Int. J. Dev. Biol. 37: 519-529 (1993)

Original Article

Involvement of the proto-oncogene c-ets 1 and the urokinase plasminogen activator during mouse implantation and placentation

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ABSTRACT **Many of the Ets proteins have been shown to be transcription activators. *In vitro*, Ets 1 proteins are involved in the transcriptional induction of genes such as *stromelysin 1*, *collagenase 1* or *urokinase type plasminogen activator*, which are proteases responsible for extracellular matrix degradation. *In vivo*, *c-ets 1* is expressed in a wide variety of embryonic tissues in migrating cells, especially in endothelial cells during blood vessel formation. *C-ets 1* is also expressed in stromal cells of invasive carcinomas. In the present work, we have investigated the expression of both *c-ets 1* and *u-PA*, a putative target gene of the Ets 1 proteins, within a biological model which includes both embryonic and tumoral aspects. Implantation and placentation of the mouse embryo display migration of the trophoblastic cells, which invade the stroma of the uterine endometrium and trigger the establishment of a new vascular frame. Using *in situ* hybridization, we show that the overlapping of expression of *c-ets 1* and *u-PA* is restricted to some maternal cell populations from the invasive front and to the endothelial cells of the endometrial vasculature. *C-ets 1* is never expressed in trophoblasts. In contrast, *u-PA* expression in trophoblasts is strong and coincides with the embryo invasive phase. In the embryo proper, *c-ets 1* displays a spatio-temporal expression pattern similar to that described in the chick embryo. Until E 10.5, *u-PA* is expressed neither in embryonic nor in extra-embryonic structures. The respective roles of *c-ets 1* and *u-PA* and their relationship during mammalian placentation are discussed.**

KEY WORDS: *c-ets 1 proto-oncogene, u-PA, trophoblast, invasion, angiogenesis*

Introduction

The *v-ets* oncogene was originally discovered as part of the genome of the avian erythroleukemia virus E 26 and later shown to be transduced from a cellular gene (Leprince *et al.*, 1983; Nunn *et al.*, 1983). About thirty related proteins have now been found in species ranging from flies to humans and resembling the v-Ets protein in the so-called "ETS domain". The ETS domain is a 85 amino acid DNA-binding domain that specifically interacts with sequences containing the common core GGAA/T. Many of the Ets-related proteins, including Ets 1, have been shown to be transcription activators (Bosselut *et al.*, 1990; Gunther *et al.*, 1990; Ho *et al.*, 1990; Wasylyk *et al.*, 1990). However, Ets-like proteins may have other functions, such as in DNA replication and a general role in transcription activation (for review see MacLeod *et al.*, 1992).

Recent studies in our laboratory have been aimed at establishing the patterns of expression of the proto-oncogene *c-ets 1* both in embryos and in a wide variety of tumors. Using *in situ* hybridization, we have shown that *c-ets 1* mRNA is expressed in a variety of embryonic tissues and organs of the mesodermal lineage, and especially in endothelial cells during blood vessel formation (Vandenbunder *et al.*, 1989; Quéva *et al.*, 1993). In humans, *c-ets 1* is expressed in the endothelia of developing vessels of the embryo and in blood vessels in the adult when angiogenesis resumes, most strikingly during the vascularization of tumors (Wernert *et al.*, 1992). Interestingly, *in vitro* studies have shown that the Ets proteins, acting cooperatively with AP1, are involved in the induction of the *collagenase 1* and *stromelysin 1* promoters (Frisch and Ruley, 1987; Wasylyk *et al.*, 1991), two metalloproteinases implicated in degradation of the extracellular matrix (ECM). The Ets binding site also participates in the induction of the transcription of the human

Abbreviations used in this paper: ECM, extracellular matrix; uPA, urokinase plasminogen activator.

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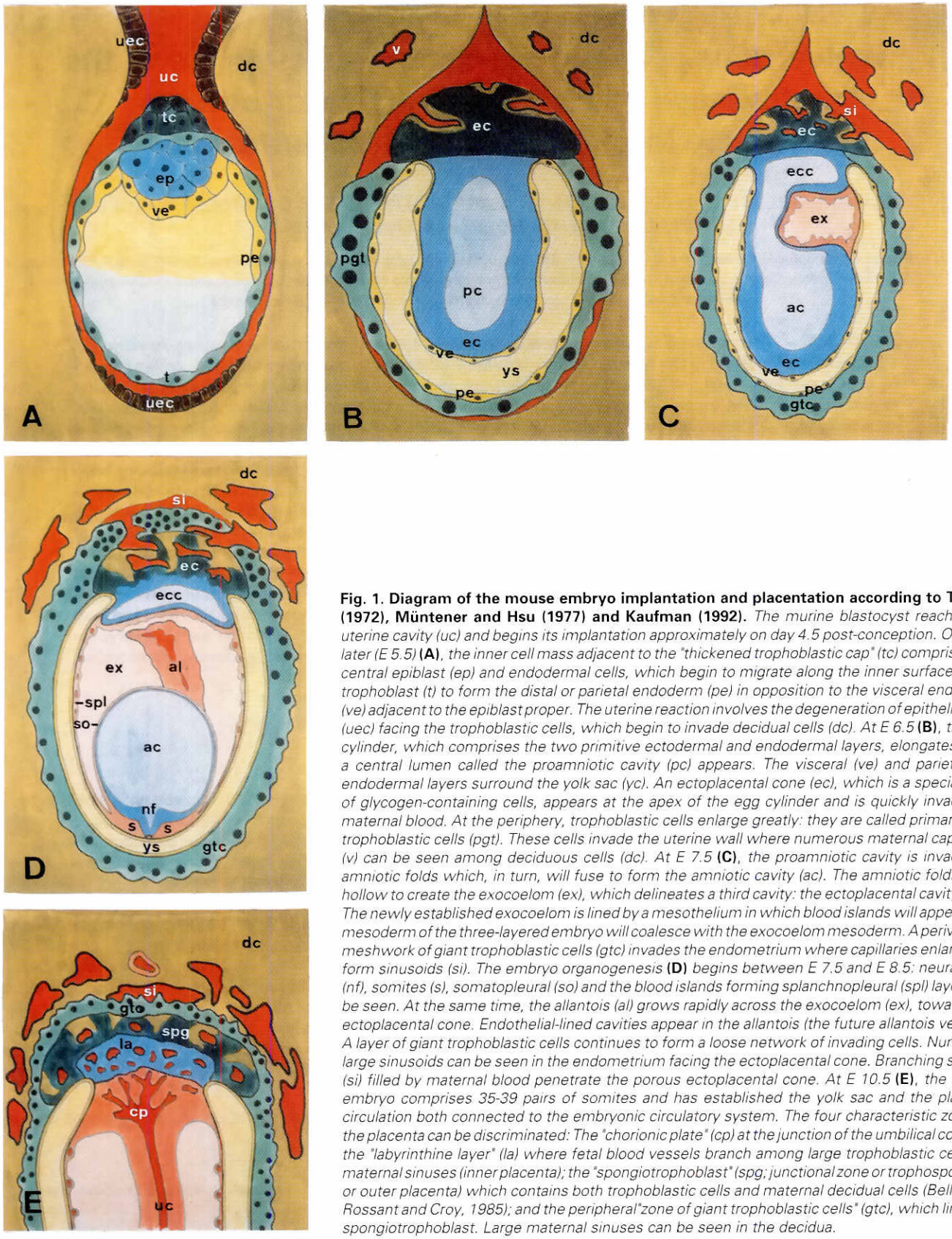

