

## Apolipoprotein expression by murine visceral yolk sac endoderm

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### SUMMARY

Apolipoprotein expression was examined in the postimplantation mouse embryo. Antibodies directed against murine Apolipoprotein AI and human low-density lipoprotein (LDL) particles specifically immunoprecipitated metabolically labelled radioactive apolipoproteins from the culture supernatant of 10.5 days *post coitum* (days *p.c.*) yolk sac visceral endoderm cultured *in vitro*. No evidence for apolipoprotein expression by other embryonic or extraembryonic tissues at this stage was obtained. Immunohistochemical staining at sectioned 10.5 days *p.c.* embryos with anti-Apolipoprotein AI antibodies revealed specific localization of immunoreactive material in the yolk sac visceral endoderm. We conclude that the yolk sac visceral endoderm is a source of lipoproteins during postimplantation embryonic development.

### INTRODUCTION

The function of lipoproteins in the adult is the transport of lipid between sites of synthesis and uptake (reviewed Jackson, Morrisett & Gotto, 1976). Associated with this transport function are lipid modification (reviewed Nilsson-Ehrle, 1980) and exchange (reviewed Barter, Hopkins & Calvert, 1982). Lipoproteins are multimolecular lipid/protein particles which can be separated into distinct classes by buoyant density ultracentrifugation (Brown, Dana & Goldstein, 1974); a) Chylomicrons and very low-density lipoproteins (VLDL); density  $< 1.006 \text{ g}\cdot\text{ml}^{-1}$ . b) Low-density lipoproteins (LDL)  $1.006 \text{ g}\cdot\text{ml}^{-1} < \text{density} < 1.063$  and c) High-density lipoproteins (HDL)  $1.063 < \text{density} < 1.21 \text{ g}\cdot\text{ml}^{-1}$ . Associated with each lipoprotein class are specific apolipoproteins, which are involved in particle structure, enzyme recognition (Fielding, 1970) and cellular recognition (reviewed Brown, Kovanen & Goldstein, 1981). The major apolipoprotein classes of mouse plasma have been recently described (LeBoeuf, Puppione, Schumaker & Lusi, 1983) and appear in many respects to resemble their human counterparts. ApoAI (relative molecular mass  $M_r$   $28 \times 10^3$ ) is the predominant apoprotein component of murine HDL, combined with trace amounts of ApoAII ( $M_r$   $8 \times 10^3$ ) and ApoAIV ( $M_r$   $46 \times 10^3$ ). LDL is principally composed of ApoB (ApoB-P1:  $M_r$   $350 \times 10^3$ , ApoB-PIII:  $M_r$   $220 \times 10^3$ ) with minor ApoC ( $M_r$   $6\text{--}12 \times 10^3$ ) components. ApoE ( $M_r$   $38 \times 10^3$ ) is the major

apolipoprotein species of VLDL. The liver and intestine are the major sites of lipoprotein synthesis in the foetus and adult (Zannis, Kurnit & Breslow, 1982; Wu & Windmueller, 1978). ApoE is also secreted by macrophages (Basu *et al.* 1982).

Our attention was drawn to the possibility that lipoproteins were synthesized in the embryo by the finding that LDL and HDL were important components in a serum-free medium developed to support the proliferation of embryo-derived murine embryonal carcinoma cells *in vitro* (Heath & Deller, 1983). We report here that the visceral yolk sac endoderm can be identified, by immunological techniques, as a major site of apolipoprotein synthesis in the 10.5 days *p.c.* (days *post coitum*) mouse embryo.

## MATERIALS AND METHODS

### *Embryos*

Embryonic tissues were obtained from natural matings of C3H×C57/b16. F1 mice. Gestational age was calculated on the assumption that mating occurred at midnight preceding the finding of a copulation plug. The visceral yolk sac was separated into component mesoderm and endoderm by exposure to 2.5 % (w/v) Pancreatin, 0.5 % (w/v) trypsin as described by Levak-Svajger & Svajger (1974) for 2 h at 4 °C, followed by transfer to Earles balanced salt solution containing 0.1 % (w/v) BSA, 30 mM-HEPES, pH 7.2 at room temperature, (EBSS) and dissection. Other embryonic tissues were dissected out in EBSS and cultured in EBSS at room temperature until required.

