

# Emilin, a Component of Elastic Fibers Preferentially Located at the Elastin-Microfibrils Interface

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**Abstract.** The fine distribution of the extracellular matrix glycoprotein emilin (previously known as glycoprotein gp15) (Bressan, G. M., I. Castellani, A. Colombatti, and D. Volpin. 1983. *J. Biol. Chem.* 258: 13262–13267) has been studied at the ultrastructural level with specific antibodies. In newborn chick aorta the protein was exclusively found within elastic fibers. In both post- and pre-embedding immunolabeling emilin was mainly associated with regions where elastin and microfibrils are in close contact, such as the periphery of the fibers. This localization of emilin in aorta has been confirmed by quantitative evaluation of the distribution of gold particles within elastic fibers. In other tissues, besides being associated with typical elastic fibers, staining for emilin was found in structures lacking amorphous elastin, but where the pres-

ence of tropoelastin has been demonstrated by immunoelectron microscopy. This was particularly evident in the oxitalan fibers of the corneal stroma, in the Descemet's membrane, and in the ciliary zonule. Analysis of embryonic aorta revealed the presence of emilin at early stages of elastogenesis, before the appearance of amorphous elastin. Immunofluorescence studies have shown that emilin produced by chick embryo aorta cells in culture is strictly associated with elastin and that the process of elastin deposition is severely altered by the presence of antiemilin antibodies in the culture medium. The name of the protein was derived from its localization at sites where elastin and microfibrils are in proximity (emilin, elastin microfibril interface located protein).

**E**LASTIC fibers are morphologically formed by a central amorphous core and surrounding microfibrils of 12–13 nm in diameter, which are variously represented in different tissues. Initial studies suggested that different molecular components constitute the two parts of the fibers, a hydrophobic protein, elastin, and a hydrophilic glycoprotein which could not be characterized (Ross and Bornstein, 1969). It is now clear that the molecular composition of elastic fibers is more complex than previously thought, and several molecules have been found to be associated with either the amorphous nucleus (Cerra et al., 1984; Baccarani-Contri et al., 1991) or the microfibrils (Breathnach et al., 1979; Gibson et al., 1986; Sakai et al., 1986) in different tissues. The function of most of these components is not known. It has been suggested that they regulate particular steps of the assembly of elastic fibers and that their relative expression could be an important factor contributing to the morphological differences of elastic fibers at different locations (Ross and Bornstein, 1969; Daga-Gordini et al., 1990). In addition, the identification of fibrillin as the defective gene in Marfan syndrome (Lee et al., 1991; Dietz et al., 1991), raises the possibility that alterations of the structure or expression of other elastin-associated components may contribute to pathological conditions of elastic fibers. For these reasons the detailed knowledge of all the molecular components is a key issue in the study of elastic fibers.

Some years ago the authors isolated from chick aorta and partially characterized a glycoprotein of 115 kD (Bressan et al., 1983a), which is named emilin in the present paper. The protein is present in the connective tissue of most organs (Colombatti et al., 1985a) from where it can be solubilized only after reduction, a property indicating a structural role (Bressan et al., 1983a). Immunolocalization at the optical microscopic level suggested that emilin is associated with elastic fibers (Colombatti et al., 1987). Biosynthetic studies (Colombatti et al., 1988a) showed that emilin is not a proteolytic product of a larger precursor, confirming that it is not related to known higher molecular weight elastic fiber-associated proteins such as fibrillin (Sakai et al., 1986).

In this work we describe the ultrastructural localization of emilin in various tissues. The results indicate that emilin is indeed associated with elastic fibers and suggest that it is particularly abundant at the elastin-microfibrils interface; hence the name emilin (elastin microfibril interface located protein).

## Materials and Methods

### Antibodies

Both monoclonal and polyclonal antibodies against emilin have been used. An antiserum was obtained from rabbits immunized with purified emilin

(Colombatti et al., 1985a) and antibodies were affinity purified from the serum following two procedures. In the first procedure, serum aliquots (5 ml) were preabsorbed on a column of chick tropoelastin-Sepharose CL4B and the flow through was applied to a column of emilin-Sepharose CL4B. After thorough washing with PBS, bound antibodies were eluted from the column with 0.1 M glycine-HCl, pH 2.8, immediately neutralized with 2 M Tris-HCl, pH 8.0, and dialyzed against PBS. The affinity columns were prepared by coupling 2 mg of purified protein to CNBr-activated Sepharose CL4B (Pharmacia Fine Chemicals, Piscataway, NJ) as recommended by the manufacturer. In the second procedure, a preparation of emilin partially purified by DEAE chromatography (Bressan et al., 1983a) and purified tropoelastin were resolved by SDS-PAGE and transferred to nitrocellulose filters. Blotted proteins were visualized by Ponceau S staining, the emilin and tropoelastin bands were cut and saturated for 1 h at room temperature in PBS containing 2% BSA. A 1:20 dilution of the anti-emilin antiserum in PBS was first incubated for 2 h at room temperature with tropoelastin-containing strips and then with the emilin-containing strips under similar conditions. The emilin-containing filter was washed with PBS-BSA and the bound antibodies eluted with 0.1 M glycine-HCl, pH 2.8, neutralized and dialyzed against PBS. Antitropoelastin antibodies were affinity purified in a similar way. The generation and characterization of mouse mAbs to emilin was reported previously (Colombatti et al., 1985b).

Two mAbs against human fibrillin were obtained from Dr. Lynn Sakai of the Shriners Hospital for Crippled Children, Portland, OR. mAb 15 is human-specific and binds to both the reduced and nonreduced protein; mAb 201 cross reacts with chick fibrillin only before reduction. The mAb to fibronectin was produced by a hybridoma supplied by the American Type Culture Collection (ATCC HB91). mAb 111A3 was used to localize type VI collagen (Colombatti et al., 1988b).

### Sample Preparation

Aorta and other organs were dissected from 2-d-old chicks. For examination of aorta in embryos of 4 d, the whole thoracic area was used. Tissue specimens to be processed through the postembedding procedure were fixed in 3% paraformaldehyde-0.25% glutaraldehyde in PBS, pH 7.2, for 30 min, washed extensively with PBS, and dehydrated through a graded series of ethanol-water mixtures. Some samples were fixed in 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.2, for 6–12 h and postfixed for 30 min in cacodylate-buffered 1% OsO<sub>4</sub> before dehydration.

Groups of tissue blocks from the different animals and organs were embedded in parallel in Epon 812, LR White, and Lowicryl K4M. Embedding in Epon 812 was performed according to standard procedures (Luft, 1961). For embedding in LR White, samples dehydrated in 75% ethanol were infiltrated with LR White resin/75% ethanol (2:1) for 1 h; immersed in undiluted resin (4 periods of 1 h each at room temperature and then overnight at 4°C); transferred into fresh LR White (1 h at room temperature) and

sealed in small gelatine capsules at 50°C for 24 h under vacuum. Lowicryl embedding was carried out following the procedure described by Carlemalm et al. (1982).

In the preembedding procedure, tissue samples were immediately processed for immunolabeling (see below).

