16. For the regressions on geographic ranges of Cretaceous molluscan species pairs, the member of each pair with the stratigraphically lower (that is, geologically older) first occurrence was assigned to the horizontal axis (independent variable), a relatively dependable procedure for taxa with a good fossil record [C. R. C. Paul, *Syst. Assoc. Spec. Vol.* **21**, 75 (1982); *Palaeont. Assoc. Spec. Paper 33* (1985)]; if both members of the pair first occurred in the same 2-m.y. interval, the species whose name came first alphabetically was assigned to the horizontal axis. Linear regression coefficients were statistically indistinguishable from the product moment correlation coefficients in Table 1, presumably reflecting nearly identical variances of putative sister species or ancestral-descendant pairs.
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Apolipoprotein B-48 Is the Product of a Messenger RNA with an Organ-Specific In-Frame Stop Codon

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The primary structure of human apolipoprotein (apo) B-48 has been deduced and shown by a combination of DNA excess hybridization, sequencing of tryptic peptides, cloned complementary DNAs, and intestinal messenger RNAs (mRNAs) to be the product of an intestinal mRNA with an in-frame UAA stop codon resulting from a C to U change in the codon CAA encoding Gln^{2153} in apoB-100 mRNA. The carboxyl-terminal Ile^{2152} of apoB-48 purified from chylous ascites fluid has apparently been cleaved from the initial translation product, leaving Met^{2151} as the new carboxyl-terminus. These data indicate that ~85% of the intestinal mRNAs terminate within ~0.1 to 1.0 kilobase downstream from the stop codon. The other ~15% have lengths similar to hepatic apoB-100 mRNA even though they have the same in-frame stop codon. The organ-specific introduction of a stop codon to a mRNA appears unprecedented and might have implications for cryptic polyadenylation signal recognition and RNA processing.

APOLIPOPROTEIN (APO) B HAS BEEN one of the most sought after proteins because of its important role in lipid metabolism and in the development of atherosclerosis. It is the largest protein species and an obligatory component of chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), and low density lipoproteins (LDL). ApoB is heterogeneous but exists primarily in two forms: apoB-100 and apoB-48. ApoB-100 is synthesized primarily by the liver and is the major protein constituent of VLDL, IDL, and LDL. ApoB-48 is synthesized by the intestine and is found in chylomicrons and chylomicron remnants (*1*). The primary structure of human apoB-100 (4536 residues) has recently been deduced from the nucleotide sequence of overlapping liver complementary DNAs (cDNAs) (*2, 3*). The structure of apoB-48 remains elusive. It does not bind to the LDL receptor and has a molecular weight ~48% of that of apoB-100. Monoclonal antibody mapping studies indicate that apoB-48 shares antigenic determinants with the NH_2-terminal half of apoB-100 (*4*). However, complete structural analysis of apoB-48 has been hampered by its huge size, its insolubility in aqueous buffers, and difficulties in obtaining large amounts of the purified protein.

We now present the primary structure of apoB-48 obtained by nucleotide sequence analysis of cloned human intestinal apoB cDNAs and direct sequencing of human intestinal messenger RNAs (mRNAs). Our findings are corroborated by direct amino acid sequence analysis of multiple tryptic peptide fragments of purified human apoB-48, including its COOH-terminal tryptic fragment.

The adult small intestine synthesizes only apoB-48. Although apoB-48 and apoB-100 are the products of a single gene (*5*), studies indicate that apoB-48 is not the product of a post-translational cleavage of apoB-100 (*6*). It would then seem that the intestine would produce only apoB-48 mRNA. Unexpectedly, however, we found sequences identical to different regions of apoB-100 cDNA, including the COOH-terminal region, in cDNA clones from a human intestinal cDNA library (*7*).

We measured the relative concentrations of apoB-100 cDNA hybridizable sequences in the human intestine with cDNA probes corresponding to various regions of human apoB-100. In adult human small intestine the 3′ half of the apoB-100 mRNA sequence is present at ~15% of that of the 5′ half (Fig. 1). The 5′ half is at a concentration of $\sim 4.7 \times 10^7$ molecules per microgram of RNA in both human liver (Hep G2 cells) and intestine. In the intestine the concentration of mRNA sequences 3′ to those that code for amino acid residues ~1900 to 2400 drops to $\sim 7 \times 10^6$ molecules per microgram of RNA, whereas in the liver the concentration stays at $\sim 4.7 \times 10^7$ molecules per microgram of RNA. This suggests that adult intestine contains mainly an mRNA species that directs the synthesis of a protein corresponding to the NH_2-terminal half of apoB-100.

