The C-terminus of the retinal homeobox (rax) gene product modulates transcription in a context-dependent manner

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Purpose: The evolutionarily conserved retinal homeobox (Rax) transcription factor is essential for normal eye development in all vertebrates. Despite Rax’s biologic significance, the molecular mechanisms underlying Rax molecular function as a transcriptional regulator are poorly defined. The rax gene encodes a conserved octapeptide motif (OP) near the N-terminus and several conserved regions in the C-terminus of unknown function, including the orthopedia, aristalless, rax (OAR) domain and the RX domain. The purpose of this study is to investigate the contribution of these conserved domains in Rax function.

Methods: N-and C-terminal deletion and point mutations were generated in Xenopus laevis rax.L (previously known as Rx1A) using PCR-based methods. We examined the ability of mutated Rax to transactivate a reporter gene consisting of a portion of a rax target gene promoter (from the Xenopus rhodopsin gene) fused to a firefly luciferase coding region and transfected into human embryonic kidney 293T (HEK293T) cells. Portions of the Rax C-terminal region were also assayed for transactivation activity in the context of a heterologous DNA binding domain with an appropriate reporter gene.

Results: Full-length Rax weakly activated the reporter. Deletion of the Rax C-terminus increased Rax activity, suggesting that the C-terminus functions to repress Rax activity. Further deletion eventually resulted in a decrease in activity, suggesting that the C-terminal region also can function to enhance Rax activity. Deletion or mutation of the OP motif resulted in a slight decrease in Rax activity. Mutation or deletion of the N-terminal OP motif resulted in a mild decrease in activity and dampened the activity levels of the C-terminal deletions. Further, fusion of the C-terminus of Rax to a heterologous DNA binding domain enhanced transactivation.

Conclusions: The present data indicate that the C-terminus of Rax can function to repress or activate transcription in a context-dependent manner. These data support our hypothesis that the highly conserved OAR domain, in combination with other regulatory elements in the Rax C-terminus, coordinates Rax activity, perhaps through functional interaction with the N-terminal OP motif. Taken together, these data provide insight into the structural features that regulate Rax activity.

The retinal homeobox (rax) gene product is essential for the normal development of the retina, where this gene product is necessary for normal development of retinal stem and progenitor cells and maintenance of photoreceptors in all vertebrates studied (reviewed in [1,2]). The rax gene is part of the paired-type homeobox gene family and encodes a protein that includes several conserved domains, including an octapeptide or engrailed-homology motif (OP), a paired-type homeodomain (HD), a Rx domain (RX), and an OAR domain, named after the first gene products with this domain, orthopedia, aristalless, and rax [3-5]. The HD is a well-characterized DNA binding domain, and the OP domain functions in transcriptional repression through interaction with Groucho family corepressors [6]. The OAR domain has been suggested to be involved in intramolecular functional regulation in a related protein, Prxl [7].

Rax is thought to primarily function as a transcriptional activator. It has been shown to bind the photoreceptor conserved element-1 (PCE-1) site, C/TAATTA, originally discovered in the transcriptional regulatory regions of several genes expressed in photoreceptors [8]. At least two such genes, rhodopsin and red cone opsin, are direct targets of rax [9]. Rax is involved in activation of expression of these genes [9,10]. Rax also activates expression of reporter genes containing PCE-1 sites [8,9,11-13]. Additionally, the rax loss-of-function phenotype can be phenocopied by overexpression of a constitutive repressor form of Rax (Rax-engrailed repressor fusion) [14,15]. However, some genes are upregulated by rax loss of function [16], suggesting that they may be normally repressed by Rax. Thus, rax functions as a transcriptional activator but may function as a repressor in some contexts.

Rax alone seems to function as a weak transcriptional activator in the reporter assays discussed above. However,
Rax can interact with other proteins to enhance reporter gene activation to a greater extent. Rax can synergistically interact with other factors that activate the rhodopsin gene promoter, such as crx and nrl, to activate reporter constructs [9,10,17]. Additionally, Rax can interact with the yap protein; in zebrafish retinal development, YAP interaction with Rx1 inhibits transactivation of photoreceptor development genes, such as otx2, crx, and rhodopsin [17]. Another paired-homeodomain transcription factor, Prx1, is a relatively weak transcriptional activator [7]. Prx1 contains potent activation domains that are repressed in an intramolecular manner by a C-terminal OAR domain. We wondered if the Rax OAR domain also functions as an intramolecular repression domain. In this work, we describe a functional analysis of Rax. We report that the Rax C-terminus suppresses Rax activity in the context of the full-length protein but can also promote transactivation in a heterologous reporter gene system. Thus, the function of the Rax C-terminus is dependent on the context.