### Immunolabeling

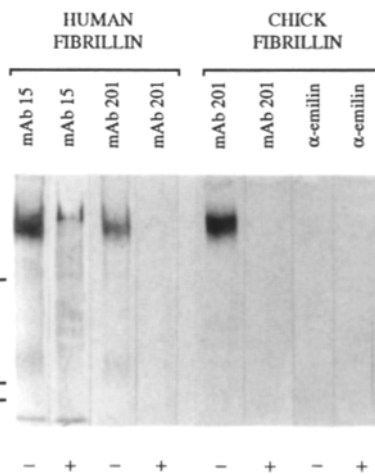
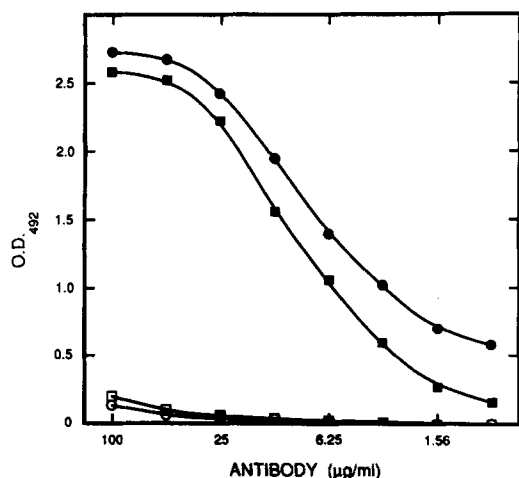
Postembedding: ultrathin sections were collected on uncoated nickel grids, saturated with 2% BSA in PBS (PBS-BSA) for 10 min and incubated with appropriate dilutions of antibodies in PBS-BSA for 1 h at room temperature or overnight at 4°C. The sections were then washed several times in PBS-BSA, incubated for 1 h with a diluted solution (1:50) of goat anti-rabbit antibodies conjugated with colloidal gold particles (Janssen Pharmaceutica, Beerse, Belgium), rinsed in PBS-BSA followed by distilled water and stained for EM (see below). Preembedding: unfixed tissue specimens were processed at 4°C starting with an overnight incubation with the first antibody. This was followed by washing in PBS-BSA (three times for 6–12 h), incubation with gold-conjugated second antibody for 12–24 h and extensive rinsing in PBS-BSA (three times for 12 h). The samples were then fixed for 2 h in 2.5% glutaraldehyde in Na cacodylate buffer, pH 7.2, postfixed in cacodylate-buffered 1% OsO<sub>4</sub> (30 min), dehydrated, and embedded in Epon 812.

### Electron Microscopy

Ultrathin sections of post- and preembedding immunolabeled specimens were stained for 15 min with 2.5% alcoholic uranyl acetate and 5 min with Reynold's lead citrate and examined in a Philips EM 400 electron microscope (Philips Technologies, Cheshire, CT).

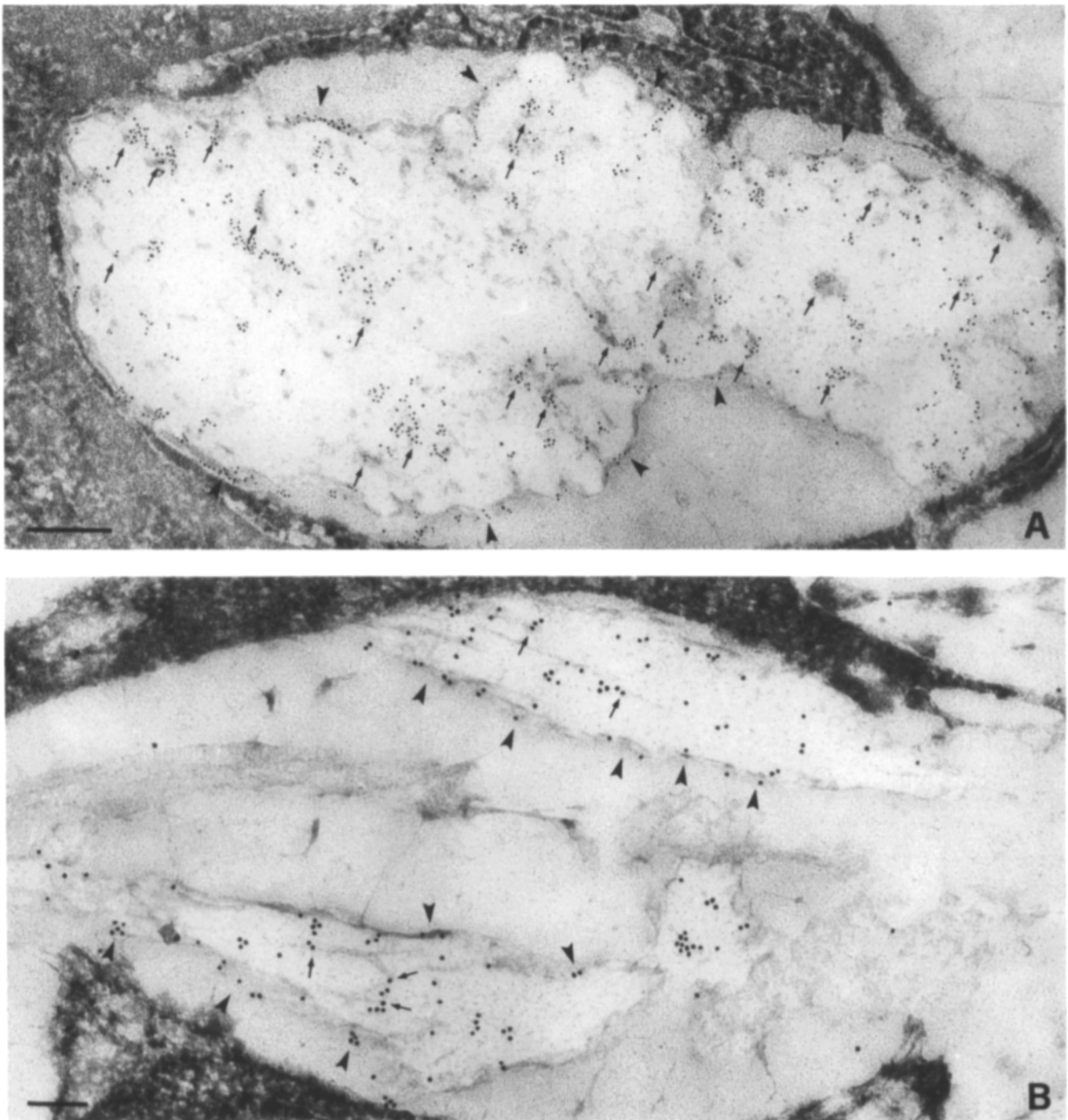
### Quantitative Evaluation of Gold Particle Distribution

The quantitative determination of the distribution of gold particles was performed on sections taken from different samples embedded in LR White and processed with rabbit polyclonal antibodies to emilin or tropoelastin and 10-nm-diameter colloidal gold as described above. Elastic fiber containing fields from randomly chosen micrographs (nine for each antibody) were printed at a magnification of 60,000 and three types of regions were identified on the fibers on the basis of the distribution of the uranyl acetate-lead citrate stain: (a) the electron-lucent area, which constitutes most of the amorphous core of the fiber; (b) the electron-dense peripheral area which includes the elastin-microfibrils interface; and (c) the electron-dense areas within the elastic fiber. The border of the different regions was delineated on a transparent sheet and the dimensions of the regions were determined using an IBAS 2000 Image Analysis System (Kontron Image Analysis Divi-



**Figure 1.** Specificity of anti-emilin polyclonal antibodies. (Left panel) ELISA on purified emilin and tropoelastin. Binding of: (■—■) anti-emilin to emilin; (□—□) anti-emilin to tropoelastin (●—●) antitropoelastin to tropoelastin; and (○—○) antitropoelastin to emilin. The antibodies against the two proteins were purified by affinity column chromatography as described in Materials and Methods. (Right panel) Western immunoblot analysis showing the lack of reactivity of antiemilin antibodies to fibrillin. mAbs 15 and 201 were raised against human fibrillin

(Sakai et al., 1986). One of the anti-human fibrillin mAbs, 201, identified a protein of the size expected for fibrillin in a fraction of the serum free medium of chick embryo aorta cells purified by DEAE chromatography. Antiemilin antibodies (α-emilin) were completely unreactive against the same fraction. Protein samples were electrophoresed under reducing (+) and nonreducing conditions (-). Markers on the left indicate the migration of protein standards of 200, 116, and 92 kD.



**Figure 2.** Immunolocalization of emilin on mature elastic fibers. Aorta embedded in LR White and reacted with the polyclonal antibody. (A) Cross-sectioned elastic fiber. Mean diameter of gold particles, 5 nm. (B) Elastic fibers cut obliquely. Mean diameter of gold particles, 10 nm. Arrows point out sites where colloidal gold is associated with electron dense areas. Arrowheads indicate staining at the border of elastic fibers. Bars, 0.3  $\mu\text{m}$ .

sion) with the IPS Program. The gold particles within each area were counted and the density calculated.

