

Independent regulation of sterol regulatory element-binding proteins 1 and 2 in hamster liver

(membrane-bound transcription factors/proteolytic processing/basic-helix-loop-helix proteins/low density lipoprotein receptor/3-hydroxy-3-methylglutaryl CoA synthase)

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ABSTRACT Two sterol regulatory element-binding proteins (SREBPs, designated SREBP-1 and SREBP-2), each ≈ 1150 amino acids in length, are attached to membranes of the endoplasmic reticulum and nuclear envelope in human and hamster tissue culture cells. In the absence of sterols, soluble fragments of ≈ 470 amino acids are released from both proteins by proteolytic cleavage. The soluble fragments enter the nucleus, where they bind to sterol regulatory elements in the promoters of genes encoding the low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl CoA synthase, thereby activating transcription. Proteolytic processing of both SREBPs is blocked coordinately by sterol overloading and enhanced coordinately when sterols are depleted by treatment with an inhibitor of cholesterol synthesis. In contrast to these findings in cultured cells, the current data show that SREBP-1 and -2 are not coordinately regulated in hamster liver. In untreated animals the soluble fragment of SREBP-1, but not of SREBP-2, was detected by immunoblotting of a liver nuclear extract. Depletion of sterols by treatment with a bile acid-binding resin (colestipol) and a cholesterol synthesis inhibitor (mevinolin) led to a marked increase in the nuclear form of SREBP-2 and a reciprocal decline in the nuclear form of SREBP-1. These findings suggest that SREBP-1 is responsible for basal transcription of the low density lipoprotein receptor and 3-hydroxy-3-methylglutaryl CoA synthase genes in hamster liver and that SREBP-2 is responsible for the increased transcription that follows sterol depletion with a bile acid-binding resin and a cholesterol synthesis inhibitor.

Two sterol regulatory element-binding proteins (SREBPs) have been proposed to control the transcription of the genes for the low density lipoprotein (LDL) receptor, 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase, and other genes whose control regions contain sterol regulatory elements (1-4). The two SREBPs, designated SREBP-1 and SREBP-2, have a similar domain structure and are believed to operate through a similar mechanism.

The SREBPs are synthesized as long polypeptide chains of ≈ 1150 amino acids, which are designated as precursor forms. Both precursors migrate on SDS/polyacrylamide gels with apparent molecular masses of ≈ 125 kDa (1-5). They are attached to membranes of the endoplasmic reticulum and nuclear envelope by means of hydrophobic sequences located between residues 470 and 570. Within the NH₂-terminal 470 residues, each protein contains a basic-helix-loop-helix-leucine zipper motif that allows the protein to form homodimers and to bind to sterol regulatory elements. The NH₂-terminal domains of both proteins also contain an acidic region that acts as a transcriptional activator (4). When human and hamster cultured cells are grown in the absence of sterols,

a protease cleaves the SREBPs between the leucine zipper and the membrane attachment domain, releasing the mature NH₂-terminal portion of the protein from the membrane (3, 4). Although the NH₂-terminal portions are ≈ 470 amino acids long, they migrate anomalously on SDS gels with molecular masses of ≈ 68 kDa (1-5). They enter the nucleus and bind to sterol regulatory elements, thereby activating transcription of the LDL receptor and HMG CoA synthase genes (3-5).

When sterols overaccumulate in cells, the cleavage of both SREBP precursors is abolished coordinately and the mature forms disappear rapidly from the nucleus (3). As a result, transcription of the LDL receptor and HMG CoA synthase genes declines. Conversely, cleavage of both SREBP precursors is enhanced coordinately when cells are incubated with compactin, an inhibitor of cholesterol synthesis, which depletes cells of cholesterol (5).

SREBP-1 can potentially exist in four isoforms as a result of the alternative use of two exons at both the 5' and 3' ends (1). Thus far, there is no evidence to suggest a functional difference between the four isoforms of SREBP-1 (1). Cultured cells, such as human HeLa cells and Chinese hamster ovary (CHO) cells, express both SREBP-1 and SREBP-2, and the processing of both proteins is regulated coordinately (3, 5). Each of the proteins can act independently when overproduced in cultured cells as a result of transfection, and there is no evidence that a heterodimer is required (2). The reason for the existence of two apparently redundant SREBPs is unknown.

