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Induction of Trophoblastic Interferon Expression in Ovine Blastocysts after Treatment with Double-Stranded RNA

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ABSTRACT

Ovine trophoblast protein-1 (oTP-1) is an interferon (IFN) related to the IFN-ω. The objectives of this research were: (i) to attempt to induce oTP-1 mRNA in day-11 ovine conceptuses with polyinosinic-polycytidylic acid (poly(I) · poly(C)), and (ii) to determine if IFN-ω mRNA is also produced on day 11 of gestation. In experiment I, conceptuses were cultured in presence of 100 μg/ml poly(I) · poly(C) ($n = 5$) or medium alone (control, $n = 3$) for up to 8 h. *In situ* hybridization was used to assess effects of treatment on mRNA concentrations for oTP-1 and actin (positive hybridization control). Poly(I) · poly(C) increased oTP-1 mRNA concentrations approximately 2.5-fold ($p < 0.01$), but had no effect on actin mRNA. In experiment II, the presence of mRNA for oTP-1 and ovine IFN-ω was determined by using reverse transcription–polymerase chain reaction (RT-PCR) analysis of conceptus total RNA coupled with Southern blot hybridization of the PCR reaction products with specific cDNA probes. oTP-1 mRNA was detectable in all poly(I) · poly(C)-treated ($n = 7$) and control ($n = 6$) conceptuses, whereas IFN-ω mRNA was detected in only three of seven poly(I) · poly(C)-treated conceptuses and not in any controls. Together these results demonstrate that expression of oTP-1 mRNA can be enhanced by treatment with poly(I) · poly(C) and that oTP-1 is the primary but not the only type I-IFN inducible in conceptuses on day 11 of gestation.

INTRODUCTION

In general, type I interferons (IFN) are induced in various cell types in response to viral infection.[1] These viral induction responses can also be mimicked by treatment of cells with a synthetic double-stranded RNA, such as polyinosinic-polycytidylic acid (poly(I) · poly(C)).[1,2] In addition to these common IFN inducers, however, other compounds such as platelet-derived growth factor and colony stimulating factor-1 have also been found to induce synthesis of type I IFN. Finally, IFN itself can "prime" its own synthesis as well as amplify its own production in response to other induction agents.[1,2]

The ovine conceptus produces proteins that have been classified as IFN-ω, based on similarities in cDNA sequence,[3] amino-terminal amino acid sequence,[3–5] and antiviral bioactivity.[6–8] These IFN were originally designated as ovine trophoblast protein-1 (oTP-1) and represent a group of variants encoded by individual mRNA.[9] Ovine TP-1 is produced between days 13 and 21 of gestation,[10] as detected by two-dimensional gel electrophoresis of radiolabeled conceptus culture products, but has been reported to be produced as early as day 8 of pregnancy based on radioimmunoassay of conceptus culture media.[11]

The mechanisms by which expression of the trophoblastic IFN is controlled during embryonic development are currently unknown. The mRNA for oTP-1 is clearly developmentally regulated, however, being present at low concentrations prior to day 13 of gestation followed by expression at high concentrations on days 13 and 14.[12–15] Based on the fact that oTP-1 is a type I IFN, it seemed possible that oTP-1 genes might be regulated in a manner similar to that for other type I IFN. Therefore, the objectives of the research presented herein were two-fold. The first was to determine if oTP-1 mRNA production in day-11 ovine conceptuses was inducible with synthetic double-stranded RNA. The second was to determine if IFN-ω distinct from oTP-1 was produced by day-11 ovine conceptuses before or after exposure to synthetic double-stranded RNA.

