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Expression of c-*onc* genes: c-*fos* transcripts accumulate to high levels during development of mouse placenta, yolk sac and amnion

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The c-*onc* genes c-*fos* and c-*fms* are expressed at high levels specifically in mouse extra-embryonal tissues. Here, we report the results of a detailed analysis of expression of these genes within the developing placenta and extra-embryonal membranes (i.e., yolk sac and amnion). (i) The c-*fos* gene is expressed at relatively high, but nearly constant levels in the undissected placenta throughout gestation. (ii) The level of c-*fos* transcripts is ≥15-fold higher in the separated outer portion of the midgestation placenta (primarily undifferentiated fetus-derived cytotrophoblast maternal decidua) relative to the inner moiety (predominantly differentiated syncytiotrophoblast). (iii) In the inner placenta and in the extra-embryonal membranes c-*fos* transcripts accumulate as gestation proceeds. The abundance of c-*fos* transcripts in the micro-surgically isolated 18th day amnion reaches a level which is two orders of magnitude greater than that in midgestation fetuses, and is thus close to the level of v-*fos* transcripts in virus-transformed cells. (iv) The distribution of c-*fos* transcripts within the developing extra-embryonal tissues is markedly different from that of the c-*fms* gene. It is suggested that the c-*fos* and c-*fms* proteins may participate in differentiation, growth or transport processes occurring in mouse extra-embryonal tissues.

Key words: mouse development/placenta/amnion/c-*onc* genes/transcription

Introduction

Cellular sequences homologous to retroviral oncogenes (v-*onc*) have been identified in the genome of a variety of species throughout the vertebrate phylum (Stehelin *et al.*, 1976; Bishop, 1981; Bishop and Varmus, 1982), and have even been detected in invertebrates (Shilo and Weinberg, 1981). Because of their evolutionary conservation, a crucial role for these cellular sequences (c-*onc*) in normal cellular metabolism has often been postulated (Bishop, 1981; Bishop and Varmus, 1982). As their viral counterparts are known to interfere with normal cell differentiation and proliferation, a widely held hypothesis is that c-*onc* genes may fulfil certain physiological functions in these processes (Graf and Beug, 1978; Bishop, 1978, 1981; Ellis *et al.*, 1981; Bishop and Varmus, 1982).

The cellular counterparts of several avian retroviral oncogenes have been shown to exhibit tissue-specific patterns of transcriptional activity in chickens (Chen, 1980; Gonda *et al.*, 1982; Shibuya *et al.*, 1982). In addition, expression of the cellular homologues c-*myb* and c-*myc* of avian myeloblastosis virus and avian myelocytomatosis virus, respectively, have been shown to be inversely correlated with differentiation of human haematopoietic cells (Westin *et al.*, 1982a, 1982b). Data obtained from a comprehensive analysis of c-*onc* transcription during mouse development (Müller *et al.*, 1982, 1983) have lent further credence to the hypothesis that c-*onc* genes play a role in normal developmental processes. These studies have shown that the cellular homologues c-*fos* (Curran *et al.*, 1982) c-*abl*(Goff *et al.*, 1980) and c-*fms* (Donner *et al.*, 1982) of the oncogenes of the FBJ murine osteosarcoma virus (FBJ-MuSV), the Abelson murine leukemia virus and the McDonough strain of feline sarcoma virus, respectively, exhibit distinct stage- and tissue-specific patterns of expression. In contrast, the cellular counterpart c-ras^{Ha} (Ellis *et al.*, 1980) of the oncogene of Harvey murine sarcoma virus was found to be expressed ubiquitously in embryos, fetuses and extra-embryonal tissues at all stages of development analyzed. Similarly, transcripts from the cellular homologue c-ras^{Ki} (Ellis *et al.*, 1981) of the oncogene of Kirsten murine sarcoma virus were observed in all tissues investigated with ≤3-fold variations in the level of expression.

Transcripts from the c-*fos* and c-*fms* genes were detected at greatly elevated levels during mouse prenatal development specifically in the placenta, and, in the case of c-*fms*, also in the extra-embryonal membranes (Müller *et al.*, 1982, 1983). Additionally, the c-*fms* gene was shown to be expressed in a stage-specific fashion in both the placenta and the extra-embryonal membranes (Müller *et al.*, 1983). In the case of c-*fos*, only two stages of prenatal development were analyzed (Müller *et al.*, 1982). High levels of transcripts were detected in day 10 and day 18 placentas, whereas expression was relatively low in day 10 and 12 extra-embryonal membranes. In addition, in previous studies only undissected placentas and combined extra-embryonal membranes were analyzed. To obtain a more detailed picture of the expression patterns of the c-*fos* and c-*fms* genes we have analyzed the tissue-specific distribution of transcripts from these c-*onc* genes within the placenta and the extra-embryonal membranes, as well as the time course of c-*fos* expression during development of these extra-embryonal tissues.

