Asymmetric distribution of pause transfer sequences in apolipoprotein B-100

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Abstract Lipoprotein assembly requires a complex and regulated set of events that includes apolipoprotein B (apoB) translocation across the endoplasmic reticulum (ER) membrane, folding, and association with lipids. Unlike simple secretory proteins which are cotranslationally translocated directly into the ER lumen, nascent apoB contains pause transfer (PT) sequences that direct the transient stopping and subsequent restarting of its translocation, a phenomenon termed translocational pausing. During one particular translocational pause in apoB, the ribosome–membrane junction and ER translocation channel have been shown to be altered in such a way as to expose the nascent polypeptide to the cytosol and direct a change in the proteins neighboring the nascent chain. In this study, we have experimentally identified the location and distribution of the translocational pauses that are present throughout apoB-100. We find that pause transfer sequences are distributed asymmetrically, clustering in three distinct domains: *a*) nine functional PT sequences appear in the amino terminal 20% of apoB, *b*) four more PT sequences occur just before the end of apoB-48, and *c*) an additional ten PT sequences are found between apoB-65–95. These clusters are interrupted by two lipid binding regions of approximately 100 kD each in which no PT sequences occur. The implications of this asymmetric distribution of PT sequences, and their correlation with previously hypothesized structural and functional domains of apoB, are discussed.—**Kivlen, M. H., C. A. Dorsey, V. R. Lingappa, and R. S. Hegde.** Asymmetric distribution of pause transfer sequences in apolipoprotein B-100. *J. Lipid Res.* 1997. **38:** 1149–1162.

Supplementary key words topogenic sequence • lipoprotein biogenesis • translocation • endoplasmic reticulum

Apolipoprotein B (apoB) is a large (~500 kD) hydrophobic protein found in chylomicrons, very low density lipoproteins (VLDL), and low density lipoprotein (LDL) particles. Lipoprotein particle assembly is a complex and regulated set of events during which apoB undergoes both covalent modifications (e.g., glycosylation and disulfide bond formation) and non-covalent modifications (e.g., chain folding and association with lipids) (1, 2). At least some of these events are likely to occur during translocation of apoB across the membrane of the endoplasmic reticulum (ER), when the apoB chain is unfolded and amenable to associations or modifications that would otherwise be sterically constrained later in biogenesis. Indeed, a variety of enzymes that catalyze modifications of proteins act at the time of translocation (3–5).

To explore the possibility that specialized mechanisms were involved in lipoprotein particle assembly, we previously examined the early biogenesis of apoB (6, 7). We found that apoB contains specific sequences, termed pause transfer (PT) sequences, that direct the transient stopping and subsequent restarting of chain translocation into the ER lumen, without significantly affecting nascent chain synthesis. By contrast, simple secretory proteins are cotranslationally translocated directly into the ER lumen, without such transient dissociation of translocation from nascent chain elongation. While other complex secretory and membrane proteins have subsequently been found to contain functional PT sequences (8) (R. S. Hegde and V. R. Lingappa, unpublished results), apoB may be unique in the large number of PT sequences that appear to be involved in its biogenesis (6).

Recently, progress has been made in understanding the molecular events of one translocational pause of apoB (9). During the translocational pause occurring at apoB-6.7, the tight seal between the ribosome and the translocation channel, which normally serves to shield nascent chains from the cytosol, is dramatically and reversibly opened. At the same time there is a coordinate alteration in the proteins with which the nascent chain associates. As a result, macromolecular interactions can occur between the nascent chain and the cytosol that are not possible during translocation of simple secretory

Abbreviations: apoB, apolipoprotein B; ER, endoplasmic reticulum; MTP, microsomal triglyceride transfer protein; PT, pause transfer; PK, proteinase K; VLDL, very low density lipoprotein.

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proteins. These findings argue strongly that, in at least some cases, translocational pausing is an example of cellular regulation of the translocation machinery (10).

In the case of apoB, such regulation seems likely to be related to events of lipoprotein particle formation and secretion or degradation. However, the precise role of this and the other specific PT sequences in apoB biogenesis remains an unsolved problem. It is possible, for example by virtue of differences in flanking sequences, that different PT sequences mediate different types of events in apoB biogenesis. These events may have in common only that they manifest by the available assays as translocational pausing. Thus, only a subset of PT sequences may actually open the ribosome–membrane junction in the manner recently described for the apoB-6.7 PT sequence (9). Furthermore, regardless of the mechanism of action of any particular PT sequence, a variety of consequences relevant to lipoprotein particle assembly or secretion might ensue, or be facilitated, during a translocational pause. Exactly which of several possible consequences actually occurs during a particular translocational pause might depend not only on the particular pause in question but also on factors involved in regulation of lipoprotein particle formation (e.g., levels of substrates, metabolites, hormones).

A first step in understanding the role of translocational pauses in apoB biogenesis would be achieved by exhaustively mapping the distribution of PT sequences throughout the entire length of apoB-100. Here we report a systematic survey designed to identify all of the functional PT sequences in apoB-100 and the correlation of their location to proposed structural and functional domains of apoB. We find 23 distinct PT sequences clustered in three regions of apoB: the N-terminal 20% which has nine distinct PT sequences; the apoB-48 junction, in whose proximity four PT sequences are found; and apoB-65–95 where ten additional PT sequences are located. Conversely, two large regions, apoB-20–44 and apoB-47–67, each encompassing approximately 100 kD or more of the apoB coding region, appear devoid of functional PT sequences. These findings have implications for the relationship of PT sequences to structural features and functional organization of apoB in lipoprotein particles and are discussed below.

EXPERIMENTAL PROCEDURES

Transcriptional, translation, and proteolysis assays

Rabbit reticulocyte lysate and microsomal membranes from either dog pancreas or rat liver were prepared exactly as described previously (11, 12). Transcription using SP6 polymerase, translation, and proteolysis were as described previously (7). All translation reactions were carried out at 25°C for 60 min and proteolysis reactions at 0°C for 30 min using 0.4 mg/ml proteinase K (PK). EDTA treatment, where indicated, was carried out by adding EDTA to 10 mM, and incubating for 10 min at 25°C. In some cases, microsomal membranes were isolated after the translation reactions by centrifugation for 4 min at 50,000 rpm in a TLA100 rotor (Beckman). The membranes were resuspended in 100 mM KCl, 250 mM sucrose, 50 mM HEPES, pH 7.4, 5 mM $MgOAc_2$ before further manipulations. Proteolysis reactions were terminated by the addition of phenylmethylsulfonyl fluoride (PMSF) to 5 mM, incubating at 0°C for 5 min, and transferring the sample to 10 volumes of boiling 0.1 M Tris, 1% SDS. This method of terminating the proteolysis reactions was found to completely inactivate the PK, leaving proteins in the lumen of the ER undigested. This was confirmed using a variety of lumenal proteins as controls, including prolactin, beta lactamase, GRP94, and BiP (data not shown). Samples were analyzed by SDS-PAGE on 15% gels, and the proteins were visualized by autoradiography.