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MATERIALS AND METHODS

Animals: Mature crossbred ewes, primarily of Rambouillet and Dorset breeding, were checked for estrus twice daily. Ewes exhibiting normal estrus cycles of 16–17 days were bred at estrus (day 0) with fertile rams. Conceptuses were flushed surgically from the uterus with modified Eagle's minimum essential medium[10] by using techniques previously described.[16]

Experiment I: Eight ovine conceptuses obtained on day 11 of pregnancy were immediately recovered from the uterine flushings and transferred into culture media consisting of complete modified Eagle's medium (MEM) containing 5,000 IU/ml penicillin, 5,000 μg/ml streptomycin, 11 mg/ml sodium pyruvate, 25 m*M* HEPES, pH 7.3, and 5% fetal bovine serum (FBS). Conceptuses were then transported to the laboratory, washed once in complete MEM without FBS, and randomized into one of two treatment groups. Conceptuses were cultured in individual wells of a 24-well plate (Corning Plastics, Corning, NY) for 1 h at 37°C in a 5% CO_2 atmosphere in the presence of either 400 μl of 100 μg/ml poly(I) · poly(C) (Lee Biomolecular, Inc., San Diego, CA) dissolved in serum-free complete MEM (poly(I) · poly(C); n = 5) or 400 μl of serum-free complete MEM alone (control, n = 3). After an initial 1-h culture period, 600 μl of complete MEM containing 5% FBS was added to each culture well (final FBS concentration of 3%). Conceptuses were cultured for an additional period of either 3 or 7 h to give a total culture period of 4 or 8 h. Relative amounts of oTP-1 and actin mRNA were assessed by *in situ* hybridization with hybridization signals detected by autoradiography after 5 days exposure at 4°C.

Experiment II: A total of 13 ovine conceptuses were processed as described for experiment I. Conceptuses were randomized into one of two treatment groups: (i) 100 μg/ml poly(I) · poly(C) (National Institutes of Health, Bethesda, MD; n = 7) or (ii) media alone (n = 6, control). Cultures and administration of treatments were performed as described for experiment I with the exception that all conceptuses were cultured for a total of 8 h. Following culture, total RNA was extracted from individual conceptuses by a modification of the protocol described by Chomczynski and Sacchi.[17] Briefly, individual conceptuses were washed in culture medium with 5% FBS and then transferred into a volume of 100 μl of 4 *M* guanindine thiocyanate, 25 m*M* potassium citrate, pH 7, 0.5% sarcosyl and 0.1 *M* 2-mercaptoethanol (GTC buffer) to which 2.75 μg of yeast tRNA had been added. Conceptuses in GTC were vortexed extensively to solubilize all cells. Sodium acetate, pH 4.0, was then added to a final concentration of 50 m*M*. Samples were extracted with sequential additions of acidified phenol (100 μl) and choloroform (20 μl), held on ice for 10 min, and then centrifuged (10,000 × *g*, 12 min). The aqueous phase was collected and total RNA was precipitated at −80°C after the addition of 2.5 volumes of ethanol. Samples were stored for 4 months as ethanol precipitates at −80°C prior to reverse transcription-polymerase chain reaction (RT-PCR) and Southern hybridization analysis.

In situ *hybridization:* All procedures for *in situ* hybridization and quantitation were performed as described previously.[13,14] Briefly, oTP-1 mRNA was detected by hybridization with random primed ^{35}S-labeled cDNA probes from either a coding plus 3′-untranslated region fragment (oTP-560) or a 266-bp fragment from the 3′-untranslated region (oTP-266).[13] For each conceptus, adjacent sections were hybridized (50% formamide, 0.6 *M* sodium chloride, 42°C) with either ^{35}S-labeled γ-actin cDNA[18] or ^{35}S-labeled pBS M13$^-$ plasmid DNA probes. Hybridization signals were detected after 5 days exposure at 4°C. The relative intensity of hybridization signals of sections from control and treated conceptuses, quantified as optical density based on the reflectance of hybridized silver grains from the cDNA, was measured under dark-field illumination by video image analysis (Bioquant System IV, R&M Biometrics, Nashville, TN).[14] In these measurements, the upper range of reflectance values did not exceed 85% of the maximum grey-level value and were within the linear range.[14] Results were tested for significance by means of a Student's t-test.