We performed direct sequence analysis of apoB-48 isolated from a patient with chylous ascites (*8*) and purified by column chromatography in SDS. The protein was found to be pure by SDS–gel electrophore-

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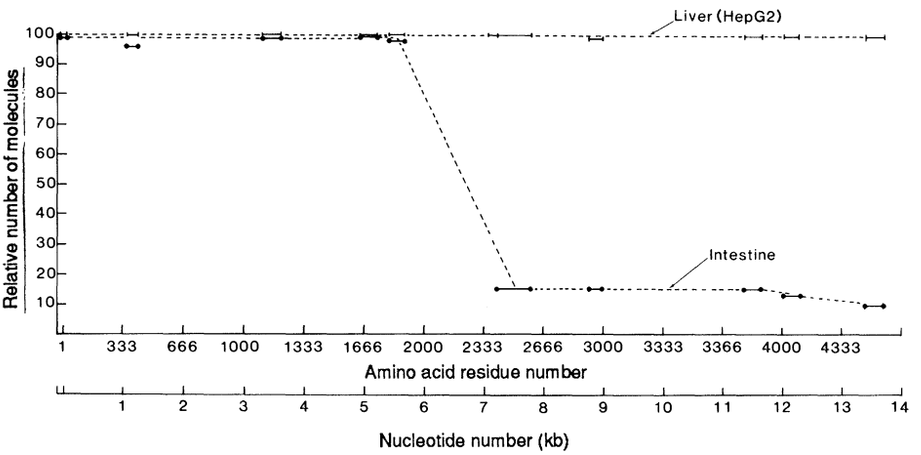

Fig. 1. Quantitative hybridization of intestinal and liver RNA to cloned apoB-100 cDNA probes. Total RNA was isolated from human small intestine and cultured human hepatoma (Hep G2) cells (*16*). Single-stranded ^{32}P-labeled cDNA probes were prepared from restriction fragments of cloned apoB-100 cDNAs, and complementary RNA sequences were assayed by DNA-excess solution hybridization (*17*). Horizontal lines bounded by small vertical bars represent values obtained from hybridization to total hepatic RNA. Those bounded by solid circles represent the corresponding values with intestinal RNA. The lengths of the horizontal lines equal the lengths of the probes. For the relative number of molecules, 100% equals 4.7×10^7 molecules per microgram of RNA. Numbering of the amino acid residues starts with the mature peptide; similarly, numbering of the nucleotides starts with the region of the mRNA coding for the mature part of apoB-100.

sis. It reacted with monoclonal antibodies that recognize plasma apoB-48 and the NH_2-terminal half of apoB-100 but not with antibodies that recognize the COOH-terminal portion of apoB-100 (*9*). The purified protein was digested with trypsin, and the peptides were fractionated on two successive high-performance liquid chromatography (HPLC) columns. Most of the peptides coming off the second HPLC were found to be pure by NH_2-terminal analysis and direct sequencing. Fifty peptides covering 539 amino acid residues were sequenced (Table 1). All the peptides match the corresponding NH_2-terminal LDL-apoB-100 peptides and the cDNA-deduced amino acid sequences from hepatic apoB-100 (*2, 3*). However, the last peptide ends at Met^{2151}, whereas in LDL-apoB-100 the same tryptic peptide extends to Lys^{2159}. Because no other tryptic peptides terminated in a residue other than Lys or Arg or were identified subsequent to this peptide, we reasoned that Met^{2151} is the COOH-terminal amino acid of chylous apoB-48.

The position of Met^{2151} as the COOH-terminus of apoB-48 matches that projected from the quantitative nucleic acid hybridization experiments (Fig. 1). This suggests that apoB synthesized in the intestine terminates in this region of the sequence. To characterize the mRNA that codes for apoB-48, we sequenced intestinal cDNA clones spanning this region of the molecule. Five independent cDNA clones covered sequences that encode the putative COOH-terminal peptide (Fig. 2, A to C). The sequences of all five clones displayed a single base substitution at nucleotide 6457 that changed the codon CAA (Gln^{2153}) to an in-frame stop codon, TAA. The sequence 3′ to the in-frame stop codon was identical to that of hepatic apoB-100 mRNA (*2*). In two of these clones a polyadenylate tail was identified 106 bases after the termination codon and 19 bases downstream from an AATTAA sequence (underlined in Fig. 2C). Three of the clones, which have the stop codon at the same position, extend well beyond this region into the 3′ half of the molecule in a region that is present at a concentration of ~15% that of its 5′ region (Fig. 1).