Plasmid constructions

All manipulations with DNA were performed by standard techniques (13). All of the constructions described below are in an SP64 derived vector (Promega) containing the 5′ untranslated region from *Xenopus* globin. The plasmid B15, which contains the coding region for the first 15% of apoB, has been described (6). The nomenclature used below refers to codon 1 as the initiating ATG of apoB. With the exception of the constructs B8-15 and B71-84, all of the constructs were made as follows. A segment of the coding region of apoB was amplified using PCR and ligated into B15 digested with Bsu36I (located at codon 108) and XbaI (located beyond the coding region). The segments (in codons) amplified for each of the clones were: B15–22, 698 to 1067; B20–34, 914 to 1565; B32–44, 1519 to 2128; B40–55, 1861 to 2512; B44–48, 2010 to 2178; B57–71, 2630 to 3271; B81–95, 3705 to 4357; B81–84, 3690 to 3857; B93–97, 4249 to 4433; B96–100, 4394 to 4563. The plasmid B8–15 was generated by digesting B15 with Bsu36I and StuI, treating with Klenow, and recircularizing the plasmid. B71–84 was made by engineering a PCR fragment encoding codons 3247 to 3857 of apoB into B15 digested with PstI (located at codon 88) and XbaI. Fidelity of constructions was confirmed by DNA sequencing across selected coding regions assayed for pausing.

Assignment of PT sequences

As described in the results, SDS-PAGE and autoradiography analysis of translation reaction protease digestion products identifies translocational pauses by three criteria: *i*) protease accessibility in the absence of detergents, *ii*) generation of a discrete transmembrane fragment upon protease digestion, and *iii*) restoration of full protease protection concomitant with loss of the transmembrane fragment, upon EDTA treatment (due to restarting of the paused chain). As the precise point of truncation is known, the approximate location of a PT sequence can be deduced from the size of its shift on SDS-PAGE upon PK digestion. As described in Results, the size of the shift of the full-length product, upon PK digestion in constructions scoring positive for PT sequences by the above assays, was quantified and interpolated to generate an approximate location, plus or minus about ten amino acid residues, for each PT sequence.

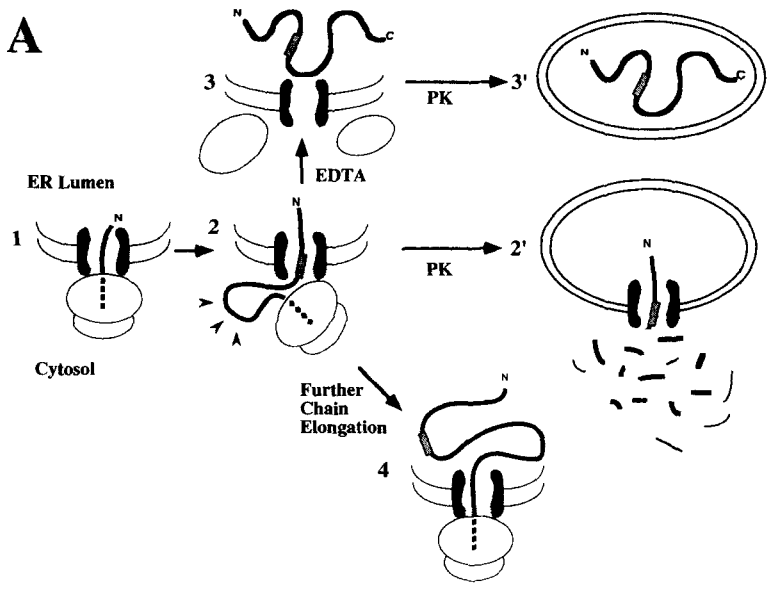
RESULTS

Detection of translocational pauses is an experimental challenge for several reasons. First, like other early events of protein biogenesis (such as targeting to the ER membrane, signal sequence cleavage, and N-linked glycosylation), translocational pausing occurs cotranslationally. That is, it occurs even before synthesis of the substrate is finished. As with other early events of protein biogenesis (14, 15), the overlap between translocational pausing and protein synthesis makes its study in living cells extremely difficult. The study of apoB assembly at the ER in living cells is further impaired by the occurrence of rapid and regulated degradation of a subpopulation of apoB assembly intermediates (16, 17). Cell-free translation systems supplemented with microsomal membranes were developed as a means by which the events of chain translocation could be studied and subjected to biochemical dissection (18). A large body of data supports the notion that events observed in these systems faithfully reflect events occurring in living cells (19–21).

An even more formidable problem for studying translocational pausing, even in cell-free systems is that, by definition, it is a transient event. A protein that pauses early in chain growth may be fully translocated to the ER lumen by the time its synthesis is completed. Thus, at the end of synthesis, the topology of the formerly paused chain is indistinguishable from that of a simple secretory protein that never paused during its translocation. As the precise roles of translocational pausing remain unknown, no simple indirect assay (e.g., of pause-dependent covalent modifications) can, at present, substitute for a direct assay of translocational pausing. Thus, the only recourse for the study of translocational pausing is to "trap" chains in the act.

Precisely this goal was achieved when it was observed that carefully chosen truncations prior to the termination codon, of a PT sequence-containing coding region, would generate a relatively homogeneous nascent chain population engaged in translocational pausing (7). Such functional intermediates can be studied and manipulated much as has been done for various translocation-associated events (7, 22–24). Using this approach, a high fidelity assay for translocational pausing has been developed in which a series of truncated mRNAs are used to examine successive translocation intermediates (see **Fig. 1A**). Three crucial properties distinguish paused from non-paused translocation intermediates. First, the translocationally paused chain is accessible to PK digestion from the cytosolic side under conditions where simple secretory proteins are fully protected by the ribosome–membrane junction from protease digestion. Second, proteolysis of a paused nascent chain from the cytosolic side of the membrane not only diminishes the amount of full length chains, it also generates a discrete lower molecular weight product that represents the domain that is in the ER lumen. Protection of that fragment from proteases is, of course, dependent on membrane integrity. Finally, translocationally paused nascent chain intermediates, but not bona fide integrated transmembrane chains, have been shown to restart translocation after EDTA treatment (7). By assessing the protease accessibility of the nascent chain after EDTA treatment, truly paused nascent chains are distinguished (by their change to full protection from proteases) from integrated nascent chains or artifacts of protease overdigestion (whose proteolytic accessibility is not changed by EDTA treatment). The fidelity of conclusions based on such chain truncation experiments have been substantiated by the study of events in "real time" using antibodies and site-specific proteases as novel probes of translocational pausing (9). In the present study, translocation intermediates generated from any given truncation were scored as translocationally paused only when all three of the above criteria were met.