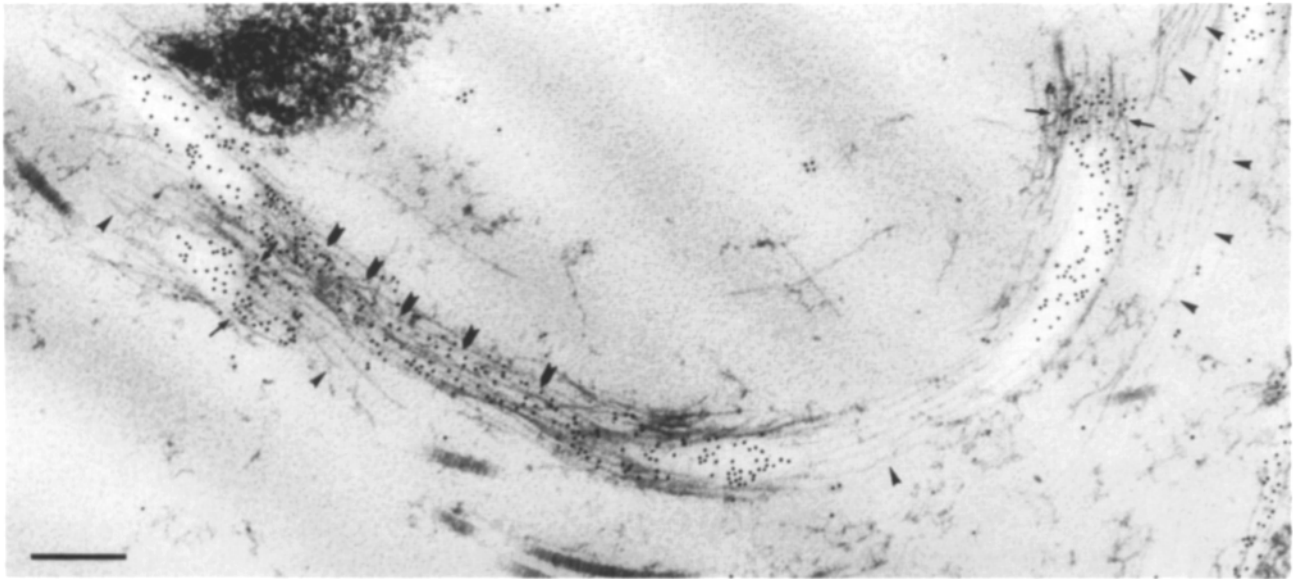
### **Cell Cultures and Immunofluorescence**

Chick embryo aorta cells were isolated as described (Marigo et al., 1992) and grown in DME supplemented with 10% FCS.

When used for immunofluorescence, second passage cells were seeded at a density of 90,000/cm<sup>2</sup> into tissue culture chamber slides (Lab-Tek, Miles Scientific, Kankakee, IL) coated with gelatin and grown for 1 wk with

one change of the medium. The slides were fixed in Bouin's mixture for 5 min, thoroughly washed, and incubated for 15 min in 2% BSA in phosphate PBS (PBS-BSA). The samples were then treated for 1 h at room temperature with the desired first antibody(ies), washed in PBS-BSA and incubated with the appropriate fluorescent second antibody(ies) for 1 h at room temperature. After washing, the slides were mounted in 90% glycerol in PBS and observed in a Zeiss Axiovert microscope equipped with epifluorescence optics.

In some experiments, the cells were plated and grown in DME with 10% FCS containing a 1:50 or 1:100 dilution of the rabbit antiserum against emilin.



**Figure 3.** Immunolocalization of emilin on Epon-embedded aorta. The polyclonal antibody was used. Strong staining is associated with microfibrils close to the amorphous core of obliquely cut fibers (*arrows*). A tangential section of an elastic fiber (*thick arrows*) showing small regions of amorphous elastin surrounded by microfibrils is also intensely labeled. Portions of microfibrils distant from the amorphous core or isolated (*arrowheads*) are not significantly decorated. Mean diameter of gold particles, 10 nm. Bar, 0.6  $\mu\text{m}$ .

### ***Fibrillins***

Chicken fibrillin was partially purified from the medium of chick embryo aorta cell cultures by DEAE chromatography. The serum-free medium was collected and processed according to Sakai et al. (1991). Purified human fibrillin was a generous gift of Dr. Lynn Sakai of the Shriners Hospital for Crippled Children.

### ***Other Methods***

ELISA, SDS-PAGE, and Western blots were performed following established procedures (Rennard et al., 1980; Studier, 1973; Towbin et al., 1979).

### ***Results***

The specificity of polyclonal antibodies used was carefully assayed against the purified proteins (emilin and tropoelastin) and crude tissue preparations. Antibodies purified by affinity binding on both columns and nitrocellulose strips were specific for the proteins they were raised against (Fig. 1, *left panel*). Antiemilin antibodies did not react with other protein species, since they recognized only one protein band by Western blotting on a crude extract from chick aorta (data not shown). In addition antiemilin antibodies were not reactive against a partially purified preparation of chick fibrillin (Fig. 1, *right panel*). In the same sample, under nonreducing conditions, mAb 201 detected a protein which comigrated with the band of purified human fibrillin. Human fibrillin was also identified by mAb 15 and, as expected (Sakai et al., 1986), reduction of the sample with mercaptoethanol decreased the migration of the band.

### ***Emilin Is Associated with Elastic Fibers in Aorta***

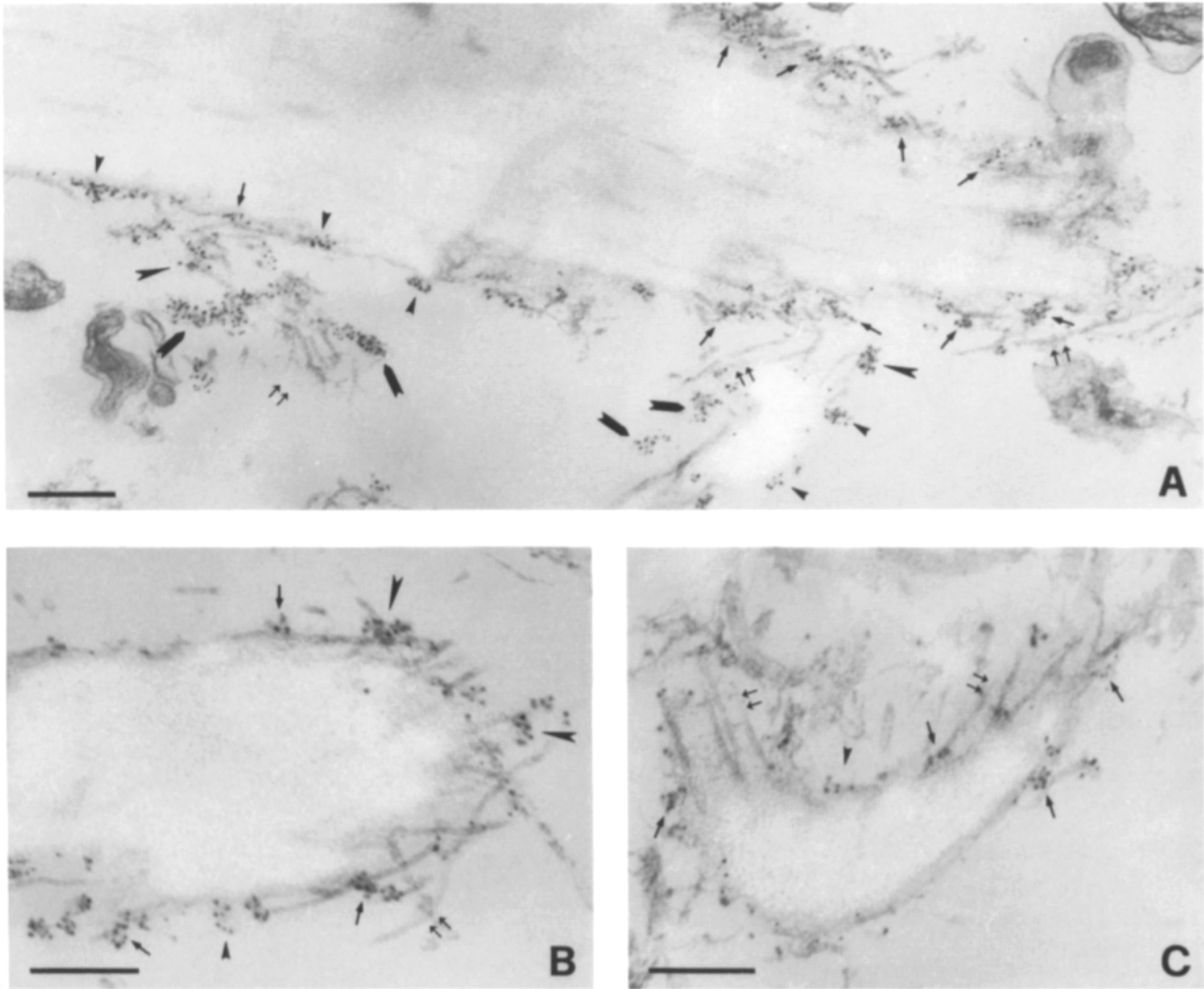
Blood vessels contain the highest concentration of emilin (Bressan et al., 1983a). For this reason the distribution of the protein was extensively investigated in the aorta. To rule out the possibility that the pattern of immunostaining was

