Cloning and Characterization of a Novel Zinc Finger Transcriptional Repressor

A DIRECT ROLE OF THE ZINC FINGER MOTIF IN REPRESSION

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We have identified a novel transcriptional repressor, AEBP2, that binds to a regulatory sequence (termed AE-1) located in the proximal promoter region of the aP2 gene that encodes the adipose fatty acid-binding protein. Sequence analysis of AEBP2 cDNA revealed that it encodes a protein containing three Cys2-His2-type zinc fingers. Northern blot analysis revealed two transcripts (4.5 and 3.5 kilobases) which were ubiquitously expressed in every mouse tissue examined. In co-transfection assays, AEBP2 repressed transcription from the homologous aP2 promoter containing multiple copies of the AE-1 sequence. Moreover, a chimeric construct encoding a fusion AEBP2 protein with the Gal4 DNA-binding domain was able to repress the transcriptional activity of a heterologous promoter containing the Gal4-binding sequence. The transcriptional repression function of AEBP2 was completely abolished when one of the conserved histidine residues and a flanking serine residue in the middle zinc finger were replaced with an arginine residue. The defective transcriptional repression function of the mutant derivative was due neither to lack of expression nor to a failure to localize to the nucleus. Moreover, both the wild-type and mutant derivative of either the histidine-tagged recombinant AEBP2 proteins or the in vitro translated Gal4-AEBP2 fusion proteins were equally able to bind to the target DNA. These results suggest that a portion of the zinc finger structure may play a direct role in transcriptional repression function, but not in DNA binding.

The adipose P2 (aP2 or 422) gene, which encodes the adipose fatty acid-binding protein, is thought to be an important gene in triglyceride metabolism during adipocyte differentiation (1–3). The abundance of aP2 mRNA is greatly enhanced during adipocyte differentiation (4). The AE-1 sequence (nucleotides −159 to −125) in the proximal promoter region of the aP2 gene functions in aP2 gene expression as either a positive or a negative regulatory element. Mutation at the AE-1 site affects its ability to bind specific nuclear factors and diminishes the promoter function of the aP2 gene in adipocytes (5). At least one protein, C/EBPα1, binds to the AE-1 sequence and functions as a transcriptional activator for aP2 gene expression during adipocyte differentiation (5, 6). Other AE-1-binding proteins (termed AEBP) in 3T3 preadipocytes have been implicated as transcriptional repressors in the regulation of aP2 gene expression (5, 7, 8). To clone a cDNA encoding a protein that interacts at the AE-1 site, we expressed cDNAs from a 3T3-L1 preadipocyte cell library with a Uni-Zap XR vector (Stratagene) and screened for the clones by the Affinity Screening procedure using random concatamers of the AE-1 sequence. Three independent phage plaques were isolated that produce fusion proteins interacting specifically with the AE-1 sequence. Further analysis revealed that one cDNA clone encodes mRNA whose expression is down-regulated during adipocyte differentiation. This cDNA and its encoded protein, AEBP1, has been previously characterized in detail. AEBP1 is a novel carboxypeptidase, and the carboxypeptidase activity of AEBP1 is involved in aP2 repression. AEBP1, by binding to the regulatory AE-1 site, acts as a negative regulator of aP2 gene expression (9). A new member of the family of AE-1-binding proteins, being reported here, is a zinc finger protein which is able to repress reporter gene expression through both homologous and heterologous promoters. Overexpression of AEBP2 in cells with a reporter construct, which is driven by the aP2 promoter containing a multiple copies of the AE-1 sequence, resulted in repression of transcriptional activity. Furthermore, an AEBP2 fusion protein with the DNA-binding domain of the yeast transcriptional activator Gal4 was able to repress the expression of a reporter gene driven by a heterologous promoter with five copies of the Gal4-binding sequence. Significantly, the repression function of AEBP2 was completely abolished, without affecting its DNA binding ability and expression level, when one of the conserved histidine residues in the middle zinc finger motif was mutated. These results indicate that the middle zinc finger motif of AEBP2 is critical for the transcriptional function, and suggest that some of the zinc finger motif may not be involved in DNA binding.