Fig. 1. Diagram of the mouse embryo implantation and placentation according to Theiler (1972), Müntener and Hsu (1977) and Kaufman (1992). *The murine blastocyst reaches the uterine cavity (uc) and begins its implantation approximately on day 4.5 post-conception. One day later (E 5.5)* **(A)**, *the inner cell mass adjacent to the "thickened trophoblastic cap" (tc) comprises the central epiblast (ep) and endodermal cells, which begin to migrate along the inner surface of the trophoblast (t) to form the distal or parietal endoderm (pe) in opposition to the visceral endoderm (ve) adjacent to the epiblast proper. The uterine reaction involves the degeneration of epithelial cells (uec) facing the trophoblastic cells, which begin to invade decidual cells (dc). At E 6.5* **(B)**, *the egg cylinder, which comprises the two primitive ectodermal and endodermal layers, elongates while a central lumen called the proamniotic cavity (pc) appears. The visceral (ve) and parietal (pe) endodermal layers surround the yolk sac (yc). An ectoplacental cone (ec), which is a special zone of glycogen-containing cells, appears at the apex of the egg cylinder and is quickly invaded by maternal blood. At the periphery, trophoblastic cells enlarge greatly: they are called primary giant trophoblastic cells (pgt). These cells invade the uterine wall where numerous maternal capillaries (v) can be seen among deciduous cells (dc). At E 7.5* **(C)**, *the proamniotic cavity is invaded by amniotic folds which, in turn, will fuse to form the amniotic cavity (ac). The amniotic folds grow hollow to create the exocoelom (ex), which delineates a third cavity: the ectoplacental cavity (ecc). The newly established exocoelom is lined by a mesothelium in which blood islands will appear. The mesoderm of the three-layered embryo will coalesce with the exocoelom mesoderm. A perivitelline meshwork of giant trophoblastic cells (gtc) invades the endometrium where capillaries enlarge and form sinusoids (si). The embryo organogenesis* **(D)** *begins between E 7.5 and E 8.5: neural folds (nf), somites (s), somatopleural (so) and the blood islands forming splanchnopleural (spl) layers can be seen. At the same time, the allantois (al) grows rapidly across the exocoelom (ex), towards the ectoplacental cone. Endothelial-lined cavities appear in the allantois (the future allantois vessels). A layer of giant trophoblastic cells continues to form a loose network of invading cells. Numerous large sinusoids can be seen in the endometrium facing the ectoplacental cone. Branching sinuses (si) filled by maternal blood penetrate the porous ectoplacental cone. At E 10.5* **(E)**, *the mouse embryo comprises 35-39 pairs of somites and has established the yolk sac and the placental circulation both connected to the embryonic circulatory system. The four characteristic zones of the placenta can be discriminated: The "chorionic plate" (cp) at the junction of the umbilical cord (uc); the "labyrinthine layer" (la) where fetal blood vessels branch among large trophoblastic cells and maternal sinuses (inner placenta); the "spongiotrophoblast" (spg; junctional zone or trophospongium or outer placenta) which contains both trophoblastic cells and maternal decidual cells (Bell, 1983; Rossant and Croy, 1985); and the peripheral "zone of giant trophoblastic cells" (gtc), which lines the spongiotrophoblast. Large maternal sinuses can be seen in the decidua.*

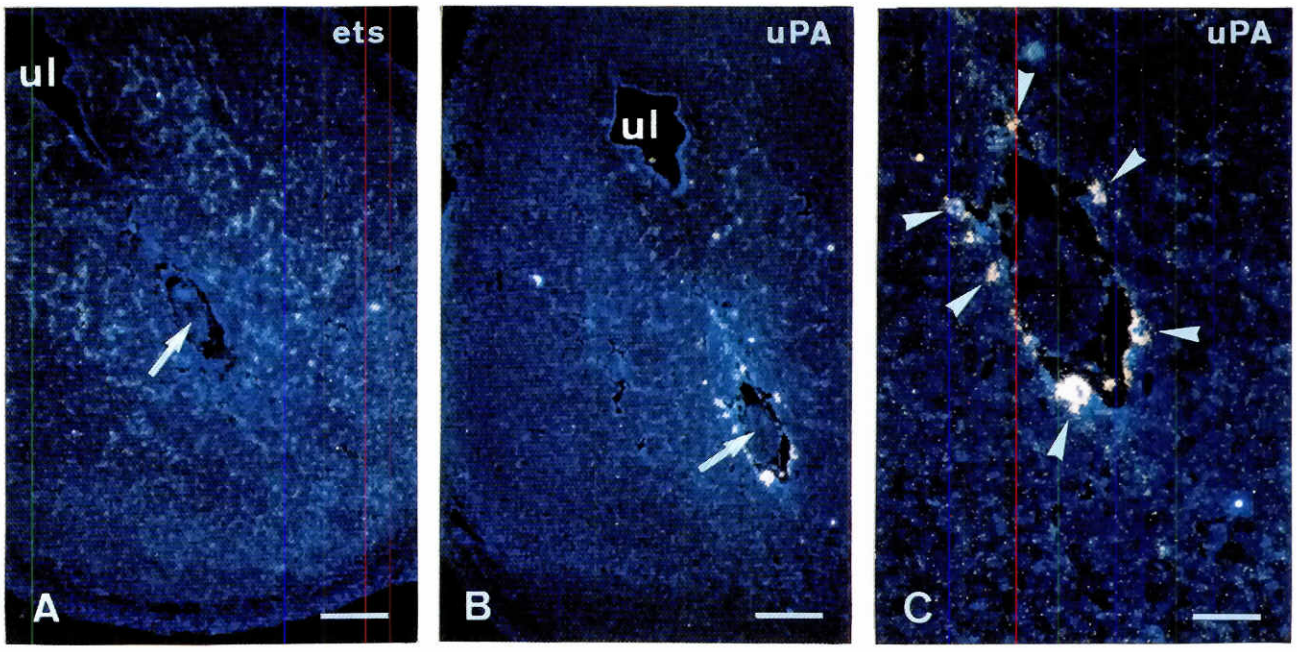

Fig. 2.Expression pattern of *c-ets 1* and *u-PA* at E 6.5. *A low magnification view shows that endothelial cells of blood vessels located within the endometrial wall express the proto-oncogene c-ets 1* **(A)**. *The embryo itself (arrow) is not labeled* **(A)**. *In contrast, the u-PA expression pattern does not overlap the ets expression pattern. Indeed, only focussed accumulations of silver grains can be seen in the vicinity of the implantation site* **(B)**. *u-PA transcripts are localized in the trophoblastic cells which are invading the decidua* (**C**, *arrow heads). Note that neither the endothelial walls within the decidua nor the egg cylinder cells are labeled. ul: uterine lumen. Bars, 250 μm for A,B and 100 μm for C.*

and murine *urokinase plasminogen activator (u-PA)* (Rorth *et al.*, 1990; Nerlov *et al.*, 1991), which binds to a membrane receptor (Blasi *et al.*, 1987) and is thought to localize the formation of active plasmin at the cell surface (Stephens *et al.*, 1989). During angiogenesis, the production of matrix degrading proteases by endothelial cells and the subsequent degradation of the basement membrane that surrounds preexisting capillaries or venules are essential to ensure the invasive properties of endothelial cells and the formation of new blood vessels (Pepper *et al.*, 1987; Alexander and Werb, 1989; Mignatti *et al.*, 1989). Therefore, it is tempting to speculate that *c-ets 1* might participate in the activation of genes encoding proteinases responsible for the degradation of the ECM. This last process is thought to play a key role in cell migration during embryonic development and tumor invasion (see Liotta *et al.*, 1991 for a detailed review).

The aim of the present work was to investigate the expression of *c-ets 1* in a biological model that replicates all the processes described above. Indeed, pre-implantation, implantation and placentation of the mammalian embryo imply migration of extraembryonic cells which in turn invade the stroma of the uterine endometrium and reorganize its vascular frame (see Fig. 1 for details). In addition, we have compared the *c-ets 1* and *u-PA* expression patterns in order to investigate *in vivo* the relation between this transcription factor and one of its putative target genes. Here, we show a superimposable pattern of expression only in some maternal cell populations. In contrast, the extraembryonic giant trophoblastic cells strongly express *u-PA* but never express *c-ets 1*. Moreover, we did not detect *u-PA* transcripts in the embryonic annexes or in the embryo proper, while *c-ets 1* displayed a spatio-temporal expression pattern similar to that described in the chick embryo.

Results

Expression of c-ets 1 and u-PA at E 6.5

The observation of serial sections which were hybridized either with the *c-ets 1* probe or the *u-PA* probe revealed clearly distinct expression patterns. The endothelial cells of the forming blood vessels within the decidua expressed the proto-oncogene, while the blastocyst did not (Fig. 2A). In contrast, *u-PA* was not seen within the maternal tissue (Fig. 2B), but was expressed by invading primary giant trophoblastic cells (Fig. 2C). Intraembryonic cells were not labeled (Fig. 2C). Our control sense probes never displayed signals over the background.

Expression of c-ets 1 and u-PA at E 7.5

The *c-ets 1* expression pattern was similar to that of E 6.5, i.e. a conspicuous signal localized on endothelial cells of the maternal blood vessels (Fig. 3A). Trophoblastic cells did not express *c-ets 1* but were strongly labeled by the *u-PA* antisense probe (Fig. 3B and D). Contrasting with the expression pattern at E 6.5, the endothelial wall of some blood vessels facing the ectoplacental cone expressed *u-PA* (Fig. 3B).

Expression of c-ets 1 and u-PA at E 8.5

C-ets 1 transcripts were observed lining the blood vessels within the whole decidua (Fig. 4A), but the most intense signal was seen in the mesometral zone of the uterus facing the ectoplacental cone. In this region, labeled sinusoids appeared numerous and strands of labeled cells extended within the decidua towards the implantation site. Individual cells lining the invasion front were also labeled (Fig. 4B and C). In these cases, labeled cells harbored a small nucleus when compared to those of decidual or trophoblastic cells.

