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Interferons as Hormones of Pregnancy*

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I. Introduction

ABOUT 5 yr ago it was discovered that a protein which was secreted in quantity by preimplantation sheep conceptuses and implicated in preventing luteolysis during the second and third weeks of pregnancy was, in fact, structurally related to type I interferons (IFN), proteins best known for their antiviral and immunomodulatory activities. This discovery attracted considerable attention because it revealed a new and completely unsuspected role for a cytokine as a reproductive hormone. Several reviews have been published on various aspects of this topic (1–10). In the more comprehensive updated article that follows, we describe how IFN came to be implicated as antiluteolytic agents in domestic ruminant species, the properties of these IFN, and some of the unusual features pertaining to their expression and transcriptional regulation. We address the questions as to whether these IFN have any unique biological and structural properties, as to how they evolved, and as to whether type I IFN in general might have a universal role in mammalian pregnancy.

A few terms are used throughout this review with which the reader may not be entirely familiar. For example, we prefer to use *conceptus* rather than embryo, because the former term encompasses the trophoblast and fetal membranes, as well as the embryo proper. In later pregnancy, *fetal-placental unit* can be appropriately substituted for conceptus. From *Section III* onward we have also chosen to name the IFN produced by the preimplantation ruminant conceptus as IFNτ to distinguish it from the related IFNα, -β, and -ω. This abbreviation became necessary in order to avoid the continued use of older terms such as trophoblast proteins (including oTP-1 and bTP-1), trophoblastin, embryonic interferons, IFNα_{II}, and IFNα2 which either have value only in a historical context or else are inappropriate or incorrect. The classification, although not yet sanctioned by any official committee on IFN nomenclature, is most likely justifiable in view of the clear-cut differences between these IFN and the other type I IFN subfamilies (see *Section V.B*).

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II. The Trophoblast Proteins, oTP-1 and bTP-1, Are Hormones of Pregnancy and Related Structurally to Type I IFN

A. Preimplantation conceptus development and maternal recognition of pregnancy

After the egg has been fertilized, the conceptus of a eutherian mammal follows a programmed path of cell cleavage and development until the blastocyst is formed (11). In some species, including mouse and human, the hatched blastocyst immediately attaches to the uterine wall, trophoblast outgrowth begins, and implantation is initiated. In others, *e.g.* rabbit, the blastocyst enlarges considerably before the trophoblast begins to intrude through the uterine epithelium. Preimplantation development of the conceptus is even more extensive within the hoofed mammals, which include the domestic ruminants, *e.g.* cattle, goats, and sheep, and many monogastric species such as pigs and horses. In all these animals, the blastocyst continues to expand after hatching until it reaches a diameter of several millimeters. At about day 12–13 in sheep or day 14–15 in cattle, this spherical structure starts to elongate until it reaches a length of 15 cm or more, 3 days later, a process which places the trophoblast in direct apposition with much of the luminal uterine epithelium. During this remarkable phase of elongation, which is largely attributable to growth of the trophoblast, the outer cell layer, or trophectoderm, eventually begins to attach to the enclosing uterine epithelium, and implantation is initiated at specific sites (12–14), the caruncles, on the uterine wall where trophoblast binucleate cells become concentrated (15). The regions of fused trophoblast and maternal caruncular tissue ultimately develop into prominent, well vascularized structures known as placentomes, which are the sites of primary gas exchange and nutrient transport after the first 3 to 4 weeks of pregnancy. They are also sites at which trophoblast binucleate cells release placental hormones, such as the placental lactogens, toward the maternal vasculature (16).

In species where implantation occurs relatively late in development, the early conceptus is clearly capable of "communicating" with the mother well before erosion of the uterine epithelium has become apparent and without the trophoblast having direct access to the maternal vasculature (3). Therefore, the molecules involved in communication must either diffuse or be transported across the intact epithelium to enter the maternal circulation. Alternatively, they must elicit their effect locally, with the endometrium acting as a transducer of whatever primary response the conceptus initiates.

The term *maternal recognition of pregnancy* has been used in its broadest sense to describe how the mother responds to the presence of a conceptus within her reproductive tract and, particularly, how such events allow the pregnancy to advance successfully (17). One important outcome of such conceptus-maternal interaction in most mammals is extension of the functional lifespan of the corpus luteum (CL), an event that ensures continued production of uterotrophic steroid hormones, and progesterone in particular. This process creates a uterine environment conducive for continued development of the conceptus. Failure of the conceptus to signal its presence within the appropriate timeframe results in loss of the pregnancy. Rates of embryonic mortality resulting from such miscommunication between the mother and conceptus at the time of maternal recognition of pregnancy are believed to be high in farm animal species (18, 19), and such losses have considerable economic implications.

The timing of maternal recognition of pregnancy varies widely among different mammals, and the methods utilized for maintaining a functional CL of pregnancy also differ from species to species. In primates, the CL is "rescued" through the actions of a luteotrophic hormone, chorionic gonadotropin (CG), released by the trophoblast as it implants (20). Production of CG is readily detectable in maternal circulation by day 8–11 of pregnancy in humans when the maternal blood supply comes in contact with the embryonic trophectoderm (21). CG acts directly on the CL and is probably essential to sustain luteal cell function for the first month of pregnancy, by which time placental progesterone production begins to be sufficient to maintain the pregnancy (22). In the absence of a developing conceptus, the CL undergoes a spontaneous structural regression through currently unknown mechanisms.

There is little evidence for a placental gonadotropin in domestic ungulates such as pigs, sheep, and cattle, at least in the early stages of pregnancy. In these animals, the initial role of the conceptus appears to be in the prevention of luteolysis rather than in luteal maintenance. Luteal regression does not occur spontaneously in cyclic females, but requires the episodic release of prostaglandin $F_{2\alpha}$ (PGF) originating from the uterine endometrium (23–26). The hormonal regulation of PGF production during the nonfertile cycle in ruminants has been extensively reviewed and likely involves control by oxytocin originating from the posterior pituitary and/or the CL (26, 27). PGF released by the endometrium travels by a counter-current mechanism via the utero-ovarian vasculature to the ovary where it interacts with specific cell surface receptors to promote rapid regression of the CL during the late luteal phase of the estrous cycle.

B. Antiluteolysins of sheep and cattle

In domestic ruminants, luteal regression is thought to be prevented in early pregnancy by a conceptus-mediated

suppression of episodic PGF production or release (24–27). The sequence of events leading up to the identification and purification of the primary ovine and bovine conceptus products involved with this process has been reviewed extensively (1–10) and will not be discussed in detail here. However, several significant contributions to their identification are worthy of mention. Early investigations indicated that this conceptus signal was proteinaceous, based on its heat lability and susceptibility to protease inactivation. It was also shown to be secreted transiently by the sheep conceptus between days 12 and 21 of pregnancy, and had to be recognized by the mother before day 13 to prevent resumption of a normal luteal cycle.

In 1982 Godkin *et al.* (28) described the purification of a low molecular weight protein released into culture medium by day 13–21 ovine conceptuses. This secretory protein, eventually designated ovine trophoblast protein-1 (oTP-1) (29), which in retrospect was probably identical to trophoblastin, a protein partially characterized 3 yr earlier by Martal *et al.* (30) from conceptus homogenates, consisted of several isoforms with mol wt of about 18,000, and isoelectric points ranging from 5.3 to 5.7 (28, 29). Shortly thereafter, it was demonstrated that bovine conceptuses produced a similar complex of proteins [bovine trophoblast protein-1 (bTP-1)] (31) that cross-reacted with oTP-1 antiserum (32), although each component was of slightly higher mol wt (22,000 to 24,000). The differences in size between oTP-1 and bTP-1 variants were later attributed to the presence of carbohydrate on the bovine, but not the ovine TP-1 (33, 34).

Infusion of oTP-1 into the uteri of nonpregnant ewes resulted in an extension of luteal lifespan comparable to that observed with total conceptus secretory products (35, 36) and led to a blunting of the pulsatile release of PGF from the uterus (37). Furthermore, infusion of conceptus secretory products from which oTP-1 had been removed by immunoadsorption failed to increase the interestrous interval (36). These observations, plus the fact that oTP-1 production was limited to the critical window of maternal recognition of pregnancy, strongly implicated oTP-1 as the ovine antiluteolysin (and by analogy, bTP-1 as the bovine antiluteolysin). More recently, the goat conceptus has also been shown to produce an abundant caprine TP-1 protein (cTP-1) (38, 39).

Unlike CG of primates, oTP-1 does not enter the maternal circulation in appreciable quantity (*Section IV.D*) and has no direct luteotrophic effects (29). Instead, it acts locally upon the uterus (24, 25). Interestingly in sheep, basal PGF production is not eliminated, and circulating levels of the PGF metabolite, 15-keto-13,14-dihydro-PGF, can be higher in pregnant ewes than in cyclic ewes (40–42). However, in pregnant animals, pulses of PGF are diminished, and the CL remains functional for much of the pregnancy. The exact mechanisms whereby the TP-1 of sheep and cattle modulate PGF production or release are not yet known, although several possible models will be discussed later in this review (*Section III.B*). Whether the conceptus produces additional factors that cause the CL to remain refractory to the tonic release of PGF by the uterus during pregnancy is also currently unknown. Recent data have suggested that the peri-implantation ovine conceptus secretes a protein that protects luteal cells from the luteolytic action of PGF, but the identity and mode of action of this product remain to be elucidated (43). In addition, low molecular weight metabolites, such as PGE, released by the conceptus, and known to be weakly luteotrophic, may play some role in luteal maintenance (44). Clearly, factors other than the TP-1 must be involved with luteal maintenance later in pregnancy since administration of oTP-1 or bTP-1 extends luteal lifespan for only days to a few weeks (35, 36), whereas the CL normally persists throughout pregnancy.

