The polypeptides and genes for ovine and bovine trophoblast protein-1

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Summary. Ovine and bovine trophoblast protein-1 (oTP-1 and bTP-1) have been strongly implicated as antiluteolytic agents and responsible for maternal recognition of pregnancy in sheep and cattle, respectively. Both are interferons (IFN) belonging to the IFN-α family, but their length (172 residues versus 166 for most IFN-α) places them in an unusual subclass (the IFN-αi). The various isoforms of oTP-1 and bTP-1 produced by trophoblast tissue appear to arise in part from translation of multiple mRNAs which are themselves the products of distinct genes. These genes, like those for other IFN-α, are without introns. However, the genes for oTP-1 and bTP-1 form a distinct subgroup within the IFN-αi on the basis of their overall primary sequences and the high conservation of the 3′-untranslated ends of their transcription units. The bTP-1 genes also differ from the bovine IFN-αi in the organization of the promoter regions upstream from the transcription start site. Nevertheless, computer-aided analysis of the primary polypeptide sequences of oTP-1 and bTP-1 indicates that the molecules are likely to have approximately the same shapes and dimensions as all other IFN-α molecules. It remains to be determined whether they have unique biological properties which distinguish them from other IFN-α molecules.

Keywords: α-interferons; trophoblast; molecular cloning; sheep; cattle

Introduction

Ovine trophoblast protein-1 (oTP-1) and bovine trophoblast protein-1 (bTP-1) have been strongly implicated as mediators of maternal recognition of pregnancy in sheep and cattle (Bazer et al., 1986). In particular, they appear to be major factors in preventing the regression of the corpus luteum in these two species. Ovine TP-1 is produced in major amounts by trophoectoderm at around the time the conceptus begins to elongate from a sphere to a longer, thread-like form, i.e. about Day 13 (Hansen et al., 1988; Farin et al., 1989). On two-dimensional polyacrylamide gels oTP-1 is evident as 3 or 4 isoforms of M, about 18 000 (Godkin et al., 1982; Imakawa et al., 1987). It is the main secretory product of conceptuses between Days 13 and 21 of pregnancy (Godkin et al., 1982) and the major translation product of Day-16 conceptus RNA (Hansen et al., 1985; Anthony et al., 1988). It can extend the length of the oestrous cycle when infused into the uteri of non-pregnant ewes (Godkin et al., 1984a; Vallet et al., 1988) and appears to act as a paracrine hormone directed locally to the uterine endometrium where it influences protein synthesis (Godkin et al., 1984b;
Vallet et al., 1987; Sharif et al., 1989) and dampens the pulsatile output of the uterine luteolysin, prostaglandin F-2α (Fincher et al., 1986).

The bovine homologue of oTP-1, bTP-1 also consists of several isoforms when analysed by two-dimensional electrophoresis, although it is a rather more basic protein than oTP-1 (Bartol et al., 1985; Helmer et al., 1987). In addition, however, there are at least two size classes of molecule (M, 22,000 and 24,000). As reviewed in the next section, the isoelectric variants are probably the products of different genes, whereas the different size classes most probably arise from differential glycosylation. Major synthesis of bTP-1 is initiated around the time that a conceptus must be present in the uterus in order to prevent regression of the corpus luteum, i.e. around Day 15 (Farin et al., 1990). As in the case of oTP-1, the induction of bTP-1 seems to be triggered at the time when the spherical blastocyst begins to elongate. However, the time of morphological transition seems less fixed in cattle than in sheep (unpublished results) so that in some conceptuses high concentrations of bTP-1 mRNA have been noted associated with elongating conceptuses as early as Day 12–13 (Farin et al., 1990). In addition, bTP-1 synthesis is spread over a much longer period of conceptus development. Godkin et al. (1988), for example, have noted bTP-1 production as late as Day 36 of pregnancy, while oTP-1 synthesis is essentially completed by Day 23 (Godkin et al., 1982; Farin et al., 1989). Like oTP-1, bTP-1 seems to function as an antiluteolysin. Injection of total conceptus secretory proteins (Knickerbocker et al., 1986a) or of purified bTP-1 (Helmer et al., 1989) into the uterine lumina of non-pregnant recipient cows can extend luteal function significantly, and this effect is most probably due to an inhibition of pulsatile PGF-2α output by the endometrium (Knickerbocker et al., 1986b). These various aspects of the biology of oTP-1 and bTP-1 are discussed in further detail by Flint et al. (1991), Salamonsen et al. (1991) and Bazer et al. (1991).