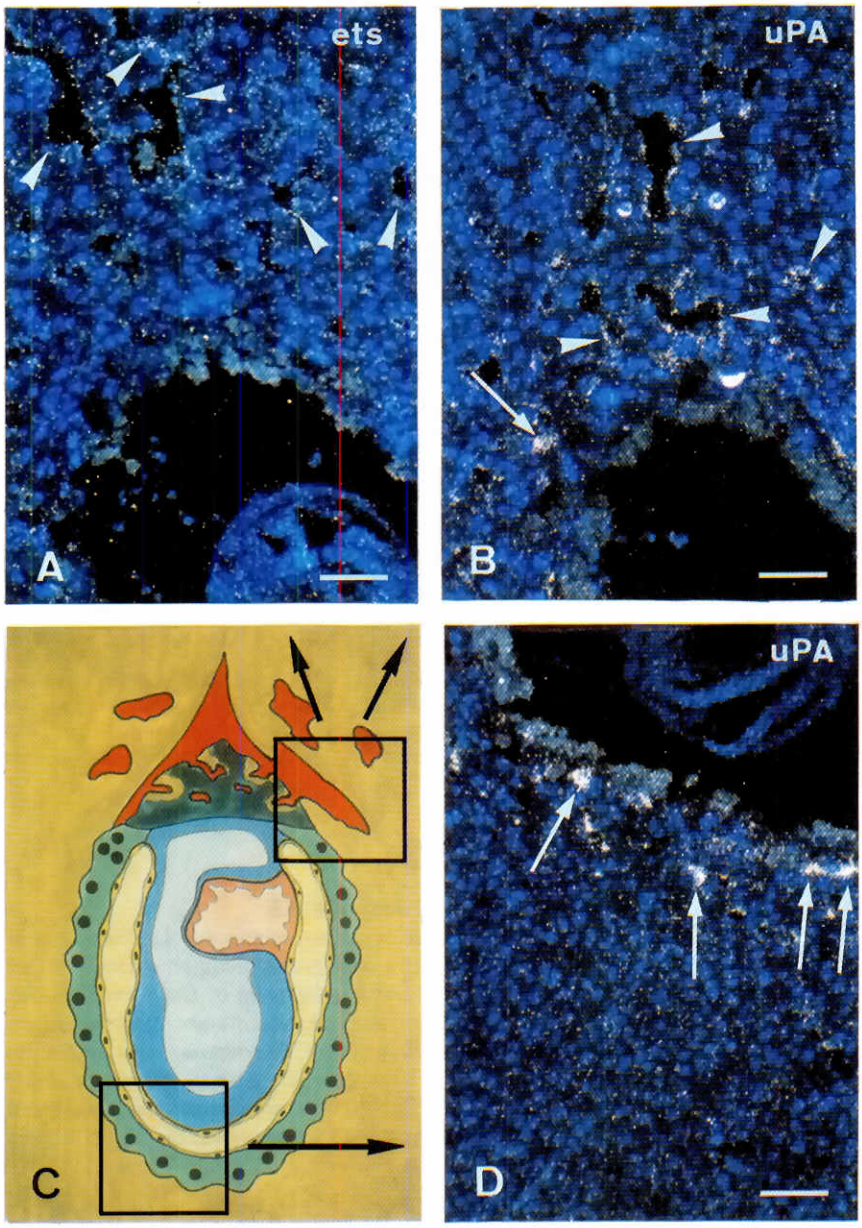

Fig. 3. Expression pattern of *c-ets 1* and *u-PA* at E 7.5. *The proto-oncogene c-ets 1 is expressed by endothelial cells of the decidual blood vessels* (**A**, arrow heads). *No signal can be seen at the invasion front attesting that trophoblastic cells do not express c-ets 1. Hybridizations performed with the u-PA probe show a conspicuous signal localized both to these trophoblastic cells* (**B-D**, arrows) *and to the endothelial cells of some blood vessels within the mesometral zone of the uterus* (**B**, arrow heads). **(C)** *shows the approximate localization of photographs illustrating this developmental stage. Bar, 100 μm for A,B,D.*

Neither the giant trophoblastic cells (Fig. 4B and C) nor the cells of the growing ectoplacental cone (Fig. 4A) where maternal blood was observed expressed the proto-oncogene *c-ets 1*. In the embryo, typical neurula stages were observed. It is worthy of note that *c-ets 1* was strongly expressed in the mesodermal lineage and more specially in the splanchnopleural mesoderm (Fig. 4A and C) which, in turn, is the site of formation of the extraembryonic blood system. Moreover, many cells within the allantois were strongly labeled (Fig. 4A).

u-PA labeling was restricted to cohorts of cells located around the ectoplacental cone (Fig. 4D). In some cases, these cells lined small maternal blood sinusoids; in other cases, they were aligned in irregular strands infiltrated between decidual cells (Fig. 4E and F). Compared to *c-ets 1* expression, *u-PA* labeling was restricted to a narrow strand around the ectoplacental cone. Giant trophoblastic cells no longer expressed *u-PA* mRNAs. Using our antisense *u-PA* probe, we never observed any signal in the embryo proper (Fig. 4D). Using an antibody directed against von Willebrand factor to identify endothelial cells, staining was positive at the margin of the large sinusoids infiltrating the decidua. However, the signal intensity decreased towards the implantation site, and cells of the invasion front did not always display a significant staining (Fig. 5A and B). Positive inclusions were seen within trophoblastic cells attesting that these cells engulf components of the maternal vasculature (Fig. 5B).

Expression of c-ets 1 and u-PA at E 10.5

At E 10.5, the placenta reaches its final configuration. *C-ets* labeling was seen within the labyrinthine layer lining the numerous fetal blood vessels. The spongiotrophoblast and the zone of giant trophoblastic cells facing the decidua never expressed *c-ets 1*, whereas peripheral cells of large maternal sinusoids filled with red