### *Isolation of lipoproteins*

Human lipoproteins were isolated from fresh human plasma (Oxford regional blood transfusion centre) as previously described (Heath & Deller, 1983). Total murine lipoproteins were isolated from 120 ml of freshly drawn mouse plasma by buoyant density ultracentrifugation. The plasma was adjusted to a density of 1.21 g·ml<sup>-1</sup> by addition of solid KBr and centrifuged at 100 000 *g* for 48 h. The floating lipoprotein fraction was removed and rebanded in KBr (density 1.21 g·ml<sup>-1</sup>) by ultracentrifugation as before. The purity of individual fractions was assessed as described (Heath & Deller, 1983). Murine lipoproteins appeared pure apart from trace contamination by a single molecular species corresponding in relative molecular mass to serum albumin.

### *Preparation of antisera*

#### 1) *Anti-ApoAI*

Total mouse lipoproteins (approximately 250 µg) were subjected to electrophoresis in 12.5 % (w/v) polyacrylamide gels containing 0.1 % (w/v) sodium lauryl sulphate using the discontinuous tris buffer system of Laemmli

(1970). Multiple sample slots were used in each electrophoretic separation. Following electrophoresis, a strip of gel corresponding to the relative molecular mass marker track plus one sample track was excized, fixed in 10 % (v/v) acetic acid, 40 % (v/v) methanol, 0.025 % (w/v) Coomassie blue R-250 and destained in 10 % (v/v) acetic acid, 40 % (v/v) methanol to visualize protein bands. The remainder of the gel was fixed in 0.5 % (v/v) glutaraldehyde in phosphate-buffered saline (PBS) at 4 °C. The region of the sample gel corresponding to the observed mobility of ApoAI (apparent relative molecular mass,  $28 \times 10^3$ ) was excized, washed in PBS, crushed and homogenized in Freund's complete adjuvant. The mixture was injected into rabbits at multiple subcutaneous sites. The animals were injected four times at 10-day intervals. The animal was bled seven days after the final immunization. Partially purified Immune Ig was prepared by 40 % saturated ammonium sulphate precipitation, followed by dialysis of the precipitate against 500 volumes of PBS at 4 °C for 24 h.

## 2) *Anti-human LDL*

Rabbits were immunized with human LDL (250 µg) homogenized in Freund's complete adjuvant using the same immunization schedule as described above. The specificity of each antiserum was determined by SDS-PAGE of mouse plasma and amniotic fluid, followed by electrophoretic transfer to nitrocellulose paper and reaction with the antibody (see below).

### *Metabolic labelling and immunoprecipitation*

Isolated embryonic tissues were cultured for 16 h in 1 ml of ECM medium (Heath & Deller, 1983) made up in a basal medium of Dulbecco's modified Eagles medium diluted 1/100 with methionine-free minimal essential medium and supplemented with 50 µCi/ml [<sup>35</sup>S]methionine (Amersham 2000 mCi/mmol). The culture supernatant was removed and centrifuged at 15 000 g for 15 min to remove cells and debris before immunoprecipitation. The labelled culture supernatants were 'precleared' by incubation with 10 % (v/v) protein A sepharose (PAS; Pharmacia, 30 mg PAS was pre-incubated with 1 ml PBS, 0.1 % (w/v) BSA (bovine serum albumin) for 90 min at 4 °C with continuous agitation to give stock PAS suspension). The PAS was removed by centrifugation at 15 000 g for 5 min and the supernatant incubated for 60 min at 4 °C with 10 % (v/v) PAS precomplexed with immune Ig. Precomplexed PAS was prepared by incubating 30 mg PAS with 1 ml of immune Ig diluted 1/10 for 60 min with continuous agitation, followed by three washes of 1 ml PBS with 0.1 % (w/v) BSA. About 90 % of the Ig was bound to PAS as judged by measurement of optical density at 280 nm. The precomplexed PAS was removed by centrifugation and replaced with a second aliquot of 10 % (v/v) precomplexed PAS. Following a further 60 min incubation at 4 °C with continuous agitation the precomplexed PAS was pelleted by centrifugation, combined with the first aliquot of precomplexed PAS and pelleted through a cushion of 20 % (w/v) sucrose overlaid by 10 % (w/v)