Results

Strategy

The anatomy of a mid-gestation mouse conceptus and our dissection strategy are shown in Figure 1A and B. The placenta was dissected into two components on the basis that a distinctive marker, alkaline phosphatase, was located specifically on the inner two-thirds of the placenta (Figure 2A, B). The inner portion of the placenta is the 'labyrinth' where exchange of nutrients between maternal and fetal circulations takes place. It consists of at least four different cell types: fetal endothelial cells (mesoderm) lining fetal capillaries, chorionic trophoblast (probably syncytial) and ectoplacental trophoblast as a syncytium and as fenestrated cells lining the maternal blood sinuses (Hernandez-Verdun, 1974). These cell types differentiate as gestation proceeds.

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The outer portion of the placenta consists largely of fetal cytotrophoblast and giant cells with some maternal decidua (Everett, 1935; Davies and Glasser, 1968; Müntener and Hsu, 1977). Figure 2C shows that expression of transferrin receptors, an additional distinct marker, was also found to be largely confined to the syncytiotrophoblastic two-thirds of the placenta. To determine the degree of cross-contamination of the dissected placental components, the separated inner and outer portions of the placenta were assayed for alkaline phosphatase activity at several stages of development. The specific activity of the outer moiety averaged 0.04 mU/mg protein on the 13th to 16th day of gestation, compared with 0.50 mU/mg protein in the inner portion. From these data we conclude that contamination of the outer placenta with tissue from the inner moiety is $<8\%$.

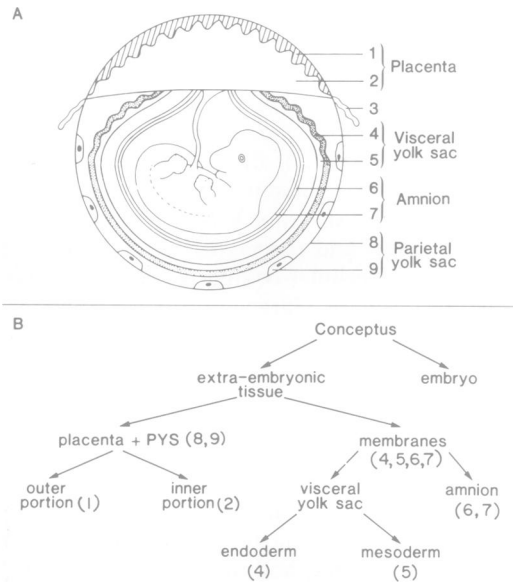

Fig. 1. Dissection of the mid-gestation conceptus. **(A)** Stylized diagram of sectioned conceptus at the 12th – 18th day of gestation. **(B)** Dissection strategy employed in the present study. 1: outer portion of the placenta; 2: inner portion of the placenta; 3: remaining trophoblast portion of parietal yolk sac (PYS); 4: endoderm of VYS; 5: mesoderm of VYS; 6: mesoderm of amnion; 7: ectoderm of amnion; 8: Reichert's membrane; 9: parietal endoderm cell of PYS.

We also dissected the extra-embryonal membranes into their components, amnion and visceral yolk sac (VYS). The latter was further separated by enzymatic and surgical means (Adamson and Ayers, 1979) into its two layers, visceral endoderm and visceral mesoderm (Figure 1A and B). The cross-contamination of dissected components was monitored by assaying all samples for alpha-fetoprotein (AFP) transcripts (Figure 6). AFP is known to be expressed specifically in the visceral endoderm and fetal liver, and exhibits a stage-specific pattern of expression in both tissues (Wilson and Zimmerman, 1976; Dziadek and Adamson, 1978). Figure 6 shows that VYS mesoderm and amnion preparations are contaminated with $<10\%$ endoderm. All other tissues appear to be largely free of contamination with endoderm.