Since all five independent cDNA clones show the C to T transition at the same position, it almost certainly reflects a change in the structure of intestinal mRNA compared to hepatic mRNA. Such a change

Table 1. Amino acid sequence of apoB-48 tryptic peptides. Contiguous tryptic peptides are presented as a single sequence. For example, the COOH-terminal sequence at the bottom is a combination of three tryptic peptides. Alignment in such cases is based on published apoB-100 sequences (*2, 3*). Many of these peptides have been sequenced more than once. None of the tryptic peptides extended beyond Met^{2151}. The underlined peptide is the only one that ends in a residue other than Lys or Arg. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Residues	Sequence
24–48	KYTYNYEAESSSGVPGTADSRSATR
74–83	EVYGFNPEGK
102–112	YELKLAIPEGK
131–147	RGIISALLVPPETEEAK
170–180	GNVATEISTER
249–260	YGMVAQVTQTLK
271–277	FFGEGTK
279–287	MGLAFESTK
499–505	AAIQALR
511–530	DKDQEVLLQTFLDDASPGDK
609–642	NYQLYKSVSLPSLDPASAKIEGNLIFDPNNYLPK
669–680	GFEPTLEALFGK
752–770	ILGEELGFASLHDLQLLGK
923–933	TEVIPPLIENR
974–997	LELELRPTGEIEQYSVSATYELQR
1001–1016	ALVDTLKFVTQAEGAK
1026–1051	YNRQSMTLSSEVQIPDFDVDLGTILR
1065–1072	LTLDIQNK
1095–1101	GVISIPR
1285–1290	MLETVR
1366–1388	ADSVVDLLSYNVQGSGETTYDHK
1403–1409	FLDSNIK
1459–1471	IDGQFRVSSFYAK
1510–1529	YEDGTLSLTSTSDLQSGIIK
1536–1544	YENYELTLK
1573–1583	SEYQADYESLR
1620–1634	IGQDGISTSATTNLK
1670–1697	FSLDGKAALTELSLGSAYQAMILGVDSK
1743–1754	LDNIYSSDKFYK
1832–1870	VQGVEFSHRLNTDIAGLASAIDMSTNYNSDSLHFSNVFR
1941–1955	VSALLTPAEQTGTWK
1978–1985	IGVELTGR
2017–2035	DAVEKPQEFTIVAFVKYDK
2124–2151	ITENDIQIALDDAKINFNEKLSQLQTYM

would involve an unprecedented mechanism. Direct sequence analysis of intestinal apoB mRNAs from two unrelated subjects (Fig. 2D) confirms that the C to U change is present in intestinal mRNAs and not a cloning artifact. The complete absence of a G band in the presence of a strong A band indicates that essentially all apoB-48 mRNAs have a U instead of a C in this position. We also compared the initiation site of human liver apoB mRNA with that of intestinal apoB mRNA (Fig. 2E) and found that both mRNAs start at the same two positions, 127 and 128 bases 5′ to the translation initiation codon.

Our studies of cloned intestinal apoB cDNAs and mRNA indicate that apoB-48 is the translation product of a mRNA that shares sequences common to apoB-100, but in which a premature in-frame termination codon ends the protein product at Ile^{2152}. Thus, there is a single amino acid difference between the cDNA-deduced and the peptide-deduced COOH-terminus; the COOH-terminal peptide ends at Met^{2151}, missing Ile^{2152}. This difference can be explained by the action of carboxypeptidase A which removes nonpolar residues from the COOH-terminus of proteins. Carboxypeptidase A activity has been reported in peritoneal mast cells and muscle (*10*) and in serum under certain circumstances (*11*). Since the apoB-48 was purified from chylous ascites fluid, it is likely that carboxypeptidase A produced by the peritoneal cells removed the COOH-terminal residue.