sucrose in PBS at 15 000 g for 15 min. The combined precomplexed PAS pellets were washed once with PBS, 0.1 % (w/v) BSA made 1 M in NaCl and once with PBS, 0.1 % (w/v) BSA, 0.1 % (w/v) SDS and then boiled for 5 min in 200  $\mu$ l of Laemmli sample buffer (Laemmli, 1970) made 50 mM in dithiothreitol. Samples were subjected to discontinuous SDS-PAGE in resolving gels containing 12 % (w/v) acrylamide and processed for fluorography according to Bonner & Laskey (1974). The dried gels were exposed to Fuji RX X-ray film at  $-70^{\circ}\text{C}$  for 10–14 days before development (Kodak DX 90) and fixation (Kodak F X40).

### *Immunoblotting*

Electrophoretic transfer and immunoblotting on nitrocellulose paper (type SM, Sartorius) was performed according to Towbin, Staehlin & Gordon (1979). The electrophoretic blots were soaked in 3 % (w/v) BSA in PBS for 2 h at room temperature, and incubated with antibody preparations (diluted 1/100 in PBS plus 3 % (w/v) BSA) at room temperature on a rotating shaker. The sheets were washed in PBS containing 0.5 M NaCl (three changes of 30 min each) and incubated with peroxidase-conjugated sheep anti-rabbit IgG (Serotec) at 1/300 dilution in PBS plus 3 % BSA (w/v) for 2 h. Following washing in PBS, 0.5 M NaCl as above, antibody binding was revealed by reaction with 10 mg diaminobenzidine freshly dissolved in 100 ml 3 mM-citrate buffer pH 5.0 containing 25  $\mu$ l 30 %  $\text{H}_2\text{O}_2$ .

### *Immunohistochemistry*

Tissue specimens were fixed in Engelhardt's solution (96 % (v/v) ethanol: galacial acetic acid in the ratio 99:1, Engelhardt, Goussev, Shipova & Abelev, 1971) at  $4^{\circ}\text{C}$  for 18 h. 7.5 days *p.c.* embryos were fixed *in utero*. 10.5 days *p.c.* embryos were removed from the uterus and a small slit was cut in the yolk sac before fixation. The fixed tissues were dehydrated at  $4^{\circ}\text{C}$  through two changes of absolute ethanol and two changes of xylene before embedding in paraffin wax at  $56^{\circ}\text{C}$ . Sections were cut at a nominal thickness of 7  $\mu\text{m}$ , mounted on slides, dried overnight at  $37^{\circ}\text{C}$  and stored at  $4^{\circ}\text{C}$  before staining. The sections were dewaxed in xylene, rehydrated through serial dilutions of ethanol and incubated in methanol containing 1 % (v/v) hydrogen peroxide for 15 min at room temperature to inactivate endogenous peroxidases. The slides were thoroughly washed with PBS, 1 % (w/v) BSA and incubated with immune Ig (1/50 dilution), or pre-immune Ig (as control), for 45 min at room temperature in a humidified chamber. The sections were washed with PBS, 1 % (w/v) BSA and further incubated with peroxidase conjugated sheep anti rabbit Ig (Serotec) at 1/50 dilution for 30 min as above. Peroxidase localization was revealed by reaction with diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M-sodium phosphate buffer, 10 mM-imidazole (Sigma) for 2–5 min at room temperature. The reaction was terminated by washing in PBS and the sections dehydrated, cleared and mounted in DPX (Gurr) before observation.