EXPERIMENTAL PROCEDURES

Molecular Cloning of AEBP2—To clone a cDNA encoding a protein that interacts at the AE-1 site (nucleotides −159 to −125 of the aP2 gene, Refs. 8 and 9) in 3T3-L1 preadipocytes, cDNAs from a 3T3-L1 preadipocytes library were expressed with a Uni-Zap XR vector (Stratagene) and screened for an expression clone by the affinity screening procedure (10) with random concatamers of the AE-1 sequence as described previously (9).

Computer Homology Searches and Sequence Analysis—The 303-residue AEBP2 sequence was compared with the non-redundant GenBank™ data base using the National Center for Biotechnology Information program tblastn. Sequences with significant matches around
protein or the protein was made by utilizing the expression vector pET-16b (Novagen) washed twice with 2X SSC, 0.1% SDS for 30 min at room temperature and twice with 0.2X SSC, 0.1% SDS for 30 min at 65 °C before exposure to x-ray film overnight at 20 °C.

Northern (RNA) Blot Analysis—Total RNA from different mouse tissues was prepared with the RNA STAT-60 Solution (TEL-TEST “B,” Inc.) according to the manufacturer’s protocol. Twenty micrograms of total RNA from each tissue were loaded on a formaldehyde denaturating 1% agarose gel and blotted onto MSi nylon transfer membrane (Micron Separations Inc.) as described (11). The filter was hybridized in the QuiHyb solution (Stratagene) with 32P-labeled AEBP2 cDNA probe in a hybridization oven (Hybaid) for 3 hr at 65 °C. The filter was washed twice with 2X SSC, 0.1% SDS for 15 min at room temperature and twice with 0.2X SSC, 0.1% SDS for 30 min at 65 °C before exposure to x-ray film at −70 °C.

Electrophoretic Mobility Shift Assay (EMSA)—Recombinant AEBP2 protein was made by utilizing the expression vector pET-16b (Novagen) as described previously (9). EMSA was carried out by incubating the recombinant protein or the in vitro produced Gal4 fusion proteins, which were made by the TNT-coupled wheat germ extract system (Promega), with 0.25 fmol of 32P-end-labeled AE-1 sequence in a buffer containing 100 mM KCl, 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, and 200 mM ZnCl2. After 30 min incubation at room temperature, the mixture was loaded on a 4% polyacrylamide nondenaturing gel in 6.75 mM Tris-HCl, pH 7.9, 1 mM EDTA, pH 8.0, 3.3 mM sodium acetate, pH 7.9, and 2.5% glycerol, and electrophoresed at 15 V/cm at 4 °C. Both the gel and the running buffer contained 100 mM ZnCl2.

Transfection and CAT Assay—The transient transfection assay was carried out by the Polybrene procedure (12) as described previously (9). All transfections also included 1 mM g of the reporter plasmid pB2/3AE-1/−120/CAT and 5 μg of the AEBP2 expression plasmid pG4-AEBP2. The transfection was also performed with 5 μg each of the reporter plasmids pGALKTCKAT or pTKCAT and the fusion Gal-AEBP2 expression plasmid pG4-AEBP2 or the control plasmid pG4-AEBP2(−). All transfections also included 1 μg of pHermesLaz, β-Galactosidase activity was assayed 48 hr after transfection to normalize for transfection efficiency, and CAT activity was assayed as described (9).

RESULTS

Isolation and Sequence Analysis of AEBP2 cDNA—A 3T3-L1 preadipocyte cDNA expression library (Uni-Zap XR vector; Stratagene) was screened with random concatamers of the AE-1 sequence by the affinity screening procedure (10) as described previously (9). Three independent phage plaques (A1, A2, and A8) produced fusion proteins interacting specifically with the AE-1 sequence. Further screening of the library with the partial cDNA sequence from A2 resulted in the isolation of a longer cDNA, which we named AEBP2 (preadipocyte enhancer-binding protein 2, different from AEBP1 (9)). Sequence analysis revealed that this cDNA contains an open reading frame of 303 amino acid residues from the first ATG codon located at nucleotide 85 to a termination codon located at nucleotide 994 (Fig. 1A). The polypeptide predicted in the open reading frame from the first ATG codon has a calculated relative molecular mass of 33.3 kDa. Coupled in vitro transcription and translation of the AEBP2 cDNA produced a protein with a molecular mass of about 35 kDa (data not shown). Moreover, a protein with a molecular mass of about 33 kDa was immunoprecipitated with antibody raised against the recombinant AEBP2 protein (data not shown). Therefore, based on the molecular weights of the endogenous and in vitro translated products and the Kozak rule for the translation initiation site (13), the first ATG codon is most likely the translation start site. The cDNA has a relatively long 3′-untranslated region with a consensus polyadenylation signal (AAUAAA) and poly(A) tail. A schematic representation of AEBP2 cDNA is shown in Fig. 1B.

