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Organ distribution of apolipoprotein gene transcripts in 6–12 week postfertilization human embryos

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SUMMARY

In the liver and the yolk sack of 6–12 week postfertilization human embryos, we have detected RNA transcripts from the following apolipoprotein genes: AI, AII, B, CII, CIII and E. The mRNA from the apolipoprotein CIII gene was relatively more abundant in the total RNA from the yolk sack than in that from the liver. The gut and adrenals contained transcripts of all these apolipoprotein genes apart from apolipoprotein AII. The kidneys and heart contained some apolipoprotein transcripts. In conjunction with previous studies, these results suggest that in the human embryo apolipoprotein genes are transcribed in a much larger range of organs than is the case in the adult. Many of these organs lack endoderm tissues.

INTRODUCTION

In the liver and the gut of adult humans there are high levels of the mRNA that codes for apolipoproteins. Apolipoproteins complex with lipids to form water-soluble lipoprotein particles, and they are required to transport water-insoluble lipids from one part of the body to another (reviewed by Mahley, Innerarity, Ralls & Weisgraber, 1984). There are at least nine genes in a haploid human genome, each of which codes for discrete apolipoproteins; these apolipoproteins are distinguished by amino acid sequence, size and function (see Discussion). Transcripts of several of these genes are found in a wider range of organs in the 5 month postfertilization human foetus than in the adult (Zannis *et al.* 1985). These observations suggest that the organ specificity of gene expression may markedly change during embryogenesis.

Here we describe the organ distribution of transcripts from six of the apolipoprotein genes. By studying earlier embryos at 6 to 12 weeks after fertilization, we have observed an even wider organ distribution. For the first time, it is also shown that the human yolk sack transcribes as wide a range of apolipoprotein genes as the liver.

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Key words: apolipoproteins, human embryo, serum proteins, gene expression, yolk sack.

MATERIALS AND METHODS

Isolation of RNA

Foetal organs were obtained (Hyldahl, 1984), and the postfertilization age was estimated (Thompson *et al.* 1984). In many experiments we combined the small organs from several embryos, whose ages fell into the same 10 day interval after fertilization. RNA extraction was started within 2 h of death.

The organs were quickly dissolved by vigorous pipetting and shaking in aqueous 4 M-guanidinium isothiocyanate (Fluka) with 5 mM-sodium citrate, 0·1 M β-mercaptoethanol and 0·5 % (v/v) Sarkosyl. Dissolved samples were either processed immediately or stored at −20 °C. The dissolved samples were layered over a 2·2 ml cushion of aqueous 5·7 M-caesium chloride in 0·1 M-EDTA (diaminoethane tetra-acetic acid) at pH 8·0 in 12 ml capacity tubes (Beckman). They were centrifuged in an SW 40 rotor at 33 000 rev. min^{-1} for 24 h at 15–20 °C (similar to Chirgwin, Przytyla, MacDonald & Rutter, 1979).

The pellets of RNA were dissolved in 100 μl of aqueous 10 mM Tris–HCl (Sigma), pH 7·4, 1 mM-EDTA (TE buffer). Next they were ethanol precipitated twice, redissolved again in 100 μl TE buffer, and stored at −20 °C. The concentration of RNA was measured by absorbance at 260 nm.

Labelling of DNA probes with ^{32}P

All the apolipoprotein probes were in the form of cDNA sequences (see Sharpe *et al.* 1984; F. E. Baralle, unpublished results). The probes for Apo A I, Apo A II, Apo C II, Apo C III and Apo E were either *Bam*HI:*Eco*RI fragments or *Bam*HI:*Hind*III fragments, which had been cloned into the *Pvu*II site of a pAT 153 vector. The Apo B probe contained the 3′ 2 kb end of clone pB4 (Shoulders *et al.* 1985), subcloned into a pSp 62 vector; the apolipoprotein B probe reacts with a large transcript which has the capacity to code for apolipoprotein B100 (Milne *et al.* 1984; Sparks & Sparks, 1985). The size of the inserts varied: Apo AI at 890 bp; Apo AII at 450 bp; Apo CII at 489 bp; Apo CIII at 580 bp; Apo E at 175 bp; Apo B at approximately 2000 bp. The probes were cut from the vectors with the appropriate restriction enzymes, and isolated following electrophoresis through low gelling temperature 1·2 % (w/v) agarose gels (FMC Seakem), stained with 1 μg ml^{-1} ethidium bromide (Sigma); they were then purified through NACS columns (BRL). All the probes were labelled with [^{32}P]dATP (Amersham International). The probes for Apo AI, Apo AII, Apo CII, Apo CIII and Apo E were nick translated (Rigby, Dieckmann, Rhodes & Berg, 1977), using the Amersham nick translation kit; the final specific activities ranged from 1 to $2·5 \times 10^8$ cts min^{-1} μg^{-1} DNA. The Apo B probe did not label to high specific activity with this method. It was labelled with oligopriming (Feinberg & Vogelstein, 1984), using random hexanucleotides (Pharmacia) and DNA polymerase 1 (Amersham International). The specific activity varied between 2 to 3×10^8 cts min^{-1} μg^{-1} DNA.