Total RNA or, whenever sufficient tissue could be obtained, poly(A) containing [poly(A)$^+$] RNA was analyzed for the presence of c-*onc*-related transcripts by a dot blot technique. Dot blot autoradiograms were quantitatively evaluated by scanning with a densitometer. Agarose gel electrophoresis followed by Northern blotting was used to confirm the results obtained by dot blot analysis, and to determine the sizes of c-*onc*-related transcripts. The latter point appears to be of particular importance since one case has been described where enhanced transcription of a c-*onc* gene was associated with a tissue-specific mRNA of a size different from those observed in other tissues (transcription of c-*abl* in mouse testes; Müller *et al.*, 1982). It has previously been shown (Müller *et al.*, 1982) that c-*ras*^{Ha} is expressed at nearly constant levels in embryos/fetuses and extra-embryonal tissues throughout prenatal development. Therefore, in the present study, the *ras*^{Ha}-specific probe served as an internal positive

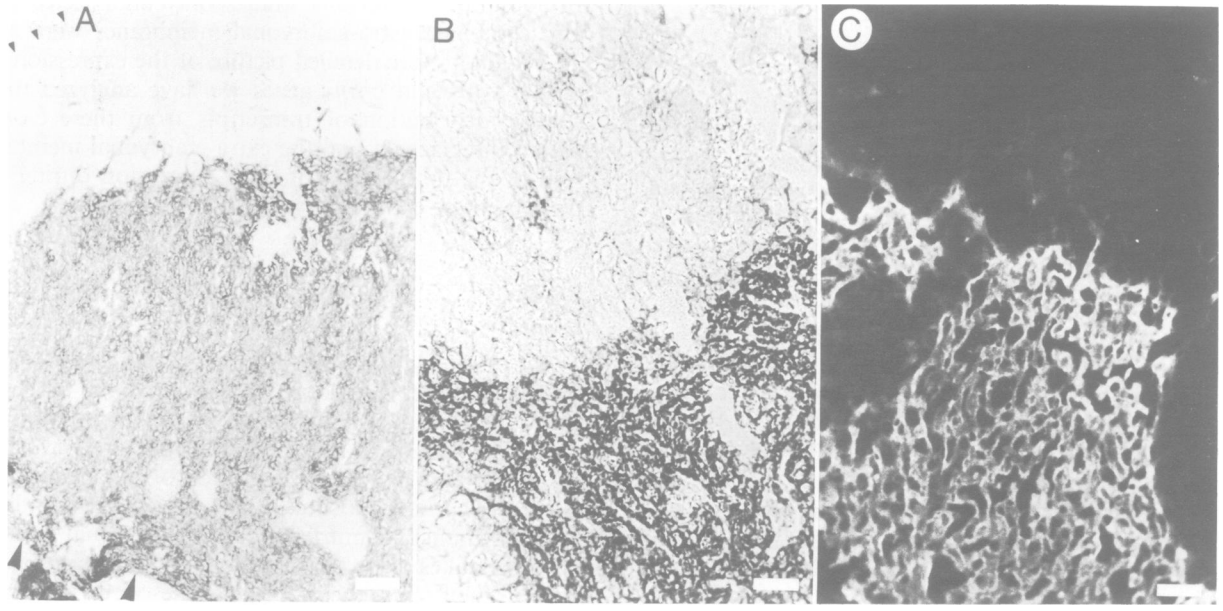

Fig. 2. Distribution of alkaline phosphatase **(A,B)** and transferrin receptors **(C)** in the inner and outer placenta. The outer portion is at the top of each panel. Bars indicate 200 μm in **(A)** and 50 μm in **(B)** and **(C)**. **(A)** Bright-field; low power ojective lens; large arrowheads show inner margin of the placenta; small arrowheads indicate the outer margin of the placenta. **(B)** Phase contrast; higher power objective lens. **(C)** Immunofluorescence (sections of placenta adjacent to those in **A** and **B**).

control to show that differential expression patterns observed with other c-*onc* genes were not due to fluctuations of RNA concentrations.

Stage-specific expression of the c-fos gene in extra-embryonal tissues

Figure 3A and B shows that expression of the c-*fos* gene was largely confined to extra-embryonal structures, where the abundance of transcripts was found to be up to 50-fold greater than in the fetus. The level of c-*fos* transcripts was constant throughout development of the placenta, but increased in the extra-embryonal membranes ~6-fold between the 12th and 18th day of gestation. In 12th day extra-embryonal membranes the concentration of c-*fos* transcripts was found to be about one-third of that observed in placenta, whereas in 18th day membranes the level of c-*fos* transcripts was ~3-fold greater than in placenta (an average value from four different experiments; e.g., Figure 3A). No variations in signal intensity were observed when the blots used for the quantitation shown in Figure 3A and B were hybridized to the v-*ras*^{Ha}-specific probe (not shown).