In livers of animals the levels of mRNA produced by sterol-regulated genes, including those for the LDL receptor and HMG CoA synthase, can be increased by treatment with mevinolin, an inhibitor of HMG CoA reductase (6, 7). By blocking cholesterol synthesis, mevinolin induces a transient state of cholesterol deprivation, and this increases transcription of the sterol-regulated genes (8). The effects of mevinolin are enhanced when the drug is given together with a bile acid-binding resin, such as colestipol. By blocking the intestinal reabsorption of bile acids, these resins stimulate an enhanced conversion of cholesterol into bile acids, thereby increasing hepatic demands for cholesterol even further (9).

The current experiments were designed to determine whether the combination of an HMG CoA reductase inhibitor and a bile acid-binding resin enhances the proteolytic processing of SREBP-1 and SREBP-2 in hamster liver. In contrast to the findings in cultured cells, the current data indicate that the mature forms of SREBP-1 and SREBP-2 are not regulated coordinately in hamster liver. The mature form of SREBP-2 increases, whereas the mature form of SREBP-1 decreases in livers of hamsters treated with mevinolin plus colestipol.

Abbreviations: HMG CoA, 3-hydroxy-3-methylglutaryl CoA; LDL, low density lipoprotein; SREBP, sterol regulatory element-binding protein.

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MATERIALS AND METHODS

Animals. Male Golden Syrian hamsters (100–120 g), obtained from Sasco (Omaha, NE), were exposed to a 12-hr light/12-hr dark cycle and fed either standard 4% mouse/rat chow diet (Teklad, Madison, WI) or the same diet supplemented with 4% (wt/wt) colestipol (Upjohn) and 0.025–0.15% (wt/wt) mevinolin (Merck, Sharp & Dohme). Hamsters were sacrificed in the middle of the light cycle without fasting.

Nuclear Extracts. Nuclear extracts of hamster liver were prepared by a modification of the method of Gorski *et al.* (10). Equal portions (1.6 g) of liver from three hamsters were excised, rinsed in cold phosphate-buffered saline, pooled (4.8 g), and suspended in 30 ml of buffer A [10 mM Hepes at pH 7.6/25 mM KCl/1 mM sodium EDTA/2 M sucrose/10% (vol/vol) glycerol/0.15 mM spermine/2 mM spermidine] supplemented with protease inhibitors (*N*-acetyl-leucyl-leucyl-norleucinal at 50 μ g/ml, 0.1 mM Pefabloc, pepstatin A at 5 μ g/ml, leupeptin at 10 μ g/ml, aprotinin at 2 μ g/ml). The livers were homogenized briefly in a Polytron and were then transferred to a Potter–Elvehjem homogenizer, where they were subjected to three strokes of a Teflon pestle. The homogenate was filtered through four layers of cheesecloth, and the sample (25 ml) was layered over 10 ml of buffer A in a Sorvall AH629 rotor and centrifuged at 24,000 rpm for 1 hr at 4°C. The resulting nuclear pellet was resuspended in 1 ml of buffer containing 10 mM Hepes at pH 7.6, 100 mM KCl, 2 mM MgCl₂, 1 mM sodium EDTA, 1 mM dithiothreitol, and 10% (vol/vol) glycerol supplemented with proteinase inhibitors, after which 0.1 vol of 4 M ammonium sulfate (pH 7.9) was added. Each mixture was agitated gently for 40 min at 4°C and then centrifuged at 85,000 rpm in a Beckman TLA-100.2 rotor for 45 min at 4°C. The supernatant was used as nuclear extract.

Membrane Fractions. Detergent-solubilized membranes were prepared from different aliquots of the same hamster livers that were used for nuclear extracts. Equal portions (0.5 g) of liver from three hamsters were pooled (1.5 g) and homogenized, and the 800- to 100,000-g pellet was isolated and solubilized with Triton X-100 as described (11), except that all buffers contained protease inhibitors consisting of 1 mM phenylmethanesulfonyl fluoride, 1 mM 1, 10-phenanthroline, leupeptin at 50 μ g/ml, pepstatin A at 1 μ g/ml, aprotinin at 0.5 μ g/ml, *N*-acetyl-leucyl-leucyl-norleucinal at 25 μ g/ml.