RT-PCR and Southern blotting: Ethanol precipitates of RNA from poly(I) · poly(C)-treated and control conceptus were centrifuged (12,000 x *g*, 10 min), washed in 70% ethanol, dried and resuspended in 10 μl of sterile, diethylpyrocarbonate (depc)-treated water. Aliquots of 5 μl of total RNA representing individual conceptus samples were digested with RNase-free DNase (900 U/μl; Bethesda Research Laboratories, Bethesda, MD) in 40 m*M* Tris · Cl (pH 7.5), 10 m*M* sodium chloride, 6 m*M* magnesium chloride at 37°C for 15 min. Reactions were stopped by heating to 90°C for 5 min. Proteins were removed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). Total RNA was precipitated at −80°C by the addition of sodium acetate to 0.3 *M* and 2.5 volumes of absolute ethanol. Precipitates were washed in 70% ethanol, vacuum dried, and resuspended in 11 μl of sterile, depc-treated water. Three microliters of DNase-treated total RNA from each conceptus were reverse-transcribed (RT) at 42°C for 45 min by using avian myeloblastosis virus (AMV) reverse transcriptase (Seikagaku America, Inc., St. Petersburg, FL) in a 20-μl reaction volume which included 50 m*M* Tris · Cl, pH 8.3, 25 m*M* potassium chloride, 3 m*M* magnesium chloride, 5 m*M* dithiothreitol, RNasin (9 units; Promega Corp., Madison, WI). 1 m*M* spermidine, 1 m*M* each of dATP, dTTP, dCTP, dGTP, and 40pM of dT_{15}-oligonucleotide primer. Reactions were stopped by heating to 95°C for 3 min. Negative control RT reactions (no AMV reverse transcriptase added) were run in parallel on 3 μl of pools of DNase-treated total RNA samples from control (n = 6) or poly(I) · poly(C)-treated (n = 7) conceptuses.

Products of the RT reactions (3-μl aliquots) were then used as templates for specific PCR reactions. The 3′-/5′-oligonucleotide primer pairs used to identify oTP-1 and bovine IFN-ω (bIFN-α_{II}) sequences subcloned into pBS M13$^-$ were as follows: oTP-1 3′ primer (GGAAATTTGTTAAGTTAC), oTP-1 5′ primer (CCACATCAGCCTCCTACACCC); bIFN-ω 3′ primer (TTATATGGAAAAATAAATATGAGG), bIFN-ω 5′ primer (GGCTGCAGACCTGAAATCACCTTGACATGA). The expected PCR amplification product lengths were 247 nucleotides for the oTP-1 primer pairs and 277 nucleotides for the bIFN-ω primer pairs. Specificity of each primer pair was verified by PCR amplification from 10 ng of ovine genomic DNA (data not shown) or by PCR amplification from full-length cDNA templates that had been inserted into plasmid vectors followed by Southern blotting and hybridization with specific radiolabeled probes. For PCR amplification of conceptus mRNA, oligo-dT_{15} was used as a 3′ primer and the respective 5′ primer was used for oTP-1 and oIFN-ω product amplification. PCR amplifications were carried out in a 20-μl volume consisting of 3 μl RT reaction product (template), 30 p*M* each of 3′ and 5′ primers, 10 m*M* Tris · Cl, pH 8.3, 50 m*M* potassium chloride, 1.5 m*M* magnesium chloride, 0.01% gelatin, 200 μ*M* each of dATP, dTTP, dCTP, and dGTP. After PCR amplification for 40 cycles (each 1 min 94°C; 1 min 50°C; 1 min 72°C), products from 50% of the reaction volume were subjected to electrophoresis in a 1.8% agarose gel with 1 μg/ml ethidium bromide added.

After electrophoresis, agarose gels were photographed under UV illumination. PCR products were then Southern blotted onto nylon membranes (Biotrans, ICN) according to standard procedures[19] by using a vacuum apparatus (Pharmacia LKB, New York) and hybridized under stringent conditions. Blots were dried 80°C for 2 h and prehybridized for 6–15 h at 42°C. Prehybridization buffer consisted of 75 m*M* sodium citrate, pH 7.0, 0.75 *M* sodium chloride, 50 m*M* sodium phosphate, pH 7.0, 0.1% (wt/vol) SDS, 0.1% (wt/vol) Ficoll, 0.1% (vol/vol) polyvinylpyrrolidine, 0.1% (wt/vol) bovine serum albumin, and 200 μg/ml denatured herring sperm DNA. Hybridizations were carried out in the same buffer to which [^{32}P]cDNA (sp. act., $\sim 9 \times 10^8$ dpm/μg) representing either oTP-1[12] or bovine IFN-α_{II}[20] were added. Blots were hybridized at 42°C for 16 h, washed in 0.3 *M* sodium chloride, 0.03 *M* sodium citrate, 0.5% (wt/vol) SDS for 20 min at 42°C, and in 0.015 *M* sodium chloride, 0.0015 *M* sodium citrate, 0.5% (wt/vol) SDS for 20 min at 60°C (oTP-1) or 42°C (boIFN-ω). Blots were exposed to Kodak XAR film for 5 days.

