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Homeostatic regulation of hepatocyte nuclear transcription factor 1 expression in cultured hepatoma cells

(Northern blot hybridization/gel-retardation analysis/colloid osmotic pressure/reporter gene expression)

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ABSTRACT **Serum colloid osmotic pressure is believed to control the hepatic output of plasma proteins, including albumin. The present study was aimed at identifying the molecular basis for feedback control of albumin gene expression in highly differentiated hepatoma cells. The steady-state level of albumin mRNA and the activity of a 282-bp albumin promoter–chloramphenicol acetyltransferase reporter gene in cells incubated in the presence of increasing amounts of serum albumin or dextran were significantly and selectively decreased. When nuclear extracts from cells exposed to 5% (wt/vol) serum albumin were tested in a gel-retardation assay with six oligonucleotide probes containing DNA elements of the albumin promoter, only the element B-retarded band, which contains the nucleotide recognition sequence for hepatocyte nuclear transcription factor 1α (HNF-1α), was consistently decreased as compared to nuclear extract from cells not exposed to serum albumin. Moreover, the activity of a reporter gene with a basal TATA-promoter driven by multiple HNF-1α recognition elements was selectively inhibited in cells incubated in the presence of 5% serum albumin. A reduction of HNF-1α mRNA appears to be responsible for this response to a change in the level of macromolecules in the incubation medium. These results indicate that activity of a dominant liver transcription factor, HNF-1α, controlling the transcription of several liver-specific genes, is modulated by a fluctuation in the level of oncotically active macromolecules.**

The liver represents the main source of serum albumin (1, 2). The main function of serum albumin is to maintain a normal serum colloid osmotic pressure (COP). The COP, in turn, seems to exert homeostatic control over the synthesis and/or secretion of proteins by the liver (3). In fact, in a variety of conditions in which the serum level of albumin falls either acutely (e.g., plasmapheresis) or chronically (e.g., nephrotic syndrome), hepatic production of albumin is increased (4, 5). In contrast, hyperglobulinemia, infusion of γ-globulin, or administration of dextran also results in decreased serum albumin levels (6–8). Recently, we reported (9) that feedback control of albumin production *in vivo* by serum colloids, including albumin, high molecular mass dextran, or γ-globulin, is regulated at the level of gene transcription in both normal and analbuminemic rats.

Molecular studies have shown that a significant component in the control of albumin gene transcription maps to the promoter-proximal region (10–12). By using both *in vivo* studies and cell-free nuclear extracts (NEs) from liver, six functionally defined and evolutionarily highly conserved domains have been identified (13–16). These cis-acting elements are bound by the following specific liver-enriched or general nuclear transcription factors: hepatocyte nuclear transcription factor 1α (HNF-1α) and -β, NF-Y, DBP, C/EBP, LAP, and an NF-1-like protein (13, 14, 17, 18). It has also been shown that specific expression of albumin is preserved in some well-differentiated hepatoma cell lines (10, 17, 19).

The present study was designed to investigate further the molecular basis for feedback control of albumin gene transcription by COP. This was accomplished by functionally dissecting activity of the albumin 5′-proximal-promoter region in highly differentiated hepatoma cell lines cultured in the absence or presence of oncotically active macromolecules. Our results indicate that oncotic pressure reduces the amount of HNF-1α in the nucleus of cultured hepatoma cells, so that transcription from the albumin promoter and another enhancer/promoter element containing an HNF-1α recognition sequence is functionally reduced. To our knowledge, these results represent the first example of molecular regulation of specific gene expression in a higher eukaryotic organism mediated by physiological factors totally external to the affected cell.

MATERIALS AND METHODS

Cell Cultures. FAO and HuH-7 hepatoma cell lines were maintained in RPMI 1640 medium with 10% (vol/vol) fetal calf serum. Cells at subconfluency were used for different experimental manipulations as specified below.