## RESULTS

### (a) *Metabolic labelling and immunoprecipitation*

Two types of polyclonal antibodies were employed to examine the expression of apolipoproteins by embryonic tissues; (a) A specific antiserum raised in rabbits against electrophoretically purified mouse apoAI. This antibody reacts with two

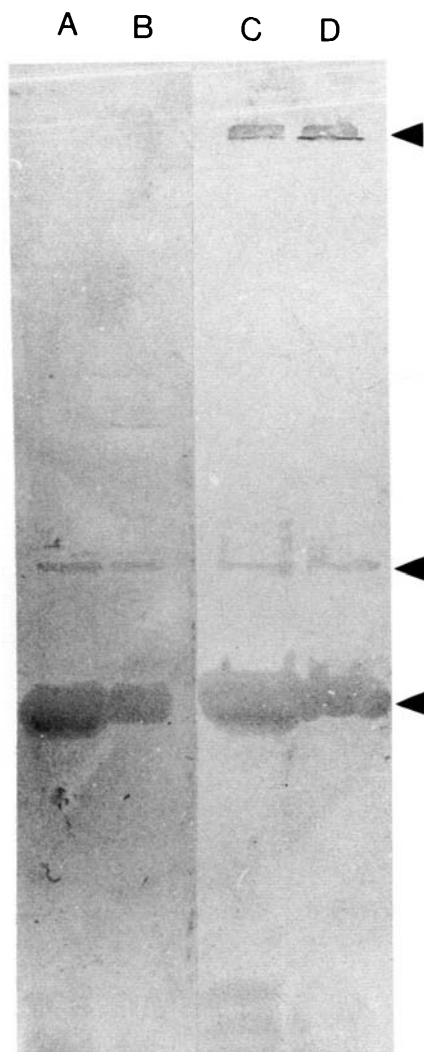


Fig. 1. Characterization of antisera by electrophoretic blotting. Adult mouse plasma (lanes A and C) and 14.5 days *p.c.* pooled amniotic fluid (lanes B and D) were separated by SDS-PAGE on a 12% acrylamide gel, transferred to nitrocellulose paper and stained with either rabbit anti-mouse ApoA1 (lanes A and B) or rabbit anti human LDL (lanes C and D), followed by localization of antibody binding by reaction with peroxidase-conjugated sheep anti-rabbit IgG. Reaction was observed with species of relative molecular masses  $28 \times 10^3$ ,  $38 \times 10^3$  and  $>200 \times 10^3$  (arrowed).