Molecular cloning of the mRNA for oTP-1 and bTP-1

Amino acid sequencing of the NH₂ terminal of oTP-1 (Imakawa et al., 1987; Stewart et al., 1987; Charpigny et al., 1988) and the cloning of oTP-1 (Imakawa et al., 1987) and bTP-1 (Imakawa et al., 1989) cDNA revealed that both proteins were related to the alpha-interferon (IFN-α) family of proteins. More specifically, they appeared to belong to a subgroup of IFN-α whose members are 172 rather than 166 residues in length. This subgroup has been named IFN-αult by Capon et al. (1985) or IFN-αβ by Hauptmann & Swetly (1985) to distinguish it from the 166-residue type (usually referred to as IFN-α1). A comparison of the nucleotide and inferred amino acid sequences of a bTP-1 and a bovine IFN-αβ cDNA is shown in Fig. 1. It should be noted that these cDNA molecules are very similar to each other and diverge most markedly in their 3'-untranslated termini.

The multiple isoforms of oTP-1 and bTP-1 which are evident during two-dimensional electrophoresis suggested that each protein may be translated from more than a single species of mRNA (Anthony et al., 1988). Indeed, the presence of multiple transcripts immediately became evident in the initial cDNA cloning studies (Imakawa et al., 1987, 1989; Stewart et al., 1989). We have obtained the sequences of 7 cDNA clones for oTP-1 that appear close to full length. The differences in nucleotide sequence and inferred amino acid sequence within the 195 codon open reading frame are summarized in Fig. 2. There appear to be two major subtypes (oTP-1A and oTP-1B). The upper 5 clones are of the B type (201, p3, p5, p7 and p8) and are very similar to each other. A total of 9 base differences accounting for 7 amino acid substitutions are evident among the 5 clones. Because some of these substitutions would result in charge alterations, e.g. gln for glu at position 5, asp for gly at position 101 and lys for glu at 107, the pI of the resulting proteins would be expected to differ.

The second group of cDNA (Group A) represented by clones p6 and p12 were very similar to each other but differed quite extensively from the B group in both nucleotide and inferred amino acid sequence. The most interesting feature of Group A was the substitution of an A for G in codon
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Fig. 1. A comparison of bTP-1 and bovine IFN-α subunit cDNA and their deduced polypeptide sequences. The deduced amino acid sequence of bTP-1 is given above the nucleotide sequence. Amino acid residues differing in bovine IFN-α are indicated below the nucleotide sequence. Asterisks mark differences between the nucleotide sequences that do not result in a change in amino acid residue. Potential polyadenylation signals are underlined. Asn^18 constitutes a potential site for N-glycosylation in bTP-1.

101 to provide an asn instead of an asp at residue 78 of the mature protein. This substitution creates a potential site for N-glycosylation on oTP-1 which is identical to the one thought to carry carbohydrate on bTP-1. However, there is no evidence that any of the forms of oTP-1 so far identified are glycosylated (Anthony et al., 1988). By contrast, all of the cDNA for bTP-1 that we have so far sequenced code for an asp at position 78, and all of the isoforms of bTP-1 appear to be glycoproteins (Anthony et al., 1988; Helmer et al., 1988). Interestingly, the 3'-ends of all 7 cDNA for oTP-1
Amino acid position: 5 6 12 16 24 25 55 67 69 72 76 78 83 100 101 102 107 112 113 114 128 154 165
Amino acid sequence: glu arg arg lys leu ser pro pro tyr tyr his ala asp glu arg gly gln gly gly asn met tyr val met
of oTP-201