Agarose gel electrophoresis followed by transfer of the RNA to nitrocellulose paper and hybridization to the v-*ras*^{Ha}-specific probe revealed heterogeneous c-*ras*^{Ha}-related transcripts of ~1.4 kb at all stages of placental development, as well as in 17th day fetus and extra-embryonal membranes (Figure 4A). Similar transcripts have previously been described in early conceptuses and mid-gestation embryos (Müller *et al.*, 1982). On a longer exposure of the blot shown in Figure 4A another transcript of ~4.3 kb could be identified, albeit at a much lower intensity of hybridization (unpublished data). Transcripts of 2.2 kb related to c-*fos* were detected at constant levels throughout placental development, and at an elevated level in 17th day extra-embryonal membranes (Figure 4B). The additional 3.5-kb transcript observed in several samples is presumably an immature mRNA precursor. This explanation is based on data obtained from nucleotide sequence analyses, which predict a size of 3397 bp for the c-*fos* (mouse) gene and the presence of three introns with a total length of 1270 bp (Van Beveren *et al.*, 1983). Density scanning of the autoradiogram showed that the 2.2-kb mRNA was ~100-fold more abundant than the 3.5-kb transcript.

Differential expression of the c-fos gene in outer and inner moiety of the placenta

Figure 5A shows the distribution of c-*onc*-encoded transcripts in the surgically separated outer and inner portions of the placenta (see Figures 1 and 2) from the 12th to the 20th day of the gestational period. No significant differences of c-*ras*^{Ha}, c-*ras*^{Ki} and c-*fms* expression could be detected in the inner and outer placenta at all stages analyzed. In contrast, the concentrations of c-*fos* transcripts were ≥15-fold higher in the outer portion of both 12th and 14th day placenta relative to the inner moiety. This finding suggests that c-*fos* transcripts in the mid-gestation placenta originate largely either from the maternally derived decidua basalis or from relatively undifferentiated fetal cytotrophoblast, the two major constituents of the outer placenta. At later stages of prenatal development, however, the concentration of c-*fos* transcripts in the inner placenta increases 5- to 10-fold, and reaches a level which is ~50% that found in the outer portion of the placenta at the 20th day of gestation (Figure 5A). This increase in the level of c-*fos* transcripts in the inner placenta

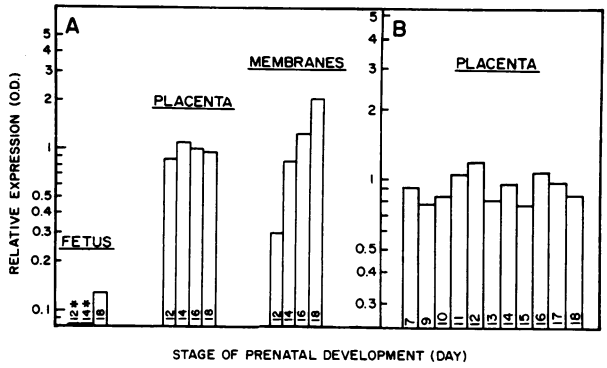

Fig. 3. Quantification of the levels of c-*fos* transcripts in the developing fetus, placenta and extra-embryonal membranes. O.D. values were determined by densitometer scanning of dot blot autoradiograms and normalized to one for the average level of expression in placenta. Relative expression (expressed as relative O.D.) was plotted against the stage of development. **(A)** Quantitative evaluation by scanning with a densitometer of dot blot autoradiograms prepared using 1 μg of poly(A)$^+$ RNA. (*), value <0.03. **(B)** 5 μg of total RNA were analyzed by dot blot analysis and the autoradiogram was quantitatively evaluated by scanning with a densitometer. (*), value <0.4.