cDNA probes used for Southern hybridizations: oTP-1 cDNA produced in PCR amplification reactions was identified by using a ^{32}P-random primed oTP-1 probe specific for the 3′-untranslated region of the oTP-1 mRNA sequence (oTP-266).[13,14] oIFN-ω cDNA PCR amplification products were identified by using a ^{32}P-random primed bovine IFN-ω probe encompassing the 3′ untranslated region only.[20] Specificity of all probes was verified by Southern blotting of PCR amplification products (see Fig. 3A,B, lanes 11, 12, and 22).

RESULTS

Experiment I

Treatment of day-11 conceptuses with poly(I) · poly(C) increased the relative intensity of hybridization signals for oTP-1 approximately 2.5-fold over that of controls when either the oTP-560 or oTP-266 cDNA probes were used (data not shown). There was a consistently lower intensity of hybridization signal associated with the oTP-266 cDNA probe, but this was probably due to a difference in the relative specific activities of the two probes.[14] Hybridization signals for actin mRNA were unaffected by treatment with poly(I) · poly(C) (Fig. 1). When hybridization signals from treated conceptuses were expressed as a percent of controls, treatment with poly(I) · poly(C) was associated with an increase ($p < 0.01$) in trophoblastic IFN mRNA levels relative to controls (Fig. 1). However, it should be noted that considerable variability was observed among conceptuses in the strength of the hybridization signals observed. In addition, among the poly(I) · poly(C)-treated conceptuses, variability also existed between embryos in the responsiveness of cells within the embryonic disc to induction of oTP-1 mRNA (Fig. 2).

Experiment II

The results of the Southern blot analysis of RT-PCR products for oTP-1 and oIFN-ω mRNA from the control and poly(I) · poly(C) treatment groups are presented in Fig. 3, A and B. As expected "mock" reverse transcription of total conceptus RNA pools and PCR amplification of water control samples (no template added) did not produce any PCR products (lanes 10, 2, and 3, respectively in Fig. 3A, B). oTP-1 RT-PCR amplification products, and thus oTP-1 mRNA, were identified in 6 of 6 control and 7 of 7 poly(I) · poly(C)-treated conceptuses (Fig. 3A, lanes 4–9 and 15–21). However, oTP-1 signals were only weakly detected in 3 of 6 control conceptuses compared with only 1 of 7 treated with poly(I) · poly(C). The increased length of the conceptus oTP-1 RT-PCR products as compared to the control plasmid containing oTP-1 sequence (Fig. 3A, lanes 11 and 22) was due to the use of oligo-dT_{15} as a 3′ PCR primer. Use of this primer would be expected to add approximately 50 additional bases between the annealing site of the 3′-oTP-1 specific primer and the termination of the poly(A)$^+$ tail.[3] The presence of at least two hybridizing bands probably relates to variability in the lengths of the oTP-1 transcripts,[21] the presence of more than one polyadenylation signal in some known trophoblast IFN genes[22] and, as indicated above, the use of the oligo-dT_{15} primer during PCR. This nonspecific 3′ primer was employed to distinguish mRNA from genomic DNA. Because all known type I IFN are intronless, a more "specific" 3′ primer

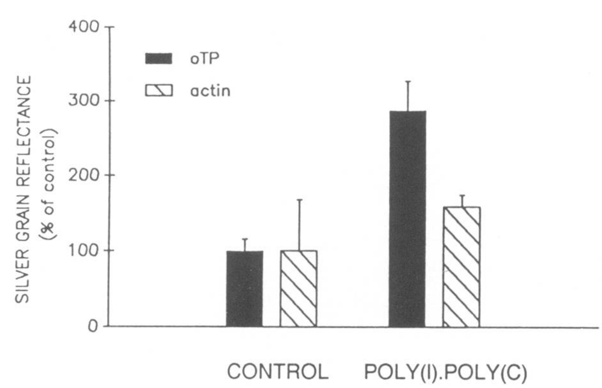

FIG. 1. Effect of treatment of day-11 ovine conceptuses with poly(I) · poly(C). Relative hybridization signals (silver grain reflectance) for oTP-1 and actin mRNA expressed as a percent of control values (mean ± SE).