Immunoblot Analysis. Aliquots of nuclear extracts (150 μ g) and membrane fractions (100 μ g) from hamster liver were mixed with SDS loading buffer (12) and subjected to SDS/PAGE on an 8% gel. Immunoblot analysis was done by using the ECL immunoblotting detection system kit (Amersham), as described (3), except that the blocking reactions were supplemented with 5% (wt/vol) dry milk without calf serum, and the washing solutions after both primary and secondary antibody incubations were supplemented with 0.1% (wt/vol) SDS, 1% (vol/vol) Nonidet P-40, and 0.5% (wt/vol) sodium deoxycholate. The following primary antibodies were used: mouse monoclonal antibody (IgG-2A4) against amino acids 301–407 of human SREBP-1a at 4 μ g/ml (4) and a 1:4000 dilution of rabbit polyclonal antiserum against amino acids 32–250 of hamster SREBP-2 (5). The primary antibodies were visualized with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (made in donkey or sheep).

RNA Blot Analysis. Poly(A)⁺ mRNA was prepared from different portions of the same hamster livers that were used for nuclear extracts and membrane fractions. Equal portions (1 g) of liver from three hamsters were pooled (3 g), homogenized, and extracted in an acid guanidinium thiocyanate/phenol/chloroform mixture (13). Poly(A)⁺ RNA was isolated from total RNA by using oligo-(dT)₃₀ beads (Qiagen, Chatsworth, CA). Blot hybridization was done as described (1) with the following random-primed ³²P-labeled cDNA fragments: for SREBP-1 mRNA, 3.9-kb *Not* I–*Sal* I full-length insert of

hamster pSREBP-1 (4); for SREBP-2 mRNA, 5-kb *Not* I–*Sal* I full-length insert of hamster pSREBP-2 (5); for HMG CoA synthase mRNA, 1.5-kb *Hind*III fragment of hamster p53K-312 (14); for LDL receptor mRNA, 2.3-kb *Kpn* I–*Xba* I fragment of mouse LDL receptor cDNA (provided by S. Ishibashi of this Department); and for glyceraldehyde-3-phosphate dehydrogenase mRNA, 1.2-kb *Hind*III–*Bam*HI fragment of rat glyceraldehyde-3-phosphate dehydrogenase cDNA (15). All hybridizations contained ³²P-labeled probe at 1–5 × 10⁶ cpm/ml. Blots were exposed to Kodak XAR-5 film at –70°C for the indicated time.

Other Assays. Plasma cholesterol was measured by the cholesterol oxidase method (Boehringer Mannheim). Protein content of liver extracts was measured by the Lowry method (16).

RESULTS

Fig. 1 shows the results of an experiment in which hamsters were fed a diet containing 4% (wt/vol) colestipol/0.05% mevinolin for various times, after which the animals were sacrificed and livers were fractionated into a crude nuclear pellet and an 800- to 100,000-g membrane fraction. Aliquots were subjected to SDS/PAGE and immunoblotted with antibodies specific for SREBP-1 or SREBP-2. In control animals (zero time), the membrane fraction contained the 125-kDa precursor forms of SREBP-1 and SREBP-2 (designated “P” in Fig. 1A and B, respectively). The nuclear fraction contained the mature 68-kDa form of SREBP-1 (designated “M” in Fig. 1A) but no detectable mature form of SREBP-2 (Fig. 1B). With increased time on the colestipol/mevinolin diet, there was a progressive decline in the mature nuclear form of SREBP-1 and a reciprocal increase in the nuclear form of SREBP-2. Fig. 1C and D provides a semiquantitative analysis of these data as determined from densitometric scans of the chemiluminescence films. As shown in Fig. 1E, the ratio of the

