Splicing pathways of the chicken apo very low density lipoprotein II (pre)messenger RNA

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The precursor-mRNA transcribed from the chicken apo very low density lipoprotein II gene was identified. This gene which is under full estrogen control and only expressed in the liver, possesses three introns. Splicing intermediates were characterized by hybridization with intron-specific probes, and by electron microscopy of R-loops. The introns appear to be excised in a non-obligatory order, but at different rates.

Apo-VLDL II *RNA splicing* *Estrogen* *Gene expression* *Splicing pathway* *Yolk protein gene* *pre-mRNA*

1. INTRODUCTION

Since most eukaryotic genes have a mosaic structure the excision of the introns and correct ligation of the exons to a functional mRNA has formed the subject of many studies [1,2]. In recent years, experiments in cell-free systems have elucidated part of the mechanism and structures involved [3,4]. However, it is still far from clear what determines the rates of removal of different introns.

In our laboratory, the structure and expression of the chicken apo very low density lipoprotein II (apo-VLDL II) gene have been investigated. Apo-VLDL II is a yolk protein made in large amounts in the liver of chickens upon estradiol stimulation. The gene, 2918 nt long [5], contains three introns [6]; the mRNA (excluding the poly(A) tail) is 656 nt long and codes for a protein of 106 amino acids [7]. The present paper shows that the excision of an intron does not require prior excision of another. However, the rates of excision are different.

This paper is dedicated to Prakash Datta as a token of gratitude and friendship

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2. MATERIALS AND METHODS

2.1. *RNA preparation*

Roosters (White Leghorn) were injected subcutaneously with estradiol (50 mg per kg body wt). Total cellular RNA was prepared by the LiCl-urea method as described by Auffray and Rougeon [8] with slight modifications. Polysomal RNA was isolated from large-scale preparations of liver polysomes [9] according to the LiCl-urea method. Polyadenylated RNA was isolated from total cellular or polysomal RNA by affinity chromatography on oligo(dT)-cellulose according to Aviv and Leder [10].

2.2. *Preparation of DNA probes*

The construction and characterization of the apo-VLDL II cDNA 3.33 have been described earlier [7]. All other probes were derived from a 3.0-kb *BglII* fragment which covers the larger part of the apo-VLDL II gene, and had been cloned in pBR322 [5]. The entire insert DNA was isolated [11,12] and nick-translated [13] to yield the so-called total probe. Various probes, specific for the

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various introns, were obtained by cloning suitable fragments of the *Bgl*II clone. A 287-bp *Pst*I fragment from intron A, and a 348-bp *Sau*3A fragment from intron C, were subcloned in M13mp9 bacteriophage DNA, as described earlier [5]. Single-stranded phage DNA was used as a template for Klenow polymerase to synthesize a probe which is complementary to RNA sequences in the intron A or intron C region. A 206-bp *Alu*I fragment from intron B was inserted into pBR322, which had been linearized with *Bam*HI, and made blunt-ended with Klenow polymerase [14]. Nucleotide sequence analysis confirmed the presence of the desired 206-bp fragment in one of the hybrid plasmids obtained. The plasmid containing the intron B insert was treated with *Taq*I. A 450-bp fragment harboring the insert was isolated, nick-translated, and used as an intron B-specific probe.

2.3. *Electrophoresis and hybridization of RNA*

RNA was treated with glyoxal and dimethyl sulfoxide according to McMaster and Carmichael [15] and run in 1.1% agarose gels. The RNA was transferred to nitrocellulose (Schleicher and Schuell BA85, or Millipore HAHY) using 20 × SSC [16]. Hybridization of the RNA blots was done essentially as described by Jeffreys and Flavell [17]. The final washing step was in 0.2 × SSC.