Fig. 2. A summary of nucleotide differences within the coding region of 7 different full-length cDNA for oTP-1 that have been cloned and sequenced in this laboratory. Two types of oTP-1 cDNA have been identified arbitrarily on the basis of degree of sequence identity and the presence of a potential codon for asn78. Sequences for 5 cDNA clones representing the B-type (201, p3, p5, p7, p8) are shown in the upper part of the diagram. Base changes and any resulting alteration in amino acid sequence are shown for each of the codons within the open reading frame of the cDNA. The single base change which should give no alteration in amino acid sequence in clone p5 is designated with an asterisk: 9 base differences giving rise to changes in 7 amino acids were noted among the 5 clones.

The A-type of cDNA (clones p6 and p12) is shown in the bottom part of the diagram. These two cDNA differed from clone 201 at 19 or more base positions within the open reading frame. The G→A substitution in the first position of codon 78 would result in the substitution of an asn(1) for asp and creates a potential site for N-glycosylation. These clones would also be expected to produce proteins of more basic pi than the A-type. Silent changes are again marked with an asterisk.

showed very little variability. A total of only 8 base changes were noted among all of the 7 clones (data not shown).

These data provide a basis to explain the multiple isoforms of oTP-1 and bTP-1. However, because the cDNA libraries were prepared from mRNA derived from several embryos, it is presently impossible to determine how many of the different cDNA shown in Fig. 2 and reported in earlier publications (Imakawa et al., 1987, 1989; Stewart et al., 1989) represent transcripts of different genes or allelic forms of the same gene.

Primary and deduced secondary structures of the trophoblast interferons

The trophoblast proteins oTP-1 and bTP-1 clearly resemble the IFN-α more than the more familiar IFN-α1 both in terms of the lengths of their polypeptides and in amino acid sequence (Imakawa et al., 1989). The similarity to the predicted sequence of a bovine IFN-α1 is 70% compared to a 47-54% identity for cattle, human, rodent and pig IFN-α1. However, oTP-1 and bTP-1 clearly resemble each other (~80% identity) more than they do the bovine IFN-α1. Nevertheless, they possess several regions of sequence conserved in all IFN-α molecules so far characterized, including the 4 cysteines which participate in intrachain disulphide bonds, and a stretch of peptide near the carboxyl terminus. Moreover, the sequence similarity of oTP-1 and bTP-1 to IFN-α occurs...
Polypeptides and genes of oTP-1 and bTP-1 throughout the lengths of their polypeptide chains and is not confined to specific regions. It should also be recognized that the IFN-α molecules are themselves a highly divergent group of proteins (Gillespie et al., 1984; DeMaeyer & DeMaeyer-Guignard, 1988). In general, they rarely show more than 60% sequence identity even between closely related species. Indeed, this diversity in sequence and multiplicity of the IFN-α genes themselves has raised questions as to whether their products might have subtly different functions.

A prediction of secondary structures by the procedure of Hopp & Woods (1981) has shown that oTP-1 and bTP-1 are likely to possess a considerable amount of alpha helical structure (Roberts et al., 1990) as do other IFN (Zoon & Wetzel, 1984). Hydrophilicity profiles for an oTP-1, a bTP-1, a bovine IFN-α, and a bovine IFN-αII are shown in Fig. 3 for the 166 residues over which these 4 proteins overlap in sequence. The profiles for each IFN are very similar, and the data suggest that the overall tertiary structures of these molecules are likely to be conserved, i.e. the molecules will have approximately the same shapes and dimensions even though minor details of their architecture may differ.

Do trophoblast IFN molecules constitute a distinct IFN subgroup?