METHODS

Gene nomenclature: The gene nomenclature for Xenopus conforms to the guidelines set out by the Xenopus Nomenclature Committee: The gene and RNA names and symbols are lowercase italic (e.g., rax), and the protein names are first letter capital, not italicized (Rax). (For more details, please refer to Xenbase.)

Plasmids: pCS2FLAG was a gift from Dr. Peter Klein (Philadelphia, PA; Addgene plasmid #16331). pCS2-mt-GFP was a gift from Dr. M. Klymkowsky (Boulder, CO; Addgene plasmid #15681). cDNA encoding the Rax (also known as Rx1A, Rax.L) or the Rax2 (also known as Rx-L) coding region, lacking the initiation codons, were inserted into pCS2FLAG with PCR-based cloning methods. Deletion and point mutants were generated with PCR-based standard cloning techniques. Luciferase assay plasmids (pRL null and pGL3 basic) were obtained from the manufacturer (Promega, Madison, WI). The Xenopus laevis rhodopsin promoter reporter gene (XOP-luciferase) was a gift from Dr. B. Knox (Syracuse, NY).

Reporter gene assays: Human embryonic kidney 293T (HEK293T) cells grown under standard conditions were obtained (ATCC, Manassas, VA). As the cells were obtained directly from ATCC and were used for assays not specific to the cell line (the assays are not dependent on the identity of the cell line and could have been performed in various lines), the cells were not verified with short tandem repeat (STR) analysis. The cells were transfected with effector and reporter plasmids using Fugene HD Transfection Reagent (Thermo-Fisher, Waltham, MA) as recommended by the manufacturer. For each transfection involving only a single effector (those shown in Figure 1, Figure 2, and Figure 3), a total of 0.92625 µg DNA was included (0.75 µg effector plasmid, 0.125 µg firefly reporter plasmid, 0.00125 µg Renilla luciferase plasmid [pRL null], and 0.05 µg green fluorescent protein (GFP) plasmid [pCS2-mt-GFP]). For transfection involving two effectors (those shown in Figure 2), a total of 1.00075 µg DNA was used (0.45 µg each effector plasmid, 0.0.075 µg firefly reporter plasmid, 0.00075 µg Renilla luciferase plasmid [pRL null], and 0.025 µg GFP plasmid). In all no-effector control transfection experiments, the effector plasmid was replaced with the corresponding empty vector (pCS2FLAG, GAL4DBD, or pCS2). Cells were observed under fluorescent light for expression of GFP as an indicator of transfection success and then harvested, lysed, and assayed for firefly and Renilla luciferase activities using the Stop & Glo Dual Luciferase Assay Kit (Promega) as recommended by the manufacturer. The firefly luciferase values were normalized to Renilla luciferase values, averaged, and expressed as fold induction relative to the effector vector-only controls. Experiments included nine replicates for each condition and were performed one to ten times (nine to 90 total replicates).

Statistical analysis: Error bars represent the standard error of the mean. Statistical analysis was performed using the Student t test with Microsoft Excel software. We applied the Bonferroni correction for multiple sampling. For reporter gene assays involving the XOP-luc reporter, 29 variables were tested (not all are included in this work) to yield a Bonferroni critical value of p<0.0017 (0.05/29). For reporter gene assays involving the GAL4 UAS-luc reporter, 16 variables were tested (not all are included in this work) to yield a Bonferroni critical value of p<0.003125 (0.05/16). We verified that all Rax proteins from mutated constructs were produced and were approximately the correct size by immunoblotting using antibodies raised against the FLAG epitope (Appendix 1).