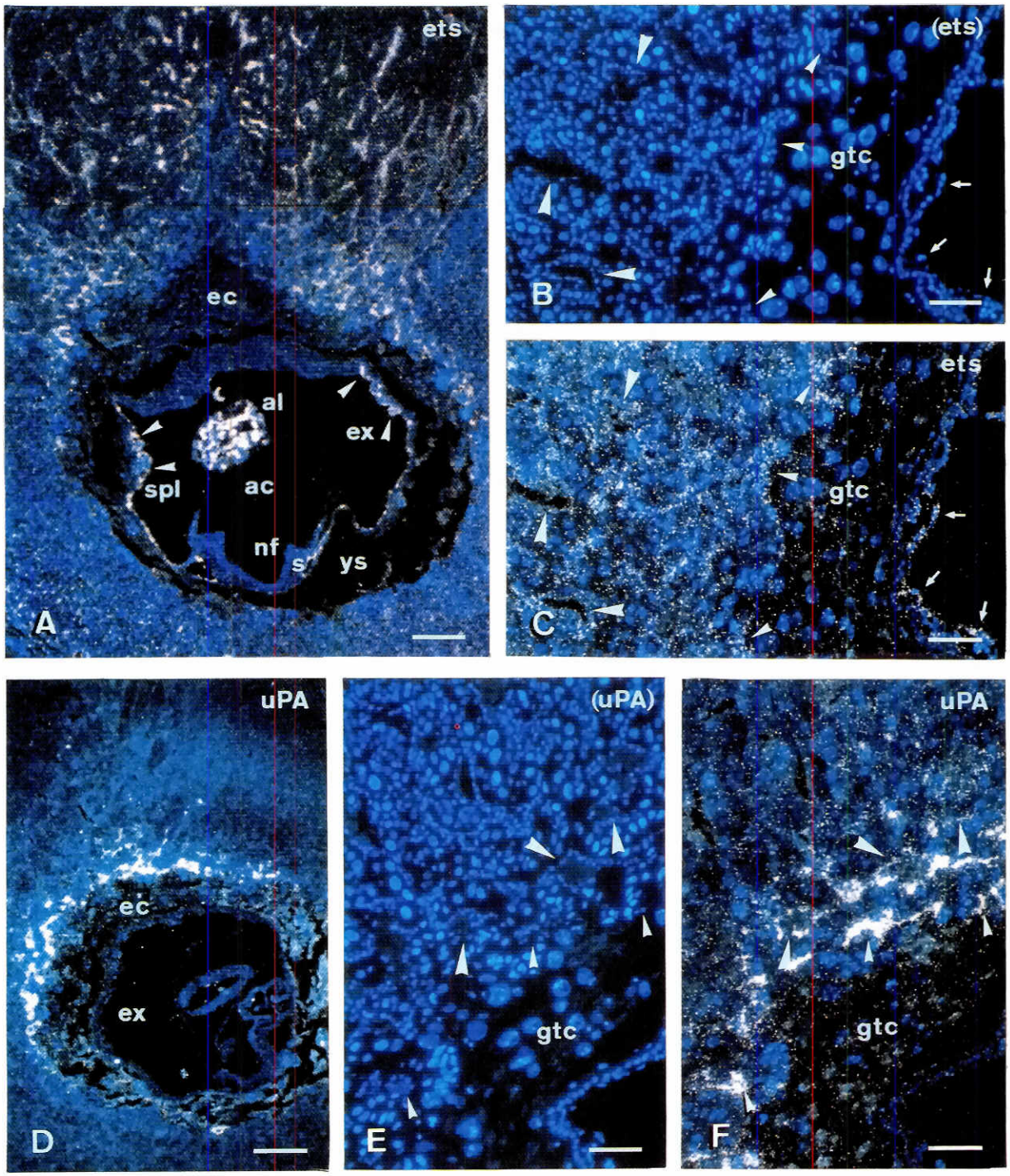

Fig. 4. Expression pattern of *c-ets 1* and *u-PA* at E 8.5. *A general view* **(A)** *demonstrates the c-ets 1 intense labeling of the blood vessels that converge towards the ectoplacental cone (ec). This transversal section of the embryo also shows the typical labeling located essentially in the mesodermal lineage of this neurula stage embryo. Conspicuous labeling can be observed in the splanchnopleural layer (spl), especially at the level of the establishing extraembryonic blood islands, which protrude into the exocoelom (arrow heads). In the exocoelom (ex) lining the amniotic cavity (ac) the allantois (al) is strongly labeled. Higher magnifications* (**B-C**, *respectively Hoechst staining and Hoechst staining plus dark field illumination of the same sections) illustrate the ets expression both in the wall of maternal blood vessels (large arrow heads) and in numerous cells of the endometrium. Maternal cells from the invasion front are also labeled (small arrow heads). Small arrows point labeled embryonic blood islands within the splanchnopleural mesoderm. In contrast, giant trophoblastic cells (gtc) do not express c-ets 1. u-PA expression is restricted to a narrow strand facing the ectoplacental cone* **(D)**. *Higher magnifications* (**E-F,** *respectively Hoechst staining and Hoechst staining plus dark field illumination of the same sections) show that giant trophoblastic cells (gtc) no longer express u-PA but that the signal is located on cells with small nuclei facing the invasion front (small arrow heads) or cells surrounding cohorts of maternal red blood cells (large arrow heads). Bars, 250 μm for A,D and 100 μm for B,C,E,F.*

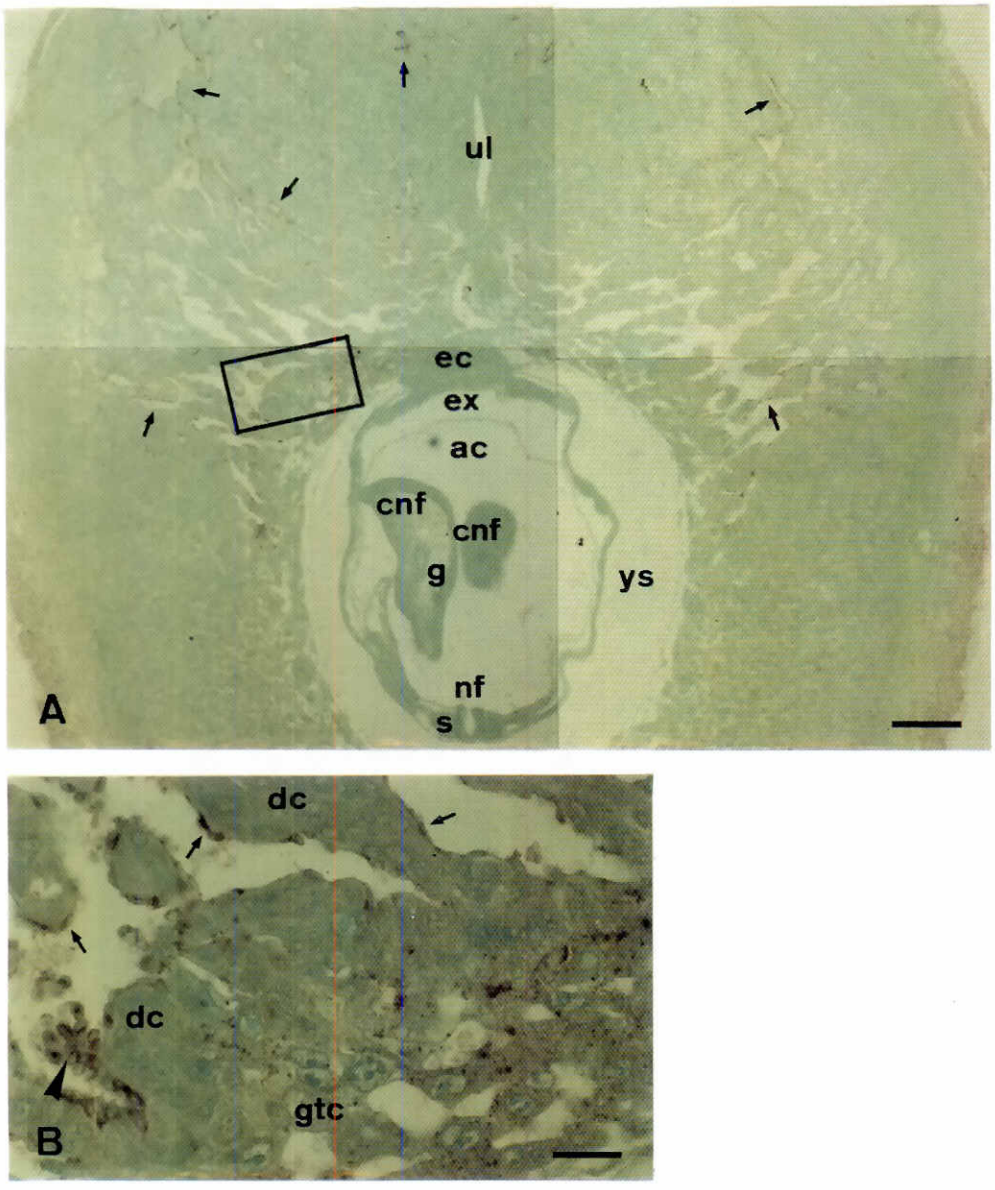

Fig. 5. Expression of the von Willebrand factor at E 8.5. *Labeling with the von Willebrand antibody decreases from the outer side of the mesometral zone towards the implantation site* (**A**, small arrows). *The embryo has reached the neurula stage and displays typical features of this stage: large cephalic neural fold (cnf), the anterior end of the gut (g). The same section shows posterior neural fold (nf) and somites (s). ul: uterine lumen; ec: ectoplacental cone; ex: exocoelom; ac: amniotic cavity; ys: yolk sac cavity.* **(B)** *is an enlarged photograph of the invasion front depicted in A. Sparse endothelial cells are labeled by the antibody (small arrows) and underline the infiltrated decidual cells (dc). A sinusoid wall is tangentially cut in this section (arrow head). Giant trophoblastic cells (gtc) display some positive inclusions, further establishing the process of maternal uterine degradation. Bars, 250 μm for A and 50 μm for B.*

blood cells expressed *ets* transcripts (Fig. 6A and B). The invasion front comprised numerous labeled cells, the nuclei of which were small or flat as those of endothelial cells (Fig. 6A and B). The expression of *u-PA* was observed in some groups of cells within the placental spongiotrophoblast (Fig. 6C and D). Neither fetal vessel walls, nor giant trophoblastic cells expressed this gene. The expression of *u-PA* in the maternal part of the placenta was confined to the sinusoid walls and apparently to subpopulations of decidual cells within the invasion front (Fig. 6C and D). Peripheral cells of the large maternal sinusoids were strongly labeled by the anti von Willebrand factor as were the major fetal blood vessels (Fig. 6E), but the cells bordering the invasion front were poorly labeled (Fig. 6E).

The main features of the *c-ets 1* expression pattern in E 10.5 mouse embryos, at the onset of their organogenesis (Fig. 7A-C), can be summarized in the following terms: mesenchymal cells surrounding the central nervous system and its peripheral extensions such as optic vesicles expressed the proto-oncogene. Moreover, a strong signal lined the external face of the neuroepithelium (Fig. 7A). *C-ets 1* transcripts were also seen in the mesenchyme of branchial arches, in the endocardium and in the wall of major blood vessels such as anterior and posterior cardinal veins, dorsal aortae or small capillaries (Fig. 7B and C). More caudal sections revealed a strong labeling in emigrating neural crest cells and mesenchymal cells within the sclerotomal component of the somites (Fig. 7C). The dermomyotome was never labeled. *C-ets 1* was also expressed in the mesentery of the hindgut (Fig. 7C). Mesenchymal cells surrounding the Wolffian ducts and the posterior cardinal vein walls were also labeled (data not shown). Mesodermal cells of the young limb buds displayed a faint labeling (Fig. 7C). We did not detect *c-ets 1* transcripts in epithelia but rather in remodeling and migrating mesenchymal cells.

In contrast, as predicted by the lack of labeling at the periphery of extraembryonic blood vessels in the labyrinthine placenta, we never detected *u-PA* transcripts in the embryo proper (Fig. 7D-F).