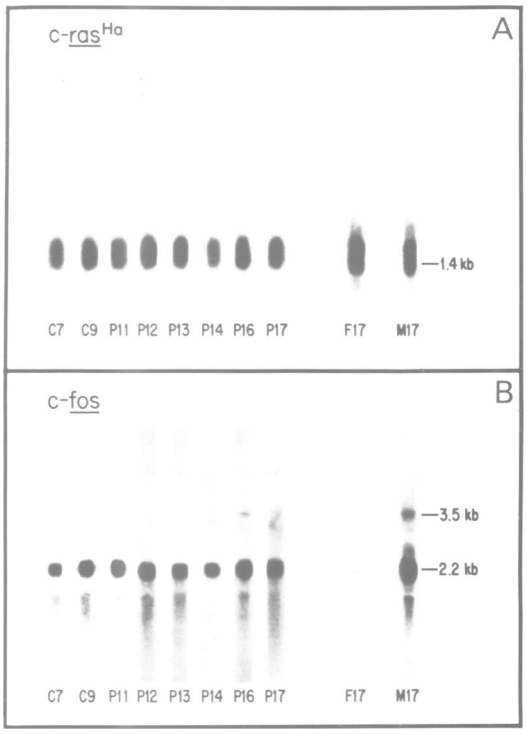

Fig. 4. Transcripts related to c-*ras*^{Ha} **(A)** and c-*fos* **(B)** in the developing placenta and in the 17th day fetus and membranes. 20 μg of poly(A)$^+$ RNA were analyzed by the Northern blotting technique as described in Materials and methods. The blot shown in **(A)** had previously been hybridized to the *fos*-specific probe and exposed **(B)**. It was then washed with 50% formamide at 68°C for 2 h to remove the probe and hybridized to the *ras*^{Ha}-specific probe **(A)**. Blots were exposed for approximately 60 h **(A)** and 36 h **(B)**, respectively. C7, C9: 7th and 9th day conceptuses; P11–P17: 11th–17th day placentas; F17: 17th day fetus; M17: 17th day membranes.

may originate from developing chorionic tissue which in the mouse is fused with the placenta. A tissue-specific distribution of c-*fos* transcripts within the mid-gestation placenta was also detected by agarose gel electrophoresis followed by blot

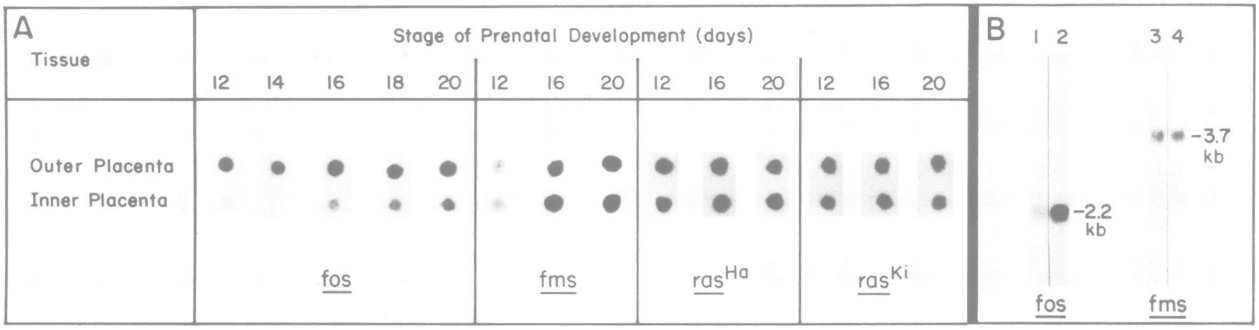

Fig. 5. Expression of c-*onc* genes in the surgically separated inner and outer portions of 12th – 20th day placentas. **(A)** Dot blot analysis of 1 μg of poly(A)$^+$ RNA. Blots were exposed for 30 h. **(B)** Northern blot analysis of 20 μg of poly(A)$^+$ RNA. Blots were exposed for 36 h. 1 and 3: inner placenta; 2 and 4: outer placenta.

analysis as shown in Figure 5B. A considerably greater concentration of 2.2-kb c-*fos*-related transcripts was observed in the outer (Figure 5B, lane 2) compared with the inner (Figure 5B, lane 1) placenta at the 12th day of the gestational period. As expected, the concentrations of 3.7-kb c-*fms*-related transcripts were found to be similar in both moieties of the 12th day placenta (Figure 5B, lanes 3, 4).

Tissue-specific transcription of c-onc genes within the extra-embryonal membranes

The extra-embryonal membranes were surgically and enzymatically dissected into their components as outlined in Figure 1 and described in detail in Materials and methods. The relative levels of c-*onc* transcripts in these separated fractions were determined and compared with those in the placenta. Figure 6A shows that the abundance of both c-*ras*^{Ha} and c-*ras*^{Ki} transcripts was similar in all extra-embryonal tissues. In contrast, a highly specific pattern of c-*fos* expression was observed. The highest level of transcripts was detected in 18th day amnion (Figure 6A), which is a minor constituent of the combined membrane fraction. Densitometer scanning of the blot autoradiograms shown in Figure 6A revealed that the concentration of c-*fos* transcripts in this tissue is ~3-fold greater than in 18th day VYS or 16th day separated endoderm and mesoderm, ~10-fold greater than in the placenta, and >200-fold greater than in mid-gestation embryos. The abundance of c-*fms* transcripts was found to be 2- to 4-fold greater in 18th day placenta than in the constituents of the extra-embryonal membranes (values of three independent experiments; e.g., Figure 6A), and >40-fold greater than in the fetus. Agarose gel electrophoresis (Figure 6B) revealed 2.2-kb c-*fos*-related transcripts at a considerably greater concentration in 18th day amnion than in VYS and placenta. Transcripts of 3.7 kb related to c-*fms* were observed at higher concentrations in 14th – 18th day placenta compared with 18th day VYS and 16th day visceral endoderm (Figure 6B).