Also depicted in Fig. 1A is the transient nature of a translocational pause. Specifically, we have observed that an increase in the number of amino acids between the point of translocational pausing and the point of truncation used to detect the pause results in a decrease in the percent of chains that are found in the paused state (R. S. Hegde, unpublished observations). At truncation points of more than approximately 150 amino acids beyond the pause, many chains are no longer



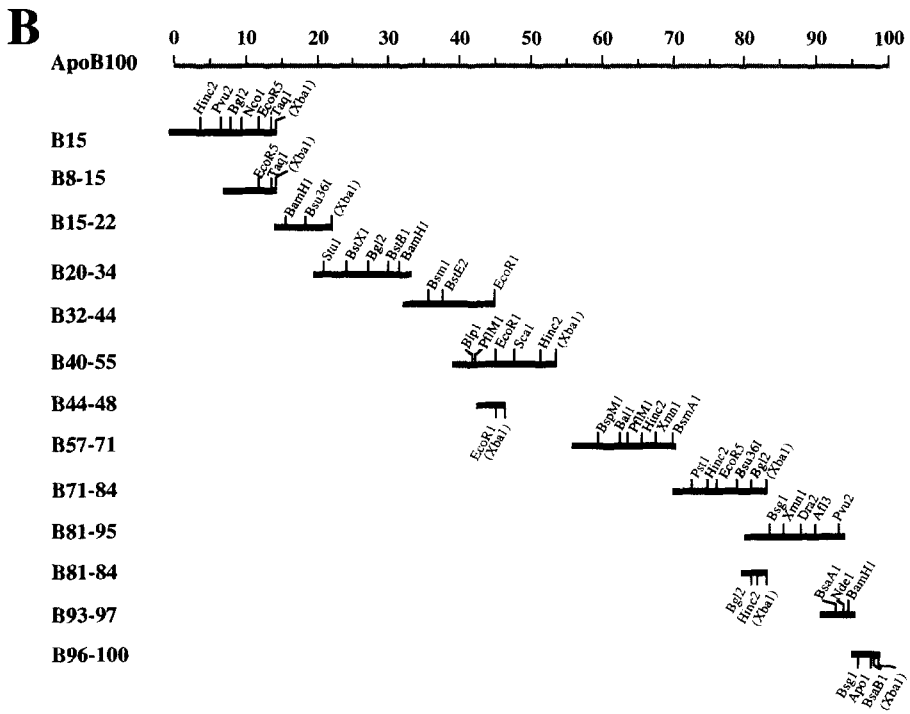


Fig. 1. (A) Demonstration of the use of serially truncated translocation intermediates to detect translocational pauses. In diagrams 1, 2, and 4, translocation intermediates of increasing length are depicted, with diagram 2 representing a translocationally paused chain directed by a discrete PT sequence (shaded box). The paused chain is accessible to proteases from the cytosolic side (arrowheads), which when treated with EDTA (diagram 3), is no longer accessible. A later translocation intermediate (diagram 4) demonstrates that the pause is transient in nature, and also is relieved by further chain growth. 2′ and 3′ show the result of PK digestion of the chains described in 2 and 3, respectively. PK digestion of 1 or 4 would show full protection from PK, comparable to that described for 3, in 3′. (B) Scale diagrams of the constructs and truncation sites used in this study. Each construct consists of the first 108 codons of apoB followed by the domain indicated by the black bars (see Experimental Procedures). Restriction sites used to scan for translocational pauses are indicated, with parentheses representing engineered sites not found in full-length apoB.

paused (7, 9). It appears that with this additional chain growth, the transient pause has largely restarted translocation. Thus, the choice of truncation points determines to some extent the amount of pausing detected, with distal truncation points revealing relatively fewer paused chains. These observations documenting the transient nature of a translocational pause partly explain the variable proportion of chains that appear not to be paused in the assays used in this and previous studies.

To survey the entire length of apoB-100 for translocational pausing, nascent chain intermediates in the process of translocating every domain of apoB need to be examined. Previously we have noted that no translocational pauses are apparent prior to apoB-2.3 (6, 7). PT sequences can be mapped by serial truncation of the full length apoB coding region through about apoB-15. Beyond this length (approximately 70 kD), a shift down in size of the truncated nascent chain upon protease-digestion is no longer a reliably sensitive assay for occurrence of a translocational pause or for localization of the implicated PT sequence. These problems were

solved by first generating a family of constructs in which defined regions of apoB were engineered (see Experimental Procedures) directly downstream of the amino terminal 108 amino acid residues of apoB (i.e., before the first detectable PT sequence, see Fig. 1B). The coding regions were then truncated at sites that are an average of approximately 90 codons apart (2% lengths of apoB) and these truncations were used to scan for translocational pauses. The choice of this interval for the truncations was based on an observation from the study of numerous pauses, namely that translocational pauses can generally be detected easily even when the point of truncation is 100 to 150 codons beyond the PT sequence (R. S. Hegde, unpublished results). These experiments allowed translocational pausing to be determined directly and without ambiguity.

It should be noted that our approach to the detection of translocational pauses in apoB-100 by using a series of constructs encoding different regions of the molecule makes the assumption that to some extent, pauses can be studied in the absence of the remainder of apoB-100. This assumption is reasonable given our previous findings that pause transfer sequences are modular and are not affected significantly by the sequence context in which they are found. This was demonstrated by showing that several pause transfer sequences, when moved into otherwise non-pausing proteins, confer predictable translocational pausing behavior (7–9). Thus, for the purposes of identification, it is likely that most, if not all, pauses will be detectable in the approach used in this study. However, it remains possible that long-range intramolecular interactions that are not recreated when specified regions of the apoB-100 molecule are studied in isolation play a yet undetermined role in some cases of translocational pausing.

Figure 2 presents the raw SDS-PAGE data for a typical experiment analyzing a sequence of apoB near the B-48 junction. In this experiment, microsomal membranes derived from dog pancreas (panel A) were compared to those from rat liver (panel B). Translation of truncated transcription products in the presence of microsomes from either source generated a translocational pause at precisely the same point. Moreover, this pause behaved identically in both membranes by all three criteria for translocational pausing as described above. Analysis of several other truncation points in multiple regions of apoB similarly demonstrated that microsomal membranes from dog pancreas and rat liver were comparable with respect to whether a translocational pause was detected or not (data not shown). Thus, it appears that within the limits of our current assays for translocational pausing, little or no difference could be ascertained between these two tissue sources. Whether the consequences of translocational pausing are dramatically different in the different tissues remains to be seen (see Discussion).