RESULTS

The rax gene product acts as a weak activator in transactivation assays but activates transcription to a greater degree in cooperation with other transcription factors [9,10]. One possible explanation for this phenomenon is that Rax may contain an intramolecular repressive domain, as is the case for the related Prx1 protein [7]. To explore this possibility, we performed a functional analysis to determine the ability of Rax containing deletions of or point mutations in the C-terminus to transactivate a Xenopus rhodopsin gene promoter (XOP) reporter (Figure 1A). We will refer to the
transactivation activity simply as Rax activity. Deletion of the OAR domain resulted in a small but statistically significant increase in Rax activity (Figure 1C, construct ΔC293), suggesting that the OAR domain is inhibitory. Adjacent to the OAR domain is a conserved PPXY (PPPY starting at residue 279 in Rax), which is recognized by the WW domains of the YAP/TAZ proteins and is important for the function of zebrafish Rx1 [17]. Deletion past this motif increased Rax activity further, to approximately three times that of full-length Rax (Figure 1C, ΔC212). Deletion of the PPXY motif alone (Figure 1D, ΔPPXY), however, impaired Rax activity compared to full-length Rax (Figure 1D, FL) suggesting that repression of Rax activity by the C-terminus requires the PPXY motif and the OAR domain.

The region between the PPXY motif and the RX domain contains a species-specific number of proline residues.

Figure 1. The Rax C-terminal region contains activation and repression domains. Full-length (FL) or mutated Rax or Rax2 was assayed for the ability to activate expression of a *Xenopus rhodopsin* gene promoter–luciferase reporter gene (C and D or E, respectively). A: Schematic representation of the Rax constructs assayed. Numbers indicate amino acid residues. Dotted lines indicate internal deletions. Point mutations are marked by an asterisk (*). B: Schematic representation of Rax2 constructs assayed. C, D: Luciferase assay results for Rax constructs diagrammed in A. Reporter gene activity is calculated as firefly luciferase activity normalized to the *Renilla* luciferase control and is presented relative to activity of a pCS2-only plasmid control. Values represent the mean of three to nine independent experiments (nine replicates per experiment). Error bars represent standard deviation from the mean. All vector-only and Rax activity values are statistically significantly different from full-length Rax activity using a corrected (Bonferroni) critical value of p<0.0017 (*p=4.06 × 10^{-48}, n=81; **p=3.25 × 10^{-4}, n=27; ***p=1.04 × 10^{-31}, n=27; ****p=6.15 × 10^{-36}, n=27; *****p=3.73 × 10^{-4}, n=27; #p=8.75 × 10^{-18}, n=54; ##p=2.42 × 10^{-10}, n=27; ###p=3.28 × 10^{-20}, n=27). E: Rax2 ΔC180 activity is not statistically significantly different from full-length Rax2 activity (Student *t* test, p=0.02). Abbreviations: HD = homeodomain, OAR = orthopedia, aristaless, rax, OP = octapeptide domain, PPXY = PPXY motif (putative YAP/TAZ binding sequence), RX = Rx domain. *in construct 3PA denotes mutated proline residues (to alanine residues).
Deletion of this region further increased Rax activity (Figure 1C, ΔC244), suggesting that regulation of Rax activity by the C-terminus may be mediated by structural proline residues. Mutation of the three consecutive proline residues in this region (P278, P279, and P280) to alanine in the context of the full-length Rax protein also resulted in an increase in Rax activity (Figure 1D, construct 3PA), despite the presence of the PPXY motif and the OAR domain. Further deletion, removal of the RX domain (ΔRX), resulted in a decrease in Rax activity to essentially the level of full-length Rax (Figure 1C). However, deletion of the RX domain in the context of full-length Rax (ΔRX) resulted in a modest increase in activity (Figure 1D). These conflicting results indicate that the Rx domain has different functions in these two contexts.
The Rax-related protein Rax2 (also known as RxL, RaxL, or QRX) contains a homeodomain, an Rx domain, and an OAR domain that are similar to those of Rax (Figure 1B), but no identifiable OP, polyproline stretch, or PPXY motif. Rax2 functions as a stronger transcriptional activator than Rax in reporter gene assays [11-13]. Deletion of the Rax2 OAR domain did not have a significant effect on Rax2 activity (Figure 1E), further supporting our hypothesis that the OAR domain functions in concert with other intra- or intermolecular elements to mediate Rax activity.