Structural Properties of AEBP2—The predicted protein contains three tandemly repeated sequence motifs related to the Gli-Krüppel (CysX2-HisX7) type zinc finger (Fig. 2A). All three zinc fingers encoded by the cDNA of AEBP2 fit the consensus sequence for this type of zinc finger (Fig. 2B). Sequence alignment of the zinc fingers of AEBP2 with those of other zinc finger proteins containing three zinc finger motifs is shown in Fig. 2C.

Outside the putative zinc finger motif, a single consensus phosphorylation site ([Ser*/Thr*]-Pro-X-Lys/Arg) for Cdc2 kinase, a highly conserved cell cycle regulatory serine/threonine kinase, exists in the open reading frame (SPSK, Fig. 1). The consensus phosphorylation site is flanked by the zinc finger domain and a region with high proportion of basic amino acids (RRK>LKNKKRR) that may be a nuclear localization signal (NLS) (Fig. 1). Phosphorylation at the SPSK site may affect the function of the NLS and regulate import of the protein into the nucleus (14).

Tissue Distribution of AEBP2 mRNA—Northern hybridization was conducted to determine the expression pattern of AEBP2 in a variety of mouse tissues. Two transcripts (~4.5 and ~3.5 kilobases) with different degrees of abundance were detected in all the tissues (Fig. 3). Relatively low levels of AEBP2 RNA were detected in the liver (lane 3). In contrast to all other tissues, the brain contained the larger form of AEBP2 RNA more abundantly (lane 6). In some tissues (e.g. skeletal muscle, brain, and heart) extra RNA with higher molecular weights was also detected (lanes 5, 6, and 8). The significance of such differential expression of AEBP2 mRNA in different tissues is not understood. AEBP2 transcripts were also detected in embryonic brain and liver tissues, and in the placenta (data not shown). The ubiquitous nature of AEBP2 expression raises the possibility that AEBP2 may have an important function in mouse development.

AEBP2, a DNA-binding Protein—We have reported previously that the AE-1 site serves as a bifunctional element in the regulation of aP2 gene expression (5, 8, 9). The AE-1 sequence binds either the transcriptional activator CEBPγ (5) or the transcriptional repressor AEBP1 (9). AEBP2, as another AE-1-binding protein, may also participate in this regulation. Histidine-tagged recombinant AEBP2 protein was used to characterize the binding properties of AEBP2 as described previously (9). Here we show that AEBP2 binds to the AE-1 oligonucleotide in a sequence-specific manner (Fig. 4). The AEBP2-AE-1 complex can be competed away by adding an excess amount of the unlabeled AE-1 oligonucleotides (Fig. 4, lane 3), but not by SP1 (lane 4) or by AP3 (lane 5) binding sequences. The binding reaction needs Zn2+, for very weak or no binding was observed in the absence of Zn2+ (data not shown). Interestingly, even without an intact second zinc finger motif, as indicated by mutating one of the conserved histidine residues (AEBP2 cDNA was digested with DraIII at nucleotides 422 to 430 and the newly created ends were blunted by exonuclease activity and ligated, replacing the histidine 114 and serine 115 residues with an arginine residue), the binding activity remained intact (data not shown). These results suggest that the second zinc finger motif is dispensable for DNA binding, and that the DNA binding activity may be localized in the other two fingers.