due to the particular method applied, different procedures of tissue processing (pre- and postembedding), various embedding media (Epon 812, LR White, and Lowicryl) and several antibody preparations (polyclonal and monoclonal) were compared.

With the postembedding procedure, the strongest labeling for emilin on mature elastic fibers was observed with samples embedded in LR White (Fig. 2). Labeling was found within amorphous elastin in the form of clusters of gold particles mainly associated with a meshwork which showed affinity for EM stains. This meshwork, which is usually found in electron micrographs of elastic fibers, is more prominent in samples embedded in LR White. Labeling also outlined the periphery of amorphous elastin at several sites. The peripheral staining was very evident in elastic fibers of small dimensions.

### ***Emilin Is Mostly Found in Regions Where Elastin and Microfibrils Are in Close Contact***

The microfibrillar coat was not apparent in samples embedded in LR White and therefore the location of emilin labeling with regard to the microfibril's coat could not be clearly determined. In Epon sections, although the internal meshwork of the amorphous core was less distinct than with LR White, the morphology of microfibrils was well preserved (Fig. 3). The association of staining with microfibrils was apparently nonhomogeneous. Microfibrils close to the amorphous nuclei were intensely labeled. This was evident in cross sections of elastic fibers (Fig. 3, *arrows*). Fig. 3 also shows a portion of a fiber which has been tangentially cut and in which small regions of amorphous elastin are surrounded by microfibrils (Fig. 3, *thick arrows*). Staining was also strong at this site. Parts of microfibrils located at some distance from the fiber's core or apparently isolated in the extracellular space were very poorly decorated (Fig. 3, *arrowheads*).



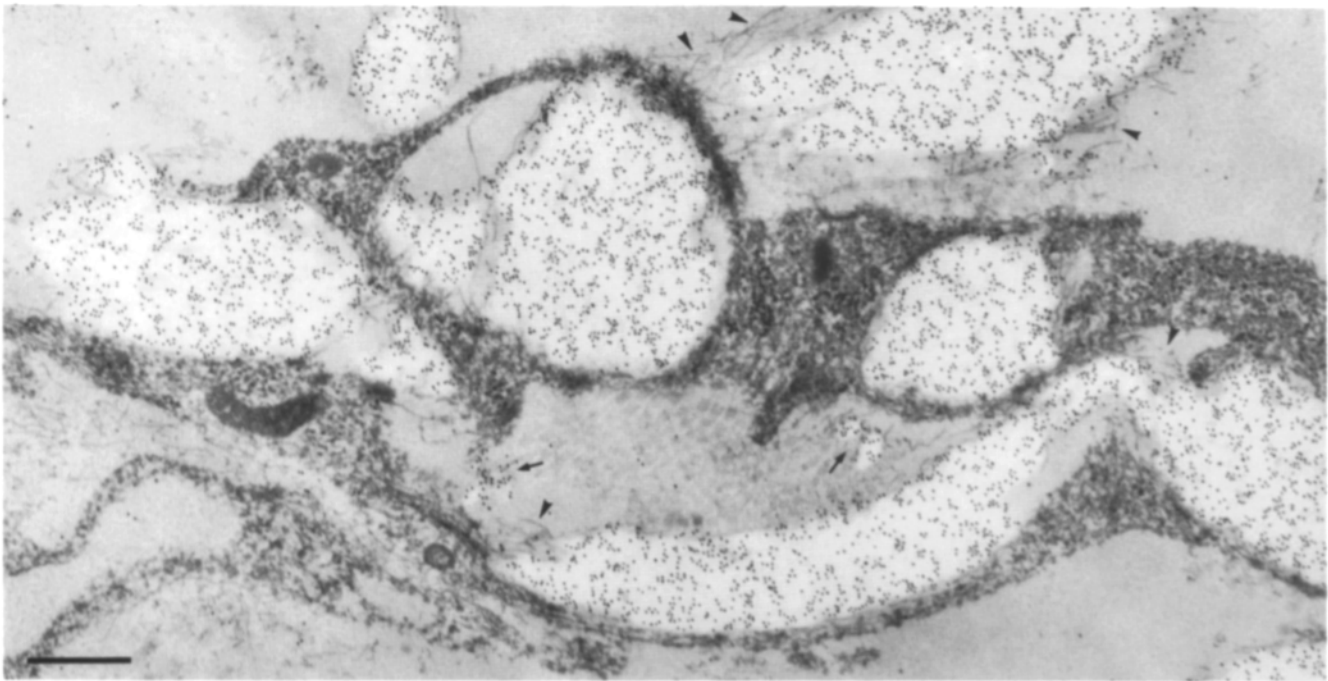
**Figure 4.** Localization of emilin in aorta by preembedding immunolabeling. Samples were incubated with the polyclonal antibodies. Most staining is associated with electron-dense, structureless material which: (a) is located at sites where microfibrils and amorphous elastin are in close contact (*arrows*); (b) covers regions of the surface of elastic fibers where microfibrils had apparently been stripped off (*small arrowheads*); (c) is isolated from the fibers (*thick arrows*); and (d) is mixed with microfibrils (*large arrowheads*). Well resolved microfibrils are usually poorly decorated (*double arrows*). Mean diameter of gold particles, 10 nm. Bars, 0.3  $\mu\text{m}$ .

To better define the association of emilin with the morphological components of elastic fibers (amorphous elastin and microfibrils), aorta samples processed through the preembedding method were also examined. It was previously noted that, when this procedure was applied, immunologic staining did not penetrate into amorphous elastin, but had access to the surface of elastic fibers (Daga-Gordini et al., 1987b). The procedure is therefore very useful for studying the association of emilin with microfibrils. Examples of preembedding immunolabeling with polyclonal antibodies are shown in Fig. 4. Intense staining was detected on patches of electron-dense structureless material which was associated exclusively with elastic fibers. Most of this material was found at sites where microfibrils contact and penetrate into amorphous elastin (Fig. 4, *arrows*). Some labeling covered regions of the surface of elastic fibers in which microfibrils had apparently been stripped off (Fig. 4, *small arrowheads*).

The electron-dense labeled material was also detected either isolated at some distance from the fibers (Fig. 4, *thick arrows*) or mixed with microfibrils (Fig. 4, *large arrowheads*). Microfibrils were not a target of the immunologic staining: in fact, except at places where the electron-dense material was present, well resolved microfibrils were usually poorly decorated (Fig. 4, *double arrows*).

Staining obtained with anti-emilin mAbs was weak with both the pre- and postembedding procedures (data not shown); its distribution, however, was similar to that described with polyclonal antibodies. A comparable labeling pattern was observed with samples embedded in Lowicryl and reacted with poly- or monoclonal antibodies (data not shown).