We next investigated the involvement of the octapeptide motif (OP) in Rax transactivation activity. The OP is found in several transcription factors and is thought to function in mediating interaction with corepressors of the Groucho family [6]. The OP contains an invariant leucine residue in the seventh position [3,18]; mutation at this position in the Rax-relative aristales-related homeobox (ARX) gene (Gene ID 170302, OMIM 300382; L38P in X. laevis Arx, Figure 2B) is associated with mental retardation in human patients [19,20] and reduces Groucho-dependent repression activity [21]. We assayed the activity of several mutations or deletions of the portion of the Rax N-terminus containing the OP (Figure 2A). Mutation in the invariant leucine (L38P in Rax, Figure 2B) resulted in a statistically significant decrease in Rax activity (Figure 2C). A similar decrease was observed when the OP was deleted. Interestingly, deletion of the N-terminal 39 amino acids (ΔN39, spanning the N-terminus through the OP) did not result in a statistically significant change in Rax activity while deletion of the N-terminal 32 amino acids (ΔN32, spanning the N-terminus to the OP) resulted in a statistically significant increase in activity.

Figure 3. The Rax C-terminal domain is sufficient to mediate reporter gene expression. Portions of the Rax C-terminal region were fused to a heterologous GAL4 DNA binding domain (GAL4 DBD) and assayed for transactivation of a GAL4-responsive firefly luciferase reporter. Data presentation and abbreviations are as described in Figure 1. The GAL4 DBD–Rax C-terminal fusions are represented schematically at the top of the figure. Values represent the mean of three to ten independent experiments (nine replicates per experiment). Error bars represent standard deviation from the mean. Transactivation activity values for all constructs tested are statistically significantly different from the activity of vector-only or GAL4 DBD alone using a corrected (Bonferroni) critical value p<0.003125 (*p=1.77 × 10^{-9}, n=90; **p=4.36 × 10^{-40}, n=63; ***p=2.29 × 10^{-51}, n=63; ****p=8.33 × 10^{-51}, n=63; ****p=3.76 × 10^{-12}, n=45).
These results suggest that the OP does not function as a repression domain in the context of an intact Rax N-terminus.

We next asked if there was a functional interaction between the Rax N- and C-terminal regions. We prepared and assayed Rax constructs containing C-terminal deletion ΔC293 or ΔC244 in combination with L38P or ΔOP (Figure 2A). These C-terminal deletions alone resulted in increased activity, as discussed above. Additional mutation of the N-terminus did not have a noticeable effect on ΔC293 but dampened the increased activity of ΔC244. ΔC244 is 2.1 times as active as ΔC293 in the context of the wild-type N-terminus (2.10±0.118 fold) but less so in the context of L38P or ΔOP (1.33±0.0683 or 1.74±0.064 fold, respectively). These results indicate the possibility of a functional interaction between the Rax N- and C-terminal regions.

We next took a similar approach to systematically fuse Rax C-terminal regions to the GAL4 DNA binding domain (GAL4 DBD; Figure 3) and assayed the transactivation activity of these proteins using a GAL4-responsive reporter gene. Fusion of a known repression domain (enR from the engrailed gene) to GAL4 DBD strongly repressed reporter gene activity while fusion of a known activation domain (the viral VP16 activation domain) strongly activated reporter gene activity (Appendix 2).

Fusion of progressively longer Rax C-terminal fragments increased reporter gene transactivation with maximum activity achieved when the region C-terminal to the RX domain, including the conserved proline residues, the PPXY motif, and the OAR domain (Figure 3, C245–322). Interestingly, inclusion of the RX domain (C213–322) resulted in less robust transactivation compared to that of C245–322. Taken together, these data suggest that the C-terminus of Rax can repress transcription in the context of full-length Rax and activate transcription in the context of a heterologous DNA binding domain. Further, although transcriptional modulation by the C-terminus of Rax is context-dependent, the C-terminal proline residues, PPXY motif, and OAR domain appear to play a significant and cooperative role in net Rax activity.