RNA Blot Analysis. Hepatoma cells were incubated in standard culture medium in the absence or presence of various amounts of rat serum albumin (RSA), human serum albumin (HSA), or high molecular mass dextran of 70 kDa (Sigma). After 48 hr, cells were washed with phosphate-buffered saline (PBS) and RNA was extracted using the acid guanidinium thiocyanate/phenol/chloroform method (20). Total RNA was then subjected to Northern blot analysis as described (9), using [^{32}P]dCTP random oligonucleotide-primed cDNA probes. The following cDNA clones were used as hybridization probes: (*i*) HSA (21), (*ii*) chicken β-actin (22), (*iii*) human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (23), and (*iv*) mouse HNF-1α (24).

Cell Transfection. Cells were plated at a density of 2×10^6 cells per 60-mm dish in standard serum-free medium without macromolecules and were cotransfected with 14 μg of supercoiled test plasmid DNA or 1 μg of pSV$_2$CAT plasmid DNA and 2 μg of pRSVluc internal control plasmid by lipofection (Lipofectin, GIBCO/BRL), by the manufacturer's protocol. After 16–18 hr, cells were washed with PBS, and fresh culture medium was added, containing different amounts of RSA, HSA, or dextran for an additional 48 hr. The plasmid pAlbCAT contains the mouse albumin promoter

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Abbreviations: COP, colloid osmotic pressure; RSA, rat serum albumin; HSA, human serum albumin; CAT, chloramphenicol acetyltransferase; HNF-1, hepatocyte nuclear transcription factor 1; NE, nuclear extract; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(positions −330 to +10) cloned into the MTEV.JT-chloroamphenicol acetyltransferase (CAT) reporter gene (25). The $(\beta 28)_3$-CAT plasmid contains three copies of the β-fibrinogen HNF-1 binding site placed upstream of the γ-fibrinogen basal promoter and the CAT gene (24, 26). pRSVluc and pSV2CAT contain the Rous sarcoma virus long terminal repeat or the simian virus 40 early-region promoter inserted upstream of the firefly luciferase (27) or CAT (28) gene, respectively. Transfected cells were washed twice with PBS and lysed on the culture plate by adding a solution containing 1% Triton X-100, 25 mM glycylglycine (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM dithiothreitol. Luciferase activity was assayed by standard chemiluminescence methods, typically using 5 μl of cell extract. CAT activity was determined by the method of Gorman *et al.* (28). After incubation at 37°C for 2 hr (pAlbCAT) or 45 min [pSV_2CAT and $(\beta 28)_3$-CAT], the reaction products were resolved by thin layer chromatography (TLC). The percent conversion of ^{14}C-labeled chloramphenicol to monoacetylated forms was calculated by determining the radioactivity by scintillation counting of the substrate and product spots cut out of the TLC plate and normalized to luciferase activity in the same extracts (to control for transfection efficiency).

Electrophoretic Mobility Shift Assay and Protein Blot Analysis. NEs were prepared from hepatoma cells cultured for 48 hr in the absence or presence of various amount of macromolecules, by using the method of Digman *et al.* (29) with minor modifications. Cells were lysed on ice in one packed cell volume of solution B and passed through a 26-gauge syringe. Nuclei were pelleted by centrifugation in a microcentrifuge at 12,000 rpm for 20 sec and lysed in a two-thirds packed cell volume of solution C. After stirring the resulting suspension for 30 min, chromatin was pelleted by centrifugation at 25,000 rpm for 30 min in an SW56 rotor. The supernatant was dialyzed for 2–4 hr against 500 vol of buffer D, and the dialysate was cleared by centrifugation at 12,000 rpm for 10 min. Aliquots were frozen in liquid nitrogen and stored at −80°C. Protein concentration (typically 2–4 mg/ml) was determined by the Bio-Rad protein assay method using bovine serum albumin as standard.

Oligonucleotides for each of the six rat albumin promoter elements, A through F (14), were annealed to an excess of the complementary strand, ^{32}P-end-labeled by the T4 DNA kinase reaction. Gel mobility shift assays were performed with 5 μg of NE in a 10-μl reaction mixture of 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris·HCl (pH 7.5), and 2 μg of poly(dI)·poly(dC). Competition assays or "supershift" experiments were performed by adding to the reaction mixture a 50- to 100-fold molar excess of unlabeled probe or 0.5–1 μl of HNF-1α-specific antisera, respectively, and preincubating for 15 min on ice prior to addition of labeled probe. After incubation at room temperature for 20 min, reaction products were resolved on 5% polyacrylamide gels in 0.5× TBE (1× TBE is 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) and exposed to autoradiography.