Although studies to date indicate that apoB-100 is not synthesized in the adult intestine (*1, 6*), we found an entire 14-kb apoB-100 mRNA-like sequence in the human small intestine. Our study indicates that all intestinal apoB mRNAs, irrespective of their length, have the in-frame premature stop codon. All translation products would then terminate at Ile^{2152}. This interpretation is consistent with all of our experimental data.

Our findings on the intestinal apoB mRNA and protein structures have implications for several aspects of cellular and molecular biology. (i) ApoB-48 shows no LDL receptor binding activity because the putative receptor binding domains in apoB-100 are beyond Ile^{2152}, the COOH-terminus of apoB-48. (ii) ApoB-100 and apoB-48 are the products of a single gene (*5*). The mechanism for the C to U change in intestinal apoB mRNA is unknown. Alternate splicing cannot account for the results since the change falls within a large exon (*12*), but it is still not clear if the change occurs at the intestinal cellular cDNA, pre-mRNA, or mRNA level. In either case, it would involve a novel mechanism without known precedent. Glickman *et al.* (*6*) found that early fetal intestine synthesizes mainly apoB-100, and that the capacity to synthesize apoB-48 is acquired during maturation of the fetus. The mechanism involved in the stop codon generation must be developmentally regulated. (iii) The introduction of an in-frame stop codon into intestinal apoB mRNA apparently allows the recognition of cryptic polyadenylation signals preexisting in apoB mRNA such that a polyadenylate tail is added 19 bases downstream from an AAUUAA sequence, the first functional variant of the classical AAUAAA signal 3′ to the stop codon (*13*). Furthermore, a G-T cluster and an oligo(T) stretch are identified 28 and 35 bases 3′ to the AAUUAA motif (underlined in Fig. 2C). These are part of the potential polyadenylation signal complex identified in eukaryotes (*13*). Northern blot analyses of intestinal RNAs showed a sharp band at ~14 kb and a broad smear at ~7 to 8 kb (*14*) suggesting that the polyadenyla-