Level of c-onc gene expression in extra-embryonal tissues

The abundance of c-*fos* transcripts in 18th day amnion is very high relative to the concentration of c-*fos* transcripts in other tissues and compared with the level of transcripts from other c-*onc* genes (Figure 6; Müller *et al.*, 1982). Therefore, it was of interest to compare the concentration of c-*fos* transcripts in this tissue with the level of v-*fos* transcripts in FBJ-MuSV-transformed cells. Figure 7 shows that concentrations of v-*fos* transcripts in the RS2 FBJ-MuSV-transformed rat fibroblastic cell line (Curran and Teich, 1982) and in an FBJ-MuSV-induced osteosarcoma are only ~2-fold higher

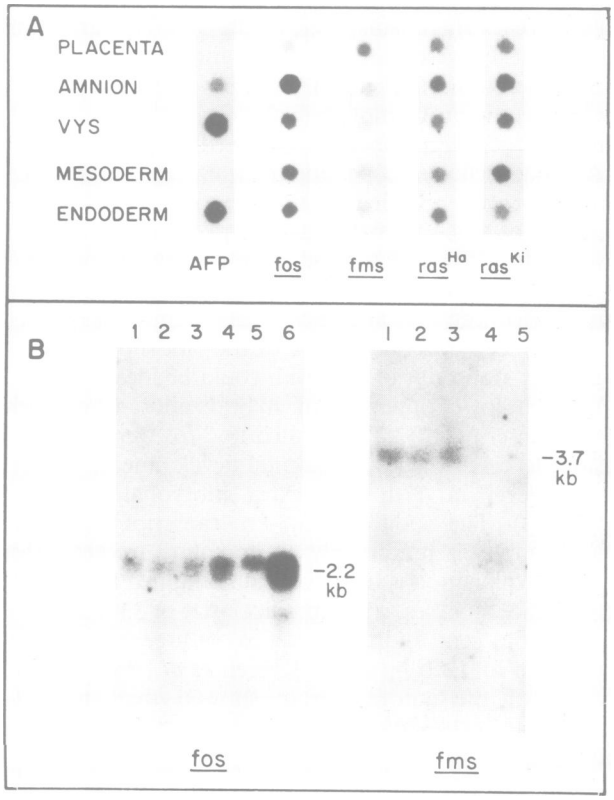

Fig. 6. Expression of AFP and c-*onc* genes in the surgically and enzymatically dissected components of the extra-embryonal membranes. **(A)** Dot blot analysis of 5 μg of total RNA. Blots were exposed for 24 h. Placenta, amnion and VYS from 18th day conceptuses; visceral mesoderm and endoderm from day 16 conceptuses. **(B)** Northern blot analysis of 50 μg of total RNA. Blots were exposed for 60 h. **1**: 14th day placenta; **2**: 16th day placenta; **3**: 18th day placenta; **4**: 18th day VYS; **5**: 16th day visceral endoderm; **6**: 18th day amnion.

than the level of c-*fos* transcripts in 18th day amnion. In contrast, *fos*-related transcripts were ~20-fold more abundant in the FBJ-MuSV-transformed cells than in placenta. In a similar experiment we found that the concentrations of both *ras*^{Ha}- and *ras*^{Ki}-related transcripts in cell lines transformed by HaSV or KiSV, respectively, were ~10- to 20-fold higher than in 12th day extra-embryonal membranes or placenta (data not shown).

Discussion

In recent years major research efforts have focused on the physiological function of the cellular homologues (c-*onc*) of

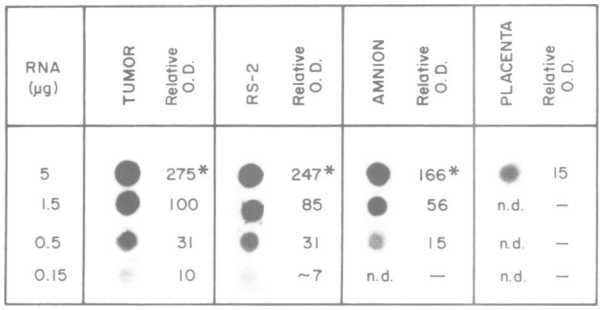

Fig. 7. Comparison of the level of *fos* transcripts in a FBJ-MSV-induced osteosarcoma (tumor), a FBJ-MSV-transformed cell line (RS-2), 18th day amnion and 12th day placenta. Dot blot analysis of varying amounts of total RNA as indicated. Blots were exposed for 36 h and quantitatively evaluated by scanning with a densitometer. O.D. values were normalized to 100 for the concentration of v-*fos* transcripts in 1.5 μg of tumor RNA. (*) Values determined from a shorter blot exposure (14 h). n.d., not done.

retroviral oncogenes. The results of several studies concerned with the expression of c-*onc* genes suggest that their encoded proteins may act during normal developmental processes. We have previously reported that two c-*onc* genes, c-*fos* and c-*fms*, are specifically expressed in mouse extra-embryonal structures (Müller *et al.*, 1982, 1983). Here, we have analyzed in detail the tissue-specific distribution and developmentally controlled accumulation of transcripts from these c-*onc* genes within the placenta and the extra-embryonal membranes.