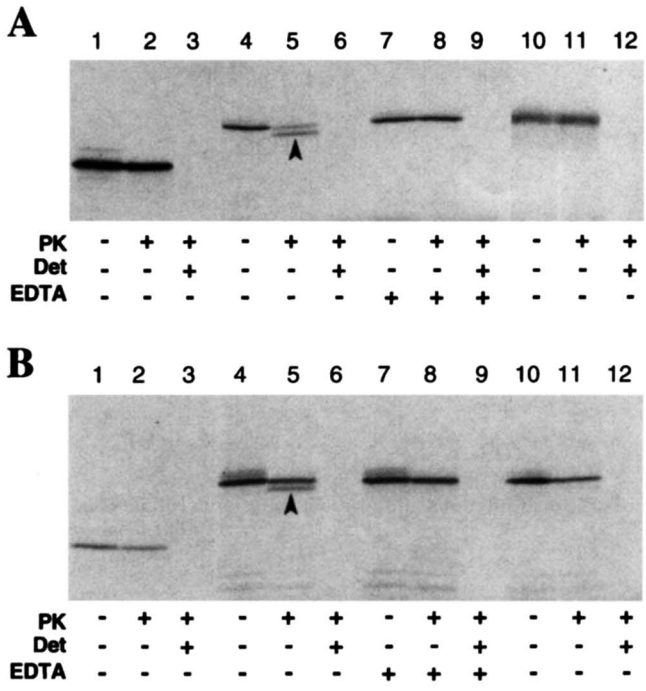


Fig. 2. Pausing during translocation across pancreas- and liver-derived microsomes. The B44–48 construct (see Fig. 1B) was truncated at either EcoR1 (lanes 1–3), Xba1 (lanes 4–9), or left untruncated (lanes 10–12). The DNA was then used in an in vitro transcription and translation reaction using reticulocyte lysate and microsomal membranes from either dog pancreas (panel A) or rat liver (panel B). After translation for 60 min, one aliquot of the translation reaction was treated with 10 mM EDTA for 10 min (lanes 7–9). The microsomal membranes were then isolated by centrifugation, resuspended, and either left untreated (lanes 1, 4, 7, 10), or subjected to digestion for 30 min at 0°C with 0.4 mg/ml proteinase K (PK) in the absence (lanes 2, 5, 8, 11) or presence (lanes 3, 6, 9, 12) of 0.5% Triton X-100 (Det). The reactions were terminated with PMSF as described in the Experimental Procedures, and the samples were analyzed by SDS-PAGE and autoradiography. The arrowhead in lane 5 points to the fragment generated by digestion of the sample with PK, and is indicative of a translocationally paused chain (see Text for details).

We next performed a thorough analysis for translocational pausing in apoB-100 using each of the clones described in Fig. 1B. For this analysis, pancreatic microsomal membranes were used as they are more readily available, have consistently more translocation activity, and appeared to be indistinguishable from rat liver microsomal membranes in mediating translocational pausing. The clones encoding different regions of apoB were truncated as indicated (see Fig. 1B), used to generate translocation intermediates by translation in the presence of pancreatic ER-derived microsomal membranes, and analyzed by PK digestion as for Fig. 2. In each case that a putative translocational pause was initially detected by shift in size with PK digestion, it was corroborated by the ability of prior treatment with

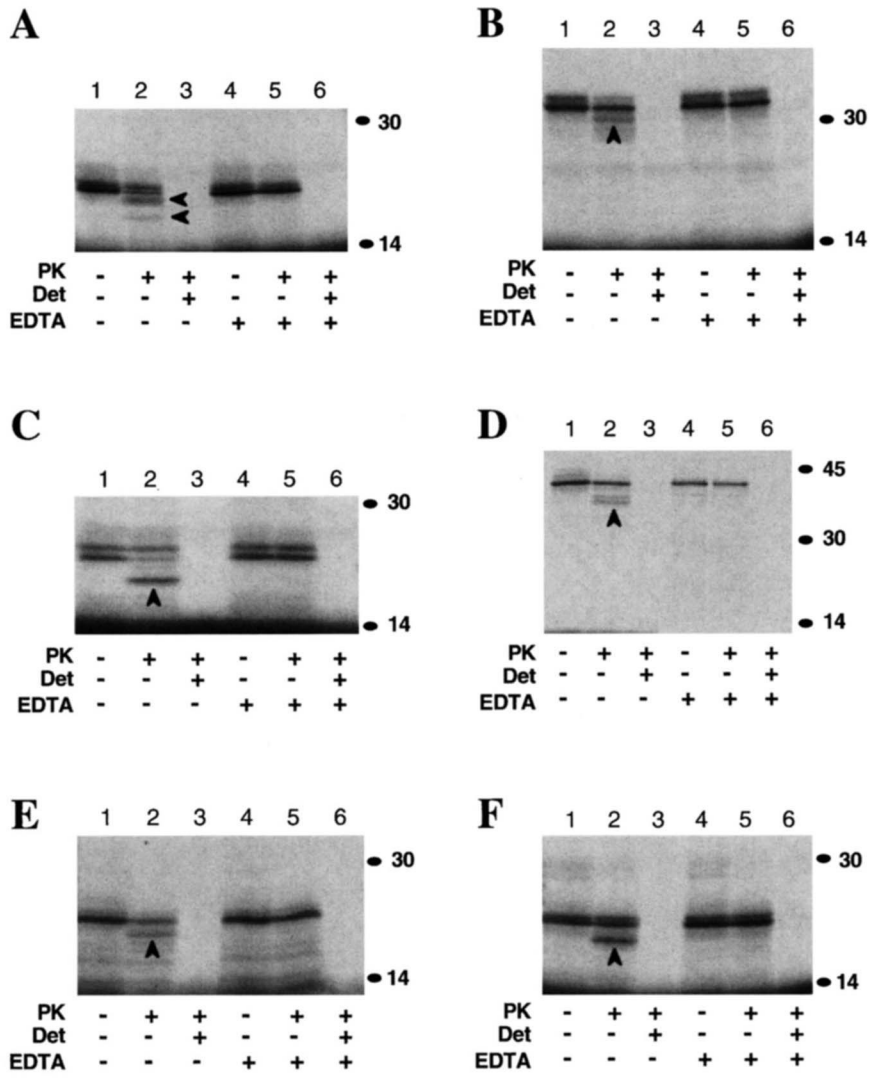


Fig. 3. Examples of translocational pausing in apoB. Selected truncated constructions of apoB were transcribed, translated in the presence of dog pancreatic rough microsomal membranes, and assayed for translocational pausing essentially as described in Fig. 2A. Treatments with PK, detergent, and EDTA were as in Fig. 2, and are indicated below the appropriate lanes. The arrowhead in each panel indicates the fragment generated by digestion of the sample with PK and is indicative of a translocationally paused chain (see text for details). The constructions shown are: (A) B15/Hinc2; (B) B15/Pvu2; (C) B40–55/PflM1; (D) B32–44/BstE2; (E) B71–84/Pst1; (F) B81–84/Hinc2.

EDTA to abolish both the shift in size and the loss of protection from PK digestion. Shown in **Fig. 3** are six independent examples of translocational pausing in apoB. These representative examples display pause transfers from all three regions of apoB in which they were found. By contrast, other regions of apoB did not display translocational pausing. Four such examples, in the B20–34 construct, are shown in **Fig. 4.**

Figure 5 graphically displays the relative positions of the 49 truncation points within the entire apoB-100 coding region, to scale, and indicates the accessibility to PK before and after EDTA treatment. Based on these data, 23 specific and distinct translocational pauses were assigned, each reflecting the action of a different PT sequence. While an examination of the SDS-PAGE data (in Figs. 2–4) allow a clear distinction to be made between truncation points that reveal a translocational pause and those that do not, the quantitative analysis in Fig. 5 reveals that some variability exists in the protease protection both before and after EDTA treatment.