C. Molecular cloning and identification of trophoblast proteins as interferons

The identification of oTP-1 and bTP-1 as type I IFN was unsuspected and was the result of molecular cloning of cDNA (45, 46) and protein sequencing (47, 48) procedures. The discovery revealed a novel role for IFN in the normal physiology of early embryonic development. As will be discussed throughout the remainder of this manuscript, these trophoblast IFN (IFNτ) possess a number of unique features which not only suit them well for their role in the establishment of pregnancy but also distinguish them from other well characterized type I IFN. Since those original reports, a large number of additional cDNA sequences have been published (49–54). All of these cDNA have certain common features: they are about 1 kilobase in length and possess 585 base pairs (bp) open reading frames that code for 195 amino acid polypeptides. Ultimately these isoforms will have to be classified as ovIFN-τ1, ovIFN-τ2, etc.

IFN were so named because they could confer resistance to cells to subsequent viral infection (55, 56). Unfortunately, such a functional definition has tended to underplay the involvement of IFN in modulating processes such as cellular differentiation, proliferation, and immune function (56–58) as well as in reproduction (59). It also led to the classifying together of quite different molecules simply because they displayed similar antiviral activity. Subsequently it has become clear that there are at least two different types of IFN, types I and II (Table 1) which bear little structural resemblance to each other.

In those mammalian species where it has been studied, type II IFN is represented by a single gene and is better

known as IFNγ (56). Although often considered to be primarily a product of T cells and to function in activating macrophage, IFNγ (or immune IFN) is released by a variety of cell types, including porcine trophoblast (60, 61) and has broad pleiotropic effects (62). There is no evidence, however, that it functions as an antiluteolysin in pigs (see *Section IV.B*).

Type I IFN, by contrast, consists of at least three distinct subtypes (α-, β-, and ω-) (Table 1). Although each of these possesses similar biological properties, presumably due to their binding to a common receptor (*Section III.C*), each subtype differs considerably in amino acid sequence and serological properties (56, 63–66). Each subtype can be encoded by multiple genes, and the different gene products within a subtype such as the IFNα, for example, can themselves differ significantly in sequence and most probably in biological potency (56). In cattle there are at least 15 known α-genes (63), 9–11 β-genes (67), and at least 15–20 genes related to the IFNω (63).

The various boIFNτ gene products of cattle have less than 30% sequence identity to IFNβ and only about 50% identity to known bovine IFNα (46). The greatest similarity (~70%) is to IFNω (previously known as IFNα_{II}) . However, boIFNτ and ovIFNτ resemble each other in both amino acid and nucleotide gene sequence more than they do the IFNω within their own species. Thus, there are compelling reasons to consider IFNτ as a distinct structural subtype (see *Section V.B*).

The sequence of one isoform of ovIFNτ, which was deduced from a complementary DNA, is shown in Fig. 1A. As in all IFNα (56, 65) and in the 172-amino acid IFNω (68), the NH_2-terminal residue is a highly conserved cysteine that is probably in an intramolecular disulfide linkage with cys^{99} (69). A second disulfide most likely links cys^{29} and cys^{139}. The latter pair of cysteines are found on all type I IFN, and the intact disulfide is essential for biological activity. In addition to these four cysteines, there are 16 additional amino acids that are highly conserved across all known IFNα, -β, and -ω (70, 71). Significantly, all 20 of these amino acids are placed identically in most IFNτ (Fig. 1A and 1B).

TABLE 1. Description of IFN subtypes

	Type I	Type II
Acid stable:	Yes	No
Inducer:	Virus	T cell activation
Cell of origin:	Several	T cell[a]
Subtypes:	Alpha (multiple) Beta (single)[b] Omega (multiple)	Gamma (single)
Genes:	Intronless	3 introns
Chromosomal location (human):	Clustered on chromosome 9	Chromosome 12

[a] Also produced by pig trophoblast.
[b] Multiple genes in ungulates.

As noted earlier, it is curious that there is so much diversity in amino acid sequence among the type I IFN. IFNα isoforms within a single species can vary by as much as 20% or more in overall sequence. Despite these apparent differences, however, hydrophilicity-hydrophobicity plots of the primary structures of any chosen type I IFN are barely distinguishable (3, 72). All seem to possess quite similar predicted secondary structures (3, 71–73). It is unfortunate that at the time of writing, precise three-dimensional conformations of IFNα and -β are not available. However, a representative murine IFNβ has been crystallized (74, 75), and preliminary data on its structure have been published as an abstract (75). Theoretical conformation analyses suggest that all type I IFN fold similarly and probably consist of five α-helices (A through E; Fig. 1B) arranged in an antiparallel manner and connected by loop regions (71–73). Although the precise packing topology of the helices is unclear, the five-helix structure shown in Fig. 1B is probably roughly correct and can provide a useful working model as to how type I IFN interact with their common receptor (*Section III.C*). The positioning of the poorly conserved carboxyl "tail," which is six to seven residues longer in IFNω and IFNτ than in IFNα or -β, is particularly difficult to predict, however, and is shown in Fig. 1B as a projection that extends downward from helix E, although it may fold back across the molecule.

The ovIFNτ molecule in Fig. 1 possesses a potential site for N-glycosylation on Asn^{78}. However, not all predicted ovIFNτ sequences have this motif (51), and there is no biochemical evidence that any of the isoforms is a glycoprotein (28, 32–34). On the other hand, boIFNτ (33, 34) and some forms of caprine TP-1 (39) are N-glycosylated, presumably on Asn^{78}, a residue that is probably close to the junction between helix B and the loop connecting helix B to C. Variability in glycosylation is not uncommon within IFN subtypes (56), but its significance is unclear. An aglycosyl recombinant form of boIFNτ has been prepared in *Escherichia coli*, is relatively stable, and possesses biological properties typical of other type I IFN (76).

III. Bioactivities of the Trophoblast IFN

A. Antiviral, antiproliferative, and immunomodulatory properties

The structural similarity of the IFNτ to other type I IFN has been born out in their biological properties. They can, for example, protect several cell types known to possess type I receptors from lysis by a range of viruses, including the commonly employed vesicular stomatis virus, with at least an equivalent potency to IFNα

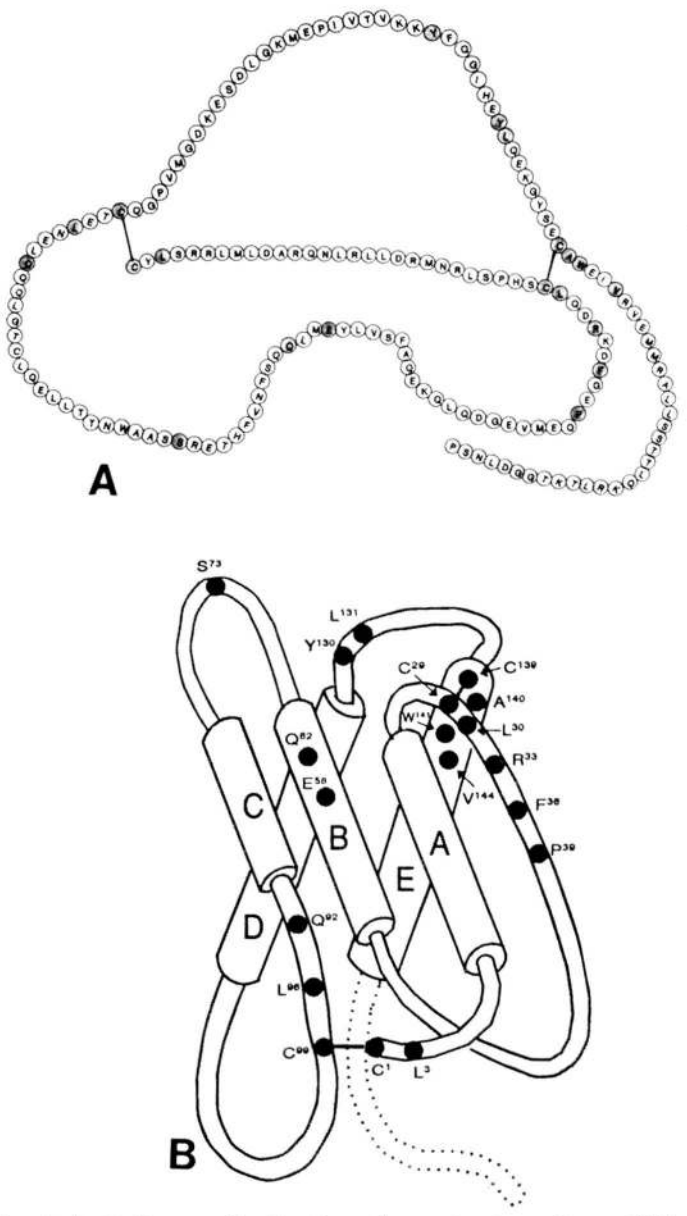

FIG. 1. A, A diagram showing the primary structure of an ovIFNτ deduced from the cDNA sequence oTP-1p6 of Klemann *et al.* (51). The *shaded circles* indicate amino acids that are conserved in most known type I IFN. Disulfide bonds are indicated by *lines*. The NH_2-terminal residue is Cys while the COOH-terminal residue is Pro.