RESULTS

To develop an *in vitro* model to study feedback regulation of mRNA biogenesis by serum COP, we used cultured human (HuH-7) and rat (FAO) hepatoma cell lines. The morphologic appearance of cells exposed to 2–5% RSA, HSA, or dextran was not different from that of control cells. However, after addition of 7–8% albumin or dextran in the culture medium, cells appeared slightly pycnotic with reduced volume, presumably due to water efflux.

Effect of Oncotically Active Macromolecules on Expression of the Albumin Gene or an Albumin Promoter-Driven Reporter Gene. To verify whether increasing COP might depress the accumulation of albumin mRNA in cultured hepatoma cells, we added various amounts of serum albumin to the culture medium and measured the steady-state levels of albumin mRNA, as compared to control mRNAs. Fig. 1*A* shows the results of a typical experiment using HuH-7 cells and 5% or 8% HSA. The steady-state level of albumin mRNA was significantly decreased upon exposure of cells to 5% or 8% HSA, whereas no change was observed in the steady-state level of transcripts for another liver-specific gene (GAPDH) or a general "housekeeping" gene (β-actin). Densitometric analysis of Northern blots from three sets of experiments documented a 35–50% and 55–70% decrease in the steady-state level of albumin mRNA in cells exposed to 5% or 8% HSA, respectively. Similar results were obtained with 5% dextran (data not shown).

Since the 170-bp sequence immediately upstream of the albumin gene transcriptional start site contains all the critical elements necessary for its tissue-specific expression in rat hepatoma cells (10–12) and this region is highly conserved between human and rodents (13), we tested whether the expression of a reporter gene driven by the albumin promoter might be modulated by exposure of hepatoma cells to oncotically active macromolecules. Transfection efficiency with the pAlbCAT construct was higher in human than in rat cell lines. As shown in Fig. 1*B*, incubation of HuH-7 cells in the presence of 5% or 8% HSA resulted in a 50–60% reduction in percentage conversion of CAT to mono- and diacetylated forms. There was no effect with 2% HSA in the culture medium (data not shown). CAT activity driven by a viral promoter (pSV_2CAT) was not influenced by 5% or 8% HSA in the incubation medium. Similar results were obtained with 5% dextran added to the culture medium.

Effect of Oncotically Active Macromolecules on HNF-1α Binding to the Albumin Promoter. Six cis-regulatory elements (elements A–F), which constitute the albumin promoter region and their cognate DNA-binding transcriptional factors (Fig. 2), have been identified and characterized (13, 17, 18). To investigate the DNA–protein interactions on the albumin promoter in cells incubated in the absence or presence of oncotically active macromolecules, we studied the gel-retardation pattern of these six albumin promoter elements

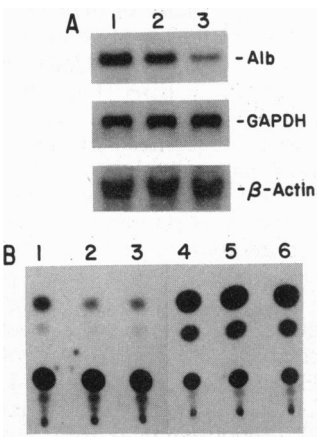

FIG. 1. Effect of serum albumin in the medium on albumin mRNA steady-state levels and albumin promoter–CAT reporter gene expression in cultured hepatoma cells. (*A*) Total cellular RNA (10 μg), obtained from HuH-7 cells incubated for 48 hr in the absence (lane 1) or presence of 5% HSA (lane 2) or 8% HSA (lane 3), was subjected to Northern blot analysis with specific cDNA probes, as noted. Alb, albumin. (*B*) HuH7-cells were cotransfected with 14 μg of supercoiled pAlbCAT plasmid (lanes 1–3) or 1 μg of pSV_2CAT plasmid (lanes 4–6) and 2 μg of pRSVluc internal control plasmid by lipofection. After 16–18 hr, cells were washed with PBS, and fresh culture medium was added containing 5% HSA (lanes 2 and 5) or 8% HSA (lanes 3 and 6) and assayed 48 hr later for CAT activity.