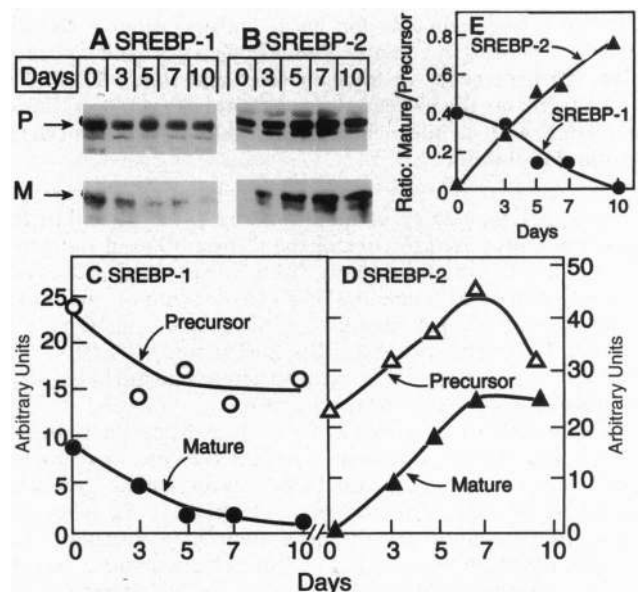


FIG. 1. Changes in the precursor and mature forms of SREBP-1 (A) and SREBP-2 (B) in membranes (Upper) and nuclear extracts (Lower) from livers of hamsters fed 0.05% mevinolin plus 4% cholesterol for various times. Immunoblots were done on pooled samples from livers of three animals as described. Blots were exposed to film for 40–120 sec. The amount of radioactivity in each band was quantified with a computing densitometer model 300A using IMAGE QUANTI software version 2.0 (Molecular Dynamics), and the data are shown in C and D. E shows the ratio of radioactivity (precursor/mature bands) plotted from the data in C and D. P, precursor form of SREBP; M, mature form of SREBP.

mature to the precursor form of SREBP-1 was high in the untreated animals and declined precipitously during treatment; this was accompanied by a marked increase in the ratio of the mature to the precursor form of SREBP-2 (>50-fold). These ratios represent relative estimates and not absolute values because different aliquots of the nuclear and membrane pellets were subjected to electrophoresis and because the 125-kDa precursor and the 68-kDa mature forms may transfer to the nitrocellulose membrane with different efficiencies. Nevertheless, changes in the ratio with time reflect valid changes in the relative amounts of precursor and mature forms of each SREBP.

Fig. 2 presents measurements of various mRNAs in the livers of the colestipol/mevinolin-treated animals as determined by RNA blot hybridization followed by phosphoimaging quantification. The drug treatment elicited a 40-fold increase in the mRNA for HMG CoA synthase and a smaller 3-fold increase in the mRNA for the LDL receptor. The mRNA for SREBP-2 rose in parallel with the rise in LDL receptor mRNA. There was no significant change in the amount of mRNA for SREBP-1.

In several experiments (data not shown), we failed to observe an effect of colestipol alone on the amounts of the mature nuclear forms of SREBP-1 or SREBP-2. Mevinolin given alone increased the nuclear form of SREBP-2 in some experiments but not in others. Consistent effects were observed only when the two drugs were given together.

Fig. 3 shows an experiment in which hamsters were fed for 7 days with a diet containing 4% colestipol with increased amounts of mevinolin up to 0.15%. In the absence of mevinolin or colestipol, the liver membranes contained the 125-kDa precursor forms of SREBP-1 (Fig. 3A) and SREBP-2 (Fig. 3C). The nuclei contained only the mature form of SREBP-1 (Fig. 3B and D). Treatment with colestipol plus a low concentration of mevinolin (0.025%) had no detectable effect on SREBP-1, but it caused a marked increase (>50-fold) in the nuclear form of SREBP-2. As the dose of mevinolin increased further, the amount of the nuclear form of SREBP-2 increased somewhat, and the nuclear form of SREBP-1 declined to barely detectable levels (Fig. 3B and D). The precursor form of SREBP-2 was present in the membranes of the untreated animals, and it increased ~3-fold as the dose of mevinolin increased (Fig. 3C). The concentration of total plasma cholesterol declined as the dose of mevinolin increased (Fig. 4).