B, A diagram showing a predicted three-dimensional structure of a type I IFN. This model is based roughly upon the theoretical conformation analysis of Zav'yalov *et al.* (71, 72) and a brief written description of the crystal structure of mouse IFNβ provided by Senda in an abstract (75). The former predicted a three-dimensional structure of five antiparallel helices similar to that of IL-2. Senda *et al.* (75), however, showed that in IFNβ, unlike IL-2, the helices A and B were parallel to each other and that helix A was considerably longer than had been predicted. The five α-helices are A (residues 6–23), B (50–65), C (79–88), D (112–129), and E (137–152). The 20-residue carboxyl tail, whose conformation on IFNω and IFNτ is unknown, is shown with a *broken line* extending below the molecule, but could well fold back across the helical core. The Cys^1-Cys^{99} disulfide bridge is not present on all IFNβ but is present on other type I IFN. Seventeen of the 18 highly conserved residues, with Y^{123} hidden on helix D behind helix B, as well as Cys^1 and Cys^{99}, are shown as *closed circles*. Note the highly conserved region where the A-B loop (24–49) and helix E are in close juxtaposition.

(76–82). The IFNτ can also markedly slow the proliferation of bovine kidney epithelial cells (78, 83) and human WISH cells (83) over roughly the same concentrations at which most IFNα are growth inhibitory. However, at high concentrations, ovIFNτ has been noted to be far less cytotoxic than either boIFNα or a huIFNα preparation (83). Consequently, IFNτ have been claimed to constitute a "cell friendly" class of IFN (83, 84). Such claims for unique properties of IFNτ, however, must be balanced against observations of striking differences in pyrogenicity, cytotoxicity, and antiproliferative properties between individual human IFNα subtypes, particularly when cross-species comparisons have been made (reviewed in Ref. 85).

The IFNτ strongly inhibit incorporation of [^{3}H]thymidine into lymphocytes after exposure to mitogens (78, 86–89) or after placement in culture with "foreign" lymphocyte populations (89). Again, however, IFNτ and IFNα provide comparable effects over a similar range of concentrations, and there has certainly been no evidence that either ovIFNτ or boIFNτ exhibit any unusual biological properties that set them aside from other type I IFN.

The IFNτ are capable of up-regulating at least one of the enzymes that has been implicated in the metabolic responses of cells to type I IFN. Endometrial synthesis of 2,5-oligoadenylate synthetase, for example, is markedly enhanced by IFNτ (90–92). However, an almost equivalent response occurs with hu- and boIFN-α.

Both boIFNα and IFNτ, with about equal potency, markedly up-regulate a highly acidic 70,000 Mr secretory protein in both ovine endometrial explants (29, 93, 94) and cultured uterine epithelial cells (95). This same protein has been observed to be a product of endometrium in contact with trophoblast only during the period of ovIFNτ production (96) and to be induced when nonpregnant ewes are injected with boIFNα (94). It remains to be identified functionally.

All the above data show that the IFNτ are bona fide type I IFN with little to distinguish their biological properties from those of IFNα.

B. Comparative effects of IFNτ and IFNα on reproductive parameters

The most striking biological effect of the IFNτ, oTP-1, and bTP-1 has been their ability to prolong the estrous cycle and lifespan of the CL when infused into the uteri of nonpregnant sheep or cattle (35, 36, 79, 97–99). Purified "natural" ovIFNτ, which consists of at least three to

four distinct isoforms, and recombinant products, which are, of course, products of single genes, appear equally effective in this regard (79, 97). The daily intrauterine infusion of either type of preparation into nonpregnant ewes was able to extend estrous cycle length from days to weeks relative to control ewes. The concentrations chosen in the sheep experiments (100–250 μg/day) had been calculated to approximate the daily amount of the mixture of isoforms of ovIFNτ secreted by conceptuses at the peak time of production (around day 15). By contrast, however, boIFNα was ineffective in ewes when used at such low concentrations (100) and was able to induce only a minimal response (2 to 3 days extension) in both cattle (101, 102) and sheep (100) when doses exceeding 1 mg/day were employed. Relatively large intramuscular injections also delayed luteolysis for a few days in both species (102, 103) but never for an extended period. Strikingly, however, such an injection regimen of boIFN-α maintained over the day 11–18 period, when IFNτ would normally be produced, has been used successfully to improve fertility in ewes in a series of breeding trials (104–106). The rationale for these experiments was that the injected IFN would supplement the activity of endogenously produced oTP-1 and ensure a complete maternal response in the mother in cases where the conceptus might be small or otherwise lagging in its development.

Although intramuscular injections of boIFNα also produced a slight increase in estrous cycle length in cattle (102, 103), a comparable beneficial outcome on fertility has not been observed, and serious side effects were noted in animals following the initial injections (103, 107, 108). The symptoms, fever and malaise, were not unlike those noted after IFNα therapy in humans (85), and the elevation in body temperature may have contributed to embryonic loss. It will be of interest if IFNτ, which can now be readily prepared by a variety of recombinant procedures (76, 79–81, 109) and in practicable amounts for animal experimentation, have similar adverse side effects when injected intramuscularly into cattle. Conceivably if they are less pyrogenic than boIFNα1, they might become useful fertility agents. As mentioned earlier, different species are known to vary markedly in their responses to foreign IFN, and individual IFN subtypes can elicit widely different pyrogenic effects even in the same species (85).

What underlies the ability of the IFNτ to prolong luteal lifespan and why are the IFNτ so much more efficient than IFNα in this regard? As outlined earlier, all evidence is consistent with the view that luteolysis in large ruminant species is prevented by intervention in the pulsatile production or release of PGF by the endometrium (reviewed in Refs. 26 and 27). Intrauterine administration of either whole conceptus secretory proteins or purified IFNτ reduces the number and amplitude of luteolytic pulses of PGF (25, 37, 110). Nevertheless, despite somewhat conflicting evidence that type I IFN, including IFNτ, can inhibit PGF synthesis (91, 111–114), the endometrium of pregnant ewes (but not of cattle) appears to produce as much or possibly even more basal amounts of PGF than cyclic ewes (26, 27). Therefore, the antiluteolytic effects of IFNτ in ewes may have more to do with the release rather than inhibition of biosynthesis of prostaglandin. In this regard, attention has now shifted to the control of PGF pulsatility and to the role of oxytocin in this process. This small peptide, by binding to its surface receptor, is believed to induce PGF release by the endometrium and is probably responsible for the pulsatile nature of the response (26, 27, 115, 116). In ewes, oxytocin receptors increase in the critical day 12–14 period preceding the onset of luteolysis but are downregulated during pregnancy and after IFNτ administration (26, 88, 115–117). As a consequence of these data, it has been suggested that the buildup of oxytocin receptors that occurs in the estrous cycle is reversed by IFNτ treatment (84).

C. Interaction of IFNτ with type I receptors

High affinity, low capacity binding of ovIFNτ to receptors in endometrium was first reported by Godkin *et al.* (29). These receptors were assumed to mediate the complex physiological responses, including the marked changes in protein synthesis that occurred when the endometrium was first exposed to ovIFNτ during early pregnancy (29). Stewart *et al.* (47) later showed that purified ovIFNτ displaced [^{125}I]huIFNα from such receptors and thereby confirmed that the former was a type I IFN. Equilibrium-binding studies have revealed dissociation constant (K_d) values in the range of 0.1×10^{-10} M to 0.4×10^{-10} M (47, 118, 119), values that seemed to vary slightly according to the stage of the estrous cycle (119). In addition, some evidence has been provided for a group of lower affinity sites ($K_d \sim 10^{-10}$ M) (118) and may correspond to the ones first noted by Godkin *et al.* (29). However, since secreted ovIFNτ is a mixture of at least four isoforms, it is possible that the noted complexity of binding was the result of varying affinities of different subtypes for a single receptor, a phenomenon well known among the IFNα (56, 120, 121). The binding constants observed were well within the broad general range of those expected for specific binding type I IFN to receptor (120).

One anomalous observation regarding IFNτ binding to its receptor needs to be explained. Hansen *et al.* (118) used the bifunctional cross-linking reagent disuccinimidyl suberate to show that [^{125}I]ovIFNτ became associated with at least two polypeptides (Mr ~ 100,000 and ~

70,000) and could be displaced from both by boIFNα. By contrast, boIFNα could only be cross-linked to the larger binding protein. Since proteolysis did not appear to be responsible for creating the two polypeptides, these experiments can be interpreted in at least two ways. Either there is a unique IFNτ receptor polypeptide (in which case the displacement by boIFNα is hard to explain),or else the oTP-1 employed possessed reactive lysine residues that were more in a position to react and form cross-links to the 70,000 Mr protein than equivalent residues in boIFNα. The latter explanation implies that the IFN receptor may always consist of more than one polypeptide. Thus, one of the two "subunits" (most likely the 100 K form) probably corresponded to the 557 amino acid transmembrane glycoprotein cloned by Uze *et al.* (122). Transfection experiments have shown that additional components are most likely required for this receptor polypeptide to assume high affinity binding and to mediate biological responses (122, 123). The second subunit (the 70 K form) may be such an accessory protein that becomes fortuitously cross-linked to one or more of the isoforms of ovIFNτ, but not to boIFNα. The two binding proteins do not constitute a unique endometrial receptor, since a similar pair of polypeptides was noted when ovIFNτ (but again not when boIFNα) became cross-linked to spleen receptors (118). Multiple subunit receptor complexes have recently been described for a broad range of cytokines (124, 125).