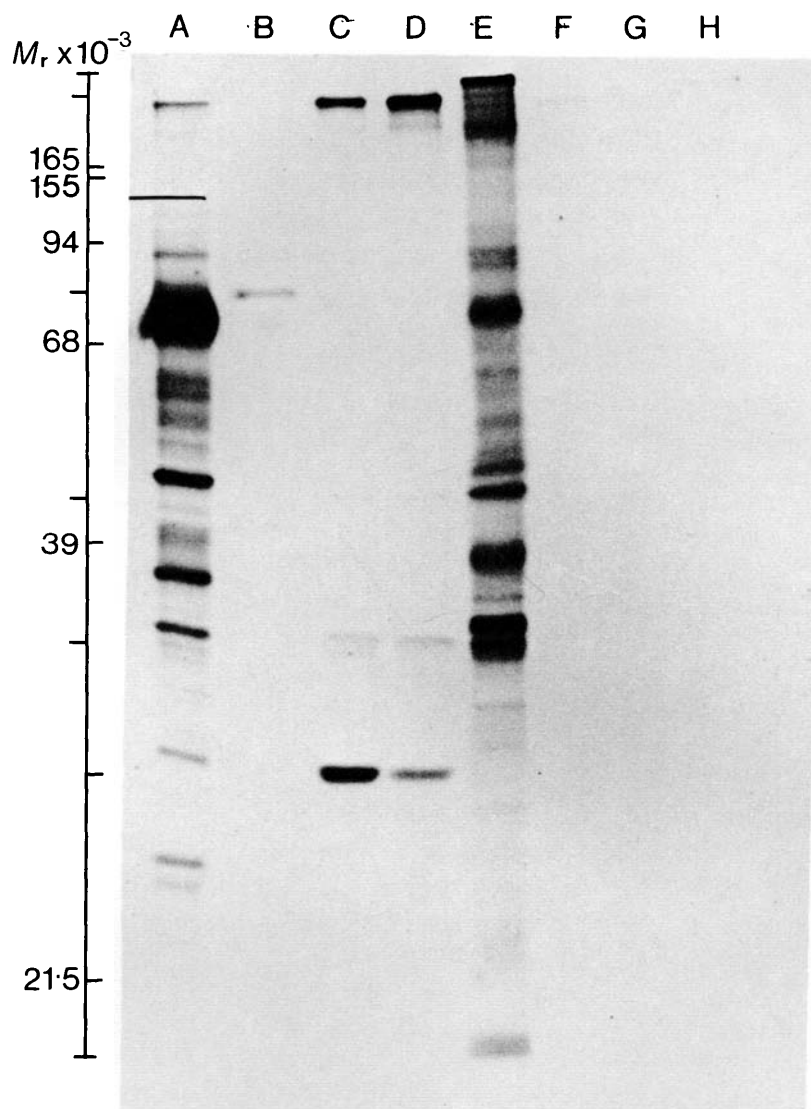


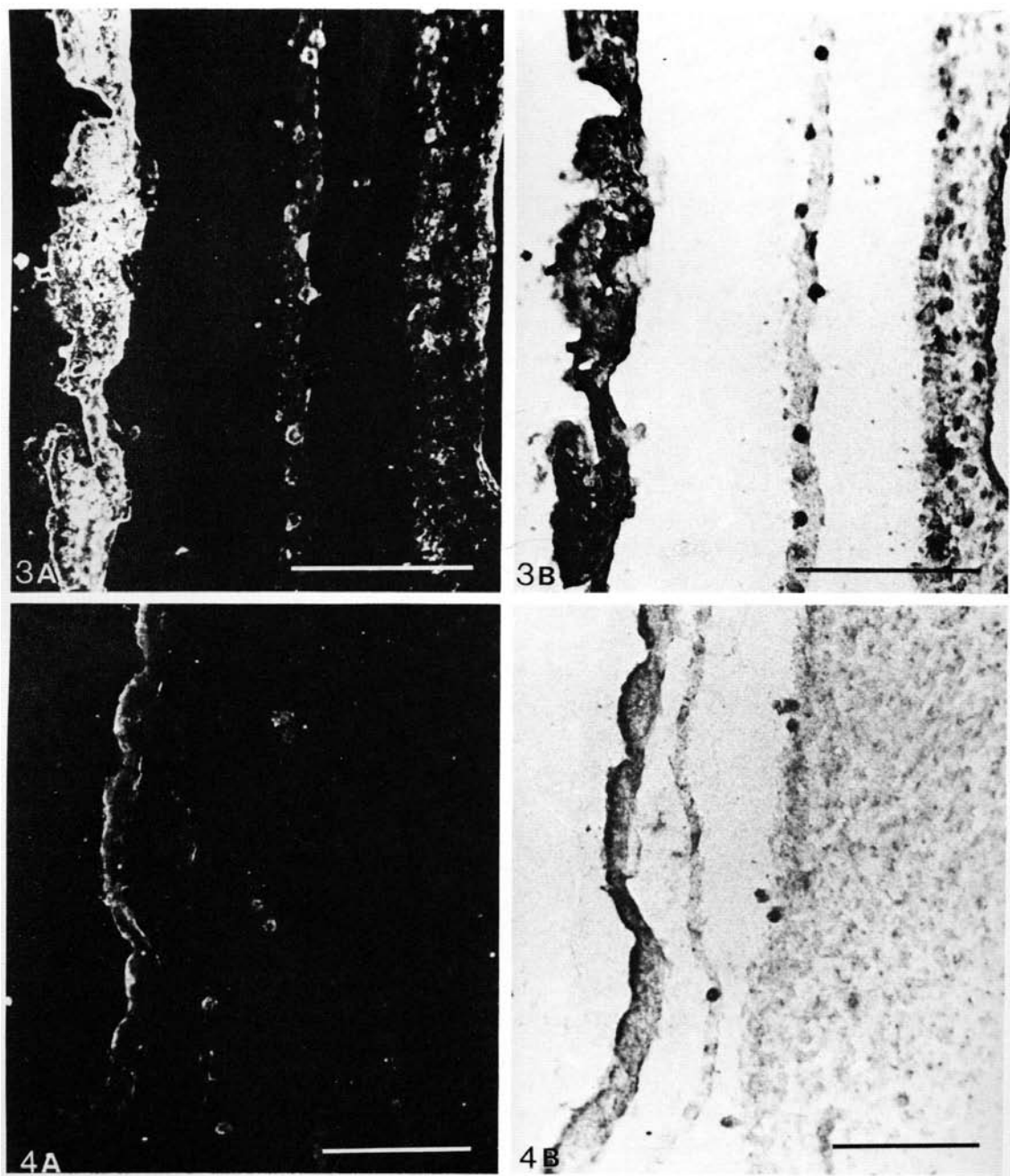
Fig. 2. SDS-PAGE slab gel analysis of immunoprecipitates from [ $^{35}\text{S}$ ]methionine labelled yolk sac culture supernatant. A–D: yolk sac endoderm. E–H: yolk sac mesoderm. A and E: total secreted proteins. B and F: immunoprecipitation by goat anti-mouse transferrin Ig. C and G: immunoprecipitation by rabbit anti-mouse apolipoprotein AI Ig. D and H immunoprecipitation by rabbit anti human LDL Ig. The mobility of relative molecular mass standards is indicated at the left.