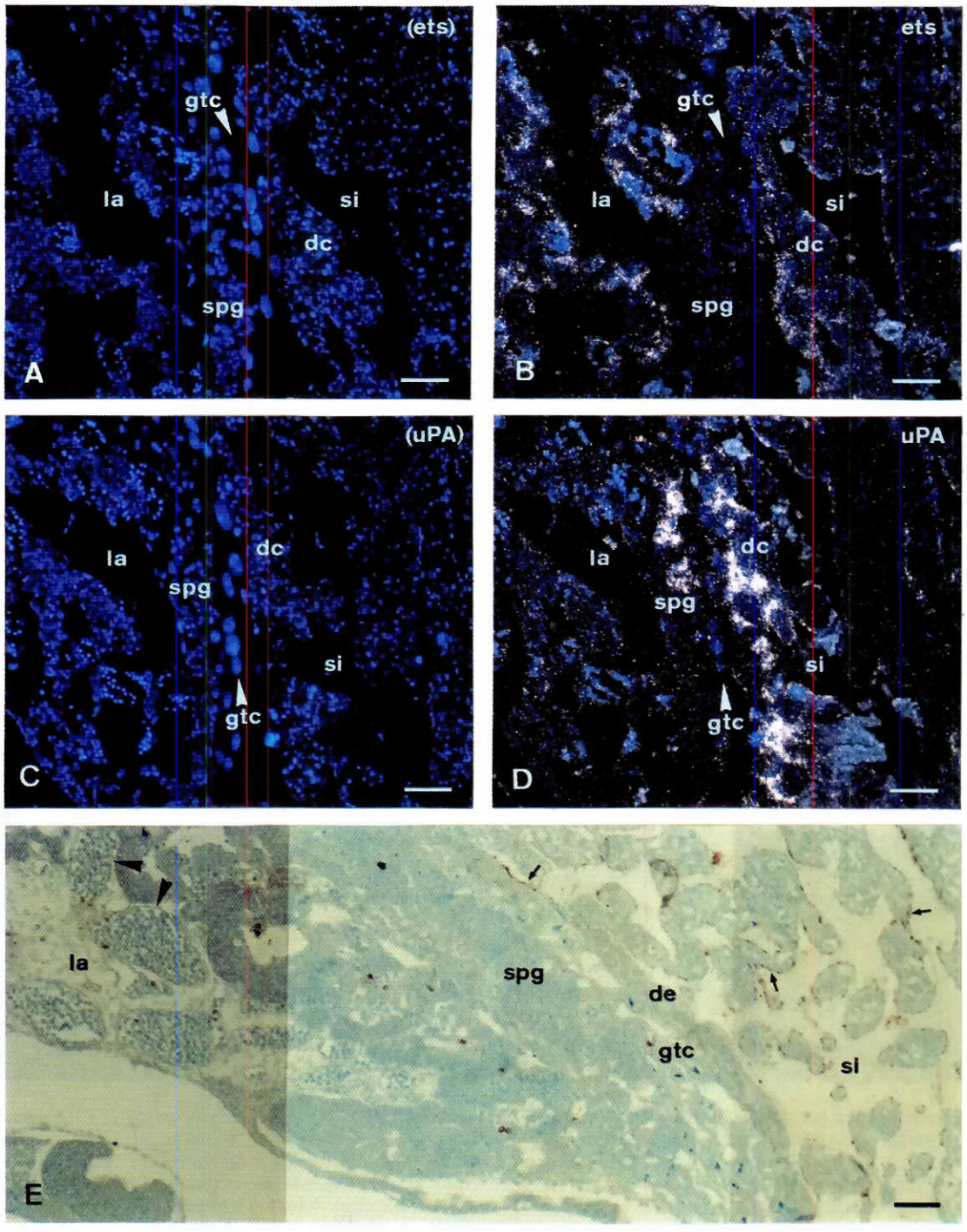

Fig. 6. Expression pattern of *c-ets 1* and *u-PA* in the placenta at E 10.5. (A and C) *Hoechst staining.* **(B and D)** *Hoechst staining plus dark field illumination of the same sections. These views were selected to illustrate the invasion front and show on the left the fetal part of the placenta comprising the labyrinthine layer (la), the spongiotrophoblast (spg), the giant trophoblastic cell layer (gtc) facing the decidua and its sinusoids on the right. C-ets 1* **(A-B)** *is strongly expressed in the endothelial cells of the fetal blood vessels within the labyrinthine layer (fetal blood cells display a strongly refringent nucleus). In contrast, no signal can be observed in either the spongiotrophoblast or the giant trophoblastic cells. The maternal part of the placenta displays labeling that is restricted to endothelial cells of large sinusoids and cells at the invasion front. A striking difference is observed when u-PA expression is studied in the fetal placenta: indeed, only some cohorts of cells within the spongiotrophoblast are labeled* **(C-D)**. *As previously seen, giant trophoblastic cells do not express u-PA. Moreover, the newly established fetal vessels are not labeled. In contrast, large maternal sinusoids are lined by u-PA labeling as cohorts of cells situated at the invasion front* **(C-D)**. *However, this expression is not superimpose on that of c-ets 1. Staining with the von Willebrand Factor antibody* **(E)** *shows the endothelial cells lining the large maternal sinusoids and sparse endothelial cells of the invasion front (small arrows). Moreover, the von Willebrand factor is present at the periphery of large embryonic vessels in the labyrinthine layer (arrow heads). Bar, 100 μm for A,B,C,D,E.*