The likely manner in which type I IFN, including IFNτ, interact with receptor polypeptides is still unclear. Large numbers of experiments have been carried out over the past decade to map regions of the IFNα molecule that are crucial for either receptor binding, biological activity, or both. Approaches have included a comparison of naturally occurring allelic subvariants that exhibit very different activities (126, 127), alterations in specific residues, or whole domains on the IFN by site-directed mutagenesis (128, 129), inhibition of binding or activity with antibodies directed to specific epitopes (130, 131), and competition binding studies with specific peptides (132). These results are best interpreted by examining the model shown in Fig. 1B and can be briefly summarized as follows. The disulfide bond 39–139 is always required for activity, while the 1–99 bond is necessary in only some IFNα (in others the entire NH_2-terminus can be eliminated). At least part of helix A and the well conserved loop between helices A and B participate in receptor binding and contribute to antiviral potency and antiproliferative activity. A carboxyl-terminal domain stretching from at least the middle of helix D to the end of helix E interacts with the receptor and is also necessary for biological activity. The carboxyl tail, from residue 152 to 162 in IFNα, appears to be largely disposable. It should be emphasized that not all substitutions within the crucial regions may necessarily have an effect. Similarly, some changes outside those discussed can measurably change receptor binding affinity, possibly by subtly altering packing or folding of the structure.

Very few complementary studies have been performed to confirm these general rules with the IFNτ. Pontzer *et al.* (133) showed that relatively high concentrations of an NH_2-terminal peptide (residues 1–37) negated antiviral activity of ovIFNτ but not that of "natural" IFNα. Antibodies to this domain also inhibited ovIFNτ receptor binding. By contrast, a carboxyl- terminal peptide (residues 139–172) effectively inhibited the antiviral activity of both kinds of IFN subtypes. It was concluded that the NH_2-terminal domain of ovIFNτ binds to a unique site on the receptor complex. However, these observations are clearly open to more than one interpretation and do not in themselves contradict the general rules of IFN-receptor interaction discussed above.

The data are consistent with the view that IFNτ binds to the same receptor or receptor complex as other type I IFN with roughly similar affinity. It remains to be confirmed whether some of the unusual properties of IFNτ, such as their ability to extend the length of the estrous cycle, depend upon interaction with a secondary accessory protein on the receptor complex.

D. Second messenger systems for the IFNτ

Type I IFN appear to initiate their effects through activation of latent transcription factors that then bind to IFN response elements on selected target genes (134, 135). Considerable controversy exists, however, about the signal transducing pathway utilized after the IFN first binds to its receptor. The one receptor polypeptide so far identified (122) carries no obvious intracellular protein kinase or phosphatase domains and bears no resemblance to the seven-helix receptors known to interact with G proteins. The signaling components may well reside on one or more of the so far uncharacterized accessory proteins and activate a completely novel signal transduction system. Evidence has been presented for IFN activation of a protein kinase C and for diacylglycerol production in human lymphoblastoid cells (136), while a transient stimulation of phospholipase A_2 with a rapid accompanying release of arachidonic acid has been reported in mouse fibroblasts (137). The latter pathway might be expected to increase rather than decrease prostaglandin synthesis in endometrial tissue.

Information on second messenger system activity in the uterus is limited. cAMP concentrations fell and cGMP increased slightly in endometrial explants from nonpregnant ewes exposed to ovIFNτ, while $(Bu)_2$cAMP antagonized the specific effects of the interferon on endometrial protein synthesis (94). The phosphatidyli-

nositol cycle has been reported to be stimulated in some experiments (138), but in others (139) ovIFNτ appeared to inhibit the rise in inositol triphosphate and total inositol phosphates that occurred when endometrial tissue from day 16 of the cycle was exposed to oxytocin, a hormone that stimulates prostaglandin release (26, 27). In summary, the signal transduction systems employed by type I IFN in general and by IFNτ in particular are unclear. This situation may remain confusing until more is known about the receptor system as a whole.

IV. The Expression of IFN During Pregnancy

A. Are Type I IFN expressed universally by embryonic and placental tissues?

The first report of IFN expression by uninduced tissues from the feto-placental unit was in mice (140). Since that time these observations have been extended to several other mammalian species including the ruminants, hamster, pig, and human and have been summarized in other reviews (3, 10). The finding that a potent cytokine is expressed at the placental-maternal interface has prompted the suggestion that IFN must play a universally important role in the establishment and/or maintenance of pregnancy (59). A likely function of these IFN has been proposed to be the modulation of the maternal immune system (141, 142), but data in support of this hypothesis are lacking, and no specific target cell type has been defined. Certainly, however, in species outside the *Bovidae* there is no evidence that type I IFN have an antiluteolytic role. In pig, for instance, infusion of whole conceptus secretory proteins failed to extend estrous cycle length in nonpregnant gilts (143). Indeed, estrogen appears to be the primary antiluteolysin in swine (25).

It should also be stressed that, while IFN may be expressed by conceptuses and placental tissues of many species, the nature and amount of the IFN released differ markedly. From a comparative standpoint, those IFN that are produced when placentation first begins have been of particular interest, because it is at this stage when the attention of the maternal immune system may be first drawn to the presence of the conceptus. Antiviral activity, suggestive of IFN, is produced by peri-implantation conceptuses of several nonruminant species including mice (144), pigs (60, 61, 145, 146), horses (147), and rabbits (148). Whereas ovine and bovine conceptuses produce several million units of antiviral activity in culture (10, 149, 150), maximal production of antiviral activity reported for these other species is 1,000- to 10,000-fold less. Such low amounts probably explain why some groups have failed to detect any production of antiviral activity by preimplantation equine (151), human (151), and mouse conceptuses (152, 153). With day 4 mouse blastocysts, for example, antiviral activity was only detected by direct coculture of conceptuses with target cells (144).

Our understanding of the antiviral activities that are produced around implantation in nonruminant species has been hampered because the factors have often been incompletely characterized both serologically and biochemically. Antisera against various IFN subtypes have been used for identification purposes, but it is clear that such data can be misleading, since these assays cannot always distinguish between legitimate IFN and other substances that may simply induce the target cells in the antiviral assay to produce IFN themselves (154). The use of DNA probes to detect and clone IFN mRNA from conceptus tissues should soon begin to advance the field greatly. At present this approach has not been successful in species other than the pig, in which the primary IFN produced by peri-implantation conceptuses is IFNγ (60, 61). Coexpression of a type I IFN also occurs, however, since an antiserum against human IFNα detected a 22,000 Mr protein on Western blots of conceptus culture medium (145), and a different antiserum against human "leukocyte IFN" partially neutralized the antiviral activity produced by conceptuses (61, 146). On the other hand, a porcine IFNα DNA probe did not detect transcripts in Northern blot analysis or cDNA library screening (Cross, J. C., and R. M. Roberts, unpublished results). Screening of a porcine genomic library with a porcine IFNω probe has identified an unusual kind of type I IFN gene that is related to IFNω but clearly distinct from IFNα and IFNτ (155). It is still unclear, however, whether this IFNω represents the type I IFN secreted by the porcine conceptus. Together these data imply that the pig may be quite different than the ruminants in the kind of IFN expressed and in the roles these IFN play, despite the tempting desire to consider all species similar and to present a unifying hypothesis (59).

Immunoneutralization antiviral assays of mouse (144) and horse (147) conceptus culture media all implicated a type I IFN as being responsible for the antiviral activity. Extensive Northern blotting and *in situ* hybridization analyses have been conducted with murine IFNα and -β, and equine IFNα and -ω probes in their respective species, as well as IFNτ probes across species, in an effort to identify transcripts related to type I IFN. To date all have failed (Ref. 144 and Farin, C. E., and R. M. Roberts, unpublished data). These experiments indicate either that the IFN produced were not the type I IFN subtypes that were addressed, or that the mRNA were present at extremely low levels.

The conclusion from studies in several mammalian species is that IFN genes are expressed during the peri-implantation period of normal pregnancies. The implication, therefore, is that cells within the conceptus at this period differentiate and produce IFN, often in very

small amounts, either as a consequence of an autonomous developmental cue or in response to a maternal stimulus that is universally present during mammalian embryogenesis. Thus, the phenomenon might be expected in some of its aspects to mimic the normal induction pathway for the IFN genes by virus, and other virus-responsive IFN genes might, therefore, be anticipated to be minimally expressed during the peri-implantation period.