Table 1 summarizes nucleotide sequence differences between the transcripts for oTP-1, bTP-1, a bovine IFN-α, and bovine IFN-αI in the 5'- and 3'-untranslated termini and within the protein coding region. These data illustrate two main points. First, it is clear that the oTP-1 and bTP-1 transcripts resemble each other more than they do the apparently related bovine IFN-αII transcript. Second, the region of greatest similarity between the oTP-1 and bTP-1 transcripts lies within the 3'-untranslated region of the molecules. This high degree of conservation in the 3'-ends persists when all the cDNA variants for oTP-1 and bTP-1 are considered (data not shown). By contrast, the same 3'-region provides the greatest degree of dissimilarity with the bovine IFN-αII transcript (see also Fig. 1). Although it is unclear why the 3'-ends of the trophoblast IFN mRNA should be so highly conserved, it seems likely to have some functional significance and suggested to us that these IFN proteins might belong to a distinct subgroup within the larger IFN-αII family.

It is possible to exploit the use of 3'-specific cDNA probes for oTP-1 and bTP-1 to distinguish mRNA for the trophoblast IFN from those for other IFN by carefully controlling hybridization conditions during in-situ hybridization procedures (Farin et al., 1989, 1990). By using such methodology the onset of oTP-1 and bTP-1 gene expression can be accurately pinpointed during
Table 1. Comparison of the base sequences within the 5' and 3' non-coding regions and within the coding regions of cDNA for bTP-1, oTP-1, a bovine IFN-α_H and a bovine IFN-α_I.

| IFN       | 5'-Non-coding (%) | Coding (%) | 3'-Non-coding (%) | Predicted amino acid sequence identity (%) |
|-----------|-------------------|------------|-------------------|-------------------------------------------|
| bTP-1     | 100               | 100        | 100               | 100                                       |
| oTP-1     | 76                | 90         | 92                | 80                                        |
| bIFN-α_H  | 72                | 85         | 70                | 72                                        |
| bIFN-α_I  | 53                | 63         | 53                | 50                                        |

The sequences compared were λbTP-509 (Imakawa et al., 1989), oTP-1 clone 201 (see Fig. 2), and the bovine IFN-α_H and -α_I genomic clones described by Capon et al. (1985). The 5'-non-coding region represents the stretch of nucleotides 5' to the ATG initiation codon. The 3'-non-coding region lies between the termination codon and the poly(A) tail. Sequence comparisons were only performed on aligned regions with gaps ignored. The last 18 nucleotides of bTP-1, oTP-1 and bIFN-α_I cDNA were compared with each other but were excluded in comparisons with α_I cDNA.

Fig. 4. In-situ localization of bTP-1 and actin mRNA in a bovine conceptus at Day 19 of pregnancy. (a) A bright-field micrograph (8 μm) stained with toluidine blue. Large arrow indicates trophoblast cells; small arrows show underlying extraembryonic endoderm. (b) A dark-field micrograph on an adjacent section which had been hybridized to [35S]oTP 266 cDNA. The latter has 92% identity with bTP-1 mRNA. Silver grains, indicative of a positive hybridization, appear white. Note that the positive signal is confined to trophoblast. (c) A section adjacent to those in (a) and (b) which had been hybridized to a [35S]α-I actin cDNA. Here a signal is also observed over the endoderm. (d) A control section which had been hybridized to 35S-labelled vector cDNA. Note the low numbers of silver grains over the tissue. For details of the procedures, including the specific activities of the probes, see Farin et al. (1990).
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Fig. 5. Southern blot analysis of restriction endonuclease-digested ovine genomic DNA with a full-length ovine cDNA for oTP-1 (a) or a cDNA representing the 3'-untranslated end of the mRNA (b). Sheep genomic DNA was digested to completion with the indicated restriction endonucleases and separated by electrophoresis along with uncut (UC) DNA. (a) A Southern blot hybridized with a full-length (FL) oTP-1 cDNA probe which probably recognizes both oTP-1 and IFN-α, and related gene sequences. (b) Hybridization was with a 3' oTP-1 probe (oTP-266; bp 650–912) which is specific for oTP-1. Densitometric scanning of the autoradiographs suggests that some of the darker hybridizing bands may represent restriction fragments containing multiple genes. Sizes of molecular weight markers are indicated in kb.

Embryonic development. These mRNA are confined to the trophoderm (Fig. 4) and begin to increase at around the time that the conceptus starts to elongate from a sphere to a longer thread-like form which in sheep is around Day 13 and in cattle around Day 15 (Farin et al., 1989, 1990).