Transcription of the c-fos gene

High levels of transcripts from both the c-*fos* and c-*fms* genes were detected specifically in the placenta and the extra-embryonal membranes (Figures 3A, 4B; Müller *et al.*, 1982, 1983). In the inner placenta as well as in the extra-embryonal membranes the concentrations of both c-*fos* and c-*fms* transcripts were found to increase gradually during prenatal development (Figures 3A, 5A). However, in contrast to the nearly constant expression of c-*fos*, the concentration of c-*fms* transcripts increases also during development of the outer placenta (Figure 5A). Analyses of the dissected components of extra-embryonal structures revealed distinctive distributions of c-*fos* and c-*fms* transcripts within the extra-embryonal tissues. Thus, in contrast to c-*fms*, significantly higher levels of c-*fos* transcripts were detected in the outer relative to the inner moiety of the mid-gestation placenta, and c-*fos* expression was considerably greater in late-gestation amnion, visceral endoderm and mesoderm compared with the placenta (Figures 5, 6).

The observed increase in the level of c-*fos* transcripts in both the extra-embryonal membranes (Figure 3A) and the inner placenta (Figure 5A) could be the consequence of: (i) an increase in the concentration of c-*fos* transcripts in a particular cell type during late gestation; or (ii) an increase in the relative number of cells containing high, but constant levels of c-*fos* transcripts. Increasing levels of c-*fos* transcripts in certain cell types could in turn be due to: (i) an increasing rate of transcriptional activity of the c-*fos* gene as gestation proceeds; or (ii) a constant rate of c-*fos* mRNA synthesis which is greater than the rate of degradation. The stability of transcripts has been shown to be an important determinant of the cytoplasmic mRNA concentration in the case of several eukaryotic genes (Harpold *et al.*, 1979). This observation may be particularly relevant in view of the fact that the stability of poly(A)$^+$ RNA in the mouse VYS has been reported to increase during late gestation (Andrews *et al.*, 1982).

The level of c-*fos* transcripts in the amnion was found to be close to the level of v-*fos* transcripts in cells neoplastically transformed by FBJ-MSV (Figure 7). Since it seems to be unlikely that the c-*fos* mRNA is not translated into protein, this finding indicates that a high level of c-*fos* expression is not sufficient to induce neoplastic transformation of amniotic cells. There are at least two explanations for this observation. The cell type(s) responsible for the high level of c-*fos* transcripts in the amnion may not be susceptible to transformation by the *fos* protein. Alternatively, the c-*fos* protein might lack oncogenic properties in any cell type. Nucleotide sequence analysis has indicated that the v-*fos* and c-*fos* (mouse and human) gene products differ at their carboxy termini (Van Beveren *et al.*, 1983; van Straaten *et al.*, 1983). The transforming potential of the v-*fos* protein might thus have arisen from a structural alteration which leads to the synthesis of an aberrant form of the normal cellular *fos* protein.

Implications of c-onc gene expression in mouse extra-embryonal tissues

The extra-embryonal tissues of the mouse comprise both the disc-shaped chorioallantoic placenta and the fetus-encircling amnion and yolk sacs (Figure 1). All these tissues have similar functions in protection and nourishment of the fetus, and have been shown, in both mouse and human, to synthesize and secrete a number of polypeptide and steroid hormones, hormone releasing factors and other compounds which assist the proper development and growth of the fetus (Gibbon *et al.*, 1975: Hertz, 1978). Furthermore, the human placeta has been reported to produce a tumor growth factor-like growth factor (Stromberg *et al.*, 1982), and to express abundant amounts of growth factor receptors (Figure 2C; Nexo *et al.*, 1979; Bhaumick *et al.*, 1981; Fox and Page Faulk, 1981; Adamson *et al.*, 1981). It is possible that c-*onc* gene products exert functions in any of these differentiative, proliferative and growth supporting processes. It is particularly tempting to speculate that the c-*fos* and c-*fms* proteins may be involved in the 'pseudo-malignant' properties of the mouse trophoblast (Kirby, 1965; Novak and Woodruff, 1974; Stromberg *et al.*, 1982). However, the fact that high levels of expression of both c-*onc* genes occur in late-gestation amnion and VYS argues against such a function, since there is no evidence that the constituents of the extra-embryonal membranes exhibit any properties characteristic of malignant cells. Interestingly though, rat yolk sac can give rise to carcinomas when allowed to proliferate through the punctured uterine wall (Wewer, 1982). Alternatively, a common function of the c-*fos* and c-*fms* gene products in processes associated with the transport of nutrients would correlate with the tissue distribution of transcripts from these c-*onc* genes.