B. Maturation of the IFN system during embryonic development

It has been recognized for several years that the ability of cells to respond to both IFN and IFN inducers is dependent on the state of differentiation of those cells. For example, chicken embryo cells from later developmental stages produce more IFN in response to an inducer than cells from earlier stages (156). Similarly, chicken embryo cells produce more IFN after being "aged" *in vitro* (157). Several mammalian embryonal carcinoma (EC) cells have been established that share features with pluripotent embryonic stem cells (158). Undifferentiated EC cells are completely nonresponsive to IFN and to IFN inducers, whereas EC cells induced to differentiate, *e.g.* with retinoic acid, gain the ability to respond to IFN and to produce IFN (159–162). The lack of responsiveness to IFN may not be due to a lack of IFN receptors on these cells (163) but rather may relate to a defect in the activation of the IFN-induced antiviral mechanisms, such as the absence of double-stranded RNA-dependent protein kinase.

The maturity of the IFN system is also linked to the developmental age of the conceptus. For example, murine conceptus tissues could be virally induced to produce antiviral activity, but expression was restricted to tissue derived from outside the inner cell mass, *e.g.* trophoblast, and the induced antiviral activity was not apparent until after day 7 of gestation, approximately 2 days after implantation (152). Preimplantation murine conceptuses were not protected by IFN from viral challenge (164). Few studies have been done with conceptuses from other mammalian species, although day 10 bovine conceptuses were not protected by IFN from viral infection *in vitro* with vesicular stomatitis virus (165). Treatment of similar stage conceptuses with the IFN inducer poly(I):poly(C) did elicit the antiviral response, however, although the nature of the IFN, *e.g.* whether it was IFNτ, was not determined (166). The apparent contradiction between these two studies suggests that maturation of the IFN system, *i.e.* the ability to produce and to respond to IFN, may occur around day 10 in the bovine conceptus.

The discovery that IFN inducibility is restricted to trophoblastic cells in the murine conceptus is intriguing in view of the highly localized expression of IFNτ to the ovine and bovine trophectoderm (*Section IV.D*) and suggests a basic difference between these cells and those of the inner cell mass. Few studies have examined IFN inducibility in trophoblast. Primary cultures of partially purified cytotrophoblast from human term placentas can produce a small amount of IFNβ in response to poly(I):poly(C) (167). Syncytiotrophoblast produced less antiviral activity than the nonfused cytotrophoblast cells, and the transformed trophoblast cell line, JAR, was even less responsive.

Experiments using mammalian conceptuses and EC cells all suggest, therefore, that the maturation of the IFN system is dependent on a certain degree of cellular differentiation, and that IFN-competent cells possess a repertoire of inducible transcription factors distinct from that in undifferentiated cells. Present information on how IFN-inducible genes and the IFN genes themselves are regulated now suggest that the two pathways are interconnected, perhaps by a common set of transcription factors that includes interferon regulatory factor-1 (IRF-1) (134). It would be anticipated, therefore, that both the onset in the responsiveness to IFN and the inducibility of IFN genes themselves would occur coincidentally during development. The details on how the IFN system matures during embryonic development are only now beginning to emerge.

The process of EC cell differentiation is associated with the loss of a nuclear protein able to bind the IFNβ promoter (168). This result suggests a model in which a dominant repressor protein complex inhibits activation of the IFNβ promoter in the undifferentiated state. Consistent with such a "dominant repressor model" is the observation that the differentiation process is associated with the development of DNase I sensitivity in the IFNβ promoter (169). The DNA element that serves as the putative repressor binding site is a degenerate form of the "octamer" sequence (170) and is present close to the upstream boundary of the virus response element of the IFNβ promoter (*Section IV.C*). The identity of the inhibitory factor is unclear, although octamer-binding proteins that are expressed in undifferentiated, but not differentiated, EC cells have been described (171).

Recently it has also become apparent that at least one of the transcriptional control systems involved in type I IFN expression matures coincidentally with EC cell differentiation (172). In undifferentiated EC cells both IRF-1 (transactivator) and IRF-2 (repressor) are absent, and type I IFN genes are not virally inducible. Overexpression of IRF-1 alone, however, induces IFN gene expression and affords virus inducibility. After differentiation, both IRF-1 and IRF-2 are expressed in EC cells, and the IFN system becomes both inducible and subject to down-regulation.

What do these observations tell us about the control of IFNτ expression during embryogenesis? They may explain why the genes are not transcribed in the inner cell mass, presumably as a result of silencing by one or more major repressors that may affect all type I IFN genes. Second, there may be an absence of potential transcriptional activators such as IRF-1. The studies also suggest that at about the time the IFNτ are first expressed constitutively, when trophoblast differentiation is being initiated, the IFN system as a whole matures and other type I IFN become potentially inducible by virus.

C. General features of transcriptional regulation of IFNα, -β and -ω

Although there are reports of low constitutive expression in a variety of tissues (173–175), type I IFN genes generally appear to be silent until induced by virus and thus play a major role in host defense against viral infection. A range of other factors, including several cytokines, can also act as inducers in certain cell types (56, 176).

Even though primary regulation of IFNα and -β is transcriptional, expression is invariably transient, rarely lasting more than a few hours even in continuous presence of inducing agent (56). Thus, the initial high rate of mRNA production is quickly repressed, and IFN synthesis slows. The short half-lives of the transcripts (177, 178), which have adenosine/uridine rich destabilizing motifs in their 3′-untranslated termini (see Refs. 46, 176, 179, and 180), presumably contribute to this sharp drop in IFN expression. These general rules do not appear to apply to IFNτ, which are only weakly inducible by virus and whose synthesis appears to be sustained over a period of days rather than hours.

Transcription of the IFNβ gene has been intensively studied (see Ref. 181) because in most species it is represented by a single copy that is inducible in cultured fibroblasts. Transcriptional control is complex, even though only the promoter region up to about position -120 above the transcriptional start site is required for high levels of virus induction. Within this region there are at least four overlapping but distinct positive regulatory domains (PRD I though IV) that have been implicated in virus inducibility. Flanking the entire virus response element are two negative response domains that maintain the gene in a silent state under noninducing conditions. Each of the PRD is capable of conferring viral inducibility when multimerized upstream of reporter genes but also appear capable of responding to various nonviral inducers (181, 182). For example, PRD I is IFN-inducible while PRD II and IV are responsive to phorbol esters and cAMP, respectively. Not surprisingly, several transcription factors have been identified which interact with the IFNβ promoter, most notably NF-κB (183), which binds PRD II, and interferon regulatory factor-1 (IRF-1) (184, 185), which interacts with at least three purine-rich regions containing the motif AA(G/T)(T/G)GA overlapping all four PRD (Fig. 2). IRF-2, a repressor, can bind to these same sequences and may, in part, be responsible for the subsequent postinduction silencing of the IFNβ gene (186, 187). Although IRF-1 has been implicated as a major participant in expression of both IFNβ and IFN-inducible genes (134, 135, 172, 181, 188), efficient induction clearly depends upon interactions among many mini-enhancer sites so as to provide a flexible and graded response to virus and various modulating stimuli.

The IFNα are often coexpressed with IFNβ and are also efficiently virus-inducible (56). The study of IFNα promoters, however, is less advanced, in part because the human and murine genomes possess at least 15 potentially functional IFNα genes which differ only subtly in sequence yet which appear to vary widely in virus inducibility (189). Nonetheless, as with the IFNβ, only about 120 bp of the 5′ promoter region are required for full virus responsiveness (190). NF-κB binding sites are not present in any known IFNα promoter, although several potential binding sites for IRF-1 do exist, and IFNα promoters effectively compete for IRF-1 binding (184). A GAAATG motif, not found in the IFNβ promoter, is present in the human IFNα1 promoter (191) (Fig. 2). It binds a factor distinct from IRF-1, the TG-protein which has not been fully characterized, and it is unclear whether it represents a transactivator, although multimerization of the TG-protein binding motif creates a virus-inducible enhancer.

Descriptions of the regulation of other type I IFN have not been made. Genes for human (63), porcine (155), ovine (52, 192, 193), and bovine (53, 63) IFNω have been cloned. They possess considerable sequence identity with

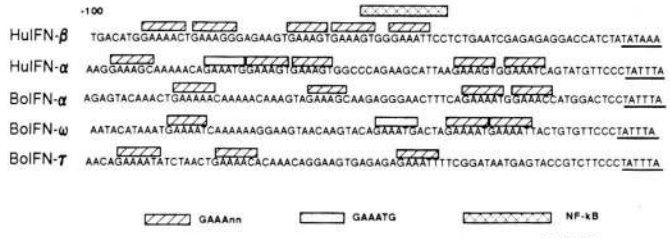

FIG. 2. A comparison of the promoter regions for type I IFN genes. The sequences upstream of the TATA box for human IFNβ and IFNα1, for the boIFNα1 and IFNω1 and for the boIFNτ genes are aligned. The position of the hexamers with the sequences AAATGA (which is thought to bind the transcription factors IRF-1 and IRF-2), GAAANN, GAAATG, and a motif that binds the NF-κB transcription factor are indicated by *shaded rectangles*. [The data are from Du *et al.* (182), Hansen *et al.* (53), Harada *et al.* (1987), Lenardo *et al.* (191), and MacDonald *et al.* (191).]

the IFNα genes upstream of the transcription start site (Fig. 2), and so it has been assumed that IFNα and -ω genes share common transcriptional regulatory mechanisms. In the human, where there is probably only one functional gene (194), IFNω is clearly virus inducible (63, 64) and constitutes 15% of the antiviral activity released by virus-induced leukocytes (68, 195).