A similar approach has more recently been used to identify specific bTP-1 and bovine IFN-α, genes. For example, we have screened a bovine genomic library with 3'-specific cDNA probes and isolated several bTP-1 and IFN-α, clones from cross-hybridizing bacteriophage plaques. The identity of each DNA insert was further confirmed by means of the polymerase chain reaction in conjunction with specific oligonucleotide primers, and the product was subcloned and sequenced. The bTP-1 genes, like those of IFN-α, and -α, were intronless. The protein coding region had between 95 and 100% sequence identities with known cDNA molecules for bTP-1. They possessed a TATA box at position −27 to −33 from the likely transcription start site and two putative viral response elements (VRE) (Fujita et al., 1987; Raj et al., 1989) 14 bp apart at base positions −69 to −74 and −88 to −93. The 5'-region of the bTP-1 gene was distinct in both overall sequence and in the placement of the VRE when compared with three IFN-α, genes. These results indicate that the bTP-1 genes have diverged significantly from related IFN-α, genes in their promoter regions.
Interestingly, however, they may still be potentially inducible by virus since they possess so-called virus response elements.

Specific 3'-end cDNA probes have also been used in Southern blotting experiments to determine the relative number of IFN-α II and trophoblast IFN genes. Genomic DNA derived from leucocytes of individual cows or sheep was digested with a range of restriction endonucleases, Southern blotted and allowed to hybridize with 32P-labelled cDNA probes representing oTP-1, bTP-1 or bovine IFN-α II. Figure 5, for example, shows that when a full-length oTP-1 cDNA was used to probe restriction endonuclease-digested ovine genomic DNA, at least 15 bands greater than 1 kb in length were detected in some of the lanes. However, when a more specific probe representing the 3'-noncoding end of the cDNA (bases 650–912) was used, a maximum of only about five cross-hybridizing bands were evident (Fig. 4B). Essentially similar results have been obtained with bovine DNA. These data again emphasize that there are several genes for oTP-1 and bTP-1 and that these genes may be distinct from the IFN-α II identified earlier by Capon et al. (1985).

Discussion

There have been several recent papers which have reviewed the evidence that the trophoblast IFN substances are mediators of maternal recognition of pregnancy in cattle and sheep (Bazer et al., 1986; Roberts et al., 1990). Although there seems little doubt that oTP-1 and bTP-1 function as antiluteolytic agents, it remains unresolved as to whether other compounds produced by the conceptuses are required to supplement the activity of oTP-1 and bTP-1 to cause an extension of the interoestrous interval for more than a few days. In addition, it is still not clear whether other IFN-α molecules can fully mimic the action of the trophoblast IFN on reproductive parameters (Plante et al., 1988; Stewart et al., 1989; Salamonsen et al., 1991). This question will be more easily answered once recombinant bTP-1 and oTP-1 become available in workable quantities.

The experiments reviewed in this paper do strongly suggest, however, that bTP-1 and oTP-1, although 172 residues long, are distinct from other IFN-α II proteins in a number of important respects. The very different organizations of IFN-α II and bTP-1 genes in the 5'-promoter regions indicate, for example, that the two types of IFN might be under distinct types of transcriptional control. Indeed, in preliminary studies we have shown that bTP-1 is very poorly expressed in Sendai virus-induced leucocytes and IFN-α I and -α II transcripts are rare in Day 19 trophoblast tissue (unpublished results). Nevertheless, the fact that the bTP-1 gene does carry putative viral response elements and that oTP-1 is inducible by synthetic double-stranded RNA in Day-11 sheep conceptuses (Roberts et al., 1990) is consistent with the hypothesis that genes for the trophoblast IFN are virally responsive, even if viral induction does not constitute the normal basis for oTP-1 and bTP-1 expression during pregnancy. It remains to be determined whether the uterine secretions of the pregnant ewe or cow contain factors responsible for the massive increase in transcription of the oTP-1 and bTP-1 genes around the time of maternal recognition of pregnancy.

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