Northern blotting

Solutions of total extractable RNA (5–20 μg) were denatured for 15 min at 60 °C. The 20 μl final reaction volume contained 20 mM-MOPS (3-(*N*-morpholino)propanesulphonic acid), 5 mM-sodium acetate, 1 mM-EDTA at pH 7·0 (1×MOPS) with 50 % (v/v) deionized formamide and 1·85 % (v/v) formaldehyde. Before gel loading, 2 μl of 6 × loading buffer was added. This contained aqueous 15 % (w/v) Ficoll 400, 0·25 % (w/v) bromophenol blue, and 0·25 % (w/v) xylene cyanol. The samples were run on a 1·5 % (w/v) denaturing agarose gel containing 1×MOPS buffer and 0·23 M-formaldehyde. Gels sized 11×14 cm were run overnight at 10 mA, and the buffer was 1×MOPS with 8·7 % (w/v) formaldehyde.

The RNA was transferred to nitrocellulose filters (Schleicher and Schull BA 85, 0·45 μm pore size), by blotting overnight in 20×SSC (1×SSC = aqueous 0·15 M-NaCl, 0·015 M-sodium citrate, pH 7·0). The blots were washed in aqueous 1 mM-EDTA at pH 8·0, air dried, and baked for 4 h at 80 °C between two sheets of Whatman 3 mm 'Chrom' paper.

The blots were prehybridized overnight with 250 μg ml^{-1} of sonicated and denatured salmon sperm DNA in 5×SSC, 50 mM-phosphate buffer at pH 6·8, 5 × Denhardt's solution (Denhardt, 1966), 0·1 % (w/v) sodium dodecyl sulphate (SDS) and 50 % (v/v) deionized formamide. They were hybridized with the labelled probes for at least 48 h at 42 °C with final probe counts in the

hybridizing buffer varying from 1 to 3×10^6 cts min^{-1} ml^{-1}. The hybridization buffer contained 5×SSC, 25 mM-phosphate buffer at pH 6·8, 2·5 × Denhardt's solution, 0·1 % (w/v) SDS, 50 % (v/v) deionized formamide, and 250 μg ml^{-1} of sonicated and denatured salmon sperm DNA. The probe was denatured before use by boiling for 15 min in water with the salmon sperm DNA. The filters were washed at high stringency with a final wash at 65°C for 30 min in 0·1×SSC. Radioactivity was detected with preflashed Kodak X-Omat.S film (Laskey & Mills, 1977).

Densitometry

To measure the relative abundance of steady state levels of apolipoprotein mRNA in the various organs, varying amounts of RNA from foetal liver were treated as above, and the exposed film scanned with a Vitatron densitometer to determine the range of liver RNA loadings that gave a linear increase in darkening of the film, with a particular probe and a particular duration of film exposure. By scanning individual films that had both been exposed to liver controls which darkened the film within this range, and that had also been exposed to other organs, it was possible to estimate roughly the relative abundance of different apolipoprotein gene transcripts in the total extractable RNA from different organs (see Table 2).

In most cases, we only scanned the single discrete darkened band that coincided in mobility with that described previously in adult tissues, namely: Apo AI, 0·9 kb; Apo AII, 0·5 kb (Sharpe *et al.* 1984); Apo B, 22 kb (Huang, Bock, Feinstein & Breslow, 1985); Apo CII, 0·5 kb (Myklebost *et al.* 1984); Apo CIII, 0·6 kb (Sharpe *et al.* 1984); Apo E, 1·2 kb (Wallis *et al.* 1983). In the case of apolipoprotein B, we only scanned the darkened band with a mobility of about 14 kb, and we did not measure the density of the lower molecular weight smear on the film.