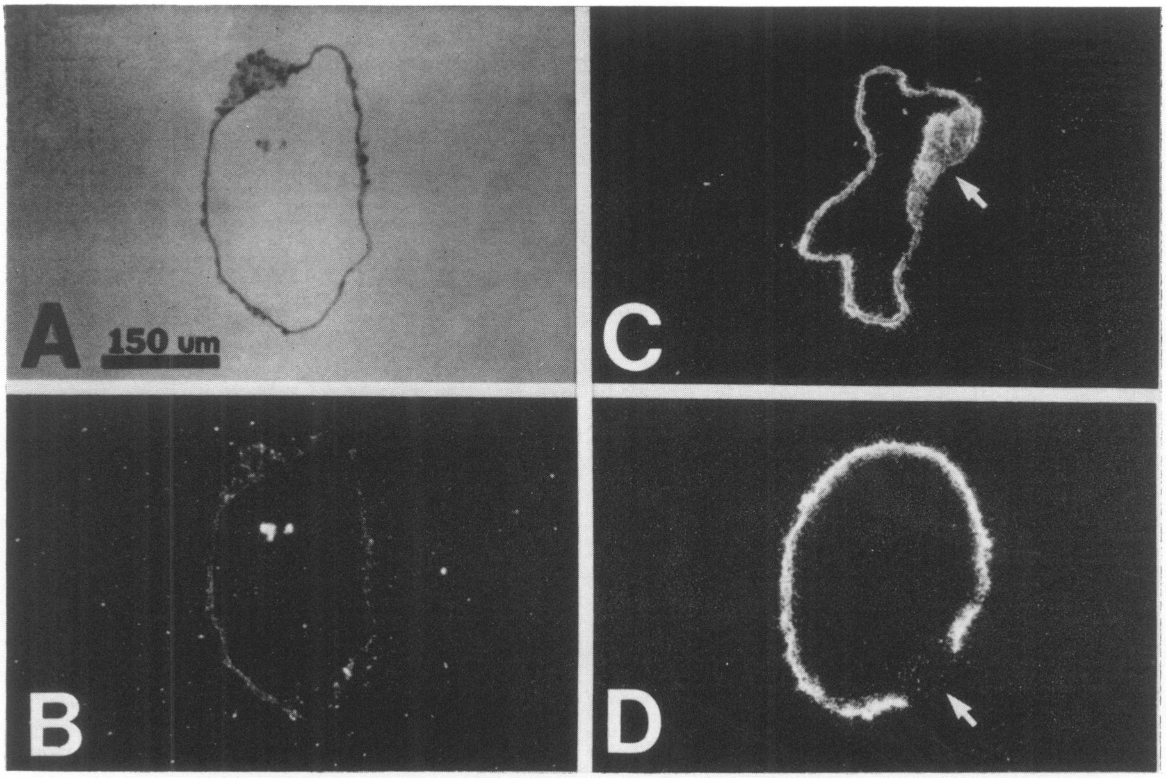

FIG. 2. *In situ* hybridization of day-11 ovine conceptuses after culture in control media (A, bright-field; B dark-field) or in media supplemented with poly(I) · poly(C) (C,D) [Experiment I; hybridization with ^{35}S-labeled oTP-560 cDNA]. Note the intense level of hybridization signal in embryos pictured in C and D compared with the embryo in B. Arrows in C and D indicate the position of the inner cell mass. Also note that in one of the two poly(I) · poly(C)-treated conceptuses illustrated, trophoblastic IFN mRNA is localized in both the inner cell mass and trophectoderm.

would allow any contaminating genomic DNA to be amplified in the PCR reaction.