Transcriptional Function of AEBP2—To examine whether
**FIG. 1.** Primary structure of mouse AEBP2. A, nucleotide and predicted amino acid sequence of mouse AEBP2. A polyadenylation signal (aataaa) in the 3'-untranslated region is underlined. Conserved histidine and cysteine residues in the zinc finger motif are also underlined. The NLS consisting of amino acids from 171 to 181 is also underlined. A Cdc2 kinase phosphorylation site (SPSK) located at amino acids 163 to 166 is in italics and underlined. A restriction enzyme DraIII site at nucleotides 422–430, which was utilized in the construction of the mutant derivative of AEBP2 (see text), is underlined. A restriction enzyme XmnI site at nucleotides 820–829, which was utilized in the construction of the Gal4-AEBP2 fusion expression plasmid (see “Experimental Procedures”), is also underlined. B, schematic representation of the AEBP2 open reading frame and cDNA. The region indicated by a vertical line adjacent to the NLS is a putative Cdc2 kinase phosphorylation site (SPSK). The thin lines depict untranslated regions of the cDNA.
AEBP2 is able to regulate transcription through interaction with the AE-1 site, we used a reporter construct in which the bacterial chloramphenicol acetyltransferase (CAT) gene expression is driven by the \( aP2 \) promoter containing an AE-1 site (9), along with an AEBP2 expression plasmid (pRc/CMVAEBP2). In preliminary transient transfection experiments, we have not observed any effect of AEBP2 on expression of the CAT gene driven by the native \( aP2 \) promoter (2168 to 1216), which contains the AE-1 site at nucleotides 2159 to 2125. This lack of effect may be due to a positive element (AP1) at nucleotides 2125 to 2118 (5). Since transcriptional activity can be influenced by increasing the extent of binding site of a transcription factor, we have analyzed the transcription function of AEBP2 with a reporter containing a multiple copies of the AE-1 sequence. Transient transfection analysis with the CAT reporter plasmid \( \text{paP2}(3\text{AE-1/2}120)\text{CAT} \), in which the CAT gene is driven by the \( aP2 \) promoter (−120 to +21) with three copies of the AE-1 sequence inserted upstream of the promoter, demonstrated that AEBP2 has repression activity. The CAT activity from cells co-transfected with pRc/CMVAEBP2 was significantly decreased compared with the CAT activity in cells co-transfected with the control plasmid pRc/CMV (Fig. 5A). From the in vitro binding assay, we concluded that the middle zinc finger is dispensable for binding to the AE-1 sequence. We therefore asked whether the mutation in the middle zinc finger has any affect on the repression function of AEBP2. We constructed an expression plasmid that expresses the mutant derivative of AEBP2, and analyzed the transcriptional repression function using the reporter plasmid \( \text{paP2}(3\text{AE-1/2}120)\text{CAT} \). As shown in Fig. 5A, the repression activity of this mutant version of AEBP2 was significantly diminished, suggesting that the middle zinc finger may be critical for the repression function.