RESULTS

We studied the organ distribution of transcripts from the following apolipoprotein genes: apolipoproteins AI, AII, B, CII, CIII and E (Fig. 1; Table 1; probes described in Materials and Methods). The stages of embryogenesis represented in the samples ranged from the stage when distinct digits were visible on the limbs to well into the foetal period, when most of the organs are in their final adult position (Moore, 1982). However, the limited comparative data from embryos of different ages preclude any conclusions about age-dependent changes in the abundance of apolipoprotein transcripts.

Digestive system and yolk sack

All the apolipoprotein gene products that we studied were present in foetal liver, thus adding apolipoproteins AII and B to the range of apolipoprotein transcripts that have been observed in liver at later stages of development (Zannis *et al.* 1985).

Apolipoprotein AI, AII, B, CII and CIII gene products were present in the yolk sack at a higher abundance than that found in any other organ except the liver (Table 2). In all yolk sack samples, apolipoprotein CIII gene transcripts were more abundant than in the liver. In some yolk sack samples apolipoproteins AI and AII were also more abundant in the yolk sack than in the liver. Variation in the relative abundance of these and other transcripts (Table 2) probably reflects variations both in foetal age and in the time between death and RNA extraction. Despite this variation between samples, both these results and the observation that the human yolk sack secretes substantial amounts of apolipoproteins AI and B (Shi *et al.* 1985)