In contrast to the positive identification of oTP-1 mRNA in the majority of day-11 conceptuses, IFN-ω RT-PCR amplification products were identified in none of the control conceptuses. However, these transcripts were detected in 3 of 7 poly(I) · poly(C)-treated conceptuses. These latter results indicate that other type I IFN can be expressed, albeit at very low levels, in day-11 conceptuses after poly(I) · poly(C) exposure.

DISCUSSION

Based on the results presented in here, expression of oTP-1 genes appears to be inducible prior to the normal onset of major expression on day 13 of pregnancy. In experiment I, for example, treatment of day-11 ovine conceptuses with poly(I) · poly(C) led to increased concentrations of oTP-1 mRNA in trophectoderm as determined by *in situ* hybridization analysis. In experiement II RT-PCR and Southern hybridization showed that a greater proportion of conceptuses gave a strong signal for oTP-1 mRNA after they had been exposed to poly(I) · poly(C). It should be noted, however, that no attempt was made to measure the actual amount of PCR product formed in the reactions. The treatment also appeared to induce mRNA for a related IFN, oIFN-ω. Together these observations indicate that expression of oTP-1 and oIFN-ω genes in conceptuses can be induced in a limited manner by exposure to double-stranded RNA. It was not, therefore, surprising that the genes for both bTP-1 and oTP-1[23–24] have recently been found to contain sequences in their 5′-upstream promoter regions which have been implicated in the inducibility of IFN-α and IFN-β genes by viruses and double-stranded RNA[25–28] in other cell types. These sequences include a pair of hexanucleotide motifs at positions −69 to −74 and −88 to −93, which resemble the viral response elements that bind the transcription factor interferon regulatory factor-1 (IRF-1)[29] and a series of GAAANN sequences found in other virus-inducible or IFN-responsive genes.[28,30] These characteristic motifs are positioned and arranged rather differently in the trophoblast IFN genes than in other IFN genes, including those of the IFN-ω.[24] Nonetheless, their presence may account for the inducibility of oTP-1 by poly(I) · poly(C) noted here and the protection conferred on day 9 bovine conceptuses against vesicular stomatitis virus following their exposure to the IFN-inducer, Newcastle disease virus.[31]