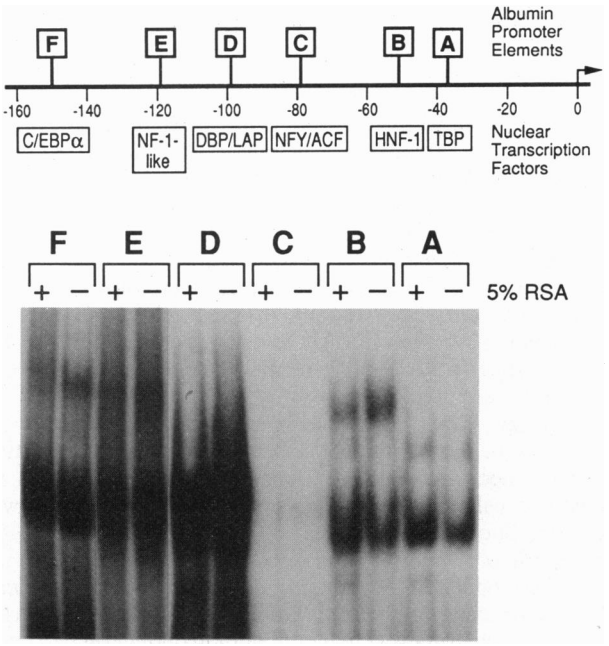

FIG. 2. Effect of serum albumin on transcription factor–albumin promoter-element binding activity. The six promoter elements and trans-acting factors controlling basal transcription activity of the albumin gene are schematically diagrammed at the top. NEs were prepared from FAO cells incubated in the absence (−) or presence (+) of 5% RSA and assayed with labeled oligonucleotides containing the various albumin promoter elements as indicated by using a gel mobility shift assay. Individual pairs of lanes show gel-shift activity with labeled oligonucleotides containing albumin promoter elements A to F.

with NEs from hepatoma cells cultured in the presence or absence of albumin (Fig. 2). The element B band shift with NE from cells incubated in the presence of 5% HSA was significantly decreased, as compared to that obtained with NE from control cells (Fig. 2). However, no significant difference in the gel-shift pattern was detected in the presence or absence of 5% HSA for any of the other albumin promoter elements. Specificity of the element B band-shift pattern was confirmed by competition experiments using a 50- or 100-fold molar excess of unlabeled element B (Fig. 3*A*) and increasing amounts of RSA (Fig. 3*B*). Similar results were obtained with 5% dextran. Since element B oligonucleotide is known to be recognized by nuclear transcription factor HNF-1α (30), an antibody to this factor (kindly provided by G. Crabtree, University of California, San Francisco) was added to the gel-retardation reaction mixture. As shown in Fig. 3*B*, addition of rabbit anti-rat HNF-1 to the reaction caused a "supershift" of the DNA–protein complex, thus identifying nuclear transcription factor HNF-1α as responsible for retardation of element B electrophoretic mobility. No "supershift" for HNF-1α was obtained with an antibody to C/EBPβ (kindly provided by M. Chojkier, University of California, San Diego). Furthermore, after transferring the DNA–protein complex to a nitrocellulose filter and processing the blot with HNF-1 antiserum, we confirmed that the amount of HNF-1 protein bound to the albumin promoter element B was reduced in NE from cells exposed to 5% HSA (data not shown).

Effect of Serum Albumin on the Transactivating Function of HNF-1α. To study the transactivating function of HNF-1 and the response to high COP in the context of a heterologous promoter, expression of a CAT reporter construct containing the basal γ-fibrinogen promoter driven by three copies of the β-fibrinogen HNF-1 binding site (24, 26) (kindly provided by G. Crabtree) was tested in HuH-7 cells. Exposure of HuH-7