**DISCUSSION**

We have presented data demonstrating that the Rax C-terminal region can modulate transcription in a context-dependent manner and may function through cooperation with the N-terminal region. These findings are consistent with the model proposed by Norris and colleagues for Prx1 [7]. In this model, the C-terminal domain, including an OAR domain, was proposed to act as an intramolecular inhibitor of Prx1 activity, and this inhibition could be alleviated in a context-dependent manner by interaction of the OAR domain with some cofactor, perhaps including neighboring transcription factors on a target gene promoter. Deletion of the OAR domain alone in the context of full-length Rax resulted in only a modest increase in Rax activity; when the OAR domain alone was fused to the GAL4 DBD, only a modest increase in transactivation was observed. Although these data imply that the OAR domain mediates Rax activity, a much greater effect was observed when the conserved proline residues, PPXY motif, and OAR domain were analyzed in combination. Additionally, deletion of the OAR domain from Rax2 did not affect Rax2 activity. Taken together, these data suggest that the OAR domain alone is a weak modulating factor in and of itself, and we propose that the OAR domain cooperates with additional regulatory elements within the Rax C-terminus, including the conserved proline residues and PPXY motif to modulate Rax activity. Finally, we found that mutation or deletion of the OP motif in the Rax N-terminal region reduced the degree of activation observed with deletion of the Rax C-terminus, consistent with the suggestion that the Prx1 OAR domain might interact with activation domains in the N-terminal portion of the peptide [7]. Future studies will focus on the biologic significance of the Rax C-terminus in vivo and evaluate its intra- and intermolecular interactions.

We found that deletion of the PPXY motif in the context of an absent OAR domain increased Rax activity, consistent with the zebrafish Rx1 result. However, we also found that deletion of the PPXY motif in the context of the intact C-terminus reduced Rax activity. Further, addition of the PPXY motif and the OAR domain to a heterologous GAL4 DNA binding domain increased activity compared to addition of the OAR domain alone. These latter results are consistent with the PPXY motif playing a positive role in enhancing Rax activity. Asoaka and colleagues demonstrated that signaling through the Hippo signaling pathway resulted in inhibition of retinal differentiation by interfering with rx1-mediated activation of crx, otx2, and rhodopsin, involving interaction between the Rx1 PPXY motif and the YAP WW domain [17]. They proposed that interaction between YAP and Rx1 was inhibitory to Rx1 target gene transcription while interaction of YAP with the TEAD transcription factor through a different domain activated transcription of proliferation-associated genes. YAP is known primarily as a transcription activator (reviewed in [22]). Interestingly, YAP/TAZ proteins can interact with Runx2 to act as transcriptional activators or repressors in a promoter-specific context [23]. This is thought to involve interactions with neighboring transcription factors or associated coregulators. It is possible that the contribution of the PPXY motif to Rax activity is dependent on interaction with cofactors that interact with different Rax...
C-terminal domains, such as the RX and OAR domains. Further, our experiments were performed in HEK293T cells using a rhodopsin promoter reporter gene, but in the absence of other rhodopsin transactivators, such as crx and nrl, while the zebrafish YAP/Rx1 experiments were performed in intact animals. It is possible that the transcriptional transactivation outcome of the Rax–YAP interaction is dependent on the promoter context, including the functions of neighboring transcription factors. Finally, the zebrafish experiments were performed using Rx1, which has a specific role in mediating photoreceptor differentiation [24]. It would be interesting to discover the nature of YAP involvement with other zebrafish rax gene products. Rx2 and Rx3 have overlapping but different roles in retinal development and photoreceptor differentiation. It is interesting to speculate that YAP may not repress Rax function in retinal progenitor cells as the protein does in differentiating photoreceptors.

We observed that the RX domain may have different functions in different contexts. The present data are consistent with the RX domain acting repressively in the context of an intact C-terminus only. We speculate that the RX domain may be involved in providing proper conformation or folding of the C-terminus. Additionally, the RX domain could be involved in promoting protein–protein interactions that modulate the activity of the Rax C-terminal region.

We observed that mutation or deletion of the OP motif resulted in a modest decrease in Rax activity, suggesting that the Rax OP motif does not function as a repression domain as is the case in other related proteins, such as Arx. It is possible that the Rax OP motif does not function as a Groucho-dependent repression domain in the context of the Rax. It is interesting to speculate that the conformation of the OP motif in the context of Rax is refractory to interaction with Groucho-family corepressors. It may be useful to assay the transactivation activity of the Rax N-terminus using a heterologous DNA binding domain and an appropriate reporter, as we did for the Rax C-terminus.

