 amino acid stretch of the human fibroblast growth factor receptor 4 ($P \approx 0.004$).

Third, we have found a significant positive correlation between sense and antisense signal from kidney sections in sheep of different ages probed with human IGF-II riboprobes (in preparation). Although antisense signal was lower than sense, the antisense:sense ratio was less in younger animals (two days to six weeks), than older animals (four months to two years). Hence there appears to be an ontogenic increase in the 'antisense' IGF-II RNA levels in sheep kidney.

However, there is a trivial alternative to the existence of a genuine IGF antisense RNA, and that is a short sequence homologous to the probe present in an abundant non-IGF RNA. When only the 'polylinker' cloning region in pGEM3 was transcribed from the pGEM3 T7 promoter and used as a probe, we detected binding to an ovarian follicle, especially in the granulosa cells (unpublished). Although our 'antisense' RNA was mostly in the thecal/interstitial zone (Figure 6) and the human sequence which detected the same 'antisense' at low stringency was transcribed from the SP6 promoter, the situation is far from resolved.

If IGF-I or IGF-II antisense RNAs do exist, a biological function remains to be established. Artificial antisense RNAs introduced into cells greatly reduce the expression of the corresponding sense transcript (Hélène and Toulmé, 1990). A natural antisense RNA sufficiently homologous to both IGF-I and IGF-II mRNAs might eliminate the potential for synthesis of either IGF in a cell.

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Note added in proof:

Witkiewitz, Bolander and Edwards (Biotechniques 14:458-463, 1993) have recently shown that part of the cloning site present in pGEM3 T7 RNA transcripts binds strongly to ribosomal RNA sequences. This could account for much of the 'antisense' RNA observed by us, but does not alter the arguments discussed here for the existence of lower levels of *bona fide* IGF antisense RNA (the unpublished experiments which detected oIGF-II antisense RNA during ontogeny were carried out with a probe missing the suspect sequence).

Image photographs were taken with a Polaroid VI-350 video printer obtained using New Zealand Lotteries Grant #022365.