2.4. *R-looping*

R-loops were formed by hybridization of 0.25 μg cloned genomic apo-VLDL II DNA (clone 12 [6]) and 15 μg polyadenylated RNA in 20 μl hybridization buffer [18] for 20 h, applying a linear temperature gradient from 50 to 48°C. The R-loops were stabilized by glyoxal fixation [19]. Double-stranded DNA and R-loops were separated from non-hybridized RNA molecules by chromatography on a Sepharose-4B column (120 × 3 mm) with the hybridization buffer as eluant. Aliquots of the fractions were spread from 50% formamide (v/v), 2 M urea, 0.12 M Tris-HCl, 10 mM EDTA, and 0.01% cytochrome *c* (pH 7.8) on triply distilled water. Further processing of specimens and electron microscopy were according to Arnberg et al. [20].

3. RESULTS

3.1. *Products of the apo-VLDL II gene transcription*

Total cellular polyadenylated RNA from the livers of estrogen-treated roosters was fractionated by agarose gel electrophoresis and transferred to nitrocellulose filters. Transcripts from the apo-VLDL II gene were visualized by hybridization with nick-translated 12Bg3.0 DNA, a genomic clone containing the major part of the apo-VLDL II gene. Knowledge of the size and the exon-intron structure of the gene (fig.1) led to an – albeit tentative – assignment of the observed bands (fig.2). Obviously, the strongly hybridizing band with the highest mobility represents the mature apo-VLDL II mRNA. It is the only band hybridizing in a blot of polysomal polyadenylated RNA (lanes 4 and 5). The band contains an RNA of about 880 nucleotides, implying that it contains a poly(A) tail of about 200 residues, besides the 656-nt exon sequence. In accordance with its length of 3200 nucleotides, the largest RNA represents the polyadenylated primary transcript. Several other detected bands are processing intermediates.

Since the probes used are unique for the apo-VLDL II region [21] we can exclude that some of the bands represent transcripts from other genes.

3.2. *Identification of splicing intermediates*

If we assume that an intron is split out as a whole in one step, and that only one species of apo-VLDL II mRNA will result from the process-

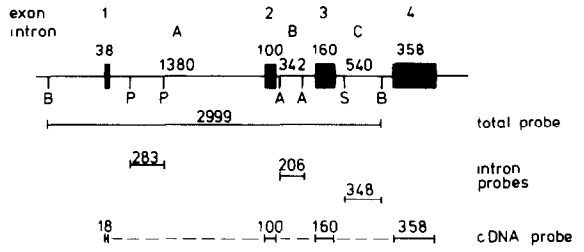

Fig.1. Map of the apo-VLDL II gene in chicken DNA and location of the probes. The exons are indicated by black boxes. The sizes of exons and introns are derived from sequence data and are given in base pairs. Restriction sites are indicated: B, *Bgl*II; P, *Pst*I; A, *Alu*I; S, *Sau*3A. The probes used are shown; their sizes are given in base pairs. Details are described in section 2.