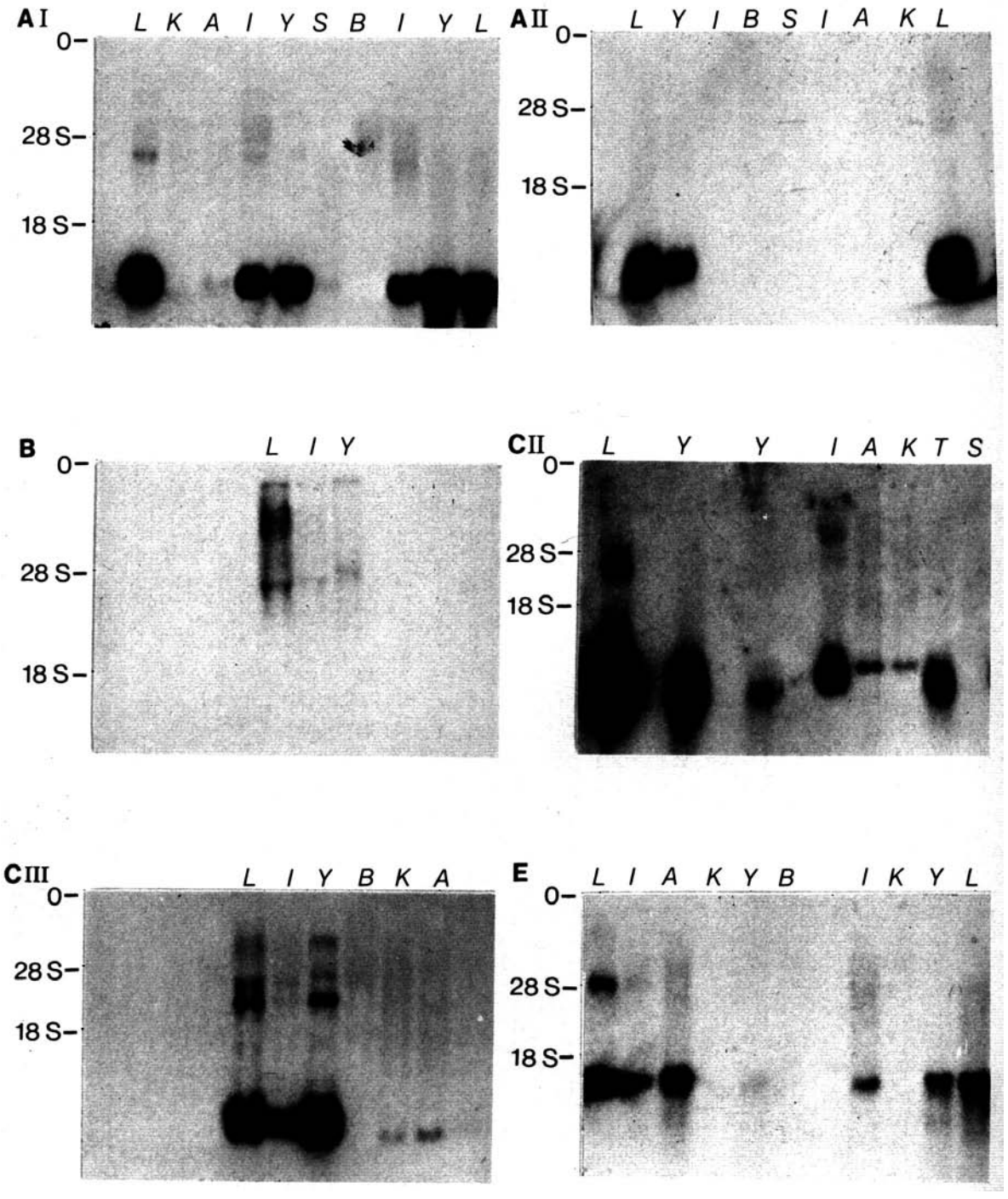

Fig. 1. Northern blot analysis of size fractionated total RNA from foetal organs. One sample of a primary human teratoma is included. All samples loaded at 15 μg, except when noted below. Apolipoprotein AI: liver samples at 5 μg, and yolk sacks at 10 μg. Apolipoprotein AII: liver samples at 5 μg, and yolk sack and stomach at 10 μg. Apolipoprotein CII: yolk sack and stomach samples at 10 μg, and human teratoma at 20 μg. Apolipoprotein E: liver samples at 5 μg, yolk sack, adrenal and brain samples at 10 μg. Unspecific binding of the probe in the region of the 28 S and 18 S ribosomal RNA markers was found in many experiments. *L*, liver; *K*, kidney; *A*, adrenal; *I*, intestine; *Y*, yolk sack; *S*, stomach; *B*, brain; *T*, teratoma.

establish the yolk sack as a potential major source of a wide variety of apolipoproteins during the second and third months of human development. The visceral yolk sack of the early mouse and rat embryo has similarly been shown to contain relatively more abundant transcripts for apolipoproteins AI and E than the foetal liver (Meehan *et al.* 1984; Elshourbagy, Liao, Mahley & Taylor, 1985).

We confirm the presence of transcripts for apolipoproteins AI, CII, CIII and E in the gut (Zannis *et al.* 1985), and we also observe apolipoprotein B transcripts in this organ. Although we did not identify the region of the gut that was included in any particular gut sample, the consistent failure to detect apolipoprotein AII gene transcripts in the large number of samples we examined suggests that stable transcription products of this gene are absent from the whole gut at these stages of development.

Abundant apolipoprotein gene transcripts were not consistently detected in the stomach, as Zannis *et al.* (1985) had previously found. The sporadic observation of AI and B apolipoprotein transcripts in the stomach is consistent with contamination of these samples by gut or liver tissue, although we consider that careful dissection and washing exclude this explanation.

Adrenal, kidney and brain

We confirm the observation of Zannis *et al.* (1985) that apolipoprotein AI and E gene transcripts are present in the adrenal, and in addition we find transcripts of apolipoproteins B, CII and CIII in this organ.

In the kidney, we detect transcripts of apolipoprotein B, CII and CIII genes, but we have not found apolipoprotein AI and there are only traces of E mRNA: the latter mRNA is clearly present in the kidneys of older foetuses and adults (Wallis *et al.* 1983; Zannis *et al.* 1985). Similarly, we do not detect apolipoprotein E transcripts in the brain, although they are present in the brain of older foetuses and

Table 1. *Frequency of detection of apolipoprotein transcripts in samples of each organ*

Organ	Apo AI	Apo AII	Apo B	Apo CII	Apo CIII	Apo E
Liver	18/18	10/10	5/6	6/6	6/6	7/7
Yolk sack	11/11	7/7	3/4	4/4	4/4	5/5
Gut	15/16	0/8	5/6	3/3	4/4	5/5
Stomach	3/5	0/2	1/2	0/1	nd	0/2
Adrenal	3/3	0/3	2/2	1/1	1/1	3/3
Kidney	0/4	0/3	2/2	1/1	2/3	4/4
Brain	0/8	0/5	1/2	0/3	0/4	0/4
Heart	2/2	nd	nd	nd	nd	1/1

In each case a sample represents one or more foetal organs combined in a single RNA extraction. The figures are the proportion of samples in which a transcript was detected; we have included cases where the band was obvious, even though its relative density was less than 1 % of the liver control. When no transcript was detected, then the densitometry of the autoradiographs allows us to conclude that the mRNA for that particular apolipoprotein gene transcript was at least 100-fold less abundant in the total RNA from that organ than in the total RNA from the liver. 'nd' means 'not done'.