To gain more insight into the function of c-*onc*-encoded proteins in the normal development of extra-embryonal structures in the mouse, it will now be of crucial importance to localize, by *in situ* hybridization techniques (e.g., Brahic and Haase, 1978), the cell types which exhibit elevated levels of c-*onc* transcription, and to identify and study the biochemical and biological properties of the c-*onc*-encoded proteins.

Materials and methods

Dissecton of mouse conceptuses

Random bred Swiss mice were mated, and the gestational period was timed by designating the day of finding the copulation plug as the 1st day of gestation. The exact stage of gestation was determined by the scheme of Rafferty

(Rafferty, 1970), which uses the size of the fetus and its morphological appearance as decisive criteria. From the 7th to 9th day, conceptuses were taken for RNA extractions as dissected from the uterine wall. Conceptuses from the 10th day of gestation onwards were separated into fetus, placenta (with part of the adhering parietal yolk sac and trophoectoderm), and extra-embryonal membranes (amnion and VYS). In some experiments, the VYS was surgically separated from the amnion, and its two layers (endoderm and mesoderm) were separated according to a published procedure (Adamson and Ayers, 1979). Placental tissue was separated into an outer and an inner portion (see Figure 2) by first trimming and discarding the perimeter with its adhering parietal yolk sac. The inner moiety was teased apart from the outer using forceps and an iridectomy knife.

Alkaline phosphatase histochemistry

Fresh 18th day placentas were frozen in OCT cryostat fluid (Tissue-Tek, Miles Laboratories) and placed so that cross sections were cut. Sections (6 μm) were dried onto gelatinized slides and fixed for 5 min in acetone at −20°C. Staining for alkaline phosphatase using the method described by Burstone (1958) gave a deep red positive reaction on the inner two-thirds of the placenta.

Alkaline phosphatase assay

The release of *p*-nitrophenol in butanol extracts of placental homogenates was measured at 410 nm after 10 min incubations with *p*-nitrophenol phosphate at 37°C and pH 10.4 (Doellgast *et al.*, 1977). The protein concentrations of the extracts were determined by the Coomassie Brilliant Blue G-250 dye-binding method (Sedmak and Grossberg, 1977). The specific activity of alkaline phosphatase was expressed as mU/mg protein. One mU of alkaline phosphatase activity liberates 1 nmol of *p*-nitrophenol/min.

Immunofluorescence test for transferrin receptor

Rat monoclonal antibodies to mouse transferrin receptor (a generous gift of I. Trowbridge and J. Lesley, Salk Institute, San Diego, CA) was applied to fresh sections of 18th day placenta cut as described above. Sections were incubated with 10 μg/ml antibody for 30 min at room temperature and subsequently washed with phosphate-buffered saline (PBS). The second antibody (fluorescein isothiocyanate-labeled goat anti-rat IgG, Miles Laboratories) was applied under the same conditions. After washing off excess antibody with PBS, the resulting fluorescence was observed in a Nikon fluorescence microscope and photographed using Kodak Tri-X Pan black and white film. For the control, normal rat serum was used instead of the anti-transferrin receptor antibody; minimal background fluorescence was observed (data not shown; but see negative portions in Figure 2C).

Isolation and blot analysis of RNA

Total RNA was extracted from mouse tissues (between 0.3 and 1.5 g) and selected for poly(A)$^+$ mRNA by oligo(dT)-cellulose chromatography as described elsewhere (Müller *et al.*, 1983). Non-selected or poly(A)$^+$ RNA was analyzed by dot blot analysis or agarose gel electrophoresis followed by blotting onto nitrocellulose paper as previously described (Thomas, 1980; Müller *et al.*, 1983). Blots were hybridized with molecularly cloned oncogene-specific probes (for details see Müller *et al.*, 1982, 1983; Curran *et al.*, 1982; Donner *et al.*, 1982; Ellis *et al.*, 1980, 1981; Tilghman *et al.*, 1979). The sizes of c-*onc* transcripts were determined relative to 18S and 28S rRNA markers, which were assumed to be 1.8 kb and 4.5 kb, respectively.

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