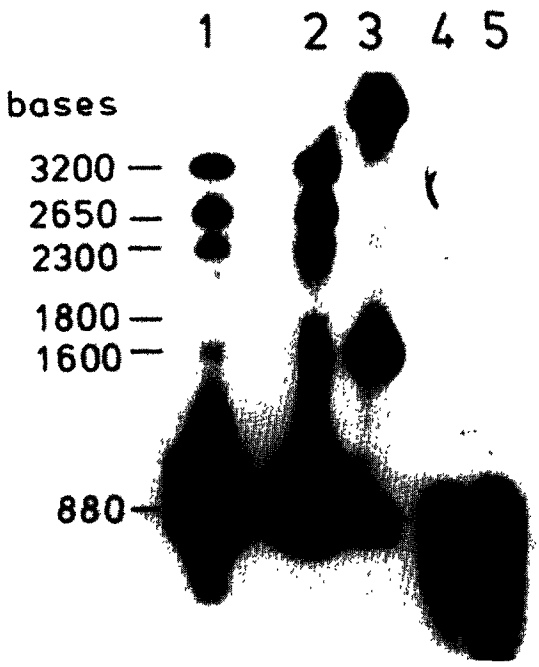

Fig.2. Electrophoretic separation of apo-VLDL II RNA sequences. (Lane 1 and 2) 50 μg total cellular polyadenylated RNA from the liver of a rooster 2 days after estrogen administration; (lane 3) marker DNA consisting of 900-, 1600- and 3900-bp *Pst*I fragments of clone 12Bg3.0; (lane 4) 5 μg, and (lane 5) 25 μg polysomal polyadenylated RNA from the liver of an estrogen-treated rooster. Blots on nitrocellulose filters were hybridized with nick-translated total probe. Some weak bands (2850, 1250 nucleotides) visible on the autoradiograph are lost in the photographic reproduction.

ing we can distinguish between two possibilities:
(i) the three introns are removed via a single obligatory pathway which thus will have only two intermediates;
(ii) the introns are excised in a random fashion yielding six intermediates (fig.3).

To identify the bands observed, we hybridized RNA blots of gels with separate probes specific for each intron as indicated in fig.1. The results of the hybridization experiments are shown in fig.4. The 3200-nucleotide band hybridizes with probes, specific for intron A, B and C, respectively, confirming that it is the intact primary transcript. The 2650-nucleotide band hybridizes with the intron A- as well as with the intron B-specific probe, but not

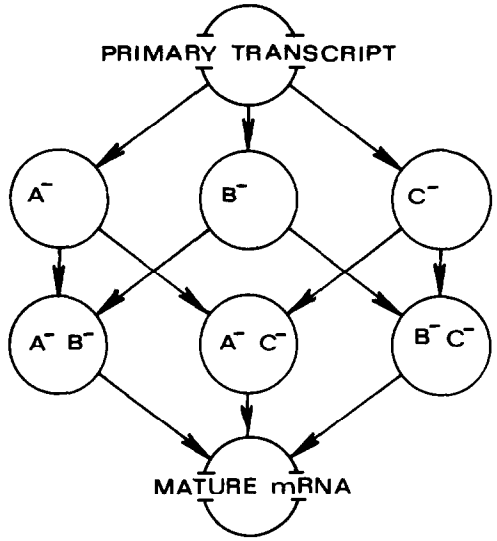

Fig.3. Possible intermediates in the processing of the apo-VLDL II primary transcript. The presumed intermediates and the introns which are lacking are indicated.

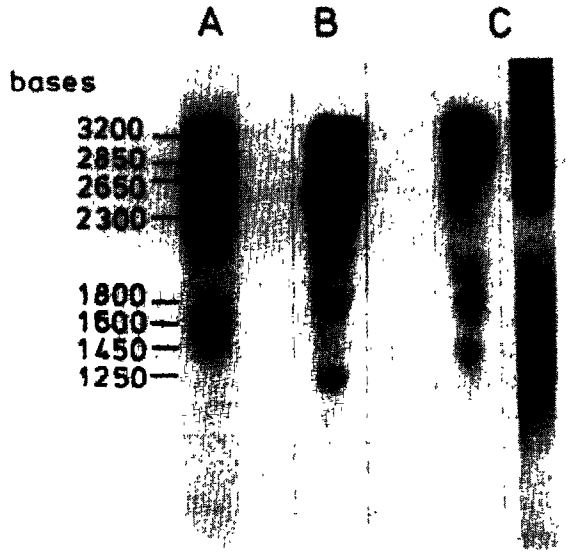

Fig.4. Characterization of apo-VLDL II splicing intermediates. 50 μg aliquots of total cellular polyadenylated RNA from the liver of a rooster 2 days after estrogen administration were treated with glyoxal, and separated by agarose gel electrophoresis. Blots were hybridized with the 283-nt intron A probe (panel A), the 206-nt intron B probe (panel B), or the 358-nt intron C probe (panel C). See further fig.1 and section 2.

with the intron C probe. This result, as well as the size of the band, 550 nucleotides shorter than the primary transcript, strongly suggests that it represents the processing intermediate from which intron C has been excised. Following a similar reasoning, the 2300-nucleotide RNA is concluded to be the intermediate lacking both intron B and intron C. Apparently, intron C is removed more quickly than intron B, and intron B before intron A, so that one route is: 3200 nt (primary transcript) $\longrightarrow$ (−C) $\longrightarrow$ 2650 $\longrightarrow$ (−B) $\longrightarrow$ 2300 $\longrightarrow$ (−A) $\longrightarrow$ 880 nt (mature mRNA).