Table 2. *Relative abundance of RNA from organs, compared to abundance in liver*

Organ	Apo AI (%)	Apo AII (%)	Apo B (%)	Apo CII (%)	Apo CIII (%)	Apo E (%)
Liver	100	100	100	100	100	100
Yolk sack	85·5 (60, 82, 90, 110)	73·3 (38, 38, 54, 163)	26·0 (22, 26, 30)	37·5 (8, 20, 57, 65)	149·0 (140, 158)	20·5 (8, 33)
Gut	29·8 (14, 28, 36, 41)	<1·0	18·0 (16, 20)	17·3 (12, 16, 19, 22)	54·0 (48, 60)	18·0 (14, 17, 23)
Stomach	13 (6, 20)	<1·0	trace	<1·0	nd	<1·0
Adrenal	7·3 (7, 7·5)	<1·0	trace	2·5 (2, 3)	7 (7, 7)	50·5 (49, 52)
Kidney	<1·0	<1·0	trace	2·0 (1·5, 2·5)	5 (5, 5)	<1·0

These figures are the mean of the abundance of mRNA in each organ, expressed as a percentage of the abundance in liver. Measurements on individual samples are recorded in brackets below the mean. The samples each contained RNA from several embryos. In the case of apolipoprotein B, we measured the relative abundance of the 14 kb transcripts, and use 'trace' to indicate lower molecular weight material which reacted with the probe in the absence of a clear 14 kb band; this material is probably degraded mRNA. 'nd' means 'not done'.

adults (Elshourbagy *et al.* 1985; Zannis *et al.* 1985). These differences probably reflect the age of the foetus, because the abundance of apolipoprotein E transcripts increases with age in the brain and the kidney of the newborn rat (Elshourbagy *et al.* 1985). Apolipoprotein E is found as a protein among all the astrocyte cell types in the rat brain (Boyles *et al.* 1985), and it is possible that astrocytes do not develop this property until the fifth month of human development.

Lack of abundant apolipoprotein transcripts in umbilical cord blood

Adult and foetal human white blood cells contain apolipoprotein E gene transcripts (Wallis *et al.* 1983; Zannis *et al.* 1985), and we wished to identify the contribution that apolipoprotein gene transcripts in circulating blood might make to the amounts of these RNA molecules in human organs. We compared the relative abundance of apolipoproteins AI and E transcripts in umbilical cord blood and in foetal liver. Densitometry of single filters, each containing different dilutions of total RNA from these two sources (see Materials and Methods), showed that these types of RNA molecules were at least 100-fold less abundant in this blood when compared to foetal liver (results not shown). We cannot exclude the possibility that particular blood cells concentrate in particular organs, but we have assumed that, when the abundance of these and other apolipoprotein gene transcripts extracted from an organ exceeds 1 % of their abundance in liver, then at least some of the tissues that constitute that organ are expressing apolipoprotein genes. We have previously failed to detect the synthesis and the secretion of apolipoproteins AI and B by umbilical cord blood (Shi *et al.* 1985).

DISCUSSION

In conjunction with the observations of Zannis *et al.* (1985) on older foetuses, these results raise several issues.

Apolipoprotein synthesis and low density lipoprotein use

First, it is a physiological curiosity that organs that depend on a supply of lipids, delivered by low density lipoproteins, should themselves contain transcripts of the genes that make up the protein part of these particles; it is known that both the foetal human adrenal and testis require this form of lipid supply to maintain a high rate of steroid synthesis in culture (Carr *et al.* 1980, 1983; Simpson, Boggaram, Funkenstein & Waterman, 1985), and both contain apolipoprotein gene transcripts (Zannis *et al.* 1985; this study). Second, there is a discrepancy between the distribution of the transcripts from these genes and the detection of their synthesized and secreted proteins; for instance, neither the foetal adrenal nor the foetal kidney has yet been shown to secrete newly synthesized apolipoproteins into organ culture medium (Shi *et al.* 1985), although both these organs secrete apolipoprotein E in adult humans and in both cynomolgus and African green monkeys

(Blue *et al.* 1983; Williams, Dawson, Newman & Rudel, 1985). If we assume that these apolipoprotein transcripts are translated into proteins in the foetus, then these two puzzles would be resolved if the local synthesis of apolipoproteins was used to shuffle lipids between the tissues within an organ (discussed by Blue *et al.* 1983; Mahley *et al.* 1984; Elshourbagy *et al.* 1985). In this situation, there might be little or no secretion of apolipoproteins into culture medium.