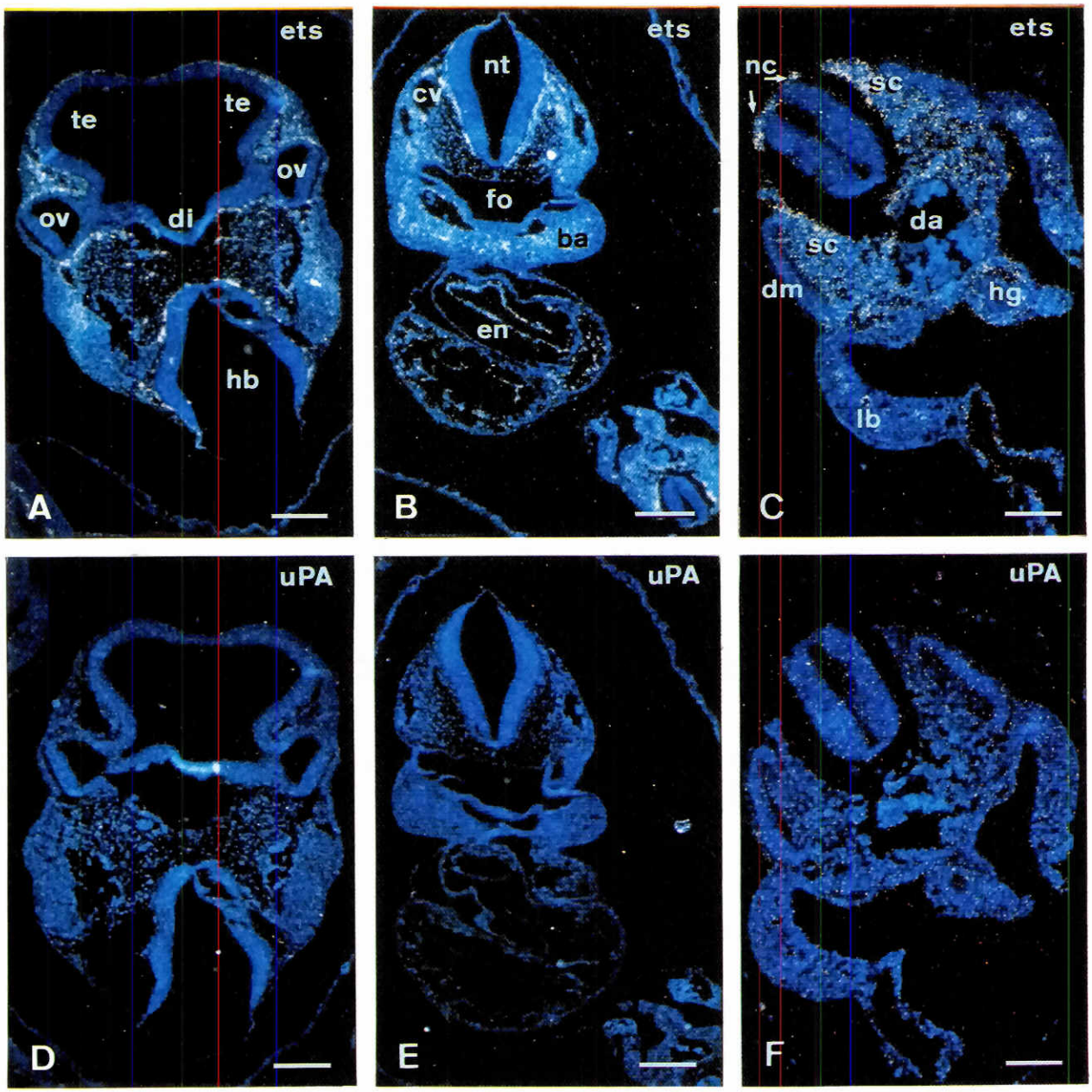

Fig. 7. Expression pattern of *c-ets 1* and *u-PA* in the E 10.5 embryo. *Transverse sections performed in the anterior part of E 10.5 embryos show strong labeling with the c-ets 1 probe* **(A)**. *The cephalic mesenchyme surrounding the central nervous system and its optic appendages, the maxillary component of the first branchial arch, express the proto-oncogene (ov: optic vesicle; di: diencephalon; te: telencephalic vesicle; hb: hindbrain). At the same level, u-PA is not expressed* **(D)**. *The light area seen in (D) at the level of the diencephalon does not represent a positive in situ hybridization signal but is due to refringence. More posteriorly* **(B)**, *a strong signal for c-ets 1 is seen around both the neural tube (nt) and the anterior cardinal vein (cv) as in the mesenchyme of the mandibular component of the first branchial arch (ba) and the pharyngeal part of the foregut (fo). The endocardium (en) is also strongly labeled. At this level, u-PA is not expressed in any embryonic structure* **(E)**. *Transverse sections performed through the caudal part of the embryo show that c-ets 1* **(C)** *is strongly expressed in neural crests (nc, small arrows), in the sclerotomal part of the somites (sc), in the wall of the dorsal aorta (da), in the mesentery surrounding the hindgut (hg) as in the growing somatopleural mesoderm responsible for the limb bud growth (lb). u-PA is not expressed in the posterior part of the embryo* **(F)**. *Bars, 250 μm for A,B,D,E and 100 μm for C,F.*

Discussion

In this report, we describe the expression of the proto-oncogene *c-ets 1* during the implantation of the mammalian embryo and the partial overlapping of expression with *u-PA* .

Involvement of both u-PA and c-ets 1 during implantation

The penetrative nature of hemochorial placentation so mimics that seen with highly invasive tumors that the normal trophoblast has been called pseudomalignant. The processes of implantation and placentation are both dependent upon the remodeling of the

uterine endometrium and vasculature by trophoblasts. Invasion through the uterine endometrium is promoted by proteases bound to the trophoblast cell surface or present in the extracellular environment (Alexander and Werb, 1989). Consistent with this finding, metalloproteinases such as collagenase or stromelysin and their inhibitors have been detected both during early mammalian development (Brenner *et al.*, 1989; Behrendtsen *et al.*, 1992) and during tumor angiogenesis (Blood and Zetter, 1990).

We have shown that the expression of *u-PA* in mouse trophoblast cells coincides temporally with the embryo's invasive phase and is localized to regions of invasion. This study confirms and extends the results of Sappino *et al.* (1989). We have found that *u-PA* expression is transient in trophoblastic cells reaching its maximal level at E 7.5 and decreasing at E 8.5. *u-PA* expression in trophospongial cells was also observed until E 10.5. Our data demonstrate a conspicuous labeling in the wall of decidual sinuses and a strong labeling over cohorts of cells at the invasion front. Although some populations exhibit features of endothelial cells, the identity of other groups cannot be ascertained. The pattern of expression of *u-PA* suggests that it might be involved in the invasive process of the trophoblast through the uterine wall.

Numerous cells lining the invasion front were also labeled by the *c-ets 1* probe. By contrast, this proto-oncogene was not expressed by the trophoblastic cells that infiltrate the uterine wall. This pattern is reminiscent of the studies of Wernert *et al.* (1992 and manuscript submitted) in which *c-ets 1* transcripts were detected in fibroblastic cells of the stroma surrounding invasive carcinomas for different human tissues, while no expression was detected in the tumoral invasive cells within the same tissues. This pattern suggests that the invasive cells might send a signal to the cells of the invasive front to trigger the *c-ets 1* transcription.

Cotransfection experiments in cultured cells have shown that *c-ets 1* is able to activate the *collagenase 1* and *stromelysin 1* promoters (Gutman and Wasylyk, 1990; Wasylyk *et al.*, 1991) and that the conserved Ets binding-site is essential for the activity of the *u-PA* promoter (Rorth *et al.*, 1990; Nerlov *et al.*, 1991). Thus, it is tempting to speculate that c-Ets 1 is a regulatory element in the events leading to ECM degradation during mammal embryo implantation. We propose that c-Ets 1, which is expressed in maternal cells of the invasive front, may be able to trigger *u-PA* expression within a subpopulation of these *c-ets 1*-positive maternal cells. This model implies that the c-Ets 1 proteins are not efficient in stimulating *u-PA* transcription in all the cells of the invasive front. The uterus must act to limit implantation. To this end, the uterine decidual response delimits the boundaries of invasion and factors exist that regulate implantation (Strickland and Richards, 1992). It is likely that some of these molecules may interact with c-Ets 1 protein and subsequently limit the effect of this transcription factor.

Involvement of both u-PA and c-ets 1 during the angiogenesis processes

Our study provides further evidence that *c-ets 1* is tightly linked to angiogenesis or vasculogenesis during embryonic implantation and development.

Indeed, during mouse embryo implantation and placentation, new capillaries form that grow towards the ectoplacental cone. At the end of this process, large maternal sinuses can be observed lining the fetal part of the placenta. Our study shows dramatically that the pattern of expression of *c-ets 1* overlaps with the regions where angiogenesis takes place. *C-ets 1* is expressed in endothelial cells during the establishment of the vascular network both in the endometrial wall and in the extra-embryonic and embryonic tissues, while *u-PA* expression is only observed in endothelial cells of some maternal blood vessels facing the ectoplacental cone at E 7.5. The *u-PA* signal is very high until the E 10.5 stage, when Sappino *et al.* (1989) describe a faint label in cells lining the maternal sinuses at the invasion front. Co-expression was seen in the endothelial wall of maternal blood vessels or sinuses from E 7.5 to E 10.5.

These results raise interesting questions about the role of both *c-ets 1* and *u-PA* in the process of angiogenesis as previously noted. The production of *u-PA in vivo*, in the context of natural angiogenic processes, has been shown neither in tumoral context nor in the embryo (Pyke *et al.*, 1991; Sappino *et al.*, 1991). In conjunction with these observations, we have not detected *u-PA* mRNA in the mouse embryos. In contrast, *u-PA* expression was detected in endothelial cells within the maternal decidua during the placentation. These results are in agreement with previous studies showing that *u-PA* is expressed in endothelial cells during neovascularization of ovarian follicles, of the corpus luteum, and the maternal decidua in mouse (Bacharach *et al.*, 1992). These results suggest that the expression of *u-PA* during the angiogenic process depends on the histological context of the endothelial cell.

In view of the detection of *u-PA* transcripts in endothelial cells, within the maternal tissues, we postulate a role for u-PA in fibrinolysis during the invasion of the maternal decidua by trophoblastic cells. Indeed, during tumoral procedure or wound healing, it has been shown that the local injury of the tissues results in the leakage of plasma and hemorrhage from damaged blood vessels. Extravasated plasma and blood clot rapidly upon contact with tissue procoagulants to form a gel that consists essentially of fibrin. Then fibrinolysis occurs to prevent migratory thrombophlebitis. This fibrinolysis is attributed to secreted plasminogen activators that catalyze the conversion of the plasma zymogen plasminogen to the actively fibrinolytic protease plasmin (Harold and Dvorak, 1986). In agreement with this knowledge and with our results, we postulate that u-PA may play a role in fibrinolysis which could prevent thrombosis of blood vessel when invaded by trophoblastic cells.

Evidence is accumulating to suggest that the Ets 1 proteins might regulate the transcription of the genes coding for matrix degrading proteinases which are necessary for both angiogenesis and tumor invasion. These proteinases are also known to be essential for both endothelial cell migration (Mignatti *et al.*, 1989) and releasing trapped growth factors via extracellular matrix degradation (Saksela and Rifkin, 1990).

Indeed, an interesting aspect of this regulatory pathway is that u-PA mediates the release of an active form of basic fibroblast growth factor-heparan sulfate complexes (bFGF-HSPG) from endothelial ECM, and that in turn bFGF added into the medium of endothelial cell cultures increases PA activity (Saksela and Rifkin, 1990). In bovine endothelial cells, there is an autocrine stimulation pathway by which the bFGF released by the cells regulates their own migration, and at the same time PA activity and DNA synthesis (Sato and Rifkin, 1988). Moreover, Mignatti *et al.* (1989) using an *in vitro* assay have shown that angiogenesis requires bFGF-induced proteinases. Placental tissues are known to contain high levels of bFGF (Moscatelli *et al.*, 1986), and a great number of high affinity binding sites for bFGF are present on cell membranes of mouse placenta at stage E 12 (Hondermarck *et al.*, 1990). Therefore, bFGF might be involved in the endometrial vascular reorganization during

placentation, along with other factors, such as vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) and placental growth factor (PlGF), which exhibit angiogenic activity and have been isolated from human term placenta (Burgos, 1983; Moscatelli *et al.*, 1986; Ishikawa *et al.*, 1989; Maglione *et al.*, 1991; Maglione *et al.*, 1993).