To establish alternative routes, the demonstration of intermediates containing intron C only, or together with A or B is necessary. RNAs from which only intron A has been excised should be 1800 nucleotides long. We indeed observe a weak band of this size, hybridizing with both the intron B and intron C probe, but not with the intron A probe. Intermediates from which only intron B has been excised should be 2850 nucleotides long. Upon hybridization with an intron C probe we observe a faint band (panel C) at the expected position which migrates definitely more slowly than the 2650-nt band observed with the intron B probe (panel B). However the absence of intron B in the 2850-nt intermediate could not be clearly established because of the vicinity of the strong 3200-nt and 2650-nt band. We have also characterized weak bands of 1450 and 1250 nucleotides. The 1450-nucleotide band hybridizes only with the intron C probe, whereas the 1250-nucleotide band hybridizes only with the intron B probe. We conclude that only intron C or intron B is present in the 1450- and 1250-nt band, respectively. All hybridization results are summarized in table 1. Our data support the view that the introns of the primary transcript of the apo-VLDL II gene are excised in a non-obligatory order. The 1600-nt band has been identified as an aberrant transcript terminating inside intron A, and is thus not a processing intermediate [22].

3.3. *Electron microscopy*

As an independent approach to the identification of the primary transcript and processing intermediates of the apo-VLDL II mRNA, total polyadenylated cellular RNA was hybridized under R-loop conditions with clone-12 DNA [6] which contains the entire apo-VLDL II gene and 8.5 kb of 5′- and 7.0 kb of 3′-flanking DNA. Several characteristic structures were observed in the electron microscope (fig.5). As expected, the most abundant R-loop structures were formed by the mature mRNA (panel D). These show two intron loops corresponding to the introns B and C. The absence of the intron A loop is due to branch migration of the short (38 bp) exon 1. Other frequently observed structures contain a continuous region of about 3 kb (panel A). We interpret these to be primary transcripts. In R-loop containing intermediates we recognize RNAs lacking intron C (panel B), and also those lacking both intron B and intron C (panel C). Most of the structures discussed above contain a small stretch of RNA protruding at the end of the hybrid region which we interpret to be the poly(A) tail.

Table 1

Summary of the hybridization signals of the apo-VLDL II intron-specific probes

	Hybridization signal			Introns present		
	A	B	C			
3200	+	+	+	A	B	C
2850			+	(A)		C
2650	+	+	−	A	B	
2300	+	−	−	A		
1800	−	+	+		B	C
1600[a]	+	−	−	A		
1450	−	−	+			C
1250	−	+	−		B	

[a] The 1600-nt RNA is not a splicing intermediate, but an aberrant transcript [22]

The presence of a hybridization signal is indicated as (+), absence as (−). The most likely interpretation, based on hybridization specificity and size, is given

4. DISCUSSION

The identification of the intermediates proves that the splicing of the apo-VLDL II primary transcript does not follow an obligatory pathway. However, some routes are clearly preferred since the concentrations of the various intermediates differ. The most simple kinetic model is one in which each intron is excised with its own rate constant, ir-