To further characterize the transcriptional function of AEBP2, we used a AEBP2 fusion protein with an added DNA-
**AEBP2 is a transcriptional repressor.** NIH 3T3 cells were plated in 60-mm dishes and transfected with the indicated plasmids by the Polybrene method (12). **A**, CAT activity from cells transfected with the reporter plasmid paP2(3AE1/-120)CAT, which contains three copies of the AE-1 sequence upstream of the aP2 promoter (-120 to +21). In each transfection 5 μg of effector plasmid (pRC/CMV, pRC/CMVAEBP2 or the mutant derivative pRC/CMVAEBP2(HS/R)) and 2 μg of paP2(3AE1/-120)CAT along with 1 μg of pHermes-lacZ, a CMV-driven β-galactosidase expressing plasmid, were used. β-Galactosidase activity was used to normalize the transfection efficiency and CAT assays was performed as described (9). The values shown represent three separate transfection experiments. **B**, transient transfection analysis was carried out with 5 μg each of the reporter plasmids pGALTKCAT (lanes 1–3) or pTKCAT (lanes 4–6) and the Gal4-fusion plasmids pG4-AEBP2, the mutant derivative pG4-AEBP2(HS/R) and the control plasmid pG4-AEBP2(-). AC and C indicate acetylated chloramphenicol and chloramphenicol, respectively. Similar results were obtained in three different transfection experiments. **C**, both the wild-type and the mutant derivative of the Gal4-AEBP2 fusion protein were equally expressed. Protein extracts from cells transfected with either pG4-AEBP2 or pG4-AEBP2(HS/R) were prepared, and 20 μg of the protein extracts were loaded on a 10% SDS-polyacrylamide gel electrophoresis, then Western immunoblot analysis was performed according to the standard protocol using anti-Gal4 (DNA-binding domain) antibody (Santa Cruz Biotechnology) and ECL detection reagents (Amersham). The fusion proteins Gal4-AEBP1 (9) and Gal4-AEBP2 or Gal4-AEBP2(HS/R) are indicated. **D**, both the wild-type and the mutant derivative of the Gal4-AEBP2 fusion protein were equally able to bind the Gal4-binding sequence. The TNT-coupled wheat germ extract system (Promega) was used to synthesize the Gal4-AEBP2 fusion proteins. EMSA was carried out with the extract from the in vitro translation reactions with pG4-AEBP2 (lane 1), pG4-AEBP2(HS/R) (lane 2) or the parental vector pG4 (lane 3). The probe was a DNA fragment containing five copies of the Gal4-binding sequence (9). The protein-DNA complex and the free probe are indicated.
binding specificity of the yeast Gal4 transcription factor as an effector, and the reporter plasmid pGALTKCAT, which contains five copies of the Gal4-binding sequence upstream of the TATA box of the thymidine kinase (TK) promoter driving the CAT gene expression (9), in the transient transfection assay. After co-transfection with pGALTKCAT, a very low CAT activity was measured from the cells transfected with pG4-AEBP2, the Gal4-AEBP2 expression plasmid (Fig. 5B, lane 2), in comparison to the activity in the control cells transfected with the control plasmid pG4-AEBP2(−), in which the AEBP2 sequence is inserted in the opposite orientation in relation to the Gal4 DNA-binding domain (Fig. 5B, lane 1). No difference in the CAT activity was observed when pTKCAT, a reporter plasmid lacking a Gal4-binding site, was used (Fig. 5B, lanes 4 and 5). Therefore, repression by AEBP2 requires localization to the promoter region, and the repression activity is specific and is not due to transcriptional “squelching” or to a general nonspecific shutdown of the RNA polymerase II machinery. Furthermore, these results suggest that the repression function of AEBP2 may be mediated by an active repression mechanism, rather than by a passive repression mechanism which usually involves by competition with a positive factor(s) in the binding to the common DNA site. As in the case of the homologous promoter assay, the mutation in the middle zinc finger of AEBP2 resulted in decreased repression activity when Gal4-AEBP2 was tested using a heterologous promoter with the Gal4-binding site (Fig. 5B, lane 3). To examine whether the wild-type and the mutant derivative of the Gal4-AEBP2 fusion proteins were equally expressed in the transfected cells, the fusion proteins were analyzed by Western blotting with the anti-Gal4 antibodies. As shown in Fig. 5C, both Gal4-AEBP2 (lane 1) and Gal4-AEBP2(HS/R) (lane 2) fusion proteins were equally detectable. The lack of repression function for the mutant derivative is not due to its failure to localize to the nucleus. Similar levels of wild-type and mutant derivative of the Gal4-AEBP2 fusion proteins were detected in the Western blot analysis of a nuclear fraction isolated from the transfected cells (data not shown). Moreover, this mutation did not affect the binding of the Gal4-AEBP2 fusion protein to the Gal4 binding sequence: both the wild-type and the mutant derivative were equally able to bind to the Gal4 binding sequence in the in vitro binding assay using the in vitro translated fusion proteins (Fig. 5D). These results further indicate that the middle zinc finger motif of AEBP2 is important for its repression function.