Recent results from our laboratory have shown that different angiogenic factors such as bFGF, TNFα or TGFß can trigger *c-ets 1* expression in cultured endothelial cells (Fafeur *et al.*, personal communication). Therefore, we suggest the existence of a regulatory pathway in which placental angiogenic factors would trigger *c-ets 1* expression, which in turn might activate *u-PA* transcription. Consequently, *u-PA* could trigger the activation of bFGF leading to increases in both PA activity and endothelial cell migration. The combined action of placental angiogenic factors and of the c-Ets 1transcription factor may allow the rapid endometrial vascular reorganization.

Roles of c-ets 1 during mouse development

C-ets 1 expression maps within the embryo proper corroborate previous results obtained in the chicken embryo (Vandenbunder *et al.*, 1989; Quéva *et al.*, 1993). *C-ets 1* is expressed at the onset of the formation of blood islands in the splanchnopleural layer lining the visceral endoderm and in the wall of the young blood vessels emerging within the allantois. Endothelial cells of intraembryonic blood vessels were also labeled with the *ets* probe. Beyond this specific pattern, *c-ets 1* was expressed in certain situations. For example, *c-ets 1* was expressed at sites where epithelio-mesenchymal transitions occur (neural crest and sclerotomal cell emigration) or when epithelio-mesenchymal interactions trigger organogenesis (branchial arches, kidneys, limb buds).

Knock-out experiments of the murine *c-ets 1* gene by means of homologous recombination are in progress to determine the significance of the observations described above. If *c-ets 1* is shown to be instrumental in the progression of the trophoblast as in angiogenic processes, it would further suggest that this oncogene is an important activator of the processes involved in promoting the invasiveness of tumor cells.

Materials and Methods

Mouse model and histological procedures

Four female mice of the Balb/c strain were caged overnight with one male. On the next morning, observation of a copulation plug determined day 0 of development (E 0). Pregnant animals were killed by cervical dislocation at E 6.5, E 7.5, E 8.5, E 10.5. Fig. 1 describes the main events occurring during this period of the embryonic life. Uterine horns were exposed by laparotomy, removed and placed in PBS. Implantation sites were swiftly trimmed free of fat and mesentery tissues and isolated before being fixed at 4°C for 16 h in 4% paraformaldehyde in PBS containing 5 mM $MgCl_2$, washed in PBS, dehydrated, embedded in paraffin and serially cut (7 µm). Tissue sections were transferred to 3-aminopropyl triethoxysilane (TESPA, Aldrich) coated slides and heated at 42°C for two days. Slides were stored at 4°C until use.

Preparation of ^{35}S RNA probes

The antisense and sense RNA probes were constructed from a 1.6 kb Sac I-Kpn I fragment of the mouse *c-ets 1* cDNA cloned in the Bluescript SK plasmid. The pSP64 *u-PA* plasmid harboring a 660 bp Hind III-Pst I fragment of the murine *u-PA* cDNA was a gift of Dominique Belin and Jean-Dominique Vassali (CMU, University of Geneva, Switzerland) and was used to prepare the antisense probes. This fragment cloned in the pSP65 plasmid provided the control sense probes (for detailed sequences, see respectively Chen, 1990 and Belin *et al.*, 1985).

In situ hybridization

In situ hybridization was a modification of the method of Cox *et al.* (1984) reported by Quéva *et al.* (1992). After deparaffinization and hydration, the slides were incubated in 0.1 M glycine, 0.2 M Tris-HCl pH 7.4 for 10 min at room temperature, treated with 1 µg ml^{-1} proteinase K (Boehringer Mannheim) for 15 min at 37°C, post-fixed in 4% paraformaldehyde, washed in PBS, acetylated by 0.25% acetic anhydride in 0.1 M triethanolamine, washed in 2x SSC and dehydrated by ethanol. Probes were denatured at 80°C and diluted in the hybridization buffer (50% formamide, NaCl 0.3 M, Tris-HCl pH 8 20 mM, EDTA 5 mM, dextran sulfate 10%, Denhardt's 1x, tRNA from E. coli 0.5 mg ml^{-1}, DTT 100 mM). Hybridization mixtures were applied to slides and siliconized coverslips were placed over the sections. Hybridization was performed at 60°C for 18 h. The slides were subsequently washed several times in 4x SSC 10 mM DTT in which the coverslips were removed. Mismatched or unhybridized probes were digested by incubation with 20 µg ml^{-1} RNAse A (type III A, Sigma) in 0.4 M NaCl, Tris-HCl pH 7.5 10 mM, EDTA 50 mM at 37°C for 1 h. The slides were washed in the RNAse buffer, then in 2x SSC at 60°C for 15 min and in 0.1x SSC at 60°C for 15 min. They were dehydrated in ethanol, dried and dipped in nuclear track emulsion (Kodak NTB2). The slides were exposed for 2 or 3 weeks at 4°C. After development, they were stained using the intercalating dye Hoechst 33258, mounted in glycergel (Sebia) and observed under a double illumination using an Olympus BH2 photomicroscope with epifluorescence for Hoechst staining and a dark-field condenser for silver grain detection.

Detection of endothelial cells

Von Willebrand factor was used as a specific marker for endothelial cells (Jaffe *et al.*, 1974; Ginsburg *et al.*, 1985). Staining was performed using rabbit antibodies to human von Willebrand factor and the biotin-extravidin kit (Sigma) according to the manufacturer specifications.

Acknowledgments

We thank Dominique Belin for the gift of the u-PA probe, Veronique Fafeur and Robert Lafyatis for critical reading, Francine Laloux for patient typing and Robert Lafyatis for improving the English translation of our manuscript. This work was supported by the CNRS, the MRT and the Association pour la Recherche sur le Cancer.

References

ALEXANDER, C.M. and WERB, Z. (1989). Proteinases and extracellular matrix remodeling. *Curr. Opin. Cell Biol. 1:* 974-982.

BACHARACH, E., ITIN, A. and KESHET, E. (1992). *In vivo* patterns of expression of urokinase and its inhibitor PAI-1 suggest a concerted role in regulating physiological angiogenesis. *Proc. Natl. Acad. Sci. USA 89:* 10686-10690.

BEHRENDTSEN, O., ALEXANDER, C.M. and WERB, Z. (1992). Metalloproteinases mediate extracellular matrix degradation by cells from mouse blastocyst outgrowths. *Development 114:* 447-456.

BELIN, D., VASSALI, J-D., COMBEPINE, C., GODEAU, F., NAGAMINE, Y., REICH, E., KOSHER, H.P. and DUVOISIN, R.M. (1985). Cloning, nucleotide sequencing and expression of cDNAs encoding mouse urokinase-type plasminogen activator. *Eur. J. Biochem. 148:* 225-232.

BELL, S.C. (1983). Decidualization and associated cell types: implications for the role of the placental bed in the materno-fetal immunological relationship. *J. Reprod. Immunol. 5:* 185-194.

BLASI, F., VASSALLI, J-D. and DANO, K. (1987). Urokinase-type plasminogen activator: proenzyme, receptor, and inhibitors. *J. Cell Biol. 104:* 801-804.

BLOOD, C.H. and ZETTER, B.R. (1990). Tumor interactions with the vasculature: angiogenesis and tumor metastasis. *Biochim. Biophys. Acta 1032:* 89-118.

BOSSELUT, R., DUVALL, J.F., GEGONNE, A., BAILLY, M., HEMAR, A., BRADY, J. and GHYSDAEL, J. (1990). The product of the c-ets 1 proto-oncogene and the related Ets-2 protein act as transcriptional activators of the long terminal repeat of human T cell leukemia virus HTLV-1. *EMBO J. 9:* 3137-3144.

BRENNER, C.A., ADLER, R.R., RAPPOLEE, D.A., PEDERSEN, R.A. and WERB, Z. (1989). Genes for extracellular matrix-degrading metalloproteinases and their inhibitor, TIMP, are expressed during early mammalian development. *Genes Dev. 3:* 848-859.

BURGOS, H. (1983). Angiogenic and growth factors in human amnio-chorion and placenta. *Eur. J. Clin. Invest. 13:* 289-296.

CHEN, J.H. (1990). Cloning, sequencing and expression of mouse c-ets 1 cDNA in baculovirus expression system. *Oncogene Res. 5:* 277-285.

COX, K.H., DE LEON, D.V., ANGERER, L.M. and ANGERER, R.C. (1984). Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Dev. Biol. 101:* 485-502.

FRISCH, S.M. and RULEY, H.E. (1987). Transcription from the stromelysin promoter is induced by interleukin-1 and repressed by dexamethasone. *J. Biol. Chem. 34:* 16300-16304.

GINSBURG, D., HANDIN, R.I., BONTHRON, D.T., DONLON, T.A., BRUNS, G.A.P., LATT, S.A. and ORKIN, S.H. (1985). Human von Willebrand factor (vWF): isolation of complementary DNA (cDNA) and chromosomal localization. *Science 228:* 1401-1406.