The distribution of staining for tropoelastin is presented in Fig. 5. It is apparent that label distribution for the two proteins is very different. In particular, with antitropoelastin an-



**Figure 5.** Staining of elastic fibers with antibodies to tropoelastin in aorta from 2-d-old chick. Note the intense labeling of amorphous cores of both large and small (arrows) elastic fibers and the poor reaction of the microfibrillar coat (arrowheads). Epon embedding. 10-nm gold particles. Bar, 1  $\mu$ m.

tibodies there was no obvious preference of gold localization at the periphery of both large and small elastic fibers and most microfibrils were unlabeled.

### Quantitative Evaluation of Labeling

The qualitative examination of the data obtained with the post- and pre-embedding procedures suggested that emilin preferentially localized at the elastin-microfibril interface and in the electron-dense areas present within the amorphous core. This conclusion was further tested by quantitative evaluation of labeling. The density of gold particles was

**Table I. Distribution of Gold Particles on Elastic Fibers\***

|                                  | Density $\pm$ SD | Significance   |
|----------------------------------|------------------|----------------|
| <b>Anti-emilin antibody</b>      |                  |                |
| Area 1                           | 60 $\pm$ 20      |                |
| Area 2                           | 250 $\pm$ 80     | $p < 0.0001$ § |
| Area 3                           | 476 $\pm$ 238    | $p < 0.0001$ § |
| Area 1 + 3                       | 88 $\pm$ 22      | $p < 0.0001$ ‡ |
| <b>Antitropoelastin antibody</b> |                  |                |
| Area 1                           | 272 $\pm$ 34     |                |
| Area 2                           | 323 $\pm$ 136    | $p > 0.1$ §    |
| Area 3                           | 638 $\pm$ 152    | $p < 0.0001$ § |
| Area 1 + 3                       | 300 $\pm$ 40     | $p > 0.5$ ‡    |

\* Elastic fibers on electron micrographs were divided into three regions: (1) the electron-lucent area of the amorphous core, (2) the electron-dense peripheral area which includes the elastin-microfibrils interface, and (3) the electron-dense areas within the amorphous core. The density of gold particles was determined for each region and the values expressed as number of gold particles/ $\mu$ m<sup>2</sup>.

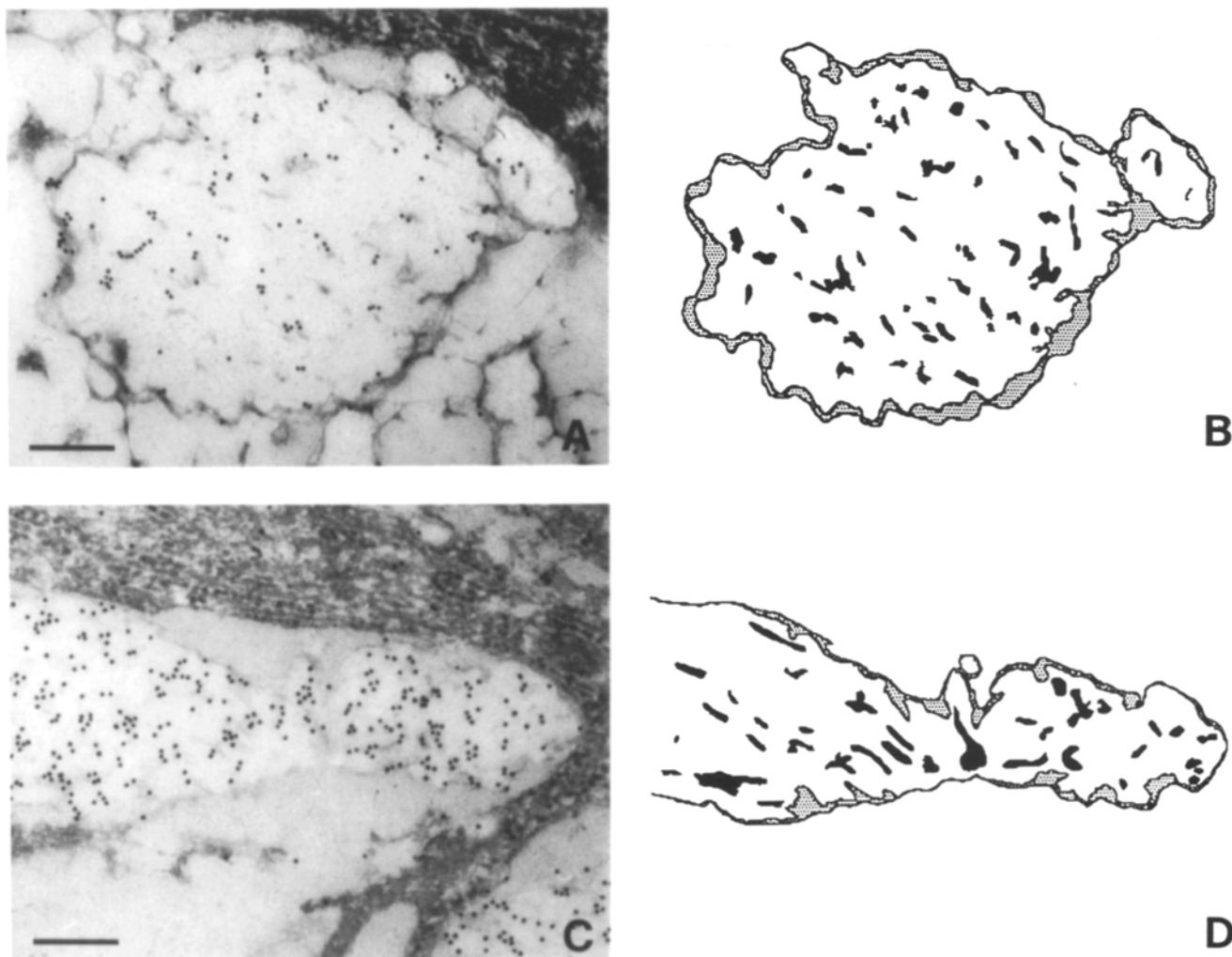
‡ Comparison with area 2 evaluated by the *t* test.

§ Comparison with area 1 evaluated by the *t* test.

determined in three regions of elastic fibers identified as: (a) the electron-lucent area of the amorphous core of the fiber; (b) the electron-dense peripheral area which includes the elastin-microfibrils interface; and (c) the electron-dense areas within the amorphous nucleus (Fig. 6). The density of label for emilin in region 1 was significantly lower than in regions 2 and 3 (Table I). This distribution of gold particles, however, could be due to staining artifacts and not to a peculiar localization of emilin. This possibility was examined by staining sections with an antibody against elastin, which should be evenly present in the fiber. As expected, the density of colloidal gold in regions 1 and 2 was not dissimilar. Surprisingly, however, the difference of density between regions 1 and 3 was significant (Table I). This suggests that the higher accessibility of antigenic sites in region 3 by antibodies is artifactual. Therefore, a more stringent criterion of evaluation of the significance of labeling of the boundary zone (area 2) is the comparison with that of the remaining parts of the fiber (area 1 + 3). When this comparison was done, the difference for emilin was still very significant, whereas that for elastin was not (Table I), indicating that emilin is preferentially located at the periphery of elastic fibers.

### Localization of Emilin in Other Tissues

Previous work has shown that emilin is a widespread component of connective tissues (Colombatti et al., 1985a). In every tissue examined by EM in the present study (skin, intestine, eye, kidney, skeletal muscle), emilin was exclusively detected in elastic fibers. In most tissues the picture was comparable with the one described in aorta, except for the fact that the overall morphology of elastic fibers varied in the different tissues. Two examples are reported in Fig. 7, A and



**Figure 6.** Identification of different areas within elastic fibers for quantitative evaluation of gold particles density. Micrographs of samples stained for emilin (*A*) and tropoelastin (*C*) are shown with the derived scheme (*B* and *D*) used for determination of the different areas with an image analysis system. The measured zones are the white region within the fiber, corresponding to amorphous elastin, referred to as area 1 in the text; the stippled region, corresponding to the electron-dense perimeter of the fiber, and identified as area 2; the black regions, corresponding to the electron-dense patches within the fiber's nucleus, and indicated as area 3. Samples were embedded in LR White and reacted with polyclonal antibodies. 10-nm-diameter colloidal gold. Bars, 0.5  $\mu\text{m}$ .