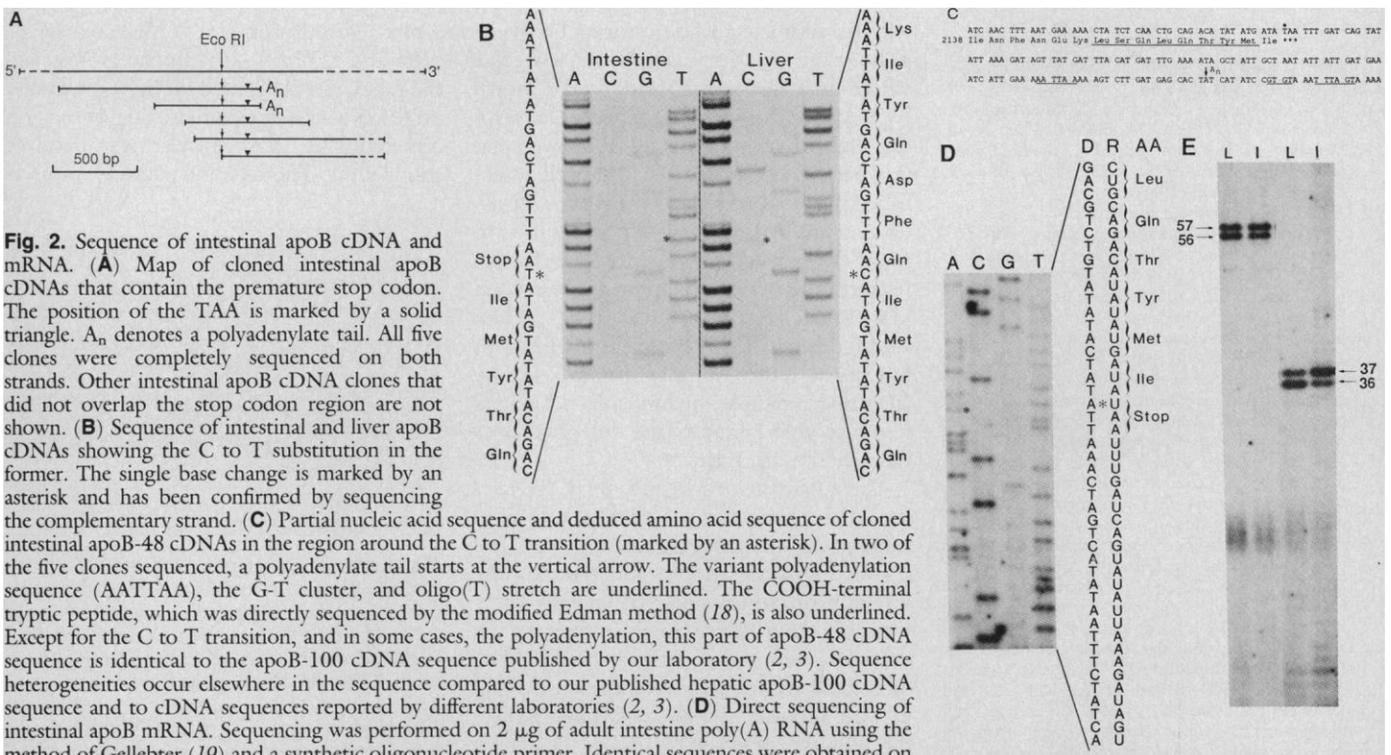

Fig. 2. Sequence of intestinal apoB cDNA and mRNA. (**A**) Map of cloned intestinal apoB cDNAs that contain the premature stop codon. The position of the TAA is marked by a solid triangle. A_n denotes a polyadenylate tail. All five clones were completely sequenced on both strands. Other intestinal apoB cDNA clones that did not overlap the stop codon region are not shown. (**B**) Sequence of intestinal and liver apoB cDNAs showing the C to T substitution in the former. The single base change is marked by an asterisk and has been confirmed by sequencing the complementary strand. (**C**) Partial nucleic acid sequence and deduced amino acid sequence of cloned intestinal apoB-48 cDNAs in the region around the C to T transition (marked by an asterisk). In two of the five clones sequenced, a polyadenylate tail starts at the vertical arrow. The variant polyadenylation sequence (AATTAA), the G-T cluster, and oligo(T) stretch are underlined. The COOH-terminal tryptic peptide, which was directly sequenced by the modified Edman method (*18*), is also underlined. Except for the C to T transition, and in some cases, the polyadenylation, this part of apoB-48 cDNA sequence is identical to the apoB-100 cDNA sequence published by our laboratory (*2, 3*). Sequence heterogeneities occur elsewhere in the sequence compared to our published hepatic apoB-100 cDNA sequence and to cDNA sequences reported by different laboratories (*2, 3*). (**D**) Direct sequencing of intestinal apoB mRNA. Sequencing was performed on 2 μg of adult intestine poly(A) RNA using the method of Gellebter (*19*) and a synthetic oligonucleotide primer. Identical sequences were obtained on adult intestinal RNA preparations isolated from two unrelated subjects. Liver poly(A) RNAs (from adult human liver and Hep G2 cells) gave an identical sequence, except that a G replaced the A marked by an asterisk. D, Complementary DNA sequence; R, RNA sequence; AA, deduced amino acid sequence. (**E**) Mapping of transcription initiation site of adult human intestinal and hepatic apoB mRNA. The sites of transcription initiation were mapped by primer extension using two oligonucleotides (d[CGGCCCTGGCTGGCTGGGCGG], left, and d[GGCTCCTCAGCGGCAGCAACC], right) that were complementary to the sequences 71 and 91 bases upstream to the translation initiation codon. The size of the two bands with each primer indicates that in both tissues the mRNAs start at two sites, −128 (an A) and −127 (a U) bases from the initiation codon; L, adult human liver poly(A) RNA; I, adult human intestine poly(A) mRNA.

tion site for the shorter mRNA may be heterogeneous, possibly the result of polyadenylation signals at more than one position in apoB mRNA. The same signals are present in the hepatic apoB-100 mRNA but evidently are not utilized since 100% of hepatic apoB mRNAs are full-length 14-kb structures (Fig. 1). The 14-kb intestinal apoB mRNAs (~15% of the total) must also extend for an additional 7 kb before a polyadenylate tail is added (*15*) even though they have the same in-frame stop codon as the more abundant, shorter mRNAs.