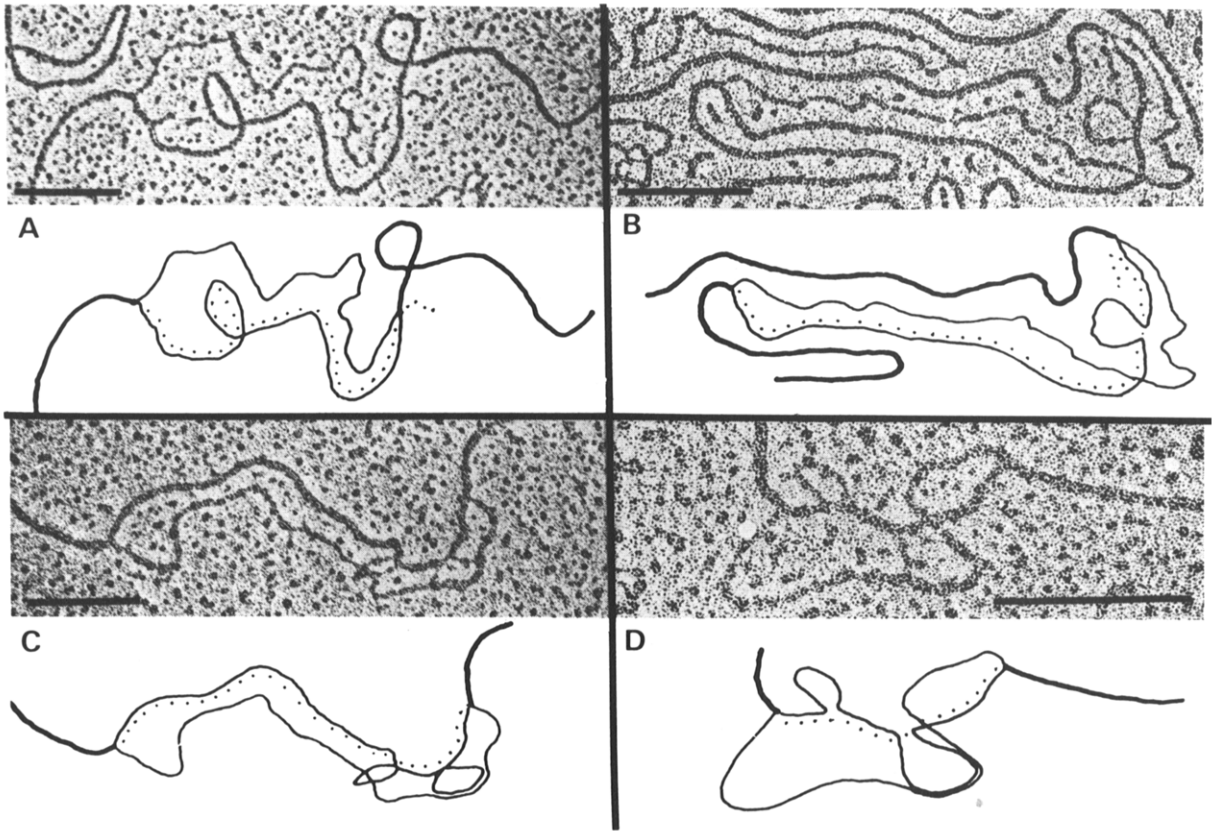

Fig.5. Electron microscopy of R-loop structures formed between genomic DNA in clone 12 and apo-VLDL II RNA. Examples are shown of R-loops formed with the primary transcript (panel A), intermediates lacking intron C (panel B), lacking intron B as well as intron C (panel C), and the mature messenger RNA (panel D). All molecules are oriented with the 5′-end of the RNA to the left. In the drawings double-stranded DNA is represented by thick lines, single-stranded DNA by thin lines, and RNA by dotted lines. The bar is 0.2 μm.

respective of the presence of other introns. In our case, excision of intron C (including the splicing of exon 3 to 4) would then proceed about 3-times as rapidly as the removal of intron B, or A, as can be calculated from the concentrations of the intermediates.

Our results are in agreement with recent studies on intron excision from β- and γ-globin in vitro [23] where a preferred, but not unique pathway was found. At this stage, we cannot infer from the nucleotide sequence which parts determine the rate of intron removal, nor whether the neighboring exons also have an influence. More work will be necessary before we fully understand the intricacies of the splicing process.

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