Such variability appears to be due to two inefficiencies inherent in the assays used. First, the percent of chains that remain paused during the time period of analysis is variable and is not only affected by the site

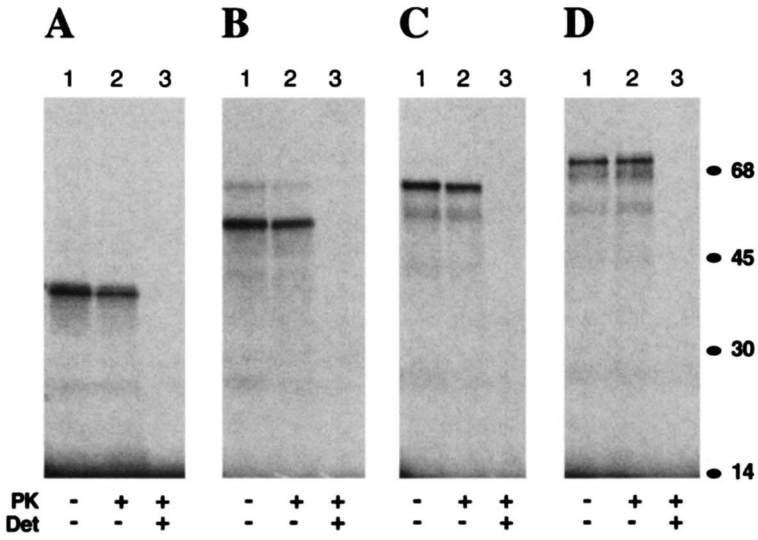


Fig. 4. Lack of translocational pauses in apoB-20–34. Selected truncated constructions of apoB-20–34 were transcribed, translated in the presence of dog pancreatic rough microsomal membranes, and assayed for translocational pausing essentially as described in Fig. 2A. Treatments with PK in the absence or presence of detergent were as in Fig. 2, and are indicated below the appropriate lanes. Analysis after EDTA treatment was not performed because a pause was not detected in the absence of EDTA. The constructions shown are: (A) B20–34/BstX1; (B) B20–34/Bg12; (C) B20–34/BstB1; (D) B20–34/BamH1.

of truncation (as discussed above), but also by a slow rate of spontaneous release of truncated nascent chain intermediates from the ribosome (9). Despite these inefficiencies that cause variation in the amount of nascent chain accessible to PK (as plotted in Fig. 5), the SDS-PAGE always shows a discrete proteolytic fragment in the case of a translocationally paused chain (as in Figs. 2 and 3), but not for non-paused chains (as in Fig. 4). Second, following EDTA treatment, nascent chain intermediates appear to be released from the transloca-

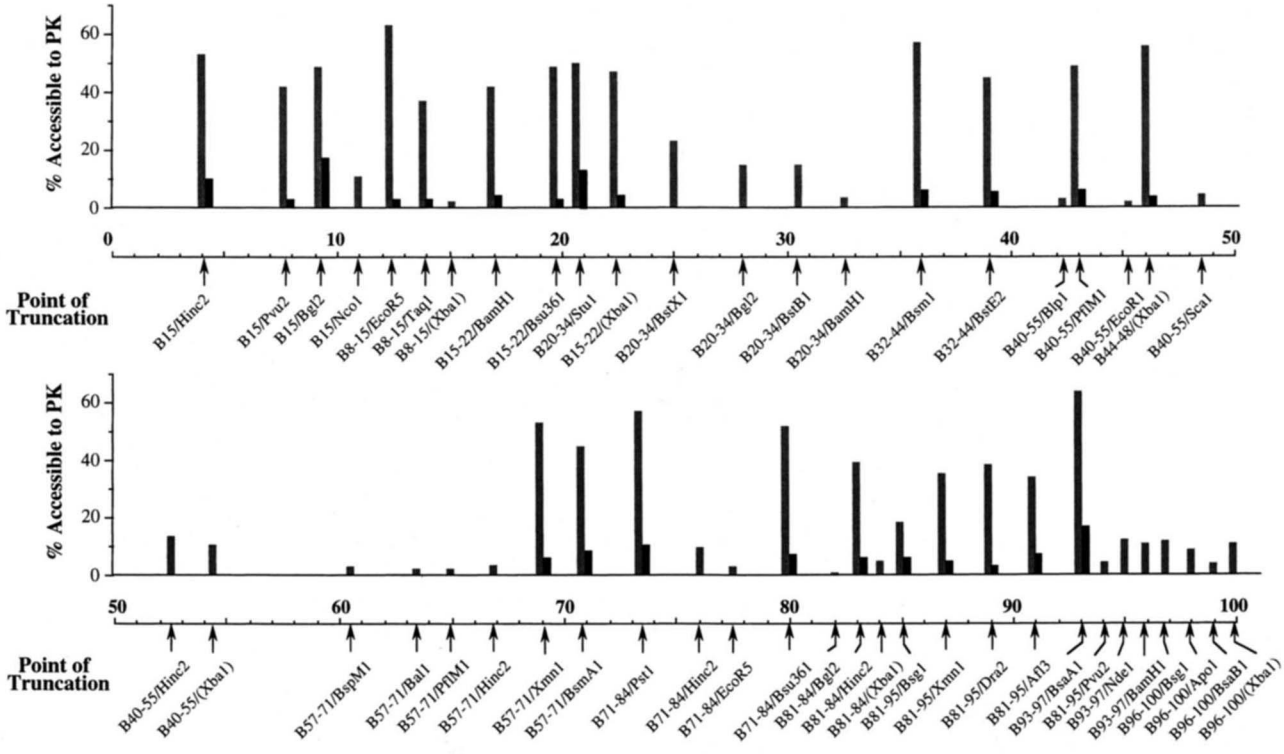


Fig. 5. Quantitative analysis of apoB for translocational pausing. The constructs diagrammed in Fig. 1B were truncated at the various sites (with their relative positions indicated by arrows on the abscissa) and used to assay translocational pausing exactly as in Fig. 2A. The percentage of the translocation intermediates that was found to be accessible to PK digestion was quantitated by densitometry and is graphed (gray bars). In each case where a potential pause was detected, the percent of chains accessible to PK after EDTA treatment was also determined and is indicated by the accompanying black bars.

TABLE 1. Analysis of apoB-100 subclones for translocational pausing

Plasmid/Site	Amino Acid in B-100	% Accessible to Protease	% Accessible after EDTA	Pause Detected
B15/Hinc2	209	52	10	+
B15/Pvu2	342	41	2	+
B15/Bgl2	401	48	18	+
B15/Nco1	471	10	—	
B8–15/EcoR5	580	62	2	+
B8–15/Taq1	661	36	2	+
B8–15/(Xba1)	692	1	—	
B15–22/BamH1	781	41	4	+
B15–22/Bsu36I	950	48	2	+
B20–34/Stu1	979	49	14	+
B15–22/(Xba1)	1036	46	4	+
B20–34/BstX1	1137	22	—	
B20–34/Bgl2	1281	14	—	
B20–34/BstB1	1405	14	—	
B20–34/BamH1	1480	2	—	
B32–44/Bsm1	1646	56	6	+
B32–44/BstE2	1797	44	5	+
B40–55/Blp1	1948	2	—	
B40–55/PflM1	1968	48	6	+
B40–55/EcoR1	2099	1	—	
B44–48/(Xba1)	2151	55	2	+
B40–55/Sca1	2217	3	—	
B40–55/Hinc2	2389	12	—	
B40–55/(Xba1)	2483	9	—	
B57–71/BspM1	2756	2	—	
B57–71/Ba11	2890	1	—	
B57–71/PflM1	2946	1	—	
B57–71/Hinc2	3037	2	—	
B57–71/Xmn1	3121	52	6	+
B57–71/BsmA1	3235	44	9	+
B71–84/Pst1	3351	56	11	+
B71–84/Hinc2	3450	8	—	
B71–84/EcoR5	3513	2	—	
B71–84/Bsu36I	3641	51	7	+
B81–84/Bgl2	3727	0	—	
B81–84/Hinc2	3770	38	5	+
B81–84/(Xba1)	3830	3	—	
B81–95/Bsg1	3852	17	5	+
B81–95/Xmn1	3939	34	4	+
B81–95/Dra2	4052	37	2	+
B81–95/Afl3	4143	33	7	+
B93–97/BsaA1	4269	62	17	+
B81–95/Pvu2	4289	3	—	
B93–97/Nde1	4315	11	—	
B93–97/BamH1	4349	10	—	
B96–100/Bsg1	4411	11	—	
B96–100/Apo1	4484	8	—	
B96–100/BsaB1	4506	3	—	
B96–100/(Xba1)	4536	10	—	