D. IFNτ genes are regulated in a cell- and developmentally specific manner

1. Trophoblast cell expression. Whereas other type I IFN genes are expressed in a variety of cell types after appropriate induction, several lines of evidence support the view that the trophoblast IFN are unique by virtue of the restricted pattern in which they are expressed. This apparent "cell specificity" was suspected when primary structure analysis revealed that the trophoblast IFN constituted a separate subtype that had not been observed in the cloning of cDNA from virally induced cells.

Before identification of oTP-1 and bTP-1 as IFN, it was known that the intrauterine transfer of isolated trophoblastic vesicles extended luteal function in cyclic sheep and cattle (196). It seemed likely, therefore, that the antiluteolytic factor was a product of the embryonic trophectoderm. This hypothesis was confirmed by the immunocytochemical localization of IFNτ to the trophectoderm, a cell layer derived from the first epithelium of the blastocyst, which forms the outer epithelial layer of the trophoblast (29, 197–199). *In situ* hybridization techniques have also permitted specific detection of IFNτ transcripts in trophectoderm (197, 200–202). This mRNA was highly localized, with transcripts entirely absent from the underlying extraembryonic endoderm, the yolk sac, the amnion, and the entire embryonic disc. Furthermore, within the trophectoderm itself, only mononucleate cells appeared to produce IFNτ (197, 198). Binucleate cells, which are scattered among mononucleate cells by day 16 in sheep (15, 16), exhibited little or no immunocytochemical staining.

The interesting observation has also been made that at the attachment sites of the trophoblast to the uterine wall, a process that begins around day 16 in sheep, ovIFNτ expression in cells adhering to uterine epithelium was reduced (197, 199). By day 22, when most of the trophoblast was attached, expression of ovIFNτ was virtually undetectable. Thus, contact between the trophoblast and the uterine epithelium appeared to inhibit ovIFNτ gene expression.

It is curious that the protooncogene, c-*fos*, exhibits an almost identical pattern of expression to IFNτ in ovine trophoblast tissue, increasing up to the time of attachment and declining in cells having contact with the uterine epithelium (199). Conceivably c-*fos* is induced by IFNτ or is itself a component of a transcriptional complex that activates IFNτ genes, even though no interferon response elements are associated with the c-*fos* gene, and no well conserved AP-1 binding sites have been found in the upstream promoter regions of IFNτ genes identified to date (see *Section IV.E*). Alternatively, both c-*fos* and IFNτ genes may be regulated by common mechanisms during this period of rapid cellular proliferation and differentiation of trophectoderm.

These observations of cell-specific expression imply that only cells of trophoblast origin transcribe the IFNτ genes, presumably because they possess unique transacting factors important for proper gene expression. A boIFNτ gene was transfected into human choriocarcinoma (JAR) cells to test this hypothesis (150). Such cells do not normally secrete detectable amounts of antiviral activity and do not possess a gene homologous to the ruminant IFNτ (*Section V.A*). Nonetheless, JAR cells transfected with the bovine gene secreted antiviral activity, whereas several nontrophoblast cell lines tested did not, supporting the notion that expression of the IFNτ genes is the result of factors unique to cells of trophoblast origin. In contrast, a similarly transfected bovine IFNω gene was not expressed, demonstrating a functional difference between these two subtypes of IFN. Even so, the expression achieved in JAR cells was only a fraction of that which occurs in bovine or ovine trophoblast, making it likely that a complete, balanced complement of required transcription factors is not present in the human cells.

2. Temporal pattern of expression. Although ovIFNτ production can be detected in cultured blastocysts as early as day 8–10 of pregnancy by using a highly sensitive immunoassay (149), large-scale production is restricted to days 13 to 21 when the conceptus is undergoing rapid expansion from a spherical to an elongated form (10, 28, 149, 203) (Fig. 3). Two-dimensional fluorographs of radiolabeled ovine conceptus secretory proteins clearly demonstrate the three to four main isoforms of ovIFNτ to be the dominant secretory products during this elongation period (28). Transcripts for ovIFNτ are detectable by *in situ* hybridization in day 10 and 11 spherical blastocysts (200–202), but increase markedly (5- to 10-fold) in concentration per cell by day 13 (200). The concentration of mRNA then declines slightly by day 15 and precipitously thereafter as judged by Northern blotting (49, 50, 204) or *in situ* hybridization (197, 200). Therefore, the increase in ovIFNτ synthesis observed between days 13 and 15 by whole conceptuses probably reflects the 20-fold increase in tissue mass that is observed during that period of extraordinary growth (205) rather than an increase in mRNA per cell. Overall, there appears to be a good correlation between the total

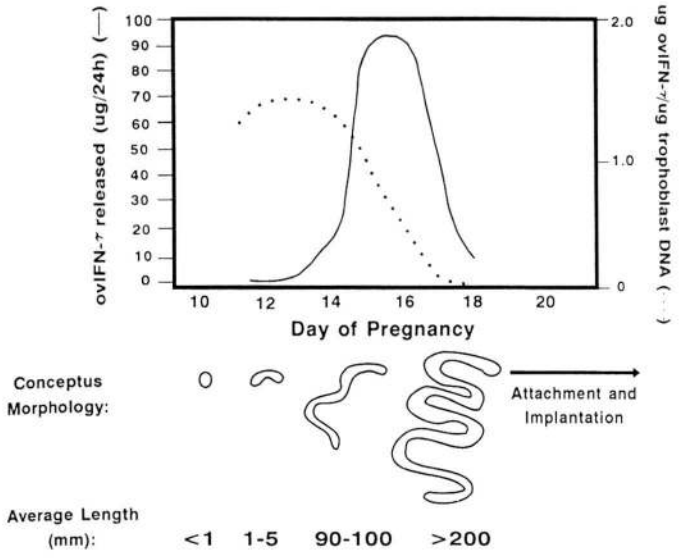

FIG. 3. A diagram showing the relationship between day of ovine conceptus development and 1) amount of ovIFNτ produced when individual conceptuses were placed in culture (S) and 2) the amount of ovIFNτ produced per μg of trophoblast DNA (*broken line*). The values for IFN produced are derived from Table 2 of Ref. 10. Antiviral units were converted to amount of IFNτ on the basis of the rough equivalency of 10^7 laboratory units and 1 mg ovIFNτ. Values for ovIFN production are consistent with those published by Ashworth and Bazer (149) who used an immunoassay to measure ovIFNτ. The amount of DNA was estimated from the data of Wales and Cunea (205) who made measurements of dry weight, and protein and DNA content per trophoblast on days 13, 15, 17, and 19. Average conceptus lengths were derived from Refs. 205 and 224.

ovIFNτ mRNA content of the tissue and the amount of protein produced.

BoIFNτ displays a similar pattern of expression as ovIFNτ, with synthesis peaking between days 17 and 19, thereby reflecting the comparatively later timeframe of maternal recognition of pregnancy in cattle (201). Although both synthesis of boIFNτ (31) and the concentration of its mRNA (201) decline after day 19, the protein continues to be produced at low levels until at least day 35 (206). The transient production of the IFNτ suggests that the initiation of expression is triggered either by an autonomous developmental change within the trophoblast cells themselves or by an external stimulus provided at the appropriate time within the uterine environment.

The initial secretion of boIFNτ antiviral activity (207) and the appearance of boIFNτ transcripts (207, 208) can first be detected at about the time the bovine blastocyst begins to expand and hatch from the zona pellucida, *i.e.* equivalent to day 8–9 *in vivo*. However, because these experiments were performed on conceptuses derived entirely under *in vitro* conditions, *i.e.* from oocytes matured and fertilized *in vitro* and cultured to the expanded blastocyst stage in the laboratory, it seems likely that this initial expression of boIFNτ, when a functional trophectoderm is first formed, is a genetically programmed event. Interestingly, although such *in vitro*-produced blastocysts continue to secrete boIFNτ in small amounts for a few days, they fail to attach to the culture dish and form outgrowths, and soon lose structural integrity (207). By contrast, when day 8 blastocysts are transferred to the uteri of synchronized cows, recovered 4 days later, and then cultured, they attach, form outgrowths, and begin to produce large amounts of boIFNτ. Therefore, while the initiation of IFNτ expression may be developmentally regulated, some factor or factors in the luteal phase environment of the maternal uterus are conceivably required either for full expression of the IFNτ or to promote trophoblast growth and development, which in turn leads to increased IFNτ production. In this regard, it has been noted with both sheep (209) and cattle (210) that an advanced luteal phase results in accelerated conceptus development and earlier production of IFNτ, and that simultaneous addition of two growth factors [insulin-like growth factor 1 (IGF-1) and IGF-2] to culture medium promotes increased synthesis of ovIFNτ (211).

The association between IFNτ expression and conceptus development is illustrated diagramatically in Fig. 3. It is intriguing that each major change in expression accompanies a significant restructuring of the conceptus. For example, initial expression is noted in association with the appearance of a definitive trophectoderm, full expression with the elongation of the blastocyst, and decline with the period of trophoblast attachment to the uterine wall. However, at present it is still not possible to determine whether IFNτ synthesis is coupled tightly to conceptus development or whether the two processes are controlled independently.