Organ specificity of serum protein synthesis

The organ distribution of apolipoprotein gene transcripts does not correspond with our current appreciation of cell lineages in mammals (mouse lineage reviewed by Gardner, 1983), or with classical notions about the organization of embryos into germ layers (criticized by Oppenheimer, 1945). For instance, the endoderm cells of the liver and the gut are thought to originate from quite different cells to those that are the precursors of yolk sack extraembryonic endoderm. Nevertheless, all six apolipoprotein genes are transcribed in both the liver and the yolk sack, extending the known functional similarities between these two organs (Gitlin & Biasucci, 1969; reviewed by Glieberman & Abelev, 1985). The generalization that the mammalian yolk sack produces serum proteins early in development is supported most recently by evidence that the genes that code for the following proteins are transcribed in this organ: albumin (mouse: Tilghman & Belayew, 1982; Meehan *et al.* 1984), alphafoetoprotein (mouse: Andrews, Dziadek & Tamaoki, 1982; Tilghman & Belayew, 1982; Dziadek & Andrews, 1983; Andrews, Adamson & Gedamu, 1984; Krumlauf, Hammer, Tilghman & Brinster, 1985), alpha-1-antitrypsin (mouse: Meehan *et al.* 1984), apolipoproteins (mouse: Meehan *et al.* 1984; rat: Elshourbagy *et al.* 1985; human: this study), insulin (rat: Muglia & Locker, 1984), insulin-like growth factor II (human: Scott *et al.* 1985), metallothioneins I and II (mouse: Andrews *et al.* 1984) and transferrin (mouse: Meehan *et al.* 1984). Most of the proteins coded by these genes had previously been shown to be secreted into culture fluid by both the rodent and the human yolk sack.

It is no longer possible to regard the synthesis of serum proteins as an exclusive property of organs that contain endoderm. The only apolipoprotein gene transcripts that are apparently restricted to organs containing endoderm are apolipoprotein AII transcripts, which are only found in the liver and the yolk sack among the organs that we have studied. Apolipoprotein AIV transcripts may also have a limited distribution, for they have only been found in the foetal intestine and adult liver in a preliminary survey (Karathanasis, 1985).

It is now common to find that serum proteins are also synthesized by organs that do not contain endoderm. For instance, apolipoprotein AI gene transcripts are found in both the kidney and the adrenal, and neither organ is believed to contain endoderm cells (human: Zannis *et al.* 1985; this study). Similarly, abundant apolipoprotein E transcripts are found away from the organs of the digestive system in both primates and rodents (Driscoll & Getz, 1984; Elshourbagy *et al.* 1985; Newman, Dawson, Rudel & Williams, 1985; Reue *et al.* 1984; Williams, Dawson, Newman & Rudel, 1985). Again, transferrin is synthesized both by the

gut, liver and the yolk sack, and by organs lacking endoderm, such as foetal spinal cord and adult ear pinna, mammary gland and the Sertoli cells of the testis (rodents: Meek & Adamson, 1985; Skinner & Griswold, 1980; Thorbecke *et al.* 1973; Wright, Musto, Mather & Bardin, 1981). Alphafoetoprotein gene transcripts are at least 50-fold more abundant per total mRNA in the mouse foetal gut, liver and yolk sack than they are in any other organs of the foetus or the adult mouse (Tilghman & Belayew, 1982; Dziadek & Andrews, 1983). However, transcripts from the alphafoetoprotein gene can also be found in the mouse foetal brain, kidney and heart (R. Krumlauf, personal communication), and a similar lack of organ specificity is shown by alphafoetoprotein synthesis in the human embryo: it is produced by the intestine, stomach and kidney in addition to the liver and yolk sack (Mackiewicz & Breborowicz, 1980).

We speculate that the elements that control the abundance of mRNA for these apolipoprotein genes must respond to the local and changing physiological demands of embryogenesis and adult life, rather than to some abstract programme of gene expression peculiar to particular cell lineages. The known functions of apolipoproteins include the solubilization of lipids, the recognition and modulation of enzymes involved in lipid metabolism and the binding of lipoproteins to their cellular receptors. Lipoproteins are also needed to sustain the rapid growth of undifferentiated human teratoma cells, and this requirement is probably shared by the multiplying stem cells of the early embryo (Engstrom, Rees & Heath, 1985; Thompson *et al.* 1984).

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