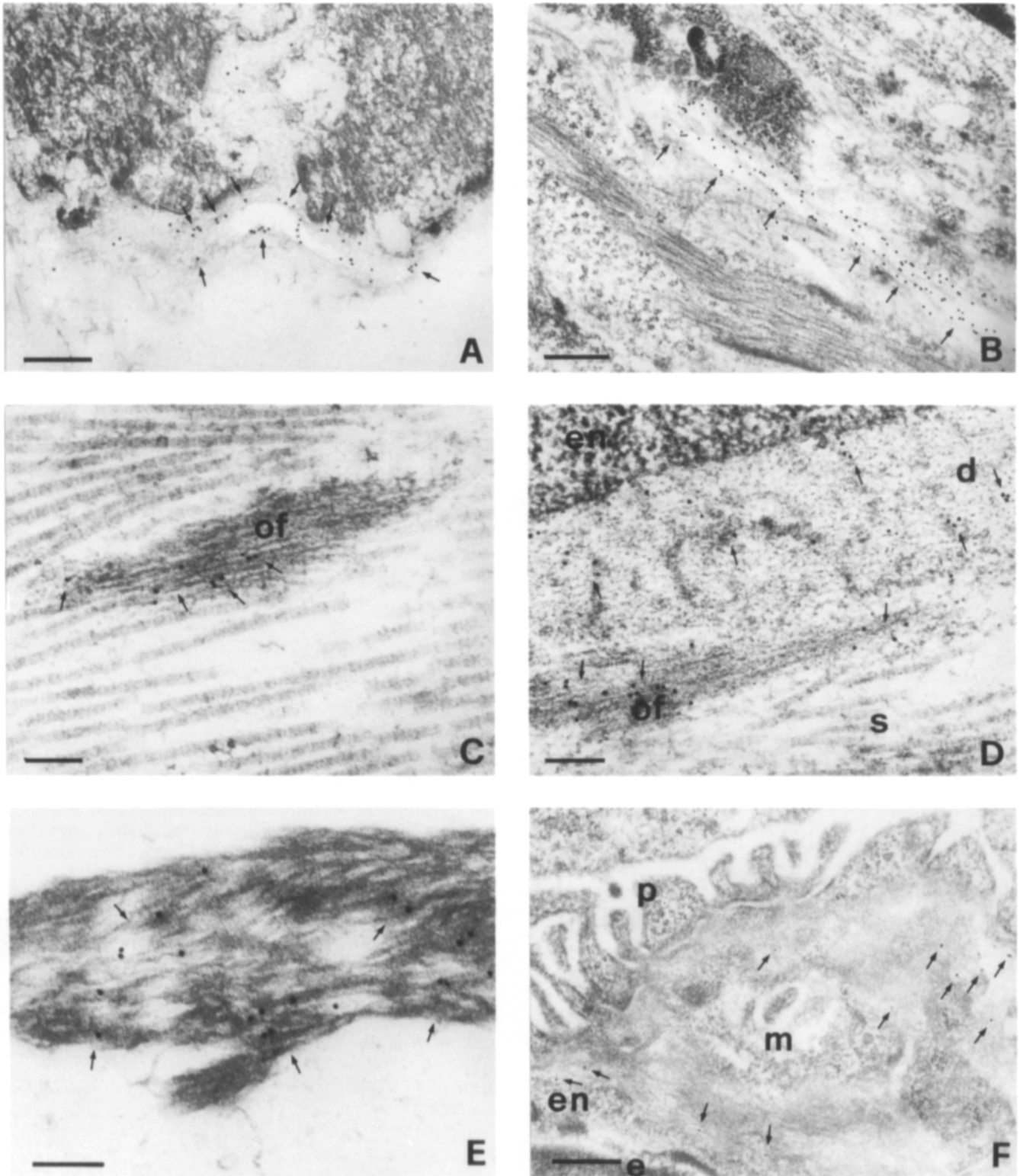
*B* for skin and intestine. In some organs, however, staining for emilin was found on structures which are considered to be related to elastic fibers, but without their typical morphology. The most notable examples were identified in the eye. In the cornea antibodies to emilin decorated oxitalan fibers, a specialized variant of elastic fibers formed by parallel bundles of microfibrils lacking amorphous elastin (Alexander and Garner, 1983) (Fig. 7, *C* and *D*). The Descemet's membrane was also labeled (Fig. 7 *D*). In addition, a faint signal was associated with the microfibrils of the Zinn's zonule (Fig. 7 *E*). Among the tissues examined, staining of microfibrils in the absence of amorphous elastin was also detected in the mesangium (Fig. 7 *F*).

#### ***Emilin in Development and Elastogenesis***

To better define the association of emilin with elastin and microfibrils, the distribution of the glycoprotein was investigated in aorta at early stages of development. Fig. 8 *A* shows a section from a 4-d-old embryo, an age at which

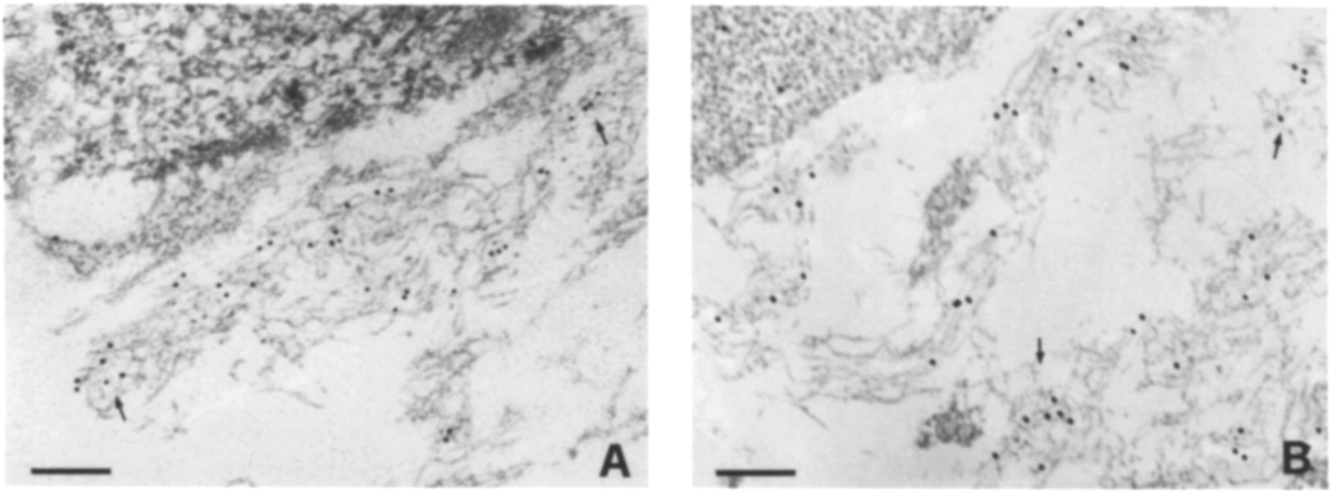
amorphous elastin deposits are absent (Daga-Gordini et al., 1987a). Emilin was localized in regions rich in fibrils whose nature could not be defined on the basis of morphological criteria. The fibrils differed from typical microfibrils surrounding elastic fibers: their diameter was smaller (8–10 instead of 12–13 nm) and variable; they often exhibited sharp bends and were irregularly arranged with frequent tangling. The same type of fibrils were labeled by antibodies against tropoelastin (Fig. 8 *B*). At later stages of development, amorphous elastin appeared within this fibrillar network and typical microfibrils became also evident (Daga-Gordini et al., 1987a).

The early appearance of emilin in development indicates that the protein may be involved in elastogenesis. The question was addressed in two additional experiments. In the first, the distribution of emilin and elastin accumulated by chick embryo aorta cells maintained in culture for one week was studied by double immunofluorescence. All the sites containing one protein also stained for the other, with a remarkable coincidence of the deposit's shape (Fig. 9, *A* and



**Figure 7.** Immunolocalization of emilin in different tissues. (A) Skin; staining of an elastic fiber of the dermis is mostly peripheral. (B) Intestine; labeling of elastic fibers of the muscular layer of the mucosa. (C) Eye; oxitalan fiber (*of*) of the corneal stroma is labeled. (D) Eye; gold particles are found on the Descemet's membrane (*d*) and on an oxitalan fiber (*of*) of the stroma of the cornea (*s*); *en*, endothelial cell. Eye; immunodecoration of fibers of the Zinn's zonule. (E) Kidney; section through the mesangium. Faint labeling is present on fibrils of the mesangial matrix. *p*, podocyte; *m*, mesangial cell; *en*, endothelial cell; and *e*, erythrocyte. Arrows in the different panels identify stained regions. Samples were embedded in Epon. Average dimension of gold particles, 10 nm. Bars: (A, B, and F) 0.5  $\mu$ m; (C-E) 0.5  $\mu$ m.