GUNTHER, C.V., NYE, J.A., BRYNER, R.S. and GRAVES, B.J. (1990). Sequence-specific DNA binding of the proto-oncoprotein ets-1 defines a transcriptional activator sequence within the long terminal repeat of the Moloney murine sarcoma virus. *Genes Dev. 4:* 667-679.

GUTMAN, A. and WASYLYK, B. (1990). The collagenase gene promoter contains aTPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites. *EMBO J. 9:* 2241-2246.

HAROLD, F. and DVORAK, M. D. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *New Engl. J. Med. 315:* 1650-1659.

HO, C.I., BHAT, N.K., GOTTSCHALK, L.R., LINDSTEN, T., THOMPSON, C.B., PAPAS, T.S. and LEIDEN, J.M. (1990). Sequence-specific binding of human ets-1 to the T cell receptor a gene enhancer. *Science 250:* 814-818.

HONDERMARCK, H., COURTY, J., LEDOUX, D., BLANCKAERT, V., BARRITAULT, D. and BOILLY, B. (1990). Evidence of high and low affinity binding sites for basic fibroblast growth factor in mouse placenta. *Biochem. Biophys. Res. Commun. 169:* 272-281.

ISHIKAWA, F., MIYAZONO, K., HELLMAN, U., DREXLER, H., WERNSTEDT, C., HAGIWARA, K., USUKI, K., TAKAKU, F., RISAU, W. and HELDIN, C.H. (1989). Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. *Nature 338:* 557-562.

JAFFE, E.A., HOYER, L.W. and NACHMAN, R.L. (1974). Synthesis of von Willebrand factor by cultured human endothelial cells. *Proc. Natl. Acad. Sci. USA 71:* 1906.

KAUFMAN, M.H. (1992). *The Atlas of Mouse Development.* Academic Press, London.

LEPRINCE, D., GEGONNE, A., COLL, J., DE TAISNE, C., SCHNEEBERGER, A., LAGROU, C. and STEHELIN, D. (1983). A putative second cell-derived oncogene of the avian leukaemia retrovirus E26. *Nature 306:* 395-397.

LIOTTA, L.A., STEEG, P.S. and STETLER-STEVENSON, W.G. (1991). Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell 64:* 327-336.

MACLEOD, K., LEPRINCE, D. and STEHELIN, D. (1992). The ets gene family. *Trends Biochem. Sci. 17:* 251-256.

MAGLIONE, D., GUERRIERO, V., VIGLIETTO, G., DELLI-BOVI, P. and PERSICO, G. (1991). Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc. Natl. Acad. Sci. USA 88:* 9267-9271.

MAGLIONE, D., GUERRIERO, V., VIGLIETTO, G., FERRARO, M.G., APRELIKOVA, O., ALITALO, K., DEL VECCHIO, S., LEI, K.J., YANG, CHOU, J. and PERSICO, G. (1993). Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PIGF), are transcribed from a single gene of chromosome 14. *Oncogene 8:* 925-931.

MIGNATTI, P., TSUBOI, R., ROBBINS, E. and RIFKIN, D.B. (1989). *In vitro* angiogenesis on the human amniotic membrane: requirement for basic fibroblast growth factor-induced proteinases. *J. Cell Biol. 108:* 671-682.

MOSCATELLI, D., PRESTA, M. and RIFKIN, D.B. (1986). Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis, and migration. *Proc. Natl. Acad Sci. USA 83:* 2091-2095.

MÜNTENER, M. and HSU, Y.C. (1977). Development of trophoblast and placenta of the mouse. A reinvestigation with regard to the *in vitro* culture of mouse trophoblast and placenta. *Acta Anat. 98:* 241-252.

NERLOV, C., RORTH, P., BLASI, F. and JOHNSEN, M. (1991). Essential AP-1 and PEA3 binding elements in the human urokinase enhancer display cell type specific activity. *Oncogene 6:* 1583-1592.

NUNN, M.F., SEEBURG, P.H., MOSCOVICI, C. and DUESBERG, P.H. (1983). Tripartite structure of the avian erythroblastosis virus E26 transforming gene. *Nature 306:* 391-395.

PEPPER, M.S., VASSALLI, J.D., MONTESANO, R. and ORCI, L. (1987). Urokinase-type plasminogen activator is induced in migrating capillary endothelial cells. *J. Cell Biol. 105:* 2535-2541.

PYKE, C., KRISTENSEN, P., RALFKIAER, E., GRONDAHL-HANSEN, J., ERIKSEN, J., BLASI, F. and DANO, K. (1991). Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. *Am. J. Pathol. 138:* 1059-1067.

QUÉVA, C., LEPRINCE, D., STEHELIN, D. and VANDENBUNDER, B. (1993). $p54^{c\text{-}ets1}$ and $p68^{c\text{-}ets1}$, the two transcription factors encoded by the c-ets 1 locus, are differentially expressed during the development of the chick embryo. *Oncogene 8:* 2511-2520.

QUÉVA, C., NESS, S.A., GRASSER, F.A., GRAF, T., VANDENBUNDER, B. and STEHELIN, D. (1992). Expression patterns of c-myb and v-myb induced myeloid-1 (mim-1) gene during the development of the chick embryo. *Development 114:* 125-133.

RORTH, P., NERLOV, C., BLASI, F. and JOHNSEN, M. (1990). Transcription factor PEA3 participates in the induction of urokinase plasminogen activator transcription in murine keratinocytes stimulated with epidermal growth factor or phorbol-ester. *Nucleic Acids Res. 18:* 5009-5017.

ROSSANT, J. and CROY, B.A. (1985). Genetic identification of tissue of origin of cellular populations within the mouse placenta. *J. Embryol. Exp. Morphol. 86:* 177-189.

SAKSELA, O. and RIFKIN, D.B. (1990). Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity. *J. Cell Biol. 110:* 767-775.

SAPPINO, A-P., HUARTE, J., BELIN, D. and VASSALI, J-D. (1989). Plasminogen activators in tissue remodeling and invasion: mRNA localization in mouse ovaries and implanting embryos. *J. Cell Biol. 109:* 2471-2479.

SAPPINO, A-P., HUARTE, J., VASSALI, J-D. and BELIN, D. (1991). Sites of synthesis of urokinase and tissue-type plasminogen activators in the murine kidney. *J. Clin. Invest. 87:* 962-970.

SATO, Y. and RIFKIN, D.B. (1988). Autocrine activities of basic fibroblast growth factor: regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. *J. Cell Biol. 107:* 1199-1205.

STEPHENS, R.W., POLLANEN, J., TAPIOVAARA, H., LEUNG, K.C., SIM, P.S., SALONEN, E.M., RONNE, E., BEHRENDT, E., DANO, N. and VAHERI, A. (1989). Activation of pro-urokinase and plasminogen on human sarcoma cells: a proteolytic system with surface bound reactants. *J. Cell Biol. 108:* 1987-1995.

STRICKLAND, S. and RICHARDS, W.G. (1992). Invasion of trophoblasts. *Cell 71:* 355-357.

THEILER, K. (1972). *The House Mouse. Development and Normal Stages from Fertilization to 4 Weeks of Age.* Springer-Verlag, Berlin.

VANDENBUNDER, B., PARDANAUD, L., JAFFREDO, T., MIRABEL, M.A. and STEHELIN, D. (1989). Complementary patterns of expression of c-ets 1, c-myb and c-myc in the blood forming system of the chick embryo. *Development 107:* 265-274.

WASYLYK, B., WASYLYK, C., FLORES, P., BEGUE, A., LEPRINCE, D. and STEHELIN, D. (1990). The c-ets proto-oncogenes encode transcription factors that cooperate with c-fos and c-jun for transcriptional activation. *Nature 346:* 191-193.

WASYLYK, C., GUTMAN, A., NICHOLSON, R. and WASYLYK, B. (1991). C-ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. *EMBO J. 10:* 1127-1134.

WERNERT, N., GILLES, F., FAFEUR, V., BOUALI, F., RAES, M.B., DUPRESSOIR, T., CESBRON, J.Y., PYKE, C., SEITZ, G., REMBERGER, K., GOSSELIN, B., VANDENBUNDER, B. and STEHELIN, D. The transcription factor c-ets 1 and its putative target genes encoding extracellular matrix degrading proteases are expressed within stromal fibroblasts of invasive human carcinomas and in cultured fibroblasts activated by TNFα and bFGF. (Submitted).

WERNERT, N., RAES, M.B., LASSALLE, P., DEHOUCK, M.P., GOSSELIN, B., VANDENBUNDER, B. and STEHELIN, D. (1992). C-ets 1 proto-oncogene is a transcription factor expressed in endothelial cells during tumor vascularization and other forms of angiogenesis in humans. *Am. J. Pathol.. 138:* 111-117.

Accepted for publication: October 1993