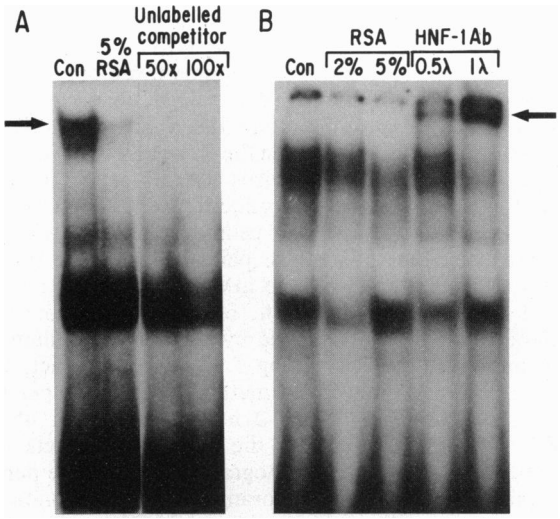

FIG. 3. Identification of HNF-1α as the transcription factor binding to albumin promoter element B and specificity of this DNA–protein interaction. NE (5 μg) from FAO cells was incubated with 10,000 cpm of labeled rat element B (5′-AGTATGGTTAATGATCTACAG-3′) in a gel mobility shift assay. (*A*) Inhibition of specific labeled DNA–protein interaction by addition of 5% RSA or excess unlabeled element B oligonucleotide (50- or 100-fold) to the reaction mixture. The specific DNA–protein complex inhibited by addition of albumin (or dextran) to the medium is shown by an arrow. (*B*) Influence of increasing amounts of RSA in the culture medium or preincubation of the NE with HNF-1α antibody. With increasing amounts of RSA, there is a progressive decrease in the amount of specific element B DNA–protein complex and with increasing amounts of HNF-1α antibody, the migration of element B DNA–protein complex is retarded (the position of this complex is also noted by an arrow).

cells to 5% HSA down-regulated CAT activity driven by this HNF-1 responsive promoter [$(\beta 28)_3$CAT] by ≈60% (Fig. 4, lanes 4 vs. 6), as assessed in three sets of experiments, with no change in the activity of a pSV$_2$CAT reporter gene (Fig. 4, lanes 1 vs. 3).

Effect of Serum Albumin on HNF-1α mRNA Synthesis. Finally, to determine whether reduced HNF-1 activity resulting from increased external COP was due to decreased expression of the transcriptionally controlled HNF-1 gene (31, 32), Northern blot analysis of RNA from cells exposed

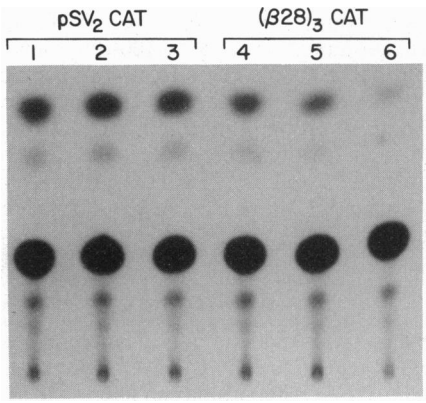

FIG. 4. Effect of serum albumin on transactivation function of the HNF-1α recognition element. HuH7 cells were cotransfected by lipofection with 1 μg of pSV$_2$CAT plasmid (lanes 1–3) or 14 g of supercoiled (β28)3CAT plasmid (lanes 4–6) and 2 μg of pRSVluc internal control plasmid. After 16–18 hr, cells were washed with PBS, and fresh culture medium was added containing 2% HSA (lanes 2 and 5) or 5% HSA (lanes 3 and 6) and assayed 48 hr later for CAT activity.

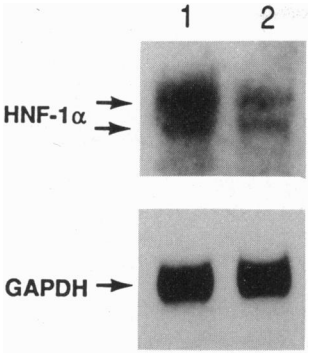

FIG. 5. Effect of serum albumin on the steady-state level of HNF-1α mRNA. Total cellular RNA was extracted from FAO cells incubated for 48 hr in the absence (lane 1) or the presence (lane 2) of 5% RSA. Northern blot analysis of 20 μg of total RNA was performed using specific cDNA probes, as noted. The arrows indicate the 3.6- and 3.2-kb HNF-1α transcripts.

or not exposed to serum albumin was performed, using a specific HNF-1α cDNA probe. Fig. 5 shows a significant decrease in the steady-state level of HNF-1α mRNA in FAO cells incubated for 48 hr in the presence of 5% RSA, with no change in the mRNA level of a control gene (GAPDH).