**Figure 8.** Localization of emilin in early elastogenesis. 4-d-old chick embryo aorta embedded in Epon 812 and stained with polyclonal antibodies against emilin (A) and tropoelastin (B). Arrows indicate regions of labeling in which the morphology of fibrils is particularly dissimilar from microfibrils. Average dimension of gold particles, 10 nm. Bars, 0.3  $\mu$ m.

B). This was not the case when the locations of elastin and type VI collagen were compared (Fig. 9, C and D). In the second experiment, the same type of cells were grown for one week in the presence of either the diluted rabbit antiserum against emilin or the preimmune serum and the distribution of emilin and elastin in the two groups of samples compared. The presence of the antiserum induced only a slight modification of the immunofluorescence pattern of emilin, consisting of a frequent presence of thicker fibers (Fig. 10, A and B). On the contrary, the distribution of elastin was deeply altered. Staining of control cultures was fibrillar (Fig. 10 C) and was very similar to that of emilin. In the presence of the antiemilin antiserum elastin deposits were formed mainly by large clumps, which were very rarely seen in controls (Fig. 10 D). The alterations were specific for elastic fibers, since the fluorescence pattern of fibronectin (Fig. 10, E and F) and type VI collagen (data not shown) were not affected.

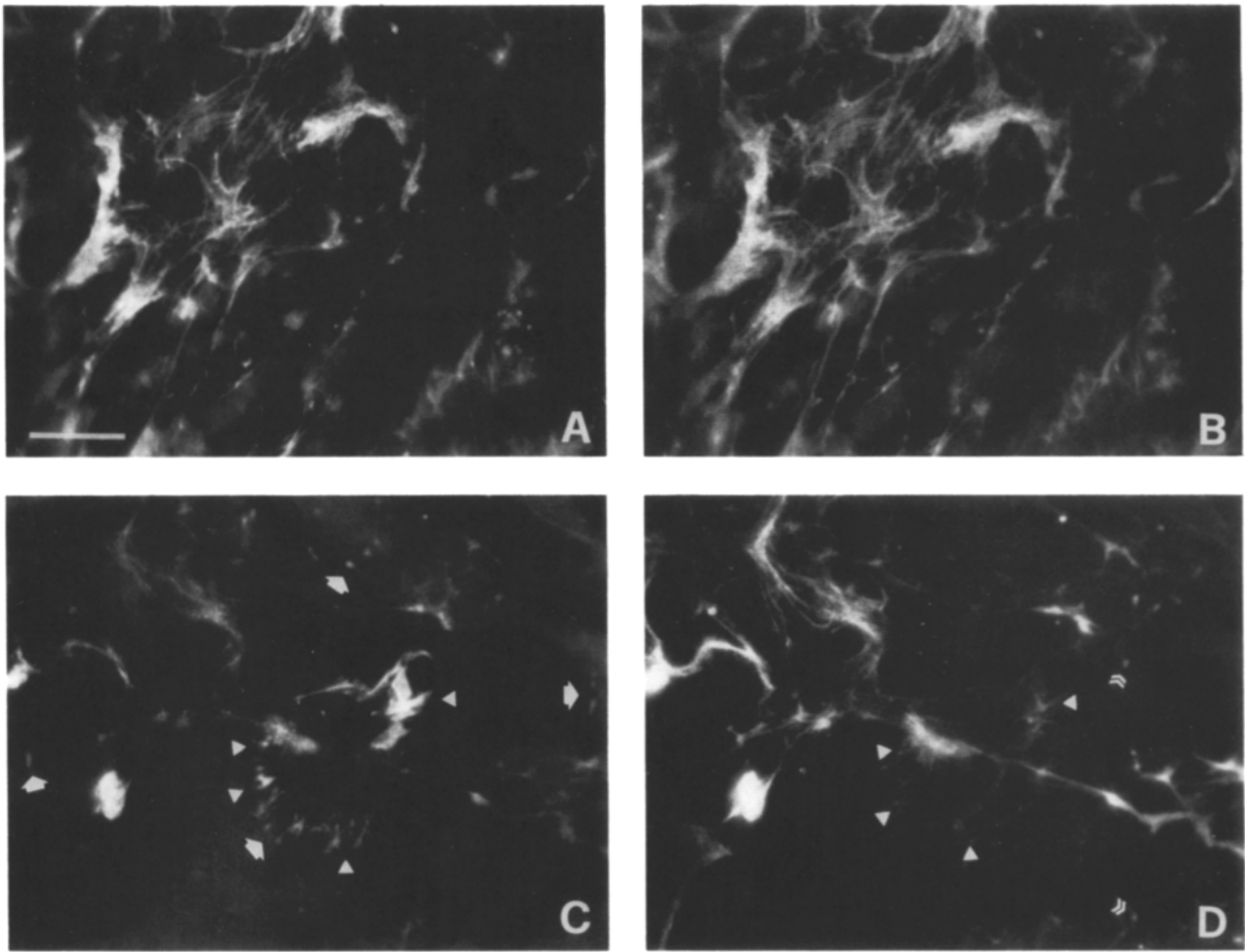
## Discussion

The results reported in this paper describe features of the ultrastructural distribution of emilin in different tissues and during elastogenesis.

The data clearly establish that emilin is a component of elastic fibers. In the last few years several molecular species were mapped within elastic fibers. Some components, such as elastin, MAGP, and fibrillin are exclusively associated with elastic fibers (Daga-Gordini et al., 1987a; Gibson et al., 1986; Sakai et al., 1986), while others, like amyloid P, vitronectin, and some proteoglycans are also found in other extracellular locations or in serum (Breathnach et al., 1979; Baccarani-Contri et al., 1991; Dahlback et al., 1989). Emilin belongs to the first group. The first evidence for this conclusion is that the distribution of the protein in vivo is restricted either within typical elastic fibers or in structures where elastin and microfibrils cannot be morphologically distinguished, but tropoelastin-derived material, and microfibrillar constituents can be revealed by immunohistology. This has been observed for oxitalan fibers of the cornea, con-

sisting of bundles of microfibrils lacking amorphous elastin, and Zinn's zonule, formed by ordered arrays of microfibrils and the Descemet's membrane, where neither amorphous elastin nor microfibrils can be morphologically detected. These structures have been shown to contain fibrillin and MAGP (Sakai et al., 1986; Kumaratilake et al., 1989), and, at least in the chick, also tropoelastin-derived material (Daga-Gordini et al., 1990). A second evidence for emilin being an integral component of elastic fibers is the remarkable coincidence of the location of the protein and elastin accumulated by aorta cells in vitro, an observation which indicates that emilin found in tissues is produced in situ and is not of blood origin.