Measurements from cultured ovine conceptuses have indicated that at around day 15 to 16 of gestation a single conceptus can release more than 100 μg IFN in 24 h (10, 149) (Fig. 3). Since synthesis in culture is not linear and tails off markedly after 6–12 h (180), this value is probably an underestimate of quantities produced in the pregnant uterus. In relationship to DNA content, synthesis is highest between days 12 and 14 and then begins to decline markedly. These data are consistent with the experiments described earlier in which *in situ* hybridization was used to detect ovIFNτ mRNA (200, 201).

On initial scrutiny, the magnitude of IFNτ expression (>100 μg daily around days 15 to 16) seems far in excess of what would be required to saturate endometrial type I receptors, which, as noted earlier, have K_d values around 10^{-11} M, *i.e.* they would be half-saturated with ovIFNτ at 0.2 ng/ml. However, expression per cell rather than per conceptus (Fig. 3) is maximal about 3 days earlier when the first pulses of PGF are detectable in the nonpregnant ewe (26, 27). At this critical juncture, over-

all production of ovIFNτ is low (probably <1 μg/day), yet still seems to be sufficient to be antiluteolytic. The apparent superfluity of ovIFNτ over the subsequent few days, when so much growth occurs, may be a vestige of this earlier very high cellular expression at days 12–13. Alternatively, the high production by elongated conceptuses may be necessary if the IFNτ has to diffuse to locations more distant than the uterine surface and glandular epithelium. In this regard, low amounts of antiviral activity (~60 international reference units/ml or ~3 ng/ml) were consistently detected in the utero-ovarian veins (but not in the peripheral circulation) of pregnant ewes at day 15 after mating, and such activity was absent in nonmated controls (105).

E. Features of IFNτ gene promoters

1. The promoter dictates cell specificity. The discovery that JAR (150) and other human choriocarcinoma cells such as BeWo (212) support constitutive expression of IFNτ genes has allowed different promoter-reporter gene constructs to be tested for expression (150). These experiments have clearly revealed that the upstream region to position −126 from the transcription start site of the bovine IFNτ gene could increase expression of a human GH reporter gene 4- to 5-fold relative to a promoterless control, and that sequences between −126 and −450 possessed additional enhancer activity. These results contrast with similar studies carried out with IFNα and IFNβ genes where sequences upstream of about −120 appeared to be dispensable for conferring viral inducibility (*Section IV.C*).

Genomic sequences have now been reported for the IFNτ of cattle (53, 213), sheep (52, 192), goat (192), and musk ox (192), all of which are related species within the *Bovidae* family. Like the genes of other known type I IFN, they lack introns as revealed by Southern blotting analysis. There are a minimum of four to five such genes in each of the above species, except the musk ox which has two. In addition to three genes that resemble known cDNA, one pseudogene (52) and a gene that codes for an ovIFNτ of 162 amino acids (192) have been described. The proximal promoter regions of all the genes are highly conserved both within and across species (Fig. 4), underscoring the likelihood that the unusual promoter organization of the IFNτ genes (see below) is an essential component of the tissue- and stage-specific patterns of their expression. Unlike other type I genes, which usually start to diverge markedly from each other beyond about 120 bases upstream of the transcription start site, sequence similarities in the IFNτ persist up to at least base position −400 both within and between species (see Ref. 192), suggesting that these more distal regions may be important for promoter regulation. Such predictions have, of course, been born out in the promotion-deletion studies discussed previously.

It has also been instructive to compare the promoter regions of the IFNτ genes with the sequences that comprise tissue-specific enhancer elements of the human CG α-subunit (CGα) (214) and placental lactogen (PL) (215) genes, both of which are expressed in human trophoblast cell lines. Enhancers for these genes have been defined that are both necessary and sufficient to direct cell-specific expression. Nevertheless, no sequences resembling either of these enhancer elements are found in the first 450 bp of the IFNτ promoter. On the other hand, the CGα and PL enhancers are themselves highly distinct and show no obvious resemblance to each other. Thus, comparisons of the trophoblast IFN, CGα, and PL genes suggest that each is regulated independently. CGα and PL genes are, in fact, not expressed coordinately *in vivo*. Rather, CGα is produced much earlier in the development of the placenta than PL. Furthermore, CGα is expressed at an earlier stage of trophoblast cell differentiation than PL *in vitro* (216). Since the IFNτ genes are probably not present in primates (192), it is difficult to judge with any precision where such genes lie in a regulatory hierarchy compared to the other trophoblast-specific genes. Nonetheless, expression of the IFNτ genes begins at a relatively early stage of trophoblast differentiation in ruminant conceptuses and probably precedes at least one member of the PL family (217). In ruminants the PL-like hormones are synthesized by the binucleate cells, which are few in number at the time of maximal IFNτ expression (16). Again, these observations make it likely that factors that influence trophoblast cell differentiation are critically important determinants of trophoblast IFN transcription.

2. The trophoblast IFN promoter is functionally distinct from other type I IFN. Production of IFN by the early ovine and bovine conceptus is unparalleled in any other system. For example, on an equivalent cell basis, bovine conceptuses at day 18 of gestation released at least 300-fold more IFN constitutively in culture than did leukocytes induced by virus (150). The difference between these IFN-producing systems appears to be due to some unique feature of IFNτ genes since at least 99% of the IFN secreted by the conceptuses is IFNτ, while the mRNA of other type I IFN are present at levels roughly equivalent to those found in virus-induced leukocytes. These data support the conclusion that the IFNτ genes are specialized in their pattern of expression and suggest a basic difference between them and other type I IFN in the means by which transcription is regulated.

Whereas the boIFNτ promoter is active in JAR cells, the boIFNω promoter is not (*Section IV.D.1*). Reporter gene activity is at least 100- to 500-fold higher when

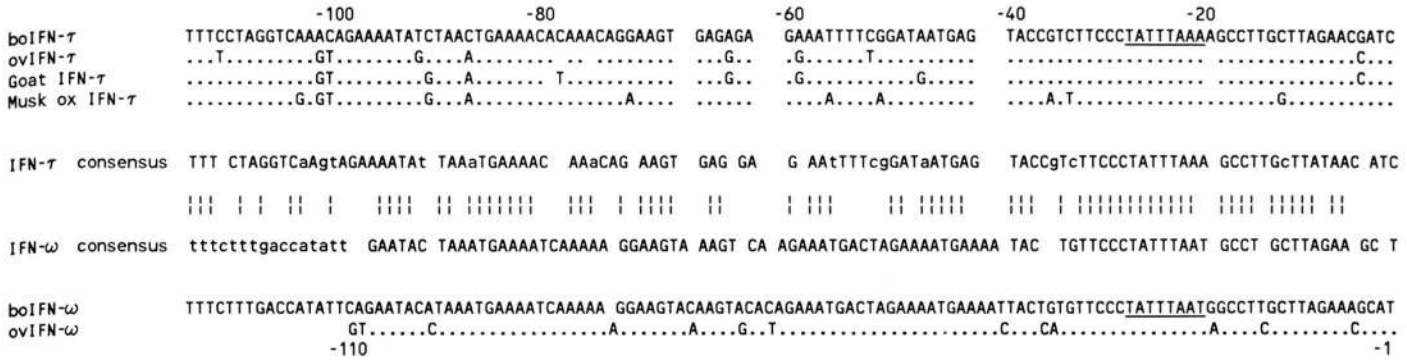

FIG. 4. Promoter sequence alignment of the genes for bovine, ovine, goat, and musk ox IFNτ, and bovine and ovine IFNω. Sequences are compared over the first ~110 bases from the transcription start site with the TATA box *underlined*. The bases that are conserved between all promoters within either the IFNτ or IFNω are indicated in *capital letters* in the consensus sequences. Those that are conserved in all but one gene promoter are shown in *lower case letters*. Sequence conservation between the two consensus sequences for IFNτ and IFNω are notated by *vertical lines*. Gaps have been introduced to provide optimal sequence alignments. Data are from Hansen *et al.* (53) and Leaman *et al.* (192).

expression from approximately 450 bp of IFNτ and IFNω promoters is compared (150). Sequence comparisons among members of the type I IFN genes reveal a high degree of superficial similarity across all members, including the IFNτ, within the proximal promoter region (53, 56, 65, 141, 192) (Fig. 2). Hexameric repeats of the sequence GAAANN (where N is any nucleotide) or AAGTGA are prevalent in all genes, although the arrangement and numbers of such sequences, thought to confer responsiveness to virus induction, are different for each gene. Upstream of the relatively well conserved proximal promoter, the similarity of the IFNτ promoter to other type I IFN promoters decreases, as does the similarity among all type I IFN genes, including different loci of a single subtype (192). This observation is not unexpected since sequences upstream of approximately position −120 are dispensible for normal regulation of both IFNα (191) and -β genes (181). As described earlier, the conservation of IFNτ gene sequences well upstream of the transcription start site seems to be an exception to this rule (192).