DISCUSSION

The current results provide a glimpse of the regulation of the proteolytic processing of SREBP-1 and SREBP-2 in livers of

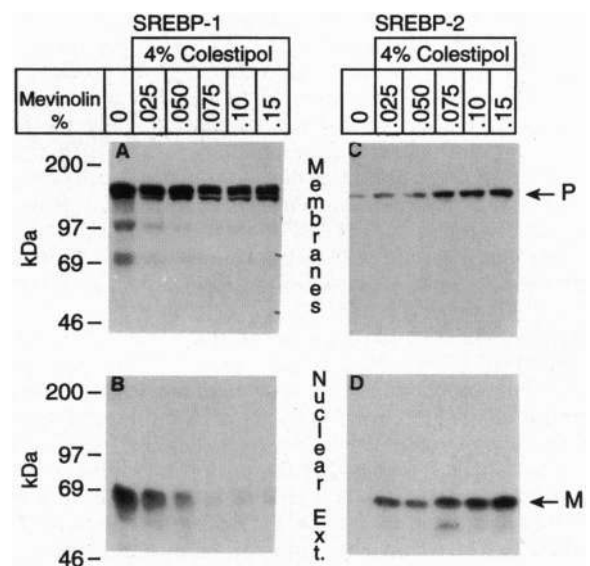


FIG. 3. Changes in the precursor and mature forms of SREBP-1 (A, B) and SREBP-2 (C, D) in membranes (A, C) and nuclear extracts (B, D) from livers of hamsters fed the indicated amount of mevinolin supplemented with 4% colestipol for 7 days. Immunoblots were done on pooled samples from livers of three animals as described. Blots were exposed to film for 20 sec. P, precursor form of SREBP; M, mature form of SREBP.

intact animals. In contrast to previous findings in cultured cells (3, 5), the data suggest that SREBP-1 and SREBP-2 are regulated independently in hamster liver, raising the possibility that the two proteins may serve different functions in this organ.

In the basal state the mature form of SREBP-1, but not SREBP-2, was easily detected in the nuclear fraction of liver cells. This result suggests that SREBP-1 is responsible for the basal level of expression of sterol-regulated genes such as HMG CoA synthase and the LDL receptor in this organ. On the other hand, SREBP-2 appears to account for the elevated level of expression of these genes in cholesterol-depleted livers, as indicated by the increase in both the precursor and mature forms of SREBP-2 after treatment with colestipol plus mevinolin. In the colestipol/mevinolin-treated animals, the efficiency of processing of SREBP-2 was much higher than that of SREBP-1, as indicated by the higher ratio of the mature nuclear form to the precursor form.

The decline in the nuclear form of SREBP-1 after colestipol/mevinolin treatment was not predicted by earlier studies in cultured cells. In these cells the proteolytic processing of

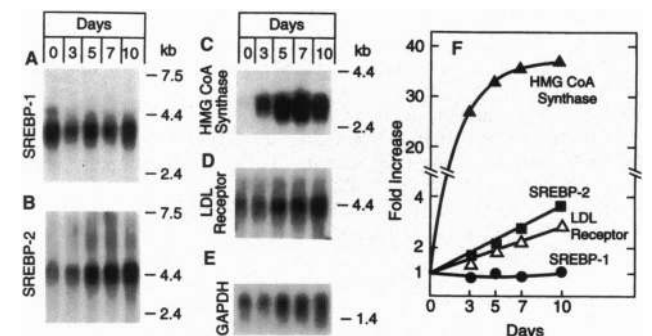


FIG. 2. Changes in the amounts of mRNA for SREBP-1 (A), SREBP-2 (B), HMG CoA synthase (C), LDL receptor (D), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (E) in livers of hamsters fed 0.05% mevinolin plus 4% colestipol for various times (same animals as Fig. 1). Blot hybridization was done on poly(A)⁺ RNA isolated from pooled samples of liver from three animals as described. The blots in A-E were exposed to film for 10, 24, 3, 16, and 20 hr, respectively. The amount of radioactivity in each band was quantified with a Bio-Imaging analyzer using BAS1000 MacBAS software (Fuji Medical Systems), and the data are shown in F.