An important question addressed in this study was the preferential localization of emilin within the mature elastic fiber. The purification of polyclonal antibodies specific for emilin and tropoelastin has been very important in solving the problem. The results obtained with these reagents were then confirmed using mAbs and the use of different embedding media and immunolabeling procedures (post- and preembedding). Finally, the distribution of labeling was quantitatively evaluated. The last step required the subdivision of elastic fibers into different areas, clearly identified in LR White sections. The electron-dense border delimiting the elastic fiber was significantly enriched in emilin, whose uneven distribution indicates that this region contains a specific domain of elastic fibers. This was supported by several observations: (a) staining with antitropoelastin antibodies revealed that elastin was present in the peripheral zone. This suggests that the domain either contains the external layer of amorphous elastin or is delimited by it at the internal side; (b) in Epon-embedded sections the portion of the microfibrillar coat proximal to amorphous elastin was intensely labeled for emilin, indicating that the electron-dense rim of elastic fibers also contains microfibrils. Because of poor labeling of microfibrils distant from the amorphous core of the fiber, the most internal elements of the microfibrillar coat either are part of the domain or constitute its external border; and (c) one prominent localization of emilin in preembedded samples was at sites where amorphous elastin and micro-



**Figure 9.** Colocalization of emilin and elastin deposited by chick embryo aorta cells in culture. (*A* and *B*) Elastin (*A*) and emilin (*B*) distribution of a sample processed for double immunofluorescence with rabbit antiserum against tropoelastin and mouse mAbs against emilin. (*C* and *D*) elastin (*C*) and type VI collagen (*D*) distribution of a sample processed for double immunofluorescence with a rabbit antiserum against tropoelastin and a mouse mAb against type VI collagen. After treatment with the first antibodies, the slides were incubated with a mixture of FITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG and then examined for rhodamine fluorescence (*A* and *C*) and fluoresceine fluorescence (*B* and *D*). Arrows point out some sites where elastin is detectable and type VI collagen is not. Arrowheads indicate regions in which fluorescence for the two proteins is present, but differs in morphology. Some sites positive only for collagen VI are also marked (*double arrowheads*). Bar, 20  $\mu\text{m}$ .

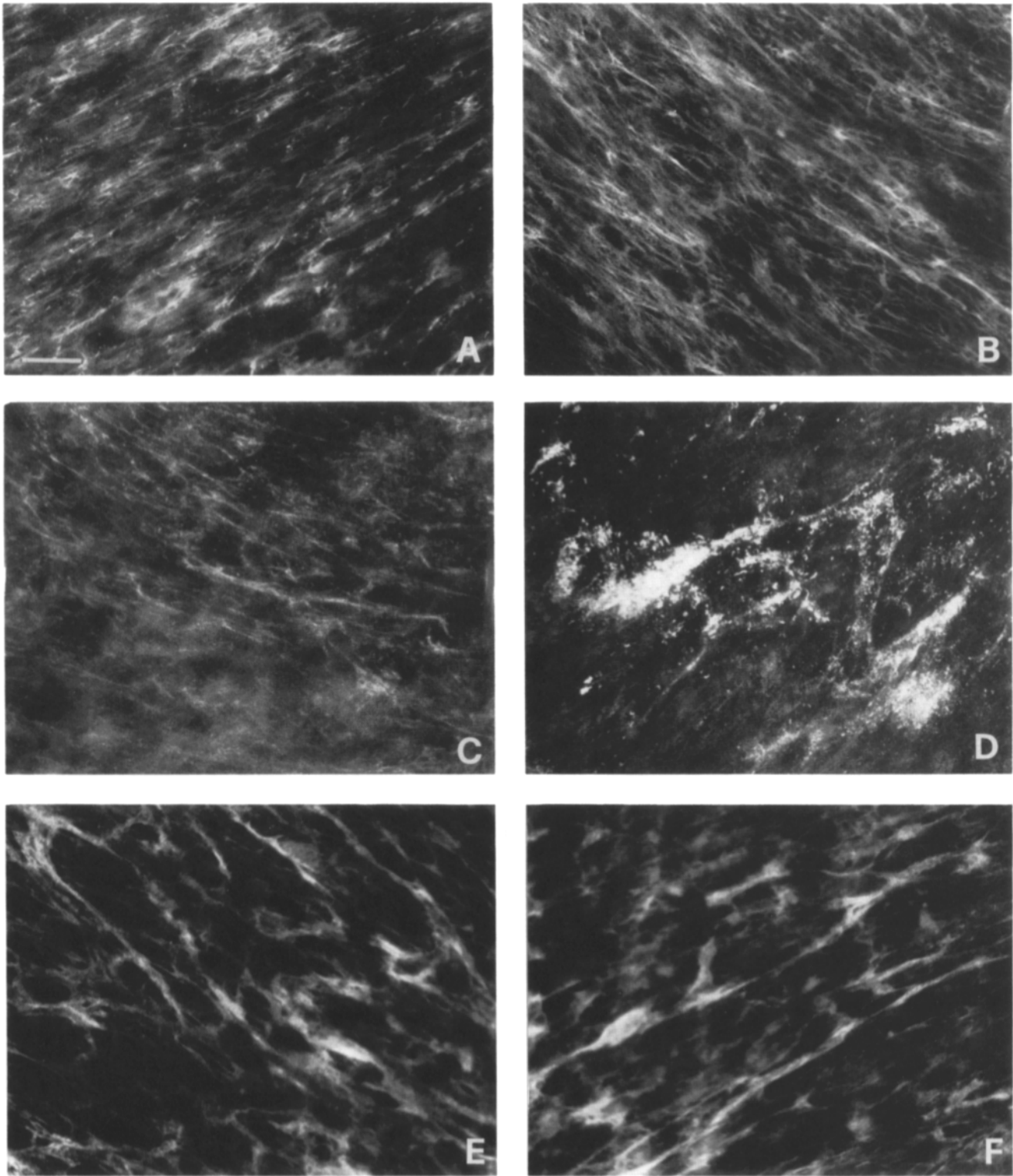
fibrils contacted each other. The conclusion is therefore that amorphous elastin and microfibrils are intimately mixed in the emilin-enriched, peripheral domain of elastic fibers. It cannot be excluded that emilin might also be concentrated in the electron-dense areas usually found within the amorphous core of elastic fibers; however, the observation that immunolabeling was high for both emilin and tropoelastin raises the possibility that any antigen is particularly accessible to antibodies in these areas.

Although often associated with microfibrils, emilin is very likely not one of their components. In fact, (*a*) well resolved and isolated microfibrils were poorly labeled by antibodies to emilin both in pre- and postembedded samples; (*b*) in sections obtained with the preembedding procedure, staining was very often detected on structureless material associated with either amorphous elastin or microfibrils. This material might well be the result of a partial redistribution of emilin during processing of the samples, however its existence still

indicates that emilin is distinct from microfibrils; and (*c*) the intensity of labeling for emilin was not related to the amount of microfibrils present in a given tissue.

Emilin was detected in early stages of aorta development in association with a network of fibrils smaller in diameter than microfibrils, likely representing maturing microfibrils. At these sites typical microfibrils and amorphous elastin appear (Daga-Gordini et al., 1987a). Thus, emilin deposition can be considered an early event in elastogenesis, suggesting a specific function of the protein in this process. This conclusion is enforced by the finding that antiemilin antibodies deeply alter the normal process of elastic fiber formation in vitro. The identification of the step of elastogenesis affected by the antibodies cannot be inferred from our data and will require the characterization of the molecular interactions of emilin with itself and with other components of the elastic fiber.

The localization of emilin emphasizes the molecular com-



**Figure 10.** Alteration of elastogenesis by antiemilin antibodies. Chick embryo aorta cells were grown for 1 wk in the presence of either rabbit preimmune serum (*A*, *C*, and *E*), or rabbit antiserum against emilin (*B*, *D*, and *F*) and stained with mouse mAbs to emilin (*A* and *B*), elastin (*C* and *D*) and fibronectin (*E* and *F*). In this particular experiment the dilution of the rabbit sera was 1:50. Similar results were obtained with a 1:100 dilution. Bar, 25  $\mu\text{m}$ .

plexity of elastic fibers. It has been known for several years (Alexander and Garner, 1983) and recently confirmed by immunoelectron microscopy (Kumaratilake et al., 1989; Daga-Gordini et al., 1990) that elastic fibers exhibit morphological variability in different tissues, that is a varying proportion of

the amorphous and fibrillar components and a distinctive architecture of the fiber network. The importance of these aspects of the structure of elastic fibers is stressed by the discovery that alterations of the fibrillin gene are responsible for the Marfan's syndrome and that genetic defect of a fibrillin-

like gene is involved in congenital contractural arachnodactyly (Lee et al., 1991; Dietz et al., 1991). The isolation and characterization of several constituents of elastic fibers, including tropoelastin, fibrillin, MAGP, and emilin will help to elucidate the morphological variability of elastic fibers at the molecular level and foster the understanding of their biology and pathology.

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