Although IFNτ genes possess promoter sequences which contain elements found in virus-inducible IFN genes (Fig. 2), they are only poorly responsive to virus or double-stranded RNA. Sendai virus stimulation of bovine leukocytes induced expression of IFNτ that was detectable only by reverse transcription-polymerase chain reaction but not by antiviral assay (150). Similarly, ovine conceptuses incubated with poly(I):poly(C) expressed IFNτ mRNA at levels only 2-fold higher than controls (202). In addition, reporter gene constructs employing the IFNτ promoter were not detectably induced in transfected nontrophoblast cells (CHO and L929) by Newcastle's disease virus (J. C. Cross, unpublished data). As another test of the similarity between trophoblast and other type I IFN promoters, cells were cotransfected with various IFN promoter-reporter gene constructs and a plasmid which overexpressed the transcriptional activator IRF-1. As discussed earlier (*Section IV.C*), IRF-1 binds purine-rich hexamer sequences found commonly in all type I IFN promoters, and its overexpression transactivates endogenous IFNα, -β, and -ω genes (185). When tested in CHO cells in which neither the IFNω nor IFNτ promoters are active, IRF-1 increased expression from a bovine IFNω promoter 3- to 4-fold, whereas it had no stimulatory effect on a boIFNτ promoter (218).

Together these data indicate that while IFNτ and other type I IFN promoters are quite similar at a sequence level, they are functionally distinct, a result that suggests that subtle differences in transcription factor binding sites have large overall effects on transcription. The organization of the "mini-enhancer" elements thought to comprise the viral responsive element appear to be distinct in the IFNτ compared to other known type I genes (see Refs. 53 and 192 for discussion) (Fig. 2). In particular, even though they are present, there is no clustering of GAAANN sequences or AAATGA and related motifs that bind the transcription factor IRF-1 into structures resembling well defined viral response elements, and it is probably for this reason that the genes are so poorly inducible by virus. In addition, there are no NF-κB binding sites, and no motifs indicative of possible cAMP or phorbol ester responsiveness.

V. The Uniqueness of the IFNτ

A. Evolution of the IFNτ genes and their restricted distribution in mammals

The type I IFN are thought to have evolved from a common ancestral gene before the time of mammalian divergence (56, 70). Sequence similarities among the type I IFN suggest that these genes continued to evolve as depicted in Fig. 5. Note that after the divergence of the IFNβ from IFN, the IFNα prototypic gene became ex-

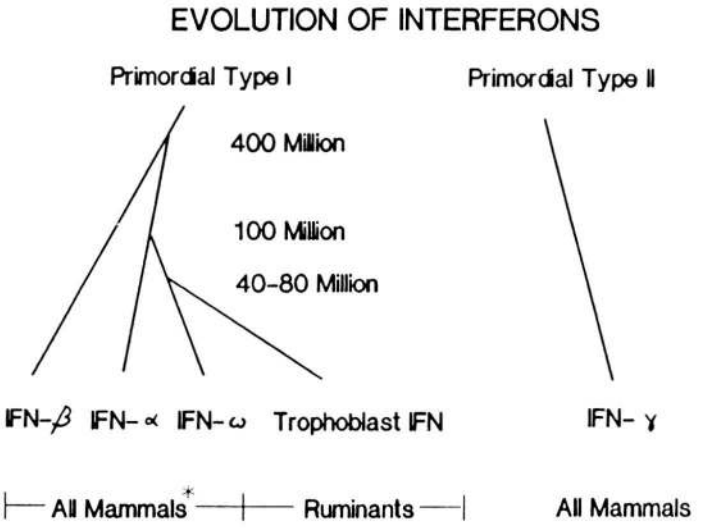

FIG. 5. A diagram showing the probable temporal sequence divergence of the various type I IFN subtypes (α, β, ω, and τ) from a single precursor gene. The IFNτ probably arose from the IFNω about 60 million years ago. All the subtypes in cattle are represented by multiple genes (not shown). *, IFNω genes have not been identified in several species including the dog and mouse.

tensively amplified. The IFNω genes are more closely related to the IFNα than IFNβ and most likely diverged from them somewhat later in evolution.

Genomic Southern blot experiments with cDNA probes designed to hybridize to IFNτ but not to related IFNω genes have indicated the apparent absence of IFNτ in all species except the large ruminants, *e.g.* the *Bovidae* mentioned above, gazelle, deer, and giraffe (192). By contrast, the genes for IFNα and -β have been found in all mammalian species where they have been sought (56, 67), although the number for both types varies widely from species to species. The IFNω are also represented in most mammals [notable exceptions being dog and rodents (192, 219)], usually as multiple gene copies (53, 63, 155, 192, 220). Certain species that are closely related evolutionarily to ruminants possess IFNω genes (155) but apparently lack IFNτ (192). For example, the pig and hippopotamus (*Suina* suborder) and the llama (*Tylopoda* suborder) are within the order *Artiodactyla* and may have diverged from the *Ruminantia* suborder as recently as 55 to 60 million years ago (221). Similarly, the horse and zebra, which also lack IFNτ genes, are members of the order *Perissodactyla*, that may have separated from the *Artiodactyla* as recently as 65 million years ago (221), although other authorities suggest a more ancient divergence (222). It may also be significant that the giraffe, which diverged from the cattle and sheep an estimated 30 million years ago, gave a single hybridizing band on Southern blots, while the *Bovidae* all possessed multiple genes (192). These data together suggest that the IFNτ must have diverged from the IFNω within the last 65 million years and duplicated several times to their present numbers in domestic ruminants quite recently. It is interesting that the amplification of IFNβ genes, which are also represented by multiple copies in the ungulates but not, as far as is known, in most other species (56, 65, 67), may have gone hand in hand with the evolution of the IFNτ. Since all the type I IFN genes, including those from cattle (223), are invariably clustered together on the same chromosome, it will be intriguing to determine their relative map positions since this information may provide a clue as to how the gene duplication events occurred. Direct sequence comparisons to determine more precisely when IFNτ divergence occurred and from which member of the IFNω these genes evolved will have to await a detailed analysis of all potential trophoblast IFN and IFNω genes within a potential link species such as the giraffe.

Why should the IFNτ genes be restricted to the *Artiodactyls*? As discussed earlier, IFN production by ruminant conceptuses during the peri-implantation period is unparalleled among other mammalian species. Nonetheless, it is also clear that only the ruminant species appear to utilize an IFN as the initiator of maternal recognition of pregnancy. Thus, while most or all species produce some form of IFN possibly as a consequence of the development of the trophoblast from the inner cell mass, it could be argued that only the ruminant species have co-opted an IFN (and also one that is produced constitutively) to ensure the establishment of pregnancy. This difference has perhaps to do with the widely variant forms of placentation among the mammalian species and suggests that the evolution of the IFNτ genes was coincident with the evolution of the syndesmochorial placentation form utilized by ruminants.

B. The IFNτ are a structurally distinct type I IFN subtype

As discussed in *Section II.C*, although the IFNτ show structural similarity to other type I IFN, the extent of their amino acid sequence identity to the subtype they most resemble, the IFNω, is only about 70%. In fact bovine, ovine, and caprine IFNτ are more similar to each other in sequence than boIFNτ is to boIFNω, and all evidence to date suggests they may constitute a serologically distinct group. Cross-species conservation of cDNA nucleotide sequence is also particularly high in the untranslated 5′ and 3′ segments of IFNτ transcripts, and the latter can be used as a highly specific probe for IFNτ mRNA and genes (53, 192, 200–202). When genomic sequences are compared, the promoter regions of the bovine, caprine, ovine, and musk ox are almost completely conserved (Fig. 4) (192).

The above structural information, combined with other unusual features of the IFNτ, *e.g.* their poor indu-

cibility by virus (*Section IV.E*), the magnitude of their production, and the trophoblast-specific nature of their expression (*Section IV.D*) provide a persuasive case for classifying the IFNτ separately from the IFNω. This separation is consistent with the evolutionary data presented in *Section V.A* and the general rules of nomenclature that have previously been applied to IFN (56). It would not be surprising if yet other subtypes emerge in view of the numerous bands that hybridize to full-length IFNω probes on Southern blots of bovine and ovine genomic DNA (50, 53, 192). Conceivably some of these as yet undefined genes either represent evolutionary intermediates between the IFNω and IFNτ or new branches of IFN divergence.

C. Do the IFNτ have unique properties?

The above question has been alluded to earlier (*Section III.B*) but is worth reconsidering in this concluding paragraph, since it is central to much of the current debate concerning the function of the IFNτ during pregnancy. Most evidence is consistent with the view that the IFNτ do not differ greatly from other type I IFN in structure or in the majority of their biological properties. They probably also bind to a common type I receptor subunit in the receptor complex, although the possibility of unique accessory subunits certainly exists. The only well authenticated difference regards effects of an IFNτ *vs.* boIFNα on estrous cycle length after the reagents are infused into the uterine horns of nonpregnant animals and where responses appear to be much greater to ovIFNτ than to boIFNα (*Section III.B*). The reason why IFNτ is more potent than IFNα is unclear but may relate to the slight differences in binding properties noted for the endometrial receptor complex (118, 119) or even to the apparently higher cytotoxicity of IFN-α (83). Tenfold or greater differences in efficacy in such processes as antiviral or antiproliferative activity are well documented among individual IFNα subtypes (56, 85), although it remains controversial as to whether all such potency differences can be attributed to relative affinity-binding constants for the receptor. Once more appropriate IFN reagents are utilized for comparative studies, *e.g.* recombinant ovIFNα for studies on sheep and boIFNτ on cattle, the issue may become properly resolved. An alternative explanation, of course, is that the IFNτ intervene in maternal recognition of pregnancy solely by virtue of their massive production in trophoblast at a particularly critical stage of gestation.

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