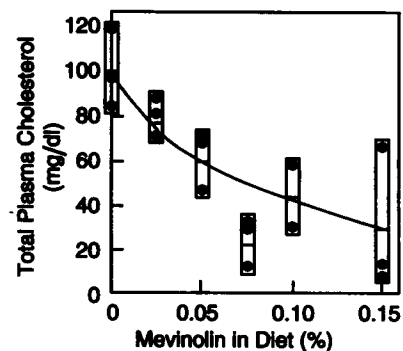


FIG. 4. Decrease in total plasma cholesterol in livers of hamsters fed various amounts of mevinolin/4% colestipol for 7 days. Plasma cholesterol was measured on each of the three animals per group (same animals as for Fig. 3), and the mean value is denoted by the horizontal bar. (In the 0.10% mevinolin group, the blood sample from one animal was spilled.)

both SREBP-1 and SREBP-2 is enhanced when cholesterol synthesis is blocked by an inhibitor of HMG CoA reductase (5). This has given rise to the idea that the two proteins are processed by the same protease and, hence, they are subject to coordinate regulation. In contrast to cultured cells, the liver may contain separate proteases that cleave SREBP-1 and SREBP-2, and these proteases may be subject to independent regulation. It is also possible that a single SREBP protease exists, but SREBP-1 and SREBP-2 respond to it differently in liver, perhaps because the two proteins occupy different membrane locations, either within a single cell or in different cells.

Previous studies in rats have shown that HMG CoA reductase and HMG CoA synthase, two sterol-regulated genes, are preferentially expressed in a subset of hepatocytes located near the portal triads (17, 18). Inhibitors of cholesterol synthesis cause the expression to spread to cells that are located near the central vein. It is possible that the periportal cells preferentially produce SREBP-1 and the pericentral cells produce SREBP-2, but only in response to sterol depletion. This hypothesis can be tested in the future by immunocytochemistry using antibodies to SREBP-1 and SREBP-2.

A precedent for sterol-independent regulation of the processing of SREBP-1 and SREBP-2 has been obtained in studies of a mutant line of cultured Chinese hamster ovary (CHO) cells that produce a truncated form of SREBP-2 as a result of a rearrangement in one copy of the SREBP-2-encoding gene (5). The truncated protein includes the basic-helix-loop-helix-leucine zipper domain and the transcriptional activation domain, but it lacks the membrane-attachment domain. The truncated protein is not attached to the membrane, and it enters the nucleus and activates transcription spontaneously without a requirement for sterol-regulated proteolysis (5). The cells that produce this truncated SREBP-2 fail to process either SREBP-1 or the SREBP-2 that is produced by the normal SREBP-2-encoding gene. These data suggest a feedback mechanism by which the active form of SREBP-2 inhibits the proteolytic processing of full-length SREBP-1 and SREBP-2 (5). If this feedback mechanism operates in liver, it raises the possibility that the increased production of SREBP-2 in response to colestipol/mevinolin inhibits the processing of SREBP-1, thus explaining the decline in the processed nuclear form of SREBP-1 in the livers of the colestipol/mevinolin-treated animals. Such a hypothesis would not explain why SREBP-2 itself continues to be processed so efficiently in these livers.

The reason for the differential regulation of SREBP-1 and SREBP-2 in hamster liver may relate to the possible involvement of SREBP-1 in regulating fatty acid synthesis as well as cholesterol synthesis. In a screen for basic-helix-loop-helix-leucine zipper proteins, Tontonoz *et al.* (19) isolated the rat homologue of SREBP-1 from an adipocyte cDNA library; the cDNA was designated ADD1. In transfection assays in NIH 3T3 cells, ADD1 cDNA was shown to activate transcription of a reporter gene driven by a multimerized "E-box" sequence that corresponds to the E-box present in the 5' flanking region of fatty acid synthetase. If SREBP-1, but not SREBP-2,

regulates the endogenous fatty acid synthetase promoter, this might explain why SREBP-2, but not SREBP-1, is induced by cholesterol deprivation *in vivo* in hamster liver.

Note Added in Proof. In rats the amounts of HMG CoA synthase protein and mRNA vary over a diurnal cycle with a peak at midpoint of the dark phase (20). Similar measurements have not been made in hamsters. In the current studies the light cycle lasted from 7 a.m. to 7 p.m., and the animals were killed at 11 a.m. If hamsters have a diurnal rhythm similar to that of rats, it is likely that the amount of mature forms of SREBP-1 and SREBP-2 vary and that 11 a.m. would represent a low point. Whether or not SREBP-1 or 2 shows a diurnal rhythm and whether or not the response to cholesterol deprivation is similar in the light and dark phases remain to be answered.

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