The amino acid number within mature apoB-100 for each truncation is indicated. A "+" indicates that all three criteria (see text) for the presence of a translocational pause were met. Only truncation points that demonstrated a putative pause were analyzed after EDTA treatment.

tionally paused state and can subsequently complete translocation into the lumen. However, this step is sometimes incomplete, resulting in a small percent of chains "falling out" of the translocation channel into the cytosol rather than translocating into the lumen. These chains score as accessible to PK, but do not generate a discrete proteolytic fragment indicative of a paused nascent chain. Thus, examination of the SDS-PAGE data shows clearly that the pause has been released (although some of the chains fail to complete translocation into the lumen), even though a quantitative analysis reveals some chains accessible to protease (as in Fig. 5). For these reasons, the SDS-PAGE data is used to unambiguously assign sites of translocational pausing, while the quantitative analysis is presented as an objective corroboration of such assignments.

The tabulated data of the entire analysis, indicating both the amino acid of apoB-100 at which truncation was carried out, and the assessment of whether or not a pause was detected, is shown in **Table 1.** The PT sequences were each named B'x, where x is the position, in percent, within apoB-100. The position of each PT sequence was estimated by the point of truncation, corrected for the length of chain that was accessible to protease. The resulting map (**Fig. 6A**) indicates the point in chain growth at which translocation actually stopped, presumably at the PT sequence.

DISCUSSION

ApoB, the protein in which translocational pausing was first observed, is also the polypeptide with the greatest number of translocational pauses identified to date. However, until the present study, the precise number and distribution of translocational pauses in apoB-100 has been a matter of speculation (6, 7). Progress in understanding the role of PT sequences in lipoprotein particle assembly has been impeded by lack of a map of their number, location, and distribution in apoB-100. Here we present the results of a systematic analysis of essentially all of apoB-100, using a stringent three-parameter assay for translocational pausing, that reveals 23 translocational pauses clustered in three regions of apoB. The remarkable number and distribution of translocational pauses observed in apoB-100 may provide important clues as to their role in lipoprotein particle assembly.

How are PT sequences defined?

In the present study, the most stringent criteria currently available were used to score for the presence of a PT sequence. In order to be designated as translocationally paused, *i*) the chain had to be accessible to PK digestion from the cytosol under conditions where simple secretory proteins are fully protected from digestion; *ii*) PK digestion had to generate a discrete lower molecular weight product, itself fully protected from further PK digestion, as long as membrane integrity is

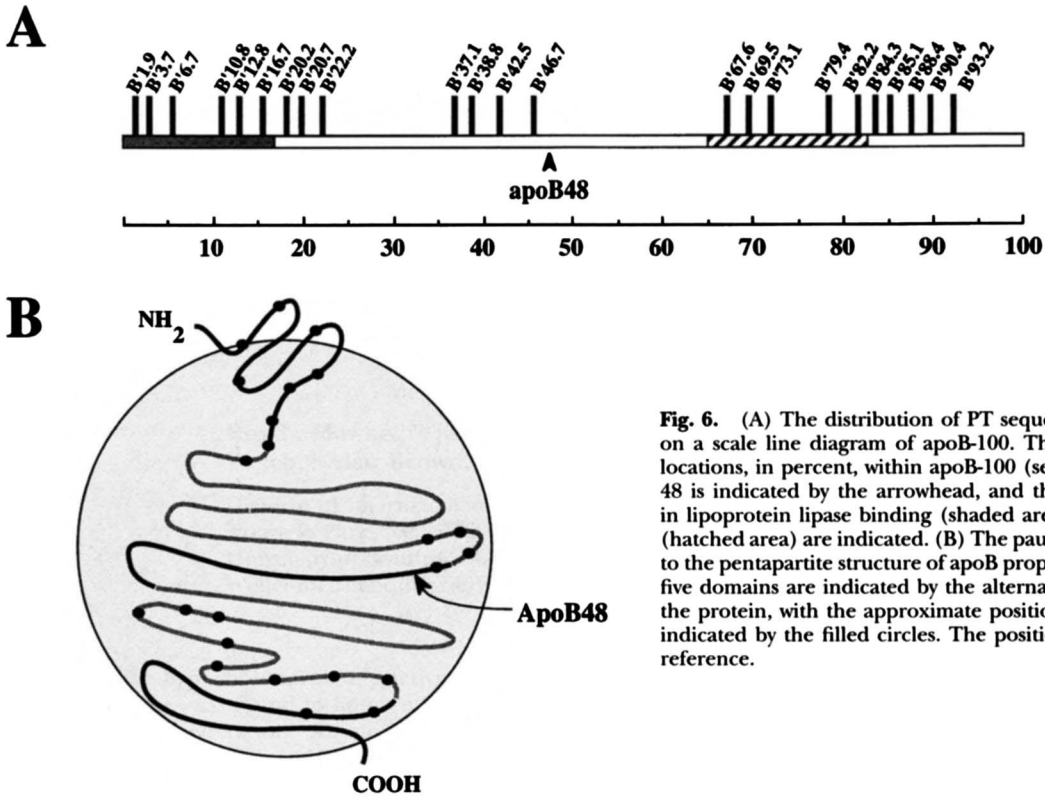


Fig. 6. (A) The distribution of PT sequences (black bars) is indicated on a scale line diagram of apoB-100. They are named based on their locations, in percent, within apoB-100 (see text). The position of apoB-48 is indicated by the arrowhead, and the regions of apoB implicated in lipoprotein lipase binding (shaded area) and LDL receptor binding (hatched area) are indicated. (B) The pauses are diagrammed in relation to the pentapartite structure of apoB proposed by Segrest et al. (44). The five domains are indicated by the alternating black and gray regions of the protein, with the approximate positions of the PT sequences being indicated by the filled circles. The position of apoB-48 is indicated for reference.

maintained; and *iii*) EDTA treatment had to abolish the appearance of the lower molecular weight fragment, as well as the digestion of the full length product, upon subsequent PK treatment. Given the stringent criteria applied, we doubt the presence of false positives among the PT sequences identified here. Likewise, while it is formally possible that some PT sequences were missed, it should be noted that none of the truncation sites that did not score as bona fide PT sequences were judged to be ambiguous in terms of fulfilling only one or two rather than all three of the operational criteria by which translocational pausing was scored. We cannot, however, rule out the possibility, for example, of multiple overlapping PT sequences in those regions in which PT sequence clusters were observed, as the limit of resolution of this analysis is approximately 20 codons. Thus, 23 may ultimately prove to be a lower estimate for the number of PT sequences present in apoB-100, and may need to be revised upward somewhat, on higher resolution analysis. Nevertheless, the markedly asymmetric distribution of PT sequences described here would still hold true regardless, as this feature would not be altered by additional PT sequences found in close proximity to those currently identified. This asymmetric distribution suggests some possible functional roles and sets the stage for future testing of hypotheses that relate individual translocational pauses to specific features of lipoprotein particle structure and function.