Although virus-like particles have been identified in both

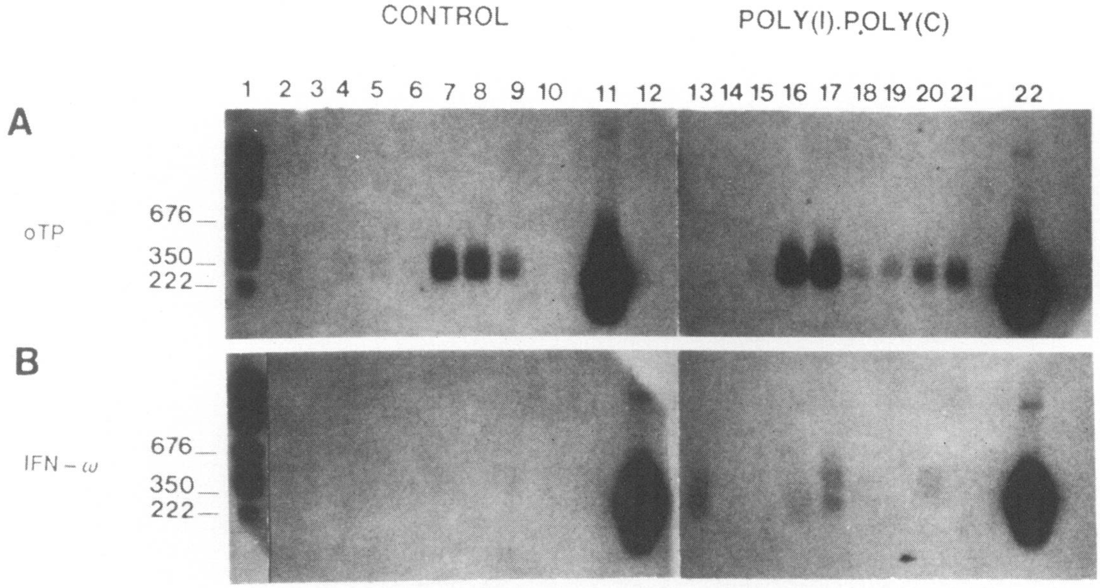

FIG. 3. Southern hybridization analysis of RT-PCR products of control and poly(I) · poly(C)-treated day-11 ovine conceptuses (see text for details). A. Analysis of oTP-1 RT-PCR products by hybridization with ^{32}P-labeled oTP-266 cDNA. Lane 1, Pgem standards, base-pair lengths of selected standard fragments indicated in left margin; lanes 2–3, mock RT reactions with pooled control (lane 2) or poly(I) · poly(C) (lane 3) day-11 conceptus total RNA (negative control); lanes 4–9, RT-PCR products from individual control conceptuses; lane 10, PCR water blank (negative control); lane 11, plasmid containing oTP-1 cDNA sequence (PCR product only); lane 12, plasmid containing boIFN-ω DNA sequence (PCR product only); lanes 13–14, RT-PCR and mock RT-PCR products of GBK2 cell total RNA (negative controls), respectively; lanes 15–21, RT-PCR products from individual poly(I) · poly(C)-treated conceptuses; lane 22, plasmid containing oTP-1 cDNA sequence (PCR product only). Standards and positive control plasmid PCR products (lanes 1, 11, 12, and 22) intentionally overexposed to allow adequate exposure of RT-PCR products from control and poly(I) · poly(C)-treated conceptus samples (lanes 4–9 and 15–21). B. Analysis of IFN-ω RT-PCR products by hybridization with ^{32}P-labeled boIFN-ω 3′-untranslated region DNA probe. All lanes are as in A except lane 22. Lane 22, Plasmid containing boIFN-ω DNA sequence (PCR product only).

conceptuses and reproductive tracts of mice and sheep early in gestation,[32–34] there is no convincing evidence to suggest that a virus is the natural inducer of trophoblast IFN around the time of maternal recognition of pregnancy in domestic ruminants. Other more likely inducers of oTP-1 include growth factors, such as colony stimulating factor-1 (CSF-1) and platelet-derived growth factor, and cytokines such as interleukin-1, interleukin-2, and tumor necrosis factor, all of which are capable of inducing IFN in other cell systems.[1] Interestingly, colony-stimulating factor-1 is known to be produced by the uterine endometrium of mice[35] but so far not of other species. Even though the precise mechanisms whereby growth factors, cytokines, and viruses influence IFN gene expression is presently unclear, it is possible that the signaling pathways converge at some stage and involve common transcriptional elements.

In view of the massive induction of trophoblast IFN relative to other type I IFN during maternal recognition of pregnancy,[36] it seems likely that there are tissue-specific enhancer elements associated with the trophoblast IFN genes that operate independently of the viral-responsive enhancer elements described earlier. Such elements might control the cell specificity, magnitude, and timing of oTP-1 expression. In this regard, it is interesting that a positive hybridization signal for oTP-1 was detected in 3 of 5 conceptuses treated with poly(I) · poly(C) in the embryonic disc as well as in the trophectoderm. In contrast, the remaining two conceptuses showed a more typical trophoblast-specific localization of oTP-1 gene expression.[13,14] Although we have no good explanation for these observations, it seems possible that variations in the true developmental age of the conceptuses recovered on day 11 of gestation gave rise to subtle differences in the state of differentiation of the cells within the embryonic disc and hence in their responsiveness of the IFN genes to poly(I) · poly(C).

In conclusion, the results presented here indicate that expression of oTP-1 mRNA may be enhanced in conceptuses by a common type I IFN inducer. Nonetheless, it should be noted that the 2.5-fold induction of oTP-1 mRNA in response to poly(I) · poly(C) is considerably less than the 10-fold or more increase per unit of tissue noted during normal development between day 11 and day 13.[12,13] Therefore, even though modest inducibility of oTP-1 genes has been demonstrated, it seems that we are far from completely understanding the tissue-specific and temporal factors involved in controlling gene expression *in vivo*.

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