molecular species in adult mouse plasma and amniotic fluid, corresponding in relative molecular mass to ApoA1 ( $28 \times 10^3$ ) and ApoE ( $38 \times 10^3$ ), by electrophoresis and immunoblotting (Fig. 1 lanes A and B). (b) The second antibody was initially raised by immunization with density-gradient-purified human LDL, but was found to cross react with murine apolipoproteins, presumably

reflecting the existence of some sequence homology between human and mouse apolipoproteins. This second antibody reacts with molecular species in adult mouse plasma and amniotic fluid corresponding in relative molecular mass to ApoA1, ApoE and ApoB ( $M_r > 200 \times 10^3$ ) by immunoblotting (Fig. 1 lanes C and D). A variety of embryonic tissues from different gestational stages was metabolically labelled *in vitro* with [ $^{35}$ S]methionine and the culture supernatant examined for the presence of immunoreactive material by immunoprecipitation. A radioactively labelled species corresponding in relative molecular mass to ApoA1 ( $28 \times 10^3$ ) was specifically immunoprecipitated from the visceral yolk sac endoderm of the 10.5 days *p.c.* mouse embryo (Fig. 2) or the 15.5 days *p.c.* liver (not shown). Specific immunoprecipitation of ApoA1 was not observed in the other tissues examined; yolk sac mesoderm, parietal endoderm, and amnion from the 10.5 days *p.c.* embryo and whole 7.5 days *p.c.* embryos. Three other radioactive yolk sac endoderm specific proteins, of approximate apparent relative molecular masses  $38 \times 10^3$ ,  $47 \times 10^3$  and  $>200 \times 10^3$  were routinely co-precipitated along with ApoAI by our anti-ApoAI sera. The sizes of these proteins correspond with the known relative molecular masses of ApoE, ApoAIV, and the ApoB family of apolipoproteins respectively. Molecules of identical apparent relative molecular mass (including ApoAI) were specifically precipitated from yolk sac endoderm by the anti human LDL sera which specifically reacts with ApoA1, ApoE and ApoB. Furthermore, these species were not precipitated by an unrelated goat anti-transferrin serum, routinely used as a positive tissue-specificity control antisera (Fig. 2 lane B), and were not observed in immunoprecipitations of radiolabelled culture supernatant from other tissues. We conclude that 10.5 days *p.c.* yolk sac visceral endoderm specifically secretes ApoAI and other major adult plasma apolipoprotein species. These apoproteins may well be packaged into lipoprotein particles which would explain the ability to precipitate the same apolipoproteins by antisera with overlapping reaction specificities.