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7. An adult human small intestinal cDNA expression library (~4×10^6 independent clones) was produced in the vector λgt11. Selection for apoB-specific clones was performed by oligonucleotide hybridization and antibody screening as described for hepatic apoB cDNAs (*2*).
8. The chylous fluid was centrifuged at a density of 1.006 g/ml at 55,000 rpm (20 hours, 10°C, Ti60 rotor) in a Beckman L5-65 ultracentrifuge. The chylomicrons were collected and further purified by centrifugation at a density of 1.006 g/ml. They were delipidated by extraction with acetone and ethanol (1:1, by volume) at −20°C for 18 hours. The precipitate was extracted with the same solvent, washed with diethyl ether, and dried under N_2 and subsequently at reduced pressure at 4°C.
9. Immunoblot analysis of the purified apoB-48 was performed against the following monoclonal antibodies: 2D8 (R. Theolis *et al.*, *Arteriosclerosis* **4**, 498 [1984]), MB47 (S. G. Young *et al.*, *ibid.* **6**, 178 [1986]), and M-34, which binds to apoB-100 and apoB-48 at a site other than the 2D8 binding site (S. R. Silberman, unpublished). Chylous and plasma chylomicron apoB-48 both reacted with 2D8 and M-34 but not with MB47, which is consistent with the finding that chylous apoB-48 is immunologically similar to plasma chylomicron apoB-48.
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Behavioral Recovery Induced by Applied Electric Fields After Spinal Cord Hemisection in Guinea Pig

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Applied electric fields were used to promote axonal regeneration in spinal cords of adult guinea pigs. A propriospinal intersegmental reflex (the cutaneous trunci muscle reflex) was used to test lateral tract function after hemisection of the thoracic spinal cord. An electrical field (200 microvolts per millimeter, cathode rostral) applied across the lesion led to functional recovery of the cutaneous trunci muscle reflex in 25 percent of experimental animals, whereas the functional deficit remained in control animals, which were implanted with inactive stimulators.

MANY ATTEMPTS HAVE BEEN MADE to induce axonal regeneration in injured mammalian spinal cord, with the ultimate goal of restoring behavioral function (*1*). Yet, even in the few cases where clear anatomical evidence of axonal regeneration has been obtained, unequivocal demonstration of functional recovery has not been possible (*2–4*). It is well established that applied electric fields affect development and regeneration of neurites in vitro (*5–7*) and in vivo (*8, 9*) and that they induce regeneration of dorsal column axons in adult mammalian spinal cord (*3*). The present study addresses the effect of electric fields on functional return in mammalian cord by analyzing a simple, quantifiable behavioral response that requires the integrity of a thoracic sensory tract.

The cutaneous trunci muscle (CTM) reflex is a contraction of the back skin in response to cutaneous stimulation (*10*). The CTM originates around the base of the forelimb on either side and spreads backward in a thin sheet, closely applied to the dermis of almost the entire back skin. The motoneurons that innervate it are contained in the cervical spinal cord and project through the brachial plexus in the lateral thoracic nerve (*11*). The reflex is driven by sensory fibers in segmental dorsal cutaneous nerves (DCN). In the rat, the reflex requires a pinch stimulation and is mediated by A-delta and C fibers (*10*). In the guinea pig, the reflex can be elicited by light touch and produces a stereotyped twitching of the skin centered 2 to 3 cm rostral to the point of stimulation. The afferent pathway projects

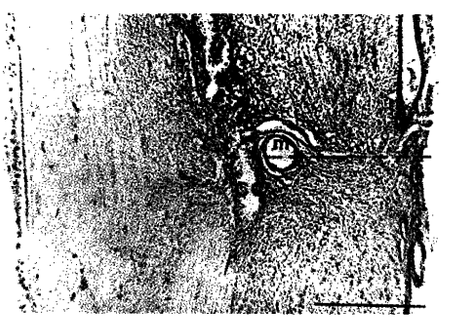

Fig. 1. Photomicrograph of a horizontal section at the level of the central canal (CC) showing a right lateral hemisection of a guinea pig spinal cord. The hole (m) in the tissue was left by a marker, a short length of Prolene monofilament inserted into the lesion immediately after section. To make the hemisection, an insect pin (000) was pushed dorsoventrally completely through the spinal cord at the midline, then the right side of the cord was cut and the locating pin removed laterally to confirm the section. The dashed line indicates the plane of section, cutting completely through the white matter of the right side. This animal was electrically treated and showed functional recovery by day 56. Rostral, top of photograph. Scale bar, 700 μm.

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