We have previously observed that Rax can synergistically interact with XLmaf and otx5b (Xenopus analogs of nrl and crx) in the context of the Xenopus rhodopsin gene promoter [9]. It is possible that one or both of these transcription factors interact with the OAR domain and/or PPXY motif, directly or indirectly, thus relieving the inhibitory activity of the Rax C-terminus. Additionally, the OAR domain may interact with other proteins to effect changes in transactivation activity. For example, HMG-17, associated with transcriptionally active chromatin, can interact with the Rax-relative Pitx2 through its HD and OAR domains, mediating activity of the Wingless (Wnt) signaling pathway [25]. We have demonstrated that the Arx C-terminus can interact with CtBP, where it interacts with Groucho-family corepressors that interact with the Arx OP [21]. We speculate that such interactions could result in recruitment of additional activators or repressors to the promoter through interaction with Rax alone or in combination with other transcription factors bound to the target promoter and result in further regulation of transcription through interaction with the basal transcription complex or changes in target gene chromatin accessibility.

**APPENDIX 1. IMMUNOBLOT OF TRANSFECTED CELL LYSATES.**

A. Immunoblot of lysates prepared from cells transfected with plasmids encoding FLAG-tagged Rax N-terminal mutations using antibodies raised against the FLAG epitope tag. Lanes: 1 – empty vector; 2 – Rax FL; 3 – L38P; 4 - ΔOP; 5 – ΔN31; 6 – ΔN39. B. Immunoblot of lysates prepared from cells transfected with plasmids encoding FLAG-tagged Rax C-terminal truncations using antibodies raised against the FLAG epitope tag. In this blot we observed a nonspecific band of approximately 60 kDa. This band was not observed in all blotting conditions. Lanes: 1 – empty vector; 2 – Rax FL; 3 - ΔC293; 4 – ΔC277; 5 – ΔC244; 6 – ΔC212. C. Immunoblot of lysates prepared from cells transfected with plasmids encoding FLAG-tagged Rax internal deletions and point mutations using antibodies raised against the FLAG epitope tag. Lanes: 1 – empty vector; 2 – Rax FL; 3 – ΔRX; 4 – ΔPPXY; 5 – 3PA. D. Immunoblot of lysates prepared from cells transfected with plasmids encoding FLAG-tagged Rax internal deletions and point mutations using antibodies raised against the FLAG epitope tag. Lanes: 1 – Rax FL; 2 – L38P + ΔC293; 3 – ΔOP + ΔC293; 4 – L38P + ΔC244; 5 – ΔOP + ΔC244. E. Immunoblot of lysates prepared from cells transfected with GAL4 DBD fusion proteins using antibody raised to GAL4 DBD. Lanes: 1 – GAL4 DBD; 2 – GAL4 DBD+enR; 3 – GAL4 DBD+VP16; 4 – GAL4 DBD+Rax C290–322; 5 – GAL4 DBD+Rax273–322; 6 – GAL4 DBD+Rax C245–322; 7 – GAL4 DBD+Rax C213–322. F. Immunoblot of lysates prepared from cells transfected with FLAG-tagged Rax and Rax2 using antibodies raised against the FLAG epitope tag. Lanes: 1 – Rax FL; 2 – Rax2 FL; 3 – Rax2 ΔC180. In all panels, numbers at the left edge of the blot represent sizes of molecular weight markers (kDa). To access the data, click or select the words “Appendix 1.”
APPENDIX 2. ACTIVITIES OF CONTROL GAL4 DBD FUSIONS.

A strong repression domain (the repression domain from the Drosophila melanogaster engrailed gene, enR) or a strong activation domain (the viral activation domain, VP16) were fused to a heterologous GAL4 DNA binding domain (GAL4 DBD) and assayed for transactivation of a GAL4-responsive firefly luciferase reporter. A. GAL4 DBD fusions used in this experiment. B. Reporter gene activity is calculated as firefly luciferase activity normalized to Renilla luciferase control and is presented relative to activity of the GAL4 DBD only construct. Values represent the mean of 9 - 10 independent experiments (9 replicates per experiment). Normalized values are shown above each bar. Error bars represent standard deviation from the mean. Transactivation activity values for all constructs tested are significantly different from the activity of GAL4 DBD using a corrected (Bonferroni) critical value p<0.003125. * - p=2.05 X 10^-4 n=90, ** - p=3.62 X 10^-13 n=90. For GAL4 DBD only, n=87. To access the data, click or select the words “Appendix 2.”

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