DISCUSSION

In this report, we have characterized a novel transcriptional repressor (termed AEBP2) containing three copies of the Gli-Krüppel (Cys2-His2)-type zinc finger motif, and demonstrated that at least one of the zinc fingers is important for the repression function. The zinc finger structure, which was originally identified as DNA binding structure in the RNA polymerase III transcription factor TFIIIA (15, 16), is one of the well known common motifs among transcription factors. Zinc finger proteins can function as either activators or repressors, and most of these proteins, in addition to having the zinc finger motif whose transcriptional role is not clearly understood, contain other types of transcriptional domains. Some well defined activation motifs have been characterized by the presence of serine/threonine-rich, proline-rich, glutamine-rich, or acidic amino acid-rich domains (17). For repression, the alanine-rich domain in the Drosophila protein Krüppel has been shown to be responsible for its repression function (18–20). The KRAB domain, an evolutionarily conserved Krüppel-associated box located in the N-terminal regions of more than one-third of all Krüppel-class zinc finger proteins, also has been characterized as a repression motif (21, 22). Other domains which have been implicated as repression motifs include a 34-amino acid element in the early growth response factor-1 transcription factor (23), the SCAN box (the term derived from the first letters of the names of the four proteins initially found to contain a common motif: SRE-ZBP, CT-fin-51, AW-1, Number 18 cDNA; Ref. 24), the POZ domain in the proto-oncogene product BCL-6 (25), a small C-terminal domain comprising the last 24 amino acids and containing several leucine-proline dipeptide repeats in the Mig1 protein (26), and a glycine-rich region in the YY1 transcription factor (27). A repression domain was also localized within the zinc finger region of YY1 (28). Unlike other zinc finger transcription factors, AEBP2 does not contain any of the repression motifs described above. Surprisingly, the repressing function of AEBP2 was totally abolished, without affecting its DNA binding ability, when the middle zinc finger was destroyed by mutating one of the conserved histidine residues. That a zinc finger motif is critical only for a transcription function has never been reported, although a functional domain which overlaps the DNA-binding domain has been localized in a zinc finger motif (28). The AEBP2 mutation in the middle zinc finger motif may alter the overall conformation of the AEBP2 protein, since the band shift of the Gal4-AEBP2-DNA complex was different between the wild-type and the mutant derivative (Fig. 5D). This apparent change in the conformation did not affect the DNA binding ability (Fig. 5D), but did severely affect the repression function of AEBP2 (Fig. 5, A and B).

Three broad mechanisms of transcriptional repression function are hypothesized (29). Repressors may directly compete with activators for binding to a common target DNA, repressors may bind simultaneously with activators but “quench” their functions, possibly by physically masking the activation domain, and the repressors may bind DNA and interact with the general transcription machinery itself, preventing the attainment of a transcriptionally competent state. For example, Krüppel-regulated transcription is in large part, through interactions with TFIIIB and TFIIIEβ, members of the general transcription machinery (30). The repression by transcription enhancer factor-1 of human chorionic somatomammotropin promoter activity is thought to be mediated by direct interaction with the TATA-binding protein (31). To interact with the general transcription machinery, this type of transcriptional repressor binds to specific sites in the promoter (30, 32). In some cases, the repressor may recruit a global regulator that functions as a co-repressor (27). AEBP2 may regulate aP2 gene expression similarly to one of those transcription factors that directly affect the general transcription machinery through an active repression mechanism. Alternatively, AEBP2 may recruit a co-repressor in the regulation of aP2 gene expression. The mutation in the middle zinc finger may cause an alteration of the overall conformation of the protein to prevent interaction with either the general transcription machinery or a co-repressor.

Previous evidence indicates that members of the Cys2-His2 zinc finger class of transcription factors are involved in the transcriptional repression of growth factor gene expression (24) and regulate cell differentiation, probably affecting the expression of the differentiation-specific genes (33, 34). At least one positive (C/EBPα, Refs. 5 and 6) and two negative (AEBP1, Ref. 9; and AEBP2) transcription factors are involved in the regulation of aP2 gene expression through the AE-1 site. In contrast to AEBP2, which is expressed both in preadipocytes and adipocytes, C/EBPα is expressed only in the late stage of adipocyte differentiation (1–3) and AEBP1 expression is abolished in

aP2

G.-P. He and H.-S. Ro, unpublished data.
the late stage of adipocyte differentiation. Among these factors, specific interactions may take place to set the proper stage in the controlling of gene expression, by either competitively or synergistically, at different stage of physiological condition.

In summary, the cloning and characterization of the structure, expression, transcriptional function, and DNA-binding property of the novel transcription factor AEBP2 have revealed that it is a new zinc finger transcriptional repressor that is able to bind specifically to the AE-1 site located in the proximal promoter region of the \( aP2 \) gene, and that the repression function, but not the DNA binding activity, requires an intact middle zinc finger motif. Two forms of AEBP2 mRNA are broadly expressed in various mouse tissues. AEBP2 may serve as a general transcriptional regulator since these transcripts were detected in both embryonic and adult tissues.

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3 S.-W. Kim, A. Muise, and H.-S. Ro, manuscript in preparation.