#### *Immunohistochemistry*

Sections of 7.5 days *p.c.* and 10.5 days *p.c.* embryos were reacted with rabbit anti-ApoAI Ig and the tissue localization of antibody binding revealed by visualization with peroxidase-conjugated second antibody followed by staining for peroxidase activity. All 10.5 days *p.c.* embryonic tissues (including gut and liver primordia) reacted weakly with the antibody but strong specific reactivity was observed with the yolk sac endoderm (Fig. 3). No obvious cellular heterogeneity of expression was observed. No specific reactivity was observed with 7.5 days *p.c.* embryonic stages under the same conditions. This method does not distinguish between sites of synthesis and sites of deposition, but confirms the finding that the yolk sac is the principal source of ApoAI in the 10.5 days *p.c.* mouse embryo and suggests that immunologically detectable lipoprotein synthesis begins in the embryo at some stage between 7.5 days *p.c.* and 10.5 days *p.c.*



Figs 3 and 4. Immunoperoxidase staining of sectioned 10.5 days *p.c.* mouse embryos.

Fig. 3. Rabbit anti-apolipoprotein AI Ig. The staining is principally localized in the endoderm of the yolk sac. (A: Dark-field illumination, B: bright-field illumination.)

Fig. 4. Normal Rabbit Ig. (A: Dark-field illumination, B: bright-field illumination.)  
Bar = 100  $\mu$ m.



#### DISCUSSION

We have shown that yolk sac visceral endoderm is the major site of Apolipoprotein AI synthesis and deposition in the midgestation mouse embryo. It was possible to specifically co-precipitate ApoAI with other metabolically labelled proteins whose apparent relative molecular masses closely resemble those of the other major rodent apolipoprotein species. It is probable therefore that embryonic lipoproteins have a, at least superficially, similar protein composition to that of their adult counterparts, and that embryonic apolipoproteins are packaged into lipoprotein particles. In a survey of secreted products of the 11.5 to 18.5-day *p.c.* mouse yolk sac Janzen, Andrews & Tamaoki (1982) identified two major secreted products (termed X and Y) whose apparent relative molecular masses and apparent isoelectric points closely resemble those reported for ApoAI and ApoE respectively (Zannis *et al.* 1982). It is likely therefore that apolipoprotein synthesis has been observed previously but passed unrecognized.

We believe the significance of these findings are threefold: Firstly, the existence of lipoprotein synthesis in the embryo was predicted from the observed requirements for the growth of embryo-derived embryonal carcinoma cells in serum-free defined culture media. The technique of defined media development may therefore prove to have useful predictive powers in identifying functionally important embryonic molecules. Secondly, like transferrin (Adamson, 1982) and alphafoetoprotein (Engelhardt *et al.* 1971) apolipoproteins are secreted by the liver in the foetus. The liver, is not however, currently thought to be related by lineage to the visceral yolk sac endoderm (Gardner, 1982). This apparent coincidence may in fact reflect an underlying functional similarity between adult liver and embryonic yolk sac. The extent to which this holds may be tested by examining the expression of other liver products in the visceral yolk sac endoderm. Thirdly, both transferrin and lipoproteins have defined nutrient transport functions and may consequently play an important part in embryonic physiology. In view of the proposal (New & Brent, 1974) that the yolk sac serves a nutrient function in embryonic and foetal development and also represents a physical barrier between the foetus and the maternal uterine environment it would be of interest to determine if the transported nutrients (namely iron and lipid) have a maternal or embryonic origin.

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