Previously, sequence homology based on a small number of putative PT sequences in several proteins suggested that translocational pausing may be governed by a consensus sequence (7). While the present study does not rule out this possibility, we believe that the existing information is insufficient to generate a meaningful consensus sequence (e.g., to use in searching databases, etc.). Of the translocational pause events identified in apoB-100, approximately half appear to occur adjacent to regions that fit the proposed consensus while the remainder do not. Until additional information allows more effective consensus sequence parameters to be determined, we conclude that PT sequences must be defined functionally and determined experimentally, e.g., by criteria such as those applied in the present study. The lack of a consensus sequence for translocational pausing is not in itself worrisome as it is consistent with the experience from study of other topogenic sequences. For example, signal sequences and stop transfer sequences, for which, at least in eukaryotes, no simple consensus sequences has ever been derived, function through the recognition of receptor proteins (25–27). Furthermore, molecular chaperones, which must be able to recognize and distinguish un-

folded proteins or domains from mature, fully folded proteins, appear to do so without recognition of any clear consensus sequences (28, 29). Whether receptor proteins or chaperones are recognizing general features of the apoB chain or discrete sequences to mediate specific aspects of the translocational pausing phenomenon remains to be determined.

Role of translation in translocational pausing

The data presented in this and previous studies (6–9) indicate that apoB is translocated into the ER lumen discontinuously, pausing at multiple discrete points. Because translocation across the mammalian ER membrane is coupled to translation, the molecular basis of translocational pausing has been an issue of some debate. It has been suggested that translational (or ribosomal) pausing accounts for the discontinuous translocation of certain domains of apoB (30). In this model, a slowing down of translation at certain points (presumably at points which we have designated as PT sequences) results in the obligatory lack of translocation. Subsequent resumption of translation, according to their model, is now artifactually uncoupled from translocation, thereby rendering the newly synthesized domain accessible to proteases in the cytosol. Several observations are cited by Pease et al. (30) as being supportive of this notion: *i*) ribosomal pausing is observed at a point (B6.7) where translocation of apoB into the ER lumen is transiently stopped, *ii*) ribosomal pausing is observed at the same point in the absence of microsomal membranes, and *iii*) translocational (and concurrent ribosomal) pausing occurs in a protein not normally translocated across the ER membrane.

Although their studies document the presence of ribosomal pausing at apoB-6.7 and in non-translocated proteins, the conclusion that this is the sole basis for translocational pausing is not justified. The observation that a discrete domain of apoB is protected from protease while the remainder is accessible on the cytosolic side necessarily indicates that the protected domain translocated into the lumen, while the remainder did not. Even if a ribosomal pause had occurred at the point where translocation stopped, such a pause in translation has clearly been alleviated as evidenced by the completion of protein synthesis. By contrast, translocation into the lumen has not yet resumed, as demonstrated by the accessibility of a portion of apoB to cytosolic protease. Thus, although a pause in translation may have resulted in an obligatory pause in translocation, an additional brake in the membrane appears to prevent further translocation for a period of time even after translation resumes. This pause is not permanent, as continued chain synthesis is sufficient to eventually release the brake to translocation and allow the entire chain to be fully protected from protease. It is on this basis that we conclude that translocational pausing is not simply a trivial consequence of ribosomal pausing, and involves events and machinery in the membrane that are able to modulate the duration of a pause. The ultimate proof of this hypothesis will be provided by the demonstration that pausing does not occur in membranes lacking in these putative components.

Pease et al. (30) have further questioned the relevance of translocational pausing based on the finding that pausing occurs when certain cytosolic proteins are made to translocate across the ER membrane. While these observations may be noteworthy, they have no bearing on the existence or possible role of translocational pausing in apoB. To illustrate, an analogy can be drawn between pausing and glycosylation, another co-translocational event occurring at the ER membrane. For example, certain cytosolic proteins can incidentally bear consensus sites for glycosylation. While such sites may even be used for glycosylation if these proteins were to be redirected to the ER lumen, it would be erroneous to conclude from such a result that glycosylation must therefore be an artifact and is unrelated to secretory protein biogenesis. Similarly, we feel it is inappropriate to draw conclusions about the role of pausing in apoB based on the results of pausing assays performed on cytosolic proteins. Thus, while the results of Pease et al. (30) raise the interesting possibility that changes in translation rate may accompany some of the events associated with translocational pausing, their data neither discount the presence of translocational pausing nor can be used to explain the mechanism by which translocational pausing occurs.

Relevance of translocational pausing to apoB biogenesis

One of the most vexing features of the study of apoB biogenesis has been trying to determine why nascent apoB undergoes translocational pausing. Circumstantial evidence suggests that pausing may be a common step in directing a wide variety of post-translocational events, including non-covalent interactions (e.g., chain folding) which are note easily assayed. What evidence then suggests that translocational pausing is a physiologically relevant step in apoB biogenesis? First, translocational pausing is not an event that occurs either at random or as a simple function of parameters such as chain length: it is sequence specific (7–9). Second, recent studies demonstrate that the mechanism of translocational pausing involves specific alterations in the translocational machinery that can be detected in "real time". Thus, translocational pausing is not an epiphenomenon of truncation or other experimental manipulations that have been used to isolate or magnify these events of apoB biogenesis (9).

The present study adds to the conclusion that translo-

cational pausing is likely to be relevant to apoB biogenesis in two respects. First, identical transmembrane protease digestion patterns were observed for translocation of nascent apoB across ER-derived microsomal membranes from both dog pancreas and rat liver. These results rule out the possibility that pausing of nascent apoB observed in microsomal vesicles from dog pancreas is an artifact (e.g., due to the simple lack of a liver-specific gene product such as microsomal triglyceride transfer protein). Second, translocational pauses were found asymmetrically distributed within the apoB coding region, with clusters of pauses occurring in regions of apoB that are of particular interest for lipoprotein particle formation (see blow). This observation suggests a relevance of PT sequences to formation of lipoprotein structure. Experiments must now be designed to test this hypothesis.