DISCUSSION

The present study addressed the molecular basis for feedback control of albumin gene transcription by serum albumin and other macromolecules in highly differentiated rat and human hepatoma cell lines. Specific studies included albumin mRNA accumulation, albumin promoter-driven reporter gene activity, and nuclear protein–albumin promoter element interactions in response to oncotically active macromolecules, including serum albumin and high molecular mass dextran. We were able to show that a sequence of 282 bp upstream of the albumin mRNA cap site, unaided by the presence of any enhancer element, determines the responsiveness of a CAT reporter gene to changes in external COP. By protein–DNA binding studies and transfection experiments, we showed that interaction of HNF-1α with its recognition element appears to be specifically affected by fluctuations in COP, regardless of the promoter context in which this recognition element is contained (i.e., the albumin promoter and another oligonucleotide cassette specifically responsive to HNF-1α were similarly affected). Finally, the production of HNF-1α mRNA appears to be selectively down-regulated by changes in COP.

HNF-1α is a dominant liver regulatory protein distantly related to the POU-homeobox family (31, 32). It binds to DNA as a dimer via a helix–turn–helix motif and, in addition to albumin, controls the transcriptional activity of several other liver-specific genes, including α- and β-fibrinogen (26), α_1-antitrypsin (26, 33), α-fetoprotein (34, 35), transthyretin (36), aldolase B (37), pyruvate kinase (38), and the hepatitis B virus large surface antigen gene (34). These features and the fact that HNF-1 is related to homeoproteins suggest that this transcription factor may be involved in the establishment of the differentiated liver phenotype.

The present study indicates that HNF-1α binding activity to its consensus sequence on albumin or other gene promoters might also respond to environmental stimuli, such as fluctuations in the COP, which may thus control the transcriptional response of these genes. In fact, although each cis element on the albumin promoter has been shown to contribute to some extent to the tissue-specific expression of this gene, occupancy of element B by HNF-1α appears to play a key role in controlling albumin gene transcription (18, 30, 39). Besides albumin, the hepatic output of other plasma proteins, whose gene promoters contain an HNF-1α recognition element, is also influenced by changes in the serum COP. This is demonstrated by a significant increase in the circulating level of these proteins during severe hypoproteinemia [e.g., nephrotic syndrome, analbuminemia, etc. (40, 41)].

The precise mechanism for the reduction of HNF-1α and/or the specific signaling pathway from the cell surface to the nucleus has not been identified. However, these findings may be related to the recently observed influence of changes in cell shape induced by extracellular matrix components on specific gene expression by hepatocytes (42). Moreover, another important transcription factor, DBP, which binds to the albumin promoter-element D and contributes to expression of the albumin gene, is specifically sensitive to still unrecognized environmental stimuli (presumably hormones) intervening during the normal circadian rhythm (43). Indeed, an increase or decrease in hepatic cell volume due to critical changes in the level of serum colloids might trigger intracellular signaling pathways leading to modified activity of specific nuclear transcription factors (e.g., HNF-1α), which in turn controls the transcriptional activity of cell-specific genes responsive to this factor. Elucidation of these pathways and the specific mechanisms by which the eukaryotic cell and/or hepatocyte sense a change in external colloid osmotic pressure represent intriguing areas for future research. It is also known that expression of the HNF-1α gene is regulated by HNF-4 (44). Whether this factor is ultimately responsible for the changes in HNF-1 expression induced by oncotically active macromolecules remains to be determined.

Note Added in Proof. While these studies were in progress, Tsutsumi *et al.* (45) reported results similar to those presented in Fig. 1.

We thank Dr. Gerald Crabtree for providing the HNF-1α cDNA probe, $(\beta 28)_3$ CAT, and HNF-1α antibody; Dr. Mario Chojkier for C/EBPβ antibody; Dr. R. Lawn for the HSA cDNA probe; and Dr. J. Wilson for pAlbCAT. Our research was supported in part by National Institutes of Health Grants DK-17609 and P30-DK-41296.

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