Translocational pausing has been viewed as a mechanism by which particular regions of the nascent chain can be isolated from the general events of translocation across the ER membrane (9). Perhaps this serves to effect covalent or non-covalent modifications or to confer aspects of regulation that do not occur in simple secretory proteins that lack PT sequences. In at least one case, translocational pausing has been shown to provide a domain of the nascent chain access to an otherwise restricted compartment, the cytosol (9). However, the precise consequence of exposure to the cytosol in that case remains unknown. In other cases, translocational pausing may also allow rearrangement of proteins in the proximity of the nascent chain, allowing a single translocation channel-associated protein (such as protein disulfide isomerase or calreticulin, see refs. 31–33) to serve multiple functions. At present it is not possible to determine how many of the 23 PT sequences identified in apoB-100 function to open the ribosome–membrane junction or whether other apoB PT sequences are involved in completely different functions. Finally, it is possible that at least in some cases, translocational pausing might simply increase the efficiency with which a particular modificational enzyme is able to find and interact with its nascent substrate, or serve to direct chain folding by a pathway that would otherwise not be kinetically favored. In these possibilities, determining the role of translocational pausing will likely prove particularly challenging. Regardless of the precise consequence(s) of any particular translocational pause for the nascent chain, recent studies examining the translocational machinery, during a translocational pause and subsequent to restarting, strongly suggest that translocational pausing is a means by which protein associations during translocation can be regulated (9).

Examination of the topology that apoB achieves in various cultured cell systems has yielded variable results. Some groups have found apoB to be located entirely in the lumen of the ER and Golgi (30, 34) while other studies demonstrated large amounts of apoB to be present as a transmembrane molecule with domains accessible to the cytoplasm (15–17, 35–37). Although translocational pausing can, in some instances, serve to expose a secretory protein to the cytosolic environment, the relationship between these transient events and the observation of cytosolically exposed apoB in cells is unclear. It is conceivable that pausing can be modulated to vary the length of time that certain domains of apoB are exposed to the cytosol such that, under some circumstances, apoB can be transmembrane, while at other times it is entirely in the lumen. The testing of this and related hypotheses will depend on the ability to modulate pausing, either by mutations within apoB or by alteration of the proposed ER machinery involved in pausing. Once pausing can be manipulated, the subsequent effects on apoB-100 topology, secretion, or degradation can be assessed. The current studies mapping the location of the pauses should be an important step in achieving this goal.

Translocational pausing and liver-specific events

It is particularly interesting that we have thus far observed no difference in the translocational pausing behavior of apoB between dog pancreas and rat liver microsomal membranes. This indicates that the actual event of a translocational pause may not be liver specific or even apoB specific. This notion is supported by the observation that other proteins, some of which are not expressed in a tissue-specific manner (such as GRP94), also undergo translocational pausing (R. S. Hegde and V. R. Lingappa, unpublished results). However, given that the consequence of a pause is to expose the nascent chain to a different cellular environment (such as cytosolic proteins or different membrane proteins), the events immediately following a translocational pause could very well be substrate- and tissue-specific. For example, it might be that in the case of apoB, certain translocational pauses allow interactions with liver-specific cytosolic factors. Such interactions would not necessarily be a prerequisite for a pause, but might have significant consequences for lipoprotein biogenesis. Thus, while our current studies have enabled us to characterize and map the translocational pauses in apoB-100, the substrate- and tissue-specific consequences of these events remain to be determined. Future studies using modified cell-free translation and translocation systems containing liver-specific components may be useful in addressing these questions.

Examination of apoB secretion from liver-derived as well as non-liver cells has implicated liver-specific gene product(s) in lipoprotein formation. Specifically, the microsomal triglyceride transfer protein (MTP), defective in abetalipoproteinemia, has been shown to be

required for the efficient secretion of apoB (38–40). Furthermore, it has been suggested recently that the MTP-dependent defect in apoB secretion lies at the level of translocation across the ER membrane. Evidence for this hypothesis comes from the observation that in MTP-deficient cells, apoB is found to be only partially translocated into the ER with large domains accessible to the cytosol (35–37, 41). By contrast, other groups have failed to detect cytosolically exposed domains of apoB in non-hepatic cells, arguing against a role for MTP in the translocation process (34). Our studies indicate that MTP, in addition to not being required for translocational pausing, is also not required for efficient translocation of apoB-15 into the ER lumen. Furthermore, other regions of the apoB molecule (see Fig. 1B) also appear to translocate efficiently into pancreas-derived microsomal membranes that lack MTP (M. H. Kivlen, R. S. Hegde, and V. R. Lingappa, unpublished observations).

While these various results appear to contradict each other, several possible explanations exist. For example, the cytosolically exposed population of apoB that has been observed by some groups (and referred to as 'incompletely translocated') may actually be representative of apoB that has been partially reverse-translocated from the ER lumen prior to degradation. This explanation is plausible given the existence of such a pathway of reverse translocation and subsequent degradation by the proteosome (42, 43). Further support comes from the observation that an inhibitor of the proteosome (ALLN) inhibits apoB degradation, with a concomitant increase in cytosolically exposed apoB (35). Thus, it may be that apoB indeed translocates quite efficiently under most circumstances, but can later be partially reverse-translocated, giving the appearance of incomplete translocation. Differences in the extent of such reverse-translocation or degradation, possibly modulated indirectly by MTP, may account for the discrepant observations concerning the topology of apoB.

Implications of PT sequence distribution in apoB-100

A line diagram of apoB-100, annotated with the positions of PT sequences, reveals a striking asymmetry in their distribution (Fig. 6A). Furthermore, data from a wide variety of sources, both experimental and theoretical, have led to the conclusion that apoB contains multiple functional domains (44–47). The correlation of the asymmetrical distribution of PT sequences with the various functional domains (Fig. 6) raises potentially significant and testable roles for translocational pausing in lipoprotein biogenesis.

The first cluster of PT sequences in apoB occurs in the amino terminal 20% of the protein. This region, which is particularly rich in disulfide bonds, displays distinct functional domains that may serve accessory functions such as lipoprotein lipase binding (45) and phospholipid binding (46). The second cluster of PT sequences occurs just prior to the junction of apoB-48. ApoB-48 is the shortened form of apoB that is found on chylomicrons made in the intestine, but not in VLDL made in the liver or on LDL derived from VLDLs, where only full-length apoB-100 is found in humans. Location of this PT sequence cluster is consistent with a role in facilitation of (currently unknown) molecular events associated with biogenesis of the region of apoB distal to B-48. The third cluster of PT sequences may, in fact, represent two contiguous clusters. The front half of the third PT sequence cluster overlaps with a domain implicated as being the LDL receptor binding domain (47). The back half occurs in a carboxy-terminal lipid binding domain (44). Equally striking as the clustering of the PT sequences are the regions between the first and second cluster, and between the second and third PT sequence cluster. These large domains of apoB, believed to be involved in lipid binding (44), appear to be entirely devoid of PT sequences. This raises the possibility that one may be able to disrupt proper lipid loading onto apoB by disrupting the PT sequences that precede them.

With a detailed map of PT sequences now in hand, it should be possible to test specific hypotheses involving possible roles for PT sequences in apoB biogenesis. Previous studies suggest that it should be possible to generate point mutants in which PT sequence function is abolished (48), thereby minimizing change in primary amino acid sequence while profoundly affecting whatever roles are played by translocational pausing in apoB biogenesis and lipoprotein particle assembly.

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